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(54)【発明の名称】周辺型ベンゾジアゼピン受容体関連タンパク質、クローン化発現及びその使用方法

(57)【要約】
本発明は、ＰＢＲ会合タンパク質をコード化する核酸に関するものであり、さらにこれをＰＡＰの製造に使用する方法に関するものであり、さらに当該ＰＡＰの使用方法に関するものである。本研究において、我々はＰＢＲタンパク質と相互作用を有するタンパク質（ＰＡＰ）を、酵母菌二ハイブリッド システムにより同定することに成功した。ＰＢＲはマウス精巣ｃＤＮＡライブラリ検索用の餌としても、これを使用した。ＰＢＲとの相互作用能力に基づいて５種類のクローンを単離した。これらのタンパク質はＰＢＲの機能調節に関与し、当該受容体の内因性リガンド又はアロステリック調節剤としても役立てることが可能である。

【特許請求の範囲】
【請求項１】
単離ＰＢＲ関連タンパク質（ＰＡＰ）のＤＮＡフラグメント又はそのタンパク質。
【請求項２】
ＰＢＲ関連タンパク質をコード化する単離精製ＤＮＡフラグメント。
【請求項３】
当該ＤＮＡフラグメントがＳＥＱ ＩＤ ＮＯ： １、ＳＥＱ ＩＤ ＮＯ： ２、ＳＥＱ ＩＤ ＮＯ： ３、ＳＥＱ ＩＤ ＮＯ： ４、及びＳＥＱ ＩＤ ＮＯ： ５の中で特定される配列或いは少なくとも３０個のヌクレオチドで構成される当該配列のポリヌクレオチド フラグメントで構成される、単離精製ＤＮＡフラグメント。
【請求項４】
当該ＤＮＡフラグメントがゲンバンク取得番号ＡＦ０２２７７０又はゲンバンク取得番号ＡＦ０２０３３８で特定される配列で構成され、或いは当該配列のポリヌクレオチド配列が少なくとも３０個のヌクレオチドで構成される、ＰＢＲ関連タンパク質のペプチドをコード化する単離精製ＤＮＡフラグメント。
【請求項５】
ＰＡＰ７の４６３個のアミノ酸、或いはＰＡＰ７の天然変異体又は合成変異体、或いは少なくとも１０個のアミノ酸で構成されるそのペプチド フラグメントをコード化する、請求項２記載の単離精製ＰＡＰ７ ＤＮＡフラグメント。
【請求項６】
ＰＡＰ８の１９０個のアミノ酸、或いはＰＡＰ８をコード化するその天然変異体又は合成変異体、或いは少なくとも１０個のアミノ酸で構成されるそのペプチド フラグメントをコード化する、請求項２記載の単離精製ＰＡＰ８ ＤＮＡフラグメント。
【請求項７】
ＰＡＰ１５の１６４個のアミノ酸、或いはＰＡＰ１５をコード化するその天然変異体又は合成変異体、或いは少なくとも１０個のアミノ酸で構成されるそのペプチド フラグメントをコード化する、請求項２記載の単離精製ＰＡＰ１５のＤＮＡフラグメント。
【請求項８】
ＰＡＰ２０の１９６個のアミノ酸、或いはＰＡＰ２０をコード化するその天然変異体又は合成変異体、或いは少なくとも１０個のアミノ酸で構成されるそのペプチド フラグメントをコード化する、請求項２記載の単離精製ＰＡＰ２０ ＤＮＡフラグメント。
【請求項９】
（ｉ）ベクター及び（ｉｉ）請求項１記載のＰＡＰ ＤＮＡフラグメントで構成される、組み替えＤＮＡ構造。
【請求項１０】
（ｉ）ベクター及び（ｉｉ）請求項３記載のＰＡＰ ＤＮＡフラグメントで構成される、組み替えＤＮＡ構造。
【請求項１１】
当該ベクターが発現ベクターである、請求項１０記載の組み替えＤＮＡ構造。
【請求項１２】
当該ベクターが原核生物のベクターである、請求項１０記載の組み替えＤＮＡ構造。
【請求項１３】
当該ベクターが真核生物のベクターである、請求項１０記載の組み替えＤＮＡ構造。
【請求項１４】
請求項１０記載の組み替えＤＮＡ構造で形質変換した宿主細胞。
【請求項１５】
当該細胞が原核生物の細胞である、請求項１４記載の宿主細胞。
【請求項１６】
当該細胞が真核生物の細胞である、請求項１４記載の宿主細胞。
【請求項１７】

請求項１５又は１６記載の細胞を、当該ＤＮＡフラグメントが発現され、当該ＰＡＰペプチドがこの方法により製造される条件下で培養することにより構成される、ＰＡＰペプチドを製造する方法。
【請求項１８】
請求項１７の方法により製造される、単離組み替えＰＡＰ。
【請求項１９】
ＳＥＱ ＩＤ ＮＯ： ７で特定されるアミノ酸配列又は少なくとも５個のアミノ酸で構成されるその一部のＰＡＰ７ポリペプチド。
【請求項２０】
ＳＥＱ ＩＤ ＮＯ： ８で特定されるアミノ酸配列又は少なくとも５個のアミノ酸で構成されるその一部のＰＡＰ８ポリペプチド。
【請求項２１】
ＳＥＱ ＩＤ ＮＯ： ９、又は少なくとも５個のアミノ酸から成るその一部として特定されるアミノ酸配列で構成される、ＰＡＰ１５ポリペプチド。
【請求項２２】
ＳＥＱ ＩＤ ＮＯ： １０、又は少なくとも５個のアミノ酸から成るその一部として特定されるアミノ酸配列で構成される、ＰＡＰ２０ ポリペプチド。
【請求項２３】
当該方法が、（ｉ）当該ＰＡＰを認識する抗体で当該サンプルを結合させ、（ｉｉ）ＰＡＰ及び当該ＰＡＰに対する抗体の間で形成された錯体の存在又は不存在を検出する、ＰＡＰ７、ＰＡＰ８、ＰＡＰ１５、ＰＡＰ２０で構成されるグループから選ばれるサンプル中でＰＡＰを検出する方法。
【請求項２４】
当該方法が二ハイブリッド分析で構成される、ＰＢＲ関連タンパク質を検出する方法。
【請求項２５】
ＳＥＱ ＩＤ ＮＯ： ６、７ 、８及び９で特定されるアミノ酸配列又はその一部を有するペプチドに対する抗体。
【請求項２６】
ＳＥＱ ＩＤ ＮＯ： １１で特定されるアミノ酸配列で構成されるペプチドに対するＰＡＰ７抗体。
【請求項２７】
当該方法が、（ｉ）当該ＰＡＰが当該細胞中で造られるように請求項１０記載の組み替えＤＮＡ構造を細胞中へ送入し、（ｉｉ）ＰＡＰ活性を低減させ又は除去する少なくとも１種類の薬剤を単独又は組み合わせて当該細胞に添加し、（ｉｉｉ） 当該薬剤の存在下に当該細胞中におけるＰＡＰ活性を検出してこれを当該薬剤を添加しなかった比較対照と比較することで構成される、ＰＡＰ活性を低減させ又はこれを除去する薬剤を検出する方法。ここに、比較対照と比較してＰＡＰ活性が減少した場合、当該減少は、薬剤がＰＡＰ活性を低減させ又は除去したことを示している。
【請求項２８】
当該方法が、（ｉ）ＰＡＰが当該細胞中で造られるように請求項１０記載の組み替えＤＮＡ構造を細胞に送入し、（ｉｉ）当該細胞に少なくとも１種類の薬剤を単独又は組み合わせて添加し、（ｉｉｉ） 当該薬剤がＰＡＰ活性を刺激するかどうかを調べる目的で、当該細胞中においてＰＡＰ活性を測定し、これを当該薬剤を添加しなかった比較対照と比べることにより検出することで構成される、ＰＡＰ活性発現促進薬剤の検出方法。ここに、当該細胞中における当該ＰＡＰ活性がと比較対照と比べて増加した場合、当該増加は当該薬剤が刺激性であることを示している。
【請求項２９】
ＰＡＰ活性の抑制能力を有する薬剤。
【請求項３０】
ＰＡＰ活性の促進能力を有する薬剤。

【請求項３１】
ＰＡＰ活性を低減又は除去することが有益な病気を治療するために使用する、請求項２９記載の薬剤で構成される治療用化合物。
【請求項３２】
ＰＡＰ活性の増加が有益な病気を治療するために使用する、請求項３０記載の薬剤で構成される治療用化合物。
【請求項３３】
サンプル中のＰＡＰ７、ＰＡＰ８、ＰＡＰ１５及びＰＡＰ２０で構成されるグループから選ばれた少なくとも１種類のＰＡＰを、ポリメラーゼ連鎖反応を使用して検出する方法。
【請求項３４】
ＰＡＰ ＲＮＡ又はｃＤＮＡへのハイブリッド化或いはＰＡＰ配列及び適切な補助薬剤の増幅もしくはそのいずれかに適した、当該ＰＡＰ ＲＮＡ又はｃＤＮＡに固有のプライマー又はオリゴヌクレオチドから成るサンプル中のＰＡＰ７、ＰＡＰ８、ＰＡＰ１５及びＰＡＰ２０で構成されるグループから選ばれた、少なくとも１種類のＰＡＰのＲＮＡ又はｃＤＮＡを検出することを目的とする診察用キット。
【請求項３５】
当該核酸を発現させ、当該細胞中でＰＡＰを造ることを目的として、当該細胞中に当該ＰＡＰをコード化するＰＡＰ核酸を導入することによる、細胞中のＰＡＰ３、ＰＡＰ７、ＰＡＰ８、ＰＡＰ１５及びＰＡＰ２０から成るグループから選ばれたＰＡＰの増加方法。
【請求項３６】
当該方法が当該治療を必要とする個人に、ＰＡＰ発現又は機能を低減させ又はこれを除去する有効量の薬剤を医薬用希釈剤に添加して投与することで構成される、細胞増殖の増加が原因で発生する病気を治療し又はその症状を改善することを目的とする治療方法。
【請求項３７】
当該病気が癌である、請求項３６記載の方法。
【請求項３８】
当該方法が、当該治療を必要とする個人にＰＡＰの発現量又はその機能を低減させ又はこれを除去する薬剤を医薬用希釈剤に添加してその有効量を投与することにより構成される、コレステロールの異常水準により発生する症状を治療又は改善することを目的とする治療方法。
【請求項３９】
当該症状が癌、神経退行性疾患、進行性疾患、ストレス、及び発作から成るグループから選ばれる、請求項３８記載の治療方法。
【請求項４０】
当該方法が、当該細胞中においてＰＡＰの水準を増減させることにより構成される、細胞中でＰＡＰの活性、機能又は標的を調節する方法。
【発明の詳細な説明】
【０００１】
（発明の分野）
本発明は、突然変異体、変異体、フラグメント及び誘導体を含む周辺型ベンゾジアゼピン受容体（ＰＢＲ）関連タンパク質（ＰＡＰ）をコード化する核酸分子に関するものである。　また本発明は、このような核酸分子で構成されるベクター及び宿主細胞、ＰＡＰの使用方法、ＰＡＰ又はＰＢＲの抑制剤及び活性化剤をスクリーニングする方法、及び本発明の組成物又はポリペプチドで構成されるキットに関するものでもある。
【０００２】
（発明の背景）
周辺型ベンゾジアゼピン受容体（ＰＢＲ）は、元来、これがベンゾジアゼピン ジアゼパムを比較的高親和力で結合させる機能を有するが故に発見されたものである（Ｐａｐａｄｏｐｏｕｌｏｓ，Ｖ；１９９３，Ｅｎｄｏｃｒ．Ｒｅｖ．１４，２２２－２４０）。ベンゾジアゼピンは、中枢神経系においてγ－アミノ酪酸受容体活性の調節による仲介

不安を緩和させる薬理作用を有し、そのために最も高い頻度で処方される薬剤の一つである（Ｃｏｓｔａ，Ｅ．及びＧｕｉｄｏｔｔｉ，Ａ．；１９７９，Ａｎｎｕ．Ｒｅｖ．Ｐｈａｒｍａｃｏｌ．Ｔｏｘｉｃｏｌ．１９，５３１－５４５）。ＰＢＲは、上記神経伝達物質受容体と区別される、もう一つの種類に属するベンゾジアゼピンの結合サイトである。より詳細な研究の結果、ベンゾジアゼピンの他にも、ＰＢＲは他の種類の有機化合物にも高親和力で結合することが判明した（Ｐａｐａｄｏｐｏｕｌｏｓ；１９９３，ｓｕｐｒａ）。ＰＢＲは、本発明において調査した全組織中に存在したが、ステロイド製造組織中に特に高濃度で存在することが明らかになった。当該組織中において、ＰＢＲは主に外部ミトコンドリア膜（ＯＭＭ）中に存在することが明らかになった（Ａｎｈｏｌｔ，Ｒ．Ｒ．Ｈ．ら；１９８６，Ｊ．Ｂｉｏｌ．Ｃｈｅｍ．２６１，５７６－５８３）。

【０００３】

１８ｋＤａのイソキノリン結合タンパク質が、クローン化することにより発現したＰＢＲであることも確認されている（Ｐａｐａｄｏｐｏｕｌｏｓ，Ｖ．１９９８，Ｐｒｏｃ．Ｓｏｃ．Ｅｘｐ．Ｂｉｏｌ．Ｍｅｄ．２１７：１３０－１４２．）。次にＰＢＲは、外部ミトコンドリア膜から内部ミトコンドリア膜へのコレステロール送入を仲介する、ステロイド生成組織（Ｐａｐａｄｏｐｏｕｌｏｓ，１９９８，ｓｕｐｒａ；Ｐａｐａｄｏｐｏｕｌｏｓ，Ｖ．ら，１９９０，Ｊ．Ｂｉｏｌ．Ｃｈｅｍ．２６５，３７７２－３７７９）の機能的構成成分であることも分かった（Ｋｒｕｅｇｅｒ，Ｋ．Ｅ．及びＰａｐａｄｏｐｏｕｌｏｓ，Ｖ．，１９９０，Ｊ．Ｂｉｏｌ．Ｃｈｅｍ．２６５，１５０１５－１５０２２）。さらに詳細な研究の結果、生体中で副腎ＰＢＲが薬理作用により減少し、これにより、グルココルチコイドの循環量が減少することが判明した（Ｐａｐａｄｏｐｏｕｌｏｓ，Ｖ．；１９９８，ｓｕｐｒａ）。さらに、ライジッヒ細胞中のＰＢＲ遺伝子を標的としてこれを攪乱し、これにより、コレステロールのミトコンドリア中への輸送及びステロイドの形成、ＰＢＲ ｃＤＮＡによるステロイド生成で突然変異細胞の形質移入が阻止されることも判明した（Ｐａｐａｄｏｐｏｕｌｏｓ，Ｖ．ら；１９９７，Ｊ．Ｂｉｏｌ．Ｃｈｅｍ．２７２，３２１２９－３２１３５）。

【０００４】

ステロイド生成細胞中にはＰＢＲが極端に豊富に存在し、これが主に外部ミトコンドリア膜上に存在することが分かった（Ａｎｈｏｌｔ，Ｒ．ら，１９８６，Ｊ．Ｂｉｏｌ．Ｃｈｅｍ．２６１，５７６－５８３）。ＰＢＲは、１８ｋＤａのイソキノリン結合タンパク質及び３４ｋＤａの孔形成電圧依存アニオン タンパク質、好ましくは外部又は内部ミトコンドリア膜の接触サイト上に存在するこれらタンパク質で構成される、マルチメーター錯体に関連するものと考えられている（ＭｃＥｎｅｒｙ，Ｍ．Ｗ．ら，Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．Ｕ．Ｓ．Ａ．８９，３１７０－３１７４；Ｇａｒｎｉｅｒ，Ｍ．ら，１９９４，Ｍｏｌ．Ｐｈａｒｍａｃｏｌ．４５，２０１－２１１；Ｐａｐａｄｏｐｏｕｌｏｓ，Ｖ．ら，１９９４，Ｍｏｌ．Ｃｅｌ．Ｅｎｄｏｃｒ．１０４，Ｒ５－Ｒ９）。

【０００５】

ＰＢＲの薬剤リガンドがその受容体に結合すると、これが生体内のステロイド生成細胞中においてステロイド合成を刺激する（Ｐａｐａｄｏｐｏｕｌｏｓ，Ｖ．ら，１９９０，Ｊ．Ｂｉｏｌ．Ｃｈｅｍ．２６５，３７７２－３７７９；Ｒｉｔｔａ，Ｍ．Ｎ．ら，１９８９，Ｎｅｕｒｏｅｎｄｏｃｒｉｎｏｌｏｇｙ４９，２６２－２６６；Ｂａｒｎｅａ，Ｅ．Ｒ．ら，１９８９，Ｍｏｌ．Ｃｅｌｌ．Ｅｍｄｏｃｒ．６４，１５５－１５９；Ａｍｓｔｅｒｄａｍ，Ａ．及びＳｕｈ，Ｂ．Ｓ．，１９９１，Ｅｎｄｏｃｒｉｎｏｌｏｇｙ１２８，５０３－５１０；Ｙａｎａｇｉｂａｓｈｉ，Ｋ．ら，１９８９，Ｊ．Ｂｉｏｃｈｅｍ．（Ｔｏｋｙｏ）１０６，１０２６－１０２９）。

【０００６】

同様にして、生体内研究の結果から、下垂体切除を行ったネズミの体内において、高親和性ＰＢＲリガンドがステロイドの血漿濃度を増加させることが分かっている（Ａｍｒｉ，Ｈ．ら，１９９６，Ｅｎｄｏｃｒｉｎｏｌｏｇｙ１３７，５７０７－５７１８）。さらに単離ミトコンドリアに関する生体内研究において、ＰＢＲリガンド、薬剤リガンド、又は内因性ＰＢＲリガンド、ポリペプチド ジアゼパム結合抑制剤（ＢＤＩ）（Ｐａｐａｄｏｐｏｕｌｏｓ，Ｖ．ら，１９９７，Ｓｔｅｒｏｉｄｓ６２，２１－２８）が、コレステロールの外部ミトコンドリア膜から内部ミトコンドリア膜への移行速度を増加させることにより、プレグネノロンの生成を刺激するという証拠が得られている（Ｋｒｕｅｇｅｒ，Ｋ．Ｅ．及びＰａｐａｄｏｐｏｕｌｏｓ，Ｖ．，１９９０，Ｊ．Ｂｉｏｌ．Ｃｈｅｍ．２６５，１５０１５－１５０２２；Ｙａｎａｇｉｂａｓｈｉ，Ｋ．ら，１９８８，Ｅｎｄｏｃｒｉｎｏｌｏｇｙ１２３，２０７５－２０８２；Ｂｅｓｍａｎ，Ｍ．Ｊ．ら，１９８９，Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．Ｕ．Ｓ．Ａ．８６，４８９７－４９０１；Ｐａｐａｄｏｐｏｕｌｏｓ，Ｖ．ら，１９９１，Ｅｎｄｏｃｒｉｎｏｌｏｇｙ１２９，１４８１－１４８８）。

【０００７】

１８ｋＤａ ＰＢＲのアミノ酸配列に基づき、三次元モデルが開発された（Ｐａｐａｄｏｐｏｕｌｏｓ，Ｖ．ら，１９９６，Ｔｈｅ Ｌｅｙｄｉｇ Ｃｅｌｌ；Ｐａｙｎｅ，Ａ．Ｈ．ら，（ｅｄｓ）Ｃａｃｈｅ Ｒｉｖｅｒ Ｐｒｅｓｓ，ＩＬ，ｐｐ５９６－６２８）。このモデルは、コレステロール分子及びチャンネル機能に適合することが示されており、コレステロールの輸送におけるＰＢＲの役割を支持している。最近、我々はステロイド生成において相同的組み替えを行ってＰＢＲが存在しない細胞を発生させ、これにステロイド製造能力が無いことを示すことにより、ＰＢＲがステロイド生成反応で演じる役割について証明した（Ｐａｐａｄｏｐｏｕｌｏｓ，Ｖ．ら，１９９７，Ｊ．Ｂｉｏｌ．Ｃｈｅｍ．２７２，３２１２９－３２１３５）。

【０００８】

しかし、コレステロールの水溶性同族体である２２Ｒ－ヒドロキシコレステロールを添加することにより、これらの細胞はステロイドの製造能力を回復した。このことは、コレステロールの輸送機構が損なわれていたことを示している。さらに１８ ｋＤａのＰＢＲタンパク質を発現した細菌中でコレステロールの輸送実験を行い、コレステロール チャンネル機能又はコレステロール輸送機能を有することに関して、その決定的な証拠を得ることができた（Ｌｉ及びＰａｐａｄｏｐｏｕｌｏｓ，１９９８，Ｅｎｄｏｃｒｉｎｏｌｏｇｙ１３９，４９９１－４９９７）。

【０００９】

神経膠腫（Ｒｉｃｈｆｉｅｌｄ，Ｅ．Ｋ．ら，１９８８，Ｎｅｕｒｏｌｏｇｙ３８，１２５５－１２６２）、結腸腺癌及び卵巣癌（Ｋａｔｚ，Ｙ．ら，１９８８，Ｅｕｒ．Ｊ．Ｐｈａｒｍａｃｏｌ．１４８，４８３－４８４；Ｋａｔｚ，Ｙ．ら，１９９０，Ｃｌｉｎｉｃａｌ Ｓｃｉ．７８，１５５－１５８）のできたネズミの脳などについて数多くの腫瘍研究を行い、正常組織と比較して周辺型ベンゾジアゼピン受容体（ＰＢＲ）の濃度が著しく増していることが判明した。

【００１０】

本明細書に引用した下記及び上記の全文書については、参考文献として完全な形でこれらを引用した。さらに、ヒトの脳神経膠腫又は星細胞腫において、正常な柔組織と比較してＰＢＲの密度が１２倍に増加していることが見いだされた（Ｃｏｒｎｕ，Ｐ．ら，１９９２，Ａｃｔａ Ｎｅｕｒｏｃｈｉｒ １１９，１４６－１５２）。

【００１１】

本発明者らは、ＰＢＲ密度は、これらの細胞中における当該受容体の増殖活性を反映している可能性を示唆した。最近、ＰＢＲが細胞の増殖に関与していることがさらに示されており（Ｎｅａｒｙ，Ｊ．Ｔ．ら，１９９５，Ｂｒａｉｎ Ｒｅｓｅａｒｃｈ ６７５，２７－３０；Ｍｉｅｔｔｉｎｅｎ，Ｈ．ら，１９９５，Ｃａｎｃｅｒ Ｒｅｓｅａｒｃｈ ５５，２６９１－２６９５）、ヒトの星細胞腫中におけるその発現が、腫瘍の悪性

度及び増殖指数に関連して起こるものであることが見いだされた（Ｍｉｅｔｔｉｎｅｎ，Ｈ．ら，ｓｕｐｒａ；Ａｌｈｏ，Ｈ．，１９９４，Ｃｅｌｌ Ｇｒｏｗｔｈ Ｄｉｆｆｅｒｅｎｃｅ ５，１００５－１０１４）。

【００１２】

ヒト乳ガンのバイオプシーにおいてＰＢＲのキャラクタリゼーションを行った結果、ヒト乳腫瘍細胞の侵襲性及び転移性はＰＢＲの発現濃度に比例することが見いだされた。即ち、これら侵襲性及び転移性細胞においては、ＰＢＲが攻撃的な腫瘍細胞の核の中に主に存在するのに対して、侵襲性ではあっても非攻撃的な細胞の細胞質中においてはＰＢＲが細胞質中に主に存在し、これら細胞中におけるＰＢＲの細胞下位組織中における局在性と相関することが見いだされた。ＰＢＲの発現におけるこれらの変化を、乳癌患者において、特に攻撃的な固形腫瘍の検出、診断、予防及び治療用のツールとして広く利用することができる。

【００１３】

ＰＢＲ及びその内因性リガンド（ポリペプチドとジアゼパムの結合抑制剤）はともに構造的にステロイド生成細胞中に発現するので、ホルモンによるＰＢＲ機能の調節は、他のタンパク質との関連により行われる。この相互作用により、ステロイドの生合成が開始される。従って、どのタンパク質がＰＢＲに関連し、ＰＢＲの機能を調節するのかを確認する必要がある。

【００１４】

（発明の要約）

本発明は、上記ニーズに適合するものである。我々は二ハイブリッド系を使用して、ＰＢＲと相互作用するＰＢＲ関連タンパク質（ＰＡＰ）を突き止めることができた。我々は、マウスの精巣ｃＤＮＡライブラリーを検索するためにＰＢＲを餌として使用した。そのＰＢＲとの相互作用能力により、これらのライブラリーからＰＢＲと相互作用する５種類のクローン（ＰＡＰ３、ＰＡＰ７、ＰＡＰ８、ＰＡＰ１５及びＰＡＰ２０）を単離した。確認されたヌクレオチド配列の中で、ＰＡＰ３は、前に単離したｍｅｇ１タンパク質（Ｄｏｎ，Ｊ．及びＷｏｌｇｅｍｕｔｈ，Ｄ．Ｊ．，１９９２，Ｃｅｌｌ Ｇｒｏｗｔｈ Ｄｉｆｆｅｒ．３，４９５；Ｅｖｅｒ，Ｌ．５，１９９９，Ｃｅｌｌ Ｇｒｏｗｔｈ Ｄｉｆｆｅｒ．１０，１９－２６）と同一の配列であった。ゲンバンク データベースを検索したところＰＡＰ７、ＰＡＰ８、ＰＡＰ１５及びＰＡＰ２０に相当する配列は見つからず、従ってこれらは新規配列であることが分かった。ＰＡＰ７及びＰＡＰ１７は、同じ新規タンパク質産物の異なるクローンである。全ＰＡＰは脂肪族アシル化（ミリストイル化）サイト及びＰＫＣホスフォリル化サイトを有している。さらに、ＰＡＰ２０はＰＫＡホスフォリル化サイトを有している。ＰＡＰの分布及び機能、並びにそのＰＢＲに対する機能的関係に関しては、現在検討中である。

【００１５】

これまで脳、精巣、卵巣、副腎、腎臓及び筋肉などのマウス主要組織中におけるＰＡＰ７の分布は、ＰＢＲのより広い発現パターンに類似のプロフィールを示し、その発現量は当該組織のステロイド生成能力に平行していた。これらのデータは、ＰＢＲの機能調節における、当該受容体の内因生リガンド又はアロステリック調節剤とし役立つこれらＰＡＰの役割を示している。

【００１６】

従って、ＰＡＰ３（ＳＥＱ ＩＤ ＮＯ：１）、ＰＡＰ７（ＳＥＱ ＩＤ ＮＯ：２）及びゲンバンク取得番号ＡＦ０２２７７０、ＰＡＰ８（ＳＥＱ ＩＤ ＮＯ：３）、ＰＡＰ１５（ＳＥＱ ＩＤ ＮＯ：４）及びＰＡＰ２０（ＳＥＱ ＩＤ ＮＯ：５）及びゲンバンク取得番号ＡＦ０２０３３８などのＰＢＲ関連タンパク質をコード化する新規ＤＮＡフラグンメントを提供することが、本発明の目的ではない。ＤＮＡフラグメントはｃＤＮＡであるかゲノムＤＮＡであるかを問わず、いずれもＰＢＲ関連タンパク質コード核酸配列の検出用診断剤として、又はＤＮＡコード化タンパク質調製用薬剤として、又はＰＡＰコード化配列調製用薬剤として、又は治療剤として使用することができる。

【００１７】
上記ＤＮＡ配列によりコード化されたＰＡＰ用アミノ酸配列を提供することが、本発明のもう一つの目的である。
【００１８】
ベクター及び上記ＤＮＡフラグメントで構成される組み替えベクターを提供することが、本発明のもう一つの目的である。
【００１９】
上記組み替えＤＮＡ構造で形質変換した宿主細胞を提供することが、本発明のもう一つの目的である。
【００２０】
宿主細胞を上記ＤＮＡフラグメントが発現されてＰＡＰが造られるような条件下で培養し、当該ＰＡＰを単離して、例えば薬剤及びＰＢＲ又はＰＡＰ自体の抑制剤検索試薬として、又は診断用試薬として、又は治療用試薬としてこれを使用することによりＰＡＰを製造する方法を提供することが、本発明のもう一つの目的である。
【００２１】
上記組み替えＰＡＰに対する抗体を提供することが、本発明のさらに他の目的である。
【００２２】
（ｉ）サンプルを、上記ＰＡＰのいずれか一つを認識する抗体と結合させ、
（ｉｉ）ＰＡＰとこれに固有の抗体との間で形成される錯体の存在又は不存在を検出することで構成される、サンプル中のＰＡＰ３、ＰＡＰ７、ＰＡＰ８、ＰＡＰ１５、又はＰＡＰ２０を検出する方法を提供することが、本発明のさらに他の目的である。
【００２３】
ＰＡＰに対する抗体及び細胞、組織又は酵母菌、哺乳類、動物、鳥、魚の血清、及び植物中にＰＡＰが存在することを検出する目的に適した補助試薬で構成される診断用キットを提供することが、本発明のさらに他の目的である。
【００２４】
ポリメラーゼ連鎖反応を使用して、サンプル中のＰＡＰを検出する方法を提供することが、本発明のさらに他の目的である。
【００２５】
ＰＡＰ ＲＮＡ又はｃＤＮＡへのハイブリッド化及びＰＡＰ配列の増幅に適したＰＡＰ ＲＮＡ又はｃＤＮＡに固有のプライマー又はオリゴヌクレオチド、及び哺乳類の組織中でＰＡＰ ＲＮＡ又はｃＤＮＡを検出する目的に適した補助試薬で構成される診断用キットを提供することが、本発明のさらに他の目的である。
【００２６】
ハイブリッド化分析により、サンプル中のＰＡＰ ＲＮＡ又はｃＤＮＡの存在又は不存在を分析することで構成される、サンプル中のＰＡＰを検出する方法を提供することが、本発明のさらに他の目的である。
【００２７】
サンプル中のＰＢＲを測定する方法を提供することが、本発明の目的の一つである。当該方法は、ＰＢＲと錯体化したＰＡＰの存在を測定することで構成される。
【００２８】
ＰＢＲとＰＡＰの相互作用を増減させることにより、ＰＢＲの機能を調節し又はＰＢＲの標的を変更する方法を提供することが、本発明のさらにもう一つの目的である。調節できるＰＢＲの機能には、コレステロールの細胞中への輸送機能、ステロイドの製造機能、細胞の増殖機能、及び胚形成機能などが含まれる。
【００２９】
ＰＢＲの機能を増減させるようなＰＡＰを細胞中へ送入することにより、細胞中におけるＰＢＲの機能又は発現量を増減させる方法を提供することが、本発明のさらにもう一つの目的である。
【００３０】

当該細胞中におけるＰＡＰ濃度を変更することにより、ステロイド生成量を増減させる方法を提供することが、本発明のさらにもう一つの目的である。

【００３１】

ＰＢＲの機能異常又は発現異常又は局在化が原因で細胞の増殖量が増え、これにより起こる病気を治療し又はその症状を改善する方法を提供することが、本発明のさらにもう一つの目的である。当該方法は、このような治療を必要とする個人に、ＰＢＲの当該異常発現、異常機能又は異常局在化を修正するような有効量のＰＡＰに医薬用希釈剤を添加し、これを投与することにより構成される。

【００３２】

細胞の増殖量が減少したために起こった病気を治療又は改善する方法を提供することが、本発明のさらにもう一つの目的である。当該方法は、このような治療を必要とする個人に、有効量のＰＡＰ又はこれに対する抗体又はＰＡＰの発現又は機能を抑制する薬剤を、医薬用賦形剤に加えてこれを投与することにより構成される。

【００３３】

ステロイド生成量の増減により起こる病気を治療又は改善する方法を提供することが、本発明のさらにもう一つの目的である。当該方法は、このような治療を必要とする個人に有効量のＰＡＰ又はこれに対する抗体或いはＰＡＰの発現量又は機能を抑制又は活性化する薬剤を医薬用賦形剤に添加して投与することにより構成される。

【００３４】

ＰＡＰ及び当該全配列又は当該配列のフラグメントを組み入れるベクターをコード化するｃＤＮＡ配列、並びに当該ベクターで形質変換され又はこれにより転写された原核生物又は真核生物の細胞を、このような細胞中においてＰＡＰ又はＰＢＲの発現又は機能を抑制する薬剤の検索に使用する目的で提供することが、本発明のさらにもう一つの目的である。

【００３５】

（詳細な説明）

本明細書に記載した５種類のＰＡＰは、二ハイブリッド化分析法を使用することにより見いだされた。当該二ハイブリッド化分析法は、生体内におけるタンパク質－タンパク質相互作用の検出を目的とする、酵母菌による遺伝子分析法である。二ハイブリッド分析法で陽性の結果が得られた場合、標的タンパク質と相互作用を有するタンパク質コード化遺伝子を迅速に同定することが可能である。さらに、当該二ハイブリッド分析法は、弱い一時的な相互作用でも検出できる高感度検出法である。このように弱い一時的な相互作用は、大型天然錯体の特徴である。最も注目すべきは、当該二ハイブリッド分析法は生体内で実施されるので、当該分析において関与するタンパク質は、天然の形態を取っている可能性が高い。

【００３６】

二ハイブリッド分析法は、多くの真核生物の転写活性化剤が、２種類の物理的に分離できるモジュール式ドメインで構成されているという事実に基づいて実施される。その一つはＤＮＡ結合ドメインとして作用し、他の一つは転写活性化ドメインとして機能する。ＤＮＡ結合ドメインは転写因子をこの因子により規制される遺伝子の上流域に存在する特定ＤＮＡ配列に局在化し、活性化ドメインは転写の開始に必要な他の転写成分に接触する。両ドメインは正常な活性化機能のために必要であり、通常、これら２つのドメインは同じタンパク質の一部を形成している。

【００３７】

我々のＰＡＰ検索実験においては、ＣＬＯＮＴＥＣＨ社から入手したＭＡＴＣＨＭＡＫＥＲ Ｔｗｏ－Ｈｙｂｒｉｄ Ｓｙｓｔｅｍを使用した。このＭＡＴＣＨＭＡＫＥＲ Ｓｙｓｔｅｍにおいては、ＧＡＬ４転写活性化剤の２つの機能ドメインをコード化する配列が、２つの異なるシャトル又は発現ベクター（ｐＧＢＴ９及びｐＧＡＤ１０）の中へクローン化されている。当該ｐＧＢＴ９ハイブリッド クローン化ベクターは、ＧＡＬ４ ＤＮＡ結合ドメインとＰＢＲタンパク質を融合させるために使用される。

【００３８】
ｐＧＡＤ１０ ハイブリッド クローン化ベクターは、ＧＡＬ４活性化ドメインとランダム タンパク質の集団を融合マウス精巣ライブラリー（ＣＬＯＮＴＥＣＨ社）の中で融合させるために使用される。両ハイブリッド タンパク質は、そのいずれかがＧＡＬ４ ＤＮＡ結合ドメインであるか又は異種起源から当該活性化ドメインに添加された核局在化配列により、酵母菌核を標的として作用する。
【００３９】
ＰＢＲタンパク質及び未知タンパク質（単数又は複数）が相互作用を及ぼし合う場合、ＧＡＬ４のＤＮＡ結合ドメインはその転写活性化ドメインに結合するので、上流のＧＡＬ４結合サイトを含む適切なレポーター遺伝子（ｌａｃＺ又はＨＩＳ３）の転写固有機能を使用して、２つのタンパク質間の相互作用が示される。これにより、２つの相互に作用し合うハイブリッド構造により形質変換されるクローンの陽性選択が可能となり、ライブラリー検索が一層便利で実用的に行えるようになる。陽性クローンの存在が確認された後、キットに備えられた配列決定用プライマーを使用して、相互に作用し合うタンパク質に対応する遺伝子の配列を決定した。
【００４０】
一つの態様において、本発明は、ＰＢＲ関連タンパク質（ＰＡＰ）をコード化するＤＮＡ又はｃＤＮＡの配列に関するものである。単離したクローンは５種類であった。即ち、ＰＡＰ３：５６８ ｂｐで構成され、ＳＥＱ ＩＤ ＮＯ： １で示される（Ｄｏｎ， Ｊ．及びＷｏｌｇｅｍｕｔｈ， Ｄ．Ｊ．， １９９２， Ｃｅｌｌ Ｇｒｏｗｔｈ Ｄｉｆｆｅｒ．３， ４９５； Ｅｖｅｒ， Ｌ．ら， １９９９， Ｃｅｌｌ Ｇｒｏｗｔｈ Ｄｉｆｆｅｒ．１０， １９－２６）。８３個のアミノ酸（ＳＥＱ ＩＤ ＮＯ： ６）から成るペプチドをコード化する。ＰＡＰ７：ＳＥＱ ＩＤ ＮＯ： ２で示される配列の６９６から１１６４まで延びる５７７ ｂｐで構成され、３６３個のアミノ酸から成り、ＳＥＱ ＩＤ ＮＯ： ７で示されるポリペプチドをコード化する。ＰＡＰ８：ＳＥＱ ＩＤ ＮＯ： ３で示される５６８ ｂｐで構成され、ＳＥＱ ＩＤ ＮＯ： ８で示される１９０個のアミノ酸から成るポリペプチドをコード化する。ＰＡＰ１５：ＳＥＱ ＩＤ ＮＯ： ４で示される４９０ ｂｐで構成され、ＳＥＱ ＩＤ ＮＯ： ９で示される１６４個のアミノ酸から成るポリペプチドをコード化する。ＰＡＰ２０：ＳＥＱ ＩＤ ＮＯ： ５で示される５８８ ｂｐで構成され、ＳＥＱ ＩＤ ＮＯ： １０で示される１９６個のアミノ酸から成るポリペプチドをコード化する。
【００４１】
ＰＡＰ３は、前に単離したｍｅｇ １と同じタンパク質であることが確認された。
【００４２】
ＰＡＰ７及びＰＡＰ１７は、同じ新規タンパク質産物の異なるクローンである。５’，３’－ＲＡＣＥ Ｓｙｓｔｅｍ （ＣＬＯＮＴＥＣＨ社）を使用することにより、その他にもＰＡＰ７配列が得られ、そのほぼ完全長遺伝子が、停止コドン及び３’端のいくつかの未翻訳配列を含め、ＳＥＱ ＩＤ ＮＯ： ２として確認された。ＤＮＡ配列によりコード化されるポリペプチドの分子量は、約５０ ｋＤと計算された。５７７ ｂｐの初期単離ＤＮＡフラグメントから造られたＰＡＰ７抗体を使用して、下記の例に示す方法により、約５２ ｋＤのタンパク質を免疫沈降させた。
【００４３】
タンパク質配列の分析を行った結果、いくつかの共通配列及び下記重要サイトの存在が明らかになった。即ち、２６２－２６７及び２７１－２７６の位置におけるＳＥＱ ＩＤ ＮＯ： ７で示される２個の潜在的ミリストイル化サイト、３９５－３９６、１１３－１１５、２５５－２５７、２８０－２８２、３３１－３３３、及び３３９－３４１におけるＳＥＱ ＩＤ ＮＯ： ７で示される５個のＰＫＣホスフォリル化サイト、位置２４－１０８におけるＳＥＱ ＩＤ ＮＯ： ７で示されるアシル－Ｃｏ－Ａサイト、位置１５０－１６７におけるＳＥＱ ＩＤ ＮＯ： ７の核局在化ドメイン、位置９８－２４７におけるＳＥＱ ＩＤ ＮＯ： ２で示されるトロポニン サイト、及び位置１２６－１５５におけるＳＥ

ＱＩＤＮＯ：７で示されるＨＳＰ９０ドメインである。

【００４４】

ＰＡＰ７の分布及び発現状況を、脳、精巣、卵巣、副腎及び腎臓などマウスの主要組織並びにマウスＣ６神経膠腫細胞、ＭＡ－１０ ライジッヒ細胞、及びＹ１副腎皮質細胞などの組織培養細胞系で調べた。ステロイドの生合成に関与するいずれの組織細胞系においても、ＰＡＰ７の発現パターンは、ＰＢＲの幅広発現プロフィールに類似していた。さらに、これら細胞系におけるＰＢＲ及びＰＡＰ７の発現量は、いずれもそのステロイド生合成能力と関係付けることができた。このことは、ＰＡＰ７がＰＢＲによるステロイドの生合成に実際に関与している可能性を示唆している。

【００４５】

ＰＡＰ６、ＰＡＰ１５及びＰＡＰ２０は新規遺伝子である。ＰＡＰ２０でコード化されるポリペプチドには、２個の潜在的ミリストイル化サイト、１個のＰＫＣホスフォリル化サイト、及び１個のＰＫＡホスフォリル化サイトが存在する。タンパク質をミリストイル化することにより、当該タンパク質を細胞膜に接触させることが可能になり、細胞信号の発信に参加できるようになる（Ｃａｓｅｙ，Ｐ．Ｊ．，１９９５，Ｓｃｉｅｎｃｅ ２６８，２２１－２２５；Ｂｏｕｔｉｎ，Ｊ．Ａ．，１１９９７，Ｃｅｌｌ Ｓｉｇｎａｌ ９，１５－３５）。ＰＡＰ２０は、主に精巣内に発現される。ＰＢＲのＰＡＰ２０との相互作用は、リガンドとしてＰＫ１１１９５を使用してリガンド結合の親和力を増大させた。従って、ＰＡＰ２０がＰＢＲの内因生リガンド ＤＢＩに対する親和力を調節して、ＰＢＲ機能の増減に役立っている可能性が高い。ＰＡＰ３及びＰＡＰ２０の組織分布を、それぞれ図７及び図８に示す。

【００４６】

従って、本発明の一つの側面は、ＰＡＰポリペプチドをコード化する、ＳＥＱ ＩＤ ＮＯ：１－９から選ばれたヌクレオチド配列のポリヌクレオチドで構成される、単離核酸分子を提供するものである。本明細書に記載したＰＡＰ遺伝子の他の部分又は完全部分をコード化するｃＤＮＡ又はゲノム配列をクローン化する目的で、本明細書に記載された配列を使用することは、当該技術分野に精通した通常技量の持ち主であれば、容易に実施し得るものであり、従って、これらの関連配列は本発明の範囲内に含まれるものである。

【００４７】

さらに本発明の単離核酸分子には、上記の配列とは実質的に異なってはいても、遺伝子コードが縮重しているために、まだＰＡＰをコード化能力を維持している配列で構成されるＤＮＡ分子が含まれる。勿論、当該遺伝子コード及び種固有コドンの指向性については、当該技術分野において良く知られるところである。従って、当該技術分野に精通した人にとっては、例えば特定宿主のためにコドンの発現を最適化する（例えばヒトｍＲＮＡ中のコドンをＥ．ｃｏｌｉなどの細菌性宿主又は植物性宿主に適したコドンに変化させる）目的で、上記縮重変異体を造るようなことは日常茶飯事である。

【００４８】

本発明の核酸分子は、ｍＲＮＡのようなＲＮＡ形態でも、或いは、例えばクローン化又は合成法により得られるｃＤＮＡ形態であっても又はゲノムＤＮＡなどのＤＮＡ形態であっても良い。当該ＤＮＡは二重鎖であっても一重鎖であっても良い。一重鎖ＤＮＡ又はＲＮＡはコード化鎖となることができ、センス鎖としても知られている。一重鎖は非コード化鎖となることもできる。当該一重鎖はアンチセンス鎖とも呼ばれている。

【００４９】

「単離」されることにより、核酸分子はその天然環境から取り出された核酸分子、ＤＮＡ又はＲＮＡとしての利用を意図することができる。例えば、ベクター中に含まれる組み替えＤＮＡ分子は、本発明においては「単離」分子であると考えられる。さらに他の「単離」ＤＮＡ分子の例として、非相同宿主細胞中に維持され又は（部分的又は実質的に）精製された溶液中の組み替えＤＮＡ分子を挙げることができる。単離ＲＮＡ分子には、本発明におけるＤＮＡ分子の生体内又は生体外ＲＮＡ転写体が含まれる。本発明における単離核酸分子には、さらに合成法により造られる分子も含まれる。

【００５０】
本発明は、さらに核酸分子のコード化部分又は本明細書に記載したヌクレオチド配列のコード化フラグメントをも指向するものである。フラグメントには、２個の整数から選ばれた、その長さが少なくとも１０個の隣接ヌクレオチド部分も含まれる。２個の整数の中の１つはヌクレオチド位５’を、他の一つはヌクレオチド位３’を示す。ここに、各ヌクレオチド配列における第一のヌクレオチドを以て位置１とする。即ちこれらの整数は、その長さが少なくとも１０個の隣接ヌクレオチド塩基或いは１０及び完全ヌクレオチド配列の長さ－１の間の整数におけるフラグメントの５’位及び３’位のヌクレオチドの全組み合わせを示している。

【００５１】
さらに本発明には、ヌクレオチドの位置ではなく、そのサイズで特定されるフラグメントで構成されるポリヌクレオチドが含まれる。本発明には、隣接ヌクレオチドに関して、１－及び完全ヌクレオチド配列の完全長－１の間の整数から選ばれた、全サイズのフラグメントが含まれる。優先的なサイズには、２０－５０個のヌクレオチド、プライマー及びプローブとして有用な５０－３００個のヌクレオチドが含まれる。代表的な配列が誘導される領域には、例えば当該配列の中で特定のエピトープ又はドメインをコード化する領域が含まれるが、当該領域はこれらに限定されるものではない。当該領域には、特に、例えばＳＥＱ ＩＤ ＮＯ： １， ２， ３， ４ 及び5で示されるＰＢＲ結合ドメイン、ＳＥＱ ＩＤ ＮＯ： ７の位置２６２－２６７及び位置２７１－２７６における潜在的ミリストイル化サイト、並びにＳＥＱ ＩＤ ＮＯ： ７の位置３９５－３９６、位置１１３－１１５、位置２５５－２５７、位置２８０－２８２、位置３３１－３３３、及び位置３３９－３４１における５個のＰＫＣホスフォリル化サイト、ＳＥＱ ＩＤ ＮＯ： ７の位置２４－１０８におけるアシル－Ｃｏ－Ａサイト、ＳＥＱ ＩＤ ＮＯ： ７の位置１５０－１６７における核局在化ドメイン、ＳＥＱ ＩＤ ＮＯ： ７の位置９８－２４７におけるトロポニン サイト、並びにＳＥＱ ＩＤ ＮＯ： ７の位置１２６－１５５ におけるＨＳＰ９０ドメインなどがある。

【００５２】
他の側面において、本発明は、厳しいハイブリッド化条件下において本発明の上記ポリヌクレオチド配列又はその特定フラグメントにハイブリッド化するようなポリヌクレオチドで構成される、単離核酸分子を提供するものである。「厳しいハイブリッド化条件」とは、下記で構成される溶液中で４２ ℃、一晩中インキュベートすることを意味している。即ち、５０％ホルムアミド、５Ｘ ＳＳＣ （１５０ ｍＭ ＮａＣｌ， １５ ｍＭクエン酸三ナトリウム）、５０ ｍＭ燐酸ナトリウム（ｐＨ ７．６）、５Ｘデンハルト溶液、１０％硫酸デキストラン、及び２０ ｇ／ｍｌの擦り混ぜた変成サケ精液ＤＮＡの溶液を使用する。インキュベート後、フィルターを約６５℃の０．１Ｘ ＳＳＣ中で洗浄する。

【００５３】
本発明のポリペプチド又はその一部をコード化する配列は、当該技術分野において良く知られているような標識配列、又は融合ポリペプチドの精製を容易にする目的でペプチドをコード化するような配列、ヘルパーＴ細胞を刺激することで知られている抗原決定基、後翻訳修飾サイトをコード化するペプチド、又は融合タンパク質を希望位置、例えば非相同リーダー配列に対して標的化するアミノ酸配列を持つペプチドなど、追加機能を付与するような他の配列にこれを融合させることができる。

【００５４】
本発明は、さらに本発明におけるＰＡＰの一部、その類似体又はその誘導体をコード化する核酸分子の変異体に関するものである。変異体は、天然対立遺伝子変異体などのように天然に存在するものもある。

【００５５】
「対立遺伝子変異体」は、ある微生物の染色体に与えられた遺伝子座を占拠するいくつかの遺伝子形態の一つを意味している。非天然変異体は、既知の突然変異生成技術によりこれを造ることができる。このような変異体には、ヌクレオチド置換、欠失、又は１個以上

のヌクレオチドをコード化又は非コード化領域或いはこれら両方の領域に付加することにより造られる変異体が含まれる。コード化領域に変更を加えると、保守的又は非保守的なアミノ酸の置換、欠失、又は付加構造をつくり出す。これらの中で特に好ましいのは、本明細書で開示したＰＡＰポリペプチド又はその一部の性質及び活性を変化させないような無症状置換、付加、及び欠失である。この点で好ましいものに、保守的置換がある。

【００５６】

上記核酸に対して少なくとも９０－９９％の同一性を有する核酸分子は、本発明のもう一つの側面である。これらの核酸は、ＰＡＰ活性を有するポリペプチドをコードかするかしないかに係わり無く、本発明の範囲に含まれる。「ＰＡＰ活性を有するポリペプチド」という語は、下記方法により測定した本発明のＰＡＰ活性に似てはいても、これと同一ではない活性を示すポリペプチドを表している。本発明におけるポリペプチドの生物学的活性又は機能は、高度の構造的同一性又は類似性を共有する他の微生物から採取したポリペプチドと類似しているか、又はこれと同一であることが期待される。

【００５７】

他の態様において、本発明は、上記のベクター及びＤＮＡ配列を含む組み替えＤＮＡ分子に関するものである。当該ベクターは、プラスミド、ファージ、コスミド、ＹＡＣ、ＤＮＡベクター Ｐｉｃｈｉａ ｐａｓｔｏｒｉｓなどの真核生物発現ベクター、又は例えばバキュロウィルス ベクター、レトロウィルス ベクター又はアデノウィルス ベクターなどのウィルス ベクター、及び当該技術分野で知られているその他ベクターの形を取ることができる。

【００５８】

クローン化遺伝子は、プロモーター配列、又は誘発可能な配列又は細胞型固有配列など、ある種の制御配列の制御下に置く（即ちこれと結合するように操作する）ことができる。当該技術分野に普通程度に精通した人には、これに適したプロモーターが何であるかは、良く知られているところである。当該発現構造は、さらに転写サイト、開始サイト、終結サイト、及び転写領域においては翻訳用リボソーム結合サイトを含有している。好んで使用されるベクターの中には、例えばｐＧＢＴ９、ｐＧＡＤ１０（ＣＬＯＮＥＴＥＣＨ社）、ＰＳＶｚｅｏ（Ｉｎｖｉｔｒｏｇｅｎ社）、ｐＢｌｕｅＳｃｒｉｐｔ（Ｓｔｒａｔａｇｅｎｅ社）、ｐＣＭＶ５（Ｉｎｖｉｔｒｏｇｅｎ社）、ｐＣＲＩＩ（Ｉｎｖｉｔｒｏｇｅｎ社）などがある。

【００５９】

当該構造の宿主細胞導入は、燐酸カルシウムの形質移入、電気泳動、感染、及びその他当該技術分野で知られており、Ｃｕｒｒｅｎｔ Ｐｒｏｔｏｃｏｌｓ ｉｎ Ｍｏｌｅｃｕｌａｒ Ｂｉｏｌｏｇｙ，Ａｕｓｕｂｅｌ，Ｆ．Ｍ．ら編，Ｗｉｌｅｙ ＆ Ｓｏｎｓ，Ｉｎｃ．など、標準的な試験室マニュアルに記載されている方法により、これを実施することができる。本明細書に引用した全文書（前記及び後記）は、参考文献としてそれらを完全な形で引用した。

【００６０】

さらに他の態様において、本発明は、安定に形質変換し、又は上記組み替えＤＮＡ構造をトランスフェクトした宿主細胞に関するものである。当該宿主細胞は原核生物（例えば細菌）の細胞であっても、低級真核生物（例えば酵母菌又は昆虫）又は高級真核生物（例えばネズミ及びヒトを含む全動物；但しこれらに限定されるものではない）であっても良い。指定宿主に適した制御配列を使用する限り、原核生物の宿主も真核生物の宿主も、ともに希望するコード化配列の発現にこれらを使用することができる。原核生物の宿主の中にあって、Ｅ．ｃｏｌｉは最も頻繁に使用される宿主である。原核生物の宿主に対する発現制御配列には、プロモーター（オペレーター部分を含んでも含まなくても良い）結合サイト及びリボソーム結合サイトが含まれる。

【００６１】

原核生物の宿主に適した伝達ベクターは、通常、例えばｐＢＲ３２２（アンピシリン及びテトラサイクリン抵抗付与オペロンを含むプラスミド）、及び種々のＰＵＣベクターから

誘導される。当該ベクターも抗生物質抵抗標識付与配列を含んでいる。これらの標識を選択使用することにより、適切な形質変換体を得ることができる。一般的なクローン化方法については、例えばＭａｎｉａｔｉｓ，Ｆｉｔｓｃｈ及びＳａｍｂｒｏｏｋ；Ｍｏｌｅｃｕｌａｒ Ｃｌｏｎｉｎｇ；実験室マニュアル（１９８２）；又はＤＮＡ Ｃｌｏｎｉｎｇ，Ｖｏｌ．Ｉ及びＩＩ（Ｄ．Ｎ．Ｇｌｏｖｅｒ編，１９８５）を参照されたい。

【００６２】

当該ＤＮＡ配列はベクター中に存在し、ＩｇＧ分子、アジュバント、担体、又はグルタチオン Ｓ－トランスフェラーゼ又は一連のヒスチジン残基（ヒスチジン タグとしても知られている）などは、ＰＡＰ生成助剤のコード化配列に結合するように操作することが可能である。当該組み替え分子は、植物細胞又は真核生物細胞、例えば哺乳類の細胞及び培養液中の酵母菌細胞をトランスフェクトする場合に適している可能性が高い。Ｓａｃｃｈｒｏｍｙｃｅｓ ｃｅｒｅｖｉｓｉａｅ、Ｓａｃｃｈａｒｏｍｙｃｅｓ ｃａｒｌｓｂｅｒｇｅｎｓｉｓ、及びＰｉｃｈｉａ ｐａｓｔｏｒｉｓは、酵母菌宿主の中で最も良く使用される細胞であり、便利な真菌性宿主である。

【００６３】

酵母菌ベクターに対する制御配列については、当該技術分野で良く知られている。発現用宿主細胞として入手可能な哺乳類細胞系も当該技術分野において良く知られており、これには多くの不死滅化細胞系が含まれている。これらの中でもＨＥＫ２９３細胞、ＮＩＨ３Ｔ３細胞、ＭＡ１０ライジッヒ細胞、マウスＣ６神経膠腫細胞、Ｙ１副腎細胞、及びＭＤＡ－２３１、ＭＣＦ－７など、乳癌細胞系などの当該不死滅化細胞系は、Ｔｈｅ Ａｍｅｒｉｃａｎ Ｔｙｐｅ Ｃｕｌｔｕｒｅ Ｃｏｌｌｅｃｔｉｏｎ（ＡＴＣＣ）からこれらを入手することができる。適切なプロモーターについても当該技術分野で良く知られており、これにはＳＶ４０、ラウス サルコマ ウィルス（ＲＳＶ）、アデノウィルス（ＡＤＶ）、ウシ パピロマ ウィルス（ＢＰＶ）、及びシトメガロウィルス（ＣＭＶ）などのウィルス性プロモーターが含まれている。

【００６４】

哺乳類の細胞もターミネーター配列及びポリＡ付加配列を必要とし、発現量を増大させるエンハンサー配列もこれに含めることができる。また、遺伝子増幅を引き起こすような配列も好ましい。これらの配列は、当該技術分野において良く知られた配列である。形質変換又はトランスフェクトした宿主細胞は、上記ＤＮＡ配列源としてこれを使用することができる。当該組み替え分子が発現系の形態を取る場合、当該形質変換細胞又はトランスフェクト細胞を下記タンパク質源として使用することが可能である。

【００６５】

他の態様において、本発明は、上記ＰＡＰポリペプチド又はその全対立遺伝子変異体に関するものである。当該変異体は、当該ポリペプチドを使用して免疫学的に同定することができる。

【００６６】

上記ポリペプチド又はアミノ酸の配列は、当該配列中でコード化されるポリペプチド又はその一部分と同一のアミノ酸配列を有するポリペプチドを意味している。ここに、当該一部分は少なくとも２－５個のアミノ酸で構成され、より好ましくは少なくとも８－１０個のアミノ酸で構成され、さらに好ましくは少なくとも１１－１５個のアミノ酸で構成されている。或いは、当該ポリペプチド又はその一部分は、当該配合中においてコード化されたポリペプチドを使用して、免疫学的にこれを同定することが可能である。

【００６７】

組み替えポリペプチド又は誘導ポリペプチドは、必ずしも指定の核酸配列から翻訳されたものである必要は無い。当該配列は、どんな方法でもこれを調製することができる。その方法には、例えば化学合成法、又は組み替え系を発現させる方法などがある。さらに当該ポリペプチドは、その抗抗原性を増大させるような他のタンパク質又はポリペプチド、例えばアジュバントにこれを融合させることもできる。

【００６８】
上記のように、本発明の方法は、上記核酸分子又はベクターを宿主細胞に挿入し、当該ポリペプチド配列を発現させて問題のポリペプチドを当該宿主細胞によりコード化し、全ての長さのポリペプチド全てを製造する場合に適した方法である。当該核酸分子又はベクターを宿主細胞中に導入して形質変換宿主細胞を造る方法は、燐酸カルシウム トランスフェクション、ＤＥＡＥ－デキストラン仲介トランスフェクション、カチオン性脂質仲介トランスフェクション、電気泳動、形質導入、感染、その他の方法によりこれを実施することができる。このような方法については、Ｄａｖｉｓら, Ｂａｓｉｃ Ｍｅｔｈｏｄｓ ｉｎ Ｍｏｌｅｃｕｌａｒ Ｂｉｏｌｏｇｙ （１９８６）など、数多くの標準的試験室マニュアルの中に記載されている。

【００６９】
一旦形質変換された宿主細胞が得られたら、ｐＨ及び温度の生理適合条件下及び宿主細胞の成長を支える同化可能な炭素源、窒素源及び必須ミネラル源を含む適切な栄養媒体中において当該細胞を培養することができる。組み替えポリペプチドの製造培養条件は、当該宿主細胞の形質変換に使用するベクターの種類により左右される。例えば、ある種の発現ベクターは、遺伝子発現を開始させて組み替えポリペプチドを得るために、特定温度において細胞が成長するような調節域で構成され、またある発現ベクターでは、特定化学薬剤又は誘発剤を細胞成長媒体に添加することが必要である。

【００７０】
このようにして、本明細書で使用される「組み替えポリペプチドの製造条件」という語は、ある一定の培養条件に限定されるべきものではない。上記宿主細胞及び宿主ベクターへの使用に適した培養媒体及び培養条件については、当該技術分野において良く知られているところである。宿主細胞中において製造を行った後、当該ポリペプチドは、いくつかの方法によりこれを単離することができる。問題のポリペプチドを当該宿主細胞から遊離させるためには、当該細胞はこれを溶解又は破裂させる。

【００７１】
この溶解は、当該細胞を低張溶液と接触させ、リソチームなどの細胞壁攪乱酵素で処理し、超音波破壊し、高圧処理し、又は上記方法の組み合わせ処理を行うことによりこれを達成することができる。細菌細胞を攪乱して溶解させるその他方法に関しては、通常良く知られた方法を使用して、これを実施することができる。

【００７２】
当該攪乱操作に続き、錯体混合物から粒子を分離するに適したいずれかの方法で、当該ポリペプチドを細胞の残骸から分離することができる。当該ポリペプチドは、次にこれを良く知られている単離方法で精製することができる。精製に適した方法として、硫酸アンモニウム又はエタノールによる沈殿法、酸抽出法、電気泳動法、免疫吸着法、アニオン又はカチオン交換クロマトグラフ法、ホスフォセルロース クロマトグラフ法、疎水性相互作用クロマトグラフ法、アフィニティ クロマトグラフ法、免疫アフィニティ クロマトグラフ法、サイズ排除クロマトグラフ法、液体クロマトグラフ法 （ＬＣ）、高性能液体クロマトグラフ法 （ＨＰＬＣ）、高速高性能液体クロマトグラフ法 （ＦＰＬＣ）、ヒドロキシル アパタイト クロマトグラフ法及びレクチン クロマトグラフ法などを挙げることができるが、当該方法はこれらに限定されるものではない。

【００７３】
当該組み替えポリペプチド又は融合タンパク質は、検出能力を付与するために標識化及び非標識化し、ＰＡＰの検出又はＰＢＲの検出及び測定を目的とする診断用ツールとして、これを使用することができる。さらにこれらポリペプチドは、ＰＢＲの発現量を調節する方法でもこれを使用することができる。さらに、当該組み替えタンパク質は、ＰＢＲ上におけるその効果を経て、細胞の死滅及び細胞の増殖を抑える治療用薬剤として、これを使用することができる。

【００７４】
形質変換した宿主細胞は、宿主タンパク質又は化学誘導薬剤又は当該細胞と相互作用して

ＰＡＰの機能又は発現量を変化させ、これによりＰＢＲの機能、発現量又は局在化を調節する他のタンパク質など、ＰＡＰの発現又は機能に関する効果を経て、ＰＢＲの機能、発現量又は標的化を調節する薬剤の有効性分析に、これを使用することができる。

【００７５】

もう一つの態様において、本発明は、上記組み替えタンパク質（又はポリペプチド）に固有のモノクローン性又はポリクローン性抗体に関するものである。例えば上記ペプチドに対して、又は少なくとも１０個のアミノ酸、好ましくは１１－１５個のアミノ酸から成るその一部分に対して、一つの抗体を構築することができる。標準的な方法を使用する当該技術分野において通常の技量を持つ人であれば、本発明のタンパク質（又はポリペプチド）或いはそのユニーク部分に対するモノクローン抗体及びポリクローン抗体を構築することが可能である。抗体製造の材料及び方法については、当該技術分野において良く知られるところである（例えば、Ｇｏｄｉｎｇ，Ｍｏｎｏｃｌｏｎａｌ Ａｎｔｉｂｏｄｉｅｓ；Ｐｒｉｎｃｉｐｌｅｓ ａｎｄ Ｐｒａｃｔｉｃｅ，第４章，１９８６を参照のこと）。

【００７６】

ＰＡＰの発現量については、いくつかの水準でこれを検出することができる。当技術分野で良く知られている標準的な方法を使用して、ＰＡＰ ＲＮＡの検出及び定量分析方法を設計することができる。これには、特にノーザン ハイブリッド化分析、原位置ハイブリッド化分析、及びＰＣＲ分析を含めることが可能である。核酸のハイブリッド化方法に関する一般的な説明については、例えば、Ｍａｎｉａｔｉｓ，Ｆｉｔｓｃｈ及びＳａｍｂｒｏｏｋ；Ｍｏｌｅｃｕｌａｒ Ｃｌｏｎｉｎｇ；試験室用マニュアル（１９８２）、又はＣｕｒｒｅｎｔ Ｐｒｏｔｏｃｏｌｓ ｉｎ Ｍｏｌｅｃｕｌａｒ Ｂｉｏｌｏｇｙ，Ａｕｓｕｂｅｌ，Ｆ．Ｍ．ら編，Ｗｉｌｅｙ ＆ Ｓｏｎｓ，Ｉｎｃ．をご参照頂きたい。ＰＡＰ ＲＮＡ検出用ポリヌクレオチド プローブは、ＳＥＱ ＩＤ ＮＯ：１－９に示した配列から、これを設計することができる。

【００７７】

例えば、サンプルから単離したＲＮＡは、その表面をニトロセルロース膜などでコーティングし、これをノーザン ハイブリッド化試験用として調製することができる。例えば生検用サンプルを原位置ハイブリッド化する場合、当該組織サンプルは、当該技術分野において広く知られている標準的方法によるハイブリッド化を目的としてこれを調製し、ＰＡＰ ＲＮＡを特に認識するポリヌクレオチド配列により、これをハイブリッド化することが可能である。サンプル ＲＮＡと当該ポリヌクレオチドの間で形成されたハイブリッドの存在は、当該技術分野で知られている放射性化学測定又は免疫化学測定など、如何なる方法によってもこれを検出することができる。

【００７８】

当技術分野に精通した者なら、対応核酸配列と高度の重複部分を有するアミノ酸配列の広い領域に亘り検索できるような、可なり長いプローブの調製が好ましいことは、容易に理解できるところであろう。その他の場合においては、２セットのプローブを同時に使用し、それぞれのプローブで別々の遺伝子領域を探索できるようにすることが望ましい。使用するプローブの正確な長さはあまり重要ではない。代表的なプローブ配列の長さはヌクレオチド５００個以下であり、２５０個以下がより代表的であり、１００個以下であることも可能であり、さらに７５個以下であることも可能である。関連標的配列を区別できるような充分な差が存在するユニークなポリヌクレオチド領域を広く覆うことが必要であり、そのためにプローブ配列の長さを増やすことも必要であろう。この理由で、プローブの長さはヌクレオチド約１０－約１００個分であることが好ましく、約２０－約５０個分であることがさらに好ましい。

【００７９】

ポリメラーゼ連鎖反応 （ＰＣＲ）又は逆転写 ＰＣＲ （ＲＴ－ＰＣＲ）を使用してＰＡＰを検出するためのプライマーを設計するには、ＰＡＰのＤＮＡ配列を使用することができる。当該プライマーは、ＰＡＰの存在又は不存在の検出、或いは標準と比較することに

よりＰＡＰの定量を行うことを目的として、特にＰＡＰ ＲＮＡの逆転写により造られたＰＡＰ ｃＤＮＡとこれを結合させる。当該プライマーは、当該ＰＡＰ配列の領域に対して相同的又は相補的であり、その長さはヌクレオチド７－４０個分、好ましくは１０－１５個分、最も好ましくは１８－２５個分の範囲内において、どんな長さであっても良い。ＰＣＲ又はＰＴ－ＰＣＲ反応に必要な試薬及び比較対照については、当技術分野において良く知られるところである。次に、例えばゲル分画法、放射線化学法、及び免疫化学法により、増幅された産物についてＰＡＰ配列が存在するかしないかを分析する。必要とする細胞数が少なくて済むので、この方法は有利である。ＰＡＰが一旦検出されたら、同じ方法を用いて得られた正常細胞に関する結果と比較することにより、当該細胞がＰＡＰを過剰発現しているか又は過少発現しているかを決定することができる。例えば、ＰＡＰ７ ＲＮＡの濃度増加は、特にステロイド生成細胞中のＰＢＲ発現濃度と関係付けることができる。ここに、当該細胞のステロイド生成能力の増加とＰＢＲ及びＰＡＰ７ ＲＮＡ量の増加との間には相関関係が存在する。

【００８０】

他の態様において、本発明は、細胞中においてＰＡＰ ＲＮＡを検出することを目的とする診断用キットに関するものである。ここに当該キットは、原位置ハイブリッド化法又はノーザン分析法により細胞中でＰＡＰ ＲＮＡを検出するために、ＰＣＲ又はＰＴ－ＰＣＲ又はＰＡＰポリヌクレオチドによりＰＡＰを検出することを目的とする、一種類以上のＰＡＰオリゴヌクレオチド プライマー容器を有するパッケージ単位で構成される。また、いくつかのキットでは、希望する方法で使用する種々の試薬を入れた容器が含まれている。当該キットは、１種類以上の下記品目もこれを含むことができる。即ち、重合酵素、緩衝液、説明書、比較対照物質、検出用標識などである。当該キットは、本発明に従って当該方法を実施するために適切な割合で混合した試薬の容器も、これを含むことができる。試薬の容器には、主題の方法を実施する場合、一々計量する必要が無いように単位量の試薬を入れておくことが望ましい。

【００８１】

さらに他の態様において、本発明は、特定の生物学的サンプル中におけるＰＡＰの存在を確認し、その濃度を定量化する方法を提供するものである。サンプル中におけるＰＡＰ濃度を確認（又は定量）する場合、この目的のために種々の方法を使用することができる。

【００８２】

ＰＡＰを検出することを目的とする診断分析は、腫瘍又は正常組織から採取した細胞吸引物から得た組織断面並びに細胞を生検又は原位置分析することでこれを構成することができる。さらに分析は、器官、組織、細胞、尿、或いは血清又は血液、もしくはその他体液又はその抽出物について、これを実施することができる。

【００８３】

生検分析を行う場合、当該分析は、分析サンプルをＰＡＰリガンド（天然又は合成）又は抗体（多クローン性又は単クローン性）に接触させることで、これを構成することができる。当該リガンド又は抗体は、ＰＡＰ或いはＰＡＰの検出能力又はＰＡＰと、サンプル中に存在するＰＡＰリガンド又は添加抗体との間で形成された錯体の検出能力を有する抗血清を認識することができる。

【００８４】

ＰＡＰリガンド又は基体には、天然及び合成リガンド及び動物又は植物の抽出物など、天然発生源から誘導される誘導体の他に、例えばＰＢＲが含まれる。

【００８５】

細胞増殖又は細胞死の減少に関連して発生する癌などの病気の診断及び予後に使用する種々の標識及び標識方法を使用し、ＰＡＰリガンド又は抗ＰＡＰ抗体、或いはＰＡＰの検出能力を有するリガンド及び抗体のフラグメントを標識化することができる。本発明で使用し得る標識の例としては、酵素標識、放射性同位元素標識、非放射性同位元素標識、及び化学ルミネセンス標識などが存在する。但し、当該標識例は、これらの例に限定されるものではない。

【００８６】
適切な酵素標識の例として、マレート デヒドロジェナーゼ、スタフィロコッカル ヌクレアーゼ、デルター５－ステロイド イソメラーゼ、酵母菌－アルコール デヒドロジェナーゼ、燐酸アルファ－グリセリン デヒドロジェナーゼ、燐酸トリオース イソメラーゼ、ペルオキシダーゼ、アルカリ性ホスファターゼ、アスパラギナーゼ、グルコース オキシダーゼ、ベーターガラクトシダーゼ、リボヌクレアーゼ、ウレアーゼ、カタラーゼ、グルコース－６－ホスフェート デヒドロジェナーゼ、グルコアミラーゼ、アセチルコリン エステラーゼなどが存在する。
【００８７】
適切な放射性同位元素標識の例としては、^{3}Ｈ、^{111}Ｉｎ、^{125}Ｉ、^{32}Ｐ、^{35}Ｓ、^{14}Ｃ、^{57}Ｔｏ、^{58}Ｃｏ、^{59}Ｆｅ、^{75}Ｓｅ、^{152}Ｅｕ、^{90}Ｙ、^{67}Ｃｕ、^{21}Ｃｉ、^{211}Ａｔ、^{212}Ｐｂ、^{47}Ｓｃ、^{109}Ｐｄ、^{11}Ｃ、^{15}Ｆ、^{123}Ｉなどが存在する。
【００８８】
適切な非放射性同位元素標識の例としては、^{257}Ｇｄ、^{55}Ｍｎ、^{163}Ｄｙ、^{62}Ｔｒ、^{46}Ｆｅなどが存在する。
【００８９】
適切な蛍光標識の例としては、^{152}Ｅｕ標識、フルオレセイン標識、イソチオシアネート標識、ローダミン標識、フィコエリスリン標識、フィコジアニン標識、アロフィコジアニン標識、フルオレサミン標識などが存在する。
【００９０】
化学発光標識の例としては、ルミナール標識、イソルミナール標識、芳香族アクリジニウム エステル標識、イミダゾール標識、アクリジニウム塩標識、蓚酸エステル標識、ルシフェリン標識、ルシフェラーゼ標識などが存在する。
【００９１】
当技術分野に精通した者であれば、本発明に使用し得る上記以外の適切な標識に関する知識を持ち合わせているものと考えられる。これら標識とリガンドとの結合並びに抗体又はそのフラグメントとの結合は、当技術分野において標準的な技術を身に付けた者であれば、一般に知られている標準的技法を使用することにより、これを達成することができる。これに関する代表的な技法については、Ｋｅｎｎｅｄｙ，Ｊ．Ｈ．ら，１９７６（Ｃｌｉｎ．Ｃｈｉｍ．Ａｃｔａ７０，１－３１）、及びＳｃｈｕｒｓ，Ａ．Ｈ．Ｗ．Ｍ．ら，１９７７（Ｃｌｉｎ．Ｃｈｉｍ．Ａｃｔａ８１，１－４０）により記載されている。後者の文献の中で述べられているカップリング技法は、グルタール アルデヒド法、過ヨウ素酸塩法、ジマレイミド法、及びその他方法である。これらの方法に関しては、全て本明細書中に引用した。
【００９２】
本発明における抗体（又は抗体のフラグメント）の検出方法は、担体を使用することにより、これを改良することができる。良く知られた担体にはガラス、ポリスチレン、ポリプロピレン、ポリエチレン、デキストラン、ナイロン、アミラス、天然及び修飾セルロース、ポリアクリルアミド、アガロース、及びマグネタイトなどがある。本発明の目的において、担体の性格は、ある程度可溶性であっても又は不溶性であっても良い。支持物質は、カップリング分子がＰＡＰに結合し得るようなものであれば、事実上如何なる構造のものであっても良い。
従って、当該支持物質の構造形状は、ビーズのように球状であっても良く、或いは試験管内面又はロッド外面のように円筒状であっても良い。或いは、当該表面はシート、試験片などのように平坦であっても良い。当技術分野に精通した人であれば、その他モノクローン性抗体を結合させる適切な担体が何であるべきかに気づき、或いは日常実験によりこれらを確認することができるであろう。
【００９３】
ＰＡＰの存在を定量的又は定性的に検出する目的で、リガンド又は抗体或いは上記ＰＡＰ

の抗体又はリガンドのフラグメントを使用することができる。このような検出操作は、種々の免疫分析法などを使用することにより、これを行うことができる。当技術分野で良く知られた標準的な方法を使用し、（固形支持体）表面、例えばミクロ滴定プレート又は膜（例えばニトロセルロース膜）、ＰＡＰ又はその一部に固有の抗体をコーティングし、これをＰＡＰに起因する病気の存在が疑われる人から採取したサンプルと接触させることにより、その診断分析方法を組み立てることができる。

【００９４】

このようにしてサンプル中のＰＡＰと当該ＰＡＰに固有の抗体との間で生じた錯体の存在は、当技術分野において公知の検出方法、例えば蛍光抗体分光分析法又は比色法などのいずれかにより、これを検出することができる。放射線免疫分析法については、Ｗｏｒｋ，Ｔ．Ｓ．ら著「分子生物学における試験室的技法及び生化学について」（Ｎｏｒｔｈ Ｈｏｌｌａｎｄ Ｐｕｂｌｉｓｈｉｎｇ Ｃｏｍｐａｎｙ，Ｎ．Ｙ．（１９７８）の中で詳しく説明されている。この文献は、本明細書にも引用した。サンドイッチ分析法については、Ｗｉｄｅが「放射線免疫分析法」（Ｋｉｒｋｈａｍ及びＨｕｎｔｅｒ；Ｅ．＆Ｓ．Ｌｉｖｉｎｇｓｔｏｎｅ，Ｅｄｉｎｂｕｒｇｈ，１９７０）の第１９９－２０６頁にその解説を行っている。

【００９５】

本発明の診断方法によれば、胆石、アテローム性動脈硬化症、ニーマンピック病、シトステロール血症、ジストロフィー、腫瘍増殖（腫瘍形成）、スナイダー皮質結晶性ジストロフィーなど、ＰＢＲが係わる病気を予測することができる。脳疾患には、コレステロール代謝及びアルツハイマー病、テルリウム毒性、スミスーレルミーオピッツ症候群、髄鞘形成、進行性異常及び脱髄、チャーコットーマリー歯病、ペリツースーメルツバッハー病、多重性硬化症、ＳＬＡなどが含まれる。或いは、当該方法及び組成物は予防治療、又は予防治療に有効な化合物の検索に役立つ可能性もある。

【００９６】

当該組み替えタンパク質は、ＰＡＰ活性の抑制剤又は活性化剤の同定にこれを使用することが可能である。この方法により、ＰＢＲ活性調節能力のある薬剤を同定することもできる。下記の例に記載した分析方法を使用することにより、或いは例えば薬剤をＰＡＰを発現している細胞内へ導入してＰＡＰ ＲＮＡ又はタンパク質の増減を検出することにより、ＰＡＰ活性を減少又は除去し或いはこれを増大させる天然又は合成薬剤を発見することができる。抑制剤又は活性化剤の作用機構に関する知識は、ＰＡＰの活性増減が検出される限り必要ではない。抑制剤には、ＰＢＲのようにＰＡＰ基質に結合するか又はこれを封鎖するような薬剤、又はその共因子、又はそれ自体が直接的に例えば非可逆的にＰＡＰに結合し、又は間接的に例えば競争化合物に結合するような薬剤をその基質に結合したＰＡＰと結合させ、このようにして抑制効果を発揮するような薬剤をこれに含めることができる。

【００９７】

活性化剤には、ＰＡＰ固有の機能に対して必要な共因子、或いは当該ＰＡＰとＰＢＲ又は特定のＰＡＰ基質との結合又は放出の転換速度を速めるような薬剤を、これに含めることもできる。本発明関連の薬剤により、ＰＡＰの部分抑制又は完全抑制を達成し、或いはＰＡＰを種々の度合いで活性化することができる。これにより、ＰＢＲの機能が調節されることもあれば、されないこともある。ストレス、癌、神経退行性疾患即ち発作、アルツハイマー病、進行性疾患、不妊症、及び免疫不全などの治療又はその改善に、ＰＡＰ活性抑制剤又はその活性化剤を使用することができる。

【００９８】

（ヒト又は動物の体内において）ＰＡＰ濃度を低下させ或いはＰＡＰ活性を低下又は抑制するような薬剤は、ＰＡＰ濃度の上昇に起因する病気の治療にこれを使用することができる。これと同様に、ＰＡＰ濃度を上昇させ又はＰＡＰ活性を増大させるような薬剤は、ＰＡＰ濃度の低下に起因する病気の治療にこれを使用することができる。ＰＡＰ濃度の増減は、ＰＡＰ濃度が正常細胞内におけるＰＡＰ濃度の約２－３倍又は約１／２－１／３にな

ったとき、或いはその約１０－１００倍又は１／１０－１／１００になった場合にその判断が行われる。

【００９９】

ＰＡＰ ＲＮＡを減少させるような薬剤には、ＰＡＰ ＲＮＡの消化能力を有する１種類以上のリボチーム、或いはＰＡＰの翻訳を抑制又は減少させ、その結果ＰＡＰ濃度を低下させるような、ＰＡＰ ＲＮＡへハイブリッド化する能力を有するアンチセンス オリゴヌクレオチドが含まれる。但し、当該薬剤の種類がこれだけに限定されるものではない。これらの薬剤は、ＤＮＡ、即ちウィルス性外皮レポーター タンパク質を含むプロテオリポソーム中に捕捉されたＤＮＡ（Ｋａｎｏｄａ，Ｙ．ら，１９８９，Ｓｃｉｅｎｃｅ ２４３，３７５）として、或いは当該ＤＮＡ又はＲＮＡが造られるように標的細胞中に発現し得るベクターの一部として、これを投与することが可能である。

【０１００】

特定の細胞種中に発現するベクターについては、当該技術分野において広く知られているところである。例えば乳腺細胞に関しては、Ｆｕｒｔｈ（１９９７）（Ｊ．Ｍａｍｍａｒｙ Ｇｌａｎｄ Ｂｉｏｌ．Ｎｅｏｐｌ．２，３７３）が、乳腺中における遺伝子発現の条件付き制御の例について記述しているので、これをご参照頂きたい。或いは、当該ＤＮＡは、これを担体とともに注入することができる。担体としてはシトキン、例えばインターロイキン２又はポリシン糖タンパク質担体などのタンパク質を使用することができる。このような担体タンパク質及びベクター並びにこれらの使用方法については、当技術分野において広く知られているところである。さらに、当該ＤＮＡはこれを小さな金のビーズ上にコーティングし、例えば遺伝子ガンを用いて当該ビーズを皮膚の中へ導入することもできる（Ｕｌｍｅｒ，Ｊ．Ｂ．ら，１９９３，Ｓｃｉｅｎｃｅ ２５９，１７４５）。

【０１０１】

もしくは抗体、或いは拮抗剤などのＰＡＰ活性を低下又は抑制（ＰＡＰの発現量、製造量又は活性のいずれかを低下又は抑制）する能力を有する化合物は、ＰＡＰを減少又は抑制するような薬剤が造られるように、単離された事実上の精製タンパク質として、或いは標的細胞中で発現能力を有する発現ベクターの一部として、これを投与することができる。同様に作動薬など、ＰＡＰ活性を増加又は活性化（即ちＰＡＰの発現量、製造量、又は活性のいずれかを増加又は活性化）する能力を有するような化合物は、ＰＡＰの効果を高め又はこれを活性化するような薬剤が造られるように、単離され、事実上精製されたタンパク質として、又は標的細胞中で発現能力を有する発現ベクターの一部として、これを投与することができる。

【０１０２】

さらに、タンパク質の安定性に影響を与える因子、及び安定性に影響を与える種々のイオン〔即ちCa^{+2}（Ｃａｌｖｏ，Ｄ．Ｊ．及びＭｅｄｉｎａ，Ｊ．Ｈ．，１９９３；Ｊ．Ｒｅｃｅｐｔ．Ｒｅｓ．１３，９７５－９８７）〕、或いはハロゲン化物又はＤＩＤＳ（４，４’－ジイソチオシアノスチルベン－２，２’－ジスルホン酸）などのアニオン経路ブロッカーなどのアニオン、イオン輸送ブロッカー（Ｓｋｏｌｎｉｃｋ，Ｐ．，１９８７，Ｅｕｒ．Ｊ．Ｐｈａｒｍａｃｏｌ．１３３，２０５－２１４）、或いは例えばリン脂質 ホスファチジルセリン及びホスファチジルイノシトールのような脂質などＰＢＲの安定性に影響を与えるような因子を投与して、ＰＡＰ及びＰＢＲの発現量及び機能を調節することもできる。ここにリン脂質の存在は、受容体活性のために必要である（Ｍｏｙｎａｇｈ，Ｐ．Ｎ．及びＷｉｌｌｉａｍｓ，Ｄ．Ｃ．，１９９２，Ｂｉｏｃｈｅｍ．Ｐｈａｒｍａｃｏｌ．４３，１９３９－１９４５）。

【０１０３】

これらの処方物は、標準的経路によりこれを投与することができる。一般に、当該処方物は、局所、皮内、腹腔内、経口、直腸、又は腸管外（例えば静脈内、皮下、又は筋肉内）経路によりこれを投与する。さらに、ＰＡＰ抑制又はＰＡＰ活性化化合物は、これを生分解性ポリマー中に組み込み、例えば腫瘍の存在部位などの希望薬剤送入部位の近傍にこれ

を埋め込む。又は、ＰＡＰ抑制又はＰＡＰ活性化化合物がゆっくりと全身に放出されるような埋め込み方をしても良い。

【０１０４】

当該生分解性ポリマー及びその使用方法に関しては、例えばＢｒｅｍら，１９９１，Ｊ．Ｎｅｕｒｏｓｕｒｇ．７４，４４１－４４６の中に詳細な記述がある。これらの化合物は、ＰＡＰの効果を充分に抑制できるような量を患者に投与するものとする。同様に、ＰＡＰの発現、製造、安定性又は機能に対して正又は負の効果を与える能力を有する薬剤は、希望する効果を充分に発揮できるような量を患者に投与するものとする。当該薬剤の投与量、投与経路などにより、このような反応に対して充分な影響が得られれば、その量はＰＡＰの抑制又は誘導を達成するに充分であったと考えることができる。

【０１０５】

本発明に係わるＰＡＰはＰＢＲと会合する能力を有し、当該ＰＢＲが適切な標的、機能、発現、又は安定性を有する場合において、始めてその役割を果たすことができる。従って、ＰＢＲの機能を抑制し、又はこれを低下させ、又はＰＢＲの存在位置を変える方法には、ＰＡＰをその受容体から解離させる方法が含まれる。これらのＰＡＰがＰＢＲと会合しているＰＢＲ上のサイトをブロックするような薬剤を使用すれば、このことが可能になる。或いは、ＰＢＲとの会合に関与しているＰＡＰ上のサイトをブロックしても良い。

【０１０６】

このような薬剤として、例えばこのようなサイトを認識し、或いはこれらサイトの形態を変化させてＰＡＰとＰＢＲの会合を抑制又は除去するような、抗体又は拮抗剤が存在する。（ヒト又は動物の体内において）ＰＢＲ濃度を減少させ、或いはＰＢＲの活性を低下させ又は抑制するような薬剤は、ＰＢＲ濃度が高まることにより発生するような病気の治療に、これを使用することができる。このような病気には、転移性癌（例えば乳癌）、又は細胞増殖速度の増大に起因する病気、又は細胞中へのコレステロール輸送速度の増大に起因する病気などがある。腫瘍細胞中のＰＢＲの濃度が正常細胞中の濃度の約２－３倍、特に約１０－１００倍に増加した場合、ＰＢＲの濃度増加があったものと判断される。

【０１０７】

抗体或いはＰＢＲとＰＡＰの会合度を低下させ又はこれを抑制する能力を有する化合物は、単離され事実上精製されたタンパク質として、或いは当該ＰＢＲ－ＰＡＰの会合を減少又は抑制する薬剤が造られるように標的細胞中で発現能力を有する発現ベクターの一部として、これを投与することができる。これらの処方物は、標準的経路によりこれを投与することができる。一般に、当該処方物は、局所、皮内、腹腔内、経口、直腸、又は腸管外（例えば静脈内、皮下、又は筋肉内）経路によりこれを投与することができる。さらにこれらの化合物は、これを生分解性ポリマー中に組み込み、これを薬剤の希望送入サイト、例えば腫瘍が存在するサイトの近傍に埋め込むか、又は当該化合物がゆっくりと全身に放出されるようにこれを埋め込む。当該生分解性ポリマー及びその使用方法に関しては、例えばＢｒｅｍら，１９９１，Ｊ．Ｎｅｕｒｏｓｕｒｇ．７４，４４１－４４６の中に詳細な記述がある。

【０１０８】

これらの化合物は、ＰＢＲ／ＰＡＰの会合を充分に抑制できるような量を患者に投与するものとする。

【０１０９】

細胞増殖におけるＰＢＲの機能に沿って、ＰＡＰとＰＢＲの会合度を増大させることによりＰＢＲの機能を刺激するような薬剤は、ＰＢＲの減少又は細胞増殖速度の低下に起因する病気の治療に、これを使用することができる。ここに、ＰＢＲはこのような増殖（例えば進行性遅滞）を増大させる能力を有している。ＰＢＲは、コレステロールの輸送に関与することも分かっており、従って、ＰＢＲ及び会合ＰＡＰの機能又は安定性を増大させるような薬剤は、細胞中へのコレステロール輸送速度を増大させる目的でこれを使用することができる。

【０１１０】

コレステロールの輸送量が不足することに起因する病気には、リポイド副腎過形成などがあり、ミエリン及び髄鞘形成などコレステロールが必要な化合物生産量を増加させる必要があるような病気には、アルツハイマー病、脊髄障害、及び脳神経障害〔Ｓｎｉｐｅｓ，Ｇ．及びＳｕｔｅｒ，Ｕ．（１９９７）；「コレステロールとミエリン」；Ｓｕｂｃｅｌｌｕｌａｒ Ｂｉｏｃｈｅｍｉｓｔｒｙ，Ｒｏｂｅｒｔ Ｂｉｔｔｍａｎ編，第２８巻，ｐｐ．１７３－２０４，Ｐｌｅｎｕｍ Ｐｒｅｓｓ，ニューヨーク〕などがある。

【０１１１】

発現量、機能、標的細胞、或いは上記したＰＡＰ又はＰＢＲの会合を調節するような薬剤を患者に投与する場合、その投与量は患者の年齢、体重、身長、性、総合的医療状態、過去の病歴などの要因により左右される。一般に、患者には約１ｐｇ／ｋｇ－１０ｍｇ／ｋｇ（患者の体重）の薬剤を投与することが望ましい。但し、これより少ない量又は多い量を投与する場合もあり得る。

【０１１２】

ある組成物の投与がその患者にとって耐えられるものである場合、当該薬剤を「薬理学的に使用可能である」と言い、その投与量が生理的に有意である場合、このような投与量を「治療有効量」と呼ぶ。ある薬剤を患者に投与し、これにより当該患者に検出し得るような変化が現れた場合、その薬剤は生理的に有意であると言う。

【０１１３】

本発明の化合物は、医薬として有用な組成物を調製するために、既知の方法に従い、これらの物質又はその機能的誘導体を医薬として使用可能なベヒクルに混合し、これを処方することができる。適切なベヒクル及びその処方（例えばヒト血清アルブミンなど、その他ヒトのタンパク質も含む）については、例えばＲｅｍｉｎｇｔｏｎ’ｓ Ｐｈａｒｍａｃｅｕｔｉｃａｌ Ｓｃｉｅｎｃｅｓ第１６版，Ｏｓｏｌ，Ａ．編，Ｍａｃｋ Ｅａｓｔｏｎ ＰＡ．（１９８０）の中にその記述がある。有効な投与に適した医薬として使用し得る組成物を処方するために、当該組成物に有効量の上記化合物を適切量の担体ベヒクルとともに添加することができる。

【０１１４】

その他薬学的な方法として、作用の永続性を制御するような方法も併せて使用することができる。当該化合物と錯体を形成し、又はこれを吸収するようなポリマーを使用することにより、薬剤の放出速度を制御することができる。被制御送入は、適切な高分子物質（例えばポリエステル、ポリアミノ酸、ポリビニルピロリドン、エチレン－酢酸ビニル共重合体、メチルセルロース、カルボキシメチルセルロース、又は硫酸プロタミン）を選択し、当該高分子物質の濃度及びその封入方法を選択して被制御放出を行うことにより、これを実施することができる。被制御放出製剤の使用により当該薬剤作用の永続性を制御する方法として、この他にポリエステル、ポリアミノ酸、ヒドロゲル、ポリ乳酸又はエチレン－酢酸ビニル共重合体などのポリマー物質の粒子中に、本発明の化合物を組み入れる方法がある。

【０１１５】

或いは、これらの薬剤をポリマー粒子中に組み込む代わりに、先ずミクロカプセルを調製してこれらの物質をその中に捕捉することもできる。例えば、界面重合を行ってこれらのミクロカプセルを調製することもできる。或いは、ヒドロキシメチルセルロース又はゼラチン製のミクロカプセル及びポリメタクリル酸メチル製ミクロカプセルをそれぞれ調製して使用する方法もある。或いは、コロイド状の薬剤送入システム（例えばリポソーム、アルブミンのミクロスフェア、ミクロエマルション、ナノ粒子、及びナノカプセル又はマクロエマルション）を使用することもできる。このような技法が、Ｒｅｍｉｎｇｔｏｎ’ｓ Ｐｈａｒｍａｃｅｕｔｉｃａｌ Ｓｃｉｅｎｃｅｓ（１９８０）の中で開示されている。

【０１１６】

本発明は、上記の診断又は治療に使用するキットを提供するものでもある。本発明の当該

側面に基づくキットは、瓶、チューブ、アンプル、ボトルなど、１個以上の容器でこれを構成し、これら容器の内容物は本発明の１種類以上の組成物でこれを構成することができる。

【０１１７】

本発明のキットは、１種類以上の下記構成成分、本発明における１種類以上の化合物、及び１種類以上の賦形剤、希釈剤又はアジュバントでこれを構成することができる。

【０１１８】

本明細書に記した方法及びその応用に適したその他の修飾及び対応方法については、当技術分野における通常技量の持ち主にとっては明らかであり、本発明又はその態様の範囲から逸脱すること無くこれを実施できることは、直ちに理解し得るところであろう。本発明に関して既にその詳細を説明したが、さらに下記の例を用いて同じ内容を具体的に分かりやすく説明する。但し、これらの例は説明を唯一の目的として使用するものであり、本発明の範囲に限定を加えることがその目的ではない。

【０１１９】

下記の例においては、下記の材料及び方法を使用する。　（材料及び方法）

（材料）

［α－^{32}Ｐ］ｄＣＴＰ（比活性：３０００　Ｃｉ／ｍｍｏｌ）、

［１，２，６，７－Ｎ－^{3}Ｈ］プロゲステロン（比活性：９４．１　Ｃｉ／ｍｍｏｌ）及び

^{3}Ｈ－１－（２－クロルフェニル）－Ｎ－メチル－Ｎ－（１－メチル－プロピル）－３－イソキノリンカルボキシアミド　（ＰＫ１１１９５；比活性　８６．９　Ｃｉ／ｍｍｏｌ）

上記材料は、ＮＥＮ　Ｌｉｆｅ　Ｓｃｉｅｎｃｅ　Ｐｒｏｄｕｃｔｓ社（マサチューセッツ州ボストン）からこれを入手した。

ＰＫ１１１９５は、Ｒｅｓｅａｒｃｈ　Ｂｉｏｃｈｅｍｉｃａｌｓ社（マサチューセッツ州ナティック）からこれを入手した。

ニトロセルロース　（０．４５　μｍ）は、Ｈｏｅｆｆｅｒ　Ｓｃｉｅｎｔｉｆｉｃ社（カリホルニア州サンフランシスコ）からこれを入手した。

２２Ｒ　ヒドロキシコレステロールは、Ｓｉｇｍａ社から購入した。

制限酵素類はＳｔｒａｔａｇｅｎｅ社（カリホルニア州ラホーヤ）及びＮｅｗ　Ｅｎｇｌａｎｄ　Ｂｉｏｌａｂｓ社（マサチューセッツ州ベバリー）から購入した。

細胞培養液は、Ｌｉｆｅ　Ｔｅｃｈｎｏｌｏｇｉｅｓ社（ニューヨーク州グランドアイランド）から購入した。

組織培養用プラスチックウエアは、Ｃｏｒｎｉｎｇ社（ニューヨーク州コーニング）から購入した。

電気泳動用の試薬及び材料は、ＢｉｏＲａｄ社から供給を受けた。

その他全薬品は分析グレードであり、種々の商業的供給源からこれらを入手した。

【０１２０】

（菌株及び媒体）

Ｓａｃｃｈａｒｏｍｙｃｅｓ　ｃｅｒｅｖｉｓｉａｅレポーター菌株　ＨＦ７ｃの遺伝子型はＭＡＴａ、ｕｒａ３－５２、ｈｉｓ３－２００、ｌｙｓ２－８０１、ａｄｅ２－１０１、ｔｒｐｌ－９０１、ｌｅｕ２－３、１１２、ｇａｌ４－５４２、ｇａｌ８０－５３８、ＬＹＳ２：：ＧＡＬ－ＨＩＳ３、ＵＲＡ３：：（ＧＡＬ４　１７－ｍｅｒｓ）$_{3}$－ＣＹＣ１－ｌａｃＺ（カリホルニア州パロアルト　ＣＬＯＮＴＥＣＨ社）である。酵母菌株は標準液体ＹＰＤ媒体中又は最小ＳＤ合成媒体（適切なアミノ酸を補足；カリホルニア州パロアルト　ＣＬＯＮＴＥＣＨ社）中、３０　℃で生育させた。

【０１２１】

（プラスミド及びその構造）

マウスのＰＢＲ　ｃＤＮＡコード化配列をｐＧＢＴ９（カリホルニア州パロアルト　ＣＬＯＮＴＥＣＨ社）の中へＥｃｏＲ　Ｉサイト及びＢａｍＨ　Ｉサイトでサブクローン化　（ｐＧＢＴ－ＰＢＲ）した。融合サイトの配列については、これを決定して検証した。ＰＢＲ

リガンド結合の分析を行い、機能的融合ＰＢＲタンパク質（酵母菌中で発現）について検証を行った。マウス精巣ｃＤＮＡライブラリを、ｐＧＡＤ１０［ＬＥＵ２、ＧＡＬ４（７６８－８８１）］（カリホルニア州パロアルト ＣＬＯＮＴＥＣＨ社）中で構築した。形質転換株をＬＢ－寒天－アンピシリン上で生育させ、プラスミドＤＮＡをＱＩＡＧＥＮ Ｐｌａｓｍｉｄ Ｇｉｇａキット（カリホルニア州バレンシア ＱＩＡＧＥＮ社）を使用して精製することにより、予め作成したライブラリを増幅した。トランスフェクションの実験において、ＰＡＰ７部分配列（１９２個のアミノ酸Ｃ末端配列を含む）をＰＳＶｚｅｏベクター（カリホルニア州カールスバド Ｉｎｖｉｔｒｏｇｅｎ社）のＥｃｏＲＩ及びＢａｍＨ１サイトに挿入した。

【０１２２】

（酵母菌二ハイブリッド検索）

Ｃｌｏｎｔｅｃｈ ＭＡＴＣＨＭＡＫＥＲ 二ハイブリッドシステムを本研究に適用した（説明書中で詳述）。これを簡単に説明すると、酵母菌レポーターの宿主株 ＨＦ７ｃをｐＧＢＴ－ＰＢＲ及びマウス精巣ｃＤＮＡとともにｐＧＡＤ１０プラスミド中で酢酸リチウム高能率法を使用して同時形質変換した（Ｇｉｅｔｚ，Ｄ．ら，１９９２，Ｎｕｃｌｅｉｃ Ａｃｉｄｓ Ｒｅｓ．２０，１４２５）。コロニー リフト フィルター分析法によりＨＩＳ陽性クローンをさらに選別し、そのβガラクトシダーゼ活性を求めた。プラスミドＤＮＡを酵母菌細胞からＥｓｃｈｅｒｉｃｈｉａ ｃｏｌｉ ＤＨ５？中へ救済した。プラスミドｐＧＢＴ－ＰＢＲを用いてプラスミドを酵母菌 ＨＦ７ｃ細胞中へ再形質変換し、ヒスチジン原栄養株及びβガラクトシダーゼ活性を試験した（クロンテック社マニュアルによる）。陽性クローンから採取したｃＤＮＡ挿入物について、その配列を決定した。５’及び３’ＲＡＣＥキット（カリホルニア州パロアルト ＣＬＯＮＴＥＣＨ社）を使用して、完全長のＰＡＰ７ ｃＤＮＡを得た。

【０１２３】

（配列分析）

ＡＢＩ ＰＲＩＳＭ™ 染料ターミネータ サイクル配列決定反応キット（カリホルニア州フォスター市 ＰＥ Ｂｉｏｓｙｓｔｅｍｓ社）及びＡｐｐｌｉｅｄ Ｂｉｏｓｙｓｔｅｍｓ配列決定キット（カリホルニア州フォスター市 Ａｐｐｌｉｅｄ Ｂｉｏｓｙｓｔｅｍｓ社）を使用して、Ｔｈｅ Ｌｏｍｂａｒｄｉ Ｃａｎｃｅｒ Ｃｅｎｔｅｒ Ｓｅｑｕｅｎｃｉｎｇ Ｃｏｒｅ Ｆａｃｉｌｉｔｙ（ジョージタウン大学）において配列決定を行った。ＧｅｎｅＢａｎｋ™ Ｄａｔａｂａｓｅに対してＥｎｔｒｅｚ ａｎｄ ＢＬＡＳＴプログラムを使用し、ＤＮＡ配列の分析を行った。

【０１２４】

（細胞培養液の一時的トランスフェクション）

前に報告した方法（Ｐａｐａｄｏｐｏｕｌｏｓ，Ｖ．ら，１９９０，Ｊ．Ｂｉｏｌ．Ｃｈｅｍ．２６５，３７７２－３７７９）により、ウマの血清１５％を含有する修飾ウェイマスＭＢ７５２／１媒体中でＭＡ－１０細胞を生育させた。マウスＣ６神経膠腫及びマウスＹ１副腎皮質細胞を、それぞれＤＭＥＭ及びＤＭＥＭ Ｆ１２中で、１０％胎児ウシ血清とともに培養した。電気泳動法（Ｅｌ Ｈｅｆｎａｗｙ，Ｔ．ら，１９９６，Ｍｏｌ．Ｃｅｌｌ Ｅｎｄｏｃｒｉｎｏｌ．１１９，２０７－２１７）によりＭＡ１０細胞を一時的にトランスフェクトした。各ジーンパルサー キュヴェット（空隙０．４ｃｍ，カリホルニア州ハーキュレス ＢｉｏＲａｄ社）には、抗生物質を含まない３５０μｌの完全ウェイマス成長媒体（上記参照）中に８ｘ10^6個の細胞、及び５０μｌの０．１ｘＴＥ中に３０μｇのプラスミドＤＮＡが含まれていた。電気泳動用キュヴェット中の細胞に、ジーンパルサー（カリホルニア州ハーキュレス ＢｉｏＲａｄ社）から発生させた電圧３３０Ｖ、静電容量９５０μＦｄの電気ショックを与えた。直ちに当該細胞を氷上で１０分間保持し、その後これを９６井のプレート上に移した。

【０１２５】

（放射性リガンドの結合分析）

我々が前に報告した方法（Ｐａｐａｄｏｐｏｕｌｏｓ，Ｖ．ら，１９９０，ｓｕｐｒ

ａ；Ｇａｒｎｉｅｒ，Ｍ．ら，１９９４；Ｍｏｌｅｃｕｌａｒ Ｐｈａｒｍａｃｏｌｏｇｙ ４５，２０１－２１１）により、^{3}Ｈ－１－（２－クロルフェニル）－Ｎ－メチル－Ｎ－（１－メチル－プロピル）－３－イソキノリンカルボキシアミド（ＰＫ１１１９５，マサチューセッツ州ボストン ＮＥＮ社）の結合試験を実施した。ＬＩＧＡＮＤプログラム（Ｍｕｎｓｏｎ，Ｐ．Ｊ．及びＲｏｄｂａｒｄ，Ｄ．，１９８０；Ａｎａｌ Ｂｉｏｃｈｅｍ．１０７，２２０－２３９）を使用して、データのスカチャード プロット分析を行うことにより、解離定数（Ｋｄ）及び結合サイト数（Ｂｍａｘ）を決定した。

【０１２６】

〔ＲＮＡ（ノーザン）ブロット分析〕

酸グアニジウム チオシアネート－フェノール－クロロホルム抽出法により、ＲＮＡ ＳＴＡＴ６０試薬（テキサス州フレンズウッド Ｔｅｌ－Ｔｅｓｔ社）を使用して、全組織及び細胞ＲＮＡを単離した。変性電気泳動法によりＲＮＡを分離し、これをＮｙｔｒａｎ膜（ニューハンプシャー州キーン Ｓｃｈｌｅｉｃｈｅｒ ＆ Ｓｃｈｕｅｌｌ社）に移した。ランダムプライミング（インジアナ州インジアナポリス Ｂｏｅｈｒｉｎｇｅｒ Ｍａｎｎｈｅｉｍ社）から^{32}Ｐを発生させ、この^{32}ＰでＰＡＰ７ ｃＤＮＡプローブを標識化し、当該ＰＡＰ７ ｃＤＮＡ プローブで当該ＲＮＡブロットをハイブリッド化した。Ｋｏｄａｋ Ｘ－Ｏｍａｔ ＡＲフィルム（ニューヨーク州ロチェスター Ｅａｓｔｍａｎ Ｋｏｄａｋ社）を－８０℃で一晩当該ブロットに暴露することにより、オートラジオグラフィーを実施した。

【０１２７】

（ステロイド生合成）

９６井のプレートを使用し、ＭＡ－１０細胞を密度２．５ｘ１０^{4}／井で一晩プレート培養した。濃度５０ ｎｇ／ｍｌのｈＣＧを使用し、０．２ ｍｌ／井の血清を含まない媒体中で当該細胞に２時間刺激を与えた。当該培養媒体を集め、ＲＴＡによりプロゲステロンの生成試験を行った。当該分析は、メーカー推奨条件に従い、抗プロゲステロン抗血清（カリホルニア州コスタメザ ＩＣＮ社）を使用してこれを実施した。当該プロゲステロン生成量は、各井中のタンパク質量を調節することにより、これを正規化した。Ｗａｌｌａｃ社（メリーランド州ガイタースバーグ ＥＧ＆Ｇ Ｗａｌｌａｃ社）が提供したソフトウェアを使用し、放射能免疫分析のデータを解析した。

【０１２８】

（抗体発生及びウェスタン分析）

ＰＡＰ７タンパク質のペプチドＳＳＤＥＥＥＥＥＥＥＮＶＴＣＥＥＫＡＫＫＮＡＮＫＰ（ＳＥＱ ＩＤ ＮＯ：１１）で系列免疫化することによりウサギ抗ＰＡＰ７抗体を調製し、これをＫＬＨにカップリングした。アガロース（テキサス州モントゴメリー Ｂｅｔｈｙｌ Ｌａｂｏｒａｔｏｒｉｅｓ社）上に固定したこれと同じペプチドを含むアフィニティ樹脂により、ＰＡＰ７抗体を精製した。ＭＡ１０細胞をサンプル緩衝液〔２５ ｍＭトリスＨＣｌ（ｐＨ ６．８），１％ ＳＤＳ，５％βメルカプトエタノール，１ ｍＭ ＥＤＴＡ，４％ グリセリン，及び０．０１％ ブロムフェノール ブルー〕中へ可溶化し、５分間煮沸し、１５％ ＳＤＳ－ＰＡＧＥミニゲル（カリホルニア州リッチモンド ＢｉｏＲａｄ社；ＭｉｎｉＰｒｏｔｅｉｎ ＩＩ Ｓｙｓｔｅｍ）上に負荷した。

【０１２９】

電流密度２５ ｍＡ／ゲルで、標準 ＳＤＳ－ＰＡＧＥランニング緩衝液（２５ ｍＭトリス，１９２ ｍＭグリシン，及び０．１％ ＳＤＳ）を使用して電気泳動を実施した。当該タンパク質を電気泳動によりニトロセルロース膜（ニューハンプシャー州キーン Ｓｃｈｌｅｉｃｈｅｒ ＆ Ｓｃｈｕｅｌｌ社）上に移した。当該膜を、１０％ カーネーション無脂肪ミルクを含むブロッキング緩衝液〔ＴＴＢＳ緩衝液（２０ ｍＭトリスＨＣｌ，ｐＨ ７．５，０．５ Ｍ ＮａＣｌ，及び０．０５％ Ｔｗｅｅｎ－２０〕中、室温で１時間インキュベートし、次にＰＡＰ７（１：２０００）に対する一次抗体で２時間インキュベートし

た。当該膜をＴＴＢＳで３回、各１０分間ずつ洗浄した。二次抗体〔ＨＲＰと共役（信号形質導入）したヤギの抗ウサギ ＩｇＧ〕で１時間インキュベートした後、当該膜をＴＴＢＳで３回、各１０分間ずつ洗浄した。ルネサンス キット （デラウェア州ウィルミントン Ｎｅｗ Ｅｎｇｌａｎｄ Ｎｕｃｌｅａｒ社）を使用し、メーカーの指示に従い、ケミルミネセンスにより固有タンパク質バンドを検出した。

【０１３０】

（免疫細胞化学処理）

４室ＳｕｐｅｒＣｅｌｌ Ｃｕｌｔｕｒｅ Ｓｌｉｄｅ （ペンシルバニア州ピッツバーグ Ｆｉｓｈｅｒ Ｓｃｉｅｎｔｉｆｉｃ社）上でＭＡ－１０細胞を培養し、４ ℃、１５分間かけてメタノールで固定した。固定した当該細胞を、ＰＡＰ７ペプチドの存在下又は不存在下において、ＰＡＰ７抗体（希釈度 １ ： ２５０）で１時間インキュベートした。洗浄後、当該細胞をＨＲＰ共役ヤギ抗ウサギ二次抗体（ケンタッキー州レキシントン Ｔｒａｎｓｄｕｃｔｉｏｎ Ｌａｂ社）で１時間インキュベートした。ＰＡＰ７染色を、クロモーゲンとしてＡＥＣ（３－アミノ－９－エチル カルバゾール）を使用し、ペルオキシダーゼで可視化して、赤色の反応生成物を得た。ヘマトキシリンで後染色した後、スライドを脱水処理し、永久固定した。

【０１３１】

（免疫組織化学処理）

マウスの組織を液体チッ素中で新しく切り取って採取した。切断直後に、試験片を冷たいメタノール中で５分間かけて固定した。次に当該スライドをH_2O_2の０．３％メタノール溶液を入れた室温の部屋の中に２０分間置き、内因性ペルオキシダーゼ活性を抑制し、次にブロッキング溶液（１０％ヤギ血清；カリホルニア州サンフランシスコ Ｚｙｍｅｄ社）中で１５分間インキュベートした。その後、当該スライドを、抗ＰＡＰ７抗体（１ ： ２５０）で２時間、室温でインキュベートし、水及びＰＢＳで洗浄し、ＨＲＰ共役ヤギ抗ウサギ二次抗体で１時間、室温でインキュベートし、次にＰＢＳで洗浄した。染色を行うためにＡＥＣ試薬で１時間、３７ ℃で処理した後、当該断面をヘマトキシリンで後染色し、脱水して永久固定した。

【０１３２】

（タンパク質の定量化及び統計学的解析）

Ｂｒａｄｆｏｒｄの染料結合分析法（Ｂｒａｄｆｏｒｄ，Ｍ．Ｍ．，１９７６，Ａｎａｌ．Ｂｉｏｃｈｅｍ．７２，２４８－２５４）により、ウシ血清アルブミンを標準物質としてタンパク質を定量した。統計学的解析として、先ずＡＮＯＶＡ法を実施し、次にＧｒａｐｈＰａｄ社（カリホルニア州サンヂエゴ）から入手したＩｎｓｔａｔ （ｖ．２．０４）パッケージを使用してＳｔｕｄｅｎｔ－Ｎｅｗｍａｎ－ｋｅｕｌｓ試験又はＤｕｎｎｅｔｔ多重比較試験を実施した。

【０１３３】

（例１：ＰＢＲ会合タンパク質の単離）

我々は、ＣＬＯＮＴＥＣＨ社のＭＡＴＣＨＭＡＫＥＲ二ハイブリッド システムを使用して、その産物がＰＢＲタンパク質と相互作用するように遺伝子をクローン化した。ＧＡＬ４ （１－１４７）－ＰＢＲ 融合（プラスミドｐＧＢＴ９＋ＰＢＲ）を餌として使用し、ｐＧＡＤ１０二ハイブリッドベクターの中に構築したマウスＭＡＴＣＨＭＡＫＥＲ精巣ＤＮＡライブラリの検索を行った。約 3×10^6 個の変移体を試験し、ＰＢＲとの相互作用能力を有する５種類の陽性クローンを得た。これらの変移体から採取したライブラリ プラスミドをＥ．ｃｏｌｉ菌株 ＤＨ５ａ中に救済した。Ｈｉｓ－及びβガラクトシダーゼの両発現型は、ｐＧＢＴ９－ＰＢＲ担持菌株ＨＦ７ｃを二ラウンドで形質変換することにより、その存在を確認した（表１）。

表１： ＰＢＲによる酵母菌二ハイブリッド マウス精巣ライブラリ検索結果の要約

クローン活性	Ｈｉｓ３	βガラクトシダーゼ
ＰＡＰ３	＋	＋＋
ＰＡＰ７	＋	＋＋

ＰＡＰ２０　＋　＋＋
陽性対照　＋　＋＋＋

【０１３４】
これら陽性クローンから採取したプラスミドについて、制限酵素消化を行って第一回目の分析を行い、次に配列分析を実施した。これにより、２種類のクローンが単一遺伝子によりコード化されていることが明らかになった。この遺伝子はこれまで知られていなかった未知の遺伝子であり、これをＰＢＲ会合タンパク質７（ＰＡＰ７）と命名した。その他３種類のクローンについては、種々の異なる産物をコード化することが明らかになった。５’ＲＡＣＥ及び３’ＲＡＣＥ試験を行うことにより、両菌株に関してＰＡＰ７ｃＤＮＡクローンの完全配列（ＳＥＱ ＩＤ ＮＯ： ２）を求めた。当該クローンは、計算分子量約５２ ｋＤａを有する４６３－アミノ酸タンパク質をコード化することが明らかになった。ＢＬＡＳＴプログラムを使用してＧｅｎｅｂａｎｋデータベースを相同体検索した結果、当該クローンは、これまで同定されたことの無い新規配列であることが明らかになった。

【０１３５】
（例２：ＭＡ－１０ライジッヒ腫瘍細胞中におけるＰＡＰ７タンパク質の発現）
ＭＡ－１０細胞タンパク質の全抽出物を、ウェスタンブロット法により、ＰＡＰ７抗体を使用して分析した。この抗体は、特に５０ ｋＤａタンパク質バンドを認識する（ 図１Ａ）。ＭＡ－１０細胞中におけるＰＡＰ７タンパク質の発現についても、免疫細胞化学的手法によりこれをチェックした。ＰＡＰ７抗体は特にＭＡ－１０細胞を染色し、当該信号の多くは細胞形質中に局在していることが判明した（図１Ｂ）。この信号は、ＰＡＰ７ペプチドによりこれを中和することができた。この方法により抗体を発生させ、これを精製した。

【０１３６】
（例３：ＰＡＰ７細胞並びにドットブロット法及びノーザンブロット法による組織発現）
ドットブロット分析法により、ＰＡＰ７が脳、眼、下顎腺、精巣及び卵巣中において高度に発現されることが観察された。興味あることに、ＰＡＰ７の発現は、初期胚段階において最高水準に到達し、出生前には減少することが判明した（図２Ａ及び２Ｂ）。ノーザンブロット分析により、ＰＡＰ７ ｍＲＮＡは一貫して副腎、脳、心臓、肝臓、精巣及び卵巣の組織に発現することが明らかになった。ＰＡＰ７には１ ｋｂの転写体が存在し、当該転写体は精巣中にのみ発現した。３ ｋｂの主要転写体は他の組織中で発現した（図３Ａ及び３Ｂ）。ＰＡＰ７は３種類の細胞系でも高度の発現量を示した。
ステロイド生合成に関する研究には、この現象を広く利用した。３種類の細胞系全てにおいて、正常組織中と同じ分子量サイズのＰＡＰ７転写体が発現した。これら細胞系におけるＰＡＰ７の発現量は、それらのステロイド生成能力と比例関係にあることが明らかになった（図３Ａ及び３Ｂ）。ＰＢＲ ｍＲＮＡの発現量についても、これらの同じ組織及び細胞系の中でこれをチェックした。ＰＢＲの発現水準は、特にこれら３種類の細胞系において、ＰＡＰ７ ｍＲＮＡの発現パターンと平行関係にあった（図４Ａ及び４Ｂ）。

【０１３７】
（例４：ＰＡＰ７細胞の分布）
種々の組織中におけるＰＡＰ７タンパク質の発現について、免疫組織化学法によりそのチェックを行った（図５）。精巣のライジッヒ細胞及び生殖細胞の中（図５Ｃ及び５Ｄ）、脳の海馬細胞及び神経細胞の中（図５Ｅ及び５Ｆ）、副腎腺の索状層細胞の中（図５Ｇ及び５Ｈ）、及び卵巣の顆粒膜細胞の中（図５Ａ及び５Ｂ）のいずれにおいても、ＰＡＰ７の存在が認められた。肝臓及び腎臓も、低濃度のＰＡＰ７タンパク質を発現した（データは示さなかった）。各試験片は、負の比較対照としてＰＡＰ７ペプチド中和抗体を使用してこれを染色した。その後の原位置ハイブリッド化研究の結果から、ＰＡＰ７ ｍＲＮＡの発現が、ＰＡＰ７タンパク質の発現と同じパターンを示すことが明らかになった。

【０１３８】
（例５：ＭＡ－１０細胞中におけるステロイド生合成に及ぼすＰＡＰ７の効果）

ＰＢＲ結合ドメインを含むＰＡＰ７部分配列をｐＳＶｚｅｏ哺乳類発現ベクター中へサブクローン化した。このｐＳＶｚｅｏＰＡＰ７ベクターを、一時的にＭＡ－１０細胞中へ形質移入した。ｐＳＶｚｅｏ空ベクターも、比較対照として細胞中へ形質移入した。両空ベクター（ｐＳＶｚｅｏ形質移入体及びｐＳＶｚｅｏＰＡＰ７形質移入体）のステロイド生合成能力は、ホルモン （ｈＣＧ）刺激に反応して起こるプロゲステロンの生産量を監視することにより、これをチェックした。ＰＡＰ７形質移入体は、ｐＳＶｚｅｏベクターの形質移入体と比較した場合、投与量及び時間に依存する形でＭＡ－１０細胞中におけるプロゲステロンの生産量を著しく低下させた（図６）。

【０１３９】

（考　察）

ステロイド生合成においてＰＢＲはコレステロールの輸送量を調節している。その機構に関してより理解を深めるために、我々は酵母菌二ハイブリッド分析を実施してＰＢＲ関連タンパク質の同定を行った。マウスＰＢＲ ｃＤＮＡをｐＧＢＴ９ ベクター中に挿入し、餌としてＧＡＬ４ ＤＮＡ結合ドメイン及びＰＢＲ融合タンパク質を発生させた。ＰＢＲリガンド（ＰＫ１１１９５）と融合したタンパク質について、その受容体リガンドの結合活性を測定した。

我々が得た結果は、酵母菌中に発現した当該融合ＰＢＲタンパク質が、天然のＰＢＲタンパク質と類似の結合親和性を有していたことを示している（データは示さなかった）。ＰＢＲは、精巣を含む種々の周辺組織中でその存在が確認されている （Ｇａｖｉｓｈ，Ｍ．及びＷｅｉｚｍａｎ，Ｒ．，１９９７，Ｃｌｉｎ．Ｎｅｕｒｏｐｈａｒｍａｃｏｌ．２０，４７３－４８１）。ステロイド生成において、精巣は重要組織であり、且つ非常に良く研究されている組織の一つでもある （Ｈｕｈｔａｎｉｅｍｉ，Ｉ．及びＴｏｐｐａｒｉ，Ｊ．，１９９５，Ａｄｖ．Ｅｘｐ．Ｍｅｄ．Ｂｉｏｌ．３７７，３３－５４）。

マウス精巣のライジッヒ細胞におけるステロイド生合成の機構及び過程におけるＰＢＲの役割に関しても、多くの報告が存在する （Ｐａｐａｄｏｐｏｕｌｏｓ Ｖ．ら、１９９７，Ｓｔｅｒｏｉｄｅｓ ６２，２１－２８；Ｐａｐａｄｏｐｏｕｌｏｓ，Ｖ．ら，１９９８，Ｅｎｄｏｃｒ．Ｒｅｓ．２４，４７９－４８７）。従って、我々はマウスの精巣ｃＤＮＡライブラリを、この二ハイブリッド検索研究に適用した。ｐＧＡＤ１０ベクターを使用し、融合Ｂａｌｂ／ｃマウスの精巣ライブラリの中にランダムにタンパク質を集めて、ＧＡＬ４活性化ドメインの融合タンパク質を発生させた。酵母菌二ハイブリッド検索により、陽性クローンの一つとして、ＰＡＰ７の存在が確認された。当該クローンは、ＰＢＲとの相互作用能力を有することが証明された（表１）。

【０１４０】

このようにして、我々は、ＰＢＲと相互作用するマウス タンパク質に、ＰＡＰ７ ｃＤＮＡをクローン化してこれをコード化した。データベースの検索結果に基づき、ＰＡＰ７は新規遺伝子産物であることが判明した。最近ＰＲＡＸ－１が、特にＰＢＲとの間に相互作用を持つ新しいタンパク質であることが報告された （Ｇａｌｉｅｇｕｅ，Ｓ．ら，１９９９，Ｊ．Ｂｉｏｌ．Ｃｈｅｍ．２７４，２９３８－２９５２）。このタンパク質との唯一の類似点は、両タンパク質がグルタミン酸基を含有していることである。ＰＡＰ７の一部は、その機能がまだ知られていないＣ．ｅｌａｇａｎｓ遺伝子と極めて高度の相同性を共有していることも知られている （Ｗｉｌｓｏｎ，Ｒ．ら，１９９４，Ｎａｔｕｒｅ ３６８，３２－３８）。

【０１４１】

事実、Ｃ．ｅｌｅｇａｎｓ細胞の培養にはコレステロールが必要とされる （Ｂｒｅｎｎｅｒ，Ｓ．，１９７４，Ｇｅｎｅｔｉｃｓ ７７，７１－９４）。コレステロールの輸送にＰＢＲが関与し、ＰＢＲ遺伝子が全ての微生物中において高度に保守的であることを考えると、このデータは、細胞の生存及び成長という基本要件を満たすには、ＰＡＰ７の発現が必要であることを示唆しているものと考えることができる。ＰＡＰ７はＲＡＬＢＰ（細胞内レチノイド輸送において機能する疎水性リガンド結合タンパク質）とも、いく

つかの相同性を共有していることが知られている（Ｏｚａｋｉ，Ｋら，１９９４，Ｊ．Ｂｉｏｌ．Ｃｈｅｍ．２６９，３８３８－３８４５）。

【０１４２】

スイスポート プロジット プロフィール走査を使用する配列モチーフ分析により、ＰＡＰ７に脂肪族アシル化（ミリストイル化）サイト、アシル－ＣｏＡ結合タンパク質のシグナチャー、及びＰＫＣホスフォリル化サイトが存在することが判明した。タンパク質をミリストイル化することにより、タンパク質は細胞膜に付着し、細胞信号の発信に参加できるようになる（Ｃａｓｅｙ，Ｐ．Ｊ．，１９９５，Ｓｃｉｅｎｃｅ２６８，２２１－２２５；Ｂｏｕｔｉｎ，Ｊ．Ａ．，１９９７，Ｃｅｌｌ Ｓｉｇｎａｌ ９，１５－３５）。ＰＢＲは疎水性タンパク質であり、外部ミトコンドリア膜と密接な関連を有している。この性質により、ＰＡＰ７がホルモン刺激信号をＰＢＲに送り、ＰＢＲとの相互作用を発生し、このようにしてコレステロール輸送においてＰＢＲ活性を調節することが可能となる。

興味あることに、アシル－ＣｏＡ結合タンパク質は、ＰＢＲ内因性リガンド（ジアゼパム結合抑制剤：ＤＢＩ）の別名でもある（Ｒｏｓｅ，Ｔ．Ｍ．ら，１９９２，Ｐｒｏｃ．Ｎａｔｌ．Ａｃａｄ．Ｓｃｉ．Ｕ．Ｓ．Ａ．８９，１１２８７－１１２９１；Ｃｏｓｔａ，Ｅ．及びＧｕｉｄｏｔｔｉ，Ａ．，１９９１，Ｌｉｆｅ Ｓｃｉ．４９，３２５－３４４；Ｓｕｋ，Ｋ．ら，１９９９，Ｂｉｏｃｈｅｍ．Ｂｉｏｐｈｙｓ．Ａｃｔａ １４５４，１２６－１３１）。この情報は、ＰＡＰ７が他のＰＢＲ内因性リガンドと配位することによりその機能を発揮することを示唆しているものと考えられる。ＰＡＰ７がタンパク質キナーゼの潜在的ホスフォリル化サイトを有するという事実は、ＰＢＲがＰＡＰ７タンパク質と相互作用してホルモン刺激により調節を行うという、もう一つの可能性を高めるものである。

【０１４３】

脳、精巣、卵巣、副腎、及び腎臓並びにその他いくつかの細胞系など、主なマウス組織についてＰＡＰ７の分布及び発現量を調べた。ＰＡＰ７の発現パターンは、これよりブロードなＰＢＲの発現プロフィールに類似している。前に行った研究（Ｐａｐａｄｏｐｏｕｌｏｓ，Ｖ．ら，１９９８，前出）によれば、副腎のｚｏｎａ ｆａｓｃｉｃｕｌａｔａ細胞により糖コルチコイドが生産されることが明らかになっている。

【０１４４】

卵巣においては、顆粒膜細胞が存在するｃｏｕｐｕｓ ｌｕｔｅｕｍがプロゲステロンを分泌する。さらに、精巣のライジッヒ細胞はテストステロンを生産する能力を有している。主要ステロイド生成組織の中ではＰＡＰ７が高度に発現され、これらのステロイド生産細胞中においてその濃度が高まるので、ＰＢＲ錯体の形成量又はその形態を変化させることにより、ステロイドの生合成又はステロイド形成量の調節に、ＰＡＰ７が関与できるようになる。

【０１４５】

マウスＣ６神経膠細胞、ＭＡ－１０ライジッヒ細胞及びＹ１副腎皮質細胞は、ステロイド生合成の研究用に選ばれる良く知られた細胞モデルである。これらの細胞系において、ＰＡＰ７の発現量はＰＢＲの発現量に比例相関する。

【０１４６】

さらに、これらの細胞系内におけるＰＢＲの発現濃度とＰＡＰ７の発現濃度は、これら物質のステロイド生成能力に対してともに平行的である。この事実も、ＰＡＰ７がＰＢＲ経由でステロイド生合成に関与していることを示唆するものである。小型ＰＡＰ７転写体は精巣中のみに発現したが、この現象は、精巣中に発現した他の遺伝子についても観察されるものである（Ｚｈａｎｇ，Ｆ．Ｐ．ら，１９９７，Ｅｎｄｏｃｒｉｎｏｌｏｇｙ １３８，２４８１－２４９０；Ｍａｕｄｕｉｔ，Ｃ．ら，１９９９，Ｊ．Ｂｉｏｌ．Ｃｈｅｍ．２７４，７７０－７７５）。ライジッヒ細胞以外の精巣中免疫染色により、小型転写体の発現量を表すことが可能である。

【０１４７】

ＰＡＰ７タンパク質はＭＡ－１０細胞中に発現し、大部分の染色は細胞形質中に局在して行われる。ＰＡＰ７の細胞レベル以下における分布研究については目下進行中である。将来その結果が、ＰＢＲ－ＰＡＰ７間の相互作用に関して、より詳細な情報を提供してくれるものと期待される。ＰＢＲをノックアウトしたマウスは子宮の中で死んでしまう。この事実は、ＰＢＲがマウスの胚成長過程において必須の役割を演じていることを示している。興味あることに、ＰＡＰ７ ｍＲＮＡは、マウスの初期胚の成長過程において高度に発現する。

【０１４８】

この結果から、マウスの初期発育過程において、ＰＢＲと会合したＰＡＰ７がマウスの成長に重要な役割を演じていることが推定される。このことは、さらに、ＰＢＲがステロイド生成以外の新しい機能を有する可能性を示唆している。

そのＰＢＲ結合ドメインを含め、ＰＡＰ７フラグメントが過剰発現すると、ＭＡ－１０細胞中において、これが飽和濃度のｈＣＧ（５０ｎｇ／ｍｌ）による刺激で生成するプロゲステロンの形成を充分に抑制し得ることが明らかになった。

前に行った研究の結果によれば、これらの細胞によるプロゲステロン生産量は、ステロイド生合成量の目安となる（Ｆｒｅｅｍａｎ，Ｄ．Ａ．，１９８７，Ｅｎｄｏｃｒｉｎｏｌｏｇｙ １２０，１２４－１３２；Ｇａｒｎｉｅｒ，Ｍ．ら，１９９４，Ｊ．Ｂｉｏｌ．Ｃｈｅｍ．２６９，２２１０５－２２１１２）。機能抑制面から考えれば、過剰発現したＰＡＰ７フラグメントは、ＭＡ－１０細胞中において、天然ＰＡＰ７に対する競争因子として作用し、ＰＢＲと競争して結合に参加する可能性がある。我々は、ＰＢＲ結合ドメインしか持っていない形質移入ＰＡＰ７フラグメントは、天然のＰＡＰ７のように完全機能することはできないものと信じている。

しかし形質移入ＰＡＰ７フラグメントは、ＰＢＲと内因性ＰＡＰ７との相互作用を競争的に阻止し、これによりＰＢＲの正常機能をブロックするものと考えられる。

【０１４９】

結論として、本明細書に記した結果から、今回同定されたＰＡＰ７は、内因生リガンド又は当該受容体のアロステリック調節剤として作用し、これによりＰＢＲの機能調節に関与していることを示唆している。

【０１５０】

（ｉ）ＰＢＲはコレステロールのチャンネル又は輸送手段として作用し、（ｉｉ）ＰＢＲはまた環境抗ステロイド生成物質の標的としても作用し、（ｉｉｉ） さらにＰＢＲは乳癌の進行及び腫瘍細胞の増殖に関与する（Ｈａｒｄｗｉｃｋ，Ｍ．ら，１９９９，Ｃａｎｃｅｒ Ｒｅｓ．５９，８３１－８４２）という観察結果を考慮し、ＰＡＰ７の同定及びキャラクタリゼーションを行うことにより、ステロイドの生成においても、又さらに広い総合的な分野においても、ＰＢＲの果たす役割をもっと広く理解し得るものと我々は信じている。

【図面の簡単な説明】

本発明に関する上記の特徴、側面、及び利点、及びその他の特徴、側面、及び利点は、下記の説明及び付属請求範囲並びに付属図面を参照することにより、より良く理解されるものと思われる。

【図１】マウスのＭＡ－１０ライジッヒ腫瘍細胞中に発現したＰＡＰ７タンパク質；（Ａ）ウェスタンブロット法；（Ｂ）免疫細胞化学法。

【図２】ドットブロット分析によるＰＡＰ７ ｍＲＮＡの組織分布測定；（Ａ）１００－５００ ｎｇのポリ（Ａ）＋マウス組織から採取したＲＮＡを含むマスターブロットを、「材料及び方法」の項で述べた方法により、高度のストリンジェンシーで^{12}Ｐ－標識ＰＡＰ７プローブとハイブリッド化した。当該オートラジオグラムを一晩暴感光させた。（Ｂ）ＰＡＰ７発現量の密度測定分析

【図３】ノーザンブロット分析によるＰＡＰ７の組織分布測定；（Ａ）各レーン毎に異なるマウス組織（採取組織名を図中に示す）から採取した２０ μｇの全ＲＮＡを使用して、ノーザンブロット分析を実施した。当該ブロットを、「材料及び方法」の項に記した方

法を使用し、$^{1\ 2}$Ｐ－標識ＰＡＰ７プローブにより、高ストリンジェンシーでハイブリッド化した。ＰＡＰ７発現量を密度測定分析により測定した。

【図４】ノーザンブロット分析によるＰＢＲの組織分布測定。（Ａ）各レーン毎に異なるマウス組織（組織名を図中に示す）から採取した２０ ｎｇの全ＲＮＡを使用して、ノーザンブロット分析を実施した。当該ブロットを、$^{3\ 2}$Ｐ－標識ＰＢＲプローブを使用し、「材料及び方法」の項に記した方法により、高ストリンジェンシーでハイブリッド化した。当該オートラジオグラムを一晩感光させた。（Ｂ）ノーザンブロット分析を行うために、ブロットを密度測定分析により定量化した。

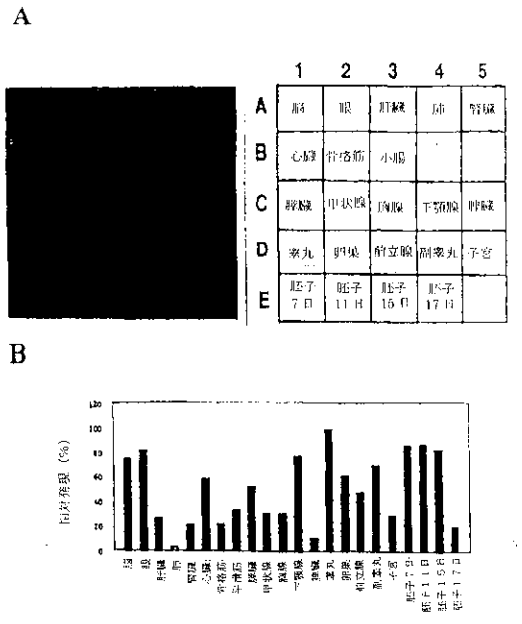
【図５】項ＰＡＰ７抗体によるマウス組織の免疫組織学的染色。

【図６】ステロイド生合成に関するＰＡＰ７の効果。ｈＣＧ刺激によるＭＡ－１０中におけるプロゲステロンの形成（Ａ）。種々の時間過程におけるプロゲステロンの形成量（Ｂ）。結果は、互いに独立な実験結果（ｎ＝２－６）の平均値＋標準偏差として示した。

【図７】ドットブロット分析によるＰＡＰ３ ｍＲＮＡの組織分布測定。（Ａ）１００－５００ ｎｇのポリ（Ａ）＋マウス組織から採取したＲＮＡを含むマスターブロットを、「実験手順」の項に記した方法を使用し、$^{3\ 2}$Ｐ－標識ＰＡＰ３プローブにより、高ストリンジェンシーでハイブリッド化した。当該オートラジオグラムを一晩感光させた。（Ｂ）ＰＡＰ３発現量の密度測定分析。

【図８】ドットブロット分析によるＰＡＰ２０ ｍＲＮＡの分布測定。（Ａ）１００－５００ ｎｇのポリ（Ａ）＋マウス組織から採取したＲＮＡを含むマスターブロットを、「実験手順」の項に記した方法を使用し、$^{3\ 2}$Ｐ－標識ＰＡＰ２０プローブにより、高ストリンジェンシーでハイブリッド化した。当該オートラジオグラムを一晩感光させた。（Ｂ）ＰＡＰ２０発現量の密度測定による分析。

【図２】



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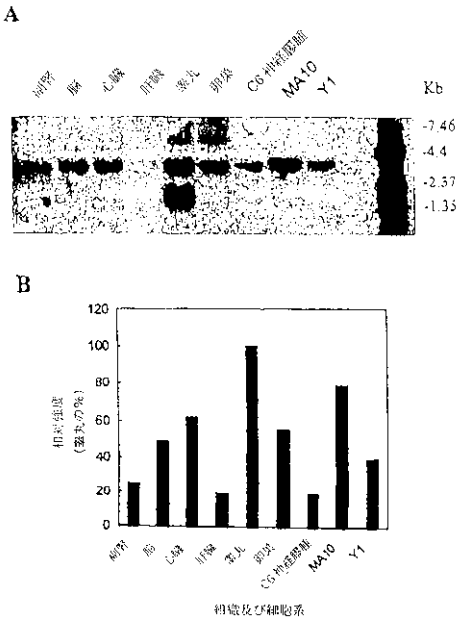

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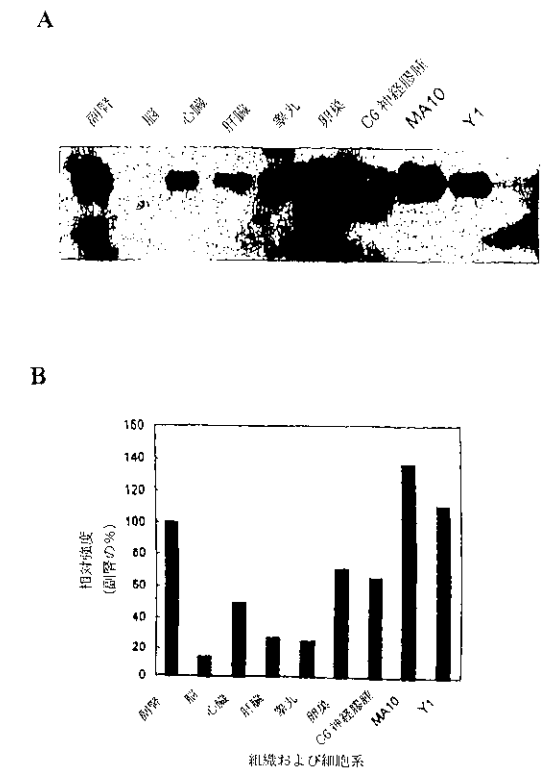

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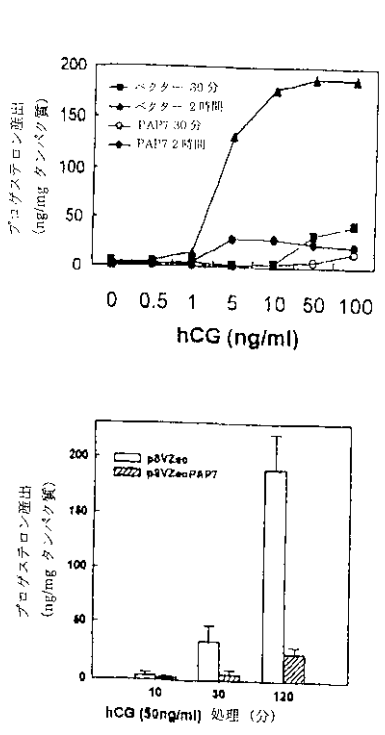

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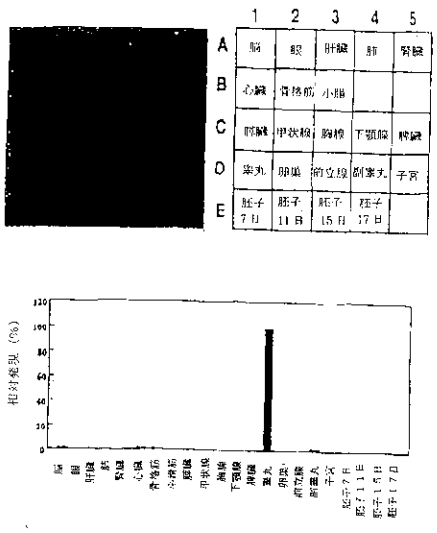

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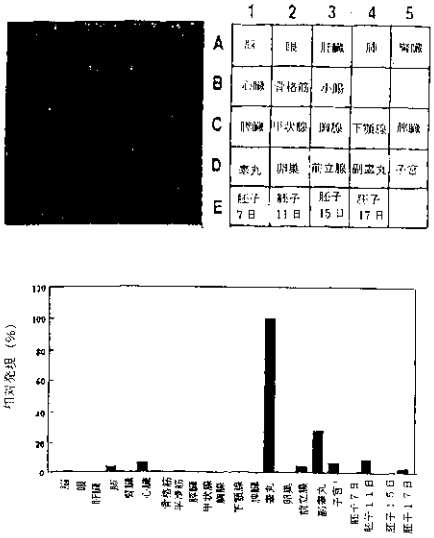

PAP3

DAD22

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(57) Abstract

The present invention relates to nucleic acids encoding PBR-associated proteins (PAPs) and methods for use in producing PAPs and methods of using PAPs. In this study we have identified proteins (PAPs) that interact with PBR protein by a yeast two-hybrid system. We used PBR as bait to screen a mouse testis cDNA library. Five clones were isolated based on their ability to interact with PBR. These proteins may be involved in the regulation of the function of PBR, serving as an endogenous ligand or allosteric modulator of the receptor.

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TITLE OF THE INVENTION

Peripheral-type Benzodiazepine Receptor Associated Proteins, Cloning, Expression and Methods of Use

Field of the Invention

The present invention relates to nucleic acid molecules encoding peripheral-type benzodiazepine receptor (PBR) associated proteins (PAPs), including mutants, variants, fragments and derivatives thereof, and to vectors and host cells comprising such nucleic acid molecules; methods of using PAPs; method for screening for inhibitors and activators of PAPs or PBR; and kits comprising the compositions or polypeptides of the invention.

Background of the Invention

The peripheral-type benzodiazepine receptor (PBR) was originally discovered because it binds the benzodiazepine diazepam with relatively high affinity (Papadopoulos, V. 1993, *Endocr. Rev.* 14:222-240). Benzodiazepines are among the most highly prescribed drugs due to their pharmacological actions in relieving anxiety mediated through modulating the activity of γ-aminobutyric acid receptors in the central nervous system (Costa, E. and Guidotti, A. 1979, *Annu. Rev. Pharmacol. Toxicol.* 19:531-545). PBR is another class of binding sites for benzodiazepines distinct from the aforementioned neurotransmitter receptors. Further studies demonstrated that in addition to benzodiazepines, PBR binds other classes of organic compounds with high affinity (Papadopoulos, 1993, *supra*). PBR, although present in all tissues examined, was found to be particularly high in steroid

producing tissues, where it was primarily localized in the outer mitochondrial membrane (OMM) (Anholt, R.R.H. *et al.* 1986, *J. Biol. Chem.* 261:576-583). An 18 kDa isoquinoline-binding protein was identified as PBR, cloned and expressed (Papadopoulos, V. 1998, *Proc Soc. Exp. Biol. Med.* 217:130-142). It was then demonstrated that PBR is a functional component of the steroidogenic machinery (Papadopoulos, 1998, *supra*; Papadopoulos V. *et al.* 1990, *J. Biol. Chem.* 265:3772-3779) mediating cholesterol delivery from the outer to the inner mitochondrial membrane (Krueger, K. E. and Papadopoulos, V. 1990, *J. Biol. Chem.* 265:15015-15022). Further studies demonstrated that pharmacologically induced reduction of adrenal PBR levels *in vivo* resulted in decreased circulating glucocorticoid levels (Papadopoulos, V. 1998, *supra*). In addition, targeted disruption of the PBR gene in Leydig cells resulted in the arrest of cholesterol transport into mitochondria and steroid formation; transfection of the mutant cells with a PBR cDNA rescued steroidogenesis (Papadopoulos, V. *et al.* 1997, *J. Biol. Chem.* 272:32129-32135).

PBR is extremely abundant in steroidogenic cells and found primarily on outer mitochondrial membranes (Anholt, R. *et al.* 1986, *J. Biol. Chem.* 261:576-583). PBR is thought to be associated with a multimeric complex composed of the 18-kDa isoquinoline-binding protein and the 34-kDa pore-forming voltage-dependent anion channel protein, preferentially located on the outer/inner mitochondrial membrane contact sites (McEnery, M. W. *et al. Proc. Natl. Acad. Sci. U.S.A.* 89:3170-3174; Garnier, M. *et al.* 1994, *Mol. Pharmacol.* 45:201-211; Papadopoulos, V. *et al.* 1994, *Mol. Cel. Endocr.* 104:R5-R9). Drug ligands of PBR, upon binding to the receptor, simulate steroid synthesis in

steroidogenic cells *in vitro* (Papadopoulos, V. *et al.* 1990, *J. Biol. Chem.* 265:3772-3779; Ritta, M. N. *et al.* 1989, *Neuroendocrinology* 49: 262-266; Barnea, E. R. *et al.* 1989, *Mol. Cell. Endocr.* 64:155-159; Amsterdam, A. and Suh, B. S. 1991, *Endocrinology* 128:503-510; Yanagibashi, K. *et al.* 1989, *J. Biochem.* (*Tokyo*) 106: 1026-1029). Likewise, *in vivo* studies showed that high affinity PBR ligands increase steroid plasma levels in hypophysectomized rats (Amri, H. *et al.* 1996, *Endocrinology* 137:5707-5718). Further *in vitro* studies on isolated mitochondria provided evidence that PBR ligands, drug ligands, or the endogenous PBR ligand, the polypeptide diazepam-binding inhibitor (BDI) (Papadopoulos, V. *et al.* 1997, *Steroids* 62:21-28), stimulate pregnenolone formation by increasing the rate of cholesterol transfer from the outer to the inner mitochondrial membrane (Krueger, K. E. and Papadopoulos, V. 1990, *J. Biol. Chem.* 265:15015-15022; Yanagibashi, K. *et al.* 1988, *Endocrinology* 123: 2075-2082; Besman, M. J. *et al.* 1989, *Proc. Natl. Acad. Sci. U.S.A.* 86: 4897-4901; Papadopoulos, V. *et al.* 1991, *Endocrinology* 129: 1481-1488).

Based on the amino acid sequence of the 18-kDa PBR, a three dimensional model was developed (Papadopoulos, V. 1996, In: The Leydig Cell. Payne, A. H. *et al.* (eds) Cache River Press, IL, pp 596-628). This model was shown to accomodate a cholesterol molecule and function as a channel, supporting the role of PBR in cholesterol transport. Recently we demonstrated the role of PBR in steroidogenesis by generating PBR negative cells by homologous recombination (Papadopoulos, V. *et al.* 1997, *J. Biol. Chem.* 272:32129-32135) that failed to produce steroids. However, addition of the hydrosoluble

analogue of cholesterol, 22R-hydroxycholesterol, recovered steroid production by these cells, indicating that the cholesterol transport mechanism was impaired. Further cholesterol transport experiments in bacteria expressing the 18-kDa PBR protein provided definitive evidence for a function as a cholesterol channel/transporter (Li and Papadopoulos, 1998, *Endocrinology* 139, 4991-4997).

Studies in a number of tumors such as rat brain containing glioma tumors (Richfield, E. K. *et al.* 1988, *Neurology* 38:1255-1262), colonic adenocarcinoma and ovarian carcinoma (Katz, Y. *et al.* 1988, *Eur. J. Pharmacol.* 148: 483-484 and Katz, Y. *et al.* 1990, *Clinical Sci.* 78:155-158) have shown an abundance of peripheral-type benzodiazepine receptors (PBR) compared to normal tissue. All documents cited herein *infra* and *supra* are hereby incorporated in their entirety by reference thereto. Moreover, a 12-fold increase in PBR density relative to normal parenchyma, was found in human brain glioma or astrocytoma (Cornu, P. *et al.* 1992, *Acta Neurochir.* 119:146-152). The authors suggested that PBR densities may reflect the proliferative activity of the receptor in these tissues. Recently, the involvement of PBR in cell proliferation was further shown (Neary, J. T. *et al.* 1995, *Brain Research* 675:27-30; Miettinen, H. *et al.* 1995, *Cancer Research* 55:2691-2695), and its expression in human astrocytic tumors was found to be associated with tumor malignancy and proliferative index (Miettinen, H. *et al. supra*; Alho, H. 1994, *Cell Growth Different.* 5:1005-1014). Characterization of PBR in human breast cancer biopsies, led to the discovery that the invasive and metastatic ability of human breast tumor cells is proportional to the level of PBR expressed, and correlates with the subcellular

localization of PBR in these cells in that PBR is found primarily in the nucleus in aggressive tumor cells whereas PBR is found primarily in the cytoplasm of invasive but non-aggressive cells. These changes in PBR expression can be used as a tool for detection, diagnosis, prevention and treatment in breast cancer patients, in particular, and in aggressive solid tumors in general.

Since both PBR and its endogenous ligand, the polypeptide diazepam binding inhibitor, are constitutively expressed in steroidogenic cells, the regulation of PBR function by hormones may be due to its association with other proteins. This interaction may result in the initiation of steroid biosynthesis. Therefore, there is a need to identify proteins which associate with PBR and may modulate PBR function.

Summary of the Invention

The present invention meets the need described above. We have identified PBR associated proteins (PAPs) that interact with PBR using the two-hybrid system. We used PBR as a bait to screen a mouse testis cDNA library. Five clones were isolated by their ability to interact with PBR: PAP3, PAP7, PAP8, PAP15, and PAP20. Among the nucleotide sequences identified, PAP3 was identical to the previously isolated meg1 protein (Don, J. and Wolgemuth, D. J., 1992, *Cell Growth Differ.* 3, 495; Ever, L. et al., 1999, *Cell Growth Differ.* 10, 19-26). PAP7, PAP8, PAP15, and PAP20 are novel sequences according to a search in the Genebank database which did not find a match for these sequences. PAP7 and PAP17 are different clones of the same novel protein product. All PAPs have fatty acylation (myristoylation) sites and PKC

phosphorylation sites. In addition, PAP20 has a PKA phosphorylation site. The distribution and function of the PAPs, as well as their functional relationship to PBR is under investigation.

So far, the distribution of PAP7 in major mouse tissues such as brain, testis, ovary, adrenal, kidney and muscle showed a profile similar to the broader expression pattern of PBR, with an expression level paralleling the steroidogenic ability of the tissue. These data imply a role for these PAPs in the regulation of PBR function, serving as endogenous ligands or allosteric modulators of the receptor.

Therefore, it is an object of the present invention to provide novel DNA fragments encoding PBR associated proteins, PAP3 (SEQ ID NO:1), PAP7 (SEQ ID NO:2) and Genbank accession no. AF022770, PAP8 (SEQ ID NO:3), PAP15 (SEQ ID NO:4), and PAP20 (SEQ ID NO:5) GenBank accession no. AF020338. The DNA fragment is useful as a diagnostic agent for detection of nucleic acid seguences encoding PBR-associated proteins, as an agent for preparation of the protein encoded by the DNA, for the preparation of sequences encoding PAPs, whether cDNA or genomic, and as therapeutic agents.

It is another object of the invention to provide an amino acid sequence for PAPs encoded by the DNA sequences described above.

It is another object of the invention to provide a recombinant vector comprising a vector and the above described DNA fragments.

It is a further object of the present invention to provide a host cell transformed with the above-described recombinant DNA construct.

It is another object of the present invention to provide a method for producing PAPs which comprises culturing a host cell under conditions such that the above-described DNA fragment is expressed and a PAP is thereby produced, and isolating the PAP for use as a reagent, for example for screening drugs and inhibitors of PER or the PAP itself, for diagnosis, and for therapy.

It is a further object of the present invention to provide an antibody to the above-described recombinant PAPs.

It is yet another object of the present invention to provide a method for detecting any of PAP3, PAP7, PAP8, PAP15, or PAP20 in a sample comprising:

(i) contacting a sample with antibodies which recognize any one of the PAPs mentioned above; and

(ii) detecting the presence or absence of a complex formed between the PAP and antibodies specific therefor.

It is a further object of the present invention to provide a diagnostic kit comprising an antibody against PAP and ancillary reagents suitable for use in detecting the presence of PAP in cells, tissue or serum from yeast, mammals, animals, birds, fish, and plants.

It is yet another object of the present invention to provide a method for the detection of PAP from a sample using the polymerase chain reaction.

It is a further object of the present invention to provide a diagnostic kit comprising primers or oligonucleotides specific for PAP RNA or cDNA suitable for hybridization to PAP RNA or cDNA and/or amplification of PAP sequences and ancillary reagents suitable for use in detecting PAP RNA/cDNA in mammalian tissue.

It is yet another object of the present invention to provide a method for the detection of a PAP in a sample which comprises assaying for the presence or absence of PAP RNA or cDNA in a sample by hybridization assays.

It is an object of the present invention to provide a method for the measurement of PBR in a sample. The method comprises measuring the presence of a PAP complexed with PBR.

It is yet another object of the present invention to provide a method for modulating the function or altering the targeting of PBR by increasing or decreasing interaction of PBR with a PAP. PBR functions which can be modulated include cholesterol transport into the cell, steroid production, cell proliferation, and embryogenesis.

It is still another object of the present invention to provide a method for increasing or decreasing PBR function or expression in a cell by providing into the cell a PAP such that PBR function is increased or decreased.

It is yet another object of the present invention to provide a method for increasing or reducing steroidogenesis by altering the level of PAP in said cell.

It is still another object of the invention to provide a therapeutic method for the treatment or amelioration of diseases resulting from increased cell proliferation resulting from aberrant PBR function or expression or localization, said method comprising providing to an individual in need of such treatment an effective amount of a PAP, in a pharmaceutically acceptable diluent, of a PAP such that the aberrant PBR expression, function or localization, is corrected.

It is another object of the present invention to provide a therapeutic method for the treatment or amelioration of diseases resulting from decreased cell proliferation, said method comprising providing to an individual in need of such treatment an effective amount of PAP or an antibody against PAP or an agent which inhibits PAP expression or function in a pharmaceutically acceptable excipient.

It is another object of the present invention to provide a therapeutic method for the treatment or amelioration of diseases resulting from decreased or increased steroidogenesis, said method comprising providing to an individual in need of such treatment an effective amount of a PAP or an antibody against PAP or an agent which inhibits or activates PAP expression or function in a pharmaceutically acceptable diluent.

It is yet a further object of the present invention to provide a cDNA sequence encoding PAPs and vectors incorporating all or a fragment of said sequence, and cells, prokaryotic and eukaryotic, transformed or transfected with said vectors, for use in screening agents and drugs which inhibit expression or function of PAPs or PBR in such cells.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features, aspects, and advantages of the present invention will become better understood with reference to the following description and appended claims, and accompanying drawings where:

Figure 1. PAP7 protein expressed in MA10 mouse Leydig tumor cells. (A) Western blot. (B), Immunocytochemistry.

Figure 2. PAP7 mRNA Tissue distribution analysis by Dot blot analysis. (A), a Master blot

containing 100-500 ng of poly(A)+ RNA from mouse tissues were hybridized at high stringency with a ^{32}P-labeled PAP7 probe as described under "Materials and Methods." The autoradiogram was exposed overnight. (B), densitometric analysis of PAP7 expression.

Figure 3. PAP7 tissue distribution analysis by Northern blot analysis. (A), Northern blot analysis was performed using 20 µg of total RNA/lane from different mouse tissues as indicated. The blot was hybridized at high stringency with a ^{32}P-labeled PAP7 probe as described under "Materials and Methods". The autoradiogram was exposed overnight. (B), Densitometric analysis of PAP7 expression.

Figure 4. PBR tissue distribution analysis by Northern blot analysis. (A), Northern blot analysis was performed using 20 µg of total RNA/lane from different mouse tissues as indicated. The blot was hybridized at high stringency with a ^{32}P-labeled PBR probe as described under "Materials and Methods". The autoradiogram was exposed overnight. (B), For Northern blot analysis, blots were quantitated by densitometry.

Figure 5. Immunohistological staining of mouse tissues with anti-PAP7 antibody.

Figure 6. The effect of PAP7 on Steroid Biosynthesis. Progesterone formation in MA-10 stimulated by hCG (A), for different time course (B). The results shown represent the means + S.D. from 2 to 6 independent experiments.

Figure 7. PAP3 mRNA Tissue distribution analysis by Dot blot analysis. (A), a Master blot containing 100-500 ng of poly(A)+ RNA from mouse tissues were hybridized at high stringency with a ^{32}P-labeled PAP3 probe as described under "Experimental

Procedures." The autoradiogram was exposed overnight. (B), densitometric analysis of PAP3 expression.

Figure 8. PAP20 mRNA Tissue distribution analysis by Dot blot analysis. (A), a Master blot containing 100-500 ng of poly(A)+ RNA from mouse tissues were hybridized at high stringency with a ^{32}P-labeled PAP20 probe as described under "Experimental Procedures." The autoradiogram was exposed overnight. (B), densitometric analysis of PAP20 expression.

DETAILED DESCRIPTION

The five PAPs described in this application were discovered using a two-hybrid assay. The two-hybrid assay is a yeast-based genetic assay used to detect protein-protein interactions *in vivo*. A positive result obtained with the two-hybrid assay allows rapid identification of genes encoding proteins that interact with a target protein. In addition the two-hybrid assay is a sensitive method for detecting weak and transient interactions, which are probably the norm in large native complexes. Most notably, because the two-hybrid assay is performed *in vivo*, the proteins involved are more likely to be in their native conformations.

The two-hybrid assay is based on the fact that many eukaryotic transcriptional activators consist of two physically separable modular domains: one acts as the DNA-binding domain, while the other functions as the transcriptional activation domain. The DNA-binding domain localizes the transcription factor to specific DNA sequences present in the upstream region of genes that are regulated by this factor, while the activation domain contacts other components of the transcription machinery required to initiate transcription. Both domains are required for normal

activation functioning, and normally the two domains are part of the same protein.

In our PAPs screening experiment, the MATCHMAKER Two-Hybrid System from CLONTECH was used. In the MATCHMAKER System, sequences encoding the two functional domains of the GAL4 transcriptional activator have been cloned into two different shuttle/expression vectors (pGBT9 and pGAD10). The pGBT9 hybrid cloning vector is used to generate a fusion of the GAL4 DNA-binding domain with PBR protein. The pGAD10 hybrid cloning vector is used to generate a fusion of the GAL4 activation domain with a collection of random proteins in a fusion mouse testis library (CLONTECH). Both hybrid proteins are targeted to the yeast nucleus by nuclear localization sequences that either are an intrinsic part of the GAL4 DNA-binding domain or have been added to the activation domain from a heterologous source. If PBR protein and an unknown protein or proteins interact with each other, the DNA-binding domain of GAL4 will be tethered to its transcriptional activation domain, and the proper function of the transcription of an appropriate reporter gene (lacZ or HIS3) containing upstream GAL4 binding sites is used to indicate interaction between the two proteins. This allows a positive selection for clones that are transformed by two interacting hybrid constructs and makes library screening more convenient and practical. After a positive clone has been identified, the gene corresponding to the interacting protein was sequenced using the sequencing primers provided in the kit.

In one embodiment, the present invention relates to a DNA or cDNA sequence encoding PBR associated proteins (PAPs). Five clones were isolated, PAP3, comprising 568 bp and identified in SEQ ID NO:1 (Don,

J. and Wolgemuth, D. J., 1992, *Cell Growth Differ.* 3, 495; Ever, L. et al., 1999, *Cell Growth Differ.* 10, 19-26) which encodes a peptide of 83 amino acids identified in SEQ ID NO:6; PAP7, comprising 577 bp extending from 696 to 1164 of the sequence identified in SEQ ID NO:2, which encodes a polypeptide of 363 amino acids, identified in SEQ ID NO:7; PAP8, comprising 568 bp identified in SEQ ID NO:3, which encode a polypeptide of 190 amino acids, identified in SEQ ID NO:8; PAP15 comprising 490 bp identified in SEQ ID NO:4, which encode a polypeptide of 164 amino acids, identified in SEQ ID NO:9; and PAP20 comprising 588 bp identified in SEQ ID NO:5, which encode a polypeptide of 196 amino acids, identified in SEQ ID NO:10.

PAP3 has been identified as the previously isolated meg 1 protein.

PAP7 and PAP17 are different clones of the same novel protein product. Additional PAP7 sequence has been obtained using the 5', 3'RACE system (CLONTECH) and the near full-length gene is identified in SEQ ID NO:2 including the stop codon and some untranslated sequence at the 3' end. The polypeptide encoded by the DNA sequence would have a calculated molecular weight of about 50 kD. Using an PAP7 antibody produced from the initial isolated DNA fragment of 577 bp, a protein of about 52 kD is immunoprecipitated as shown in the Examples below. Analysis of the protein sequence indicates several concensus sequences and important sites such as: two potential myristoylation sites at positions 262-267 and 271-276 of SEQ ID NO:7 and five PKC phosphorylation sites at 395-396, 113-115, 255-257, 280-282, 331-333, and 339-341 of SEQ ID NO:7, an Acyl-Co-A site at position 24-108 of SEQ ID NO:7, a nuclear localization domain at position 150-

167 of SEQ ID NO:7, a troponin site at position 98-247 of SEQ ID NO:2 and an HSP90 domain at position 126-155 of SEQ ID NO:7. The distribution and expression of PAP7 were examined in major mouse tissues such as brain, testis, ovary, adrenal, and kidney, as well as in tissue culture cell lines such as mouse C6 glioma cells, MA-10 Leydig cells, and Y1 adrenal cortical cells. The PAP7 expression pattern is similar to the broader expression profile of PBR in both tissues and cell lines involved in steroid biosynthesis. Additionally, both PBR and PAP7 expression level in the cell lines correlated with their steroidogenic biosynthesis ability, which suggests that PAP7 may be involved in steroid biosynthesis through PBR.

PAP 8, PAP15, and PAP20, are novel genes. The polypeptide encoded by PAP20 has two potential myristoylation sites, one PKC phosphorylation site and one PKA phosphorylation site. Protein myristoylation enables the protein to attach to the cellular membrane and thus take part in cell signaling (Casey, P.J. 1995, Science 268, 221-225; Boutin, J. A. 11997, *Cell Signal* 9, 15-35). PAP20 is predominantly expressed in the testis. Interaction of PBR with PAP20 increased the affinity of ligand binding using PK11195 as a ligand. Therefore, it is likely that PAP20 serves to increase or decrease PBR function by modulating PBR's affinity to its endogenous ligand, DBI. The tissue distribution of PAP3 and PAP20 is shown in Figure 7 and Figure 8, respectively.

Thus, one aspect of the invention provides an isolated nucleic acid molecule comprising a polynucleotide having a nucleotide sequence selected from SEQ ID NO:1-9 encoding PAPs polypeptides. It is within the skill of a person with ordinary skill in the art to use the sequences provided herein for the

purpose of cloning cDNA or genomic sequences which encode other parts or complete portions of the PAP genes described herein and therefore, these related sequences are encompassed within the present invention.

In addition, isolated nucleic acid molecules of the invention include DNA molecules which comprise a sequence substantially different from those described above but which, due to the degeneracy of the genetic code, still encode PAPs. Of course, the genetic code and species-specific codon preferences are well known in the art. Thus, it would be routine for one skilled in the art to generate the degenerate variants described above, for instance, to optimize codon expression for a particular host (e.g., change codons in the human mRNA to those preferred by a bacterial host such as *E.coli* or plant host).

Nucleic acid molecules of the present invention may be in the form of RNA, such as mRNA, or in the form of DNA, including, for instance, cDNA and genomic DNA obtained by cloning or produced synthetically. The DNA may be double-stranded or single-stranded. Single-stranded DNA or RNA may be the coding strand, also known as the sense strand, or it may be the non-coding strand, also referred to as the antisense strand.

By "isolated" nucleic acid molecule(s) is intended a nucleic acid molecule, DNA or RNA, which has been removed from its native environment. For example, recombinant DNA molecules contained in a vector are considered isolated for the purposes of the present invention. Further examples of isolated DNA molecules include recombinant DNA molecules maintained in heterologous host cells or purified (partially or substantially) DNA molecules in solution. Isolated

RNA molecules include *in vivo* or *in vitro* RNA transcripts of the DNA molecules of the present invention. Isolated nucleic acid molecules according to the present invention further include such molecules produced synthetically.

The present invention is further directed to nucleic acid molecules encoding portions or fragments of the nucleotide sequences described herein. Fragments include portions of the nucleotide sequences of at least 10 contiguous nucleotides in length selected from any two integers, one of which representing a 5' nucleotide position and a second of which representing a 3' nucleotide position, where the first nucleotide for each nucleotide sequence is position 1. That is, every combination of a 5' and 3' nucleotide position that a fragment at least 10 contiguous nucleotide bases in length or any integer between 10 and the length of an entire nucleotide sequence minus 1.

Further, the invention includes polynucleotides comprising fragments specified by size, in nucleotides, rather than by nucleotide positions. The invention includes any fragment size, in contiguous nucleotides, selected from integers between 1- and the entire length of an entire nucleotide sequence minus 1. Preferred sizes include 20-50 nucleotides, 50-300 nucleotides useful as primers and probes. Regions from which typical sequences may be derived include but are not limited to, for example, regions encoding specific epitopes or domains within said sequence, for example, the PER binding domain extending in SEQ ID NO:1, 2, 3, 4, and 5, potential myristoylation sites at positions 262-267 and 271-276 of SEQ ID NO:7 and five PKC phosphorylation sites at 395-396, 113-115, 255-257, 280-282, 331-333, and 339-341 of SEQ ID NO:7,

an Acyl-Co-A site at position 24-108 of SEQ ID NO:7, a nuclear localization domain at position 150-167 of SEQ ID NO:7, a troponin site at position 98-247 of SEQ ID NO:7 and an HSP90 domain at position 126-155 of SEQ ID NO:7, among others.

In another aspect, the invention provides isolated nucleic acid molecules comprising polynucleotides which hybridize under stringent hybridization conditions to a polynucleotide sequence of the present invention described above, or a specified fragment thereof. By "stringent hybridization conditions" is intended overnight incubation at 42°C in a solution comprising: 50% formamide, 5X SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5X Denhardt's solution, 10% dextran sulfate, and 20 g/ml denatured sheared salmon sperm DNA, followed by washing the filters in 0.1X SSC at about 65°C.

The sequences encoding the polypeptides of the present invention or portions thereof may be fused to other sequences which provide additional functions known in the art such as a marker sequence, or a sequence encoding a peptide which facilitates purification of the fused polypeptide, peptides having antigenic determinants known to provide helper T-cell stimulation, peptides encoding sites for post-translational modifications, or amino acid sequences which target the fusion protein to a desired location, e.g. a heterologous leader sequence.

The present invention further relates to variants of the nucleic acid molecules of the present invention, which encode portions, analogs or derivatives of the PAPs of the present invention. A variant may occur naturally, such as a natural allelic variant. By an "allelic variant" is intended one of

several alternate forms of a gene occupying a given locus of a chromosome of an organism. Non-naturally occuring variants may be produced by known mutagenesis techniques. Such variants include those produced by nucleotide substitution, deletion, or addition of one or more nucleotides in the coding or noncoding regions or both. Alterations in the coding regions may produce conservative or nonconservative amino acid substitutions, deletions, or additions. Especially preferred among these are silent substitutions, additions, and deletions which do not alter the properties and activities of PAPs polypeptides disclosed herein or portions thereof. Also preferred in this regard are conservative substitutions.

Nucleic acid molecules with at least 90-99% identity to a nucleic acid identified above is another aspect of the present invention. These nucleic acids are included irrespective of whether they encode a polypeptide having PAP activity. By "a polypeptide having PAP activity" is intended polypeptides exhibiting activity similar, but not identical, to an activity of the PAP of the invention, as measured in the assays described below. The biological acitivity or function of the polypeptides of the present invention are expected to be similar or identical to polypeptides from other organisms that share a high degree of structural identity/similarity.

In another embodiment, the present invention relates to a recombinant DNA molecule that includes a vector and a DNA sequence as described above. The vector can take the form of a plasmid, phage, cosmid, YAC, eukaryotic expression vector such as a DNA vector, *Pichia pastoris*, or a virus vector such as for example, baculovirus vectors, retroviral vectors or adenoviral vectors, and others known in the art. The

cloned gene may optionally be placed under the control of (i.e., operably linked to) certain control sequences such as promoter sequences, or sequences which may be inducible and/or cell type-specific. Suitable promoters will be known to a person with ordinary skill in the art. The expression construct will further contain sites for transcription initiation, termination and, in the transcribed region, a ribosome binding site for translation. Among the vectors preferred for use include pGBT9, PGAD10 (Clonetech), PSVzeo (Invitrogen), pBlueScript (Stratagene), pCMV5 (Invitrogen), pCRII (Invitrogen) to name a few.

Introduction of the construct into the host cell can be effected by calcium phosphate transfection, electroporation, infection, and other methods known in the art and described in standard laboratory manuals such as Current Protocols in Molecular Biology, Ausubel, F. M. *et al.* (Eds), Wiley & Sons, Inc. All documents cited herein supra and infra are hereby incorporated in their entirety by referece thereto.

In a further embodiment, the present invention relates to host cells stably transformed or transfected with the above-described recombinant DNA constructs. The host cell can be prokaryotic (for example, bacterial), lower eukaryotic (for example, yeast or insect) or higher eukaryotic (for example, all mammals, including but not limited to rat and human). Both prokaryotic and eukaryotic host cells may be used for expression of desired coding sequences when appropriate control sequences which are compatible with the designated host are used. Among prokaryotic hosts, *E. coli* is most frequently used. Expression control sequences for prokaryotes include promoters, optionally containing operator portions,

and ribosome binding sites. Transfer vectors compatible with prokaryotic hosts are commonly derived from, for example, pBR322, a plasmid containing operons conferring ampicillin and tetracycline resistance, and the various pUC vectors, which also contain sequences conferring antibiotic resistance markers. These markers may be used to obtain successful transformants by selection. Please see e.g., Maniatis, Fitsch and Sambrook, Molecular Cloning: A Laboratory Manual (1982) or DNA Cloning, Volumes I and II (D. N. Glover ed. 1985) for general cloning methods. The DNA sequence can be present in the vector operably linked to a sequence encoding an IgG molecule, an adjuvant, a carrier, or an agent for aid in purification of PAPs, such as glutathione S-transferase, or a series of histidine residues also known as a histidine tag. The recombinant molecule can be suitable for transfecting plant cells or eukaryotic cells, for example, mammalian cells and yeast cells in culture systems. *Saccharomyces cerevisiae*, *Saccharomyces carlsbergensis*, and *Pichia pastoris* are the most commonly used yeast hosts, and are convenient fungal hosts. Control sequences for yeast vectors are known in the art. Mammalian cell lines available as hosts for expression are known in the art and include many immortalized cell lines available from the American Type Culture Collection (ATCC), such as HEK293 cells , and NIH 3T3 cells, MA10 Leydig cells, mouse C6 glioma cells, Y1 adrenal cells, and breast cancer cell lines such as MDA-231, MCF-7, to name a few. Suitable promoters are also known in the art and include viral promoters such as that from SV40, Rous sarcoma virus (RSV), adenovirus (ADV), bovine papilloma virus (BPV), and cytomegalovirus

(CMV). Mammalian cells may also require terminator sequences and poly A addition sequences; enhancer sequences which increase expression may also be included, and sequences which cause amplification of the gene may also be desirable. These sequences are known in the art. The transformed or transfected host cells can be used as a source of DNA sequences described above. When the recombinant molecule takes the form of an expression system, the transformed or transfected cells can be used as a source of the protein described below.

In another embodiment, the present invention relates to the PAP polypeptides described above or any allelic variation thereof which is immunologically identifiable with the polypeptides.

A polypeptide or amino acid sequence derived from the amino acid sequences mentioned above, refers to a polypeptide having an amino acid sequence identical to that of a polypeptide encoded in the sequence, or a portion thereof wherein the portion consists of at least 2-5 amino acids, and more preferably at least 8-10 amino acids, and even more preferably at least 11-15 amino acids, or which is immunologically identifiable with a polypeptide encoded in the sequence.

A recombinant or derived polypeptide is not necessarily translated from a designated nucleic acid sequence; it may be generated in any manner, including for example, chemical synthesis, or expression of a recombinant expression system. In addition the polypeptide can be fused to other proteins or polypeptides which increase its antigenicity, such as adjuvants for example.

As noted above, the methods of the present invention are suitable for production of any

polypeptide of any length, via insertion of the above-described nucleic acid molecules or vectors into a host cell and expression of the nucleotide sequence encoding the polypeptide of interest by the host cell. Introduction of the nucleic acid molecules or vectors into a host cell to produce a transformed host cell can be effected by calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection or other methods. Such methods are described in many standard laboratory manuals, such as Davis et al., Basic Methods In Molecular Biology (1986). Once transformed host cells have been obtained, the cells may be cultivated under any physiologically compatible conditions of pH and temperature, in any suitable nutrient medium containing assimilable sources of carbon, nitrogen and essential minerals that support host cell growth. Recombinant polypeptide-producing cultivation conditions will vary according to the type of vector used to transform the host cells. For example, certain expression vectors comprise regulatory regions which require cell growth at certain temperatures, or addition of certain chemicals or inducing agents to the cell growth medium, to initiate the gene expression resulting in the production of the recombinant polypeptide. Thus, the term "recombinant polypeptide-producing conditions," as used herein, is not meant to be limited to any one set of cultivation conditions. Appropriate culture media and conditions for the above-described host cells and vectors are well-known in the art. Following its production in the host cells, the polypeptide of interest may be isolated by several techniques. To liberate the polypeptide of interest from the host cells, the cells

are lysed or ruptured. This lysis may be accomplished by contacting the cells with a hypotonic solution, by treatment with a cell wall-disrupting enzyme such as lysozyme, by sonication, by treatment with high pressure, or by a combination of the above methods. Other methods of bacterial cell disruption and lysis that are known to one of ordinary skill may also be used.

Following disruption, the polypeptide may be separated from the cellular debris by any technique suitable for separation of particles in complex mixtures. The polypeptide may then be purified by well known isolation techniques. Suitable techniques for purification include, but are not limited to, ammonium sulfate or ethanol precipitation, acid extraction, electrophoresis, immunoadsorption, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, immunoaffinity chromatography, size exclusion chromatography, liquid chromatography (LC), high performance LC (HPLC), fast performance LC (FPLC), hydroxylapatite chromatography and lectin chromatography.

The recombinant polypeptide or fusion protein can be used, detectably labeled and unlabeled, as a diagnostic tool for the detection of PAPs or for the detection and measurement of PBR. Additionally, these polypeptides can be used in a method for modulating PBR expression. In addition, the recombinant protein can be used as a therapeutic agent to reduce cell death and/or increase cell proliferation via its effect on PBR function. The transformed host cells can be used to analyze the effectiveness of drugs and agents which modulate PBR function, expression or

targeting via their effect on the expression or function of PAPs, such as host proteins or chemically derived agents or other proteins which may interact with the cell to alter the PAP function or expression, thereby modulating PBR function, expression or localization.

In another embodiment, the present invention relates to monoclonal or polyclonal antibodies specific for the above-described recombinant proteins (or polypeptides). For instance, an antibody can be raised against a peptide described above, or against a portion thereof of at least 10 amino acids, perferrably, 11-15 amino acids. Persons with ordinary skill in the art using standard methodology can raise monoclonal and polyclonal antibodies to the protein (or polypeptide) of the present invention, or a unique portion thereof. Material and methods for producing antibodies are well known in the art (see for example Goding, in, Monoclonal Antibodies: Principles and Practice, Chapter 4, 1986).

The amount of PAP expression can be detected at several levels. Using standard methodology well known in the art, assays for the detection and quantitation of PAP RNA can be designed, and include northern hybridization assays, *in situ* hybridization assays, and PCR assays, among others. Please see e.g., Maniatis, Fitsch and Sambrook, Molecular Cloning; A Laboratory Manual (1982) or DNA Cloning, Volumes I and II (D. N. Glover ed. 1985), or Current Protocols in Molecular Biology, Ausubel, F. M. *et al.* (Eds), Wiley & Sons, Inc. for general description of methods for nucleic acid hybridization. Polynucleotide probes for the detection of PAP RNA can be designed from the sequences described in SEQ ID NO: 1-9. For example,

RNA isolated from samples can be coated onto a surface such as a nitrocellulose membrane and prepared for northern hybridization. In the case of *in situ* hybridization of biopsy samples for example, the tissue sample can be prepared for hybridization by standard methods known in the art and hybridized with polynucleotide sequences which specifically recognize PAP RNA. The presence of a hybrid formed between the sample RNA and the polynucleotide can be detected by any method known in the art such as radiochemistry, or immunochemistry, to name a few.

One of skill in the art may find it desirable to prepare probes that are fairly long and/or encompass regions of the amino acid sequence which would have a high degree of redundancy in the corresponding nucleic acid sequences. In other cases, it may be desirable to use two sets of probes simultaneously, each to a different region of the gene. While the exact length of any probe employed is not critical, typical probe sequences are no greater than 500 nucleotides, even more typically they are no greater than 250 nucleotides; they may be no greater than 100 nucleotides, and also may be no greater than 75 nucleotides in length. Longer probe sequences may be necessary to encompass unique polynucleotide regions with differences sufficient to allow related target sequences to be distinguished. For this reason, probes are preferably from about 10 to about 100 nucleotides in length and more preferably from about 20 to about 50 nucleotides.

The DNA sequence of PAPs can be used to design primers for use in the detection of PAPs using the polymerase chain reaction (PCR) or reverse transciption PCR (RT-PCR). The primers can specifically bind to the PAP cDNA produced by reverse

transcription of PAP RNA, for the purpose of detecting the presence, absence, or quantifying the amount of PAP by comparison to a standard. The primers can be any length ranging from 7-40 nucleotides, preferably 10-15 nucleotides, most preferably 18-25 nucleotides homologous or complementary to a region of the PAP sequence. Reagents and controls necessary for PCR or RT-PCR reactions are well known in the art. The amplified products can then be analyzed for the presence or absence of PAP sequences, for example by gel fractionation, by radiochemistry, and immunochemical techniques. This method is advantageous since it requires a small number of cells. Once PAP is detected, a determination whether the cell is overexpressing or underexpressin PAP can be made by comparison to the results obtained from a normal cell using the same method. For example, increased PAP7 RNA levels correlate with PBR expression levels, especially in steroidogenic cells, wherein, an increase in steroidogenic capability of the cells correlates with an increase in PBR and PAP7 RNA.

In another embodiment, the present invention relates to a diagnostic kit for the detection of PAP RNA in cells, said kit comprising a package unit having one or more containers of PAP oligonucleotide primers for detection of PAP by PCR or RT-PCR or PAP polynucleotides for the detection of PAP RNA in cells by *in situ* hybridization or northern analysis, and in some kits including containers of various reagents used for the method desired. The kit may also contain one or more of the following items: polymerization enzymes, buffers, instructions, controls, detection labels. Kits may include containers of reagents mixed together in suitable proportions for performing the

methods in accordance with the invention. Reagent containers preferably contain reagents in unit quantities that obviate measuring steps when performing the subject methods.

In a further embodiment, the present invention provides a method for identifying and quantifying the level of PAP present in a particular biological sample. Any of a variety of methods which are capable of identifying (or quantifying) the level of PAP in a sample can be used for this purpose.

Diagnostic assays to detect PAPs may comprise a biopsy or *in situ* assay of cells from an organ or tissue sections, as well as an aspirate of cells from a tumor or normal tissue. In addition, assays may be conducted upon cellular extracts from organs, tissues, cells, urine, or serum or blood or any other body fluid or extract.

When assaying a biopsy, the assay will comprise, contacting the sample to be assayed with a PAP ligand, natural or synthetic, or an antibody, polyclonal or monoclonal, which recognizes PAP, or antiserum capable of detecting PAP, and detecting the complex formed between PAP present in the sample and the PAP ligand or antibody added.

PAP ligands or substrates include for example, PBR, in addition to natural and synthetic classes of ligands and their derivatives which can be derived from natural sources such as animal or plant extracts.

PAP ligands or anti-PAP antibodies, or fragments of ligand and antibodies capable of detecting PAP may be labeled using any of a variety of labels and methods of labeling for use in diagnosis and prognosis of disease associated with increased cell proliferation, such as cancer, or reduced cell death. Examples of types of labels which can be used in the

present invention include, but are not limited to, enzyme labels, radioisotopic labels, non-radioactive isotopic labels, and chemiluminescent labels.

Examples of suitable enzyme labels include malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast-alcohol dehydrogenase, alpha-glycerol phosphate dehydrogenase, triose phosphate isomerase, peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase, acetylcholine esterase, etc.

Examples of suitable radioisotopic labels include ^{3}H, ^{111}In, ^{125}I, ^{32}P, ^{35}S, ^{14}C, ^{51}To, ^{58}Co, ^{59}Fe, ^{75}Se, ^{152}Eu, ^{90}Y, ^{67}Cu, ^{217}Ci, ^{211}At, ^{212}Pb, ^{47}Sc, ^{109}Pd, ^{11}C, ^{13}F, ^{123}I, etc.

Examples of suitable non-radioactive isotopic labels include ^{157}Gd, ^{55}Mn, ^{162}Dy, ^{52}Tr, ^{56}Fe, etc.

Examples of suitable fluorescent labels include a ^{152}Eu label, a fluorescein label, an isothiocyanate label, a rhodamine label, a phycoerythrin label, a phycodyanin label, an allophycocyanin label, a fluorescamine label, etc.

Examples of chemiluminescent labels include a luminal label, an isoluminal label, an aromatic acridinium ester label, an imidazole label, an acridinium salt label, an oxalate ester label, a luciferin label, a luciferase label,etc.

Those of ordinary skill in the art will know of other suitable labels which may be employed in accordance with the present invention. The binding of these labels to ligands and to antibodies or fragments thereof can be accomplished using standard techniques commonly known to those of ordinary skill in the art. Typical techniques are described by Kennedy, J. H., *et al.*,1976 (*Clin. Chim. Acta* 70:1-31), and Schurs, A. H.

W. M., *et al.* 1977 (*Clin. Chim Acta* 81:1-40). Coupling techniques mentioned in the latter are the glutaraldehyde method, the periodate method, the dimaleimide method, and others, all of which are incorporated by reference herein.

The detection of antibodies (or fragments of antibodies) of the present invention can be improved through the use of carriers. Well-known carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agaroses, and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention. The support material may have virtually any possible structural configuration so long as the coupled molecule is capable of binding to PAP. Thus, the support configuration may be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc. Those skilled in the art will note many other suitable carriers for binding monoclonal antibody, or will be able to ascertain the same by use of routine experimentation.

The ligands or antibodies, or fragments of antibodies or ligands of PAPs discussed above may be used to quantitatively or qualitatively detect the presence of PAP. Such detection may be accomplished using any of a variety of immunoassays known to persons of ordinary skill in the art such as radioimmunoassays, immunometic assays, etc. Using standard methodology well known in the art, a diagnostic assay can be constructed by coating on a surface (i.e. a solid support) for example, a microtitration plate or a membrane (e.g.

nitrocelluolose membrane), antibodies specific for PAP or a portion of PAP, and contacting it with a sample from a person suspected of having a PAP related disease. The presence of a resulting complex formed between PAP in the sample and antibodies specific therefor can be detected by any of the known detection methods common in the art such as fluorescent antibody spectroscopy or colorimetry. A good description of a radioimmune assay may be found in Laboratory Techniques and Biochemistry in Molecular Biology, by Work, T.S., *et al.* North Holland Publishing Company, N.Y. (1978), incorporated by reference herein. Sandwich assays are described by Wide at pages 199-206 of Radioimmune Assay Method, edited by Kirkham and Hunter, E. & S. Livingstone, Edinburgh, 1970.

The diagnostic methods of this invention can be predictive of diseases involving PBR including gallstones, atherosclerosis, Niemann-Pick C, Sitosterolemia, Dystrophy, Tumor proliferation (tumorigenesis), Schnyder's corneal crystalline dystrophy. Brain disorders include cholesterol metabolism and Alzheimer's disease, Tellurium toxicity, Smith-Lemli-Opitz syndrome, myelinization, developmental abnormalities and demyelization : Charcot-Marie-Tooth disease; Pelizaeus-Merzbacher disease, Multiple sclerosis, SLA, to name a few. Alternatively, the methods and compositions may be useful as prophylactic treatment, or in screening for compounds effective in prophylactic treatments.

The recombinant protein can be used to identify inhibitors or activators of a PAP activity which allows the identification of drugs or agents which modulate PBR activity. Using an assay as described below in the Examples, or for example, introducing a

drug or agent to a cell expressing a PAP and detecting a increase or decrease in the level of PAP RNA or protein, natural and synthetic agents and drugs can be discovered which result in a reduction or elimination, or increase in a PAP activity. Knowledge of the mechanism of action of the inhibitor or activator is not necessary as long as a decrease or increase in the activity of a PAP is detected. Inhibitors may include agents or drugs which either bind or sequester the PAP's substrate(s), such as PBR, or cofactor(s), or inhibit PAP itself, directly, for example by irreversible binding of the agent or drug to the PAP, or indirectly, for example by introducing an agent which binds the competes with PAP binding to its substrate. Activators may include cofactors necessary for proper PAP function or agents which allow a higher turnover rate of binding or release of the PAP to/from PBR or the particular PAP substrate. Agents or drugs related to this invention may result in partial or complete inhibition or various degrees of activation of PAP which may or may not result in modulation of PBR function. Inhibitors or activators of PAP activity may be used in the treatment or amelioration of conditions such as stress, cancer, neurodegenerative disorders, i.e. stroke, Alzheimer's, developmental disorders, infertility, and immune disorders.

Agents which decrease the level of PAP (i.e. in a human or an animal) or reduce or inhibit PAP activity may be used in the therapy of any disease associated with the elevated levels of PAP. Similarly, agents which increase the level of PAP or activate PAP activity may be used in the therapy of any disease associated with reduced levels of PAP. An increase or decrease in the level of PAP is determined when the

change in the level of PAP is about 2-3 fold higher or lower than the level of PAP in the normal cell, up to about 10-100 fold higher or lower than the amount of PAP in a normal cell. Agents which decrease PAP RNA include, but are not limited to, one or more ribozymes capable of digesting PAP RNA, or antisense oligonucleotides capable of hybridizing to PAP RNA such that the translation of PAP is inhibited or reduced resulting in a decrease in the level of PAP. These agents can be administered as DNA, as DNA entrapped in proteoliposomes containing viral envelope receptor proteins (Kanoda, Y. *et al.*, 1989, *Science* 243, 375) or as part of a vector which can be expressed in the target cell such that the DNA or RNA is made. Vectors which are expressed in particular cell types are known in the art, for example, for the mammary gland, please see Furth, (1997) (*J. Mammary Gland Biol. Neopl.* 2, 373) for examples of conditional control of gene expression in the mammary gland. Alternatively, the DNA can be injected along with a carrier. A carrier can be a protein such as a cytokine, for example interleukin 2, or polylysine-glycoprotein carrier. Such carrier proteins and vectors and methods of using same are known in the art. In addition, the DNA could be coated onto tiny gold beads and said beads introduced into the skin with, for example, a gene gun (Ulmer, J. B. *et al.*, 1993, *Science* 259, 1745).

Alternatively, antibodies, or compounds capable of reducing or inhibiting PAP activity, that is reducing or inhibiting either the expression, production or activity of PAP, such as antagonists, can be provided as an isolated and substantially purified protein, or as part of an expression vector capable of being expressed in the target cell such

that the PAP-reducing or inhibiting agent is produced. Similarly, compounds capable of increasing or activating PAP activity, that is increasing or activating either the expression, production, or activity of PAP, such as agonists, can be provided as an isolated and substaially purified protein, or as part of an expression vector capable of being expressed in the target cell such that the PAP-elevating or activating agent is produced. In addition, factors which affect the stability of the protein, and co-factors such as various ions, i.e. Ca2+ (Calvo, D. J. and Medina, J. H., 1993, *J. Recept. Res.* 13:975-987), or anions, such as halides or anion channel blockers such as DIDS (4,4'diisothiocyanostilbene-2,2'-disulfonic acid), an ion transport blocker (Skolnick, P., 1987, *Eur. J. Pharmacol.* 133:205-214), or factors which affect the stability of PBR such as lipids, for example, the phospholipids phosphatidylserine and phosphatidylinositol whereby the presence of the phospholipids is required for receptor activity (Moynagh, P. N. and Williams, D.C., 1992, *Biochem. Pharmacol.* 43:1939-1945) can be administered to modulate the expression and function of the PAPs and PBR. These formulations can be administered by standard routes. In general, the combinations may be administered by the topical, transdermal, intraperitoneal, oral, rectal, or parenteral (e.g. intravenous, subcutaneous, or intramuscular) route. In addition, PAP-inhibiting or PAP-activating compounds may be incorporated into biodegradable polymers being implanted in the vicinity of where drug delivery is desired, for example, at the site of a tumor or implanted so that the PAP-inhibiting or PAP-activating compound is slowly released systemically.

The biodegradable polymers and their use are described, for example, in detail in Brem *et al.* (1991) *J. Neurosurg.* 74, 441-446. These compounds are intended to be provided to recipient subjects in an amount sufficient to effect the inhibition of PAP. Similarly, agents which are capable of negatively or positively effecting the expression, production, stability or function of PAP, are intended to be provided to recipient subjects in an amount sufficient to result in the desired effect. An amount is said to be sufficient to "effect" the inhibition or induction of PAP if the dosage, route of administration, etc. of the agent are sufficient to influence such a response.

The PAPs identified in this application were discovered due to their ability to associate with PBR, and may play a role in the proper targeting, function, expression, or stability of PBR. Therefore, a method for inhibiting or reducing PBR function, or altering the localization of PBR, would include a method for dissociating PAPs from the receptor. This is possible using agents which block the site on PBR at which these PAPs associate with PBR, or alternatively, blocking the site on the PAPs which is involved in PBR-association. Such agents would include antibodies or antogonists which recognize such sites or which alter the conformation of these sites such that PAP and PBR association is inhibited or eliminated. Agents which decrease the level of PBR (i.e. in a human or an animal) or reduce or inhibit PBR activity may be used in the therapy of any disease associated with the elevated levels of PBR such as metastatic cancer, for example breast cancer, or diseases associated with increased cell proliferation or increased cholesterol transport into the cell. An increase in the level of PBR is determined when the

level of PBR in a tumor cell is about 2-3 times the level of PBR in the normal cell, up to about 10-100 times the amount of PBR in a normal cell.

Antibodies or compounds capable of reducing or inhibiting the association between PBR and PAPs can be provided as an isolated and substantially purified protein, or as part of an expression vector capable of being expressed in the target cell such that the PBR-PAP-association reducing or inhibiting agent is produced. These formulations can be administered by standard routes. In general, the combinations may be administered by the topical, transdermal, intraperitoneal, oral, rectal, or parenteral (e.g. intravenous, subcutaneous, or intramuscular) route. In addition, these compounds may be incorporated into biodegradable polymers being implanted in the vicinity of where drug delivery is desired, for example, at the site of a tumor or implanted so that the compound is slowly released systemically. The biodegradable polymers and their use are described, for example, in detail in Brem *et al.* (1991) *J. Neurosurg.* 74, 441-446.

These compounds are intended to be provided to recipient subjects in an amount sufficient to effect the inhibition of PBR/PAP association.

In line with the function of PBR in cell proliferation, agents which stimulate the function of PBR by increasing the association of PAPs to PBR, may be used in the therapy of any disease associated with a decrease of PBR, or a decrease in cell proliferation, wherein PBR is capable of increasing such proliferation, e.g. developmental retardation. PBR has also been shown to be involved in cholesterol transport, therefore, an agent or drug which results in an increase in function or stability of PBR and its associated PAPs can be used to increase cholesterol

transport into cells. Diseases where cholesterol transport is defficient include lipoidal adrenal hyperplasia, and diseases where there is a requirement for increased production of compounds requiring cholesterol such as myelin and myelination including Alzheimer's disease, spinal chord injury, and brain development neuropathy [Snipes, G. and Suter, U. (1997) Cholesterol and Myelin. *In*: Subcellular Biochemistry, Robert Bittman (ed.), vol. 28, pp.173-204, Plenum Press, New York], to name a few.

In providing a patient with any agent which modulates the expression, function, targeting, or association of PAP or PBR as discussed above, the dosage of administered agent will vary depending upon such factors as the patient's age, weight, height, sex, general medical condition, previous medical history, etc. In general, it is desirable to provide the recipient with a dosage of agent which is in the range of from about 1 pg/kg to 10 mg/kg (body weight of patient), although a lower or higher dosage may be administered.

A composition is said to be "pharmacologically acceptable" if its administration can be tolerated by a recipient patient. Such an agent is said to be administered in a "therapeutically effective amount" if the amount administered is physiologically significant. An agent is physiologically significant if its presence results in a detectable change in the physiology of a recipient patient.

The compounds of the present invention can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby these materials, or their functional derivatives, are combined in admixture with a pharmaceutically

acceptable carrier vehicle. Suitable vehicles and their formulation, inclusive of other human proteins, e.g., human serum albumin, are described, for example, in Remington's Pharmaceutical Sciences [16th ed., Osol, A. ed., Mack Easton PA. (1980)]. In order to form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain an effective amount of the above-described compounds together with a suitable amount of carrier vehicle.

Additional pharmaceutical methods may be employed to control the duration of action. Control release preparations may be achieved through the use of polymers to complex or absorb the compounds. The controlled delivery may be exercised by selecting appropriate macromolecules (for example polyesters, polyamino acids, polyvinyl, pyrrolidone, ethylenevinylacetate, methylcellulose, carboxymethylcellulose, or protamine sulfate) and the concentration of macromolecules as well as the method of incorporation in order to control release. Another possible method to control the duration of action by controlled release preparations is to incorporate the compounds of the present invention into particles of a polymeric material such as polyesters, polyamino acids, hydrogels, poly(lactic acid) or ethylene vinylacetate copolymers. Alternatively, instead of incorporating these agents into polymeric particles, it is possible to entrap these materials in microcapsules prepared, for example, interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly(methylmethacrylate)microcapsules, respectively, or in colloidal drug delivery systems, for example, liposomes, albumin microspheres, microemulsions,

nanoparticles, and nanocapsules or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences (1980).

The present invention also provides kits for use in the diagnostic or therapeutic methods described above. Kits according to this aspect of the invention may comprise one or more containers, such as vials, tubes, ampules, bottles and the like, which may comprise one or more of the compositions of the invention.

The kits of the invention may comprise one or more of the following components, one or more compounds or compositions of the invention, and one or more excipient, diluent, or adjuvant.

It will be readily apparent to one of ordinary skill in the relevant arts that other suitable modifications and adaptations to the methods and applications described herein are obvious and may be made without departing from the scope of the invention or any embodiment thereof. Having now described the present invention in detail, the same will be more clearly understood by reference to the following examples, which are included herewith for purposes of illustration only and are not intended to be limiting of the invention.

The following Materials and Methods were used in the Examples desribed below.

Material and Methods

Materials

[α-^{32}P]dCTP (specific actifity, 3000 Ci/mmol), [1,2,6,7-N-^{3}H]progesterone (specific activity, 94.1 Ci/mmol) and ^{3}H-1-(2-chlorophenyl)-N-methyl-N-(1-methyl-propyl)-3-isoquinolinecarboxamide (PK 11195) (specific activity, 86.9 Ci/mmol) were obtained from

NEN Life Science Products (Boston, MA). PK11195 was obtained from Research Biochemicals, Inc. (Natick, MA). Nitrocellulose (0.45 μm) was from Hoeffer Scientific (San Francisco, CA). 22R Hydroxycholesterol was purchased from Sigma. Restriction enzymes were from Stratagene (La Jolla, CA) and New England Biolabs (Beverly, MA). Cell culture supplies were purchased from from Life Technologies, Inc. (Grand Island, NY). Tissue culture plasticware was from Corning (Corning, NY). Electrophoresis reagents and materials were supplied from BioRad. All other chemicals used were of analytical grade and were obtained from various commercial sources.

Strains and media

The genotype of the *Saccharomyces cerevisiae* reporter strain HF7c is *MATa, ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3, 112, gal4-542, gal80-538, LYS2::GAL-HIS3, URA3::(GAL4 17-mers)$_3$-CYC1-lacZ* (CLONTECH, Palo Alto, CA). Yeast strains were grown at 30°C in standard liquid YPD medium or minimal SD synthetic medium with appropriate supplement amino acids (CLONTECH, Palo Alto, CA).

Plasmids and construction

The mouse PBR cDNA coding sequence was subcloned into pGBT9 (CLONTECH, Palo Alto, CA) at *EcoR I* and *BamH I* sites (pGBT-PBR). The fusion site was verified by sequencing. Functional fusion PBR protein, expressed in yeast cells, was verified by PBR ligand binding assay. Mouse testis cDNA library was constructed in pGAD10 [LEU2, GAL4 (768-881)] (CLONTECH, Palo Alto, CA). Amplification of premade libraries was performed by growing the transformants on LB-agar-ampicillin and purifying the plasmids DNA

with QIAGEN Plasmid Giga kit (QIAGEN, Valencia, CA). In the transfection experiments, PAP7 partial sequence (including 192 Amino acids C-terminal sequence) was inserted into pSVzeo vector (Invitrogen, Carlsbad , CA) at *EcoRI* and *BamH1* sites.

Yeast two-hybrid screening

The Clontech MATCHMAKER two-hybrid system was applied in this study (detailed in manufacturer's instruction book). Briefly, the yeast reporter host strain HF7c was simultaneously cotransformed with both pGBT-PBR and the mouse testis cDNA library in pGAD10 plasmid by using lithium acetate high-efficiency method (Gietz, D. et al., 1992, *Nucleic Acids Res.* 20, 1425). HIS positive clones were further selected by colony lift filter assay for β-galactosidase activity. Plasmid DNA was rescued in Escherichia coli DH5? from yeast cells. Plasmids were retransformed into yeast HF7c cells with plasmid pGBT-PBR to test for histidine prototrophy and β-galactosidase activity (Clontech manual). The cDNA inserts from the positive clones were sequenced. The full length PAP7 cDNA was obtained by using 5' and 3' RACE kit from kit (CLONTECH, Palo Alto, CA).

Sequence analysis

The ABI PRISMTM dyes terminator cycle sequencing ready reaction kit (PE Biosystems, Foster City , CA) and an Applied Biosystems sequencer were used for sequencing (Applied Biosystems, Foster City , CA) at the Lombardi Cancer Center Sequencing Core Facility (Georgetown Unviersity). DNA sequences were analyzed by using Entrez and BLAST program against GeneBankTM Database.

Cell culture transient transfection

MA-10 cells were grown in modified Waymouth's MB752/1 medium containing 15% horse serum, as described previously (Papadopoulos, V. et al., 1990, *J. Biol. Chem.* 265, 3772-3779). Mouse C6 glioma and mouse Y1 adrenal cortical cells were cultured in DMEM and DMEM F12 repectively, with 10% fetal bovine serum. MA10 cells were transiently transfected by electroporation (El Hefnawy, T. et al., 1996, *Mol. Cell Endocrinol.* 119, 207-217). Each Genepulser cuvette (0.4 cm-gap, BioRad, Hercules, CA) contained $8x10^6$ cells in 350 µl antibiotic-free complete Waymouth's growth medium (see above), plus 30µg plasmids DNA in 50 µl of 0.1 x TE. Cells in electroporation cuvettes were electro-shocked at 330 V and at a capacitance of 950 µFd generated from Genepulser (BioRad, Hercules, CA). The cells were kept immediately on ice for 10 min before plated into 96 well plates.

Radioligand binding assays

^{3}H-1-(2-chlorophenyl)-N-methyl-N-(1-methyl-propyl)-3-isoquinolinecarboxamide (PK 11195) (NEN, Boston, MA) binding studies were performed as we previously described (Papadopoulos, V. et al., 1990, supra; Garnier, M. et al., 1994, *Molecular Pharmacology* 45, 201-211). The dissociation constant (Kd) and the number of binding sites (Bmax) were determined by Scatchard plot analysis of the data using the LIGAND program (Munson, P. J. and Rodbard, D., 1980, *Anal. Biochem.* 107, 220-239).

RNA (Northern) blot analysis

Total tissue and cellular RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method using RNA STAT60 reagent (Tel-Test

Inc., Friendswood, TX). RNA was separated by denature electrophoresis and transfered to Nytran membrane (Schleicher & Schuell Inc., Keene, NH). The RNA blots were hybridized with ^{32}P labeled PAP7 cDNA probe generated from random priming (Boehringer Mannheim, Indianapolis, IN). Autoradiography was performed by exposing Kodak X-Omat AR films (Eastman Kodak, Rochester, NY) to the blots at -80°C overnight.

Steroid biosynthesis

MA-10 cells were plated into 96-well plate at the density of $2.5x10^4$/well for overnight. The cells were stimulated with 50 ng/ml hCG in 0.2 ml/well serum-free medium for 2 hours. The culture medium was collected and tested for progesterone production by RIA. The assay was carried out by using anti-progesterone antisera (ICN, Costa Mesa, CA), following the conditions recommended by the manufacturer. The progesterone production was normalized by the amount of protein in each well. Radioimmunoassay data was analyzed using the software provided by Wallac (EG&G Wallac, Gaithersburg, MD).

Antibody generation and Western analysis

Rabbit anti-PAP7 antibody was prepared by sequential immunization with a peptide SSDEEEEEEENVTCEEKAKKNANKP (SEQ ID NO:11) of PAP7 protein, which was coupled to KLH. PAP7 antibodies were purified by an affinity resin containing the same peptide immobilized onto agarose (Bethyl Laboratories, Montgomery, TX). MA10 cells were solubilized in sample buffer (25 mM Tris-HCl (pH6.8), 1% SDS, 5% β-mercaptoethanol, 1 mM EDTA, 4% glycerol, and 0.01% bromophenol blue), boiled for 5 min, and loaded onto a 15% SDS-PAGE minigel (MiniProtein II System, BioRad, Richmond, CA). Electrophoresis was performed at 25

mA/gel using a standard SDS-PAGE running buffer (25 mM Tris, 192 mM glycine, and 0.1% SDS). The proteins were electrophoretically transferred to nitrocellulose membrane (Schleicher & Schuell Inc., Keene, NH). The membrane was incubated in blocking buffer (TTBS buffer (20 mM Tris/HCl, pH 7.5, 0.5M NaCl, and 0.05% Tween-20) containing 10% Carnation nonfat milk) at room temperature for 1 hour, followed by incubation with a primary antibody against PAP7 (1:2000) for 2 hours. The membrane was washed with TTBS three times for 10 min each time. After one-hour incubation with the secondary antibody, goat anti-rabbit IgG conjugated with HRP (Signal Transduction), the membrane was washed with TTBS three times for 10 min each time. Specific protein bands were detected by chemiluminescence using the Renaissance Kit (DuPont-New England Nuclear, Wilmington, DE) according to manufacturer's directions.

Immunocytochemistry

MA-10 cells were cultured on four-chambered SuperCell Culture Slides (Fisher Scientific, Pittsburgh, PA) and fixed with methanol at 4°C for 15 min. The fixed cells were incubated with PAP7 antibody (1:250 dilution) with or without PAP7 peptide for 1 hour. After washing, the cells were incubated with HRP conjugated goat anti-rabbit secondary antibody (Transduction Lab, Lexington, KY) for 1 hour. PAP7 staining was visualized with peroxidase using AEC (3-amino-9-ethyl carbazole) as a chromogen to yield a red reaction product. After counterstaining with hematoxylin, slides were dehydrated and permanently mounted.

Immunohistochemistry

Mouse tissues were freshly snapped in liquid nitrogen. Specimens were fixed in cold methanol right after sectioning for 5 min. The slides were then placed in a chamber containing 0.3% H_2O_2 solution in methanol for 20 min at room temperature to inhibit the endogenous peroxidase activity and then incubated in blocking solution (10% goat serum) (Zymed, South San Francisco, CA) for 15 min. Subsequently, the slides were incubated with anti-PAP7 antibody (1:250) for 2 h at room temperature, washed with water and PBS, incubated with HRP conjugated goat anti-rabbit secondary antibody for 1 hour at room temperature, and then washed with PBS. After treatment with AEC reagent for 1 hr at 37°C for color staining, the sections were counterstained with hematoxylin, dehydrated and permanently mounted.

Protein quantification ans Statistical analysis

Proteins were quantified by dye-binding assay of Bradford (Bradford, M. M., 1976, *Anal. Biochem.* 72, 248-254) with bovine serum albumin as the standard. Statistical analysis was performed by ANOVA followed by the Student-Newman-Keuls test or the Dunnett multiple comparisons test using the Instat (v.2.04) package from GraphPad, Inc. (San Diego, CA).

Example 1

Isolation of PBR Associated Proteins

We have used the MATCHMAKER Two-Hybrid System from CLONTECH in order to clone genes whose products interact with PBR protein. GAL4 (1-147)-PBR fusion (plasmid pGBT9 + PBR) was used as a bait to screen a mouse MATCHMAKER testis cDNA library constructed into the pGAD10 two-hybrid vector. About $3x10^6$ transformants were tested, and five positive clones were obtained for their ability to interact with PBR.

Library plasmids from these transformants were rescued in E. coli strain DH5α. Both the His+ phenotype and the expression of ß-galactosidase were confirmed by a second-round transformation of strain HF7c carrying pGBT9-PBR (Table 1).

Table 1: Summary of yeast two hybrid screen of mouse testis library by PBR

Clone	His3	β-Galactosidase activity
PAP3	+	++
PAP7	+	++
PAP20	+	++
Positive control	+	+++

Plasmids from these positive clones were first analyzed by restriction enzyme digestion and followed by sequence analysis. Two clones were shown to be coded by single gene, which was an unknown gene and was named as PBR associated protein 7 (PAP7). The other three clones encoded different products. After 5' RACE and 3' RACE, PAP7 cDNA clone was completely sequenced on both strands (SEQ ID NO:2) and it encoded a 463-amino acid protein with a calculated molecular weight of about 52 kDa. A homology search in the Genebank database using the BLAST program showed that this is a new sequence, previously unidentified.

Example 2

PAP7 protein expression in MA-10 Leydig tumor cells

The total MA-10 cell protein extracts were analyzed by western blot using PAP7 antibody. This antibody specifically recognizes a 50 kDa-protein band (Fig. 1A). The PAP7 protein expression in MA-10 cell

was also checked by immunocytochemistry. PAP7 antibody specifically stained MA-10 cell, with the signal mostly localized in the cytoplasm (Figure 1B). This signal can be neutralized by PAP7 peptide, which was used to generate and purify this antibody.

Example 3

PAP7 cells and tissue expression by Dot and Northern Blot

By dot bot analysis, PAP7 was observed to be highly expressed in brain, eye, submax gland, testis, and ovary. Interestingly, PAP7 expression was at its highest level at early embryonic stage, and decreased before birth (Fig.2A and 2B). Consistently, PAP7 mRNA was expressed in adrenal, brain, heart, liver, testis and ovarian tissues by Northern blot analysis. PAP7 had a 1 kb transcript which was only expressed in testis and a 3 Kb major mRNA transcript in the other tissues (Fig. 3A and 3B). PAP7 was also highly expressed in three cell lines, C6 glioma, MA-10 Leydig cells and Y1 adrenal cells, which have been widely used for studying steroid biosynthesis. All three cell lines expressed PAP7 transcript of the same molecular weight size as in normal tissues. The PAP7 expression level in these cell lines was proportionally correlated with their steroidogenic capability (Fig. 3A and 3B). The PBR mRNA expression level was also checked in these same tissues and cell lines. The PBR expression level was parallel with PAP7 mRNA expression pattern, especially in those three cell lines (Fig. 4A and 4B).

Example 4

PAP7 Cellular distribution

PAP7 protein expression in different tissues was checked by immunohistochemistry (Fig 5). PAP7 was

present in both Leydig and germ cells in testis (Figure 5C and 5D), in hypocampus and neuronal cells in brain (Figure 5E and 5F), in fasciculata cells in adrenal gland (Figure 5G and 5H), and in granulosa cells in ovary (Figure 5A and 5B). Liver and kidney expressed low level of PAP7 protein (data not shown). Each specimen was stained with PAP7 peptide neutralized antibody as a negative control. Subsequent in situ hybridization studies showed that PAP7 mRNA followed PAP-7 protein expression.

Example 5

The effect of PAP7 on steroid biosynthesis in MA-10 cell

PAP7 partial sequence including PBR binding domain was subcloned into pSVzeo mammalian expression vector. This pSvzeoPAP7 vector was transiently transfected into MA-10 cells. pSVzeo empty vector was also transfected into cells as control. The capability of steroid biosynthesis of both empty vector pSVzeo transfectants and pSVzeoPAP7 transfectants was checked by monitoring the progesterone production in response to hormonal (hCG) stimulation. PAP7 transfectants had significantly reduced the level of progesterone production in MA-10 cells as compared with pSVzeo vector transfectant at a dose and time dependent manner (Fig 6).

Discussion

In order to better understand the mechanism of how PBR regulates cholesterol transport activity in steroid biosynthesis, we performed yeast two-hybrid assay to identify the PBR associated protein(s). Mouse PBR cDNA was inserted into the pGBT9 vector to generate a GAL4 DNA-binding domain and PBR fusion

protein as bait. The receptor ligand binding activity of the fusion protein with PBR ligand, PK11195, was tested. Our result indicated that the fusion PBR protein expressed in yeast possessed the similar binding affinity as the native PBR protein (data not shown). PBR has been identified in various peripheral tissues (Gavish, M. and Weizman, R., 1997, *Clin. Neuropharmacol.* 20, 473-481) including testis. Testis is one of the important and very well studied tissues for steroidogenesis (Huhtaniemi, I, and Toppari, J., 1995, *Adv. Exp. Med. Biol.* 377, 33-54). The steroid biosynthesis in the mouse testicular Leydig cell and the role of PBR in this process are also well documented(Papadopoulos, V. et al., 1997, *Steroids* 62, 21-28; Papadopoulos, V. et al., 1998, *Endocr. Res.* 24, 479-487). Therefore, we applied mouse testis cDNA library in this two-hybrid screen study. The pGAD10 vector was used to generate a fusion protein of the GAL4 activation domain with a collection of random proteins in the fusion Balb/c mouse testis library. Through the yeast two hybrid screen, PAP7 was identified as one of the positive clones, which demonstrated its ability to interact with PBR (Table 1). Thus, we cloned the PAP7 cDNA coding for a mouse protein that interacted with PBR. Based on the database search, PAP7 is a novel gene product. Recently, PRAX-1 was reported as a new protein that specifically interates with PBR (Galiegue, S. et al., 1999, *J. Biol. Chem.* 274,2938-2952). The only similarity is that both proteins contain glutamic-acid streches. Part of PAP7 shares quite high homology with a *C. elegans* gene that has an unknown function (Wilson, R. et al., 1994, *Nature* 368, 32-38). In fact, cholesterol is required for *C. elegans* cell culture (Brenner, S., 1974, *Genetics* 77, 71-94).

Considering that PBR is involved in cholesterol transport and PBR gene is highly conservative in all type of organisms, this data suggests that PAP7 expression may be needed to meet basic requirements for cell survival and growth. PAP7 also shares some homologies with RALBP, a hydrophobic ligand-binding protein that functions in intracellular retinoid transport (Ozaki, K. et al., 1994, *J. Biol. Chem.* 269, 3838-3845).

By sequence motif analysis using Swiss-Port Prosite profile scan, PAP7 has fatty acylation (myristoylation) sites, Acyl-CoA-binding protein signature and PKC phosphorylation sites. Protein myristoylation enables protein to attach to the cellular membrane and thus take part in cell signaling (Casey, P.J., 1995, Science 268, 221-225; Boutin, J. A., 1997, *Cell Signal* 9, 15-35). PBR is a hydrophobic protein and tightly associated with the outer mitochondrial membrane. This property could enable PAP7 passing hormone stimulation signal to and interacting with PBR thus regulating PBR activity in cholesterol transport. Interestingly, Acyl-CoA-binding protein is the other name of PBR endogenous ligand, diazepam binding inhibitor (DBI)(Rose, T. M. et al., 1992, *Proc. Natl. Acad. Sci. U.S.A.* 89, 11287-11291; Costa, E. and Guidotti, A., 1991, Life Sci. 49, 325-344; Suk, K. et al., 1999, *Biochim. Biophys. Acta* 1454, 126-131). This information suggests that PAP7 may coordinate with other PBR endogenous ligands to fulfill its function. The fact that PAP7 has the potential protein kinase phosphorylation sites raises another possibility that PBR could be regulated by hormone stimulation through the interaction with PAP7 protein.

The distribution and expression of PAP7 were examined in major mouse tissues such as brain, testis, ovary, adrenal, and kidney, as well as some cell lines. The PAP7 expressing pattern is similar to the broader expression profile of PBR. According to the previous studies (Papadopoulos, V. et al., 1998, supra), glucocorticoids are produced by zona fasciculata cells in adrenal. In the ovary, coupus luteum, where the granulosa cells are located, secretes progesterone. In addition, the testicular Leydig cells are able to produce testosterone. Since PAP7 is highly expressed in major steroidogenic tissues and is more concentrated in these steroid producing cells, PAP7 may be involved in steroid biosynthesis or the regulation of steroid formation by changing the formation or the conformation of the PBR complex. Mouse C6 glioma cells, MA-10 Leydig cells and Y1 adrenal cortical cells are popular cell models selected for studying steroid biosynthesis. PAP7 expression is proportionally correlated to the PBR expression in these cell lines. Additionally, both PBR and PAP7 expression level in these cell lines are parallel with their steroidogenic capability, which also suggests that PAP7 may be involved in steroid biosynthesis through a PBR pathway. A small PAP7 transcript was expressed only in testis, a phenomenon observed for other genes expressed in testis (Zhang, F. P. et al., 1997, Endocrinology 138, 2481-2490; Mauduit, C. et al., 1999, *J. Biol. Chem.* 274, 770-775). The immunostaining in the testis other than in Leydig cells could represent the expression of the smaller transcript.

The PAP7 protein is expressed in MA-10 cells and most of the staining is localized in the cytoplasm. The study of PAP7 subcellular distribution is ongoing,

the results of which may provide more detailed information about the interaction between PBR and PAP7. Since PBR knockout mice die in uterus indicates an essential role for PBR essential in mouse embryonic development. Interestingly, PAP7 mRNA is highly expressed during mouse early embryonic development. This result may suggest that PAP7, associated with PBR, could play an important role during early mouse development. This further implies that PBR may have new functions beyond steroidogenesis. Overexpression of PAP7 fragment including its PBR binding domain significantly inhibited the progesterone formation stimulated by saturating concentrations of hCG (50 ng/ml) in MA-10 cells. According to previous studies, progesterone production of these cells represented the index of steroid biosynthesis (Freeman, D. A., 1987, *Endocrinology* 120, 124-132; Garnier, M. et al., 1994, *J. Biol. Chem.* 269, 22105-22112). Based on the inhibitory manner, we assume that the overexpressed PAP7 fragment might act as a competitor of the native PAP7 in MA-10 cells and competitively bind to PBR. We believe that the tranfected PAP7 fragment having only the PBR binding domain and is not fully functional as a native PAP7, however, it competitively prevents PBR from interacting with the endogenous PAP7 and thereby blocks the normal function of PBR.

In conclusion, the results presented herein suggest that the identified PAP7 is involved in the regulation of the PBR function, serving as an endogenous ligand or allosteric modulator of the receptor.

Considering the findings tha i) PBR is a channel/transporter of cholesterol, ii) PBR is the target of environmental antisteroidogenic hazards, and (iii) PBR is involved in breast cancer aggression and

tumor cell proliferation (Hardwick, M. et al., 1999, *Cancer Res.* 59, 831-842), we believe that the identification and characterization of PAP7 will greatly contribute to the understanding of the role of PER in steroidogenesis and even in more general areas.

What is claimed is:

1. An isolated PBR-associated protein (PAP) DNA fragment or any portion thereof.

2. An isolated and purified DNA fragment which encodes a PBR-associated protein.

3. An isolated and purified DNA fragment which encodes a peptide of PBR-associated protein, said DNA fragment comprising a sequence specified in any of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5, or polynucleotide fragment of said sequence comprising at least 30 nucleotides.

4. An isolated and purified DNA fragment which encodes a peptide of PBR-associated protein, said DNA fragment comprising a sequence specified in Genbank Accession no. AF022770, or GenBank Accession no. AF020338, or a polynucleotide fragment of said sequence comprising at least 30 nucleotides.

5. An isolated and purified PAP7 DNA fragment according to claim 2 which encodes 463 amino acids of PAP7 or a natural variant or synthetic variant thereof encoding PAP7, or a peptide fragment thereof comprising at least 10 amino acids.

6. An isolated and purified PAP8 DNA fragment according to claim 2 which encodes 190 amino acids of PAP8 or a natural variant or synthetic variant thereof encoding PAP8, or a peptide fragment thereof comprising at least 10 amino acids.

7. An isolated and purified PAP15 DNA fragment according to claim 2 which encodes 164 amino acids of PAP15 or a natural variant or synthetic variant thereof encoding PAP15, or a peptide fragment thereof comprising at least 10 amino acids.

8. An isolated and purified PAP20 DNA fragment according to claim 2 which encodes 196 amino acids of PAP20 or a natural variant or synthetic variant

thereof encoding PAP20, or a peptide fragment thereof comprising at least 10 amino acids.

9. A recombinant DNA construct comprising:

(i) a vector, and

(ii) the PAP DNA fragment of claim 1.

10. A recombinant DNA construct comprising:

(i) a vector, and

(ii) the PAP DNA fragment of claim 3.

11. A recombinant DNA construct according to claim 10, wherein said vector is an expression vector.

12. The recombinant DNA construct according to claim 10, wherein said vector is a prokaryotic vector.

13. The recombinant DNA construct according to claim 10, wherein said vector is a eukaryotic vector.

14. A host cell transformed with a recombinant DNA construct according to claim 10.

15. A host cell according to claim 14, wherein said cell is prokaryotic.

16. A host cell according to claim 14, wherein said cell is eukaryotic.

17. A method for producing PAP peptide which comprises culturing the cells according to either claim 15 or 16, under conditions such that said DNA fragment is expressed and said PAP peptide is thereby produced.

18. An isolated recombinant PAP produced by the method of claim 17.

19. A PAP7 polypeptide comprising the amino acid sequence specified in SEQ ID NO:7 or a portion thereof of at least 5 amino acids.

20. A PAP8 polypeptide comprising the amino acid sequence specified in SEQ ID NO:8 or a portion thereof of at least 5 amino acids.

21. A PAP15 polypeptide comprising the amino acid sequence specified in SEQ ID NO:9 or a portion thereof of at least 5 amino acids.

22. A PAP20 polypeptide comprising the amino acid sequence specified in SEQ ID NO:10 or a portion thereof of at least 5 amino acids.

23. A method for detecting a PAP in a sample chosen from the group consisting of: PAP7 , PAP8, PAP15, PAP20, said method comprising

(i) contacting said sample with antibodies which recognize said PAP; and

(ii) detecting the presence or absence of a complex formed between PAP and antibodies specific therefor.

24. A method for detecting a PBR-associated protein, said method comprising the two hybrid assay.

25. An antibody to a peptide having the amino acid sequence specified in SEQ ID NO:6, 7, 8 and 9, or any portion thereof.

26. A PAP7 antibody to a peptide comprising the amino acid sequence specified in SEQ ID NO:11.

27. A method for detecting agents or drugs which reduce or eliminate PAP activity, said method comprising:

(i) delivering a recombinant DNA construct according to claim 10 into a cell such that PAP is produced in said cell;

(ii) adding at least one drug or agent to said cell alone or in combination; and,

(iii) detecting PAP activity in said cell in the presence of said agent or drug and comparing it to a control which did not receive said drug or agent wherein a decrease in PAP activity as compared to control indicates an drug or agent which reduces or eliminates PAP activity.

28. A method for detecting agents or drugs which promote PAP activity, said method comprising:

(i) delivering a recombinant DNA construct according to claim 10 into a cell such that PAP is produced in said cell;

(ii) adding at least one drug or agent to said cell alone or in combination; and,

(iii) detecting whether or not said drug or agent stimulates PAP activity by measuring PAP acitivity in said cell and comparing it to a control which did not receive said drug or agent wherein an increase in the activity of said PAP in said cell as compared to control indicates a stimulatory drug or agent.

29. An agent or drug capable of inhibiting PAP activity.

30. An agent or drug capable of promoting PAP activity.

31. A therapeutic compound comprising said agent or drug according to claim 29 for use in a disease wherein a decrease or elimination of PAP activity is beneficial.

32. A therapeutic compound comprising said agent or drug according to claim 30 for use in a disease wherein an increase o PAP activity is beneficial.

33. A method for detecting at least one PAP selected from the group consisting of PAP7, PAP8, PAP15, and PAP20 in a sample using the polymerase chain reaction.

34. A diagnostic kit for detecting RNA/cDNA of at least one PAP chosen from the group consisting of PAP7, PAP8, PAP15 and PAP20, in a sample comprising primers or oligonucleotides specific for said PAP RNA or cDNA suitable for hybridization to PAP RNA or cDNA and/or amplification of PAP sequences and suitable ancillary reagents.

35. A method for increasing a PAP selected from the group consisting of PAP3, PAP7, PAP8, PAP15 and PAP20 in a cell by introducing into said cell a PAP nucleic acid encoding said PAP such that said nucleic acid is expressed and PAP is produced in said cell.

36. A therapeutic method for the treatment or amelioration of diseases resulting from an increase in cell proliferation, said method comprising providing to an individual in need of such treatment an effective amount of an agent or drug which reduces or eliminates PAP expression or function in a pharmaceutically acceptable diluent.

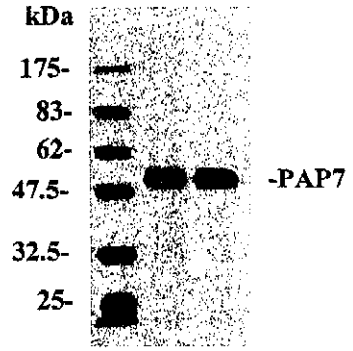
37. The method of claim 36 wherein said disease is cancer.

38. A therapeutic method for the treatment or amelioration of conditions resulting from abnormal cholesterol level, said method comprising providing to an individual in need of such treatment an effective amount of an agent or drug which reduces or eliminates PAP expression or function in a pharmaceutically acceptable diluent.

39. The method of claim 38 wherein said condition is selected from the group consisting of: cancer, neurodegenerative disorders, developmental disorders, stress, and stroke.

40. A method for modulating PBR activity, function or targeting in a cell, said method comprising increasing or decreasing level of PAP in said cell.

A



B

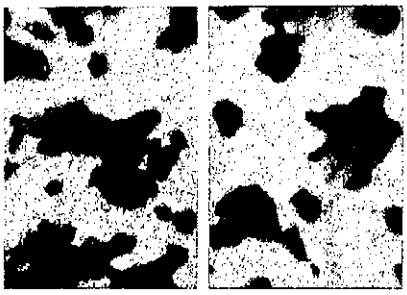
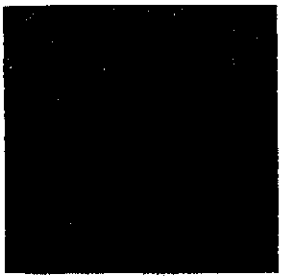


FIGURE 1/8

A



	1	2	3	4	5
A	brain	eye	liver	lung	kidney
B	heart	skeletal muscle	small intest.		
C	pancreas	thyroid	thymus	submax. gland	spleen
D	testis	ovary	prostate	epidi-dymus	uterus
E	embryo 7 days	embryo 11 days	embryo 15 days	embryo 17 days	

B

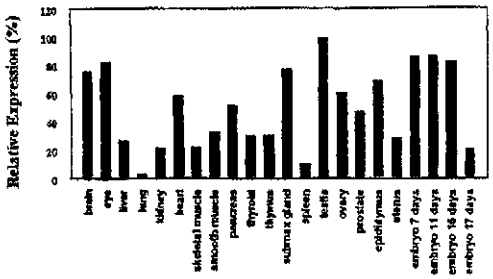
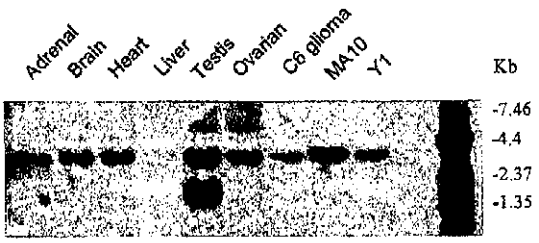


FIGURE 2/8

A


Adrenal
Brain
Heart
Liver
Testis
Ovarian
C6 glioma
MA10
Y1
Kb
-7.46
-4.4
-2.37
-1.35

B

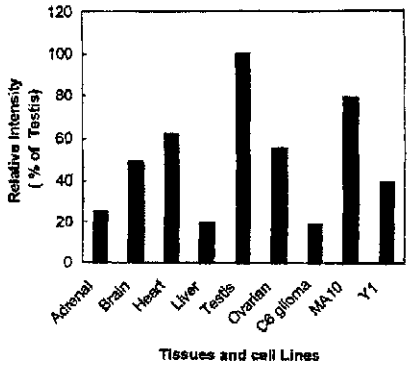

120
100
80
60
40
20
0
Relative Intensity
(% of Testis)
Adrenal
Brain
Heart
Liver
Testis
Ovarian
C6 glioma
MA10
Y1
Tissues and cell Lines

FIGURE 3/8

A


Adrenal
Brain
Heart
Liver
Testis
Ovarian
C6 glioma
MA10
Y1

B

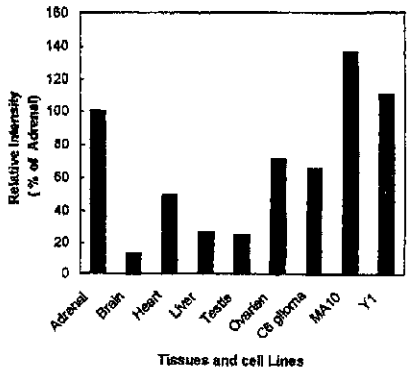

160
140
120
100
80
60
40
20
0
Relative Intensity (% of Adrenal)
Adrenal
Brain
Heart
Liver
Testis
Ovarian
C6 glioma
MA10
Y1
Tissues and cell Lines

FIGURE 4/8

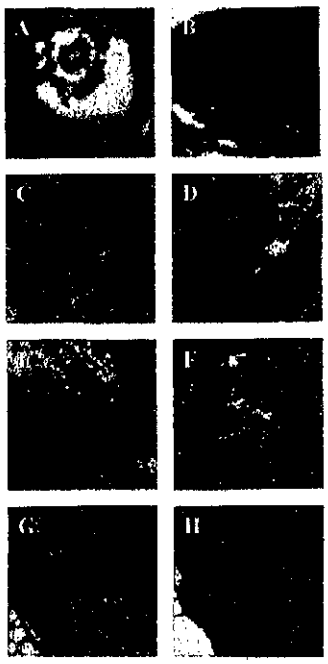
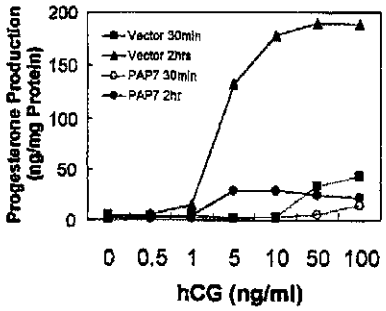


FIGURE 5/8

A


Progesterone Production (ng/mg Protein)
200
150
100
50
0
Vector 30min
Vector 2hrs
PAP7 30min
PAP7 2hr
0 0.5 1 5 10 50 100
hCG (ng/ml)

B

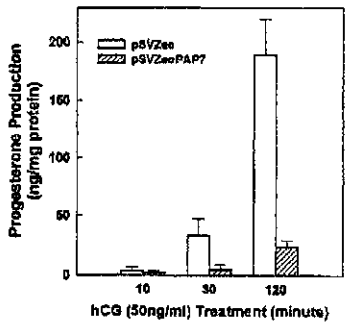
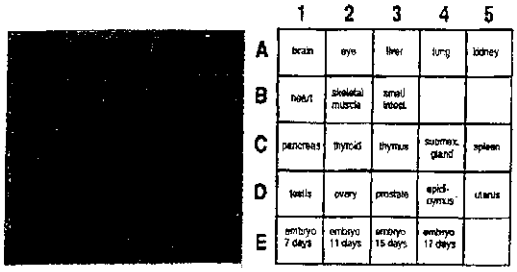

Progesterone Production (ng/mg protein)
200
150
100
50
0
pSVZeo
pSVZeoPAP7
10
30
120
hCG (50ng/ml) Treatment (minute)

FIGURE 6/8



	1	2	3	4	5
A	brain	eye	liver	lung	kidney
B	heart	skeletal muscle	small intest.		
C	pancreas	thyroid	thymus	submax. gland	spleen
D	testis	ovary	prostate	epid-dymus	uterus
E	embryo 7 days	embryo 11 days	embryo 15 days	embryo 17 days	

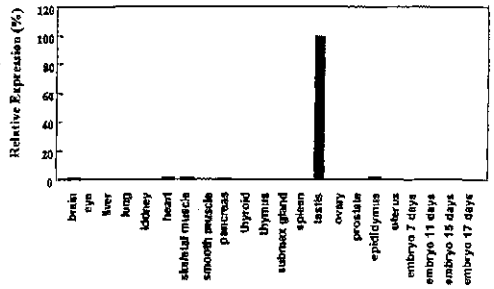


FIGURE 7/8

PAGE 3

	1	2	3	4	5
A	brain	eye	liver	lung	kidney
B	heart	skeletal muscle	small intest.		
C	pancreas	thyroid	thymus	submax. gland	spleen
D	testis	ovary	prostate	epid-dymus	uterus
E	embryo 7 days	embryo 11 days	embryo 15 days	embryo 17 days	

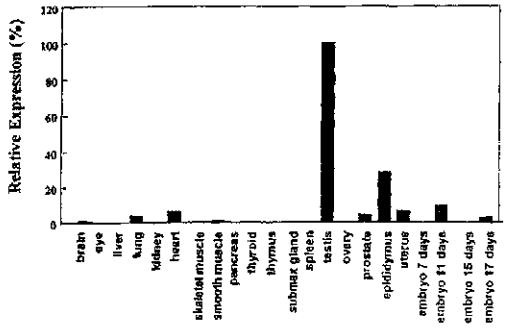


FIGURE 8/8

DAD20

SEQ ID NO:1
PAP3 nucleic acid

GAATTCGCGGCCGCGTCGACCACCGCTGCGCCCTCCTGCGAGGCCGGCTGAACGAGGAAATA
ATTGCTAATAAGGCCTCTGTAGCCATGGCTACTTCTGACGTGAAACCAAAATCAATAAGTCGTG
CCAAGAAATGGTCAGAGGAAATAGAAAATCTGTACAGATTTCAACAAGCAGGATATCGGGATG
AAATTGAATATAAACAAGTGAAACAAGTTGCCATGGTCGACCGATGGCCAGAGACAGGGTACG
TGAAGAAACTTCAGCGGAGGGACAATACTTTCTTCTACTACAACAAAGAGAGGGAGTGCGA
GGACAAGGAGGTCCACAAAGTGAAGGTTTACGTCTACTGACCTTTTCCTTTCTTCGGCTTG
GCAATGCTCCTTTAAGAATTGGTTGTTTACATTCTTCCATCGTGTAAATGTCATTTTACAA
AACAATTCACAATTCTGTCTTTAATTCATGGTGTCTTACACAACATAAACACCCACCTTGA
AACCCAAAAA

SEQ ID NO:2
PAP7 sequence:

GAATTCGCGGCCGCGTCGACCTAAAGTTGAGTTGTTCACTGTAGTGACCCGTGTGAAGGTAGTT
TTATTTTTAAATCAACTTTCATTGTGCAAACTAGTAAAAGATGGCAAAGCCTTTCATCCAACTTA
TGAAGAAAAACTGAAGTTCGTGGCACTGCATAAGCAAGTTCTTTTGGGCCCATATAACCCAGA
CACGTCCCCTGAGGTTGGATTCTTTGATGTGTTGGGGAATGATAGGAGGAGAGAATGGGCAGCT
CTGGGAAACATGTCCAAGGAGGATGCCATGGTAGAGTTTGTGAAGCTTCTAAATAAGTGTTGTC
CTCTCCTCTGGGCATATGTTGCGTCCCACAGAATAGAGAAGGAAGAAGAAGAGAAAAGAAGAA
AGGCGGAGGAGGAGCGAAGGCAGCGTGAAGAGGAAGAACGAGAGCGGCTGCAAAAGGAAGAA
GAGAAGCGGAAGCGAGAGGAGGAAGACCGGCTGAGACGGGAGGAGGAAGAGAGGCGGCGGAT
AGAGGAAGAGAGGCTTCGGCTGGAACAGCAAAAGCAGCAGATAATGGCAGCTTTAAACTCGCA
GACTGCCGTGCAATTCCAGCAGTATGCAGCCCAGCAGTATCCAGGGAACTACGAACAACAGCA
GATTCTCATCCGCCAGCTGCAGGAGCAGCACTATCAGCAGTATAAACACCAGGCAGAGCAAAC
CCAACCTGCACAACAACAGGCAGCATTACAGAAACAGCAAGAAGTAGTGATGGCTGGGGCAT
CATTGCCTGCATCATCAAAGGTGAACACAGCTGGAGCAAGTGATACACTGTCAGTTAATGGAC
AGGCCAAAACCCACACTGAAAATTCCGAAAAAGTCCTTGAGCCAGAAGCTGCAGAAGAAGCC
TTGGAAAATGGACCAAAAGACTCTCTTCCAGTGATTGCAGCTCCATCCATGTGGACAAGACCA
CAAATCAAAGACTTTAAAGAGAAGATTCGGCAGGATGCAGATTCTGTGATTACAGTACGTCGA
GGAGAAGTCGTCACCGTCCGAGTCCCGACTCATGAGGAAGGATCATACCTATTTTGGGAATTTG
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TGAAGAAAAAGCAAAAAAGAACGCCAACAAGCCTCTGCTGGATGAGATTGTACCTGTGTACCG
GCGGGACTGTCACGAGGAAGTATATGCAGGCAGCCACCAGTATCCAGGGAGGGGAGTCTATC
TCCTCAAGTTTGATAATTCCTACTCTCTGTGGAGGTCCAAGTCCGTCTACTACAGAGTCTA
TTATACTAGATAGAGCTGCTGTTCCAAGGTCCGGAGTCCAGGGTTGAGCACAACATGACGT
TTAATTTCCTTT

SEQ ID NO:3
PAP8 nucleic acid

GAATTCGCGGCCGCGTCGACGCTGGACACAAGCGTGGAGCGAAGAGCCCTTGGAGAGATTCAG
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AACCTGGGGGAGCAGACGCAGAGCGGACCCCAGGGAGGAAGGTGTCAGCGTCGGGAGAGGCA
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GCCTGCAGAGCAGCCCTGCCATGGAGATCAAGTGACTGGACTGACCCAGGACCTGGGAGAGA

ACCGCCGTTCCTGCGGCATCATGCACATGCCTGCCATCCCCGGAATTCAGCTCTGAATCCT
CTC

SEQ ID NO:4
PAP15 nucleic acid
GAATTCGCGGCCGCGTCGACCTCGGGGGTAACCCTTGGGATCATTATGATGTCACCTCTTCAAT
CTCTTGAGACCAGTGATTTATGAACTTGACATTTGGTGCCTGGTGTGGGCTTTGGAAAGCAGAG
GCCACCTTTGTCTGTAGAGGATACTGAGCGGCTGGATGGCAGGAATCCAAAGAGAAGCCAGCC
TCTCGTAGTCGCCCTGGGACAGTGGAAGAGAGGGAGGACCGGCAAAGGGGAATCTGTCTTTCT
CCTAGGCCCGAGCATGTCCCCTGTGGGACATGCTCTGTGACAGCTGAGCCTGCCCAGCCTG
CCTTTCTGAAGTTGGGTGTCTCCTGCCCACAACCAAGCCAGCAATCGGTCTGTTTTCCGAC
AACCTCAGAGCCAGACCTCACAAGCCTATTTTGGTGGTTCCCAAAATTTCTCTCAGATCTC
CATGTCTATCCCTCCACTCCCTCCAAAAGAGAAAGAAAAGAATTGAGAAAGAA

SEQ ID NO:5
PAP20 nucleic acid
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TTGTACAGAAAACCAGACTAGAGAGAAAAAAACAAGAAGCGTCTGTATCTAAAGATGCTCCAG
TGCCTACAAATACTAAAAGGGCAAAGAAACAAGAGAAGTCTCCAGGTAGAATTGCCTCACAGT
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TCATCAGATGGCCAAAAAGATGGACTTAACCTAAGTACATCTCAACAAGAAAGTATTTCAATG
ATTCCTAAAGGTCCTCCTGAAAACTCAGTTATCAGCTGTGACTCCCAGGCCCTAAATATGTTAG
CTGATCTGGCATTAAGTTCTGCTGCTGCTTCTATACCATCTTGTAAGCCCAGGAACCTTCCCTG
CGTCTCTGATTTGCCACGAAACAATGTCTTACTCACTAAAGAAAATCCATTGCTTGGTGCCT
CTGACCATGAATATCATAAGGG

SEQ ID NO:6
PAP3 amino acid sequence
Met Ala Thr Ser Asp Val Lys Pro Lys Ser Ile Ser Arg Ala Lys
Lys Trp Ser Glu Glu Ile Glu Asn Leu Tyr Arg Phe Gln Gln Ala
Gly Tyr Arg Asp Glu Ile Glu Tyr Lys Gln Val Lys Gln Val Ala
Met Val Asp Arg Trp Pro Glu Thr Gly Tyr Val Lys Lys Leu Gln
Arg Arg Asp Asn Thr Phe Phe Tyr Tyr Asn Lys Glu Arg Glu Cys
Glu Asp Lys Glu Val His Lys Val Lys Val Tyr Val Tyr

SEQ ID NO:7
PAP7 amino acid sequence
Arg Pro Arg Arg Pro Lys Val Glu Leu Phe Thr Val Val Thr Arg
Val Lys Val Val Leu Phe Leu Asn Gln Leu Ser Leu Cys Lys Leu
Val Lys Asp Gly Lys Ala Phe His Pro Thr Tyr Glu Glu Lys Leu
Lys Phe Val Ala Leu His Lys Gln Val Leu Leu Gly Pro Tyr Asn
Pro Asp Thr Ser Pro Glu Val Gly Phe Phe Asp Val Leu Gly Asn
Asp Arg Arg Arg Glu Trp Ala Ala Leu Gly Asn Met Ser Lys Glu
Asp Ala Met Val Glu Phe Val Lys Leu Leu Asn Lys Cys Cys Pro

Leu Leu Ser Ala Tyr Val Ala Ser His Arg Ile Glu Lys Glu Glu
Glu Glu Lys Arg Arg Lys Ala Glu Glu Glu Arg Arg Gln Arg Glu
Glu Glu Glu Arg Glu Arg Leu Gln Lys Glu Glu Glu Lys Arg Lys
Arg Glu Glu Glu Asp Arg Leu Arg Arg Glu Glu Glu Glu Arg Arg
Arg Ile Glu Glu Glu Arg Leu Arg Leu Glu Gln Gln Lys Gln Gln
Ile Met Ala Ala Leu Asn Ser Gln Thr Ala Val Gln Phe Gln Gln
Tyr Ala Ala Gln Gln Tyr Pro Gly Asn Tyr Glu Gln Gln Gln Ile
Leu Ile Arg Gln Leu Gln Glu Gln His Tyr Gln Gln Tyr Lys His
Gln Ala Glu Gln Thr Gln Pro Ala Gln Gln Gln Ala Ala Leu Gln
Lys Gln Gln Glu Val Val Met Ala Gly Ala Ser Leu Pro Ala Ser
Ser Lys Val Asn Thr Ala Gly Ala Ser Asp Thr Leu Ser Val Asn
Gly Gln Ala Lys Thr His Thr Glu Asn Ser Glu Lys Val Leu Glu
Pro Glu Ala Ala Glu Glu Ala Leu Glu Asn Gly Pro Lys Asp Ser
Leu Pro Val Ile Ala Ala Pro Ser Met Trp Thr Arg Pro Gln Ile
Lys Asp Phe Lys Glu Lys Ile Arg Gln Asp Ala Asp Ser Val Ile
Thr Val Arg Arg Gly Glu Val Val Thr Val Arg Val Pro Thr His
Glu Glu Gly Ser Tyr Leu Phe Trp Glu Phe Ala Thr Asp Ser Tyr
Asp Ile Gly Phe Gly Val Tyr Phe Glu Trp Thr Asp Ser Pro Asn
Ala Ala Val Ser Val His Val Ser Glu Ser Ser Asp Glu Glu Glu
Glu Glu Glu Glu Asn Val Thr Cys Glu Glu Lys Ala Lys Lys Asn
Ala Asn Lys Pro Leu Leu Asp Glu Ile Val Pro Val Tyr Arg Arg
Asp Cys His Glu Glu Val Tyr Ala Gly Ser His Gln Tyr Pro Gly
Arg Gly Val Tyr Leu Leu Lys Phe Asp Asn Ser Tyr Ser Leu Trp
Arg Ser Lys Ser Val Tyr Tyr Arg Val Tyr Tyr Thr Arg

SEQ ID NO:8
PAP8 amino acid sequence
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Ala Leu Gly Glu Ile Gln Asn Val Gly Glu Gly Ser Ser Thr Ser
Gln Gly Thr Trp Gln Ser Ser Glu Ser Ser Gln Ser Asn Leu Gly
Glu Gln Thr Gln Ser Gly Pro Gln Gly Gly Arg Cys Gln Arg Arg
Glu Arg His Asn Arg Met Glu Arg Asp Arg Arg Arg Arg Ile Arg
Ile Cys Cys Asp Glu Leu Asn Leu Leu Val Pro Phe Cys Asn Ala
Glu Thr Asp Lys Ala Thr Thr Leu Gln Trp Thr Thr Ala Phe Leu
Lys Tyr Ile Gln Glu Arg His Gly Asp Ser Leu Lys Lys Glu Phe
Glu Ser Val Phe Cys Gly Lys Thr Gly Arg Arg Leu Lys Leu Thr
Arg Pro Glu Ser Leu Val Thr Cys Pro Ala Gln Gly Ser Leu Gln
Ser Ser Pro Ala Met Glu Ile Lys

SEQ ID NO:9
PAP15 amino acid sequence
Ala Ala Gly Trp Gln Glu Ser Lys Glu Lys Pro Ala Ser Arg Ser
Arg Pro Gly Thr Val Glu Glu Arg Glu Asp Arg Gln Arg Gly Ile
Cys Leu Ser Pro Arg Pro Glu His Val Pro Cys Gly Thr Cys Ser
Val Thr Ala Glu Pro Ala Gln Pro Ala Phe Leu Lys Leu Gly Val
Ser Cys Pro Gln Pro Ser Gln Gln Ser Val Cys Phe Pro Thr Thr
Ser Glu Pro Asp Leu Thr Ser Leu Phe Trp Trp Phe Pro Lys Phe
Leu Ser Asp Leu His Val Tyr Pro Ser Thr Pro Ser Lys Arg Glu
Arg Lys Glu Leu Arg Lys Lys

SEQ ID NO:10
PAP20 amino acid sequence
Asn Ser Arg Pro Arg Arg Pro Gln Lys Arg Lys Arg Gly Ala Glu
Val Leu Ala Ala Gln Ile Val Gln Lys Thr Arg Leu Glu Arg Lys
Lys Gln Glu Ala Ser Val Ser Lys Asp Ala Pro Val Pro Thr Asn
Thr Lys Arg Ala Lys Lys Gln Glu Lys Ser Pro Gly Arg Ile Ala
Ser Gln Ser Lys Pro Pro Met Lys Lys Ser Pro Gln Lys Arg Lys
Val Asn Val Ala Arg Gly Arg Arg Asn Thr Arg Lys Gln Leu Gln
Pro Ala Glu Lys Glu Ile Ala Leu His Leu Gln Ser Glu Ile Ser
Ser Asp Gly Gln Lys Asp Gly Leu Asn Leu Ser Thr Ser Gln Gln
Glu Ser Ile Ser Met Ile Pro Lys Gly Pro Pro Glu Asn Ser Val
Ile Ser Cys Asp Ser Gln Ala Leu Asn Met Leu Ala Asp Leu Ala
Leu Ser Ser Ala Ala Ala Ser Ile Pro Ser Cys Lys Pro Arg Asn
Leu Pro Cys Val Ser Asp Leu Pro Arg Asn Asn Val Leu Leu Thr
Lys Glu Asn Pro Leu Leu Gly Ala Ser Asp His Glu Tyr His Lys
Gly

SEQ ID NO:11
Ser Ser Asp Glu Glu Glu Glu Glu Glu Glu Asn Val Thr Cys Glu
Glu Lys Ala Lys Lys Asn Ala Asn Lys Pro

【国際公開パンフレット（コレクトバージョン）】

CORRECTED VERSION*

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(57) Abstract

The present invention relates to nucleic acids encoding PBR- associated proteins (PAPs) and methods for use in producing PAPs and methods of using PAPs. In this study we have identified proteins (PAPs) that interact with PBR protein by a yeast two-hybrid system. We used PBR as bait to screen a mouse testis cDNA library. Five clones were isolated based on their ability to interact with PBR. These proteins may be involved in the regulation of the function of PBR, serving as an endogenous ligand or allosteric modulator of the receptor.

*(Referred to in PCT Gazette No. 31/2000, Section II)

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TITLE OF THE INVENTION

Peripheral-type Benzodiazepine Receptor Associated Proteins, Cloning, Expression and Methods of Use

Field of the Invention

The present invention relates to nucleic acid molecules encoding peripheral-type benzodiazepine receptor (PBR) associated proteins (PAPs), including mutants, variants, fragments and derivatives thereof, and to vectors and host cells comprising such nucleic acid molecules; methods of using PAPs; method for screening for inhibitors and activators of PAPs or PBR; and kits comprising the compositions or polypeptides of the invention.

Background of the Invention

The peripheral-type benzodiazepine receptor (PBR) was originally discovered because it binds the benzodiazepine diazepam with relatively high affinity (Papadopoulos, V. 1993, *Endocr. Rev.* 14:222-240). Benzodiazepines are among the most highly prescribed drugs due to their pharmacological actions in relieving anxiety mediated through modulating the activity of γ-aminobutyric acid receptors in the central nervous system (Costa, E. and Guidotti, A. 1979, *Annu. Rev. Pharmacol. Toxicol.* 19:531-545). PBR is another class of binding sites for benzodiazepines distinct from the aforementioned neurotransmitter receptors. Further studies demonstrated that in addition to benzodiazepines, PBR binds other classes of organic compounds with high affinity (Papadopoulos, 1993, *supra*). PBR, although present in all tissues examined, was found to be particularly high in steroid

producing tissues, where it was primarily localized in the outer mitochondrial membrane (OMM) (Anholt, R.R.H. *et al.* 1986, *J. Biol. Chem.* 261:576-583). An 18 kDa isoquinoline-binding protein was identified as PBR, cloned and expressed (Papadopoulos, V. 1998, *Proc Soc. Exp. Biol. Med.* 217:130-142). It was then demonstrated that PBR is a functional component of the steroidogenic machinery (Papadopoulos, 1998, *supra*; Papadopoulos V. *et al.* 1990, *J. Biol. Chem.* 265:3772-3779) mediating cholesterol delivery from the outer to the inner mitochondrial membrane (Krueger, K. E. and Papadopoulos, V. 1990, *J. Biol. Chem.* 265:15015-15022). Further studies demonstrated that pharmacologically induced reduction of adrenal PBR levels *in vivo* resulted in decreased circulating glucocorticoid levels (Papadopoulos, V. 1998, *supra*). In addition, targeted disruption of the PBR gene in Leydig cells resulted in the arrest of cholesterol transport into mitochondria and steroid formation; transfection of the mutant cells with a PBR cDNA rescued steroidogenesis (Papadopoulos, V. *et al.* 1997, *J. Biol. Chem.* 272:32129-32135).

PBR is extremely abundant in steroidogenic cells and found primarily on outer mitochondrial membranes (Anholt, R. *et al.* 1986, *J. Biol. Chem.* 261:576-583). PBR is thought to be associated with a multimeric complex composed of the 18-kDa isoquinoline-binding protein and the 34-kDa pore-forming voltage-dependent anion channel protoin, preferentially located on the outer/inner mitochondrial membrane contact sites (McEnery, M. W. *et al. Proc. Natl. Acad. Sci. U.S.A.* 89:3170-3174; Garnier, M. *et al.* 1994, *Mol. Pharmacol.* 45:201-211; Papadopoulos, V. *et al.* 1994, *Mol. Cel. Endocr.* 104:R5-R9). Drug ligands of PBR, upon binding to the receptor, simulate steroid synthesis in

steroidogenic cells *in vitro* (Papadopoulos, V. *et al.* 1990, *J. Biol. Chem.* 265:3772-3779; Ritta, M. N. *et al.* 1989, *Neuroendocrinology* 49: 262-266; Barnea, E. R. *et al.* 1989, *Mol. Cell. Endocr.* 64:155-159; Amsterdam, A. and Suh, B. S. 1991, *Endocrinology* 128:503-510; Yanagibashi, K. *et al.* 1989, *J. Biochem.* (*Tokyo*) 106: 1026-1029). Likewise, *in vivo* studies showed that high affinity PBR ligands increase steroid plasma levels in hypophysectomized rats (Amri, H. *et al.* 1996, *Endocrinology* 137:5707-5718). Further *in vitro* studies on isolated mitochondria provided evidence that PBR ligands, drug ligands, or the endogenous PBR ligand, the polypeptide diazepam-binding inhibitor (DBI) (Papadopoulos, V. *et al.* 1997, *Steroids* 62:21-28), stimulate pregnenolone formation by increasing the rate of cholesterol transfer from the outer to the inner mitochondrial membrane (Krueger, K. E. and Papadopoulos, V. 1990, *J. Biol. Chem.* 265:15015-15022; Yanagibashi, K. *et al.* 1988, *Endocrinology* 123: 2075-2082; Besman, M. J. *et al.* 1989, *Proc. Natl. Acad. Sci. U.S.A.* 86: 4897-4901; Papadopoulos, V. *et al.* 1991, *Endocrinology* 129: 1481-1488).

Based on the amino acid sequence of the 18-kDa PBR, a three dimensional model was developed (Papadopoulos, V. 1996, In: The Leydig Cell. Payne, A. H. *et al.* (eds) Cache River Press, IL, pp 596-628). This model was shown to accomodate a cholesterol molecule and function as a channel, supporting the role of PBR in cholesterol transport. Recently we demonstrated the role of PBR in steroidogenesis by generating PBR negative cells by homologous recombination (Papadopoulos, V. *et al.* 1997, *J. Biol. Chem.* 272:32129-32135) that failed to produce steroids. However, addition of the hydrosoluble

analogue of cholesterol, 22R-hydroxycholesterol, recovered steroid production by these cells, indicating that the cholesterol transport mechanism was impaired. Further cholesterol transport experiments in bacteria expressing the 18-kDa PBR protein provided definitive evidence for a function as a cholesterol channel/transporter (Li and Papadopoulos, 1998, *Endocrinology* 139, 4991-4997).

Studies in a number of tumors such as rat brain containing glioma tumors (Richfield, E. K. *et al.* 1988, *Neurology* 38:1255-1262), colonic adenocarcinoma and ovarian carcinoma (Katz, Y. *et al.* 1988, *Eur. J. Pharmacol.* 148: 483-484 and Katz, Y. *et al.* 1990, *Clinical Sci.* 78:155-158) have shown an abundance of peripheral-type benzodiazepine receptors (PBR) compared to normal tissue. All documents cited herein *infra* and *supra* are hereby incorporated in their entirety by reference thereto. Moreover, a 12-fold increase in PBR density relative to normal parenchyma, was found in human brain glioma or astrocytoma (Cornu, P. *et al.* 1992, *Acta Neurochir.* 119:146-152). The authors suggested that PBR densities may reflect the proliferative activity of the receptor in these tissues. Recently, the involvement of PBR in cell proliferation was further shown (Neary, J. T. *et al.* 1995, *Brain Research* 675:27-30; Miettinen, H. *et al.* 1995, *Cancer Research* 55:2691-2695), and its expression in human astrocytic tumors was found to be associated with tumor malignancy and proliferative index (Miettinen, H. *et al. supra*; Alho, H. 1994, *Cell Growth Different.* 5:1005-1014). Characterization of PBR in human breast cancer biopsies, led to the discovery that the invasive and metastatic ability of human breast tumor cells is proportional to the level of PBR expressed, and correlates with the subcellular

localization of PBR in these cells in that PBR is found primarily in the nucleus in aggressive tumor cells whereas PBR is found primarily in the cytoplasm of invasive but non-aggressive cells. These changes in PBR expression can be used as a tool for detection, diagnosis, prevention and treatment in breast cancer patients, in particular, and in aggressive solid tumors in general.

Since both PBR and its endogenous ligand, the polypeptide diazepam binding inhibitor, are constitutively expressed in steroidogenic cells, the regulation of PBR function by hormones may be due to its association with other proteins. This interaction may result in the initiation of steroid biosynthesis. Therefore, there is a need to identify proteins which associate with PBR and may modulate PBR function.

Summary of the Invention

The present invention meets the need described above. We have identified PBR associated proteins (PAPs) that interact with PBR using the two-hybrid system. We used PBR as a bait to screen a mouse testis cDNA library. Five clones were isolated by their ability to interact with PBR: PAP3, PAP7, PAP8, PAP15, and PAP20. Among the nucleotide sequences identified, PAP3 was identical to the previously isolated meg1 protein (Don, J. and Wolgemuth, D. J., 1992, *Cell Growth Differ.* 3, 495; Ever, L. et al., 1999, *Cell Growth Differ.* 10, 19-26). PAP7, PAP8, PAP15, and PAP20 are novel sequences according to a search in the Genebank database which did not find a match for these sequences. PAP7 and PAP17 are different clones of the same novel protein product. All PAPs have fatty acylation (myristoylation) sites and PKC

phosphorylation sites. In addition, PAP20 has a PKA phosphorylation site. The distribution and function of the PAPs, as well as their functional relationship to PBR is under investigation.

So far, the distribution of PAP7 in major mouse tissues such as brain, testis, ovary, adrenal, kidney and muscle showed a profile similar to the broader expression pattern of PBR, with an expression level paralleling the steroidogenic ability of the tissue. These data imply a role for these PAPs in the regulation of PBR function, serving as endogenous ligands or allosteric modulators of the receptor.

Therefore, it is an object of the present invention to provide novel DNA fragments encoding PBR associated proteins, PAP3 (SEQ ID NO:1), PAP7 (SEQ ID NO:2) and Genbank accession no. AF022770, PAP8 (SEQ ID NO:3), PAP15 (SEQ ID NO:4), and PAP20 (SEQ ID NO:5) GenBank accession no. AF020338. The DNA fragment is useful as a diagnostic agent for detection of nucleic acid sequences encoding PBR-associated proteins, as an agent for preparation of the protein encoded by the DNA, for the preparation of sequences encoding PAPs, whether cDNA or genomic, and as therapeutic agents.

It is another object of the invention to provide an amino acid sequence for PAPs encoded by the DNA sequences described above.

It is another object of the invention to provide a recombinant vector comprising a vector and the above described DNA fragments.

It is a further object of the present invention to provide a host cell transformed with the above-described recombinant DNA construct.

It is another object of the present invention to provide a method for producing PAPs which comprises culturing a host cell under conditions such that the above-described DNA fragment is expressed and a PAP is thereby produced, and isolating the PAP for use as a reagent, for example for screening drugs and inhibitors of PBR or the PAP itself, for diagnosis, and for therapy.

It is a further object of the present invention to provide an antibody to the above-described recombinant PAPs.

It is yet another object of the present invention to provide a method for detecting any of PAP3, PAP7, PAP8, PAP15, or PAP20 in a sample comprising:

(i) contacting a sample with antibodies which recognize any one of the PAPs mentioned above; and

(ii) detecting the presence or absence of a complex formed between the PAP and antibodies specific therefor.

It is a further object of the present invention to provide a diagnostic kit comprising an antibody against PAP and ancillary reagents suitable for use in detecting the presence of PAP in cells, tissue or serum from yeast, mammals, animals, birds, fish, and plants.

It is yet another object of the present invention to provide a method for the detection of PAP from a sample using the polymerase chain reaction.

It is a further object of the present invention to provide a diagnostic kit comprising primers or oligonucleotides specific for PAP RNA or cDNA suitable for hybridization to PAP RNA or cDNA and/or amplification of PAP sequences and ancillary reagents suitable for use in detecting PAP RNA/cDNA in mammalian tissue.

It is yet another object of the present invention to provide a method for the detection of a PAP in a sample which comprises assaying for the presence or absence of PAP RNA or cDNA in a sample by hybridization assays.

It is an object of the present invention to provide a method for the measurement of PBR in a sample. The method comprises measuring the presence of a PAP complexed with PBR.

It is yet another object of the present invention to provide a method for modulating the function or altering the targeting of PBR by increasing or decreasing interaction of PBR with a PAP. PBR functions which can be modulated include cholesterol transport into the cell, steroid production, cell proliferation, and embryogenesis.

It is still another object of the present invention to provide a method for increasing or decreasing PBR function or expression in a cell by providing into the cell a PAP such that PBR function is increased or decreased.

It is yet another object of the present invention to provide a method for increasing or reducing steroidogenesis by altering the level of PAP in said cell.

It is still another object of the invention to provide a therapeutic method for the treatment or amelioration of diseases resulting from increased cell proliferation resulting from aberrant PBR function or expression or localization, said method comprising providing to an individual in need of such treatment an effective amount of a PAP, in a pharmaceutically acceptable diluent, of a PAP such that the aberrant PBR expression, function or localization, is corrected.

It is another object of the present invention to provide a therapeutic method for the treatment or amelioration of diseases resulting from decreased cell proliferation, said method comprising providing to an individual in need of such treatment an effective amount of PAP or an antibody against PAP or an agent which inhibits PAP expression or function in a pharmaceutically acceptable excipient.

It is another object of the present invention to provide a therapeutic method for the treatment or amelioration of diseases resulting from decreased or increased steroidogenesis, said method comprising providing to an individual in need of such treatment an effective amount of a PAP or an antibody against PAP or an agent which inhibits or activates PAP expression or function in a pharmaceutically acceptable diluent.

It is yet a further object of the present invention to provide a cDNA sequence encoding PAPs and vectors incorporating all or a fragment of said sequence, and cells, prokaryotic and eukaryotic, transformed or transfected with said vectors, for use in screening agents and drugs which inhibit expression or function of PAPs or PBR in such cells.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features, aspects, and advantages of the present invention will become better understood with reference to the following description and appended claims, and accompanying drawings where:

Figure 1. PAP7 protein expressed in MA10 mouse Leydig tumor cells. (A) Western blot. (B), Immunocytochemistry.

Figure 2. PAP7 mRNA Tissue distribution analysis by Dot blot analysis. (A), a Master blot

containing 100-500 ng of poly(A)+ RNA from mouse tissues were hybridized at high stringency with a ^{32}P-labeled PAP7 probe as described under "Materials and Methods." The autoradiogram was exposed overnight. (B), densitometric analysis of PAP7 expression.

Figure 3. PAP7 tissue distribution analysis by Northern blot analysis. (A), Northern blot analysis was performed using 20 µg of total RNA/lane from different mouse tissues as indicated. The blot was hybridized at high stringency with a ^{32}P-labeled PAP7 probe as described under "Materials and Methods". The autoradiogram was exposed overnight. (B), Densitometric analysis of PAP7 expression.

Figure 4. PBR tissue distribution analysis by Northern blot analysis. (A), Northern blot analysis was performed using 20 µg of total RNA/lane from different mouse tissues as indicated. The blot was hybridized at high stringency with a ^{32}P-labeled PBR probe as described under "Materials and Methods". The autoradiogram was exposed overnight. (B), For Northern blot analysis, blots were quantitated by densitometry.

Figure 5. Immunohistological staining of mouse tissues with anti-PAP7 antibody.

Figure 6. The effect of PAP7 on Steroid Biosynthesis. Progesterone formation in MA-10 stimulated by hCG (A), for different time course (B). The results shown represent the means + S.D. from 2 to 6 independent experiments.

Figure 7. PAP3 mRNA Tissue distribution analysis by Dot blot analysis. (A), a Master blot containing 100-500 ng of poly(A)+ RNA from mouse tissues were hybridized at high stringency with a ^{32}P-labeled PAP3 probe as described under "Experimental

Procedures." The autoradiogram was exposed overnight. (B), densitometric analysis of PAP3 expression.

Figure 8. PAP20 mRNA Tissue distribution analysis by Dot blot analysis. (A), a Master blot containing 100-500 ng of poly(A)+ RNA from mouse tissues were hybridized at high stringency with a ^{32}P-labeled PAP20 probe as described under "Experimental Procedures." The autoradiogram was exposed overnight. (B), densitometric analysis of PAP20 expression.

DETAILED DESCRIPTION

The five PAPs described in this application were discovered using a two-hybrid assay. The two-hybrid assay is a yeast-based genetic assay used to detect protein-protein interactions *in vivo*. A positive result obtained with the two-hybrid assay allows rapid identification of genes encoding proteins that interact with a target protein. In addition the two-hybrid assay is a sensitive method for detecting weak and transient interactions, which are probably the norm in large native complexes. Most notably, because the two-hybrid assay is performed *in vivo*, the proteins involved are more likely to be in their native conformations.

The two-hybrid assay is based on the fact that many eukaryotic transcriptional activators consist of two physically separable modular domains: one acts as the DNA-binding domain, while the other functions as the transcriptional activation domain. The DNA-binding domain localizes the transcription factor to specific DNA sequences present in the upstream region of genes that are regulated by this factor, while the activation domain contacts other components of the transcription machinery required to initiate transcription. Both domains are required for normal

activation functioning, and normally the two domains are part of the same protein.

In our PAPs screening experiment, the MATCHMAKER Two-Hybrid System from CLONTECH was used. In the MATCHMAKER System, sequences encoding the two functional domains of the GAL4 transcriptional activator have been cloned into two different shuttle/expression vectors (pGBT9 and pGAD10). The pGBT9 hybrid cloning vector is used to generate a fusion of the GAL4 DNA-binding domain with PBR protein. The pGAD10 hybrid cloning vector is used to generate a fusion of the GAL4 activation domain with a collection of random proteins in a fusion mouse testis library (CLONTECH). Both hybrid proteins are targeted to the yeast nucleus by nuclear localization sequences that either are an intrinsic part of the GAL4 DNA-binding domain or have been added to the activation domain from a heterologous source. If PBR protein and an unknown protein or proteins interact with each other, the DNA-binding domain of GAL4 will be tethered to its transcriptional activation domain, and the proper function of the transcription of an appropriate reporter gene (lacZ or HIS3) containing upstream GAL4 binding sites is used to indicate interaction between the two proteins. This allows a positive selection for clones that are transformed by two interacting hybrid constructs and makes library screening more convenient and practical. After a positive clone has been identified, the gene corresponding to the interacting protein was sequenced using the sequencing primers provided in the kit.

In one embodiment, the present invention relates to a DNA or cDNA sequence encoding PBR associated proteins (PAPs). Five clones were isolated, PAP3, comprising 568 bp and identified in SEQ ID NO:1 (Don,

J. and Wolgemuth, D. J., 1992, *Cell Growth Differ.* 3, 495; Ever, L. et al., 1999, *Cell Growth Differ.* 10, 19-26) which encodes a peptide of 83 amino acids identified in SEQ ID NO:6; PAP7, comprising 577 bp extending from 696 to 1164 of the sequence identified in SEQ ID NO:2, which encodes a polypeptide of 363 amino acids, identified in SEQ ID NO:7; PAP8, comprising 568 bp identified in SEQ ID NO:3, which encode a polypeptide of 190 amino acids, identified in SEQ ID NO:8; PAP15 comprising 490 bp identified in SEQ ID NO:4, which encode a polypeptide of 164 amino acids, identified in SEQ ID NO:9; and PAP20 comprising 588 bp identified in SEQ ID NO:5, which encode a polypeptide of 196 amino acids, identified in SEQ ID NO:10.

PAP3 has been identified as the previously isolated meg 1 protein.

PAP7 and PAP17 are different clones of the same novel protein product. Additional PAP7 sequence has been obtained using the 5', 3'RACE system (CLONTECH) and the near full-length gene is identified in SEQ ID NO:2 including the stop codon and some untranslated sequence at the 3' end. The polypeptide encoded by the DNA sequence would have a calculated molecular weight of about 50 kD. Using an PAP7 antibody produced from the initial isolated DNA fragment of 577 bp, a protein of about 52 kD is immunoprecipitated as shown in the Examples below. Analysis of the protein sequence indicates several concensus sequences and important sites such as: two potential myristoylation sites at positions 262-267 and 271-276 of SEQ ID NO:7 and five PKC phosphorylation sites at 395-396, 113-115, 255-257, 280-282, 331-333, and 339-341 of SEQ ID NO:7, an Acyl-Co-A site at position 24-108 of SEQ ID NO:7, a nuclear localization domain at position 150-

167 of SEQ ID NO:7, a troponin site at position 98-247 of SEQ ID NO:2 and an HSP90 domain at position 126-155 of SEQ ID NO:7. The distribution and expression of PAP7 were examined in major mouse tissues such as brain, testis, ovary, adrenal, and kidney, as well as in tissue culture cell lines such as mouse C6 glioma cells, MA-10 Leydig cells, and Y1 adrenal cortical cells. The PAP7 expression pattern is similar to the broader expression profile of PBR in both tissues and cell lines involved in steroid biosynthesis. Additionally, both PBR and PAP7 expression level in the cell lines correlated with their steroidogenic biosynthesis ability, which suggests that PAP7 may be involved in steroid biosynthesis through PBR.

PAP 8, PAP15, and PAP20, are novel genes. The polypeptide encoded by PAP20 has two potential myristoylation sites, one PKC phosphorylation site and one PKA phosphorylation site. Protein myristoylation enables the protein to attach to the cellular membrane and thus take part in cell signaling (Casey, P.J. 1995, Science 268, 221-225; Boutin, J. A. 11997, *Cell Signal* 9, 15-35). PAP20 is predominantly expressed in the testis. Interaction of PBR with PAP20 increased the affinity of ligand binding using PK11195 as a ligand. Therefore, it is likely that PAP20 serves to increase or decrease PBR function by modulating PBR's affinity to its endogenous ligand, DBI. The tissue distribution of PAP3 and PAP20 is shown in Figure 7 and Figure 8, respectively.

Thus, one aspect of the invention provides an isolated nucleic acid molecule comprising a polynucleotide having a nucleotide sequence selected from SEQ ID NO:1-9 encoding PAPs polypeptides. It is within the skill of a person with ordinary skill in the art to use the sequences provided herein for the

purpose of cloning cDNA or genomic sequences which encode other parts or complete portions of the PAP genes described herein and therefore, these related sequences are encompassed within the present invention.

In addition, isolated nucleic acid molecules of the invention include DNA molecules which comprise a sequence substantially different from those described above but which, due to the degeneracy of the genetic code, still encode PAPs. Of course, the genetic code and species-specific codon preferences are well known in the art. Thus, it would be routine for one skilled in the art to generate the degenerate variants described above, for instance, to optimize codon expression for a particular host (e.g., change codons in the human mRNA to those preferred by a bacterial host such as *E.coli* or plant host).

Nucleic acid molecules of the present invention may be in the form of RNA, such as mRNA, or in the form of DNA, including, for instance, cDNA and genomic DNA obtained by cloning or produced synthetically. The DNA may be double-stranded or single-stranded. Single-stranded DNA or RNA may be the coding strand, also known as the sense strand, or it may be the non-coding strand, also referred to as the antisense strand.

By "isolated" nucleic acid molecule(s) is intended a nucleic acid molecule, DNA or RNA, which has been removed from its native environment. For example, recombinant DNA molecules contained in a vector are considered isolated for the purposes of the present invention. Further examples of isolated DNA molecules include recombinant DNA molecules maintained in heterologous host cells or purified (partially or substantially) DNA molecules in solution. Isolated

RNA molecules include *in vivo* or *in vitro* RNA transcripts of the DNA molecules of the present invention. Isolated nucleic acid molecules according to the present invention further include such molecules produced synthetically.

The present invention is further directed to nucleic acid molecules encoding portions or fragments of the nucleotide sequences described herein. Fragments include portions of the nucleotide sequences of at least 10 contiguous nucleotides in length selected from any two integers, one of which representing a 5' nucleotide position and a second of which representing a 3' nucleotide position, where the first nucleotide for each nucleotide sequence is position 1. That is, every combination of a 5' and 3' nucleotide position that a fragment at least 10 contiguous nucleotide bases in length or any integer between 10 and the length of an entire nucleotide sequence minus 1.

Further, the invention includes polynucleotides comprising fragments specified by size, in nucleotides, rather than by nucleotide positions. The invention includes any fragment size, in contiguous nucleotides, selected from integers between 1- and the entire length of an entire nucleotide sequence minus 1. Preferred sizes include 20-50 nucleotides, 50-300 nucleotides useful as primers and probes. Regions from which typical sequences may be derived include but are not limited to, for example, regions encoding specific epitopes or domains within said sequence, for example, the PBR binding domain extending in SEQ ID NO:1, 2, 3, 4, and 5, potential myristoylation sites at positions 262-267 and 271-276 of SEQ ID NO:7 and five PKC phosphorylation sites at 395-396, 113-115, 255-257, 280-282, 331-333, and 339-341 of SEQ ID NO:7,

an Acyl-Co-A site at position 24-108 of SEQ ID NO:7, a nuclear localization domain at position 150-167 of SEQ ID NO:7, a troponin site at position 98-247 of SEQ ID NO:7 and an HSP90 domain at position 126-155 of SEQ ID NO:7, among others.

In another aspect, the invention provides isolated nucleic acid molecules comprising polynucleotides which hybridize under stringent hybridization conditions to a polynucleotide sequence of the present invention described above, or a specified fragment thereof. By "stringent hybridization conditions" is intended overnight incubation at 42°C in a solution comprising: 50% formamide, 5X SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5X Denhardt's solution, 10% dextran sulfate, and 20 g/ml denatured sheared salmon sperm DNA, followed by washing the filters in 0.1X SSC at about 65°C.

The sequences encoding the polypeptides of the present invention or portions thereof may be fused to other sequences which provide additional functions known in the art such as a marker sequence, or a sequence encoding a peptide which facilitates purification of the fused polypeptide, peptides having antigenic determinants known to provide helper T-cell stimulation, peptides encoding sites for post-translational modifications, or amino acid sequences which target the fusion protein to a desired location, e.g. a heterologous leader sequence.

The present invention further relates to variants of the nucleic acid molecules of the present invention, which encode portions, analogs or derivatives of the PAPs of the present invention. A variant may occur naturally, such as a natural allelic variant. By an "allelic variant" is intended one of

several alternate forms of a gene occupying a given locus of a chromosome of an organism. Non-naturally occuring variants may be produced by known mutagenesis techniques. Such variants include those produced by nucleotide substitution, deletion, or addition of one or more nucleotides in the coding or noncoding regions or both. Alterations in the coding regions may produce conservative or nonconservative amino acid substitutions, deletions, or additions. Especially preferred among these are silent substitutions, additions, and deletions which do not alter the properties and activities of PAPs polypeptides disclosed herein or portions thereof. Also preferred in this regard are conservative substitutions.

Nucleic acid molecules with at least 90-99% identity to a nucleic acid identified above is another aspect of the present invention. These nucleic acids are included irrespective of whether they encode a polypeptide having PAP activity. By "a polypeptide having PAP activity" is intended polypeptides exhibiting activity similar, but not identical, to an activity of the PAP of the invention, as measured in the assays described below. The biological acitivity or function of the polypeptides of the present invention are expected to be similar or identical to polypeptides from other organisms that share a high degree of structural identity/similarity.

In another embodiment, the present invention relates to a recombinant DNA molecule that includes a vector and a DNA sequence as described above. The vector can take the form of a plasmid, phage, cosmid, YAC, eukaryotic expression vector such as a DNA vector, *Pichia pastoris*, or a virus vector such as for example, baculovirus vectors, retroviral vectors or adenoviral vectors, and others known in the art. The

cloned gene may optionally be placed under the control of (i.e., operably linked to) certain control sequences such as promoter sequences, or sequences which may be inducible and/or cell type-specific. Suitable promoters will be known to a person with ordinary skill in the art. The expression construct will further contain sites for transcription initiation, termination and, in the transcribed region, a ribosome binding site for translation. Among the vectors preferred for use include pGBT9, PGAD10 (Clonetech), PSVzeo (Invitrogen), pBlueScript (Stratagene), pCMV5 (Invitrogen), pCRII (Invitrogen) to name a few.

Introduction of the construct into the host cell can be effected by calcium phosphate transfection, electroporation, infection, and other methods known in the art and described in standard laboratory manuals such as Current Protocols in Molecular Biology, Ausubel, F. M. *et al.* (Eds), Wiley & Sons, Inc. All documents cited herein supra and infra are hereby incorporated in their entirety by referece thereto.

In a further embodiment, the present invention relates to host cells stably transformed or transfected with the above-described recombinant DNA constructs. The host cell can be prokaryotic (for example, bacterial), lower eukaryotic (for example, yeast or insect) or higher eukaryotic (for example, all mammals, including but not limited to rat and human). Both prokaryotic and eukaryotic host cells may be used for expression of desired coding sequences when appropriate control sequences which are compatible with the designated host are used. Among prokaryotic hosts, *E. coli* is most frequently used. Expression control sequences for prokaryotes include promoters, optionally containing operator portions,

and ribosome binding sites. Transfer vectors compatible with prokaryotic hosts are commonly derived from, for example, pBR322, a plasmid containing operons conferring ampicillin and tetracycline resistance, and the various pUC vectors, which also contain sequences conferring antibiotic resistance markers. These markers may be used to obtain successful transformants by selection. Please see e.g., Maniatis, Fitsch and Sambrook, Molecular Cloning; A Laboratory Manual (1982) or DNA Cloning, Volumes I and II (D. N. Glover ed. 1985) for general cloning methods. The DNA sequence can be present in the vector operably linked to a sequence encoding an IgG molecule, an adjuvant, a carrier, or an agent for aid in purification of PAPs, such as glutathione S-transferase, or a series of histidine residues also known as a histidine tag. The recombinant molecule can be suitable for transfecting plant cells or eukaryotic cells, for example, mammalian cells and yeast cells in culture systems. *Saccharomyces cerevisiae*, *Saccharomyces carlsbergensis*, and *Pichia pastoris* are the most commonly used yeast hosts, and are convenient fungal hosts. Control sequences for yeast vectors are known in the art. Mammalian cell lines available as hosts for expression are known in the art and include many immortalized cell lines available from the American Type Culture Collection (ATCC), such as HEK293 cells , and NIH 3T3 cells, MA10 Leydig cells, mouse C6 glioma cells, Y1 adrenal cells, and breast cancer cell lines such as MDA-231, MCF-7, to name a few. Suitable promoters are also known in the art and include viral promoters such as that from SV40, Rous sarcoma virus (RSV), adenovirus (ADV), bovine papilloma virus (BPV), and cytomegalovirus

(CMV). Mammalian cells may also require terminator sequences and poly A addition sequences; enhancer sequences which increase expression may also be included, and sequences which cause amplification of the gene may also be desirable. These sequences are known in the art. The transformed or transfected host cells can be used as a source of DNA sequences described above. When the recombinant molecule takes the form of an expression system, the transformed or transfected cells can be used as a source of the protein described below.

In another embodiment, the present invention relates to the PAP polypeptides described above or any allelic variation thereof which is immunologically identifiable with the polypeptides.

A polypeptide or amino acid sequence derived from the amino acid sequences mentioned above, refers to a polypeptide having an amino acid sequence identical to that of a polypeptide encoded in the sequence, or a portion thereof wherein the portion consists of at least 2-5 amino acids, and more preferably at least 8-10 amino acids, and even more preferably at least 11-15 amino acids, or which is immunologically identifiable with a polypeptide encoded in the sequence.

A recombinant or derived polypeptide is not necessarily translated from a designated nucleic acid sequence; it may be generated in any manner, including for example, chemical synthesis, or expression of a recombinant expression system. In addition the polypeptide can be fused to other proteins or polypeptides which increase its antigenicity, such as adjuvants for example.

As noted above, the methods of the present invention are suitable for production of any

polypeptide of any length, via insertion of the above-described nucleic acid molecules or vectors into a host cell and expression of the nucleotide sequence encoding the polypeptide of interest by the host cell. Introduction of the nucleic acid molecules or vectors into a host cell to produce a transformed host cell can be effected by calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection or other methods. Such methods are described in many standard laboratory manuals, such as Davis et al., Basic Methods In Molecular Biology (1986). Once transformed host cells have been obtained, the cells may be cultivated under any physiologically compatible conditions of pH and temperature, in any suitable nutrient medium containing assimilable sources of carbon, nitrogen and essential minerals that support host cell growth. Recombinant polypeptide-producing cultivation conditions will vary according to the type of vector used to transform the host cells. For example, certain expression vectors comprise regulatory regions which require cell growth at certain temperatures, or addition of certain chemicals or inducing agents to the cell growth medium, to initiate the gene expression resulting in the production of the recombinant polypeptide. Thus, the term "recombinant polypeptide-producing conditions," as used herein, is not meant to be limited to any one set of cultivation conditions. Appropriate culture media and conditions for the above-described host cells and vectors are well-known in the art. Following its production in the host cells, the polypeptide of interest may be isolated by several techniques. To liberate the polypeptide of interest from the host cells, the cells

are lysed or ruptured. This lysis may be accomplished by contacting the cells with a hypotonic solution, by treatment with a cell wall-disrupting enzyme such as lysozyme, by sonication, by treatment with high pressure, or by a combination of the above methods. Other methods of bacterial cell disruption and lysis that are known to one of ordinary skill may also be used.

Following disruption, the polypeptide may be separated from the cellular debris by any technique suitable for separation of particles in complex mixtures. The polypeptide may then be purified by well known isolation techniques. Suitable techniques for purification include, but are not limited to, ammonium sulfate or ethanol precipitation, acid extraction, electrophoresis, immunoadsorption, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, immunoaffinity chromatography, size exclusion chromatography, liquid chromatography (LC), high performance LC (HPLC), fast performance LC (FPLC), hydroxylapatite chromatography and lectin chromatography.

The recombinant polypeptide or fusion protein can be used, detectably labeled and unlabeled, as a diagnostic tool for the detection of PAPs or for the detection and measurement of PBR. Additionally, these polypeptides can be used in a method for modulating PBR expression. In addition, the recombinant protein can be used as a therapeutic agent to reduce cell death and/or increase cell proliferation via its effect on PBR function. The transformed host cells can be used to analyze the effectiveness of drugs and agents which modulate PBR function, expression or

targeting via their effect on the expression or function of PAPs, such as host proteins or chemically derived agents or other proteins which may interact with the cell to alter the PAP function or expression, thereby modulating PBR function, expression or localization.

In another embodiment, the present invention relates to monoclonal or polyclonal antibodies specific for the above-described recombinant proteins (or polypeptides). For instance, an antibody can be raised against a peptide described above, or against a portion thereof of at least 10 amino acids, perferrably, 11-15 amino acids. Persons with ordinary skill in the art using standard methodology can raise monoclonal and polyclonal antibodies to the protein (or polypeptide) of the present invention, or a unique portion thereof. Material and methods for producing antibodies are well known in the art (see for example Goding, in, Monoclonal Antibodies: Principles and Practice, Chapter 4, 1986).

The amount of PAP expression can be detected at several levels. Using standard methodology well known in the art, assays for the detection and quantitation of PAP RNA can be designed, and include northern hybridization assays, *in situ* hybridization assays, and PCR assays, among others. Please see e.g., Maniatis, Fitsch and Sambrook, Molecular Cloning; A Laboratory Manual (1982) or DNA Cloning, Volumes I and II (D. N. Glover ed. 1985), or Current Protocols in Molecular Biology, Ausubel, F. M. *et al.* (Eds), Wiley & Sons, Inc. for general description of methods for nucleic acid hybridization. Polynucleotide probes for the detection of PAP RNA can be designed from the sequences described in SEQ ID NO: 1-9. For example,

RNA isolated from samples can be coated onto a surface such as a nitrocellulose membrane and prepared for northern hybridization. In the case of *in situ* hybridization of biopsy samples for example, the tissue sample can be prepared for hybridization by standard methods known in the art and hybridized with polynucleotide sequences which specifically recognize PAP RNA. The presence of a hybrid formed between the sample RNA and the polynucleotide can be detected by any method known in the art such as radiochemistry, or immunochemistry, to name a few.

One of skill in the art may find it desirable to prepare probes that are fairly long and/or encompass regions of the amino acid sequence which would have a high degree of redundancy in the corresponding nucleic acid sequences. In other cases, it may be desirable to use two sets of probes simultaneously, each to a different region of the gene. While the exact length of any probe employed is not critical, typical probe sequences are no greater than 500 nucleotides, even more typically they are no greater than 250 nucleotides; they may be no greater than 100 nucleotides, and also may be no greater than 75 nucleotides in length. Longer probe sequences may be necessary to encompass unique polynucleotide regions with differences sufficient to allow related target sequences to be distinguished. For this reason, probes are preferably from about 10 to about 100 nucleotides in length and more preferably from about 20 to about 50 nucleotides.

The DNA sequence of PAPs can be used to design primers for use in the detection of PAPs using the polymerase chain reaction (PCR) or reverse transciption PCR (RT-PCR). The primers can specifically bind to the PAP cDNA produced by reverse

transcription of PAP RNA, for the purpose of detecting the presence, absence, or quantifying the amount of PAP by comparison to a standard. The primers can be any length ranging from 7-40 nucleotides, preferably 10-15 nucleotides, most preferably 18-25 nucleotides homologous or complementary to a region of the PAP sequence. Reagents and controls necessary for PCR or RT-PCR reactions are well known in the art. The amplified products can then be analyzed for the presence or absence of PAP sequences, for example by gel fractionation, by radiochemistry, and immunochemical techniques. This method is advantageous since it requires a small number of cells. Once PAP is detected, a determination whether the cell is overexpressing or underexpressin PAP can be made by comparison to the results obtained from a normal cell using the same method. For example, increased PAP7 RNA levels correlate with PBR expression levels, especially in steroidogenic cells, wherein, an increase in steroidogenic capability of the cells correlates with an increase in PBR and PAP7 RNA.

In another embodiment, the present invention relates to a diagnostic kit for the detection of PAP RNA in cells, said kit comprising a package unit having one or more containers of PAP oligonucleotide primers for detection of PAP by PCR or RT-PCR or PAP polynucleotides for the detection of PAP RNA in cells by *in situ* hybridization or northern analysis, and in some kits including containers of various reagents used for the method desired. The kit may also contain one or more of the following items: polymerization enzymes, buffers, instructions, controls, detection labels. Kits may include containers of reagents mixed together in suitable proportions for performing the

methods in accordance with the invention. Reagent containers preferably contain reagents in unit quantities that obviate measuring steps when performing the subject methods.

In a further embodiment, the present invention provides a method for identifying and quantifying the level of PAP present in a particular biological sample. Any of a variety of methods which are capable of identifying (or quantifying) the level of PAP in a sample can be used for this purpose.

Diagnostic assays to detect PAPs may comprise a biopsy or *in situ* assay of cells from an organ or tissue sections, as well as an aspirate of cells from a tumor or normal tissue. In addition, assays may be conducted upon cellular extracts from organs, tissues, cells, urine, or serum or blood or any other body fluid or extract.

When assaying a biopsy, the assay will comprise, contacting the sample to be assayed with a PAP ligand, natural or synthetic, or an antibody, polyclonal or monoclonal, which recognizes PAP, or antiserum capable of detecting PAP, and detecting the complex formed between PAP present in the sample and the PAP ligand or antibody added.

PAP ligands or substrates include for example, PBR, in addition to natural and synthetic classes of ligands and their derivatives which can be derived from natural sources such as animal or plant extracts.

PAP ligands or anti-PAP antibodies, or fragments of ligand and antibodies capable of detecting PAP may be labeled using any of a variety of labels and methods of labeling for use in diagnosis and prognosis of disease associated with increased cell proliferation, such as cancer, or reduced cell death. Examples of types of labels which can be used in the

present invention include, but are not limited to, enzyme labels, radioisotopic labels, non-radioactive isotopic labels, and chemiluminescent labels.

Examples of suitable enzyme labels include malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast-alcohol dehydrogenase, alpha-glycerol phosphate dehydrogenase, triose phosphate isomerase, peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase, acetylcholine esterase, etc.

Examples of suitable radioisotopic labels include ^{3}H, ^{111}In, ^{125}I, ^{32}P, ^{35}S, ^{14}C, ^{51}To, ^{58}Co, ^{59}Fe, ^{75}Se, ^{152}Eu, ^{90}Y, ^{67}Cu, ^{217}Ci, ^{211}At, ^{212}Pb, ^{47}Sc, ^{109}Pd, ^{11}C, ^{13}F, ^{153}I, etc.

Examples of suitable non-radioactive isotopic labels include ^{157}Gd, ^{55}Mn, ^{162}Dy, ^{52}Tr, ^{56}Fe, etc.

Examples of suitable fluorescent labels include a ^{152}Eu label, a fluorescein label, an isothiocyanate label, a rhodamine label, a phycoerythrin label, a phycodyanin label, an allophycocyanin label, a fluorescamine label, etc.

Examples of chemiluminescent labels include a luminal label, an isoluminal labol, an aromatic acridinium ester label, an imidazole label, an acridinium salt label, an oxalate ester label, a luciferin label, a luciferase label,etc.

Those of ordinary skill in the art will know of other suitable labels which may be employed in accordance with the present invention. The binding of these labels to ligands and to antibodies or fragments thereof can be accomplished using standard techniques commonly known to those of ordinary skill in the art. Typical techniques are described by Kennedy, J. H., *et al.*,1976 (*Clin. Chim. Acta* 70:1-31), and Schurs, A. H.

W. M., *et al.* 1977 (*Clin. Chim Acta* 81:1-40). Coupling techniques mentioned in the latter are the glutaraldehyde method, the periodate method, the dimaleimide method, and others, all of which are incorporated by reference herein.

The detection of antibodies (or fragments of antibodies) of the present invention can be improved through the use of carriers. Well-known carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agaroses, and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention. The support material may have virtually any possible structural configuration so long as the coupled molecule is capable of binding to PAP. Thus, the support configuration may be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc. Those skilled in the art will note many other suitable carriers for binding monoclonal antibody, or will be able to ascertain the same by use of routine experimentation.

The ligands or antibodies, or fragments of antibodies or ligands of PAPs discussed above may be used to quantitatively or qualitatively detect the presence of PAP. Such detection may be accomplished using any of a variety of immunoassays known to persons of ordinary skill in the art such as radioimmunoassays, immunometic assays, etc. Using standard methodology well known in the art, a diagnostic assay can be constructed by coating on a surface (i.e. a solid support) for example, a microtitration plate or a membrane (e.g.

nitrocelluolose membrane), antibodies specific for PAP or a portion of PAP, and contacting it with a sample from a person suspected of having a PAP related disease. The presence of a resulting complex formed between PAP in the sample and antibodies specific therefor can be detected by any of the known detection methods common in the art such as fluorescent antibody spectroscopy or colorimetry. A good description of a radioimmune assay may be found in Laboratory Techniques and Biochemistry in Molecular Biology. by Work, T.S., *et al.* North Holland Publishing Company, N.Y. (1978), incorporated by reference herein. Sandwich assays are described by Wide at pages 199-206 of Radioimmune Assay Method, edited by Kirkham and Hunter, E. & S. Livingstone, Edinburgh, 1970.

The diagnostic methods of this invention can be predictive of diseases involving PBR including gallstones, atherosclerosis, Niemann-Pick C, Sitosterolemia, Dystrophy, Tumor proliferation (tumorigenesis), Schnyder's corneal crystalline dystrophy. Brain disorders include cholesterol metabolism and Alzheimer's disease, Tellurium toxicity, Smith-Lemli-Opitz syndrome, myelinization, developmental abnormalities and demyelization : Charcot-Marie-Tooth disease; Pelizaeus-Merzbacher disease, Multiple sclerosis, SLA, to name a few. Alternatively, the methods and compositions may be useful as prophylactic treatment, or in screening for compounds effective in prophylactic treatments.

The recombinant protein can be used to identify inhibitors or activators of a PAP activity which allows the identification of drugs or agents which modulate PBR activity. Using an assay as described below in the Examples, or for example, introducing a

drug or agent to a cell expressing a PAP and detecting a increase or decrease in the level of PAP RNA or protein, natural and synthetic agents and drugs can be discovered which result in a reduction or elimination, or increase in a PAP activity. Knowledge of the mechanism of action of the inhibitor or activator is not necessary as long as a decrease or increase in the activity of a PAP is detected. Inhibitors may include agents or drugs which either bind or sequester the PAP's substrate(s), such as PBR, or cofactor(s), or inhibit PAP itself, directly, for example by irreversible binding of the agent or drug to the PAP, or indirectly, for example by introducing an agent which binds the competes with PAP binding to its substrate. Activators may include cofactors necessary for proper PAP function or agents which allow a higher turnover rate of binding or release of the PAP to/from PBR or the particular PAP substrate. Agents or drugs related to this invention may result in partial or complete inhibition or various degrees of activation of PAP which may or may not result in modulation of PBR function. Inhibitors or activators of PAP activity may be used in the treatment or amelioration of conditions such as stress, cancer, neurodegenerative disorders, i.e. stroke, Alzheimer's, developmental disorders, infertility, and immune disorders.

Agents which decrease the level of PAP (i.e. in a human or an animal) or reduce or inhibit PAP activity may be used in the therapy of any disease associated with the elevated levels of PAP. Similarly, agents which increase the level of PAP or activate PAP activity may be used in the therapy of any disease associated with reduced levels of PAP. An increase or decrease in the level of PAP is determined when the

change in the level of PAP is about 2-3 fold higher or lower than the level of PAP in the normal cell, up to about 10-100 fold higher or lower than the amount of PAP in a normal cell. Agents which decrease PAP RNA include, but are not limited to, one or more ribozymes capable of digesting PAP RNA, or antisense oligonucleotides capable of hybridizing to PAP RNA such that the translation of PAP is inhibited or reduced resulting in a decrease in the level of PAP. These agents can be administered as DNA, as DNA entrapped in proteoliposomes containing viral envelope receptor proteins (Kanoda, Y. *et al.*, 1989, *Science* 243, 375) or as part of a vector which can be expressed in the target cell such that the DNA or RNA is made. Vectors which are expressed in particular cell types are known in the art, for example, for the mammary gland, please see Furth, (1997) (*J. Mammary Gland Biol. Neopl.* 2, 373) for examples of conditional control of gene expression in the mammary gland. Alternatively, the DNA can be injected along with a carrier. A carrier can be a protein such as a cytokine, for example interleukin 2, or polylysine-glycoprotein carrier. Such carrier proteins and vectors and methods of using same are known in the art. In addition, the DNA could be coated onto tiny gold beads and said beads introduced into the skin with, for example, a gene gun (Ulmer, J. B. *et al.*, 1993, *Science* 259, 1745).

Alternatively, antibodies, or compounds capable of reducing or inhibiting PAP activity, that is reducing or inhibiting either the expression, production or activity of PAP, such as antagonists, can be provided as an isolated and substantially purified protein, or as part of an expression vector capable of being expressed in the target cell such

that the PAP-reducing or inhibiting agent is produced. Similarly, compounds capable of increasing or activating PAP activity, that is increasing or activating either the expression, production, or activity of PAP, such as agonists, can be provided as an isolated and substaially purified protein, or as part of an expression vector capable of being expressed in the target cell such that the PAP-elevating or activating agent is produced. In addition, factors which affect the stability of the protein, and co-factors such as various ions, i.e. Ca2+ (Calvo, D. J. and Medina, J. E., 1993, *J. Recept. Res.* 13:975-987), or anions, such as halides or anion channel blockers such as DIDS (4,4'diisothiocyanostilbene-2,2'-disulfonic acid), an ion transport blocker (Skolnick, P., 1987, *Eur. J. Pharmacol.* 133:205-214), or factors which affect the stability of PBR such as lipids, for example, the phospholipids phosphatidylserine and phosphatidylinositol whereby the presence of the phospholipids is required for receptor activity (Moynagh, P. N. and Williams, D.C., 1992, *Biochem. Pharmacol.* 43:1939-1945) can be administered to modulate the expression and function of the PAPs and PBR. These formulations can be administered by standard routes. In general, the combinations may be administered by the topical, transdermal, intraperitoneal, oral, rectal, or parenteral (e.g. intravenous, subcutaneous, or intramuscular) route. In addition, PAP-inhibiting or PAP-activating compounds may be incorporated into biodegradable polymers being implanted in the vicinity of where drug delivery is desired, for example, at the site of a tumor or implanted so that the PAP-inhibiting or PAP-activating compound is slowly released systemically.

The biodegradable polymers and their use are described, for example, in detail in Brem *et al.*(1991) *J. Neurosurg.* 74, 441-446. These compounds are intended to be provided to recipient subjects in an amount sufficient to effect the inhibition of PAP. Similarly, agents which are capable of negatively or positively effecting the expression, production, stability or function of PAP, are intended to be provided to recipient subjects in an amount sufficient to result in the desired effect. An amount is said to be sufficient to "effect" the inhibition or induction of PAP if the dosage, route of administration, etc. of the agent are sufficient to influence such a response.

The PAPs identified in this application were discovered due to their ability to associate with PBR, and may play a role in the proper targeting, function, expression, or stability of PBR. Therefore, a method for inhibiting or reducing PBR function, or altering the localization of PBR, would include a method for dissociating PAPs from the receptor. This is possible using agents which block the site on PBR at which these PAPs associate with PBR, or alternatively, blocking the site on the PAPs which is involved in PBR-association. Such agents would include antibodies or antogonists which recognize such sites or which alter the conformation of these sites such that PAP and PBR association is inhibited or eliminated. Agents which decrease the level of PBR (i.e. in a human or an animal) or reduce or inhibit PBR activity may be used in the therapy of any disease associated with the elevated levels of PBR such as metastatic cancer, for example breast cancer, or diseases associated with increased cell proliferation or increased cholesterol transport into the cell. An increase in the level of PBR is determined when the

level of PBR in a tumor cell is about 2-3 times the level of PBR in the normal cell, up to about 10-100 times the amount of PBR in a normal cell.

Antibodies or compounds capable of reducing or inhibiting the association between PBR and PAPs can be provided as an isolated and substantially purified protein, or as part of an expression vector capable of being expressed in the target cell such that the PBR-PAP-association reducing or inhibiting agent is produced. These formulations can be administered by standard routes. In general, the combinations may be administered by the topical, transdermal, intraperitoneal, oral, rectal, or parenteral (e.g. intravenous, subcutaneous, or intramuscular) route. In addition, these compounds may be incorporated into biodegradable polymers being implanted in the vicinity of where drug delivery is desired, for example, at the site of a tumor or implanted so that the compound is slowly released systemically. The biodegradable polymers and their use are described, for example, in detail in Brem *et al.*(1991) *J. Neurosurg.* 74, 441-446.

These compounds are intended to be provided to recipient subjects in an amount sufficient to effect the inhibition of PBR/PAP association.

In line with the function of PBR in cell proliferation, agents which stimulate the function of PBR by increasing the association of PAPs to PBR, may be used in the therapy of any disease associated with a decrease of PBR, or a decrease in cell proliferation, wherein PBR is capable of increasing such proliferation, e.g. developmental retardation. PBR has also been shown to be involved in cholesterol transport, therefore, an agent or drug which results in an increase in function or stability of PBR and its associated PAPs can be used to increase cholesterol

transport into cells. Diseases where cholesterol transport is defficient include lipoidal adrenal hyperplasia, and diseases where there is a requirement for increased production of compounds requiring cholesterol such as myelin and myelination including Alzheimer's disease, spinal chord injury, and brain development neuropathy [Snipes, G. and Suter, U. (1997) Cholesterol and Myelin. *In*: Subcellular Biochemistry, Robert Bittman (ed.), vol. 28, pp.173-204, Plenum Press, New York], to name a few.

In providing a patient with any agent which modulates the expression, function, targeting, or association of PAP or PBR as discussed above, the dosage of administered agent will vary depending upon such factors as the patient's age, weight, height, sex, general medical condition, previous medical history, etc. In general, it is desirable to provide the recipient with a dosage of agent which is in the range of from about 1 pg/kg to 10 mg/kg (body weight of patient), although a lower or higher dosage may be administered.

A composition is said to be "pharmacologically acceptable" if its administration can be tolerated by a recipient patient. Such an agent is said to be administered in a "therapeutically effective amount" if the amount administered is physiologically significant. An agent is physiologically significant if its presence results in a detectable change in the physiology of a recipient patient.

The compounds of the present invention can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby these materials, or their functional derivatives, are combined in admixture with a pharmaceutically

acceptable carrier vehicle. Suitable vehicles and their formulation, inclusive of other human proteins, e.g., human serum albumin, are described, for example, in Remington's Pharmaceutical Sciences [16th ed., Osol, A. ed., Mack Easton PA. (1980)]. In order to form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain an effective amount of the above-described compounds together with a suitable amount of carrier vehicle.

Additional pharmaceutical methods may be employed to control the duration of action. Control release preparations may be achieved through the use of polymers to complex or absorb the compounds. The controlled delivery may be exercised by selecting appropriate macromolecules (for example polyesters, polyamino acids, polyvinyl, pyrrolidone, ethylenevinylacetate, methylcellulose, carboxymethylcellulose, or protamine sulfate) and the concentration of macromolecules as well as the method of incorporation in order to control release. Another possible method to control the duration of action by controlled release preparations is to incorporate the compounds of the present invention into particles of a polymeric material such as polyesters, polyamino acids, hydrogels, poly(lactic acid) or ethylene vinylacetate copolymers. Alternatively, instead of incorporating these agents into polymeric particles, it is possible to entrap these materials in microcapsules prepared, for example, interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly(methylmethacrylate)microcapsules, respectively, or in colloidal drug delivery systems, for example, liposomes, albumin microspheres, microemulsions,

nanoparticles, and nanocapsules or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences (1980).

The present invention also provides kits for use in the diagnostic or therapeutic methods described above. Kits according to this aspect of the invention may comprise one or more containers, such as vials, tubes, ampules, bottles and the like, which may comprise one or more of the compositions of the invention.

The kits of the invention may comprise one or more of the following components, one or more compounds or compositions of the invention, and one or more excipient, diluent, or adjuvant.

It will be readily apparent to one of ordinary skill in the relevant arts that other suitable modifications and adaptations to the methods and applications described herein are obvious and may be made without departing from the scope of the invention or any embodiment thereof. Having now described the present invention in detail, the same will be more clearly understood by reference to the following examples, which are included herewith for purposes of illustration only and are not intended to be limiting of the invention.

The following Materials and Methods were used in the Examples desribed below.

Material and Methods

Materials

[α-^{32}P]dCTP (specific actifity, 3000 Ci/mmol], [1,2,6,7-N-^{3}H]progesterone (specific activity, 94.1 Ci/mmol) and ^{3}H-1-(2-chlorophenyl)-N-methyl-N-(1-methyl-propyl)-3-isoquinolinecarboxamide (PK 11195) (specific activity, 86.9 Ci/mmol) were obtained from

NEN Life Science Products (Boston, MA). PK11195 was obtained from Research Biochemicals, Inc. (Natick, MA). Nitrocellulose (0.45 μm) was from Hoeffer Scientific (San Francisco, CA). 22R Hydroxycholesterol was purchased from Sigma. Restriction enzymes were from Stratagene (La Jolla, CA) and New England Biolabs (Beverly, MA). Cell culture supplies were purchased from from Life Technologies, Inc. (Grand Island, NY). Tissue culture plasticware was from Corning (Corning, NY). Electrophoresis reagents and materials were supplied from BioRad. All other chemicals used were of analytical grade and were obtained from various commercial sources.

Strains and media

The genotype of the *Saccharomyces cerevisiae* reporter strain HF7c is *MATa, ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3, 112, gal4-542, gal80-538, LYS2::GAL-HIS3, URA3::(GAL4 17-mers)$_3$-CYC1-lacZ* (CLONTECH, Palo Alto, CA). Yeast strains were grown at 30°C in standard liquid YPD medium or minimal SD synthetic medium with appropriate supplement amino acids (CLONTECH, Palo Alto, CA).

Plasmids and construction

The mouse PBR cDNA coding sequence was subcloned into pGBT9 (CLONTECH, Palo Alto, CA) at *EcoR I* and *BamH I* sites (pGBT-PBR). The fusion site was verified by sequencing. Functional fusion PBR protein, expressed in yeast cells, was verified by PBR ligand binding assay. Mouse testis cDNA library was constructed in pGAD10 [LEU2, GAL4 (768-881)] (CLONTECH, Palo Alto, CA). Amplification of premade libraries was performed by growing the transformants on LB-agar-ampicillin and purifying the plasmids DNA

with QIAGEN Plasmid Giga kit (QIAGEN, Valencia, CA). In the transfection experiments, PAP7 partial sequence (including 192 Amino acids C-terminal sequence) was inserted into pSVzeo vector (Invitrogen, Carlsbad , CA) at *EcoRI* and *BamHI* sites.

Yeast two-hybrid screening

The Clontech MATCHMAKER two-hybrid system was applied in this study (detailed in manufacturer's instruction book). Briefly, the yeast reporter host strain HF7c was simultaneously cotransformed with both pGBT-PBR and the mouse testis cDNA library in pGAD10 plasmid by using lithium acetate high-efficiency method (Gietz, D. et al., 1992, *Nucleic Acids Res.* 20, 1425). HIS positive clones were further selected by colony lift filter assay for β-galactosidase activity. Plasmid DNA was rescued in Escherichia coli DH5? from yeast cells. Plasmids were retransformed into yeast HF7c cells with plasmid pGBT-PBR to test for histidine prototrophy and β-galactosidase activity (Clontech manual). The cDNA inserts from the positive clones were sequenced. The full length PAP7 cDNA was obtained by using 5' and 3' RACE kit from kit (CLONTECH, Palo Alto, CA).

Sequence analysis

The ABI PRISMTM dyes terminator cycle sequencing ready reaction kit (PE Biosystems, Foster City , CA) and an Applied Biosystems sequencer were used for sequencing (Applied Biosystems, Foster City , CA) at the Lombardi Cancer Center Sequencing Core Facility (Georgetown Unviersity). DNA sequences were analyzed by using Entrez and BLAST program against GeneBankTM Database.

Cell culture transient transfection

MA-10 cells were grown in modified Waymouth's MB752/1 medium containing 15% horse serum, as described previously (Papadopoulos, V. et al., 1990, *J. Biol. Chem.* 265, 3772-3779). Mouse C6 glioma and mouse Y1 adrenal cortical cells were cultured in DMEM and DMEM F12 repectively, with 10% fetal bovine serum. MA10 cells were transiently transfected by electroporation (El Hefnawy, T. et al., 1996, *Mol. Cell Endocrinol.* 119, 207-217). Each Genepulser cuvette (0.4 cm-gap, BioRad, Hercules, CA) contained $8x10^6$ cells in 350 µl antibiotic-free complete Waymouth's growth medium (see above), plus 30µg plasmids DNA in 50 µl of 0.1 x TE. Cells in electroporation cuvettes were electro-shocked at 330 V and at a capacitance of 950 µFd generated from Genepulser (BioRad, Hercules, CA). The cells were kept immediately on ice for 10 min before plated into 96 well plates.

Radioligand binding assays

^{3}H-1-(2-chlorophenyl)-N-methyl-N-(1-methyl-propyl)-3-isoquinolinecarboxamide (PK 11195) (NEN, Boston, MA) binding studies were performed as we previously described (Papadopoulos, V. et al., 1990, supra; Garnier, M. et al., 1994, *Molecular Pharmacology* 45, 201-211). The dissociation constant (Kd) and the number of binding sites (Bmax) were determined by Scatchard plot analysis of the data using the LIGAND program (Munson, P. J. and Rodbard, D., 1980, *Anal. Biochem.* 107, 220-239).

RNA (Northern) blot analysis

Total tissue and cellular RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method using RNA STAT60 reagent (Tel-Test

Inc., Friendswood, TX). RNA was separated by denature electrophoresis and transfered to Nytran membrane (Schleicher & Schuell Inc., Keene, NH). The RNA blots were hybridized with ^{32}P labeled PAP7 cDNA probe generated from random priming (Boehringer Mannheim, Indianapolis, IN). Autoradiography was performed by exposing Kodak X-Omat AR films (Eastman Kodak, Rochester, NY) to the blots at -80°C overnight.

Steroid biosynthesis

MA-10 cells were plated into 96-well plate at the density of $2.5x10^4$/well for overnight. The cells were stimulated with 50 ng/ml hCG in 0.2 ml/well serum-free medium for 2 hours. The culture medium was collected and tested for progesterone production by RIA. The assay was carried out by using anti-progesterone antisera (ICN, Costa Mesa, CA), following the conditions recommended by the manufacturer. The progesterone production was normalized by the amount of protein in each well. Radioimmunoassay data was analyzed using the software provided by Wallac (EG&G Wallac, Gaithersburg, MD).

Antibody generation and Western analysis

Rabbit anti-PAP7 antibody was prepared by sequential immunization with a peptide SSDEEEEEEENVTCEEKAKKNANKP (SEQ ID NO:11) of PAP7 protein, which was coupled to KLH. PAP7 antibodies were purified by an affinity resin containing the same peptide immobilized onto agarose (Bethyl Laboratories, Montgomery, TX). MA10 cells were solubilized in sample buffer (25 mM Tris-HCl (pH6.8), 1% SDS, 5% β-mercaptoethanol, 1 mM EDTA, 4% glycerol, and 0.01% bromophenol blue), boiled for 5 min, and loaded onto a 15% SDS-PAGE minigel (MiniProtein II System, BioRad, Richmond, CA). Electrophoresis was performed at 25

mA/gel using a standard SDS-PAGE running buffer (25 mM Tris, 192 mM glycine, and 0.1% SDS). The proteins were electrophoretically transferred to nitrocellulose membrane (Schleicher & Schuell Inc., Keene, NH). The membrane was incubated in blocking buffer (TTBS buffer (20 mM Tris/HCl, pH 7.5, 0.5M NaCl, and 0.05% Tween-20) containing 10% Carnation nonfat milk) at room temperature for 1 hour, followed by incubation with a primary antibody against PAP7 (1:2000) for 2 hours. The membrane was washed with TTBS three times for 10 min each time. After one-hour incubation with the secondary antibody, goat anti-rabbit IgG conjugated with HRP (Signal Transduction), the membrane was washed with TTBS three times for 10 min each time. Specific protein bands were detected by chemiluminescence using the Renaissance Kit (DuPont-New England Nuclear, Wilmington, DE) according to manufacturer's directions.

Immunocytochemistry

MA-10 cells were cultured on four-chambered SuperCell Culture Slides (Fisher Scientific, Pittsburgh, PA) and fixed with methanol at 4°C for 15 min. The fixed cells were incubated with PAP7 antibody (1:250 dilution) with or without PAP7 peptide for 1 hour. After washing, the cells were incubated with HRP conjugated goat anti-rabbit secondary antibody (Transduction Lab, Lexington, KY) for 1 hour. PAP7 staining was visualized with peroxidase using AEC (3-amino-9-ethyl carbazole) as a chromogen to yield a red reaction product. After counterstaining with hematoxylin, slides were dehydrated and permanently mounted.

Immunohistochemistry

Mouse tissues were freshly snapped in liquid nitrogen. Specimens were fixed in cold methanol right after sectioning for 5 min. The slides were then placed in a chamber containing 0.3% H_2O_2 solution in methanol for 20 min at room temperature to inhibit the endogenous peroxidase activity and then incubated in blocking solution (10% goat serum) (Zymed, South San Francisco, CA) for 15 min. Subsequently, the slides were incubated with anti-PAP7 antibody (1:250) for 2 h at room temperature, washed with water and PBS, incubated with HRP conjugated goat anti-rabbit secondary antibody for 1 hour at room temperature, and then washed with PBS. After treatment with AEC reagent for 1 hr at 37°C for color staining, the sections were counterstained with hematoxylin, dehydrated and permanently mounted.

Protein quantification ans Statistical analysis

Proteins were quantified by dye-binding assay of Bradford (Bradford, M. M., 1976, *Anal. Biochem.* 72, 248-254) with bovine serum albumin as the standard. Statistical analysis was performed by ANOVA followed by the Student-Newman-Keuls test or the Dunnett multiple comparisons test using the Instat (v.2.04) package from GraphPad, Inc. (San Diego, CA).

Example 1

Isolation of PBR Associated Proteins

We have used the MATCHMAKER Two-Hybrid System from CLONTECH in order to clone genes whose products interact with PBR protein. GAL4 (1-147)-PBR fusion (plasmid pGBT9 + PBR) was used as a bait to screen a mouse MATCHMAKER testis cDNA library constructed into the pGAD10 two-hybrid vector. About $3x10^6$ transformants were tested, and five positive clones were obtained for their ability to interact with PBR.

Library plasmids from these transformants were rescued in E. coli strain DH5α. Both the His+ phenotype and the expression of ß-galactosidase were confirmed by a second-round transformation of strain HF7c carrying pGBT9-PBR (Table 1).

Table 1: Summary of yeast two hybrid screen of mouse testis library by PBR

Clone	His3	ß-Galactosidase activity
PAP3	+	++
PAP7	+	++
PAP20	+	++
Positive control	+	+++

Plasmids from these positive clones were first analyzed by restriction enzyme digestion and followed by sequence analysis. Two clones were shown to be coded by single gene, which was an unknown gene and was named as PBR associated protein 7 (PAP7). The other three clones encoded different products. After 5' RACE and 3' RACE, PAP7 cDNA clone was completely sequenced on both strands (SEQ ID NO:2) and it encoded a 463-amino acid protein with a calculated molecular weight of about 52 kDa. A homology search in the Genebank database using the BLAST program showed that this is a new sequence, previously unidentified.

Example 2

PAP7 protein expression in MA-10 Leydig tumor cells

The total MA-10 cell protein extracts were analyzed by western blot using PAP7 antibody. This antibody specifically recognizes a 50 kDa-protein band (Fig. 1A). The PAP7 protein expression in MA-10 cell

was also checked by immunocytochemistry. PAP7 antibody specifically stained MA-10 cell, with the signal mostly localized in the cytoplasm (Figure 1B). This signal can be neutralized by PAP7 peptide, which was used to generate and purify this antibody.

Example 3

PAP7 cells and tissue expression by Dot and Northern Blot

By dot bot analysis, PAP7 was observed to be highly expressed in brain, eye, submax gland, testis, and ovary. Interestingly, PAP7 expression was at its highest level at early embryonic stage, and decreased before birth (Fig.2A and 2B). Consistently, PAP7 mRNA was expressed in adrenal, brain, heart, liver, testis and ovarian tissues by Northern blot analysis. PAP7 had a 1 kb transcript which was only expressed in testis and a 3 Kb major mRNA transcript in the other tissues (Fig. 3A and 3B). PAP7 was also highly expressed in three cell lines, C6 glioma, MA-10 Leydig cells and Y1 adrenal cells, which have been widely used for studying steroid biosynthesis. All three cell lines expressed PAP7 transcript of the same molecular weight size as in normal tissues. The PAP7 expression level in these cell lines was proportionally correlated with their steroidogenic capability (Fig. 3A and 3B). The PBR mRNA expression level was also checked in these same tissues and cell lines. The PBR expression level was parallel with PAP7 mRNA expression pattern, especially in those three cell lines (Fig. 4A and 4B).

Example 4

PAP7 Cellular distribution

PAP7 protein expression in different tissues was checked by immunohistochemistry (Fig 5). PAP7 was

present in both Leydig and germ cells in testis (Figure 5C and 5D), in hypocampus and neuronal cells in brain (Figure 5E and 5F), in fasciculata cells in adrenal gland (Figure 5G and 5H), and in granulosa cells in ovary (Figure 5A and 5B). Liver and kidney expressed low level of PAP7 protein (data not shown). Each specimen was stained with PAP7 peptide neutralized antibody as a negative control. Subsequent in situ hybridization studies showed that PAP7 mRNA followed PAP-7 protein expression.

Example 5

The effect of PAP7 on steroid biosynthesis in MA-10 cell

PAP7 partial sequence including PBR binding domain was subcloned into pSVzeo mammalian expression vector. This pSvzeoPAP7 vector was transiently transfected into MA-10 cells. pSVzeo empty vector was also transfected into cells as control. The capability of steroid biosynthesis of both empty vector pSVzeo transfectants and pSVzeoPAP7 transfectants was checked by monitoring the progesterone production in response to hormonal (hCG) stimulation. PAP7 transfectants had significantly reduced the level of progesterone production in MA-10 cells as compared with pSVzeo vector transfectant at a dose and time dependent manner (Fig 6).

Discussion

In order to better understand the mechanism of how PBR regulates cholesterol transport activity in steroid biosynthesis, we performed yeast two-hybrid assay to identify the PBR associated protein(s). Mouse PBR cDNA was inserted into the pGBT9 vector to generate a GAL4 DNA-binding domain and PBR fusion

protein as bait. The receptor ligand binding activity of the fusion protein with PBR ligand, PK11195, was tested. Our result indicated that the fusion PBR protein expressed in yeast possessed the similar binding affinity as the native PBR protein (data not shown). PBR has been identified in various peripheral tissues (Gavish, M. and Weizman, R., 1997, *Clin. Neuropharmacol.* 20, 473-481) including testis. Testis is one of the important and very well studied tissues for steroidogenesis (Huhtaniemi, I. and Toppari, J., 1995, *Adv. Exp. Med. Biol.* 377, 33-54). The steroid biosynthesis in the mouse testicular Leydig cell and the role of PBR in this process are also well documented(Papadopoulos, V. et al., 1997, *Steroids* 62, 21-28; Papadopoulos, V. et al., 1998, *Endocr. Res.* 24, 479-487). Therefore, we applied mouse testis cDNA library in this two-hybrid screen study. The pGAD10 vector was used to generate a fusion protein of the GAL4 activation domain with a collection of random proteins in the fusion Balb/c mouse testis library. Through the yeast two hybrid screen, PAP7 was identified as one of the positive clones, which demonstrated its ability to interact with PBR (Table 1). Thus, we cloned the PAP7 cDNA coding for a mouse protein that interacted with PBR. Based on the database search, PAP7 is a novel gene product. Recently, PRAX-1 was reported as a new protein that specifically interates with PBR (Galiegue, S. et al., 1999, *J. Biol. Chem.* 274,2938-2952). The only similarity is that both proteins contain glutamic-acid streches. Part of PAP7 shares quite high homology with a *C. elegans* gene that has an unknown function (Wilson, R. et al., 1994, *Nature* 368, 32-38). In fact, cholesterol is required for *C. elegans* cell culture (Brenner, S., 1974, *Genetics* 77, 71-94).

Considering that PBR is involved in cholesterol transport and PBR gene is highly conservative in all type of organisms, this data suggests that PAP7 expression may be needed to meet basic requirements for cell survival and growth. PAP7 also shares some homologies with RALBP, a hydrophobic ligand-binding protein that functions in intracellular retinoid transport (Ozaki, K. et al., 1994, *J. Biol. Chem.* 269, 3838-3845).

By sequence motif analysis using Swiss-Port Prosite profile scan, PAP7 has fatty acylation (myristoylation) sites, Acyl-CoA-binding protein signature and PKC phosphorylation sites. Protein myristoylation enables protein to attach to the cellular membrane and thus take part in cell signaling (Casey, P.J., 1995, Science 268, 221-225; Boutin, J. A., 1997, *Cell Signal* 9, 15-35). PBR is a hydrophobic protein and tightly associated with the outer mitochondrial membrane. This property could enable PAP7 passing hormone stimulation signal to and interacting with PBR thus regulating PBR activity in cholesterol transport. Interestingly, Acyl-CoA-binding protein is the other name of PBR endogenous ligand, diazepam binding inhibitor (DBI)(Rose, T. M. et al., 1992, *Proc. Natl. Acad. Sci. U.S.A.* 89, 11287-11291; Costa, E. and Guidotti, A., 1991, Life Sci. 49, 325-344; Suk, K. et al., 1999, *Biochim. Biophys. Acta* 1454, 126-131). This information suggests that PAP7 may coordinate with other PBR endogenous ligands to fulfill its function. The fact that PAP7 has the potential protein kinase phosphorylation sites raises another possibility that PBR could be regulated by hormone stimulation through the interaction with PAP7 protein.

The distribution and expression of PAP7 were examined in major mouse tissues such as brain, testis, ovary, adrenal, and kidney, as well as some cell lines. The PAP7 expressing pattern is similar to the broader expression profile of PBR. According to the previous studies (Papadopoulos, V. et al., 1998, supra), glucocorticoids are produced by zona fasciculata cells in adrenal. In the ovary, coupus luteum, where the granulosa cells are located, secretes progesterone. In addition, the testicular Leydig cells are able to produce testosterone. Since PAP7 is highly expressed in major steroidogenic tissues and is more concentrated in these steroid producing cells, PAP7 may be involved in steroid biosynthesis or the regulation of steroid formation by changing the formation or the conformation of the PBR complex. Mouse C6 glioma cells, MA-10 Leydig cells and Y1 adrenal cortical cells are popular cell models selected for studying steroid biosynthesis. PAP7 expression is proportionally correlated to the PBR expression in these cell lines. Additionally, both PBR and PAP7 expression level in these cell lines are parallel with their steroidogenic capability, which also suggests that PAP7 may be involved in steroid biosynthesis through a PBR pathway. A small PAP7 transcript was expressed only in testis, a phenomenon observed for other genes expressed in testis (Zhang, F. P. et al., 1997, Endocrinology 138, 2481-2490; Mauduit, C. et al., 1999, *J. Biol. Chem.* 274, 770-775). The immunostaining in the testis other than in Leydig cells could represent the expression of the smaller transcript.

The PAP7 protein is expressed in MA-10 cells and most of the staining is localized in the cytoplasm. The study of PAP7 subcellular distribution is ongoing,

the results of which may provide more detailed information about the interaction between PBR and PAP7. Since PBR knockout mice die in uterus indicates an essential role for PBR essential in mouse embryonic development. Interestingly, PAP7 mRNA is highly expressed during mouse early embryonic development. This result may suggest that PAP7, associated with PBR, could play an important role during early mouse development. This further implies that PBR may have new functions beyond steroidogenesis. Overexpression of PAP7 fragment including its PBR binding domain significantly inhibited the progesterone formation stimulated by saturating concentrations of hCG (50 ng/ml) in MA-10 cells. According to previous studies, progesterone production of these cells represented the index of steroid biosynthesis (Freeman, D. A., 1987, *Endocrinology* 120, 124-132; Garnier, M. et al., 1994, *J. Biol. Chem.* 269, 22105-22112). Based on the inhibitory manner, we assume that the overexpressed PAP7 fragment might act as a competitor of the native PAP7 in MA-10 cells and competitively bind to PBR. We believe that the tranfected PAP7 fragment having only the PBR binding domain and is not fully functional as a native PAP7, however, it competitively prevents PBR from interacting with the endogenous PAP7 and thereby blocks the normal function of PBR.

In conclusion, the results presented herein suggest that the identified PAP7 is involved in the regulation of the PBR function, serving as an endogenous ligand or allosteric modulator of the receptor.

Considering the findings tha i) PBR is a channel/transporter of cholesterol, ii) PBR is the target of environmental antisteroidogenic hazards, and (iii) PER is involved in breast cancer aggression and

tumor cell proliferation (Hardwick, M. et al., 1999, *Cancer Res.* 59, 831-842), we believe that the identification and characterization of PAP7 will greatly contribute to the understanding of the role of PBR in steroidogenesis and even in more general areas.

What is claimed is:

1. An isolated PBR-associated protein (PAP) DNA fragment or any portion thereof.

2. An isolated and purified DNA fragment which encodes a PBR-associated protein.

3. An isolated and purified DNA fragment which encodes a peptide of PBR-associated protein, said DNA fragment comprising a sequence specified in any of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5, or polynucleotide fragment of said sequence comprising at least 30 nucleotides.

4. An isolated and purified DNA fragment which encodes a peptide of PBR-associated protein, said DNA fragment comprising a sequence specified in Genbank Accession no. AF022770, or GenBank Accession no. AF020338, or a polynucleotide fragment of said sequence comprising at least 30 nucleotides.

5. An isolated and purified PAP7 DNA fragment according to claim 2 which encodes 463 amino acids of PAP7 or a natural variant or synthetic variant thereof encoding PAP7, or a peptide fragment thereof comprising at least 10 amino acids.

6. An isolated and purified PAP8 DNA fragment according to claim 2 which encodes 190 amino acids of PAP8 or a natural variant or synthetic variant thereof encoding PAP8, or a peptide fragment thereof comprising at least 10 amino acids.

7. An isolated and purified PAP15 DNA fragment according to claim 2 which encodes 164 amino acids of PAP15 or a natural variant or synthetic variant thereof encoding PAP15, or a peptide fragment thereof comprising at least 10 amino acids.

8. An isolated and purified PAP20 DNA fragment according to claim 2 which encodes 196 amino acids of PAP20 or a natural variant or synthetic variant

thereof encoding PAP20, or a peptide fragment thereof comprising at least 10 amino acids.

9. A recombinant DNA construct comprising:
(i) a vector, and
(ii) the PAP DNA fragment of claim 1.

10. A recombinant DNA construct comprising:
(i) a vector, and
(ii) the PAP DNA fragment of claim 3.

11. A recombinant DNA construct according to claim 10, wherein said vector is an expression vector.

12. The recombinant DNA construct according to claim 10, wherein said vector is a prokaryotic vector.

13. The recombinant DNA construct according to claim 10, wherein said vector is a eukaryotic vector.

14. A host cell transformed with a recombinant DNA construct according to claim 10.

15. A host cell according to claim 14, wherein said cell is prokaryotic.

16. A host cell according to claim 14, wherein said cell is eukaryotic.

17. A method for producing PAP peptide which comprises culturing the cells according to either claim 15 or 16, under conditions such that said DNA fragment is expressed and said PAP peptide is thereby produced.

18. An isolated recombinant PAP produced by the method of claim 17.

19. A PAP7 polypeptide comprising the amino acid sequence specified in SEQ ID NO:7 or a portion thereof of at least 5 amino acids.

20. A PAP8 polypeptide comprising the amino acid sequence specified in SEQ ID NO:8 or a portion thereof of at least 5 amino acids.

21. A PAP15 polypeptide comprising the amino acid sequence specified in SEQ ID NO:9 or a portion thereof of at least 5 amino acids.

22. A PAP20 polypeptide comprising the amino acid sequence specified in SEQ ID NO:10 or a portion thereof of at least 5 amino acids.

23. A method for detecting a PAP in a sample chosen from the group consisting of: PAP7 , PAP8, PAP15, PAP20, said method comprising

(i) contacting said sample with antibodies which recognize said PAP; and

(ii) detecting the presence or absence of a complex formed between PAP and antibodies specific therefor.

24. A method for detecting a PBR-associated protein, said method comprising the two hybrid assay.

25. An antibody to a peptide having the amino acid sequence specified in SEQ ID NO:6, 7, 8 and 9, or any portion thereof.

26. A PAP7 antibody to a peptide comprising the amino acid sequence specified in SEQ ID NO:11.

27. A method for detecting agents or drugs which reduce or eliminate PAP activity, said method comprising:

(i) delivering a recombinant DNA construct according to claim 10 into a cell such that PAP is produced in said cell;

(ii) adding at least one drug or agent to said cell alone or in combination; and,

(iii) detecting PAP activity in said cell in the presence of said agent or drug and comparing it to a control which did not receive said drug or agent wherein a decrease in PAP activity as compared to control indicates an drug or agent which reduces or eliminates PAP activity.

28. A method for detecting agents or drugs which promote PAP activity, said method comprising:

(i) delivering a recombinant DNA construct according to claim 10 into a cell such that PAP is produced in said cell;

(ii) adding at least one drug or agent to said cell alone or in combination; and,

(iii) detecting whether or not said drug or agent stimulates PAP activity by measuring PAP acitivity in said cell and comparing it to a control which did not receive said drug or agent wherein an increase in the activity of said PAP in said cell as compared to control indicates a stimulatory drug or agent.

29. An agent or drug capable of inhibiting PAP activity.

30. An agent or drug capable of promoting PAP activity.

31. A therapeutic compound comprising said agent or drug according to claim 29 for use in a disease wherein a decrease or elimination of PAP activity is beneficial.

32. A therapeutic compound comprising said agent or drug according to claim 30 for use in a disease wherein an increase o PAP activity is beneficial.

33. A method for detecting at least one PAP selected from the group consisting of PAP7, PAP8, PAP15, and PAP20 in a sample using the polymerase chain reaction.

34. A diagnostic kit for detecting RNA/cDNA of at least one PAP chosen from the group consisting of PAP7, PAP8, PAP15 and PAP20, in a sample comprising primers or oligonucleotides specific for said PAP RNA or cDNA suitable for hybridization to PAP RNA or cDNA and/or amplification of PAP sequences and suitable ancillary reagents.

35. A method for increasing a PAP selected from the group consisting of PAP3, PAP7, PAP8, PAP15 and PAP20 in a cell by introducing into said cell a PAP nucleic acid encoding said PAP such that said nucleic acid is expressed and PAP is produced in said cell.

36. A therapeutic method for the treatment or amelioration of diseases resulting from an increase in cell proliferation, said method comprising providing to an individual in need of such treatment an effective amount of an agent or drug which reduces or eliminates PAP expression or function in a pharmaceutically acceptable diluent.

37. The method of claim 36 wherein said disease is cancer.

38. A therapeutic method for the treatment or amelioration of conditions resulting from abnormal cholesterol level, said method comprising providing to an individual in need of such treatment an effective amount of an agent or drug which reduces or eliminates PAP expression or function in a pharmaceutically acceptable diluent.

39. The method of claim 38 wherein said condition is selected from the group consisting of: cancer, neurodegenerative disorders, developmental disorders, stress, and stroke.

40. A method for modulating PBR activity, function or targeting in a cell, said method comprising increasing or decreasing level of PAP in said cell.

FIG.1A

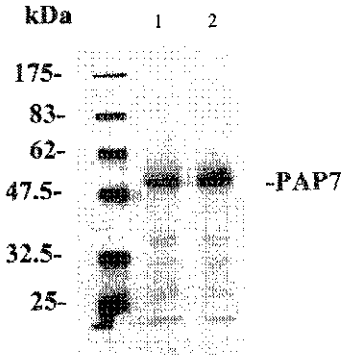


FIG.1B

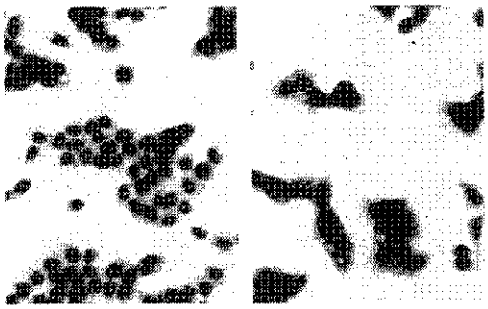
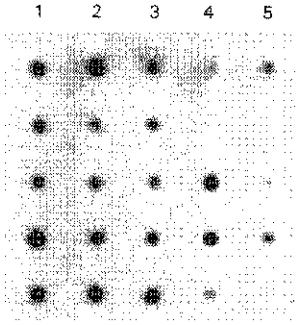


FIG.2A



	1	2	3	4	5
A	brain	eye	liver	lung	kidney
B	heart	skeletal muscle	small intes.		
C	pancreas	thyroid	thymus	submax. gland	spleen
D	testis	ovary	prostate	epidi-dymus	uterus
E	embryo 7 days	embryo 11 days	embryo 15 days	embryo 17 days	

FIG.2B

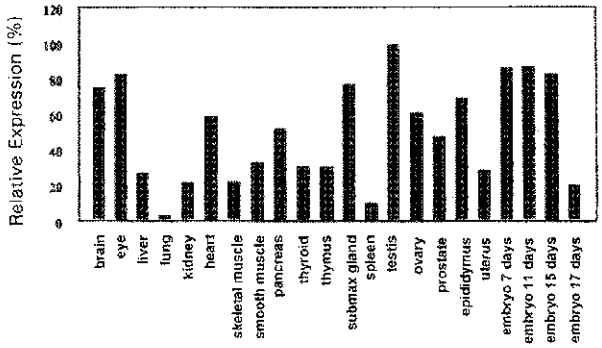


FIG.3A

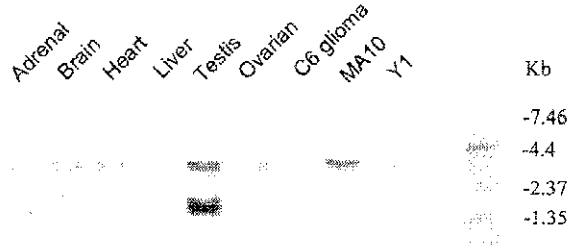

Adrenal
Brain
Heart
Liver
Testis
Ovarian
C6 glioma
MA10
Y1
Kb
-7.46
-4.4
-2.37
-1.35

FIG.3B

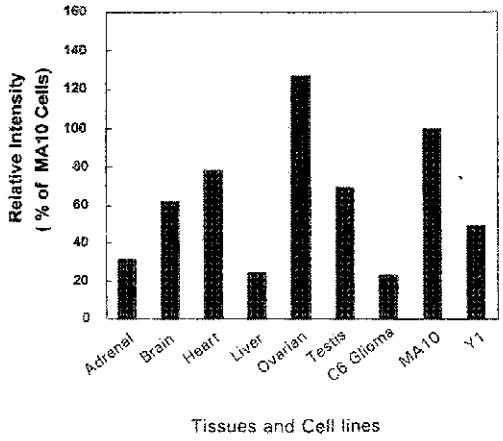

Relative Intensity
(% of MA10 Cells)
160
140
120
100
80
60
40
20
0
Adrenal
Brain
Heart
Liver
Ovarian
Testis
C6 Glioma
MA10
Y1
Tissues and Cell lines

FIG.4A

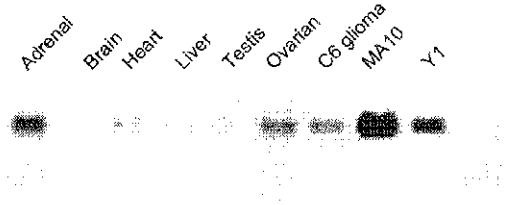

Adrenal
Brain
Heart
Liver
Testis
Ovarian
C6 glioma
MA10
Y1

FIG.4B

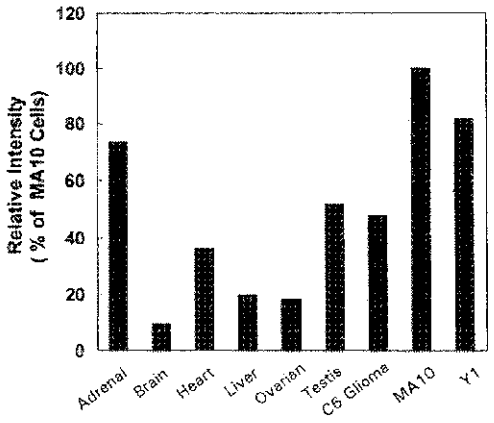

Relative Intensity
(% of MA10 Cells)
120
100
80
60
40
20
0
Adrenal
Brain
Heart
Liver
Ovarian
Testis
C6 Glioma
MA10
Y1
Tissues and Cell lines

FIG. 5A

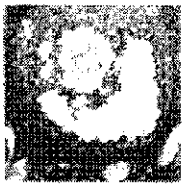


FIG. 5B

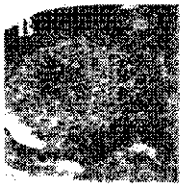


FIG. 5C

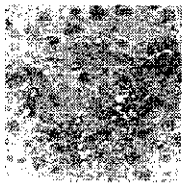


FIG. 5D

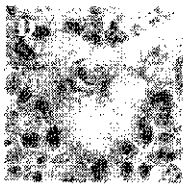


FIG. 5E

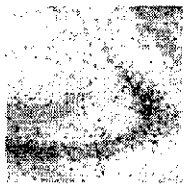


FIG. 5F

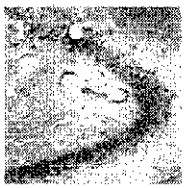


FIG. 5G

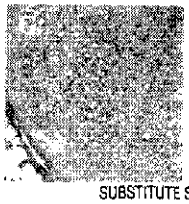


FIG. 5H

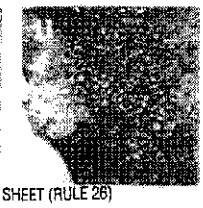


FIG. 6A

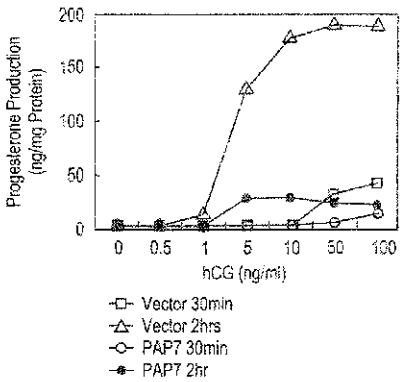

Progesterone Production (ng/mg Protein)
200
150
100
50
0
0 0.5 1 5 10 50 100
hCG (ng/ml)
Vector 30min
Vector 2hrs
PAP7 30min
PAP7 2hr

FIG. 6B

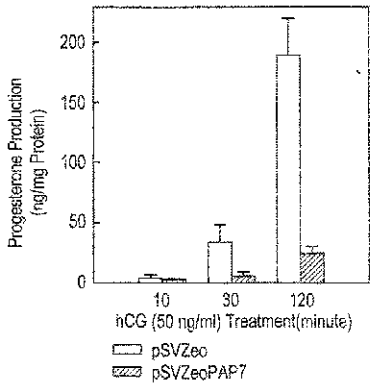
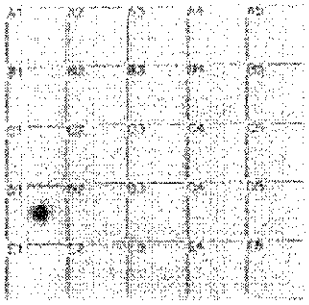

Progesterone Production (ng/mg Protein)
200
150
100
50
0
10 30 120
hCG (50 ng/ml) Treatment(minute)
pSVZeo
pSVZeoPAP7

FIG. 7A



	1	2	3	4	5
A	brain	eye	liver	lung	kidney
B	heart	skeletal muscle	small intes.		
C	pancreas	thyroid	thymus	submax gland	spleen
D	testis	ovary	prostate	epidi-dymus	uterus
E	embryo 7 days	embryo 11 days	embryo 15 days	embryo 17 days	

FIG. 7B

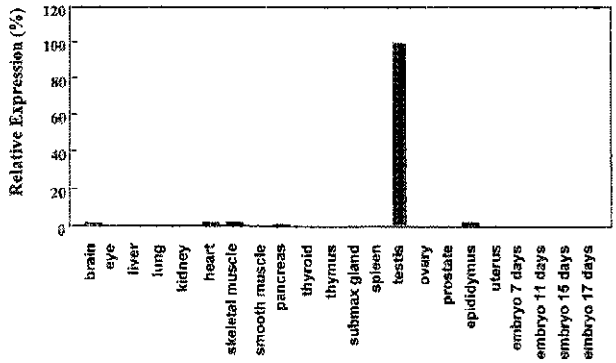
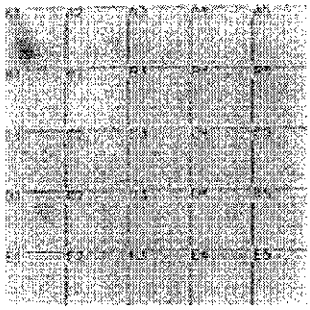
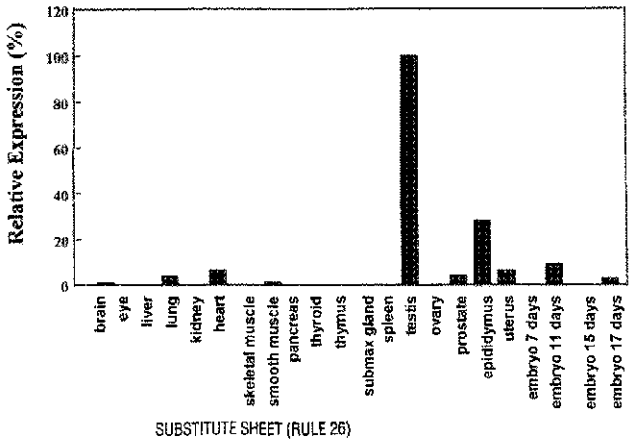


FIG.8A



	1	2	3	4	5
A	brain	eye	liver	lung	kidney
B	heart	skeletal muscle	small intes.		
C	pancreas	thyroid	thymus	submax gland	spleen
D	testis	ovary	prostate	epidi-dymus	uterus
E	embryo 7 days	embryo 11 days	embryo 15 days	embryo 17 days	

FIG.8B



SEQ ID NO:1
PAP3 nucleic acid
GAATTCGCGGCCGCGTCGACCACCGCTGCGCCCTCCTGCGAGGCCGGCTGAACGAGGAAATA
ATTGCTAATAAGGCCTCTGTAGCCATGGCTACTTCTGACGTGAAACCAAAATCAATAAGTCGTG
CCAAGAAATGGTCAGAGGAAATAGAAAATCTGTACAGATTTCAACAAGCAGGATATCGGGATG
AAATTGAATATAAACAAGTGAAACAAGTTGCCATGGTCGACCGATGGCCAGAGACAGGGTACG
TGAAGAAACTTCAGCGGAGGGACAATACTTTCTTCTACTACAACAAAGAGAGGGAGTGCGA
GGACAAGGAGGTCCACAAAGTGAAGGTTTACGTCTACTGACCTTTTCCTTTCTTCGGCTTG
GCAATGCTCCTTTAAGAATTGGTTGTTTACATTCTTCCATCGTGTAAATGTCATTTTACAA
AACAATTCACAATTCTGTCTTTAATTCATGGTGTCTTACACAACATAAACACCCACCTTGA
AACCCAAAAA

SEQ ID NO:2
PAP7 sequence:
GAATTCGCGGCCGCGTCGACCTAAAGTTGAGTTGTTCACTGTAGTGACCCGTGTGAAGGTAGTT
TTATTTTTAAATCAACTTTCATTGTGCAAACTAGTAAAAGATGGCAAAGCCTTTCATCCAACTTA
TGAAGAAAAACTGAAGTTCGTGGCACTGCATAAGCAAGTTCTTTTGGGCCCATATAACCCAGA
CACGTCCCCTGAGGTTGGATTCTTTGATGTGTTGGGGAATGATAGGAGGAGAGAATGGGCAGCT
CTGGGAAACATGTCCAAGGAGGATGCCATGGTAGAGTTTGTGAAGCTTCTAAATAAGTGTTGTC
CTCTCCTCTCGGCATATGTTGCGTCCCACAGAATAGAGAAGGAAGAAGAAGAGAAAAGAAGAA
AGGCGGAGGAGGAGCGAAGGCAGCGTGAAGAGGAAGAACGAGAGCGGCTGCAAAAGGAAGAA
GAGAAGCGGAAGCGAGAGGAGGAAGACCGGCTGAGACGGGAGGAGGAAGAGAGGCGGCGGAT
AGAGGAAGAGAGGCTTCGGCTGGAACAGCAAAAGCAGCAGATAATGGCAGCTTTAAACTCGCA
GACTGCCGTGCAATTCCAGCAGTATGCAGCCCAGCAGTATCCAGGGAACTACGAACAACAGCA
GATTCTCATCCGCCAGCTGCAGGAGCAGCACTATCAGCAGTATAAACACCAGGCAGAGCAAAC
CCAACCTGCACAACAACAGGCAGCATTACAGAAACAGCAAGAAGTAGTGATGGCTGGGGCAT
CATTGCCTGCATCATCAAAGGTGAACACAGCTGGAGCAAGTGATACACTGTCAGTTAATGGAC
AGGCCAAAACCCACACTGAAAATTCCGAAAAAGTCCTTGAGCCAGAAGCTGCAGAAGAAGCC
TTGGAAAATGGACCAAAAGACTCTCTTCCAGTGATTGCAGCTCCATCCATGTGGACAAGACCA
CAAATCAAAGACTTTAAAGAGAAGATTCGGCAGGATGCAGATTCTGTGATTACAGTACGTCGA
GGAGAAGTCGTCACCGTCCGAGTCCCGACTCATGAGGAAGGATCATACCTATTTTGGGAATTTG
CCACAGACAGTTATGACATTGGGTTTGGGGTTTATTTTGAATGGACAGACTCTCCAAATGCTGC
TGTCAGTGTGCATGTCAGTGAGTCCAGTGACGAGGAGGAGGAGGAGGAAGAAAATGTCACTTC
TGAAGAAAAAGCAAAAAAGAACGCCAACAAGCCTCTGCTGGATGAGATTGTACCTGTGTACCG
GCGGGACTGTCACGAGGAAGTATATGCAGGCAGCCACCAGTATCCAGGGAGGGGAGTCTATC
TCCTCAAGTTTGATAATTCCTACTCTCTGTGGAGGTCCAAGTCCGTCTACTACAGAGTCTA
TTATACTAGATAGAGCTGCTGTTCCAAGGTCCGGAGTCCAGGGTTGAGCACAACATGACGT
TTAATTTCCTTT

SEQ ID NO:3
PAP8 nucleic acid
GAATTCGCGGCCGCGTCGACGCTGGACACAAGCGTGGAGCGAAGAGCCCTTGGAGAGATTCAG
AATGTAGGTGAAGGCTCTTCAACATCACAGGGCACCTGGCAGTCTTCAGAGTCCTCACAGTCA
AACCTGGGGGAGCAGACGCAGAGCGGACCCCAGGGAGGAAGGTGTCAGCGTCGGGAGAGGCA
TAACCGAATGGAACGAGATAGGAGGCGCAGAATCCGCATTTGCTGTGATGAGCTGAATCTTTTA
GTTCCATTCTGCAATGCGGAGACAGATAAAGCAACAACCCTTCAGTGGACCACAGCATTCCTG
AAGTACATTCAGGAAAGACATGGGGACTCTCTTAAAAAGGAATTTGAGAGCGTGTTTTGCGGTA
AAACTGGCAGAAGGCTAAAGCTGACTAGACCCGAATCCCTGGTGACCTGCCCTGCACAGGGCA
GCCTGCAGAGCAGCCCTGCCATGGAGATCAAGTGACTGGACTGACCCAGGACCTGGGAGAGA

ACCGCCGTTCCTGCGGCATCATGCACATGCCTGCCATCCCCGGAATTCAGCTCTGAATCCT
CTC

SEQ ID NO:4
PAP15 nucleic acid
GAATTCGCGGCCGCGTCGACCTCGGGGGTAACCCTTGGGATCATTATGATGTCACCTCTTCAAT
CTCTTGAGACCAGTGATTTATGAACTTGACATTTGGTGCCTGGTGTGGGCTTTGGAAAGCAGAG
GCCACCTTTGTCTGTAGAGGATACTGAGCGGCTGGATGGCAGGAATCCAAAGAGAAGCCAGCC
TCTCGTAGTCGCCCTGGGACAGTGGAAGAGAGGGAGGACCGGCAAAGGGGAATCTGTCTTTCT
CCTAGGCCCGAGCATGTCCCCTGTGGGACATGCTCTGTGACAGCTGAGCCTGCCCAGCCTG
CCTTTCTGAAGTTGGGTGTCTCCTGCCCACAACCAAGCCAGCAATCGGTCTGTTTTCCGAC
AACCTCAGAGCCAGACCTCACAAGCCTATTTTGGTGGTTCCCAAAATTTCTCTCAGATCTC
CATGTCTATCCCTCCACTCCCTCCAAAAGAGAAAGAAAAGAATTGAGAAAGAA

SEQ ID NO:5
PAP20 nucleic acid
GAATTCGCGGCCGCGTCGACCTCAGAAGAGGAAAAGAGGTGCAGAAGTGCTGGCGGCACAAA
TTGTACAGAAAACCAGACTAGAGAGAAAAAAACAAGAAGCGTCTGTATCTAAAGATGCTCCAG
TGCCTACAAATACTAAAAGGGCAAAGAAACAAGAGAAGTCTCCAGGTAGAATTGCCTCACAGT
CTAAGCCACCCATGAAAAAGTCTCCACAAAAACGGAAGGTAAATGTAGCAAGAGGCCGTCGG
AATACCAGAAAGCAGCTCCAACCTGCCGAAAAAGAAATTGCTTTACATCTTCAATCAGAAATT
TCATCAGATGGCCAAAAAGATGGACTTAACCTAAGTACATCTCAACAAGAAAGTATTTCAATG
ATTCCTAAAGGTCCTCCTGAAAACTCAGTTATCAGCTGTGACTCCCAGGCCCTAAATATGTTAG
CTGATCTGGCATTAAGTTCTGCTGCTGCTTCTATACCATCTTGTAAGCCCAGGAACCTTCCCTG
CGTCTCTGATTTGCCACGAAACAATGTCTTACTCACTAAAGAAAATCCATTGCTTGGTGCCT
CTGACCATGAATATCATAAGGG

SEQ ID NO:6
PAP3 amino acid sequence
Met Ala Thr Ser Asp Val Lys Pro Lys Ser Ile Ser Arg Ala Lys
Lys Trp Ser Glu Glu Ile Glu Asn Leu Tyr Arg Phe Gln Gln Ala
Gly Tyr Arg Asp Glu Ile Glu Tyr Lys Gln Val Lys Gln Val Ala
Met Val Asp Arg Trp Pro Glu Thr Gly Tyr Val Lys Lys Leu Gln
Arg Arg Asp Asn Thr Phe Phe Tyr Tyr Asn Lys Glu Arg Glu Cys
Glu Asp Lys Glu Val His Lys Val Lys Val Tyr Val Tyr

SEQ ID NO:7
PAP7 amino acid sequence
Arg Pro Arg Arg Pro Lys Val Glu Leu Phe Thr Val Val Thr Arg
Val Lys Val Val Leu Phe Leu Asn Gln Leu Ser Leu Cys Lys Leu
Val Lys Asp Gly Lys Ala Phe His Pro Thr Tyr Glu Glu Lys Leu
Lys Phe Val Ala Leu His Lys Gln Val Leu Leu Gly Pro Tyr Asn
Pro Asp Thr Ser Pro Glu Val Gly Phe Phe Asp Val Leu Gly Asn
Asp Arg Arg Arg Glu Trp Ala Ala Leu Gly Asn Met Ser Lys Glu
Asp Ala Met Val Glu Phe Val Lys Leu Leu Asn Lys Cys Cys Pro

Leu Leu Ser Ala Tyr Val Ala Ser His Arg Ile Glu Lys Glu Glu
Glu Glu Lys Arg Arg Lys Ala Glu Glu Glu Arg Arg Gln Arg Glu
Glu Glu Glu Arg Glu Arg Leu Gln Lys Glu Glu Glu Lys Arg Lys
Arg Glu Glu Glu Asp Arg Leu Arg Arg Glu Glu Glu Glu Arg Arg
Arg Ile Glu Glu Glu Arg Leu Arg Leu Glu Gln Gln Lys Gln Gln
Ile Met Ala Ala Leu Asn Ser Gln Thr Ala Val Gln Phe Gln Gln
Tyr Ala Ala Gln Gln Tyr Pro Gly Asn Tyr Glu Gln Gln Gln Ile
Leu Ile Arg Gln Leu Gln Glu Gln His Tyr Gln Gln Tyr Lys His
Gln Ala Glu Gln Thr Gln Pro Ala Gln Gln Gln Ala Ala Leu Gln
Lys Gln Gln Glu Val Val Met Ala Gly Ala Ser Leu Pro Ala Ser
Ser Lys Val Asn Thr Ala Gly Ala Ser Asp Thr Leu Ser Val Asn
Gly Gln Ala Lys Thr His Thr Glu Asn Ser Glu Lys Val Leu Glu
Pro Glu Ala Ala Glu Glu Ala Leu Glu Asn Gly Pro Lys Asp Ser
Leu Pro Val Ile Ala Ala Pro Ser Met Trp Thr Arg Pro Gln Ile
Lys Asp Phe Lys Glu Lys Ile Arg Gln Asp Ala Asp Ser Val Ile
Thr Val Arg Arg Gly Glu Val Val Thr Val Arg Val Pro Thr His
Glu Glu Gly Ser Tyr Leu Phe Trp Glu Phe Ala Thr Asp Ser Tyr
Asp Ile Gly Phe Gly Val Tyr Phe Glu Trp Thr Asp Ser Pro Asn
Ala Ala Val Ser Val His Val Ser Glu Ser Ser Asp Glu Glu Glu
Glu Glu Glu Glu Asn Val Thr Cys Glu Glu Lys Ala Lys Lys Asn
Ala Asn Lys Pro Leu Leu Asp Glu Ile Val Pro Val Tyr Arg Arg
Asp Cys His Glu Glu Val Tyr Ala Gly Ser His Gln Tyr Pro Gly
Arg Gly Val Tyr Leu Leu Lys Phe Asp Asn Ser Tyr Ser Leu Trp
Arg Ser Lys Ser Val Tyr Tyr Arg Val Tyr Tyr Thr Arg

SEQ ID NO:8
PAP8 amino acid sequence
Glu Phe Ala Ala Ala Ser Thr Leu Asp Thr Ser Val Glu Arg Arg
Ala Leu Gly Glu Ile Gln Asn Val Gly Glu Gly Ser Ser Thr Ser
Gln Gly Thr Trp Gln Ser Ser Glu Ser Ser Gln Ser Asn Leu Gly
Glu Gln Thr Gln Ser Gly Pro Gln Gly Gly Arg Cys Gln Arg Arg
Glu Arg His Asn Arg Met Glu Arg Asp Arg Arg Arg Arg Ile Arg
Ile Cys Cys Asp Glu Leu Asn Leu Leu Val Pro Phe Cys Asn Ala
Glu Thr Asp Lys Ala Thr Thr Leu Gln Trp Thr Thr Ala Phe Leu
Lys Tyr Ile Gln Glu Arg His Gly Asp Ser Leu Lys Lys Glu Phe
Glu Ser Val Phe Cys Gly Lys Thr Gly Arg Arg Leu Lys Leu Thr
Arg Pro Glu Ser Leu Val Thr Cys Pro Ala Gln Gly Ser Leu Gln
Ser Ser Pro Ala Met Glu Ile Lys

SEQ ID NO:9
PAP15 amino acid sequence
Ala Ala Gly Trp Gln Glu Ser Lys Glu Lys Pro Ala Ser Arg Ser
Arg Pro Gly Thr Val Glu Glu Arg Glu Asp Arg Gln Arg Gly Ile
Cys Leu Ser Pro Arg Pro Glu His Val Pro Cys Gly Thr Cys Ser
Val Thr Ala Glu Pro Ala Gln Pro Ala Phe Leu Lys Leu Gly Val
Ser Cys Pro Gln Pro Ser Gln Gln Ser Val Cys Phe Pro Thr Thr
Ser Glu Pro Asp Leu Thr Ser Leu Phe Trp Trp Phe Pro Lys Phe
Leu Ser Asp Leu His Val Tyr Pro Ser Thr Pro Ser Lys Arg Glu
Arg Lys Glu Leu Arg Lys Lys

SEQ ID NO:10
PAP20 amino acid sequence
Asn Ser Arg Pro Arg Arg Pro Gln Lys Arg Lys Arg Gly Ala Glu
Val Leu Ala Ala Gln Ile Val Gln Lys Thr Arg Leu Glu Arg Lys
Lys Gln Glu Ala Ser Val Ser Lys Asp Ala Pro Val Pro Thr Asn
Thr Lys Arg Ala Lys Lys Gln Glu Lys Ser Pro Gly Arg Ile Ala
Ser Gln Ser Lys Pro Pro Met Lys Lys Ser Pro Gln Lys Arg Lys
Val Asn Val Ala Arg Gly Arg Arg Asn Thr Arg Lys Gln Leu Gln
Pro Ala Glu Lys Glu Ile Ala Leu His Leu Gln Ser Glu Ile Ser
Ser Asp Gly Gln Lys Asp Gly Leu Asn Leu Ser Thr Ser Gln Gln
Glu Ser Ile Ser Met Ile Pro Lys Gly Pro Pro Glu Asn Ser Val
Ile Ser Cys Asp Ser Gln Ala Leu Asn Met Leu Ala Asp Leu Ala
Leu Ser Ser Ala Ala Ala Ser Ile Pro Ser Cys Lys Pro Arg Asn
Leu Pro Cys Val Ser Asp Leu Pro Arg Asn Asn Val Leu Leu Thr
Lys Glu Asn Pro Leu Leu Gly Ala Ser Asp His Glu Tyr His Lys
Gly

SEQ ID NO:11
Ser Ser Asp Glu Glu Glu Glu Glu Glu Glu Asn Val Thr Cys Glu
Glu Lys Ala Lys Lys Asn Ala Asn Lys Pro

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CORRECTED VERSION*

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(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
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16 November 2000 (16.11.00)

(54) Title: PERIPHERAL-TYPE BENZODIAZEPINE RECEPTOR ASSOCIATED PROTEINS, CLONING, EXPRESSION AND METHODS OF USE

(57) Abstract

The present invention relates to nucleic acids encoding PBR-associated proteins (PAPs) and methods for use in producing PAPs and methods of using PAPs. In this study we have identified proteins (PAPs) that interact with PBR protein by a yeast two-hybrid system. We used PBR as bait to screen a mouse testis cDNA library. Five clones were isolated based on their ability to interact with PBR. These proteins may be involved in the regulation of the function of PBR, serving as an endogenous ligand or allosteric modulator of the receptor.

*(Referred to in PCT Gazette No. 31/2000, Section II)

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【国際調査報告】

INTERNATIONAL SEARCH REPORT

International A, cation No
PCT/US 99/18507

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/12 C07K14/47 C12N1/21 G01N33/53 C07K16/18
C12Q1/68 A61P35/00 A61P3/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K G01N C12Q A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE EMBL 'Online! AC AF022770, 1 October 1997 (1997-10-01) LI H & V PAPADOPOULOS: "M. musculus peripheral benzodiazepine receptor associated protein" XP002133903 cited in the application 94.9% identity in 178 aa overlap with SEQ ID No:7 the whole document --- -/--	1-5, 9-16,19

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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'P' document published prior to the international filing date but later than the priority date claimed

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Date of the actual completion of the international search	Date of mailing of the international search report
15 June 2000	28.06.00

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Nichogiannopoulou, A

3

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/18507

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE EMBL 'Online! AC X64455, 21 February 1992 (1992-02-21) DON J: "M. musculus mRNA megl" XP002133904 cited in the application 99% identity in 490 nt overlap with SEQ ID No:1 (17-505:28-517) the whole document	3,9-16
X	DATABASE EMBL 'Online! AC AA174581, 31 December 1996 (1996-12-31) MARRA M ET AL: "mo98e12.r1 Stratagene mouse testis (#937308) Mus musculus cDNA" XP002133905 EST with 97.9% identity in 421 nt overlap (17-435:79-498) with SEQ ID No:1 the whole document	3,9-16
E	WO 00 01821 A (INCYTE PHARMA INC) 13 January 2000 (2000-01-13) Incyte clone with 88.7% identity to SEQ ID NO:2 table 2	3-5, 9-16,19
P,A	GALIÈGUE S ET AL: "Cloning and characterisation of PRAX-1. A new protein that specifically interacts with the peripheral benzodiazepine receptor" J. BIOL. CHEM, vol. 274, no. 5, 29 January 1999 (1999-01-29), page 2938-2952 XP002122735 cited in the application the whole document	
A	KRUEGER K E ET AL: "Peripheral-type benzodiazepine receptors mediate translocation of cholesterol from outer to inner mitochondrial membranes in adrenocortical cells" JOURNAL OF BIOLOGICAL CHEMISTRY., vol. 265, no. 25, 5 September 1990 (1990-09-05), pages 15015-15022, XP002133901 ISSN: 0021-9258 cited in the application the whole document	38,39

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3

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International A...cation No
PCT/US 99/18507

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MIETTINEN H ET AL: "Expression of peripheral-type benzodiazepine receptor and diazepam binding inhibitor in human astrocytomas: relationship to cell proliferation" CANCER RESEARCH, vol. 55, 15 June 1995 (1995-06-15), pages 2691-2695, XP002133902 cited in the application the whole document	36,37

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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US 99/18507

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely

Although claims 35-40 -in as far as they concern in vivo methods- are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.: 29-32
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

The claimed agents have not been described in the application and have as such not been the subject of search.

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [X] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

1-4, 9-18, 23-25, 27-40 partially and 5, 19, 26 completely

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[X] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1998)

International Application No. PCT/US 99/18507

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box 1.2

Claims Nos.: 29-32

The claimed agents have not been described in the application and have as such not been the subject of search.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

International Application No. PCT/US 99/18507

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-3, 9-18, 24, 25, 27-32, 35-40 all partially

An isolated DNA fragment encoding PBR-associated protein 3 (PAP3) and portions of said DNA, including the DNA of SEQ ID No:1 and fragments of said sequence of at least 30 nucleotides. Recombinant DNA constructs, host cells and production methods relating to PAP3. Recombinant PAP3 and detection methods comprising the two hybrid assay. Antibodies to the PAP3 polypeptide of SEQ ID No:6. Methods for detecting agents which reduce, eliminate or promote PAP3 activity, agents and therapeutic compounds that inhibit or promote PAP activity and therapeutic methods for the treatment of disease states.

2. Claims: 5, 19, 26 completely and 1-4, 9-18, 23-25, 27-40 all partially

As invention 1 but relating to PAP7 (SEQ ID Nos: 2 and 7)

3. Claims: 6, 20 completely and 1-3, 9-18, 23-25, 27-40 all partially

As invention 1 but relating to PAP8 (SEQ ID Nos: 3 and 8)

4. Claims: 7, 21 completely and 1-3, 9-18, 23-25, 27-40 all partially

As invention 1 but relating to PAP15 (SEQ ID Nos: 4 and 9)

5. Claims: 8, 22 completely and 1-4, 9-18, 23, 24, 27-40 all partially

As invention 1 but relating to PAP20 (SEQ ID Nos: 5 and 10)

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0001821 A	13-01-2000	AU 4857999 A	24-01-2000

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QX02 QX07
4B064 AG20 AG26 AG27 CA06 CA10 CA19 CC24 DA01 DA14
4B065 AA80X AA92X AA93Y AB01 BA02 CA24 CA25 CA44 CA46
4C084 AA02 AA13 AA16 ZA012 ZA362 ZB262 ZC422
4H045 AA10 AA11 AA20 AA30 BA10 CA40 DA50 DA75 EA51 FA72
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