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The tyrosine phosphatase HD-PTP is regulated by FGF-2 through proteasome degradation

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1. ABSTRACT

Angiogenesis is essential in development and wound healing and contributes to the pathogenesis of many diseases. The signalling pathways activated in angiogenesis are, in part ,known and the overall tyrosine phosphorylation of cellular proteins plays a relevant role. By RNA fingerprinting, we isolated a tyrosine phosphatase, HD-PTP, modulated in human endothelial cells exposed to human immunodeficiency virus type 1 Tat, a viral protein known to be angiogenic. For the first time, we describe HD-PTP at the protein level. HD-PTP, a 185 kDa cytosolic protein which is expressed in endothelial cells of different origin. We show that HD-PTP is upregulated by Tat at the mRNA but not at the protein level. HD-PTP protein is differentially modulated by two angiogenic growth factors. While Vascular Endothelial Growth Factor does not affect protein levels, Fibroblast Growth Factor-2 induces HD-PTP degradation via the proteasome system.

2. INTRODUCTION

Angiogenesis is a highly ordered and regulated process driving quiescent endothelial cells into a series of events culminating with the organization of a vascular network that responds to the demands of the growing or healing tissues (1). Uncontrolled angiogenesis contributes to a number of pathological processes, including rheumatoid arthritis, blindness, tumour growth and metastasis (2). Angiogenic factors bind to tyrosine kinase receptors, triggering the phosphorylation of cytoplasmic signalling proteins which modulate endothelial cell behaviour, ultimately leading to the formation of new blood vessels. The overall protein tyrosine phosphorylation is modulated by both tyrosine kinases (PTK) and tyrosine phosphatases (PTP). Interestingly, a PTP has been shown to strictly regulate Vascular Endothelial Growth Factor (VEGF) and Fibroblast Growth Factor (FGF) receptor-mediated signal transduction and biological activity (3-4).

Human HD-PTP, a member of the PTP family, has been isolated and located on the short arm of chromosome 3 (5), an area frequently deleted in many types of human tumours (6,7). It is interesting to note that the rat homologue of HD-PTP was reported to inhibit activated Ha-ras-mediated NIH-3T3 transformation (8). HD-PTP predicted sequence contains a PTP-like domain and a PEST motif (rich in proline, glutamine, serine and threonine) in the C-terminal region. In the N-terminal region, it is homologous to a yeast protein, BRO1, involved in the mitogen-activated protein kinase signalling pathway. However, HD-PTP protein has not yet been characterized.

In this report we show that HD-PTP is differentially expressed in endothelial cells in response to HIV-1 Tat (Tat). Apart from enhancing viral transcription, Tat also affects the expression of strategic host genes and it has angiogenic properties. Indeed, Tat binds the VEGF receptor type 2 (VEGF-R2) (9) with consequent transient activation of mitogen-activated protein kinase, MAPK/ERK(1/2). The interaction with VEGF-R2 is required for Tat-induced endothelial growth and migration *in vitro* and for neovascularization *in vivo*. Tat also increases endothelial permeability, an early event in angiogenesis, through tyrosine kinases and MAPK pathways (10). Interestingly, Tat has a role in the pathogenesis of Kaposi's sarcoma (KS), a highly vascularized skin lesion characterized by marked endothelial proliferation and migration, resulting in the formation of new capillaries (11). Accordingly, Tat transgenic mice develop highly vascularized lesions which closely resemble KS as well as tumours of different histotypes (12). It is noteworthy that a modulation of tyrosine phosphatase activity by Tat has been described in several cell types (13,14).

Since Tat modulates endothelial function, we assumed that the isolation of differentially expressed genes in Tat-treated endothelial cells would yield insights into the molecular regulatory mechanisms of early events in the angiogenic process. By RNA fingerprinting, we found that Tat up-regulates HD-PTP mRNA. In this manuscript, we show for the first time that HD-PTP is modulated by Fibroblast Growth Factor-2 (FGF-2) through a proteasome dependent pathway in endothelial cells. Since Vascular Endothelial Growth Factor (VEGF) does not modulate HD-PTP at the protein level, we speculate that its regulation requires the activation of different intracellular pathways by FGF and VEGF.

3. MATERIALS AND METHODS

3.1. Isolation of HD-PTP RNA

Fingerprinting on 1 μg of total RNA from endothelial cells treated with recombinant Tat (Intracel, Cambridge, MA) was performed as described (15). The bands corresponding to differentially expressed genes were excised from the polyacrylamide gel, electroeluted, reamplified by PCR, sub-cloned in Bluescript and sequenced. The 2.5 Kb fragment of HD-PTP was obtained by screening a human umbilical vein endothelial cell (HUVEC) cDNA library (Clontech) with the 700-bp fragment obtained by DNA fingerprinting. The DNA sequence was analyzed by the Analyze and Interpret programs of the Mac Molly Suite (Berlin). Deduced protein sequences were compared and aligned using BLASTX with ClustalW 1.7 programs made available by Baylor College of Medicine.

3.2. Cell Culture

ECV304 cells (16) were cloned to obtain a monoclonal population (17) and maintained by serial passage in M199 supplemented with 10% fetal bovine serum (FBS). Human umbilical vein endothelial cells (HUVEC) were from American Type Culture Collection and cultured in medium M199 containing 10% fetal calf serum, endothelial cell growth supplement (150 μg/ml), and heparin (5 units/ml) on gelatin-coated dishes. Human dermal microvascular cells (HMEC-1) were obtained from CDC (Atlanta) and grown in MCDB131 containing epidermal growth factor (10 ng/ml), hydrocortisone (1 μg/ml) and 10% FBS. Nuclear and cytoplasmic extracts were obtained as previously described (18).

3.3. Western blot analysis

A rabbit polyclonal antibody was generated by standard procedures immunizing rabbits with a peptide (N-SIRPPGGLESPVASLPGPAEP-C) contained within the C-terminus (amino acids 1506-1526) of human HD-PTP. This epitope is not conserved in other species. While the preimmune serum does not recognize any band, the antibody against the peptide recognizes a protein band of 185 kDa which is consistent with the predicted size of human HD-PTP. IgGs against HD-PTP were purified on a protein A-Sepharose column. For Western blot, cell extracts (75 μg/lane) were resolved by 8% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose sheets at 150 mA for 16 h, and probed with anti-HD-PTP IgGs (10 μg/ml). PPARgamma and actin antibodies were from Santa Cruz (Tebu-bio). Secondary antibodies were labelled with horseradish peroxidase (Pierce). The SuperSignal chemiluminescence kit (Pierce) was used to detect immunoreactive proteins.

3.4. Purification of RNA, Northern blot and RT-PCR

Cells were rinsed with phosphate-buffered saline and lysed in 4 M guanidinium isothiocyanate. RNA was purified as described (19). RNA was electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde, capillary-blotted onto nylon membranes, and UV-cross-linked. HD-PTP and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNAs were labelled with a random primer labelling kit (Ambion Inc.). Filters were hybridized in 0.5 M sodium phosphate (pH 7.2) containing 7% SDS, 1 mM EDTA, and 20% formamide at 65 °C for 20 h and extensively washed at high stringency. mRNAs were visualized by autoradiography. For RT-PCR, 1 μg of RNA was reverse transcribed and PCR amplification was carried out using 1/50 of the final RT reaction. Each amplification cycle consisted of 30 sec at 95°C, 30 sec at 52°C and 1 min at 72°C using 30 pmol of each primer. The reaction was stopped after 30 cycles. One fifth of the reaction mix was separated on a 1% agarose gel. The sequences of the HD-PTP primers are the following: sense 5'-GCTGCAGCAGCTACGGGAGTGG-3' and antisense 5'-CTCCTTTTACAGGCTGAAGAGTGTC-3'.

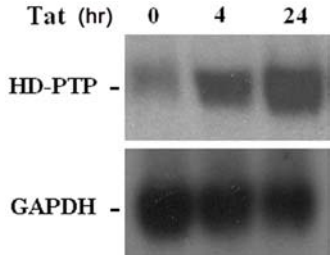

Figure 1. Modulation of HD-PTP mRNA by Tat. ECV cells were exposed to 100 ng/ml Tat for 4 and 24 h. Total RNA (10 μg/lane) was analyzed by Northern blotting as described. The blot was rehybridized to GAPDH to verify equal amounts of RNA loading among the lanes.

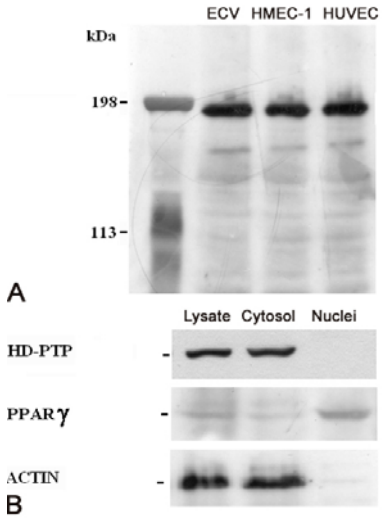

Figure 2. Expression and localization of HD-PTP in human endothelial cells. A, western blot was performed on cell extracts (75 μg) from ECV cells, HMEC-1 and HUVEC using immunopurified IgGs against HD-PTP. B, cell fractionation was performed on ECV cells by standard protocols. Unfractionated, cytosolic and nuclear extracts were analyzed by western blot. PPARgamma and actin were utilized as nuclear and cytosolic markers, respectively.

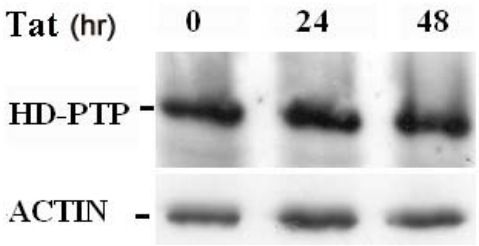

Figure 3. Expression of HD-PTP in ECV cells in response to Tat. ECV cells were exposed to 100 ng/ml Tat for 24 and 48 h. Western blot was performed on 75 μg of cell extracts. Actin was used to show that equal amounts of proteins were loaded per lane.

4. RESULTS

4.1. Modulation of HD-PTP RNA by Tat

By RNA fingerprinting on ECV cells exposed to Tat, we obtained several differentially expressed cDNA fragments. Sequence analysis and sequence homology search (NCBI-Genbank) showed that one clone up-regulated by Tat encoded for the C-terminal region of HD-PTP (data not shown), a tyrosine phosphatase located on human chromosome 3p21.3 (5). Sequence comparison also showed a 70% identity at the nucleotide level to rat PTP-TD14 (8).

The up-regulation of HD-PTP by Tat detected by RNA fingerprinting was confirmed by Northern blot analysis in ECV cells exposed to Tat (100 ng/ml). Tat increased HD-PTP mRNA with a maximal stimulation observed after 4 h (3.0 fold induction) and maintained up to 24 h (3.5 fold induction) (Figure 1).

4.2. Expression of HD-PTP in endothelial cells.

A rabbit polyclonal antibody against HD-PTP was generated against a hydrophilic C-terminal epitope of the human HD-PTP protein. The antibody specifically recognized a ~185 kDa protein which corresponds to the predicted molecular mass of HD-PTP. Using our antibody, ECV, human dermal microvascular endothelial cells (HMEC-1) and HUVEC were tested for the total amount of HD-PTP by western blot analysis. Figure 2A shows that HD-PTP was expressed to a similar extent in the three endothelial cell types tested. We also evaluated the subcellular localization of HD-PTP in ECV cells after cell fractionation. HD-PTP is a cytosolic protein (Figure 2B). Similar results were obtained in other cell types, including HUVEC and carcinoma cell lines like HeLa and KB (data not shown).

4.3. Modulation of HD-PTP in response to Tat and angiogenic growth factors

In spite of the up-regulation of HD-PTP mRNA levels in response to Tat, we did not detect any increase of HD-PTP protein by western analysis in ECV cells treated with Tat (100 ng/ml) (Figure 3), suggesting a possible regulation at the post-transcriptional level.

Since Tat binds the VEGF-R2 thus inducing angiogenesis, we evaluated whether HD-PTP could be modulated also by VEGF. RNA was extracted from ECV cells treated with VEGF (100 ng/ml) for 4, 12 and 24 h. Figure 4 A shows that VEGF up-regulated HD-PTP mRNA as detected by RT-PCR. However, we did not detect any modulation of HD-PTP at the protein level after 24 and 48 h incubation in the presence of VEGF (100 ng/ml) (Figure 4B).

We also exposed ECV cells to FGF-2 (100 ng/ml) for 4, 12 and 24 h. By RT-PCR we show that no modulation of HD-PTP occurs at the mRNA level (fig. 5A). However, we detected a marked reduction (3 fold) of HD-PTP in ECV cells treated with FGF-2 for 24 and 48 h (Figure 5B) as detected by western blot.

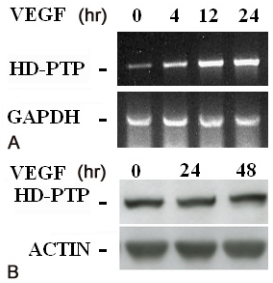

Figure 4. Expression of HD-PTP in ECV cells in response to VEGF.A, ECV cells were exposed to 100 ng/ml VEGF for 4, 12 and 24 h. RT-PCR was performed using specific primers for HD-PTP. GAPDH was utilized to show that equal amounts of RNA had been reverse-transcribed. B, ECV cells were exposed to 100 ng/ml VEGF for 24 and 48 h and western blot was performed as above. Actin was used to show that equal amounts of proteins were loaded per lane.

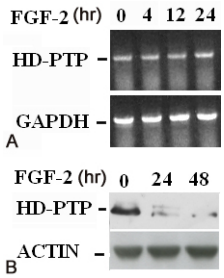

Figure 5. Expression of HD-PTP in ECV cells in response to FGF-2.A, ECV cells were exposed to 100 ng/ml FGF for 4, 12 and 24 h. RT-PCR was performed using specific primers for HD-PTP. GAPDH was utilized to show that equal amounts of RNA had been reverse-transcribed.B, ECV cells were exposed to 100 ng/ml FGF-2 for 24 and 48 h. Western blot was performed on 75 μg of cell extracts. Actin was used to show that equal amounts of proteins were loaded per lane.

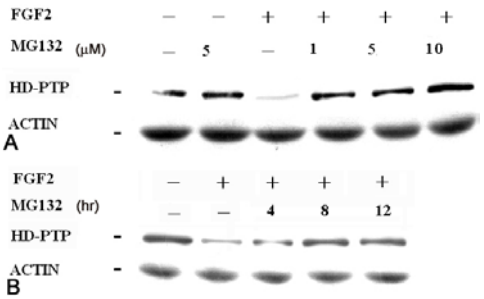

Figure 6. Involvement of the proteasome in HD-PTP down-regulation by FGF-2. A, ECV cells were exposed to FGF-2 (100 ng/ml) and different concentrations of MG132 (1, 5 and 10 μM). Western blot was performed on 75 μg of cell extracts. B, ECV cells were exposed to FGF-2 (100 ng/ml) and MG132 (5 μM) for different times. Western blot was performed on 75 μg of cell extracts. In A and B, actin was used to show that equal amounts of proteins were loaded per lane.

4.4. Inhibition of HD-PTP down-regulation by MG132

We evaluated the down-regulation of HD-PTP by FGF-2 in more detail. An important pathway to degrade cytoplasmic proteins is via the proteasome (20). The proteasome inhibitor MG132 has been widely used to turn off the proteasome (21). We first exposed ECV cells to FGF-2 (100 ng/ml) in the presence of different concentrations of MG132. We found that HD-PTP down-regulation after exposure to FGF-2 was prevented by co-incubating FGF-2 with MG132 (Figure 6A). The effect was detected with 1 μM MG132 being maximal at 10 μM. We then performed a time course experiment using 5 μM MG132 which was added for 4, 8 and 12 h. As shown in Figure 6B, HD-PTP was upregulated within 8 h from the exposure to MG132. Similar results were obtained with another proteasome inhibitor, i.e. epoxomicin (data not shown).

On the contrary, the protein levels of HD-PTP remained unchanged after treatment with Tat in the presence of MG132 (data not shown), suggesting that a different pathway is used by Tat to modulate HD-PTP.

5. DISCUSSION

Tyrosine phosphorylation is a fundamental regulatory mechanism in eukaryote physiology (22). PTPs and PTKs regulate the reversible phosphorylation of tyrosine residues in proteins, thereby controlling cell growth and differentiation, gene transcription and metabolism (22). Indeed, interference with the delicate balance between PTKs and PTPs is involved in the development of human diseases such as autoimmunity, diabetes and cancer (23). While the role of PTKs has been largely addressed, recent findings have led to the emerging recognition that PTPs play specific and active, even dominant, roles in setting the levels of tyrosine phosphorylation in cells (22,24). One hundred and seven PTP genes have been found in the human genome (25). Of these, 85 are thought to be catalytically active and there is a growing experimental evidence that PTPs tend to be very specific (24).

For the first time, we report the initial characterization of a recently isolated putative PTP, HD-PTP, and its regulation both at the RNA and protein level in endothelial cells exposed to angiogenic factors. Tat, a transactivator of both viral and host gene, up-regulates HD-PTP mRNA, while no modulation is detected at the protein level. In ECV cells, we also show that FGF-2 markedly down-modulates HD-PTP protein, whereas VEGF does not. The down-regulation of HD-PTP in FGF-2 treated cells is intriguing since an interesting feature of HD-PTP is the presence of a PEST motif located at the C-terminus. PEST sequences are found in many short-lived eukaryotic proteins and play a relevant role in their degradation (26). In particular, many PEST-containing proteins are degraded through the proteasome (27). Accordingly, the proteasome inhibitor MG132 prevents FGF-2 induced down-regulation of HD-PTP, thus suggesting that HD-PTP levels are post-transcriptionally regulated via the proteasome. Since the proteasome represents an important mechanism to degrade

many proteins implicated in signal transduction (26), it is worth noting that HD-PTP is the first described PEST-containing tyrosine phosphatase regulated by the proteasome.

The up-regulation of HD-PTP mRNA by Tat does not result in an increase of its protein levels. On the basis of the results obtained with FGF-2, we anticipated that the proteasome could be involved as well. However, MG132 does not modulate the levels of HD-PTP in ECV cells treated with Tat. It is possible that proteases other that the proteasome are involved in the regulation of HD-PTP and more studies are required to test this possibility. Since Tat binds the VEGF-R2, it is noteworthy that both Tat and VEGF do not affect the total amount of HD-PTP protein, while they both modulate its mRNA. The different effect on HD-PTP elicited by FGF-2 and VEGF is intriguing. It should be recalled that FGF-2 and VEGF activate the angiogenic program in endothelial cells through the activation of different pathways (28). FGF receptor-1 activation, but not VEGF-R1 or VEGF-R2, stimulates Crk kinase and Grb2/Sos complex which induce the activation of Ras (29). Interestingly, the rat homologue of HD-PTP, denominated PTP-TD14, suppresses Ha-Ras-mediated transformation by ~3 fold in NIH-3T3 cells (8). Taken together, these data suggest that HD-PTP negatively controls Ras pathway. Consequently, FGF-2 induced down-regulation of HD-PTP might participate in promoting the activation of Ras, which instead is not induced by Tat and VEGF. Since Ras is central in a variety of cellular functions such as proliferation and migration as well as cancer progression, HD-PTP could contribute to their regulation. Further studies are necessary to address HD-PTP role as well as its enzymatic activity and its regulation in endothelial cells.

The data presented in this study suggest that HD-PTP might participate in modulating endothelial response to angiogenic factors and, therefore, play a role in regulating the complex events leading to the formation of new vessels.

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