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PSM/SH2B1 Splice Variants: Critical Role in Src Catalytic Activation and the Resulting STAT3-Mediated Mitogenic Response

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Abstract A role of PSM/SH2B1 had been shown in mitogenesis and extending to phenotypic cell transformation, however, the underlying molecular mechanism remained to be established. Here, four alternative PSM splice variants and individual functional protein domains were compared for their role in the regulation of Src activity. We found that elevated cellular levels of PSM variants resulted in phenotypic cell transformation and potentiated cell proliferation and survival in response to serum withdrawal. PSM variant activity presented a consistent signature pattern for any tested response of highest activity observed for gamma, followed by delta, alpha, and beta with decreasing activity. PSM-potentiated cell proliferation was sensitive to Src inhibitor herbimycin and PSM and Src were found in the same immune complex. PSM variants were substrates of the Src Tyr kinase and potentiated Src catalytic activity by increasing the V_{max} and decreasing the K_m for ATP with the signature pattern of variant activity. Dominant-negative PSM peptide mimetics including the SH2 or PH domains inhibited Src catalytic activity as well as Src-mediated phenotypic cell transformation. Activation of major Src substrate STAT3 was similarly potentiated by the PSM variants in a Src-dependent fashion or inhibited by PSM domain-specific peptide mimetics. Expression of a dominant-negative STAT3 mutant blocked PSM variant-mediated phenotypic cell transformation. Our results implicate an essential role of the PSM variants in the activation of the Src kinase and the resulting mitogenic response—extending to phenotypic cell transformation and involving the established Src substrate STAT3. J. Cell. Biochem. 104: 105–118, 2008.

Key words: cell-permeant peptide; mitogenesis; phenotypic cell transformation; Pro-rich; PH; SH2; survival; SH2-B

An SH2 domain-containing sequence originally termed SH2-B had been identified in the rat [Osborne et al., 1995] and the corresponding mouse and rat proteins, respectively, based on their association with the activated catalytic insulin receptor (IR) domain [Riedel et al., 1997] or as substrates of activated JAK2 [Rui et al., 1997]. Pro-rich putative SH3 domain binding sequences, a pleckstrin homology region (PH) and a src homology 2 (SH2) domain implicated a role as a signaling mediator also termed PSM [Riedel et al., 1997] or SH2B1 [Maures et al., 2006]. A total of four alternative splice variants of PSM/SH2B1 have been reported in the mouse termed alpha, beta, gamma, and delta [Riedel et al., 1997; Rui et al., 1997; Nelms et al., 1999; Yousaf et al., 2001] and in the human genome [Nishi et al., 2005]. A single SH2B1 gene has been mapped to the distal arm of mouse chromosome 7 in a region linked to obesity in mice [Nelms et al., 1999]. In particular, the beta variant was described as a substrate and as a potent cytoplasmic activator of JAK2 in response to growth hormone signaling [Rui et al., 1997, 2000;

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Rui and Carter-Su, 1999; Carter-Su et al., 2000a,b; Kurzer et al., 2004; Miquet et al., 2005]. Activation of the tyrosine kinase receptors for insulin, insulin-like growth factor-I (IGF-I), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), or TrkA for nerve growth factor (NGF) resulted in their association with the SH2 domain of PSM/SH2B1, suggesting a putative role in the respective signaling pathways [Wang and Riedel, 1998; Riedel et al., 2000]. Tyrosine phosphorylation of PSM/SH2B1 was observed in response to insulin [Kotani et al., 1998], IGF-I, and PDGF [Yousaf et al., 2001]. PDGF stimulated PDGF receptor association with and phosphorylation of PSM/SH2B1 on tyrosine, serine, and threonine [Rui and Carter-Su, 1998; Riedel et al., 2000; Yousaf et al., 2001]. cDNA expression of the four PSM/SH2B1 variants differentially stimulated the mitogenic response to PDGF, IGF-I, and insulin whereas introduction of cell-permeant, putatively dominant-negative SH2 domain or Pro-rich peptide mimetics interfered with the mitogenic response [Riedel et al., 2000; Yousaf et al., 2001]. Microinjection of an SH2 domain peptide mimetic into transformed fibroblasts partially restored a normal actin stress fiber pattern suggesting a requirement of PSM/SH2B1 in phenotypic cell transformation [Riedel et al., 2000]. PSM/SH2B1 beta is required for growth hormone-induced actin rearrangement [Herrington et al., 2000] and regulates cellular motility [Diakonova et al., 2002] and membrane ruffling [O'Brien et al., 2003]. PSM/SH2B1 interacts with the activation loop of TrkA [Koch et al., 2000] or TrkB [Suzuki et al., 2002] and plays a specific role in TrkA-mediated differentiation [Eggert et al., 2000; Chen and Carter-Su, 2004] with a specific role of PSM/SH2B1 shown in the Akt/Forkhead signaling pathway [Wang et al., 2004]. In response to fibroblast growth factor receptor 3 (FGFR3) activation, PSM/SH2B1 associates and undergoes tyrosine phosphorylation with a putative role in the activation and nuclear translocation of Stat5 [Kong et al., 2002]. PSM/SH2B1 beta is a substrate as well as a key enhancer of RET tyrosine kinase activity and potentiates the resulting differentiation and neoplastic transformation signal apparently by protecting RET from dephosphorylation [Donatello et al., 2007]. In response to leptin PSM/SH2B1 promotes IRS1- and IRS2-mediated activation of the PI 3-kinase pathway [Duan et al., 2004a]. PSM/SH2B1 is a key regulator of adipogenesis by regulating the insulin/IGF-I receptor-Akt-Foxo1-PPARγ pathway [Yoshiga et al., 2007]. SH2B1 gene polymorphism correlates with serum leptin and body fat [Jamshidi et al., 2007]. Disruption of the SH2B1 gene suggests a critical role in the IGF-I-mediated reproductive pathway in mice [Ohtsuka et al., 2002] and as a physiologic enhancer of IR activation as well as a role in the maintenance of normal insulin sensitivity and glucose homeostasis during ageing [Duan et al., 2004b]. PSM/SH2B1 shares a high degree of structural similarity with scaffold proteins, Lnk and APS that participate in B cell receptor and T cell receptor signaling, respectively [Huang et al., 1995; Yokouchi et al., 1997; Iseki et al., 2000; Rudd, 2001; Velazquez et al., 2002; Hu and Hubbard, 2006], and display distinct cellular functions [Kubo-Akashi et al., 2004]. APS and PSM/SH2B1 variants engage in cross-regulation in the activation of JAK family tyrosine kinases and in insulin signaling [O'Brien et al., 2002; Li et al., 2007]. In contrast to the SH2B1 gene knockout [Duan et al., 2004b], disruption of the APS gene in mice results in increased insulin sensitivity and hypoinsulinemia [Minami et al., 2003]. In a direct comparison in mice, disruption of the APS gene did not display alterations in adiposity, energy balance, glucose metabolism, insulin or glucose tolerance as opposed to disruption of the SH2B1 gene which was interpreted to suggest a specific role of PSM/SH2B1 as regulator of energy and glucose metabolism in mice [Li et al., 2006] that is extended to neurons [Ren et al., 2007]. PSM/SH2B1 and APS display distinct affinities when compared for binding to IR or JAK2 [Hu and Hubbard, 2006]. In response to insulin both APS [Moodie et al., 1999] and PSM/SH2B1 undergo phosphorylation on Tyr, interact with the activation loop of IR, enhance IR autophosphorylation, and enhance (independently of PSM/SH2B1 phosphorylation) ERK and Akt activation [Ahmed and Pillay, 2001, 2003]. PSM/SH2B1 and APS play a role in neuronal differentiation [Qian et al., 1998; Rui et al., 1999; Koch et al., 2000]. Both proteins have been reported as homo- or heteropentamers, mediated through the amino terminus in an analogous mechanism and to directly modulate Trk autophosphorylation [Qian and Ginty, 2001]. The activating mechanism has been proposed as homo- or hetero-dimerization via a phenylalanine zipper at the amino terminus which in turn results in

the dimerization and activation of associated Tyr Kinase JAK2 [Dhe-Paganon et al., 2004; Nishi et al., 2005]. In addition, an alternative mechanism to support an active conformation of JAK2 involving only the SH2 domain of PSM/SH2B1 and independent of the dimerization domain has been proposed [Kurzer et al., 2006]. PSM/SH2B1 plays a role in the assembly of distinct mitogenic signaling complexes that in cultured normal fibroblasts involves PI 3-kinase only in response to PDGF when compared to insulin or IGF-I [Deng et al., 2007]. The PSM/SH2B1 variants are essential components of the insulin receptor signaling complex, necessary for its catalytic activation and the resulting insulin responses, and act as internal insulin receptor ligands that in addition to potentiating the insulin response stimulate insulin receptor catalytic activation even in the absence of insulin [Zhang et al., 2007].

Signal transducers and activators of transcription (STATs) are established mediators in Src family kinase-mediated tumorigenesis [Silva, 2004]. STATs were originally identified in the signaling pathway activated by cytokine receptors. Here STATs are activated by phosphorylation via JAK Tyr-kinases that are recruited into the signaling complex in response to cytokine stimulation [Darnell, 1997]. In addition, STATs are prominently activated by growth factor receptors in particular the erbB family as well as by Src and by related tyrosine kinases [Hayakawa and Naoe, 2006]. In particular, STAT3 was found to be fully activated by Src at the level of Tyr phosphorylation, DNA binding and transcriptional activation [Cao et al., 1996] and required for v-Src transformation [Turkson et al., 1998; Bromberg and Darnell, 2000; Odajima et al., 2000]. JAK1 has been proposed to recruit STAT3 to the PDGF receptor complex with Src for maximum STAT3 activation by Src; however, Src also activates STATs in insect cells in the absence of JAK family members [Zhang et al., 2000]. STAT3 gene ablation experiments in fibroblasts demonstrated its essential role in Src-induced cell transformation whereas STAT3 was dispensable in the support of full normal cell growth and survival [Schlessinger and Levy, 2005]. STAT3 appears to maintain focal adhesion kinase activity in the absence of cell adhesion by suppressing an inhibitory phosphatase [Schlessinger and Levy, 2005]. Constitutive STAT3 activation involving Src and JAK proteins resulting in enhanced STAT3 DNA binding activity has been observed in breast tumors when compared to normal breast tissue [Garcia et al., 2001]. In contrast, dominant-negative STAT3 led to growth arrest and apoptosis in breast cancer cells [Garcia et al., 2001]. This inhibitory potential for cell proliferation or transformation points to STAT3 as a promising therapeutic target [Silva, 2004].

In this study four PSM/SH2B1 alternative splice variants and individual functional PSM protein domains were compared for their role in the regulation of Src activity. Our results implicate an essential role of the PSM variants in the activation of the Src kinase and the resulting mitogenic response—extending to phenotypic cell transformation and involving the established Src substrate STAT3.

MATERIALS AND METHODS

All presented data are based on repeated experiments with the error between multiple measurements shown in bar graphs or with one representative experiment shown for halftone figures.

Cell-Permeant PSM/SH2B1 Domain-Specific Fusion Peptides

Cell-permeant peptides representing the PSM/SH2B1 amino-terminal Pro-rich sequence, pleckstrin homology (PH) region, or SH2 domain had been prepared by fusion with a sequence of the *Drosophila melanogaster* antennapedia homeoprotein as described earlier [Riedel et al., 2000; Deng et al., 2007]. The PH region or the SH2 domain had been produced as fusion peptides in *Escherichia coli* and the Pro-rich region was produced as a synthetic peptide (American Peptide Company) composed of the transduction sequence (RQIKIWFQNRRMKWKK) of the *D. melanogaster* antennapedia homeodomain followed by the Pro-rich amino terminal PSM sequence FPSPPALPPPPPPSWQ [Riedel et al., 2000]. An additional synthetic (Genscript, Inc.) cell-permeant peptide was composed of a fragment FCESHARAAALDFA of the amino terminal PSM dimerization domain (DD) [Nishi et al., 2005] fused with the transduction sequence RKKRRQRRR [Wadia and Dowdy, 2005] derived from the HIV TAT protein at the amino terminus via a linker sequence AA. A synthetic transduction peptide lacking PSM sequences or a dialyzed column eluate of

a control *E. coli* cell extract served as the respective peptide control. Peptides were prepared as reported earlier [Deng et al., 2007].

Antibodies, Peptide Hormones and Cell Culture

Rabbit polyclonal antibody against Src was obtained from Cell Signaling Technology, STAT3 and PSM/SH2B1 antibodies were from Santa Cruz Biotechnology, phospho-Tyr antibody from BD Biosciences, and horseradish peroxidase-coupled immunoglobulin G (IgG) antibody from Kirkegaard and Perry Laboratories. Human recombinant PDGF-BB, IGF-I, and insulin were obtained from Upstate Cell Signaling Solutions. Normal NIH3T3 fibroblasts (for experiments involving insulin stimulation NIH 3T3 fibroblasts stably expressing human IR were used) or HEK 293 human embryo kidney cells were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% (v/v) penicillin/streptomycin solution in a 5% CO_2 environment.

Transient cDNA Expression

Mouse PSM/SH2B1 alternative splice variants alpha, beta, gamma, or delta were expressed transiently from a plasmid carrying a constitutively active CMV transcriptional promoter [Yousaf et al., 2001]. v-Src [Bromberg et al., 1998], c-Src (pM src) [Johnson et al., 1985], or a dominant-negative STAT3 deletion mutant (kindly made available by Dr. Richard Jove, University of South Florida, Tampa) [Niu et al., 2002] were transiently expressed from their respective expression plasmids. Cells were transfected as reported [Deng et al., 2007] and subsequently analyzed as described below.

Immunoprecipitation and Immunoblotting, Cell Proliferation and Focus Formation, Apoptosis and Cell Detachment

Experiments were carried out as reported earlier [Deng et al., 2007].

Src Activation Kinetics and PSM Phosphorylation

HEK 293 human embryonic kidney cells were cultured to 60% confluence in 8 cm culture plates and transfected with c-Src expression plasmid. After 48 h cells were rinsed twice with ice-cold PBS and solubilized in 2 ml lysis buffer before Src protein was immunoprecipitated with specific antibody [Deng et al., 2007]. Immune complexes were rinsed twice with lysis buffer, twice with kinase buffer (25 mM Tris–HCl, pH 7.5, 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$), and were re-suspended in 500 μl kinase buffer. In parallel, HEK 293 cells cultured in 8 cm plates were transfected with individual PSM/SH2B1 variant or control expression plasmid. PSM protein was immunoprecipitated from detergent cell extracts with PSM goat polyclonal antibody and immune complexes were rinsed and re-suspended in 30 μl kinase buffer.

To assay Src catalytic activity in vitro, 120 μl of re-suspended Src immune complex and 40 μl of re-suspended PSM immune complex were mixed and 20 μl aliquots were analyzed. Src kinase was activated with varying ATP concentrations of 0, 0.32, 1.6, 8, 40, 100, and 200 μM in kinase buffer at 22°C for 1 h in a total volume of 30 μl. Src protein was immunoprecipitated with specific antibody in 200 μl cell lysis buffer containing protease and phosphatase inhibitors [Deng et al., 2007]. The immune complex was re-suspended in 30 μl 2× Laemmli loading buffer and separated by 10% SDS–PAGE. Src activity was evaluated by immunoblotting with phospho-Tyr antibody and visualized by enhanced chemiluminescence detection (ECL, Amersham). Src activity was quantified by scanning densitometry of ECL bands on film in the linear range with UN-SCAN-IT software (Silk Scientific). The net density increase was calculated for each band and the Michaelis–Menten activation kinetics of Src was presented with Prizm 4.0 software (GraphPad).

To test for PSM phosphorylation 20 μl re-suspended PSM/SH2B1 individual variant immune complex was mixed with an equal volume of re-suspended Src immune complex and the Src kinase reaction was initiated by adding 200 μM ATP for 1 h at 22°C. Subsequently, PSM variants proteins were immunoprecipitated with PSM-specific antibody in 200 μl cell lysis buffer containing protease and phosphatase inhibitors. The immune complex was re-suspended in 30 μl 2× Laemmli loading buffer and separated by 8% SDS–PAGE. PSM variant protein phosphorylation was evaluated by immunoblotting with phospho-Tyr antibody and visualized by enhanced chemiluminescence detection (ECL, Amersham).

RESULTS AND DISCUSSION

In this study we have compared the role of the four PSM/SH2B1 alternative splice variants

in mitogenesis in the context of Src regulation by specifically altering PSM function in fibroblasts with positive as well as negative experimental strategies. We have used a combination of approaches including cDNA expression as well as dominant-negative, cell-permeant peptide mimetics representing individual PSM domains such as the amino terminal Pro-rich, dimerization (DD), PH, and SH2 domain. This approach has been shown to be fully equivalent to PSM siRNA strategies confirming the activity and selectivity of the employed cell-permeant peptides [Zhang et al., 2007].

Loss of Cell Contact Inhibition

We found PSM variant regulation to extend beyond the control of mitogenesis and cell proliferation [Yousaf et al., 2001] to phenotypic cell transformation as represented by the observed cell focus formation as a result of loss of cell contact inhibition (Fig. 1). NIH 3T3 fibroblasts were individually transfected with expression plasmids representing any of the four PSM variants. Cells were propagated with culture medium changes twice a week and stained with crystal violet after 3 weeks. Loss of contact inhibition was observed as an indicator for phenotypic cell transformation for any of the variants in contrast to control transfected cells (Fig. 1A). PSM variant expression was demonstrated based on their relative migration in immunoblots of detergent cell extracts with PSM antibody and comparable variant protein levels were confirmed (Fig. 1B). The resulting formation of cell foci was observed in a signature pattern of variant activity with the highest level found for gamma and decreasing levels for delta, alpha, and lowest levels for beta (Fig. 1A). This signature pattern of relative variant activity was consistently observed for any of the measured mitogenic responses as shown below in this study. The same signature pattern had been earlier observed for the mitogenic impact with two alternative expression plasmids that produced comparable variant protein levels [Yousaf et al., 2001]. We conclude that the signature pattern we observe is a result of the individual variant proteins present at comparable levels and not of variations in the expression system.

Support of Cell Survival

To address whether the observed PSM-mediated loss of cell contact inhibition would be

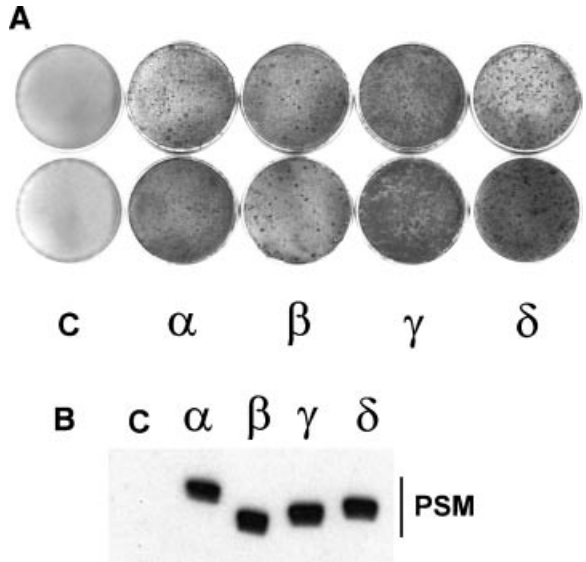

Fig. 1. PSM variant expression and the resulting loss of cell contact inhibition. Mouse NIH 3T3 fibroblasts were transfected with expression plasmids carrying cDNAs of PSM alpha, beta, gamma, or delta or with control plasmid (C). **A**: Cells (10^5) were seeded on 6-cm culture plates and propagated in DMEM containing 10% FBS with repeated changes for 3 weeks. Cells were fixed in formaldehyde, stained with 0.5% crystal violet and photographed. **B**: Detergent cell extracts were analyzed by PAGE followed by immunoblotting with PSM antibody. The position of the PSM variants proteins is indicated.

complemented by a supportive role in cell survival, NIH 3T3 fibroblasts were transfected with any of the four variant cDNAs and apoptosis was induced by serum withdrawal. DNA fragmentation (TUNEL) as a measure of apoptosis was substantially reduced by variant transfection in the signature pattern of highest activity for gamma and decreasing activity for delta, alpha, and beta (Fig. 2A). The impact on cell survival was addressed in more detail in response to specific peptide growth factors by evaluating the fraction of cells that detached from the culture plate and provided a close measure for cells undergoing apoptosis as reported earlier [Deng et al., 2007]. For this purpose NIH 3T3 fibroblasts were transfected with any of the four variant cDNAs and subsequently cultured in minimal serum in the presence of increasing doses of PDGF (Fig. 2B), IGF-I (Fig. 2C), or insulin (Fig. 2D). PSM variants strongly potentiated cell survival—increasingly with elevated hormone doses. Variant activities consistently followed the signature pattern. Highest levels of cell survival was observed in the presence of PDGF

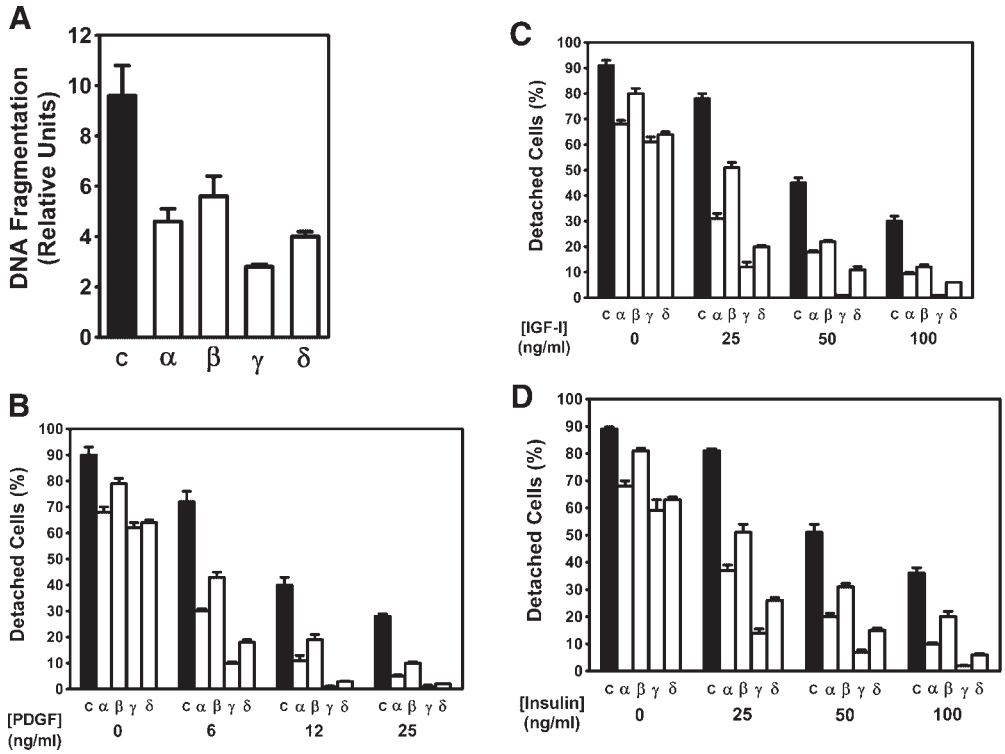

Fig. 2. PSM-potentiated cell survival. Mouse NIH 3T3 fibroblasts were transfected with expression plasmids carrying cDNAs of PSM alpha, beta, gamma, or delta or with control plasmid (C). After 4 days (**A**) DNA fragmentation was determined (TUNEL), and (**B**–**D**) attached and detached cells were quantified. Cells were cultured in DMEM with 0.5% FBS and, where indicated, with increasing concentrations of (**B**) PDGF (0, 6, 12, or 25 ng/ml), (**C**) IGF-I (0, 25, 50, or 100 ng/ml), or (**D**) insulin (0, 25, 50, or 100 ng/ml; using cells stably expressing human IR). DNA fragmentation shown in relative units and the fraction of detached cells are presented. All data points were measured in triplicate as represented by the error bar.

(Fig. 2B) followed by IGF-I (Fig. 2C) and insulin (Fig. 2D). For the highest dose of PDGF about 30% of cells were found detached whereas this fraction was substantially reduced by PSM variant expression in the signature pattern most strongly by PSM gamma to as little as 1%, followed by delta (3%), alpha (5%), and beta (10%). Overall, the PSM variants strongly potentiated cell survival in response to PDGF, IGF-I, or insulin. A potential role of Src in this mechanism would be consistent with its reported role in cell survival [Johnson et al., 2000; Westhoff et al., 2004].

Src-Mediated Cell Proliferation

The impact of elevated PSM variant levels was also confirmed on cell proliferation in the presence of PDGF, IGF-I, or insulin. For this purpose NIH 3T3 fibroblasts were transfected with any of the four variant cDNAs, cultured in minimal serum in the presence of any of the three hormones and final cell numbers were quantified (MTT). Potentiation of cell proliferation in the signature pattern of variant activity was observed for each hormone. The potentiation of cell proliferation was substantially reduced by Src inhibitor herbimycin frequently to less than half of untreated levels (Fig. 3). In combination, our findings show that the PSM variants potentiate various mitogenic responses including cell proliferation, survival, and loss of contact inhibition. The observed sensitivity to herbimycin suggests a role of cellular Src in PSM action.

Src Association and Regulation

To address the potential role of Src in PSM signaling further we tested whether association could be demonstrated between Src and PSM proteins and whether PSM would regulate Src activity. NIH 3T3 fibroblasts were transfected with Src and PSM gamma cDNA, stimulated with IGF-I, and treated with cell-permeant, dominant-negative peptide mimetics of the

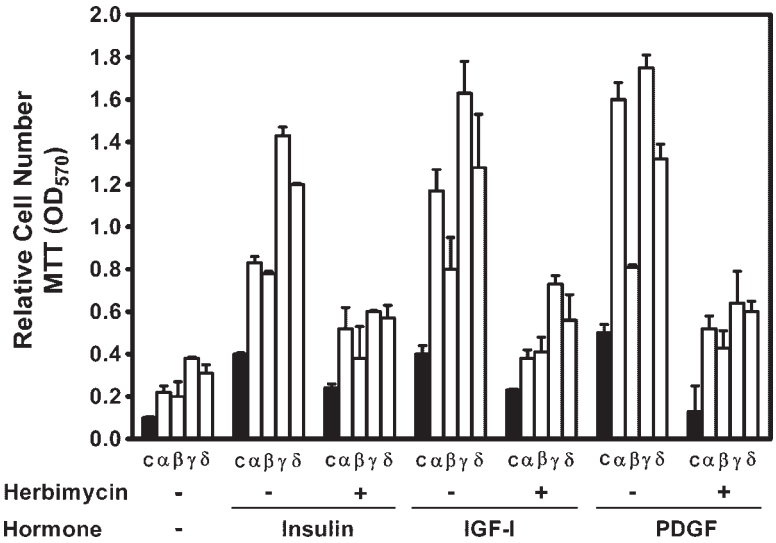

Fig. 3. PSM-potentiated cell proliferation involving Src. Mouse NIH 3T3 fibroblasts were transfected with expression plasmids carrying cDNAs of PSM alpha, beta, gamma, or delta or with control plasmid (C). Cells were incubated with 25 ng/ml PDGF, 100 ng/ml IGF-I, 100 ng/ml insulin (using cells stably expressing human IR), or were left untreated (Hormone: −). In parallel, 20 mM Src inhibitor herbimycin was added as indicated (−/+). After 3 days of culture in DMEM containing 1% FBS, cell numbers were quantified biochemically based on mitochondrial succinate dehydrogenase activity through the colorimetric change of MTT. The OD_{570} is presented as a reflection of relative cell number. All data points were measured in duplicate as represented by the error bar.

PSM amino terminal Pro-rich, dimerization (DD), PH, or SH2 domain. Immunoprecipitates with Src antibody from detergent cell extracts were analyzed by PAGE followed by immunoblotting with PSM antibody. Hormone-dependent association was observed between Src and PSM that was largely eliminated by treatment with DD or SH2 domain peptide (Fig. 4A). To test for potential regulation of Src activity by PSM the impact of PSM variant transfection or PSM dominant-negative peptide mimetics was measured. NIH 3T3 fibroblasts treated accordingly were stimulated with PDGF or IGF-I and Src was immunoprecipitated from detergent cell extracts and analyzed by PAGE followed by immunoblotting with phospho-Tyr antibody. Tyr phosphorylation of Src was stimulated by IGF-I and to a lessor degree by PDGF that was significantly potentiated for either hormone by PSM variant expression in the standard signature pattern. Src Tyr phosphorylation was largely eliminated by PSM DD or SH2 domain peptide mimetics and reduced by the Pro-rich peptide but remained unaffected by the PH domain peptide mimetic (Fig. 4B). These findings indicate a hormone-stimulated functional association between PSM and Src (direct or indirect) that results in the potentiation of Src catalytic activity in the signature pattern of variant activity. PSM appears to be an essential component of the active Src signaling complex that is displaced by dominant-negative PSM domains. Src catalytic regulation may represent the molecular basis of mitogenic signaling by the PSM variants.

Regulation of Src Kinase Kinetics and PSM Tyr Phosphorylation

PSM variant regulation of the Src kinase catalytic characteristics was addressed in more detail. Src autophosphorylation was measured in vitro with partially purified Src and PSM variants at 100 nM IGF-I and varying concentrations of ATP. Src protein was immunoprecipitated with specific antibody and its activation analyzed by immunoblotting with phospho-Tyr antibody and quantified by densitometry (Fig. 5A). Both V_{max} and K_m for ATP binding were substantially affected in the same variant-specific signature pattern as observed for all other responses (Table I). Compared to control samples at a K_m (ATP) of 119 μM, variant gamma lowered the K_m most profoundly to 12 μM followed by delta at 17 μM, alpha at 41 μM and beta with the lowest impact at 62 μM. Compared to control samples with a V_{max} set to

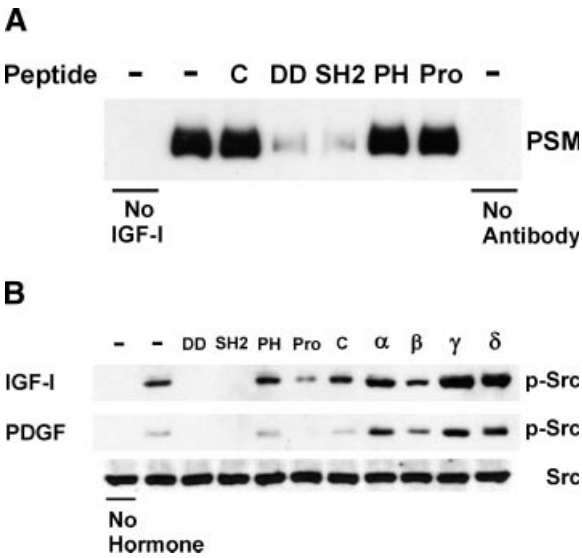

Fig. 4. PSM-Src association and regulation. Mouse NIH 3T3 fibroblasts were transfected with expression plasmids carrying cDNAs of (**A**) PSM gamma or (**B**) PSM alpha, beta, gamma, or delta or with control plasmid (C). Mouse NIH 3T3 fibroblasts were stimulated (**A**) with IGF-I or (**B**) IGF-I or PDGF (−/+) as indicated and treated with cell-permeant peptide mimetics representing the PSM amino terminal Pro-rich sequence (Pro), dimerization domain (DD), PH region, SH2 domain, or with peptide control (C), or left untreated (−). (**A**) Immunoprecipitate from detergent cell extracts with Src antibody was analyzed by immunoblotting with PSM antibody. (**B**) Detergent cell extracts were immunoprecipitated with Src antibody and were analyzed by immunoblotting with phospho-Tyr antibody or Src antibody. The position of (**A**) PSM protein, or (**B**) p-Src and total Src protein is indicated.

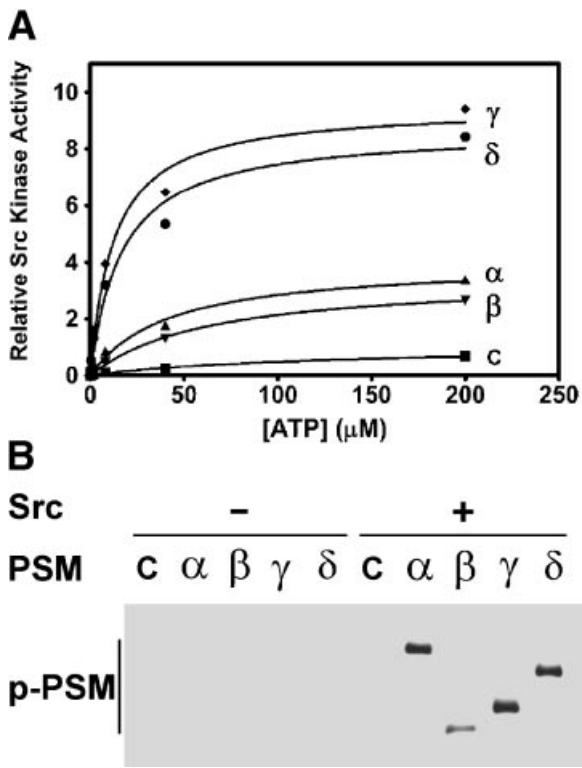

Fig. 5. PSM Tyr phosphorylation and regulation of Src catalytic kinetics. Human embryonic kidney HEK293 cells were transfected with expression plasmids carrying cDNAs of c-Src, PSM alpha, beta, gamma, or delta or with control plasmid (C). Expressed proteins were concentrated by immunoprecipitation with Src or PSM antibody, respectively. Enriched Src and individual PSM variant proteins were mixed and Tyr phosphorylation was assayed. **A**: Src kinase activity was measured with ATP concentrations varying from 0 to 200 μM as indicated. Proteins were separated by PAGE and Src catalytic activity was detected by immunoblotting with phospho-Tyr antibody and quantified by densitometry of the exposed film. The Michaelis–Menten kinetics of the reaction (Prizm 4, GraphPad Software, Inc.) has been presented (see Table I for more details). **B**: PSM variant protein phosphorylation was evaluated with 200 μM ATP in the presence or absence of c-Src cDNA transfection (+/−). Variant proteins were immunoprecipitated with PSM antibody, separated by PAGE and Tyr phosphorylation was visualized by immunoblotting with phospho-Tyr antibody. The position of the PSM variant proteins is indicated.

1, variant gamma increased the V_{max} most profoundly to 10, followed by delta at 8.7, alpha at 4.0 and beta with the lowest impact at 3.5. Both of these changes are expected to result in an increase in Src activity as routinely observed in our experiments shown above and should explain any of the PSM variant potentiation of the mitogenic response at various levels.

When we immunoprecipitated the PSM variants from the same reactions with specific antibody followed by immunoblotting with phospho-Tyr antibody we observed Src-specific PSM Tyr phosphorylation (Fig. 5B). This suggests an intrinsic regulatory partnership between the PSM variants and Src. PSM binding is necessary for Src catalytic activity and the variants potentiate Src catalytic characteristics. At the same time the PSM variants are phosphorylated on Tyr as Src kinase substrates in the PSM variant signature pattern of activity.

TABLE I. Src Kinase Kinetic Parameters in Response to PSM

Variant	K_m (±SD) ATP (μM)	V_{max} (±SD) (relative activity)
C	119.1 ± 26.1	1.0 ± 0.1
α	40.8 ± 12.3	4.0 ± 0.4
β	61.5 ± 10.4	3.5 ± 0.2
γ	12.3 ± 3.3	10.0 ± 0.6
δ	16.5 ± 5.4	8.7 ± 0.7

Regulation of Src-Mediated Loss of Cell Contact Inhibition

Since dominant-negative PSM peptide mimetics had indicated an essential role of PSM in the activation of Src catalytic activity we tested whether this could be confirmed at the cellular level in the form of contact inhibition in cell culture. Src-transfected NIH 3T3 fibroblasts were cultured in the presence of cell-permeant PSM peptides and loss of cell contact inhibition was evaluated by scoring the formation of cell foci as described for Figure 1A. Src-induced cell foci were reduced by any of the tested peptides, most strongly by the PSM SH2 domain and with decreasing activity for the Pro-rich region and least activity for the PH domain (Fig. 6). These findings support a critical role of PSM in Src catalytic activation that extends to the cellular responses to Src.

Regulation of Src Substrate STAT3 Activation

To investigate the Src-mediated signaling pathway involved in this process we tested the role of the established Src substrate STAT3 that has been implicated as an important mediator of malignant cell transformation at the transcriptional level [Cao et al., 1996]. NIH 3T3 fibroblasts were transfected with Src or with any of the PSM variants or treated with dominant-negative cell-permeant PSM peptide mimetics and stimulated with IGF-I. STAT3 was immunoprecipitated with specific antibody from detergent cell extracts, analyzed by PAGE followed by immunoblotting with phospho-Tyr antibody. STAT3 activity was strongly stimulated by v-Src as well as by PSM variant expression with the signature pattern of variant activity (Fig. 7A) but was not observed in control cells in the absence of IGF-I (not shown). The PSM variant stimulation was largely inhibited by Src inhibitor herbimycin confirming the role of Src in PSM action. Dominant-negative peptide mimetics of the PSM Pro-rich, DD, or SH2 domain inhibited STAT3 activity (Fig. 7A). These findings indicate that the observed PSM variant regulation of Src catalytic activity fully extends to Src substrate STAT3.

STAT3-Dependent Regulation of Cell Contact Inhibition

To address the role of STAT3 in the cellular response to PSM cell contact inhibition was scored in the presence of a dominant-negative STAT3 deletion mutant [Niu et al., 2002]. NIH 3T3 fibroblasts were transfected with any of the PSM variants and with the dominant-negative STAT3 deletion mutant and loss of cell contact inhibition was evaluated by scoring the formation of cell foci as described for Figure 1A. PSM variant transfection resulted in the formation of cell foci with the variant signature pattern as shown in Figure 1A whereas co-transfection with the dominant-negative STAT3 mutant abolished the response (Fig. 7B). These findings support the requirement of functional Src substrate STAT3 for the cellular response to PSM consistent with the observed PSM variant regulation of Src catalytic activity.

PSM-Regulation of the JAK2 and the Insulin Receptor Kinase

In addition to the essential role of PSM in the activation of the Src kinase a comparable role has been reported in the catalytic activation of tyrosine kinases JAK2 [Nishi et al., 2005] and the insulin receptor [Zhang et al., 2007]. In both

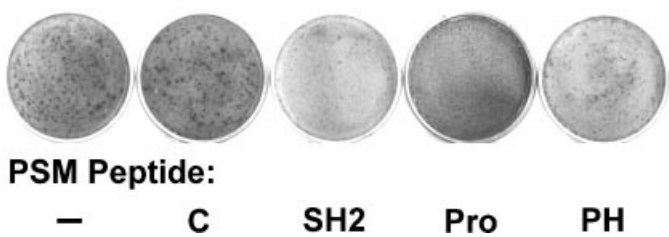

Fig. 6. PSM-regulation of Src-mediated loss of cell contact inhibition. Mouse NIH 3T3 fibroblasts were transfected with expression plasmid carrying v-Src cDNA. Cells (10^5) were seeded on 6-cm culture plates and were incubated with cell-permeant peptide mimetics representing the PSM amino terminal Pro-rich sequence (Pro), PH region, SH2 domain, or with peptide control (C), or remained untreated (–). Cultures were propagated in DMEM containing 10% FBS with repeated changes for 3 weeks. Cells were fixed in formaldehyde, stained with 0.5% crystal violet and photographed.

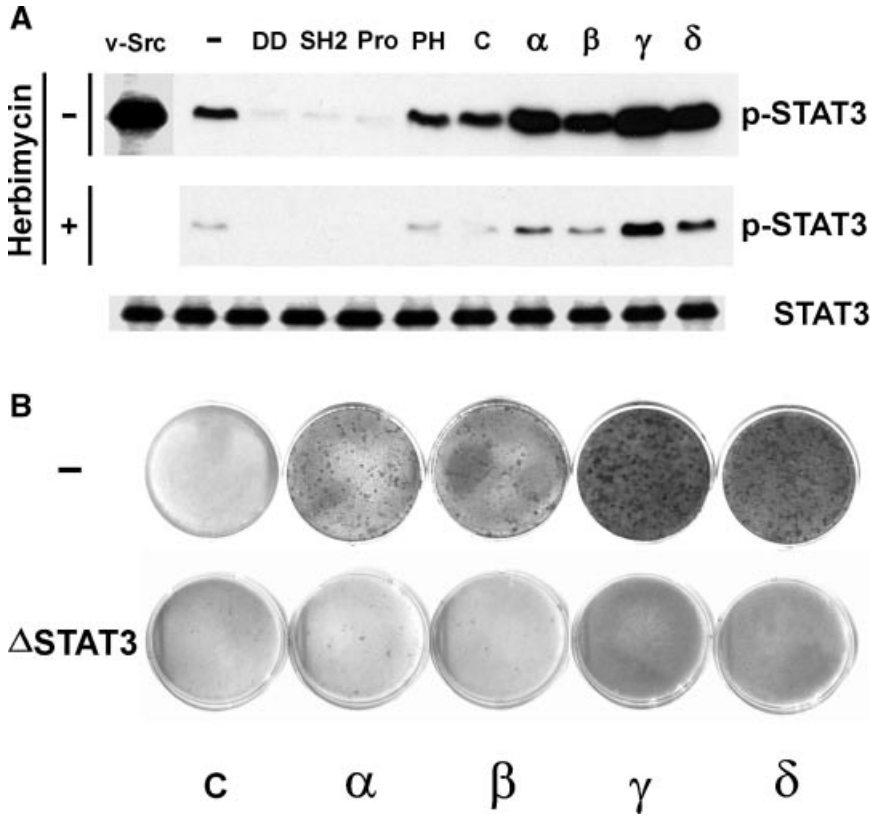

Fig. 7. PSM signaling via Src substrate STAT3. **A**: PSM-regulation of STAT3 phosphorylation. Mouse NIH 3T3 fibroblasts were transfected with expression plasmids carrying cDNAs of v-Src, PSM alpha, beta, gamma, or delta or with control plasmid (C) or remained untransfected (−). Alternatively, fibroblasts were incubated with cell-permeant peptide mimetics representing the PSM amino terminal Pro-rich sequence (Pro), dimerization domain (DD), PH region, SH2 domain, or were left untreated (−). In parallel, 20 mM Src inhibitor herbimycin was added as indicated (−/+), and cells were stimulated with IGF-I. Subsequently, immunoprecipitate from detergent cell extracts with STAT3 antibody was analyzed by immunoblotting with phospho-Tyr antibody or with STAT3 antibody and the position of p-STAT3 and total STAT3 is indicated. **B**: STAT3-dependent PSM-regulation of cell contact inhibition. Mouse NIH 3T3 fibroblasts (10^5) were seeded on 6-cm culture plates and were transfected with expression plasmids carrying cDNAs of a dominant-negative STAT3 deletion mutant (ΔSTAT3) and PSM alpha, beta, gamma, or delta or control plasmid (C). Cultures were propagated in DMEM containing 10% FBS with repeated changes for 3 weeks. Cells were fixed in formaldehyde, stained with 0.5% crystal violet and photographed.

cases a critical role has been implicated for the PSM SH2 domain as well as the dimerization domain DD at the amino terminus that mediates PSM dimerization through a Phe zipper motif [Nishi et al., 2005]. As a model the resulting PSM dimer has been proposed to interact via the SH2 domain with two units of the target Tyr kinase JAK2 [Nishi et al., 2005]. As a result either PSM SH2 domain mimetics or DD domain mimetics would be predicted to disrupt the activated PSM-kinase complex and interfere with catalytic activity. This is what we observed for the insulin receptor kinase [Zhang et al., 2007] and in this study for the Src kinase. Consequently, PSM appears to form a complex with and activate various Tyr kinases in a comparable fashion including JAK2, Src, and the insulin receptor.

Role of Individual PSM Domains

A role of an additional PSM domain, the amino terminal Pro-rich region has been implicated in Src and STAT3 activation. This is suggested by the impact of the Pro-rich PSM peptide mimetic on Src (Fig. 4B) and on STAT3 Tyr-phosphorylation (Fig. 7A). In contrast, typically no impact of the PSM PH domain has been observed except for the physiologic endpoint of Src-mediated cell focus formation (Fig. 6) where the respective peptide mimetic led to a reduced response. Consequently, the PSM SH2 and DD domains appear to be involved in all measured Src responses beginning at the level of PSM-Src association (Fig. 4A), whereas the Pro-rich region only acts at the level of Src activation (Fig. 4B) with an

impact on STAT3 (Fig. 7A). Instead, the role of the PSM PH domain appears to be limited to alternative signals (Fig. 6) possibly involving membrane association and receptors [Deng et al., 2007] that play a role in the Src-mediated cellular response. The more detailed dissection of PSM domain function will be left to a future study.

PSM-Regulation of Src/STAT3 Function

Eight lines of evidence support Src/STAT3 function as the molecular mechanism underlying PSM variant activity. (1) PSM variant potentiation of PDGF-, IGF-I-, or insulin-stimulated cell proliferation was highly sensitive to Src inhibitor herbimycin (Fig. 3). (2) Src and PSM form a complex in response to IFG-I stimulation that is dependent on PSM SH2 and DD domain function (Fig. 4A). (3) The four PSM variants potentiate Src catalytic activity in response to PDGF or IGF-I in the signature pattern of variant activity and the underlying mechanism is dependent on PSM SH2 and DD domain function (Fig. 4B). (4) The PSM variants are Src kinase substrates phosphorylated on Tyr in the signature pattern of PSM variant activity (Fig. 5B). (5) The PSM signature pattern potentiating Src catalytic activity is represented by an increase in the V_{max} and decrease in the K_m for ATP binding (Fig. 5A; Table I). (6) Src-induced cell focus formation is increasingly suppressed by peptide mimetics of the PSM PH, Pro-rich, or SH2 domains (Fig. 6). (7) The four PSM variants potentiate Tyr phosphorylation of major Src substrate STAT3 in the signature pattern of variant activity and the underlying mechanism is dependent on PSM SH2, DD, and Pro-rich domain function and sensitive to Src Inhibitor herbimycin (Fig. 7A). (8) PSM variant-induced cell focus formation is inhibited by a dominant-negative STAT3 deletion mutant indicating its dependence on functional cellular STAT3 (Fig. 7B).

Src Signaling Pathways

PSM regulates mitogenesis by potentiating the p44/42 MAP kinase pathway in response to PDGF, IGF-I, or insulin and by potentiating the PI 3-kinase pathway selectively in response to PDGF [Deng et al., 2007]. The PSM-mediated potentiation of STAT3 signaling presented in this study can easily be consolidated with this finding through the observed PSM variant-mediated potentiation of the central signaling mediator Src that regulates any of the above-mentioned signaling pathways. Src action is known to regulate various signaling pathways involving receptor Tyr kinases [Bromann et al., 2004]. In particular, v-Src can overcome the requirements for serum growth factors and anchorage that restrain normal cell growth which includes the induction of quiescent cells to enter S phase in the absence of serum mitogens [Riley et al., 2001]. For full oncogenic activation of Src signaling several pathways are required simultaneously that result in factor-independent cell proliferation [Odajima et al., 2000]. Specifically, v-Src causes constitutive activation of STAT3, PI 3-kinase, and the Ras/MAP kinase signaling cascade [Johnson et al., 2000; Odajima et al., 2000]. The Ras-mediated pathway is required for survival and together with STAT3 controls the cell cycle machinery in distinct but partly overlapping transcriptional regulatory mechanisms [Odajima et al., 2000]. Under low serum conditions v-Src appears to protect from apoptosis by activating PI 3-kinase [Johnson et al., 2000]. All these reported functions of Src are routinely altered by modulating PSM variant function in our study consistent with a critical role of PSM in Src catalytic activation.

It is not surprising to find PSM action to be dependent on STAT3 function since STAT3 is one of the best-established cellular substrates of Src. The observation that STAT3 activation is specifically required for cell transformation but dispensable for normal cell growth [Schlessinger and Levy, 2005], links the PSM variants as upstream signaling mediators to a mechanism that should offer a promising window for therapeutic intervention and to four new potential targets.

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