synergy site for α5β1 binding to osteopontin.

Integrin αvβ6 is abundant in developing epithelial organs, but expression is limited in healthy adult epithelia (Breuss et al., 1995). In response to tissue injury or inflammation αvβ6 is commonly highly induced (Hakkinen et al., 2004; Miller et al., 2001; Sawada et al., 2004). Osteopontin is also expressed at low levels in healthy adult organs, but dramatically induced in the setting of injury (Iguchi et al., 2004; Isoda et al., 2002; Takahashi et al., 2004; Wang et al., 2000). There is some evidence that expression of osteopontin and αvβ6 might be coordinately regulated in response to injury. For example, treatment of mice with intratracheal bleomycin, a drug that causes acute lung injury and inflammation, dramatically induces pulmonary expression of both osteopontin (Kaminski et al., 2000) and αvβ6 (Munger et al., 1999). However, whereas osteopontin was among the most highly induced genes in wild type mice treated with bleomycin, osteopontin was not induced in mice homozygous for a null mutation in the β6 gene, suggesting that induction of osteopontin may, in some cases, be regulated by the αvβ6 integrin. At the very least, osteopontin and αvβ6 are often coordinately expressed in developing, injured or inflamed epithelial organs.

Osteopontin has been suggested to contribute to a wide array of biological and pathological responses (Denhardt et al., 2001; Diao et al., 2004; Gravallese, 2003; Khan and Kok, 2004; Kyriakides and Bornstein, 2003; O'Regan, 2003). From this paper and several others, it is now clear that several members of the integrin family can serve as osteopontin receptors, including αvβ1, αvβ3, αvβ5, αvβ6, α4β1, α5β1, α8β1 and α9β1. Thus, some of the diversity of osteopontin function might be regulated by the integrin repertoire of the responding cells. In addition, as we have shown, the two proteolytic cleavage sites within the region of osteopontin containing all of the apparent integrin recognition sequences is likely to allow for further regulation of osteopontin function through extracellular processing by proteases. Finally, osteopontin is a highly flexible molecule (Fisher et al., 2001; Helluin et al., 2000), which might be able to adopt different conformations depending on interactions with additional proteins in the extracellular space. It is thus conceivable that the distinct structural requirements we have identified for interaction with several members of the integrin family could provide an additional level for regulation of osteopontin function.

EXPERIMENTAL PROCEDURES

Cell Lines, Antibodies and Reagents

Stably transfected SW480 cells (human colon carcinoma) with either the expression plasmids pcDNAIneoβ6 (Weinacker et al., 1994), pcDNAIneoβ3 (Yokosaki et al., 1996), pcDNAIneoα9 (Yokosaki et al., 1994) or the empty vector pcDNAIneo, CHO cells that secrete truncated integrin αvβ6 (Weinacker et al., 1994) and human lung cancer cells UCLA P3 cells were from Dean Sheppard (UCSF, San Francisco, CA). Anti-αvβ5 mAb P1F6 (Weinacker et al., 1994), anti-αvβ6 E7P6 (Weinacker et al., 1994), 10D5 (Huang et al., 1998) anti-α5β1 P3D10 (Setty et al., 1998) and anti-α9β1 Y9A2 (Wang et al., 1996) were also gifts from Dean Sheppard. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Grand Island, NY) supplemented with 1 mg/ml of the neomycin analog, G418 (Invitrogen, Grand Island, NY). GRGDSP peptide was purchased from Invitrogen (Grand Island, NY), thrombin from Amersham Biosciences (Piscataway, NJ), MMP-3 from Sigma (St. Louis, MO). Antibody LM609 against αvβ3 was from Chemicon (Temecula, CA). Phycoerythrin-conjugated anti-α5, IIA1, was from BD Biosciences (San Jose, CA). cDNA encoding osteopontin (Saitoh et al., 1995) was from Yoshiki Saitoh (Kumamoto University, Kumamoto, Japan). [^{35}S]methionine and [^{35}S]cysteine cell labeling mixture was purchased from Amersham Biosciences (Piscataway, NJ).

Cell adhesion assays

Wells of non-tissue culture treated polystyrene 96-well flat-bottom microtiter plates (Nunc Inc., Naperville, IL) were coated by incubation with 100 µl of osteopontin in phosphate buffered saline at 37°C for 1 hour. For blocking experiments cells were incubated in the presence or absence of soluble peptide or monoclonal antibody on ice for 15 minutes before plating. Wells were washed with phosphate buffered saline, then blocked with 1% bovine serum albumin in DMEM. 50,000 cells were added to each well in 200 µl of serum-free DMEM containing 0.5% bovine serum albumin. Plates were centrifuged at 10 x *g* for 1 minute, then incubated for 1 hour at 37°C in a humidified atmosphere with 5% CO_2. Non-adherent cells were removed by centrifugation topside down at 48 x *g* for 5 minutes. The attached cells were fixed with 1% formaldehyde, stained with 0.5% crystal violet, and excess dye was washed off with phosphate

buffered saline. The cells were solubilized in 50 ml of 2% Triton-X-100 and quantified by measuring the absorbance at 595 nm in a Microplate Reader (TECAN, Maennedorf, Switzerland). In each experiment wells were coated with BSA only of which absorbance values were subtracted from those of test wells. The subtracted values were not over 0.08 throughout the experiment.

Expression of recombinant osteopontin fragments

Recombinant osteopontin fragments were produced as previously described (Yokosaki et al., 1998). Briefly, cDNA encoding thrombin-cleaved N-terminal osteopontin fragment (nOPN) was amplified from the full length cDNA in pCRII by Polymerase Chain Reaction with restriction site-tagged primers that amplify the same region as previously described (Smith et al., 1996), and then cloned between the *Bam*H1 and *Eco*RI sites within the multiple cloning site of pGEX6P2 plasmid (Amersham Bioscience, Piscataway, NJ). cDNA for full length osteopontin (fOPN) was amplified with the same forward primer as above and reverse encompassing the coding region, and cloned between the *Bam*H1 and *Xho*1 sites of the pGEX6P2. One isoform, OPNa, that contains each exon (Yokosaki et al., 1999) was used throughout this study. Wild type or variant recombinant osteopontin proteins were prepared by bacterial expression as recommended by the manufacturer. Briefly, competent DH5α cells were transformed by heat shock and grown on ampicillin-containing plates. Individual colonies were picked and propagated overnight in 2 ml of 2 x YT medium with 100 μg/ml of ampicillin at 37°C. 100 ml of 2 x YT medium was inoculated with 1 ml of the bacteria, and incubated at 30°C until OD600 reached 0.5-2, at which time isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM. Cultures were grown for several more hours, cells were collected and sonicated, and glutathione S-transferase fusion proteins were affinity purified with glutathione Sepharose 4B beads and then cleaved off from glutathione S-transferase with PreScission protease (Amersham Bioscience, Piscataway, NJ) into Tris-buffer or phosphate buffered saline (PBS). Concentrations of recombinant proteins were determined by the Bradford assay (Pierce, Rockford, IL) using Bovine serum albumin (BSA) as a standard. Purity of the product was confirmed by 12.5% SDS-Polyacrylamide gel electrophoresis followed by Comassie Blue staining.

Mutagenesis

Site directed mutagenesis was performed with the QuickChange Site-Directed Mutagenesis Kit (Stratagene, San Diego, CA) as previously described (Yokosaki et al., 1998). Both strands of the expression plasmid were replicated by PCR using pfuDNA polymerase with two complementary primers designed to introduce the desired mutation. The amplification product was treated with *Dpn*I endonuclease, specific for methylated DNA, to digest the parental DNA template. Then DH5α competent cells were transformed with the PCR-generated nicked plasmid. Plasmids from several isolated colonies were prepared by QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany), and inserts were sequenced by ABI3100 sequencer (Applied Biosystems, Foster City, CA) with primers flanking the polylinker of the pGEX vector. The verified mutated inserts were subcloned into pGEX vector that had not been amplified by PCR.

Affinity Chromatography

BSA or recombinant nOPN was coupled to Sepharose beads (Amersham Bioscience, Piscataway, NJ) for 4 hours at 4 °C. Secreted αvβ6 was metabolic-labeled and produced by CHO cells transfected with truncated αv and β6 cDNAs as described (Weinacker et al., 1994). After affinity matrices were blocked with 0.5 M monoethanolamine and 0.5 M NaCl buffer with PH 8.3, and washed with 0.1 M sodium acetate and 0.5 M NaCl, followed by PBS, culture medium of the αvβ6-secreting CHO cells was passed thorough the affinity columns. The bound protein was washed with column buffer then eluted with 20 mM ethylenediamine tetraacetic acid (EDTA) in PBS. The eluted fraction was run on the 7.5 % polyacrylamide gel followed by a fluorography.

Flow cytometry

Expression of integrins αvβ3, αvβ5, αvβ6, α5β1 or α9β1 was analyzed by flow cytometry. Cells were incubated with goat serum and washed with PBS. Then cells were incubated with either antibody LM609, P1F6, E7P6 or Y9A2 for 20 minutes at 4 °C for staining αvβ3, αvβ5, αvβ6, or α9β1, respectively, followed by incubation with secondary phycoerythrin-conjugated goat anti-mouse IgG. α5β1 was stained with phycoerythrin-conjugated IIA1 for 20 minutes at 4 °C. After cells were washed with

PBS, the expressions were then quantified on 10^5 cells with a FACSCalibur (BD Biosciences, San Jose, CA).

Enzyme cleavage of osteopontin

fOPN was subjected to enzyme reaction with thrombin or MMP-3 at 37°C for 15 to 120 minutes in cleavage buffer (200 mM of NaCl, 50 mM of Tris-HCl, pH7.6, 5mM $CaCl_2$) as previously described (Agnihotri et al., 2001). The products were separated by SDS-PAGE and stained with Comassie Blue.

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REFERENCES

Agnihotri, R., Crawford, H. C., Haro, H., Matrisian, L. M., Havrda, M. C., and Liaw, L. (2001). Osteopontin, a novel substrate for matrix metalloproteinase-3 (stromelysin-1) and matrix metalloproteinase-7 (matrilysin). J Biol Chem *276*, 28261-28267.

Aota, S., Nomizu, M., and Yamada, K. M. (1994). The short amino acid sequence Pro-His-Ser-Arg-Asn in human fibronectin enhances cell-adhesive function. J Biol Chem *269*, 24756-24761.

Ashkar, S., Weber, G. F., Panoutsakopoulou, V., Sanchirico, M. E., Jansson, M., Zawaideh, S., Rittling, S. R., Denhardt, D. T., Glimcher, M. J., and Cantor, H. (2000). Eta-1 (osteopontin): an early component of type-1 (cell-mediated) immunity. Science *287*, 860-864.

Barry, S. T., Ludbrook, S. B., Murrison, E., and Horgan, C. M. (2000). A regulated interaction between $\alpha 5\beta 1$ integrin and osteopontin. Biochem Biophys Res Commun *27*, 764-769.

Boeshore, K. L., Schreiber, R. C., Vaccariello, S. A., Sachs, H. H., Salazar, R., Lee, J., Ratan, R. R., Leahy, P., and Zigmond, R. E. (2004). Novel changes in gene expression following axotomy of a sympathetic ganglion: a microarray analysis. J Neurobiol *59*, 216-235.

Breuss, J. M., Gallo, J., DeLisser, H. M., Klimanskaya, I. V., Folkesson, H. G., Pittet, J. F., Nishimura, S. L., Aldape, K., Landers, D. V., Carpenter, W., *et al.* (1995). Expression of the $\beta 6$ integrin subunit in development, neoplasia and tissue repair suggests a role in epithelial remodeling. J Cell Sci *108*, 2241-2251.

Danen, E. H., and Sonnenberg, A. (2003). Integrins in regulation of tissue development and function. J Pathol *200*, 471-480.

Denda, S., Reichardt, L. F., and Muller, U. (1998). Identification of osteopontin as a novel ligand for the integrin $\alpha 8\beta 1$ and poteintial roles for this integrin-ligand interactrion in kidney morphogenesis. Mol Biol Cell *9*, 1425-1435.

Denhardt, D. T., and Chambers, A. F. (1994). Overcoming obstacles to metastasis--defenses against host defenses: osteopontin (OPN) as a shield against attack by cytotoxic host cells. J Cell Biochem *56*, 48-51.

Denhardt, D. T., Noda, M., O'Regan, A. W., Pavlin, D., and Berman, J. S. (2001). Osteopontin as a means to cope with environmental insults: regulation of inflammation, tissue remodeling, and cell survival. J Clin Invest *107*, 1055-1061.

Diao, H., Kon, S., Iwabuchi, K., Kimura, C., Morimoto, J., Ito, D., Segawa, T., Maeda, M.,

Hamuro, J., Nakayama, T., *et al.* (2004). Osteopontin as a mediator of NKT cell function in T cell-mediated liver diseases. Immunity *21*, 539-550.

Fisher, L. W., Torchia, D. A., Fohr, B., Young, M. F., and Fedarko, N. S. (2001). Flexible structures of SIBLING proteins, bone sialoprotein, and osteopontin. Biochem Biophys Res Commun *280*, 460-465.

Gravallese, E. M. (2003). Osteopontin: a bridge between bone and the immune system. J Clin Invest *112*, 147-149.

Hakkinen, L., Koivisto, L., Gardner, H., Saarialho-Kere, U., Carroll, J. M., Lakso, M., Rauvala, H., Laato, M., Heino, J., and Larjava, H. (2004). Increased expression of β6-integrin in skin leads to spontaneous development of chronic wounds. Am J Pathol *164*, 229-242.

Helluin, O., Chan, C., Vilaire, G., Mousa, S., DeGrado, W. F., and Bennett, J. S. (2000). The activation state of αvβ3 regulates platelet and lymphocyte adhesion to intact and thrombin-cleaved osteopontin. J Biol Chem, 18337-18343.

Hu, D. D., Lin, E. C., Kovach, N. L., Hoyer, R., and Smith, J. W. (1995). A biochemical characterization of the binding of osteopontin to integrins αvβ1 and αvβ5. J Biol Chem *270*, 26232-26238.

Huang, X., Wu, J., Spong, S., and Sheppard, D. (1998). The integrin αvβ6 is critical for keratinocyte migration on both its known ligand, fibronectin, and on vitronectin. J Cell Sci *111*, 2189-2195.

Hynes, R. O. (2002). Integrins:bidirectional allosteric signaling machines. Cell *110*, 673-687.

Iguchi, S., Nishi, S., Ikegame, M., Hoshi, K., Yoshizawa, T., Kawashima, H., Arakawa, M., Ozawa, H., and Gejyo, F. (2004). Expression of osteopontin in cisplatin-induced tubular injury. Nephron Exp Nephrol *97*, 96-105.

Isoda, K., Nishikawa, K., Kamezawa, Y., Yoshida, M., Kusuhara, M., Moroi, M., Tada, N., and Ohsuzu, F. (2002). Osteopontin plays an important role in the development of medial thickening and neointimal formation. Circ Res *106*, 2720-2726.

Kaminski, N., Allard, J. D., Pittet, J. F., Zuo, F., Griffiths, M. J., Morris, D., Huang, X., Sheppard, D., and Heller, R. A. (2000). Global analysis of gene expression in pulmonary fibrosis reveals distinct programs regulating lung inflammation and fibrosis. Proc Natl Acad Sci U S A *97*, 1778-1783.

Kang, Y., Siegel, P. M., Shu, W., Drobnjak, M., Kakonen, S. M., Cordon-Cardo, C., Guise, T. A., and Massague, J. (2003). A multigenic program mediating breast cancer metastasis to bone. Cancer cell *3*, 537-549.

Khan, S. R., and Kok, D. J. (2004). Modulators of urinary stone formation. Front Biosci *9*, 1450-1482.

Kyriakides, T. R., and Bornstein, P. (2003). Matricellular proteins as modulators of wound healing and the foreign body response. Thromb Haemost *90*, 986-992.

Liaw, L., Skinner, M. P., Raines, E. W., Ross, R., Cheresh, D. A., Schwartz, S. M., and Giachelli, C. M. (1995). The adhesive and migratory effects of osteopontin are mediated via distinct cell surface integrins. Role of αvβ3 in smooth muscle cell migration to osteopontin in vitro. J Clin Invest *95*, 713-724.

Miller, L. A., Barnett. , N. L., Sheppard, D., and Hyde, D. M. (2001). Expression of the β6 integrin subunit is associated with sites of neutrophil influx in lung epithelium. J Histochem Cytochem *49*, 41-48.

Miyauchi, A., Alvarez, J., Greenfield, E.M., Teti, A., Grano, M., Colucci, S., Zanbinin-Zallone, A., Ross, F.P., Teitelbaum, S.L. and Cheresh, D. (1991). Recognition of osteopontin and related peptides by αvβ3 integrin stimulates immediate cell signals in osteoclasts. J Biol Chem *266*, 20369-20347.

Mould, A. P., Askari, J. A., and Humphries, M. J. (2000). Molecular Basis of Ligand Recognition by Integrin a5b1 I. Spscificity of ligand binding is deteermined by aminoacid sequences in the secaond and third NH_2-terminal repeats of the subunit. J Biol Chem *275*, 20324-20336.

Munger, J. S., Huang, X., Kawakatsu, H., Griffiths, M. J., Dalton, S. L., Wu, J., Pittet, J. F., Kaminski, N., Garat, C., Matthay, M. A., *et al.* (1999). The integrin αvβ6 binds and activates latent TGF beta 1: a mechanism for regulating pulmonary inflammation and fibrosis. Cell *96*, 319-328.

Nasu, K., Ishida, T., Setoguchi, M., Higuchi, Y., Akizuki, S., and Yamamoto, S. (1995). Expression of wild-type and mutated rabbit osteopontin in Escherichia coli, and their effects on adhesion and migration of P388D1 cells. Biochem J *307*, 257-265.

O'Regan, A. (2003). The role of osteopontin in lung disease. Cytokine Growth Factor Rev *14*, 479-488.

Obara, M., Kang, M. S., and Yamada, K. M. (1988). Site-directed mutagenesis of the cell-binding domain of human fibronectin: separable, synergistic sites mediate adhesive function. Cell *53*, 649-657.

Pierschbacher, M. D., and Ruoslahti, E. (1984). Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. Nature *309*, 30-33.

Redick, S. D., Settles, D. L., Briscoe, G., and Erickson, H. P. (2000). Defining fibronectin's cell

adhesion synergy site by site-directed mutagenesis. J Cell Biol *149*, 521-527.

Ruoslahti, E. (1996). RGD and other recognition sequences for integrins. Ann Rev Cell Dev Biol *12*, 697-715.

Saitoh, Y., Kuratsu, J., Takeshima, H., Yamamoto, S., and Ushio, Y. (1995). Expression of osteopontin in human glioma. Its correlation with the malignancy. Lab Invest *72*, 55-63.

Sawada, T., Abe, M., Kai, K., Kubota, K., Fuchinoue, S., and Teraoka, S. (2004). β6 integrin is up-regulated in chronic renal allograft dysfunction. Clin Transplant *18*, 525-528.

Senger, D. R., Perruzzi, C.A., Papadopoulous, S.A. and Van de Water, L. (1994). Adhesive properties of osteopontion: Regulation by a naturally occurring thrombin-cleavage in close proximity to the GRDGS cell-binding domain. Mol Biol Cell *5*, 565-574.

Setty, S., Kim, Y., Fields, G. B., Clegg, D. O., Wayner, E. A., and Tsilibary, E. C. (1998). Interactions of Type IV collagen and Its domains with human mesangial cells. J Biol Chem *273*, 12244-12249.

Sheppard, D. (2000). In vivo functions of integrins: lessons from null mutations in mice. Matrix Biology *19*, 203-209.

Smith, L. L., Cheung, H. K., Ling, L. E., Chen, J., Sheppard, D., Pytela, R., and Giachelli, C. M. (1996). Osteopontin N-terminal domain contains a cryptic adhesive sequence recognized by α9β1 integrin. J Biol Chem *271*, 28485-28491.

Takahashi, F., Takahashi, K. , Shimizu, K., Cui, R., Tada, N., Takahashi, H., Soma, S., Yoshioka, M., and Fukuchi, Y. (2004). Osteopontin is strongly expressed by alveolar macrophages in the lungs of acute respiratory distress syndrome. Lung *182*, 278-287.

Wang, A., Yokosaki, Y., Ferrando, R., Balmes, J., and Sheppard, D. (1996). Differential regulation of airway epithelial integrins by growth factors. Am J Respir Cell Mol Biol *15*, 664-672.

Wang, Y., Mochida, S., Kawashima, R., Inao, M., Matsui, A., YouLuTuZ, Y., Nagoshi, S., Uede, T., and Fujiwara, K. (2000). Increased expression of osteopontin in activated Kupffer cells and hepatic macrophages during macrophage migration in Propionibacterium acnes-treated rat liver. J Gastroenterol *35*, 696-701.

Weber, G. F., and Cantor, H. (1996). The immunology of Eta-1/osteopontin. Cytokine Growth Factor Rev *7*, 241-248.

Weinacker, A., Chen, A., Agrez, M., Cone, R. I., Nishimura, S., Wayner, E., Pytela, R., and Sheppard, D. (1994). Role of the integrin αvβ6 in cell attachment to fibronectin. Heterologous expression of intact and secreted forms of the receptor. J Biol Chem *269*,

6940-6948.

Ye, Q. H., Qin, L. X., Forgues, M., He, P., Kim, J. W., Peng, A. C., Simon, R., Li, Y., Robles, A. I., Chen, Y., *et al.* (2003). Predicting hepatitis B virus-positive metastatic hepatocellular carcinomas using gene expression profiling and supervised machine learning. Nat med *9*, 416-423.

Yokasaki, Y., and Sheppard, D. (2000). Mapping of the cryptic integrin-binding site in osteopontin suggests a new mechanism by which thrombin can regulate inflammation and tissue repair. Trends Cardiovasc Med *10*, 155-159.

Yokosaki, Y., Matsuura, N., Higashiyama, S., Murakami, I., Obara, M., Yamakido, M., Shigeto, N., Chen, J., and Sheppard, D. (1998). Identification of the ligand binding site for the integrin α9β1 in the third fibronectin type III repeat of tenascin-C. J Biol Chem *273*, 11423-11428.

Yokosaki, Y., Matsuura, N., Sasaki, T., Murakami, I., Schneider, H., Higashiyama, S., Saitoh, Y., Yamakido, M., Taooka, Y., and Sheppard, D. (1999). The integrin α9β1 binds to a novel recognition sequence (SVVYGLR) in the thrombin-cleaved amino-terminal fragment of osteopontin. J Biol Chem *274*, 36328-36334.

Yokosaki, Y., Monis, H., Chen, J., and Sheppard, D. (1996). Differential effects of the integrins α9β1, αvβ3, and αvβ6 on cell proliferative responses to tenascin. Roles of the beta subunit extracellular and cytoplasmic domains. J Biol Chem *271*, 24144-24150.

Yokosaki, Y., Palmer, E. L., Prieto, A. L., Crossin, K. L., Bourdon, M. A., Pytela, R., and Sheppard, D. (1994). The integrin α9β1 mediates cell attachment to a non-RGD site in the third fibronectin type III repeat of tenascin. J Biol Chem *269*, 26691-26696.

FIGURE LEGENDS

Fig. 1 Affinity chromatography. [^{35}S]methionine- and [^{35}S]cycteine-labeled secreted αvβ6 was passed over either bovine serum albumin-(left) or nOPN-(right) Sepharose column. Bound proteins were eluted with 20 mM EDTA and analyzed by 7.5% polyacrylamide gel under nonreducing conditions. Lane 1 was the final fraction eluted with column buffer, lanes 2-6 were eluted with EDTA. The positions of molecular mass markers (in kDa) are shown to the left.

Fig. 2 Flow cytometry of SW480 transfectants. Four cell lines, mock-, β3-, β6- and α9-transfected SW480, were analyzed for the expression of integrins αvβ3, αvβ5, αvβ6, α5β1 and α9β1. Filled histogram indicates control, unstained cells. Antibodies used were LM609 against αvβ3, P1F6 against αvβ5, E7P6 against αvβ6, IIA1 against α5β1 and Y9A2 against α9β1. Histograms positive for staining are labeled with the name of the antibody used for staining.

Fig.3 Adhesion of SW480 transfectants to NH2-terminal osteopontin fragment. Mock-, β3-, β6- or α9–transfected SW480 cells were plated on osteopontin fragment (nOPN, 60 nM) in the presence (*gray*) or absence (*white*) of antibody against αvβ5 (P1F6), α5β1 (P3D10), αvβ3 (LM609), αvβ6 (10D5), and α9β1 (Y9A2). Antibodies were used either alone or in combination and indicated under each bar. Adhesion is expressed as absorbance at 595 nm. Mean value of adhesion to BSA coated control well was subtracted. Each bar represents the mean (± s.d.) of triplicate wells.

Fig. 4 Role of the RGD site in the adhesion of β6-transfected SW480 cells to NH2-terminal osteopontin fragment. *Panel A*-Mock (*open circle*) or β6-transfected (*closed circle*) cells were plated onto wells coated with increasing concentration of nOPN, in the presence of antibodies against αvβ5 and α5β1. β6-transfectants were also studied in the presence of additional antibody against αvβ6 (*open triangle*). *Panel B*-β6-transfectant were plated on nOPN (60 nM) in the presence of antibodies against αvβ5 and α5β1. Cells were pre-incubated with (*gray*) or without (*white*) GRGDSP peptide (100 μM), or plated on a nOPN in which the RGD sequence was

mutated to RAA (*diagonal*). *Panel C*-Expression of integrins on UCLA P3 cells was analyze by FACS with antibodies IIA1 (α5β1), P1F6 (αvβ5) and E7P6 (αvβ6). *Panel D*-UCLA P3 cells were plated onto wells coated with 200nM osteopontin in the presence (*gray*) or absence (*white*) of antibody. Antibodies used are indicated under each bar. Adhesion is expressed as absorbance at 595 nm. Mean value of adhesion to BSA coated control well was subtracted. Each bar represents the mean (± s.d.) of triplicate wells.

Fig. 5 Map of the integrin-binding region of osteopontin. nOPN; N-terminal fragment of thrombin-cleaved, osteopontin. fOPN; full length osteopontin. Asparatic acid residues replaced with alanine are shown in gray. RGD sequence recognized by integrins αvβ6, αvβ3, αvβ5 and α5β1 is in a bold type. SVVYGLR sequence recognized by integrin α9β1 is in underlined Italic. MMP-3 or MMP-7 cleaves between Gly and Leu (arrow head) within the SVVYGLR. Thrombin cleavage site is indicated by an arrowhead.

Fig. 6 αvβ6, αvβ3, αvβ5, α5β1 and α9β1-mediated adhesion to mutant nOPN. Adhesion of integrins to wild type nOPN (*white*), nOPN-D154A (*gray*), nOPN-D154,157A double mutant (*black*) or nOPN-Y165A (*diagonal*). Indicated under each graph is the integrin serving as the principal osteopontin receptor under the conditions being studied. αvβ5 or α5β1-mediated adhesions was tested as adhesion of mock-transfectant in the presence of anti-α5β1 or anti-αvβ5, respectively. αvβ6, αvβ3 or α9β1 mediated-adhesion was observed as adhesion of β6-, β3-, or α9-transfected SW480 cells, respectively in the presence of anti-α5β1 and anti-αvβ5. The osteopontin coating concentration was 60 nM. Adhesion is expressed as absorbance at 595 nm. Mean value of adhesion to BSA coated control wells was subtracted. Each bar represents the mean (± s.d.) of triplicate wells.

Fig. 7 Integrins αvβ6, αvβ3, αvβ5, α5β1 and α9β1-mediated adhesion to protease-processed fragments. *Panel A*-integrin mediated adhesion to recombinant full length osteopontin (*black*), nOPN (*white*), or nOPN-dLR (*gray*). nOPN-dLR lacks 2 residues Leu and Arg at C-terminus, therefore corresponds to N-terminal fragment of MMP-3 or MMP-7 cleaved form. The osteopontin coating concentration was 60 nM.

Panel B- integrin mediated adhesion to recombinant full length osteopontin (*black*), thrombin-cleaved full length osteopontin (*white*), or MMP-3-cleaved full length osteopontin (*gray*). Indicated under each graph is the integrin serving as the principal osteopontin receptor under the conditions being studied. αvβ5 or α5β1-mediated adhesions was tested as adhesion of mock-transfectant in the presence of P3D10 or P1F6, respectively. αvβ6, αvβ3 or α9β1 mediated adhesions was observed as adhesion of β6-, β3-, or α9-transfected SW480 cells, respectively in the presence of P3D10 and P1F6. Adhesion is expressed as absorbance at 595 nm. Mean value of adhesion to BSA coated control well was subtracted. Each bar represents the mean (± s.d.) of triplicate wells. *Panel C*-Enzymatic cleavage of full length osteopontin. Recombinant full length osteopontin, N-terminal osteopontin fragment and N-terminal osteopontin fragment that lacks C-terminal Leu and Arg residues (nOPN-dLR), were analyzed by 12 % SDS-polyacrylamide gel, along with thrombin-cut and MMP3-cut full length osteopontin. Thrombin cleavage yields two fragments of essentially the same relative molecular mass. MMP-3 cleavage was incomplete.

Fig. 1

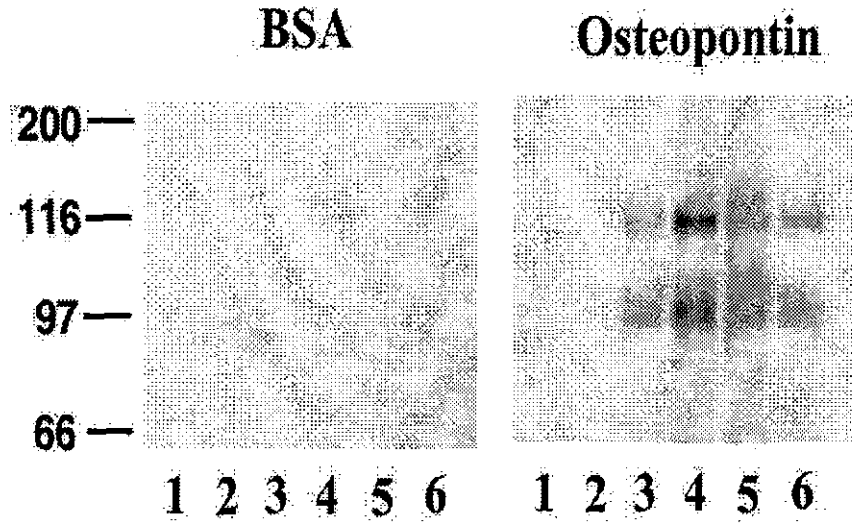


Fig. 2

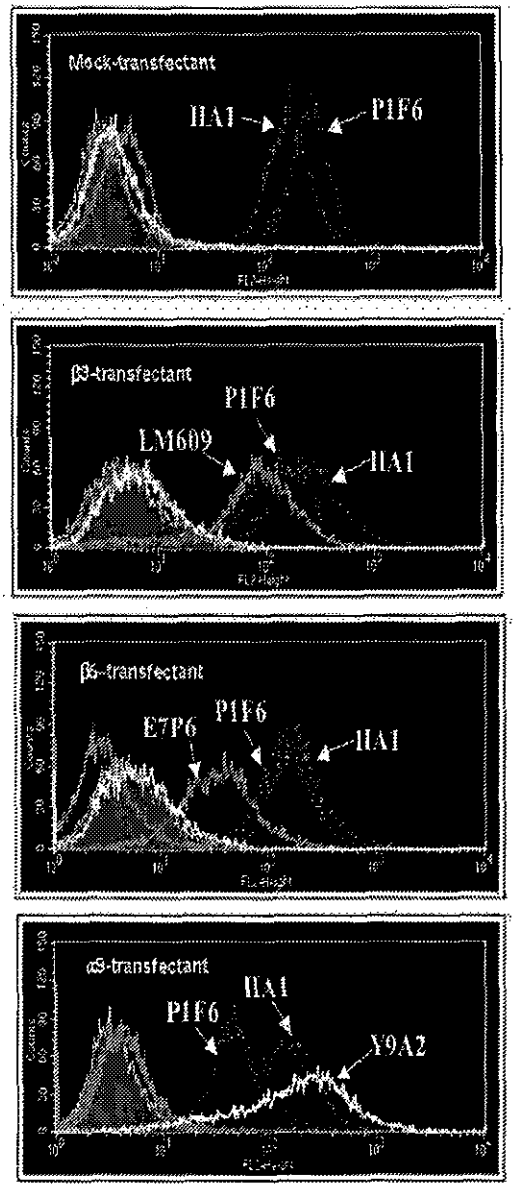

Mock-transfectant
IIA1
P1F6
β3-transfectant
P1F6
LM609
IIA1
β6-transfectant
E7P6
P1F6
IIA1
αS-transfectant
IIA1
P1F6
X9A2

Fig. 3

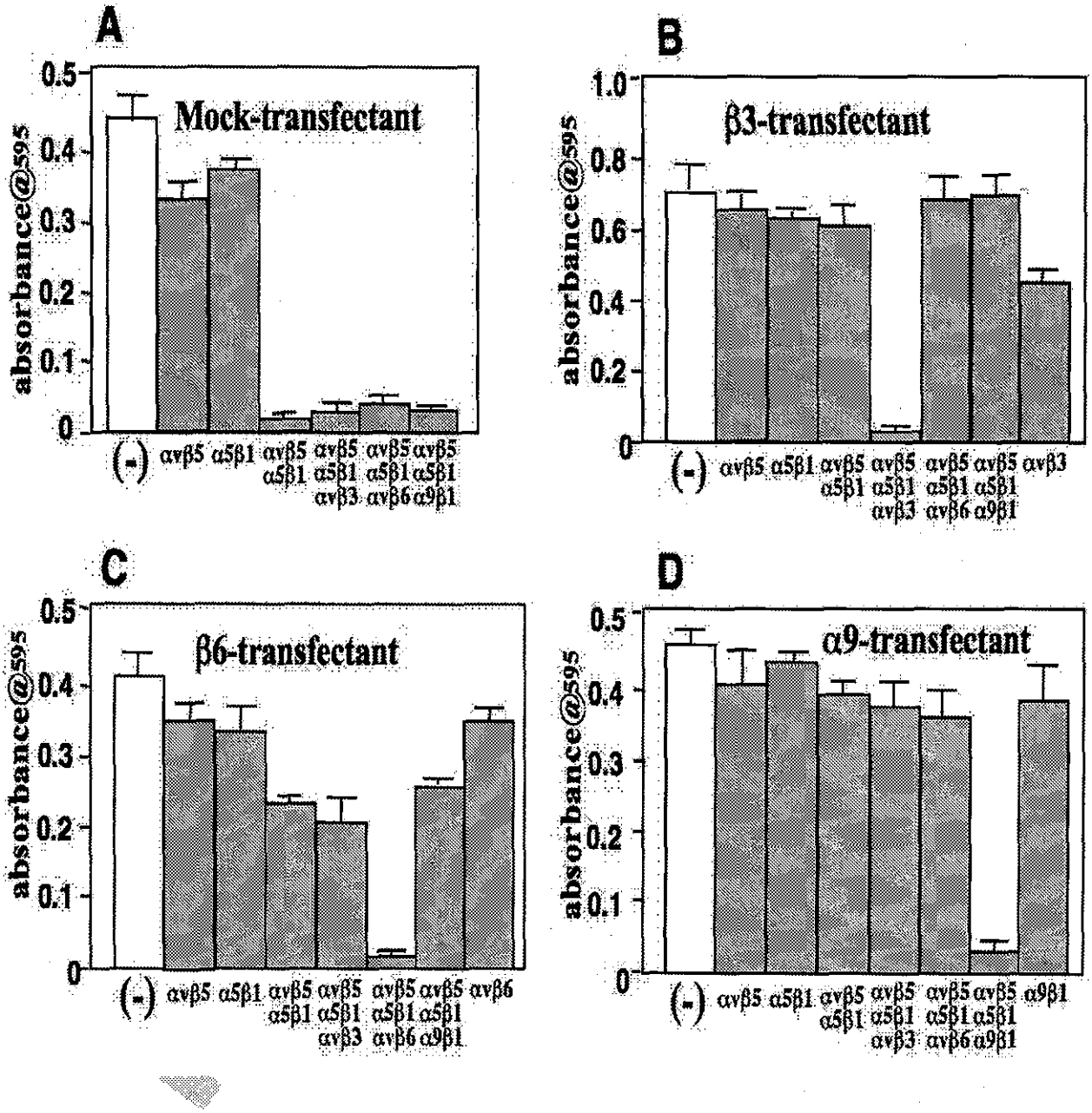

A
Mock-transfectant
absorbance@595
0.5
0.4
0.3
0.2
0.1
0
(-)
αvβ5
α5β1
αvβ5 α5β1
αvβ5 α5β1 αvβ3
αvβ5 α5β1 αvβ6
αvβ5 α5β1 α9β1
B
β3-transfectant
absorbance@595
1.0
0.8
0.6
0.4
0.2
0
(-)
αvβ5
α5β1
αvβ5 α5β1
αvβ5 α5β1 αvβ3
αvβ5 α5β1 αvβ6
αvβ5 α5β1 α9β1
αvβ3
C
β6-transfectant
absorbance@595
0.5
0.4
0.3
0.2
0.1
0
(-)
αvβ5
α5β1
αvβ5 α5β1
αvβ5 α5β1 αvβ3
αvβ5 α5β1 αvβ6
αvβ5 α5β1 α9β1
αvβ6
D
α9-transfectant
absorbance@595
0.5
0.4
0.3
0.2
0.1
0
(-)
αvβ5
α5β1
αvβ5 α5β1
αvβ5 α5β1 αvβ3
αvβ5 α5β1 αvβ6
αvβ5 α5β1 α9β1
α9β1

Fig. 4

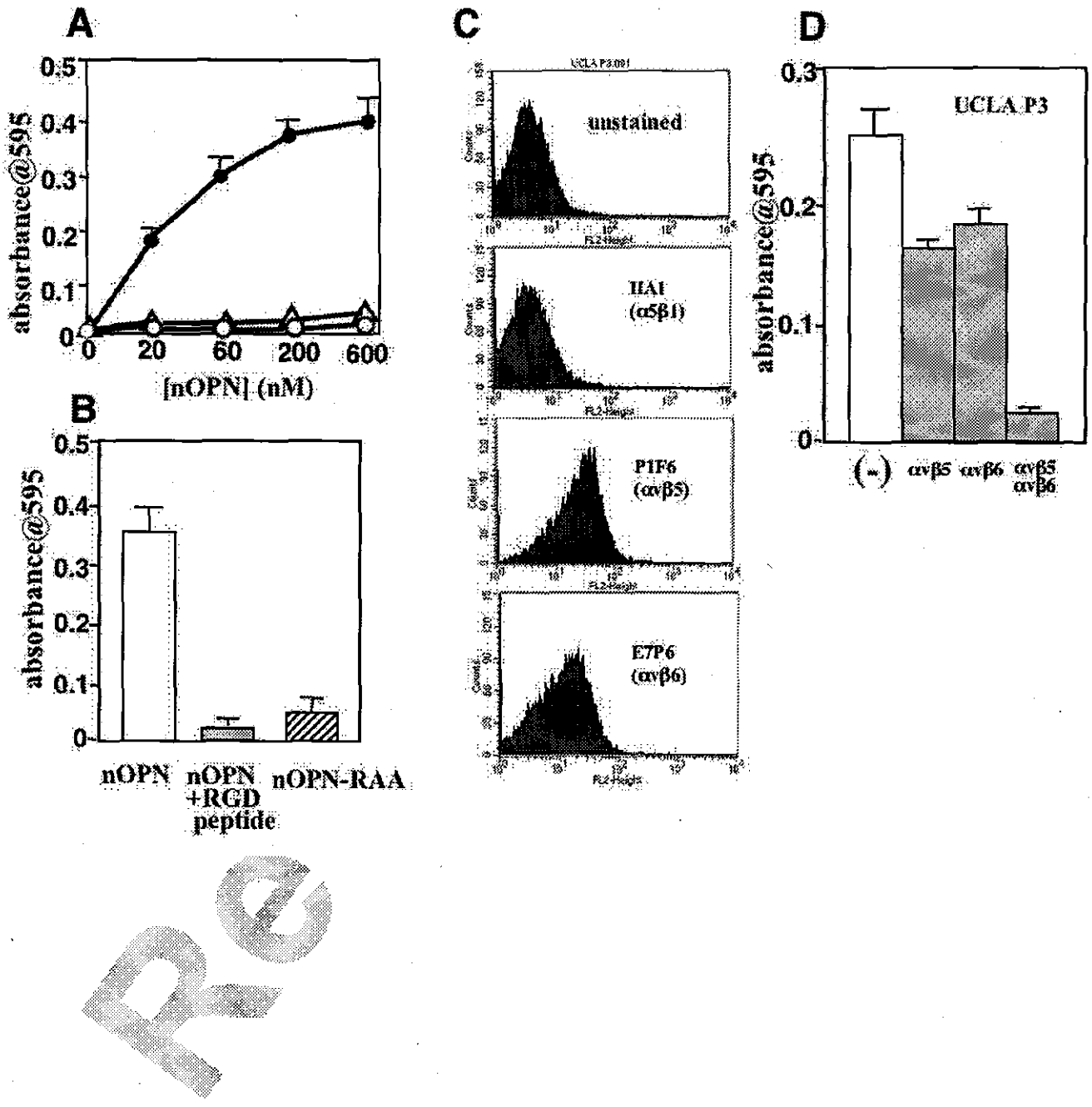

A
absorbance@595
0.5
0.4
0.3
0.2
0.1
0
0
20
60
200
600
[nOPN] (nM)
B
absorbance@595
0.5
0.4
0.3
0.2
0.1
0
nOPN
nOPN +RGD peptide
nOPN-RAA
C
unstained
IIA1 (α5β1)
P1F6 (αvβ5)
E7P6 (αvβ6)
D
absorbance@595
0.3
0.2
0.1
0
UCLA.P3
(-)
αvβ5
αvβ6
αvβ5 αvβ6

Fig. 5

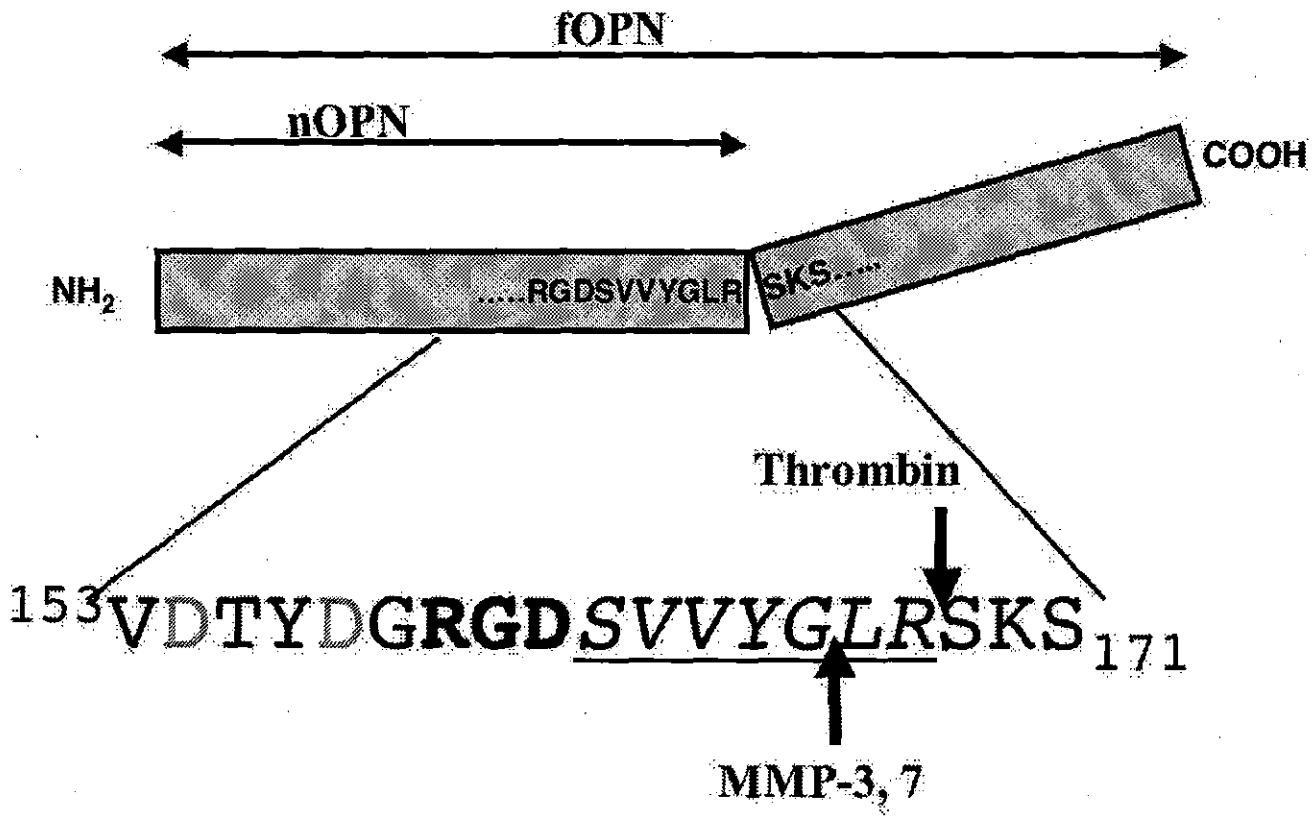

fOPN
nOPN
COOH
NH2
....RGDSVVYGLR
SKS....
Thrombin
153VDTYDGRGDSVVYGLRSKS171
MMP-3, 7

Fig. 6

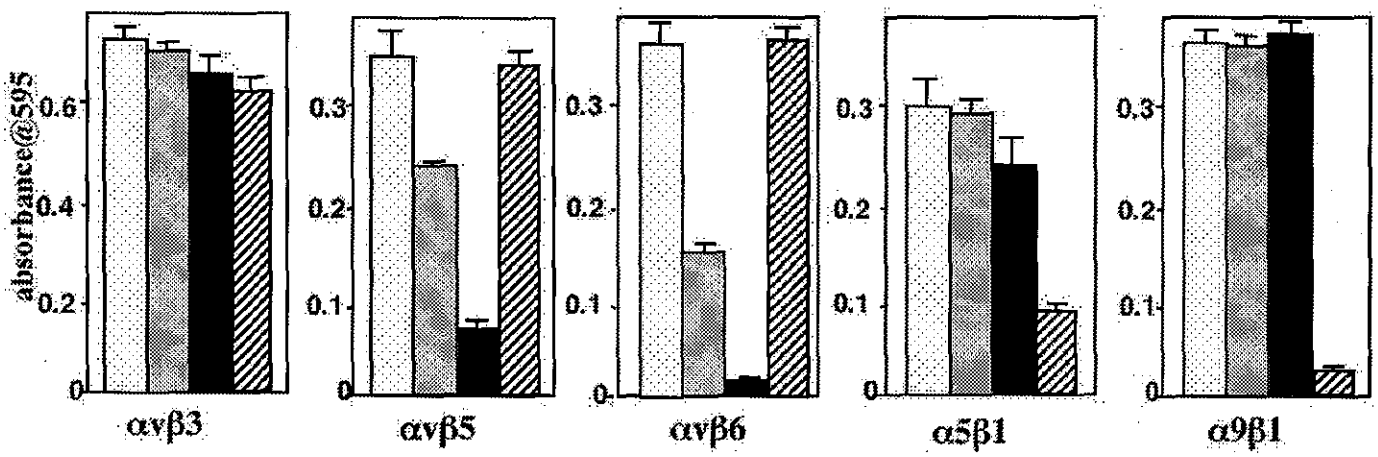
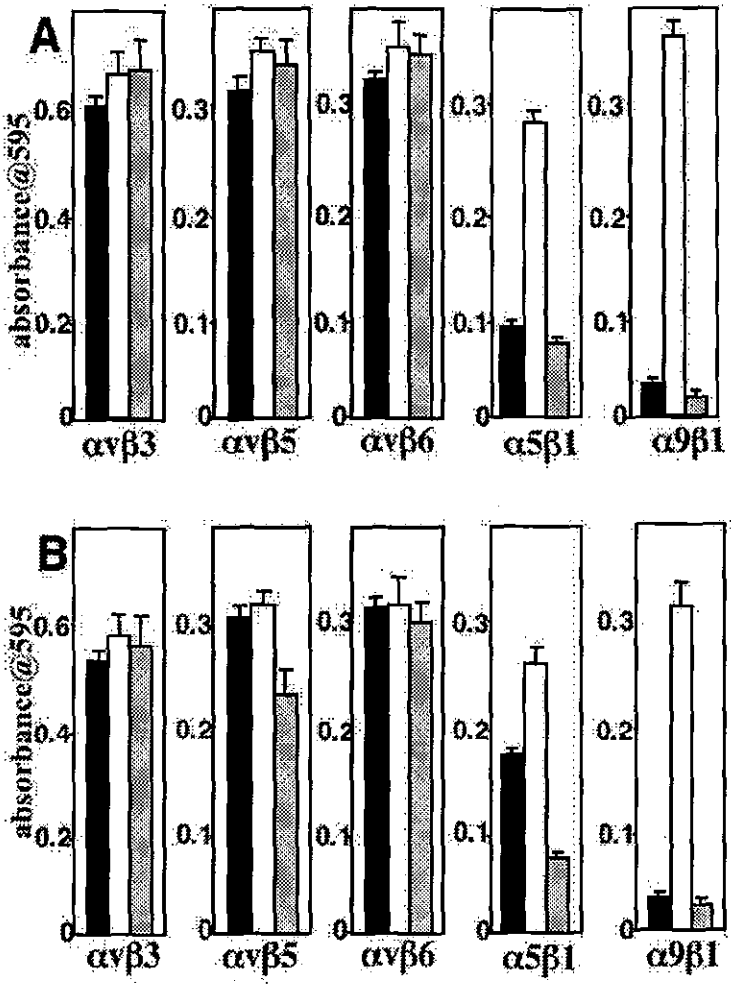
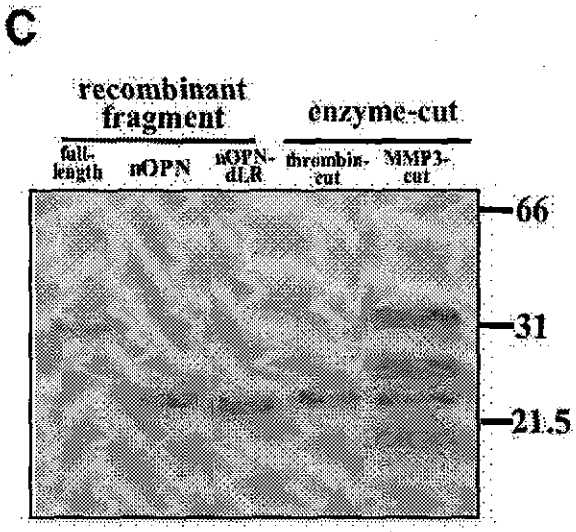

absorbance@595
0.6
0.4
0.2
0
αvβ3
0.3
0.2
0.1
0
αvβ5
0.3
0.2
0.1
0
αvβ6
0.3
0.2
0.1
0
α5β1
0.3
0.2
0.1
0
α9β1

Fig. 7


A
absorbance@595
0.6
0.4
0.2
0.3
0.2
0.1
αvβ3
αvβ5
αvβ6
α5β1
α9β1
B
absorbance@595
αvβ3
αvβ5
αvβ6
α5β1
α9β1


C
recombinant fragment
enzyme-cut
full-length
nOPN
nOPN-dLR
thrombin-cut
MMP3-cut
66
31
21.5