Identification of a Novel Serum and Growth Factor-inducible Gene in Vascular Smooth Muscle Cells*

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We have used subtraction cloning to isolate a cDNA (PS4) that identified a serum-inducible mRNA of 1.9 kilobases in rabbit vascular smooth muscle cells. DNA sequence analysis revealed one major open reading frame encoding a 9,442 M, protein. Comparison of the DNA as well as the putative protein sequence with various data bases revealed no homology with other sequences. In vitro translation of synthesized PS4 mRNA generated a major polypeptide of 12 kDa. Serum stimulation of quiescent smooth muscle cells in culture induced a rapid increase in the level of PS4 mRNA. Expression of this message was detected by 1 h, peaked at approximately 4 h, and became undetectable by 12 h. The induction of PS4 by serum was completely blocked by cycloheximide, indicating its expression required prior protein synthesis. Epidermal growth factor, acidic fibroblast growth factor, and transforming growth factor-β1 also induced a strong increase in PS4 expression. By contrast, platelet-derived growth factor-BB was only able to mildly stimulate the level of PS4 mRNA and insulin-like growth factor-I was unable to enhance PS4 expression. There was a high level of PS4 mRNA in rabbit fetal muscle, esophagus, kidney, and lung, a low level in fetal aorta and heart, and an undetectable level in fetal liver, brain, as well as, in the placenta. The expression of PS4 in the corresponding adult tissues was low or undetectable. Our analysis indicate that PS4 expression is developmentally regulated and tightly controlled by growth factors, suggesting this novel gene has a role in cell growth and differentiation.

Unscheduled vascular smooth muscle cell (SMC) division has a central role in diseases such as atherosclerosis and hypertension (Ross, 1986; Schwartz et al., 1986). It is also the major reason for the failure of surgical interventions such as coronary angioplasty, aorto-coronary bypass surgery, and tissue transplants (DeFeudis, 1991). While much is still not clear with regard to the role of different mitogens in stimulating SMC proliferation in vivo, the transition from the growth-arrested state to a proliferative state has been well characterized. For example, c-fos expression increases dramatically within 30 min after vessel wall injury, thrombospondin expression is observed by 1 h, ornithine decarboxylase activity is maximal at about 6 h, and histone synthesis as well as DNA synthesis begins 24 h after the initial stimulus (Clowes et al., 1983; Majesky et al., 1990; Raugi et al., 1990; Nishida et al., 1990). The kinetics of gene activation in vivo is nearly identical to that observed when SMCs in culture are arrested at the G0 phase of the cell cycle and subsequently stimulated with serum or growth factors (Kindy et al., 1986; Thyberg and Fredholm, 1987; Majack et al., 1987; Janat and Liau, 1992). These results indicate that arterial wall cells are blocked at the G0 phase of the cell cycle and that cultured SMCs provide an important model to study the proliferative response of these cells in vivo.

A number of genes have been identified which are rapidly induced when cultured quiescent cells are stimulated to proliferate (Denhardt et al., 1986; Nathans et al., 1988; Bravo et al., 1988; Herschman, 1991). They include transcription factors, secreted polypeptides with autocrine or paracrine functions, and cytoskeletal and extracellular matrix components. The majority of these have been identified in mitogen-treated fibroblasts and lymphocytes. The expression of several of these growth factor-inducible genes in cultured SMCs has been characterized, and they are regulated similarly to that in fibroblasts (Kindy and Sonenshein, 1986; Gadeau et al., 1991). What is unclear is whether there are growth factor-stimulated responses that are restricted to SMCs. In addition, different growth factors appear to elicit unique responses both in vivo and in cultured SMCs, but it is unclear how such responses are mediated (Lindner and Reidy, 1991; Ferns et al., 1991; Majesky et al., 1991; Janat and Liau, 1992; Janat et al., 1992; Hwang et al., 1992). In this report, we describe the identification and characterization of a novel serum-inducible gene in vascular SMCs and detail the expression of this gene after stimulation by specific growth factors and in fetal and adult tissues.

EXPERIMENTAL PROCEDURES

Materials—Defined fetal bovine serum (FBS) was purchased from Hyclone Laboratories, Inc. (Logan, UT). Bovine insulin and transferrin were obtained from Sigma. Human recombinant epidermal growth factor (EGF) was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Human recombinant platelet-derived growth factor-BB (PDGF-BB) and insulin-like growth factor-I (IGF-I) were obtained from Bachem California (Torrance, CA). Porcine transforming growth factor-β1 (TGF-β1) was obtained from R & D System (Minneapolis, MN). Human recombinant acidic fibroblast growth
factor (aFGF) was generously provided by Dr. W. H. Burgess (American Red Cross, Rockville, MD).

**Cell Culture**—Rabbit vascular smooth muscle cells were isolated by enzymatic digestion as previously described (Liu and Chan, 1989). The cells were routinely grown in medium 199 supplemented with 10% FBS, 4 μM L-glutamine, 100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B. Cells were rendered quiescent by incubation for 48 h in medium containing 0.5% FBS, 10 μM insulin, and 5 μg/ml transferrin and stimulated by feeding with medium containing 20% FBS or by adding growth factor directly to the medium at the appropriate concentration. [3H]Thymidine incorporation assays were performed as previously described (Janat and Liau, 1992).

**cDNA Library Construction and Screening**—Poly(A)+ mRNA was isolated from rabbit vascular SMC and SMC stimulated with 10% FBS for 2 h. Double stranded cDNAs were primed with oligo(dT) and synthesized using avian myeloblastosis virus reverse transcriptase and RNase H/DNA polymerase I (Gubler and Hoffman, 1983). The cDNA was blunt-ended using T4 DNA polymerase and ligated to BstXI linkers. The cDNA was subsequently size fractionated using Bio-Gel A-1.5m, ligated to EcoRI adaptors, and cloned into the Lambda Zap I1 phage (Stratagene). This 2.6-kbp cDNA fragment was converted to double stranded DNA using the Klenow enzyme and transcribed with T3 RNA polymerase using reagents and the method described in the Invitrogen subtraction kit. The cDNA-containing plasmids were first transferred to E. coli Xs127 and single stranded cDNA was subsequently obtained using the M13 helper phage, R408. Subtraction was performed with 20 μg of photo-biotinylated quiescent SMC cDNA and 2.5 μg of serum-stimulated cDNA. The biotinylated DNA and cDNAs that hybridized to this were separated from unbound cDNA by incubating with streptavidin and subsequent extraction with phenol/chloroform.

**RESULTS**

PS4 mRNA Is Rapidly and Transiently Induced by Serum but Is Not a Member of the Immediate-Early Gene Family—We constructed a cDNA library enriched in sequences expressed by cultured rabbit vascular SMC stimulated with FBS. This subtracted cDNA library was screened by RNA blot analysis and a number of cDNA clones that encoded genes displaying increased expression in serum-treated cells were isolated. One of these cDNA clones, PS4, was chosen for further analysis since the mRNA size and the pattern of serum induction was unlike serum-inducible genes previously described (Herschman, 1991; Lau and Nathans, 1991). The expression of PS4 following serum addition is shown in Fig. 1. The PS4 cDNA hybridized to a mRNA with a size of approximately 1.9 kb. Expression of PS4 was undetectable in growth-arrested SMCs and was observed by 2 h after serum addition. In other time course analyses, we found that the earliest detectable expression of this gene was by 1 h after serum addition (results not shown). Peak level of PS4 occurred around 4 h and its expression was nearly undetectable by 12 h. Glyceraldehyde-3-phosphate-dehydrogenase mRNA expression was monitored as a control and showed no appreciable change.

We next examined whether induction of PS4 mRNA expression by serum was a primary or secondary response. SMCs were stimulated with 20% FBS for 4 h in the presence and absence of 10 μg/ml cycloheximide and harvested for Northern analysis. The results, shown in Fig. 2, indicate that this protein synthesis inhibitor completely blocked the serum induced increase in PS4 mRNA level. Cycloheximide alone slightly induced the expression of PS4. This may be due to enhanced stabilization of PS4 mRNA or cycloheximide may inhibit the synthesis of transcription factor(s) that normally repress PS4 transcription. Our results indicate that PS4 mRNA induction by serum requires prior protein synthesis and therefore, is not a member of the family of immediate-early genes (Nathans et al., 1988).

**DNA Sequence Analysis and in Vitro Translation Revealed That PS4 Encodes a Novel 9.4-kDa Polypeptide**—We next examined whether PS4 was related to other serum-inducible genes that have been described (Denhardt et al., 1986; Na...
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Fig. 2. Analysis of PS4 mRNA expression in the presence of the protein synthesis inhibitor, cycloheximide (CHX). Quiescent SMCs were treated for 4 h with fresh medium containing 20% FBS in the presence or absence of 10 μg/ml cycloheximide and harvested for Northern analysis. The protein synthesis inhibitor was added 30 min prior to serum addition. Ethidium bromide staining of the Northern gel visualized the 28 S and 18 S ribosomal RNAs and revealed that there was no RNA degradation due to the addition of cycloheximide and that the amount of RNA loaded in each lane was approximately equivalent.

Fig. 3. DNA sequence and predicted protein sequence of PS4E cDNA. The sequencing was performed by dyeodeoxy chain termination. The complete DNA sequence as well as the largest ORF beginning from the first AUG are shown. The dibasic amino acid clusters in the ORF are marked by asterisks. The four ATTA motif which may confer mRNA instability are underlined. A consensus polyadenylation signal immediately upstream of the poly(A) sequence is capitalized and underlined.

thans et al., 1988; Bravo et al., 1988; Herschman, 1991). The original cDNA isolated from the subtracted library was approximately 700 bp and comprised a third of the mRNA size. We constructed a second cDNA library and used the 700-bp cDNA to isolate a 1.8-kb cDNA (PS4E) that was similar in size to the mRNA. Since the 1.9-kb mRNA presumably includes a poly(A) length of around 200 bases, PS4E likely encompasses the entire mRNA. Both PS4 and PS4E were sequenced and the 1,873 bases are shown in Fig. 3. The first AUG (translational start site) begins at nucleotide 29, is embedded within an optimal nucleotide context, and defines an open reading frame (ORF) that can potentially encode a polypeptide of 87 amino acids with a deduced molecular weight of 9442. This deduced polypeptide contains a number of potential serine/threonine phosphorylation sites and four dibasic clusters. In addition, examination of the sequence revealed an internal repeat of TVXTXG. This motif was used to search the Swiss-Pro Protein Data Bank and only four proteins were found to contain this motif. Interestingly, the sequence encompasses a cleavage site in at least two of these proteins being the junction between VP1 and VP2 coat protein of poliovirus type 2 and the signal sequence junction of the α chain of the T-cell receptor. There are a number of other AUGs scattered throughout the sequence but most of these can encode only very short polypeptides. The only other substantial ORF begins at nucleotide 845 and can potentially encode a protein of 62 amino acids. In addition, we cannot absolutely rule out the possibility that an upstream AUG exists and this could encode a polypeptide of at least 50 amino acids. The nucleotide sequence of PS4 was compared to sequences in the GenBank data base and no substantial similarity was detected to other nucleotide sequences. We also compared the three ORFs described above with the PIR and Swiss-Pro protein data banks and found that none of the potential polypeptide sequences were similar to previously described sequences. Based on these results, we conclude that PS4 is a novel gene and that the 9.4-kDa polypeptide initiated from the first AUG is the most likely translated product.

We next performed in vitro translation analysis to characterize the polypeptide product encoded by the PS4 mRNA. RNA transcripts were synthesized that encompassed the entire PS4E cDNA (+1937) as well as encompassing only the first ORF (+428). These transcripts were translated in a wheat germ translation system and analyzed on a 15% SDS-polyacrylamide gel. The results indicate that both the full-length transcript and the truncated transcript directed the translation of a major polypeptide with a molecular mass of approximately 12 kDa (Fig. 4). In addition, less prominent bands of 5.5 and 16 kDa were also detected. Since the truncated...
cated, direct transcription products identical to the full-length transcript. The translation must initiate and end prior to base +428 (+344 of the PS4 sequence shown in Fig. 3). Furthermore, the size of the major band corresponds well to the 9.4-kDa polypeptide deduced from the first AUG. We conclude from these results that the major in vitro translation product of 12 kDa initiates from the first AUG at +29.

**PS4 Expression Is Regulated by Growth Factors in a Complex Manner**—We also examined the expression of PS4 in response to stimulation with specific growth factors. Quiescent SMCs were treated with PDGF-BB, TGF-β1, aFGF, and EGF for various times and analyzed for PS4 mRNA expression. We found that both aFGF and EGF were rapid and potent inducers of PS4 mRNA level with peak induction occurring between 2 and 4 h (Fig. 5A). By contrast, TGF-β1 treatment caused an increase in PS4 that was considerably delayed, peaking well after 6 h while PDGF-BB elicited a much smaller increase in PS4 mRNA level (Fig. 5A). In other experiments, we treated SMC with different amounts of these factors and found that SMC treated with up to 20 ng/ml PDGF-BB still exhibited only a small increase in PS4 mRNA level (results not shown). By contrast, 1 ng/ml aFGF was able to enhance PS4 mRNA expression greater than 3-fold over 20 ng/ml PDGF-BB. Finally, PS4 mRNA expression was completely unresponsive to the addition of up to 50 ng/ml IGF-I (results not shown). The rapid expression of PS4 in response to aFGF contrasted distinctly with the delayed induction seen with TGF-β1. It was possible that PS4 induction by aFGF may be an immediate-early response of SMCs. We, therefore, determined whether the induction of PS4 by aFGF and by TGF-β1 required prior protein synthesis. The results, shown in Fig. 5B, indicated that cycloheximide can completely abrogate the enhanced PS4 mRNA level due to aFGF or TGF-β1. The expression of PS4 in response to serum and growth factors was not restricted to SMCs. PS4 mRNA was also induced in fibroblasts treated with serum or with aFGF (results not shown). However, we have been unable to detect PS4 induction in endothelial cells treated with aFGF or in BC3H1 cells (a nonfusing myogenic cell line) treated with serum or induced to differentiate.

**Expression of PS4 Is Developmentally Regulated and Tissue Restricted**—To gain further insight into the regulation of this novel serum-inducible gene, we examined the tissue distribution of PS4 mRNA in 4-week-old rabbits and in 25-day gestation rabbit fetus. Northern blot analysis indicated that the same 1.9-kb mRNA size existed in all the tissues that expressed PS4 and that fetal skeletal muscle, esophagus, kidney, and lung contained the highest level of this mRNA (Fig. 6). Results of further quantitative slot blot analysis are summarized in Table I. PS4 mRNA was undetectable in placenta or in fetal and 4-week-old adult liver or brain. A low level of PS4 mRNA was detected in the heart and aorta, irrespective of age. We conclude that PS4 expression is both tissue-restricted and developmentally regulated.

**DISCUSSION**

We report here the identification and characterization of a novel serum-inducible gene. Although the biological function of PS4 is unknown, the ability of polypeptide growth factors to regulate the expression of this gene as well as its tissue restricted and developmentally modulated expression indicate it may have an important role in cell growth and/or differentiation. Since the induction of PS4 by serum and growth factors requires prior protein synthesis, PS4 can be classified with a group of growth factor responsive genes commonly termed the "delayed early-response genes" (reviewed in Lau and Nathans, 1991). To date, only a limited number of these delayed early-response genes have been identified. They, predictably, include a number of biosynthetic enzymes important for DNA synthesis (Lau and Nathans, 1991; Farnham and Schimke, 1985). They also include secreted proteases, potential cytokines, and transcription factors (Lau and Nathans, 1991). In addition, proliferating cell nuclear antigen, p53, Ras, cyclin-like 1, HMG1(Y), and HMG1(C) are also members of this family (Lau and Nathans, 1991; Lanahan et al., 1992). Although, the function of many of these genes is still unknown, they clearly are part of a complex network of events that determine the cell's response to exogenous signals.

We have previously demonstrated that in rabbit SMCs, DNA synthesis does not occur until approximately 18 h after serum stimulation (Janat and Liau, 1992). However, PS4 mRNA level is nearly undetectable by 12 h after serum addition. Therefore, unlike the majority of the delayed early genes which continue to be expressed at elevated levels through S phase, PS4 expression is not directly associated with S phase, suggesting that PS4 may not have a direct role in DNA replication. Interestingly, the regulation of PS4 message and the parathyroid hormone-related protein (PTH-rP) mRNA by serum and by growth factors are very similar (Hongo et al., 1991). The time course of induction of PTH-rP mRNA in vascular SMCs is identical to PS4 induction and this expression is also repressed by cycloheximide (Hongo et al., 1991). In addition, PTH-rP mRNA expression, like PS4, is not significantly stimulated by PDGF and IGF-I. A comparison of the deduced protein sequence of PS4 and the PTH-rP sequence revealed no similarities. However, it is possible that PS4 and PTH-rP may be regulated through a common pathway. Experiments are in progress to determine whether
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Table I

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*NA, not applicable.

there are additional similarities in the regulation of PS4 and PTH-rP expression. In smooth muscle, PTH-rP is a vasorelaxant and may also affect calcium homeostasis (Mok et al., 1989). It will be interesting to explore the possibility that the polypeptide product of PS4 may also be involved in these events.

We believe that the deduced polypeptide sequence shown in Fig. 3 is the translated product of PS4 since it uses the first AUG, and this translational start site contains flanking sequences that provide both the optimal purine in position -3 and the G in position +4 (Kozak, 1991). In vitro translation, which shares many of the requirements of translation in vivo, synthesized a polypeptide of the appropriate size, and truncation analysis indicate that translation must begin at the proposed translational start site (Kozak, 1989). Nevertheless, we presently cannot fully eliminate the possibility that translation in vivo may be initiated at another AUG. A second feature in the PS4 cDNA sequence is the presence of 4 AUUUA motifs in the 3′ presumed nontranslated region. Interestingly, two of the AU motifs are in tandem. The presence of such AU-rich sequences has been shown to be the optimal purine in position -3 and the G in position +4. The deletion of these motifs in the PS4 cDNA sequence is growth factor-dependent. For example, PS4 mRNA returns to basal level more rapidly after peak induction by aFGF than by TGF-β1. It is presently unclear whether PS4 may be involved in mediating the ability of TGF-β1 to further stimulate PDGF-BB-mediated DNA synthesis.

That PS4 is highly responsive to aFGF and TGF-β1, but only weakly responsive to PDGF-BB and not responsive to IGFl is particularly interesting. In vivo PDGF appears to stimulate the first wave of SMC migration after blood vessel injury while FGF may be the primary initiator of early SMC replication (Lindner and Reidy, 1991; Ferns et al., 1991). These results indicate that SMCs have distinct responses to specific growth factors. There is also evidence for differences in second messenger signals transmitted through the receptors for PDGF and FGF. For example, the PDGF receptor tyrosine kinase is able to phosphorylate the guanosine triphosphatase-activating protein and the phosphatidylinositol 3′-kinase, but the FGF receptor is unable to do so (Molloy et al., 1989; Aaronson, 1991). It is likely that PS4 induction represents part of a pathway strongly receptive to stimulation by aFGF and TGF-β1, weakly receptive to PDGF-BB, but not at all to IGFl. Further elucidation of the regulation of PS4 may provide additional insight into the distinct response of SMCs to these potent biological modifiers.

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