The 67-kDa Enzymatically Inactive Alternatively Spliced Variant of β-Galactosidase Is Identical to the Elastin/Laminin-binding Protein*

(Received for publication, November 5, 1997, and in revised form, January 8, 1998)

Salvatore Privitera‡§, Catherine A. Prody‡§, John W. Callahan†, and Aleksander Hinek‡§

From the Divisions of ‡Cardiovascular Research and §Neurosciences, The Hospital for Sick Children and the †Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario M5G 1X8, Canada

Our previous studies showed immunological and functional similarities, as well as partial sequence homology, between the enzymatically inactive alternatively spliced variant of human β-galactosidase (S-gal) and the 67-kDa elastin/laminin-binding protein (EBP) from sheep. To define the genetic origin of the EBP further, a full-length human S-gal cDNA clone was constructed and subjected to in vitro transcription/translation. The cDNA was also transfected into COS-1 cells and into the EBP-deficient smooth muscle cells (SMC) from sheep ductus arteriosus (DA). In vitro translation yielded an unglycosylated form of the S-gal protein, which immunoreacted with anti-β-galactosidase antibodies and bound to elastin and laminin affinity columns. S-gal cDNA transfections into COS-1 and DA SMC increased expression of a 67-kDa protein that immunolocalized intracellularly and to the cell surface and, when extracted from the cells, bound to elastin. The S-gal-transfected cells displayed increased adherence to elastin-covered dishes, consistent with the cell surface distribution of the newly produced S-gal-encoded protein. Transfection of DA SMC additionally corrected their impaired elastic fiber assembly. These results conclusively identify the 67-kDa splice variant of β-galactosidase as EBP.

Virtually all cell types, including tumor cells, interact with the extracellular matrix (ECM) during certain stages of their development. Such contacts may be strictly adhesive or can transduce signals from the ECM to the intracellular machinery. These significant cell matrix interactions are mediated through specialized cell surface receptors (2, 3). Interactions between cells and elastin are mediated by a non-integrin cell surface receptor complex consisting of three protein subunits (4–6). Two of these subunits (61- and 55-kDa subunits) are cell membrane-associated proteins that immobilize the third, a 67-kDa peripheral subunit called the elastin-binding protein (EBP). The EBP binds predominantly to the repeating VGVAPG hydrophobic domains on elastin, but it may also bind to other similar hydrophobic domains on elastin (7), and to the LGTIPG sequence on laminin (5, 8). Moreover, the EBP also interacts with moieties containing β-galactosugars through a separate “lectin-like” binding domain. However, binding of β-galactosugar-bearing moieties to the lectin domain of the EBP causes such conformational changes in the 67-kDa protein that it loses its affinity for elastin and separates from the other subunits of the elastin receptor. Thus, the EBP can be shed from the cell surface by interactions with galactosugars (galactose, lactose) or with N-acetylgalactosamine-containing glycosaminoglycans (chondroitin sulfate, dermatan sulfate), which bind to its lectin site (9–11). The EBP appears to be directly involved in the generation of intracellular signal transmission after contact with its matrix ligands (6). Binding of elastin-derived peptides to the EBP, when present on the cell surface, resulted in a rapid and transient increase in free intracellular calcium (12, 13), whereas displacement of EBP with either galactose or lactose prevented such an influx. It has been suggested that the elastin receptor-mediated signal transduction involves the G1 protein and the chain activation of phospholipase C and phosphokinase C (14–16).

We found that SMC from intimal cushions of the fetal ductus arteriosus (DA) (a shunt between the fetal aorta and the pulmonary artery, which, in preparation for closure shortly after birth, develops intimal “cushions” as a result of massive migration of medial SMC into the subendothelium; see Ref. 19), as well as SMC from atherosclerotic vascular lesions are deficient in EBP (9). This indicated that the lack of this protein may be responsible for SMC detachment from the elastic matrix, hence linking it to transformation of these cells to a synthetic migratory phenotype that underlies vascular thickening and luminal narrowing common in atherosclerosis (9, 10). Moreover, we have determined that an unoccupied cell surface EBP interferes with binding of interleukin-1β (IL-1β) to its cell surface receptor on arterial SMC and modifies the response of the vascular cells to the endogenous and exogenous cytokine (20, 21).

Our previous studies aimed at detailed characterization of the EBP have established that the EBP isolated from sheep SMC displays immunological, functional, and partial sequence homology to the 67-kDa alternatively spliced variant of human β-galactosidase described by Morreau and colleagues (22) as a protein with undefined function. This form of β-galactosidase arises through the splicing out of two non-contiguous protein-
S-gal Is an Elastin-binding Protein

encoding regions in such a way that the first deletion introduces a frameshift, which is restored by the deletion of the second region. The net result of these deletions is a shortened protein with the introduction of a 32-amino acid sequence unique to the alternatively spliced form of β-galactosidase (Fig. 1A). Due to the loss of protein domains encoded by the spliced out exons 3, 4 (region 1), and 6 (region 2), S-gal does not display any enzymatic activity of β-galactosidase and is not targeted to the lysosomes. Furthermore, we have established that the unique domain of S-gal, encoded by the frameshift-generated sequence, contains an elastin/lamin-binding motif (11).

Our further studies showed that an antibody (anti-S-gal) made to a synthetic oligopeptide corresponding to the elastin/lamin-binding motif of human S-gal showed an identical immunolocalization to the cell surfaces and extracellular elastic fibers as the antibody to EBP (11). Moreover, anti-S-gal co-localized intracellularly with tropoelastin (17). Affinity chromatography of human placenta extract, as well as extracts from sheep aorta SMC on immobilized elastin and laminin, demonstrated that the 67-kDa protein-bound to these affinity columns could be eluted by lactose or γ-d-galactacto lactone and immunoreacted with a panel of antibodies recognizing the spliced variant of β-galactosidase (anti-S-gal, anti-C-gal, and anti-P-gal) and with BCZ (anti-EBP) antibody raised to bovine EBP. Thus, in addition to extensive homology, a similar molecular weight, and immunological cross-reactivity, this enzymatically inactive form of β-galactosidase also shows elastin- and laminin-binding properties that are identical to those of EBP (11). These data suggested that these proteins are similar and, in fact, may be identical in the same species.

Since the sheep β-gal gene has not yet been characterized, the conclusive proof of the genetic origin of the EBP remained to be established. Therefore, the major objective of the present study was to provide evidence of the identity between S-gal and EBP. Thus, the full-length cDNA clone encoding the alternatively spliced variant of human β-galactosidase was constructed and used in a series of in vitro transcription/translation experiments and transfected into COS-1 cells, which express very low levels of β-gal and S-gal (24), and into the EBP-deficient sheep DA SMC (9) to overexpress the S-gal-encoded protein and to determine whether its structural and functional features will match those of the 67-kDa EBP.

EXPERIMENTAL PROCEDURES

Materials—Kits, chemicals, DNA, and reagents were obtained as follows. The plasmids pSVL SV40 was obtained from Pharmacia Biotech Inc. (Baie d’Urfe, Quebec, Canada). All restriction enzymes were from New England Biolabs (Mississauga, ON, Canada). Gel purification was conducted using the Geneclean II Kit from BIO 101 Inc. (Vista, CA). DNA and Protein A-Sepharose (Pharmacia), as well as anti-Protein A antibodies, were purchased from Perkin-Elmer thermal cycler using an annealing temperature of 55 °C. The fragments were gel-purified and ligated into the EcoRV site of pBluescript SK−. The respective fragments contained a 115-bp overlapping sequence at their respective 3′ and 5′ ends (see Fig. 1B). In addition, the absence of the initial 27 bp located at the 5′ end of the 5′ fragment and 119 bp at the 3′ end of the 3′ fragment was detected (attributed to primer positioning in the initial PCR reaction). Final assembly of the full-length clone eliminated the overlapping segment by employing a common PuclI site found in the overlapping region between the two portions of S-gal. Complete double digestion of the 5′ clone using enzymes KpnI and PstI yielded a 316-bp fragment representing the 5′ segment (i.e., 5′ to the PuclI site) of S-gal, which included an additional 57 bp of vector at its 5′ end. The KpnI digestion created a 3′ overhang, which was blunt-ended by using Fnu DNA polymerase. The 316-bp 5′ fragment and a PstI-digested 3′ clone were then both agarose gel-purified, gene-cleaned, and ligated. The ligation products were transformed into bacterial cells (27) and grown on X-gal/A plates. Restriction digestes with XbaI and PstI confirmed the correct orientation of the short 5′ segment of S-gal in the new construct.

To avoid sequencing the newly constructed clone in its entirety and to insert the missing 5′ 27 bp and the 3′ 119 bp, segments on either side of the frameshifted region were replaced with their wild type counterparts. These segments are identical to those found in the wild type β-gal cDNA, which was the source of these fragments. Using EcoRI and XbaI sites, located within and 3′ to the insert respectively, a large segment was removed from the constructed clone and replaced by the corresponding fragment (which included the missing 119-bp segment of the 3′-untranslated region) isolated from wild type β-gal. From this modified clone, another double digest was conducted to remove an even larger fragment that included the entire frameshifted region along with the 3′ long portion of S-gal. The corresponding fragment was excised from the wild type β-gal clone using the restriction enzymes Cfr101 (BsrFI) and XbaI and was replaced by this S-gal cDNA segment. This ensured that the segment 5′ to the frameshifted region is error-free and complete (the 27-bp missing fragment is also added here yielded the full-length S-gal cDNA). Sequencing was done to ensure that insertion and orientation were correct.

In Vitro Transcription/Translation of the S-gal cDNA Clone—In vitro transcription/translation was done in accordance to the protocols provided by Promega. S-gal cDNA (5 μg) in pGEM-3Z was linearized (digested with Xbal), and in vitro transcription was conducted. This was followed by in vitro translation using 2 μl of RNA substrate in a nuclelease-treated rabbit reticulocyte lysate (minus microsomal mem-
S-gal Is an Elastin-binding Protein

6321

branes and protease inhibitors) in the presence of 0.8 mCi/ml [35S]methionine ([35S]Met). The translation mix (minus mRNA) was used as control. In vitro transcription/translation of the cDNA clone encoding the wild type β-galactosidase precursor (24) was also conducted for comparison. The supernatants were directly analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by autoradiography to detect for the presence of [35S]Met-labeled reaction products and to compare molecular size. The reaction products were further characterized using immunoprecipitation with antibodies recognizing β-gal, S-gal, and EBP, and then by elasin and laminin affinity columns.

Immunoprecipitation—Immunoprecipitation (IP) of the [35S]Met-labeled translation product was assessed with our well characterized anti-S-gal, anti-C-gal (11, 25), and anti-P-gal antibodies (24). The precipitated rabbit serum was used as control. Briefly, the supernatants from the in vitro translation reactions (one volume) were mixed with two volumes of IP buffer (10 mM Tris-buffered saline, pH 7.4, containing 0.2% bovine serum albumin, 0.3% Nonidet P-40, 0.3% sodium deoxycholate, and 0.02% sodium azide) and preincubated for 1 h at 4 °C with normal rabbit serum (2 μg/ml) and then, for 30 min, with two volumes of Protein A-Sepharose beads (0.1 ml of beads/A-Sepharose beads) to remove any nonspecific immunocomplexes. The supernatants were then incubated at 4 °C with either antibody (2 μg/ml) for 2 h and subsequently with two volumes of Protein A beads for 1 h. The beads were washed three times in IP buffer by centrifugation, and then the final pellets were resuspended in SDS sample buffer with 20 mM DTT, boiled for 5 min, resolved by 10% SDS-PAGE, and detected by autoradiography.

Elastin/Laminin Affinity Columns—To assess the ECM-binding properties of the [35S]Met-labeled in vitro translation product, one volume of the incubation product was mixed with two volumes of IP buffer containing a 1 mg/ml suspension of each of powdered insoluble elastin, insoluble collagen type I, and soluble laminin, and incubated for 30 min at 4 °C. The slurry of insoluble elastin and collagen was then pelleted by centrifugation. The soluble laminin was immunoprecipitated using consecutive incubations with 2 μg/ml polyclonal anti-laminin antibody and two volumes of Protein A-Sepharose beads, as described above. The resulting pellets containing the affinity-bound ligands were rinsed in IP buffer, resolved by SDS-PAGE, and analyzed by autoradiography as above.

Transfections of COS-1 Cells with the Full-length S-gal cDNA and Characterization of the S-gal-encoded Protein Product—Transient transfections of the S-gal cDNA clone into COS-1 cells, which express low levels of β-gal and S-gal (24), were conducted in accordance with a DEAE-dextran protocol (28) modified to include adenosine addition. The full-length S-gal cDNA was excised from pGEM-3Z using SalI digestion and ligated into the mammalian expression vector pSVL SV40 previously digested with XhoI. A series of digestions with the site-specific restriction enzymes (Smal and DraIII) confirmed frame and orientation of the S-gal clone. COS-1 cells were plated evenly in the 100-mm dishes (10 × 106 cells/dish) and in 20-mm dishes containing coverslips (1 × 106/dish) and maintained in α-MEM supplemented with 1% antibiotics/antimycotics and 10% (v/v) fetal calf serum for 2 days before transfections were carried out. The transfections consisted of 5% (0.25 ml) adenovirus deletion-325 (approximately 2 × 106 plaque-forming units/ml), 80% (4 ml) serum-free α-MEM, 8 μg of S-gal cDNA in pSVL, 8 μg of TKSL, 10% (0.5 ml) α-MEM + 10% fetal calf serum, and 1.6% (80 μl) DEAE-dextran (5 mg/ml). Control cultures (no DNA, vector alone, wild type β-gal alone; each including TKSL) were treated in the same manner. The cells were then harvested from 72-h cultures, and a luciferase assay was conducted to monitor transfection efficiency (29). The production of the S-gal encoded protein in control and S-gal-transfected cells was assessed by immunohistochemistry and immunoblotting, while its elastin-binding capability was tested by affinity chromatography and an in vitro adhesion assay.

Immunostaining—The presence and distribution of S-gal in the control and S-gal-transfected cells was then compared 72 h after transfection using immunostaining with anti-P-gal, anti-S-gal, and anti-EBP (BCZ) antibodies. Cultures of transfected cells from each experimental group (maintained in small dishes with coverslips) were fixed at −20 °C in 100% methanol for 30 min, rinsed with water and PBS, after which the separate coverslips were incubated for 1 h with PBS-diluted primary antibodies (2 μg/ml) followed by a 1-h incubation with the appropriate fluorescein isothiocyanate-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies. An additional 10-min incubation with propidium iodide (0.1 μg/ml) assured nuclear counterstaining. The coverslips were mounted on glass slides with eukonal and analyzed using an Olympus Vanox AH BT3 fluorescence microscope.

Western Blotting of COS-1 Cell Lysates—72 h after transfection, the cells were maintained in 100-mm dishes were dissolved and harvested by scraping. The pelleted cells were then lysed in 200 μl of PBS containing 0.25% N-ethyl-β-D-glucopyranoside, 0.1% lactate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 20 μM leupeptin, and freeze-thawed (10 times). The insoluble remnants were pelleted by centrifugation (14,000 rpm/25 min), and the supernatants were resolved by 10% SDS-PAGE, 100 μg of total protein was loaded into each well. The proteins were then electrotransferred to Immobilon P membranes and analyzed by autoradiography as above.
by Western blotting with 2 μg/ml anti-P-gal antibody, which also recognizes EBP (11) followed by a donkey anti-rabbit F(ab')2 (horseradish peroxidase-labeled) secondary antibody diluted 1:5000 and ECL.

**Elastin Affinity Columns**—To assess the elastin-binding capability of the S-gal-encoded protein produced in COS-1 cells, equal amounts of lysate (300 μg of total protein) (same lysate as used in the Western blot above) were incubated with 5 mg of insoluble elastin (1 mg/ml in PBS) for 2 h at 4 °C. The elastin slurry was then pelleted by centrifugation, rinsed with PBS, and then resuspended in SDS sample buffer with 20 mM DTT. The elastin-bound proteins released to the buffer were resolved by 10% SDS-PAGE and analyzed by Western blotting using anti-P-gal antibody, as described above.

**Adhesion Assay**—The elastin-binding capabilities of S-gal-transfected and control cells were tested in an *in vitro* assay using elastin coated wells on 96-well plates.

Cells transfected with vector alone, with wild type β-gal, or with S-gal constructs were gently scraped from their culture dishes 72 h after transfection, quickly centrifuged (1000 rpm), resuspended in 2 ml of medium, and counted using a hemocytometer. Equal numbers of cells (three trials ranging from 1500 to 7000 cells/well) from all groups were suspended in 200 μl of medium and plated in sextuplets in 96-well culture dishes that were either uncoated or coated with bovine albumin, with collagen type I, or with ₛα1-I collagen in accordance with a calcium phosphate protocol (30). The S-gal-protein with collagen type I, or with ₛα1-I collagen, was assessed after immunostaining with specific antibodies. The exact number of attached cells was then calculated from an *A*540 standard curve constructed with serial dilutions of untreated COS-1 cells.

**Transfections of DA SMC with the Full-length S-gal cDNA**—Since SMC from the late gestation ductus arteriosus have been shown to be deficient in EBP and incapable of normal elastic fiber assembly (despite the normal production of tropoelastin) and attachment to elastic fibers (9), we speculated that effective transfection of these cells with the S-gal clone may confer EBP expression and restore a normal SMC phenotype.

Transient transfections (vector alone, wild type β-gal, and S-gal; each with TKSL) of passage two SMC isolated from 100-day sheep DA (10 × 10<sup>4</sup> cells/100-mm dish and 1 × 10<sup>5</sup> cells/20-mm dish) were carried out, in accordance with a calcium phosphate protocol (30). The S-gal-protein expression was then assessed 72 h after transfection by immunostaining and by Western blotting, as described above and indicated in the figure legends. The transfected cells (30,000 cells/well plated in quadruplicate on a 24-well dish) were also tested for their ability to adhere to plastic, albumin, collagen type I, and elastin (all in a concentration of 500 μg/ml), 4 h after the initial plating, as described above. Separate cultures maintained for 7 days after transfection to allow for production of extracellular matrix were fixed in 100% methanol and production of elastic fibers, as well as deposition of extracellular fibronectin were assessed after immunostaining with specific antibodies as described previously (9, 18, 21).

**Results**

*In Vitro Transcription/Translation of the S-gal cDNA Resulted in the Synthesis of an Elastin/Laminin-Binding Protein*—In *in vitro* transcription/translation of the full-length S-gal cDNA resulted in the synthesis of a protein with a molecular weight of approximately 60,000 (Fig. 2A), consistent with the primary sequence deduced by Morreau and colleagues (22). In addition to the 60-kDa [35S]Met-labeled reaction product, autoradiography also detected several lower molecular weight bands, probably representing degradation products and/or incomplete translation products. The control reaction (i.e. no DNA and no cell lysate) did not show any species corresponding to a labeled product encoded by the S-gal cDNA clone.

Additional immunoprecipitations of the S-gal cDNA-encoded protein with anti-S-gal (raised to a synthetic peptide mimicking the elastin/laminin-binding sequence of human S-gal), anti-C-gal (raised to a synthetic peptide reflecting the C-terminal end of the β-gal precursor), and anti-P-gal (raised to the human β-galactosidase precursor) antibodies, also yielded a 60-kDa metabolically labeled protein (Fig. 2B) and confirmed its relationship to β-galactosidase. Further immunoprecipitation of the *in vitro* transcription/translation products with the anti-P-gal antibody clearly documented that the S-gal and the wild type β-gal cDNA clones encode distinct (60 and 76 kDa), but immunologically related, proteins, which correspond to the unglycosylated version of S-gal and the β-gal precursor, respectively. D, SBS-PAGE/autoradiography shows that the 60-kDa protein produced by *in vitro* transcription/translation of the S-gal cDNA binds to elastin and laminin but not to collagen type I. The supernatants containing the [35S]methionine-labeled products of *in vitro* translation were chromatographed and described under “Experimental Procedures.” The elastin- and laminin-bound proteins were released from their association with those ECM ligands by boiling in sample buffer containing SDS and DTT.

*The S-gal cDNA Clone Transfected into COS-1 Cells Increased Expression of the Elastin-binding Protein*—Although the 60-kDa S-gal cDNA-encoded product from *in vitro* transcription displayed both properties of S-gal and EBP, it was necessary to define further if the S-gal protein would hold these properties after final processing in these primate (COS-1) cells. To achieve this, we transiently expressed the S-gal cDNA in primate (COS-1) cells. A luciferase assay confirmed successful transfection and control cells were tested in an *in vitro* assay using elastin coated wells on 96-well plates.

**FIG. 2.** The *in vitro* transcription/translation of the S-gal cDNA produces a 60-kDa protein. A, an autoradiogram of SDS-PAGE shows a [35S]methionine-labeled protein product of the *in vitro* transcription/translation of the S-gal cDNA. The uppermost band corresponds to a 60-kDa protein. Lower bands are possibly due to S-gal degradation and/or incomplete S-gal translation products. In the control, no mRNA was added to the translation reaction. B, SBS-PAGE/autoradiography indicates that anti-P-gal, anti-C-gal, and anti-S-gal antibodies immunoprecipitate an identical 60-kDa [35S]methionine-labeled product of *in vitro* transcription/translation encoded by the S-gal cDNA clone. C, an autoradiogram of SBS-PAGE shows the [35S]methionine-labeled products of the *in vitro* transcription/translation of the S-gal and β-gal cDNA clones, which were immunoprecipitated with an anti-P-gal antibody. S-gal and β-gal cDNA clones encode distinct (60 and 76 kDa), but immunologically related, proteins, which correspond to the unglycosylated version of S-gal and the β-gal precursor, respectively. D, SBS-PAGE/autoradiography shows that the 60-kDa protein produced by *in vitro* transcription/translation of the S-gal cDNA binds to elastin and laminin but not to collagen type I. The supernatants containing the [35S]methionine-labeled products of *in vitro* translation were chromatographed as described under “Experimental Procedures.” The elastin- and laminin-bound proteins were released from their association with those ECM ligands by boiling in sample buffer containing SDS and DTT.
showed very low cell surface immunoreactivity with all three antibodies.

Increased expression of the S-gal-encoded protein was also confirmed when the extracts of S-gal cDNA-transfected cultures were analyzed by immunoblotting with the anti-P-gal antibody and compared with controls (Fig. 3A). The immunoreactive overexpressed protein had a molecular size of 67 kDa. Densitometric assessments of the 67-kDa anti-P-gal-immunoreactive bands additionally normalized to RLU (reflecting the transfection efficiency in particular COS-1 cultures) indicated its approximate 8–12-fold increase, as compared with all controls. The molecular size of this protein certainly corresponds to a mature S-gal (22) and to the EBP (4). In contrast, transfection of COS-1 cells with the wild type β-gal cDNA clone resulted in a substantial increase in expression of an anti-P-gal immunoreactive 85-kDa protein, corresponding to the β-gal precursor, and a less abundant 64-kDa mature β-gal (24) (Fig. 3A).

Moreover, elastin affinity of transfected COS-1 cell extracts revealed that the insoluble elastin retained the 67-kDa immunoreactive protein from the extracts of S-gal-transfected cells, but not from controls (Fig. 3B). These results clearly indicate that the overexpressed 67-kDa protein produced by the S-gal-transfected cells has an elastin-binding capability, whereas the wild type β-gal transfection product does not.

Since cellular attachment to elastic matrices depends on the presence of EBP on the cell surface (10), an in vitro adhesion assay was conducted to determine whether transient expression of S-gal would increase the ability of COS-1 cells to adhere to elastin-coated dishes. Three parallel experiments showed that approximately 4 times more of the S-gal-transfected cells attached to elastin-coated dishes (4 h after initial plating) than cells transfected with the vector alone or those transfected with the β-gal clone (Fig. 4). This S-gal-dependent increase in cellular attachment to elastin did not depend on the initial density of plating, in the tested range 1500–7000 cells/well (96-well dish). The specificity of the S-gal dependent attachment to elastin was illustrated by the fact that S-gal cDNA-transfected cells did not increase their attachment when plated on plastic, albumin, or collagen type I (Fig. 4).

These results (together with cell surface immunostaining) further suggest that this overexpressed S-gal-encoded protein is properly transported to the cell surface and facilitates cellular adhesion to elastin-coated plates. Therefore, this S-gal-encoded protein fulfills the role that, to date, has only been attributed to EBP (6).

S-gal cDNA Transfections of DA SMC Increases Expression of EBP and Affects Deposition of Extracellular Matrix—A luciferase assay indicated an efficient and rather uniform transfection in all experimental groups. Immunostaining with anti-P-gal antibody of the S-gal cDNA-transfected DA SMC cultures showed numerous cells with a vividly increased expression of immunoreactive material, localized predominantly to the cell surface. As depicted in Fig. 5 (upper panel), immunostaining with anti-P-gal antibody detected mostly intracellular epitopes localized to the lysosomes and cisternas in cells transfected with the wild type β-gal clone, while cells transfected with the S-gal clone expressed a cell surface localized protein immunoreactive with the same antibody. These data were consistent with the fact that the lysate of the S-gal-transfected cells demonstrated a substantial increase in the expression of a 67-kDa protein immunoreactive with the anti-S-gal antibody on a Western blot (Fig. 6). Densitometric assessments of the 67-kDa bands depicted in Fig. 6, additionally normalized to RLU (reflecting the transfection efficiency), indicated an approximate 10-fold increase in expression, as compared with controls. Furthermore, the S-gal-transfected DA SMC demonstrated a 5-fold increase in the number of cells attached to elastin-coated plates (4 h after initial plating), as compared with controls (Fig. 7). There was no statistically significant difference in adhesion between vector-, β-gal-, and S-gal-transfected cells when plated on plastic alone, albumin, or collagen type I (data not shown). Most interestingly, the immunocytochemistry of 7-day-old cultures revealed that S-gal-transfected DA SMC were capable of normal elastic fiber assembly, while the vector- (data not shown) and β-gal-transfected cells did not deposit elastic fibers in the newly produced matrix (Fig. 5, middle panel). Moreover, the extracellular matrix produced by the S-gal-transfected DA SMC showed very little immunodetectable fibronecin, while β-gal-transfected cells deposited an abundance of fibronecin (Fig. 5, lower panel). This result is consistent with the previously described mechanism implicating the cell surface EBP as a blocker of the adjacent IL-1 receptor type I, which transduces signals evoked by endogenous IL-1β, leading to the up-regulation of fibronecin synthesis and secretion in vascular SMC (20, 21).

**DISCUSSION**

Despite comprehensive functional characterization of the elastin-binding protein (EBP) (4–6, 9, 10), its structure has not been determined. In 1993, after tryptic digestion of sheep EBP,
alternatively spliced catalytically inactive variant of human α unique sequence (encoded by the frameshifted exon 5) of the which could be substituted without change in net charge) with 50% direct homology (and an additional three amino acids, quence, obtained from cyanogen bromide digestion, contained tures of antibody shows that extracellular matrix produced by 7-day-old cul-crease in the expression of anti-P-gal-immunoreactive material on the cell surface. Middle panel, immunostaining with an anti-tropoelastin antibody shows that extracellular matrix produced by 7-day-old cultures of β-gal-transfected DA SMC does not contain any organized elastin, while S-gal-transfected cells demonstrate normal assembly of elastic fibers. Lower panel, the extracellular matrix produced by β-gal-transfected DA SMC contains an abundance of fibronectin (FN) detected with a monoclonal anti-fibronectin antibody. Production of fibronecin in cultures of S-gal-transfected cells is greatly reduced.

we noted (11) that in addition to several oligopeptides fully homologous to human β-galactosidase, a 14-amino acid sequence, obtained from cyanogen bromide digestion, contained 50% direct homology (and an additional three amino acids, which could be substituted without change in net charge) with a unique sequence (encoded by the frameshifted exon 5) of the alternatively spliced catalytically inactive variant of human β-galactosidase (S-gal) originally described by Morreau et al. (22) and confirmed by Yamamoto et al. (32). Subsequent studies showed further immunological and functional similarities between these two proteins (6, 11, 17, 18, 31). We have shown that both extracts of human placenta and extracts of sheep arterial SMC contained a 67-kDa protein which bound to elastin and laminin affinity columns and could be eluted from those columns with several galactosugars. These elastin/laminin-binding proteins, isolated from both human and sheep tissues, were equally immunoreactive with anti-P-gal, anti-C-gal, and anti-S-gal antibodies, as well as with the monoclonal BCZ antibody raised to bovine EBP (11). In addition to identical patterns of immunostaining assessed under light and electron microscopy, we also found that anti-S-gal antibody co-localized with intracellular tropoelastin and with extracellular elastic fibers in ovine arteries (17, 18). Moreover, the treatment of cultured ovine aortic SMC with anti-S-gal antibody (recognizing and blocking to elastin-binding domain of S-gal) caused impaired assembly of elastic fibers (18).

To demonstrate that S-gal and EBP are indeed the same protein, the cDNA clone for S-gal was required. Thus, the full-length clone was constructed after precise trimming and ligation of two separate and partially overlapping cDNA fragments pulled out from human fibroblast mRNA. Sequencing of the final PCR product showed that the resulting clone was in frame and error-free as compared with the described original S-gal cDNA (22).

In attempting to determine if S-gal and EBP are really the same protein, we undertook a series of studies to characterize the properties of the S-gal-cDNA encoded protein and to determine the degree to which it displayed the features attributed to date to EBP. As a first step, we employed in vitro transcription/translation and obtained a protein of approximately 60,000 (Fig. 2), which corresponded well to the 60-kDa unglycosylated version of S-gal, as deduced from its primary sequence described by Morreau et al. (22). For comparison, we also tested the wild type β-gal cDNA clone. SDS-PAGE/autoradiography revealed that both proteins were immunoprecipitable with polyclonal anti-P-gal antibody (which recognizes common epitopes in both spliced variants of β-gal), but differed in their molecular size (Fig. 2C). The S-gal-encoded protein had a molecular mass of 60 kDa, whereas the 76-kDa, β-gal-encoded product was consistent with the deduced size of an unglycosylated and unprocessed precursor of β-gal (22). Moreover, only the 60-kDa S-gal cDNA-encoded product immunoprecipitated with an anti-S-gal antibody, raised to an unique sequence present in the frameshift-generated domain of S-gal. In addition, the 60-kDa protein produced by in vitro translation was also immunoprecipitable with anti-C-gal antibody, which recognizes a C-terminal domain present in the β-gal precursor and S-gal, but cleaved off from the mature form of the active enzyme (Fig. 2B).

The most important test for the S-gal-encoded protein was its relation to EBP, as judged by its functional ability to bind to
elastin and laminin. We demonstrated that the 60-kDa, S-gal-encoded product efficiently bound to these two matrix ligands but not to collagen type I (Fig. 2D). This result confirmed the tight protein-protein associative character of the elastin-S-gal and laminin-S-gal binding, suggested by our previous studies (11), and additionally showed that such an association does not require glycosylation or other post-translational modification of S-gal.

Since these data indicated that the S-gal produced is immunologically the same as EBP, and has the ability to bind both elastin and laminin, subsequent experiments were aimed at determining whether the protein, when expressed in mammalian cells, displayed additional properties of EBP. First, we tested whether transfection of the primate COS-1 cells with the S-gal cDNA clone would increase expression of the fully processed 67-kDa S-gal protein and then whether this overexpressed protein would localize to the cell surface and serve as a functional EBP by mediating interactions of the transfected cells with elastin. Indeed, affinity chromatography confirmed a substantial increase in the amount of the 67-kDa elastin-bound protein retained from extracts of the S-gal-transfected COS-1 cells as compared with controls (Fig. 3B). The 67-kDa protein overexpressed by S-gal-transfected COS-1 cells was immunoreactive with anti-P-gal antibody, which also recognized the 85-kDa β-gal precursor overexpressed in the β-gal-transfected cells, on Western blots (Fig. 3A). We therefore concluded that the S-gal CDNA-encoded protein, which resolves with an identical (67-kDa) molecular mass when produced in COS-1 cells, likely undergoes post-translational modifications comparable to the spliced variant of β-gal synthesized in normal cells (22).

It also has the elastin-binding capabilities identical to EBP isolated from sheep vascular SMC or from human placenta (11). Immunostaining with anti-P-gal, anti-S-gal, and BCZ antibodies (each recognizes EBP) revealed that cultures of S-gal-transfected COS-1 cells contained clusters of cells with increased expression of intracellular and cell surface-localized immunoreactive protein, as compared with controls. Moreover, patterns of immunostaining were identical to those of EBP in normal aortic SMC (9, 11).

Although both β-gal and S-gal contain identical signal peptides, which targets these proteins to the endoplasmic reticulum, the cell surface localization of the S-gal encoded protein indicates that this variant, directly translated in COS-1 cells, does not contain the putative lysosomal targeting domain (mannose 6-phosphate receptor-binding domain) present in the precursor of active β-gal. Since the exact location of this lysosomal targeting domain in the precursor of active β-gal has not been conclusively determined, one can speculate that such a critical aspect of this targeting requirement must be supplied by one, or a combination, of the exons (exons 3, 4, or 6) that are spliced out in the S-gal variant. Our results confirm that the newly produced S-gal proceeds through the secretory pathways of transfected COS-1 cells, where it is prepared for its release to the cell surface. Currently, we do not know whether the S-gal protein overexpressed in COS-1 cells associates with the two other 55-kDa and 61-kDa subunits of the elastin receptor. Immobilization of S-gal on the cell surface suggests, however, that such an association, which was recently documented by double and triple immunostaining of vascular SMC, is most probable. The increased expression of the functional elastin-binding protein was additionally confirmed by an adhesion assay, which showed significant increases in the number of S-gal-transfected cells attached to elastin-covered plates, as compared with controls (Fig. 4). An increase in specific adher-

**REFERENCES**

1. Juliano, R. L., and Haskill, S. (1993) *J. Cell Biol.* 120, 577–585
2. Lin, C. Q., and Bissel, M. (1993) *FASEB J.* 7, 737–743

---

A. Hinek and J. W. Callahan, unpublished results.
3. Abelda, M. S. (1993) Lab. Invest. 68, 4–17
4. Hinek, A., Wrenn, D. S., Mecham, R. P., and Barondes, S. H. (1988) Science 239, 1539–1541
5. Mecham, R. P., Hinek, A., Entwistle, R., Wrenn, D. S., Griffin, G. L., and Senior, R. M. (1989) Biochemistry 28, 3716–3722
6. Hinek, A. (1996) Biol. Chem. Hoppe-Seyler 377, 471–480
7. Gross, L. E., and Scott, M. (1993) Biochemistry 32, 13369–13374
8. Mecham, R. P., Hinek, A., Entwistle, R., Wrenn, D. S., Griffin, G. L., and Senior, R. M. (1989) Biochemistry 28, 3716–3722
9. Hinek, A., Mecham, R. P., Keeley, F. W., and Rabinovitch, M. (1991) J. Clin. Invest. 88, 2083–2094
10. Hinek, A., Boyle, J., and Rabinovitch, M. (1992) Exp. Cell Res. 203, 344–353
11. Jacob, M. P., Fulop, T., Foris, G., and Robert, L. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 995–999
12. Jacob, M. P., Fulop, T., Foris, G., and Robert, L. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 995–999
13. Faury, G., Ristori, M. T., Verditti, J., Jacob, M. P., and Robert, L. (1995) J. Vasc. Res. 32, 112–119
14. Fulop, T., Jacob, M. P., Foris, G., Varga, Z., and Robert, L. (1989) Cell Calcium Metabolism, Plenum Publishing Corp., New York
15. Robert, L., and Jacotot, B. (1994) J. Jap. Atheroscler. Soc. 21, 613–618
16. Varga, Z., Jacob, M. P., Robert, L., and Fulop, T. (1989) FEBS Lett. 258, 5–8
17. Hinek, A., and Rabinovitch, M. (1994) J. Cell Biol. 126, 563–574
18. Hinek, A., Klee, E., and Callahan, J. W. (1995) Exp. Cell Res. 220, 1–13
19. Boudreau, N., and Rabinovitch, M. (1991) Lab. Invest. 64, 187–199
20. Molossi, S., Clausel, N., and Rabinovitch, M. (1995) J. Cell Physiol. 163, 19–29
21. Hinek, A., Molossi, S., and Rabinovitch, M. (1996) Exp. Cell Res. 225, 122–131
22. Morreau, H., Galjart, N. J., Gillemans, N., Willemse, R., van der Horst, G. T. J., and d’Azzo, A. (1989) J. Biol. Chem. 264, 29655–29663
23. Prosser, I. W., Whitehouse, L. A., Parks, W. C., Hinek, A., Park, P. W., Mecham, R. P. (1991) Connect. Tissue Res. 25, 265–279
24. Zhang, S., McCarter, J. D., Okamura-Oho, Y., Yaghi, F., Hinek, A., Withers, S. G., and Callahan, J. W. (1994) Biochem. J. 304, 281–288
25. Okamura-Oho, Y., Zhang, S., Hilsen, W., Hinek, A., and Callahan, J. W. (1996) Biochem. J. 313, 787–794
26. Mecham, R. P., Hinek, A., Cleary, E. G., Kurich, U., Lee, S. J., and Rosen-bloom, J. (1988) Biochem. Biophys. Res. Commun. 151, 822–826
27. Hanahan, D. (1983) J. Mol. Biol. 166, 557–580
28. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (eds) (1996) Current Protocols In Molecular Biology, Suppl. 36, pp. 9.2.1–9.2.3, John Wiley & Sons, New York
29. Elsholtz, H. P., Lew, A. M., Albert, P. K., and Sundmark, V. C. (1991) J. Biol. Chem. 266, 22919–22925
30. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., pp. 16.32–16.36, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
31. Hinnek, A. (1994) Cell Adhes. Commun. 2, 185–193
32. Yamamoto, Y., Kake, C. A., Martin, B. M., Kretz, K. A., Ahern-Rindell, A. J., Naylor, N. L., Mudd, M., and O’Brien, J. S. (1993) DNA Cell Biol. 9, 119–127
33. Suzuki, Y., Sakuraba, H., and Oshima, A. (1995) Metabolic Basis of Inherited Disease, Vol. 2, McGraw-Hill, New York