Serum Stimulates *Pleiotrophin* Gene Expression in an AP-1-dependent Manner in Human Endothelial and Glioblastoma Cells

EVANGELIA POIMENIDI, MARIA HATZIAPOSTOLOU* and EVANGELIA PAPADIMITRIOU

Laboratory of Molecular Pharmacology, Department of Pharmacy, University of Patras, GR 26504 Patras, Greece

Abstract. *Background: Despite the fact that pleiotrophin (PTN) exhibits important biological activities related to tumor growth and angiogenesis, little is known about the regulation of its expression. In the present work, the effect of serum on PTN expression and secretion in the culture medium of human umbilical vein endothelial (HUVECs) and glioblastoma M059K cells was studied. Materials and Methods: The protein levels of PTN were estimated by Western blot and ptn transcription was estimated by measuring luciferase activity of a reporter gene vector carrying the wild-type or mutated full length promoter of the ptn gene. Results: Serum induced PTN protein secretion in both types of cells and up-regulated luciferase activity of the ptn promoter in a time- and concentration-dependent manner. Use of a mutant construct that lacked the serum response element (SRE) showed that serum-induced luciferase activity was partially abolished in HUVECs but not affected in M059K cells. Transfection with a construct mutated at both AP-1 binding sites led to complete abolishment of the serum-induced ptn transcription in both types of cells. Conclusion: The two AP-1 binding sites of the ptn promoter are involved in the serum stimulation of its expression, while SRE involvement seems to be partial and cell type-specific.*

Pleiotrophin (PTN), also called heparin affin regulatory peptide or heparin-binding growth-associated molecule, is an 18-kDa secreted growth factor that displays high affinity for heparin. PTN is highly conserved among species and its gene is expressed in a highly restricted temporal and spatial pattern during development, suggesting that it may contribute to a number of different functions. A growing body of evidence indicates that PTN is involved in cell proliferation, migration and differentiation of several different types of cells. It is also detected in many cancer cell lines and tumor specimens of different origin and seems to play significant roles in tumor progression, angiogenesis and metastasis (reviewed in 1-3). When the promoter region of the *ptn* gene was first cloned in 1992, no binding sites for known general transcription factors in the immediate promoter region were detected. However, in the distal 5'-region of the *ptn* promoter, two putative binding sites for the transcription factor activator protein-1 (AP-1), four binding sites for MyoD, one for the transcription factor Sp-1 and a serum response element (SRE) were found, but were not studied for their functional implication in *ptn* transcription (4).

SRE is contained in the promoter of several genes, including immediate early genes, neuronal genes, muscle-specific genes and the angiogenic modulator thrombospondin I (5). SRE sequences are responsive to signals elicited by diverse growth factors acting through different receptor types, *via* protein kinase C, intracellular cyclic AMP levels and activated forms of c-src, c-Raf and Ras (6). SRE is specifically bound by the transcription factor serum response factor (7), which seems to regulate vital cellular functions, such as cell growth, differentiation (5, 7), cytoskeletal organization and migration (8), and can be activated by a variety of agents, including serum, growth factors, neurotrophins, neurotransmitters, stress agents and viral activators (5).

The sequence corresponding to SRE in the promoter of the human *ptn* gene is CCAAAAAAGG and is found in the distal 5'-region, from nucleotide -568 to -559 (4). Since the functional activity of the SRE in the promoter of the human *ptn* gene has not yet been elucidated, the aim of the present study was to determine the effect of serum on the expression and secretion of PTN and to investigate the possible involvement of SRE in human endothelial and glioblastoma cells.

**Current address:* Molecular Oncology Research Institute, Tufts-New England Medical Centre, 750 Washington Street, Boston, MA 02111, U.S.A.

Correspondence to: E. Papadimitriou, Ph.D., Laboratory of Molecular Pharmacology, Department of Pharmacy, University of Patras, GR 26504, Greece. Tel/Fax: +30 2610969336, e-mail: epapad@upatras.gr

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Materials and Methods

Cell culture. Human umbilical vein endothelial cells (HUVECs) were isolated and cultured as described elsewhere (9) and used at passages 1-4. Human M059K glioblastoma cells were cultured in DMEM/HAM'S F12 medium supplemented with 10% fetal bovine serum (FBS). All cultures were maintained at 37˚C, 5% CO_2 and 100% humidity.

Western blot analysis for PTN. The presence of PTN in the cell culture medium was investigated as described elsewhere (10). Briefly, the conditioned medium of the cells was incubated overnight with 100 μl of heparin-Sepharose (Amersham Biosciences, New Jersey, USA) at 4˚C with continuous agitation. Bound proteins were eluted with 50 μl of Laemmli sample buffer under reducing conditions, fractionated on 17.5% SDS-PAGE and transferred to Immobilon P membranes (Millipore, Billerica, MA, USA). Blocking was performed by incubating the polyvinylidene difluoride membranes with 3% bovine serum albumin in Tris-buffered saline (TBS) containing 0.05% Tween 20 (TBS-T) overnight at 4˚C. The membranes were then incubated with 45 ng/ml affinity purified anti-PTN antibody (Santa-Cruz Biotechnology Inc., Santa Cruz, CA, USA) in TBS-T for 1 h at room temperature under continuous agitation and then with horseradish peroxidase-conjugated rabbit anti-goat IgG (Sigma-Aldrich Ltd, Athens, Greece) at a dilution of 1:7,500 in TBS-T for 1 h at room temperature under continuous agitation. Detection of PTN was performed by the ChemiLucent™ detection system kit (Millipore Biosciences, Temecula, CA, USA) according to the manufacturer's instructions. The protein levels that corresponded to PTN were quantified using ImagePC image analysis software (Scion Corporation, Frederick, MD, USA).

Total RNA isolation and reverse transcriptase-polymerase chaim reactions (PCR). Total RNA was extracted from M059K cells using the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. Reverse transcriptase-PCRs for human *ptn* and *β-actin* were performed using the Access reverse transcriptase-PCR system (Promega, Madison, WI, USA) as described elsewhere (10). The reverse transcriptase-PCR products were subjected to electrophoresis on 2% agarose gels containing 0.5 μg/ml ethidium bromide and photographed using a digital camera. The mRNA levels that corresponded to *ptn* and *β-actin* were quantified using the ImagePC image analysis software and the ratio of electrophoretic band values of *ptn versus β-actin* represents the relative expression of *ptn* gene after different treatments of cells.

Generation of human PTN luciferase mutant constructs. To produce deletion of the SRE from the human *ptn* promoter, wild-type hPTNpro2.3-Luc reporter plasmid (10) was used for oligonucleotide-directed mutagenesis with the Quik-Change site-directed mutagenesis kit (Stratagene, Cedar Creek, TX, USA). To produce the desired deletion from position -568 to -559, two pairs of mutagenic primers, 5'-GCT AAA GGC ACC AAG GAA AAA AGA GCT CTC TCC C-3' (sense) and 5'-GGG AGA GAG CTC TTT TTT CCT TGG TGC CTT TAG C-3' (antisense), were used. After the annealing of the mutagenic primers, the strands were completed with *Pfu* Turbo DNA polymerase (Stratagene), and the parental double-stranded DNA was digested by DpnI (Stratagene). The resultant DNA digest was transformed into XL1-Blue super competent bacteria cell line (Stratagene). Colonies were expanded, and the plasmid DNA was harvested using the Nucleospin plasmid kit (Macherey-Nagel) according to the manufacturer's instructions. Mutation of the insert was verified by DNA sequence analysis (Macrogen, Seoul, Korea).

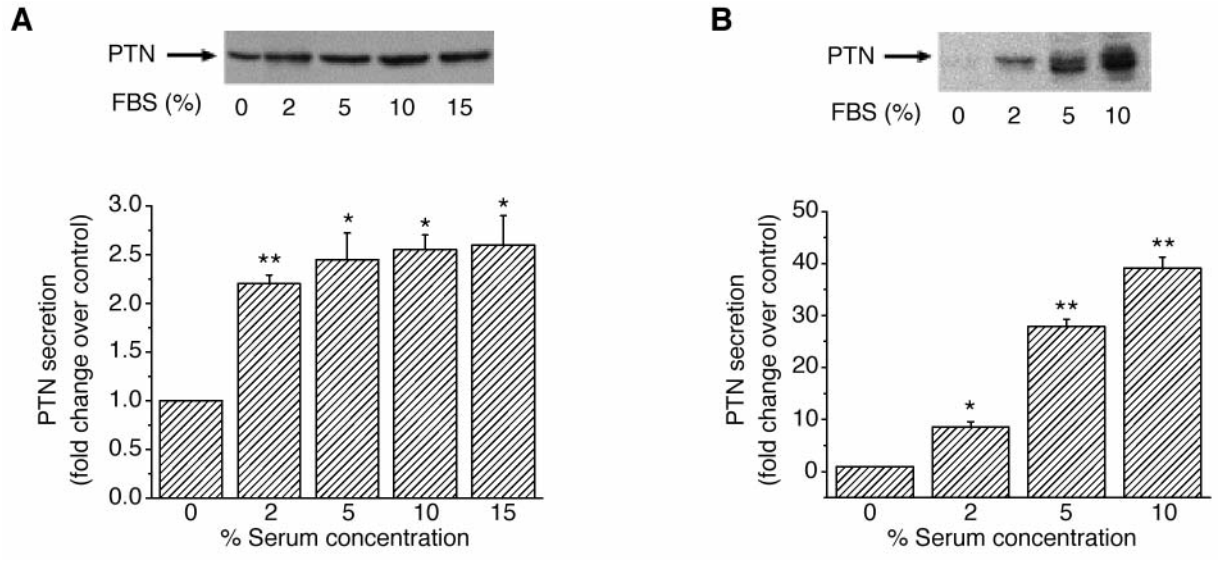
Transient transfection and luciferase assay. A total of 4×10^5 M059K cells and 5×10^5 HUVECs were seeded in 12 mm well-plates and were left overnight. Cells were then incubated with 1.5 μg of DNA and 4 μl of jetPEI™ (Polyplus Transfection, Illkirch, France) transfection reagent in 1 ml of serum-free medium for M059K cells or 1 ml of medium containing 5% FBS for HUVECs, for 4 h at 37˚C. After removing the transfection medium, corresponding fresh medium was added and 16 h later, the culture medium was replaced with medium containing different concentrations of FBS. Cells were harvested and luciferase activity was determined using the luciferase reporter gene assay from Roche Applied Science (Indianapolis, IN, USA), according to the manufacturer's instructions. Cell lysates were analyzed for protein content using the Bradford method, and luminescence units were normalized for total protein content (10).

Statistical analysis. The significance of variability between the results of each group and its corresponding control was determined by unpaired *t*-test. Each experiment included triplicate measurements for each condition tested. All results are expressed as mean±S.E. from at least three independent experiments.

Results

Serum increased expression and secretion of PTN by both HUVECs and M059K cells. Initially it was studied whether serum affects secretion of PTN in the culture medium of HUVECs and M059K cells. As shown in Figure 1A and B, serum significantly increased PTN secretion in a concentration-dependent manner in both types of cells. Although HUVECs seemed to reach maximal PTN secretion at lower serum concentrations compared with M059K cells, this may be due to the fact that before addition of different serum concentrations, M059K cells were starved in serum-free medium, while HUVECs were cultured in medium containing 5% FBS, a treatment necessary to avoid HUVEC apoptosis due to prolonged culture in serum-free conditions (data not shown). This may also explain the fact that at 0% FBS, the levels of PTN secreted by HUVECs were higher compared with those secreted by M059K cells (see upper panels in Figure 1) and the percentage increase induced by serum was lower in HUVECs compared with M059K cells. Similarly to the effect at the protein level, *ptn* mRNA levels were also increased by serum in a concentration-dependent manner (Figure 1C).

Serum induced transcriptional activation of the ptn gene. To evaluate whether the effect of serum on PTN was also observed at the transcriptional level, a plasmid construct containing the full-length promoter of the *ptn* gene fused to a luciferase reporter gene was used to transfect HUVECs or M059K cells as described in Materials and Methods.



Reporter activity was increased in a concentration-dependent manner in response to serum in both types of cells. The increase appeared from the first hour following the addition of serum into the cell culture medium and was maximal at 6 h (Figure 2).

SRE partially mediated serum-induced transcriptional activation of the ptn gene in HUVECs but not M059K cells. Since the human *ptn* promoter contains an SRE (4), we investigated the possible role of SRE in the serum-induced PTN up-regulation. For this reason, a construct was made that lacked SRE, as described in Materials and Methods and shown in Figure 3A. The reporter activities of the mutant construct in response to serum stimulation were lower compared with those of the wild-type construct in HUVECs, yet not reaching the levels of unstimulated cells (Figure 3B). On the contrary, the reporter activities of the mutant construct in the presence or absence of serum were similar to those of the wild-type construct in M059K cells (Figure 3C).

AP-1 mediated serum-induced transcriptional activation of the ptn gene in both HUVECs and M059K cells. Since SRE only partially mediates serum-induced transcriptional activation of the *ptn* gene only in HUVECs, we investigated the possible involvement of the two AP-1 binding motifs on the human *ptn* promoter (4). A construct with point mutations in both AP-1-like motifs was used, as previously described (10). Our analysis revealed that serum had no effect on the reporter activity of this construct in either type of cells (Figure 4), suggesting a pivotal role of the two AP-1 motifs in serum-stimulated transcription of the *ptn* gene in both HUVECs and M059K cells.

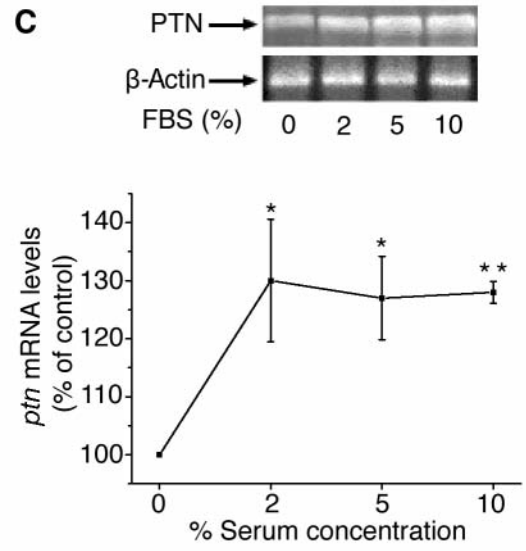


Figure 1. *Effect of serum on the secretion of PTN by HUVECs and M059K cells. The upper panel shows representative images (from three independent experiments) of Western blot analyses of cell culture media for PTN from HUVECs (A) and M059K cells (B). PTN protein amounts were quantified by densitometric analysis of the corresponding band in each lane. Results are expressed as mean±S.E. of the percentage change of the amounts of PTN protein in cells treated with different concentrations of FBS compared with untreated cells. (C) The upper panel shows a representative image (from three independent experiments) of reverse transcriptase-PCR analyses for ptn and β-actin mRNA in M059K cells. The levels of mRNA were quantified by densitometric analysis of the corresponding bands, and the ratio of PTN to β-actin mRNAs was calculated for each lane. Results are expressed as mean±S.E. of the percentage change of PTN mRNA relative amounts in cells treated with different concentrations of serum compared with the untreated cells. Asterisks denote a statistically significant difference from untreated cells: *p<0.05, **p<0.01.*

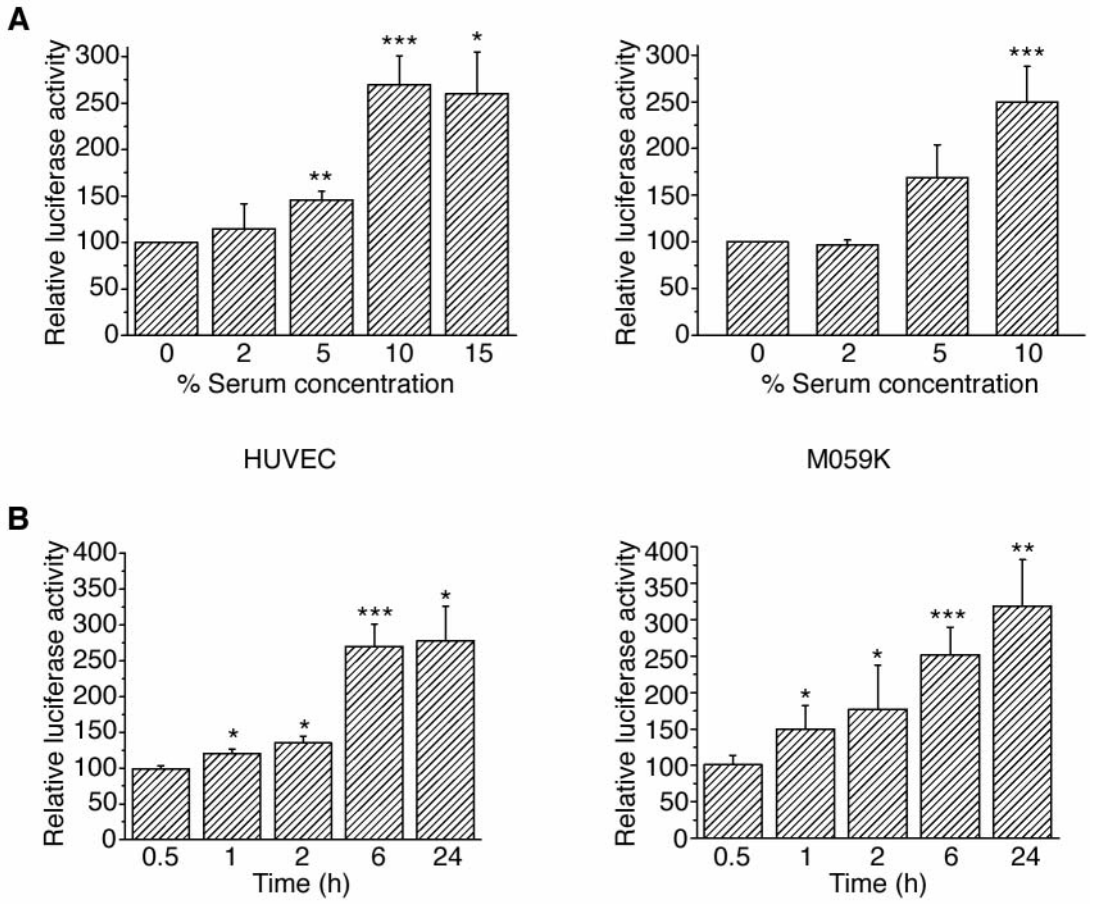


Figure 2. *Effect of serum in the transcriptional activation of the ptn gene. Relative luciferase activity in HUVECs and M059K cells after addition of different concentrations of serum (A), or at different time points after addition of serum (B) in the cell culture medium. Results are expressed as mean±S.E. of the percentage change of relative luciferase activity in treated compared with the corresponding untreated cells (control). Asterisks denote a statistically significant difference from control: *p<0.05, **p<0.01, ***p<0.001.*

Discussion

In the present work, we showed that serum up-regulates *ptn* transcription and secretion in an AP-1-dependent manner, while the SRE of the *ptn* promoter seems to be partially involved in this process in human endothelial but not glioblastoma cells. To our knowledge, this is the first work that studies the possible involvement of SRE in the transcriptional regulation of the human *ptn* gene. The difference observed between endothelial and glioblastoma cells may be attributed to differences in the accessory proteins required to form multiprotein complexes on the SRE between the two different types of cells, which in other cases have been shown to affect promoter efficiency (11, 12). These data suggest that in other types of human cells, such as neurons or smooth muscle cells, SRE may have a more significant role in *ptn* gene regulation, something that needs further investigation.

In contrast to SRE, the AP-1-binding sites of the human *ptn* promoter seem to significantly affect serum-induced *ptn* gene transcription and their mutation leads to complete abolishment of the serum induction in both types of cells. We have previously shown that these sites are implicated in *ptn* gene transcription in the basal, non-induced state (10), as well as after stimulation with hydrogen peroxide (10), fibroblast growth factor 2 (13), nitric oxide (14) and aprotinin (15). Although in the present study the molecular mechanisms involved were not studied in more detail, it is well known that serum contains several mitogens that can activate signaling molecules, such as NAD(P)H oxidase (16), nitric oxide synthase (17) and mitogen-activated protein kinases (18, 19), which can all activate AP-1, leading to *ptn* gene transcription and PTN protein secretion. Serum can also activate early response genes, such as *c-fos* (20, 21), *c-jun* (20) and *fra-1* (22), which can secondarily lead to increased *ptn* gene transcription (10).

Interestingly, the effect of exogenous PTN on cell migration in the presence of serum is different or even opposite to its effect in serum-free conditions. For example, PTN induces endothelial cell migration in serum-free

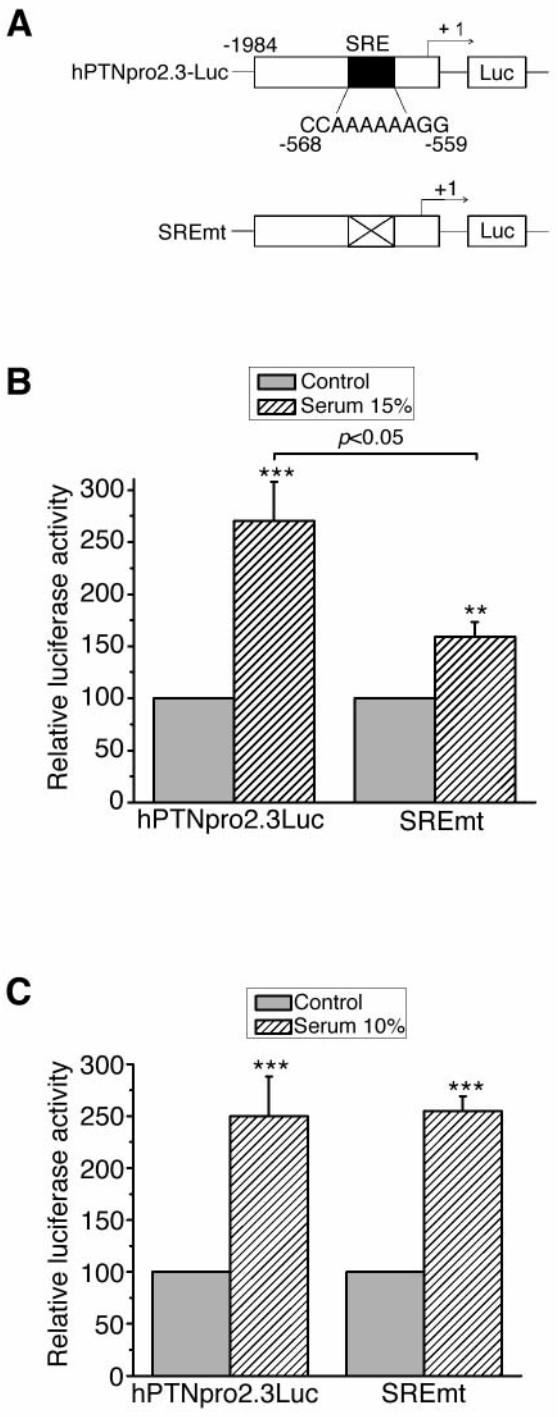


Figure 3. *Involvement of SRE of the human ptn promoter in the serum-induced transcriptional activation of the ptn gene in HUVECs and M059K cells. A, Diagram of the reporter plasmid containing 2.3kb of the human ptn 5'-flanking region (hPTNpro2.3-Luc) lacking the nucleotides from -568 to -559 (SREmt). Reporter activities of the SREmt construct were evaluated in untreated and serum-treated (for 6 h) HUVECs (B) and M059K cells (C) and expressed as mean±S.E. of the percentage change of relative luciferase activity in treated compared with the corresponding untreated cells (control). Asterisks denote a statistically significant difference from controls: **p<0.01, ***p<0.001.*

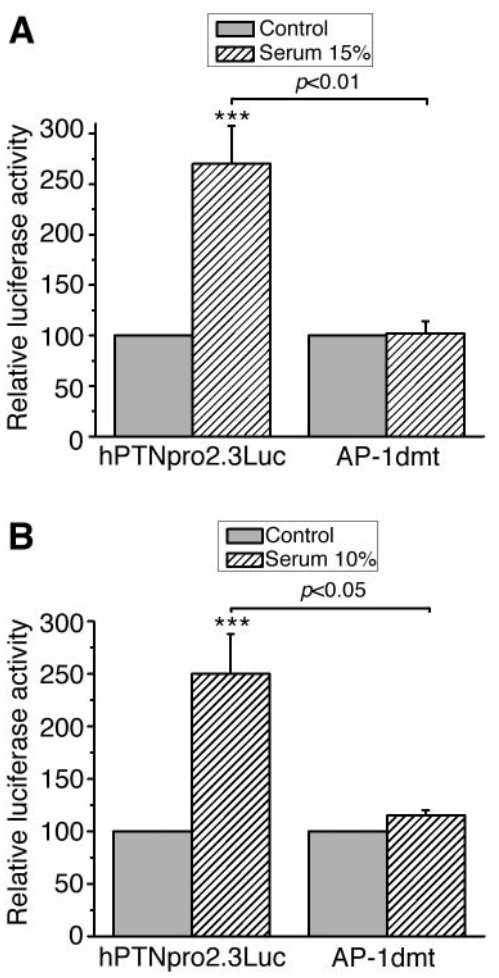


Figure 4. *Involvement of the two AP-1-binding sites of the human ptn promoter in the serum-induced transcriptional activation of the ptn gene in HUVECs and M059K cells. Reporter activities of the AP-1 mutated construct of the ptn promoter (AP-1dmt) were evaluated in untreated and serum-treated (for 6 h) cells and expressed as mean±S.E. of the percent change of relative luciferase activity in treated HUVECs (A) and M059K cells (B) compared with the corresponding untreated cells (control). Asterisks denote a statistically significant difference from controls: ***p<0.001.*

conditions (23) but has no significant effect in the presence of serum (9, 24) and this has been attributed to inhibition by PTN of vascular endothelial growth factor (24, 25) or other growth factors present in the serum. Based on the results of the present study, another explanation could be that in the presence of serum, endogenous levels of PTN are increased and cell migration cannot be further stimulated by the addition of exogenous growth factor.

In conclusion, the results of the present study suggest that in human endothelial and glioblastoma cells, transcription factor AP-1 seems to have predominant role in serum-induced up-regulation of PTN expression and secretion.

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