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Cloning, expression and characterisation of a new human low M_r phosphotyrosine protein phosphatase originating by alternative splicing

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Abstract RT-PCR experiments on RNA from K562 and HepG2 cells and from human placenta led to the isolation of a novel cDNA, a further alternative splicing product of the primary transcript of low M_r phosphotyrosine phosphatase (LMW-PTP), already known to produce isoforms 1 and 2. This new transcript represents 15–20% of the total LMW-PTP mRNA in the cell. This novel cDNA codifies for a protein that we have named SV3 (splicing variant 3): the deduced protein sequence presents the first 49 residues identical to those of isoform 1, followed by 24 unrelated amino acids, due to a frameshift introduced at the novel exon-exon boundary. The SV3 protein, expressed in *E. coli* is enzymatically inactive, most probably because unfolded, as suggested by far-UV circular dichroism (CD) experiments. SV3 protein appears to possess the characteristics of an unstructured polypeptide chain lacking the packing of side chain residues and the secondary structure level that are typical of globular proteins. This protein could represent an inactive variant of the human LMW-PTP.

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Key words: LMW-PTP; Alternative splicing; Circular dichroism

1. Introduction

Protein tyrosine phosphorylation plays a key role in the regulation of many cellular processes in eukaryotes such as cellular metabolism, proliferation, differentiation and oncogenic transformation [1]. While activation of protein tyrosine kinases (PTKs) appears to be a mandatory prerequisite for cell growth stimulation, the phosphorylated state of a protein in the cell is the result of a balance of kinase and phosphatase activity: a heterogeneous family of protein tyrosine phosphatases (PTPases) has been proposed as key regulatory elements of cellular phosphorylation levels [2]. There is evidence that PTKs and PTPases cooperate in cell growth control [3].

Low M_r phosphotyrosine phosphatase (LMW-PTP) is an 18-kDa cytosolic enzyme widely distributed in eukaryotic cells. It does not share significant sequence homology with the other two classes of PTPases, with the only important exception of the C X₅ R motif, typical of the active site of all these enzymes [4].

Two isoforms of LMW-PTP, isoforms 1 and 2 (IF1 and IF2, respectively) have been isolated from various mammalian tissues [5]. The two isoforms have been previously named

PTP_{fast} and PTP_{slow} (in human) and ACP1 and ACP2 (in rat). Genomic DNA sequencing has shown that, in man, 6 exons are present in the primary transcript and that the two isoforms originate through an alternative splicing mechanism [6], in which two exons (3F or 3S) are alternatively incorporated in the mRNA sequence. As a consequence of this phenomenon, two proteins (IF1 and IF2) are produced, presenting differences (41% of identity) only in the sequence spanning residues 40–73, codified by exon 3 [7]. It is not yet clear if the two isoforms play different physiological roles in the cell.

Alternative splicing is a feature common to many different genes. The fibronectin (FN) alternative splicing is one of the best characterised: with this mechanism, several FN variants, playing specific roles, are produced [8]. In other cases the presence of some variants derived from an alternative splicing mechanism correlates with the tumoural phenotype, as demonstrated for estrogen receptor [9].

Recently Chiarugi et al. [10] showed that LMW-PTP is involved in the negative regulation of the mitogenic stimulus starting from the activated PDGF receptor. The interaction between LMW-PTP and the PDGF receptor results in the dephosphorylation of activated PDGF-R phosphotyrosine(s) and in a negative regulation of the mitogenic signal. It is very likely that PDGF receptor is not the unique substrate of LMW-PTP, since the involvement of LMW-PTP in the regulation of the signals starting from the activated insulin receptor has also been shown [11].

In this paper we describe the isolation of a novel LMW-PTP mRNA originating via alternative splicing and the characterisation of the corresponding polypeptidic product.

2. Materials and methods

2.1. Isolation of LMW-PTP SV3 cDNAs

Total RNA was isolated from placenta, HepG2 and K562 by the guanidium isothiocyanate method [12]. RT-PCR experiments were performed under standard conditions, using degenerated oligonucleotides supplied by Pharmacia: 5'-TTTGT(C/G)TG(C/T)CT(G/C)GG(G/C)AACAT(T/C)TG as 'direct primer' and 5'-CC(A/G)-TA(A/G)TAGGGGTC(T/C)TC(A/G)AT(A/G)AT as 'reverse primer'. PCR products were cloned into PCR II tailing vector using TA Cloning System (Invitrogen). Primers 1D (5'-GTGTCTC-GGCGCCTCTGC) and 1R (5'-ACCGACTGAGAAATGCAGGA) were used for an RT-PCR experiment in order to clone the complete SV3 coding sequence, together with the Bmix (5'-ACTTC-CGGTGGGTCATTG) oligonucleotide for Southern blot experiments in order to isolate the SV3 harbouring clones.

2.2. Determination of LMW-PTP IF1, IF2 and SV3 mRNA amounts

RT-PCR was performed in 100 μ l: 10 μ l of each sample was separated on 1.5% agarose gel, alkaline blotted to nylon Hybond N⁺

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membrane filter (Amersham) and separately hybridised with ³²P-labelled oligonucleotide specific for IF1, IF2 and SV3, respectively: BFF (5'-CAACGTGGCTCATGGGA), BSr (5'-GAGAAATTGGGT-CATTGACAG) and Bmix as above. Each hybridisation was carried out in standard conditions at a temperature of 5°C below the calculated *T_m*. Hybridisation efficiency was checked using identical quantity of complementary DNA spotted on nitrocellulose. The probed nitrocellulose filters were exposed to X-ray film and the relative bands acquired by UMAX VISTA Speedy scanner were quantified by computer program NIH-IMAGER 1.61 run on a Macintosh Performa 6500.

2.3. Cloning and expression of the new LMW-PTP

The SV3 coding sequence was cloned in frame with glutathione S-transferase in the pGEX-2T bacterial expression vector (Pharmacia). The recombinant fusion protein was purified from the transformed *E. coli* TB1 strain and digested with thrombin in order to obtain the SV3 isoform alone, as already described [13].

2.4. Equilibrium dialysis experiments

Equilibrium dialysis was performed using the apparatus described by Reinard and Jacobsen [14]. As already described [15], 250 μM of recombinant IF1, IF2 or SV3 of LMW-PTP were separately placed in one of the two different compartments. After the equilibrium was reached, the radioactivity was measured in each compartment. Scatchard analysis of the data was performed as previously reported [16].

2.5. Circular dichroism

CD experiments were performed on a Jasco Model J720 spectropolarimeter equipped with a thermostated cell holder. Far UV spectra were recorded at 25°C in 50 mM acetate buffer, pH 5.5, at a protein concentration of 0.06 mg/ml using a 0.5-cm path-length quartz cell [17].

2.6. Urea titration curves

Urea titration curves were obtained at 25°C by a spectrofluorimeter Perkin Elmer LS 50 B. The fluorescence of 30 independent samples,

with different urea concentrations, containing 0.01 mg/ml of protein in 50 mM acetate buffer, pH 5.5, was recorded by using excitation and emission wavelengths of 280 and 340 nm [18].

3. Results

3.1. Isolation of LMW-PTP SV3 cDNA

Human LMW-PTP is known to be present in two isoforms originating by an alternative splicing process of a common primary transcript of a single gene [6]. At the beginning of this work we were interested in searching for different human cDNA products that could codify for LMW-PTP-like proteins, sharing common characteristic sequences, like those already recognised to be important for the catalysis. For this purpose two degenerate oligonucleotides ('direct' and 'reverse') were prepared with sequences corresponding to the parts of the protein very well conserved through evolution; the two oligonucleotides were then used for an RT-PCR experiment on total RNA from human placenta. This experiment led to the isolation of a new LMW-PTP cDNA species, 30 bases longer with respect to the already known *fast* (IF1) and *slow* (IF2) isoform cDNAs.

In order to obtain the entire coding region of this new mRNA, amplification was performed with two primers complementary to the beginning and to the end of the coding sequence (1D and 1R); DNA sequencing of the product of this PCR experiment led to the determination of the entire coding sequence of this putative new LMW-PTP. Inspection of the cDNA sequence, presented in Fig. 1, panel A, revealed that much likely this new mRNA species is derived from an alternative splicing phenomenon of the same LMW-PTP pri-

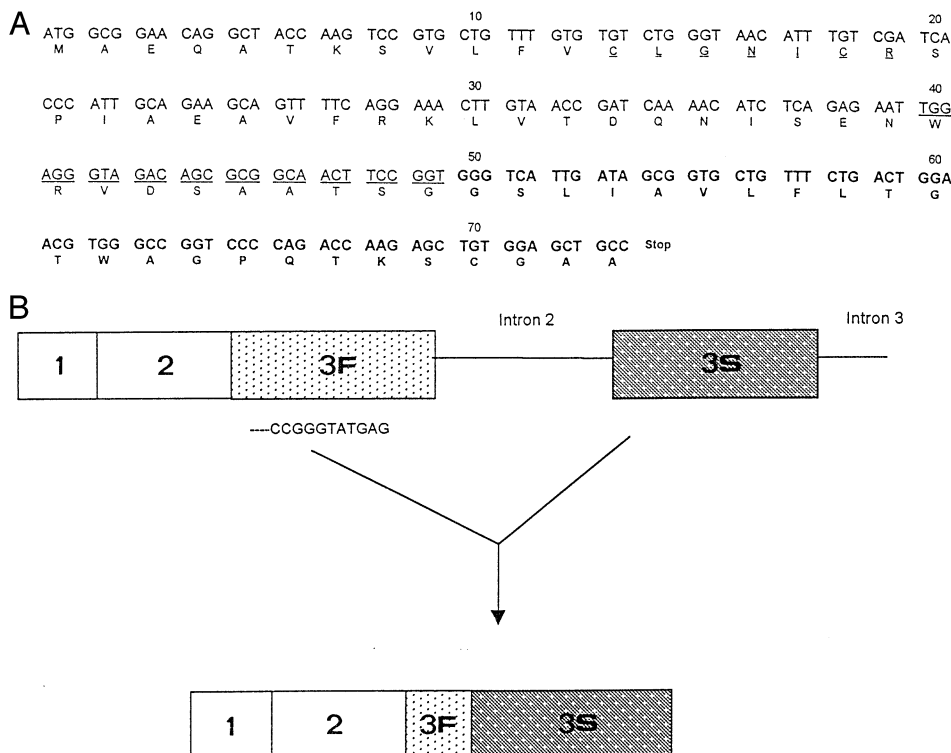


Fig. 1. A: The coding sequence of SV3. Underlined: amino acids of the catalytic loop and triplets codifying for the first 10 amino acids of the IF1 specific sequence deriving from the 3F exon (see text). In bold: nucleotide sequence deriving by the 3S exon, together with the deduced protein sequence. B: Proposed splicing mechanism producing the SV3 mRNA. The cryptic splicing site sequence, representing a possible additional acceptor site, is indicated.

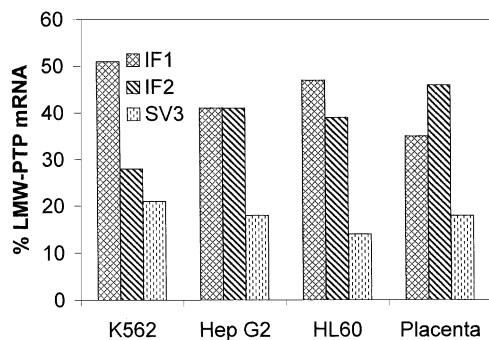


Fig. 2. Determination of the level of the mRNA specific for each LMW-PTP isoform. RT-PCR products, generated with primers complementary to sequences common to all LMW-PTP mRNAs, were used for Southern blot analysis, performed with primers specific for each mRNA species. Quantitation was obtained by computer program NIH-IMAGER 1.61. Quantities are expressed as percentage of the total LMW-PTP transcript. Sources of the different RNA are indicated.

mary transcript that produces also the IF1 and IF2 mRNAs; for this reason we have named this new LMW-PTP 'splicing variant 3' (SV3). While in the case of IF1 and IF2 exon 3F or exon 3S are alternatively incorporated in the mRNA sequence, SV3 LMW-PTP mRNA contains the first 30 bases of the 3F exon and the entire 3S exon sequence. After this point the splicing mechanism follows the same pattern of IF1 and IF2. The result is that SV3 mRNA is 30 bases longer with respect to IF1 and IF2 transcripts. Analysis of the cDNA sequence revealed that this splicing mechanism introduces a frame shift in correspondence to the 3F/3S-exon boundary. As a consequence of this fact, the deduced SV3 protein sequence is identical to the IF1 isoform up to the 49th residue; after that residue the sequence totally diverges from that of IF1 and arrives to an end in correspondence to a stop codon, giving a 73 amino acids long polypeptide, with a calculated M_r of 7676.8 Da. In Fig. 1, panel B, the probable splicing mechanism generating the SV3 isoform is described; analysis of the DNA sequence revealed the presence of a putative splice site within the 3F exon sequence (as indicated in the figure) that could represent an additional donor site in the splicing mechanism leading to the production of the SV3 mRNA.

3.2. Evaluation of the different LMW-PTP mRNA levels

The relative amounts of IF1, IF2 and SV3 mRNAs were evaluated in human placenta and in different human cell lines. RT-PCR experiments were performed on total RNA, followed by Southern blot hybridisation of the RT-PCR products with primers specific for every single isoform (BFR, BSd and Bmix for IF1, IF2 and SV3, respectively). In the RT-PCR experiments a pair of oligonucleotides was used, the first one (1D) complementary to a sequence immediately upstream the ATG start codon and the second (1R) complementary to a sequence 50 nucleotides after the stop codon; these sequences are common to all isoforms, so that the use of 1D and 1R oligonucleotides allows the amplification of all isoforms. In this way the relative amount of the different products obtained with RT-PCR will be proportional to the relative amount of the mRNA of each isoform in the starting materials. The experiments were performed on total RNA purified from human placenta and from three different human cell lines: HepG2 (hepatoma), K562 (erythroleukemia) and

HL60 (promyelocytic leukemia). The results are shown in Fig. 2, expressed as relative amount of every single mRNA species with respect to the total LMW-PTP mRNA (considered as 100%). In all cases examined the SV3 mRNA represents 15–20% of the total LMW-PTP mRNA. These results indicate that SV3 mRNA could always be present in the cell as a further product of the alternative splicing of the LMW-PTP gene transcript, although at a lower extent with respect to IF1 and IF2 mRNAs.

3.3. Expression and characterisation of the SV3 protein

The SV3 coding sequence was cloned in pGEX-2T vector and expressed in *E. coli*. The protein was expressed as a fusion with glutathione-S-transferase and then purified as native form; SDS-PAGE analysis confirmed that this product has an approximate molecular weight of 7.7 kDa, consistent with the calculated size of the polypeptide. Routinely, about 2 mg/l of the protein was obtained. This quite low yield is due to a partial precipitation of the protein, probably as a consequence of its high hydrophobicity.

In order to gain insight into the structure of the protein, the

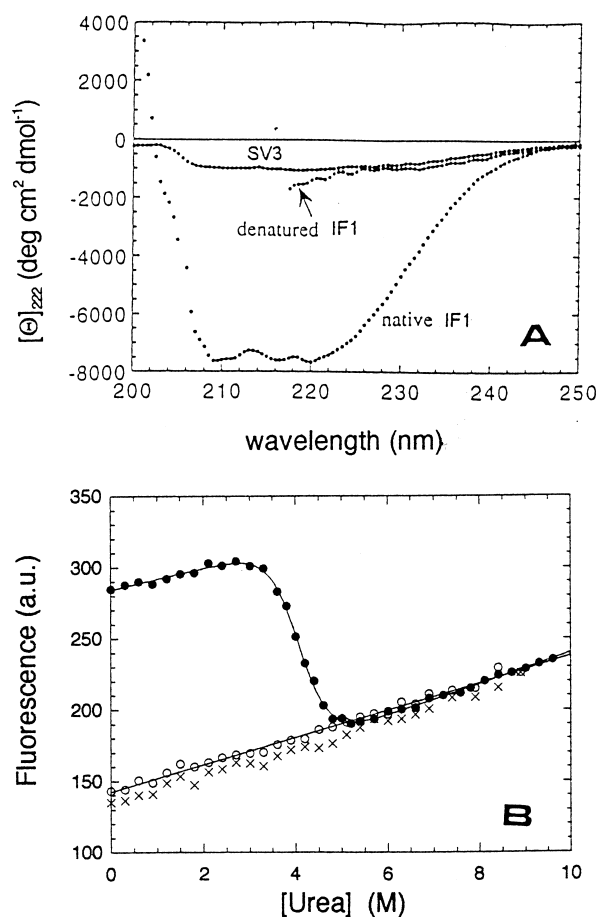


Fig. 3. A: The far UV CD spectra of SV3 and of denatured and native IF1 LMW-PTP. The spectra were acquired at 25°C in 50 mM acetate buffer, pH 5.5. Denaturation of IF1 was performed in 9.6 M urea. B: The urea titration curve of SV3 (○), SV3 in the presence of Pi (×) and IF1 LMW-PTP (●) monitored by intrinsic fluorescence emission (excitation at 280 nm, emission at 340 nm). The urea titration curve was recorded at 25°C. The SV3 data were linearly fitted, whereas the solid line through the IF1 data represents the result of the fitting procedure of Santoro et al. [22], characteristic of a two-state transition. The protein concentration was 0.01 mg/ml.

far-UV CD spectrum of the SV3 LMW-PTP was acquired and appeared to be typical of a denatured protein, the molar ellipticity being very low in the far-UV region between 200 and 250 nm. Moreover, the far-UV CD spectrum of the SV3 LMW-PTP is very similar to that of IF1 denatured in 9.6 M urea and very different to that of native IF1 (Fig. 3, panel A). In the urea titration curve of SV3, the fluorescence was found to increase linearly with urea concentration without any visible transitions. This phenomenon is usual for unfolded proteins [17] and although a linear relationship is also characteristic of native proteins, it is very unlikely that such proteins maintain their fully folded conformation up to 9 M urea. In the same experiment the IF1 form exhibits a normal fluorescence-detected transition between 3 and 5 M urea, as shown in Fig. 3, panel B, which is suggestive of a urea-induced denaturation process from a highly structured protein. The fact that this denaturation process is not evident in SV3 LMW-PTP strongly supports the hypothesis that the protein is unfolded even in the absence of denaturant. It should be underlined that while IF1 and IF2 undergo nearly total refolding after the removal of the denaturant agent, this is not true for SV3, which in the same conditions does not show any change in its unfolded conformation.

LMW-PTP is known to bind specifically the inorganic phosphate ion [15]. Generally the specific binding of ligands is a powerful expedient to stabilise the native form of a protein [18]. The urea titration curve was then repeated in the presence of 20 mM inorganic phosphate as an attempt to detect the unfolding transition upon adding denaturant. The persistent linearity of the plot still suggests the absence of any residual structure even in stabilising conditions as shown in Fig. 3, panel B.

Because of the fact that the catalytic loop characteristic of LMW-PTP (residues 12–18) is also present in SV3, determination of the enzymatic activity of this isoform was performed, using pNPP as a substrate; the protein showed no measurable enzymatic activity. Moreover, in order to investigate whether SV3 could represent a naturally occurring dominant negative variant of LMW-PTP, its possible phosphate binding capacity was evaluated; this is a main feature of LMW-PTP, essentially due to the presence in the catalytic loop of the Arg¹⁸ residue, present also in SV3. Equilibrium dialysis experiments were performed using [³²P]-inorganic phosphate, a competitive inhibitor of LMW-PTP. As far as IF1 or IF2 is concerned, a dissociation constant of about 0.5 mM was calculated by Scatchard analysis, in agreement with previous data [16]. Similar experiments performed with SV3 did not allow the calculation of any affinity constant, demonstrating that SV3, in contrast with IF1 and IF2, is not able to bind the substrate.

4. Discussion

In this paper we describe for the first time the characterisation of SV3, a new human LMW-PTP variant. SV3 mRNA clearly originated by an alternative splicing mechanism on the same primary transcript also generating the IF1 and IF2 mRNAs (Fig. 1, panel B).

The SV3 mRNA is quite abundant in human placenta and in different human cell lines that we have tested (Fig. 2). Investigating the levels of the different mRNAs in K562 cells we found that IF1 and IF2 represent 50 and 30% of the

LMW-PTP transcripts, respectively (SV3 being the residual 20%). We have noticed that the results concerning IF1 and IF2 are partially in contrast with those of Taylor et al. [19], where no IF1 is detected; this discrepancy could possibly be due to differences in distinct clones of K562 cells.

Recently we have cloned the murine LMW-PTP cDNA (manuscript in preparation). RT-PCR analysis performed on RNA from several different mouse tissues never demonstrated the presence of the SV3 transcripts, probably due to the absence, in the mouse LMW-PTP mRNA, of the cryptic splicing site that we find in the human 3F exon.

SV3 protein was expressed in *E. coli*; the protein appeared to be enzymatically inactive and not able to binding the substrate, most probably because the protein is unfolded. In fact SV3 has the characteristics of an unstructured polypeptide chain lacking the cooperative secondary and tertiary structure possessed by the native 18-kDa IF1. The absence of the long C-terminal fragment in SV3 is most probably responsible for the lack of sufficient conformational free energy to induce any level of structure in this protein. The fact that SV3 does not acquire any folded conformation after removal of high urea concentrations, in contrast to what is observed for IF1, supports the idea that the protein could have the same unfolded conformation also in the cell. On the other hand, it is difficult to evaluate whether the lack of enzymatic activity of SV3 is only due to the absence of protein structure or rather to the fact that SV3, although presenting the catalytic loop, lacks several amino acids that have been demonstrated to participate to the catalysis in the active isoforms of LMW-PTP. In fact it has been demonstrated that Asp¹²⁹ is also involved in the catalytic mechanism [16], as well as His⁶⁶ and His⁷² [20]. Moreover, the role of Tyr¹³¹ and Tyr¹³² is still under discussion; these two sites, necessary for a correct enzymatic activity can also undergo phosphorylation, causing enzyme activation [19]. Taken together, all these results suggest the possibility that SV3, once synthesised, has no enzymatic activity in the cell. It should be underlined that the protein is very hydrophobic and, possibly, in unfolded conformation. All these findings make possible the hypothesis that SV3, once produced, may be promptly degraded in the cell. The fact that the SV3 mRNA isoform is abundantly expressed in all the cells or tissues we have tested leaves open the possibility that this isoform could play other roles in the cell, different from those of IF1 and IF2.

Recently it has been proposed that many protein tyrosine phosphatases may act as tumour suppressors [21], namely those acting as negative regulators of cell proliferation. This seems to be the case for LMW-PTP [10], and we could hypothesise that a reduction of active LMW-PTP concentration in the cell might lead to cell proliferation. A mechanism that could control such levels might be a different regulation of the relative abundance of the three mRNA isoforms generated by alternative splicing. A switch towards an increased production of SV3 mRNA could result in a reduction of active enzyme and lead to cell proliferation increase. This hypothesis is also suggested by the fact that variations of alternative splicing patterns have already been correlated with tumour [9].

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