

67-kD Elastin-binding Protein Is a Protective "Companion" of Extracellular Insoluble Elastin and Intracellular Tropoelastin

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Abstract. The 67-kD elastin-binding protein (EBP) mediates cell adhesion to elastin and elastin fiber assembly, and it is similar, if not identical, to the 67-kD enzymatically inactive, alternatively spliced β -galactosidase. The latter contains an elastin binding domain (S-GAL) homologous both to the aorta EBP and to NH₂-terminal sequences of serine proteinases (Hinek, A., M. Rabinovitch, F. W. Keeley, and J. Callahan. 1993. *J. Clin. Invest.* 91:1198–1205). We now confirm the functional importance of this homology by showing that elastolytic activity of a representative serine elastase, porcine pancreatic elastase, was prevented by an antibody (anti-S-GAL) and by competing with purified EBP or S-GAL peptide. Immunohistochemistry of adult aorta indicates that the EBP exists as a permanent component of mature elastic fibers. This observation, together with the *in vitro* studies, suggests that the EBP could protect insoluble elastin from ex-

tracellular proteolysis and contribute to the extraordinary stability of this protein. Double immunolabeling of fetal lamb aorta with anti-S-GAL and antitropoelastin antibodies demonstrated, under light and electron microscopy, intracellular colocalization of the proteins in smooth muscle cells (SMC). Incubation of SMC with galactosugars to dissociate tropoelastin from EBP caused intracellular aggregation of tropoelastin. A tropoelastin/EBP complex was extracted from SMC lysates by coimmunoprecipitation and cross-linking, and its functional significance was addressed by showing that its dissociation by galactosugars caused degradation of tropoelastin by endogenous serine proteinase(s). This suggests that the EBP may also serve as a "companion" to intracellular tropoelastin, protecting this highly hydrophobic protein from self-aggregation and proteolytic degradation.

THE extracellular assembly of tropoelastin into mature elastic fibers on the microfibrillar scaffold is mediated by a cell surface 67-kD elastin-binding protein (EBP)¹ that binds the VGVAPG hydrophobic repeat on tro-

poelastin, the cell membrane, and galactosugars via three separate sites (Hinek et al., 1988; Mecham et al., 1989, 1991). The coordinated release of both tropoelastin and the EBP from the cell surface under the influence of galactosugars was discovered to be the central mechanism regulating the extracellular assembly of elastic fibers (Hinek et al., 1988, 1991). We have recently established that the 67-kD EBP expressed by sheep aorta smooth muscle cells (SMC), which also binds laminin, demonstrates immunological and functional similarity, as well as sequence homology, and may be identical to the 67-kD, catalytically inactive variant of human β -galactosidase produced by alternative splicing (Morreau et al., 1988). We further showed that the VVG-SPSAQDEASPL domain, corresponding to a frame-shift-generated sequence unique to the alternatively spliced variant (S-GAL), is responsible for its elastin/laminin binding properties (Hinek et al., 1993). In addition, sequence homology among this domain and NH₂-terminal sequences of several serine elastases indicated that these proteins might share a common ligand-binding motif (Table I). Since electron microscopic analysis of immunogold labeling localized the EBP to mature elastic fibers (Hinek et al., 1993), we hypothesized that this protein, which mediates the proper extracel-

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1. **Abbreviations used in this paper:** anti-C-GAL, polyclonal antibody to synthetic peptide reflecting a carboxy-terminal sequence of human β -galactosidase; anti-C-term, polyclonal antibody to carboxy-terminal sequence of tropoelastin; anti-S-GAL, polyclonal antibody to the synthetic peptide reflecting an elastin-binding domain on alternatively spliced form of human β -galactosidase; BA-4, monoclonal antibody to VGVAPG hydrophobic sequence on elastin representing the elastin-binding protein-binding domain; BCZ, antibody to 67-kD elastin-binding protein isolated from bovine ciliary zonule; C-GAL, synthetic peptide PPQKNKDSWLDHV corresponding to the COOH-terminal sequence common for both alternatively spliced forms of human β -GAL; DTSSP, dithio-bis-sulfosuccinimidylpropionate; EBP, elastin-binding protein; GAM, goat anti-mouse; GAR, goat anti-rabbit; H1-20, monoclonal antibody to tropoelastin; Hsp, heat shock protein; HLE, human leukocyte elastase; PPE, porcine pancreatic elastase; PVDF, polyvinylidene difluoride; S-GAL, synthetic peptides VVGSPSAQDEASPL corresponding to a sequence of alternatively spliced form of human β -GAL containing EBP; SMC, smooth muscle cells.

Table I. Homology between S-GAL, EBP, and Serine Proteinases

Proteins	Sequences
Human S-GAL	<u>VVGSPSAQDEASPLS</u>
Sheep EBP	<u>VVGGTEAQRNSWPLQ</u>
Porcine pancreatic elastase	<u>VVGGTEAQRNSWPSQ</u>
Human prekallikrein	<u>IVGGTNSSWGEPWPQ</u>
Human plasminogen	<u>VVGGCVAHPHSWPWQ</u>
Human Factor IX	<u>VVGGEDAKPGQFPWQ</u>

Comparison of the unique elastin binding sequence from the spliced variant of human β -galactosidase (Morreau et al., 1989) with the sequence determined for CNBr fragment of purified sheep elastin EBP (Hinek et al., 1993). 7 of 14 residues are identical, and a further three substitutions have no effect on net charge. NH₂-terminal sequences of porcine pancreatic elastase and human prekallikrein, human plasminogen, and human Factor IX are also included for comparison (Shotton and Hartley, 1970; Doolittle and Feng, 1987). Regions of sequence identity between these proteins are underlined.

lular assembly of insoluble elastin, may also protect it by competing with extracellular serine proteinases. Moreover, preliminary immunohistochemical colocalization of the EBP and tropoelastin in intracellular compartments of aortic SMC allowed us to speculate that the EBP might bind to tropoelastin intracellularly (Hinek and Mecham, 1990). To further investigate the close relationship between these two proteins and, particularly, to establish whether the EBP might protect soluble tropoelastin and its insoluble polymer from degradation by serine proteinases, we developed the necessary reagents: a synthetic peptide reflecting the elastin binding motif (S-GAL) and an antibody raised to this sequence (anti-S-GAL).

In this report, we first showed in an *in vitro* assay that anti-S-GAL binds to porcine pancreatic elastase and, presumably, by occupying the NH₂-terminal of this representative serine elastase, prevented its association with elastin and precluded its elastolytic activity as judged by degradation of an [³H]elastin substrate. Conversely, blocking the EBP-binding domain on elastin by a monoclonal antibody to the VGVAPG hydrophobic sequence also prevented the elastolytic activity of PPE, as did preincubation of insoluble [³H]elastin with purified EBP or with S-GAL peptides. We next used immunogold labeling of fetal sheep aorta and confirmed coimmunolocalization of the EBP and insoluble elastin over elastic fibers. Moreover, double immunogold labeling, as well as immunofluorescence, showed intracellular colocalization of these two proteins in cultured aortic SMC, which actively synthesize elastin. Further biochemical studies were then carried out to show that the newly synthesized EBP and tropoelastin coimmunoprecipitated as a complex from lysates of metabolically labeled SMC, and that an excess of galactosugars that can dissociate this complex (Hinek et al., 1989, 1991) caused aggregation of intracellular tropoelastin and also resulted in its degradation by endogenous serine proteinases. Together, our morphological and biochemical data indicate that the EBP may serve as a protective companion protein for tropoelastin during its secretion and extracellular assembly and may also protect the longevity of insoluble elastin.

Materials and Methods

Materials

Chemicals and reagents were obtained as follows: bovine ligamentum

nuchae insoluble elastin, bovine tropoelastin, κ -elastin, α -elastin, BA-4 monoclonal antibody to the VGVAPG sequence on the tropoelastin molecule (Wrenn et al., 1986), and monospecific polyclonal antibody to bovine tropoelastin (Prosser et al., 1991) were purchased from Elastin Products (Pacific, MO). Porcine pancreatic elastase, glucosamine, galactosamine, lactose, proteinase inhibitors, protein A-Sepharose, and all reagent grade chemicals were purchased from Sigma Immunochemicals (St. Louis, MO). Media, fetal bovine serum, and other tissue culture reagents were obtained from Gibco Laboratories (Burlington, Ontario, Canada). All SDS-PAGE reagents were purchased from Bio-Rad Laboratories (Richmond, CA). The chemical cross-linker 3,3'-dithio-bis-sulfosuccinimidylpropionate (DTSSP) was purchased from Pierce Chemical Co. (Rockford, IL). The Immobilon P transfer membranes were supplied by Millipore Ltd. (Mississauga, Ontario, Canada). The N-acetyl-VVGSPSAQDEASPL oligopeptide (S-GAL), corresponding to the elastin binding domain of the alternatively spliced form of human β -galactosidase, and N-Ac-(C)PPQKNKDSWLDHV oligopeptide (C-GAL), corresponding to the COOH-terminal sequence of β -galactosidase, were synthesized using an automated oligopeptide synthesizer (BioLynx; Pharmacia, Uppsala, Sweden). Polyclonal antibodies to these peptides and the polyclonal antibody to human β -galactosidase were gifts from Dr. John Callahan (University of Toronto). The HI-20 monoclonal antibody was raised to 52-kD truncated tropoelastin isolated from ovine ductus arteriosus (Hinek and Rabinovitch, 1992). The BCZ monoclonal antibody to the bovine 67-kD elastin-binding protein (Mecham et al., 1988) was a gift from Dr. R. P. Mecham (Washington University, St. Louis, MO). The specific polyclonal antibody raised against a synthetic peptide representing the carboxy-terminal sequence of tropoelastin encoded by exon 36 (Rosenbloom et al., 1986) was kindly supplied by Dr. J. Rosenbloom (University of Pennsylvania, Philadelphia, PA). Species and type-specific secondary antibodies, goat anti-rabbit (GAR), and goat anti-mouse (GAM) conjugated with gold particles for EM immunolocalization were obtained from Janssen Life Science Products (Piscataway, NJ). HRP-conjugated secondary antibodies used in Western immunoblotting were supplied by Boehringer Mannheim Corp. (Indianapolis, IN). FITC-conjugated GAM and GAR secondary antibodies were obtained from ICN Immuno-Biologicals (Lisle, IL). The Affi-gel 10 and polyvinylidene difluoride (PVDF) membranes were from Bio-Rad Laboratories. The enhanced chemiluminescence Western blotting detection kit was from Amersham Canada Ltd. (Oakville, Ontario, Canada). The [³H]elastin substrate was prepared according to the methods used by Takahashi et al. (1973) to radiolabel insoluble elastin. Radioactive [³H]NaBH₄, [³H]serine, [³H]valine, and [¹⁴C]valine were supplied by New England Nuclear (Boston, MA).

Interactions between [³H]Elastin, Pancreatic Elastase, and the EBP

PPE is a serine proteinase in which the NH₂-terminal sequence displays the strongest homology with the elastin-binding domain of the EBP (Table I). In an attempt to test the functional significance of this sequence homology, we investigated whether an antibody (anti-S-GAL) raised to the elastin binding domain of the EBP may block or modify the putative elastin-binding sequence present at the NH₂-terminal of PPE. We first showed, however, that anti-S-GAL does indeed bind to PPE and does not react with the elastin substrate. Since the BA-4 antibody recognizing the receptor binding domain on elastin was also used in further experiments, specificity of this antibody was also tested by confirming that it binds to elastins and not to elastases.

The relative specificities of both anti-S-GAL and BA-4 antibodies was tested first by dot blot analysis and then by affinity chromatography. 200- μ g samples of PPE, human leukocyte elastase (HLE), κ -elastin, and α -elastin were dot blotted on PVDF membranes and incubated with either anti-S-GAL or with BA-4 antibodies (2 mg/ml diluted 1:100). The blots were then visualized with appropriate HRP-conjugated secondary antibodies.

Interactions between PPE and the above-mentioned antibodies were also tested in an additional experiment in which 2-mg samples of both anti-S-GAL and BA-4 antibodies were immobilized in triplicate on Affi-gel 10 as described previously (Hinek et al., 1991) and incubated for 1 h on ice with 100-ng aliquots of PPE dissolved in 50 mM Tris-HCl buffer. The affinity resins were then spun down, and the elastolytic activity of supernatants containing the unbound enzyme was assessed. Elastolytic activity of PPE was assessed in an *in vitro* assay measuring degradation of an insoluble [³H]elastin substrate as previously described (Leake et al., 1983; Banda and Werb, 1991). To eliminate possible residues of the EBP that might exist in the elastin preparation, the insoluble elastin used in all assays was extracted with 2 M sodium chloride and 0.2 M lactose before being labeled with tritium.

To block or modify the putative elastin-binding sequences present at the NH₂-terminal of PPE, 50-ng samples of this enzyme were dissolved in 200 μ l of assay buffer (50 mM Tris-HCl, pH 8, containing 150 mM NaCl, 10 mM CaCl₂, 0.02% Brij, and 0.02% sodium azide) and preincubated in an Eppendorf vial for 1 h with different concentrations of an anti-S-GAL (1 ng–100 μ g) or with 10 μ g of preimmune rabbit immunoglobulin. For comparison, PPE was also preincubated with 10 μ g of IgG from an antibody raised to synthetic peptides reflecting the COOH-terminal of β -galactosidase (anti-C-GAL). At the end of a 1-h preincubation, 200- μ g aliquots of [³H]elastin substrate (specific activity-1200 cpm/1 μ g) were added to each vial, and samples were incubated at 37°C for 6 h. At the end of the incubation, all samples were microcentrifuged (8,000 g for 4 min), and 100- μ l aliquots of supernatant containing the solubilized degradation products were mixed with 4 ml scintillation fluid and counted in triplicates in a liquid scintillation counter (Rackbeta model 1219; LKB Wallac, San Francisco, CA).

We also assessed the effect of blocking the hydrophobic domains on elastin that are responsible for binding to the EBP (Senior et al., 1984; Hinek et al., 1988; Mecham et al., 1989) and, presumably, also to elastases sharing NH₂-terminal sequences homologous to the EBP (Hinek et al., 1993). 200- μ g aliquots of [³H]elastin substrate suspended in 200 μ l of assay buffer were preincubated for 1 h with 0.01–10 μ g of IgG fraction isolated from a monoclonal antibody raised to the VGVAPG domain of elastin (Wrenn et al., 1986; Wrenn and Mecham, 1987). For comparison, the [³H]elastin substrate was also incubated with 10 μ g IgG from an antibody raised against a synthetic peptide representing the sequence at the COOH-terminus of tropoelastin, which does not bind the EBP (Rosenbloom et al., 1986), and with 10 μ g of normal mouse IgG. In an additional control experiment, the 200- μ g samples of [³H]elastin were also preincubated with 10 μ g of anti-S-GAL IgG. The substrate was then washed with TBS buffer before PPE addition. After a 1-h preincubation, all samples were mixed with 50-ng aliquots of PPE dissolved in 200 μ l of assay buffer and incubated for 6 h at 37°C.

To determine directly whether the EBP may also protect elastin, in separate experiments, 200- μ g samples of [³H]elastin substrate suspended in assay buffer were preincubated for 1 h with 50 μ g of S-GAL synthetic peptide or with 50 μ g of purified EBP in the presence or absence of 100 mM lactose before a 6-h incubation with 50-ng aliquots of PPE. As controls, the radioactive elastin substrate was also preincubated with 100 mM lactose or with 50 μ g of C-GAL or 10 μ g S-GAL synthetic peptides.

In all studies involving comparison of different treatments, the radioactivity (cpm/sample) released into the supernatant reflecting the degradation of [³H]elastin was assessed as described above, and the mean and standard deviations were calculated from triplicate assessments from three different experiments. The data were compared by ANOVA, and a Duncan test of multiple comparison was used to establish which of the groups were significantly different. Results in the graphs are expressed as percentages of control samples, reflecting values for degradation of radioactive elastin by PPE.

Immunoelectron Microscopy

For immunoelectron microscopy, a postembedding method was used (Hinek et al., 1991). Thin sections of fetal and adult ovine aorta, as well as adult ovine auricular cartilage and cultured fetal lamb aorta SMC, were placed on nickel grids and blocked with 1% BSA, 1% normal goat serum, and 0.5% Tween-20 in TBS. Extracellular elastin and intracellular tropoelastin were detected with HI-20, a monoclonal antibody to tropoelastin (Hinek and Rabinovitch, 1993), while the EBP present in the extracellular matrix and in intracellular compartments was detected with anti-S-GAL, an affinity-purified polyclonal antibody to the elastin/laminin-binding domain of the alternatively spliced variant of human β -galactosidase (Hinek et al., 1993). Both antibodies were used in a concentration of 5 μ g/ml, either separately on adjacent serial sections or in combination. We have previously shown that anti-S-GAL recognizes sheep EBP and does not cross-react with elastin (Hinek et al., 1993). The immune reaction was visualized with the appropriate GAR or GAM secondary antibodies conjugated with 15-, 10-, or 5-nm gold particles (as indicated in the figure legends). Sections were then stained with uranyl acetate and lead citrate. In all immunostaining procedures, controls involved substitution of nonimmune ascites fluid or normal rabbit serum for the primary antibody.

Immunofluorescence Microscopy

For immunofluorescent intracellular localization of the EBP and tropoelastin, fetal lamb aorta SMC cultured in Medium 199 with 10% FBS for 3 d in four-well plastic slides were washed in PBS, quickly permeabilized with acetone, cooled to –20°C, and were then incubated in the presence

or absence of 50 mM lactose in PBS for 15 min or 1 h. Smooth muscle cells were then washed in PBS, fixed for 2 min with 100% methanol, washed in PBS, blocked with 1% BSA and 5% normal goat serum in PBS, and incubated with the anti-S-GAL polyclonal antibody recognizing EBP and with HI-20 monoclonal antibody to tropoelastin, both in concentrations of 10 μ g/ml. The secondary antibodies were GAR fluorescein-conjugated immunoglobulin and rhodamine-labeled GAM antibody, both diluted 1:100. As a control for studies using a monoclonal antibody, normal ascitic fluid was used and, as a control for studies using a polyclonal antibody, normal rabbit serum was substituted for the primary antibody. Additional controls included secondary antibody alone.

Metabolic Labeling and Immunoprecipitation

To establish the intracellular relationship between tropoelastin and the EBP, metabolic labeling was carried out on confluent aorta SMC cultures at passage 2. The cells (50 \times 10⁶/25-ml flask) were washed and preincubated in serum-free Medium 199 for 4 h, then labeled with [³H]valine and [³H]serine (both 10 μ Ci/ml) in valine- and serine-free medium with 5% dialyzed FBS for 6 h at 37°C. At the end of the pulse period, the cell layers were washed in PBS, scraped, centrifuged, resuspended in 300 μ l of PBS with or without 5 μ l of the chemical cross-linker DTSSP, vortexed, and placed on ice (Nakai et al., 1992). After 30 min, the DTSSP-treated cells were rinsed with 2 mM glycine in PBS to block the cross-linker activity and were then rinsed with PBS. The cells were then lysed on ice for 15 min with 1% NP-40 in 50 mM TBS, pH 8, containing proteinase inhibitors in the following final concentrations: 2 mM benzamide, 2 mM EACA, 2 mM PMSF, 1 mM EDTA, 2 mM leupeptin, and 1 mg/ml Trasylol. After centrifugation at 12,000 g for 15 min, the supernatants were precleaned by 30 min incubation with 50 μ l of protein A-Sepharose beads, then divided into equal aliquots that were separately incubated with polyclonal antibody to tropoelastin and with the BCZ monoclonal antibody to bovine EBP or with anti-S-GAL for 1 h at 4°C, and then precipitated by mixing with 50 μ l of protein A-Sepharose beads. The precipitated immune complexes were washed four times in 50 mM TBS, pH 8, with proteinase inhibitors and once with 10 mM TBS, pH 6.8. Then they were resuspended in 2 \times Laemmli's sample buffer (Laemmli, 1970) containing DTT, boiled for 5 min, and analyzed by SDS-PAGE, followed by autoradiography and by Western immunoblotting with HI-20 monoclonal antibody to tropoelastin and with anti-S-GAL or BCZ antibodies recognizing the 67-kD EBP (all in concentrations of 2 μ g/ml). The reactions were visualized using the peroxidase-conjugated goat anti-mouse or peroxidase-conjugated goat anti-rabbit antibodies diluted 1:1,000 and developed with enhanced chemiluminescence detection kit according to the manufacturer's instruction.

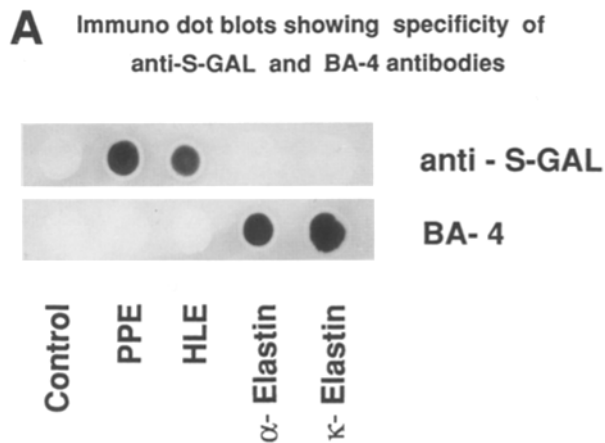
Effect of Galactosugars on Integrity of Tropoelastin

To establish whether the EBP protects intracellular tropoelastin against proteolysis, densely plated aorta SMC (50 \times 10⁶ cells/flask) at passage 2 were incubated for 18 h with 15 μ Ci/ml [¹⁴C]valine in valine-free medium with 5% dialyzed FCS. The cells were then scraped with a rubber policeman, placed on ice, rinsed well, and quickly sonicated for 10 s in water. Cell debris was spun down by microcentrifugation (8,000 g 2 min, at 4°C), and the cell lysate supernatant was mixed with an equal volume of 2 \times 0.1 M TBS, pH 8. 100- μ l aliquots were then incubated for 30 min or 3 h at 37°C, with and without 10 mM glucosamine, 10 mM galactosamine, 20 mM lactose, or 400 μ g/ml of chondroitin sulfate in the presence or absence of 5 mM EDTA, 2 mM PMSF, or 2 μ g/ml leupeptin, inhibitors of metalloproteinases, serine proteinases, and cysteine proteinases, respectively. The lysates were then immunoprecipitated with antitropoelastin antibody as described above. The integrity of labeled immunoprecipitated tropoelastin was established by SDS-PAGE followed by autoradiography and by Western blotting with the antielastin antibody as described above.

Results

Modification of Elastolytic Activity of Serine Elastases

Our experiments aimed at addressing the relative specificities of antibodies used in our studies showed that anti-S-GAL, an antibody raised to the elastin binding-domain of the EBP (Hinek et al., 1993), also recognized serine elastases (PPE and HLE) but not elastins immobilized on PVDF membranes. BA-4 antibody raised to the VGVAPG sequence



B PPE binds to immobilized anti-S-GAL

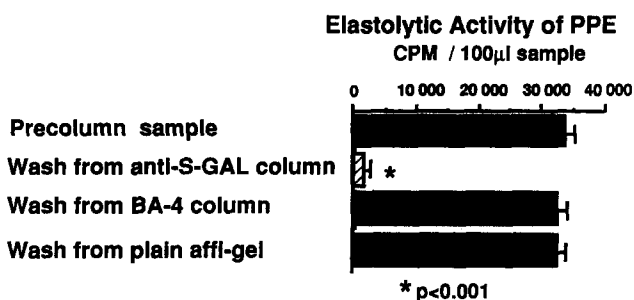


Figure 1. (A) Dot immunoblots showing that anti-S-GAL, an antibody raised to the elastin-binding domain of the EBP recognizes PPE and HLE, but does not recognize κ -elastin or α -elastin immobilized on PVDF membranes. BA-4 antibody raised to the VGV-APG sequence reflecting the receptor binding domain of elastin reacts with κ -elastin and α -elastin but not with PPE nor HLE. Control represents the blots incubated with preimmune sera and HRP labeled secondary antibodies. (B) The elastolytic activity of PPE assessed in precolumn samples and in the washes from the affinity columns made of Affi-gel immobilized anti-S-GAL and BA-4 antibodies incubated with this enzyme as described in Materials and Methods. Anti-S-GAL affinity resin but not immobilized BA-4 retained virtually all the PPE activity present in the precolumn samples. Radioactivity of degradation products (cpm/100- μ l sample) proteolytically released from the insoluble [3 H]elastin substrate to the supernatants was measured in triplicate; the mean and standard deviations were calculated.

reflecting the receptor-binding domain of elastin (Wrenn et al., 1986) strongly reacted with κ -elastin and α -elastin, but not with PPE nor HLE (Fig. 1 A). Moreover, anti-S-GAL but not BA-4 immobilized on Affi-gel retained all elastolytic activity of the PPE applied to the column (Fig. 1 B). These results indicate that anti-S-GAL binds to PPE, but not to elastin, and they further confirm the functional significance of the homology between the EBP and serine elastases suggested in our previous study (Hinek et al., 1993).

An [3 H]elastin degradation assay showed that preincubation of PPE with an anti-S-GAL IgG that recognizes its NH₂-terminal domain homologous to the elastin-binding motif of the EBP precluded degradation of this substrate in

Anti-S-GAL inhibits elastolytic activity of PPE

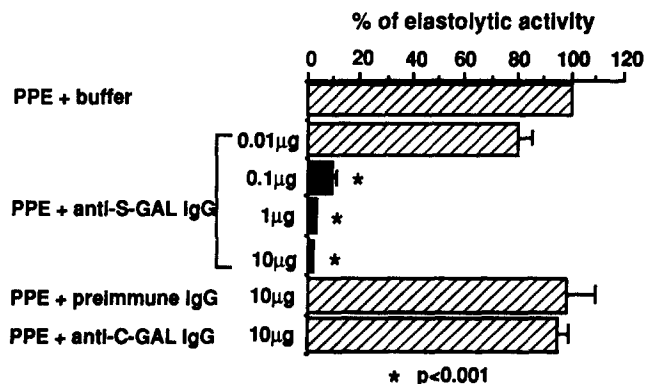


Figure 2. 1 h preincubation of 50 ng samples of PPE with different concentrations of an anti-S-GAL antibody that recognizes its NH₂-terminal domain homologous to the elastin-binding motif of the EBP precluded degradation of a 200- μ g aliquot of [3 H]elastin in a dose-dependent manner, presumably by blocking binding of this serine proteinase to the substrate. The elastolytic activity of the PPE was not affected by preincubation of the enzyme with a preimmune rabbit IgG or with anti-C-GAL IgG, an antibody that recognizes the COOH-terminal domain of the EBP, but not its elastin-binding domain. Radioactivity of degradation products (cpm/sample) proteolytically released from insoluble [3 H]elastin substrate was measured in triplicate; the mean and standard deviations were calculated. Results of three separate experiments are expressed as percentages of control samples where the PPE preincubated with TBS buffer was added to radioactive elastin substrate.

a dose-dependent manner, presumably by blocking binding of this serine proteinase to [3 H]elastin. The elastolytic activity of the PPE was not affected by preincubation of the enzyme with a preimmune rabbit IgG or with anti-C-GAL IgG, an antibody that recognizes the COOH-terminal domain of the EBP, but not its elastin-binding domain (Fig. 2).

The [3 H]elastin was also protected from PPE-dependent degradation if the substrate was preincubated with the IgG fraction of BA-4 antibody raised to VGVAPG, a peptide reflecting the EBP-binding domain of elastin. Preincubation of the substrate with an antibody to the carboxy-terminal of tropoelastin (anti-C-term) did not protect it from proteolysis, presumably because it does not block the binding of the PPE to the elastin substrate. An additional control showed that anti-S-GAL antibody has not been retained by the [3 H]elastin substrate and that such a pretreatment did not protect it from subsequent PPE-dependent degradation (Fig. 3).

The protective effect of EBP was also observed when, before the addition of PPE, the [3 H]elastin substrate was preincubated with purified EBP or with S-GAL synthetic peptides, but not with C-GAL synthetic peptides (Fig. 4). The EBP-dependent protection of the substrate was abolished, however, in the presence of lactose, which prevents the association of these two proteins. An additional control in which [3 H]elastin preincubated with lactose alone was subjected to PPE showed the same degradation rate of the substrate as in samples preincubated with TBS buffer. This indicates that this galactosugar alone does not interfere with direct association between elastin and PPE.

Blocking of VGVAPG sequences on elastin inhibits its PPE dependent degradation

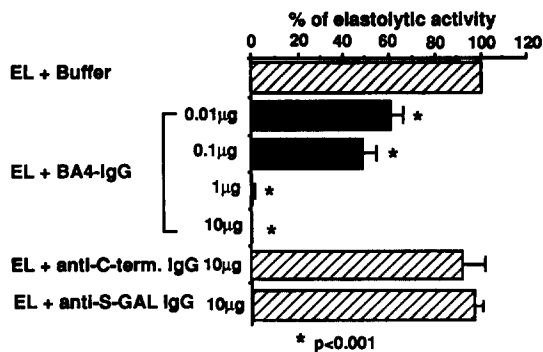


Figure 3. Preincubation of 200- μ g aliquots of [3 H]elastin substrate with different concentrations of a BA-4 monoclonal antibody that blocks the EBP binding domain on elastin (VGVAPG) protects the substrate from PPE-dependent degradation in a dose-dependent manner. Anti-C-term-IgG, an antibody to a synthetic peptide at the COOH-terminus of tropoelastin, does not influence degradation of elastin by PPE (50 mg/sample) because it does not influence binding between enzyme and substrate. Additional control shows that anti-S-GAL does not bind to [3 H]elastin and does not affect its proteolysis while preincubated with this substrate and then washed with TBS buffer before PPE addition. Results of three separate experiments are expressed as percentages of control samples where the [3 H]elastin was preincubated with TBS buffer before incubation with PPE.

Elastin and 67-kD EBP Colocalize over Elastic Fibers and in Intracellular Compartments

Electron microscopic immunogold labeling using anti-S-GAL demonstrated extracellular localization of the EBP over elastic fibers, both in cultures of fetal lamb aorta containing cells actively producing elastin, as well as in aorta from adult sheep where active elastin synthesis is no longer taking place (Fig. 5, A and B, respectively). Moreover, the EBP also localized over elastic fibers in the auricular cartilage of adult sheep, suggesting that its incorporation takes place in other tissues where elastin is deposited (Figure 5 C).

Double immunogold labeling of fetal lamb aorta not only confirmed association of elastin and the EBP over extracellular elastic fibers, but also showed that these two proteins colocalize intracellularly. While we cannot be certain, the site of colocalization would likely be the rough endoplasmic reticulum and the secretory vesicles of smooth muscle cells actively producing elastin (Fig. 6).

Immunofluorescent staining of cultured aorta SMC with anti-S-GAL revealed a strong fibrillar pattern over the entire cell surface of lightly fixed and nonpermeabilized cultured fetal aorta SMC similar to that previously described with the BCZ antibody (Hinek et al., 1991). In permeabilized cells, anti-S-GAL localizes to the perinuclear region. Moreover, the polyclonal anti-S-GAL antibody shows a pattern of intracellular localization similar to that seen using the H1-20 monoclonal antibody to tropoelastin (Fig. 7, A and B, respectively). The overlapping of the immunostaining with both antibodies detected with two different fluorescent labels was apparent on double-exposed microphotographs (Fig. 8 B) and strongly suggested intracellular colocalization of EBP and tropoelastin.

EBP and S-GAL peptide protect elastin from PPE-dependent degradation

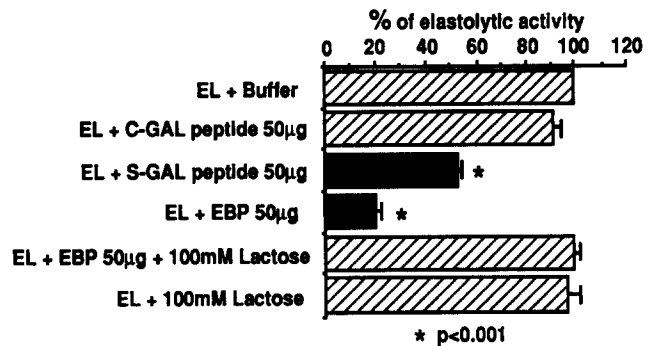


Figure 4. Binding to [3 H]elastin of purified sheep EBP or of the synthetic S-GAL peptide reflecting the elastin binding domain of the spliced variant of β -galactosidase, but not C-GAL peptide reflecting the carboxy-terminal sequence of β -galactosidase (all in concentration 50 μ g/sample) protect the 200- μ g samples of the substrate from degradation by 50-ng aliquots of PPE. The EBP does not protect the substrate in the presence of 100 mM lactose since lactose prevents association of the EBP and elastin (Hinek et al., 1988). Lactose itself does not affect elastolytic activity of PPE. Radioactivity of degradation products (cpm/sample) proteolytically released from the insoluble [3 H]elastin substrate to the supernatants was measured in triplicate; the mean and standard deviations were calculated. Results of three separate experiments are expressed as percentages of control samples where the [3 H]elastin was preincubated with TBS buffer before incubation with PPE.

Permeabilized aortic SMC incubated for 15 min with 100 mM lactose showed irregular punctate and focal distribution of the EBP. This was in contrast to the nontreated cells showing condensed perinuclear immunostaining. Permeabilized cells incubated for 1 h with PBS showed perinuclear localization of tropoelastin (Fig. 8 A), while in cells exposed to lactose, tropoelastin accumulated in dense aggregates that were located mostly at the cell periphery, suggesting a process of intracellular coacervation. (Fig. 8 C).

Tropoelastin and EBP Form an Intracellular Complex

Biochemical studies were next carried out to determine whether the intracellular colocalization of tropoelastin and EBP reflects complex formation. Labeling of cultured aortic SMC with [3 H]valine and [3 H]serine followed by cell lysate immunoprecipitation with a polyclonal antibody to tropoelastin, revealed a 67-72-kD broad band of metabolically labeled product that migrated on SDS-PAGE and was detected by autoradiography. Western immunoblotting revealed, however, that there was a heterogenous product reacting either with H1-20 monoclonal antibody to tropoelastin or with anti-S-GAL and BCZ antibodies, both of which recognize the EBP (Fig. 9 A). This suggested that tropoelastin and EBP may form an intracellular complex that is immunoprecipitated with antitropoelastin, but is separated by SDS-PAGE chromatography. This was further confirmed in experiments where the immunoprecipitation of proteins present in SMC lysates was preceded by exposure of metabolically labeled cells to the water-soluble chemical cross-linker DTSSP. We observed that both an antitropoelastin antibody, as well as

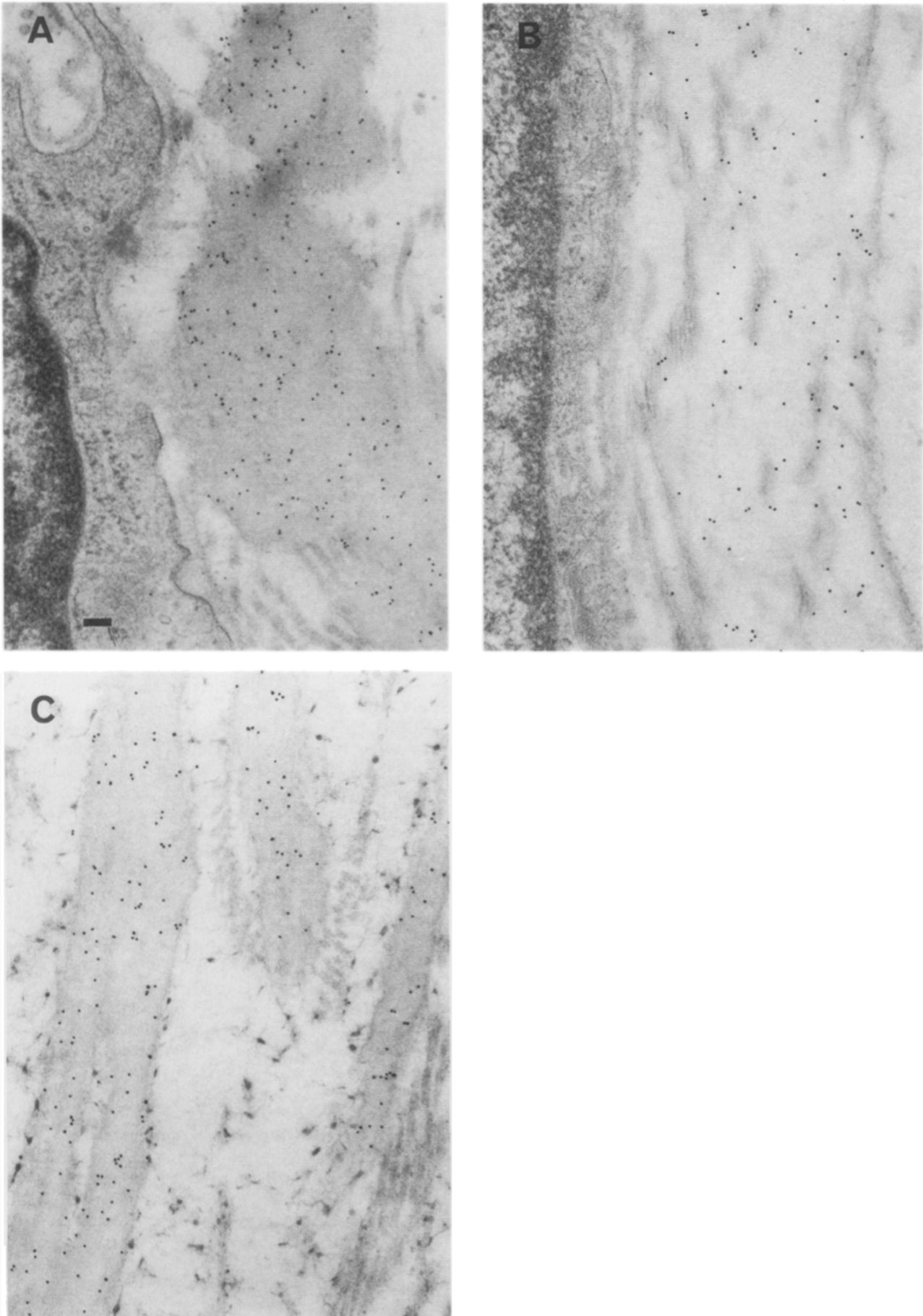


Figure 5. Anti-S-GAL, an antibody (marked by 10-nm gold particles) recognizing the EBP, localizes exclusively to elastic fibers present in the extracellular matrix of fetal lamb aorta (A), and in matrices of adult sheep aorta (B) and auricular cartilage (C). Bar, 0.1 μm .

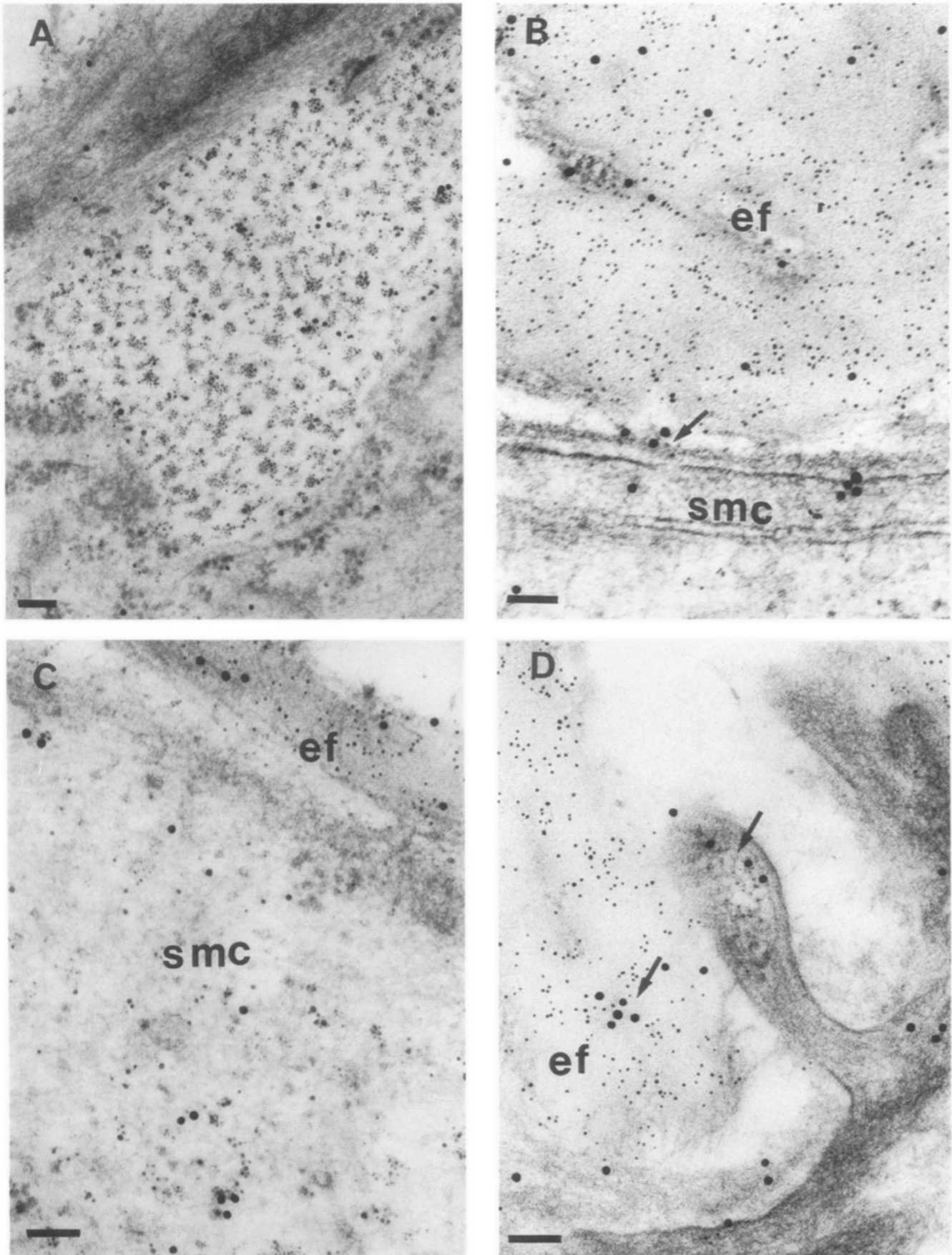


Figure 6. Representative immunoelectron micrographs with double immunogold labeling of 138-d gestation (term = 145 d) fetal lamb aorta demonstrating intracellular and extracellular colocalization of tropoelastin and EBP. Anti-S-Gal recognizing 67-kD EBP is detected with 15-nm gold particles, while a monoclonal antibody to tropoelastin is marked with 5-nm gold particles. Bar, 0.1 μm . (A) Tropoelastin and EBP colocalize in cisterna of rough endoplasmic reticulum of a smooth muscle cell. (B) Some of the EBP also colocalizes to newly produced microfibrils (*arrow*) and in the extracellular elastic fiber (*ef*). (C) In culture of smooth muscle cells (*smc*) from the same gestation aorta, the EBP colocalizes with tropoelastin intracellularly and over newly assembled *ef*. (D) A cell process of cultured smooth muscle cell is seen adjacent to a newly forming *ef*. Tropoelastin (5-nm) and the EBP (15-nm) appear to be secreted together and then incorporated into the elastic fiber (*arrows*).

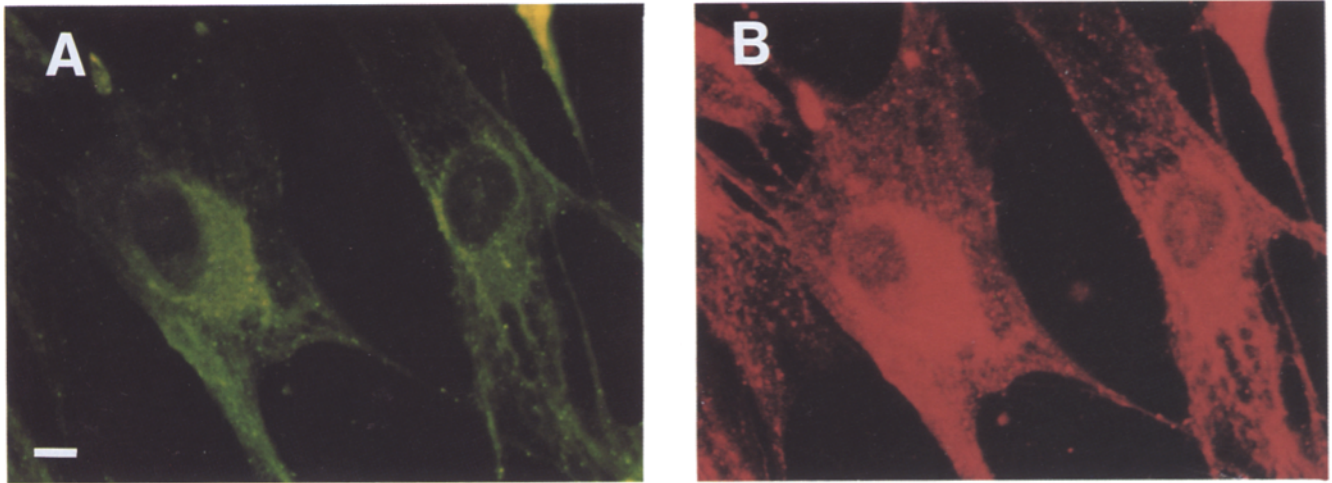


Figure 7. In permeabilized aortic SMC, double immunostaining with a fluorescein-marked polyclonal anti-S-GAL antibody recognizing the 67-kD EBP (A) and with a rhodamine-labeled HI-20 monoclonal antibody to tropoelastin (B) shows similar intracellular localization of these two proteins. Bar, 10 μ m.

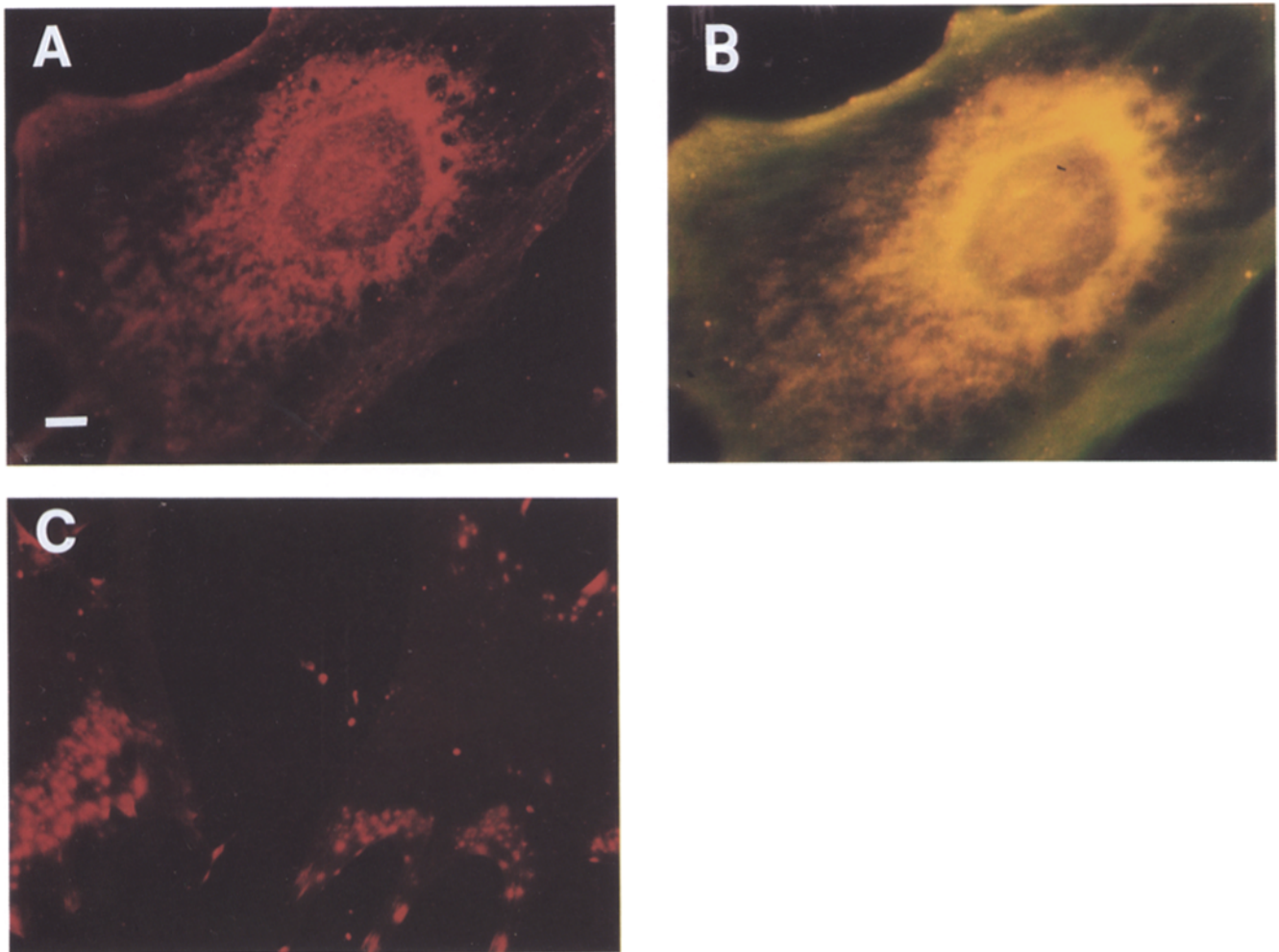


Figure 8. (A) In permeabilized aortic smooth muscle cell incubated for 1 h with PBS, tropoelastin detected with monoclonal HI-20 antibody followed by rhodamine conjugated GAM secondary antibody localizes mostly in perinuclear regions (ER and Golgi) and in the secretory vesicles. Bar, 5 μ m. (B) Double-exposed micrograph of the same cell double stained with a polyclonal anti-S-GAL and with monoclonal HI-20 antitropoelastin antibody (detected respectively with fluorescein and with rhodamine) show yellow-colored, overlapping immunostaining, suggesting intracellular colocalization of EBP and tropoelastin. (C) In permeabilized aortic smooth muscle cells incubated for 1 h with 100 mM lactose, tropoelastin localizes to dense aggregates accumulated in the cell periphery (which suggests its intracellular coacervation). Immunostaining with a monoclonal antitropoelastin antibody was followed by rhodamine-conjugated GAM secondary antibody.

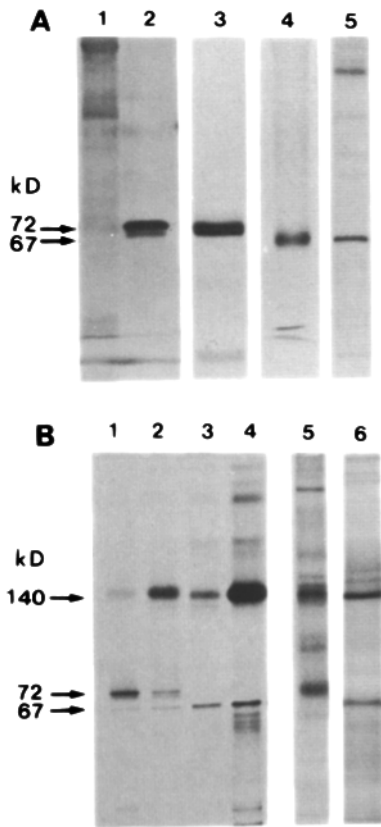


Figure 9. (A) A polyclonal antibody to tropoelastin precipitates a 67-72-kD metabolically labeled product from lysates of [^3H]valine and [^3H]serine pulsed aortic SMC as detected by SDS-PAGE followed by autoradiography (lane 2). Consecutive Western blotting with an HI-20 monoclonal antibody to tropoelastin (lane 3) and with anti-S-GAL and BCZ antibodies recognizing the 67-kD elastin binding protein (lanes 4 and 5, respectively) revealed a heterogeneous character of this species, suggesting that tropoelastin and EBP form a complex that can be immunoprecipitated with the single antibody. Lane 1 shows that the control preimmune rabbit serum does not precipitate any of these proteins from SMC lysates. (B) SDS-PAGE followed by autoradiography shows that when the immunoprecipitation of proteins present in SMC lysates was preceded by exposure of metabolically labeled cells to the water-soluble chemical cross-linker DTSSP for 15 min (lanes 1 and 3) or for 30 min (lanes 2 and 4), both antitropoelastin antibody (lane 1 and 2) and BCZ antibody recognizing the lectin binding site of the EBP (lanes 3 and 4) precipitate a 140-kD species in addition to 67- and 72-kD proteins. The longer exposure to chemical cross-linker (30 min) caused increased accumulation of the 140-kD species immunoprecipitable with both antibodies (lanes 2 and 4). The additional Western blot revealed that this 140-kD band also reacts with HI-20 monoclonal antibody to tropoelastin (lane 5) and/or with a polyclonal antibody to human β -galactosidase that also recognizes its 67-kD spliced variant and the sheep EBP (lane 6).

the BCZ antibody that recognizes the lectin-binding site of the EBP but not the S-GAL antibody, precipitated a 140-kD species in addition to 67- and 72-kD proteins. Since the Western blot revealed that the 140-kD band reacted either with HI-20 monoclonal antibody to tropoelastin or with a polyclonal antibody to human β -galactosidase that also recognizes its 67-kD spliced variant and the sheep EBP (Hinek et al., 1993) (Fig. 9 B), this indicates that a complex be-

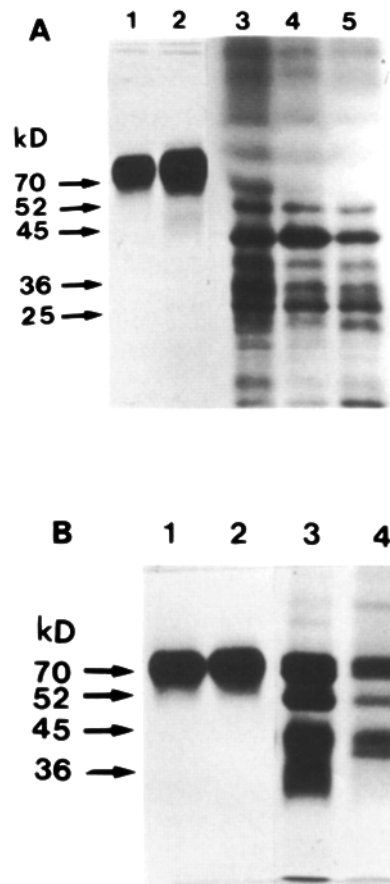


Figure 10. (A) SDS-PAGE followed by autoradiography shows that the SMC lysates incubated for 3 h with control buffer or with 10 mM glucosamine (lanes 1 and 2, respectively) contain the 68-72-kD tropoelastin (product of immunoprecipitation with a polyclonal antibody to tropoelastin). In contrast, the SMC lysates treated with 10 mM galactosamine, 20 mM lactose, or 400 $\mu\text{g}/\text{ml}$ of chondroitin sulfate (lanes 3, 4, and 5, respectively) contain multiple low molecular weight degradation products of tropoelastin, while the 68-72-kD tropoelastin is no longer apparent. (B) The SMC lysate incubated for 3 h with control TBS buffer containing 68-72-kD tropoelastin (lane 1). The lysate incubated with 20 mM lactose in the presence of 2 mM PMSF still contains the undegraded tropoelastin (lane 2), while the lysates treated with 20 mM lactose in the presence of 0.2% leupeptin or 5 mM EDTA (lanes 3 and 4, respectively) show the apparent tropoelastin degradation products.

tween these two proteins was indeed formed. The presence of immunoprecipitated 67- and 72-kD proteins indicates that some of the tropoelastin and EBP may not complex intracellularly.

It is interesting to note that the anti-S-GAL antibody did not recognize the 140-kD complex and precipitated only the 67-kD protein (data not shown). This probably resulted from the fact that anti-S-GAL, which recognizes the elastin-binding domain of the EBP, can only bind to those EBP molecules that are not already bound to tropoelastin. The same epitope present in EBP molecules engaged in the complex was saturated by tropoelastin and was not accessible to anti-S-GAL.

Effect of Galactosugars on the Integrity of Intracellular Tropoelastin

Smooth muscle cell lysates treated in the absence of proteinase inhibitors with galactosugars that dissociated the EBP-tropoelastin complex contained multiple low molecular weight species immunoprecipitable with the antitropoelastin antibody. This apparent degradation of tropoelastin was in contrast to the single 68–72-kD product of immunoprecipitation observed in untreated or glucosamine-treated lysates. As assessed by SDS-PAGE followed by autoradiography, even a 30-min exposure to lactose or galactosamine but not to glucosamine caused degradation of 68–72-kD tropoelastin synthesized by aortic SMC. The 3-h exposure was usually sufficient for complete disappearance of 68–72-kD tropoelastin and appearance of 52, 46, 36, 30, and 25 kD degradation products that were precipitable with antielastin antibody (Fig. 10 A). Cell lysates incubated for longer time periods contained even smaller fragments that appeared as a smear below 25 kD on SDS-PAGE (data not shown). The galactosugar-induced accelerated degradation of tropoelastin was inhibited by addition of PMSF, but not by leupeptin or EDTA (Fig. 10 B). This suggested that tropoelastin released from its complex with the EBP is subjected to enzymatic degradation by endogenous serine proteinase(s) present in the cell lysate.

Discussion

Recent evidence from our laboratory suggests that the 67-kD EBP is functionally and immunologically similar and may, in fact, be identical to an alternatively spliced form of β -galactosidase that has lost its catalytic activity and lysosomal targeting, but has retained its ability to bind galactosugars, and has acquired a frameshift generated sequence allowing it to bind elastin and laminin (Hinek et al., 1993). An antibody (anti-S-GAL) prepared to a synthetic peptide corresponding to this elastin/laminin-binding motif recognized the 67-kD EBP isolated from sheep aorta on Western blotting and had the same distribution of immunolocalization as the EBP on SMC and elastic fibers of sheep aortic tissue. We then established homology between the elastin/laminin-binding sequence (VVGSPSAQDEASPL) from the spliced variant of human β -galactosidase and a sequence (VVGTEAQRNSWPL) from sheep EBP, as well as a sequence (VVGTEAQRNSWPS) present at the NH₂-terminal of porcine pancreatic elastase (Hinek et al., 1993). Indeed, homologous sequences are also found at the NH₂-terminals of a wide variety of other serine proteases, most of which do not cleave insoluble elastin but do, however, digest tropoelastin (Shotton and Hartley, 1970; McKenzie and White, 1991; Doolittle and Feng, 1987; Doolittle, 1989). This homology suggested that the sequence might represent a common elastin-binding motif on EBP and these elastolytic enzymes. Moreover, since the EBP binds to the VGVAPG hydrophobic domains on elastin, we speculated that serine elastases should also bind to these domains. Therefore, saturation of the VGVAPG domains by the EBP might prevent binding of serine proteinases to tropoelastin, hence protect the substrate against degradation. In the present study, we used purified EBP, a synthetic peptide reflecting its elastin-binding motif, and an antibody raised to this sequence in a series of experiments

aimed at determining whether the EBP may bind and protect insoluble extracellular insoluble elastin, as well as intracellular soluble tropoelastin, from proteolytic degradation.

The results of our experiments showing the ability of anti-S-GAL to bind to PPE and block the elastolytic activity of this serine elastase validated our hypothesis. The fact that the elastolytic activity of PPE was not affected by exposure to an antibody (anti-C-GAL) that also recognizes EBP but not its elastin-binding domain seems to rule out nonspecific IgG-mediated interactions. Moreover, anti-S-GAL did not react with immobilized soluble preparations of elastin (Fig. 1) or with insoluble elastin (Hinek et al., 1993). The latter was also confirmed by the fact that this antibody incubated with tritiated elastin was easily washed away from this insoluble substrate and did not prevent its subsequent PPE-dependent degradation (Fig. 3).

The proposed mechanism of elastin-elastase interaction was further confirmed by the fact that blocking the EBP-binding hydrophobic VGVAPG sequence on elastin, either by the BA-4-specific monoclonal antibody, by purified EBP, or by an S-GAL synthetic peptide, appeared to prevent binding of active PPE to the substrate. The dose dependence of this phenomenon indicated that complete saturation of VGVAPG hydrophobic domains on elastin by the EBP or complete blocking by monoclonal antibody is necessary to fully prevent the enzyme-substrate interaction.

Our immunomorphological studies of fetal aorta indicate that some of the EBP is secreted together with tropoelastin and becomes incorporated into fully cross-linked elastic fibers. A highly regulated process of tropoelastin molecule deposition onto microfibrils appears to be necessary for appropriate cross-linking of tropoelastin and assembly of the mature elastin fiber (Mecham and Heuser, 1991). We have previously shown that either a deficiency in EBP or shedding of the EBP from cell surfaces by excess galactosugars disrupts assembly of elastic fibers by auricular chondrocytes and vascular SMC (Hinek et al., 1988, 1991; Hinek and Rabinovitch, 1993). This allowed us to speculate that the EBP presents tropoelastin molecules on the cell surface, and may then use its lectin site to dock to a galactosylated microfibrillar protein(s) (Gibson et al., 1989) and successively release the bound tropoelastin onto the microfibrillar scaffold.

The presence of the EBP in fully cross-linked elastic fibers from mature aorta and auricular cartilage suggests that this protein should be considered as a permanent component of those fibers. Since the results of our *in vitro* experiments showed that the EBP protects insoluble elastin from enzymatic degradation, one can speculate that the EBP, whether "trapped" by the rapidly polymerizing elastin or strategically located, may protect insoluble elastin from proteolysis and contribute to the extraordinary stability of elastic fibers *in vivo*. During development, the EBP may modulate the reciprocal interaction between elastin and elastases, controlling the dynamic remodeling processes that regulate the dimension of elastic fibers. There can be both endogenous vascular (Hornebeck et al., 1975; Robert and Robert, 1980; Todorovich-Hunter et al., 1992; Zhu et al., 1993), as well as extravascular sources of serine elastolytic activity. The EBP would not, however, protect insoluble elastin from metalloelastases or cysteine proteinases.

Results of immunofluorescent and electron microscopic

immunolocalization showing that EBP and tropoelastin colocalize intracellularly led to the suggestion that these proteins may not only coexist in the same intracellular compartment, but may actually form a complex at an early stage in the secretory pathway. This assumption was confirmed by the fact that a polyclonal antibody to tropoelastin precipitated both EBP and tropoelastin from SMC lysates as judged by immunoreactivity on Western blots with a monoclonal antibody to tropoelastin, as well as with two different antibodies that recognize the EBP. Subsequent treatment of radiolabeled SMC with DTSSP, a water-soluble chemical cross-linker, followed by immunoprecipitation with an anti-tropoelastin or with EBP antibodies resulted in a 140-kD complex recognized by both antibodies. The presence of additional 67- and 72-kD proteins immunoprecipitated with these antibodies indicates, however, that some of the tropoelastin and EBP may not complex intracellularly and may be secreted in an uncomplexed form.

Once we established that most of the EBP and tropoelastin form an intracellular complex, we were eager to elucidate the functional significance of this phenomenon. Our previous studies on ductus arteriosus SMC (Hinek and Rabinovitch, 1993) showed that those cells, deficient in EBP, deposit little insoluble elastin when compared with aortic SMC, and they secrete an abundant soluble 52-kD truncated form of tropoelastin that lacks the COOH-terminal and appears to be a product of intracellular degradation. We speculated that the EBP deficiency is responsible for the proteolytic intracellular degradation of tropoelastin in ductus SMC. The present studies confirmed that there is proteolytic degradation of the newly produced tropoelastin when there is dissociation of the EBP from tropoelastin as a result of exposure of the SMC lysates to lactose (Hinek et al., 1988, 1991). Since we showed that lactose is not itself elastolytic, and that it does not accelerate elastolytic activity of exogenous PPE, this treatment appears to allow newly produced tropoelastin to become more accessible to endogenous serine proteinase(s) present in the lysate. Moreover, the fact that PMSF but not EDTA or leupeptin prevents the degradation of newly synthesized tropoelastin indicates that endogenous serine proteinase(s) are responsible, and that intracellular metalloproteinases or cysteine proteinases do not participate in the initial stages of tropoelastin degradation.

We also speculate that EBP-tropoelastin complex formation may protect tropoelastin against premature self-aggregation (coacervation). Because of its extreme hydrophobicity, tropoelastin in solution tends to undergo self-aggregation under physiological conditions (Urry et al., 1969; Volpin et al., 1976). The maximal coacervation of tropoelastin occurs in or close to its isoelectric point (Partridge et al., 1980), and modification of this protein by blockage of free amino groups resulting in a decrease in the isoelectric point has limited tropoelastin coacervation in vitro (Podrazky and Jackson, 1976). While coacervation may be an important aspect of extracellular fiber assembly, the premature intracellular aggregation of this protein may be detrimental and irreversibly inhibit secretion of tropoelastin. On the other hand, the EBP as a "companion" protein that binds and blocks the exposed hydrophobic VGVAGG-like domains on tropoelastin would block its final folding and ensure that aggregation does not occur intracellularly until tropoelastin is released from the correct site on the cell surface.

This is in accord with the general theory of intracellular chaperoning of unfolded proteins by the heat shock proteins (Hsp) (Agard, 1993). The blockade of exposed hydrophobic patches on numerous secreted proteins requires a factor that can reversibly bind to them and protect against premature aggregation (Langer et al., 1992; Landry et al., 1992). The 67-kD EBP, like Hsp-70 and its relatives, meets this requirement. In the case of Hsp-70, the release of bound protein may require stimulation of endogenous ATPase, while the EBP can dissociate from tropoelastin under the influence of galactosugars that bind to the lectin site of this 67-kD chaperone. This process would appear to unmask its elastin binding domain (S-GAL). Our immunofluorescent localization of tropoelastin and EBP showed that even a brief exposure to lactose caused dissociation of the intracellular complex and led to aggregation of tropoelastin in the cell periphery. On the other hand, we have previously shown (Hinek et al., 1992) that binding of the purified EBP or S-GAL synthetic peptides to soluble elastin delays its temperature-dependent coacervation as monitored in an in vitro spectrophotometric assay.

On the whole, our results suggest that the 67-kD EBP homologous to alternatively spliced β -galactosidase binds tropoelastin intracellularly, escorting this highly hydrophobic protein through the secretory pathways and protecting it from premature self-aggregation and premature proteolytic degradation. In point of fact, it may represent another molecular chaperone distinct from the family of heat shock proteins. Moreover, some of this "companion protein," which is secreted as a complex with tropoelastin and mediates its orderly extracellular assembly upon the microfibrillar scaffold, gets incorporated as a permanent component of elastic fibers, which may protect the longevity of insoluble elastin.

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