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The Protein Encoded by a Growth Arrest-Specific Gene (*gas6*) Is a New Member of the Vitamin K-Dependent Proteins Related to Protein S, a Negative Coregulator in the Blood Coagulation Cascade

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A set of growth arrest-specific genes (*gas*) whose expression is negatively regulated after serum induction has previously been described (C. Schneider, R. M. King, and L. Philipson, Cell 54:787–793, 1988). The detailed analysis of one of them, *gas6*, is reported here. *gas6* mRNA (2.6 kb) is abundantly expressed in serum-starved (48 h in 0.5% fetal calf serum) NIH 3T3 cells but decreases dramatically after fetal calf serum or basic fibroblast growth factor stimulation. The human homolog of *gas6* was also cloned and sequenced, revealing a high degree of homology and a similar pattern of expression in IMR90 human fibroblasts. Computer analysis of the protein encoded by murine and human *gas6* cDNAs showed significant homology (43 and 44% amino acid identity, respectively) to human protein S, a negative coregulator in the blood coagulation pathway. By using an anti-human Gas6 monospecific affinity-purified antibody, we show that the biosynthetic level of human Gas6 fully reflects mRNA expression in IMR90 human fibroblasts. This finding thus defines a new member of vitamin K-dependent proteins that is expressed in many human and mouse tissues and may be involved in the regulation of a protease cascade relevant in growth regulation.

Interactions between serine proteases, their substrates, and their inhibitors have largely been exploited during evolution. Protease cascades are not confined to the classical blood coagulation or complement cascade. A network of proteases that control the synthesis or activity of a ligand appears an ideal and finely regulatable mechanism to trigger a rapid response to an extracellular event, with the inherent advantage of powerful amplification. Thrombin, in addition to catalyzing fibrin polymerization, can act as a novel ligand for the recently identified thrombin receptor (61), a member of the seven-transmembrane domain receptor family, possibly mediating other known effects of thrombin, including its role as a mitogen for lymphocytes and fibroblasts (8, 9). Hepatocyte growth factor (scatter factor), which promotes cell division (53) and epithelial morphogenesis (47), is similar in structure to serine proteases (38% amino acid sequence identity with plasminogen), although it lacks proteolytic activity as a result of mutation of two residues in the catalytic triad (31, 48). Hepatocyte growth factor is the ligand for the c-*met* proto-oncogene product (5, 49), a transmembrane 190-kDa heterodimer with tyrosine kinase activity that is widely expressed in normal epithelial tissues (20).

Recently, a protease pathway has been shown to play a crucial role in the dorsoventral patterning of *Drosophila* embryos (36). At least three genes (*snake*, *gastrulation defective*, and *easter*) appear to encode extracellular proteases (7, 36). Easter appears to be the ultimate protease that processes spätzle that binds and activates its receptor Toll (36). The ultimate function of this pathway is to promote the translocation of dorsal protein to the nucleus, where it regulates the transcription of a number of target genes, including *tolloid*. The product of *tolloid* contains a protease domain, homologous to human bone morphogenetic protein 1 (55), that is involved in the activation of decapentaplegic (dpp), a member of the transforming growth factor β1 family (50).

In analogy to this finely dissected developmental system, a considerable body of evidence has pointed to a set of different proteases, including serine, cysteine, and metalloproteases, as prime candidates in the regulation of tumor invasion and angiogenesis (41, 46). The activities of these proteases are strictly regulated at the levels of both gene expression and zymogen activation (45). Furthermore, the activities of most of these proteases appear to be enhanced when the enzymes are cell membrane associated. Cell-bound proteases are subject to negative regulation by natural protease inhibitors (10). Although current knowledge of protease cascades relates to tissue remodeling during tumor invasion and angiogenesis, other cells must perform similar functions. In fact, normal tissue homeostasis is dependent on balanced rates of cell division, extracellular matrix (ECM) synthesis, and degradation. Recent evidence has demonstrated a close link between cytokines and growth factors that directly modulate the three processes. The ECM acts as a reservoir for several growth factors and modulates their activities (26). There is evidence that a number of proteases is involved in growth factor mobilization from the ECM (3, 25).

To dissect the mechanism that controls growth arrest in mammalian cells, a set of genes that are highly expressed during serum starvation in NIH 3T3 mouse fibroblasts was

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previously cloned (54). In this report, we characterize a growth arrest-specific gene, *gas6*, whose expression is negatively regulated during growth induction. The product of *gas6* represents a new member of the vitamin K-dependent family that is homologous to protein S, a cofactor of the activated protein C pathway that leads to the proteolytic inactivation of factors Va and VIIIa and ultimately to an effective anticoagulant action (23, 24). This finding may thus uncover a general important contribution of growth-regulated protease cascades to cell proliferation.

MATERIALS AND METHODS

Cell lines and cell culture conditions. NIH 3T3 cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 μg/ml). For serum starvation, NIH 3T3 cells plated at $10^4/cm^2$ were shifted to 0.5% FCS for 48 h. Under these conditions, incubation with 50 μM bromodeoxyuridine (BUdR) for 3 h resulted in labeling of less than 2% of the nuclei. For induction of DNA synthesis, fresh medium containing 20% FCS was added to growth-arrested cells. Cells were harvested at various times for RNA isolation. After 18 h of BUdR incorporation, more than 90% of the nuclei scored positive for DNA synthesis. For density-dependent inhibition, cells were plated at $10^4/cm^2$ in 10% FCS. Twenty-four hours after plating, the medium was changed every 2 days. After 4 days in culture, incubation with BUdR for 2 h yielded less than 1% of labeled nuclei. DNA synthesis was determined with a mouse monoclonal antibody against BUdR as described elsewhere (6). Human IMR90 fibroblasts were obtained from the Genetic Mutant Repository (Camden, N.J.) and grown as recommended. For serum starvation, subconfluent cells were shifted to 0.5% FCS for 72 h. Under these conditions, incubation with BUdR for 3 h resulted in labeling of less than 3% of the nuclei. DNA synthesis was induced as described above; after 18 h of BUdR incorporation, more than 45% of the nuclei stained positive. Basic fibroblast growth factor (bFGF) was kindly supplied by C. Grassi-Farmitalia, Milano, Italy, and used at a concentration of 100 ng/ml.

RNA preparation and Northern (RNA) blotting analysis. For extraction of total RNA from cells, the cultures were washed twice with phosphate-buffered saline, and lysis buffer (4 M guanidine isothiocyanate, 25 mM sodium citrate, 0.1 M 2-mercaptoethanol, 0.5% *N*-laurylsarcosine) was added. RNA from mouse or human tissues was extracted by disrupting the tissue in lysis buffer with a Polytron homogenizer. RNA was then isolated as described previously (11). Total RNA (20 μg) was separated on 1% agarose gels containing 6.7% formaldehyde (40) and transferred to Duralon-UV nylon membranes (Stratagene) by using a 2016 Vacugene apparatus (Pharmacia). RNA was cross-linked by exposure to UV light (Stratalinker; Stratagene). Hybridization was performed in 1 M NaCl–1% sodium dodecyl sulfate (SDS) at 65°C, using the corresponding probes labeled with ^{32}P by random-primer synthesis (Pharmacia).

Isolation of full-length cDNA clones for *gas6*. The original *gas6* clone (54) was used to screen two cDNA libraries generated from G_0 NIH 3T3 fibroblasts and mouse kidney mRNA (34) and cloned by an orientation-specific strategy (22) in the lambda vector T7-T3/E-H (33). The murine cDNA clone was used to screen a human lung fibroblast cDNA library (Stratagene) at low stringency, and a partial cDNA clone was isolated. To isolate the complete cDNA, a HeLa cDNA library generated in the lambda vector 1149 (53a) was screened under high-stringency conditions, using the partial human *gas6* (h*gas6*) insert.

DNA sequencing and sequence analysis. All DNA fragments, obtained by using appropriate restriction enzymes, were subcloned in the Bluescript KS+ plasmid (Stratagene). Plasmid and lambda DNAs were isolated and sequenced (18) with the T7 sequencing Kit (Pharmacia). Specific synthetic oligonucleotides were also used as primers for the sequencing reactions. The sequence of each nucleotide was determined three times on average, and the entire sequence was read on both strands. The sequence of the h*gas6* cDNA clone was obtained by using the EMBL-ALF sequencer. Sequence analysis was performed by using the Intelligenetics software package.

Polyclonal antibody preparation. The cDNA of h*gas6* was digested with *Pvu*II, and the resulting fragment from nucleotides (nt) 1209 to 1788 was ligated to *Bam*HI adaptors and inserted into the *Bam*HI site of the pAR 3038 vector, which carries the promoter of the Ψ 10 gene of T7 bacteriophage (58). Expression of T7 RNA polymerase was performed by infection of host cells (*Escherichia coli* Q358) with bacteriophage λΦCEG, carrying the bacteriophage T7 gene, at a multiplicity of infection of 5 to 7. Protein expression and purification were performed as described previously (6). Rabbits were injected with 200 μg of purified bacterial hGas6 protein mixed with an equal volume of complete Freund's adjuvant. The animals were then injected with the same amount of protein in incomplete Freund's adjuvant every 3 weeks for 2 months. Specific antibodies were affinity purified by using 0.5 mg of hGas6 protein covalently coupled to Affi-Prep 10 (Bio-Rad Laboratories, Cambridge, Mass.) as described previously (6).

In vitro translation of h*gas6*. In vitro translatable h*gas6* RNA was generated from the pCITE-1 vector (Novagene, Madison, Wis.), which contains an RNA capping-independent translation enhancer sequence downstream of the T7 polymerase promoter. pCITE-h*gas6* contains a cDNA fragment from the ATG (nt 135) to the end of the clone and was cloned in pCITE in two steps. First, the h*gas6* cloned in pBluescript KS+ was digested with *Nco*I, which cuts twice in h*gas6* at position 134, corresponding to the initial methionine, and at position 1260; the resulting fragment was inserted in the same site of the pCITE vector. Second, h*gas6* pBluescript KS+ was digested with *Sac*I, which cuts at nt 698 of the cDNA, and *Sal*I, which is present in the polylinker of the plasmid 3′ to the cDNA. The resulting fragment was inserted into pCITE/*Nco*I-containing fragment digested with the same enzymes. The pCITE-h*gas6* was then linearized with *Sal*I, transcribed, and translated as described previously (43). For immunoprecipitation, 5 μl of the reticulocyte translation mixture was mixed with 0.1 ml of Nonidet P-40 (NP-40) buffer (50 mM triethanolamine [TEA; pH 7.5], 0.1% NP-40, 150 mM NaCl) and incubated for 1 h on ice with anti-hGas6 affinity-purified antibody; 50 μl of a 10% (wt/vol) suspension of protein A-Sepharose (Pharmacia Fine Chemicals) was added, and incubation was prolonged for 30 min at 4°C with rocking. After three washes with NP-40 buffer, the immunocomplex was resolved by SDS-polyacrylamide gel electrophoresis (PAGE). The gel was fixed in methanol-acetic acid and treated for fluorography with Enlightening (DuPont).

In vivo biosynthesis of hGas6. Human IMR90 fibroblasts were labeled under different growth conditions for 3 h in 0.7 ml of methionine-free Dulbecco's modified Eagle medium containing [^{35}S]methionine (ICN-TRANS ^{35}S label; 1,133 Ci/mmol) at ~500 μCi/ml. At the end of the labeling period,

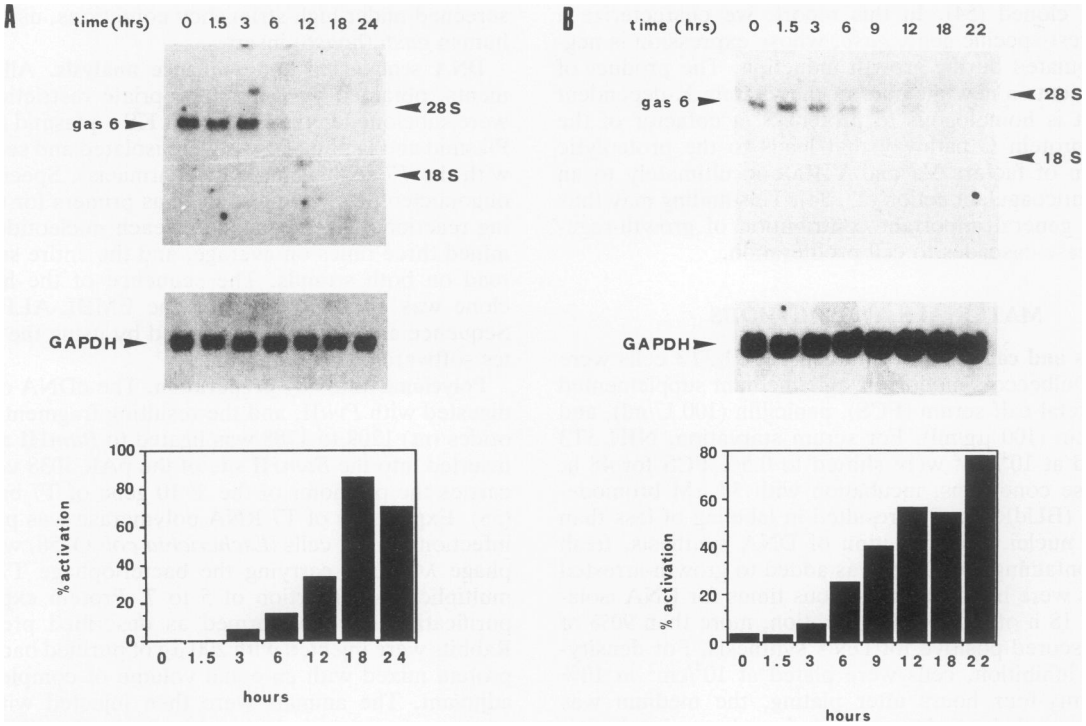

FIG. 1. Growth cycle regulation of *gas6* gene expression in NIH 3T3 cells. RNA was extracted from arrested NIH 3T3 cells (48 h in 0.5% FCS) and at the indicated times after addition of 20% FCS (A) or bFGF (B). Equal amounts (20 μg) of total RNA were analyzed by Northern blotting. The same blots were also probed with a *gapdh* cDNA probe. The histograms show the relative level of DNA synthesis for each time point analyzed on the Northern blots.

the medium was collected and supplemented with 50 mM TEA (pH 7.4), 150 mM NaCl, and 0.8% SDS (final concentrations). The cell monolayer was lysed with 0.5 ml of lysis buffer (150 mM NaCl, 50 mM TEA [pH 7.5], 0.1% NP-40) on ice for 3 min and then added with 0.8% SDS (final concentration). Both cell lysate and culture supernatant were then boiled for 4 min. After boiling, an equal volume of SDS quench buffer (150 mM NaCl, 50 mM TEA [pH 7.5], 4% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg each of aprotinin, leupeptin, antipain, and pepstatin per ml) was added. After clearing by centrifugation at 12,000 rpm for 2 min, the supernatants were incubated with 30 μl of normal rabbit serum for 1 h on ice. Samples were then transferred to an Eppendorf tube containing 20 μl of prewashed staphylococcal protein A and incubated at 4°C for 30 min with continuous rocking. After centrifugation for 2 min, the resulting supernatant was similarly treated once more and finally centrifuged for 5 min. At the end of these two preclearing steps, samples were immunoprecipitated by incubation with the affinity-purified anti-hGas6 antibody for 3 h at 4°C with rocking; 80 μl of protein A-Sepharose (10% [wt/vol]) suspension was added, and incubation was continued for 30 min at 4°C with rocking. Protein A-Sepharose was recovered by centrifugation, washed three times with 0.5% Triton X-100–20 mM TEA–150 mM NaCl–1 mM phenylmethylsulfonyl fluoride, and resuspended in SDS sample buffer. Immune complexes were released by boiling for 5 min and analyzed by SDS-PAGE as described above.

Nucleotide sequence accession numbers. The nucleic acid sequences of the murine and human *gas6* cDNAs have been submitted to the EMBL, GenBank, and DDBJ data banks under accession numbers X59846 and L13720, respectively.

RESULTS

Regulation of *gas6* mRNA expression by serum and bFGF at growth arrest and during the cell cycle. *gas6* belongs to a category of genes previously identified as growth arrest specific, because their expression is down-regulated after growth induction in arrested NIH 3T3 cells. Figure 1 shows a Northern blot analysis of *gas6* expression at various times after a synchronous cell division cycle induced either with FCS (Fig. 1A) or bFGF (Fig. 1B) in NIH 3T3 cells arrested for 48 h in 0.5% FCS (time 0). The mRNA identified by the *gas6* murine cDNA is about 2.6 kb in size and is abundantly expressed at growth arrest (time 0 in Fig. 1A). Six hours after addition of either 20% FCS (Fig. 1A) or 100 ng of bFGF per ml (Fig. 1B), *gas6* mRNA is already down-regulated. After 6 h, its level is undetectable in the case of serum stimulation, while it steadily decreases to an undetectable level after bFGF addition.

The same Northern blot was normalized for the amount of total RNA with the *gapdh* cDNA probe, known to remain constant throughout the growth cycle. The percentage of cells in S phase, from each time point analyzed on the Northern blots, is shown in the histograms in Fig. 1.

Figure 2 shows the kinetics of *gas6* mRNA accumulation during growth arrest by serum starvation (Fig. 2A) or increased cell density (Fig. 2B). *gas6* mRNA is detectable after 12 to 24 h in medium containing low serum and reaches the highest level at 48 h (Fig. 2A). Normalization of RNA amount was similarly performed on the same blot with the

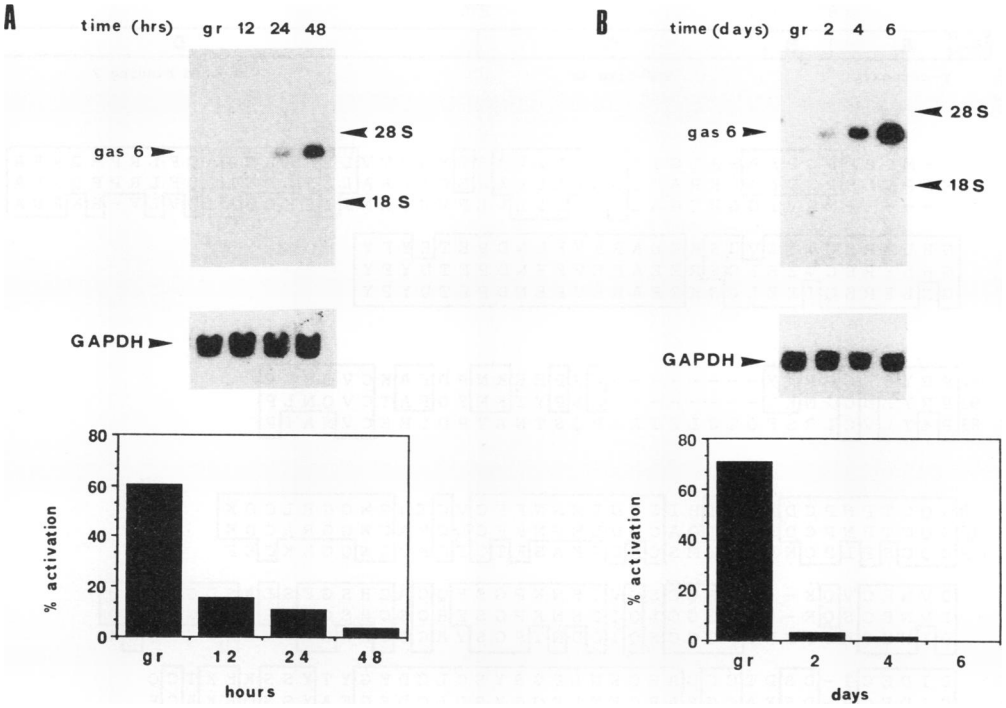

FIG. 2. Induction of *gas6* gene expression upon serum starvation and density-dependent inhibition in NIH 3T3 fibroblasts. (A) RNA was isolated from actively growing NIH 3T3 cells (gr = 24 h after seeding in 10% FCS) and at the indicated times after serum starvation in 0.5% FCS; (B) RNA was isolated from actively growing NIH 3T3 cells (gr = 24 h after seeding in 10% FCS) and at every 2 days before seeding with fresh culture medium (containing 10% FCS). Equal amounts (20 μg) of total RNA were analyzed by Northern blotting. The same blots were also probed with the *gapdh* cDNA probe. The histograms show the relative level of DNA synthesis for each time point analyzed on the Northern blots.

gapdh probe, and the percentage of cells in S phase was assessed by BUdR incorporation at each time point (shown in the histograms).

To analyze the expression of *gas6* mRNA in relation to growth arrest induced by density-dependent inhibition, NIH 3T3 cells were seeded in 10% FCS, and the medium was changed every 2 days. Figure 2B shows that *gas6* mRNA is significantly increased 2 days after seeding, and its accumulation levels off after 6 days. The same blot was normalized with *gapdh*. Under the same conditions, DNA synthesis was significantly decreased as soon as 2 days after seeding.

Mouse and human *gas6* cDNA sequences. The cDNA sequence of murine *gas6* is 2,556 nt long and encodes a protein of 673 amino acids (Gas6). Several full-length clones were analyzed both from the NIH 3T3 cDNA library and from a mouse kidney cDNA library, and all showed the same sequence and restriction pattern. The predicted protein sequence of murine *gas6* was compared against the entire protein sequence data bank, using FastDB (1). A significant homology with bovine and human vitamin K-dependent protein S (15, 42) emerged, with 43% identity between the 673 residues of murine Gas6 and the 677 residues of human protein S. The residue identity is 42% between murine Gas6 and the 676 residues of bovine protein S. To assess whether Gas6 was the murine homolog of human protein S or a related but different protein, we screened a human lung fibroblast cDNA library with murine *gas6*. A partial cDNA clone representing the human homolog was isolated and used to screen a HeLa cDNA library. A full-length cDNA clone was thus isolated and sequenced. The clone analyzed is 2,461 nt long and encodes a protein of 678 amino acids with 81% residue identity to murine Gas6 and 44% amino acid identity to human protein S. This comparison shows that hGas6 is related to but different from protein S. This finding thus identifies a new member of the vitamin K-dependent family of proteins.

Both murine and human Gas6 primary structures were compared with that of human protein S. Figure 3 shows the alignments and for the sake of clarity is divided into four regions. Region A includes the amino terminus, which contains a very conserved hydrophobic stretch typically resembling a signal peptide. This structure is consistent with protein S being a secreted protein and suggests a similar fate for Gas6. This region also contains the γ-carboxyglutamic acid (Gla) domain (16, 42) of protein S fully maintained in both murine and human Gas6. A pair of cysteines, fully conserved in region A, are known to form disulfide bonds in the human protein S (16). The Gla domain, present within the family of vitamin K-dependent proteins, is required for the calcium-dependent phospholipid binding that mediates the interaction of these proteins with cellular membranes (28, 59). A similar Gla domain-dependent interaction of Gas6 with cellular membranes may indicate a strict requirement for its compartmentalization in the regulation of a protease cascade (44). The short region B is known as the thrombin-sensitive segment of protein S (13). A Leu-Arg-Ser span represents the two thrombin cleavage sites in protein S. The comparable amino acid spans are Met-Arg-Lys and Phe-Ala-Lys in murine Gas6 or Ile-Gln-Lys and Phe-Ala-Thr in hGas6. The missing consensus may suggest that the Gas6 region B is not susceptible to the proteolytic attack by thrombin required for the negative feedback loop of the

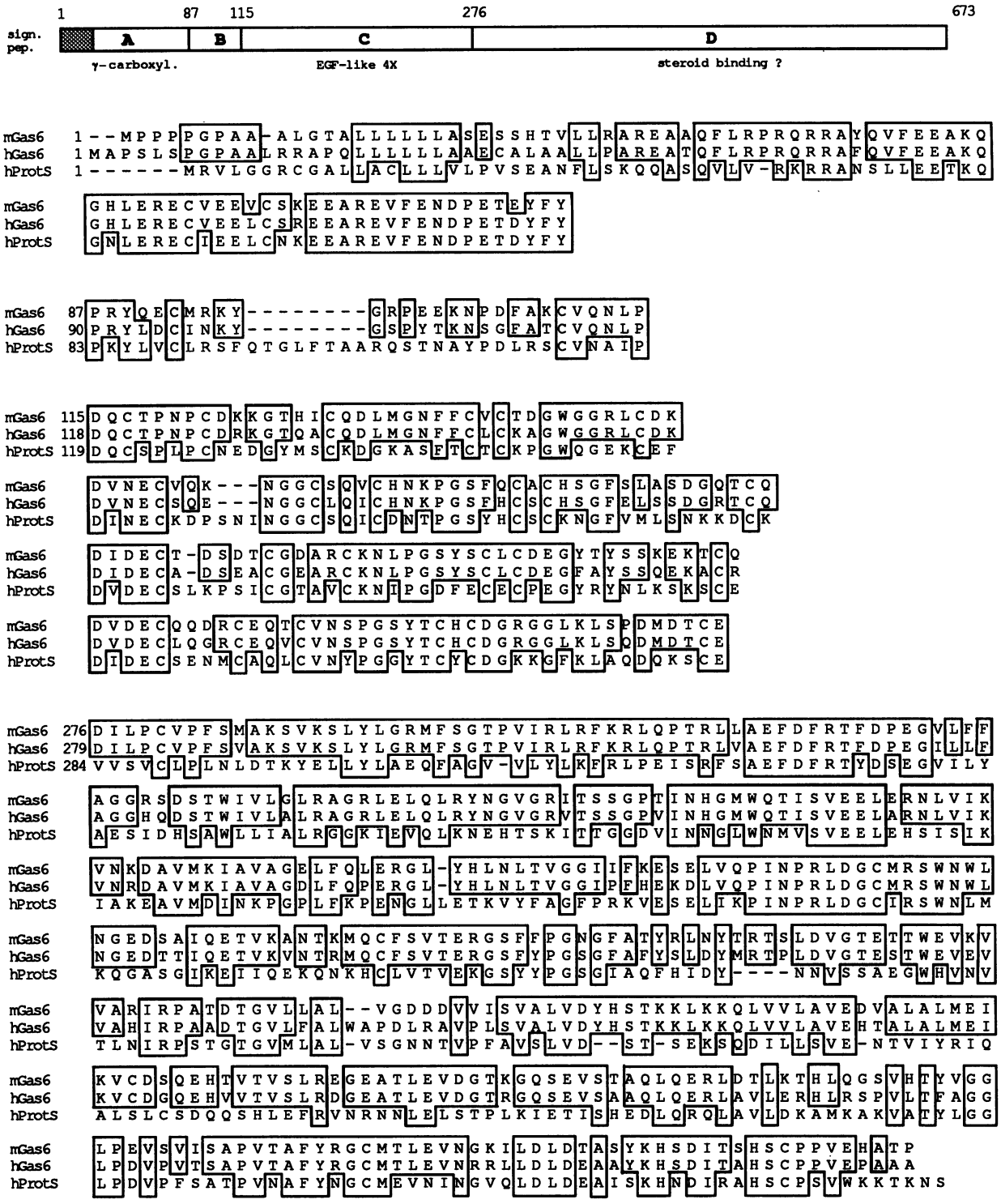

FIG. 3. Analysis of the *gas6* cDNA-encoded protein. The diagram at the top shows the overall organization of the predicted Gas6 sequence and the relative sizes of the four regions in the protein. Comparison of the predicted amino acid sequences of mGas6, hGas6, and human protein S is shown below. A, B, C, and D refer to the four regions present in these proteins (see Results for details).

coagulation cascade (13, 60). It is noteworthy that this region presents the lowest degree of homology to protein S (16% identity) relative to the other regions.

Region C includes four epidermal growth factor (EGF)-like repeats (21, 67), each containing six cysteines. A consensus sequence for β-hydroxylation of Asp and Asn residues is contained in each of these domains, as is the case for protein S (56). Hydroxylated Asp and Asn play a role in the

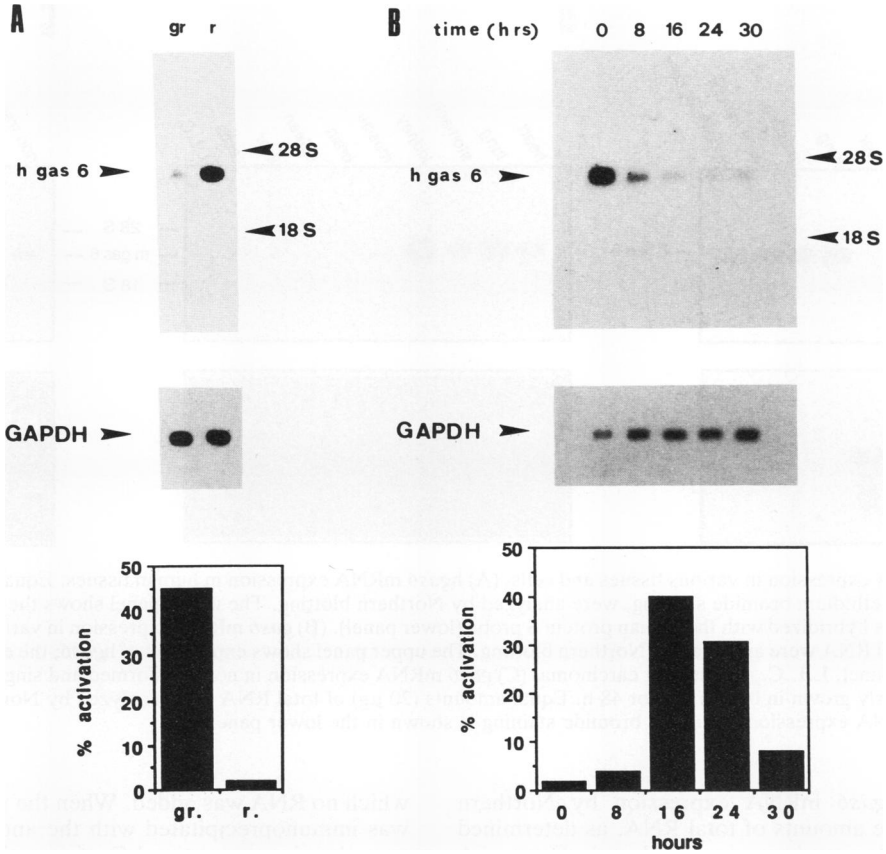

FIG. 4. Analysis of h*gas6* mRNA expression. RNA was extracted from actively growing and 72-h serum-starved human IMR90 fibroblasts (A) or serum-starved cells at different times after 20% FCS serum induction (B). The same Northern blots were probed with *gapdh*. The histograms show the relative percentage of DNA synthesis for each time point.

high-affinity binding of Ca^{2+}, as recently shown by nuclear magnetic resonance spectroscopy for the first EGF-like domain of factor IX (35), and are involved in high-affinity protein-protein interactions (51). The first EGF-like domain of Gas6 is the one possessing the lowest homology (42% amino acid identity) to the corresponding domain of human protein S relative to the other EGF domains (domains II [48% identity], III [45% identity], and IV [51% identity]).

The C-terminal region D is the most extensive in length and, like human protein S, does not show any resemblance to serine proteases. As is the case for human protein S, region D of Gas6 shows similarity to the human sex hormone-binding protein (SHBP) and rat androgen-binding proteins (2, 30). The portion of hGas6 containing the highest amino acid identity (30%) and the minimum number of gaps with human SHBP is included within amino acids 315 to 457. This similarity suggests that region D could be involved in steroid hormone binding. Other ECM components, including laminin A chain and agrin, show homology to SHBP within the same region as Gas6 (4). However, there are no experimental data to indicate that these proteins are capable of binding steroid-derived molecules. The carboxy-terminal part of mGas6 has two potential glycosylation sites at positions 417 and 488, the first being conserved also in hGas6 very close to the positions of similar potential glycosylation sites found in the other two species of protein S. The domain that interacts with the C4b-binding protein (C4BP) (17), a high-molecular-weight plasma protein involved in activation of the classical pathway of the complement system (27, 32), is thought to lie within region D of protein S. In particular, the peptide (Gly-605–Ile-614) proximal to its C terminus specifically competes for interaction with C4BP (64). The C4BP domain involved in the interaction with protein S lies within a short consensus repeat (SCR) domain (12) of the α chain and possibly of the β chain of C4BP (38, 39). The corresponding region of Gas6 shows 50% amino acid identity to the peptide (Gly-605–Ile-614) of protein S.

Regulation of h*gas6* in human fibroblast growth arrest and during the cell cycle. The human cDNA clone of *gas6* was used as a probe in Northern analysis of total RNA extracted from human IMR90 fibroblasts cultured under different growth conditions. Figure 4A shows h*gas6* expression in growing and serum-starved IMR90 human fibroblasts. The level of h*gas6* is significantly increased at growth arrest. Figure 4B shows h*gas6* expression during a synchronous cell cycle reinduction of serum-starved IMR90 fibroblasts. h*gas6* mRNA level is significantly decreased at 8 h after serum addition, reaching its lowest level at 16 h, which is maintained thereafter. The same Northern blots were probed with *gapdh* cDNA (histograms in Fig. 4), and the percentage of cells in S phase was also determined. Altogether, these results indicate that the expression of h*gas6* mRNA in IMR90 human fibroblasts is similar to that described for m*gas6* in NIH 3T3 mouse fibroblasts.

Analysis of *gas6* mRNA expression in tissues and cell lines. Total RNA isolated from different human and mouse tissues

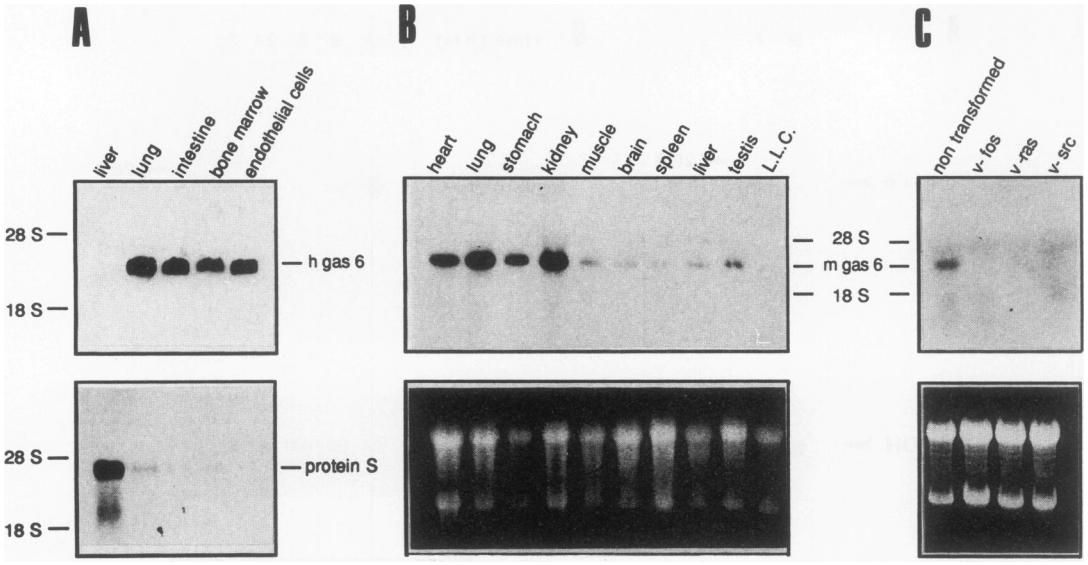

FIG. 5. *gas6* mRNA expression in various tissues and cells. (A) h*gas6* mRNA expression in human tissues. Equal amounts (20 μg) of total RNA, as estimated by ethidium bromide staining, were analyzed by Northern blotting. The upper panel shows the expression of h*gas6*; the same Northern blot was hybridized with the human protein S probe (lower panel). (B) *gas6* mRNA expression in various mouse tissues. Equal amounts (20 μg) of total RNA were analyzed by Northern blotting. The upper panel shows expression of h*gas6*; the ethidium bromide staining is shown in the lower panel. L.L.C., Lewis lung carcinoma. (C) *gas6* mRNA expression in nontransformed and single-oncogene-transformed NIH 3T3 cells previously grown in 0.5% FCS for 48 h. Equal amounts (20 μg) of total RNA were analyzed by Northern blotting; the upper panel shows *gas6* mRNA expression; ethidium bromide staining is shown in the lower panel.

was analyzed for *gas6* mRNA expression by Northern blotting. Comparable amounts of total RNA, as determined by ethidium bromide staining, were analyzed. Figure 5A shows that h*gas6* mRNA (with a relative size of 2.6 kb) is expressed in all tissues analyzed at comparable levels except in liver, where it is apparently undetectable. The same Northern blot was also probed with human protein S cDNA (kindly provided by B. Dahlbäck). The lower panel of Fig. 5A shows that protein S mRNA (relative size of 3.5 kb) is expressed in liver and at a very low level (almost undetectable) in the other tissues analyzed. Figure 5B shows an analysis of *gas6* mRNA expression in several mouse tissues, using equal relative amounts of total RNA, as determined by ethidium bromide staining (lower panel). *gas6* seems to be expressed in many tissues analyzed, with higher levels in heart, lung, stomach, and kidney. RNA from a lung tumor (Lewis lung carcinoma) showed no detectable *gas6* mRNA. Since *gas6* expression is dramatically reduced in at least one tumor in vivo, we analyzed *gas6* mRNA expression in various NIH 3T3 cell lines transformed by single oncogenes. These lines were grown in low serum (0.5% FCS) for 48 h, condition that promotes *gas6* mRNA expression in nontransformed NIH 3T3 cells. Figure 5C shows that under these conditions, the normal NIH 3T3 cells express a significant level of *gas6* mRNA, while the single-oncogene-transformed lines do not present a detectable level of *gas6*.

hGas6 biosynthesis under different growth conditions. An antibody to the hGas6 (amino acids 359 to 551) expressed in *E. coli* was affinity purified as described in Materials and Methods. To confirm that this antibody specifically recognizes the hGas6 product, we first immunoprecipitated the hGas6 primary in vitro translation product. The in vitro-transcribed hGas6 RNA was used to program a rabbit reticulocyte extract; the protein obtained (Fig. 6A, lane 1) has an apparent molecular mass of 75 kDa, as expected from the cDNA sequence. Lane 2 represents the mock control in which no RNA was added. When the total hGas6 translation was immunoprecipitated with the anti-hGas6 antibody, the same band representing hGas6 is visible (lane 4), while no band is detected when preimmune serum was used (lane 3). The hGas6 immunoprecipitated both from cell extracts (lane 5) and from conditioned medium (lane 6) has an apparent molecular weight similar to that of the in vitro primary translation product. The presence of hGas6 in conditioned medium indicates that it is secreted, as suggested from the presence of a signal sequence in cDNA sequence analysis. We therefore immunoprecipitated biosynthetically labeled hGas6 from exponentially growing and serum-starved cells. The same number of trichloroacetic acid-precipitable counts was used in the following comparative experiments.

As shown in Fig. 6B, growth arrest induced by low serum increases the level of hGas6 both in cell extracts (lane 2) and in the conditioned medium (lane 5) relative to the level of the protein synthesized by exponentially growing cells (lanes 1 and 4). To characterize hGas6 synthesis during the $G_0 \rightarrow S$ transition, 20% FCS was added for 8 h to serum-starved cells and [^{35}S]methionine was added for a further 3-h labeling period. The amount of hGas6 immunoprecipitated both from cell extracts (lane 3) and from culture medium (lane 6) is clearly decreased relative to the result for serum-starved cells. Thus, the levels of hGas6 protein are consistent with mRNA expression.

DISCUSSION

It is becoming increasingly clear that cell proliferation is modulated by a complex network of interactions mediated by extracellular, cell-matrix, and cell-cell adhesion factors (66). A potential approach to identify negative control elements has been the isolation and characterization of genes expressed during growth arrest (*gas*) (6, 43, 54). We have recently shown that one of these genes, *gas1*, is directly

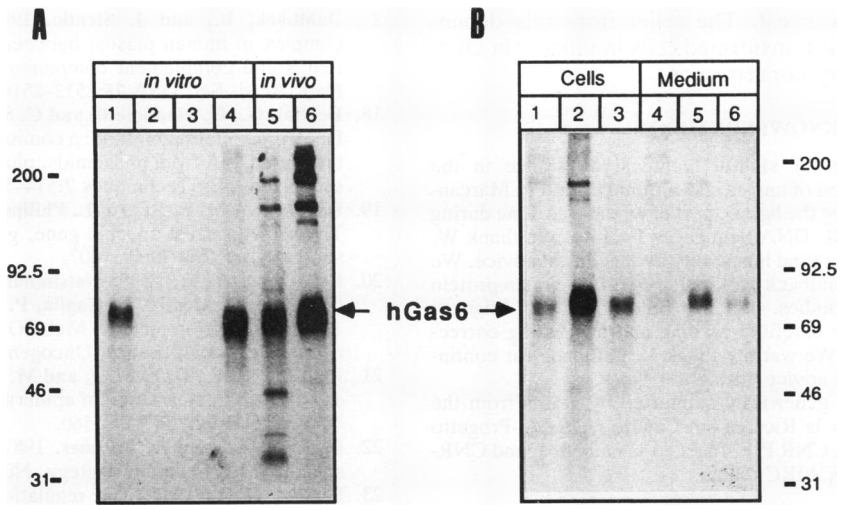

FIG. 6. Immunoprecipitation analysis of hGas6. (A) Shown are in vitro translation of h*gas6* mRNA (lane 1), mock translation (lane 2), immunoprecipitation of the in vitro-translated h*gas6* mRNA by using preimmune antiserum (lane 3) and anti-hGas6 affinity-purified antibodies (lane 4), and immunoprecipitation of hGas6 from serum-starved cellular lysates of IMR90 fibroblasts (lane 5) and the respective culture medium (lane 6) after [^{35}S]methionine in vivo labeling for 14 h. (B) Immunoprecipitation analysis of hGas6 from IMR90 fibroblasts. Conditions were as follows: exponentially growing, 24 h after seeding in 10% FCS, and 3 h of [^{35}S]methionine labeling (lanes 1 [cellular lysate] and 4 [culture medium]), serum starved, 72 h of 0.5% FCS incubation and 3 h of [^{35}S]methionine labeling (lanes 2 [cellular lysate] and 5 [culture medium]), and serum starved after 8 h of incubation with 20% FCS and an additional 3 h of [^{35}S]methionine labeling (lanes 3 [cellular lysate] and 6 [culture medium]). Equal numbers of trichloroacetic acid-precipitable counts from the respective cellular lysate (lanes 1 to 3) or culture medium (lanes 4 to 6) were processed for immunoprecipitation.

involved in growth suppression (19). In this report, we have described the structure and expression of mouse and human *gas6* cDNAs. *gas6* expression is induced after serum starvation but decreases dramatically after induction to reenter the cell cycle both in mouse NIH 3T3 cells and in human IMR90 fibroblasts. By making use of a polyclonal antibody raised against hGas6, we have also shown that the protein is secreted and that its biosynthetic level fully reflects mRNA expression.

Sequence comparisons of both murine and human cDNA have revealed that *gas6* is a new member of the family of vitamin K-dependent proteins homologous to protein S. Protein S acts as a molecular schatchen (52) in both the blood coagulation and complement cascades. During blood clotting, protein S interacts with activated protein C, and the complex catalyzes the proteolytic inactivation of factors Va and VIIIa, which are involved in thrombin activation (62, 63, 65). This protein thus acts as an important negative regulator of the blood-clotting cascade (23, 24). The involvement of protein S in the complement cascade is demonstrated by the finding that approximately half of the protein S in human plasma is bound to C4BP (17). Although its role in the complement cascade is not clear, protein S represents a unique link between the two protease pathways that regulate the complement and coagulation systems (37).

As shown by sequence comparison, the regions with the highest homology between Gas6 and protein S are region A (Gla domain) and region C (EGF domain). Region B is the most divergent, having lost the thrombin-sensitive sites. It has been proposed that the region of protein S involved in the interaction with activated protein C includes the first EGF domain (which in Gas6 bears the lowest homology with protein S relative to the four EGF-like domains) and the thrombin-sensitive segment (14) (whose corresponding region B in Gas6 shows the lowest homology relative to all the other regions). This finding suggests that a putative interaction of Gas6 with protein C, and consequently a specific involvement in the control of the coagulation cascade, is rather unlikely.

On the basis of the known involvement of protein S in the complement cascade, an interaction of Gas6 with C4BP also appears unlikely, since its putative binding region has only 50% identity to the corresponding region of protein S. However, because the target of this interaction appears to reside in the SCR domains (also known as complement-related protein domains) of C4BP, Gas6 might interact with other SCR-containing proteins. SCR domains are present in the rapidly expanding family of LEC-CAM adhesion molecules (57) as well as in some proteoglycans such as versican (68).

We can speculate that the putative function of Gas6 is likely to be different from the more restricted role of protein S in the blood coagulation and complement cascades. This would not, however, exclude the possibility that protein S itself, given the functional promiscuity in protease networks, has other functions. In fact, protein S has recently been reported to be a potent mitogen for smooth muscle cells (29). In the light of this finding, *gas6* also might have a role in cell proliferation. This hypothesis is supported by the fact that its expression seems to be less restricted than that of human protein S, being detectable in many human and mouse tissues. However, it remains to be determined whether *gas6* is expressed in different cell types or its expression is restricted to a subset of cells (endothelial cells) ubiquitously present in the tissues analyzed.

It is generally accepted that a complex equilibrium of interactions links ECM and the cell surface, both of which play an active role by focalizing growth factors, proteases, and protease inhibitors close to the site of action. We hypothesize that Gas6 may be a component of a protease cascade involved in growth regulation. The rationale behind this hypothesis originates from the strict dependence of its

expression on growth arrest. The relief from this dependence, as is the case for transformed cells in vitro or in vivo, should be the necessary consequence.

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