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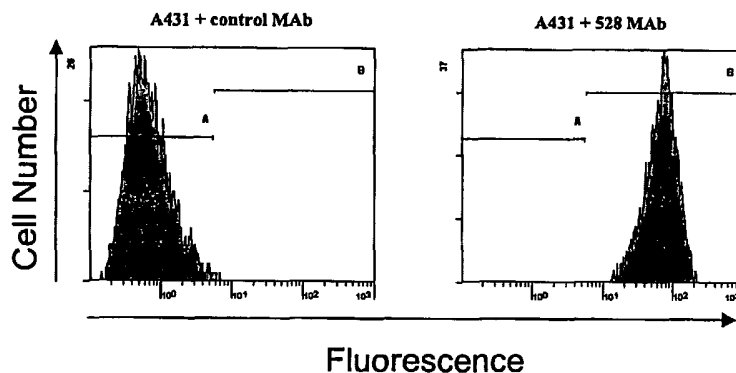
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(54) Title: ISOLATION OF MEMBRANE BOUND LIGAND-SPECIFIC COMPLEXES

Detection of EGFR Expression on the Surface of A431 Cells



(57) **Abstract:** A method to recover or "deracinate" receptors from intact membranes in their native configuration without removing microenvironmental components associated with them is described. The membranes containing the receptors are first associated with ligand-coupled solid supports to achieve association of the membranes through a receptor/ligand complex which includes the microenvironmental to the solid support and then removing the membrane from the receptor and its microenvironment by shear forces. The thus isolated receptors and their microenvironments can then be analyzed for their role in cellular differentiation, apoptosis, transformation and the like.

ISOLATION OF MEMBRANE BOUND LIGAND-SPECIFIC COMPLEXES

Cross-Reference to Related Applications

This application is a continuation-in-part of U.S. Serial No. 09/932,166 filed 17 August 2001, which claims priority under 35 U.S.C. § 119(e) to Serial No. 60/308,655 filed 27 July 2001. The contents of these applications are incorporated herein by reference.

Field of the Invention

The invention relates to methods to isolate membrane-bound complexes wherein the microenvironment of a membrane-bound protein that is bound by a specific ligand is preserved. More specifically, the invention concerns non-destructive preparation and isolation of membrane-bound, especially cell surface, receptors, along with the molecules with which they are associated.

Background Art

A multiplicity of procedures for isolation of proteins is available and practiced in the art. For soluble proteins such as those that occur in the cytoplasm, direct application of these techniques is possible, as it is unnecessary to take steps to free these proteins from their surroundings. However, proteins or lipids that are membrane-bound, have required harsher treatment to release them from the membrane. One commonly employed technique is solubilization using detergents, or other harsh reagents such as chaotropic agents or other denaturing compounds such as urea. See, for example, Molloy, M.P., *et al.*, *Electrophoresis* (1998) 19:837-834; Chevallet, M., *et al.*, *Electrophoresis* (1998) 19:1901-1909. A drawback of such methods is that the isolation process goes too far, *i.e.*, the protein is recovered essentially by itself, and is unaccompanied by the cellular membrane components that normally are associated with it and which are often crucial to its biological function. Elucidation of the biological role of such proteins requires knowledge of the substances involved in transduction of signals resulting from binding of the protein to its specific ligand. Thus, it would be extremely valuable to recover these membrane proteins along with their corollary substances, so that the nature of these substances and the biological role of the protein and its associated substances can be

determined. It will be noted that the corollary substances may be associated themselves with the membrane or may be associated with the receptor in the cytoplasm.

Another drawback of the prior art methods is that in some instances, the membranes are sufficiently disrupted by the harsh reagents that cryptic receptors become exposed and coincidentally bind to ligands of interest. Thus, the practitioner is misled into analyzing a receptor with respect to a ligand when in fact the receptor is irrelevant to the biological function of the ligand in question.

A description of one method to obtain lectin receptors from erythrocytes without the use of detergents was described by Jakobovits, A., *et al.*, *Biochem. Biophys. Res. Commun.* (1981) 100:1484-1490. In this method, erythrocytes were treated with neuraminadase and then with lectin-coated, *i.e.*, peanut agglutinin-coated sepharose beads, thus resulting in coupling the erythrocytes to the beads through an interaction between lectin and the cognate receptors on the cell surface. Unbound cells were removed and the pellet of erythrocyte-covered beads was pipetted vigorously with a Pasteur pipette. The beads were then washed to remove any erythrocytes that had been sheared, and it was determined that most erythrocytes could be removed by repetition of this treatment. Any remaining bound cells could be removed by quick vortexing and subsequent wash. While the erythrocytes were removed, the receptors coupled to lectin remained bound to the sepharose beads. These "plucked" receptors were then released from the beads by boiling or by competitive elution with galactose.

The elution of the bound components resulted in recovery of the lectin binding components designated asialoglycophorins. This work showed that cells lacking nuclei, when treated to expose the relevant sugars, could be bound through carbohydrates on their surfaces to agglutinins (*i.e.*, lectins) and that the carbohydrate-containing moieties bound to the agglutinins could be removed from the cell membranes by mechanical disruption. No description of any non-covalently associated components removed along with the glycoprotein receptors *per se* was provided. Further, there is no description of cells which had not been treated to expose the relevant sugars, nor was there a suggestion to employ ligands other than lectins. As noted below, lectins are relatively non-specific and thus will bind to a multiplicity of glycosylated proteins some of whose biological functions are irrelevant to the specific nature of the lectin.

An additional experiment wherein lectin receptors were “plucked” from mouse thymocytes was described in a chapter included in a thesis submitted in support of the award to the inventor herein of a Ph.D. to the Feinberg Graduate School and approved by the Scientific Council of the Weizmann Institute of Science on 6 June 1983. In this
5 experiment, thymocytes were labeled in various ways and treated with lectin derivatized beads according to the general procedure described above. This resulted in recovery of a complex pattern of labeled proteins, many of which represented receptors that include sugars bound by the lectins employed, thus demonstrating a relative lack of specificity of extracting relevant receptors using lectin as a binding agent.

10 While effective binding of cells can more readily be achieved through the use of lectins than through the specific ligands of the present invention, the resultant does not provide information regarding biologically relevant receptors due to the non-specificity of lectin binding. Some lectins do engender biological responses, and lectins are specific with respect to the carbohydrates to which they bind, but the individual carbohydrate targets are
15 promiscuous with respect to the receptors in which they reside. Thus, as regards individual receptors, specificity of binding is lost.

Disclosure of the Invention

It has now been found that surface receptors comprising protein, in particular in nucleated cells, can be removed along with non-covalently associated complexed
20 components by specific binding of these receptors to counterpart ligands and subsequent shearing of the bulk of the cell or of the relevant membrane from the ligand/receptor complex. In general, this process may be referred to as “deracination” of the receptor and its microenvironment. If desired, the receptor, along with its associated cellular components, can be dissociated from the specific ligand using, for example, competitive
25 binding by the receptor itself or its analogs or a number of other techniques.

The invention provides a method to rapidly and specifically isolate membrane-bound components such as cell surface receptors in their native conformation and in their native microenvironments, under conditions wherein the status of the component or receptor is preserved, thus permitting insights into the function of the receptor that are
30 unavailable when conventional methods for isolation are used. Such conventional methods typically involve detergents or chaotropic agents which distort and/or disrupt the membrane

and cytoplasmic environment surrounding the receptor and thus obliterate information as to the environment and function of the receptor itself. The invention method thus provides information which identifies and characterizes cell surface targets that interact with stimuli/ligands and for the identification and design of molecules that interact with these targets. The identification and design of molecules that interact with these targets, including molecules that inhibit them, is further informed by the understanding made available by the methods of the invention which elucidate the manner of function and additional cellular components associated with the targets.

Thus, in one aspect, the invention is directed to a method to isolate a membrane-bound receptor such as a cell surface receptor along with its microenvironment which method comprises providing a solid support coupled to a ligand specific for the desired receptor, to which membranes bearing the receptor have been bound through association with the ligand to form a ligand/receptor complex, and then shearing the cells from the ligand/receptor complex. The "ligand" may be, for example, a hormone or growth factor or may be an antibody or relevant fragment thereof so long as it is specific for the targeted protein. The receptor thus recovered will remain associated with its microenvironment. The microenvironment, of course, may in fact not include any associated cellular components and the receptor will be recovered alone. This, too, is valuable information concerning the nature of the receptor. This may optionally be followed by dissociating the ligand/receptor complex (comprising the receptor and its microenvironment) from the solid support and/or dissociating the ligand from the receptor/microenvironment to recover the receptor/microenvironment itself. In any event, as the microenvironment remains associated with the receptor, it is an additional step that can be included within the invention to analyze the microenvironment to determine what, if any, components it contains. Numerous methods are available for such analysis, including, for example, chromatographic analysis, mass spectroscopy, 2-dimensional gels and Western blots.

In the method of the invention, the ligand is specific for the receptor *per se*, and does not cross-react with receptors irrelevant to biological activity of the target receptor. As defined hereinbelow, in one embodiment ligands specific for receptors do not include lectins, for the most part. Lectins, in general, lack the requisite ability to bind only to unique biologically significant receptors. The lectins, generally, are able to bind to carbohydrates associated with proteins, and thus are cross-reactive with glycosylated

proteins in general. Lectins fall within the definition of "ligands" herein only if they are sufficiently specific to effect deracination of a particular receptor, as opposed to their typically generic behavior. In general, lectins bind to higher densities of receptors on cells as compared to more specific ligands, such as antibodies. It should be noted that antibodies or fragments thereof used as ligands can, but need not themselves result in any biological response; however, they specifically target receptors whose ligands do elicit a response.

Further, lectins, in order to bind may require pretreatment of the surface of the membrane to expose the carbohydrate moieties which they bind. A preferred method of the invention utilizes membranes which have not been subjected to any surface treatment that modifies the carbohydrates exposed on the cell surface. In a preferred embodiment, an advantage is that the membrane is employed in a more realistic native state. In this preferred method, there is no surface treatment of the membranes prior to contacting them with the specific ligand.

However, in one preferred embodiment, lectins can be employed as at least part of the regimen. Where it is understood that carbohydrate distribution will be different in a particular type of cell, it may be possible to provide lectins that are specific to a cell surface receptor, and in this special case, such lectins may be used as ligands. For example, it is understood that receptors associated with tumor cells have different glycosylation patterns from those associated with normal cells. Where the difference in carbohydrate association is sufficient to differentiate a particular receptor, for example, on a tumor cell, even if cell treatment is needed to expose the differentiating carbohydrate, a lectin specific for this carbohydrate may be used as a sufficiently specific ligand.

In still another preferred embodiment, the membranes are associated with or derived from a nucleated cell or a bacterial cell, preferably a nucleated cell. Nucleated cells are representative of virtually all typical eukaryotic cells with the exception of red blood cells. The membranes on the surface of, or contained in nucleated cells are inherently more complex than those associated with, for example, red blood cells. Further, nucleated cells perform more complex metabolic functions than red blood cells and thus offer a more fertile substrate for retrieval of receptors.

In still another preferred embodiment, the method of the invention includes the active steps described above to analyze the microenvironment in which the receptor is contained. The microenvironment may reside in the membrane itself or in the proximity of

the receptor in the cytoplasm. As stated above, the microenvironment may not contain any closely associated components, and thus the method of the invention will result in isolation of the receptor *per se*.

In another aspect, the invention is directed to a method to obtain a library of membrane-bound receptors, which method comprises providing solid supports coupled to a multiplicity of ligands; contacting the solid supports with a sample of membranes, preferably from or associated with nucleated cells, under conditions whereby receptors in said membranes or on said cells are coupled to one or more of the ligands on said solid supports, so that a ligand forms a complex with its cognate receptor. The membranes or cells are then dissociated from the ligand/receptor complexes which comprise the receptor and microenvironment. The membrane-derived receptors (with microenvironments) are optionally recovered, either alone or along with their ligands, from the solid support. The receptor/microenvironment can then be analyzed to determine what, if any, components are associated with the isolated receptor.

In additional aspects, the invention is directed to isolated receptors associated with microenvironments prepared by the method of the invention and to novel receptors identified thereby. The recovered receptors can be used in screening assays to identify compounds which agonize or antagonize them. The recovered receptors can further, if the receptors are identified as associated with undesired or diseased cells, be used in the design of antibodies, pharmaceuticals or vaccines to target these cells. The receptors can be recovered from both normal and diseased cells.

The invention also includes receptor profiles of membrane or cell samples. As further described below, the method of the invention can be used in a high throughput system to determine patterns of receptors at the cell surface or contained in the membrane.

Thus, an important aspect of the invention is the ability to characterize interactions with receptors and the cellular networks associated with their functions.

Brief Description of the Drawings

Figure 1 shows the results of flow cytometry to detect EGFR on the surface of A431 cells. A431 cells were stained with 1 μ g of either control mouse IgG or 528 anti-EGFR mouse IgG2 κ antibody for 30 minutes at 4°C. The cells were then washed in PBS + 1% fetal bovine serum, stained with FITC-conjugated secondary anti-mouse antibody,

and analyzed by flow cytometry. Results show strong expression of EGFR on the cell surface of A431 cells.

Figure 2 shows Western blots of EGFR deracinated by anti-EGFR Mab and WGA lectin. The 170 kDa EGFR protein was readily detected in the deracinated products from A431 cells using the anti-EGFR- and the WGA-coated beads.

Figure 3 shows a Western blot of deracinated activated EGFR and its associated phosphoproteins detected by anti-phosphotyrosine. The band at 170 kDa corresponds to activated EGFR. (The band at 50 kDa corresponds to cross-reactivity of the secondary antibody with mouse heavy chain (mIgH) of the mouse 528 antibody released from the beads.) The remaining bands represent associated proteins.

Figures 4A and 4B show Western blots of proteins associated with deracinated EGFR, *i.e.*, Vav2 and Grb2. This shows both EGFR-associated Vav-2 and Grb2 in the 528 Mab deracination materials. (The band detected at molecular weight 50kDa corresponds to cross-reactivity of the secondary antibody with mouse heavy chain (mIgH) of the 528 antibody released from the beads.)

Figure 5 shows a Western blot of EGFR-associated Shc proteins deracinated by anti-EGFR 528 Mab.

Figures 6A-6F are flow cytometry diagrams showing binding of anti-PSCA 1G8 Mab and WGA to the surface of PC3 cells transfected with a PSCA expression system. The cells were stained with control antibody or 1 μ g of either 1G8 anti-PSCA mouse IgG1 antibody followed by FITC-conjugated secondary anti-mouse antibody, or with FITC-conjugated WGA lectin for 30 minutes at 4°C. The cells were then analyzed by flow cytometry. 1G8 Mab binds to PC3-PSCA cells only; WGA lectin binds to both PC3 and PC3-PSCA cells.

Figures 7A and 7B are Western blots showing specific deracination of PSCA but not EGFR, by anti-PSCA 1G8 Mab as ligand. WGA mediated deracination of both PSCA and EGFR from PC3-PSCA and EGFR from PC3, PC3-PSCA and A431 cells, but deracination with 1G8 anti-PSCA Mab deracinated only PSCA (not EGFR) from PC3-PSCA. (The bands detected at molecular weight 50kDa and 25kDa correspond to cross-reactivity of the secondary antibody with mouse heavy chain (mIgH) and light chain (mIgL) of the 1G8 antibody released from the beads.)

Figures 8A and 8B are Western blots showing deracination of PSCA with WGA lectin. The results show WGA-mediated deracination of PSCA on PC3-PSCA cells, but the deracinate does not include the intracellular Grb2 protein.

Modes of Carrying Out the Invention

5 The invention provides, for the first time, a method whereby at least three important goals are achieved. First, the method permits the isolation of membrane-bound receptors that bind specifically to a ligand directly from intact cells in a form which preserves their native conformations and associated moieties, thus providing a wealth of information about the receptors and the components with which the receptors are associated
10 and thus the functions and roles of the recovered receptors that has previously been unavailable. By ascertaining the nature of moieties that are natively associated with the receptors, the role of the receptor in cellular pathways, such as proliferation, differentiation, survival, apoptosis, transformation and the like can be determined. Once such roles are determined, the receptor itself can be employed in the design of appropriate strategies to
15 control the relevant function. Second, and related to the first goal, is the assurance that the receptors retrieved are not artifacts of the experiment by avoiding the use of agents that would reveal cryptic molecules which are not of biological significance, but which coincidentally bind the ligand used. Third, the method of the invention offers a high throughput system for rapid identification of membrane-bound receptors through a facile
20 system of recovering these receptors from membranes. Some receptors identified may be previously unknown.

 The identification and characterization of protein-protein, protein-lipid, or any other type of interaction exhibited by the receptors is significant in evaluating the biological role of the receptor, and thus the biological role of ligands to which it may be
25 bound.

 As used herein, "receptor" refers to any moiety which specifically binds to a cognate ligand. Typically, the receptors are proteins and may or may not be further derivatized such as through glycosylation, phosphorylation, lipidation, coupling to additional structural components and the like. "Receptors" is used herein as a generic term
30 and is not limited to proteins that are necessarily associated with a cellular pathway. Membrane-associated receptors include, for example, G-protein coupled receptors,

cytokine receptors, ion channels, opioid receptors, hormone receptors, retinoid receptors, steroid receptors, growth factor receptors, integrins, immunoglobulins, T-cell receptors, MHC class I and II and, generally, any membrane-bound protein which comprises sufficient exposure at the surface of the membrane to bind specifically to a ligand.

5 The “ligand” may be any cognate binding agent other than a non-specific lectin which agent is specific for a membrane-bound receptor component where the component is characteristic of one or, in some cases, several receptors. (A characteristic component may be shared by several receptors, not all of which need have the same biological function, though this is thought to be the exception.) That is, the ligand should interact only with
10 receptors that it specifically recognizes as an agonist or antagonist or, in the case of antibodies or their fragments or other mimics of the native ligand, only the receptor activated by the native ligand that is mimicked. In general, lectins are excluded from the definition of ligands because they are relatively non-specific. As many proteins are glycosylated, lectins bind large numbers of proteins non-specifically. In addition, the use
15 of lectins may require treatment of the membranes prior to contact in order to expose carbohydrate residues that are masked, for example, by sialic acid residues. Thus, “ligand” as herein defined includes a range of substances which bind highly specifically to a particular receptor as opposed to lectins which may bind a multiplicity of receptors. While lectins bind carbohydrates, not all proteins that bind carbohydrates are lectins. For
20 example, antibodies may bind specific carbohydrate epitopes.

 The ligand may be a ligand which is native to the receptor - for example, a cytokine which normally binds a cytokine receptor, but may also be a synthetic compound which is designed for specific binding, including antibodies or fragments thereof. In terms of their chemical nature, the ligand may be a protein, a lipid, a sugar or a small molecule.
25 The ligand may have other characterizing features such as comprising an immunoglobulin structure or fragment thereof, activity as a toxin, enzyme, or other biological activity.

 Thus, with the exception of lectins, as noted above, as used herein, “receptor” and “ligand” refer simply to a cognate binding pair wherein the receptor is associated with a membrane and the ligand, typically, is not.

30 “Protein,” “peptide,” and “polypeptide” are used interchangeably, regardless of the length of amino acid sequence included.

As used herein, the term "microenvironment" of the receptor refers to the molecules in the immediate surroundings of the receptor and with which it is non-covalently associated. These molecules may be involved in the transduction of signals from interaction of the receptor with its ligand, or may be molecules that are activated or deactivated by interaction with the receptor. These molecules may interact constitutively with the receptor or may be interactive only upon its binding to a ligand. These molecules may reside in the membrane itself, or may be associated with the receptor in the cytoplasm. It is the nature of these molecules in the immediate microenvironment of the receptor which provide important information as to the role, mechanism of action, and/or kinetics of the receptor in cellular functioning. This assessment may be critical in a decision as to whether the action of the receptor should be agonized or antagonized, and as to whether it is useful to construct antibodies, proteins, cells, pharmaceuticals or vaccines which specifically interact with the receptor or elicit an immune response thereto. In addition, an understanding of molecules associated with the receptor will aid in the design of screening assays for identification of useful therapeutic and prophylactic agents.

The membranes used as the starting material in the invention may retain their association with their cells of origin or may be separated therefrom. For use as starting materials in the method of the invention, it will have been unnecessary to subject the membranes to any "surface treatment," as would, for example, expose shielded carbohydrates or cryptic receptors. "Surface treatment" means treatment designed to expose portions of the receptors not natively displayed by the membrane. Such surface treatments may include, for example, treatment with neuraminadase to expose carbohydrate residues; such treatment would be unnecessary within the context of the present invention as lectins are excluded from the ligands specifically binding receptors as described above. Indeed, one of the drawbacks of prior art methods to recover membrane-displayed receptors is that treatment with detergents or other harsh reagents may expose cryptic moieties which bind the ligand used in the method, thus creating an artifact uncharacteristic of the native function of the ligand. Thus, "surface treatment" is avoided so as to ensure that the membrane displays only portions of the receptors that are displayed in the native state of the membrane.

However, the "microenvironment" may in fact be devoid of any interacting substances. In this case, the receptor will be isolated absent any associated materials; *i.e.*,

the microenvironment is a void. Thus, "microenvironment" should not be understood to mandate the presence of additional substances when the receptor is recovered by the method of the invention. Where the "receptor" is unassociated in any meaningful way with additional substances, it will be isolated by itself and this, too, provides valuable
5 information.

Typically, the membrane with which the receptor is associated is a cell surface membrane. However, the invention is also applicable to recovery of receptors which are associated with other cellular membranes, such as nuclear membranes, endoplasmic reticulum, golgi apparatus, mitochondria or other cellular organelles. In applying the
10 method to intracellular receptors, nuclei or other organelles are first isolated and subjected to the method of the invention in place of intact cells. Cell surface membranes may also be isolated from the cells; however, in the case of cell surface membranes, intact cells may also be used.

Any type of cell or tissue which contains exposed receptors can be used in the
15 method of the invention. Such cells include, generally, eukaryotic cells, such as yeast cells, fungal cells in general, cells derived from invertebrates, and bacterial cells. However, the cells employed in the invention are preferably nucleated cells and can include any cell which displays receptors at its surface. Preferably, such cells are vertebrate cells, and most preferably mammalian cells.

A wide variety of such cells is employed, including, without limitation, cells
20 from adipose, areolar, connective, elastic, epithelial, neural, mucous, reticular, etc. tissues. Cells can be from alimentary, breast, cardiovascular, cutaneous, endocrine, gastrointestinal, hematopoietic, hepatobiliary, lymphoid, lymphoreticular, muscle, male or female reproductive, respiratory, skeletal, or urinary tract tissues. Thus, cells can comprise
25 hematopoietic cells, including lymphocytes of various types, progenitor cells, stem cells, macrophage, granulocytes. Cells can comprise endothelial cells, myeloid cells, tumor cells, cells derived from a various of cell lines, and cells derived from a variety of organs such as urinary bladder, brain, breast, colon, heart, kidney, liver, lung, bone marrow, ovary, pancreas, prostate, skin, stomach, testes, and the like. Highly tissue-specific cells, such as
30 those derived from the retina, cornea or choroid can also be used. Specialized cells of the immune system, or cells associated with tumors which contain tumor-associated antigens at their surfaces can also be employed.

Thus, receptors associated with membranes contained in or on tumor cells, such as those derived from benign or malignant tumor cells of prostate, bladder, kidney, colon, breast, pancreas, bone, ovary, lung, brain or non-solid tumors such as lymphomas and leukemias are available for retrieval and study according to the method of the invention. In addition, membranes derived from cells associated with various disease conditions, including infected cells, cells exhibiting an autoimmune response, or cells with other abnormalities are made available for elucidation of pathways and receptors unique to these cells.

An advantage of the invention is that a wide variety of cell types can be assessed for the types of receptors displayed and thus the role of these receptors in cell differentiation and growth further elucidated. The methods of the invention also permit profiling of the receptor pattern on particular types of cells and affords the ability to contrast the membrane receptor profile in cells which are associated with particular tissues, or at particular stages of differentiation, aging, or which are considered abnormal, such as diseased cells or tumor cells. As an example of such application, lectins can be used to profile the receptors expressed on a particular type of normal tissue, and contrast it to the receptor profile of a tumor tissue from the same type. Thus, leading to the identification of those receptors differentially expressed in the diseased tumor tissues.

In one application of the invention, a single receptor along with its microenvironment is removed from the cell surface or other cellular membrane of choice. In this embodiment, there is first provided a solid support to which is bound a ligand for the desired receptor. If the receptor is known, a convenient ligand is an antibody or fragment thereof that is immunospecific for the receptor. Such antibodies can be prepared by immunization with the previously isolated receptor (such previous isolation generally having destroyed the microenvironment of the receptor) or with cells displaying the receptor using standard immunization techniques or using phage display technology. Polyclonal antibodies which are immunospecific for the receptor can then be recovered from the sera of an immunized subject, or monoclonal antibodies may be prepared from the subject. Antibodies can also be prepared recombinantly in various forms such as single-chain antibodies. Fragments of the antibodies, such as Fab, Fab' and F(ab')₂ may also be used. Any portion of the antibody which is immunospecific can be used as the ligand for recovery of the receptor.

Even if the receptor is not known, antibodies can be prepared which result in the isolation of the previously unknown receptor along with its microenvironment containing whatever components the microenvironment may include. For example, cells may be used for immunization and monoclonal antibodies prepared from the subject of immunization and produced by hybridoma technology or using standard recombinant techniques including phage display. The individual monoclonal antibodies can then be used as ligands to retrieve previously unknown receptors from the cell surfaces or from membranes derived from these cells.

In addition to antibodies, aptamers which specifically bind the receptor can be obtained using SelexTM procedures as is understood in the art. Alternatively, the ligand may be a ligand native to the receptor or may be an arbitrarily chosen ligand recovered, for example, from a combinatorial library, including libraries which contain small molecules, peptides, antibodies and the like.

The now selected ligand is coupled to a solid support so that a receptor to which the ligand is complexed is recoverable. The solid support may be in any convenient form, including, for example, beads composed of polystyrene, latex, Sephadex, polyacrylamide and the like. Also employable are magnetic beads, fibers and other geometric configurations. Particularly convenient are multi-well plates.

The ligand is coupled to the solid support by any convenient means wherein the binding is sufficiently tight to withstand disruption by shearing. Preferably, but not necessarily, the ligand is covalently bound to the support. Depending on the choice of ligand and the chemical nature of the support, linking techniques are selected to obtain the desired binding. Such linking techniques include, for example, coupling through CNBr, or coupling through linkers, such as those available from Pierce Chemical Co., Rockford, IL.

Coupling can be through peptide linkages, disulfide linkages, cross-linking through glutaraldehyde, biotin/avidin binding and the like. The linkers can also include means for dissociating the ligand from the solid support. For example, the linker may include a photocleavable portion or a portion which is cleavable by specific enzymes or in response to a predetermined reagent which is harmless to the ligand or the receptor to be retrieved. Protein A can also be used to bind antibodies, optionally followed by a further linking procedure. The selection of linking techniques will be apparent to the skilled artisan.

The solid support, having been provided with, preferably, covalently bound ligand, is then treated with the cells, tissues or membranes containing the desired receptor. The membrane may be included in an intact cell or tissue, an intact nucleus or organelle, or may be prepared as an isolated membrane *per se*. The ligand-derivatized solid support is then treated with the membrane that contains receptor under conditions wherein a complex is formed between the receptor and the coupled ligand. These conditions are typically physiological conditions of neutral pH, room temperature or slightly higher or lower, and, if intact cells are used, preferably appropriate osmotic pressure. The contact time varies but may be as short as one minute or as long as several hours or overnight. After sufficient time has elapsed that a complex between ligand and receptor is formed, the solid support is removed from the sample that contains the membranes and optionally washed to remove unbound membrane, cells or organelles. Washing is generally under standard physiological temperature and pH as well. The now-recovered solid support thus contains ligand complexed to receptor wherein the receptor remains embedded in the membrane of the intact cell, nucleus, or membrane preparation.

The next step is to dissociate the receptor/ligand complex from the membrane in which the receptor resides. In order to perform this step, a gentle force, such as a shear force, is applied. This force can be obtained in a variety of ways, including vortexing, extrusion, such as through vigorous repetitive pipetting, shaking, sonication under mild conditions, stirring, or any other method to impose a gentle shear force. The shear is such that the receptor/ligand complex remains intact while the receptor, along with associated moieties is removed gently from the membrane in which it has resided. The receptor, thus, continues to be associated with the molecules it interacts with *in situ*. If intact cells or tissues have been used, the cells or tissues may remain intact and viable. The fact that they are not lysed permits avoidance of contamination with debris released from lysed cells or tissues. This step may be referred to as "deracination."

After the receptor along with its microenvironment has thus been removed from the membrane, the receptor and its associated components can be analyzed and identified either on the solid support or after removal therefrom. Removal of the receptor from the solid support can be effected, for example, by competitively dissociating the ligand/receptor complex, by cleavage of the entire ligand/receptor complex from the support by releasing the ligand from the support, or by providing dissociation conditions

whereby the ligand/receptor complex is disrupted. For example, if the ligand is an antibody, an antigen which binds the antibody in competition with the receptor can be used to compete the receptor away from binding to the solid support. Such dissociation can also be achieved by altering the conditions so that the ligand no longer binds to a receptor, for example, by altering the pH. If the linkage used to couple the ligand to the solid support has been designed to include a photocleavable portion, the entire complex can be removed by exposure to the appropriate wavelength of light. If the linkage of ligand has been designed to include an enzyme cleavage site, the solid support is then treated with the appropriate enzyme. Alternative ways to dissociate the ligand/receptor complex to free the receptor and its microenvironment alone would include, as well as competitive binding, treatment with high temperatures or with solvents or pH conditions which result in the dissociation. However, competitive interaction is preferred as alternative methods for dissociation of the complex may also dissociate the receptor from the components of its microenvironment.

The further analysis and identification of the receptor and its associated components is then conducted by means generally known in the art. The protein may be sequenced, and the protein, its post transcriptional modifiers and the accompanying materials of the microenvironment may be analyzed by chromatographic methods, NMR, mass spectrometry, infrared spectrometry and the like. A variety of analytical tools is now available for characterizing the protein itself and its microenvironment. Two dimensional electrophoresis, Western blot, and mass spectrometry, and by analytical means which discern protein-protein interactions may be used. See, for example, Rudert, F., *et al.*, *Biotechnol. Ann. Rev.* (2000) 5:45-86; Link, A.J., *et al.*, *Nature Biotechnol.* (1999) 17:676-682; Neubauer, G., *et al.*, *Proc. Natl. Acad. Sci. USA* (1997) 94:385-390. The contents of these documents is incorporated herein by reference to demonstrate the nature of these techniques.

In outline, one illustrative embodiment of the invention as follows: Beads are coated with an antibody specific for an antigen expressed at a cellular surface. The antibody-coated beads are exposed to the antigen-expressing cells, resulting in formation of complexes between the antibody and the antigens at the cell surface. The cells that are bound to beads are then removed using, for example, shearing, leaving behind the antibody complexed to the antigen and its associated membrane components which are thus

deracinated from the cells. The cells which are sheared from the complex are typically fully viable. The antigen with its associated membrane components is then eluted from the antibody/antigen complex and subjected to analysis, for example, by mass spectrometry to identify the antigen and the components associated with it, as described above.

5 The method of the invention can be used for the recovery and analysis of both receptors whose presence was already generally known and to recover receptors which were previously unidentified. To recover unidentified receptors, the specific ligand may be arbitrarily chosen, and can comprise a plurality of arbitrarily chosen receptors. For example, it may be desirable to recover any membrane-embedded receptor which can
10 complex with a particular steroid or with a particular cytokine or with a particular opioid or with a particular antigen or antibody.

 Alternatively, it may be desirable to recover a multiplicity of receptors non-specifically. This can be accomplished using the method of the invention by providing the portion of the receptor exposed on the membrane with one member of a specific binding
15 pair, such as generically biotinylating the proteins at the membrane surface and then utilizing avidin as the ligand. The recovered receptors are then separated by electrophoresis, and analyzed as described herein.

 The method of the invention can also be adapted to high throughput screening to permit identification of receptors to a multiplicity of ligands. For example, separate
20 portions of a solid support may be coupled to a multiplicity of ligands and the multiplicity of ligands then interacted with a sample of membranes which, as before, may comprise intact cells, intact nuclei, intact organelles, or membrane preparations *per se*. The receptor profile of the membrane preparation can then be ascertained in detail.

 Initially, the array of ligands bound by the membrane preparation can be
25 ascertained by labeling the membranes (or cells or nuclei) and detecting the presence of label on a solid support. This can be done, for example, by utilizing fluorescent, colorimetric, radiolabels, or other well known techniques to render the membranes detectable, or by secondarily labeling the membranes by reaction with labeled reagent, such as labeled antibody. The method of the invention of recovering the receptor containing its
30 microenvironment from the membranes can then be employed further to elucidate not only the array of ligand-binding receptors, but also the nature of the role of the displayed receptor in the life of the cell. Characteristic profiles can thus be obtained for a variety of

cells and distinctions made between various differentiated cells, normal cells, diseased cells, tumor cells and the like.

A particularly useful application of the method of the invention is the recovery and identification of peptides associated with HLA class I and class II presentation. In this application, cognate ligands, such as antibodies, directed to antigens encoded by specific MHC haplotypes are coupled to the solid support and incubated with antigen-presenting cells. As a result, a complex is formed between the MHC class I or class II antigen bearing a peptide and the cognate ligand. The complex-containing solid support is then subjected to mechanical disruption to dissociate the complex from the cells, leaving behind the complex of HLA and bound peptide on the support; the peptide can then conveniently be removed from the complex and analyzed. This method can also be applied to, for example, tumor cells to identify peptides presented at the surface which then may be used to elicit a cytotoxic T-cell response to the tumor, since tumor-associated antigens are restricted by MHC.

Other applications include recovery of antigens specific to particular cells, such as Prostate Stem Cell Antigen (PSCA), Prostate Membrane-Specific Antigen (PMSA), various lymphocyte markers such as CD34, CD8, CD4, CD10 and the like, immunoglobulins characteristic of B cells, retinoid receptors, opioid receptors growth factor receptors, ion channels, integrins and the like.

Thus, the method of the invention can be used to identify cellular components associated with signal transduction pathways, such as that associated with EGF activation of the EGF receptor. Antibodies are used to obtain the EGF receptor along with its microenvironment and the components associated with the EGF receptor are analyzed to identify and characterize them.

The following examples are intended to illustrate but not to limit the invention.

Example 1

Deracination of EGF Receptor (EGFR) by Anti-EGFR Mab and Wheat Germ Agglutinin (WGA) lectin

Deracination of EGFR in its native configuration directly from the cell surface of human tumor cells was achieved using beads coupled with anti-EGFR Mab and, as a

control, with Wheat-Germ Agglutinin (WGA) lectin. Deracination was performed on the A431 human epidermoid carcinoma cells, which express high levels of EGFR in the order of 1×10^6 receptors per cell, as shown by flow cytometry (Figure 1) and by previous studies (Sato, *et al.*, *Mol Biol Med* (1983) 1:511-529; Gill, *et al.*, *J Biol Chem* (1984) 259(12):7755-7760).

Specific deracination of EGFR was done using the mouse IgG2ak anti-EGFR 528 Mab (Santa Cruz Biotechnology Inc.), which has an affinity of about 1×10^{-9} M to EGFR displayed on cells. For comparison, WGA, a lectin that binds to N-acetylglucosamine carbohydrate moieties of cell surface proteins including EGFR (Zeng, *et al.*, *Mol Cell Biochem* (1995) 142(2):117) was also used.

Cyanobromide (CNBr)-activated Sepharose 6MB (Amersham Pharmacia Biotech) beads were coupled with the mouse anti-EGFR antibody 528 (IgG2ak), according to the manufacturer's instructions. Approximately 0.1 grams of either 528-coated beads or WGA-coated beads (Amersham Pharmacia Biotech) were incubated with 5×10^6 A431 tumor cells, with or without prior stimulation with $1 \mu\text{g/ml}$ EGF for 5 minutes. After 15 minutes incubation at room temperature, excess unbound cells were washed 3 times with PBS by settling the beads and discarding the cells in the supernatant.

Cells bound to beads were removed by pipetting with a pasteur pipette 5-6 times. The beads were allowed to settle, and the released cells in the supernatant were discarded. The process was repeated 4-5 times. The leftover cells bound to beads were removed by gentle vortexing. The removed cells were shown to be viable. The beads were allowed to pellet and the supernatant was discarded.

The beads were visualized under light microscopy to confirm complete removal of cells, resuspended in sample buffer (0.06M Tris-HCL pH 6.8, 10% glycerol, 2%SDS, 5% β -ME, 0.001% bromophenol blue), boiled for 3 minutes, and the supernatant loaded on an SDS-PAGE polyacrylamide gel. The gel was transferred unto nitrocellulose membrane and blotted.

Blots were stained with rabbit anti-EGFR antibody, followed by HRP-conjugated anti-rabbit antibody, and developed using the ECL chemiluminescence kit (Amersham Pharmacia) (Figure 2). The 170 kDa EGFR protein was readily detected as a sharp band in the material deracinated from A431 cells using the anti-EGFR. In the material from WGA-coated beads, the bands were more diffuse since more protein is

deracinated. These results indicated successful deracination of EGFR from A431 cells by the 528 Mab. As expected, similar levels of EGFR proteins were deracinated from cells cultured in absence or presence of EGF.

Example 2

5 Deracination of EGFR-associated proteins by Anti-EGFR Mab and WGA lectin

The binding of EGF to its receptor, EGFR, triggers a cascade of cellular biochemical events including the receptor tyrosine autophosphorylation as well as phosphorylation of several cytoplasmic molecules (Pandey, *et al.*, *PNAS* (2000) 97:179-184). EGFR autophosphorylation leads to its interaction with cytoplasmic
10 signaling proteins containing src homology domains (SH2), and phosphotyrosine interaction domains (PID), such as Grb2 and Shc proteins (O'Bryan, *et al.*, *J Biol Chem* (1998) 273(32):20431). Association of Grb2 and Shc proteins with EGFR was demonstrated by fluorescence resonance energy transfer analysis (Sorkin, *et al.*, *Current Biology* (2000) 10:1395), by immunoprecipitation (Hashimoto, *et al.*, *J Bio Chem* (1999)
15 274(29):20139-10143; Kimoto, *et al.*, *Genes Cells* (2000) 5:749-764) and by mass spectrometry (Pandey, *et al.*, *PNAS* (2000) 97:179-184). Combination of immunoprecipitation with mass spectrometry identified another EGFR-interacting protein, Vav-2, to be tyrosine phosphorylated upon EGF-mediated stimulation (Pandey, *et al.*, *PNAS* (2000) 97:179-184).

20 To isolate activated EGFR and EGFR-associated proteins in their native configuration, deracination on A431 cells stimulated with EGF was performed, using anti-EGFR Mab coated beads as described in Example 1. The deracination products were run on an SDS-PAGE gel and the membranes were stained with the mouse anti-phosphotyrosine antibody (Santa Cruz Biotechnology Inc.), followed with
25 HRP-conjugated anti-mouse antibody.

As shown in Figure 3, deracination of EGFR by 528 Mab from EGF-activated A431 cells led to the isolation of highly tyrosine phosphorylated EGFR as compared to the samples deracinated from unstimulated cells, and to A431 cell lysate. The detection of low level EGFR phosphorylation in unstimulated cells corroborates with previous publications
30 showing that anti-EGFR 528 Mab itself is capable of stimulating EGFR phosphorylation and activation (Gill, *et al.*, *J Biol Chem* (1984) 259:7755-7760). The analysis also detected

additional tyrosine phosphorylated proteins with approximate molecular weights of 95, 90, 85, 70, 65 and 60 kDa, most of which (90, 85, 70, 65 and 60 KDa) were significantly enriched in the deracination products of EGF-stimulated cells as compared to the unstimulated cells. Most of these tyrosine phosphorylated proteins are in agreement with those previously identified to be associated with EGFR by immunoprecipitation studies, including Grb2, Vav-2, and Shc proteins (25, 110, and 46/52/66 kDa, respectively). (The band detected at molecular weight of ~50kDa corresponds to cross-reactivity of the secondary antibody with the mouse heavy chain (mIgH) of 528 Mab released from the beads.)

Further characterization of the deracinated samples was carried out by Western blot analysis using detecting antibodies specific to Vav-2 (Figure 4A), Grb2 (Figure 4B), and Shc proteins (Figure 5).

Western blot analysis of 528 Mab-deracinated samples using anti-Vav-2 antibody readily detected Vav-2 protein (approximately 110kDa) indicating its association with EGFR in both stimulated and unstimulated A431 cells (Figure 4A). Similarly, Grb2 protein (25 kDa) was also readily detected in the anti-EGFR Mab deracinated materials (Figure 4B). Of note, the band detected at molecular weight ~50kDa corresponds to cross-reactivity of the secondary antibody with mouse heavy chain (mIgH) of the 528 antibody released from the beads.

The isolation of EGFR-associated protein Vav-2 and Grb2 in both unstimulated and EGF-stimulated cells could be explained by the observation that anti-EGFR itself is capable of activating EGFR, which can lead to interaction with Vav-2 in absence of EGF stimulation (Gill, *et al.*, *J Biol Chem* (1984) 259:7755-7760).

Shc proteins can exist in 3 isoforms, P46, P52 and P66. They are adaptor proteins shown to associate with EGFR and mediate downstream signaling leading to JNK activation (Hashimoto, *et al.*, *J Bio Chem* (1999) 274(29):20139-10143). The association of Shc proteins with EGFR in the deracinated samples was examined using anti-Shc antibodies (Santa Cruz Biotechnology Inc.). Figure 5 shows that deracination with 528 Mab resulted in the isolation of the 3 isoforms of Shc. Interestingly, the P46 and P66 isoforms of Shc were detected at similar levels in the deracination products and whole cell lysate. However, the P52 product was less abundant in the deracination products of unstimulated cells compared to whole cell lysate, and even less abundant in the stimulated cells

compared to unstimulated cells. This observation suggests differential binding of Shc proteins to the EGFR receptor following EGF stimulation.

Altogether, these data show that deracination using a receptor specific Mab or a lectin (WGA) leads to the isolation of the receptor together with its specific interacting proteins. The ligand-specific deracination of EGFR using 528 Mab leads to specific isolation of the EGFR receptor in its native configuration, without use of detergents, as well as to its interacting adaptor proteins.

Example 3

Deracination of PSCA using anti-PSCA antibodies and WGA lectin

Prostate Stem Cell Antigen (PSCA) (Reiter, *et al.*, *PNAS* (1998) 95:1735-1740; Wu, *et al.*, *Oncogene* (2000) 19:1288-1296) is immunoreactive with 1G8 Mab (Reiter, *et al.*, *PNAS* (1998) 95:1735-1740). The 1G8 Mab binds to the PSCA receptor with an affinity of approximately 1×10^{-9} M, whereas WGA binds specifically to N-acetyl glucosamine moieties of PSCA, as well as such moieties on other cell surface molecules such as EGFR. The experiments were performed using the prostate tumor cell line PC3, which does not express the PSCA receptor, or with PC3 stably transfected with the cDNA encoding PSCA (PC3-PSCA) (Wu, *et al.*, *Oncogene* (2000) 19:1288-1296).

Figure 6 shows flow cytometry of untreated PC3 and PC3-PSCA cells and these cells treated with antibody or lectin. PSCA is shown to be expressed on the surface of PC3-PSCA cells (Figure 6E) but not on PC3 cells (Figure 6B) by staining with 1G8 Mab. Binding of WGA to soluble PSCA was demonstrated by ELISA (data not shown), and binding to both PC3-and PC3-PSCA cells was demonstrated by flow cytometry (Figure 6C and 6F).

Deracination was performed on PC3, PC3-PSCA, or the control PSCA-negative A431 cells using either 1G8 Mab or WGA coated beads as described in Example 1. The deracination products were run on an SDS-PAGE gel and the membranes were stained with the mouse anti-PSCA antibody 3C5 (IgG2 α ; Wu *et al.*, *Oncogene* 2000, 19:1288-96), followed by HRP-conjugated anti-mouse IgG2 α antibody and then developed using the ECL chemiluminescence kit. As shown in Figure 7A, the highly glycosylated PSCA protein, similar to that detected in PC3-PSCA lysates, was only detected in the products

deracinated from PC3-PSCA by both 1G8 MAb and WGA, but not from the parental cell line PC3 or the A431 cells.

Development of the blot with rabbit anti-EGFR antibody showed that deracination of EGFR was incidentally obtained only using WGA lectin, but not by anti-PSCA coated beads (Figure 7B). The higher level of EGFR protein deracinated from A431 cells as compared to PC3 and PC3-PSCA cells is in agreement with the higher cell surface levels of EGFR on A431 cells as compared to PC3 cells. Deracination of PSCA but not EGFR, using 1G8 anti-PSCA Mab, demonstrates specificity of deracination using the Mab-coated beads, and lack of non-specific contamination of additional membranes proteins not associated with PSCA.

Results of deracination performed on PC3 and PC3-PSCA cells using WGA coated beads are shown in Figures 8A and 8B. Western blots of the deracination products were stained with either mouse anti-PSCA 3C5 Mab (Figure 8A), or rabbit anti-Grb2 antibody (Figure 8B). PSCA was detected in the deracination products of PC3-PSCA but not PC3. Development of the blot with anti-Grb2 antibody detected the cytoplasmic protein Grb2 in the lysates of both PC3 and PC3-PSCA cells, but not in the products deracinated from WGA-coated beads. This indicates that, unlike EGFR, Grb2 is not associated with PSCA, a GPI- linked protein (Reiter, *et al.*, *PNAS* (1998) 95:1735-40).

Claims

1. A method to isolate and characterize a membrane-bound receptor along with its microenvironment which method comprises

(a) providing a solid support coupled to a ligand which specifically binds said
5 receptor;

(b) treating said solid support with a sample comprising nucleated cells or organelles thereof comprising said membrane-bound receptors, and which cells or organelles have not been surface-treated,

wherein a complex is formed between said membrane-bound receptor and the
10 ligand thus generating a ligand/receptor complex comprising the receptor and its microenvironment, which complex is coupled to solid support through the ligand;

(c) separating the solid support from the remainder of the sample;

(d) subjecting the separated solid support to a force sufficient to dissociate the receptor and its microenvironment from the membrane but insufficient to disrupt the

15 ligand/receptor complex;

thus obtaining solid support coupled to a ligand/receptor complex whereby the receptor retains its microenvironment but is separated from the membrane, and, optionally,

(e) analyzing the microenvironment of the receptor.

2. The method of claim 1 wherein step (e) is performed.

20 3. The method of claim 1 which further comprises removing the ligand/receptor complex comprising the receptor and its microenvironment from the solid support.

4. The method of claim 1 which further comprises removing the receptor and its microenvironment without ligand from the solid support.

25 5. The method of claim 1 wherein, in step (d), the cells remain intact.

6. The method of claim 1 wherein the cells are vertebrate cells.

7. The method of claim 6 wherein the cells are tumor cells or diseased cells..

8. The method of claim 6 wherein the cells are hematopoietic cells, or cells from adipose, areolar, connective, elastic, epithelial, endothelial, neural, mucous or reticular tissues.

5 9. The method of claim 1 wherein the ligand is an antibody or an immunospecific portion thereof.

10. The method of claim 1 wherein the receptor comprises an HLA antigen.

11. The method of claim 1 wherein the receptor comprises a tumor associated antigen.

10 12. The method of claim 1 wherein the receptor is a cytokine receptor, a hormone receptor, an opioid receptor, or a steroid receptor.

13. The method of claim 1 wherein the force is achieved through extrusion.

14. The method of claim 1 wherein the force is achieved through vortexing or shaking.

15 15. The method of claim 1 wherein the force is achieved through sonication.

16. The method of claim 1 wherein the solid support comprises beads.

17. The method of claim 14 wherein said beads are polyacrylamide beads, polystyrene beads, Sephadex beads, or latex beads.

18. The method of claim 1 wherein the solid support is a multi-well plate.

20 19. The method of claim 3 wherein the ligand is coupled to the solid support through a linker containing a photocleavable portion and said removing is effected by exposing the linker to light.

20. The method of claim 3 wherein the ligand is coupled to solid support through a linker containing a portion cleavable by an enzyme and said removing is effected by exposing said linker to said enzyme.

21. The method of claim 4 wherein said removing is achieved by treating the
5 complexed solid support with a substance that binds said ligand in competition with the receptor.

22. A method to recover a multiplicity of receptors along with their microenvironments which method comprises:

(a) providing a multiplicity of solid support portions each coupled to a different
10 ligand;

(b) treating a sample comprising nucleated cells that comprise at least two cell surface receptors, wherein said cells are not surface treated with said multiplicity of solid support portions under conditions wherein ligand/receptor complexes are formed between said ligands and receptors at said cell surfaces;

15 (c) removing the solid support portions from the sample; and

(d) subjecting the solid support portions to forces sufficient to remove the receptors and their microenvironments from the surface of said cells but insufficient to disrupt the ligand/receptor complexes, and

(e) analyzing the respective microenvironments of at least two receptors.

20 23. The method of claim 22 which further comprises removing the ligand/receptor complexes or the receptors and their microenvironments from the solid support.

24. The method of claim 22 which further includes identifying the receptors.

25. The method of claim 23 which further includes identifying the receptors.

25 26. The method of claim 24 identifying components in said micro-environment.

27. The method of claim 25 identifying components in said micro-environment.

28. The method of claim 24 which further includes organizing the identified receptors into a profile characteristic of the membrane sample.

29. The method of claim 25 which further includes organizing the identified receptors into a profile characteristic of the membrane sample.

5 30. The method of claim 22 wherein each of said multiplicity of portion is coupled to a different ligand.

31. The method of claim 30 wherein said ligands are monoclonal antibodies.

Figure 1: Detection of EGFR Expression on the Surface of A431 Cells

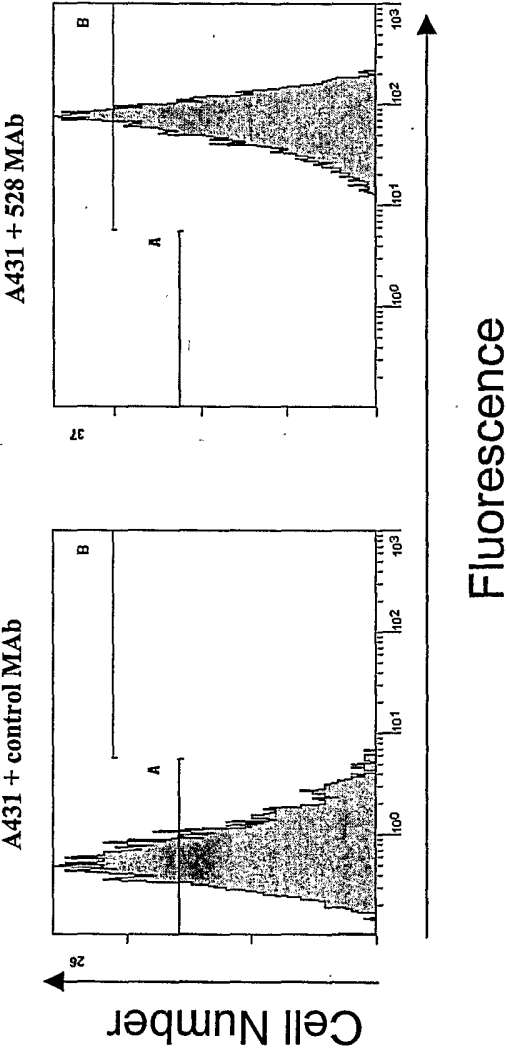
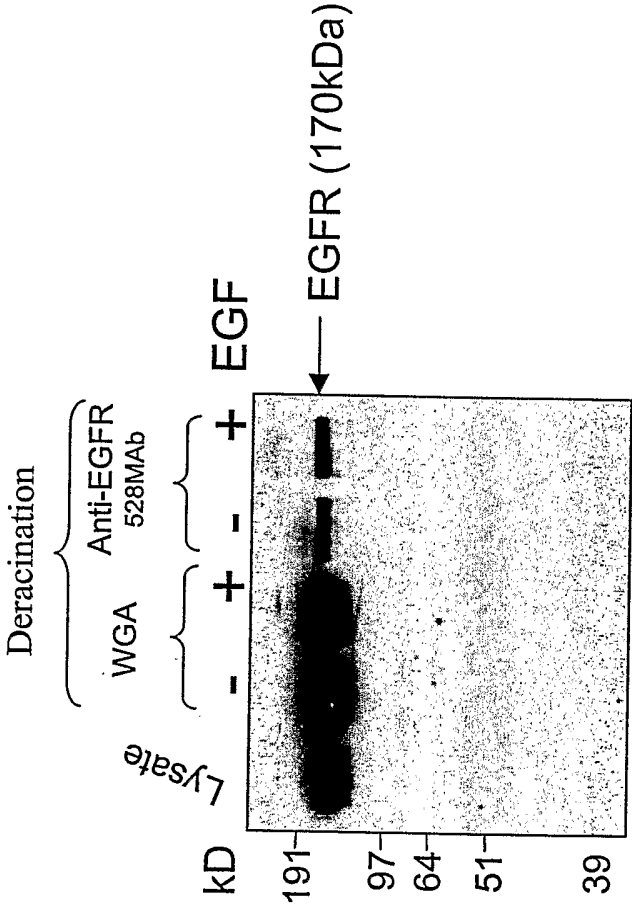
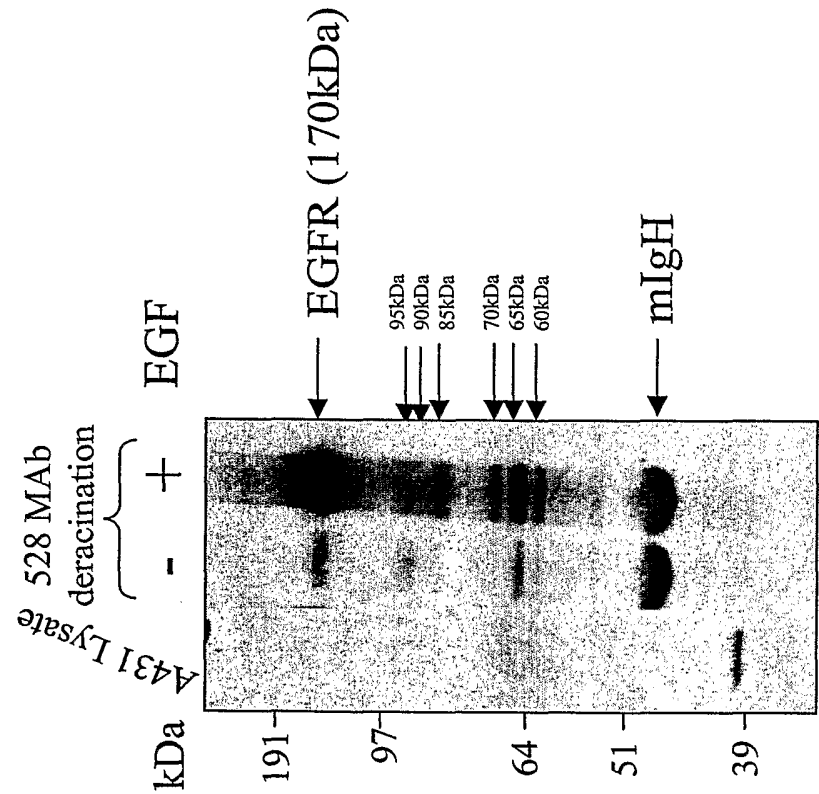


Figure 2: Deracination of EGFR by Anti-EGFR MAb and WGA Lectin



Detection: Rabbit anti-EGFR

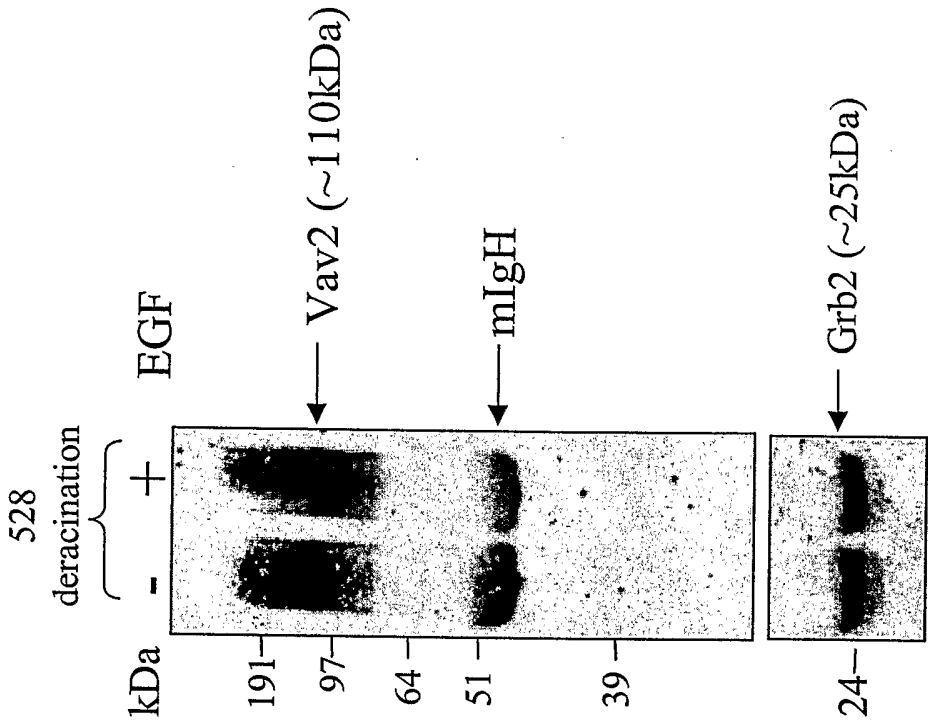
Figure 3: Deracination of activated EGFR and its Associated Phosphoproteins by Anti-EGFR 528 MAb



Detection: Anti-phosphotyrosine

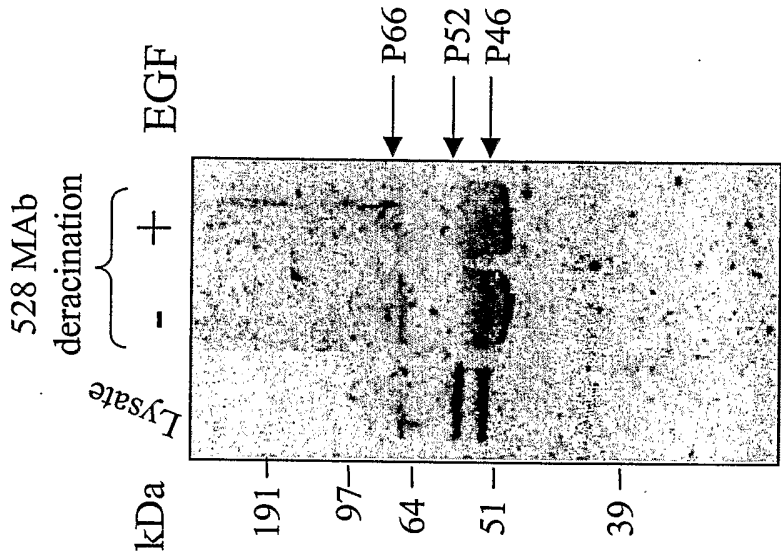
Figure 4: Deracination of EGFR-Associated Vav2 and Grb2 by Anti-EGFR MAb

A. Detection: Anti-Vav-2



B. Detection: Anti-Grb2

Figure 5: Deracination of EGFR-Associated Shc Proteins by Anti-EGFR 528 MAb



Detection: Anti-Shc

Figure 6: Binding of anti-PSCA 1G8 MAb and WGA to the surface of PC3 Cells

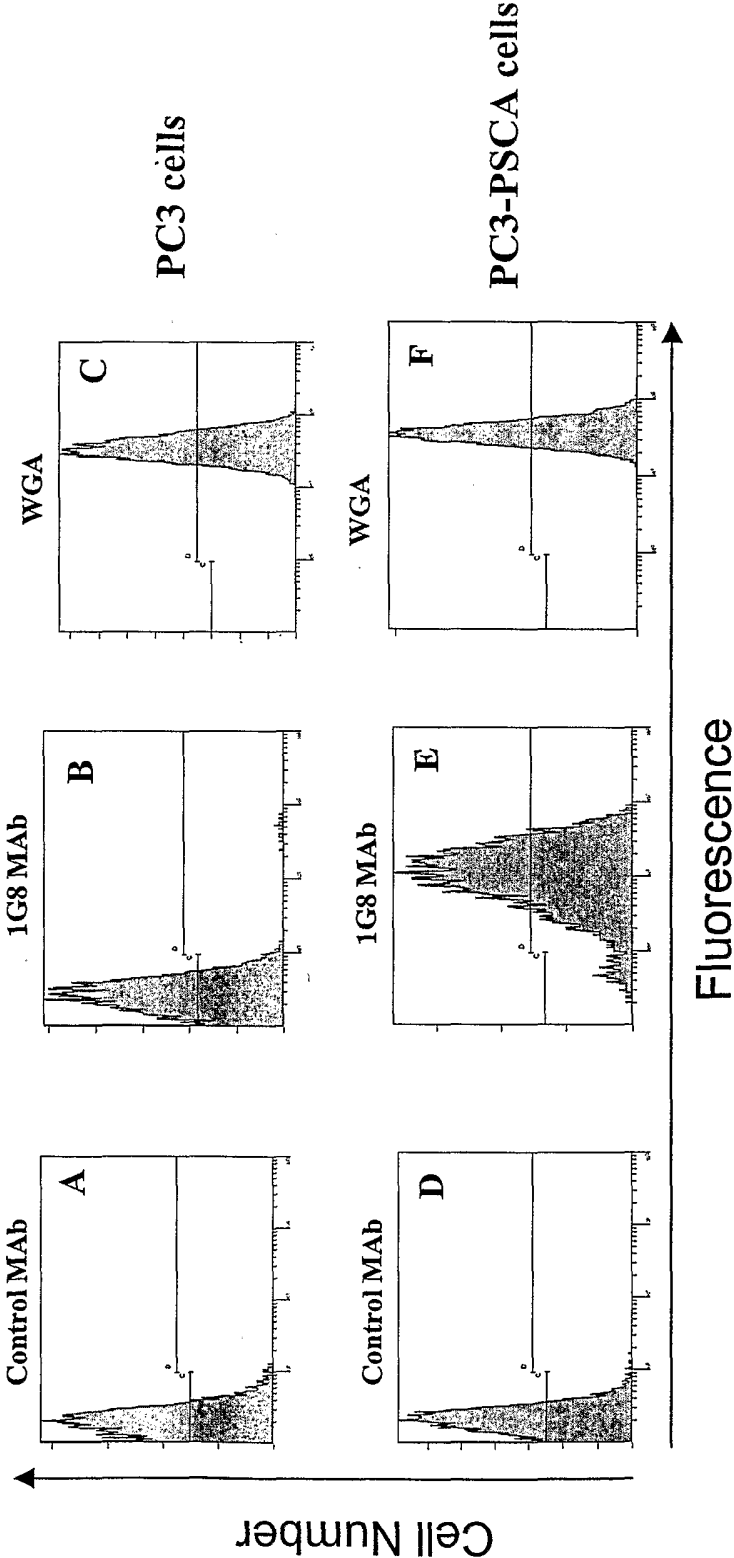


Figure 7: Specific Deracination of PSCA but not EGFR by Anti-PSCA 1G8 MAb

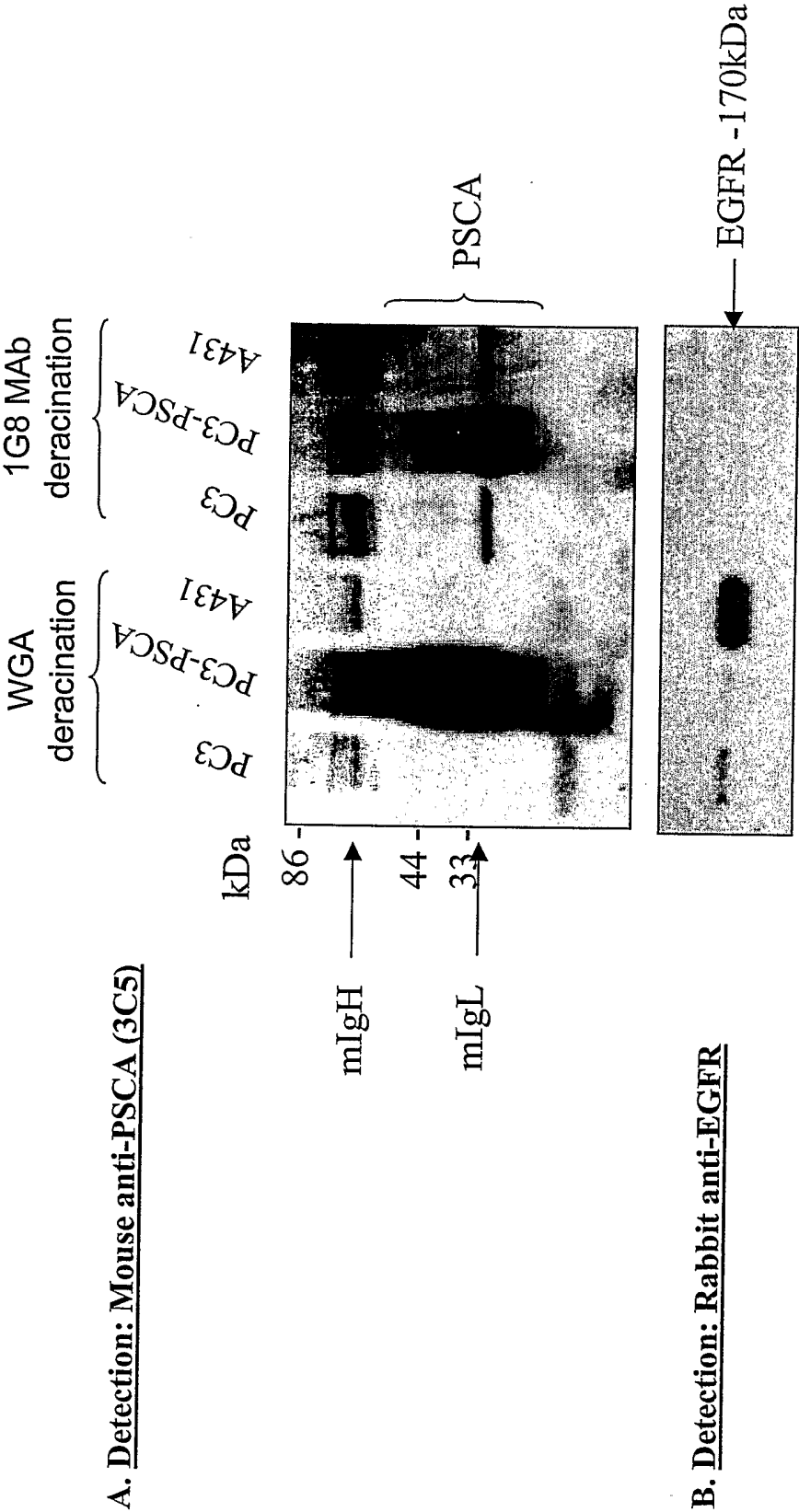
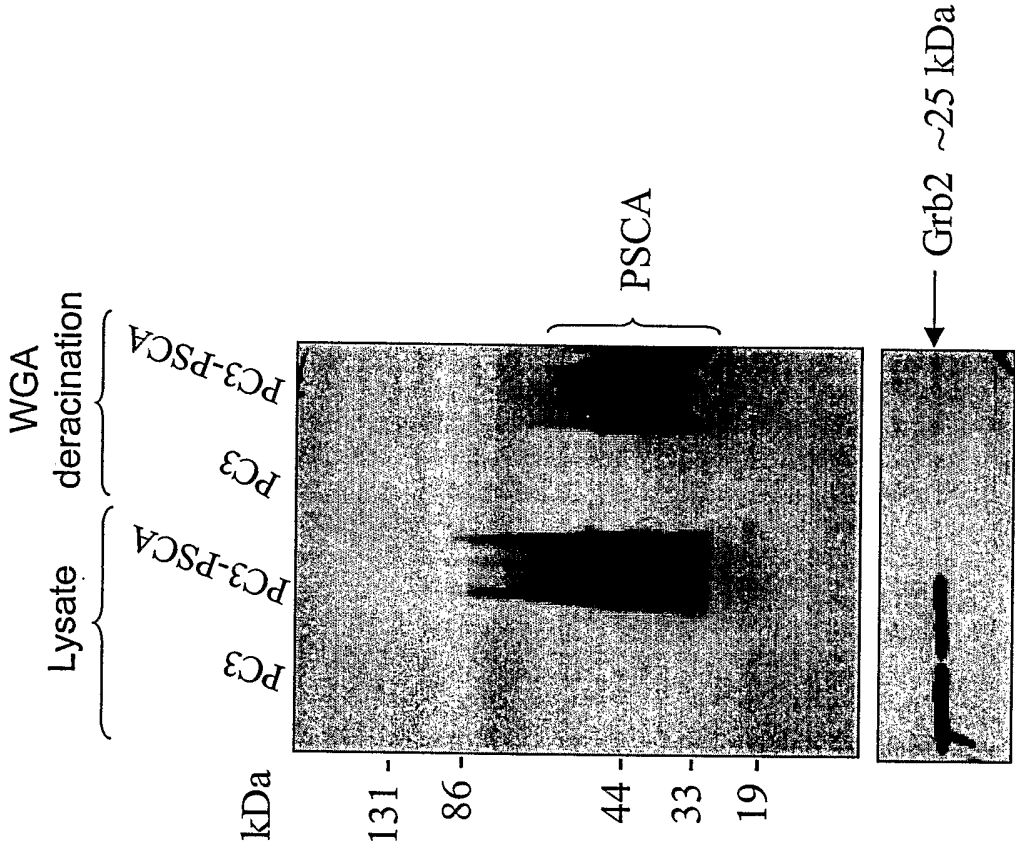


Figure 8: Deracination of PSCA with WGA Lectin



A. Detection: Anti-PSCA (3C5)

B. Detection: Anti-Grb2