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(19) **United States**(12) **Patent Application Publication**
CRINE et al.(10) **Pub. No.: US 2016/0052968 A1**(43) **Pub. Date: Feb. 25, 2016**(54) **BONE DELIVERY CONJUGATES AND
METHOD OF USING SAME TO TARGET
PROTEINS TO BONE**filed on Jul. 23, 2004, provisional application No.
60/614,984, filed on Oct. 4, 2004.(71) Applicant: **ALEXION PHARMACEUTICALS,
INC.**, Cheshire, CT (US)(72) Inventors: **Philippe CRINE**, Outremont (CA); **Guy
Boileau**, Brossard (CA); **Isabelle
Lemire**, Montreal (CA); **Thomas P.
Loisel**, Montreal (CA)(21) Appl. No.: **14/931,310**(22) Filed: **Nov. 3, 2015****Related U.S. Application Data**(63) Continuation of application No. 12/638,527, filed on
Dec. 15, 2009, which is a continuation of application
No. 11/111,664, filed on Apr. 21, 2005, now Pat. No.
7,763,712.(60) Provisional application No. 60/563,828, filed on Apr.
21, 2004, provisional application No. 60/590,347,**Publication Classification**(51) **Int. Cl.****C07K 7/06** (2006.01)**C12N 9/16** (2006.01)(52) **U.S. Cl.**CPC ... **C07K 7/06** (2013.01); **C12N 9/16** (2013.01);
C07K 2319/01 (2013.01); **A61K 38/00**
(2013.01)

(57)

ABSTRACT

A bone delivery conjugate having a structure selected from the group consisting of: A) X-D_n-Y-protein-Z; and B) Z-protein-Y-D_n-X, wherein X is absent or is an amino acid sequence of at least one amino acid; Y is absent or is an amino acid sequence of at least one amino acid; Z is absent or is an amino acid sequence of at least one amino acid; and D_n is a poly aspartate wherein n=10 to 16. Compositions comprising same and methods of use thereof.

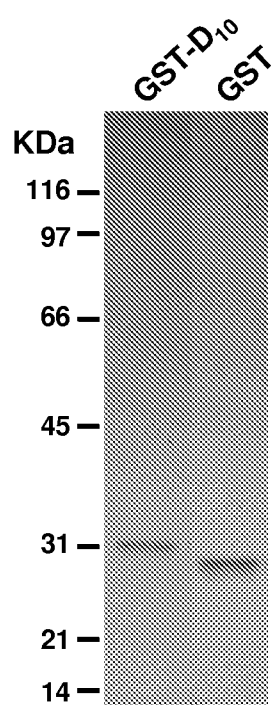


Fig. 1

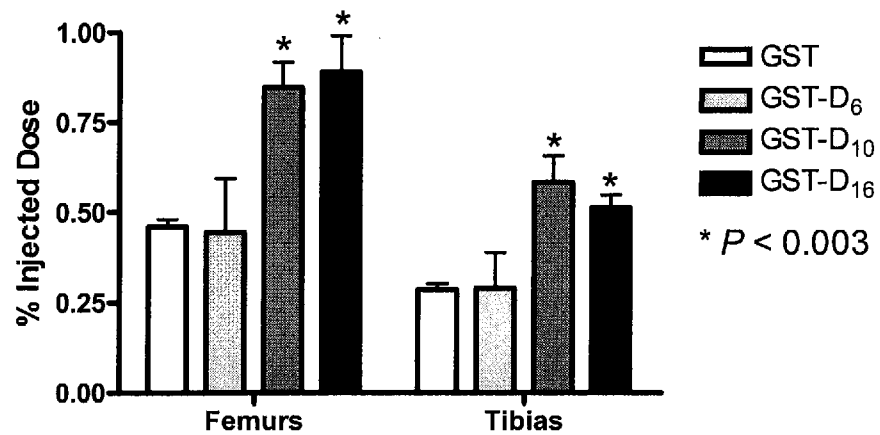


Fig. 2

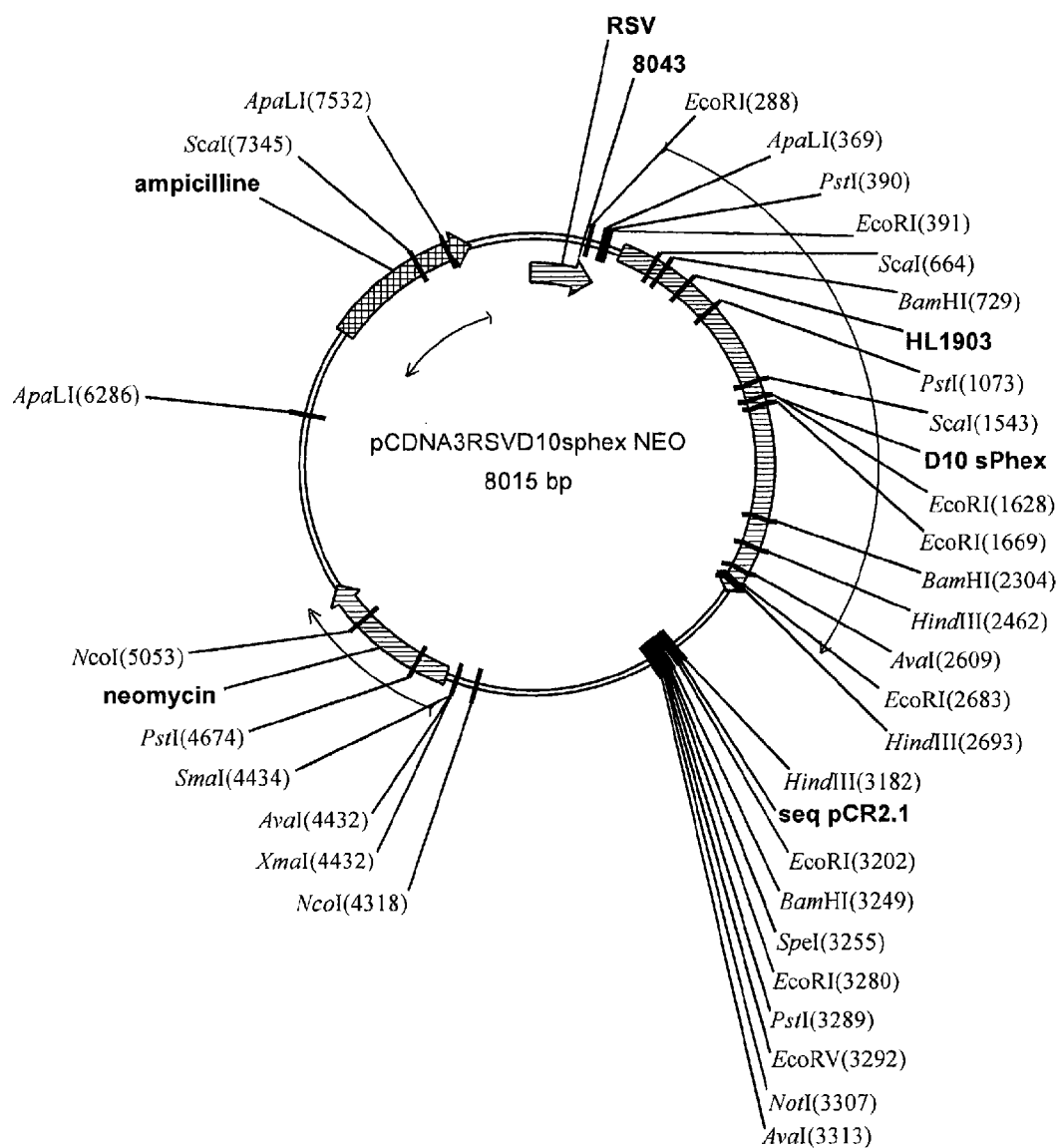


Fig. 3

Fig. 4A

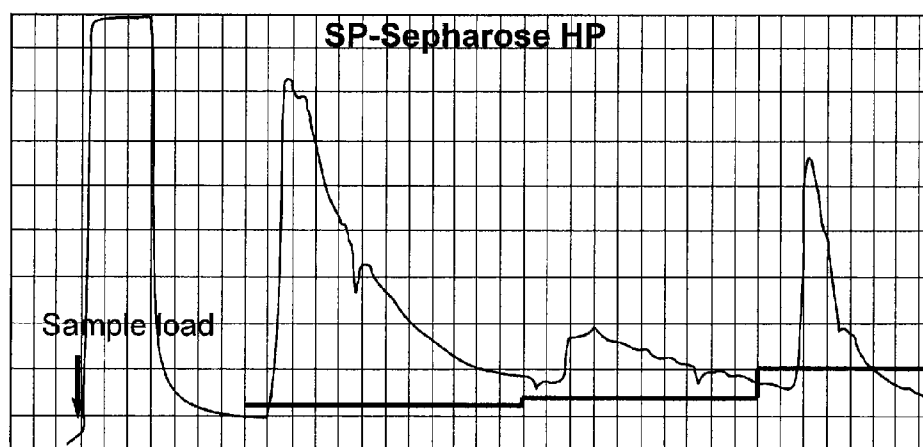
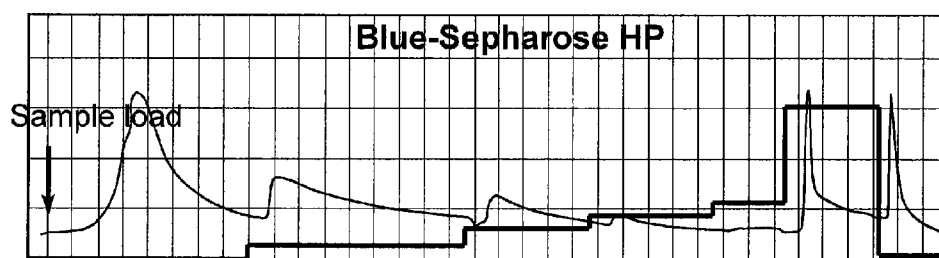


Fig. 4B



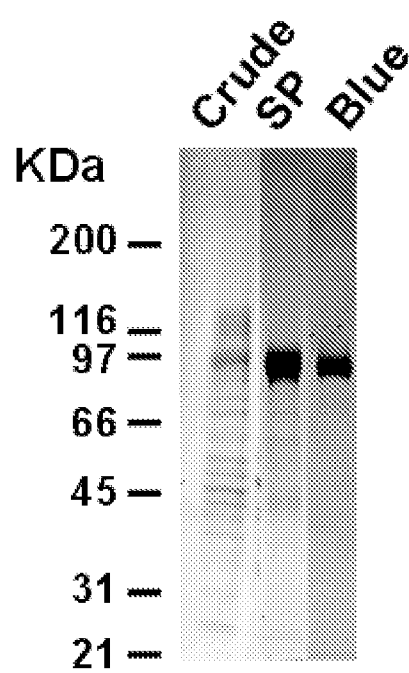


Fig. 5

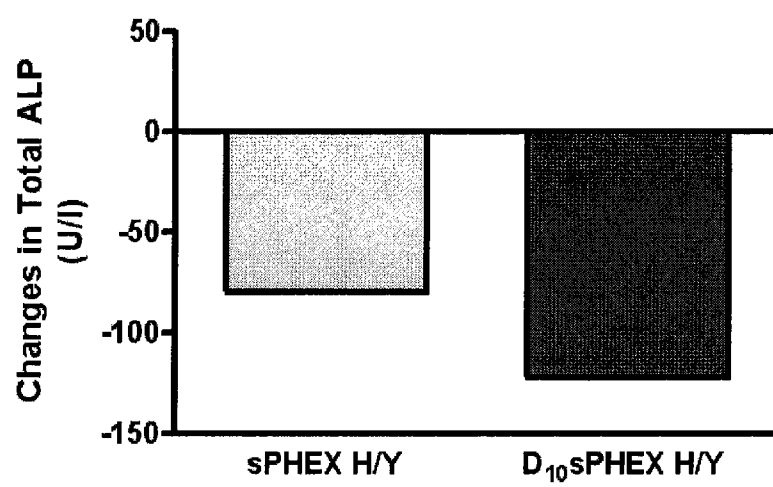


Fig. 6

atggaagcagaaacagggagcagcgtggagactggaaagaaggccaacagaggcactcgaattgccttg
tcgtgtttgtcctgacagtgatcgtcaacaaacaaccagtcagggtctcttatccggagatgacgatga
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tgccctttacaagttcatgggtgatactgcccgtgcttttaggagctaacagttccagagcagagcatgac
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agtttaggggtcaatgggtgcaattagtaactttgaagaattccagaaagcttttaactgtccaccaattc
cacgatgaacagaggcatggactcctgccgactctggtag

Fig. 7

MEAETGSSVETGKKANRGTRIALVVFVLTVIAQQTTSQGLLSGDDDDDDDDDDSGSLQAKQEYCLKPECI
EAAAAILSKVNLSVDPNFFRFACDGIWISNNPIPEDMPSYGVYPWLRHNVDLKLKELLEKSISRRTDTE
AIQKAKILYSSCMNEKAIEKADAKPLLHILRHSPFRWPVLESNIGPEGVWSEKFSLLQTLATFRGQYSN
SVFIRLYVSPDDKASNEHILKLDQATLSLAVREDYLDNSTEAKSYRDALYKFMVDTAVLLGANSRAEHD
MKSVLRLLEIKIAEIMIPHENRTSEAMYNKMNISELSAMIPOFDWLGYYKKVIDTRLYPHLKDISPENVV
VRVPQYFKDLFRILGSEKKTIANYLVRMVSRIPNLSRRFQYRWLEFSRVIQGTTLTPQWDKCVNFI
ESALPYVVGKMFVDVYFQEDKKEMMEELVEGVRWAFIDMLEKENEWMDAGTKRKAKEKARAVLAKVGYPE
FIMNDTHVNEDLKAIFSEADYFGNVLQTRKYLAQSDFFWLKAVPKTEWFTNPTTVNAFYASTNQIRF
PAGELQKPPFWGTEYPRSLSYGAIGVIVGHEFTHGFDNNGRKYDKNGNLDPPWSTEESEKFKETKCMIN
QYSNYYWKKAGLNVKGKRTLGENIADNGGLREAFRAYRKWINDRRQGLEEPLLPGITFTNNQLFFLSYAH
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Fig. 8

Fig. 9A

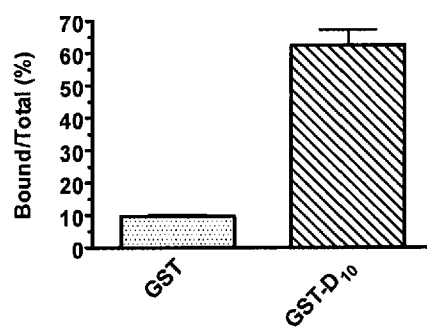
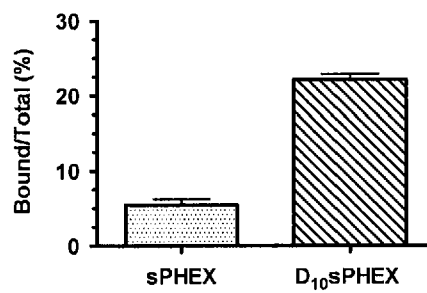


Fig. 9B

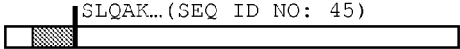
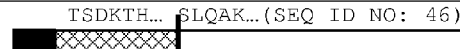
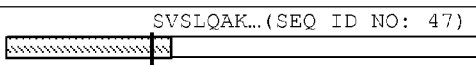
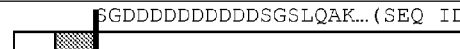
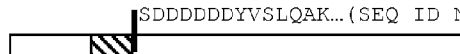


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1 MEAETGSSVE TGKKANRGTR IALVVFVGGT LVLGTILFLV SQGLLSLQAK QEYCLKPECI
61 EAAAAILSKV NLSVDPCDNF FRFACDGIWIS NNPIPEDMPS YGVYPWLRHN VDLKLKELLE
121 KSISRRRDTE AIQKAKILYS SCMNEKAIEK ADAKPLLHIL RHSPFRWPVL ESNIGPEGVW
181 SERKFSLLQT LATFRGQYSN SVFIRLYVSP DDKASNEHIL KLDQATLSLA VREDYLDNST
241 EAKSYRDALY KFMVDTAVLL GANSSRAEHD MKSVLRLEIK IAEIMIPHEN RTSEAMYNKM
301 NISELSAMIP QFDWLGYIKK VIDTRLYPHL KDISPSENVV VRVPQYFKDL FRILGSEKKE
361 TIANYLVWRM VYSRIPNLSR RFQYRWLEFS RVIQGTITLL PQWDKCVNFI ESALPYVVGK
421 MFVDVYFQED KKEMMEELVE GVRWAFIDML EKENEWMDAG TKRKAKEKAR AVLAKVGYPE
481 FIMNDTHVNE DLKAIKFSEA DYFGNVLQTR KYLAQSDFFW LRKAVPKTEW FTNPTTVNAF
541 YSASTNQIRF PAGELQKPFF WGTEYPRSL S YGAIGVIVGH EFTHGFDNNG RKYDKNGNLD
601 PWWSTESEEEK FKEKTKCMIN QYSNYWYKKA GLNVKGKRTL GENIADNGGL REAFRAYRKW
661 INDRRQGLEE PLLPGITFTN NQLFFLSYAH VRCNSYRPEA AREQVQIGAH SPPQFRVNGA
721 ISNSEEFQKA FNCPPNSTMN RGMDSCLW
```

Fig. 10

SGDDDDDDDDSGSLQAKQEYCLKPECIEAAAAILSKVNLSVDFCDNFFRFACDGWISNNPIPEDMPSY
GVYPWLRHNVDLKLKELLEKSISRRTDEAIQKAKILYSSCMNEKAIEKADAKPLLHILRHSPFRWPVLE
SNIGPEGVWSEKFSLLQTLATFRGQYSNSVFIRLYVSPDDKASNEHILKLDQATLSLAVREDYLDNSTE
AKSYRDALYKFMVDTAVLLGANSSRAEHDMKSVLRLEIKIAEIMIPHENRTSEAMYNKMNISELSAMIPQ
FDWLGYIKKVIDTRLYPHLKDISPENVVVRVPQYFKDLFRILGSEKKTIANYLWVRMVYSRIPNLSRR
FQYRWLEFSRVIQGTTLTPQWDKCVNFIESALPYVVGKMFVDVYFQEDKKEMMEELVEGVRWAFIDMLE
KENEWMDAGTKRKAKEKARAVLAKVGYPEFIMNDTHVNEDLKAIKFSEADYFGNVLQTRKYLAQSDFFWL
RKAVPKTEWFTNPTTVNAFYASTNQIRFPAGELQKPF~~FW~~GTEYPRSLSYGAIGVIVGHEFTHGFDNNGR
KYDKNGNLDPPWSTEEKFKEKTKCMINQYSNYYWKKAGLNVKGKRTLGENIADNGGLREAFRAYRKWI
NDRRQGLEEPLLPGITFTNNQLFFLSYAHVRCNSYRPEAAREQVQIGAHSPQFRVNGAISNFEEFQKAF
NCPPNSTMNRGMDSCRLW

Fig. 11

	Protein construct	Specific activity (UAF/sec/μg)	Schematic representation
1	sPHEX	12.3	
2	Fc-sPHEX	5.75	
3	pCDNA3-RSV-NL1furin-sPHEX	N/A	
4	D ₁₀ sPHEX	12.0	
5	D ₆ sPHEX	8.55	

: Represents the mutated transmembrane domain of PHEX.

: Represents the human IgG1 signal peptide.

: Represents the human IgG1 heavy chain domains hinge, CH2 and CH3.

: 1 N-terminal sequence with the furin cleavage site (RTVVKRISV; SEQ ID NO: 50).

|: Furin cleavage site.

|:peptidase signal cleavage site

Fig. 12

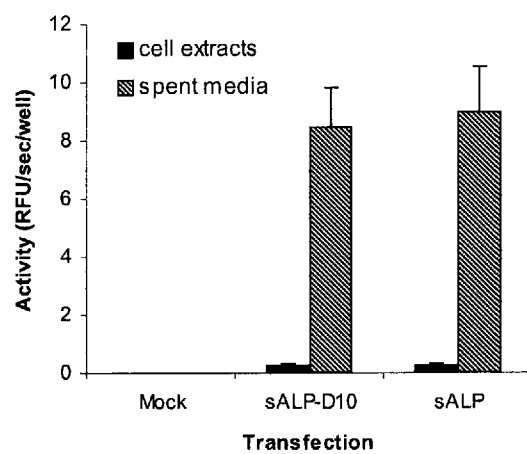
**Fig. 13**

Fig. 14A

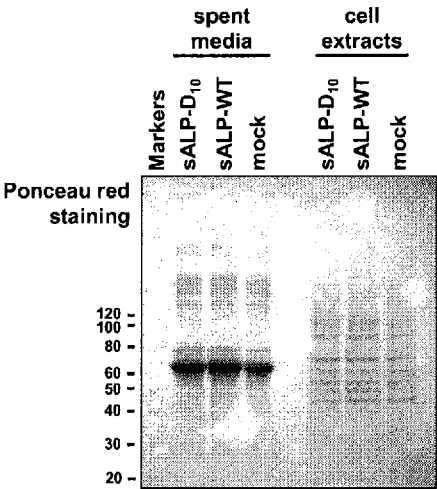
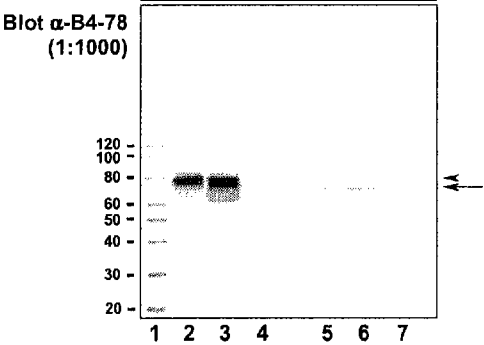


Fig. 14B



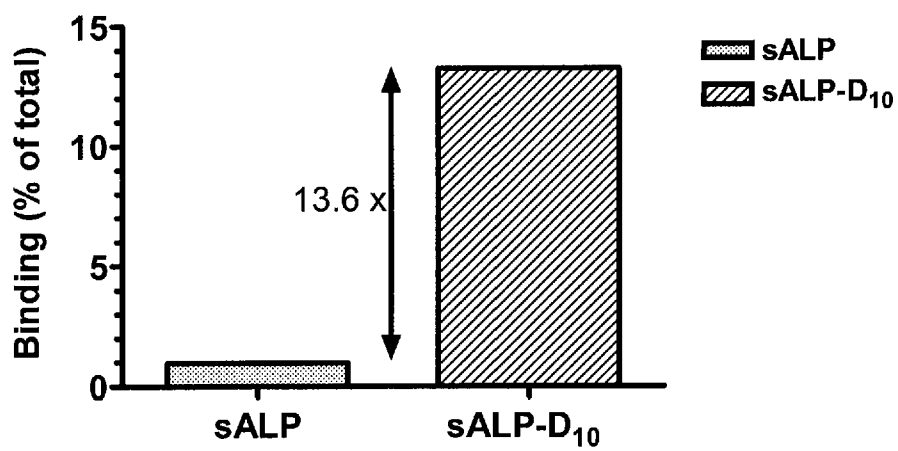


Fig. 15

Fig. 16A

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|      |             |            |            |            |            |            |             |
|------|-------------|------------|------------|------------|------------|------------|-------------|
| 1    | GGATCCACCA  | TGATTTCACC | ATTCTTAGTA | CTGGCCATTG | GCACCTGCCT | TACTAACTCC | TTAGTGCCAG  |
| 71   | AGAAAGAGAA  | AGACCCCAAG | TACTGGCGAG | ACCAAGCGCA | AGAGACACTG | AAATAIGCCC | TGGAGCTTCA  |
| 141  | GAAGCTCAAC  | ACCAACGTGG | CTAAGAATGT | CATCATGTTT | CTGGGAGATG | GGATGGGTGT | CTCCACAGTG  |
| 211  | ACGGCTGCCC  | GCATCCTCAA | GGGTGAGTTC | CACCACAACC | CTGGGGAGGA | GACCAGGTG  | GAGATGGACA  |
| 281  | AGTTCCCTTT  | CGTGGCCCTC | TCCAAGACGT | ACAACACCAA | TGCCCAGGTC | CCTGACAGCG | CCGGCACCGC  |
| 351  | CACCGCCTAC  | CTGTGTGGGG | TGAAGGCCAA | TGAGGGCACC | GTGGGGGTAA | GCGCAGCCAC | TGAGCGTTCC  |
| 421  | CGGTGCAACA  | CCACCCAGGC | GAACCAAGTC | ACCTCCATCC | TGCGCTGGGC | CAAGGACGCT | GGGAAATCTG  |
| 491  | TGGGCATTGT  | GACCACCACG | AGAGTGAACC | ATGCCACCCC | CAGCGCCGCC | TACGCCCACT | CGGCTGACCG  |
| 561  | GGACTGGTAC  | TCAGACAACG | AGATGCCCCC | TGAGGCCCTT | AGCCAGGGCT | GTAAGGACAT | CGCCTACCAG  |
| 631  | CTCATGCATA  | ACATCAGGGA | CATTGACGTG | ATCATGGGGG | GTGGCCGGAA | ATACATGTAC | CCCAAGAATA  |
| 701  | AAACTGATGT  | GGAGTATGAG | AGTGACGAGA | AAGCCAGGGG | CACGAGGCTG | GACGGCCTGG | ACCTCGTTGA  |
| 771  | CACCTGGAAG  | AGCTTCAAAC | CGAGATACAA | GCACTCCAC  | TTCATCTGGA | ACCGCACGGA | ACTCCTGACC  |
| 841  | CTTGACCCCC  | ACAAATGTGA | CTACCTATTG | GGTCTCTTCG | AGCCAGGGGA | CATGCAGTAC | GAGCTGAACA  |
| 911  | GGAAACAACGT | GACGGACCCG | TCACTCTCCG | AGATGGTGGT | GGTGGCCATC | CAGATCCTGC | GGAGAAGCCC  |
| 981  | CAAAGGCTTC  | TTCTTGCTGG | TGGAAGGAGG | CAGAATTGAC | CACGGGCACC | ATGAAGGAAA | AGCCAAGCAG  |
| 1051 | GCCCTGCATG  | AGGCGGTGGA | GATGGACCGG | GCCATCGGGC | AGGCAGGCAG | CTTGACCTCC | TCCGAAGACA  |
| 1121 | CTCTGACCGT  | GGTCACTGCG | GACCATTCCC | ACGTCTTCAC | ATTTGGTGGA | TACACCCCCC | GTGGCAACTC  |
| 1191 | TATCTTTGGT  | CTGGCCCCCA | TGCTGAGTGA | CACAGACAAG | AAGCCCTTCA | CTGCCATCCT | GTATGGCAAT  |
| 1261 | GGGCTTGGCT  | ACAAGGTGGT | GGGCGGTGAA | CGAGAGAATG | TCTCCATGGT | GGACTATGCT | CACAACAACCT |
| 1331 | ACCAGGCGCA  | GTCTGCTGTG | CCCCTGCGCC | ACGAGACCCA | CGGCGGGGAG | GACGTGGCCG | TCTTCTCCAA  |
| 1401 | GGGCCCCATG  | GCGCACCTGC | TGCACGGCGT | CCACGAGCAG | AACTACGTCC | CCCACGTGAT | GGCGTATGCA  |

XbaI  
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1471	GCCTGCATCG	GGGCCAACCT	CGGCCACTGT	GCTCCTGCCA	GCTCGTAGTC	TAGA
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Fig. 16B

1	MISPFVLVLA	GTCLTNSLVP	EKEKDPKYWR	DQAQETLKYA	LLEQLNNTNV
51	AKNVIMFLGD	GMGVSTVTAA	RILKGQLHHN	PGEETRLEMD	KFPFVALSKT
101	YNTNAQVPDS	AGTATAYLCG	VKANEGTVGV	SAATERSRCN	TTQNEVTSI
151	LRWAKDAGKS	VGIVTTTRVN	HATPSAAYAH	SADRDWYSDN	EMPPEALSQG
201	CKDIAYQLMH	NIRDIDVIMG	GGRKYMYPKN	KTDVREYSEDE	KARGTRLDGL
251	DLVDTWKSFK	PRYKHSHFIW	NRELLTLDP	HNVDYLLGLF	EPGDMQYELN
301	RNNVTDPSLS	EMVVVAIQIL	RKNPKGFFLL	VEGGRIDHGH	HEGKAKQALH
351	EAVEMDRAIG	QAGSLTSSSD	TLTVVTADHS	HVFTFGGYTP	RGNSIFGLAP
401	MLSDTDKKPF	TAILYGNPGP	YKVVGGEREN	VSMVDYAHNN	YQAQSAVPLR
451	HETHGGEDVA	VFSKGPMABL	LHGVHEQNYV	PHVMAYACI	GANLGHCAPA
501	SS				

Fig. 17A

BamHI

1 GGATCCACCA TGATTTCACC ATTCTTAGTA CTGGCCATTG GCACCTGCCT TACTAACTCC TTAGTGCCAG
71 AGAAAGAGAA AGACCCCAAG TACTGGCGAG ACCAAGCGCA AGAGACACTG AAATATGCCC TGGAGCTTCA
141 GAAGCTCAAC ACCAACGTGG CTAAGAATGT CATCATGTTC CTGGGAGATG GGATGGGTGT CTCCACAGTG
211 ACGGCTGCCC GCATCCTCAA GGCCTCAGCTC CACCACAACC CTGGGGAGGA GACCAGGCTG GAGATGSACA
281 AGTTCCCTT CCGTGGCCCTC TCCAAGACGT ACAACACCAA TGCCCAAGTC CCTGACAGCG CCGGCACCGC
351 CACCGCCTAC CTGTGTGGGG TGAAGGCCAA TGAGGGCACC GTGGGGGTAA GCGCAGCCAC TGAGCGTTCC
421 CGGTGCAACA CCACCCAGGG GAACGAGGTC ACCTCCATCC TGGCTGGGC CAAGGACGCT GGGAAATCTG
491 TGGGCATTGT GACCACCACG AGAGTGAACC ATGCCACCCC CAGCGCCGCC TACGCCCCT CGGCTGACCG
561 GGACTGGTAC TCAGACAACG AGATGCCCCC TGAGGCCTTG AGCCAGGGCT GTAAGGACAT CGCTACCAG
631 CTCATGCATA ACATCAGGGA CATTGACGTG ATCATGGGGG GTGGCCGGAA ATACATGTAC CCCAAGAATA
701 AAAGTATGAT GGAGTATGAG AGTGACGAGA AAGCCAGGGG CACGAGGCTG GACGCCCTGG ACCTCGTTGA
771 CACCTGGAAG AGCTTCAAAC CGAGATACAA GCACTCCAC T'CATCTGGA ACCGCACGGA ACTCCTGACC
841 CTTGACCCCC ACAATGTGGA CTACCTATTG GGTCTCTTCG AGCCAGGGGA CATGCAGTAC GAGCTGAACA
911 GGAACAACGT GACGGACCCG T'CACTCTCCG AGATGGTGGT GGTGGCCATC CAGATCCTGC GGAAGAACCC
981 CAAAGGCTTC T'CTTGGCTGG TGGAAAGAGG CAGAATTGAC CACGGGCACC ATGAAGGAAA AGCCAACGAG
1051 GCCCTGCATG AGGCGGTGGA GATGGACCGG GCCATCGGGC AGGCAGGCAG CTTGACCTCC TCGGAAGACA
1121 CTCTGACCGT GGTCACTGCG GACCATTCCC ACGTCTTCAC ATTTGGTGGG TACACCCCCC GTGGCAACTC
1191 TATCTTTGGT CTGGCCCCCA TGCTGAGTGA CACAGACAAG AAGCCCTTCA CTGCCATCCT GTATGGCAAT
1261 GGGCCTGGCT ACAAGGTGGT GGGCGGTGAA CGAGAGAATG TCTCCATGGT GGACTATGCT CACAACAAC
1331 ACCAGCGCGA GTCTGCTGTG CCCCTGCGCC ACGAGACCCA CGCGCGGGAG GACGTGGCCG TCCTCTCCAA
1401 GGGCCCCATG CCGCACCTGC TGCACGGCGT CCACGAGCAG AACTACGTC CCCACGTGAT GGCATATGCA
1471 GCCTGCATCG GGGCCAACCT CGGCCACTGT GCTCCTGCCA GCTCGGATGA GCACGATGAT GACGATGATG
XbaI

1541 ACGACTAGTC TAGA

Fig. 17B

1 MISPFVLVLA GTCLTNSLVP EKEKDPKYWR DQAQETLKYA LELQKINTNV
51 AKNVIMFLGD GMGVSTVTAA RILKGQLHHN PGBETRLEMD KFPFVALSKT
101 YNTNAQVPDS AGTATAYLCG VKANEGTVGV SAATERSRCN TTQNEVTSI
151 LRWAKDAGKS VGIVTTTRVN HATPSAAYAH SADRDWYSDN EMPPEALSQG
201 CKDIAYQLMH NIRDIDVIMG GGRKMYPKN KTDVEYESDE KARGTRLDGL
251 DLVDTWKSFK PRYKSHFIW NRTELLTLDL HNVLYLLGLF EPGDMQYELN
301 RNNVTDPSLS EMVVVAIQIL RKNPKGPFLL VEGGRIDHGH HEGKAKQALH
351 EAVEMDRAIG QAGSLTSSD TLTVVTADHS HVFTFGGYTP RGNSIFGLAP
401 MLSDTDKKPF TAILYGNPFG YKVVGGEREN VSMVDYAHNN YQAQSAVPLR
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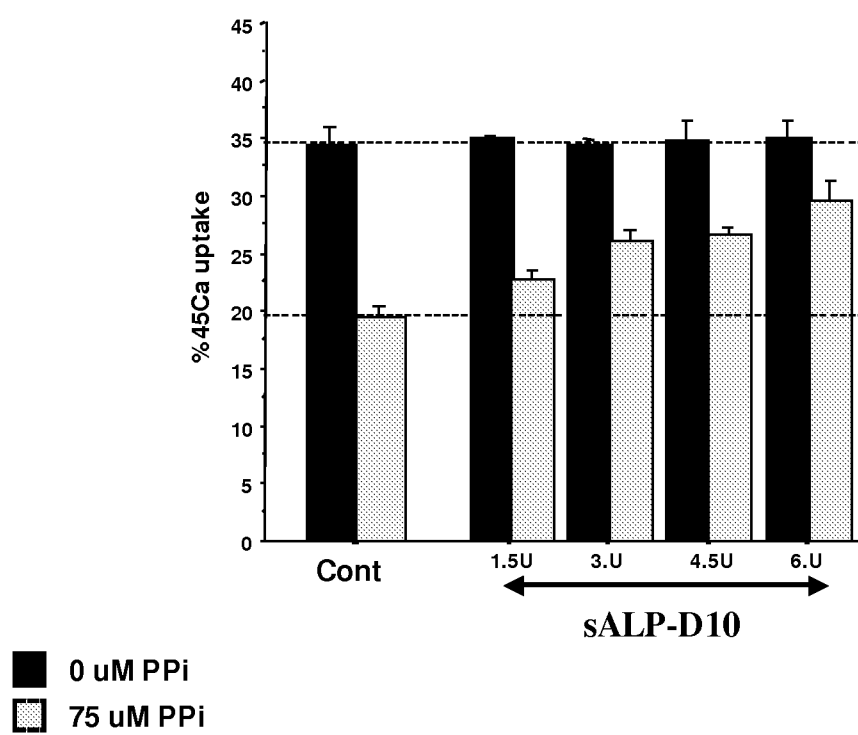


Fig. 18

BONE DELIVERY CONJUGATES AND METHOD OF USING SAME TO TARGET PROTEINS TO BONE

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application is a continuation of U.S. application Ser. No. 11/111,664, filed Apr. 21, 2005, which claims the benefit of the filing dates of U.S. Provisional Application Nos. 60/563,828 filed Apr. 21, 2004, 60/590,347 filed Jul. 23, 2004, and 60/614,984 filed Oct. 4, 2004, each of which is incorporated herein by reference.

FIELD OF THE INVENTION

[0002] The present invention relates to bone delivery conjugates and method of using same to target proteins to bone. More specifically, the present invention relates to bone delivery compositions comprising peptide motifs, engineered within the structure of a protein through recombinant DNA technology to promote binding to bone matrix.

BACKGROUND OF THE INVENTION

[0003] Technological advances in molecular biology, recombinant protein production and large scale protein purification have allowed the production of large amounts of proteins now used as biopharmaceuticals. For example, monoclonal antibodies and soluble forms of the TNF- α receptor have been used in the treatment of autoimmune diseases such as Crohn's disease or severe forms of psoriasis (1). Another example of use of recombinant protein is enzyme replacement therapy (ERT). ERT has been used to treat lysosomal storage diseases. This group of genetic disorders is characterized by the loss of function of lysosome enzymes resulting in severe somatic, and sometimes neuronal, pathologies. In ERT for these diseases, the patients are infused with large doses of normal enzymes. These infused enzymes are then internalized from circulation via cell surface receptors (mannose-6 phosphate receptor) and enter the endocytic pathway on their way to their site of action, the lysosome. Not all attempts to treat genetic disorders through ERT have been successful.

[0004] Hypophosphatasia is a rare, heritable type of rickets or osteomalacia that occurs with an incidence of 1 per 100,000 births for the more severe form of the disease. Milder forms are more prevalent. In this inborn metabolism defect, mutations inactivate the gene that encodes the tissue-nonspecific isoenzyme of alkaline phosphatase. It is characterized biochemically by subnormal alkaline phosphatase activity in serum. Alkaline phosphatase deficiency in osteoblasts and chondrocytes impairs skeletal mineralization, leading to rickets or osteomalacia.

[0005] There is a very broad range of expressivity of hypophosphatasia, spanning from a perinatal form often causing stillbirth from an unmineralized skeleton, to a milder form featuring only premature loss of teeth. Severely affected infants and children inherit hypophosphatasia as an autosomal recessive trait. There are four main forms of the disease: perinatal, infantile, childhood and adult. Perinatal hypophosphatasia manifests during gestation and most affected newborns survive only briefly. Infantile hypophosphatasia becomes clinically apparent before 6 months of age. About 50% of patients die within a year. Childhood hypophosphatasia varies greatly in severity but most of these patients will

suffer from skeletal symptoms throughout their life. Adult hypophosphatasia appears during middle age, with symptoms such as painful recurrent stress fractures having poor healing.

[0006] Osteoblasts and chondrocytes are normally rich in tissue-nonspecific alkaline phosphatase where it is attached to the cell surface. In hypophosphatasia, the lack of alkaline phosphatase activity results in the extracellular accumulation of three phosphorus-compounds believed to be substrates of the enzyme: phosphoethanolamine (PEA), inorganic pyrophosphate (PPi) and pyridoxal 5'-phosphate (PLP). PPi is an inhibitor of hydroxyapatite crystal growth, and PPi build-up in the disease accounts for the impaired skeletal mineralization. Consequently, providing active enzyme to patients suffering from hypophosphatasia will decrease extracellular PPi levels and improve skeletal mineralization.

[0007] Currently, there is no established medical therapy for hypophosphatasia. Trials of enzyme replacement using intravenous infusions of alkaline phosphatase have failed. It appears that alkaline phosphatase activity must be increased not in circulation but in the skeleton itself. This hypothesis was confirmed recently by bone marrow transplantation. Unfortunately, the benefits of the transplantation lasted only for a short period of time due to poor engraftment.

[0008] There is a therefore a need to provide enzyme replacement therapy approach to provide active enzyme to the skeleton of patients suffering from hypophosphatasia.

[0009] Bone-targeted proteins could be useful not only for the treatment or prevention of hypophosphatasia (loss of function of alkaline phosphatase) but also for the treatment or prevention of other genetic diseases characterized by defective enzymatic activity involved in bone metabolism, such as X-linked hypophosphatemic rickets (XLH) (loss of function of phosphate regulating gene with homology to endopeptidases on the X chromosome (PHEX)).

[0010] XLH is the most prevalent of the familial hypophosphatemicias (OMIM 307800, 307810). It is characterized by reduced phosphate reuptake in the kidney, hypophosphatemia, normocalcemia, normal to low plasma 1,25-dihydroxyvitamin D3 (1,25(OH)₂D, calcitriol) levels, normal parathyroid gland function and elevated plasma alkaline phosphatase activity. These changes are associated with growth retardation, lower extremity deformity, radiologic and histomorphometric evidence of rickets and osteomalacia. This disease appears to result from combined renal defects in tubular phosphate reabsorption and vitamin D metabolism, as well as a functional disorder in bone and teeth. XLH results from inactivating mutations in the PHEX gene, a member of the zinc metalloproteinase family of type II integral membrane glycoproteins. These mutations prevent the expression of a functional PHEX enzyme at the cell surface of osteoblasts. As of now, treatment of XLH patients is restricted to supplementation with oral inorganic phosphate (Pi) supplements in four or five divided doses per day, and co-administration of 1,25(OH)₂D to compensate for the inappropriate synthesis of 1,25(OH)₂D. Such high doses of phosphate frequently cause gastrointestinal intolerances, particularly diarrhea, leading to patient non-compliance. On the one hand, the phosphate load carries the risk of provoking secondary hyperparathyroidism (which may be severe enough to necessitate parathyroidectomy) while on the other hand, administration of excess 1,25(OH)₂D may lead to hypercalciuria, hypercalcemia and nephrocalcinosis.

[0011] Useful ERT for XLH would therefore seek to replace the defective PHEX enzyme in XLH patients with a functional enzyme obtained through recombinant DNA technology. As the normal PHEX enzyme is anchored in osteoblast plasma membrane by a hydrophobic peptide, the natural form of PHEX cannot be produced and purified in sufficient quantities to be used in a pharmaceutical preparation. To circumvent the problem, a soluble form of recombinant PHEX (or sPHEX) was engineered and produced in cell cultures, purified and formulated for intravenous (IV) administration (WO 00/50580). sPHEX was then injected in Hyp mice, a mouse model for XLH, as described in co-pending U.S. application Ser. No. 10/362,259. Improvement of several bone related serum parameter were observed including a reduction of the abnormally high levels of serum alkaline phosphatase. Although these experiments were successful, it was believed that the efficacy of therapeutic sPHEX might be enhanced if the recombinant protein was modified so as to promote its binding to bone minerals.

[0012] There is therefore a need for means to successfully target proteins to bone matrix.

[0013] Biphosphonates are known to present high affinity binding to hydroxyapatite (HA), and has been used to target small molecules (4) and proteins (5) to bones. However this strategy requires chemical modifications of the purified proteins, and presents several disadvantages including possible interference with protein activity and additional purification steps.

[0014] Another strategy to target small molecules to bone has been to conjugate these entities to acidic peptides such as poly-Asp (6). This strategy was developed after the observation that several proteins synthesized by osteoblasts, the bone forming cells, bind to bone matrix through sequences particularly rich in acidic amino acid residues (Asp and Glu). This is the case of osteopontin (7) and bone sialoprotein, two non-collagenous proteins. Hence acidic peptides (E_{2-10} and D_{2-10}) were used to target small molecules (i.e. methotrexate, FITC, Fmoc, biotin, estradiol) to hydroxyapatite in vitro. Acidic peptides (E_6 and D_{6-10}) were used to target small molecules (i.e. FITC, Fmoc, estradiol) to hydroxyapatite in vivo. Finally, was shown to confer to BSA, hemoglobin and IgG the ability to bind hydroxyapatite in vitro. In all the above cases, linking of the acidic sequence was performed chemically.

[0015] The present invention seeks to meet these needs and other needs.

[0016] The present description refers to a number of documents, the content of which is herein incorporated by reference in their entirety.

SUMMARY OF THE INVENTION

[0017] The present invention shows that large and complex molecules such as proteins can be fused with acidic peptides to successfully target bone in vivo.

[0018] According to a specific embodiment of the present invention there is provided a bone delivery conjugate having a structure selected from the group consisting of: A) X-Dn-Y-protein-Z; and B) Z-protein-Y-Dn-X, wherein X is absent or is an amino acid sequence of at least one amino acid; Y is absent or is an amino acid sequence of at least one amino acid; Z is absent or is an amino acid sequence of at least one amino acid; and Dn is a poly aspartate wherein $n=10$ to 16. In an other specific embodiment of the present invention, the protein in the bone delivery conjugate is a soluble phosphate regulating gene with homology to endopeptidases on the X

chromosome (sPHEX). In an other specific embodiment of the present invention, the structure of the conjugate is: X-Dn-Y-sPHEX-Z. In other specific embodiment of the present invention, the sPHEX has a sequence selected from the group consisting of amino acids 46 to 749 of FIG. 10; 47 to 749 of FIG. 10; 48 to 749 of FIG. 10; 49 to 749 of FIG. 10; 50 to 749 of FIG. 10; 51 to 749 of FIG. 10; 52 to 749 of FIG. 10; 53 to 749 of FIG. 10; and 54 to 749 of FIG. 10. In a specific embodiment of these bone delivery conjugates, n is 10. In an other specific embodiment of this bone delivery conjugate, n is 11. In an other specific embodiment of this bone delivery conjugate, n is 12. In an other specific embodiment of this bone delivery conjugate, n is 13. In an other specific embodiment of this bone delivery conjugate, n is 14. In an other specific embodiment of this bone delivery conjugate, n is 15. In an other specific embodiment of this bone delivery conjugate, n is 16. In a more specific embodiment of the present invention, the sPHEX consists of the sequence of amino acids 46 to 749 of FIG. 10 and $n=10$.

[0019] In another specific embodiment of the present invention, the protein in the conjugate is a soluble alkaline phosphatase (sALP). In an other specific embodiment, the structure of the conjugate is: Z-sALP-X-Dn-Y. In an other specific embodiment, sALP is encoded by the sequence as set forth in FIG. 16A. In an other specific embodiment, sALP has the sequence as set forth in FIG. 16B. In a specific embodiment of these bone delivery conjugates, n is 10. In an other specific embodiment of this bone delivery conjugate, n is 11. In an other specific embodiment of this bone delivery conjugate, n is 12. In an other specific embodiment of this bone delivery conjugate, n is 13. In an other specific embodiment of this bone delivery conjugate, n is 14. In an other specific embodiment of this bone delivery conjugate, n is 15. In an other specific embodiment of this bone delivery conjugate, n is 16. In a more specific embodiment, $n=10$.

[0020] There is also provided an isolated nucleic acid molecule comprising a polynucleotide sequence selected from the group consisting of: a polynucleotide encoding a polypeptide comprising an amino acid sequence as set forth in FIG. 8; a polynucleotide encoding a polypeptide comprising an amino acid sequence as set forth in FIG. 11; a polynucleotide comprising the nucleotide sequence as set forth in FIG. 7; a nucleotide sequence completely complementary to any of the nucleotide sequences in (a), (b) or (c); and a nucleotide sequence which hybridizes under high stringency conditions to any of the nucleotide sequences in (a), (b), (c) or (d), wherein the high stringency conditions comprise: pre-hybridization and hybridization in $6\times$ SSC, $5\times$ Denhardt's reagent, 0.5% SDS and 100 mg/ml of denatured fragmented salmon sperm DNA at 68°C .; and washes in $2\times$ SSC and 0.5% SDS at room temperature for 10 min; in $2\times$ SSC and 0.1% SDS at room temperature for 10 min; and in $0.1\times$ SSC and 0.5% SDS at 65°C . three times for 5 minutes.

[0021] There is also provided a recombinant vector comprising said sequence. There is also provided a recombinant host cell comprising said vector.

[0022] There is also provided an isolated nucleic acid molecule comprising a polynucleotide sequence selected from the group consisting of: a polynucleotide comprising the nucleotide sequence as set forth in FIG. 17A; a polynucleotide encoding a polypeptide comprising an amino acid sequence as set forth in FIG. 17B; a nucleotide sequence completely complementary to any of the nucleotide sequences in (a) or (b); and a nucleotide sequence which

hybridizes under high stringency conditions to any of the nucleotide sequences in (a), (b) or (c), wherein the high stringency conditions comprise: pre-hybridization and hybridization in 6×SSC, 5×Denhardt's reagent, 0.5% SDS and 100 mg/ml of denatured fragmented salmon sperm DNA at 68° C.; and washes in 2×SSC and 0.5% SDS at room temperature for 10 min; in 2×SSC and 0.1% SDS at room temperature for 10 min; and in 0.1×SSC and 0.5% SDS at 65° C. three times for 5 minutes.

[0023] There is also provided an isolated nucleic acid molecule encoding a functional soluble PHEX comprising a polynucleotide sequence selected from the group consisting of: a polynucleotide encoding a sPHEX comprising amino acids 54 to 749 as set forth in FIG. 10; a polynucleotide encoding a sPHEX comprising amino acids 53 to 749 as set forth in FIG. 10; a polynucleotide encoding a sPHEX comprising amino acids 52 to 749 as set forth in FIG. 10; a polynucleotide encoding a sPHEX comprising amino acids 51 to 749 as set forth in FIG. 10; a polynucleotide encoding a sPHEX comprising amino acids 50 to 749 as set forth in FIG. 10; a polynucleotide encoding a sPHEX comprising amino acids 49 to 749 as set forth in FIG. 10; a polynucleotide encoding a sPHEX comprising amino acids 48 to 749 as set forth in FIG. 10; a polynucleotide encoding a sPHEX comprising amino acids 47 to 749 as set forth in FIG. 10; a polynucleotide encoding a sPHEX comprising amino acids 46 to 749 as set forth in FIG. 10; a nucleotide sequence completely complementary to any of the nucleotide sequences in (a) to (j); and a nucleotide sequence which hybridizes under high stringency conditions to any of the nucleotide sequences in (a) to (j), wherein the high stringency conditions comprise: pre-hybridization and hybridization in 6×SSC, 5×Denhardt's reagent, 0.5% SDS and 100 mg/ml of denatured fragmented salmon sperm DNA at 68° C.; and washes in 2×SSC and 0.5% SDS at room temperature for 10 min; in 2×SSC and 0.1% SDS at room temperature for 10 min; and in 0.1×SSC and 0.5% SDS at 65° C. three times for 5 minutes. In an other embodiment, the isolated nucleic acid molecule further comprises at its 5' end, a polynucleotide encoding a poly-aspartate selected from the group consisting of D₁₀ to D₁₆.

[0024] There is also provided an isolated sPHEX polypeptide comprising a sequence selected from the group consisting of: amino acids 54 to 749 as set for in FIG. 10; amino acids 53 to 749 as set for in FIG. 10; amino acids 52 to 749 as set for in FIG. 10; amino acids 51 to 749 as set for in FIG. 10; amino acids 50 to 749 as set for in FIG. 10; amino acids 49 to 749 as set for in FIG. 10; amino acids 48 to 749 as set for in FIG. 10; amino acids 47 to 749 as set for in FIG. 10; and amino acids 46 to 749 as set for in FIG. 10.

[0025] There is also provided a bone delivery composition comprising a bone delivery conjugate of the present invention, and a pharmaceutically acceptable carrier.

[0026] There is also provided a method of delivering a protein to bone tissue of a mammal comprising administering to said mammal an effective amount of a bone delivery conjugate as recited of the present invention.

[0027] There is also provided a method of delivering sPHEX to bone tissue of a mammal comprising administering to said mammal an effective amount of a bone delivery conjugate of the present invention.

[0028] There is also provided a method of delivering ALP to bone tissue of a mammal in need thereof comprising

administering to said mammal an effective amount of a bone delivery conjugate of the present invention.

[0029] There is also provided a method of treating a condition or disease related to a bone defect characterized by a lack of or an insufficient amount of functional phosphate regulating gene with homology to endopeptidases on the X chromosome (PHEX) comprising administering to a mammal in need thereof a conjugate of the present invention, said conjugate being in a pharmaceutically acceptable carrier. In specific embodiments, the condition or disease is X-linked hypophosphatemic rickets (XLH).

[0030] There is also provided a method of treating a condition or disease related to a bone defect characterized by a lack of or an insufficient amount of functional alkaline phosphatase comprising administering to a mammal in need thereof a conjugate of the present invention, said conjugate being in a pharmaceutically acceptable carrier. In specific embodiments, the condition or disease is hypophosphatasia.

[0031] There is also provided a method of screening peptides for use in a bone delivery protein-peptide conjugate comprising the steps of: fusing a candidate peptide to a reporter protein to form a protein-peptide conjugate; contacting the conjugate with bone tissue or mineral phase of bone; and wherein the candidate peptide is selected when the presence of the reporter protein on bone tissue or mineral phase of bone is higher when it is conjugated with the candidate peptide than when it is not.

[0032] According to a specific embodiment of the present invention there is provided a bone delivery conjugate of a protein fused to a peptide selected from the group consisting of deca-aspartate (D₁₀) to hexadeca-aspartate (D₁₆).

[0033] In specific embodiments of conjugates of the present invention, the sPHEX is fused at its N-terminal to D₁₀. In an other specific embodiment, the sPHEX is fused at its N-terminal to D₁₁. In an other specific embodiment, the sPHEX is fused at its N-terminal to D₁₂. In an other specific embodiment, the sPHEX is fused at its N-terminal to D₁₃. In an other specific embodiment, the sPHEX is fused at its N-terminal to D₁₄. In an other specific embodiment, the sPHEX is fused at its N-terminal to D₁₅. In an other specific embodiment, the sPHEX is fused at its N-terminal to D₁₆.

[0034] According specific embodiments of conjugates of the present invention, the sALP is fused at its C-terminal to D₁₀. In an other specific embodiment, the sALP is fused at its C-terminal to D₁₁. In an other specific embodiment, the sALP is fused at its C-terminal to D₁₂. In an other specific embodiment, the sALP is fused at its C-terminal to D₁₃. In an other specific embodiment, the sALP is fused at its C-terminal to D₁₄. In an other specific embodiment, the sALP is fused at its C-terminal to D₁₅. In an other specific embodiment, the sALP is fused at its C-terminal to D₁₆.

[0035] It is understood that any functional soluble protein may be used in the conjugate of the present invention. Although results for conjugates comprising one specific sPHEX or sALP of the present invention are presented herein, it is understood that any other functional sPHEX or sALP may be so used.

sPHEX

[0036] As used herein sPHEX means any soluble biologically active fragment of PHEX or mutein thereof. Those of skill in the art may prepare expression constructs other than those expressly described herein for optimal production of sPHEX in suitable cell lines transfected therewith. Moreover, skilled artisans may design fragments of cDNA encoding

soluble biologically active fragments and muteins of the naturally occurring PHEX which possess the same or similar biological activity to the naturally occurring full-length enzyme.

[0037] To create a recombinant source for sPHEX, a large series of expression vectors may be constructed and tested for expression of a PHEX cDNA. Based on transient transfection experiments, as well as stable transfections, an expression construct may be identified that provides a particularly high level of expression.

[0038] Without being so limited, any sPHEX comprising at least a native PHEX ectodomain portion starting with the cysteine at position 54 of the sequence presented at FIG. 10 is encompassed by the present invention.

[0039] The conjugates according to specific embodiments of the present invention thus are any sPHEX comprising this 54-749 fragment of the native PHEX, preferably the 53-749 native fragment, more preferably the native 52-749 fragment, more preferably the native 51-749 fragment, more preferably the 50-749 native fragment, more preferably the 49-749 native fragment, more preferably the 48-749 native fragment, more preferably the 47-749 native fragment, and more preferably the 46-749 native fragment, along with a poly-aspartate selected from the group consisting of D₁₀ to D₁₆ fused immediately upstream of this fragment.

[0040] The conjugate may further optionally comprise one or more additional amino acids 1) upstream from the poly-aspartate; and/or 2) between the poly-aspartate and the native fragment or functional equivalent. These amino acids may be any amino acid. According to specific embodiments, they may be selected independently from the group consisting of any amino acid except for cysteine, proline and tryptophan namely those amino acids known to induce disulfide bond formation or changes in conformation.

[0041] These amino acids may be present in the conjugate when for instance the cloning strategy used to produce it introduces them in these locations.

[0042] According to specific cloning strategies, amino acids located upstream of the poly-aspartate in the recombinant cleavable PHEX can be selected according to known parameters so as to provide an adequate substrate for specific enzymes of the secretory pathway (e.g. furin or signal peptidase) of the host cell that will be used to cleave the produced recombinant cleavable PHEXs into a secreted bone targeting sPHEX. The likelihood of a designed sequence being cleaved by the signal peptidase of the host cell can be predicted by an appropriate computer algorithm such as that described in Bendtsen et al. (J Mol Biol. 2004 Jul. 16; 340(4):783-95) and available on the Web at <http://www.cbs.dtu.dk/services/SignalP/> which takes into account parameters including the following: the amino acids at position -3 and -1 from the cleavage site by the signal peptidase desirably have small and non charged side chains. Preferably, at position -1: Ala, Ser, Gly, Cys, Thr and occasionally Gln, Pro, and Leu. Similarly those at position -3 should preferably be: Ala, Ser, Gly, Cys, Thr, Ile, Leu, Val. Moreover, amino acids in position -6 and -4 from the cleavage site are desirably those capable of inducing the formation of a beta-turn (such as Pro) residues.

[0043] The present invention hence encompasses conjugates comprising additional amino acids that may be selected on the basis of the cloning strategy used to produce a cleavable recombinant PHEX. Hence the cleavable recombinant PHEX disclosed in Examples 3 and 4 below contains such additional amino acids upstream of the poly-aspartate and

between the poly-aspartate and the native ectodomain sequence. Also, the present invention encompasses a conjugate comprising the secPHEX disclosed in co-pending application no. WO 02/15918 prepared by fusing NL-1 N-terminal fragment comprising a furin site to the PHEX native ectodomain with the vector pCDNA3/RSV/NL-1-PHEX, and a secPHEX comprising an immunoglobulin fragment at its N-terminal. More particularly, FIG. 12 schematically presents the structure of secPHEXs that comprise additional amino acids upstream of the native 46-749 PHEX ectodomain fragment. Constructs no. 1 to 3 and 5 could be fused to a poly-aspartate and be used as conjugates of the present invention. Construct no. 4 constitutes a conjugate of the present invention: it comprises a D₁₀ poly-aspartate and a native ectodomain fragment.

[0044] The conjugates of the present invention further also encompass sPHEXs comprising deletions at their C-terminal non detrimental to their enzymatic activity.

[0045] Furthermore, the present invention comprises conjugates wherein the poly-aspartate would be attached at the C-terminal of the native PHEX ectodomain fragment.

sALP

[0046] ALP is a membrane-bound protein anchored through a glycolipid to its C-terminal. This glycolipid anchor (GPI) is added post translationally after removal of a hydrophobic C-terminal end which serves both as transitional membrane anchor and as a signal for the addition of the GPI. Hence the sALP used in Example 6 herein is constituted of an ALP wherein the first amino acid of the hydrophobic C-terminal sequence, namely alanine, is replaced by a stop codon. The soluble ALP so formed contains all amino acids of the native and thus active anchored form of ALP.

[0047] The sALP conjugates according to specific embodiments of the present invention thus are any sALP along with a poly-aspartate selected from the group consisting of D₁₀ to D₁₆ fused immediately downstream of this fragment.

[0048] The conjugate may further optionally comprise one or more additional amino acids 1) upstream from the poly-aspartate; and/or 2) between the poly-aspartate and the native sALP fragment or functional equivalent. This is the case for instance when the cloning strategy used to produce the bone targeting conjugate introduces exogenous amino acids in these locations. However the exogenous amino acids should be selected so as not to provide an additional transamination site. The likelihood of a designed sequence being cleaved by the transaminase of the host cell can be predicted as described by Ikezawa (Biol Pharm. Bull. 2002, 25(4) 409-417).

[0049] The conjugates of the present invention further also encompass sALPs comprising deletions at their N-terminal non detrimental to their enzymatic activity.

[0050] Furthermore, the present invention comprises conjugates wherein the poly-aspartate would be attached at the N-terminal of the native ALP anchored fragment or its biologically active fragment.

[0051] The term "recombinant protein" is used herein to refer to a protein encoded by a genetically manipulated nucleic acid inserted into a prokaryotic or eukaryotic host cell. The nucleic acid is generally placed within a vector, such as a plasmid or virus, as appropriate for the host cell. Although *E. coli* has been used as a host for expressing the conjugates of the present invention in the Examples presented herein, a person of ordinary skill in the art will understand that a number of other hosts may be used to produce recombinant proteins according to methods that are routine in the art.

Representative methods are disclosed in Maniatis, et al. Cold Springs Harbor Laboratory (1989). "Recombinant cleavable protein" as used herein is meant to refer to a recombinant protein that may be cleaved by a host's enzyme so as to produce a secreted/soluble protein.

[0052] The term "ectodomain fragment" is meant herein when used in relation to PHEX is meant to refer to PHEX's fragment that is located outside of the cellular membrane when found in its native form.

[0053] The term "bone tissue" is used herein to refer to tissue synthesized by osteoblasts composed of an organic matrix containing mostly collagen and mineralized by the deposition of hydroxyapatite crystals.

[0054] The fusion proteins comprised in the bone delivery conjugates of the present invention are useful for therapeutic treatment of bone defective conditions by providing an effective amount of the fusion protein to the bone. The fusion protein is provided in the form of a pharmaceutical composition in any standard pharmaceutically acceptable carrier, and is administered by any standard procedure, for example by intravenous injection.

[0055] The term "pharmaceutically acceptable carrier" is used herein to refer, when parenteral administration is elected as the route of administration, to pharmaceutically acceptable sterile aqueous or non-aqueous solvents, suspensions or emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oil, fish oil, and injectable organic esters. Aqueous solvents include water; water-alcohol solutions; physiological saline; buffered medical parenteral vehicles including sodium chloride solution, Ringer's dextrose solution, dextrose plus sodium chloride solution, fluid and nutrient replenishers; electrolyte replenishers; Ringer's solution containing lactose, or fixed oils.

[0056] The term "effective amount" is used herein to refer to the minimal amount of a pharmaceutical composition that should be administered to a mammal in order to achieve a significant therapeutic effect. The dosages will depend on many factors including the mode of administration. Typically, the amount of protein contained within a single dose will be an amount that effectively prevents, delays or treats bone related undesired condition without inducing significant toxicity. In particular, an effective amount of the conjugate and compositions of the present invention will comprise an amount of fusion protein which will cause a significant alleviation of clinical symptoms of the condition.

[0057] The effective amount may be given daily, weekly, monthly or fractions thereof. Typically, a pharmaceutical composition of the invention can be administered in an amount from about 0.001 mg up to about 500 mg per kg of body weight per day (e.g., 10 mg, 50 mg, 100 mg, or 250 mg). Dosages may be provided in either a single or multiple dosage regimen. For example, in some embodiments the effective amount is a dose that ranges from about 1 mg to about 25 grams of the conjugate to be targeted to bone per day, from about 50 mg to about 10 grams of the conjugate to be targeted to bone per day, from about 100 mg to about 5 grams of the conjugate to be targeted to bone per day, about 1 gram of the conjugate to be targeted to bone per day, about 1 mg to about 25 grams of the conjugate to be targeted to bone per week, about 50 mg to about 10 grams of the conjugate to be targeted to bone per week, about 100 mg to about 5 grams of the conjugate to be targeted to bone every other day, and about 1 gram of the conjugate to be targeted to bone once a week.

[0058] These are simply guidelines since the actual dose must be carefully selected and titrated by the attending physician based upon clinical factors unique to each patient. The optimal daily dose will be determined by methods known in the art and will be influenced by factors such as the age of the patient and other clinically relevant factors. In addition, patients may be taking medications for other diseases or conditions. The other medications may be continued during the time that the protein for delivery to bone is given to the patient, but it is particularly advisable in such cases to begin with low doses to determine if adverse side effects are experienced.

[0059] The term "high stringency conditions" are meant to refer to conditions enabling sequences with a high homology to bind. Without being so limited, examples of such conditions are listed in the handbook "*Molecular cloning, a laboratory manual*", second edition of 1989 from Sambrook et al.: 6xSSC or 6xSSPE, Denhardt's reagent or not, 0.5% SDS and the temperature used for obtaining high stringency conditions is most often in around 68° C. (see pages 9.47 to 9.55 of Sambrook) for nucleic acid of 300 to 1500 nucleotides. Although the optimal temperature to be used for a specific nucleic acid probe may be empirically calculated, and although there is room for alternatives in the buffer conditions selected, within these very well known condition ranges, the nucleic acid captured will not vary significantly. Indeed, Sambrook clearly indicates that the "choice depends to a large extent on personal preference" (see page 9.47). Sambrook specifies that the formula to calculate the optimal temperature which varies according to the fraction of guanine and cytosine in the nucleic acid probe and the length of the probe (10 to 20° C. lower than T_m wherein $T_m = 81.5^\circ \text{C.} + 16.6(\log_{10}[\text{Na}^+]) + 0.41(\text{fraction G+C}) - 0.63 (\% \text{ formamide} - (600/\text{l}))$ (see pages 9.50 and 9.51 of Sambrook).

[0060] Other objects, advantages and features of the present invention will become more apparent upon reading of the following non-restrictive description of preferred embodiments thereof, given by way of example only with reference to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0061] In the appended drawings:

[0062] FIG. 1 presents the purity status of GST and GST-D₁₀ proteins on an SDS polyacrylamide gel after CL-4B chromatography;

[0063] FIG. 2 shows the promotion of GST binding to bone by D₆, D₁₀ and D₁₆ peptide motifs through the percentage of the injected dose of recombinant GST found associated with specific tissues;

[0064] FIG. 3 provides a schematic representation of the plasmid pCDNA3-RSV-D₁₀sPHEX-NEO vector;

[0065] FIG. 4 presents a chromatographic profile of 280 nm detection of PHEX flow for the SP-Sepharose™ HP (A) and the blue-Sepharose HP (B). Straight line represents buffer ratio;

[0066] FIG. 5 presents a Sypro-ruby™ stained SDS-PAGE analysis of the different fractions collected throughout D₁₀sPHEX purification procedure;

[0067] FIG. 6 shows the variation in serum alkaline phosphatase levels (ALP) observed in Hyp mice injected daily with i.v. doses of sPHEX and D₁₀-sPHEX for 14 days. U/I values represent the decrease observed between day -3 (corresponding in the graphic to 0 U/I) and day 15, of the injection regimen and are the mean of measures made on 6 animals;

[0068] FIG. 7 shows the nucleotide sequence of a recombinant DNA sequence encoding a protein cleavable so as to produce D₁₀-sPHEX (SEQ ID NO: 1);

[0069] FIG. 8 shows the amino acid sequence encoded by the D₁₀-sPHEX of FIG. 7 (SEQ ID NO: 2);

[0070] FIG. 9 compares the binding to the mineral phase of bone of proteins (A. GST B. sPHEX) with that of their deca-aspartate fused counterparts;

[0071] FIG. 10 shows the nucleotide sequence of a native (or membrane-bound) PHEX (SEQ ID NO: 3);

[0072] FIG. 11 shows the amino acid sequence (SEQ ID NO: 4) of a D₁₀-sPHEX conjugate produced by cleavage of the recombinant cleavable protein of FIG. 8;

[0073] FIG. 12 schematically illustrates the structure and activities of various secPHEX constructs;

[0074] FIG. 13 graphically illustrates through fluorimetric measurement of the alkaline phosphatase activity in the soluble cell extract and spent medium of HEK293 transiently transfected with expression vectors encoding sALP-D₁₀ and sALP;

[0075] FIG. 14 graphically illustrates the detection of sALP and sALP-D₁₀ by Western blotting with the specific B4-78 antibody in the spent media and cell extract of HEK-293 after transient transfection. (Panel A: Ponceau red staining; Panel B: Blot a-B4-78). Shown on the left are the sizes of the molecular weight markers;

[0076] FIG. 15 graphically shows the binding to bone mineral phase of a deca-aspartate fused to secreted alkaline phosphatase;

[0077] FIG. 16 shows A. the nucleotidic sequence (SEQ ID NO: 5) of a soluble alkaline phosphatase; and B. the amino acid sequence (SEQ ID NO: 6) of that soluble alkaline phosphatase;

[0078] FIG. 17 shows A. the nucleotidic sequence (SEQ ID NO: 7) encoding a conjugate of the present invention, namely sALP-D₁₀; and B. the amino acid sequence (SEQ ID NO: 8) of that conjugate; and

[0079] FIG. 18 graphically shows the effect of D10-sALP on PPI-mediated mineralization inhibition.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

[0080] The present invention showed that specific poly-aspartic peptides fused in frame to a protein, as exemplified herein by the glutathione-S-transferase protein (GST), used as a reporter protein, by sPHEX and by sALP, can significantly increase the bone binding capacity of these proteins.

[0081] The present invention is illustrated in further details by the following non-limiting examples.

[0082] Table 1 presents the sequence of oligonucleotides used in Examples 1 to 7.

TABLE 1

SEQUENCE OF SYNTHETIC OLIGONUCLEOTIDES USED IN EXAMPLES 1 TO 7		
D ₆	SEQ ID NO: 9.	5' - GATCCGATGACGATGACGATG ACGC - 3'
	SEQ ID NO: 10.	5' - GGCCGCGTCATCGTCATCGTC ATCG - 3'
D ₁₀	SEQ ID NO: 11.	5' - GATCCGATGACGATGACGATG ACGATGACGATGACGC - 3'
	SEQ ID NO: 12.	5' - GGCCGCGTCATCGTCATCGTC ATCGTCATCGTCATCG - 3'
D ₁₆	SEQ ID NO: 13.	5' - GATCCGATGACGATGACGATG ACGATGACGATGACGATGACGATG ACGATGACGC - 3'

TABLE 1 -continued

SEQUENCE OF SYNTHETIC OLIGONUCLEOTIDES USED IN EXAMPLES 1 TO 7		
	SEQ ID NO: 14.	5' - GGCCGCGTCATCGTCATCGTC ATCGTCATCGTCATCGTCATCGTC ATCGTCATCG - 3'
hMEPE	SEQ ID NO: 15.	5' - GATCCGATGACAGTAGTGAGT CATCTGACAGTGGCAGTTCAAGTG AGAGCGATGGTGACGC - 3'
	SEQ ID NO: 16.	5' - GGCCGCGTCACCATCGCTCTC ACTTGAAGTGCCTGTCAGATGA CTCACTACTGTCATCG - 3'
hStatherin	SEQ ID NO: 17.	5' - GATCCGATTCTATGGAAGAGA AATTTTTCGCTAGAATTGGAAGAT TCGGTGC - 3'
	SEQ ID NO: 18.	5' - GGCCGCACCGAATCTTCCAAT TCTACGCAAAATTTCTCTTCAGA TGAATCG - 3'
hMGP	SEQ ID NO: 19.	5' - GATCCTGTTATGAATCACATG AAAGCATGGAATCTTATGAACCTTA ATCCCTTCATTGC - 3'
	SEQ ID NO: 20.	5' - GGCCGCAATGAAGGATTAAG TTCATAAGATTCCATGCTTTCATG TGATTCTAATACAG - 3'
hOsteopontin	SEQ ID NO: 21.	5' - GATCCGAAATGCTGTGTCCT CTGAAGAAACCAATGACTTTAAAG C - 3'
	SEQ ID NO: 22.	5' - GGCCGCTTTAAAGTCATTGGT TTCTTCAGAGGACACAGCATTCTG G - 3'
hBSP2	SEQ ID NO: 23.	5' - GATCCGCGAGTAGTGACTCAT CCGAAGAAATGGAGATGACAGTT CAGAAGAGGAGGAGGAAGC - 3'
	SEQ ID NO: 24.	5' - GGCCGCTTCTCTCTCTCTTC TGAAGTGTCTCTCTCTCTCTCTC GGATGAGTCACTACTGCCG - 3'
hIGFBP5	SEQ ID NO: 25.	5' - GATCCCGCAAAGGATTCTACA AGAGAAAGCAGTGCAACCTTTCCC GTGGCCGCAAGCGTGC - 3'
	SEQ ID NO: 26.	5' - GGCCGCGAGCTTGCGGCCACG GGAAGGTTTGCACTGCTTTCTCTT GTAGAATCCTTTGCCG - 3'
M81736 CBS	SEQ ID NO: 27.	5' - AGTCGGGATCCGGAACAAGCA GCGTGTCTAC - 3'
	SEQ ID NO: 28.	5' - AGATCGCGGCCGCTCAATTGT GCACGGTGTGATTAAAG - 3'
D ₁₀	SEQ ID NO: 29.	5' - CCGGAGATGACGATGACGATG ACGATGACGATGACT - 3'
	SEQ ID NO: 30.	3' - TCTACTGCTACTGCTACTGCT ACTGCTACTGAGGCC - 5'

Example 1

Bone Binding of GST-D₆, GST-D₁₀ and GST-D₁₆

[0083] Recombinant DNA technology was used to generate a plasmid containing a nucleic acid encoding GST followed in frame by a nucleic acid encoding a D₆, D₁₀ or D₁₆ acidic peptide. To obtain the GST-D₆, GST-D₁₀ and GST-D₁₆ conjugates, the oligonucleotide of SEQ ID NO:9 (see Table 1) was first mixed with the oligonucleotide of SEQ ID NO:10,

oligonucleotide of SEQ ID NO:11 mixed with oligonucleotide of SEQ ID NO:12, and oligonucleotide of SEQ ID NO:13 mixed with oligonucleotide of SEQ ID NO:14. This procedure generated duplex oligonucleotides coding for D₆, D₁₀, and D₁₆, respectively, and having extremities compatible with cloning in the pGEX3T-4 plasmid (Pharmacia biotechnology) pre-digested with restriction endonucleases BamHI and NotI. pGEX3T-4 vectors were transformed into AP401 protease minus *E. coli* bacteria strain (lon::mini tetR ara-Δlac-pro naIA argEam rifR thiI [F' pro AB lacIq Z M15]).

[0084] Positive bacterial colonies were used to seed a 10 ml pre-culture of double YT media and 100 mg/litre ampicillin. Bacteria were grown overnight at 37° C. in an orbital shaker set at 250 rpm. The pre-culture was added to 500 ml of fresh double YT ampicillin media in a 2 litres Erlenmeyer flask. Bacteria were let to grow at 37° C. under orbital shaking until a 595 nm optical density of 0.7 was reached. Protein expression was then induced by adding 500 μl of 0.1 M IPTG solution and the bacteria put back to incubation for 2 hours. Bacteria were spun down at 8000×g for 10 minutes at 4° C. The pellet was suspended in 25 ml of ice-cold PBS containing Complete-EDTA caplet protease inhibitor (Boehringer Mannheim) and frozen at -20° C.

[0085] Bacteria cells were thawed and disrupted on ice with 6 pulses of sonication every 50 seconds prior to centrifugation at 12000×g for 10 minutes at 4° C. Supernatant was mixed with 500 μl of GS-4B wet resin (Amersham Pharmacia Biotech) equilibrated with PBS. The resin was kept as a suspension during the overnight incubation at 4° C. The resin was rinsed with PBS until 280 nm optical density was below 0.01. Resin was then laid on an empty column and proteins eluted with 10 mM glutathione dissolved in PBS. The pooled elution fractions were dialyzed against 1 mM sodium PO₄ pH 7.4 and 150 mM NaCl. Dialyzed proteins were filtered in a sterile environment on 0.22 μm PES membrane and kept at 4° C. Typically 40 and 60 mg of pure proteins were recovered per litre of culture respectively. FIG. 1 shows an example of an SDS-PAGE analysis of the purified GST and GST-D₁₀. Purified proteins were iodinated using Iodo-Beads Iodination Reagent (Pierce).

[0086] GST and peptide-fused GST were dialyzed against PBS and concentration set to 2 mg/ml. Iodination reaction was initiated by adding 2 PBS-rinsed Iodo-Beads to 2 mCi of Na¹²⁵I (100 μCi/μl, ICN) dissolved in 500 μl of PBS. Beads were incubated at room temperature for five minutes before adding 1 mg of dialyzed protein. The iodination reaction proceeded for 15 minutes before the bead was removed and rinsed in 500 ml of PBS. To the final 1.5 ml of iodinated protein solution, 15 μl of 6 mM NaI was added to dilute non-specific radioactivity. The mixture was then desalted using PD-10 gel filtration columns (Amersham Pharmacia Biotech) equilibrated with PBS. Proteins eluted in the void volume. They were concentrated and dialysed against the in vivo buffer (1 mM sodium PO₄ pH 7.4 and 150 mM NaCl) using Centriprep-YM10™ cartridges (Amicon). Radioactivity was measured using a gamma radiation counter, protein concentration was assessed by the Bradford assay and ¹²⁵I chemical linkage to proteins was revealed by autoradiography of dried SDS-PAGE. Iodinated samples were kept at 4° C. Bone Binding Ability of GST-Poly-Aspartic Peptides Fusion Proteins Compared to that of GST Alone

[0087] The iodinated GST-fusion proteins were injected to mice under isoflurane anesthesia as an intravenous bolus through the subclavian vein. A dose of 1 mg of iodinated

protein/per kg body weight was injected. The maximum dose volume was set at 10 ml/kg. Duration of treatment was sixty minutes. Ten and sixty minutes after injection, blood samples (0.1 to 0.2 ml) were collected via the subclavian vein under anesthesia into serum/gel clotting activator Microvette™ tubes (Sarstedt, #20. 1291). At necropsy, blood samples were collected and animals were sacrificed by exsanguination from the heart under isoflurane anesthesia. Organs (kidneys, liver, femurs, tibias and thyroid) were collected, rinsed in saline 0.9% USP, blotted on gauze and transferred into gamma counter tubes. Serum samples and organs were weighted and radioactivity was measured. Results were expressed as percentage of injected dose. Neither D₁₀-GST nor D₁₆-GST promoted binding to other organs than bone. This showed the specificity of these conjugates to bone (Data not shown).

[0088] FIG. 2 shows that GST-D₆ fusion protein did not bind more to tibia or femur than GST alone. In contrast, D₁₀ and D₁₆ peptide motifs promoted GST binding to bones.

[0089] The fact that D₆, a peptide shown to successfully deliver small molecules to bone could not successfully deliver a protein, namely GST, to bone shows that it is not predictable whether a specific acidic peptide known to effectively deliver a small molecule to bone will also be effective in delivering a protein to bone.

Example 2

Binding Ability of GST Fused with Various Peptides

[0090] Human matrix extracellular phosphoglycoprotein (hMEPE) is a protein synthesized by osteoblasts that shows major similarities to a group of bone and teeth mineral matrix phosphor-glycoproteins, proteins known to naturally bind to bone matrix (8). Of particular importance, hMEPE presents at its carboxy-terminus a sequence of 18 amino acid residues (DDSSSESDSGSSSESDDGD) (SEQ ID NO: 31) similar to acidic peptides found in dentin phosphorin and dentin sialophosphoprotein, both known to bind to bone matrix (8).

[0091] Human Statherin (hStatherin) is a protein synthesized by salivary glands, which similarly to histatin directly modulates hydroxyapatite nucleation and/or growth. Of particular importance, hStatherin presents a sequence of 15 amino acid residues at positions 20 to 34 (DSSEKFLRRIGRFG) (SEQ ID NO: 32) that was shown to bind tightly to hydroxyapatite (9).

[0092] Human Matrix Gla Protein (hMGP) is a protein synthesized by vascular smooth muscle cells and chondrocytes that functions as an inhibitor of hydroxyapatite polymerization by binding to crystal nuclei. Of particular importance, hMGP presents at its amino-terminus a sequence of 17 amino acid residue at positions 19 to 35 of the open reading frame (CYESHESMESYELNPF1) (SEQ ID NO: 33) similar to phosphorylated gamma carboxyglutamic acidic peptides found in osteocalcin known to bind to bone matrix, and thought to promote binding to bone matrix (10).

[0093] Human osteopontin (hOPN) is a protein synthesized by osteoblasts that regulates hydroxyapatite crystal growth. This protein belongs to the bone sialophosphoprotein family. Of particular importance, hOPN presents a sequence of 13 amino acid residue (QNAVSSSEETNDFK) (SEQ ID NO: 34) at positions 58 to 70 of the open reading frame. This sequence shows a high level of homology among mammal species. Secondary structure prediction makes this sequence appropriate to solvent exposure and this sequence was shown to be

phosphorylated at its serine residues. This latter characteristic is thought to affect binding to bone matrix (11).

[0094] Human Bone Sialoprotein II (hBSP2) is a protein synthesized by osteoblasts that shows major similarities to a group of bone and teeth mineral matrix phosphor-glycoproteins, proteins known to naturally bind to bone matrix. Of particular importance, hBSP2 presents at its amino-terminus a sequence of 18 amino acid residues at positions 62 to 79 of the open reading frame (GSSDSSEENGDDSEEEEE) (SEQ ID NO: 35) similar to acidic peptides found in dentin phosphorin and MEPE, and thought to promote binding to bone matrix (8).

[0095] Human Insulin-like Growth Factor binding protein-5 (hIGFBP5) is synthesized by osteoblasts. This protein, similarly to proteins of the IGFBP family, is thought to regulate osteoblast function in the bone remodeling process. Of particular importance, hIGFBP5 presents a sequence of 18 amino acid residues at positions 221 to 238 of the open reading frame (RKGIFYKRKQCKPSRGRKR) (SEQ ID NO: 36) that was shown to bind tightly to hydroxyapatite (12).

[0096] *Staphylococcus aureus* collagen adhesin (M81736) is a protein expressed at the surface of *S. aureus* that promotes bacteria binding to collagen matrix of mammalian bone and cartilageneous tissues. Such a binding was reported to be instrumental in the development of pathogenesis such as osteomyelitis and infectious arthritis. Of particular importance, the collagen binding domain (CBS) of this adhesin was reported to encompass 151 amino acid residues (G168 to N318) of the open reading frame of the protein (13, 14). The amino acid primary sequence being the following:

(SEQ ID NO: 37)
GTSSVFYYKTGDMLPEDTTHRWFLNNNEKSYVSKDITIKDQI
GGGQQLDLSTLNLINVTGTHSNYYSGQSAITDFEKAFFGSKITVDNKTNTI
DVTIPQGYGSYNSFSINYKTKITNEQQKEFVNNSQAWYQEHGKEEVNGKS
FNHTVHN.

[0097] Plasmids containing the acidic peptide sequences derived from hMEPE, hStatherin, hMGP, hOPN, hBSP2, hIGFBP5 and CBS following GST in frame were constructed to determine whether they could promote bone targeting of a recombinant protein. Recombinant DNA technology as described in Example 1 was used to generate plasmids for hMEPE, hStatherin, hMGP, hOPN, hBSP2 and hIGFBP5 derived peptides. The oligonucleotide pairs identified in Table 1 for each of these peptides were mixed to obtain the corresponding GST-acidic peptide fusion protein. This procedure generated duplex oligonucleotides coding for these acidic peptides and having extremities compatible with cloning in the pGEX3T-4 (Pharmacia biotechnology) plasmid pre digested with restriction endonucleases BamHI and NotI.

[0098] A CBS-containing plasmid was constructed as follows. A synthetic gene corresponding to the CBS sequence was obtained from Bio S&T (Montreal) and inserted in plasmid pLIV Select. Oligonucleotides of SEQ ID NO: 27 and 28 were used as primers in PCR reactions with plasmid pLIV Select containing the CBS gene to amplify the CBS specific sequences. pGEX-4T-3 vectors were transformed into AP401 protease minus *E. coli* bacteria strain (lon::mini tetR ara-Δlac-pro nlaA argEam rifR thiI [F' pro AB lacIq Z M15]).

[0099] Protein production and purification, and pharmacodistribution of the iodinated fusion protein were performed as described in Example 1.

[0100] None of these GST-acidic peptides was shown to bind to bones (result not shown).

[0101] The fact that the peptide derived from statherin, a peptide shown to successfully deliver a small portion of osteopontin to bone, could not successfully deliver the GST protein to bone shows that it is not predictable whether a specific acidic peptide known to effectively deliver a small peptide to bone will also be effective in delivering a protein to bone.

Example 3

D₁₀ Increases sPHEX's Ability to Correct Alkaline Phosphatase Levels in Mice

[0102] PHEX is a metallopeptidase that is widely believed to control the level of bone peptide factors involved in the regulation of mineralization and kidney phosphate homeostasis. PHEX is expressed at the surface of osteoblasts and osteocytes in contact with or imbedded in the bone matrix. This example provides data on the design, production and purification of an extended form of sPHEX containing at its N-terminus a sequence of 10 aspartic acid residues designed to anchor itself to the bone matrix.

D₁₀sPHEX Expression Vector

[0103] A BspEI endonuclease restriction site was inserted by site directed mutagenesis (QuickChange, Stratagene) into the pCDNA3-RSV-sPHEX-NEO vector (Boileau G. et al., Biochem. J. (2001) 355, 707-13) using the following oligonucleotide primers:

(SEQ ID NO: 38)
5' - CAGTCAAGGTCTCTTATCCGGAAGTCTCCAAGCTAACAGG-3'
and
(SEQ ID NO: 39)
5' - CTGTTTAGCTTGGAGACTTCCGGATAAGAGACCTTGACTGG-3'.

[0104] The hexamer BspEI sequence (underlined) was inserted in frame with and upstream of the sPHEX DNA sequence. This construct encodes a recombinant protein which is cleavable between the leucine and serine at positions 41 and 42, respectively in FIG. 8. It is constituted therefore of two exogenous amino acids, followed downstream by a deca-aspartate, which is in turn followed by two additional exogenous amino acids. These 4 exogenous amino acids derive from the cloning strategy used to produce the conjugate. These exogenous amino acids were shown not to defeat the enzymatic activity of the conjugate (See FIG. 12 showing the specific activity of this construct) but may be dispensed with. Downstream of these exogenous amino acids is an ectodomain fragment of the native PHEX starting therefore with the serine at position 46 of the sequence presented in FIG. 10. The modified pCDNA3-RSV-NEO vector was cleaved with BspEI and then digested with alkaline phosphatase to remove the 5' phosphate moieties. An oligonucleotide duplex coding for deca-aspartate: [5'-CCGGAGATGACGATGACGATGACGATGACGATGACT-3' (SEQ ID NO: 29) and 3'-TCTACTGCTACTGCTACTGCTACTGCTACTGAGGCC-5' (SEQ ID NO: 30)] was first phosphorylated on its 5' ends with T4 polynucleotide kinase and ligated to the BspEI digested vector. This yielded the pCDNA3-RSV-D₁₀sPHEX-NEO vector (FIG. 3). This vector comprised the

sequence presented in FIG. 7 which encodes the recombinant cleavable PHEX having the amino acid sequence presented in FIG. 8.

Expression of Recombinant D₁₀sPHEX

[0105] To induce the stable expression of the D₁₀sPHEX protein, the pCDNA3-RSV-D₁₀sPHEX-NEO vector was transfected in LLC-PK1 cells (Porcine Kidney cells; ATCC No. CRL-1392) using the Lipofectamine-Plus™ liposome transfection kit (Invitrogen). Transfected cells were selected by adding 400 µg/ml G-418 (Life Technologies) to the medium. Clones of G-418 resistant cells were screened for D₁₀sPHEX expression using the PHEX fluorescent enzymatic assay [Campos M. et al. *Biochem. J.* (2003) 373, 271-9]. The apparent molecular weight of the protein recovered in the spent medium was estimated by immunoblotting using a monoclonal antibody raised against a recombinant human PHEX fragment (K121-E294) as described previously (Ruchon A F et al. *J. Bone Miner. Res.* (2000) 15, 1440-1450). A G-418 resistant clone expressing 1 to 2 mg of D₁₀sPHEX per litre was used for protein production. Cells were seeded in Cellstack-10™ (Corning) at a density of 7×10⁷ in 1.75 litres of media (199 media, 6% FBS, 1 mM NaPyruvate, Penicillin 1×10⁵ U/litre, Streptomycin 100 mg/litre and 1% G-418). D₁₀sPHEX expression was increased by incubating the cells in 1.75 litre of DMEM+10 mM sodium butyrate for four days at 37° C. and 5% CO₂ prior to harvest of the spent medium.

Purification and Characterization

[0106] Cell supernatant was centrifuged at 500×g for 5 minutes at 4° C. and filtered on fiberglass (Fisher, APFC09050) and concentrated 10 to 40 times using an Ultra-Sette™ 30 tangential flow filtration device (Pall Canada). The pH of the solution was brought to 5.6 with 1 M acetic acid before an overnight dialysis at 4° C. against 50 mM sodium acetate, 100 mM NaCl pH 5.6 (SP-buffer). The dialyzed supernatant was loaded, at a flow rate of 4 ml/min, on a 20 ml SulfoPropyl-Sepharose cation-exchange column (Amersham Pharmacia Biotech) previously equilibrated with SP-buffer. The column was washed with the same buffer at the same flow rate until 280 nm absorbance baseline was reached. Most of the contaminant proteins were then eluted with a 226 mM NaCl step in the SP buffer. D₁₀sPHEX was then eluted with a 280 mM NaCl step (FIG. 4A). Fractions were analyzed by SDS-PAGE and with the PHEX enzymatic activity assay. Fractions containing sPHEX were pooled and extensively dialyzed against 20 mM MOPS pH 7, 250 mM NaCl prior to loading on a 5 ml Blue-Sepharose™ HP (Amersham Pharmacia) column at 5 ml/min. The column was rinsed, at the same flow rate with the same buffer and most of the D₁₀sPHEX protein was recovered by increasing the NaCl concentration stepwise to 350 mM (FIG. 4B). Purity of the final fraction was greater than 95%. Alternatively, the Blue-Sepharose™ could be replaced by Heparin-Sepharose™ (Amersham Pharmacia) on which D₁₀sPHEX binds tightly over a range of pH (5 to 8). D₁₀sPHEX was eluted by using NaCl gradient. Purity was determined to be above 90%. D₁₀sPHEX was concentrated and dialyzed against 1 mM sodium PO₄ pH 7.4, 150 mM NaCl using Centriprep-50™ cartridges. Dialyzed sample was filtered in a sterile environment on 0.22 µm membrane. Purified D₁₀sPHEX was shown to remain stable over months at 4° C. Protein concentrations were determined using the Bradford method (DC protein assay kit; Biorad) with bovine serum albumin (BSA) as a standard. Protein purity was assessed by Sypro-Ruby™ (Mo-

lecular Probes) staining of proteins resolved on SDS-PAGE 4-12% (FIG. 3). D₁₀sPHEX enzymatic activity was determined using the fluorogenic substrate.

Effect of sPHEX and D₁₀sPHEX Injections on Circulating Levels of Alkaline Phosphatase in Hyp Mice

[0107] The X-linked Hyp mice harbors a large deletion in 3' region of the PHEX gene and is the murine homologue of human X-linked hypophosphatemia (XLH). These mice therefore represent a useful model to study the pathophysiology of XLH as well as a to test the efficacy of therapeutic agents in preclinical studies.

[0108] The potential therapeutic effect of D₁₀sPHEX and sPHEX was thus investigated with bolus intravenous injection to Hyp/Y mice over a 2 week period.

[0109] D₁₀sPHEX and sPHEX were dialyzed against vehicle and the solutions were filtered through 0.22 µm low binding protein filter. The solutions were aliquoted and re-assayed for enzymatic activity and concentration by fluorogenic enzymatic assay and Bradford method, respectively.

[0110] Each mouse was anesthetized with vaporized Isoflurane (2%) and D₁₀sPHEX, or sPHEX were injected as an intravenous bolus through the subclavian vein. The dose was 5 mg/kg of body weight for each group. The animals were treated once daily for 14 consecutive days. Blood samples (0.1-0.2 ml) were collected via the subclavian vein under anesthesia on study days -3 and +15 (before necropsy, 24 hours after last injection). Total Alkaline phosphatase (ALP) levels were assayed in diluted serum (30 µl of serum sample with 90 µl of 0.9% saline USP). Although, appropriate dosages for human patients are not proportional to those used in mice, these dosages are predictive of the dosages ranges that could be suitable in humans using published tables.

[0111] As seen in FIG. 6 the D₁₀-extended form of sPHEX induced a larger decrease in alkaline phosphatase levels than the normal sPHEX form.

Example 4

D₁₀ Fusion to Recombinant GST Increases its Binding to the Mineral Phase of Bone In Vitro

Fluorescein Labelling of Purified Proteins

[0112] Recombinant purified proteins were labelled with fluorescein-isothiocyanate (FITC, Molecular Probes F143). Reaction was carried out by adding proteins to 10 mM sodium phosphate, 50 mM NaCl buffer pH 7 at a final protein concentration of 1 mg/ml. Labelling reaction was started by adding FITC dissolved in DMSO at a concentration of 20 mg/ml to reach 20:1 molar ratio with respect to the protein concentration. The mixture was left to react at room temperature for an hour. Labelled protein was separated from the free fluorescein on a PD-10™ column (Pharmacia) prior to dialysis in the binding buffer (1 mM sodium phosphate 150 mM NaCl, pH 7.4).

Preparation of the Mineral Phase of Bones

[0113] Long bones were dissected from a rat and crushed to powder in a liquid nitrogen cooled mortar. The powder was either kept at -80° C. or directly used. An aliquot of the powder (300 mg) was washed 3 times with 8 ml of PBS and 8 ml of 1 M HCl were added. The mixture was kept in suspension on a rotating mixer for 1 hour at room temperature. The insoluble fraction was spun down and the clear

[0114] Aliquots of 20 μl of the acidic bone extract were mixed with 2 μl of 10 M NaOH and the precipitate was pelleted 10,000 $\times g$ for 3 minutes at room temperature. The pellet was rinsed twice by resuspending in 100 μl of binding buffer. The bone extract was then mixed with 100 μl of a solution containing 5 to 45 μg of fluorescein-labelled protein in the binding buffer to which phosphate was added to reach a final concentration of 80 mM. The samples were incubated for 30 minutes at room temperature on the rotating wheel to keep the mineral phase in suspension. The samples were then centrifuged for 3 minutes at room temperature. The pellet containing the bound protein was dissolved in 200 μl of 0.5 M EDTA pH 8. To estimate the amount of free protein present, 100 μl of 0.5 M EDTA pH 8 was added to the supernatant. Fluorescence of the different samples was measured on a 96 wells plate reader set at 494 nm for excitation and 516 nm for emission.

[0115] Samples containing 50 µg of fluorescein-labelled GST and GST-D₁₀ were used in the binding assay described above. FIG. 9 A shows that fusion of the D₁₀ sequence to GST caused a 6-fold increase in binding to the mineral phase of bone.

D₁₀ Fusion to sPHEX Increases its Binding to Bone

[0116] Using a procedure analogous to that described in Example 4 above, samples containing 50 µg of fluorescein-labelled sPHEX and D₁₀sPHEX were used in a binding assay. FIG. 9 B shows that fusion of the D₁₀ sequence to sPHEX caused a 4.3 increase in binding to the mineral phase of bone.

[0117] In contrast, D₆-SPHEX was constructed and tested after *in vivo* injection in animals (as described in Example 1 above) and did not promote binding of recombinant proteins to bone (Data not shown).

D₁₀ Fusion to a Soluble Form of Alkaline Phosphatase Increases its Targeting to the Mineral Phase of Bone

[0118] Construction of Expression Vectors Encoding Human Recombinant Soluble Phosphatase Alkaline, sALP and sALP-D₁₀

[0119] The human full length cDNA encoding tissue non-specific alkaline phosphatase (ALP) was obtained from bone marrow polyA RNA (Clontech) by RT-PCR. Briefly, 20 ng of polyA was reverse transcribed with SuperscriptII™ and an oligo dT₁₂₋₁₈ using the First Strand Synthesis System (Invitrogen). An aliquot representing 1/20th of the RT step was used directly in a PCR reaction with ALP specific oligos (forward 5'-gataaagcagggtcttgggggtgcc-3' (SEQ ID NO: *); reverse 5'-gtggcatctgtcacgggcttgg-3' (SEQ ID NO: *)) and the Expand High Fidelity Enzyme Kit™ (Roche). The resulting ALP specific product (1644 bp) was separated on and purified from an agarose gel (1%) using the Qiaquick Gel Extraction Kit™ (QIAGEN). The ALP cDNA was then ligated into the

pCR4-blunt-TOPO™ vector (Invitrogen), transformed into Top10™ bacteria (Invitrogen), and a positive clone identified by colony PCR. The identity of the cDNA was verified by automated DNA sequencing.

[0120] Secreted forms of ALP (sALP) having the GPI anchor signal removed were constructed by PCR using Expand High Fidelity Enzyme Kit™. They comprised residues 1-502 followed by either a stop codon (sALP) or a decarboxylase targeting motif and a stop codon (sALP-D10). In both cases the forward primer (5'-ttgcatccaccatgatttcaccattcttagtac-3' (SEQ ID NO: 40)) covered the initiator methionine (underlined) and included a BamHI site (italicized). The reverse primers (sALP: 5'-tctcatgctacgagctggcaggagcacagtgccg-3' (SEQ ID NO: 41); sALP-D10: 5'-tctcatgctacgagctggcaggagcacagtgccg-3' (SEQ ID NO: 42)) contained a stop codon (underlined) and an XbaI site (italicized). The PCR products were digested with BamHI and XbaI and cloned into the pCDNA3.1-RSV that had been pre-digested with the same enzymes. Plasmid DNA was sequenced.

[0121] Enzymatic activity of sALP and sALP-D₁₀ was assayed using 4-methylumbelliferyl phosphate (MUP, Molecular Probes, M8425) as a fluorogenic substrate according to Gee K R et al. (Anal. Biochem. 273, 41-48 (1999)). Typically, the assay was carried out at 37° C. in 96-well plates in a final volume of 200 µl with 10 µM of MUP. Readings were recorded using a Spectramax Gemini™ (Molecular Devices) plate reader every minute for 30 minutes at 450 nm upon excitation at 360 nm. Emission wavelength cut-off was set at 435 nm. ALP initial speed rate was estimated by linear regression fitting (with r^2 equal or greater than 0.98).

Expression of Recombinant sALP and sALP-D₁₀ Proteins

[0122] In order to determine whether the recombinant sALP and sALP-D₁₀ proteins were secreted, each construct (pCDNA3-RSV-sALP-NEO and pCDNA3-RSV-sALP-D₁₀-NEO) was transiently transfected in HEK-293S cells (Human Embryonic Kidney cells; ATCC No. CRL-1392) using the Lipofectamine-Plus liposome transfection KitTM (Invitrogen). HEK-293S cells were also mock-transfected as a negative control. The day after transfection, cells were incubated for 24 h in serum-free DMEM. The conditioned media were collected and centrifuged at 14000 RPM for 5 min at 4° C. to remove dead cells and debris. The supernatants were assayed for sALP or sALP-D₁₀ enzymatic activity and expression using the ALP fluorescent enzymatic assay and Western blotting respectively. For Western blotting, the spent media were precipitated for 1 h on ice with trichloroacetic acid (final concentration 10% (v/v)). The precipitated proteins were spun down at 14000 RPM for 20 min at 4° C., washed once with chilled acetone, dried, and resuspended in 60 µl 1× Laemmli sample buffer with DTT and boiled for 5 min.

[10123] To evaluate the intracellular content of sALP and sALP-D₁₀ the cells were washed 3 times with PBS and lysed with 200 µl Tris-HCl 50 mM (pH 8) containing 150 mM NaCl and 1% NP-40 on ice for 20 min. The lysates were spun down and the supernatant was assayed for sALP or sALP-D₁₀ enzymatic activity and expression using the ALP fluorescent enzymatic assay and Western blotting, respectively. For Western blotting, 50 µl aliquots were mixed with 10 µl 6× Laemmli sample buffer with DTT and boiled for 5 min.

[0124] Samples were loaded on a Novex Precast™ 4-12% Tris-Glycine polyacrylamide gel (Invitrogen) and transferred onto 0.45 µm nitrocellulose (Protran, Schleicher&Schuell, Keene, N.H.) with Tris-glycine containing 10% methanol. The membrane was stained with Ponceau red and blocked for 1 h at room temperature with PBS containing 0.05% Tween 20™ (PBST) and 5% dried milk. The membrane was then sequentially incubated at room temperature with the anti-hBAP antibody (mAb 4B-78, Developmental Studies Hybridoma Bank) (1:1000 in PBST with 5% dried milk) and a rabbit anti-mouse IgG coupled to horseradish peroxidase (Sigma) (1:12000 in PBST with 5% dried milk). The signal was developed with the Western Lightning Chemiluminescence Reagent Plus™ (PerkinElmer).

[0125] The ALP enzymatic activity measured in the conditioned media of HEK293 after transient transfection was very high and of similar magnitude for pCDNA3-RSV-sALP-NEO (sALP) and pCDNA3-RSV-sALP-D₁₀-NEO (sALP-D₁₀) (FIG. 13). This activity was specific to the plasmid DNA transfected as it was undetectable in mock-transfected cells (mock). The relative activity measured in the media was 35-times greater than that measured in the cell extracts thus attesting to the secretory nature of sALP and sALP-D₁₀. Accordingly, for both sALP and sALP-D₁₀, immunoblotting using a monoclonal antibody raised against recombinant tissue non-specific human alkaline phosphatase (mAb 4B-78, Developmental Studies Hybridoma Bank) revealed a much stronger signal in the conditioned media than in the cell extracts (FIG. 14B, compare lanes 2, 3 vs. 5, 6). No signal was visualized in the mock-transfected samples (FIG. 14B, lanes 4 and 7). The signal appearing in the mock-transfected cells consists of BSA trace. The apparent molecular weight of the protein detected was estimated to be 70 kDa in the cell extracts (arrow) and slightly higher in the conditioned media (arrowhead). Ponceau red staining of the membrane was performed to monitor the uniform loading of samples (FIG. 14A).

Generation of HEK293 Cells Constitutively Secreting sALP and sALP-D₁₀

[0126] To induce the stable expression of the sALP and sALP-D₁₀ proteins, the pCDNA3-RSV-sALP-NEO and pCDNA3-RSV-sALP-D₁₀-NEO vectors was transfected separately in HEK-293S cells using the Lipofectamine-Plus liposome transfection Kit™ (Invitrogen). Transfected cells were selected by adding 800 µg/ml G418 (Life Technologies) to the medium. For each transfection a pool of G-418 resistant cells were analyzed for sALP or sALP-D₁₀ expression in the spent culture media using the ALP fluorescent enzymatic assay. The conditioned media collected from the stable cell lines were used for the binding assay study on the bone mineral.

Binding to Reconstituted Mineral Phase of Bone

[0127] Aliquots of 20 µl of the acidic bone extract were mixed with 2 µl of 10 M NaOH and the precipitate was pelleted at 10,000×g for 3 minutes at room temperature. The pellet was rinsed twice in 100 µl of buffer (1 mM sodium phosphate pH 7.4+150 mM NaCl). The resultant mineral phase of bone (equivalent to 0.37 mg of dried powder) was then mixed with 100 µl of a solution containing sALP or sALP-D₁₀ proteins in the binding buffer (80 mM sodium phosphate pH 7.4+150 mM NaCl). The samples were incubated for 30 minutes at room temperature on the rotating wheel to keep the mineral phase in suspension. The samples

were then centrifuged for 3 minutes at room temperature. The pellet containing the bound protein was mixed with 180 µl of the ALP enzymatic assay buffer containing 0.1% BSA and the reaction initiated by adding 20 µl of 100 µM MUP. To allow for more homogeneous assay, conditions the 96 wells plate was shaken for 10 seconds every minute for the duration of the assay.

[0128] Enzymatic activity retained on reconstituted mineral bone phase was compared to the equivalent enzymatic activity added in the binding assay. Values of 0.98% and 13.3% of total protein activity bound to the bone mineral phase were calculated for sALP and sALP-D₁₀ respectively. A binding difference of more than 13 times in favour of sALP-D₁₀ suggests that the C-terminal fused deca-aspartate sequence directly targets sALP to the mineral phase of bone. Furthermore, the fact that it was possible to measure directly ALP activity bound to the mineral phase of bone indicates that the enzyme is bound in a catalytically competent form to hydroxyapatite crystals.

[0129] Such fusion protein can be targeted directly to bones where the accumulation of PPi inhibits skeletal mineralization.

Example 7

D₁₀-ALP Decreases Inhibitory Effect of Pyrophosphate on Bone Mineralization

[0130] UMR106 cells were grown to confluence. They were then cultured for a further 7 days in media containing 10 mM β-glycerophosphate to induce mineralization. Throughout this 7-day culture period, cells were treated with or without 75 µM pyrophosphate (PPi), a mineralization inhibitor and a alkaline phosphatase substrate. To assess the ability of alkaline phosphatase to rescue the PPi-induced mineralization inhibition, cells treated with or without PPi were cultured with varying concentrations of semi-purified D₁₀-sALP produced from HEK293, human embryonic kidney cells. Mineralization was assessed by ⁴⁵Ca uptake. Parameters used for this experiment are presented in table 2 below.

TABLE 2

PARAMETERS USED IN D ₁₀ -ALP ON PPI-INDUCED MINERALIZATION INHIBITION				
ALP	[ALP] (Units/well)	µl ALP/well	β-GP (mM)	PPi (µM)
—	0	0	10	0
—	0	0	10	75
D ₁₀ -sALP	1.5	0.5	10	0
D ₁₀ -sALP	1.5	0.5	10	75
D ₁₀ -sALP	3	1.0	10	0
D ₁₀ -sALP	3	1.0	10	75
D ₁₀ -sALP	4.5	1.5	10	0
D ₁₀ -sALP	4.5	1.5	10	75
D ₁₀ -sALP	6	2	10	0
D ₁₀ -sALP	6	2	10	75

[0131] 7-days of treatment with PPi resulted in a 43% decrease in mineralization. Co-treatment of cultures with D₁₀-sALP resulted in a dose-responsive rescue of this mineralization inhibition. Treatment with 1.5 units of D₁₀-sALP resulted in a 30% decrease, 3 and 4.5 units a 24% decrease and 6 units resulted in a 15% decrease in mineralization, corresponding to a 65% rescue of PPi-induced mineralization inhibition.

[0132] These results show that the treatment of mineralizing osteoblast with D₁₀-sALP dose-responsively rescues mineralization inhibition induced by PPI.

[0133] The above Examples shows that a poly-aspartate fusion to recombinant proteins increases their binding to the mineral phase of bone or to bone tissue and increases the ability of the protein to perform its biological activity as compared to when it is administered alone.

[0134] Although the present invention has been described hereinabove by way of preferred embodiments thereof, it can be modified, without departing from the spirit and nature of the subject invention as defined in the appended claims.

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Ser	Pro	Asp	Asp	Lys	Ala	Ser	Asn	Glu	His	Ile	Leu	Lys	Leu	Asp	Gln
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Ala	Thr	Leu	Ser	Leu	Ala	Val	Arg	Glu	Asp	Tyr	Leu	Asp	Asn	Ser	Thr
225					230					235					240
Glu	Ala	Lys	Ser	Tyr	Arg	Asp	Ala	Leu	Tyr	Lys	Phe	Met	Val	Asp	Thr
				245					250					255	
Ala	Val	Leu	Leu	Gly	Ala	Asn	Ser	Ser	Arg	Ala	Glu	His	Asp	Met	Lys
		260						265					270		
Ser	Val	Leu	Arg	Leu	Glu	Ile	Lys	Ile	Ala	Glu	Ile	Met	Ile	Pro	His
		275					280					285			
Glu	Asn	Arg	Thr	Ser	Glu	Ala	Met	Tyr	Asn	Lys	Met	Asn	Ile	Ser	Glu
	290					295					300				
Leu	Ser	Ala	Met	Ile	Pro	Gln	Phe	Asp	Trp	Leu	Gly	Tyr	Ile	Lys	Lys
305					310					315					320
Val	Ile	Asp	Thr	Arg	Leu	Tyr	Pro	His	Leu	Lys	Asp	Ile	Ser	Pro	Ser
				325					330					335	
Glu	Asn	Val	Val	Val	Arg	Val	Pro	Gln	Tyr	Phe	Lys	Asp	Leu	Phe	Arg
			340					345					350		
Ile	Leu	Gly	Ser	Glu	Arg	Lys	Lys	Thr	Ile	Ala	Asn	Tyr	Leu	Val	Trp
		355					360					365			
Arg	Met	Val	Tyr	Ser	Arg	Ile	Pro	Asn	Leu	Ser	Arg	Arg	Phe	Gln	Tyr
	370					375					380				
Arg	Trp	Leu	Glu	Phe	Ser	Arg	Val	Ile	Gln	Gly	Thr	Thr	Thr	Leu	Leu
385					390					395					400
Pro	Gln	Trp	Asp	Lys	Cys	Val	Asn	Phe	Ile	Glu	Ser	Ala	Leu	Pro	Tyr
				405					410					415	
Val	Val	Gly	Lys	Met	Phe	Val	Asp	Val	Tyr	Phe	Gln	Glu	Asp	Lys	Lys

-continued

420				425				430			
Glu Met Met Glu Glu Leu Val Glu Gly Val Arg Trp Ala Phe Ile Asp	435	440	445								
Met Leu Glu Lys Glu Asn Glu Trp Met Asp Ala Gly Thr Lys Arg Lys	450	455	460								
Ala Lys Glu Lys Ala Arg Ala Val Leu Ala Lys Val Gly Tyr Pro Glu	465	470	475								
Phe Ile Met Asn Asp Thr His Val Asn Glu Asp Leu Lys Ala Ile Lys	485	490	495								
Phe Ser Glu Ala Asp Tyr Phe Gly Asn Val Leu Gln Thr Arg Lys Tyr	500	505	510								
Leu Ala Gln Ser Asp Phe Phe Trp Leu Arg Lys Ala Val Pro Lys Thr	515	520	525								
Glu Trp Phe Thr Asn Pro Thr Thr Val Asn Ala Phe Tyr Ser Ala Ser	530	535	540								
Thr Asn Gln Ile Arg Phe Pro Ala Gly Glu Leu Gln Lys Pro Phe Phe	545	550	555								
Trp Gly Thr Glu Tyr Pro Arg Ser Leu Ser Tyr Gly Ala Ile Gly Val	565	570	575								
Ile Val Gly His Glu Phe Thr His Gly Phe Asp Asn Asn Gly Arg Lys	580	585	590								
Tyr Asp Lys Asn Gly Asn Leu Asp Pro Trp Trp Ser Thr Glu Ser Glu	595	600	605								
Glu Lys Phe Lys Glu Lys Thr Lys Cys Met Ile Asn Gln Tyr Ser Asn	610	615	620								
Tyr Tyr Trp Lys Lys Ala Gly Leu Asn Val Lys Gly Lys Arg Thr Leu	625	630	635								
Gly Glu Asn Ile Ala Asp Asn Gly Gly Leu Arg Glu Ala Phe Arg Ala	645	650	655								
Tyr Arg Lys Trp Ile Asn Asp Arg Arg Gln Gly Leu Glu Glu Pro Leu	660	665	670								
Leu Pro Gly Ile Thr Phe Thr Asn Asn Gln Leu Phe Phe Leu Ser Tyr	675	680	685								
Ala His Val Arg Cys Asn Ser Tyr Arg Pro Glu Ala Ala Arg Glu Gln	690	695	700								
Val Gln Ile Gly Ala His Ser Pro Pro Gln Phe Arg Val Asn Gly Ala	705	710	715								
Ile Ser Asn Ser Glu Glu Phe Gln Lys Ala Phe Asn Cys Pro Pro Asn	725	730	735								
Ser Thr Met Asn Arg Gly Met Asp Ser Cys Arg Leu Trp	740	745									

<210> SEQ ID NO 4

<211> LENGTH: 718

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Soluble PHEX conjugate

<400> SEQUENCE: 4

Ser Gly Asp Asp Asp Asp Asp Asp Asp Asp Asp Ser Gly Ser Leu	1	5	10	15
---	---	---	----	----

Gln Ala Lys Gln Glu Tyr Cys Leu Lys Pro Glu Cys Ile Glu Ala Ala

-continued

20							25					30				
Ala	Ala	Ile	Leu	Ser	Lys	Val	Asn	Leu	Ser	Val	Asp	Pro	Cys	Asp	Asn	
	35						40					45				
Phe	Phe	Arg	Phe	Ala	Cys	Asp	Gly	Trp	Ile	Ser	Asn	Asn	Pro	Ile	Pro	
	50					55					60					
Glu	Asp	Met	Pro	Ser	Tyr	Gly	Val	Tyr	Pro	Trp	Leu	Arg	His	Asn	Val	
65					70					75				80		
Asp	Leu	Lys	Leu	Lys	Glu	Leu	Leu	Glu	Lys	Ser	Ile	Ser	Arg	Arg	Arg	
				85					90					95		
Asp	Thr	Glu	Ala	Ile	Gln	Lys	Ala	Lys	Ile	Leu	Tyr	Ser	Ser	Cys	Met	
			100					105					110			
Asn	Glu	Lys	Ala	Ile	Glu	Lys	Ala	Asp	Ala	Lys	Pro	Leu	Leu	His	Ile	
		115					120					125				
Leu	Arg	His	Ser	Pro	Phe	Arg	Trp	Pro	Val	Leu	Glu	Ser	Asn	Ile	Gly	
	130					135					140					
Pro	Glu	Gly	Val	Trp	Ser	Glu	Arg	Lys	Phe	Ser	Leu	Leu	Gln	Thr	Leu	
145					150					155					160	
Ala	Thr	Phe	Arg	Gly	Gln	Tyr	Ser	Asn	Ser	Val	Phe	Ile	Arg	Leu	Tyr	
				165					170					175		
Val	Ser	Pro	Asp	Asp	Lys	Ala	Ser	Asn	Glu	His	Ile	Leu	Lys	Leu	Asp	
			180					185					190			
Gln	Ala	Thr	Leu	Ser	Leu	Ala	Val	Arg	Glu	Asp	Tyr	Leu	Asp	Asn	Ser	
	195						200					205				
Thr	Glu	Ala	Lys	Ser	Tyr	Arg	Asp	Ala	Leu	Tyr	Lys	Phe	Met	Val	Asp	
	210					215					220					
Thr	Ala	Val	Leu	Leu	Gly	Ala	Asn	Ser	Ser	Arg	Ala	Glu	His	Asp	Met	
225					230					235				240		
Lys	Ser	Val	Leu	Arg	Leu	Glu	Ile	Lys	Ile	Ala	Glu	Ile	Met	Ile	Pro	
			245						250					255		
His	Glu	Asn	Arg	Thr	Ser	Glu	Ala	Met	Tyr	Asn	Lys	Met	Asn	Ile	Ser	
		260						265					270			
Glu	Leu	Ser	Ala	Met	Ile	Pro	Gln	Phe	Asp	Trp	Leu	Gly	Tyr	Ile	Lys	
	275						280					285				
Lys	Val	Ile	Asp	Thr	Arg	Leu	Tyr	Pro	His	Leu	Lys	Asp	Ile	Ser	Pro	
	290					295					300					
Ser	Glu	Asn	Val	Val	Val	Arg	Val	Pro	Gln	Tyr	Phe	Lys	Asp	Leu	Phe	
305					310					315				320		
Arg	Ile	Leu	Gly	Ser	Glu	Arg	Lys	Lys	Thr	Ile	Ala	Asn	Tyr	Leu	Val	
			325						330					335		
Trp	Arg	Met	Val	Tyr	Ser	Arg	Ile	Pro	Asn	Leu	Ser	Arg	Arg	Phe	Gln	
		340						345					350			
Tyr	Arg	Trp	Leu	Glu	Phe	Ser	Arg	Val	Ile	Gln	Gly	Thr	Thr	Thr	Leu	
	355						360					365				
Leu	Pro	Gln	Trp	Asp	Lys	Cys	Val	Asn	Phe	Ile	Glu	Ser	Ala	Leu	Pro	
	370					375					380					
Tyr	Val	Val	Gly	Lys	Met	Phe	Val	Asp	Val	Tyr	Phe	Gln	Glu	Asp	Lys	
385					390					395				400		
Lys	Glu	Met	Met	Glu	Glu	Leu	Val	Glu	Gly	Val	Arg	Trp	Ala	Phe	Ile	
			405						410					415		
Asp	Met	Leu	Glu	Lys	Glu	Asn	Glu	Trp	Met	Asp	Ala	Gly	Thr	Lys	Arg	
		420						425					430			

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Lys Ala Lys Glu Lys Ala Arg Ala Val Leu Ala Lys Val Gly Tyr Pro
 435 440 445
 Glu Phe Ile Met Asn Asp Thr His Val Asn Glu Asp Leu Lys Ala Ile
 450 455 460
 Lys Phe Ser Glu Ala Asp Tyr Phe Gly Asn Val Leu Gln Thr Arg Lys
 465 470 475 480
 Tyr Leu Ala Gln Ser Asp Phe Phe Trp Leu Arg Lys Ala Val Pro Lys
 485 490 495
 Thr Glu Trp Phe Thr Asn Pro Thr Thr Val Asn Ala Phe Tyr Ser Ala
 500 505 510
 Ser Thr Asn Gln Ile Arg Phe Pro Ala Gly Glu Leu Gln Lys Pro Phe
 515 520 525
 Phe Trp Gly Thr Glu Tyr Pro Arg Ser Leu Ser Tyr Gly Ala Ile Gly
 530 535 540
 Val Ile Val Gly His Glu Phe Thr His Gly Phe Asp Asn Asn Gly Arg
 545 550 555 560
 Lys Tyr Asp Lys Asn Gly Asn Leu Asp Pro Trp Trp Ser Thr Glu Ser
 565 570 575
 Glu Glu Lys Phe Lys Glu Lys Thr Lys Cys Met Ile Asn Gln Tyr Ser
 580 585 590
 Asn Tyr Tyr Trp Lys Lys Ala Gly Leu Asn Val Lys Gly Lys Arg Thr
 595 600 605
 Leu Gly Glu Asn Ile Ala Asp Asn Gly Gly Leu Arg Glu Ala Phe Arg
 610 615 620
 Ala Tyr Arg Lys Trp Ile Asn Asp Arg Arg Gln Gly Leu Glu Glu Pro
 625 630 635 640
 Leu Leu Pro Gly Ile Thr Phe Thr Asn Asn Gln Leu Phe Phe Leu Ser
 645 650 655
 Tyr Ala His Val Arg Cys Asn Ser Tyr Arg Pro Glu Ala Ala Arg Glu
 660 665 670
 Gln Val Gln Ile Gly Ala His Ser Pro Pro Gln Phe Arg Val Asn Gly
 675 680 685
 Ala Ile Ser Asn Phe Glu Glu Phe Gln Lys Ala Phe Asn Cys Pro Pro
 690 695 700
 Asn Ser Thr Met Asn Arg Gly Met Asp Ser Cys Arg Leu Trp
 705 710 715

<210> SEQ ID NO 5

<211> LENGTH: 1524

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Encoding soluble alkaline phosphatase

<400> SEQUENCE: 5

```

ggatccacca tgatttcacc attcttagta ctggccattg gcacctgcct tactaactcc      60
ttagtgccag agaaagagaa agacccaag tactggcgag accaagcgca agagacactg      120
aaatatgccc tggagcttca gaagctcaac accaacgtgg ctaagaatgt catcatgttc      180
ctgggagatg ggatgggtgt ctccacagtg acggtgccc gcatectcaa gggtcagctc      240
caccacaacc ctggggagga gaccaggctg gagatggaca agttcccctt cgtggccctc      300
tccaagacgt acaacaccaa tgcccaggtc cctgacagcg ccggcaccgc caccgcctac      360

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ctgtgtgggg tgaaggccaa tgagggcacc gtgggggtaa gcgcagccac tgagcgttcc 420
cgggtgaaca ccaccaggg gaacgaggtc acctccatcc tgcgctgggc caaggacgct 480
gggaaatctg tgggcattgt gaccaccacg agagtgaacc atgccacccc cagcgccgcc 540
tacgcccact cggctgaccg ggactggtac tcagacaacg agatgcccc tgaggccttg 600
agccagggct gtaaggacat cgcctaccag ctcatgcata acatcaggga cattgacgtg 660
atcatggggg gtggccggaa atacatgtac cccaagaata aaactgatgt ggagtatgag 720
agtgcagaga aagccagggg cagcaggtg gacggcctgg acctcgttga cacctggaag 780
agcttcaaac cgagatacaa gcactccac ttcactctgga accgcacgga actcctgacc 840
cttgaccccc acaatgtgga ctacctattg ggtctcttcg agccagggga catgcagtac 900
gagctgaaca ggaacaacgt gacggaccgg tcaactctcg agatgggtgg ggtggccatc 960
cagatcctgc ggaagaaccc caaaggcttc ttcttgctgg tgaaggagg cagaattgac 1020
cacgggcacc atgaaggaaa agccaagcag gccctgcatg aggcgggtgga gatggaccgg 1080
gccatcgggc aggcaggcag cttgacctcc tcggaagaca ctctgaccgt ggtcactgcg 1140
gaccattccc acgtcttcac atttggtgga tacaccccc gtggcaactc tatctttggt 1200
ctggccccc tgctgagtga cacagacaag aagcccttca ctgccatcct gtatggcaat 1260
gggctgggt acaagtggtt gggcggtgaa cgagagaatg tctccatggt ggactatgct 1320
cacaacaact accaggcgca gtctgctgtg cccctgcgcc acgagacca cgcgggggag 1380
gacgtggccg tcttctccaa gggcccatg gcgcacctgc tgcacggcgt ccacgagcag 1440
aactacgtcc cccacgtgat ggcgtatgca gctgcatcg gggccaaact cggccactgt 1500
gctcctgcca gctcgtagtc taga 1524

```

```

<210> SEQ ID NO 6
<211> LENGTH: 502
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Soluble alkaline phosphatase

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<400> SEQUENCE: 6

```

```

Met Ile Ser Pro Phe Leu Val Leu Ala Ile Gly Thr Cys Leu Thr Asn
1             5             10            15

Ser Leu Val Pro Glu Lys Glu Lys Asp Pro Lys Tyr Trp Arg Asp Gln
20            25            30

Ala Gln Glu Thr Leu Lys Tyr Ala Leu Glu Leu Gln Lys Leu Asn Thr
35            40            45

Asn Val Ala Lys Asn Val Ile Met Phe Leu Gly Asp Gly Met Gly Val
50            55            60

Ser Thr Val Thr Ala Ala Arg Ile Leu Lys Gly Gln Leu His His Asn
65            70            75            80

Pro Gly Glu Glu Thr Arg Leu Glu Met Asp Lys Phe Pro Phe Val Ala
85            90            95

Leu Ser Lys Thr Tyr Asn Thr Asn Ala Gln Val Pro Asp Ser Ala Gly
100           105           110

Thr Ala Thr Ala Tyr Leu Cys Gly Val Lys Ala Asn Glu Gly Thr Val
115           120           125

Gly Val Ser Ala Ala Thr Glu Arg Ser Arg Cys Asn Thr Thr Gln Gly

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-continued

130	135	140
Asn Glu Val Thr Ser Ile Leu Arg Trp Ala Lys Asp Ala Gly Lys Ser		
145	150	155 160
Val Gly Ile Val Thr Thr Thr Arg Val Asn His Ala Thr Pro Ser Ala		
	165	170 175
Ala Tyr Ala His Ser Ala Asp Arg Asp Trp Tyr Ser Asp Asn Glu Met		
	180	185 190
Pro Pro Glu Ala Leu Ser Gln Gly Cys Lys Asp Ile Ala Tyr Gln Leu		
	195	200 205
Met His Asn Ile Arg Asp Ile Asp Val Ile Met Gly Gly Gly Arg Lys		
	210	215 220
Tyr Met Tyr Pro Lys Asn Lys Thr Asp Val Glu Tyr Glu Ser Asp Glu		
	225	230 235 240
Lys Ala Arg Gly Thr Arg Leu Asp Gly Leu Asp Leu Val Asp Thr Trp		
	245	250 255
Lys Ser Phe Lys Pro Arg Tyr Lys His Ser His Phe Ile Trp Asn Arg		
	260	265 270
Thr Glu Leu Leu Thr Leu Asp Pro His Asn Val Asp Tyr Leu Leu Gly		
	275	280 285
Leu Phe Glu Pro Gly Asp Met Gln Tyr Glu Leu Asn Arg Asn Asn Val		
	290	295 300
Thr Asp Pro Ser Leu Ser Glu Met Val Val Val Ala Ile Gln Ile Leu		
	305	310 315 320
Arg Lys Asn Pro Lys Gly Phe Phe Leu Leu Val Glu Gly Gly Arg Ile		
	325	330 335
Asp His Gly His His Glu Gly Lys Ala Lys Gln Ala Leu His Glu Ala		
	340	345 350
Val Glu Met Asp Arg Ala Ile Gly Gln Ala Gly Ser Leu Thr Ser Ser		
	355	360 365
Glu Asp Thr Leu Thr Val Val Thr Ala Asp His Ser His Val Phe Thr		
	370	375 380
Phe Gly Gly Tyr Thr Pro Arg Gly Asn Ser Ile Phe Gly Leu Ala Pro		
	385	390 395 400
Met Leu Ser Asp Thr Asp Lys Lys Pro Phe Thr Ala Ile Leu Tyr Gly		
	405	410 415
Asn Gly Pro Gly Tyr Lys Val Val Gly Gly Glu Arg Glu Asn Val Ser		
	420	425 430
Met Val Asp Tyr Ala His Asn Asn Tyr Gln Ala Gln Ser Ala Val Pro		
	435	440 445
Leu Arg His Glu Thr His Gly Gly Glu Asp Val Ala Val Phe Ser Lys		
	450	455 460
Gly Pro Met Ala His Leu Leu His Gly Val His Glu Gln Asn Tyr Val		
	465	470 475 480
Pro His Val Met Ala Tyr Ala Ala Cys Ile Gly Ala Asn Leu Gly His		
	485	490 495
Cys Ala Pro Ala Ser Ser		
	500	

<210> SEQ ID NO 7

<211> LENGTH: 1554

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

-continued

<220> FEATURE:

<223> OTHER INFORMATION: Encoding soluble alkaline phosphatase conjugate

<400> SEQUENCE: 7

```

ggatccacca tgatttcacc attcttagta ctggccattg gcacctgcct tactaactcc      60
ttagtgccag agaaagagaa agacccaag tactggcgag accaagcgca agagacactg      120
aaatatgccc tggagcttca gaagctcaac accaacgtgg ctaagaatgt catcatgttc      180
ctgggagatg ggatgggtgt ctccacagtg acggctgccc gcatcctcaa gggtcagctc      240
caccacaacc ctggggagga gaccaggctg gagatggaca agttccccctt cgtggccctc      300
tccaagacgt acaacaccaa tgcccaggtc cctgacagcg ccggcaccgc caccgcctac      360
ctgtgtgggg tgaaggccaa tgagggcacc gtggggggtaa gcgcagccac tgagcgttcc      420
cggtgcaaca ccaccagggy gaacgaggtc acctccatcc tgcgctgggc caaggacgct      480
gggaaatctg tgggcattgt gaccaccacg agagtgaacc atgccacccc cagcgccgcc      540
tacgcccact cggctgaccg ggactggtac tcagacaacg agatgcccc tgaggccttg      600
agccagggct gtaaggacat cgcctaccag ctcatgcata acatcaggga cattgacgtg      660
atcatggggg gtggccggaa atacatgtac cccaagaata aaactgatgt ggagtatgag      720
agtgcagaga aagccagggy cacgaggctg gacggcctgg acctcgttga cacctggaag      780
agcttcaaac cgagatacaa gcactccac ttcatctgga accgcacgga actcctgacc      840
cttgaccccc acaatgtgga ctacctattg ggtctcttcg agccagggga catgcagtac      900
gagctgaaca ggaacaacgt gacggaccgg tcactctccg agatgggtggg ggtggccatc      960
cagatcctgc ggaagaaccc caaaggcttc ttcttgctgg tggaaggagg cagaattgac     1020
cacgggcacc atgaaggaag agccaagcag gccctgcatg aggcgggtgga gatggaccgg     1080
gccatcgggc aggcaggcag cttgacctcc tcggaagaca ctctgaccgt ggtcactgcg     1140
gaccattccc acgtcttcac atttggtgga tacaccccc gtggcaactc tatctttggt     1200
ctggccccc tgetgagtga cacagacaag aagcccttca ctgccatcct gtatggcaat     1260
gggcctggct acaaggtggt gggcggtgaa cgagagaatg tctccatggt ggactatgct     1320
cacaacaact accaggcgca gtctgctgtg ccctgcgcc acgagacca cggcggggag     1380
gacgtggccg tcttctccaa gggccccatg gcgcacctgc tgcacggcgt ccacgagcag     1440
aactacgtcc cccacgtgat ggcgtatgca gcctgcatcg gggccaacct cggccactgt     1500
gctcctgcca gctcggatga cgacgatgat gacgatgatg acgactagtc taga         1554

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<210> SEQ ID NO 8

<211> LENGTH: 512

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Soluble alkaline phosphatase conjugate

<400> SEQUENCE: 8

```

Met Ile Ser Pro Phe Leu Val Leu Ala Ile Gly Thr Cys Leu Thr Asn
1           5           10          15

Ser Leu Val Pro Glu Lys Glu Lys Asp Pro Lys Tyr Trp Arg Asp Gln
20          25          30

Ala Gln Glu Thr Leu Lys Tyr Ala Leu Glu Leu Gln Lys Leu Asn Thr
35          40          45

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Asn	Val	Ala	Lys	Asn	Val	Ile	Met	Phe	Leu	Gly	Asp	Gly	Met	Gly	Val
50						55					60				
Ser	Thr	Val	Thr	Ala	Ala	Arg	Ile	Leu	Lys	Gly	Gln	Leu	His	His	Asn
65					70					75					80
Pro	Gly	Glu	Glu	Thr	Arg	Leu	Glu	Met	Asp	Lys	Phe	Pro	Phe	Val	Ala
				85					90					95	
Leu	Ser	Lys	Thr	Tyr	Asn	Thr	Asn	Ala	Gln	Val	Pro	Asp	Ser	Ala	Gly
			100					105					110		
Thr	Ala	Thr	Ala	Tyr	Leu	Cys	Gly	Val	Lys	Ala	Asn	Glu	Gly	Thr	Val
		115					120					125			
Gly	Val	Ser	Ala	Ala	Thr	Glu	Arg	Ser	Arg	Cys	Asn	Thr	Thr	Gln	Gly
	130					135					140				
Asn	Glu	Val	Thr	Ser	Ile	Leu	Arg	Trp	Ala	Lys	Asp	Ala	Gly	Lys	Ser
145					150					155					160
Val	Gly	Ile	Val	Thr	Thr	Thr	Arg	Val	Asn	His	Ala	Thr	Pro	Ser	Ala
			165						170					175	
Ala	Tyr	Ala	His	Ser	Ala	Asp	Arg	Asp	Trp	Tyr	Ser	Asp	Asn	Glu	Met
			180					185					190		
Pro	Pro	Glu	Ala	Leu	Ser	Gln	Gly	Cys	Lys	Asp	Ile	Ala	Tyr	Gln	Leu
		195					200					205			
Met	His	Asn	Ile	Arg	Asp	Ile	Asp	Val	Ile	Met	Gly	Gly	Gly	Arg	Lys
	210					215					220				
Tyr	Met	Tyr	Pro	Lys	Asn	Lys	Thr	Asp	Val	Glu	Tyr	Glu	Ser	Asp	Glu
225					230					235					240
Lys	Ala	Arg	Gly	Thr	Arg	Leu	Asp	Gly	Leu	Asp	Leu	Val	Asp	Thr	Trp
				245					250					255	
Lys	Ser	Phe	Lys	Pro	Arg	Tyr	Lys	His	Ser	His	Phe	Ile	Trp	Asn	Arg
			260					265					270		
Thr	Glu	Leu	Leu	Thr	Leu	Asp	Pro	His	Asn	Val	Asp	Tyr	Leu	Leu	Gly
		275					280					285			
Leu	Phe	Glu	Pro	Gly	Asp	Met	Gln	Tyr	Glu	Leu	Asn	Arg	Asn	Asn	Val
	290					295					300				
Thr	Asp	Pro	Ser	Leu	Ser	Glu	Met	Val	Val	Val	Ala	Ile	Gln	Ile	Leu
305					310					315					320
Arg	Lys	Asn	Pro	Lys	Gly	Phe	Phe	Leu	Leu	Val	Glu	Gly	Gly	Arg	Ile
				325					330					335	
Asp	His	Gly	His	His	Glu	Gly	Lys	Ala	Lys	Gln	Ala	Leu	His	Glu	Ala
			340					345					350		
Val	Glu	Met	Asp	Arg	Ala	Ile	Gly	Gln	Ala	Gly	Ser	Leu	Thr	Ser	Ser
		355					360					365			
Glu	Asp	Thr	Leu	Thr	Val	Val	Thr	Ala	Asp	His	Ser	His	Val	Phe	Thr
	370					375					380				
Phe	Gly	Gly	Tyr	Thr	Pro	Arg	Gly	Asn	Ser	Ile	Phe	Gly	Leu	Ala	Pro
385					390					395					400
Met	Leu	Ser	Asp	Thr	Asp	Lys	Lys	Pro	Phe	Thr	Ala	Ile	Leu	Tyr	Gly
				405					410					415	
Asn	Gly	Pro	Gly	Tyr	Lys	Val	Val	Gly	Gly	Glu	Arg	Glu	Asn	Val	Ser
			420					425					430		
Met	Val	Asp	Tyr	Ala	His	Asn	Asn	Tyr	Gln	Ala	Gln	Ser	Ala	Val	Pro
		435					440					445			
Leu	Arg	His	Glu	Thr	His	Gly	Gly	Glu	Asp	Val	Ala	Val	Phe	Ser	Lys

-continued

450	455	460	
Gly Pro Met Ala His Leu Leu His Gly Val His Glu Gln Asn Tyr Val			
465	470	475	480
Pro His Val Met Ala Tyr Ala Ala Cys Ile Gly Ala Asn Leu Gly His			
	485	490	495
Cys Ala Pro Ala Ser Ser Asp Asp Asp Asp Asp Asp Asp Asp Asp			
	500	505	510

<210> SEQ ID NO 9
 <211> LENGTH: 25
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 9

gatccgatga cgatgacgat gacgc	25
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<210> SEQ ID NO 10
 <211> LENGTH: 25
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 10

ggccgcgtca tcgtcatcgt catcg	25
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<210> SEQ ID NO 11
 <211> LENGTH: 37
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 11

gatccgatga cgatgacgat gacgatgacg atgacgc	37
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<210> SEQ ID NO 12
 <211> LENGTH: 37
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 12

ggccgcgtca tcgtcatcgt catcgtcac gtcacg	37
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<210> SEQ ID NO 13
 <211> LENGTH: 55
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 13

gatccgatga cgatgacgat gacgatgacg atgacgatga cgatgacgat gacgc	55
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<210> SEQ ID NO 14
 <211> LENGTH: 55
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence

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<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 14

ggccgcgtca tcgtcatcgt catcgtcac gtcacgtca tcgtcatcgt catcg 55

<210> SEQ ID NO 15
<211> LENGTH: 61
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 15

gatccgatga cagtagtgag tcacttgaca gtggcagttc aagtgagagc gatggtgacg 60
c 61

<210> SEQ ID NO 16
<211> LENGTH: 61
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 16

ggccgcgtca ccacgcctct cacttgaact gccactgtca gatgactcac tactgtcac 60
g 61

<210> SEQ ID NO 17
<211> LENGTH: 52
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 17

gatccgattc atctgaagag aaatttttgc gtagaattgg aagattcggg gc 52

<210> SEQ ID NO 18
<211> LENGTH: 52
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 18

ggccgcaccg aatcttccaa ttctaagcaa aaatttctct tcagatgaat cg 52

<210> SEQ ID NO 19
<211> LENGTH: 58
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 19

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<210> SEQ ID NO 20
<211> LENGTH: 58
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence

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<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 20

ggccgcaatg aagggattaa gttcataaga ttccatgctt tcatgtgatt cataacag 58

<210> SEQ ID NO 21
<211> LENGTH: 46
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 21

gatcccgaa tgctgtgtcc tctgaagaaa ccaatgactt taaagc 46

<210> SEQ ID NO 22
<211> LENGTH: 46
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 22

ggccgcttta aagtcattgg tttcttcaga ggacacagca ttctgg 46

<210> SEQ ID NO 23
<211> LENGTH: 64
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 23

gatccggcag tagtgactca tccgaagaaa atggagatga cagttcagaa gaggaggagg 60
aagc 64

<210> SEQ ID NO 24
<211> LENGTH: 64
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 24

ggccgcttcc tctctctctt ctgaactgtc atctccattt tcttcggatg agtcactact 60
gccg 64

<210> SEQ ID NO 25
<211> LENGTH: 61
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 25

gatcccgcaa aggattctac aagagaaagc agtgcaaacc ttcccggtggc cgcaagcgtg 60
c 61

<210> SEQ ID NO 26
<211> LENGTH: 61

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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 26

ggcgcgcacg ttgcggccac gggaagggtt gcactgcttt ctctttaga atcctttgcg 60
g 61

<210> SEQ ID NO 27
<211> LENGTH: 32
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 27

agtcgggatc cggaacaagc agcgtgttct ac 32

<210> SEQ ID NO 28
<211> LENGTH: 39
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 28

agatcgcggc cgctcaattg tgcacggtgt gattaaagg 39

<210> SEQ ID NO 29
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 29

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<210> SEQ ID NO 30
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide

<400> SEQUENCE: 30

tctactgcta ctgctactgc tactgctact gaggcc 36

<210> SEQ ID NO 31
<211> LENGTH: 18
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Human fragment of MEPE

<400> SEQUENCE: 31

Asp Asp Ser Ser Glu Ser Ser Asp Ser Gly Ser Ser Ser Glu Ser Asp
1 5 10 15

Gly Asp

<210> SEQ ID NO 32

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<211> LENGTH: 15
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Human fragment of Statherin

<400> SEQUENCE: 32

Asp Ser Ser Glu Glu Lys Phe Leu Arg Arg Ile Gly Arg Phe Gly
1 5 10 15

<210> SEQ ID NO 33
<211> LENGTH: 17
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Human fragment of MGP

<400> SEQUENCE: 33

Cys Tyr Glu Ser His Glu Ser Met Glu Ser Tyr Glu Leu Asn Pro Phe
1 5 10 15

Ile

<210> SEQ ID NO 34
<211> LENGTH: 13
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Human fragment of OPN

<400> SEQUENCE: 34

Gln Asn Ala Val Ser Ser Glu Glu Thr Asn Asp Phe Lys
1 5 10

<210> SEQ ID NO 35
<211> LENGTH: 18
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Human fragment of BSP2

<400> SEQUENCE: 35

Gly Ser Ser Asp Ser Ser Glu Glu Asn Gly Asp Asp Ser Ser Glu Glu
1 5 10 15

Glu Glu

<210> SEQ ID NO 36
<211> LENGTH: 18
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Human fragment of IGFBP5

<400> SEQUENCE: 36

Arg Lys Gly Phe Tyr Lys Arg Lys Gln Cys Lys Pro Ser Arg Gly Arg
1 5 10 15

Lys Arg

<210> SEQ ID NO 37
<211> LENGTH: 151
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Collagen binding domain of staphilococcus

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aureus collagen adhesin M81736

<400> SEQUENCE: 37

Gly Thr Ser Ser Val Phe Tyr Tyr Lys Thr Gly Asp Met Leu Pro Glu
 1 5 10 15
 Asp Thr Thr His Val Arg Trp Phe Leu Asn Ile Asn Asn Glu Lys Ser
 20 25 30
 Tyr Val Ser Lys Asp Ile Thr Ile Lys Asp Gln Ile Gln Gly Gly Gln
 35 40 45
 Gln Leu Asp Leu Ser Thr Leu Asn Ile Asn Val Thr Gly Thr His Ser
 50 55 60
 Asn Tyr Tyr Ser Gly Gln Ser Ala Ile Thr Asp Phe Glu Lys Ala Phe
 65 70 75 80
 Pro Gly Ser Lys Ile Thr Val Asp Asn Thr Lys Asn Thr Ile Asp Val
 85 90 95
 Thr Ile Pro Gln Gly Tyr Gly Ser Tyr Asn Ser Phe Ser Ile Asn Tyr
 100 105 110
 Lys Thr Lys Ile Thr Asn Glu Gln Gln Lys Glu Phe Val Asn Asn Ser
 115 120 125
 Gln Ala Trp Tyr Gln Glu His Gly Lys Glu Glu Val Asn Gly Lys Ser
 130 135 140
 Phe Asn His Thr Val His Asn
 145 150

<210> SEQ ID NO 38

<211> LENGTH: 41

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Oligonucleotide primer

<400> SEQUENCE: 38

cagtcagggt ctcttatccg gaagtcctcca agctaaacag g

41

<210> SEQ ID NO 39

<211> LENGTH: 41

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Oligonucleotide primer

<400> SEQUENCE: 39

ctgttttagct tggagacttc cggataagag accttgactg g

41

<210> SEQ ID NO 40

<211> LENGTH: 25

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Oligonucleotide primer

<400> SEQUENCE: 40

gataaagcag gtcttgggggt gcacc

25

<210> SEQ ID NO 41

<211> LENGTH: 25

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

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<223> OTHER INFORMATION: Oligonucleotide primer

<400> SEQUENCE: 41

gttggcatct gtcacgggct tgtgg 25

<210> SEQ ID NO 42

<211> LENGTH: 32

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Oligonucleotide primer

<400> SEQUENCE: 42

tggatccacc atgatttcac cattcttagt ac 32

<210> SEQ ID NO 43

<211> LENGTH: 35

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Oligonucleotide primer

<400> SEQUENCE: 43

ttctagacta cgagctggca ggagcacagt ggccg 35

<210> SEQ ID NO 44

<211> LENGTH: 65

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Oligonucleotide primer

<400> SEQUENCE: 44

ttctagacta gtcgtcatca tcgtcatcat cgtcgtcatc cgagctggca ggagcacagt 60

ggccg 65

<210> SEQ ID NO 45

<211> LENGTH: 5

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Fragment surrounding cleavage site

<400> SEQUENCE: 45

Ser Leu Gln Ala Lys
1 5

<210> SEQ ID NO 46

<211> LENGTH: 11

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Fragment surrounding cleavage site

<400> SEQUENCE: 46

Thr Ser Asp Lys Thr His Ser Leu Gln Ala Lys
1 5 10

<210> SEQ ID NO 47

<211> LENGTH: 7

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

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<223> OTHER INFORMATION: Fragment surrounding cleavage site

<400> SEQUENCE: 47

Ser Val Ser Leu Gln Ala Lys
1 5

<210> SEQ ID NO 48

<211> LENGTH: 19

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Fragment surrounding cleavage site

<400> SEQUENCE: 48

Ser Gly Asp Asp Asp Asp Asp Asp Asp Asp Ser Gly Ser Leu
1 5 10 15

Gln Ala Lys

<210> SEQ ID NO 49

<211> LENGTH: 14

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Fragment surrounding cleavage site

<400> SEQUENCE: 49

Ser Asp Asp Asp Asp Asp Asp Tyr Val Ser Leu Gln Ala Lys
1 5 10

<210> SEQ ID NO 50

<211> LENGTH: 8

<212> TYPE: PRT

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Fragment surrounding cleavage site

<400> SEQUENCE: 50

Arg Thr Val Val Lys Arg Ser Val
1 5

1-11. (canceled)

12. A bone delivery conjugate comprising sALP-Y-D_n, wherein

Y is an amino acid sequence of at least one amino acid;

D_n is a poly aspartate wherein n=10; and

sALP is a secreted soluble tissue non-specific alkaline phosphatase of SEQ ID NO: 6;

wherein said bone delivery conjugate is catalytically competent to allow formation of hydroxyapatite crystals in bone.

13. The bone delivery conjugate of claim 12, wherein said sALP is capable of catalyzing the cleavage of inorganic pyrophosphate (PPi).

14. The bone delivery conjugate of claim 13, wherein said bone delivery conjugate is encoded by a nucleic acid molecule which hybridizes under high stringency conditions to a nucleic acid molecule having a nucleic acid sequence complementary to at least one of SEQ ID NO: 5 and SEQ ID NO: 7.

15. The bone delivery conjugate of claim 14, wherein said high stringency conditions comprise pre-hybridization and hybridization in 6×SSC, 5×Denhardt's reagent, 0.5% SDS

and 100 mg/ml of denatured fragmented salmon sperm DNA at 68° C.; and washes in 2×SSC and 0.5% SDS at room temperature for 10 minutes; in 2×SSC and 0.1% SDS at room temperature for 10 minutes; and in 0.1×SSC and 0.5% SDS at 65° C. three times for five minutes.

16. A composition comprising the bone delivery conjugate of claim 12 and at least one pharmaceutically acceptable carrier.

17. The composition of claim 16, wherein said sALP is capable of catalyzing the cleavage of inorganic pyrophosphate (PPi).

18. A method of delivering a protein to bone tissue of a mammal to treat a bone related undesired condition in said mammal, comprising administering to said mammal an effective amount of a bone delivery conjugate comprising sALP-Y-D_n, wherein

Y is an amino acid sequence of at least one amino acid;

D_n is a poly aspartate wherein n=10;

sALP is a secreted soluble tissue non-specific alkaline phosphatase of SEQ ID NO: 6; and

said bone delivery conjugate is catalytically competent to allow formation of hydroxyapatite crystals in bone.

19. The method of claim **18**, wherein said bone delivery conjugate is encoded by a nucleic acid molecule which hybridizes under high stringency conditions to a nucleic acid molecule having a nucleic acid sequence complementary at least one of SEQ ID NO: 5 and SEQ ID NO: 7; wherein said high stringency conditions comprise pre-hybridization and hybridization in 6×SSC, 5×Denhardt's reagent, 0.5% SDS and 100 mg/ml of denatured fragmented salmon sperm DNA at 68° C.; and washes in 2×SSC and 0.5% SDS at room temperature for 10 minutes; in 2×SSC and 0.1% SDS at room temperature for 10 minutes; and in 0.1×SSC and 0.5% SDS at 65° C. three times for five minutes.

20. The method of claim **18**, wherein said mammal is a human, and the bone delivery conjugate further comprises at least one pharmaceutically acceptable carrier.

21. The method of claim **20**, wherein said effective amount is administered to said human more than once at an interval ranging from daily to weekly.

22. The method of claim **21**, wherein said effective amount is from about 1 to about 50 milligrams per kilogram of body weight.

23. The method of claim **22**, wherein said effective amount is from about 1 to about 10 milligrams per kilogram of body weight.

24. The method of claim **23**, wherein said effective amount is about 1 milligram per kilogram of body weight.

25. The method of claim **20**, wherein said bone related undesired condition is at least one of hypophosphatasia (HPP), hypophosphatemic rickets, osteomalacia, a lack or insufficient amount of functional alkaline phosphatase, impaired skeletal mineralization, growth retardation, lower extremity deformity, hyperparathyroidism, parathyroidectomy, hypercalciuria, hypercalcemia, and nephrocalcinosis.

26. The method of claim **25**, wherein the HPP is perinatal HPP, infantile HPP, childhood HPP, or adult HPP.

27. The method of claim **20**, wherein the pharmaceutically acceptable carrier is saline.

28. The method of claim **18**, wherein said sALP is capable of catalyzing the cleavage of inorganic pyrophosphate (PPi).

29. The composition of claim **16**, wherein the pharmaceutically acceptable carrier is saline.

* * * * *