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Elastin Binds to a Multifunctional 67-Kilodalton Peripheral Membrane Protein[†]

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ABSTRACT: Elastin binding proteins from plasma membranes of elastin-producing cells were isolated by affinity chromatography on immobilized elastin peptides. Three proteins of 67, 61, and 55 kDa were released from the elastin resin by guanidine/detergent, soluble elastin peptides, synthetic peptide VGVAPG, or galactoside sugars, but not by synthetic RGD-containing peptide or sugars not related to galactose. All three proteins incorporated radiolabel upon extracellular iodination and contained [³H]leucine following metabolic labeling, confirming that each is a synthetic product of the cell. The 67-kDa protein could be released from the cell surface with lactose-containing buffers, whereas solubilization of the 61- and 55-kDa components required the presence of detergent. Although all three proteins were retained on elastin affinity columns, the 61- and 55-kDa components were retained only in the presence of 67-kDa protein, suggesting that the 67-kDa protein binds elastin and the 61- and 55-kDa proteins bind to the 67-kDa protein. We propose that the 67-, 61-, and 55-kDa proteins constitute an elastin-receptor complex that forms a trans-membrane link between the extracellular matrix and the intracellular compartment.

Many macromolecules of the extracellular matrix interact with receptors of binding proteins on the cell surface (Hynes, 1987; Ruoslahti & Pierschbacher, 1987). These matrix receptors are thought to play a role in cell movement and in mediating extracellular signals that influence cellular metabolism in development and tissue repair. Earlier studies in our laboratory have shown that both interstitial and inflammatory cells detect and move toward elastin fragments in chemotaxis assays (Senior et al., 1982, 1984), providing suggestive evidence for a functional elastin receptor on these cells. Direct evidence for an elastin binding protein was provided by the demonstration of specific and saturable binding of tropoelastin to protease-sensitive, high-affinity ($K_d = 8$ nM) sites on ligamentum nuchae fibroblasts (Wrenn et al., 1988). The appearance of active receptor on ligament cells occurred coincident with expression of the elastin phenotype (Mecham et al., 1984a,b), and chemotaxis studies have shown that a hydrophobic repeating hexapeptide in elastin, VGVAPG,¹ defines a receptor binding site on the molecule (Senior et al., 1984; Wrenn et al., 1988). Interestingly, the elastin receptor has lectin-like properties that are important for ligand binding as well as elastin fiber assembly in the extracellular space (Hinek et al., 1988).

In this report, we show that a 67-kDa peripheral membrane protein binds elastin via the VGVAPG repeat and confirm that the protein's lectin properties play an important role in ligand and cell binding. The 67-kDa component has distinct binding sites for elastin and carbohydrate and a cell binding site that interacts with subunits associated with the cell surface. This receptor differs substantially from other matrix receptors in

which ligand and cell binding properties belong to transmembrane proteins (Hynes, 1987; Ruoslahti & Pierschbacher, 1987).

EXPERIMENTAL PROCEDURES

Reagents. Affi-Gel 10 was purchased from Bio-Rad Laboratories (Richmond, CA). Colloidal gold and silver enhancement kits were purchased from Janssen Life Science Products (Piscataway, NJ), and species- and type-specific second antibodies conjugated with peroxidase were from Boehringer Mannheim (Indianapolis, IN). Antibodies to chicken muscle actin and bovine serum albumin were from Miles Scientific, Naperville, IL. Iodobeads were from Pierce Chemical (Rockford, IL). Thermolysin-derived 110-kDa cell binding fragment of human fibronectin coupled to Sepharose was provided by Dr. John A. McDonald. Synthetic peptides VGVAPG, VSLSPG, REGDPS, GRGDSP, and LREGDPSSS were a generous gift of Dr. George Wilner. PDGF was a gift from Dr. Thomas Deuel. All other chemicals were from Sigma Chemical Co. (St. Louis, MO). Human neutrophil elastase and bovine elastin from ligamentum nuchae were purchased from Elastin Products Co. (Pacific, MO).

Cell Isolation and Culture. Fibroblasts from ligamentum nuchae were grown from explants of fetal bovine tissue as described (Mecham et al., 1981). Chondroblasts from ear cartilage of a 150-day bovine fetus were isolated following collagenase digestion of the cleaned tissue (Mecham, 1987). All experiments were performed on first through third passage cells. Cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) containing high glucose and high bicarbonate and supplemented with antibiotics, nonessential amino acids, and 10% (v/v) bovine calf serum.

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¹ Peptide abbreviations: VGVAPG, Val-Gly-Val-Ala-Pro-Gly; VSLSPG, Val-Ser-Leu-Ser-Pro-Gly; REGDPS, Arg-Glu-Gly-Asp-Pro-Ser; LREGDPSSS, Leu-Arg-Glu-Gly-Asp-Pro-Ser-Ser-Ser; GRGDSP, Gly-Arg-Gly-Asp-Ser-Pro.

Labeling of Cells and Plasma Membranes. Chondroblasts in 100-mm² culture dishes were iodinated by using Iodobeads as described in Wrenn et al. (1988). Metabolic labeling of receptor was accomplished by incubating cultured chondroblasts for 18 h in leucine-deficient DMEM supplemented with 10 μ Ci/mL [³H]leucine (specific activity 42 Ci/mmol, ICN Radiochemicals) and 3% bovine calf serum.

Chemotaxis to Elastin Peptides. Ligamentum nuchae fibroblasts (first passage) were assayed for directed migration to elastin peptides according to a modified Boyden chamber chemotaxis assay as described (Senior et al., 1984). The effects of lactose on cell movement were determined by incubating cells overnight in normal growth medium supplemented with 20 mM lactose and by including lactose in both top and bottom compartments of the chamber during the chemotaxis assay. Chemotaxis to PDGF served as the positive control. All experiments were done at least twice in triplicate with five readings at each test concentration.

Affinity Chromatography and Elastin Receptor Isolation. Elastin affinity resin was prepared from human leukocyte elastase derived peptides of bovine aorta elastin (Senior et al., 1982). Elastin peptides (20 mg/mL of resin) were dissolved in coupling buffer (0.1 M sodium bicarbonate buffer, pH 8) and reacted overnight with Affi-Gel 10 resin as described by the manufacturer. Active ester sites on the resin were blocked by a 1-h incubation with 0.1 M ethanolamine (pH 8). Affi-Gel coated with bovine serum albumin served as a control.

Plasma membranes, prepared from cultured cells as described previously (Wrenn et al., 1988), were extracted with membrane solubilization buffer [3 M guanidine hydrochloride (Gdn-HCl), 10 mM Hepes, pH 8, 0.1 M dithiothreitol (DTT), 0.5% (w/v) octyl β -glucoside (OBG), and protease inhibitors²]. Extraction was overnight at 4 °C with constant stirring, and insoluble material was pelleted by centrifugation. The supernatant was dialyzed exhaustively at 4 °C against 0.1 M sodium bicarbonate, pH 8, containing protease inhibitors and mixed with the various affinity resins for 2–4 h. The resins and supernatants were transferred to a siliconized glass column, and unbound material was removed by washing with 0.1 M sodium bicarbonate buffer, pH 8, until the A_{280} of the eluent returned to the background level. Protein bound to the immobilized elastin was eluted with either (A) 3 M guanidine containing 0.5% octyl β -glucoside, (B) 0.5 mg/mL elastin peptides generated from bovine aortic elastin using neutrophil elastase (Senior et al., 1982), (C) 0.5 mg/mL synthetic peptide VGVAPG, VSLSPG, REGDPS, LREGDPSSS, or GRGDSP, or (D) 100 mM lactose. All reagents were dissolved in 0.1 M sodium bicarbonate buffer, pH 8, and all procedures were at 4 °C. Affinity isolation on immobilized asialofetuin was as described previously (Hinek et al., 1988).

Amino Acid Analysis of Cell and Tissue Receptor. Sixty-seven-kilodalton elastin binding protein was prepared from lung as described (Cerra et al., 1984; Hinek et al., 1988). Lactose extracts of late-stage bovine fetal lung tissue were dialyzed against 0.1 M sodium bicarbonate buffer (4 °C) and chromatographed on elastin affinity columns. Bound protein was eluted with 100 mM lactose, dialyzed against water, and purified further on a Beckman HPLC system using a Spherogel TSK 3000-SW column (7.5 mm \times 30 cm) with water as the solvent at a flow rate of 1 mL/min. SDS-PAGE of the collected peak showed a band at 67 kDa. An aliquot of the isolated protein together with 67-kDa protein from cell

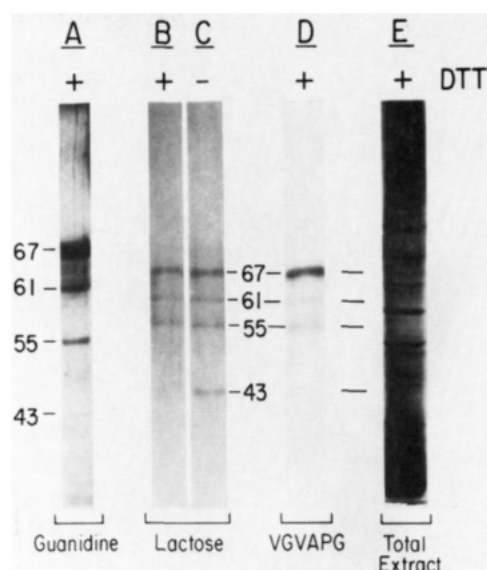


FIGURE 1: SDS-PAGE of elastin binding proteins in plasma membranes from auricular chondroblasts. Plasma membrane extracts from auricular chondroblasts were chromatographed on elastin affinity columns and retained proteins eluted with 3 M guanidine hydrochloride (Gdn-HCl), 10 mM Hepes, and 0.5% octyl β -glucoside (lane A); 0.1 M lactose (lanes B and C); or 5 μ g/mL synthetic VGVAPG (lane D). Lane E shows the total membrane extract. Proteins were visualized by silver staining. Molecular mass assignments were made from standards run on each gel.

membranes (Figure 3A) was hydrolyzed overnight at 105 °C in constant-boiling HCl and the amino acid composition determined by using a Beckman 119C amino acid analyzer.

Electrophoresis and Immunoblot Analysis. Proteins eluted from the affinity columns were dialyzed exhaustively against water at 4 °C and concentrated by lyophilization. Concentrated samples were suspended in SDS sample buffer with or without DTT and analyzed by SDS-polyacrylamide gel electrophoresis on 0.45 mm thick, 7%–12% gradient gels (Wrenn et al., 1986). Protein bands were visualized by silver staining and molecular weight assignments made from standards included on each gel. For fluorography, gels were impregnated with EN³HANCE and exposed to Kodak XAR-5 film at –70 °C. Proteins were transferred from SDS gels to nitrocellulose at 2 mA overnight (4 °C) and the blots developed with appropriate antibodies according to Wrenn et al. (1987). Antibodies containing immunogold were visualized by amplification using a silver enhancement kit as described by the manufacturer.

RESULTS

Affinity Isolation of Elastin Binding Proteins from Cell Membranes. Elastin binding proteins from the plasma membrane of auricular chondroblasts were isolated by affinity chromatography using elastin-Affi-Gel. SDS-PAGE of proteins released from the elastin column by guanidine/OBG buffer or lactose showed four bands at molecular masses of 67, 61, 55, and 43 kDa (Figure 1). The 43-kDa band was not detected when gels were run under reducing conditions, suggesting that this protein may have been partially degraded during purification. No protein material was retained from the membrane extracts by a bovine serum albumin column (Wrenn et al., 1988).

The elastin binding proteins from chondroblast membranes eluted from the elastin affinity column as a broad peak over numerous column fractions, but only after approximately two column volumes of elution buffer had passed through the affinity resin. Inclusion of low levels of nonionic detergent

² Protease inhibitors included 5 mM EDTA, 5 mM benzamidine, 0.1 M ϵ -aminocaproic acid, and 2 mM PMSF.

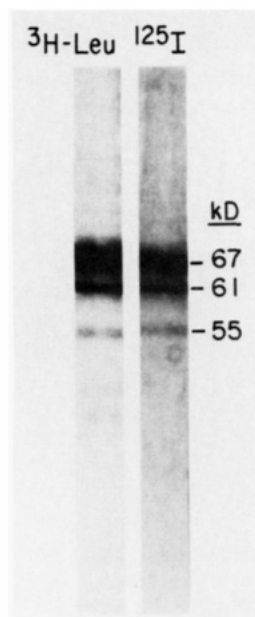


FIGURE 2: Radiolabeling of elastin binding proteins. Proteins from membrane extracts of chondroblasts grown in the presence of [^3H]leucine (lane A) or following extracellular iodination with [^{125}I] (lane B) were isolated by elastin affinity chromatography and separated by SDS-PAGE under reducing conditions. Gel A was exposed to Kodak XAR-5 film for 3 weeks; gel B, for 2 days.

(0.1% OBG) facilitated release of the protein as a sharper peak. A similar observation has been made for elution of the 67-kDa laminin receptor from laminin (von der Mark & Risse, 1987; Wewer et al., 1986). Immunoblot analysis indicated that the 67-kDa protein was not bovine serum albumin or tropoelastin. The 43-kDa band was identified as actin by reactivity with antibody to chicken muscle actin. None of the four bands reacted with anti-fibronectin (not shown). All four proteins contained [^3H]leucine following metabolic labeling of chondroblasts (Figure 2), confirming that each is a synthetic product of the cell. To determine the orientation of the elastin binding proteins in the cell membrane, intact chondroblasts were iodinated by using Iodobeads, and elastin binding proteins in the plasma membrane were isolated by affinity chromatography. As shown in Figure 2, the 67-, 61-, and 55-kDa proteins incorporated radioisotope.

In previous studies we established that the 67-kDa elastin binding protein has lectin properties that enable its extraction from cell membranes or from tissues such as lung with buffers containing high concentrations of galactose-like sugars (Hinek et al., 1988). Figure 3 shows that the 67-kDa component is the only elastin binding protein removed from cell membranes with lactose extraction. In contrast, extraction of the 61- and 55-kDa proteins requires the presence of detergent. Table I compares the amino acid composition of 67-kDa protein purified from bovine lung and from plasma membranes of chondroblasts and fibroblasts. The three proteins have similar if not identical amino acid compositions characterized by an abundance of serine, glycine, and glutamate-glutamine and absence of hydroxyproline and hydroxylysine. The composition differs substantially from bovine serum albumin, the most likely contaminant in the 67-kDa molecular mass range.

Elution of Receptor from Elastin Column with Elastin Peptides and with VGVAPG Peptide. The same three proteins released from the elastin affinity column by guanidine/OBG buffer or lactose could also be eluted with elastin peptides or with the synthetic peptide VGVAPG (Figure 1). These binding proteins could be released with as little as 5 $\mu\text{g}/\text{mL}$

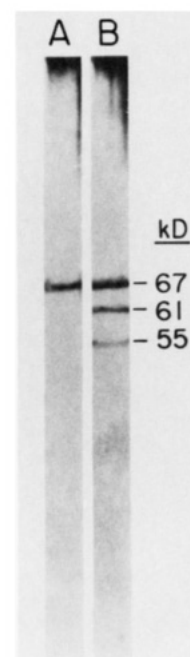


FIGURE 3: Selective extraction and purification of 67-kDa protein. Plasma membranes from cultured chondroblasts were extracted with buffer containing 100 mM lactose or 3 M Gdn-HCl, 10 mM Hepes, 0.5% octyl β -glucoside, and 0.1 M DTT. Extracts were dialyzed against water, lyophilized, dissolved in bicarbonate buffer containing protease inhibitors, and chromatographed on an elastin affinity column. Elution of bound protein with lactose showed a single band at 67-kDa in the lactose extract (lane A) and the characteristic three proteins in the detergent extract (lane B). Proteins were visualized by silver staining. The dark material at the top of lanes A and B was a staining artifact present throughout the top of the gel.

Table I: Amino Acid Composition of 67-kDa Elastin Binding Proteins

	residues/1000			
	chondroblast ^a	fibroblast ^a	lung ^b	BSA ^c
Cys	7	5	7	60
Hyp	0	0	0	0
Asx	71	69	70	93
Thr	40	44	43	58
Ser	210	205	200	48
Glx	171	187	176	136
Pro	32	29	28	48
Gly	204	195	201	28
Ala	78	82	80	79
Val	28	31	32	62
Met	8	3	5	7
Ile	19	21	20	24
Leu	34	31	36	105
Tyr	13	12	12	33
Phe	13	16	18	46
Hyl	0	0	0	0
His	36	34	30	29
Lys	30	27	30	101
Trp	nd ^d	nd	nd	3
Arg	8	11	12	40

^a From plasma membranes from bovine auricular chondroblasts and ligament fibroblasts. ^b From lactose extract of fetal bovine lung.

^c Composition of bovine serum albumin derived from Peters (1985).

^d nd = not determined.

elastin peptides and 50 $\mu\text{g}/\text{mL}$ VGVAPG, but to assure complete elution, buffers containing 0.5 mg/mL elastin peptides or VGVAPG were used routinely in this study. The affinity of the binding proteins for elastin was not affected by synthetic peptides REGDPS or LREGDPSSS, sequences in human elastin (Indik et al., 1987) that do not have chemotactic activity for fibroblasts, nor by VSLSPG, a variant of the

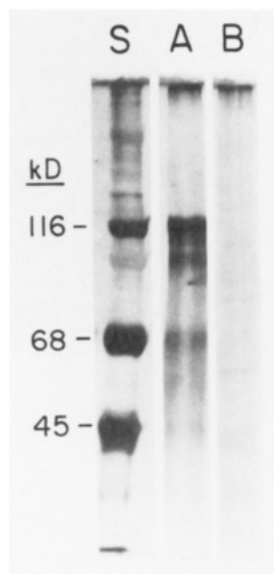


FIGURE 4: Elastin receptor does not interact with fibronectin. A detergent extract of chondroblast plasma membranes was passed over an affinity column containing the 110-kDa cell binding fragment of fibronectin. Bound proteins were eluted with guanidine/DBG buffer and analyzed by SDS-PAGE under reducing conditions (gel A). No proteins corresponding to the elastin-receptor complex were observed. A portion of the same membrane extract was fractionated over elastin affinity resin and the column eluted with 500 μ g/mL synthetic GRGDS. No proteins were released with this synthetic peptide (gel B), suggesting that the fibronectin receptor does not bind to elastin and that the elastin receptor does not recognize the fibronectin cell binding sequence. The same membrane extract passed over an elastin affinity column is shown in Figure 3. Molecular mass standards (lane S) include β -galactosidase (116 kDa), bovine serum albumin (68 kDa), and egg albumin (45 kDa). Proteins were visualized by silver staining.

VGAPG receptor binding sequence in elastin. These results rule out a nonspecific elution by peptides generally. No protein bands were eluted from the column with guanidine/DBG buffer following elution with elastin peptides or VGAPG, suggesting that bound proteins had been removed quantitatively by elastin peptides. Further evidence that binding involves the VGAPG domain on elastin was provided by the finding that preincubation of the elastin affinity resin with a monoclonal antibody specific for VGAPG (Wrenn et al., 1986) blocked binding of all three receptor proteins. This antibody did not block binding of 67-kDa protein to the asialofetuin column (not shown).

To determine whether the elastin binding proteins might be related to the fibronectin receptor, membrane extracts were passed through a column of immobilized 110-kDa cell binding fragment of fibronectin (Zardi et al., 1985). Elution of bound proteins demonstrated a broad band on SDS-PAGE at approximately 120 kDa (Figure 4) and fainter bands at smaller molecular masses. There was, however, no evidence for interaction of the elastin binding proteins with the fibronectin peptide. Synthetic GRGDSP, the binding sequence of the fibronectin and vitronectin receptors, did not elute elastin binding proteins from elastin affinity columns (Figure 4).

The 67-kDa Protein Contains the Elastin Binding Site. The association of 67-, 61-, and 55-kDa proteins with elastin affinity resin raised the question of whether the three proteins interact with elastin individually or as a complex. To investigate this question, we removed the 67-kDa component from plasma membranes with lactose and then extracted the 55- and 61-kDa proteins from the plasma membrane with guanidine/detergent. The isolated 67-kDa component bound to the elastin affinity column in the absence of 55- or 61-kDa subunits whereas the 55- and 61-kDa proteins did not bind

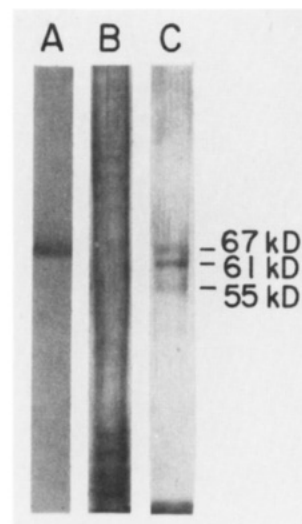


FIGURE 5: The 67-kDa protein contains the elastin binding site. Sixty-seven-kilodalton component was separated from 61- and 55-kDa proteins by extracting auricular chondroblast plasma membranes with 100 mM lactose. The lactose-extracted membranes were then solubilized with 3 M Gdn-HCl, 10 mM Hepes, 0.5% octyl β -glucoside, and 0.1 M DTT and, following dialysis against bicarbonate buffer, mixed with immobilized asialofetuin to absorb residual 67-kDa protein. Chromatography of the dialyzed lactose and detergent extracts over elastin peptides and elution of bound protein with lactose in 0.1 M bicarbonate buffer, pH 8, containing 0.1% OBG and 0.1 M DTT, showed the expected 67-kDa protein in the lactose extract (lane A), but the 61- or 55-kDa proteins in the 67-kDa-depleted membrane extract did not bind to the column (lane B). When 67-kDa protein from lane A was added to material in lane B and then rechromatographed on immobilized elastin peptides, the 67-kDa as well as 61- and 55-kDa proteins were retained on the column (lane C). Gel samples were run under reducing conditions, and proteins were visualized by silver staining. Lane B was developed twice as long as lanes A and C to confirm that 55- and 61-kDa proteins do not bind to elastin in the absence of 67-kDa protein.

in the absence of 67-kDa protein (Figure 5, lane B). Binding of the 55- and 61-kDa proteins to elastin was restored, however, following reconstitution with 67-kDa protein (Figure 5, lane C).

The elastin binding properties of the 67-kDa protein were confirmed by passing a tropoelastin-enriched extract (Wrenn et al., 1987) through an affinity column containing purified 67-kDa protein. Figure 6 shows that two proteins of approximately 65 and 75 kDa bound and were eluted with lactose. The 65-kDa protein reacted positively on immunoblot analysis with a monoclonal antibody to elastin (Wrenn et al., 1986), confirming that the isolated 67-kDa protein retains elastin binding activity and, consistent with its extracellular location, does not require reconstitution into liposomes to bind tropoelastin. The nature of the 75-kDa protein is unknown.

Chemotaxis to Elastin Peptides. Fibroblasts from ligamentum nuchae show directed migration to elastin peptides (Senior et al., 1982). To determine whether the elastin binding proteins identified in this study might mediate this process, ligament cells were cultured for 18 h in lactose-containing medium to dissociate the 67-kDa elastin binding component from the cell membrane. Figure 7 shows that pretreatment of cells with lactose, and the inclusion of lactose in the top and bottom compartments of the assay chamber, abolished the chemotactic response to elastin but did not alter directed movement to PDGF, a growth factor chemoattractant that binds to a different receptor. The inhibition of directed cell movement to elastin but not to PDGF indicates that lactose does not have a generalized inhibitory effect upon cell migration.

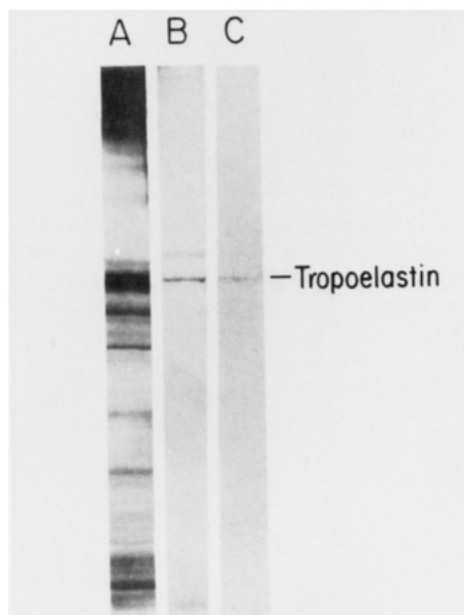


FIGURE 6: Binding of tropoelastin by immobilized purified 67-kDa protein. A tropoelastin-enriched extract from ligamentum nuchae (lane A) was passed through an affinity column containing purified 67-kDa elastin binding protein (Figure 3, lane A), and bound proteins were eluted with 0.1 M lactose (lane B). The lower molecular weight band was identified as tropoelastin by immunoblot analysis with a monoclonal antibody to elastin (lane C). Proteins in lanes A and B are visualized by silver stain.

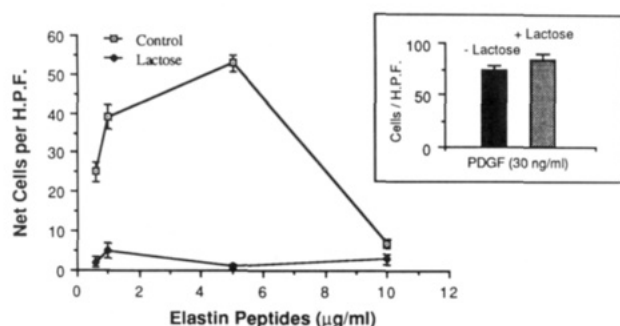


FIGURE 7: The effects of lactose on fibroblast chemotaxis to elastin peptides. Chemotaxis to elastin peptides was assessed for ligamentum nuchae fibroblasts cultured for 18 h in the presence or absence of 20 mM lactose. Movement toward 1 nM PDGF (insert) served as a positive control for both cell populations. Results are expressed as net cells per high power field (mean \pm SEM, $n = 15$).

DISCUSSION

The present study confirms our earlier findings (Hinek et al., 1988; Wrenn et al., 1988) that three proteins of molecular masses 67, 61, and 55 kDa can be isolated by elastin affinity chromatography from plasma membranes of elastin-producing cells. Our current results suggest that the 61- and 55-kDa proteins do not bind elastin directly but combine with the 67-kDa protein to form a "reconstituted" complex when detergent is removed from the extraction buffer. Evidence that the 67-kDa component contains the elastin binding activity was provided by the observation that 67-kDa protein binds elastin in the absence of 61- and 55-kDa components, but the 55- and 61-kDa proteins do not bind elastin in the absence of 67-kDa protein. Thus, of the three proteins that associate with the elastin column only the 67-kDa component is a true elastin binding protein.

The 61- and 55-kDa proteins have properties of integral membrane proteins since they can only be extracted from cells or membranes with buffers that contain detergents. Both proteins incorporate radiolabel after extracellular iodination,

indicating that part of their structure is exposed to the external surface, but it is not known whether one or both proteins span the plasma membrane and interact with the intracellular compartment. The 67-kDa protein, in contrast, displays properties of a peripheral membrane protein associated with the external surface of the cell (Hinek et al., 1988). In a previous study we showed that antibodies that react with the 67-kDa protein do not recognize either the 61- or 55-kDa proteins on immunoblots (Hinek et al., 1988). These findings argue, but do not prove, that the 61- and 55-kDa components are unique proteins that do not arise through degradation of the 67-kDa protein.

The binding properties of the 67-kDa component demonstrate the specificity one would expect for an elastin binding protein. In addition to interacting with peptides from insoluble elastin, the 67-kDa protein binds tropoelastin, the soluble elastin precursor molecule. Confirmation that binding involves the elastin recognition sequence VGVAPG was obtained by the specific elution of receptor from elastin affinity columns with synthetic VGVAPG peptide. Importantly, our finding that a hexapeptide with a similar sequence (VSLSPG) did not elute the receptor from elastin affinity columns minimizes the possibility that the 67-kDa protein binds nonspecifically to any hydrophobic sequence. The absence of RGD sequences in bovine elastin and the inability of synthetic RGD-containing peptides to elute bound proteins from elastin affinity columns illustrates that binding of elastin to the cell does not involve the same sequence that has been described for binding of fibronectin to its receptor. Blood et al. (1988) have recently identified a 59-kDa protein on tumor cells that recognizes the VGVAPG sequence. How this protein relates to the 67-kDa elastin binding protein identified in our study is unclear, but together, these results provide strong support for the characterization of VGVAPG as a specific recognition sequence for cell surface receptors.

We found earlier that the 67-kDa protein has lectin properties that greatly influence both ligand and cell binding (Hinek et al., 1988). The significance of this observation was at first puzzling since elastin is not a glycoprotein and hence could not interact with the 67-kDa protein via a sugar-mediated mechanism (this was confirmed by inhibition of binding by synthetic VGVAPG). Nevertheless, interactions between elastin and the 67-kDa protein, and interactions between the 67-kDa protein and the cell surface, are disrupted specifically by galactose-like sugars (Hinek et al., 1988). We interpret these results as evidence for separate protein and carbohydrate binding sites on the 67-kDa protein and propose that binding of carbohydrate diminishes the affinity of the 67-kDa component for elastin. There is precedent for proteins containing binding sites for both protein and carbohydrate ligands. Recently, binding of mannose 6-phosphate to the insulin-like growth factor II (IGF-II)/mannose 6-phosphate receptor has been shown to act synergistically to increase receptor affinity for IGF-II (MacDonald et al., 1988). In a similar way, the 67-kDa protein shows multivalent regulation, except that binding of carbohydrate reduces binding affinity at the protein site and abolishes its specific interaction with elastin.

Because the 67-kDa component is not an integral membrane protein, how it associates with the cell surface is of great importance in explaining its functional properties. We have shown in this and in a previous study (Hinek et al., 1988) that the 67-kDa protein can be removed from the plasma membrane by lactose-containing buffers, suggesting that interaction with the cell surface is mediated directly or indirectly by the sugar binding site on the 67-kDa protein. One possible

mechanism suggested by this finding is that the 67-kDa component interacts as a lectin with a glycoconjugate (either glycoprotein or glycolipid) on the cell surface. We feel, however, that the carbohydrate binding domain is unlikely to mediate such an interaction because occupancy of the carbohydrate site greatly diminishes the protein's affinity for elastin. Yet we have shown previously that tropoelastin binds with high affinity to receptors on the cell surface (Wrenn et al., 1988). For such binding to occur, the sugar site on receptor protein attached to the cell surface must be unoccupied.

The propensity for the 61- and 55-kDa proteins to associate with the 67-kDa component suggests another model for providing specific attachment of the 67-kDa protein to the cell surface. Because the 61- and 55-kDa components demonstrate properties of integral membrane proteins, we propose that one or both proteins provide an anchoring or membrane-attachment site on the cell surface that is recognized specifically by a cell binding domain on the 67-kDa protein. Binding to one or both of these proteins could in turn be regulated by the carbohydrate site on the 67-kDa component in much the same way that affinity for elastin binding is regulated. As long as the carbohydrate site is unoccupied, the 67-kDa component can associate with anchoring protein(s) on the plasma membrane. Binding of carbohydrate, however, decreases the affinity for the anchoring subunits resulting in the release of the 67-kDa protein from the cell surface.

The 61- and 55-kDa proteins may serve functions other than simply providing an attachment site for the 67-kDa component. One role might be signal transduction arising from interactions with the 67-kDa protein or from elastin binding to the 67-kDa protein. The implication of this model is that the 67-, 61-, and 55-kDa proteins form a cell surface complex that has properties of an elastin receptor. The distinction between a "receptor" and "binding protein" is sometimes difficult, but implicit in the definition of a receptor is a functional involvement in signal transduction. There is, in fact, accumulating evidence that binding of elastin to the cell surface initiates a biological response within the cell. Jacob et al. (Jacob et al., 1987; Varga et al., 1988) have shown that extracellular elastin peptides alter ion fluxes in mononuclear cells, fibroblasts, and smooth muscle cells, and our own studies have demonstrated that elastin peptides provide chemotactic signals to numerous cell types. The strongest evidence that the three proteins function as a receptor complex is the failure of lactose-treated ligament fibroblasts to respond to elastin peptides in a chemotaxis assay. As shown in Figure 3, lactose removes the 67-kDa component from the cell surface but does not extract the membrane-associated proteins. Even though the 61- and 55-kDa components remain associated with these lactose-treated cells, it is clear from the results in Figure 7 that cellular recognition of elastin peptides, as assessed by a positive chemotactic response, does not occur, a finding in agreement with the observation that 61- and 55-kDa proteins do not directly bind elastin. The chemotactic response shown in Figure 7 supports a signaling role for the elastin binding complex which must involve the 67-kDa component and one or both of the membrane-associated proteins. In this context, the 67-kDa protein by itself is an elastin "binding protein" but becomes a component of an elastin "receptor" when complexed with the 61- and 55-kDa components. In addition to inducing a chemotactic response, we have shown in previous publications (Wrenn et al., 1988) that elastin binding to the cell surface is saturable, occurs with a nanomolar binding coefficient (gives a straight line on Scatchard analysis), and shows specific competition with unlabeled protein. Furthermore, we have

shown that monocyte chemotaxis to VGVAPG can be selectively blocked by preexposing the cells to elastin peptides (Senior et al., 1984), a response typical of receptor desensitization. Together, these observations make a strong case for calling the elastin binding protein a "receptor".

The elastin, carbohydrate, and cell binding properties of the 67-kDa protein suggest several possible functions for the proposed receptor. One biological role relates to elastin fiber assembly. We have shown previously (Hinek et al., 1988) that addition of lactose or galactose to cultures of elastin-producing cells alters the distribution of 67-kDa protein on the cell surface and interrupts the formation of mature elastin fibers in the extracellular space. Similarly, agarose has been shown to inhibit elastin fiber formation in transplants of smooth muscle cells and elastic cartilage chondrocytes (Hinek et al., 1984). Organization of a functional elastin fiber is a complex process involving interactions between tropoelastin and glycoprotein microfibrils. How this interaction occurs is not known, but it has long been presumed that tropoelastin is secreted from the cell and somehow finds its way through the extracellular matrix to the growing elastic fiber where it interacts with microfibrils and becomes oriented in the proper alignment for cross-linking. Repeated experiments over a period of years, however, have failed to provide convincing evidence to support this view. Our previous studies (Hinek et al., 1988) suggest that the assembly process is not random but is mediated at the cell surface by the elastin receptor. With multiple binding sites, the 67-kDa protein is ideally suited to direct the specific association between tropoelastin and highly glycosylated microfibrillar components.

A second possible function concerns cell movement or attachment. Cells using this receptor to adhere to, or move through, elastin- or laminin- (see below) rich matrices could alter cell matrix binding through interactions with glycoconjugates interacting at the carbohydrate site. This interaction could release the receptor from the protein ligand or "shed" the receptor from the cell-anchoring subunit. An advantage of this mechanism is that shedding the receptor by reducing its binding affinity to the cell surface does not require proteolytic cleavage of a transmembrane protein. Our finding that lactose inhibits fibroblast chemotaxis to elastin peptides reinforces the possible role of this receptor in mediating cell movement and suggests an association of one or both of the membrane-associated components with the cytoskeleton. The copurification of actin with the three elastin binding proteins is suggestive of cytoskeletal involvement, although a specific interaction between actin and receptor has not been demonstrated.

We have found a high degree of similarity between the 67-kDa elastin binding protein and the 67-72-kDa laminin receptor from tumor and muscle cells (unpublished results). The elastin receptor binds both laminin and elastin with similar affinities, can be eluted from elastin columns with laminin and from laminin columns with elastin peptides, and can be eluted from both elastin and laminin with lactose and synthetic VGVAPG peptide. It is of interest to note that Hall et al. (1988) have recently identified a 67-kDa laminin binding protein from muscle that, like the 67-kDa elastin binding protein described in this study, has properties of a peripheral membrane protein. It will be of great interest to determine how the two proteins are related.

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Structural and Functional Studies on the Sodium- and Chloride-Coupled γ -Aminobutyric Acid Transporter: Deglycosylation and Limited Proteolysis[†]

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ABSTRACT: The sodium- and chloride-coupled γ -aminobutyric transporter, an 80-kDa glycoprotein, has been subjected to deglycosylation and limited proteolysis. The treatment of the 80-kDa band with endoglycosidase F results in its disappearance and reveals the presence of a polypeptide with an apparent molecular mass of about 60 kDa, which is devoid of ¹²⁵I-labeled wheat germ agglutinin binding activity but is nevertheless recognized by the antibodies against the 80-kDa band. Upon limited proteolysis with papain or Pronase, the 80-kDa band was degraded to one with an apparent molecular mass of about 60 kDa. This polypeptide still contains the ¹²⁵I-labeled wheat germ agglutinin binding activity but is not recognized by the antibody. The effect of proteolysis on function was examined. The transporter was purified by use of all steps except that for the lectin chromatography [Radian, R., Bendahan, A., & Kanner, B. I. (1986) *J. Biol. Chem.* 261, 15437-15441]. After papain treatment and lectin chromatography, γ -aminobutyric transport activity was eluted with *N*-acetylglucosamine. The characteristics of transport were the same as those of the pure transporter, but the preparation contained instead of the 80-kDa polypeptide two fragments of about 66 and 60 kDa. The ability of the anti-80-kDa antibody to recognize these fragments was relatively low. The observations indicate that the transporter contains exposed domains which are not important for function.

The γ -aminobutyric acid (GABA)¹ transporter from rat brain is thought, just like other neurotransmitter transporters, to terminate the overall process of synaptic transmission (Iversen, 1971, 1973; Kuhar, 1973; Bennet et al., 1974). The protein which catalyzes cotransport of sodium, chloride, and GABA (Kanner, 1978, 1983; Kanner & Schuldiner, 1987;

Radian & Kanner, 1983; Keynan & Kanner, 1988) has been purified to near homogeneity with a rapid reconstitution assay

¹ Abbreviations: GABA, γ -aminobutyric acid; WGA, wheat germ agglutinin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, 135 mM NaCl, 2.5 mM KCl, 1.5 mM KH₂PO₄, 8 mM Na₂HPO₄, pH 7.4; buffer A, 0.1 M NaP_i, pH 6.8, 300 mM NaCl, 0.2% Triton X-100, 50 mM octyl β -glucoside; buffer B, 25 mM sodium citrate, 50 mM NaP_i, pH 5, 25 mM NaCl, 0.01% SDS; buffer C, 0.2 M NaP_i, pH 6.1, 100 mM EDTA, 2% NP-40, 2% β -mercaptoethanol.

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