In this report we demonstrate that soluble peptides, elastin degradation products stimulate proliferation of arterial smooth muscle cells. We show that these effects are due to generation of intracellular signals transduced through the cell surface elastin receptor, which consists of peripheral 67-kDa elastin-binding protein (EBP) (spliced variant of β-galactosidase), immobilized to the transmembrane sialidase and the protective protein. We found that elastin receptor-transduced signaling triggers activation of G proteins, opening of L-type Ca2+ channels, and a sequential activation of tyrosine kinases: FAK, c-Src, platelet-derived growth factor-receptor kinase and then Ras-Raf-MEK1/2-ERK1/2 phosphorylation cascade. This, in turn, causes an increase in expression of cyclins and cyclin-dependent kinases, and a consequent increase in cellular proliferation. The EBP-transduced signals also induce tyrosine kinase-dependent phosphorylation of β-tubulin, LC3, microtubule-associated protein 1, and α-actin and troponin-T, which could be linked to reorganization of cytoskeleton. We have also disclosed that induction of these signals can be abolished by anti-EBP antibody or by galactosugars, which cause shedding of EBP from the cell surface. Moreover, elastin-derived peptides did not induce proliferation of EBP-deficient cells derived from patients bearing a nonsense mutation of the β-galactosidase gene or sialidase-deficient cells from patients with congenital sialidosis.

It has been well established that formation of neointima in vascular diseases is associated with impaired assembly of tropoelastin into insoluble elastin (1–6) and with extensive degradation of the elastin-rich extracellular matrix by numerous proteinases leaking from the serum and secreted from the infiltrating platelets, leukocytes, and activated vascular cells (7–10). It has also been suggested that local accumulation of non-assembled tropoelastin and small elastin-derived peptides may constitute an important factor in the activation of the normally quiescent medial SMC1 into the proliferative and migratory phenotype, which participates in the formation of the occlusive neointima in vascular diseases (2, 3, 11–15). Results of in vitro studies have also established that elastin-derived peptides can stimulate proliferation of human skin fibroblasts (16), lymphoblasts (17), and several types of cancer cells (18, 19) as well as cellular chemotaxis and chemokinesis (18, 20–24).

Elastin does not contain the RGD sequence and does not interact with cell surface integrins. Our previous studies demonstrated that numerous cell types, including vascular myocytes, express the cell surface elastin receptor complex, which consists of three subunits (25, 26), and that the average cell contains ~2 × 105 elastin binding sites with the binding affinity (Kd) of 8 nM (27). We found that two of those subunits (55- and 61-kDa) are anchored to the plasma membrane, whereas the third, a peripheral 67-kDa protein, actually binds elastin (25). This major functional component of the receptor complex was named the elastin binding protein (EBP). The repeat heptapeptide in tropoelastin, VGVAPG, has been identified as a chief ligand for high affinity binding to this cell surface receptor (25–27). It has been later established that diverse peptides maintaining GXXPG sequence, including the LGTIPG sequence present on the domain V of B1 chain of laminin, can also bind to the EBP and induce similar cellular effects (28–32). Importantly, we found that the EBP has additional galactolentin properties (25), hence, it may also bind moieties containing galactosugars through a separate binding domain. We have learned, however, that binding of these carbohydrate-bearing moieties causes conformational changes of the 67-kDa protein, so that it loses its ability to bind elastin and separates from the complex with other receptor subunits (1, 25). Sequencing of the EBP isolated from arterial SMC and molecular cloning led us to the discovery that this protein is identical to the 67-kDa, enzymatically inactive, alternatively spliced variant of human β-galactosidase (S-GAL) and that it binds elastin and laminin through the domain located in the unique frameshift-generated sequence (33, 34). We also characterized the 61-kDa subunit of the elastin receptor as sialidase (neuraminidase) (EC 3.2.1.18) and the 55-kDa subunit as the protective protein, also called carboxypeptidase A or cathepsin A (EC 3.4.16.1) (35).

Previous studies lead only to a partial disclosure of the signaling pathways that may be linked to the EBP. We, and others, show that interaction between elastin-derived peptides and EBP residing on the surface of several cell types results in a rapid and transient increase in free intracellular Ca2+ (18, 29), tracellular signal-regulated protein kinase; FAK, focal adhesion kinase; FCS, fetal calf serum; FBS, fetal bovine serum; LC3, light chain 3 microtubule-associated protein 1; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; PDGF, platelet-derived growth factor; S-GAL, spliced variant of β-galactosidase; PBS, phosphate-buffered saline; JNK, c-Jun N-terminal kinase.
36) and that the EBP-mediated opening of calcium channels involves pertussis toxin-sensitive G proteins and activation of phospholipase C and protein kinase C in fibroblasts and lymphocytes (21, 38). Studies by Kamisato and colleagues (39, 40) additionally revealed that the EBP-dependent chemotactic response of macrophages to elastin-derived peptides also involves

![A](image1.png)

**Fig. 1.** A, representative photomicrographs depicting immunostaining with anti-BrdUrd antibody in 3-day-old cultures of CA SMC maintained in medium with 5% FBS in the absence and presence of 50 μg/ml κ-elastin. B, growth curves of CA SMC maintained in serum-free medium for 1–7 days in the presence or absence of κ-elastin. Estimation of cell number and total DNA assay in quadruplicate SMC cultures (initially plated at 50,000 cells/dish) indicated that κ-elastin stimulates cellular proliferation rate and that such an effect can be abolish when cells were additionally treated with lactose, which causes release of EBP molecules from the cell surface. C, CA SMC cultured in 5% FBS medium demonstrate a dose-dependent up-regulation of [3H]thymidine incorporation after exposure κ-elastin or to synthetic VGVAPG peptide reflecting the major EBP ligand. Cells that were serum-starved for 48 h and further maintained in the serum-free medium also demonstrate a proportional increase in incorporation of [3H]thymidine in response to κ-elastin. Cells treated simultaneously with 10 ng/ml PDGF-BB and 50 μg/ml κ-elastin incorporated more [3H]thymidine than cells treated with 10 ng/ml PDGF-BB alone. D, the mitogenic response to κ-elastin was practically abolished when the CA SMC were preincubated and then simultaneously treated with lactose, which causes shedding of EBP from the cell surface, or with anti-EBP antibody, which blocks the elastin binding domain of the EBP. The κ-elastin-induced up-regulation of [3H]thymidine incorporation was also abolished when cultured SMC were preincubated and then simultaneously treated with inhibitors of G protein (pertussis toxin), L-type calcium channels (nisoldipine), tyrosine kinases (genistein), c-Src (PP2), Ras (radicicol), or MEK1/2 kinase (PD98059). Moreover, pretreatment with inhibitor of PDGF receptor kinase (AG1295), but not with EGF receptor kinase inhibitor (AG1498), caused a significant decrease in the magnitude of the κ-elastin-induced up-regulation of [3H]thymidine incorporation in SMC cultures.
stimulation of cGMP and cGMP-dependent protein kinase. Brassart and colleagues (32) reported that pertussis toxin-sensitive G proteins and tyrosine kinase are involved in the EBP-mediated up-regulation of matrix metalloproteinase-2 in HT-1080 cancer cells. Because the abovementioned data clearly suggest that the cell surface EBP functions as a subunit of a true receptor capable of transmitting physiological signals from the extracellular matrix into the cell interior, the present studies have been aimed at a detailed disclosure of the intracellular signaling pathways generated by the association between elastin-derived peptides and the EBP.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Culture media, fetal bovine serum, and other tissue culture reagents were obtained from Invitrogen (Burlington, Ontario, Canada). Lactose, VGVAPG peptides, proteinase inhibitors, genistein, pertussis toxin, radicicol, PD98059, AG1295, AG1498, agarse-linked protein A, and all reagent grade chemicals were purchased from Sigma (ST. Louis, MO). Preparation of soluble elastin degradation products, c-elastin was obtained from Elastin Product Co. (Owensville, MO). AG1478, PD2, and PD 98059 were purchased from Calbiochem Co. (San Diego, CA). Endoproteinase Lys-C was obtained from Roche Molecular Biochemicals (Laval, Quebec, Canada). Monoclonal antibodies against phosphotyrosine (PY20), polyclonal antibodies against FAK, cyclin A, cyclin D1, cdk2, and cdk4 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibody (p44/42) to phosphorylated ERK 1/2 was obtained from Cell Signaling Co. (Beverly, MA). Polyclonal antibody to phospho-Src was obtained from Upstate Biotechnology (Lake Placid, NY). Polyclonal antibody to LC3 microtubule-associated protein 1 was a gift of Dr. J. Hammerback from Winston-Salem, NC. Species and type-specific secondary antibodies conjugated to horseradish peroxidase, enhanced chemiluminescence kit, BrdUrd-based cell proliferation kit, RN 20, and "3H"thymidine were all purchased from Amersham Biosciences Canada Ltd. (Oakville, Ontario, Canada). Fibrinase-conjugated goat-anti-rabbit secondary antibody was obtained from ICN Immuno-Biologicals (Lisle, IL). The platelet-derived growth factor BB (PDGF-BB) was from Collaborative Research Inc. (Bedford, MA).

**Cell Culture**—SMC were isolated by enzymatic digestion of porcine coronary arteries (CA) and cultured in Medium 199 supplement with 10% FBS as previously described (41). Second passage of CA SMC was then used in all experiments. Normal human skin fibroblasts, EBP-deficient fibroblasts derived from patients with GM1-gangliosidosis bearing a nonsense mutation of the β-galactosidase gene (42), and sialidase-deficient fibroblasts from patients with sialidosis (43) were also used.

**Cell Proliferation Assays**—The mitogenic effects of the soluble elastin degradation products (c-elastin) or VGVAPG peptide on cultured CA SMC were determined by immunodetection of BrdUrd with the Ameersham Biosciences cell proliferation kit, according to the manufacturer's instructions and by the incorporation of "3H"thymidine (14, 19). Cells were plated in six-well dishes (1 × 10⁶ cells/dish) and maintained for 24 h in Medium 199 containing 10% FBS. Cells were then transferred to serum-free medium for synchronization of their cell cycle. Seventy-two hours later, cells were transferred again, either to serum-free medium or to medium with 5% FBS and maintained in the presence or absence of 50 μg/ml c-elastin and 2 μg/ml "3H"thymidine for the next 48 h. Additionally, estimation of the growth curves in quadruplicate cultures of CA SMC maintained for 1–7 days in the presence or absence of c-elastin were also performed. At the end of each incubation period, cells from individual cultures (initially plated at 50,000 cells/dish) were trypsinized and counted in a cell counter. Total DNA was assayed at each end point using the DNeasy tissue system. To determine whether the mitogenic signal of c-elastin was transmitted through the EBP, quadruplicate cultures of CA SMC were preincubated for 3 h with 50 mM lactose, which causes release of the EBP from the cell surface (1, 18, 25–29), or with 5 μg/ml anti-S-GAL antibody, which blocks its elastin binding domain (33). They were then incubated with 2 μg/ml "3H"thymidine for the next 48 h, in the presence or absence of these reagents and 50 μg/ml c-elastin as described above. To determine whether other factors commonly involved in intracellular mitogenic signaling might also be required for the c-elastin-induced mitogenic response, parallel quadruplicate cultures of CA SMC maintained for 48 h in the presence and absence of 50 μg/ml c-elastin were also pretreated for 1 h and further exposed to 100 ng/ml G protein inhibitor, pertussis toxin (44), 10 μg/ml protein kinase inhibitor, genistein (45), 10 μM L-type calcium channel-blocker, nisoldipine (46), 10 μM inhibitor of C-Src PFP (47), 5 μM inhibitor of Ras, radicicol (48), 50 μM MEK1/2 inhibitor, and PD98059 (49, 50) or with 10 μM PDGF receptor kinase inhibitor, AG1295 (51), and 10 μM EGF receptor kinase inhibitor, AG1498 (51). At the end of each experiment, the amount of thymidine incorporation was determined by liquid scintillation counting. All the above listed inhibitors were used in concentrations that were proven to be non-cytotoxic, when tested in a series of pilot experiments, in which SMC were treated with the increased concentrations of each inhibitor for different times ranging from 30 min to 12 h. In those experiments cell viability was assessed by the trypan blue exclusion test ( marking dead cells) and by the neutral red incorporation into the living cells. The appearance of 20% more dead cells in the inhibitor-treated cultures than in untreated controls was considered as cytotoxic. The selected concentrations of inhibitors also allowed for reversibility of their anti-mitogenic effect 24 h after cessation of treatment and transfer to the media containing c-elastin alone (tested by "3H"thymidine incorporation). To definitively demonstrate involvement of EBP and sialidase in the transduction of mitogenic signals, EBP-deficient cells derived from patients with GM1-gangliosidosis bearing a nonsense mutation of the β-galactosidase gene (42) and sialidase-deficient cells from sialidosis patients (43) were also exposed to c-elastin and "3H"thymidine as described above.

**Immunostaining**—To demonstrate the lack of EBP expression in GM1-gangliosidosis fibroblasts bearing a nonsense mutation of the β-galactosidase gene, subconfluent cultures of these cells and normal fibroblasts were immunostained with anti-S-GAL antibody and counterstained with propidium iodide to visualize nuclei, as previously described (42).

**Immunoprecipitation**—Cultures of CA SMCs (initially plated 1×10⁵ cells/dish) incubated for 72 h in serum-free medium were exposed for different periods of time ranging from 15 min to 24 h to 50 μg/ml c-elastin in the presence or absence of the various reagents inhibiting steps of intracellular signaling, as specified in the figure legends. At the end of each experiment cells were lysed by boiling in 62.5 mM Tris-HCl buffer, pH 6.8, containing 2% SDS, 10% β-mercaptoethanol, and 0.001% bromphenol blue. Proteins were resolved by 12% SDS-PAGE, transferred to nitrocellulose membranes, and then immunoblotted with anti-p-Tyr-PY20 antibody, with anti-FAK antibody, with anti-LC3 antibody, or with antibodies recognizing phosphorylated forms of c-Src and ERK 1/2 (anti-p-Src and anti-p-ERK 1/2) (all at 0.5 μg/ml). Cyclin A, cdk2, and cdk4 were detected with specific anti-human polyclonal antibodies (all at 0.2 μg/ml). Expression of the EBP was also detected with anti-SGAL antibody in lysates of normal and GM1 fibroblasts. All blots were then treated with the appropriate secondary antibodies and examined using the enhanced chemiluminescence detection system. The degree of expression or phosphorylation of EBP-nudetected signaling molecules was measured by densitometry.
agarose as previously described (33). Immunoprecipitated proteins were then resolved at 12% SDS-PAGE, transferred to nitrocellulose membranes, and probed with 2.5 μg/ml monoclonal PY20 antibody recognizing phosphotyrosine. The reverse experiment was also performed.

Isolation and Partial Sequencing of the Tyrosine-phosphorylated Proteins—The lysates of κ-elastin-treated cells were resolved at 12% SDS-PAGE and transferred to nitrocellulose membranes. Parallel membranes were then immunoblotted with anti-p-Tyr antibody or stained with Coomassie Blue. The blue bands matching those recognized with anti-p-Tyr antibody were dissected from the membranes, and proteins were extracted with a 1:1 mixture of 10% formic acid and 100% acetonitrile as described before (52). Extracted individual proteins were then dissolved in 50 mM of 25 mM Tris, pH 8.5, containing 1 mM EDTA and additionally subjected to further cleavage with 1 μg of endoproteinase Lys-C. The obtained cleavage products were separated by C-18 reversed-phase high-performance liquid chromatography, and the major isolated peptide peaks were sequenced by gas phase protein sequencing (Porton Instruments, Tarzana, CA). The sequences obtained were then subjected to homology search by the Advanced BLAST at NCBI protein data base.

Detection of Ca²⁺ Influxes in Single Cells—Subconfluent cultures of SMC plated on cover slips were preincubated for 30 min in PBS containing 5 μM of Ca²⁺ ion-binding fluorochrome, Fura-2AM according to the manufacturer’s instructions. Cultures were then washed extensively, covered with Medium 199 (containing 1% FBS, 1.8 mM Ca²⁺), transferred to the incubation chamber, and monitored under an inverted fluorescent microscope (516-nm emission filter) connected to a charge-coupled device camera and a computerized video analysis system (Image-Pro Plus software for Macintosh, Media Cybernetics, Silver Spring, MD) allowing the real-time analysis of the captured images. Images of 20 single cells were then captured in real-time after addition of 50 μg/ml κ-elastin to the incubation chamber.

Intracellular influxes of free calcium ions were also analyzed by the perforated-patch method in voltage-clamp experiments. L-type Ca²⁺ currents were recorded at 37°C from single SMCs and analyzed using
Intracellular signaling indicated that 15-min treatment of CA SMC with 50 μM nisoldipine, or 100 ng/ml pertussis toxin, with 10 ng/ml anti-S-GAL antibody, lactose, anti-S-GAL antibody, or 10 mM lactose) did not increase their \[^{3}H\]thymidine incorporation in response to \(\kappa\)-elastin or VGVAPG peptides (Fig. 2).

Real-time fluorescence microscopy and patch clamping confirmed that SMC exposed to \(\kappa\)-elastin demonstrated a rapid transient influx of \(Ca^{2+}\) into the cytoplasm (Fig. 3, A and B). Fluorometry additionally indicated that such \(\kappa\)-elastin-induced \(Ca^{2+}\) influxes were inhibited by nisoldipine and were not observed in cells deprived of cell surface EBP (pretreatment with lactose) or after blocking of G proteins with pertussis toxin (Fig. 3C).

Western blot analysis with anti-phosphotyrosine antibody indicated that even 5-min exposure of SMC to \(\kappa\)-elastin led to tyrosine phosphorylation of multiple proteins and that such \(\kappa\)-elastin-induced phosphorylation did not occur in cells preincubated with anti-EBP antibody, lactose, pertussis toxin, nisoldipine, or genistein (Fig. 4A). Immunoprecipitation with the anti-phosphotyrosine antibody followed by immunoblotting with several antibodies recognizing proteins involved in the intracellular signaling further demonstrated that treatment with \(\kappa\)-elastin up-regulated levels of tyrosine phosphorylation of 125-kDa tyrosine-phosphorylated foci-related kinase (FAK), 60-kDa c-Src, and 42- to 44-kDa ERK1/2. Treatment with \(\kappa\)-elastin did not cause an increase in phosphorylated JNK or P38 MAPKs. The degree of phosphorylation of immunodetected signaling molecules was measured by densitometry (right panel).

Microelectrode amplifiers (Axopatch 200B, Axon Instruments, Union City, CA) under software control (pCLAMP 7.0, Axon Instruments, Union City, CA), before and after addition of 50 μg/ml elastin to the external solution as previously described (53).

**Measurement of \(Ca^{2+}\) Influxes in Cell Suspension**—Cultured SMC were scraped from the plastic dishes and suspended in PBS (pH 7.4) containing 5% bovine serum albumin and preincubated for 30 min with the fluorescent, \(Ca^{2+}\)-binding dye Fluo-4-AM diluted to a final concentration 3 μM. Cells were then washed in PBS, and separate aliquots containing 4 × 10⁵ cells/ml were re-suspended in Medium 199 (containing 1.8 mM \(Ca^{2+}\) ) and incubated for 10 min in the presence or absence of 0.1 mM lactose, 100 ng/ml pertussis toxin, or the calcium channel blocker nisoldipine (1 μM). Influxes of \(Ca^{2+}\), marked by fluorescence, were monitored by a spectrometer at an emission wavelength of 516 nm and excitation wavelength of 488 nm.

**RESULTS**

All parameters, immunodetection of incorporated BrdUrd, assessment of growth curves, and measurements of \[^{3}H\]thymidine incorporation (Fig. 1), demonstrated that CA SMC significantly increased their proliferation rate after exposure to \(\kappa\)-elastin or to the synthetic VGVAPG peptide, mimicking a major ligand of the elastin receptor. The magnitude of elastin peptide-induced increase in cellular proliferation was more visible in cultures maintained in FBS-containing medium than in cultures maintained in serum-free medium. Treatment with \(\kappa\)-elastin also enhanced the magnitude of mitogenic response of CA SMC maintained in serum-free medium to PDGF-BB (Fig. 1C). Conversely, the proliferative response to \(\kappa\)-elastin was significantly decreased when cells were both treated with lactose, which causes release of EBP molecules from the cell surface, or with anti-S-GAL antibody, which blocks the elastin binding domain of the EBP (Fig. 1, B and D).

Further experiments demonstrated that \(\kappa\)-elastin-stimulated \[^{3}H\]thymidine incorporation was blocked by preincubation of cultured SMC with inhibitors of G protein (pertussis toxin), \(L\)-type calcium channels (nisoldipine), tyrosine kinases (genistein), c-Src (PP2), Ras (radicicol), or MEK1/2 kinase (PD98059), and all abolished the \(\kappa\)-elastin-induced up-regulation (Fig. 1D). This suggested that EBP-induced mitogenic signaling requires G protein, \(L\)-type calcium channels, certain tyrosine kinase(s), and ERK1/2 MAPKs. We have also established that inhibitor of PDGF receptor kinase (AG1295), but not with EGF receptor kinase inhibitor (AG1498), caused a significant decrease in the magnitude of the \(\kappa\)-elastin-induced up-regulation of \[^{3}H\]thymidine incorporation.

Involvement of the EBP in the transduction of proliferative signals was additionally confirmed by the fact that EBP-deficient fibroblasts derived from patients with the congenital GM1-gangliosidosis (bearing a nonsense mutation of the \(\beta\)-galactosidase gene) did not increase their \[^{3}H\]thymidine incorporation in response to \(\kappa\)-elastin or VGVAPG peptides (Fig. 2).

Additional data confirmed that phosphorylation of ERK1/2 is a critical event in the elastin peptide-dependent mitogenic signaling. We found that elastin peptide-induced phosphorylation of ERK1/2 requires the presence of functional EBP (inhibited by lactose), including activation of G proteins (inhibited by pertussis toxin), influx of \(Ca^{2+}\) ions (inhibited by nisoldipine), and activation of such phosphotyrosine kinases as c-Src (inhibited by genistein and by PP2). These steps were followed by phosphorylation of Ras-Raf (inhibited by radicicol) and phos-
Signaling through the Elastin Receptor

**FIG. 5.** A. Western blots with antibody to phosphorylated tyrosine (anti-P-Tyr) and to phosphorylated c-Src (anti-P-c-Src) show that 5-min exposure of CA SMC to 50 μg/ml κ-elastin induced an increase in the level of 60-kDa-phosphorylated protein. Such a strong κ-elastin-dependent phosphorylation of 60-kDa c-Src did not occur, however, in cells pretreated with 100 mM lactose, 100 ng/ml pertussis toxin, or with 10 μM genistein. The pretreatment of SMC with 5 μM radicicol, with 10 μM PD98059, or with 10 μM AG1295 did not inhibit κ-elastin-induced phosphorylation of 60 kDa c-Src. B. Western blot with anti-phospho-ERK1/2 antibody shows that 15-min incubation of CA SMC with 50 μg/ml κ-elastin resulted in a significant increase in phosphorylation of ERK1/2. This response was also apparent after treatment with 10 ng/ml VGVAPG hexapeptide reflecting the EBP binding domain in elastin. The phosphotyrosine phosphorylation of ERK1/2 was completely inhibited in cells pretreated for 30 min with 100 mM lactose, 100 ng/ml pertussis toxin, 10 μM genistein, 10 μM nisoldipine, 10 μM PD2, 5 μM radicicol, and 10 μM PD98059. C. CA SMC simultaneously treated for 15 min with 50 μg/ml κ-elastin and with 10 μM PDGF receptor kinase inhibitor, AG1295, demonstrated much lower levels of phosphorylated ERK1/2 than cells treated with κ-elastin alone. In contrast, addition of 10 μM EGF receptor kinase inhibitor, AG1498, did not diminish the high levels of phosphorylated ERK1/2 induced by simultaneous treatment of CA SMC with 50 μg/ml κ-elastin. The degree of phosphorylation of immunodetected signaling molecules was measured by densitometry (lower panels). In B and C, when phosphorylation of ERK1/2 was estimated, separate readings for the 44-kDa (left) and 42-kDa (right) bands resulted.

The degree of phosphorylation of MEK1/2 (inhibited by PD89059) (Fig. 5B). Moreover, treatment of CA SMC with the inhibitor of PDGF receptor kinase (AG1295), but not with EGF receptor kinase inhibitor (AG1498), caused a significant decrease in κ-elastin-induced phosphorylation of ERK1/2 (Fig. 5C), indicating that κ-elastin-dependent mitogenic signals, which include phosphorylation of Src, may also lead to trans-activation of the PDGF receptor and subsequent activation of the Ras-Raf-MEK1/2-ERK1/2 phosphorylation cascade. This was consistent with data presented in Fig. 1C showing that inhibition of the PDGF receptor kinase caused a significant decrease (but not complete inhibition) in the magnitude of cellular mitogenic response to κ-elastin.

Further analysis revealed that serum-starved CA SMC treated for 24 h with 50 μg/ml κ-elastin or with 10 ng/ml VGVAPG demonstrated up-regulation in their expression of cyclins D1, A, and E, as well as expression of cyclin-dependent kinases, cdk4 and cdk2 (Fig. 6A). This up-regulation in expression of cyclins D, A, and E and both cdk4s has been abolished in cells simultaneously treated with κ-elastin and PD98059, indicating that phosphorylation of ERK1/2 consists of a crucial step preceding an increase in expression of these factors controlling progression of the cell cycle (Fig. 6B). The 24-h-old cultures of CA SMC maintained in medium containing 5% FBS also demonstrated up-regulation in their expression of cyclins A and E as well as cdk2 when treated with 50 μg/ml κ-elastin. Treatment with κ-elastin did not, however, produce any increase in their already high expression of cyclin D1 and cdk4 induced by FBS (data not shown).

Results depicted in Fig. 7 also indicate that pretreatment of SMC with G protein inhibitor, pertussis toxin, inhibitor of l-type calcium channels, nisoldipine, and, with the phosphotyrosine kinase inhibitor genistein, prevented the κ-elastin-induced phosphorylation of the 125-kDa FAK. In contrast, pretreatment of cells with the PDGF receptor kinase inhibitor, AG1295, or the MEK1/2 inhibitor, PD98059, did not prevent the κ-elastin-dependent phosphorylation of FAK.

Sequencing of purified proteins matching the molecular weight of tyrosine-phosphorylated species detected in SMCs after treatment with κ-elastin identified them as cytoskeletal components: 54-kDa β-tubulin, 42-kDa α-actin, and 16-kDa troponin-T (Fig. 8). Moreover, immunoprecipitation with anti-phosphotyrosine antibody followed by Western blotting with the antibody recognizing 15-kDa LC3 microtubule-associated protein 1, showed significant up-regulation of this protein (data not shown), which is also known as a factor facilitating translation of fibronectin mRNA (54).

**DISCUSSION**

Results presented in this report showed that, similar to other cell types (15–19), cultured SMC isolated from porcine coronary...
arteries also up-regulate their proliferation after exposure to soluble elastin-derived peptides. Because proliferative response of CA SMC to soluble elastin-derived peptides was practically abolished in cells treated with lactose, which causes release of 67-kDa EBP molecules from the cell surface, or with anti-S-Gal antibody, which blocks the elastin binding domain of the 67-kDa EBP (33, 34), we conclude that this protein is a crucial component of the cell surface elastin receptor that transduces intracellular signals. The most definitive confirmation of EBP involvement in the transduction of mitogenic signals came from experiments demonstrating that EBP-deficient human fibroblasts derived from patients with congenital GM1-gangliosidosis, bearing a nonsense mutation of the β-galactosidase gene (42), did not up-regulate their expression of cyclins D1, A, and E, as well as expression of cyclin-dependent kinases, cdk4 and cdk2. This up-regulation in expression of cyclins D, A, and E and both cdks has been abolished in cells simultaneously treated with 50 μg/ml κ-elastin and with 50 μM PD98059, indicating that phosphorylation of ERK1/2 consists of a crucial step preceding the increase in expression of these factors controlling progression of the cell cycle. The degree of expression of immunodetected molecules was measured by densitometry (right panels).

Results of further experiments aimed at identifying mitogenic signaling pathways transduced by the EBP (Fig. 1 D) strongly suggested that EBP-induced mitogenic signaling involves activation of G proteins and opening of L-type calcium channels. Further analysis confirmed that activation of G proteins must be an immediate effect of stimulation of the elastin receptor that is followed by intracellular influx of Ca^{2+} (Fig. 3) and a downstream phosphorylation of numerous proteins, including such tyrosine kinases as FAK, c-Src, and ERK1/2 MAPK, but not phosphorylation of two other members of the MAPK family, neither JNK or p38 MAPK (Fig. 4). Because the κ-elastin-induced and ERK1/2 phosphorylations were inhibited by the tyrosine kinase inhibitor, genistein, an inhibitor of Ras, radicicol, and an inhibitor of MEK1/2, PD98059 (Fig. 5 B), we concluded that the entire Ras-Raf-MEK1/2-ERK1/2 cascade was involved in a final activation of this MAPK. Our conclusion is consistent with previous reports showing that, in many cellular systems, the receptor-mediated proliferation and differ-

Fig. 6. A and B, Western blots with specific antibodies recognizing cyclins and cdks in the whole cell extracts of CA SMC, which were serum-starved for 24 h and then maintained in serum-free medium for another 24 h in the presence or absence of 50 μg/ml of κ-elastin or 10 ng/ml VGVAPG peptide. Cells treated with κ-elastin or VGVAPG demonstrated up-regulation in their expression of cyclins D1, A, and E, as well as expression of cyclin-dependent kinases, cdk4 and cdk2. This up-regulation in expression of cyclins D, A, and E and both cdks has been abolished in cells simultaneously treated with 50 μg/ml κ-elastin and with 50 μM PD98059, indicating that phosphorylation of ERK1/2 consists of a crucial step preceding the increase in expression of these factors controlling progression of the cell cycle. The degree of expression of immunodetected molecules was measured by densitometry (right panels).

Fig. 7. A, Western immunoblot with antibody to phosphorylated tyrosine (anti-P-Tyr) shows that 5-min treatment of CA SMC with 50 μg/ml κ-elastin or with 10 ng/ml VGVAPG caused up-regulation in tyrosine phosphorylation of 125-kDa protein, which was also recognized by the parallel blot with anti-FAK antibody. Cells pretreated for 30 min with 100 mM lactose, 100 ng/ml pertussis toxin, or 10 μM genistein and then treated with 50 μg/ml κ-elastin did not reveal any increase in tyrosine-phosphorylated FAK, as compared with untreated controls. B, proteins immunoprecipitated from CA SMC lysates with the anti-FAK antibody were separated by polyacrylamide gel electrophoresis and then visualized with antibody to phosphorylated tyrosine (anti-P-Tyr) on Western blots. Results demonstrate that, in contrast to control cultures, CA SMC treated for 5 min with 50 μg/ml κ-elastin or with 10 ng/ml VGVAPG demonstrated strong tyrosine phosphorylation of 125-kDa FAK. Treatment of parallel CA SMC cultures with 50 μg/ml κ-elastin did not induce tyrosine phosphorylation of 125-kDa FAK when cells were preincubated for 30 min with 100 mM lactose (causing shedding of the EBP), 100 ng/ml pertussis toxin, 10 μM genistein, and 10 μM nisoldipine. The pretreatment of CA SMC with 50 μM PD98059 or 10 μM AG1295 did not inhibit κ-elastin-induced phosphorylation of 125-kDa FAK. The degree of phosphorylation and expression of immunodetected FAK was measured by densitometry (lower panels).
growth factors, differentiation signals, are part of a multikinase module through which a variety of extracellular stimuli (growth factors, differentiation signals, and cellular stress) are transmitted into the cell (57). Receptor tyrosine kinases, upon autophosphorylation and activation of adaptor proteins, recruit Ras and subsequently engage the Raf/MEK/ERK cascade.

Our finding, that the positions of elastin peptide-induced activation of G proteins and opening of L-type calcium channels are located upstream of the tyrosine kinase activation pathways, seems to be consistent with accumulating evidence indicating that G proteins may interact with numerous and diverse cell surface receptors and act as a common molecular switch transducing extracellular stimuli into intracellular responses (58) and that Ca^{2+} influxes (blocked by nifedipine) are involved in mitogenic signaling in neonatal SMC, mediated by phosphorylated MEK1/2 and ERK 1/2 (59). At the present time, we can only speculate on the nature of molecular mechanism leading to the elastin-dependent involvement of G proteins.

Our results, demonstrating that exposure of serum-starved cell surface elastin receptor, which consists of peripheral elastin-binding protein (EBP), connected to the protective protein (SASE), and the transmembrane sialidase (SASE). A very early step of the elastin receptor-dependent signaling is the activation of G proteins, which is followed by the opening of the L-type calcium channels and a rapid Ca^{2+} influx into the cytosol. These events are followed by activation of FAK and c-Src, trans-activation of PDGF receptor kinase, and a consecutive activation of the Ras-Raf-MEK1/2-ERK1/2 phosphorylation cascade, leading to an increase in expression of factors facilitating cell cycle progression (cyclins and cdks). The elastin receptor-activated signals also involve tyrosine phosphorylation of cytoskeletal components.

Moreover, because β-elastin-induced phosphorylation of EBP1/2 was substantially diminished in cells simultaneously treated with the inhibitor of the PDGF receptor kinase, AG1295, but not with the inhibitor of the EGF receptor kinase, AG1498 (Fig. 5C), we conclude that the EBP-transduced signaling pathway also includes trans-activation of PDGF receptor kinase and, from this point, imitates the PDGF-induced mitogenic signaling, even in the absence of this growth factor. We have to stress, however, that EBP ligands (β-elastin and VGVAPG) did not bind PDGF in our in vitro co-immunoprecipitation assays (data not shown). Moreover, the second passage test of smooth muscle cells, which were quickly isolated from coronary arteries by the enzymatic method, represent a prerequisite upstream step in the elastin-dependent up-regulation of these factors.

The notion about EBP-mediated trans-activation of the PDGF receptor is consistent with observations indicating that stimulation of a single cell surface receptor can induce intracellular signaling pathways dependent on trans-activation of other adjacent receptors (61–63). The PDGF-independent trans-activation of the PDGF receptor (64–67) and ligand-independent activation of other growth factor receptors, includ-
ing EGF and insulin-like growth factor receptors, have been described previously by factors (endothelin, angiotensin II, 5-hydroxytryptamine, and dopamine) that interact with G protein-coupled receptors (68–72).

Our observation, that the elastin peptide-induced signaling pathway includes activation of G protein and consecutive phosphorylation of 60-kDa c-Src, seems to be consistent with the report of Nebigil and colleagues (73) who demonstrated that c-Src plays an upstream role in the trans-activation of the PDGF receptor in the mitogenic signaling propagated by the G protein-coupled receptor interacting with 5-hydroxytryptamine. The possibility of the interaction of c-Src with G protein-coupled receptors (endothelin, angiotensin II, 5-hydroxytryptamine, and dopamine) that interact with G protein-coupled receptors (68–72), indicates that a lack of elastic fibers, itself, may be instrumental in stimulating a heightened proliferation of arterial SMC. Indeed, we have recently extended this notion by showing that a higher than normal proliferation of human aortic SMCs, isolated from patients with haploinsufficiency of elastin genes (SVAS and WBS), could be reversed by exogenous insoluble elastin added to the culture media. We have additionally demonstrated that insoluble elastin exercises its anti-mitogenic effect by sequestering growth factors present in serum added to the culture media (37). We believe that these data are complementary to the results presented in the present report and, taken together, shed new light on the mechanism responsible for developing occlusive arterial lesions. We suggest that common growth factors, which freely access their cell surface receptors (in the absence of insoluble elastin), act in concert with the soluble elastin degradation products in stimulating SMC proliferation. Our results may explain the empirical observation that both enzymatic degradation of the existing elastic fibers (which creates elastin-derived peptides) (7–10) and impaired assembly of new elastic fibers (1–6) coincide with formation of the hypertensive neointima.

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