Four PSM/SH2-B alternative splice variants and their differential roles in mitogenesis

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Running title: PSM/SH2-B variants and mitogenesis

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SUMMARY

An SH2 domain originally termed SH2-B had been identified as a direct cellular binding target of a number of mostly mitogenic receptors. The complete cellular protein, termed PSM, and respective sequence variants share additional Pro-rich and PH regions, as well as similarities with APS and Lnk. A role of these mediators has been implicated in signaling pathways such as downstream of growth hormone receptor and receptor tyrosine kinases including the insulin, IGF-I, PDGF, NGF, HGF, and FGF receptors. As a result of this report a total of four PSM/SH2-B sequence variants termed alpha, beta, gamma, and delta have now been identified in the mouse and have been compared to the available rat and human sequences. Variant differences are based on alternative splicing and define distinct last exons 7, 8, and 9 that result in reading frame shifts and unique carboxyl terminal amino acid sequences. Variant sequences have been identified from cDNA libraries and directly by RT-PCR. Sequence analysis predicts four distinctly sized protein products that have been demonstrated after cDNA expression. All were found phosphorylated on tyrosine specifically in response to IGF-I and PDGF stimulation. cDNA expression of the four variants caused distinct levels of stimulation of IGF-I- and PDGF-induced mitogenesis. Stimulation was inducer dose dependent in experiments involving ecdysone-regulated expression plasmids. With two alternative sets of expression plasmids, the most pronounced increase in mitogenesis was consistently observed for the gamma variant followed by delta, alpha, and beta with decreasing responses. In contrast, the mitogenic response to EGF consistently remained unaffected. The variants are expressed in most mouse tissues, typically, most strongly in pairs of alpha and delta or beta and gamma. Our findings implicate differential roles of the SH2-B/PSM splice variants in specific mitogenic signaling pathways.
INTRODUCTION

PSM has been identified as a mouse protein based on its association with the activated catalytic insulin receptor (IR) domain in a yeast two-hybrid screen (1, 2). PSM carries a number of domains including Pro-rich putative SH3 domain binding regions, a pleckstrin homology domain (PH), a src homology 2 (SH2) domain, and many potential Tyr and Ser phosphorylation motifs which suggest a role as a putative signaling mediator. Such a role is also supported by the observation that PSM associates with activated but not with inactive forms of IR (2). A related sequence termed SH2-B had been identified in the rat based on its association with the Fc epsilon RI receptor that represents a rat homologue of PSM (3). A variant form of PSM/SH2-B, termed beta, has been reported which carries an additional 100 bp of coding region downstream of the SH2 domain and is predicted to result in a shortened protein product (2, 4). This variant was described as a substrate and as a potent cytoplasmic activator of the tyrosine kinase JAK2 in response to growth hormone signaling (4-6). The interaction with JAK2 involves the SH2 domain and amino terminal region of SH2-B beta (7). An association of PSM/SH2-B was established with the insulin-like growth factor-I (IGF-I) receptor (IGF-IR) that depends on receptor activation and was mapped to phospho-tyrosine sites that are conserved between the insulin and IGF-I receptors (8). An association with platelet-derived growth factor receptor (PDGFR) in response to PDGF activation was described for PSM/SH2-B that stimulated its phosphorylation on tyrosine, serine, and threonine (9, 10). In response to insulin, tyrosine phosphorylation had originally not been observed for any form of PSM (4, 8) but has more recently been reported in an independent study (11). With regard to its physiologic role PSM cDNA expression has been shown to stimulate the mitogenic response to PDGF-BB, IGF-I, and insulin whereas introduction of a cell-membrane-permeable, putatively dominant-negative SH2 domain peptide interfered with the same response (10). In addition, microinjection of the SH2 domain into

1The abbreviations used are: IR, insulin receptor; IGF-I, insulin-like growth factor; PDGF, platelet-derived growth factor; NGF, Nerve growth factor; HGF, hepatocyte growth factor; FGF, fibroblast growth factor; EGF, epidermal growth factor; SH2 and SH3, Src homology-2 and -3, respectively; PH, pleckstrin homology.
transformed fibroblasts partially restored a normal actin stress fiber pattern suggesting a stimulatory role of PSM in normal and malignant cell proliferation (10). Independently, SH2-B beta was shown to be required for growth hormone-induced actin reorganization (12). The SH2-B gene structure and alternative splice sites have been reported in comparison with a third variant termed gamma that specifically binds to Y1146 of IR (13). A single gene has been mapped to the distal arm of mouse chromosome 7 in a region linked to obesity in mice (13).

SH2-B shares a high degree of structural similarity with adaptor proteins, APS and Lnk, that have been shown to participate in signaling events initiated by B cell receptor and T cell receptor, respectively (14, 15, 16). Thus, the common structural relationship between PSM/SH2-B, APS and Lnk may suggest functional similarities between these three proteins. APS is also expressed in insulin-responsive tissues such as skeletal muscle and fat and becomes phosphorylated in 3T3-L1 adipocytes in response to insulin (17). The SH2 domain of APS, which bears 80% identity with the SH2 domain of SH2-B, associates directly with tyrosyl-phosphorylated insulin receptor (17). APS also associates with activated PDGFR or with c-Cbl and inhibits PDGF-induced mitogenesis (18). SH2-B (19) and APS have been implicated as substrates of Trk nerve growth factor receptors TrkA and TrkB/C, respectively, with a role in developing neurons and neuronal differentiation (20, 21). SH2-B and APS exist as homo- or heteropentamers mediated through the amino terminus and influence neurotropin signaling through direct modulation of Trk autophosphorylation (22). SH2-B interacts with the activation loop of TrkA (19) and plays a specific role in TrkA-mediated differentiation in human neuroblastoma cells (23).

The present study has focussed on the identification of additional mouse sequence variants, the comparison of the involved alternative splice junctions, the predicted differences in protein primary structure when compared to human and rat, and on the implicated putative biochemical and physiologic differences. As a result of this report a total of four PSM/SH2-B sequence variants termed alpha, beta, gamma, and delta have now been identified in the mouse and have been compared to the available rat and human sequences. Variant differences are based on alternative splicing and define distinct last exons 7, 8, and 9 that result in reading frame shifts and unique carboxyl terminal amino acid sequences. Variant sequences have been identified from cDNA libraries and directly by RT-PCR. Sequence analysis predicts four distinctly sized protein products that have been demonstrated after cDNA expression. All were found...
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most mouse tissues, typically, most strongly in pairs of alpha and delta or beta and gamma. Our findings 
implicate differential roles of the SH2-B/PSM splice variants in specific mitogenic signaling pathways. In 
combination, the existing data suggest that PSM/SH2-B represent a family of adapters of several known 
and possibly additional unknown members that belong to a superfamily of signaling mediators including 
APS and Lnk. Their physiologic roles include the control of mitogenesis, neuronal differentiation and 
development and likely other mechanisms of cell regulation.

EXPERIMENTAL PROCEDURES

PSM/SH2-B cDNA isolation

The isolation of complete PSM/SH2-B cDNA has been described earlier (2 ). A 529 bp fragment 
containing the SH2 domain of PSM/SH2-B identified by yeast two-hybrid screening served as a 
hybridization probe to screen a mouse brain lambda Uni-Zap XR library (Stratagene). Positive clones were 
isolated and the resulting plasmids analyzed by fluorescence automated DNA sequencing. The 5’ end of 
the cDNA was isolated by 5’ RACE-PCR (Gibco BRL) using mouse brain total RNA. The RACE-PCR 
products were inserted into a TA vector (Invitrogen) and a 1.5 kb insert was released by digestion with 
EcoR I. Five isolated clones were sequenced. One, TA3, was used for the assembly of the cDNA 
containing the complete protein-coding region of the PSM variants. A 1.2 kb Drd I - EcoRI fragment 
containing part of the 5’ untranslated region (-130 bp) and of the coding region was isolated from TA3. 
The 5’ Drd I site was end filled and the resulting fragment joined with the Hinc II and EcoRI cloning sites 
of pBluescript KS’ (Stratagene). The resulting plasmid (clone 2) was digested with BspE I, at a unique 
site in the insert, and with Sma I within the multiple cloning site region of KS’ for subsequent assembly.
described below.

**Complete cDNA assembly of four PSM/SH2-B variants**

Clone 5-1 was originally obtained from a mouse brain lambda Uni-Zap XR library and carries a 1.9 kb PSM cDNA insert, and a unique *Bsp*E I site at its 5’ end that was used to join the two parts of the PSM/SH2-B cDNA. This clone also carried the 153 bp insert representing the gamma variant. A 1.5 kb 5’ *Bsp*E I - 3’ *Sca* I fragment containing almost half of the protein coding region was isolated from clone 5-1 and inserted into *Bsp*E I - *Sma* I sites in clone 2 carrying the 5’ end of the PSM/SH2-B coding sequence (described earlier). With this approach a subclone PSM/SH2-Bγ/KS⁺ (clone B23) was prepared, that carried the complete protein coding cDNA for the gamma isoform. A 2.5kb *Kpn* I - *Bam*H I fragment containing the complete PSM/SH2-B gamma variant cDNA was released from clone B23 and inserted into *Kpn* I and *Bam*H I cloning sites of the ecdysone-inducible expression vector pIND (Invitrogen). The resulting plasmid PSM/SH2-B gamma/IND was used to generate the construct for the alpha variant cDNA.

Clone 20 obtained from the mouse brain lambda Uni-Zap XR library which carries a 1.1 kb PSM/SH2-B cDNA insert and corresponds to the alpha variant. An 884 bp *Eco*RI - *Bsp*H I fragment was isolated from clone 20, end filled at the *Bsp*H I site and subcloned into the *Eco*RI and *Sma* I sites of KS⁺. From the resulting plasmid a 591 bp *Bst*X I - *Not* I fragment was released and joined with a *Bst*X I - *Not* I fragment of PSM/SH2-B gamma/IND. This step exchanged the region of the gamma-specific cDNA with alpha variant sequences and resulted in an expression construct for PSM/SH2-B alpha/IND. Since the PSM/SH2-B alpha/IND plasmid had lost a unique *Bst*X I site within the multiple cloning site region of the vector, the insert-specific *Bst*X I site was used for the construction of complete protein coding cDNA of the beta and delta variants of PSM/SH2-B.

Clones 34 and 33 were originally identified in the mouse brain lambda Uni-Zap XR library with 1.1 kb PSM cDNA inserts that corresponded to the beta and delta variants, respectively. Two *Bst*X I - *Bst*E II fragments, a 500 bp fragment from clone 34 containing beta variant specific sequences and a 453 bp fragment from clone 33 carrying delta variant specific sequences were individually exchanged with the corresponding alpha variant specific 400 bp fragment in PSM/SH2-B alpha/IND. These exchanges resulted in PSM/SH2-B beta/IND and PSM/SH2-B delta/IND constructs.
At every step of the cDNA construction and assembly all resulting plasmids were analysed by restriction analysis and all ligation junctions were confirmed by DNA sequencing. All four variant constructs carry 91 bp of 5' untranslated nucleotides upstream of the ATG translation start codon. The lengths of 3' untranslated sequences after the stop codon differ between all variants with 149 bp for PSM/SH2-B alpha/IND, 505 bp for PSM/SH2-B beta/IND, 361 bp for PSM/SH2-B gamma/IND, and 298 bp for PSM/SH2-B delta/IND. 2 kb *NheI* - *BamHI* restriction fragments of each variant carrying the complete protein coding region were released and inserted into the *XbaI* and *BamHI* sites of an alternative expression plasmid (CVN) under control of constitutive early simian virus 40 transcriptional promoter elements (24).

**RT-PCR**

The region containing the variant-related inserts was amplified by reverse transcription-polymerase chain reaction (RT-PCR). Complementary DNA was synthesized from 0.5 µg mouse brain polyA⁺ RNA (Clontech) using oligo(dT) or from 2 ug total RNA from mouse tissues. Moloney Murine Leukemia Virus reverse transcriptase (Boehringer, Mannheim) was employed in buffer containing 5 mM MgCl₂ in a total volume of 20 µl with the following parameters for one cycle: 42°C for 60 min, 99°C for 5 min and 4°C for 5 min. Following reverse transcription, PCR was carried out (where indicated at varying Mg²⁺ concentrations between 1mM - 2mM) using *Taq* DNA polymerase and primers flanking the insert, 5’ TTCGATATGCTTGAGCACTTCCGG 3’ including nucleotide positions 2081-2104, and 5’ GCCTCTTCTGCCCCAGGATGT 3’ including nucleotide positions 2345-2365 (Genbank accession number AF020526) (2). Each cycle consisted of denaturation at 94°C for 40 s, annealing at 66°C for 40 s and primer extension at 72°C for 1 min for 30 cycles, with a final extension step at 72°C for 10 min. For the experiment shown in Fig. 1C, the PCR products from mouse brain polyA⁺ RNA were cloned into pCR2.1 TA cloning vector or TOPO TA vector (Invitrogen). The resulting plasmids were analysed by *EcoRI* digestion and fluorescence automated DNA sequencing. For the experiment shown in Fig. 2, PCR products from mouse tissue RNA were separated on a DNA agarose gel. β-actin as internal control is shown using the same amplification protocol and upstream primer 5’ CGTCTGGACCTGGCTGGCCGGACC 3’ and downstream primer 5’ CTAGAAGCATTTGCGGTGGACGATG 3’.
Complete PSM/SH2-B cDNA expression in mammalian cells

Mouse NIH 3T3 cells were transiently transfected with each of the four PSM/SH2-B variants in ecdysone inducible pIND or constitutive CVN expression plasmids. Subconfluent cultures of NIH 3T3 fibroblasts were rinsed with serum- and antibiotic-free medium and incubated for 5 h at 37°C in 2 ml transfection solution. This contained 2 µg of each plasmid, the variant-specific pIND and pVgRXR of the two-plasmid expression system, 10 µl lipofectamine and 8 µl Plus reagent, according to the instructions of the manufacturer (Life Technologies, Gaithersburg, MD). One ml of complete culture medium was added, cells were incubated for 12 h, and subsequently for 5 h in fresh complete medium. For subsequent biochemical and mitogenic analysis cells were treated as described in the next sections.

Immunoprecipitation and analysis of tyrosine phosphorylation

For protein expression analysis, cells were cultured in fresh complete medium for an additional 36 h in the presence of the ecdysone analog ponasterone A (10µM). Subsequently, cells were rinsed twice with PBS and harvested in ice-cold lysis buffer containing 50 mM HEPES pH 7.4, 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EDTA, 10 mM NaF, 100 mM Na3VO4, 10 mM Na pyrophosphate, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 1 mM PMSF. Proteins (300 µg) were first mixed with PSM antiserum (10) and co-precipitated with protein A sepharose for 1 h. Precipitated proteins were rinsed with lysis buffer, separated by 8% SDS-PAGE, and analyzed by immunoblotting with PSM antiserum using the ECL detection system (Amersham).

Alternatively, to elucidate tyrosine phosphorylation, transfected cells were incubated in normal culture medium for 24 h before reaching quiescence in serum-free medium after 20 h. During both steps the ecdysone analog ponasterone A was present at the various indicated concentrations. Subsequently, IGF-I or EGF at 100 ng/ml or alternatively PDGF-BB at 25 ng/ml were added for 15 minutes. Cells were rinsed twice with PBS and harvested in ice-cold lysis buffer. Proteins were first mixed with PSM antiserum and co-precipitated with protein A sepharose. Precipitates were washed with lysis buffer, separated by 8% SDS-PAGE and immunoblotted with anti-phospho Tyr antibody PY 20 (Transduction Laboratories).
DNA synthesis

To assay for DNA synthesis transfected cells were split into 24 well plates and incubated in complete medium in the presence or absence of the ecdysone analog ponasterone A at various concentrations for 24 h before they were starved for 20 h. Subsequently, IGF-I or EGF at 100 ng/ml or alternatively PDGF-BB at 25 ng/ml were added for 18 h, and finally 0.1µCi (methyl-³H thymidine) at 3 T bq/mmol for 5 h. Cells were rinsed three times in ice-cold PBS and incubated in 10% trichloracetic acid for 1 h at 4°C. Precipitates were rinsed and 0.5 ml of 0.2 N NaOH, 0.1% SDS were added at 37°C for lysis 1 h before the pH was neutralized by addition of 0.5 ml 2 M Tris pH 6.8. Two ml of cocktail (ScintiSafe econo-1, Fisher) were added to quantify the incorporated radioactivity by liquid scintillation spectroscopy.

Cell proliferation

To measure cell proliferation transfected cells were split into 24 well plates and incubated in complete medium for 24 h. Cells were rinsed once with ice-cold PBS and incubated with 100 ng/ml IGF-I or 25 ng/ml PDGF-BB in medium with 0.5% serum. After 3 days, 200 ul 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was added for 4 h. The resulting formazan was dissolved in isopropyl alcohol and quantified colorimetrically at O.D. 570 (25). The result has been presented as a measure of cell number.

RESULTS

Identification of four PSM/SH2-B sequence variants.

The SH2 domain encoding region had earlier been identified in a yeast two-hybrid screen (1). It was used to screen a mouse brain Uni-Zap XR library (Stratagene) in order to isolate the complete PSM/SH2-B protein coding cDNA (2). A number of independent clones were identified and eight were sequenced and compared with the originally reported sequence termed alpha (2-4). One clone represented the same alpha sequence, one clone carried a 100 bp insertion representing a variant termed beta (2, 4), five clones carried a 153 bp insert representing a variant termed gamma (13), and one clone carried a 53 bp
insert representing a variant termed delta. The 153 bp insert was composed of both the 100 bp and 53 bp inserts. All insertions were located downstream near the SH2 domain coding sequence after nucleotide 1894 (when A of the initiation codon is defined as 1) of the complete mouse PSM/SH2-B alpha cDNA sequence (Genbank accession number AF020526) (2, 3). Starting from this position a comparison of the four variant sequences (Fig. 1a) suggested that from the longest sequence carrying the 153 bp insertion (gamma) the other isoforms are generated by alternative splicing of the primary PSM/SH2-B transcript (13). Since the observed sequence inserts result in reading frame shifts, all variant sequences predict unique protein carboxyl termini that carry specific motifs including Pro-rich for alpha, gamma, and delta; as well as Ser/Trp-rich and a nuclear localization signal for delta (Fig. 1a). A comparison with listed sequences representing human variants alpha (Genbank accession number AF 227967), beta (AF 227968), and gamma (AF 227969), as well as rat variants alpha (Genbank accession number RNU 57391) and beta (AF 047577) (2, 3) indicates highly conserved alternative splice variants and implicates functional differences of the existing isoforms (Fig. 1b).

Expression of PSM/SH2-B variants in mouse brain.

To address whether mRNA representing the observed variant sequences could be independently demonstrated in mouse brain poly A+ RNA sequences were amplified by the reverse transcription polymerase chain reaction (RT-PCR) using a set of diagnostic primers (see Experimental Procedures) to distinguish between the variant sequences by size. On agarose gels all resulting PCR products correlated well with the fragment sizes expected for the four variants, 338 bp for gamma, 285 bp for beta, 238 bp for delta, and 185 bp for alpha (Fig. 1c). Fragments were introduced into a TA cloning vector either directly or after additional PCR amplification and numerous clones were characterized by DNA sequence analysis. These studies independently established identical variant nucleotide sequences compared to those that were earlier observed in the cDNA library. Variant sequences were identified repeatedly in independent clones for delta (9), beta (19), alpha (17), except for gamma. This is likely explained by the low abundance and diffuse pattern for this band shown as the largest size at the top of the gel (Fig. 1c). It may reflect a low expression level of this isoform that is also suggested in a comparison of various tissues (Fig. 2) and/or reduced stability of the respective mRNA that has not been further investigated.
Variant exon-intron structure and alternative splicing.

Based on the reported genomic organization of the SH2-B gene (13) the SH2 domain is encoded by exons 6 and 7 (Fig. 3a). In addition, exon 7 carries the 53 bp insert that is unique to the gamma and delta variants (Figs. 1a, 4a). Exon 8 carries the 100-nucleotide insert unique to both beta and gamma variants but is absent from both alpha and delta transcripts. Based on the reported genomic sequence (13) all identified splice sites including those of the delta variant (Fig. 3b) match reported consensus sequences for mammalian splice donor and acceptor sequences. When compared to the alpha variant protein coding region, the sequence insertions as a result of alternative splicing cause reading frame shifts. These lead to termination codons and variant proteins that are shorter than the PSM/SH2-B alpha isoform (Fig. 1a, 4a) (2). All variants are identical through Glu 631 the last shared aa. The subsequent carboxyl terminal sequences are unique for all variants, except for the first 18 aa that are shared by both gamma and delta (Fig. 4a). Accordingly, the 53 nucleotides at the 5’end of the 153 bp insert found in gamma, constitute the delta insert (Fig 1a).

Protein variants exhibit different carboxyl termini.

Based on the deduced primary structure all four variant proteins carry Pro-rich and Ser-rich regions as well as a PH domain in addition to SH2 domain (Figs. 1a, 4a). Numerous potential phosphorylation target motifs are found for Tyr and in particular for Ser/Thr kinases (2). In the unique carboxyl terminal regions a Pro-rich putative SH3 domain interacting site is present in the alpha (aa 645-654), gamma (aa 668-676) and delta (aa 653-663) variants (Figs. 1a, 4a). In addition, the delta variant carries two highly basic regions (aa 675-678 and 692-700) that closely match the consensus sequence for nuclear localization signals (NL), K/R K/R X K/R (26), and a third Ser/Trp-rich region (S/W aa 708-718) (Figs. 1a, 4a). All observed sequence motifs are compatible with a role of the variant proteins as adapter/mediators in signalling pathways.

Expression of variant proteins from cDNA.

To directly demonstrate that the variant sequences will be expressed into distinct protein products...
cDNA expression plasmids were prepared. We chose an ecdysone-inducible expression system (pIND, Invitrogen) to allow us to correlate varying doses of cDNA expression with putative physiologic responses in subsequent experiments. Mouse NIH 3T3 cell cultures were transiently transfected with each of the four variant plasmids and expression was induced with ecdysone. Proteins from detergent cell lysates were immunoprecipitated, separated by SDS-PAGE, and identified by immunoblotting with PSM-specific rabbit antiserum (8). When compared to control-transfected cells a single distinct major protein band was observed for each variant. Relative migrations corresponded to approximately 90 kD for alpha, 85 kD for delta, and 80 kD or less for gamma and beta (Fig. 4b). The beta variant typically migrated slightly faster than gamma but the observed size differences were minimal. The observed relative sizes are fully compatible with the primary structures predicted by the variant sequences (Figs. 1b, 4a) and suggest that each sequence variant encodes a distinct protein product with putatively unique functional aspects.

**Variant tissue distribution in the mouse.**

To evaluate the distribution of the four variants in different tissues total RNA from mouse tissues was analyzed by RT-PCR. Northern analysis was not feasible since the four variants cannot be distinguished by unique DNA sequences as hybridization probes. Primers flanking the 100 and 53 bp sequence inserts (see top of Fig. 1a) which distinguish between the four variants were used to amplify short PCR fragments from different tissues that were compared on an agarose gel (Fig. 2). The amplification of actin sequences under the same conditions served as an internal control. Expression of alpha and delta variants was clearly demonstrated in thymus, skeletal muscle, kidney, spleen, lung, and particularly strongly in brain and 10 day embryonal tissue, typically with a significantly higher signal for delta when compared to alpha, except in testes where the ratio was inverted. Only marginal amounts were found in ovary or heart and none of any variants was convincingly demonstrated in liver. The beta and gamma variants were prominently detected in ovary and heart, complementary to the presence of the delta and alpha isoforms. In addition, beta and gamma were detected in testis and minimally in skeletal muscle, brain, lung, and thymus. Typically beta was significantly more prominent than gamma. Our results suggest a possible co-operative action between delta and alpha as well as between beta and gamma; but typically only one of either pair was prominently present in any tested tissue. The variants will likely exhibit some
functional redundancy in addition to specific roles.

Ecdysone dose-responsive variant protein-mediated differential mitogenic effects.

To address the putative functional differences between the variant proteins we investigated their effects on three mitogenic signals since we had previously established a mitogenic role for PSM (10). For this purpose mouse NIH 3T3 fibroblasts were individually transfected with each variant plasmid, starved to quiescence, and stimulated with IGF-I, PDGF, or EGF. In parallel, increasing doses of ecdysone allowed us to raise variant protein expression levels and observe the resulting physiologic consequences. DNA synthesis was measured by exposing transfected cells to $^3$H-thymidine and quantifying acid precipitated radioactivity. In the absence of ecdysone, a modest stimulation of DNA synthesis was observed for each growth factor up to about two-fold over control cell levels (Fig. 5a). IGF-I- and PDGF-stimulated DNA synthesis was significantly increased by each variant at 2 uM ecdysone and dramatically further at the highest employed dose of 10 uM ecdysone. Maximum stimulation reached 9-fold over control cell levels for the gamma variant that consistently displayed the highest level of activity (Fig. 5a). The delta, alpha, and beta variants followed with decreasing activity consistently in this order. Relative activities were consistently observed in this order also at lower ecdysone concentrations of 2 uM with overall reduced effects. No significant effect was observed for any of the variants in the absence of growth factor or after EGF stimulation. This implicates specific roles of the variants in the signaling mechanisms downstream of the IGF-I and PDGF receptors but not of the EGF receptor. It is consistent with earlier observations that implicated a mitogenic function of PSM in the IGF-I and PDGF receptor pathways but not in the EGF receptor pathway (10).

Variant protein-mediated differential mitogenic responses with constitutive expression plasmids.

To exclude any potential impact of individual expression plasmid preparations on our observations experiments were carried out with additional preparations of ecdysone-inducible plasmids as well as with a constitutive expression plasmid. In the latter plasmid variant protein expression was controlled by early simian virus 40 transcriptional promoter elements. Upon transfection all variant proteins resulted in mitogenic responses (Fig. 5b) that were very similar compared to those observed at the highest
ecdysone concentration (Fig. 5a). These experiments confirmed that the observed mitogenic responses are not plasmid-specific.

**Variant protein-mediated differential levels of cell proliferation.**

We evaluated whether the observed impact on DNA synthesis could also be measured at the level of cell proliferation and the resulting cell numbers. For this purpose mouse NIH 3T3 fibroblasts were individually transfected with each (constitutive) variant plasmid. Cells were subsequently stimulated with IGF-I or PDGF and cell proliferation was measured with the MTT assay. A specific increase in cell proliferation was observed in response to IGF-I and PDGF that was differentially increased by the variant proteins. As previously shown in Figures 5a and 5b the highest level of stimulation was observed with the gamma variant followed by delta, alpha, and beta with decreasing levels (Fig. 5c). In combination, these data consistently implicate differential mitogenic roles for each variant protein.

**Variant protein tyrosine phosphorylation.**

To begin to dissect the molecular mechanisms involved in PSM function we evaluated phosphorylation of the four variant proteins on Tyr in response to mitogenic signals. Transfected NIH 3T3 fibroblasts were incubated with various concentrations of ecdysone, starved to quiescence, and stimulated with IGF-I, PDGF, or EGF. Detergent cell lysates were immunoprecipitated with PSM antiserum, proteins were separated by SDS PAGE and analysed by immunoblotting with a phospho Tyr-specific antibody. Specifically in response to IGF-I and PDGF all four variants were detected as distinct individual protein bands (Fig. 6) of similar migration as shown in Fig. 4b. The appearance was properly correlated with the dose of ecdysone used to induce expression. In addition, the IGF-I and PDGF receptors were observed in amounts proportional to the variant proteins as expected based on co-immunoprecipitation with PSM after growth factor and ecdysone stimulation. No signal was observed in response to EGF; the presence of the EGF receptor had been confirmed in control lysates (Fig. 6). The data show that all four variants are phosphorylated on Tyr in response to IGF-I and PDGF receptor activation. Since significant differences were not detected in the signal between the variants and since putative target phosphorylation motifs are only found in the sequences shared between all variants this modification is unlikely to play a role in the
differential mitogenic responses observed in Fig. 5. However, it may play an important role in PSM activation that will be the focus of future experiments.

**DISCUSSION**

In the present study we have compared the characteristic features of the four PSM/SH2-B variants including the newly identified isoform delta. Our data are consistent with a common gene from which all variants are derived by alternative splicing (Nelms et al., 1999). We have identified unique carboxyl-terminal sequences for each variant containing specific functional motifs that are predicted to define functional differences. Compatible with these structural differences we observed distinct levels of mitogenic stimulation for the four variants similarly for PDGF- and IGF-I –induced DNA synthesis and cell proliferation. Functional differences of the variants are also supported by the differential expression pattern found in many tissues.

When compared with the initially discovered variant now termed alpha, the beta, gamma and delta isoforms contain insertions of 100bp, 153bp and 53bp, respectively, just downstream of their shared SH2 domain coding region (Fig. 1a). Nucleotide sequence analysis of the inserts suggests that in addition to the longest gamma form containing the complete 153 bp insert, alternative splicing results in the removal of 53 bp, 100 bp, or the complete 153 bp insert to result in the beta, delta, or alpha variants, respectively. The genomic organization of the SH2-B gene indicates that the 53 nucleotide segment of the insert is located at the 3’ end of exon 7 and provides the alternative splice sites to produce the alpha, beta and gamma variants (13). The alpha and beta transcripts (Fig. 3a) use the alternative donor splice site located 53 nucleotides upstream of the normal exon 7/intron 7 boundary, thereby excluding 53 nucleotides at the 3’ end of exon 7 (13). The alpha isoform also excludes the following exon 8 that is included in the beta transcript (13). In contrast, both gamma and delta isoforms use the downstream splice site at the exon 7/intron 7 boundary. Exon 8 is included in the gamma transcript (13) but excluded from the delta variant (Fig. 1a). Following the differential use of exon 8 (termed exon 8beta/8gamma) all variants use the next downstream exon (termed exon 9beta/8alpha) (13). The donor splice site from exon 8 and the acceptor
splice site from exon 9 beta create the termination codon (Fig. 3b) in the gamma transcript (13).

The splicing of pre-mRNA for the removal of intervening introns is a crucial post-transcriptional process requiring a co-ordinated action of several small nuclear ribonucleoprotein particles (snRNPs) and non-snRNP factors which assemble on pre-mRNA into a large multicomponent complex, the spliceosome (27). Among the essential non-snRNP splicing factors are the highly conserved Ser/Arg-rich SR proteins that participate not only in the early stages of spliceosomal assembly and affect the selection of alternative splice sites (28, 29). Different members of the SR protein family display distinct functions associated with preferential use of 5′ splice sites in alternative pre-mRNA splicing in vitro, in addition to exhibiting differential expression in a variety of mammalian cell types and tissues (30, 31). Splicing enhancers located within exons of pre-mRNA can facilitate splicing of upstream introns that are subject to alternative splicing (32-35). SR proteins recognize these exonic enhancer signals with distinct specificities (29, 31, 36-39), that may strongly effect splice site selection (31, 40). The combination of SR proteins displaying different specificities for pre-mRNA subclasses and the regulated levels of SR proteins in different cell types contribute to the modulation of cell specific splice site selection (29, 31, 41). It was tempting to speculate that the expression of the variants would dependent on tissue and/or cell type based on the presence of alternative differential splicing mechanisms. To evaluate the distribution of the four variants in different tissues northern analysis was not feasible since specific sequences are not available that would distinguish the variants by hybridization. With RT-PCR (Fig.2) we typically observed a complementary expression pattern of either delta and alpha in most tissues or beta and gamma in others. These findings suggest that both alternative splice sites at the 3’ end of exon 7 of the SH2-B gene (13) are used together with preferential exclusion of exon 8 (termed exon 8 beta/8 gamma) for the generation of delta and alpha isoforms. Interestingly, both beta and gamma use exon 8 (exon 8beta/8gamma) (13). Such differential splicing and exon selecting events may be regulated at the tissue and/or cell level to determine the expression of the variants. In our experiments the signal was typically significantly higher for delta when compared to alpha or for beta when compared to gamma. Our results suggest a possible co-operative action between delta and alpha as well as between beta and gamma; typically only one of either pair was prominently present in any tested tissue. The variants will likely exhibit some functional redundancy in
Of considerable interest is the finding that all four protein variants exhibit unique carboxyl termini (Figs. 1a, 4a) which implicate functional differences in signalling pathways. All variants share the PH and SH2 domains and are identical up to residue Gln 631 (Fig. 4a). The beta isoform was shown to be localized at the plasma membrane of PC12 cells (21). If this subcellular localization occurs via the shared PH domain (21), the other variants may share similar intracellular localizations. Notable is the presence of nuclear localization signals in the unique carboxyl terminus of delta (Fig.1a) which may implicate a nuclear role of this variant in gene expression, a promising area of future investigation. Alpha constitutively interacts with Grb2, primarily via the SH3 domain of Grb2 and the Pro-rich region located between the PH and the SH2 domain of alpha (20) implicating the other variants in similar associations. Major features in the unique carboxyl termini of alpha and gamma include the presence of Pro-rich motifs (Figs. 1a and 4a) that may recruit other SH3 domain containing signalling mediators. Other structural features within the carboxyl terminal of alpha include a sequence SFV (aa 754-756) (Fig. 1a) that matches the consensus recognition motif (S/TXV) for PDZ domains that are found in a variety of signalling proteins (42). Alpha also contains a sequence RSTSRDP (aa 633-639), which resembles the proposed Raf consensus sequence (R)SXpSXP where serine is phosphorylated, as a binding motif for 14-3-3 proteins (43), yet, peptides lacking Arg still interact with 14-3-3 proteins. 14-3-3 epsilon binds phosphorylated serine of both the IGF-1 receptor and IRS-1 (44). The beta variant carries a sequence PDASSTLLP (aa 650-658) (Fig. 1a) similar to the 14-3-3 binding sequence found in the carboxyl terminus of the IGF-1 receptor (44). The distinct carboxyl termini result in widely differing isoelectric points for the variants, pI 8.1 for gamma, 5.6 for delta, 5.5 for beta, and 5.0 for alpha (45). The fact that PSM/SH2-B contains a PH domain, an SH2 domain in addition to several potential phosphorylation sites as well as Pro-rich and Ser-rich regions together with unique features associated with the carboxyl termini of the four variants suggests multiple interactions with other signalling mediators to define differential roles in various signaling pathways.

Various reports implicated different motifs of IR in the interaction with PSM/SH2-B (8, 11, 13). In the absence of information about the family of four SH2-B splice variants these differences remained
unresolved. It is conceivable that the variant-specific carboxyl termini influence the interaction of the adjacent SH2 domain with receptor Tyr kinases. For the alpha variant the IR activation loop was implicated in the interaction with a GST fusion protein including the SH2 domain of alpha (11). Earlier studies in this lab with the (at that time unrecognized) beta variant found that a GST fusion protein containing the SH2 domain and carboxyl terminal sequences of PSM beta no longer bound to IR mutants lacking Tyr 960 in the juxtamembrane region or Tyr 1322 at the IR carboxyl terminus (8). These findings were supported with IGF-IR mutants lacking the homologous Tyr 950 or Tyr 1316 and by competition experiments with specific IR phosphopeptides representing Tyr 960 and Tyr 1322 (8). For the gamma variant Tyr 1146 in the catalytic loop of IR has been implicated in the association in yeast two-hybrid interactions studies and by some biochemical data (13). An association has been reported with the catalytic loop of TrkA (20, 19; which shares similarity with the catalytic loop of IR) for an unspecified SH2-B form that correlates with the reported observation for gamma (13). It is still possible that the differing observations are in part explained by the alternative experimental approaches taken in the various reports. However, it is compelling to speculate that PSM/SH2-B alpha and gamma variants may indeed share target motifs in their interaction with IR and other receptor Tyr kinases such as TrkA whereas the beta variant may display distinct binding preferences. The exact receptor binding preferences of all four PSM variants remain to be verified in controlled, comparative experiments. These are now feasible and should include the new variant delta.

We have begun to address the functional differences of the variants in signal transduction by comparing their impact on mitogenesis in fibroblasts. All variants stimulated DNA synthesis in response to IGF-I or PDGF but not to EGF (Fig. 5a, b), consistent with earlier studies focused on alpha (10). Gamma consistently stimulated DNA synthesis most strongly followed by delta, alpha, and beta with decreasing impact. For all variants DNA synthesis was responsive to the dose of ecdysone that controlled transcription of the cDNA (Fig. 5a). To address any role of the expression plasmid and the individual cDNA transfection, experiments were repeatedly carried out and in addition with constitutive expression plasmids (Fig. 5b). All experiments showed the same most pronounced mitogenic stimulation by gamma followed by delta, alpha, and beta with decreasing responses. The consistent absence of any observed
modulation of the mitogenic response to EGF confirmed the specificity of the experimental approach. To address whether these observation could be generalized to the level of cell proliferation, experiments were repeated by scoring cell numbers with the MTT assay. Cell proliferation was similarly stimulated in response to IGF-I or PDGF most prominently by gamma, followed by delta, alpha, and beta with decreasing impact (Fig. 5c). In combination, these results implicate differential roles of the variants in IGF-I- and PDGF-mediated mitogenesis that remain to be elucidated at the molecular level.

We have begun to investigate potential molecular mechanisms of PSM/SH2-B activation by evaluating phosphorylation on Tyr. We found all variants to be phosphorylated on Tyr specifically in response to IGF-I and PDGF but not to EGF (Fig.6). However, unlike the differential effect on mitogenesis, Tyr phosphorylation was similar between all variants. This is consistent with the presence of potential receptor target motifs only in the shared (2) but not in the unique variant sequences. Whereas Tyr phosphorylation is unlikely to explain differential roles of the variants in mitogenesis, it may be important in the molecular activation of PSM that certainly warrants further investigation. Future experiments to elucidate the specific functional differences between the variants will focus on preferences for cellular partners and downstream mediators in established mitogenic signaling mechanisms.

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Legends to Figures

Figure 1a. Comparison of PSM/SH2-B mouse variant nucleotide sequences and deduced protein primary structures. Carboxyl terminal mouse nucleotide sequences for all four variants alpha, beta, gamma, and delta (indicated on the right) have been shown starting and aligned at nucleotide 1891 (A of the initiation codon has been defined as nucleotide 1). Amino acids are shown underneath and have been aligned starting from the corresponding codon for the last shared aa Q 631. Nucleotide sequence inserts unique to the gamma and delta variants have been underlined, sequences unique to the gamma and beta variants double underlined. Absent nucleotides have been represented by a hatched line, identical nucleotides when compared to the previous variant by a vertical line if they remain untranslated. Deduced aa for each variant have been aligned below the respective first nucleotide of the encoding triplet and indicate the altered reading frame for each variant. Translation termination codons for each variant have been indicated by a star at the end of each aa sequence. Specific aa sequence motifs, Pro-rich (hatched), Ser/Trp-rich (dotted), nuclear localization signals (waved) have been underlined.

Figure 1b. Comparison of predicted PSM/SH2-B variant primary structures between human, mouse, and rat. The reported predicted variant aa sequences from human (46), mouse (2, 13), and rat (3, 4) have been aligned for maximum similarity and aa numbers are shown starting with the initiator Met (defined as 1). Human sequences (in bold) are shown above and rat sequences below the mouse sequence only where they differ. Complete human, mouse, and rat variant sequences have been compared as far as sequences are available. Variant specific carboxyl terminal sequences have been compared individually starting with aa 631 as indicated by their greek letters on the right. Where sequence information was not available this has been indicated in the left margin by (-). For each of the species aa that are absent have been represented by (-) in the respective sequence. The Genbank accession numbers are: Human alpha (AF 227967), Human beta (AF 227968), Human gamma (AF 227969), Mouse alpha (AF 020526), Mouse beta (AF 020526), Mouse gamma (AF 074329), Mouse delta (AF380422), Rat alpha (RNU 57391), and Rat beta (AF 047577).
Figure 1c. **Comparison of mouse variant sequences by RT-PCR.** After reverse transcription of mouse brain mRNA PCR was carried out with specific diagnostic primers to amplify variant-specific fragments of carboxyl terminal coding regions. Amplification products have been presented after electrophoresis on an ethidium bromide stained agarose gel based on reactions with varying Mg$^{2+}$ concentrations as indicated. The predicted fragment sizes for the alpha, beta, gamma and delta variants, respectively, are 185 bp, 285 bp, 338 bp and 238 bp. The position of specific size markers (in bp) is shown on the left.

Figure 2. **Comparison of variant distribution in mouse tissues.** After reverse transcription of mRNA from various mouse tissues, PCR was carried out with specific diagnostic primers (see Experimental Procedures) to amplify variant-specific fragments of carboxyl terminal coding regions and in parallel to amplify a control beta-actin fragment. Products were compared on an ethidium bromide-stained agarose gel with specific size markers (in bp) shown on the left. The presented mouse tissues are thymus (Th), skeletal muscle (Mu), 10 day embryo (Em), kidney (Ki), ovary (Ov), testis (Te), spleen (Sp), lung (Lu), heart (He), liver (Li), and brain (Br).

Figure 3a. **Exon-intron structure of mouse variant sequences.** Exon and intron boundaries are shown based on the information reported by Nelms et al. (13). All variants have been aligned for comparison with the new delta sequences. Exons (numbered underneath 1-9 in bold) have been represented by heavy lines. Protein coding regions have been indicated by heavy boxes and their aa boundaries by vertically-oriented numbers (the initiator Met has been defined as 1). Amino acids that are encoded by two exons have been assigned to the exon that carries two nucleotides of the respective codon. Introns have been represented by light horizontal lines and their length in nucleotides has been shown underneath. The location of termination codons has been indicated (STOP). Shared sequences including exons 1-6 that are identical for each variant at the beginning of the gene have only been shown once at the top. As indicated by an arrow they precede each variable sequence that is shown individually only starting with exon 7.

Figure 3b. **Mouse variant-specific alternative splice boundaries.** Sequence boundaries are shown based on the information reported by Nelms et al. (13). All variants have been aligned for comparison with
the new delta sequences. Exon sequences are represented by upper-case letters and straight, horizontal, bold lines, intron sequences by lower-case letters and tented bold lines, and splice junctions by light vertical lines. Complete intron sizes have been shown in bp for each variant. All sequences start with the 3′ boundary of exon 7 and represent the splice events to exon 9 of beta or to exon 8 of the remaining variants. The length of the protein-coding region in aa has been shown in bold for each variant on the right. The availability (+) of sequence data for each variant from a cDNA library or from RT-PCR (brain mRNA) is indicated on the far right.

**Figure 4a. Comparison of variant protein domain structure.** Shared sequences of the variants have been aligned and represented by hatched boxes including regions amino terminal (vertical hatches) and carboxyl terminal (tilted hatches) to the SH2 domain (dotted and labelled SH2). Sequences unique to each variant have been shown by open boxes labelled with the respective variant letter, and sequences shared between gamma and delta by horizontal hatches. Numbers represent aa positions in each variant (initiator Met has been defined as 1). Capital letters below the sequences indicate specific aa motifs including Pro-rich (P), Ser-rich (S), Ser/Trp-rich (S/W), putative Tyr phosphorylation sites (Y), and putative nuclear localization signals (NL).

**Figure 4b. Protein products of ecdysone-regulated variant cDNA transient expression.** NIH 3T3 fibroblasts were transfected with variant-specific plasmids or control plasmid (-) and expression was induced with ecdysone. Protein products were immunoprecipitated from detergent cell lysates with PSM-specific rabbit antiserum, separated by PAGE and visualized in immunoblots with PSM-specific rabbit antiserum. Approximate sizes of the respective bands shown on the right in kD are based on MW markers.

**Figure 5a, b. Variant-dependent differential stimulation of IGF-I- and PDGF-mediated mitogenesis.** Responses were individually tested in NIH 3T3 fibroblasts to each of the four variants, represented by distinctly labelled bars. Responses were compared after transfection with variant-specific plasmids or control plasmid. (a) For ecdysone-inducible plasmids expression was induced with varying doses of ecdysone (0, 2, 10) indicated in uM or alternatively (b), constitutive expression plasmids were
used. Cells were starved to quiescence, and stimulated with IGF-I, PDGF, EGF, or left untreated (-) as indicated in the figure. Acid precipitated $^3$H-thymidine was quantified by liquid scintillation spectroscopy. All data points were measured in duplicate in each experiment as represented by the error bar and one representative of several experiments has been shown.

**Figure 5c.** Variant-dependent differential stimulation of IGF-I- and PDGF-mediated cell proliferation. Individual variants are represented by distinctly labelled bars as indicated in the figure. Responses were compared in NIH 3T3 fibroblasts after transfection with constitutive variant-specific plasmids or control plasmid. Cells in minimal serum were stimulated with IGF-I, PDGF, or were left untreated (-) as indicated in the figure. Cell proliferation was evaluated by quantifying cell numbers biochemically based on mitochondrial succinate dehydrogenase activity through the colorimetric change of MTT. All data points were measured in duplicate in the experiment as represented by the error bar.

**Figure 6.** PDGF- and IGF-I-specific stimulation of variant protein tyrosine phosphorylation. Normal (PDGF) or EGF receptor over-expressing (EGF) NIH 3T3 fibroblasts, or IGF-I receptor over-expressing mouse embryo fibroblasts (IGF-I) were transfected with ecdysone-inducible variant expression plasmids or control plasmid. Cells were starved for 20 h in the presence of various doses of ecdysone (0, 2, 10 uM) and stimulated with 25 ng/ml PDGF BB, 100 ng/ml EGF or 100 ng/ml IGF-I (respectively, for the cell lines indicated above) for 15 min or were left untreated (-). Detergent cell extracts were immediately separated by SDS PAGE (indicated by W/O IP) or were typically immunoprecipitated with PSM/SH2-B antiserum prior to SDS PAGE. Proteins were identified in immunoblots with phosphotyrosine antibodies.
Figure 1a
Figure 4a

Figure 4b
Figure 6

[Image of a gel blot with bands labeled PDGFR, EGFR, IGF-IR, and Peptide Hormone with various concentrations indicated]

WO IP

EGF

α β γ δ α β γ δ 

10 10 10 0 2 10 2 10 0 2 10 2 10

Ecdysone
Four PSM/SH2-B alternative splice variants and their differential roles in mitogenesis
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