(54) Title: METHOD FOR CELL ADHESION AND WOUND HEALING
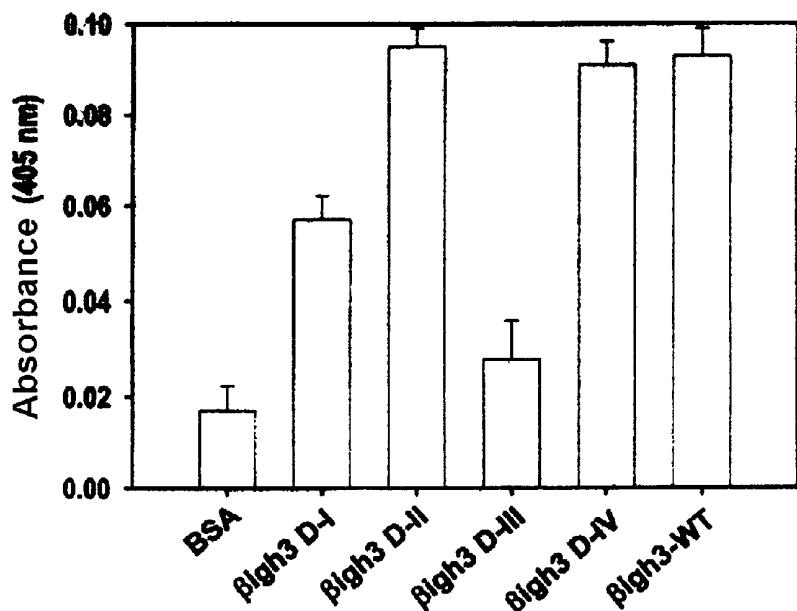
(57) Abstract: The present invention relates to a method for cell adhesion and wound healing with internal domains of βig-h3. Particularly, the present invention relates to the method of using recombinant proteins containing one or more of 2nd or 4th internal domain of βig-h3 for cell adhesion and wound healing, wherein the 2nd or 4th internal domain of βig-h3 has aspartic acid and isoleucine essential for interaction with integrin which represent a high homology in base sequence of βig-h3 internal domains. The recombinant proteins containing one or more 2nd_ or 4th_ internal domain of βig-h3 are effective for cell adhesion and wound healing by itself and can be used for developing cell culture medium and wound healing agent.

# METHOD FOR CELL ADHESION AND WOUND HEALING

## FIELD OF THE INVENTION

5      The present invention relates to peptides for use in cell adhesion and wound healing.  More particularly, the present invention relates to the use in cell adhesion and wound healing of peptides containing one or more copies of the $2^{nd}$ and/or $4^{th}$ fas-1 domain of βig-h3, said $2^{nd}$ and $4^{th}$

10   domains sharing a high homology in two amino acids, aspartic acid and isoleucine, essential for binding to integrin and thus mediating cell adhesion.  Also, the present invention is concerned with an expression system for the peptides useful in cell adhesion and wound healing.

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## BACKGROUND OF THE INVENTION

βig-h3 is an extracellular matrix protein whose expression is induced in various cell lines, including human

20   melanoma cells, mammary ephithelial cells, keratinocytes, and lung fibroblasts, following signaling by active TGF-β (Skonier, J. et al., DNA Cell Biol. 13, 571, 1994).  The βig-h3 gene was first isolated by differential hybridization screening of a cDNA library made from a human lung

25   adenocarcinoma cell line that had been treated with TGF-β.

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βig-h3 gene encodes a 683-amino acid protein that is highly conserved between species. It contains an N-terminal secretory signal peptide and an Arg-Gly-Asp (RGD) motif at the C-terminus. The RGD motif is found in many extracellular matrix proteins modulating cell adhesion and serves as a ligand recognition sequence for several integrins (Stonier, J. et al., DNA Cell Biol., 11, 511, 1992).

According to several studies, βig-h3 is known to be involved in cell growth and proliferation, wound healing, and cell adhesion, although the underlying mechanisms for these functions are still unclear. However, βig-h3 seems to play an important role in the morphogenesis and interactions with cells and extracellular matrix proteins in various tissues.

Some evidence related to the role of βig-h3 in mediating cell attachment and detachment is provided by several studies. For example, purified βig-h3 protein is found to promote the attachment and spreading of skin fibroblasts while inhibiting the adhesion of A549, HeLa and Wi-38 cells in serum-free media. Particularly, βig-h3 is known to have inhibitory activity against tumor cell growth, and to affect colony formation and morphology. The inhibitory activity was demonstrated by the report in which transfection of βig-h3 expression plasmids into CHO (Chinese

hamster ovary) cells led to marked decreases in cell proliferation and the ability of these cells to form tumors in nude mice. Further, a wound healing method was developed on the basis of the finding that application of a

5    pharmaceutically effective amount of βig-h3 to wounds makes cells, especially fibroblasts, spread over and adhere to the wound site. Consequently, βig-h3, a cell adhesion molecule induced by TGF-β in various cell lines, plays a very important role in cell growth, cell differentiation, wound

10   healing, morphogenesis and cell adhesion (Rawe, I. M. et al., Invest. Ophthalmol. Vis. Sci. 38, 893, 1997; Lebaron, R. G. et al., J. Invest. Dermatol. 104, 844, 1995).

βig-h3 contains four 140 amino acid repeats with internal homology, namely fas-1 domains. The internal

15   repeat domains have highly conserved sequences found in secretory proteins or membrane proteins of various species, including mammals, insects, sea urchins, plants, yeasts, and bacteria. Proteins containing the conserved sequence are exemplified by periostin, fasciclin I, sea urchin HLC-2,

20   algal-CAM and mycobacterium MPB70. The conserved domain in these proteins (hereinafter referred to as "fas-1") consists of about 110 to 140 amino acids with two highly conserved branches, H1 and H2, of about 10 amino acids each (Kawamoto, T. et al., Biochem. Biophys. Acta. 1395, 288, 1998).

25         Four fas-1 domains are found in βig-h3, periostin, and

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fasciclin I, two fas-1 domains in HLC-2, and only one fas-1
domain in MPB70.  Although the functions of the proteins are
not elucidated clearly, some of them are known to act as
cell adhesion molecules.  For instance, βig-h3, periostin,
5  and fasciclin 1 are reported to mediate the adhesion of
fibroblasts, osteoblasts, and nerve cells, respectively.
Also, it is disclosed that algal-CAM is a cell adhesion
molecule present in embryos of the algae Volvox (LeBaron, R.
G., et al., J. Invest. Dermatol. 104, 844, 1995; Horiuchi, K.
10  et al., J. Bone Miner. Res. 14, 1239, 1999; Huber, O. et al.,
EMBO J. 13, 4212, 1994).

At first, it was believed that the cell attachment
activity of βig-h3 would be mediated by the C-terminal RGD
motif.  However, some research results revealed that the RGD
15  motif is not necessary for promoting the spreading of
chondrocytes and that the mature soluble βig-h3 whose RGD
motif is deleted by carboxyl-terminus processing is able to
inhibit cell adhesion, leading to the conclusion that the
RGD motif of βig-h3 is dispensable for mediating the cell
20  attachment activity of βig-h3.  In addition, it has been
recently reported that βig-h3 promotes the spreading of
fibroblasts via integrin α1β1 whereas the RGD motif of βig-
h3 is not necessary for mediating the cell adhesion property
of βig-h3.  According to a recent report, βig-h3 binds
25  specifically to integrin to enhance the cell adhesion and

spreading of cells irrespective of RGD motif (Ohno, S. et al., Biochm. Biophys. Acta 1451, 196, 1999). Further, the conserved peptides H1 and H2 of βig-h3 were found to have no influence on βig-h3-mediated cell adhesion. These results, taken together, indicate that amino acids indispensable for the cell attachment activity of βig-h3 exist somewhere other than the H1 and H2 regions. A computer search based on homologies not only among the repeated fas-1 domains of βig-h3 but also among fas-1 domains of other proteins revealed that there are a few highly conserved amino acids in addition to H1 and H2 peptides, suggesting the possibility of the involvement of the conserved amino acid sequences in the cell attachment activity.

Of the domains of βig-h3, known to play an important role in cell adhesion, either of the 2nd or 4th domain is identified as a minimum domain essential for the cell adhesion of the molecule in accordance with the present invention. Based on these findings, recombinant proteins containing the essential functional domains are also identified as being effective for wound healing, in accordance with the present invention.

Recent research for wound healing has been subdivided into cell biology and molecular biology and the promotion of wound healing has had increasing applications in various clinical fields. However, cell biological and molecular

biological mechanisms of wound healing still remain unclear. According to findings disclosed thus far, wound healing is a tissue response to trauma, leading to tissue repair through complex biological processes, including chemotaxis, cell
5    differentiation and replication, matrix protein synthesis, angiogenesis, and wound reconstitution (Steed, D. L., et al., Clin. Plast. Surg. 25, 397, 1998).

Growth factors are representative materials that appear in the early stage of the wound healing process and
10   control the subsequent wound healing process. Having strong influence over all stages of wound healing, growth factors act to control the growth, differentiation and metabolism of cells and reorganize the environs of the wound by their chemotactic properties which attract various cells types
15   that are involved in inflammation and tissue repair, cellular proliferation, stimulating angiogenesis and the synthesis and degradation of the extracellular matrix. PDGF (platelet-derived growth factor) attracts fibroblasts to the wound and stimulates them to proliferate, and transforming
20   growth factor-beta (TGF-β) causes them to make collagen. PDGF is chemotactic for most cells involved in wound healing, stimulates angiogenesis, remodeling and contraction, and activates wound healing cells (Mustoe, T. A. et al., J. Clin. Invest. 87, 694, 1991; Lepisto, J. et al., J. Surg. Res. 53,
25   596, 1992).    EGF (epidermal growth factor) stimulates

keratinocyte migration, angiogenesis and granulation tissue
development and activates mitogenesis of keratinocyets and
fibroblasts (Franklin, J. D. et al., Plast. Recsonst. Surg.
64, 766, 1979; Buckly, A. et al., Proc. Natl. Acad. Sci. USA,
5    82, 7340, 1985).    bFGF (basic fibroblast growth factor)
stimulates angiogenesis, epithelialization, and collagenous
fiber deposition, and associates with heparin in various
forms to perform relevant functions (Tsuboi, R. et al., J.
Exp. Med. 172, 245, 1990; Kinsnorth, A. N. et al., Br. J.
10    Surg. 77, 409, 1990). IGF (insulin-like growth factor)
enhances cell differentiation. VEGF (vascular endothelial
growth factor) increases vasopermeability and promotes
endothelial mitogenesis.

        Of the growth factors and cytokines involved in wound
15    healing, TGF-β is the most representative.    Existing in
three forms (TGF-β1, TGF-β2 and TGF-β3) in mammals, the
cytokine plays important roles in the growth and
differentiation of various cells and has various complex
functions, including control of cell growth, regulation of
20    immune responses, stimulation of osteogenesis, induction of
cartilage specific macromolecules, and promotion of wound
healing (Bennett, N. T. et al., Am. J. Surg. 165, 728, 1993).
Appearing in the ephithelium during wound healing, TGF-β is
believed to stimulate the expression of integrin within
25    keratinocytes during re-epithelialization.    In recent

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research into TGF-β expression, it was revealed that TGF-β3
mRNA is expressed in the epithelia of normal skin and acute
and chronic wounds, while TGF-β1 mRNA is not expressed in
normal skin and chronic wounds, but expressed in the
5    epithelial layer regenerated from acute wounds, and nowhere
is expressed TGF-β2 mRNA (Schmid, P. et al., J. Pathol. 171,
191, 1993). Based on the effects, even though their
mechanisms are not firmly established, TGF-β is expected to
play a major role in re-epithelialization.

10        Expression of βig-h3 is up-regulated by TGF-β,
suggesting that βig-h3 is involved in the mediation of some
signals of TGF-β. CHO (Chinese hamster ovary) cells
transformed with βig-h3 expression plasmids are reported to
show decreased tumorigenic ability (Skonier, J. et al., DNA
15   Cell Biol. 13, 571, 1994). In contrast, βig-h3 expression
is down-regulated in dexamethasone-treated stem cells, some
tumor cells and the fibroblasts cultured from the skin
lesion sites afflicted with localized hyperostosis of
melorheostosis. βig-h3 is also reported to serve as a
20   negative regulator of osteogenesis (Genini, M. et al., Int.
J. Cancer 66, 571, 1996; Schenker, T. et al., Exp. Cell. Res.
239, 161, 1998; Kim, J. et al., J. Cell Biochem. 77, 169,
2000). In addition to these functions, βig-h3, known as a
cell adhesion molecule, promotes the adhesion and spreading
25   of fibroblasts in the dermis. According to studies into the

distribution of βig-h3 in eye tissues, it is reported that the adhesion molecule is expressed in corneal epithelia of normal adults, intracorneal fetal stromal cells, and the endothelial and stromal cells in the process of wound healing. In addition, βig-h3 is expressed in the juxtaglomerular apparatus and proximal tubules of the kidneys, and its expression is increased in diabetes mellitus. Further, it is found in subendothelial smooth muscles of the coronary arteries of normal persons, and its amount is increased in the endometria of blood vessels in the case of arteriosclerosis. However, the expression of βig-h3 in normal dermal tissues and dermal wounds has not yet been firmly established (Klintworth, G. K. et al., Am. J. Pathol. 152, 743, 1998; Munier, F. L. et al., Nature Genetics 15, 247, 1997; Streeten B. W. et al., Arch. Ophthalmol. Vis. Sci. 38, 893, 1997). As mentioned above, the distribution and expression of βig-h3 in normal human tissues remains unclear. Particularly, there are no reports regarding expression patterns of βig-h3 in dermal wounds. However, some research groups have reported that βig-h3 functions to promote the adhesion and spreading of dermal fibroblasts, so that it is expected to make a contribution to the promotion of wound healing.

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## SUMMARY OF THE INVENTION

With the background in mind, the intensive and thorough research on βig-h3-mediated cell adhesion, leading to the present invention, resulted in the finding that there exist highly conserved amino acid sequences, in addition to H1 and H2 motifs, among fas-1 domains of βig-h3 and among fas-1 domains of other peptides, as analyzed by computer search, and particularly, high homology is detected at aspartic acid and isoleucine residues at positions near the H2 region. In addition, the $2^{nd}$ and $4^{th}$ domains of βig-h3, each containing the conserved amino acid residues, were found to induce cell adhesion through α3β1 integrin. Further, recombinant proteins which were designed to have the $2^{nd}$ and/or $4^{th}$ fas-1 domain of βig-h3 were identified as being identical to wild type βig-h3 in cell attachment and spreading activity and wound healing effect.

Therefore, it is an object of the present invention to provide peptides which contain conserved amino acid sequences essential for cell attachment, spreading and detachment activity.

It is another object of the present invention to provide the use of the peptides in cell adhesion and wound healing.

It is a further object of the present invention to

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provide an expression system for the peptides.

It is still a further object of the present invention to provide a method for attaching cells.

It is still another object of the present invention to
5   provide a method for healing wounds.

In accordance with an aspect of the present invention, there is provided a recombinant protein, comprising a portion of domains of βig-h3, useful in mammalian cell attachment.

10   In accordance with another aspect of the present invention, there are provided expression vectors pβig-h3 D-II, pβig-h3 D-IV, and pβig-h3 D-IV 4X , capable of expressing the $2^{nd}$ and $4^{th}$ fas-1 domain of βig-h3 corresponding to amino acids 237-377 and 498-637,
15   respectively.

In accordance with a further aspect of the present invention, there are provided novel E. coli strains, transformed with the expression vectors pβig-h3 D-II, pβig-h3 D-IV, and pβig-h3 D-IV 4X, identified as E. coli
20   BL21/Hisβ-g (accession No. KCTC 0905BP), E. coli BL21/Hisβ-e (accession No. KCTC 0904BP) and E. coli BL21/Hisβ-e4x (accession No. KCTC 0906BP), respectively.

In accordance with still a further aspect of the present invention, there is provided a method for attaching
25   cells, comprising the steps of: preparing a recombinant

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protein containing one or more copies of the 2$^{nd}$ and/or 4$^{th}$ domain of βig-h3, by use of an expression vector; coating the recombinant protein onto a solid support; and applying cells to the protein-coated solid support.

5        In accordance with still another aspect of the present invention, there is provided the use of the recombinant protein in cell attachment.

In accordance with yet another aspect of the present invention, there is provided the use of the recombinant
10   protein in wound healing.

In accordance with still yet another aspect of the present invention, there is provided a method for healing wounds, comprising the steps of: coating a solid support with a recombinant protein containing one or more copies of
15   the 2$^{nd}$ and/or the 4$^{th}$ domain of βig-h3; attaching skin cells to the solid support; and applying the solid support to wounds.


## BRIEF DESCRIPTION OF THE DRAWINGS

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Fig. 1a is a schematic diagram showing recombinant proteins βigh3-WT and βigh3-ΔRGD, wherein conserved regions are represented by ▨ and ▩, and RGD motif by ®.

Fig. 2 is a photograph showing SDS-PAGE results of
25   recombinant proteins βigh3-WT and βigh3-△RGD.

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Fig. 3 is a microphotograph showing HCE cell adhesion and spreading effects of recombinant proteins βigh3-WT and βigh3-△RGD after dying with crystal violet.

Fig. 4 shows curves in which the HCE cell adhesion and
5   spreading activities of the recombinant proteins βigh3-WT and βigh3-△RGD are found to be concentration-dependent as measured by the count (A) and surface area (B) of attached cells.

Fig. 5 shows histograms in which the HCE cell adhesion
10  activities of the recombinant proteins βigh3-WT and βigh3-△RGD are compared in terms of count (A) and surface area (B) of attached cells.

Fig. 6a is a histogram showing effects of various compounds on the HCE cell adhesion activities of the
15  recombinant proteins βigh3-WT and βigh3-△RGD.

Fig. 6b is a histogram showing effects of divalent cations on the HCE cell adhesion activities of the recombinant protein βigh3-WT.

Fig. 6c is a histogram showing the inhibition effect
20  of anti-integrin monoclonal antibody on the HCE cell adhesion activity of the recombinant protein βigh3-WT.

Fig. 6d is a histogram showing the inhibitory effect of anti-integrin monoclonal antibody on the HCE cell adhesion activities of various proteins.

25  Fig. 6e is a histogram showing adhesion specificity of

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K562 cells for the recombinant protein βigh3-WT and matrix proteins.

Fig 7 is a schematic diagram showing recombinant proteins having each of the fas-1 domains of βig-h3.

Fig. 8 is a photograph showing SDS-PAGE results of recombinant proteins containing fas-1 domains of βig-h3.

Fig. 9 is a histogram showing HCE cell adhesion activities of recombinant proteins containing fas-1 domains of βig-h3.

Fig. 10 is a histogram showing the inhibitory effects of anti-integrin antibodies on HCE cell adhesion activities of the recombinant proteins containing fas-1 domains of βig-h3.

Fig. 11 shows parts of amino acid sequences of various matrix proteins containing fas-1 domains.

Fig. 12 is a schematic diagram showing substitution mutants of the 4$^{th}$ domain of βig-h3.

Fig. 13 is a photograph showing SDS-PAGE results of recombinant substitution mutants of the 4$^{th}$ domain of βig-h3.

Fig. 14 is a histogram showing cell adhesion activities of substitution mutants of the 4$^{th}$ domain of βig-h3.

Fig. 15 is a schematic diagram showing recombinant proteins βigh3-D-IV, βigh3-D-IV 2X, 3X and 4X containing one, two, three and four copies of the 4$^{th}$ domain of βig-h3.

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Fig. 16 shows photographs of the recombinant proteins βigh3-D-Ⅳ, βigh3-D-Ⅳ 2X, 3X and 4X run on 10% SDS-PAGE (A) and 8% nondenaturing PAGE (B), which are purified with the aid of Ni-NTA agarose resin

5    Fig. 17 shows optical photographs of wounds to which an ointment base is applied alone (A) and in combination with fibronectin (B), His-β-b (C), and βig-h3-D-IV (D).

Fig. 18 shows microphotographs of wounds which are in the process of re-epithelialization after being treated with

10   an ointment base alone (A) and in combination with fibronectin (B), His-β-b (C), and βig-h3-D-IV (D).

Fig. 19 shows optical photographs of wounds which have collagenous fibers formed after being treated with an ointment alone (A) and in combination with fibronectin (B),

15   His-β-b (C), and βig-h3-D-IV (D).

Fig. 20 is a histogram showing HCE cell adhesion activities of the recombinant proteins βigh3-D-Ⅳ, βigh3-D-Ⅳ 2X, 3X and 4X, which contain at least one copy of the 4th domain of βig-h3.

20   Fig. 21 shows optical photographs of wounds whose areas are reduced after being treated with a chitosan base alone (A) and in combination with fibronectin (B), βig-h3 3X (C), and βig-h3 4X (D).

## DETAILED DESCRIPTION OF THE INVENTION

In the present invention, recombinant proteins are prepared on the basis of the 2$^{nd}$ and 4$^{th}$ fas-1 domains of βig-

5   h3 and used alone or in combination, for cell adhesion and spreading. To select the 2$^{nd}$ and 4$^{th}$ domains, the domains of βig-h3 active in cell adhesion and spreading was identified. To this end, the C-terminal sequence Arg-Gly-Asp (RGD), known as a ligand recognition sequence for several integrins,

10   was examined for its effect on the cell adhesion property of βig-h3. The cell attachment activity was measured using the number and surface area of attached cells. As a result, βig-h3 was found to promote cell adhesion and spreading, independent of the RGD motif.

15   Based on this finding, chemical reagents were used to address the specificity of cell adhesion activity of βig-h3 and to get further clues about the nature of the cell surface receptor for βig-h3. The data obtained from the use of chemical reagents suggest that the cell surface receptor

20   for βig-h3, which is involved in the cell adhesion activity of βig-h3, could be one of the RGD-dependent integrins, which require divalent cations for interaction with βig-h3.

Next, to identify minimum domains essential for the cell adhesion function of βig-h3, an examination was made of

25   the ability of each fas-1 domain to mediate cell adhesion.

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This examination was based on the fact that fas-1 domains are found in various cell adhesion molecules, such as βig-h3, periostin, fasciclin I, HLC-2, and algal-CAM and the number of fas-1 domains present in such adhesion molecules varies from protein to protein. This fact led to the inference that all four fas-1 domains might not be required for the cell adhesion activity of βig-h3 and in an extreme case, only one domain could mediate the cell adhesion activity of βig-h3. In the present invention, the either of $2^{nd}$ or $4^{th}$ fas-1 domain of βig-h3 is revealed to be sufficient for the cell adhesion function of βig-h3. These results lead to the conclusion that the H1 and H2 sequences, common in the four domains of βig-h3, are not essential for the mediation of the cell adhesion activity of βig-h3. Additionally, two amino acids, that is, aspartic acid and isoleucine at positions near the H2 region within the $2^{nd}$ and $4^{th}$ fas-1 domains, were found to be highly conserved, implying that these amino acid residues constitute a cell adhesion-related motif. The indispensability of the two conserved amino acids for cell adhesion was identified using substitution mutants of the $4^{th}$ fas-1 domain of βig-h3.

In another embodiment of the present invention, a wound healing method is provided in which the $2^{nd}$ and $4^{th}$ fas-1 domains of βig-h3 are used, individually or in combination.

An comparison was made of wound healing effects of

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mutant βig-h3 proteins containing cell adhesion-active domains only and those of wild type βig-h3 (βig-h3-WT) containing domains a portion of the domains. In this regard, recombinant proteins containing the cell adhesion-active

5    domains were applied to rats.

When a recombinant protein containing the 4th fas-1 domain of βig-h3 was used a pharmaceutically effective ingredient for an ointment, wound shrinkage was observed, in addition to re-epithelialization and collagenous fiber

10   formation. Ultimately, these results mean that one of the 2nd or 4th fas-1 domain of βig-h3, in which the conserved aspartic acid and isoleucine exist, is useful for wound healing and thus can be utilized for the development of therapeutics for wounds.

15       Also, excellent cell adhesion and wound healing effects were obtained using a recombinant protein containing the 2nd fas-1 domain of βig-h3 or 2nd and 4th domains.

Over the protein containing all of the domains, recombinant proteins containing parts of the domains have an

20   advantage in that they can be produced in larger quantities because they are synthesized in water-soluble forms and thus do not undergo denaturation.


## EXAMPLES

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A better understanding of the present invention may be obtained in light of the following examples which are set forth to illustrate, but are not to be construed to limit the present invention.

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**EXAMPLE 1: Identification of Cell Adhesion Activity of RGD-Independent βig-h3 Proteins**

**1-1: Production of recombinant βig-h3 protein**

10          In order to find the domains of βig-h3 which have, in practice, cell adhesion and spreading activity, the C-terminal sequence Arg-Gly-Asp (RGD), known as a ligand recognition sequence for several integrins, was examined for effect on the cell adhesion property of βig-h3. In this

15     regard, an RGD-deleted recombinant βig-h3 protein (βigh3-ΔRGD) and a wild-type recombinant βig-h3 protein (βigh3-WT) were prepared.

First, the full-length human βig-h3 cDNA cloned in pBluescript (pBsβig-h3) was digested with NdeI and BglII.

20     The DNA fragment was subcloned into the EcoRV-EcoRI site of pET-29b(+) (Novagen Inc.). βigh3-WT was prepared by introducing a 1351 bp NcoI fragment excised from βig-h3 cDNA into the NcoI site of this clone. βigh3-ΔRGD was derived from βigh3-WT by cutting out a 3'-fragment of the βigh3-WT

25     plasmid with AoCI and NotI followed by blunting and self

ligation, as shown in Fig. 1.

After being transformed with each recombinant plasmid, E.coli BL 21 DE3 was cultured in LB medium containing 50 µg/ml kanamycin at 37 °C until the optical density (OD) at

5   595 nm reached 0.5-0.6. The recombinant βig-h3 proteins were induced using 1 mM isopropyl-β-D-(-)- thiogalactopyranoside (IPTG) at 37 °C for 3 hours. The pellet thus obtained was resuspended in a lysis buffer (50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 1 % Triton X-

10  100, 1 mM PMSF, 0.5 mM DTT) and then sonicated. The inclusion bodies were dissolved in a denaturation buffer of 8 M urea containing 20 mM, followed by the purification of the denatured proteins with the aid of Ni-NTA resin (Qiagen). The recombinant proteins were eluted with 200 mM imidazole

15  solution and then dialyzed sequentially from high to low urea in 20 mM Tris-HCl buffer containing 50 mM NaCl. These recombinant proteins were analyzed using SDS-PAGE, as shown in Fig. 2.


20  **1-2: Assay for the cell adhesion activity of recombinant βig-h3 fas-1 domain proteins**


Human corneal epithelial (HCE) cells used in this assay were cultured in DMEM (EMEM/F-12, Gibco BRL)

25  supplemented with 15% fetal bovine serum, Gentamicin (40

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μg/ml), insulin (5 μg/ml), cholera toxin (0.1 μg/ml) and human epidermal growth factor (hEGF) at 37 °C in 5 % $CO_2$.

The cell adhesion assay was performed as follows. First, the recombinant βig-h3 proteins or other

5    extracellular matrix proteins were let to adhere to the bottoms of 96-well microculture plates (Falcon) by incubation at 37 °C for 1 hour and blocked with PBS containing 0.2% BSA. The coated extracellular matrix proteins were human plasma vitronectin (Promega), purified

10   human plasma fibronectin (pFN), chicken collagen types I and II (Chemicon International Inc.), bovine collagen types IV and VI (Chemicon), mouse laminin (Chemicon), and bovine serum albumin (BSA) (Sigma). Cells were trypsinized and suspended in the culture media at a density of $2 \times 10^5$

15   cells/ml. 0.1 ml of the cell suspension was added to each well of the plates coated with the recombinant proteins.

Following incubation at 37 °C for 1 hour, unattached cells were removed by rinsing with PBS. Attached cells were incubated for 1 hour at 37 °C in 50 mM citrate buffer, pH

20   5.0, containing 3.75 mM p-nitrophenol-N-acetyl 1-β-D-glycosaminide as a hexosaminidase substrate and 0.25 % Triton X-100, followed by the addition of 50 mM glycine buffer, pH 10.4, containing 5 mM EDTA to block the enzyme activity. A measurement was made of absorbance at 405 nm in

25   a Multiskan MCC/340 microplate reader.

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To determine cell area as an index for cell adhesion activity, $4 \times 10^4$ cells were applied to substrates in 48-well culture plates. The attached cells were fixed weith 8 % glutaraldehyde (Sigma) and then stained with 0.25 % Crystal
5   Violet (Sigma) in 20 % methanol. Measurement of cell areas was performed by Image-Pro plus software (Media Cybernetics). Experiments were repeated in triplicate with 200 or 300 measurements per site for each experiment. Data is reported as the mean area at specific time points ± standard error of
10  mean.

As a result of the measurement of cell adhesion and spreading activity using βigh3-WT and βigh3-ΔRGD, the numbers and surface areas of HCE cells which adhered to βigh3WT were clearly greater than those attached to albumin
15  serving as a negative control, and were comparable to those of cells which adhered to fibronectin as shown in Fig. 3. The cell adhesion and spreading activities of βig-h3 were concentration-dependent, as shown in Figs. 4A and 4B). βig-h3ΔRGD lacking the RGD motif was almost equally effective at
20  supporting cell adhesion and spreading (Fig. 5A and 5B). These results, taken together, confirm that βig-h3 supports cell adhesion and spreading, independent of the RGD motif.


**EXPERIMENTAL   EXAMPLE   1:   Identification   of   Cell   Surface**
25  **Receptor   of   βig-h3   Involved   in   Cell   Adhesion   Activity   of**

<u>βig-h3</u>

## 1-1: Identification of cell adhesion activity using matrix peptide and reagent

In order to identify cell surface receptors involved

5   in the cell adhesion activity of βig-h3 protein, an inhibition assay was performed using various reagents.

Initially, plastic culture dishes were coated with 10 μg/ml fibronectin, βigh3-WT or βigh3-ΔRGD. HCE cells were preincubated for 30 min in media containing 5 mM EDTA, 100

10  μg/ml βigh3-WT, 100 μg/ml βigh3-ΔRGD, 1 mM RGD, 1 mM RGE or 100 μg/ml fibronectin, or none of them, and then assayed for cell adhesion as in Example 1.

Cell adhesion to βig-h3 was significantly inhibited by βig-h3 itself, RGD peptide and EDTA, and partially inhibited

15  by fibronectin and EGTA, while being not inhibited by RGE peptide.    Cell    adhesion    to    fibronectin    was    also significantly inhibited by fibronectin itself, RGD peptide and EDTA, and partially inhibited by βig-h3 and EGTA, but not by RGE peptide, as shown in Fig. 6A.   These results

20  indicate that the cell surface receptor for βig-h3, which is involved in the cell adhesion activity of βig-h3, could be one of the RGD-dependent integrins.


## 1-2: Effect of divalent cations on cell adhesion activity

25       To analyze the divalent cation sensitivity of βig-h3-

mediated adhesion, cells were suspended in HEPES-buffered

saline (HBS) (150 mM NaCl, 25 mM HEPES, 2 mM EDTA, pH 7.4)

at a density of $2 \times 10^5$ cells/ml and incubated at 37 °C for 30

min.  Then, they were washed twice in HBS and resuspended in

5    the same buffer.  Aliquots of cells (50 µl) were then added

to the microculture plate wells and incubated for 30 min at

37 °C  in  a  humidified  atmosphere  of  5 %  $CO_2$  with  50  µl

aliquots of HBS containing divalent cations ($MnCl_2$, $MgCl_2$ or

$CaCl_2$)  at  a  concentration  twice  as  large  as  the  final

10   concentration.   Subsequently,  they  were  plated  on  ligand-

coated dishes to perform the adhesion assay.

Cell adhesion to βig-h3 was strongly promoted by $Mn^{2+}$,

and to a lesser extent by $Mg^{2+}$, but only marginally by $Ca^{2+}$,

as  shown  in  Fig.  6B.    taken  together,  the  results

15   demonstrate  that  the  cell  surface  receptor  of  βig-h3  is  a

kind  of  RGD-dependent  integrin  which  requires  divalent

cations for interaction with βig-h3.


## 1-3: Identification of cell surface receptor of βig-h3 using

20  **monoclonal antibody against integrin**

To  identify  receptors  for  βig-h3,  function-blocking

monoclonal antibodies to integrin subunits were examined for

their  effect  on  the  adhesion  of  HCE  cells  to  a  surface

coated with βig-h3.  In this regard, initially, HCE ($3 \times 10^5$

25   cells/ml)  were  preincubated  in  an  incubation  solution    in

the presence of each of the monoclonal antibodies (5 µg/ml) against different types of integrins at 37 °C for 30 min. The preincubated cells were transferred onto plates precoated with βig-h3 proteins and then incubated further at 5   37 °C for 1 hour, followed by the quantitative analysis of βig-h3 binding with hexosaminidase substrate. The values are expressed as percentages of the number of cells adhering to βig-h3 in the absence of monoclonal antibodies.

Adhesion to the βig-h3 coated surface was specifically 10  inhibited by antibody against α3 subunit. Because the integrin α3 subunit is known to couple with the integrin β1 subunit, anti-β1 antibody significantly blocked cell adhesion to βig-h3, as shown in Fig. 6C. Similar results were observed using HT1080 cells.

15     As a control experiment for the function-blocking antibodies, fibronectin, vitronectin, laminin and type I collagen were employed as substrata. HCE cells were preincubated with function-blocking monoclonal antibodies to integrin subunits and then transferred onto wells coated 20  with 10 µg/ml fibronectin, vitronectin, type I collagen or laminin. Following incubation, cell counts of adhered cells were analyzed.

Cell adhesion to fibronectin was shown to be clearly inhibited by antibodies to integrins α3 and α5. Adhesion to 25  vitronectin and type I collagen was blocked by antibodies to

25

integrin αv and α2, respectively, whereas cell adhesion to laminin was inhibited by antibodies to integrins α3 and α6, as shown in Fig. 6D. On the other hand, antibody to β1 integrin efficiently inhibited cells from adhering to all

5 ligands mentioned above.

For another control experiment, K562 cells, known to express α5, but not α3 integrin, were used. K562 cells were inoculated onto plates coated with βigh3-WT, fibronectin, laminin, or type I collagen and incubated for 1 hour,

10 followed by the hexosaminidase analysis. K562 cells did not adhere to βig-h3, but adhered to fibronectin and vitronectin. Taken together, these results suggest integrin α3β1 is a specific receptor for βig-h3 in HCE cells, as shown in Fig. 6E.

15

## EXAMPLE 2: Identification of Domains Essential to Cell Adhesion Activity of βig-h3

In an attempt to identify essential amino acids

20 conferring cell adhesion activity of βig-h3, an examination was made to determine whether each repeat domain is capable of mediating cell adhesion.

Four recombinant proteins corresponding respectively to four repeat domain were prepared: four βig-h3 cDNA

25 fragments encoding amino acids 129-241, 237-377, 368-506,

and 498-637, respectively, were amplified by PCR and cloned into the EcoRV-XhoI site of pET-29b(+) and the resulting four expression vectors, named pβig-h3 D-I, pβig-h3 D-II, pβig-h3 D-III, and pβig-h3 D-IV, were used to prepare the

5  recombinant proteins, as shown in Fig. 7. E. coli transformants with the expression vectors pβig-h3 D-II and pβig-h3 D-IV were designated E. coli BL21/Hisβ-g and E.coli BL21/Hisβ-e and deposited in the Korean Collection for Type Culture of Korea Research Institute of Bioscience and

10  Biotechnology (KRIBB) with accession Nos. KCTC 0905BP and KCTC 0904BP, respectively, on Dec. 4, 2000.

Expression and purification of the recombinant proteins βig-h3 D-1, βig-h3 D-II, βig-h3 D-III, and βig-h3 D-IV followed the procedure described in Example 1-1 and

15  they were identified by SDS-PAGE, as shown in Fig. 8.

In regard to the mediation of cell adhesion, the $2^{nd}$ and $4^{th}$ fas-1 domains were equally active compared to the wild type βig-h3 whereas the $1^{st}$ fas-1 domain was weak and the $3^{rd}$ fas-1 domain was not active at all, as shown in Fig.

20  9.

In experiments with function-blocking antibodies to integrin subunits, both $2^{nd}$ and $4^{th}$ fas-1 domain-mediated cell adhesion were almost fully blocked by antibodies to α3 and β1 integrins, suggesting that both $2^{nd}$ and $4^{th}$ fas-1 domains

25  have amino acids essential for interacting with α3β1

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integrin, as shown in Fig. 10. These results also support the conclusion that neither H1 nor H2 sequence mediates cell adhesion activity of βig-h3 because the 1$^{st}$ and 3$^{rd}$ domains are not active in cell adhesion, although they have H1 or H2

5      sequence.


**EXAMPLE 3: Identification of Conserved Amino Acid Sequence Essential for Cell Adhesion of βig-h3**

**3-1: Identification of Conserved Motif by Amino Acid**
10     **Sequence alignment**

To find the amino acid sequence responsible for cell adhesion in 2$^{nd}$ and 4$^{th}$ fas-1 domains of βig-h3, which independently show cell attachment, a computer search based on homologies not only among the repeated fas-1 domains of
15     βig-h3, but also among fas-1 domains of other proteins was carried out. As a result, two amino acids, aspartic acid and isoleucine, near the H2 region, were found to be highly conserved among various proteins, as shown in Fig. 11. In addition, it was found that aspartic acid and isoleucine are
20     both conserved in the 2$^{nd}$ and 4$^{th}$ fas-1 domains of βig-h3, which are of high cell attachment activity, while only aspartic acid is conserved in the 1$^{st}$ fas-1 domain, which shows intermediate cell attachment activity. As for the 3$^{rd}$ fas-1 domain which shows no cell attachment activity, it has
25     neither of the two amino acids. This fact is further

28

evidence that the aspartic acid and isoleucine residues near the H$_2$ region are indispensable for mediating the cell attachment and spreading activity.

5    **3-2:  Identification  of  cell  adhesion  activity  of  the**
**conversed amino acid sequence using substitution mutants**

To further confirm that the two amino acids are essential for cell adhesion, the recombinant protein containing the 4$^{th}$ fas-1 domain of βig-h3 was mutated by
10   substitution as shown in Fig. 12.  The substitution mutant of βig-h3 D-IV was prepared by PCR and its sequence was confirmed by base sequencing.  The mutant protein was isolated and purified in the same manner as in Example 1-1 and confirmed on SDS-PAGE, as shown in Fig. 13.
15   Examination was made of the cell attachment activity of the mutated proteins wherein the Pro616, Asp617 and Ile618 of βigh3 D-IV were, in combination, substituted with Ser, Ala and Ser, respectively.  The mutant protein having Ala instead of Asp617, named D617A (βigh3 D-IV-PaI) and the
20   mutant protein having Ser instead of Ile618, named I618S (βigh3 D-IV-PDs) significantly blocked cell adhesion whereas the mutant protein having Ser instead of Pro616, named P616S (βigh3 D-IV-sDI) was found to have no influence on cell adhesion activity.  As for the mutant protein in which the
25   three amino acids were mutated, named P616S/D617A/I618S

(βigh3 DIV-sas), it also blocked cell adhesion, as shown in Fig. 14.

The nearly complete loss of the 1$^{st}$ fas-1 domain-mediated cell attachment activity in the 1$^{st}$ fas-1 domain
5  mutated at Asp617 and Ile618 proved that the aspartic acid at position 617 and isoleucine at position 618 are very important in mediating the cell attachment activity of βigh3.


## EXAMPLE 4: Identification of βigh3 Domains Effecting Wound
10 **Healing**

### 4-1: Expression and purification of recombinant βigh3 protein

To examine whether only the βig-h3 domains active in cell adhesion show the same wound healing function as that
15 of the native βig-h3 containing all four fas-1 domains, various recombinant βigh3 proteins were prepared as shown in Fig. 15: His-β-b containing all of 4 fas-1 domains; βigh3-D-IV containing the 4$^{th}$ domain alone; and βigh3-D-IV, 2X, 3X and 4X, each containing at least one 4$^{th}$ domain. Showing the
20 same cell adhesion activity as in βigh3-WT prepared in Example 1, the recombinant βig-h3 protein His-β-b was prepared from the recombinant expression vector pET-29β anchoring at its EcoRV-EcoRI site an Asp718-BglII fragment which was obtained by deleting a some amino-terminal region
25 from βig-h3 cDNA.  The recombinant proteins His-β-b and

βigh3-D-IV were expressed and purified in the same manners as in Example 1-1 and 3.

The recombinant proteins containing at least one 4th domain, such as βig3-D-IV, 2X, 3X and 4X were prepared as follows. A DNA fragment encoding to amino acid 498-637 corresponding the 4th domain was obtained by PCR and the PCR products were blunt-ended by Klenow enzyme. This blunt-ended cDNA fragment was inserted to the EcoRV site of the pβig-h3 D-IV, which contained the 4th domain of βig-h3, and the resulting expression vector was named pβI-h3 D-IV 2X. The insert of the pβig-h3 D-IV 2X was excised by digestion with EcoRV and XhoI and blunt-ended by treatment with Klenow, followed by inserting the blunt-ended fragment into EcoRV sites of pβig-h3 D-IV and pβig-h3 D-IV 2X. The resulting expression vectors were named pβig-h3 D-IV 3X and pβig-h3 D-IV 4X. Expression of all recombinant proteins was induced for 3 hours in the presence of 1 mM IPTG and isolated by use of Ni-NTA resin (Qiagen). Isolated recombinant proteins were purified by elution with 20 mM Tris-HCl comprising 50 mM NaCl and 300 mM imidazole. βig-h3 D-IV 2X, 3X and 4X can be produced in large amounts because they are synthesized as soluble forms, unlike βig-h3 recombinant proteins containing all of the four domains, and do not undergo denaturation, as shown in Fig. 16A. Electrophoresis using non-denaturing gel revealed that βig-h3 D-IV did not form polymers while 2X

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partially formed polymers and 3X and 4X each readily formed polymers , as shown in Fig. 16B.

E. coli BL21/His β-e4X, which harbors the expression vector pβig-h3 D-IV 4X containing four 4th domains of βig-h3,
5   was deposited in the Korean Collection for Type Culture of Korea Research Institute of Bioscience and Biotechnology (KRIBB) with accession No. KCTC 0906BP on Dec. 4, 2000.

Fibronectin, serving as a positive control, was purified from citrated rat plasma by affinity chromatography
10  using gelatin-sepharose 4B. The plasma was filtered at room temperature through non-substituted sepharose 4B and the eluate was loaded onto gelatin sepharose 4B equilibrated with 0.05 M Tris-Cl containing 0.05 M EACA (ε-amino caproic acid), 0.02 M sodium citrate and 0.02 % sodium azide. After
15  being eluted, most plasma proteins were washed with a buffer containing 1 M sodium chloride. Then, absorbed fibronectin was eluted with 3M uric acid isotonic buffer which was subsequently dialyzed for about 48 hours against PBS, pH 7.2, to purify fibronectin. Its concentration was determined by
20  UV absorbance at 280 nm and freeze-dried before being stored at -20 °C.


**4-2: Assay for wound healing activity of βig-h3 D-IV containing the 4th domain**
25      To    compare    wound    healing    activity    between    the

recombinant βig-h3 protein His-β-b, which contains all of the four fas-1 domains, like native βig-h3 protein, and the recombinant βig-h3 protein βigh3-D-IV, which contains the 4th domain alone, ointment bases comprising the recombinant

5    proteins were tested as follows.

Four dermal whole layer wounds, each 2 cm in diameter, were made on the backs of rats and divided into test groups 1-A, 1-B, 1-C and 1-D according to the ointment applied thereto.

10   1-A: coated at a dose of 1 gm per day with an ointment base combined with no materials.

1-B: coated at a dose of 1 gm per day with an ointment in which fibronectin was combined at a concentration of 100 µg/ml with a base.

15   1-C: coated at a dose of 1 g per day with an ointment in which Hisβ-b protein was combined at a concentration of 100 µg/ml with a base.

1-D: coated at a dose of 1 g per day with an ointment in which βigh3-D-IV protein was combined at a concentration

20   of 100 µg/ml with a base.

The backs of etherized rats were shaved, followed by sterilizing the shaved region with betadin solution.  In test group 1, the back of each rat was cut by use of a No. 15 surgical blade to form four circular wounds with a

25   diameter of 2 cm penetrating the whole dermal layers.

Ointments for test groups 1-A, 1-B, 1-C and I-D were applied
at an amount of about 1 g to the wounds which were then
covered with a synthetic dressing (Tegaderm® 3M) and lightly
bandaged.  Application of ointments was performed once every
5    day.

With a base of an aqueous material (SamA base), each
of the ointments contained, per 1 g, spermaceti 38 mg,
stearyl alcohol 116 mg, polyethylglycol 38 mg, conc.
glycerin 192 mg, ethanol 23 mg, lauryl sodium sulfate mg,
10   ethyl paraoxybenzoate 0.87 mg, butyl paraoxybenzoate 0.12 mg,
and purified water.

First, morphologies of wounds were observed.  The same
scale was positioned near each wound and pictures were taken
at the same distance from each wound.  Pictures were scanned
15   in a computer and used to measure areas of the wounds with
the aid of NIH image analysis system (Bio-Optics).  To take
the pictures, the muscle was completely relaxed by
etherizing the rats.  Measurements were performed once every
other day until the 22nd day.  For comparing test groups, the
20   measured values were analyzed according to ANOVA test and
Scheffer's test.

In all test groups, wound areas were observed to be
gradually reduced just after the formation of wounds.  The
test groups to which fibronectin and the recombinant βig-h3
25   protein were applied were measured to be more quickly

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reduced in wound area than the test group to which the
ointment base alone was applied.   A significant difference
in wound area was seen after 7 days of ointment application.
Statistically, there were significant differences (p<0.05)
5   between wounds of group I-A and the other groups, which did
not show a significant difference therebetween.   The results
are given in Table 1, below and Fig. 17.


### TABLE 1: Healing Effect of Ointment Bases Combined with Recombinant βig-h3 Proteins on Wounds

10

| Group | Day | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 2* | 4 | 6 | 8* | 10* | 12 | 14 | 16 | 18 | 20 | 22 |
| I-A (Control) | 3.15 ±0.49 | 3.09$^a$ ±0.31 | 2.45 ±0.39 | 2.17 ±0.46 | 1.64$^a$ ±0.50 | 1.80$^a$ ±0.11 | 0.84 ±0.32 | 0.56 ±0.31 | 0.34 ±0.07 | 0.19 ±0.04 | 0.17 ±0.05 | 0.08 ±0.01 |
| I-B (Fibronectin) | 3.17 ±0.78 | 2.38$^b$ ±0.55 | 2.01 ±0.54 | 1.83 ±0.42 | 1.39$^b$ ±0.38 | 1.26$^b$ ±0.18 | 0.54 ±0.11 | 0.39 ±0.12 | 0.25 ±0.12 | 0.16 ±0.09 | 0.12 ±0.09 | 0.06 ±0.03 |
| I-C (His-β-b) | 3.14 ±0.46 | 2.58$^b$ ±0.47 | 1.89 ±0.26 | 1.71 ±0.33 | 1.42$^b$ ±0.45 | 1.37$^b$ ±0.71 | 0.46 ±0.06 | 0.36 ±0.13 | 0.26 ±0.09 | 0.15 ±0.10 | 0.11 ±0.03 | 0.03 ±0.01 |
| I-D (βig-h3-D-IV) | 3.15 ±0.43 | 2.62$^b$ ±0.52 | 2.37 ±0.45 | 1.98 ±0.52 | 1.51$^b$ ±0.21 | 0.98$^b$ ±0.69 | 0.44 ±0.24 | 0.22 ±0.09 | 0.20 ±0.09 | 0.18 ±0.10 | 0.13 ±0.07 | 0.06 ±0.01 |

Value : mean±SD

*: p<0.05 by ANOVA and Scheffe's test

a, b: vertically significant difference of data in statistics

15

   Histological analysis was conducted under an optical
microscope.   Biopsies of wound sites were taken at days 3, 7,
10, 14 and 20, and fixed in 10 % formalin and solidified
with paraffin.   6 μm slices of the samples were dyed with
20   hematoxylin-eosin   (H&E)   and   Masson's   trichrome   before

observation under a microscope. Wound healing effects according to time of each test material were evaluated through re-epithelialization and formation of collagenous fibers. In the case of re-epithelialization, epithelial

5  formation was semi-quantified in such a way that zero was set for the formation of no epithelial layers, 1+ for initiation of epithelialization, 2+ for incomplete epithelial layer structure, and 3+ for complete epithelial layer structure. Regarding comparison among test groups and

10 differences according to time within each group, measured values were statistically analyzed using ANOVA test and Scheffe's test. As for collagenous fiber formation, it was graded as 1+ for insignificant formation of collagenous fibers as observed with trichrome dye, 2+ for scatteringly

15 spaced collagenous fibers, and 3+ for dense collagenous fibers.

As observed by an optical microscope, re-epithelialization appeared to start at day 7 to 10 in test groups 1-B, 1-C and 1-D and be completed at day 20. In the

20 case of the control group 1-A, on the other hand, re-epithelialization was not yet initiated even at day 14 and was not completed at day 20. The results are given in Table 2, below and Fig. 18.

25 **TABLE 2: Re-ephithelialization of Wound**

| Group | Day | | | | |
|---|---|---|---|---|---|
| | 3 | 7 | 10 | 14 | 20 |
| 1-A (Control) | 0 | 0 | 0 | 0 | 2+ |
| 1-B Fibronectin) | 0 | 1+ | 1+ | 2+ | 3+ |
| 1-C (His-β-b) | 0 | 0 | 1+ | 2+ | 3+ |
| 1-D (βigD-IV) | 0 | 0 | 1+ | 2+ | 3+ |

Results for formation of collagenous fibers are given in Table 3, below. As seen in Table 3, collagenous fibers were not significantly formed in test groups 1-A and 1-D until day 7 with maintenance of grade +1, whereas test groups 1-B and 1-C were graded as 2+. However, all test groups were graded as 2+ at day 10 with relatively rich collagenous fibers. At day 14, it was observed that collagenous fibers were densely formed and well arranged with grade +3, as shown in Fig. 19. Naturally, denser collagenous fibers reflect more improved wound healing progress.

**TABLE 3: Formation Behavior of Collagenous Fibers at Wound**

| Group | Day | | | | |
|---|---|---|---|---|---|
| | 3 | 7 | 10 | 14 | 20 |
| 1-A (Control) | 1+ | 1+ | 2+ | 3+ | 3+ |
| 1-B (Fibronectin) | 1+ | 2+ | 2+ | 3+ | 3+ |
| 1-C (His-β-b) | 1+ | 2+ | 2+ | 3+ | 3+ |
| 1-D (βigD-IV) | 1+ | 1+ | 2+ | 3+ | 3+ |

Formation grade of collagenous fibers

   0 : negative,        1+ : insignificant,

   2+ : scatteringly formed,   3+ : very dense

**4-3: Wound healing effect of at least one 4<sup>th</sup> domain-containing recombinant proteins βigh3-D-IV, βig-h3 D-IV 2X, 3X and 4X**

5          Based on the finding that βigh3-D-IV containing only the 4<sup>th</sup> domain is efficient for wound healing, βigh3-D-IV 2X, 3X and 4X, which contained the 4<sup>th</sup> domain in duplicate, triplicate and quadruplicate, respectively, were prepared in order to assay for wound healing activity.

10          The recombinant proteins were assayed for HCE cell adhesion activity in the same manner as in Example 1-2. The results are given in Fig. 20. As seen, the recombinant proteins βig-h3 D-IV 2X, 3X and 4X were all found to effectively induce the adhesion of HCE cells.

15          In order to examine wound healing effects of the recombinant proteins, the following experiments were conducted.

           Adult Spraque-Dawley lineage rats with a body weight of 250-300 gm were raised with standard feedstuff at a
20    constant temperature and humidity.

           In test group 2, four circular dermal whole layer wounds were made on the back of each rat and coated with chitosan bases combined with materials of interest:

           2-A: wound coated with chitosan base only

25          2-B: wound coated with chitosan base in combination

with 500 µg/ml of fibronectin

2-E:wound coated with chitosan base in combination with 500 µg/ml of βig-h3 D-IV 3X protein

2-F: wound coated with chitosan base in combination with 500 µg/ml of βig-h3 D-IV 4X protein

The composites based on chitosan were prepared as follows. 1 g of water soluble chitosan (poly(1-4) 2-amino-2-deoxy-β-D-glucan) with a molecular weight of 600,000 Da was dissolved in 100 ml of sterile distilled water and the resulting 1% solution was dispensed in aliquot of 2 ml to to 12-well plate (Corning, USA), followed by the addition of 100 µg of gentamycin per well. Fibronectin, βigh3-D-IV 3X, and βigh3-D-IV 4X were individually added to a concentration of 500 µg/ml and frozen at -70 °C, followed by freeze-drying in a freeze drier (Ilshin) for 12 hours to give disc-shaped composites.

The backs of etherized rats were shaved, followed by sterilizing the shaved region with betadin solution. Penetrating the whole dermal layers, four circular wounds with a diameter of 7 mm were formed on the back of each rat. The wounds were covered with composites used for test groups 2-A, 2-B, 2-E and 2-F, respectively, and then with Tegaderm® (3M) and lightly bandaged. The composites were changed with fresh ones every three days.

Wound healing effects were evaluated by determining

appearances of the wounds as in Example 4-2.

A high wound healing effect was obtained from the composite containing the recombinant protein βig-h3 D-IV 3X or 4X.

All rats, except for all members in the test group 2-A, one in the test group 2-B and two in test groups 2-E and 2-F each, were completely recovered from the wound at day 12 to 15. All wound areas reduced in size just after the formation of wound. As for the test group 2-A, its wound area was observed to be reduced at a relatively slow rate throughout the period of healing time. In the other test groups, the wound areas were reduced greatly in the first three days, gradually to day 9, and then greatly again. Turning to comparison among wounds, there were more significant differences ($p<0.05$) for the whole period of 15 days in the test groups 2-B, 2-E and 2-F, than in the test group 2-A, as shown in Table 4 and Fig. 21.

**TABLE 4: Reduction of Wound Area (mm²)**

| Group | Day | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 3* | 6* | 9* | 12* | 15* |
| 2-A (Chitosan) | 49.3±4.0 | 34.5±0.6[a] | 26.1±0.5[a] | 13.8±0.5[a] | 10.8±0.3[a] | 3.4±0.2[a] |
| 2-B (Chitosan+Fibronectin) | 49.2±0.5 | 24.1±0.6[b] | 12.9±0.6[b] | 9.7±0.8[b] | 3.2±0.4[b] | 0.8±0.2[b] |
| 2-C (Chitosan+βig-h3 3X) | 49.2±1.5 | 25.3±0.7[b] | 16.6±0.6[b] | 11.2±0.5[b] | 5.0±0.8[b] | 1.2±0.2[b] |
| 2-D (Chitosan+βig-h3 4X) | 48.5±0.4 | 24.5±0.6[b] | 14.1±0.7[b] | 9.6±0.6[b] | 4.2±0.3[b] | 1.1±0.2[b] |

Value: Mean±S.D

$^+$: p<0.05 by ANOVA test

a, b : significant difference of data in statistics

5        Consequently, the recombinant proteins of the present invention, which contain the 2$^{nd}$ and 4$^{th}$ domains of βig-h3, alone or incombination, or in multiplicate are effective for cell adhesion and wound healing and ultimately can be utilized in developing cell culture and wound healing agents.

10

## INDUSTRIAL APPLICABILITY

In the present invention, there are provided recombinant proteins containing at least one of the 2$^{nd}$ and

15  4$^{th}$ domains of βig-h3 in which one aspartic acid and one isoleucine residue, known to be essential for association with integrin, are highly conserved. Also, the recombinant proteins themselves are useful for cell adhesion and wound healing, making a contribution to the development of. cell

20  culture methods and wound healing agents.

The present invention has been described in an illustrative manner, and it is to be understood that the terminology used is intended to be in the nature of description rather than of limitation.  Many modifications

25  and variations of the present invention are possible in

light of the above teachings. Therefore, it is to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.

INTERNATIONAL FORM

# RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1

TO : KIM, In-San
   Dongseotown 106-908, #1040, Maeho-dong, Soosung-ku, Daegu 706-140,
   Republic of Korea

| I. IDENTIFICATION OF THE MICROORGANISM | |
|---|---|
| Identification reference given by the DEPOSITOR:<br><br>*Escherichia coli*<br>BL21/His β -e | Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:<br><br>**KCTC 0904BP** |

| II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION |
|---|
| The microorganism identified under I above was accompanied by:<br>[ x ] a scientific description<br>[ - ] a proposed taxonomic designation<br>(Mark with a cross where applicable) |

| III. RECEIPT AND ACCEPTANCE |
|---|
| This International Depositary Authority accepts the microorganism identified under I above, which was received by it on **December 04 2000.** |

| IV. RECEIPT OF REQUEST FOR CONVERSION |
|---|
| The microorganism identified under I above was received by this International Depositary Authority on                    and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on |

| V. INTERNATIONAL DEPOSITARY AUTHORITY | |
|---|---|
| Name: Korean Collection for Type Cultures<br><br>Address: Korea Research Institute of<br>Bioscience and Biotechnology<br>(KRIBB)<br>#52, Oun-dong, Yusong-ku,<br>Taejon 305-333,<br>Republic of Korea | Signature(s) of person(s) having the power to represent the International Depositary Authority of authorized official(s):<br><br>BAE, Kyung Sook, Director<br>Date: **December 07 2000** |

INTERNATIONAL FORM

# RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1

TO : KIM, In-San
Dongseotown 106-908, #1040, Maeho-dong, Soosung-ku, Daegu 706-140,
Republic of Korea

| I. IDENTIFICATION OF THE MICROORGANISM | |
|---|---|
| Identification reference given by the DEPOSITOR:<br><br>*Escherichia coli*<br>BL21/His β -g | Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:<br><br>**KCTC 0905BP** |

| II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION |
|---|
| The microorganism identified under I above was accompanied by:<br>[ x ] a scientific description<br>[   ] a proposed taxonomic designation<br>(Mark with a cross where applicable) |

| III. RECEIPT AND ACCEPTANCE |
|---|
| This International Depositary Authority accepts the microorganism identified under I above, which was received by it on **December 04 2000.** |

| IV. RECEIPT OF REQUEST FOR CONVERSION |
|---|
| The microorganism identified under I above was received by this International Depositary Authority on _____ and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on |

| V. INTERNATIONAL DEPOSITARY AUTHORITY | |
|---|---|
| Name: Korean Collection for Type Cultures<br><br>Address: Korea Research Institute of Bioscience and Biotechnology (KRIBB)<br>#52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea | Signature(s) of person(s) having the power to represent the International Depositary Authority of authorized official(s):<br><br><br>BAE, Kyung Sook, Director<br>Date: **December 07 2000** |

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INTERNATIONAL FORM

# RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1

TO : KIM, In-San
Dongseotown 106-908, #1040, Maeho-dong, Soosung-ku, Daegu 706-140,
Republic of Korea

| I. IDENTIFICATION OF THE MICROORGANISM | |
|---|---|
| Identification reference given by the DEPOSITOR: *Escherichia coli* BL21/His β -e4X | Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCTC 0906BP |

| II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION |
|---|
| The microorganism identified under I above was accompanied by: [ x ] a scientific description [   ] a proposed taxonomic designation (Mark with a cross where applicable) |

| III. RECEIPT AND ACCEPTANCE |
|---|
| This International Depositary Authority accepts the microorganism identified under I above, which was received by it on **December 04 2000.** |

| IV. RECEIPT OF REQUEST FOR CONVERSION |
|---|
| The microorganism identified under I above was received by this International Depositary Authority on                      and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on |

| V. INTERNATIONAL DEPOSITARY AUTHORITY | |
|---|---|
| Name: Korean Collection for Type Cultures  Address: Korea Research Institute of Bioscience and Biotechnology (KRIBB) #52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea | Signature(s) of person(s) having the power to represent the International Depositary Authority of authorized official(s): BAE, Kyung Sook, Director Date: **December 07 2000** |

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**What is Claimed is:**

1. A method of for attaching mammalian cell with a recombinant protein comprising a portion of domains of βig-
5    h3.

2. The method according to claim 1, wherein the recombinant protein comprises the $2^{nd}$ fas-1 domain of βig-h3, the $4^{th}$ fas-1 domain of βig-h3, or both of the $2^{nd}$ and $4^{th}$
10   domains of βig-h3.

3. The method according to claim 2, wherein the $2^{nd}$ or $4^{th}$ domain of βig-h3 exists as a single copy or in multiple copies.

15

4. The method according to claim 2, wherein the $2^{nd}$ fas-1 domain of βig-h3 comprises the amino acid sequence 237-377 of βig-h3 and is expressed from the expression vector pβig-h3 D-II.

20

5. The method according to claim 2, wherein the $4^{th}$ fas-1 domain of βig-h3 comprises the amino acid sequence 498-637 of βig-h3 and is expressed from the expression vector pβig-h3 D-IV.

25

6. The method according to claim 3, wherein the recombinant protein comprises the amino acid sequence 502-637 of βig-h3 in tetraplicate and is expressed from the expression vector pβig-h3 D-IV 4X.

5

7. An expression vector pβig-h3 D-II, comprising amino acids 237-377 of βig-h3 and capable of expressing the 2nd fas-1 domain of βig-h3.

10        8. A novel E. coli strain, transformed with the expression vector of claim 7, identified as E. coli BL21/Hisβ-g (accession No. KCTC 0905BP).

9. An expression vector pβig-h3 D-IV, comprising amino 15 acids 498-637 of βig-h3 and capable of expressing the 4th fas-1 domain of βig-h3.

10. A novel E. coli strain, transformed with the expression vector of claim 9, identified as E. coli 20 BL21/Hisβ-e (accession No. KCTC 0904BP).

11. An expression vector pβig-h3 D-IV 4X, expressing recombinant protein which comprises the amino acid sequence 502-637 of βig-h3 in tetraplicate and multiple copies of the 25 2nd or the 4th fas-1 domain of βig-h3.

12. A novel E. coli strain, transformed with the expression vector of claim 11, identified as E. coli BL21/Hisβ-e4x (accession No. KCTC 0906BP).

13. The method according to claim 1, comprising the steps of:

1) preparing a recombinant protein comprising one or more copies of the 2$^{nd}$ and/or 4$^{th}$ domain of βig-h3, by use of an expression vector;

2) coating the recombinant protein onto a solid support; and

3) applying cells to the protein-coated solid support.

14. A method for healing mammalian wound with a recombinant protein comprising a portion of domains of βig-h3.

15. The method according to claim 14, comprising the steps of:

1) coating a solid support with a recombinant protein containing one or more copies of the 2$^{nd}$ and/or the 4$^{th}$ domain of βig-h3;

2) attaching cells to the solid support; and

3) applying the solid support to wounds.

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FIG. 1

FIG. 2

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FIG. 3

A



B
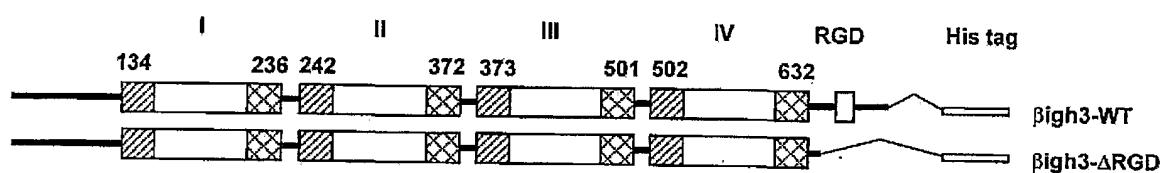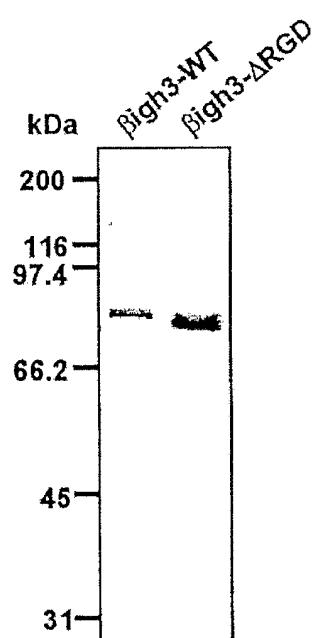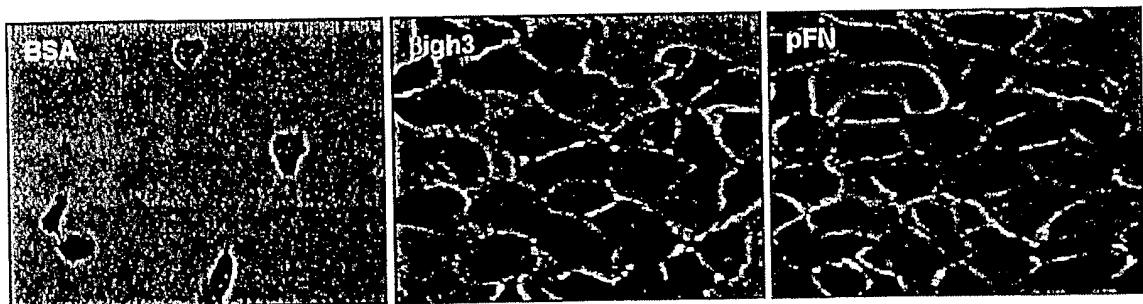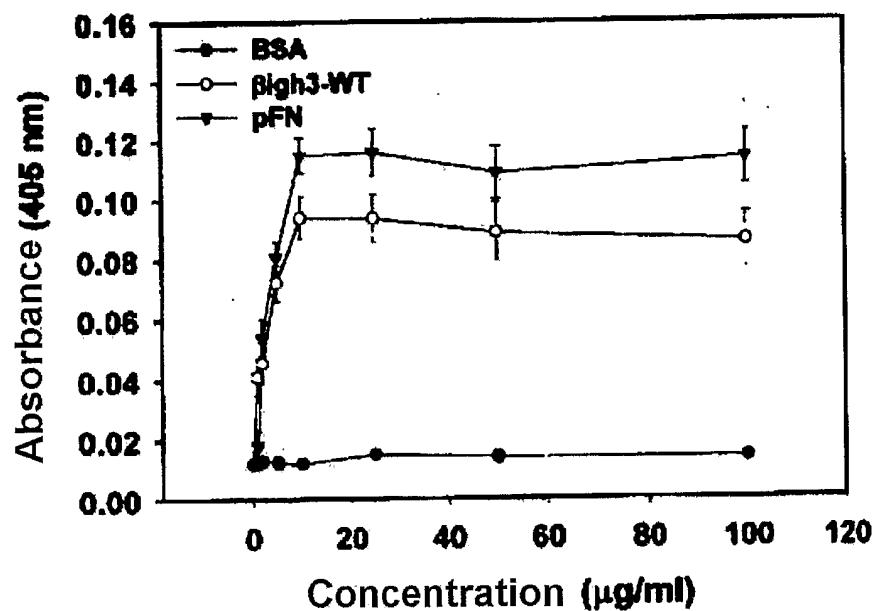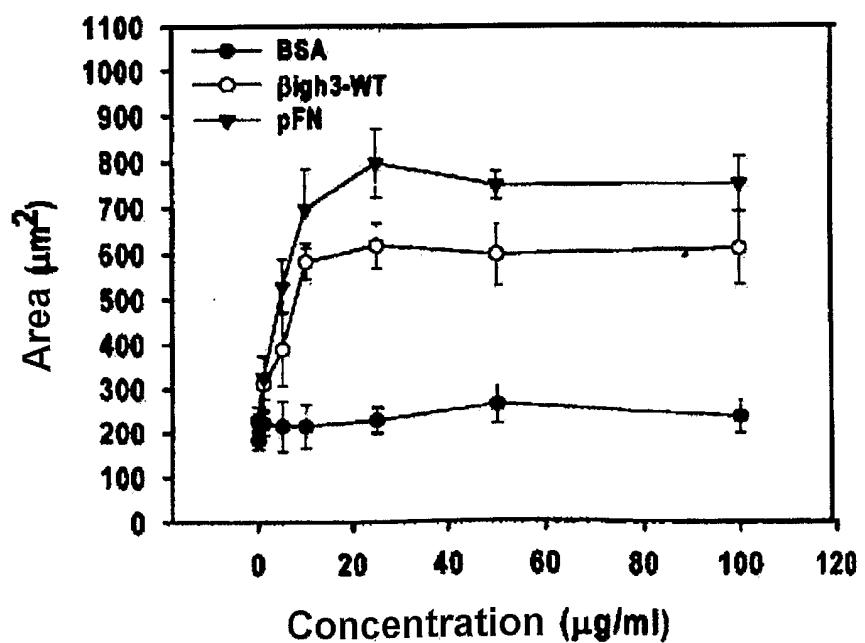
FIG. 5

A



B

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FIG. 6a



FIG. 6b

FIG. 6c



FIG. 6d

FIG. 6e

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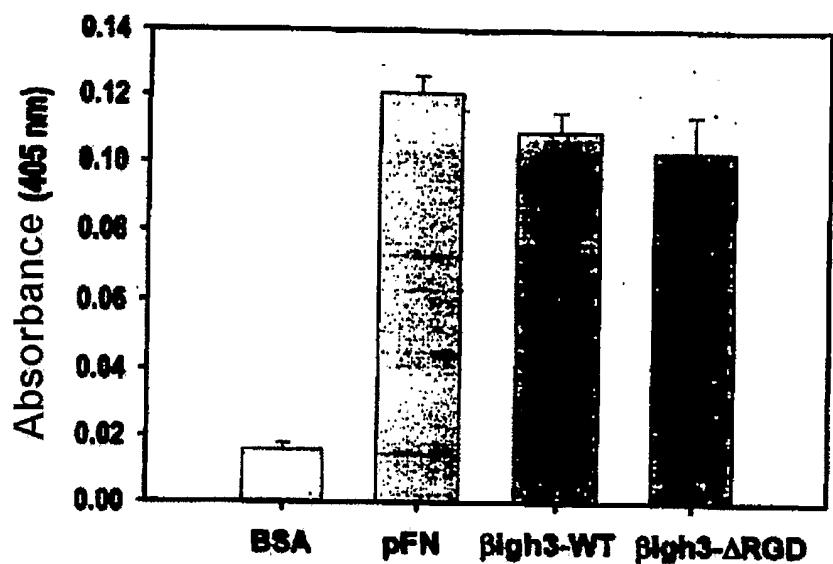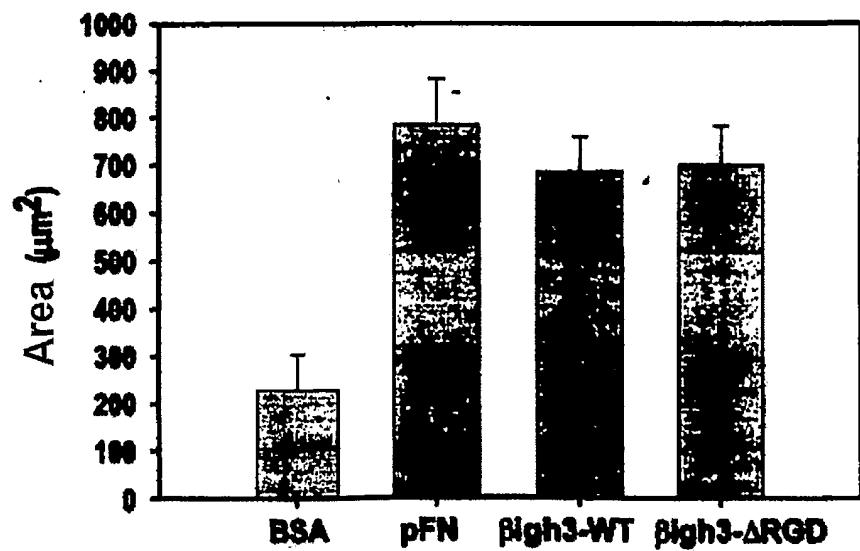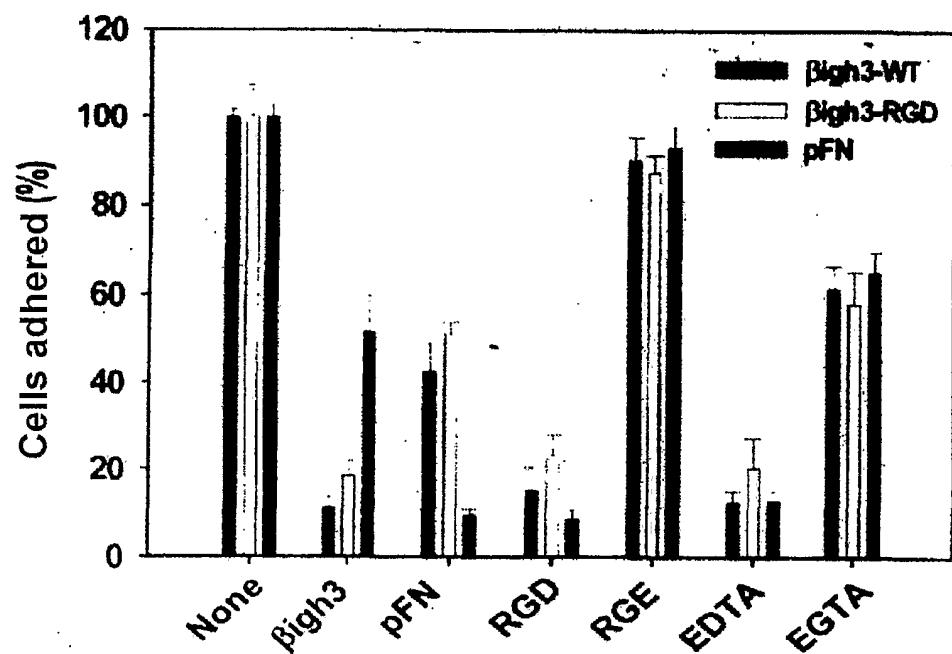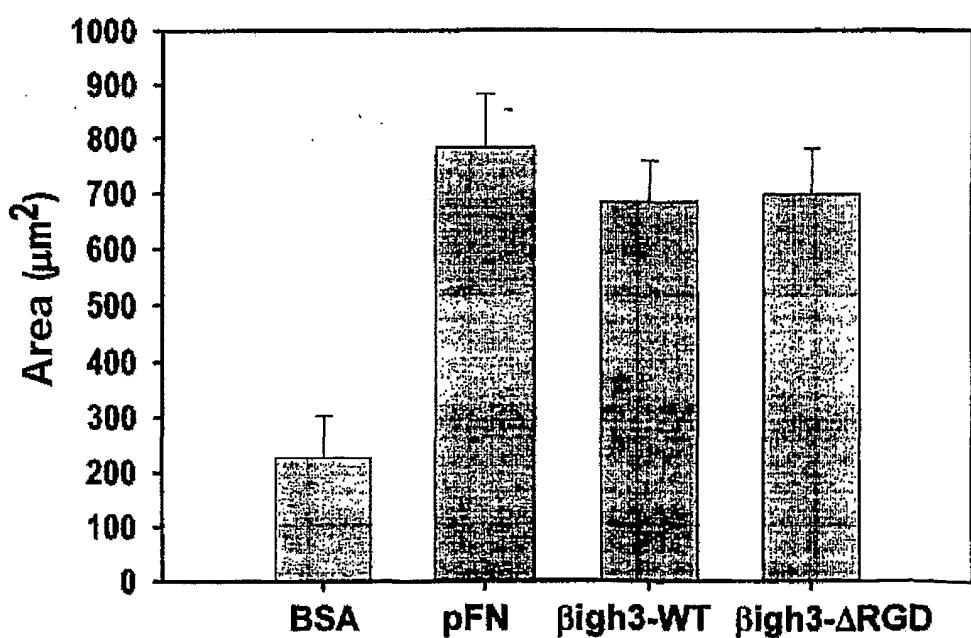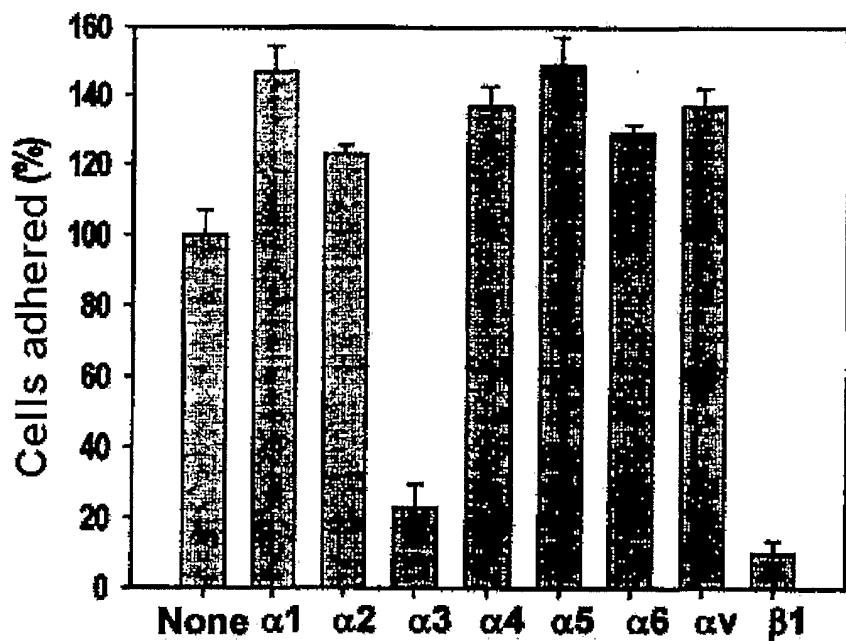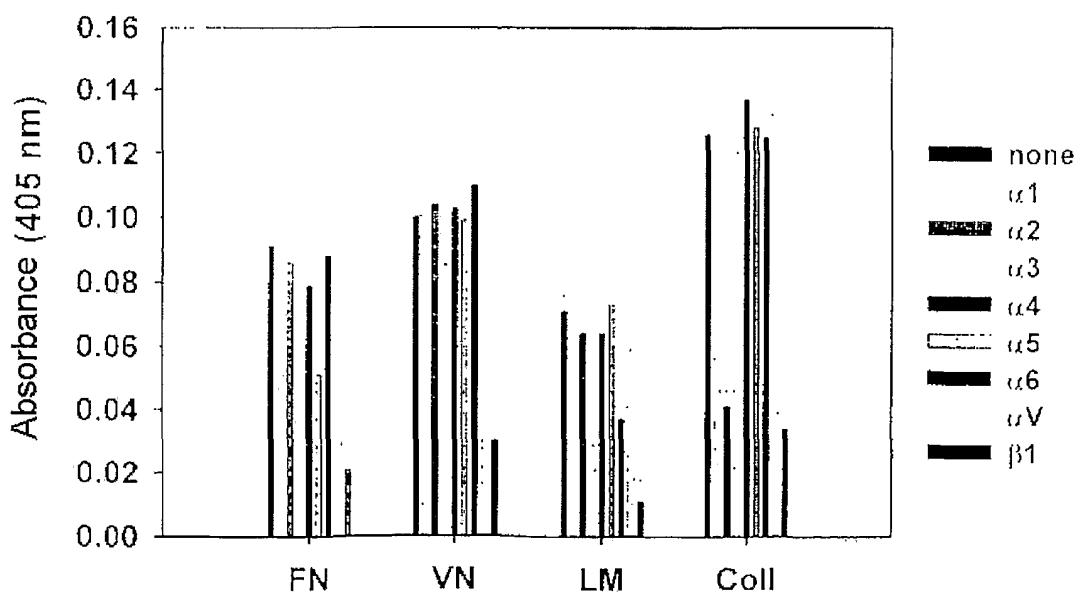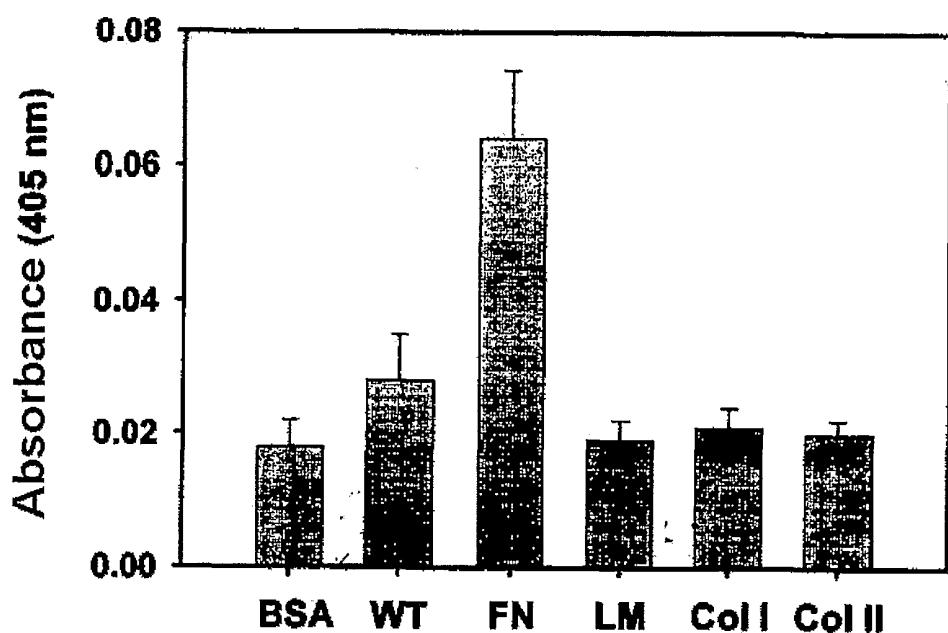
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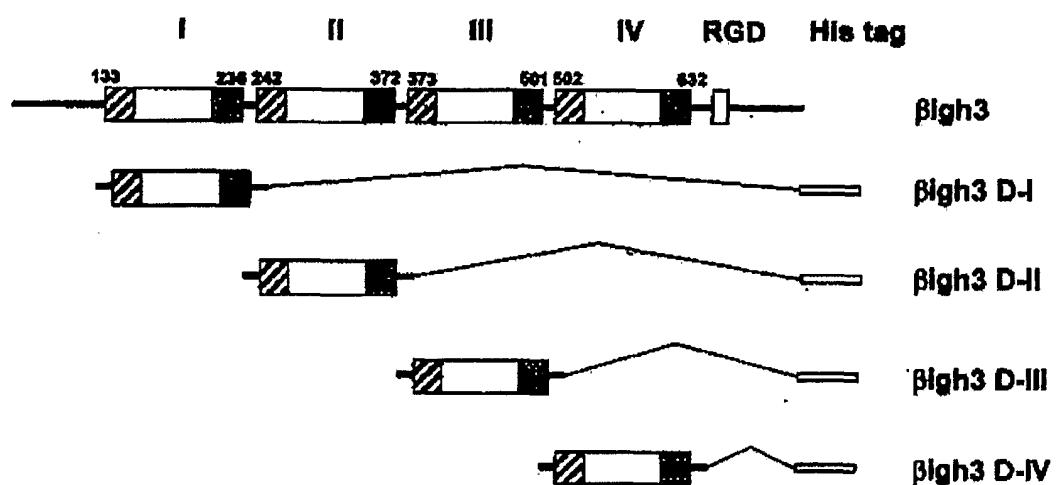
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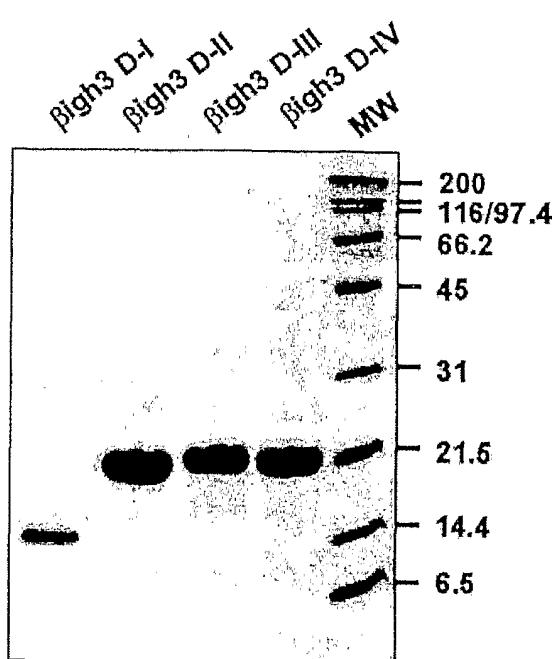
FIG. 9
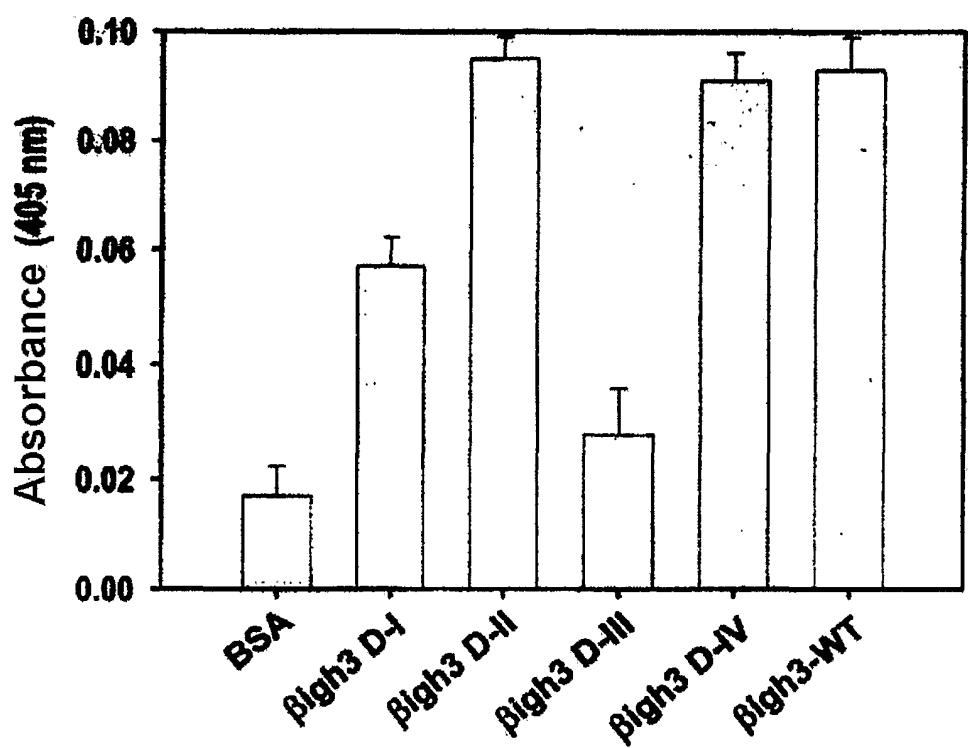
12/23

FIG. 10
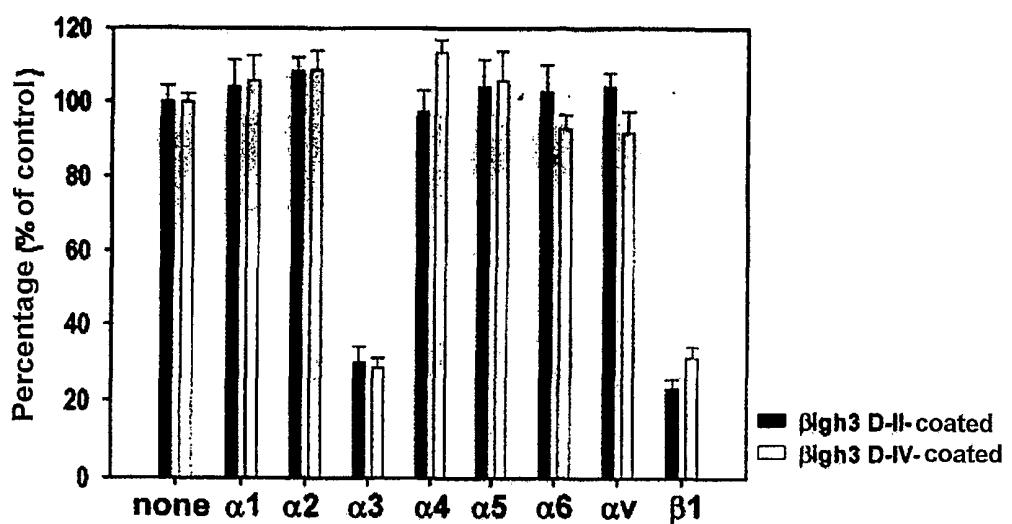
13/23

FIG. 11

H2

| | | | |
|---|---|---|---|
| BIGH3_HUMAN | 211 | TVNCARLLKADHHATNGVVHLIDKVI... | 236 |
| BIGH3_PIG | 211 | TVNCARLLKADHHATNGVVHLIDKVI... | 236 |
| BIGH3_CHICK | 201 | TVNCARLLKADHHATNGVVHVIDKVI... | 228 |
| BIGH3_HUMAN | 346 | INGKAIISNKDILATNGVIHYIDELLI.. | 372 |
| BIGH3_PIG | 346 | INGKPIISNKDVLATNGVIHFIDELLI.. | 372 |
| BIGH3_CHICK | 338 | LNGRAIIANKDILATNGVVHFVNELLI.. | 364 |
| OSF2_HUMAN | 340 | VNGIKMVNKKDIVTNNGVIHLIDQVLI.. | 366 |
| OSF2_MOUSE | 342 | INGIKMVNKKDIVTKNGVIHLIDEVLI.. | 368 |
| BIGH3_HUMAN | 608 | VNKEPVAE-PDIMATNGVVHVITNVL... | 632 |
| BIGH3_PIG | 608 | VNKEPVAE-ADIMATNGVVHTINTVL... | 632 |
| BIGH3_CHICK | 600 | VNKEPVAE-SDIMATNGVIHAVSSVL... | 624 |
| SLL1735 homolog | 106 | VKNATVLA-ADIEADNGIIHVIDNVILMG | 133 |
| SLL1735 | 106 | VKNATVII-PDIEADNGIIHVIDNVILMG | 133 |
| SLL1483 | 152 | VNKATVIS-ADVDASNGVIHVIDQVIL.. | 177 |
| OSF2_HUMAN | 604 | VNELKSKE-SDIMTTNGVIHVVDKLL... | 628 |
| OSF2_MOUSE | 606 | VNELKSKE-SDIMTTNGVIHVVDKLL... | 630 |
| Midline Fasciclin | 543 | INNLAKIIDADIMGTNGVLHVIDTIL... | 568 |
| HLC-32 | 341 | -SKASRVILRDIPTTNGVIQVIDRVIL.. | 366 |
| Midline Fasciclin | 825 | KIENAGVTKCDVVATNGILHEINDIIV.. | 851 |
| HLC-32 | 196 | TANGARVVEADRKASSGLIHVVDKVI... | 221 |

consensus        VNNAARVVKADIHATNGVIHVIDKVLIMG

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FIG. 12
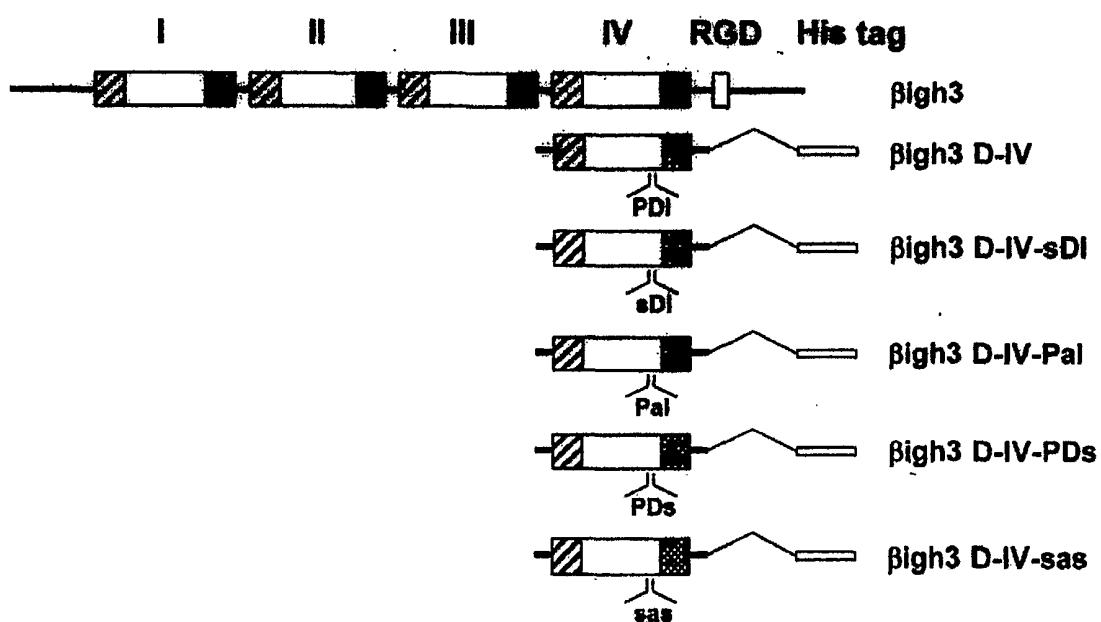
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FIG. 13
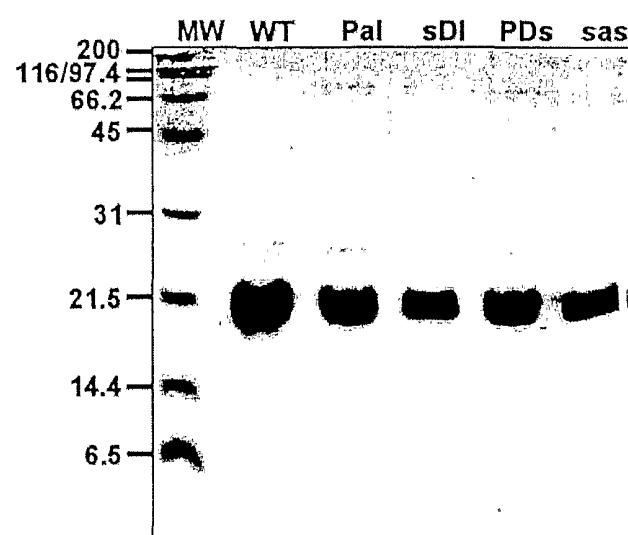
16/23

FIG. 14
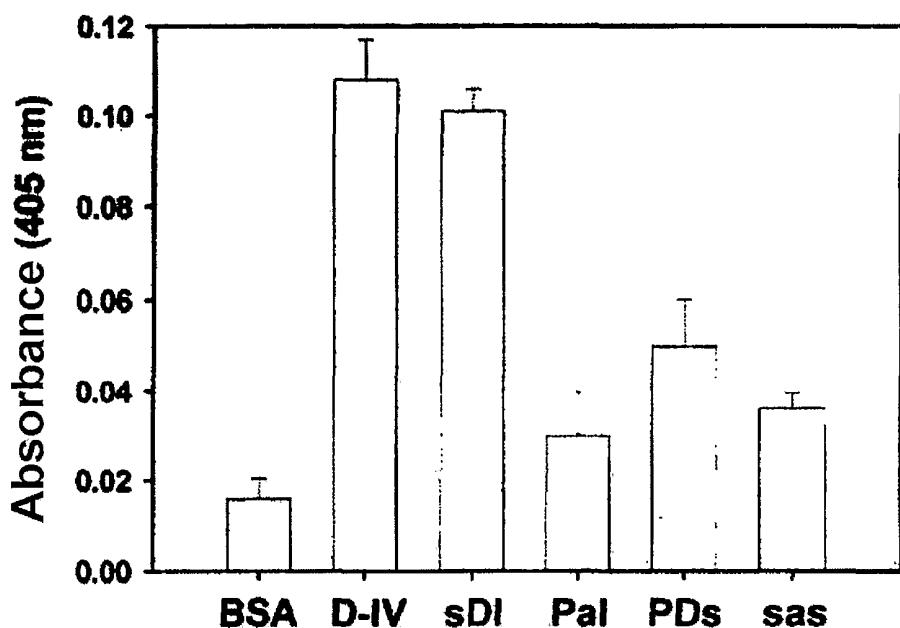
17/23

FIG. 15
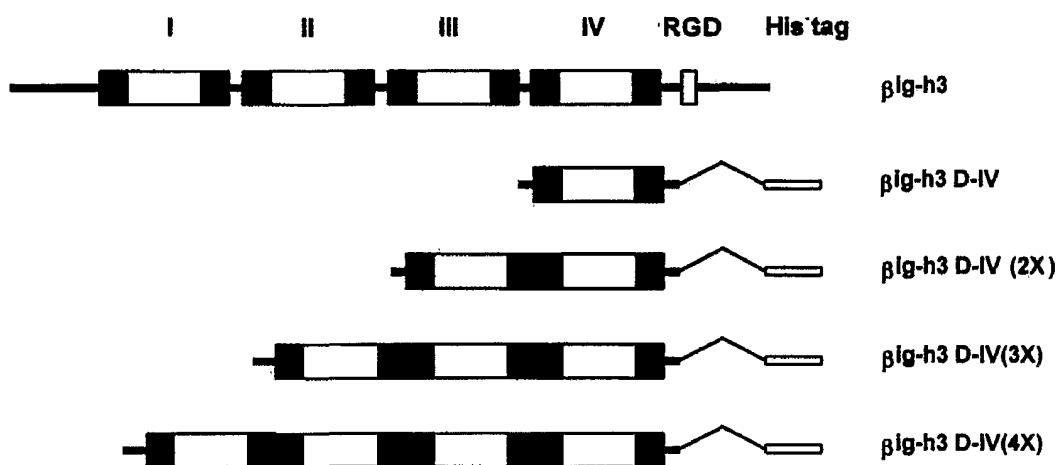
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FIG. 16
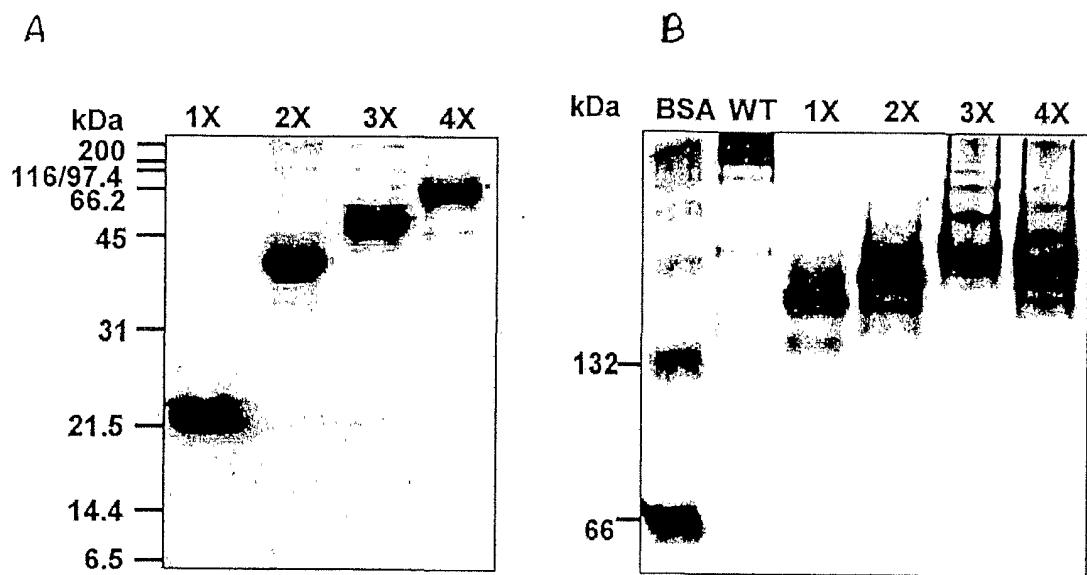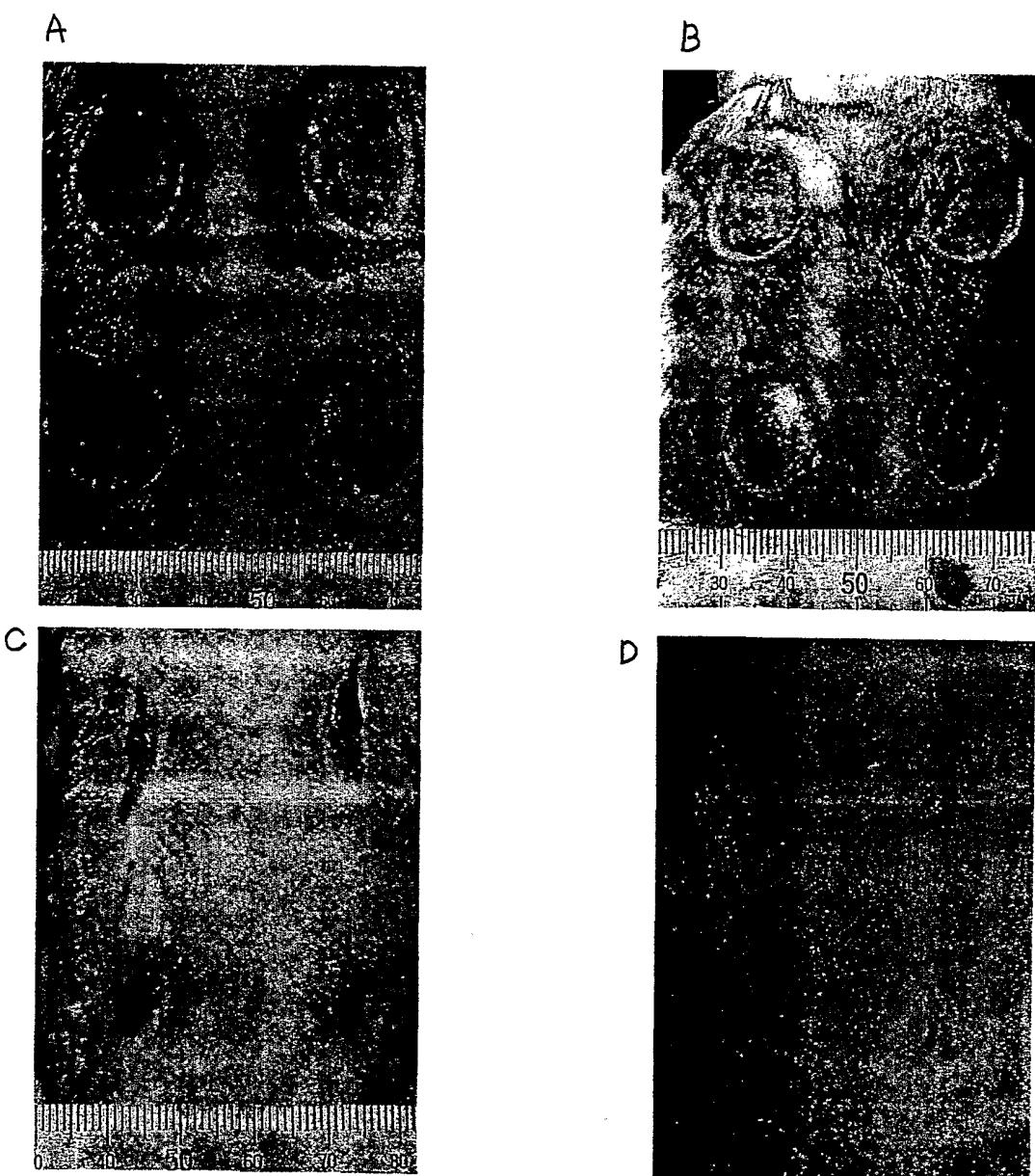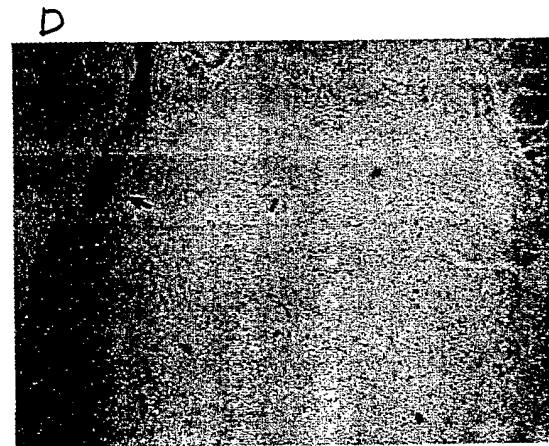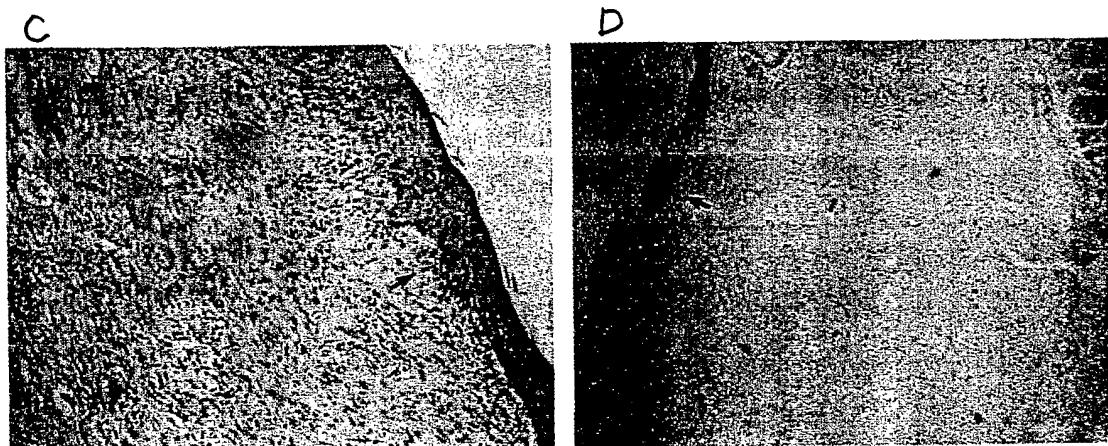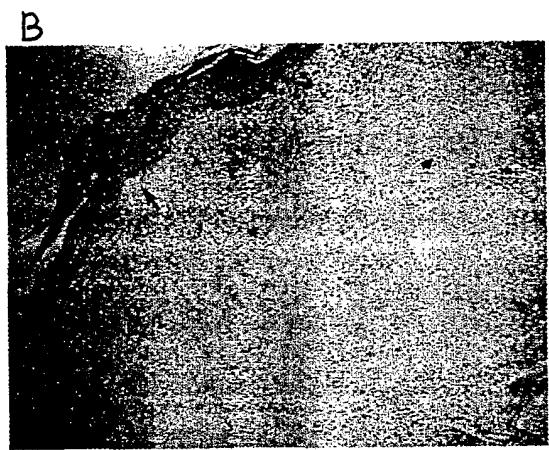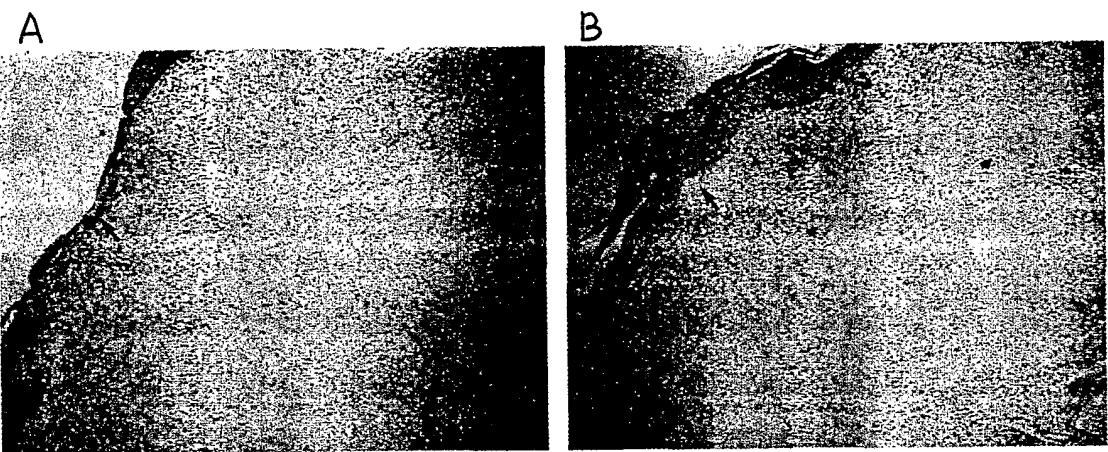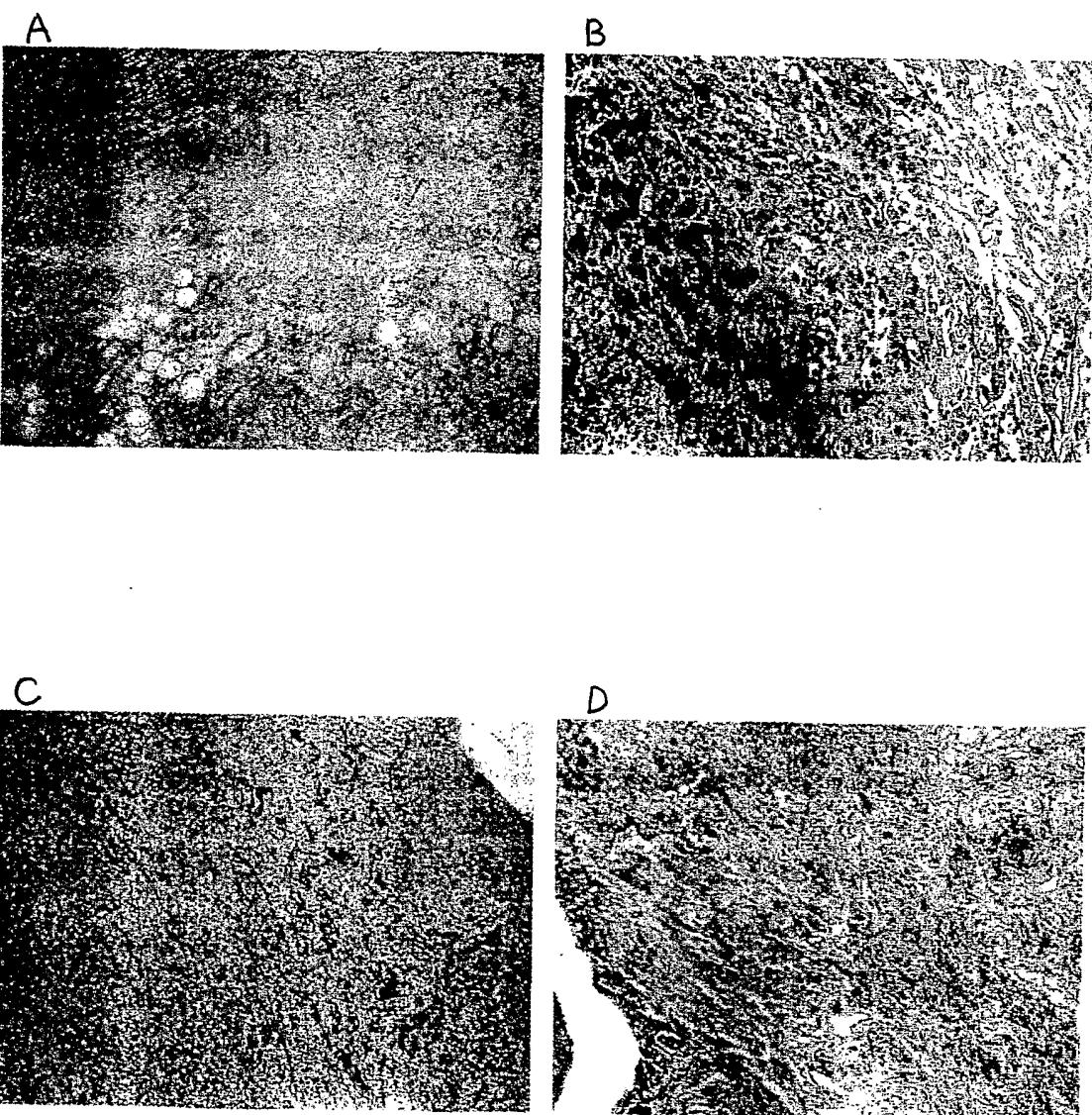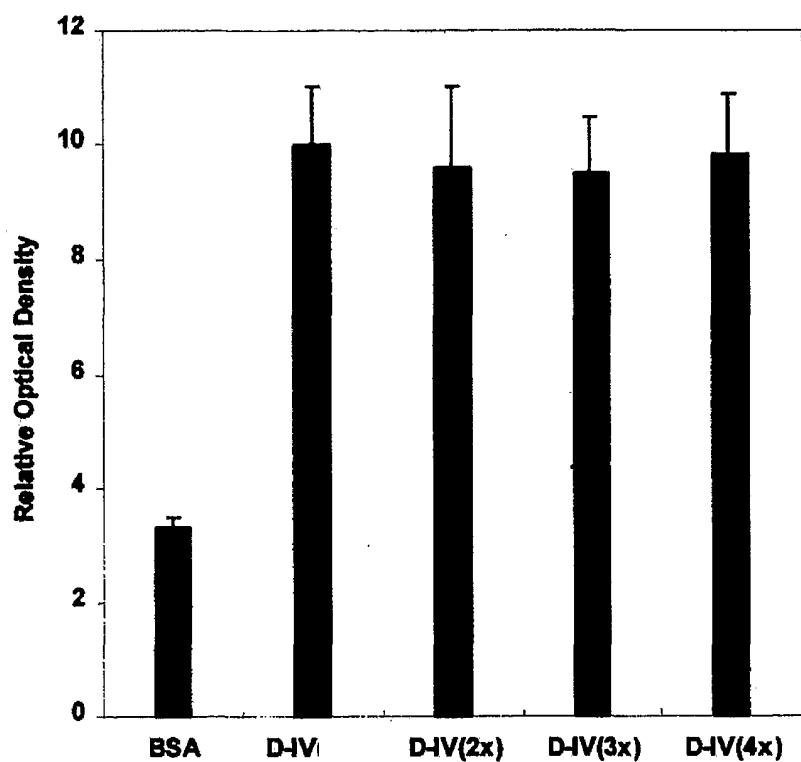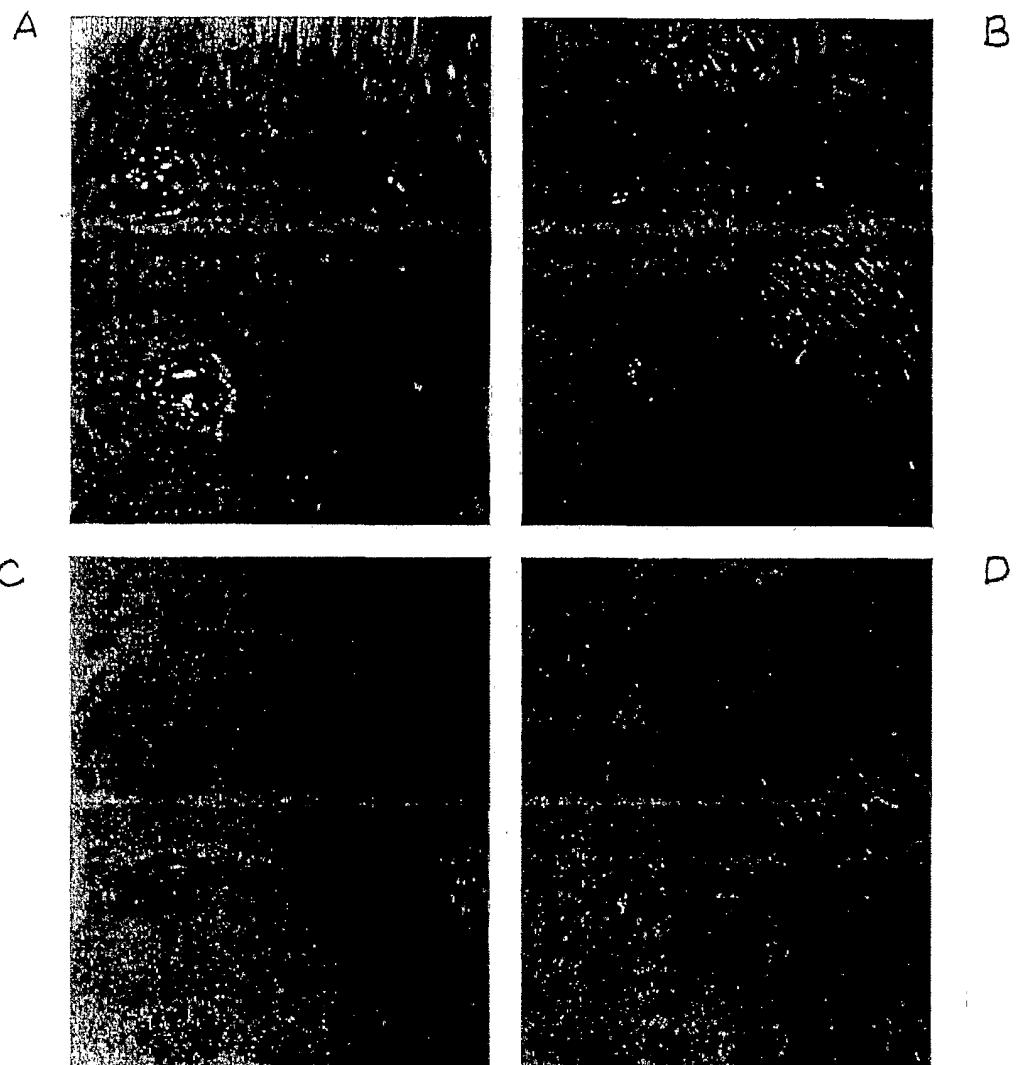
A



B

FIG. 17

A


B


C


D

FIG. 19

FIG. 20

FIG. 21

## A. CLASSIFICATION OF SUBJECT MATTER

### IPC7 A61K 38/17, C07K 14/435

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

## B. FIELDS SEARCHED

Minimun documentation searched (classification system followed by classification symbols)

IPC 7: A61K C07K C12N C07H

Documentation searched other than minimun documentation to the extent that such documents are included in the fileds searched

Korean Patents and applications for inventions since 1975

Electronic data base consulted during the intertnational search (name of data base and, where practicable, search trerms used)
CAS ON LINE, MEDLINE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X<br>A | WO 96/01102 A (Advanced Tissue Sciences) 18 January 1996<br>page 3-5, page 13-15, page 17 | 1, 7-12, 14<br>2-6, 13, 15 |
| X | US 5,444,164 (Bristol-Myers Squibb Company) 22 August 1995<br>Fig. 5 | 7-12 |
| X<br>A | LeBaron RG et al. 'Beta IG-H3, a novel secretory protein inducible by transforming growth factor-beta, is present in normal skin and promotes the adhesion and spreading of dermal fibroblasts in vitro' In J. Invest. Dermatol. 1995, Vol. 104(5), p844-849. | 1, 14<br>2-6, 13, 15 |
| X | Skonier J. et al. 'cDNA cloning and sequence analysis of beta ig-h3, a novel gene induced in a human adenocarcinoma cell line after treatment with transforming growth factor-beta' In DNA Cell Biol. 1992, Vol. 11(7), p511-522 | 7-12 |

☐ Further documents are listed in the continuation of Box C.     ☒ See patent family annex.

| * | Special categories of cited documents: |
|---|---|
| "A" | document defining the general state of the art which is not considered to be of particular relevence |
| "E" | earlier application or patent but published on or after the international filing date |
| "L" | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified) |
| "O" | document referring to an oral disclosure, use, exhibition or other means |
| "P" | document published prior to the international filing date but later than the priority date claimed |

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevence; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevence; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 30 MARCH 2001 (30.03.2001) | 30 MARCH 2001 (30.03.2001) |

| Name and mailing address of the ISA/KR | Authorized officer |
|---|---|
| Korean Industrial Property Office<br>Government Complex-Taejon, Dunsan-dong, So-ku, Taejon Metropolitan City 302-701, Republic of Korea | CHO, Myung Sun |
| Facsimile No. 82-42-472-7140 | Telephone No. 82-42-481-5605 |

# INTERNATIONAL SEARCH REPORT

Information on patent family members

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 9601102 A | 18.01.1996 | CA 2194057 A | 18.01.1996 |
| | | EP 768869 A | 23.04.1997 |
| | | US 5599788 A | 04.02.1997 |
| | | US 5714588 A | 03.02.1998 |
| US 5444164 A | 22.08.1995 | CA 2088804 A | 06.08.1993 |
| | | EP 555989 A | 18.08.1993 |
| | | EP 555989 B | 24.05.1995 |
| | | JP 7133296 A | 23.05.1995 |

Form PCT/ISA/210 (patent family annex) (July 1998)