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PSM/SH2-B Distributes Selected Mitogenic Receptor Signals to Distinct Components in the PI3-Kinase and MAP Kinase Signaling Pathways

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Abstract The Pro-rich, PH, and SH2 domain containing mitogenic signaling adapter PSM/SH2-B has been implicated as a cellular partner of various mitogenic receptor tyrosine kinases and related signaling mechanisms. Here, we report in a direct comparison of three peptide hormones, that PSM participates in the assembly of distinct mitogenic signaling complexes in response to insulin or IGF-I when compared to PDGF in cultured normal fibroblasts. The complex formed in response to insulin or IGF-I involves the respective peptide hormone receptor and presumably the established components leading to MAP kinase activation. However, our data suggest an alternative link from the PDGF receptor via PSM directly to MEK1/2 and consequently also to p44/42 activation, possibly through a scaffold protein. At least two PSM domains participate, the SH2 domain anticipated to link PSM to the respective receptor and the Pro-rich region in an association with an unidentified downstream component resulting in direct MEK1/2 and p44/42 regulation. The PDGF receptor signaling complex formed in response to PDGF involves PI 3-kinase in addition to the same components and interactions as described for insulin or IGF-I. PSM associates with PI 3-kinase via p85 and in addition the PSM PH domain participates in the regulation of PI 3-kinase activity, presumably through membrane interaction. In contrast, the PSM Pro-rich region appears to participate only in the MAP kinase signal. Both pathways contribute to the mitogenic response as shown by cell proliferation, survival, and focus formation. PSM regulates p38 MAP kinase activity in a pathway unrelated to the mitogenic response. J. Cell. Biochem. 100: 557–573, 2007.

Key words: cell proliferation; cell-permeant peptide; Pro-rich; PH; SH2; signaling complex

An SH2 domain-containing sequence termed SH2-B had been identified in the rat [Osborne et al., 1995] and the corresponding mouse protein subsequently based on its association with the activated catalytic insulin receptor (IR) domain [Hansen et al., 1996; Riedel et al., 1997]. Pro-rich putative SH3 domain binding regions, a pleckstrin homology domain (PH) and a src homology 2 (SH2) domain implicated a role as a signaling mediator, termed PSM. Three additional alternative splice variants of PSM/SH2-B have been reported in the mouse termed beta [Riedel et al., 1997; Rui et al., 1997], gamma [Nelms et al., 1999], and delta [Yousaf et al., 2001], in addition to the originally identified form termed alpha, and as well in the human genome [Nishi et al., 2005]. A single *SH2-B* 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; Rui and Carter-Su, 1999; Carter-Su et al., 2000a, 2000b; Kurzer et al., 2004; Miquet et al., 2005]. Activation

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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/SH2-B, suggesting a putative role in the respective signaling pathways [Riedel et al., 2000]. The association of PSM/SH2-B was mapped to conserved phosphotyrosine sites between the insulin and IGF-I receptors [Wang and Riedel, 1998]. Tyrosine phosphorylation of PSM/SH2-B was observed in response to insulin [Kotani et al., 1998], IGF-I, and PDGF [Yousaf et al., 2001]. PDGF stimulated PDGF receptor association with PSM/SH2-B including its phosphorylation on tyrosine, serine, and threonine [Rui and Carter-Su, 1998; Riedel et al., 2000; Yousaf et al., 2001]. cDNA expression of the four PSM/SH2-B 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/SH2-B in phenotypic cell transformation [Riedel et al., 2000]. SH2-B 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]. SH2-B 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] by regulating the Akt/Forkhead signaling pathway [Wang et al., 2004]. In response to FGF receptor 3 (FGFR3) activation, PSM/SH2-B associates and undergoes tyrosine phosphorylation with a putative role in the activation and nuclear translocation of Stat5 [Kong et al., 2002]. In response to leptin SH2-B promotes IRS1- and IRS2-mediated activation of the PI 3-kinase pathway [Duan et al., 2004a]. Disruption of the *SH2-B* 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 in the maintenance of normal insulin sensitivity and glucose homeostasis during ageing [Duan et al., 2004b].

PSM/SH2-B 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], and display distinct functions [Kubo-Akashi et al., 2004]. SH2-B beta and APS differentially regulate JAK family tyrosine kinases [O'Brien et al., 2002]. In contrast to the SH2-B 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 *SH2-B* gene and pointing to a specific role of SH2-B as regulator of energy and glucose metabolism in mice [Li et al., 2006]. In response to insulin both APS [Moodie et al., 1999] and SH2-B undergo phosphorylation on Tyr, interact with the activation loop of IR, enhance IR autophosphorylation, and enhance (independently of SH2-B phosphorylation) ERK and Akt activation [Ahmed and Pillay, 2001, 2003]. SH2-B and APS play a role in neuronal differentiation [Qian et al., 1998; Rui et al., 1999; Koch et al., 2000]. SH2-B and APS have been reported as homo- or heteropentamers, mediated through the amino terminus in an analogous mechanism and directly modulate Trk autophosphorylation [Qian and Ginty, 2001]. The activating mechanism involves homo- or heterodimerization via a phenylalanine zipper at the amino terminus which in turn results in the dimerization and activation of associated signaling partners, such as JAK2 [Dhe-Paganon et al., 2004; Nishi et al., 2005].

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 immunoblots.

Cell-Permeant PSM Domain Fusion Peptides

Cell permeant fusion peptides representing the PSM/SH2-B amino-terminal Pro-rich region

or SH2 domain had been prepared as described earlier fused with a sequence of the *Drosophila melanogaster* (*D. melanogaster)* antennapedia homeoprotein [Wang et al., 1999]. The SH2 domain had been expressed as a fusion peptide in *E. coli* and the Pro-rich region was represented by a synthetic peptide mimetic (synthesized by American Peptide Company). A synthetic peptide lacking PSM sequences or a dialyzed column eluate of a control *E. coli* cell extract served as peptide controls. A cell-permeant peptide mimetic representing the PSM PH domain was designed with codons optimized for expression in *E. coli* using oligonucleotides that contained a 5′-trityl group for column purification (Fisher Scientific). First, an expression construct for a cell permeant Pro-rich fusion peptide had been prepared as follows in which the Pro-rich sequence was subsequently replaced by the PSM PH domain in a second step as described below. Oligonucleotides included the coding region for a 16-aa cell membrane transfer sequence (RQIKIWFQNRRMKWKK) of the *D. melanogaster* antennapedia homeodomain (5′-GGC GGC AGC CAT ATG CGT CAG ATC AAA ATG TGG TTC CAG AAC CGT CGT ATG AAA TGG AAA AAA GGA TCC-3′ [membrane transfer sequence surrounded by 5′ *Nde*I and 3′ *Bam*HI sites] and 5′-CCC CAA GCT TCA CTT AAT TAA GAG CTC TTA CTG CCA AGA CGG CGG CGG CGG CGG CAG CGC CGG CGG AGA CGG GAA GGA TCC TTT TTT-3′ [Pro-rich sequence [Riedel et al., 2000] with 5′ *Hin*dIII and 3′ *Bam*HI sites]) were hybridized via a 12-nucleotide overlap and extended by mutually primed DNA synthesis. *Nde*I and *Hin*dIII restriction sites had been introduced at the 5′ and 3′ ends, respectively. A *Bam*HI restriction site had been introduced at the 3′ end of the membrane transfer sequence. The complete sequence was inserted into the *Nde*I and *Hin*dIII sites of plasmid pET 28a(+) (Novagen, WI). This expression plasmid utilizes the strong T7 transcriptional promoter, confers kanamycin resistance, and expresses an amino-terminal 6 aa His-tag peptide to allow affinity purification of the recombinant protein. The PSM PH domain coding region was amplified between aa 272 and 371, using PCR primers (5′-TAT ATA GGA TCC GCA GCT GGG CTG ACC TCA GGA GGA G-3′ and 5′-CCC ACC AAG CTT TTA TTC CTG GAT GTC AGA CAC CC-3′) which introduced a 5′ *Bam*HI site and a 3′ *Hin*dIII site into the final PCR product. The PCR product was digested with *Bam*HI and *Hin*dIII, isolated by electrophoresis followed by electroelution, and inserted into the *Bam*HI and *Hin*dIII sites of plasmid pET 28a(+) (Novagen) including the antennapedia homeodomain and Pro-rich sequence as described above to replace the Pro-rich sequence.

Recombinant plasmids were confirmed by restriction and DNA sequence analysis and introduced into *E. coli* BL21 (DE3), which carries the *lacUV5* repressor to control induction of the T7 transcriptional promoter by isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were grown in Luria-Bertani (LB) medium containing kanamycin (30 μg/ml) at 37°C to OD_{600} 0.2–0.3 followed by 1 mM IPTG induction for 5 h. Cells were sedimented, resuspended in 0.5 M Tris-HCl (pH 6.8), and lyzed by French Press (SLM Instruments, Inc.) treatment. Following centrifugation at 15,000*g* for 20 min at 4°C the cleared lysate was analyzed by SDS–PAGE (15%). Cell permeant PSM fusion peptides were purified by nickel column affinity chromatography (Novagen) and eluted with 0.4 M imidazole in the presence of 6 M urea. The peptide was dialyzed with a 3.5 kDa molecular weight cut-off (Slide-A-Lyzer, Pierce). Precipitated peptide was resolubilized in 50 mM Tris-HCl (pH 6.8) by addition of DMSO to a final concentration of 0.1%. Protein concentration was determined by Bradford analysis and the peptide was stored for up to several months at 4°C and subsequently at −80°C.

Antibodies, Peptide Hormones, and Cell Culture

Rabbit polyclonal antibodies against p44/42 MAP kinas, and phospho-specific Raf-1 or MEK1/2 were obtained from Cell Signaling Technology, p85 PI 3-kinase-specific antibody was from Upstate Cell Signaling Solutions, and horseradish peroxidase-coupled immunoglobulin G (IgG) antibody from Kirkegaard and Perry Laboratories. PSM/SH2-B antiserum had been custom-produced by Hazelton Research Products, Inc. (Denver, PA) in rabbits against a glutathione *S*-transferase (GST) fusion protein containing the SH2 domain of mouse PSM [Riedel et al., 1997]. Human recombinant PDGF-BB, IGF-I, and insulin were obtained from Upstate Cell Signaling Solutions. Normal or (specifically for insulin stimulation) IR stable expressing NIH 3T3 fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM)

supplemented with 10% FBS, 1% (v/v) penicillin/streptomycin solution in a 5% CO_2 environment.

Transient cDNA Expression

Mouse PSM/SH2-B alpha was expressed transiently from a plasmid [Yousaf et al., 2001] carrying a constitutively active CMV transcriptional promoter. Sub-confluent, normal, or (for insulin stimulation) IR stable expressing mouse NIH 3T3 fibroblasts cultured on 8-cm plates were rinsed with antibiotic-free DMEM before 3 ml transfection mix including 5–6 μg of PSM expression plasmid or control plasmid lacking PSM sequences, 30 μl Lipofectamine, and 20 μl Plus reagent were added according to the instructions of the manufacturer (Invitrogen). After 5 h the transfection medium was replaced with DMEM including 10% FBS or in the case of subsequent peptide hormone induction cells were cultured to quiescence for about 20 h. Cells were subsequently analyzed as described below. We estimate that about three out of four cells are transfected consistent with our results including those described in Figure 2.

Cell Proliferation

Normal or (for insulin stimulation) IR stable expressing mouse NIH 3T3 fibroblasts (5×10^5) were seeded and cultured on 24-well plates for 24 h in normal DMEM with 10% FBS. Cells were rinsed once and incubated with 100 ng/ml insulin, 100 ng/ml IGF-I 100 ng/ml EGF, or 25 ng/ml PDGF-BB in DMEM containing 1% FBS, and simultaneously with cell-permeant PSM SH2, Pro-rich, PH domain or control peptide mimetics at 10 μg/ml, or with inhibitors 10 μM LY294002 against PI 3-kinase, 20 μM PD098059 against MEK1/2, or 20 μM SB203580 against p38 MAP kinase. After 3 days, 200 μl of 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) was added for 4 h. The resulting product formazan was dissolved in isopropyl alcohol and quantified colorimetrically at OD_{570} [Denizot and Lang, 1986]. The obtained value has been presented as a measure of relative cell number.

Apoptosis and Cell Detachment

Normal or (for insulin stimulation) IR stable expressing NIH 3T3 fibroblasts were transiently transfected with PSM/SH2-B or control plasmid and cultured on 6-well plates. Cells were propagated in DMEM with 0.5% FBS for 4 days in various concentrations of insulin, IGF-I, or PDGF. Detached cells and adherent cells after trypsinization were collected, and both were quantified microscopically. The number of detached cells was displayed as the percent of the total cell number [Webb et al., 2000].

For TUNEL analysis the 3′ ends of DNA fragments generated by apopotosis-associated endonucleases were labeled with a DeadEnd apoptosis detection kit (Promega). Cells were permeabilized with 0.2% Triton X-100 for 5 min at 21°C and incubated with terminal transferase and biotinylated nucleotides for 60 min at 37°C. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in PBS, pH 7.2. Horseradish-peroxidase-labeled streptavidin solution was added for 30 min at 21°C. DNA labeling was measured with hydrogen peroxide as peroxidase substrate and diaminobenzidine as a stable chromogen. Cells were mounted and labeling was evaluated by light microscopy. Cells were defined as apoptotic if the whole nuclear area labeled positively. The number of apoptotic cells was presented as percent of the total cell number.

Cell Focus Formation

NIH 3T3 fibroblasts (10^5) cultured on 6-cm plates were transfected with PSM/SH2-B expression plasmid or control plasmid. Cultures were propagated in DMEM with 10% FBS which was replaced twice a week. Cells were fixed in formaldehyde and stained with 0.5% crystal violet in methanol after 3 weeks in culture.

Immunoprecipitation and Immunoblotting

For hormone induction typically 10^6 normal or (for insulin stimulation) IR stable expressing NIH 3T3 fibroblasts, untransfected or transiently transfected with PSM/SH2-B expression plasmid, were propagated to quiescence for 20 h in serum-free DMEM supplemented with 0.1% BSA. Cell-permeant peptides at 10 μg/ml were added to the medium for 1 h before 100 ng/ml insulin, 100 ng/ml IGF-I, or 25 ng/ml PDGF were supplemented for 15 min. Cultures were rinsed twice with ice-cold PBS and detergent cell extracts were prepared with lysis buffer containing 1% Triton X-100, 50 mM HEPES, pH 7.4, 10% glycerol, 137 mM NaCl, 10 mM NaF, 100 mM Na_3VO_4, 10 mM $Na_4P_2O_7$, 2 mM EDTA, 10 μg/ml leupeptin, and 1 mM PMSF. Extract containing 200–500 μg of total protein was incubated at 4°C with specific antibody and

subsequently with an additional 25 μl of protein A-Sepharose slurry (50% in the same buffer). Immune complexes were collected by centrifugation at 4°C and rinsed three times. Proteins were resuspended in Laemmli loading buffer, boiled, separated by SDS–PAGE, and transferred to a nitrocellulose membrane. Proteins were identified by immunoblotting with specific antibodies, and visualized using the Amersham ECL (enhanced chemiluminescence) detection system.

PI 3-Kinase Activity

A protocol was followed as summarized below similar to the procedure described by [Vlahos et al., 1994]. Quiescent NIH 3T3 fibroblasts (10^6) were lyzed after treatment with hormone and/or cell-permeant peptides. Five-hundred micrograms of total protein was incubated with 3 μl p85 antibody at 4°C (Upstate Cell Signaling Solutions) for 2 h and further for 1 h after addition of 25 μl of protein A-Sepharose slurry (50%, in the same buffer). Immune complexes were collected by centrifugation at 4°C and rinsed once with ice-cold lysis buffer and twice with 20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM Na_3VO_4, 10 μg/ml aprotinin, 1 mM PMSF. The precipitate was resuspended in 50 μl kinase buffer (20 mM HEPES, pH 7.4, 1 mM EGTA, 10 mM $MgCl_2$, 1 mM Na_3VO_4) including 0.2 mg/ml sonicated phosphatidylinositol (Sigma). Two to four microliters of [gamma-^{32}P] ATP (5 mCi/ml, 3,000 Ci/mmol, Perkin Elmer Life Sciences) was added to a final concentration of 50 μM. The kinase reaction was carried out for 20 min at 25°C and terminated by adding 20 μl of 6 M HCl. Phosphatidylinositol was extracted with 200 μl of $CHCl_3$/MeOH (1:1). The organic phase was washed with 80 μl of MeOH/HCl (1:1) and spotted on a Silica Gel 60 thin layer chromatography plate (Merck) pretreated with 1% potassium oxalate. Phospholipids were separated in $CHCl_3$/MeOH/H_2O_2/NH_3 (75/100/25/15) and associated radioactivity was visualized by autoradiography.

Ras Activity Assay

A Ras Activation Assay Kit (Upstate Cell Signaling Solutions) was used essentially according to the instructions of the manufacturer. Quiescent NIH 3T3 fibroblasts (5×10^6) were lyzed after hormone induction in 1 ml magnesium lysis buffer (MLB) (25 mM HEPES, pH 7.5, 150 mM NaCl, 25 mM NaF, 10 mM $MgCl_2$, 10% glycerol, 1 mM EDTA, 1% Igepal CA-630, 1 mM Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin). The lysate was precleared by centrifugation after incubation with glutathione agarose. Supernatant containing about 500 μg of total protein was incubated for 1 h under agitation at 4°C with 5 μg GST-RBD agarose to specifically bind activated Ras. Complexes were precipitated by centrifugation and rinsed three times with 1 ml MLB. Samples were resuspended, in 25 μl Laemmli loading buffer, boiled, and proteins were separated by SDS–PAGE (12.5%) and immunoblotted with Ras-specific antibody.

Raf-1 and MEK1/2 Phosphorylation

Quiescent cells (10^6) were lyzed after hormone treatment and 25 μg of total protein was separated by SDS–PAGE (8%) and immuoblotted with phospho-specific Raf-1 or MEK1/2 antibody (Cell Signaling Technology).

p44/42 MAP Kinase Assay

A p44/42 MAP kinase assay kit (Cell Signaling Technology) was used according to the instructions of the manufacturer. Quiescent NIH 3T3 fibroblasts (10^6) were lyzed after hormone and/or cell-permeant peptide treatment and 200 μl of cell lysate (about 200 μg total protein) were incubated with immobilized phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody at 4°C under agitation for 16 h. Immunocomplexes were precipitated by centrifugation, rinsed, and suspended in kinase buffer to assay MAP Kinase activity with 200 μM ATP and 2 μg Elk-1 as a p44/42 substrate. Reactions were terminated by adding 25 μl of Laemmli loading buffer, boiled, and proteins were separated by 12.5% SDS–PAGE. Phosphorylated substrate was detected on immunoblots with anti-phospho-Elk-71 (Ser 383) antibody.

p38 MAP Kinase Activity

A p38 MAP kinase assay kit (Cell Signaling Technology) was used largely according to the instructions of the manufacturer. Quiescent NIH 3T3 fibroblasts (10^6) were solubilized in 500 μl lysis buffer after hormone treatment. About 200 μg of total cellular protein was incubated with immobilized phospho-p38 MAP kinase (Thr180/Tyr182) antibody for 16 h at 4°C. Immunocomplexes were collected by centrifugation repeatedly rinsed, resuspended in kinase buffer, and incubated for 30 min at

30°C with 200 μM ATP and 2 μg ATF-2 as a substrate. Reactions were terminated by adding 25 μl Laemmli loading buffer, boiled, and proteins were separated by SDS–PAGE (12.5%). Phosphorylated substrate was detected in immunoblots with phospho-ATF-2 antibody.

RESULTS

Individual PSM Domains and Cell Proliferation

To investigate the role of PSM and some of its functional domains individually in distinct peptide hormone signaling pathways we compared proliferation of NIH 3T3 fibroblasts in response to insulin, IGF-I, PDGF, or EGF by marker enzyme activity (MTT assay) and evaluated the impact of dominant-negative peptide mimetics representing the PSM amino-terminal Pro-rich region, PH, and SH2 domains. Peptides had been rendered cell-permeant by addition of a *Drosophila* antennapedia homeoprotein sequence motif [Riedel et al., 2000] and were produced in *E. coli* in mg amounts or (if representing the Pro-rich region) were chemically synthesized. For the tested peptide hormones typically a 2.5 to 3-fold increase in relative cell number was observed (Fig. 1). None of the domain-specific peptides was found to affect EGF-stimulated cell proliferation, consistent with lack of evidence for any role of PSM in the EGF signaling pathway [Riedel et al., 2000] and in support of the specificity of our observations. In contrast, the cell proliferation response to insulin or IGF-I was significantly inhibited by the Pro-rich and SH2 domain peptides in a dose-dependent fashion whereas no effect was observed for the PH domain peptide (Fig. 1). The cell proliferation response to PDGF was inhibited by any of the three peptides in a dose-dependent fashion, most significantly by the SH2 domain and partially by the Pro-rich and PH regions. These observations implicate specific roles of the individual PSM domains in distinct peptide hormone signaling pathways that were further investigated at the level of selected downstream mediators as shown below.

Cell Survival

To address whether PSM would function as survival factor, programmed cell death was induced by serum withdrawal and survival of NIH 3T3 fibroblasts was assayed at increasing doses of peptide hormones in response to PSM cDNA transfection. Cell survival mediated by increasing doses of insulin, IGF-I, or PDGF was prominently enhanced by elevated levels of PSM (Fig. 2). Cell survival was evaluated by quantifying attached cells versus cells that were detached from the culture plate as well as by determining the fraction of cells that displayed fragmented DNA (TUNEL). Cell detachment was overall comparable to apoptosis as quantified based on DNA fragmentation (TUNEL) and both assays supported a significant role of PSM as a survival factor. At the highest hormone concentration PSM cDNA transfection was found to reduce the number of detached cells up to fivefold for PDGF with somewhat lower levels observed for IGF-I or insulin (Fig. 2). In combination, our findings support a mitogenic role of PSM that correlates with a role as a survival factor in response to insulin, IGF-I, and PDGF.

Cell Focus Formation

The observed proliferative, stimulative role of PSM was potentially indicative of aberrant cell proliferation in response to PSM cDNA transfection. This was directly tested by transfection of NIH 3T3 fibroblasts with PSM expression plasmid followed by continuous cell culture with regular changes of medium over a 3-week period. When compared to control-transfected cell cultures, PSM cDNA transfection induced the formation of hundreds of cell foci that were absent in controls (Fig. 3A). This indicates loss of contact inhibition in PSM-transfected cells, a prerequisite for the aggregation of cell foci. In addition, we had shown that microinjection of a PSM SH2 domain peptide mimetic into transformed fibroblasts partially restored a normal actin stress fiber pattern [Riedel et al., 2000]. A mitogenic role of PSM that may extend to cell transformation is supported by these findings consistent with our observations shown in Figures 1 and 2.

Pathway-Specific Inhibitors

The observed mitogenic role of PSM suggested its participation in key candidate signaling pathways that was initially addressed with pathway-specific chemical inhibitors. Inhibitors of MEK 1/2 (PD098059), p38 MAP kinase (SB203580), and PI 3-kinase (LY294002) were tested for their impact on cell proliferation in response to insulin, IGF-I, or PDGF in NIH 3T3

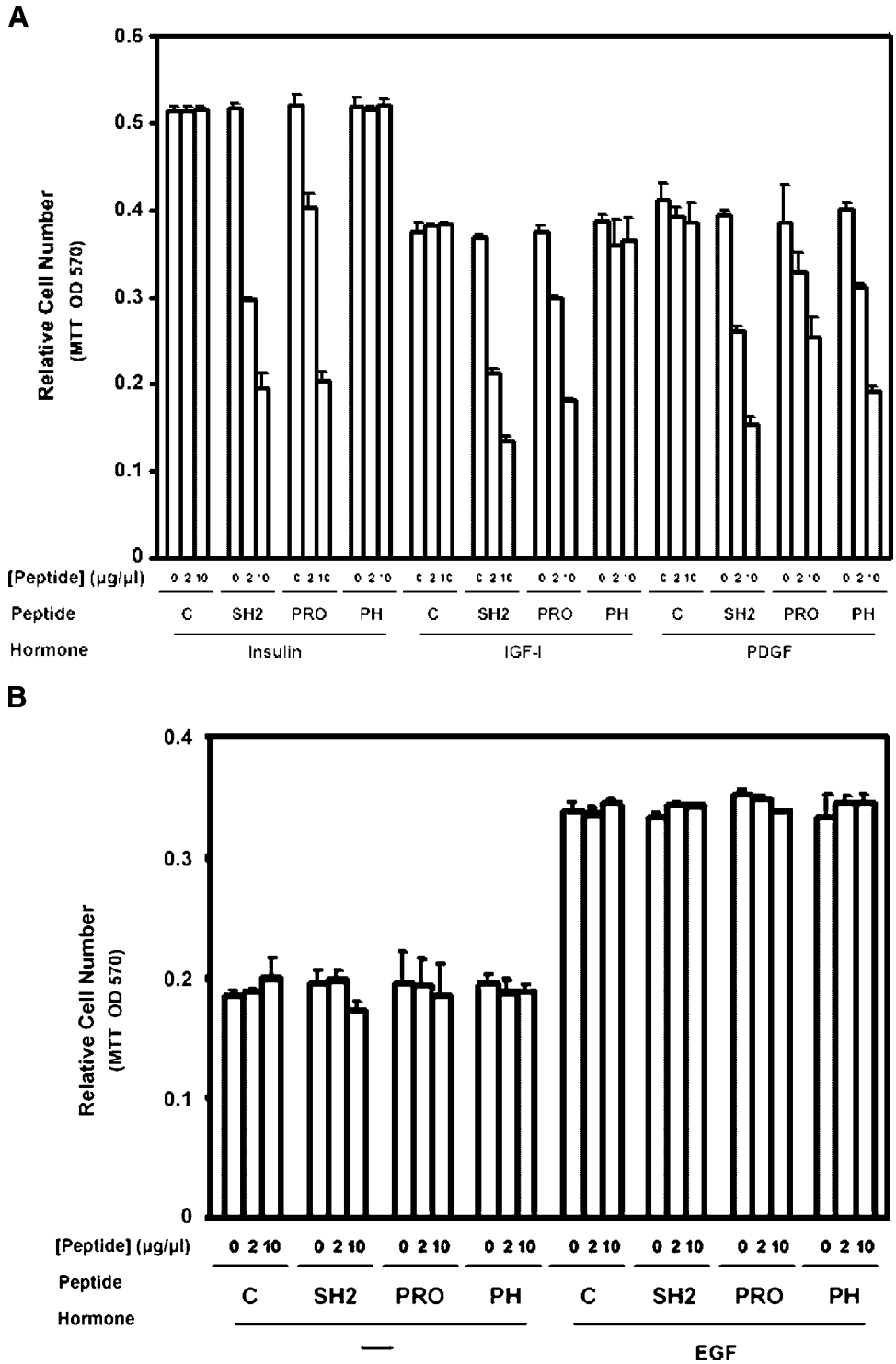

Fig. 1. Regulation of insulin-, IGF-I-, or PDGF-mediated cell proliferation by cell-permeant PSM fusion peptide mimetics of the SH2 domain, Pro-rich region, or PH domain. **A**: Mouse NIH 3T3 fibroblasts in DMEM containing 1% FBS were incubated with 100 ng/ml insulin (using cells over-expressing human IR), 100 ng/ml IGF-I, or 25 ng/ml PDGF. **B**: Mouse NIH 3T3 fibroblasts in DMEM containing 1% FBS minimal serum were incubated with 100 ng/ml EGF or were left untreated as a control (−). Simultaneously, peptide mimetics of either the PSM SH2 domain, Pro-rich region, PH domain, or control samples (Peptide: SH2, Pro, PH, C) were added to the culture medium at the indicated concentrations (0, 2, or 10 μg/ml). After 3 days cell proliferation was evaluated by quantifying cell numbers 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.

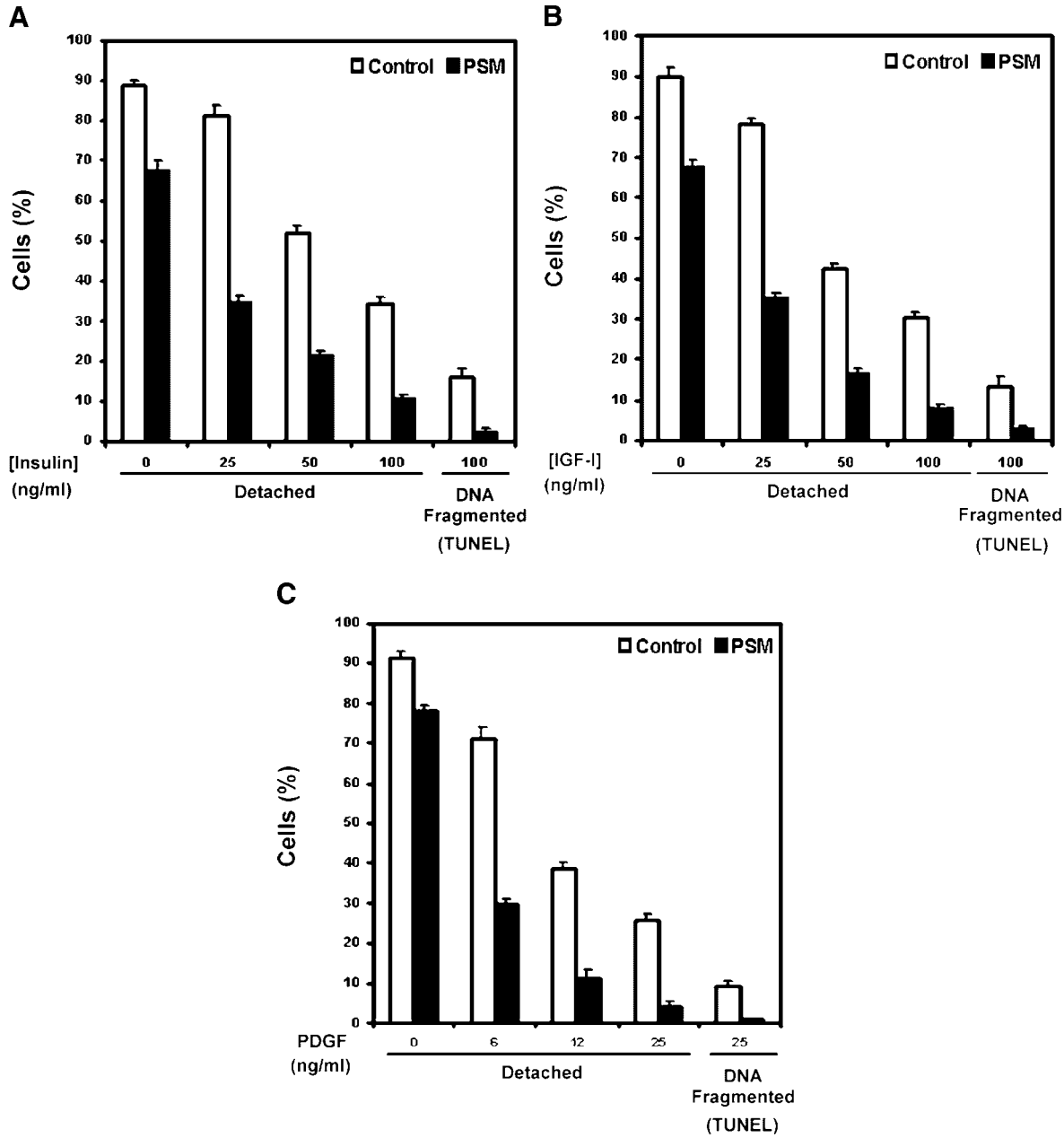

Fig. 2. Regulation of insulin-, IGF-I-, or PDGF-mediated cell survival by PSM cDNA transfection. Mouse NIH 3T3 fibroblasts were transfected with PSM or control expression plasmid. Cells were cultured in DMEM with 0.5% FBS and increasing, indicated concentrations of (**A**) insulin (0, 25, 50, or 100 ng/ml, using cells over-expressing human IR), (**B**) IGF-I (0, 25, 50, or 100 ng/ml), or (**C**) PDGF (0, 6, 12, or 25 ng/ml). After 4 days attached and detached cells were individually quantified and in parallel DNA fragmentation was determined (TUNEL) at the highest hormone dose. The percentage of detached or DNA-fragmented cells was calculated and presented. All data points were measured in triplicate as represented by the error bar.

fibroblasts transfected with PSM expression plasmid. Cell proliferation was stimulated about threefold by each hormone (Fig. 3B). PSM transfection increased this response 2.5 to 3-fold consistent with its mitogenic role described above. For PSM-transfected cells in the absence of hormone (not shown) about 0.2 OD_{570} was measured [Yousaf et al., 2001]. Inhibitor PD098059 significantly interfered with PSM stimulation in response to any of the three hormones (Fig. 3B) indicative of a role of the p44/42 MAP kinase pathway in the observed PSM stimulation of cell proliferation. In contrast, no effect was observed for inhibitor SB203580 for any response suggesting against any role of p38 MAP kinase in PSM-stimulated

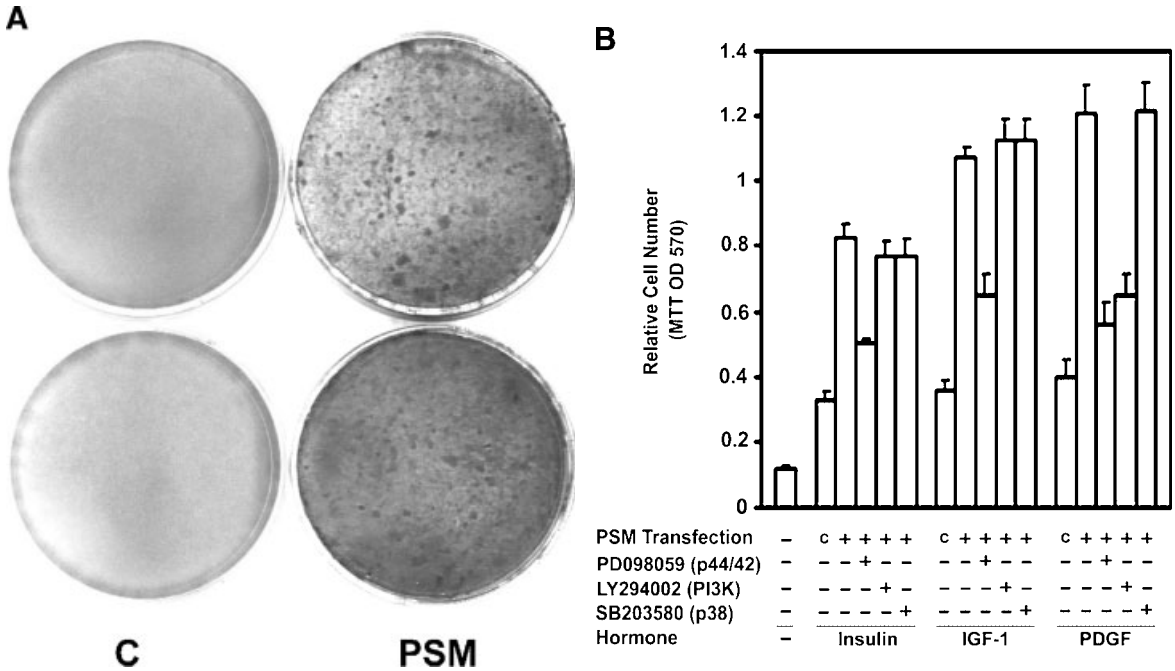

Fig. 3. **A**: Cell focus formation in response to PSM cDNA transfection. Mouse NIH 3T3 fibroblasts were transfected with PSM or control (C) expression plasmid. Cells (10^5) were seeded on 6-cm culture plates and propagated in DMEM and 10% FBS with repeated changes for 3 weeks. Cells were fixed, stained with 0.5% crystal violet, and photographed. **B**: Interference of pathway-specific inhibitors with PSM-mediated cell proliferation. Mouse NIH 3T3 fibroblasts were transfected with PSM (+), control (C) expression plasmid, or remained untransfected (−). Cells were incubated with 100 ng/ml insulin (using cells over-expressing human IR), 100 ng/ml IGF-I, 25 ng/ml PDGF, or were left untreated (Hormone:−). In parallel, 20 μM MEK inhibitor (PD098059), 10 μM PI 3-kinase inhibitor (LY294002), or 20 μM p38 MAP kinase inhibitor (SB203580) were added (+) as indicated. After 3 days of cell 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.

cell proliferation (Fig. 3B). For inhibitor LY294002 interference was exclusively observed with PSM stimulation of the PDGF response. This indicates a specific role of PI 3-kinase in PSM action that is limited to the PDGF response when compared to the insulin and IGF-I signaling pathways (Fig. 3B).

MAP Kinase Pathway

The potential regulatory role of PSM in the MAP kinase signaling pathway was addressed more specifically. The activation of various components in the involved signaling cascade including Ras, Raf-1, MEK1/2, p44/42, and p38 MAP kinase was evaluated in response to insulin, IGF-I, or PDGF after PSM cDNA transfection of NIH 3T3 fibroblasts. Activation of any of the mediators was tested individually in response to any of the peptide hormones either via phosphorylation-specific antibodies or specific substrates (Fig. 4A). Ras and Raf-1 activation remained unaffected by PSM cDNA transfection in response to PDGF and were only mildly affected by PSM cDNA transfection in response to insulin or IGF-I. In contrast, MEK1/2 and p44/42 activation was highly stimulated by PSM cDNA transfection in response to all three peptide hormones while comparable levels of cellular p44/42 protein had been demonstrated in each lane as a control (Fig. 4A). These findings point to an alternative link in response to PDGF via PSM from the PDGF receptor directly to MEK1/2 and p44/42, apparently bypassing activation of the upstream mediators Ras and Raf-1 in the established signaling cascade. A role of PSM was also implicated in the activation of p38 MAP kinase in response to insulin, IGF-I, or PDGF. Phosphorylation of its specific substrate ATF-2 was stimulated by any of the three peptide hormones and this response was substantially elevated by PSM cDNA transfection in each case (Fig. 4A).

Specific PSM Domains in MAP Kinase Regulation

To identify individual PSM domains that may be involved in the underlying signaling mechanism, activation of p44/42 MAP kinase was evaluated in the presence of cell-permeant

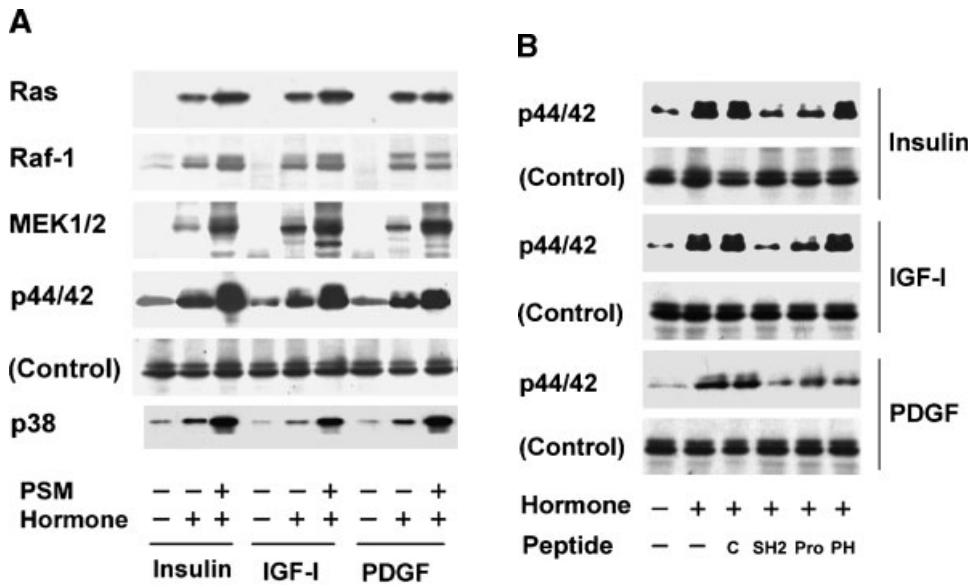

Fig. 4. Regulation of specific components of the MAP kinase pathway by complete PSM or domain-specific peptide mimetics. Mouse NIH 3T3 fibroblasts were incubated with 100 ng/ml insulin (using cells over-expressing human IR), 100 ng/ml IGF-I, or 25 ng/ml PDGF as indicated for 15 min. (Hormone: +/−) (**A**) Cells were transfected with PSM expression plasmid or remained untransfected (PSM: +/−). **B**: Cells were treated with peptide mimetics of the PSM SH2 domain, Pro-rich region, PH domain, or control samples (Peptide: SH2, Pro extracts, PH, C). Detergent cell lysates were analyzed for activation of the various signaling mediators (indicated on the left) either by immunoblotting with phosphorylation-specific antibodies (after PAGE for Raf-1 or MEK1/2) or by using specific substrates/interacting peptides (p38: ATF-2, p44/42: Elk-1; followed by PAGE) subsequent to immunoprecipitation with phosphorylation-specific antibodies. Specific substrates were recognized by phosphorylation-specific antibodies or activated Ras was precipitated with a peptide representing the interacting Ras-binding domain of Raf-1. Proteins were visualized with the ECL detection system (Amersham). The presented level of p44/42 protein in each experiment was determined in immunoblots with specific p44/42 antibody (Control).

peptide mimetics of selected PSM domains in response to insulin, IGF-I, or PDGF. For each hormone, activation of p44/42 MAP kinase was substantially inhibited by mimetics of the PSM SH2 domain and to a lesser degree of the Pro-rich region (Fig. 4B). Insulin- and IGF-I-mediated p44/42 MAP kinase activation remained unaffected by the PSM PH domain whereas the PDGF response was strongly reduced. Similar levels of cellular p44/42 protein had been demonstrated in each lane (Fig. 4B). A similar PSM domain-specific modulation had been observed in the cell proliferation response to the three hormones (Fig. 1A) that implicates p44/42 MAP kinase as a prime candidate mediator in the PSM-mediated cell proliferation response.

Specific PSM Domains in PI 3-Kinase Regulation

A role of PI 3-kinase had been implicated in PSM-stimulated cell proliferation specifically in response to PDGF by inhibitor LY294002 (Fig. 3B). A putative regulation of PI 3-kinase activity was directly addressed by interference with individual domain-specific PSM peptide mimetics and by PSM cDNA transfection. As expected, PI 3-kinase activity was found strongly stimulated by any of the three hormones. Insulin- or IGF-I-stimulated PI 3-kinase activity remained unaffected by any tested peptide mimetic or by increased PSM expression (Fig. 5A). In contrast, PDGF-stimulated PI 3-kinase activity was elevated by increased levels of PSM and inhibited most significantly by the PSM SH2 or PH domains, but not significantly by the Pro-rich region. This observation implicates a role of PSM in the PI 3-kinase response to PDGF in contrast to the insulin or IGF-I responses (Fig. 5A). This putative role was addressed by testing the interaction between PSM and p85, the regulatory subunit of PI 3-kinase in a coimmunoprecipitation experiment using PSM antiserum followed by immunoblotting with p85 antibody. Association between PSM and p85 was demonstrated specifically in response to PDGF but was not observed in response to insulin or IGF-I (Fig. 5B). Consequently, our combined

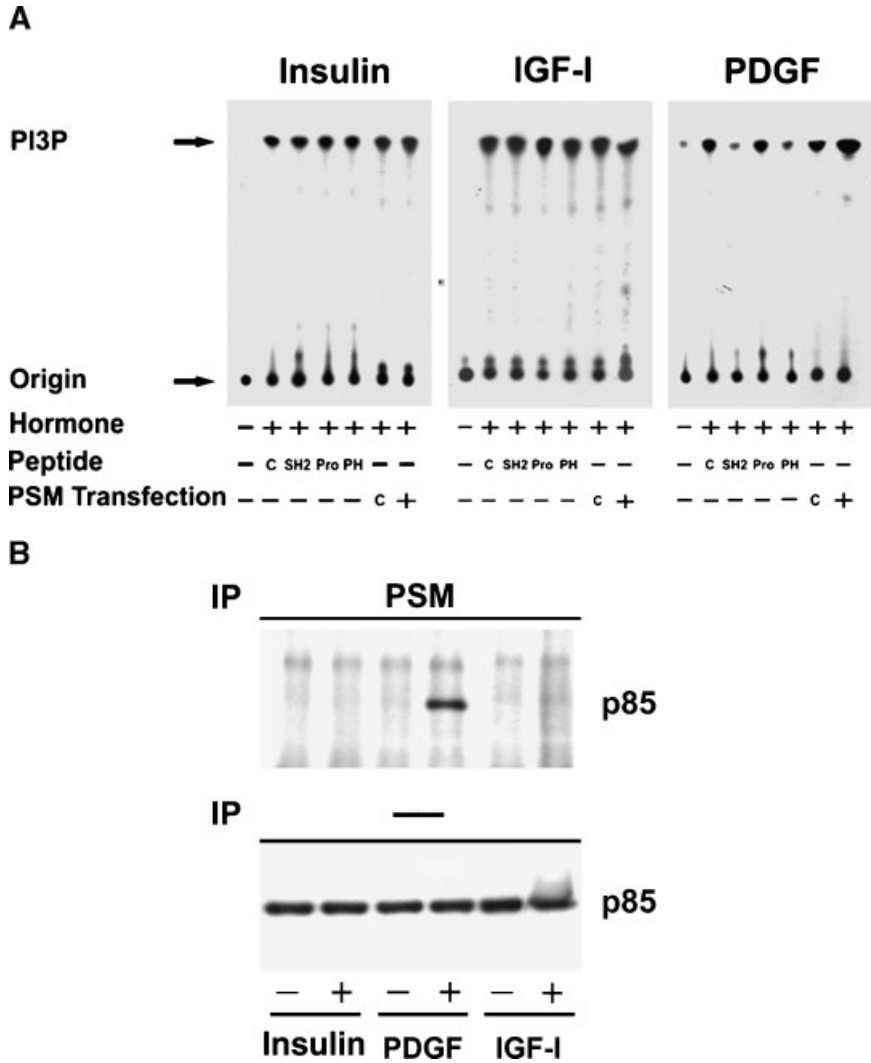

Fig. 5. **A**: Regulation of PI 3-kinase by elevated complete PSM or domain-specific peptide mimetics. Mouse NIH 3T3 fibroblasts were transfected with PSM, control (C) expression plasmid or remained untransfected (−). Cells were incubated with 100 ng/ml insulin (using cells over-expressing human IR), 100 ng/ml IGF-I, or 25 ng/ml PDGF as indicated. Simultaneously, peptide mimetics of either the PSM SH2 domain, Pro-rich region, PH domain, or control samples (Peptide: SH2, PRO, PH, C) were added to the culture medium. p85 PI 3-kinase was immunoprecipitated from detergent cell extracts and incubated with phosphatidylinositol in the presence of [gamma-^{32}P] ATP. Synthesis of phosphatidylinositol 3-phosphate (PI3P) was analyzed by thin layer chromatography and is shown in an autoradiograph (arrow). **B**: Hormone-specific association of PSM with p85 PI 3-kinase. Untransfected mouse NIH 3T3 fibroblasts were incubated with 100 ng/ml insulin (using cells over-expressing human IR), 100 ng/ml IGF-I, or 25 ng/ml PDGF as indicated. Detergent cell extracts were immediately analyzed by SDS–PAGE (IP:−) or were first immunoprecipitated with PSM-specific antiserum (IP: PSM) and protein complexes were subsequently resolved by SDS–PAGE. Proteins were analyzed in immunoblots with p85 antibody and were visualized with the ECL detection system (Amersham). The position of p85 is indicated on the right.

observations point to a role of PSM in the PI 3-kinase response to PDGF that involves the PSM SH2 and PH domains, whereas no evidence was observed for any role of PSM in the PI 3-kinase responses to insulin or IGF-I (Fig. 6). This pattern is consistent with the observed PSM regulation of the PDGF-mediated cell proliferation response (Fig. 1A) and implicates a role of PI 3-kinase in addition to p44/42 MAP kinase (Fig. 3B).

DISCUSSION

As strategies to define the role of PSM in mitogenesis we either increased the cellular level of PSM by cDNA transfection where we reached about three out of four cells or we interfered with the function of individual domains of cellular PSM by introducing cell-permeant peptide mimetics representing its

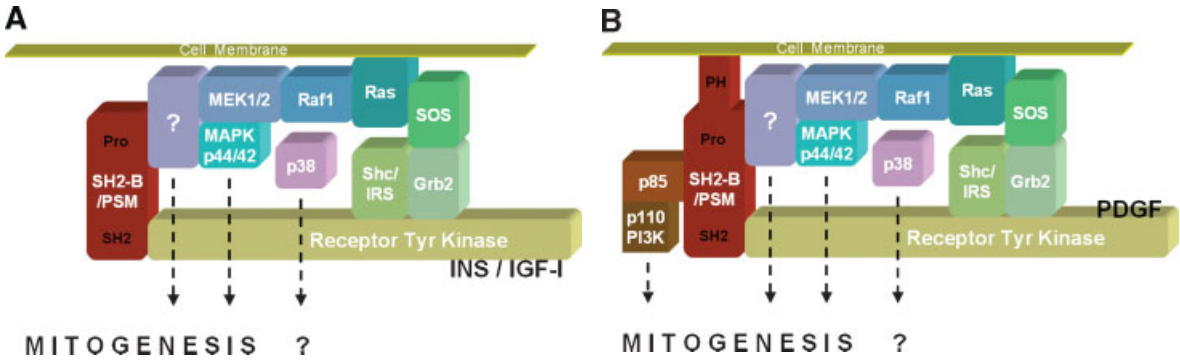

Fig. 6. Model of PSM/SH2-B signaling interactions in the mitogenic response to (**A**) insulin/IGF-I when compared to (**B**) PDGF. Individual signaling mediators are represented by rectangular shapes identified by specific name in white letters. Components with a role specific to the mitogenic response to PDGF are represented by sharp edges, components shared in the mitogenic responses to any of the three hormones by rounded edges. PSM/SH2-B is shown as a composite of its functionally implicated domains including the Pro-rich, PH, and SH2 domains (indicated in black letters). Direct contact between objects represents a postulated direct cellular association. The plasma membrane is represented by a horizontal strip in contact with membrane-associated mediators. The transmembrane association of the involved receptor tyrosine kinase has not been displayed. Relative sizes and shapes are arbitrary and have been chosen exclusively to visualize putative interactions in two dimensions.

amino terminal Pro-rich region, or its PH or SH2 domain. We have shown cell-permeant PSM domain-specific peptide mimetics to enter cells with high efficiency in a dose-dependent fashion and to specifically interfere with PSM-mediated stimulation of mitogenesis and cell proliferation in response to insulin, IGF-I, or PDGF but not in response to EGF [Riedel et al., 2000]. In our hands cell-permeant peptide mimetics were shown to represent hormone, mediator, and domain-specific physiologic responses [Wang et al., 1999]. In particular the specificity and selectivity of Pro-rich peptide mimetics was demonstrated through their distinct hormone and receptor-specific functions when comparing respective Pro-rich peptide mimetics of Grb10 [Wang et al., 1999] versus PSM [Riedel et al., 2000]. Our findings are consistent with the specific dominant-negative action routinely observed by others for isolated SH2 domains including the PSM SH2 domain [Nishi et al., 2005]. A dominant-negative and specific role of PSM peptide mimetics is also supported in a direct comparison with PSM knockdown by siRNA with comparable results (not shown).

We have demonstrated a stimulatory role of PSM in insulin-, IGF-I-, or PDGF-mediated cell survival that potentiated the established dose-dependent survival action of any of the three peptide hormones. Cell survival had been addressed by evaluating the fraction of detached versus total cells [Webb et al., 2000] or DNA fragmentation directly (TUNEL) with comparable results (Fig. 2). PSM beta had been shown to be required for growth hormone-induced actin rearrangement [Herrington et al., 2000]. Our earlier observation that microinjection of an SH2 domain peptide mimetic into transformed BALB-3T3 fibroblasts partially restored a normal actin stress fiber pattern suggested a requirement of PSM/SH2-B for phenotypic cell transformation [Riedel et al., 2000]. We now demonstrate that an increased level of PSM by cDNA transfection of NIH 3T3 fibroblasts results in the formation of cell foci and is consistent with a role of PSM in aberrant cell proliferation (Fig. 3A).

To identify the signaling pathways involved in PSM action, cell proliferation was measured in the presence of pathway-specific inhibitors (Fig. 3B). Both the MAP kinase [Welch et al., 2000] and PI 3-kinase [Palmarini et al., 2001] signaling pathways play important roles in malignant cell transformation. The stimulation of cell proliferation observed by PSM transfection was substantially reduced by MEK1/2 inhibitor PD098059 for any of the three peptide hormones whereas PI 3-kinase inhibitor LY294002 only inhibited PSM stimulation of the PDGF response. The observed lack of an impact of p38 MAPK inhibitor SB203580 sug-

gested against any role of p38 in PSM-mediated cell proliferation. This is consistent with the common view in the field against a role of p38 in insulin-, IGF-I-, or PDGF-mediated mitogenesis [Matsumoto et al., 1999; Gousseva et al., 2001]. However, PSM cDNA transfection increased p38 MAP kinase activity in response to any of the three hormones (Fig. 4A) implicating p38 in other unidentified physiologic functions of PSM such as cell motility. PSM plays a role in actin reorganization [Herrington et al., 2000] and p38 MAP kinase in PDGF-induced cell motility and actin reorganization [Matsumoto et al., 1999].

The Ras/Raf-1/MEK/ERK (p44/42 MAP kinase) signaling cascade represents an important pathway in insulin- [Cobb et al., 1994; Mastick et al., 1994], IGF-I- [Chung et al., 1991; Williams et al., 1993], and PDGF-mediated mitogenesis and cell proliferation [Roche et al., 1996]. PSM-stimulated cell proliferation in response to insulin, IGF-I, or PDGF was substantially reduced by PD098059, a pharmacological inhibitor of MEK activity (Fig. 3B), suggesting that the MEK1/2-p44/42 MAP kinase pathway plays a role in PSM-mediated cell proliferation. Regulation of NGF-induced p44/42 MAP kinase activity was not observed upon PSM cDNA transfection but PD098059 had been shown to interfere with NGF-induced phosphorylation of PSM [Rui et al., 1999]. While this may point to a potential function of PSM downstream of MEK in NGF signaling, our current observation of substantial MEK1/2 and p44/42 activation upon PSM cDNA transfection in response to insulin, IGF-I, or PDGF places a role of PSM upstream of MEK for these three peptide hormone signals. This is also supported by the reported PSM stimulation of insulin-induced p44/42 MAP kinase activity [Ahmed and Pillay, 2003].

MAPK is normally activated via the established signaling cascade downstream of the receptor involving a sequence of Grb2, SOS, Ras, Raf-1, and MEK. Intriguingly, upon PSM cDNA transfection, stimulation of Ras or Raf-1 activity was not observed in response to PDGF and only mildly in response to insulin or IGF-I (Fig. 4A) in contrast to the high level of activation observed for MEK1/2 and p44/42 MAP kinase. In response to PDGF, PSM appears to provide an alternative link from the PDGF receptor directly to MEK apparently bypassing Ras and Raf-1 activation. PDGF-stimulation of MEK1/2 and p44/42 MAP kinase activation independently of Ras and Raf-1 activation [Kivinen and Laiho, 1999; Takeda et al., 1999; Tsakiridis et al., 2001] and Ras-independent mechanisms in PDGF-mediated mitogenesis [Roche et al., 1996] have already been reported. We have attempted to investigate a potential association between PSM and MEK1/2 or p44/42 that may underlie such a mechanism but have not been able to obtain an answer which could point to an indirect association. PSM has been shown to activate JAK2 directly by dimerization [Nishi et al., 2005] but it is unknown whether such a mechanism would extend to other Tyr kinases.

PI 3-kinase plays an important role in insulin- [Ruderman et al., 1990; Roche et al., 1998; Poy et al., 2002], IGF-I- [Yamamoto et al., 1992; Lawlor et al., 2000], and PDGF- [Yu et al., 1994; Khwaja, 1999] mediated mitogenesis, cell proliferation, or survival. PI 3-kinase inhibitor LY294002 specifically interfered with PSM-stimulated cell proliferation in response to PDGF but not to insulin or IGF-I (Fig. 3B). Consistently, neither PSM cDNA transfection nor any of the dominant-negative PSM domain-specific peptide mimetics affected insulin-, or IGF-I-induced PI 3-kinase activity. In contrast, PSM PH and SH2 domain peptide mimetics inhibited while PSM cDNA transfection stimulated PDGF-mediated PI 3-kinase activation (Fig. 5A). Consistently, in coimmunoprecipitation experiments PSM association (that could be indirect) was observed with p85 in response to PDGF but not in response to insulin or IGF-I stimulation (Fig. 5B). Direct association of PSM with PDGFR [Riedel et al., 2000] as well as with IR [Riedel et al., 1997] and IGF-IR [Wang and Riedel, 1998] has been shown earlier. Comparable PSM phosphorylation has been observed in response to PDGF when compared to IGF-I [Yousaf et al., 2001] and thus cannot explain the PDGF-specificity of PSM-p85 association. In combination, these results implicate a role of PI 3-kinase in PSM-mediated cell proliferation specifically in the PDGF response that involves an association between PSM and p85. Both the PSM SH2 and PH domains are involved in PI 3-kinase regulation, the SH2 domain expectedly based on its upstream interaction with PDGFR and the PH domain presumably through an association with the cell membrane (Fig. 6B). A PI 3-kinase-dependent localization has been

described for other signaling mediators including $p62^{dok}$ where membrane localization via the PH domain has been observed in response to PDGF [Zhao et al., 2001]. PH domain-mediated PSM localization may be similarly important in the regulation of p44/42 MAP kinase activity in response to PDGF, consistent with our findings (Fig. 4B).

p44/42 activity in response to any of the three tested peptide hormones was significantly reduced by PSM Pro-rich or SH2 domain peptide mimetics. This may reflect the association of PSM with the respective receptor via its SH2 domain and potentially with the SH3 domain of an unknown mediator via its Pro-rich region. In contrast, a PSM PH domain peptide mimetic exclusively interfered with PDGF-stimulated but not with insulin- or IGF-I-stimulated p44/42 activity. The pattern observed for the regulation of p44/42 activity was matched by the regulation of cell proliferation (Fig. 1) indicating an essential role of p44/42 in PSM-mediated mitogenesis. Both Pro-rich and SH2 domain peptide mimetics were found to inhibit cell proliferation in response to any of the tested hormones whereas the PH domain peptide only interfered with cell proliferation in response to PDGF (Fig. 1). No effect was observed on EGF-mediated cell proliferation for any of the tested peptides as expected given the lack of a reported role of PSM in EGF-mediated mitogenesis [Riedel et al., 2000].

CONCLUSION

In combination, our findings suggest a role of PSM in the assembly of distinct mitogenic signaling complexes in response to insulin or IGF-I when compared to PDGF in cultured normal fibroblasts (Fig. 6). Various mediators have been implicated in defining the differences in the signaling complexes formed in response to PDGF when compared to insulin or IGF-I [Pukac et al., 1998; Taddei et al., 2000]. The complex formed in response to insulin or IGF-I involves the respective peptide hormone receptor and presumably the established components leading to MAP kinase activation. However, our data suggest a short cut from the receptor via PSM directly to MEK1/2 and consequently also to p44/42 MAP kinase activation potentially involving additional proteins. At least two PSM domains participate, the SH2 domain anticipated to link PSM to the respective receptor and the Pro-rich region in an association with an unidentified downstream component resulting in direct MEK1/2 and p44/42 regulation. The PDGF receptor signaling complex formed in response to PDGF differs by involving PI 3-kinase in addition to the same components and interactions as described for insulin or IGF-I. PSM associates with PI 3-kinase via p85 and the PSM PH domain participates in the regulation of PI 3-kinase activity presumably through membrane interaction. In contrast, the PSM Pro-rich region appears to participate only in the MAP kinase signal. Both pathways contribute to the mitogenic response as shown by cell proliferation, survival, and phenotypic transformation (Fig. 6). PSM also regulates p38 MAP kinase activity, however, in a pathway unrelated to the mitogenic response.

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