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PSM, an Insulin-Dependent, Pro-Rich, PH, SH2 Domain Containing Partner of the Insulin Receptor[1]

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Insulin stimulation results in a considerable spectrum of cellular responses, only part of which have been firmly correlated with the activation of established insulin receptor (IR) targets such as IRS-1, IRS-2, and Shc. Many responses may be transduced by alternative direct IR targets, some of which may still be unknown, may act in parallel to but independently of IRS-1, IRS-2, and Shc, and may be members of the growing family of SH2 domain-containing signaling adaptors. An SH2 domain-coding region of a protein termed PSM was cloned based on its interaction with an activated IR cytoplasmic fragment in a yeast two-hybrid screen. When used as a hybridization probe this region led to the isolation of a protein-coding cDNA which is expressed with a wide tissue distribution and exists in several variant forms. A pleckstrin homology domain and three Pro-rich regions including a putative SH3 domain binding site were identified in addition to the SH2 domain in the deduced 756 amino acid sequence. They imply a role of PSM in tyrosine kinase and phosphatase-mediated signaling pathways. A similar sequence termed SH2-B had been reported in an earlier study, which may represent the rat homolog of PSM. A role of PSM specifically in insulin action is suggested by the interaction of its SH2 domain with an activated but not with an inactive catalytic fragment of the IR in the yeast two-hybrid system *in vivo*, by the insulin-dependent association of a glutathione S-transferase (GST) PSM SH2 domain fusion protein with purified IR *in vitro*, and by the insulin-dependent association of GST PSM SH2 with the IR in cell extracts. In contrast, PSM was not found to associate with the established IR substrate IRS-1 under any conditions and appears to act independently of IRS-1. All of our findings are compatible with a putative role of PSM in insulin action.

Key words: cDNA cloning, insulin receptor, SH2 domain association, splice variants, yeast two-hybrid system.

The insulin receptor (IR) is probably the best understood receptor tyrosine kinase and the elucidation of its mechanism of action has greatly contributed to the molecular dissection of receptor tyrosine kinase signaling in general (*1-3*). The early and long-term metabolic and mitogenic consequences of responsive cells to insulin stimulation include glucose uptake, amino acid uptake, glycogen, amino acid, and fatty acid synthesis, the induction of specific genes, the inhibition of apoptosis, the stimulation of DNA synthesis, and cell proliferation (*4*). Cellular substrates of the IR include a 120 kDa hepatocyte plasma membrane ecto-ATPase (*5-7*), several 60 kDa proteins shown to associate with phosphatidylinositol (PI) 3′-kinase (*8*) or Ras-GAP (*9*), a 15 kDa myelin homologue 442 (aP2) (*10, 11*) and others which have only been characterized at the protein level (*12*). Other cellular signaling mediators that associate with the IR in response to insulin stimulation include the proto-oncogene product Vav which is phosphorylated on tyrosine (*13*), the adapter protein Gab1 (*14*), the adapter protein Grb10/IR (*15-19*), and the protein phospho Tyr phosphatases PTP1B (*20*) and possibly SHP-2 (*21*). The role of any of the above-listed putative signaling mediators in insulin action remains to be established.

The major cellular substrates of the IR are the first discovered substrate IRS-1 (*22*), the related IRS-2 (*23*) and the mitogenic signaling mediator Shc (*24*), all of which also play established roles in other signaling pathways. In

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Abbreviations: Grb10/IR, growth factor receptor binding protein 10 (also termed Grb-IR); GST, glutathione S-transferase; IR, insulin receptor; IRS-1, insulin receptor substrate-1; PAGE, polyacrylamide gel electrophoresis; PDGF, platelet-derived growth factor; PH, pleckstrin homology; PI 3′-kinase, phosphatidylinositol 3′-kinase; PKC delta, protein kinase C delta; PSM, *P*ro-rich, *P*H, *S*H2 domain containing *S*ignaling *M*ediator; SH2, Src homology-2; SH3, Src homology-3; 5′-RACE, Rapid Amplification of 5′ cDNA Ends.

particular, the most extensively-studied IR substrate IRS-1 has been shown to play important roles in the mitogenic and proliferative response to insulin involving PI 3′-kinase which IRS-1 associates with and activates *via* the regulatory domain p85 (*4, 25*). p85 has also been shown to directly associate with the IR in response to insulin stimulation (*26, 27*) but the physiological relevance of this association remains unclear. A role of IRS-1 and PI 3′-kinase has also been implicated in the control of protein synthesis by insulin, however, in a pathway (*28*) that parallels the regulation of mRNA 5′-capping *via* the phosphorylation of PHAS-1. The PHAS-1 pathway has established some of the molecular events that mediate the regulation of protein synthesis by insulin (*29, 30*). A role for IRS-1 and PI 3′-kinase has been proposed in the insulin-stimulated glucose uptake by GLUT4 glucose transporter translocation (*31, 32*), however these mechanisms were found by other approaches to depend on IRS-1 or PI 3′-kinase (*33, 34*). Some of the metabolic responses to insulin must be mediated by mechanisms that parallel PI 3′-kinase which suggest a role of an undefined alternative IR signaling pathway (*35*).

The differences reported in several studies likely result from varying insulin signaling pathways in different tissues (*36, 37*). They may in part be a consequence of varying levels of IRS-2 in different cells and tissues which may determine to what extent IRS-2, which is not functionally interchangeable with IRS-1 (*38*), can compensate for some of the normal functions of IRS-1 (*39*). This is clearly suggested by the relatively mild phenotype of mice lacking IRS-1 due to gene disruption which display hyperinsulinemia, impaired glucose tolerance, and growth retardation (*40-42*). In these mice insulin responses in skeletal muscle were more severely impaired than in liver which correlates with a reduced expression level of IRS-2 in muscle compared to liver and may explain the more severe effects of IRS-1 gene disruption in muscle (*39*). In contrast, mice lacking the IR due to gene disruption develop diabetic ketoacidosis, a marked post-natal growth retardation, skeletal muscle hypotrophy and fatty infiltration of the liver and die within one week after birth (*43, 44*). Mice heterozygous for IR and IRS-1 null alleles become diabetic with age and represent a new animal model for the disease (*45*).

In particular, an IRS-1 and PI 3′-kinase-independent, unidentified pathway has been proposed in the insulin stimulation of glucose uptake (*33, 34*). Similarly, insulin activates glycogen synthase in CHO cells by a Ras and PI 3′-kinase- (and wortmannin-) independent mechanism (*46*). Despite normal Ras binding to Raf-1, truncation of the IR carboxyl terminus impairs Raf-1, MEK, and MAPK activity, glucose transport, glycogen synthesis, PI 3′-kinase, and phosphoprotein phosphatase-1 activity whereas mitogenic responses remain largely unimpaired (*47, 48*). Binding of Ras to Raf-1 serves to translocate Raf-1 to the plasma membrane where an unidentified mechanism, involving Tyr phosphorylation, leads to its activation (*49*). This mechanism may involve undefined signaling mediators that associate with IR in a pathway parallel to IRS-1. Overexpression of IRS-1 was shown to restore the mitogenic response of a defective IR (Y960A, which fails to activate IRS-1) including activation of PI 3′-kinase but restored only partial glycogen synthesis and failed to restore MAPK/ERK activation (*50*). IR mutants in the tyrosine kinase region (R1174Q and L1178P) suggest a role of unidentified signaling mediators in the impaired glycogen synthesis, DNA synthesis and MAPK activation response to insulin in CHO cells since IRS-1 appears to be normally activated (*51*). These mediators may act in parallel to IRS-1 and IRS-2 and may resemble other adapters known to associate with other receptor tyrosine kinases such as Grb2 which are often not phosphorylated on tyrosine (*52*).

We employed the yeast two-hybrid system to identify signaling mediators for alternative pathways of the IR analogous to signals emerging from other receptor tyrosine kinases. Here we describe the identification and cloning of a *P*ro-rich, *P*H, and *S*H2 domain containing putative *S*ignaling *M*ediator, termed PSM, as an IRS-1-independent but insulin-dependent cellular partner of the IR.

EXPERIMENTAL PROCEDURES

Antibodies—Rabbit polyclonal antibody directed against the insulin receptor beta-subunit and monoclonal anti-phospho Tyr antibody PY20 had been obtained from Transduction Laboratories, anti-GAL4 and anti-IRS-1 rabbit polyclonal antibodies from Upstate Biotechnology, and horseradish peroxidase-coupled anti-IgG antibody from Kirkegaard and Perry Laboratories.

Yeast Two-Hybrid Screen—A *Bgl*I-*Pst*I cDNA fragment encoding the carboxyl terminal 402 amino acids of the insulin receptor beta-subunit (*53*) was ligated to the *Eco*RI and *Pst*I sites of pGBT9 (*54*) using *Eco*RI-*Bgl*I adaptor oligonucleotides 5′-AATTCGCGAGCAGAAAGAGGCAG-CCAGAT-3′ and 5′-TGGCTGCCTCTTTCTGCTCGCG-3′ The mutation IR K1018A was introduced by exchanging a *Bgl*I-*Pst*I fragment of the insulin receptor bait with the corresponding fragment of the mutated receptor (*55*). A *Bsp*EI (end filled)-*Hin*dII (partially filled) cDNA fragment encoding the protein coding region of rat IRS-1 (*23*) excluding the four most amino terminal aa was fused with the *Bam*HI (endfilled) and *Sal*I sites of pBTM116 (kindly provided by Paul Bartel and Stanley Fields). A *Fsp*I-*Bam*HI fragment (*Bam*HI is located in a multiple cloning site region downstream of the termination codon) encoding the protein coding region of PKC delta (*56*) excluding the five most amino terminal aa was joined with the *Sma*I and *Bam*HI sites of the bait plasmid pBTM116 (kindly provided by Paul Bartel and Stanley Fields). For the two-hybrid screen, yeast strain Y153 (*57*) was co-transformed with the insulin receptor bait and a 10.5 day post conception mouse embryo cDNA library in plasmid pVP16 (*58*). The specificity of interaction of the identified sequences was tested by mating the parental strain with strains carrying various test baits followed by the *LacZ* color assay (*58*).

Northern Analysis—A 529 bp cDNA insert encoding the PSM SH2 domain and downstream coding sequences was released from the pVP16 library plasmid at its *Not*I cloning site, labeled using nick translation with ^{32}P alpha-dNTP, and used as a hybridization probe to identify PSM-specific transcripts on a mouse multiple tissue Northern blot (MTN Blot, Clontech) as described by the manufacturer.

Cloning of the Complete Protein-Coding PSM cDNA—The PSM hybridization probe used in the Northern analysis was subsequently employed to isolate clones from a mouse

brain lambda Uni-Zap XR library (Stratagene). Positive clones were identified by secondary screening and plasmids were excised *in vivo* by ExAssist helper phage (Stratagene). The complete 5′ end of the protein-coding cDNA was isolated by PCR (5′ RACE, Gibco BRL) with mouse whole brain RNA as a template. PCR products were ligated into a pCR 2.1 TA cloning vector (Invitrogen). The complete protein-coding region was established by dideoxy fluorescence automated sequencing.

Preparation of GST Fusion Protein—A 529 bp cDNA insert encoding the PSM SH2 domain and downstream coding sequences was released from the pVP16 library plasmid at its *Not*I cloning site and inserted into the *Not*I cloning site of pGEX-4T-1 (Pharmacia). *Escherichia coli* DH5 alpha were transformed and transcription was induced with 0.5 mM isopropyl-beta-D-thiogalactoside. The expressed GST PSM SH2 domain fusion and control GST protein were purified on a glutathione-Sepharose column (Pharmacia), eluted in 10 mM reduced glutathione in 50 mM Tris-HCl pH 8.0 and stored after addition of 10 mM dithiothreitol, 1 mM EDTA.

Insulin Receptor Purification and In Vitro Binding—Insulin receptors were wheatgerm agglutinin (Vector) purified and stored in 100 mM *N*-acetylglucosamine, 50 mM HEPES, 0.1% Triton X-100 at −80°C. For activation and autophosphorylation receptors were incubated with 100 ng/ml insulin in 50 mM HEPES, pH 7.4, 10 mM $MnCl_2$, 50 mM ATP for 25 min at 25°C. Activated receptors were added to 25 μg of GST PSM SH2 domain fusion protein in 0.1% Triton X-100, 50 mM HEPES, 2 mM Na_3VO_4, and varying concentrations of NaCl. After 1 h of incubation at 4°C GST Sepharose was added for an additional 3 h incubation. The sepharose was sedimented in a benchtop microfuge, washed, and associated insulin receptors were separated by SDS PAGE and identified by immunoblotting.

Cell Cultures, Immunoprecipitation, and Immunoblotting—Subconfluent insulin receptor overexpressing chinese hamster ovary (CHO, $\sim 10^6$ receptors/cell) fibroblasts (*59*) were incubated for 16 h without serum and stimulated with 100 ng/ml insulin for 15 min. Culture dishes (150 mm) were washed twice with PBS and cells were lysed in 1% Triton X-100, 50 mM HEPES pH 7.4, 10% glycerol, 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM EDTA, 10 mM NaF, 100 mM Na_3VO_4, 10 mM beta-glycerophosphate, 2 mM EDTA, 1 mM PMSF. Proteins were directly subjected to SDS PAGE or were first mixed with GST PSM SH2 fusion protein, and co-precipitated with glutathione sepharose and after washing analyzed by SDS PAGE. Proteins were electrophoretically transfered to nitrocellulose and identified in immunoblots in the presence of 0.2% gelatin, 0.1% Tween 20 with insulin receptor, IRS-1, or phospho Tyr-specific antibodies and a horseradish peroxidase labeled secondary antibody.

RESULTS

Two-Hybrid Screening Strategy—Our rationale to find alternative IR targets was based on the assumption that they should associate with the cytoplasmic IR domain, most likely only after it had been activated. The yeast two-hybrid system was chosen as a powerful interaction trap approach (*54*) to identify proteins based on their association with the IR. Since this association takes place in the yeast nucleus where insulin stimulation of a complete IR would not be feasible we capitalized on the observation that removal of the IR extracellular insulin binding domain leads to activation of the cytoplasmic IR domain (*60*). Consequently, a bait construct was prepared as a fusion between the GAL4 DNA-binding domain and the complete IR cytoplasmic domain (*16*). To identify associating sequences we chose a random-primed mouse embryo cDNA library which should contain sequences evenly distributed over the length of the mRNA as well as a broad spectrum of the messages expected to be expressed in mouse (*58*). The insert size had been selected to range from 350 to 700 bp to ensure that a typical insert would contain a single domain, isolated from other potentially interfering sequences. After several sequences had been identified great emphasis was placed on the analysis of the specificity of the underlying interactions (*58*). Sequences were tested for their association with an activated compared to an inactive IR bait construct as a negative control. In addition, a variety of non-specific control baits were used to confirm the specificity of the underlying association. Only the combination of these strategies allowed us to identify and isolate specifically-interacting sequences from the background of non-specific, positive clones.

Isolation of the PSM SH2 Domain with the Yeast Two-Hybrid System—A two-hybrid cDNA library from 10.5 day post conception mouse embryos and fused to the transcription-activating domain of VP16 (*58*) was screened with a bait plasmid encoding a fusion protein of the GAL4 DNA-binding domain with the carboxyl terminal 402 amino acids of the insulin receptor beta-subunit (*53*). This includes the complete intracellular region of the insulin receptor which had been shown to be constitutively activated by removal of the extracellular domain (*60*). *Saccharomyces cerevisiae* strain Y153 (*57*) was co-transformed with bait and library, and colonies that grew on medium selective for *HIS3* expression were isolated. Colonies were tested for expression of the *lacZ* reporter gene in a blue color assay and negatives were eliminated. The remaining colonies were cured of the bait plasmid and *lacZ* positive colonies were eliminated (*58*).

Colonies displaying *His3* and *lacZ* positive phenotypes were chosen for cDNA sequence analysis. Library plasmids were transfered to *E. coli* and sequence analysis of the short (<700 bp) plasmid inserts revealed that most encoded protein fragments containing Src homology-2 (SH2) domains (*61*). An exception was a sequence, termed Y, that did not display an SH2 or phosphotyrosine binding (PTB) domain, which remains to be investigated. The carboxyl terminal SH2 domain of the regulatory subunit p85 of PI 3′-kinase, the SH2 domain of the signaling mediator Grb10/IR, and the SH2 domain of the transforming protein Vav were repeatedly identified. All of these proteins have also been shown to associate with the insulin receptor by alternative approaches (*13, 15-19, 26, 27*) suggesting that the employed experimental strategy identifies targets of the insulin receptor. In addition, the SH2 domain of a protein termed PSM was identified which is the focus of this report. When introduced into yeast strains carrying different test bait plasmids, all identified sequences listed above specifically associated with the activated insulin receptor cytoplasmic domain but not with control baits including the

Ser/Thr protein kinase C delta, the insulin receptor substrate IRS-1, and the commonly-used test protein lamin C (Fig. 1). Similarly, a GAL4 control plasmid and a kinase-inactivating insulin receptor mutation K1018A scored negative, demonstrating the requirement of an activated insulin receptor tyrosine kinase for any of the SH2 domain associations (Fig. 1). Expression of the various test baits in *S. cerevisiae* was confirmed in immunoblots with antibodies against the GAL4 DNA-binding domain (not shown).

Northern Analysis and Hybridization Cloning of the Complete Mouse PSM Protein Coding Region—To begin to address the tissue distribution of PSM, the cloned fragment including the SH2 domain-coding sequence was used to identify PSM transcripts by Northern analysis. A single message of about 4 kb was detected in most mouse tissues analyzed, however it was particularly prominent in liver, brain, and heart (Fig. 2). Lower levels were detected in skeletal muscle, kidney, testes, and lung, but not in spleen.

The cloned PSM SH2 domain coding fragment subsequently served as a hybridization probe to identify and isolate the complete PSM protein-coding region. Several overlapping clones were isolated and confirmed from a mouse brain cDNA lambda gt10-derived phage library and converted to phagemid by *in vivo* excision. The 5′ end of the protein-coding region was isolated by 5′ RACE. The complete protein-coding region was established from overlapping sequences (Fig. 3A). During the course of the cDNA cloning sequence variants were repeatedly isolated from the phage library. One variant form had also been independently isolated from the two-hybrid library. In multiple independent clones it contained a 100 bp sequence insert when compared to the shorter master sequence (Fig. 3B). The deduced protein product carries a distinct carboxyl terminus due to the additional sequence and the resulting reading frame shift at its insertion point in the master sequence (Fig. 3, A and B). We speculate that this sequence variant is generated by differential splicing which is supported by its boundary sequence (*62*).

PSM Sequence Analysis—The analysis of the deduced PSM primary structure with the Wisconsin Sequence Analysis Package (Genetics Computer Group, Madison, WI) including BLAST (*63*) and PROFILE (*64*) revealed a Pro-rich putative SH3 domain binding site (*65*), two additional Pro-rich sequences, and a pleckstrin homology (PH) domain (*66, 67*), in addition to the originally isolated SH2 domain (*61*) as shown in Figs. 3 and 4. Ten Tyr residues were identified, however, none of them was found in a YMXM or YXXM IR Tyr phosphorylation substrate motif (*22, 23*). In addition, a YVPS motif (*68*), two YXXL motifs (*69, 70*), and a motif (S S) displaying significant similarity to the major IR Ser phosphorylation sites 1074 and 1078 (*71*) were identified (Figs. 3 and 4).

Interaction between Purified Insulin Receptors and the PSM SH2 Domain—Originally, the association of the PSM SH2 domain with IR and its dependence on IR kinase activation had been demonstrated by yeast molecular genetics in the two-hybrid system (Fig. 1). We investigated the association biochemically by preparing GST PSM SH2 domain fusion protein and testing for its association with IR *in vitro*. GST PSM SH2 fusion protein was partially purified from *E. coli* and mixed with wheatgerm-agglutinin affinity-purified IR from overexpressing CHO cells. IR co-precipitated with glutathione Sepharose beads as detected in immunoblots (Fig. 5). The association was dependent on an activated IR tyrosine kinase and was stable also at high salt concentrations (300 mM NaCl) as expected for a specific non-covalent PSM-IR association. In addition, the PSM SH2 domain association in the two-hybrid screen was only observed with an active but not with an inactivated insulin receptor cytoplasmic domain under *in vivo* conditions in the yeast nucleus. The association observed by both experimental strategies suggests that PSM binds directly to IR without the involvement of an intermediate protein.

Interaction of PSM SH2 Domain Fusion Proteins with Insulin Receptors from Cell Lysates—Having shown the

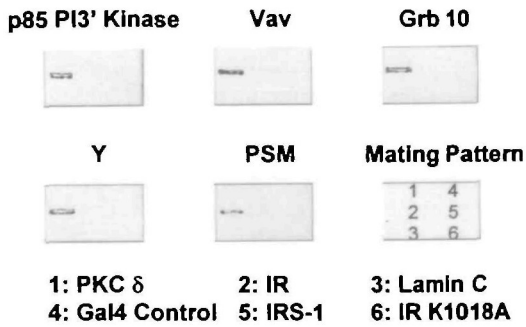

Fig. 1. **Two-hybrid prey-bait interaction tests.** Library (prey) plasmids were isolated from His prototroph and beta-galactosidase positive yeast colonies based on their interaction with cytoplasmic insulin receptor (IR) baits. Plasmids were introduced into *E. coli*, sequenced and re-introduced into *Saccharomyces cerevisiae*. Transformed L40 strains were individually crossed with five defined bait-expressing AMR70 yeast strains of the opposite mating type, and diploid strains were selected to test for the specificity of the underlying prey-bait interactions (*58*). Each plate represents five bait constructs which were tested against one prey. Diploid cells were transferred to nitrocellulose, tested for blue beta-galactosidase-mediated color and photographed after 1 h. The tested baits are: 1, protein kinase C delta (PKC delta); 2, insulin receptor cytoplasmic fragment (IR); 3, test bait lamin C; 4, Gal4 bait control plasmid, 5, IRS-1; 6, catalytically inactive mutant insulin receptor cytoplasmic fragment (IR K1018A). The isolated and tested library plasmids (preys) contained cloned SH2 domains (except for Y) of the following proteins: (a) the regulatory subunit p85 (C-terminal SH2 domain) of phosphatidylinositol 3′-kinase (p85 PI 3′-kinase), (b) the transforming protein Vav, (c) the signaling mediator Grb10/IR, (d) a novel sequence Y lacking SH2 or PTB domains and similarity to other proteins. (e) PSM.

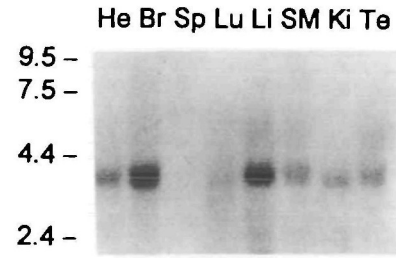

Fig. 2. **Northern analysis of PSM expression in mouse tissues.** Poly A$^+$ mouse mRNA separated by electrophoresis and transferred to a nylon membrane was probed with the ^{32}P-labeled PSM SH2 domain encoding two-hybrid library insert. The employed tissues are heart (He), brain (Br), spleen (Sp), lung (Lu), liver (Li), skeletal muscle (SM), kidney (Ki), and testis (Te). Size markers for the transcripts are indicated in kb.

A

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  1 MNGAPSPEDGVFPSPPALPPPPPSWQEFCESHARAAALDLARRFRLYLA 50
 51 SHPQYAEPGAEAAFSGRFAELFLQHFEAEVARASGSLSPPVLAPLSPGVE 100
101 IPPSHDLSLESCRVGGPLAVLGPSRSSEDLAGPLPSSVPSSTTSSKPKLK 150
151 KRFSLRSVGRSVRGSVRGILQWRGAVDSPSQAGPLETTSGPPVLGGNSNS 200
201 NSSGGAGTVGRALANDGTSPGERWTHRFERLRLSRGGGTLKDGAGMIQRE 250
251 ELLSFMGAEEAAPDPAGVGRGGGAAGLTSGGGGQPQWQKCRLLLRSEGEG 300
301 GGGSRLEFFVPPKASRPRLSIPCSTITDVRTATALEMPDRENTFVVKVEG 350
351 PSEYILETSDALHVKAWVSDIQECLSPGPCPAISPRPMTLPLAPGTSFFT 400
401 KDNTDSLELPCLNHSESLPSQDLLLGPSESNDRLSQGAYGGLSDRPSASF 450
451 SPSSASIAASHFDSMELLPPELPPRIPIEEGPPAGTVHPLSTPYPPLDTP 500
501 EAATGSFLFQGESEGGEGDQPLSGYPWFHGMLSRLKAAQLVLEGGTGSHG 550
551 VFLVRQSETRRGEYVLTFNFQGKAKHLRLSLNEEGQCRVQHLWFQSIFDM 600
601 LEHFRVHPIPLESGGSSDVVLVSYVPSQRQQERSTSRDPAQPSEPPPWTD 650
651 DASSTLLPFGASDCVTEHLP 670   VARIANT: GREQAGSHAGVCEGDRCYP 650
651 PPHPGAEEASGAPEVAAATAAAAKERQEKEKAGSGGVQEELVPVAELVPM 700
701 VELEEAIAPGTEAQGGAGSSGDLEVSLMVQLQQLPLGGNGEEGGHPRAIN 750
751 NQYSFV 756
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B

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..CAG CAG GAA CGG AGC ACC TCC CGT GA..
              |
              GC CGG GAG CAG GCT GGG AGC CAT GCA
GGG GTG TGC GAG GGC GAC CGA TGC TAC CCC GAT
GCC TCC TCC ACC CTC CTG CCC TTC GGA GCG AGT
GAC TGT GT
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Fig. 3. **A: Amino acid sequence of two mouse PSM variants.** The cloned two-hyrbrid library insert including the PSM SH2 domain was used as a hybridization probe to screen a mouse brain lambda gt10-derived cDNA library. The PSM protein-coding region was isolated from several overlapping clones and the deduced amino acid sequence is displayed from the putative initiation codon (aa 1) to the last aa (756) before the termination codon. For comparison only those aa of the related rat sequence SH2-B (*73*) that differ from mouse PSM have been indicated above the PSM aa sequence while identical aa have been omitted. An identified mouse sequence variant (VARIANT) is shown in italics which carried an additional 100 bp coding sequence and resulted in a reading frame shift and premature termination of the variant protein sequence (670 aa). Putative Tyr or Ser phosphorylation sites have been double-underlined and Pro-rich sequence motifs have been underlined. The SH2 domain is indicated by a dotted line and the PH domain by a hatched line. **B: Nucleotide sequence of the mouse PSM variant region.** The master PSM coding region is shown in the top line with its reading frame indicated by blank spaces. The inserted variant sequence is shown below the horizontal line and its position of insertion is marked by the vertical line. The altered reading frame of the master sequence downstream of the inserted variant sequence is indicated by alternating dotted and continuous underlining until it reaches a termination codon. The Genbank accession number is AF020526.

association between the PSM SH2 domain and the activated IR kinase *in vivo* in the yeast two-hybrid system and *in vitro* between partially purified proteins, we determined whether an association could be demonstrated in whole cell extracts and whether the major insulin receptor substrate IRS-1 would participate in such a putative complex. Detergent extracts of CHO cells expressing about 10^6 human insulin receptors per cell were prepared and the presence and insulin-dependent Tyr phosphorylation of IR and IRS-1 was demonstrated by a combination of IR, IRS-1, and phospho Tyr-specific antibodies in immunoblots (Fig. 6). In these experiments IR and IRS-1 were shown to be Tyr phosphorylated only in response to insulin stimulation while another phosphoprotein of slightly larger MW than IRS-1 was found to be Tyr phosphorylated independently of insulin stimulation (Fig. 6). For IRS-1 an increased MW was observed after insulin stimulation which has been noted earlier and is explained by its Tyr phosphorylation on multiple sites by the IR (*22*). When GST PSM SH2 domain

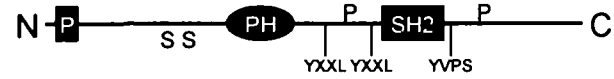

Fig. 4. **Domain structure of PSM.** The primary structure of PSM has been analyzed for sequence motifs between the amino (N) and carboxyl (C) termini of the protein. A Pro-rich putative SH3 domain binding site (P), a pleckstrin homology (PH) region, and the Src homology-2 (SH2) domains have been indicated by white letters on black spaces. In black letters, additional Pro-rich sequences are represented by P, putative Tyr phosphorylation motifs by YXXL and YVPS, and a putative two-Ser phosphorylation target motif of the insulin-dependent and insulin receptor-associated Ser kinase has been indicated by S S.

fusion protein was added and upon insulin stimulation, co-precipitation of Tyr phosphorylated IR with glutathione Sepharose was demonstrated while IRS-1 was not detected under any circumstances (Fig. 6). This is compatible with a functional mechanism of PSM which is IRS-1 independent

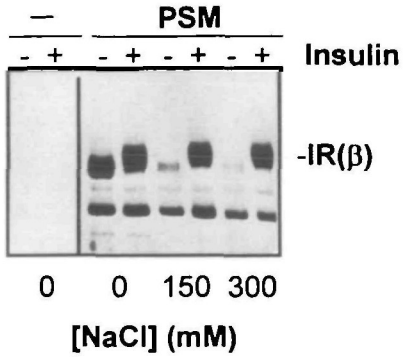

Fig. 5. **Direct association between PSM SH2 domain and insulin receptor *in vitro*.** Glutathione Sepharose-purified GST PSM SH2 (PSM) or control GST (—) was incubated with insulin-stimulated (+) or inactive (−) wheatgerm agglutinin affinity-purified IR. Complexes formed in the presence of varying concentrations of NaCl (as shown) were precipitated with glutathione Sepharose. Proteins were separated by SDS PAGE, transferred to nitrocellulose and identified by immunoblotting with specific antibodies directed against the insulin receptor beta subunit. The position of the insulin receptor β subunit [IR(β)] has been marked on the right.

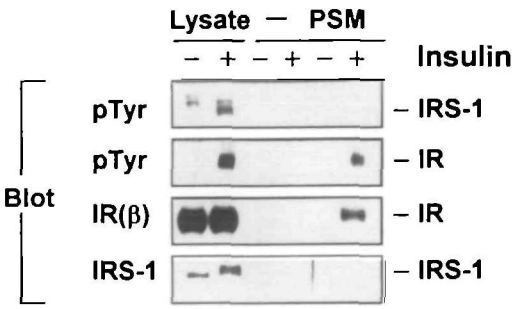

Fig. 6. **Insulin receptor association with PSM in the absence of IRS-1.** CHO fibroblasts overexpressing the insulin receptor were stimulated with insulin (+) or were left untreated (−). Detergent extracts (Lysate) were immediately separated by SDS PAGE or were first mixed with control GST (—) or GST PSM SH2 (PSM) and complexes were precipitated with glutathione Sepharose prior to SDS PAGE. Proteins were transferred to nitrocellulose and identified by immunoblotting (Blot) with phosphotyrosine antibodies (pTyr) or with specific antibodies directed against the insulin receptor β subunit [IR(β)] or against IRS-1. The position of the insulin receptor β subunit (IR) and of IRS-1 have been marked on the right.

and may act in parallel to IRS-1. The future mapping of the IR site that associates with PSM should address whether there may be competition between PSM and IRS-1 for insulin receptor binding (*72*).

DISCUSSION

With an activated cytoplasmic fragment of the IR as a bait we identified several SH2 domain encoding sequences in a yeast two-hybrid screen (Fig. 1). Most of the identified proteins including p85 PI 3′-kinase, Vav, and Grb10/IR had also been shown to associate with the IR by alternative experimental strategies (*13, 15-19, 26, 27*) which demonstrated the feasibility of our approach to identify direct IR targets. An SH2 domain-encoding sequence which had not previously been described to interact with the IR was identified and found to associate with an active but not with an inactivated IR cytoplasmic bait (Fig. 1). The cloned sequence identified a 4 kb mRNA by Northern analysis which was most abundant in mouse liver, brain, heart, and detectable in skeletal muscle, testis, kidney, and lung (Fig. 2). This is overall compatible with a putative role of the protein product in insulin action. However, the increased abundance of the message in liver and brain is notable and may help to establish its cellular functions in future experiments.

The cloned sequence served as a hybridization probe to isolate several overlapping clones from a mouse lambda gt10-derived cDNA library and to deduce the primary structure of the complete protein-coding sequence (Fig. 3). The pleckstrin homology (PH) region (*66, 67*) and the Pro-rich putative SH3 domain binding region (*65*), which were found in addition to the SH2 domain (*61*), may mediate the association of the cloned protein with other SH3 domain containing signaling mediators and cellular membranes, respectively (Fig. 4). It may function as a mediator of insulin action by acting as an adapter in the IR signaling complex. It has consequently been termed PSM. The lack of any YXXM or YMXM motifs among the ten Tyr found in the primary structure suggests that PSM may not be phosphorylated on Tyr by the insulin receptor Tyr kinase (*22, 23*) similar to many signaling adapter proteins which may function by forming complexes with other proteins and by directing their intracellular localization (*52*). Initial experiments with phosphotyrosine-specific antibodies have failed to correlate PSM candidate bands with phosphotyrosine-containing protein bands in response to insulin stimulation (not shown). However, the identity of cellular PSM remains to be established. A sequence motif containing two Ser in a context with high similarity to the major phosphorylation site of the insulin-dependent and insulin receptor-associated Ser kinase suggests that insulin-stimulated phosphorylation on Ser may play a role in PSM function (*71*).

PSM is likely to participate in alternative signaling pathways which have also been demonstrated for any other established insulin receptor target. A YVPS motif has been identified in a sequence context which shares high similarity with identified PDGF receptor phosphorylation sites in particular at the +1, +2, and +3 positions (*68*) to suggest that PSM may be a substrate of the PDGF receptor Tyr kinase. The unknown tertiary structure of PSM may bring the two identified YXXL motifs which resemble immunoreceptor Tyr-based activation motifs (ITAMs) into close proximity. It is possible that they are, like ITAMs, phosphorylated by Src family Tyr kinases (*69, 70*) and may subsequently serve as binding sites for other signaling mediators such as Zap-70 in immunoreceptor signaling mechanisms (*73*). Two Pro-rich sequences have been identified in the PSM primary structure in addition to the putative SH3 domain binding site, the significance of which remains to be determined.

During the cloning of the PMS protein-coding region a sequence termed SH2-B was reported in the rat which had been identified in a modified two-hybrid screen based on its association with a Tyr phosphorylated Fc epsilon RI receptor gamma chain (*73*). The small number of 12 aa differences observed between PSM and SH2-B, many of which represent conservative exchanges may be explained by

species-specific differences of mouse *versus* rat and suggest that PSM may represent the mouse homolog to SH2-B. This would also support the idea of an additional role for PSM in other signaling pathways as outlined above. The high level of sequence similarity between mouse and rat suggests an important functional role of PSM in the cell. The reported Northern analysis of the rat sequence SH2-B (*73*) differed significantly from the PSM Northern analysis in the mouse (Fig. 2). This could suggest that PSM and SH2-B do not in fact represent homologous genes or alternatively that the observed differences in tissue distribution are explained by species variations, potential differences in developmental stages of the employed tissues, or by differences in the distribution of sequence variants.

Several distinct PSM sequence variants were repeatedly identified at a site downstream of the SH2 domain (Fig. 3B). One sequence variant carried an additional 100 nucleotide sequence at this site. This variant was found in the two-hybrid mouse embryo library as well as in multiple independent isolates in the mouse brain lambda gt10-derived library. The 5′ end of the sequence insert displays the nucleotides G at +1, C at +2, and G at +5 sequence positions (Fig. 3B). These nucleotide positions have been described with 100% frequency at the 5′ insert boundary of documented variant splice sites whereas no consensus sites have been established for the 3′ boundary of the insert (*62*). When combined these findings suggest that the sequence presented in Fig. 3B represents a PSM splice variant. When inserted, the additional sequence results in a distinct carboxyl terminus and in a shortened protein product of 670 aa with potentially altered functional characteristics due to the altered carboxyl terminus (Fig. 3B). Given that PSM was cloned from whole embryo and brain libraries which have been derived from a variety of cell types it appears that distinct forms of PSM exist, which may correlate with distinct cell types and likely result from differential splicing events. Our data are compatible with the idea that PSM and its variants represent a new class of signaling adaptors which are expected to play roles in various signaling pathways and lack substantial overall sequence similarity to other signaling mediators.

The association observed between the activated catalytic fragment of the IR and the PSM SH2 domain *in vivo* in the yeast nucleus (Fig. 1) and *in vitro* between partially purified IR and GST PSM SH2 fusion protein (Fig. 5) indicates a direct, non-covalent association, which is stable at high (300 mM NaCl) salt concentration, and dependent on the activation of the IR kinase. While the presence of intermediate protein which may mediate the PSM-IR association indirectly cannot be ruled out in either cell context, it is unlikely that the same specific mediator would be present and active at either experimental condition. The association was also demonstrated in detergent whole cell extracts in the presence of most cellular components (Fig. 6). The PSM-IR association was dependent on the kinase activation of the IR while the major insulin receptor substrate IRS-1 was not detected in the PSM-IR complex, independent of its state of activation.

Given that IRS-1 has been shown to participate in various insulin actions, PSM appears to be involved in a parallel signaling mechanism. Future experiments will address the specific site of PSM association on the IR primary structure which, if it involves the juxtamembrane region, may overlap with the IRS-1 binding site and result in a possible competition of both molecules for IR binding (*72*). PSM may participate in insulin responses which are not or only partially IRS-1 dependent (see "Introduction"). Alternatively, PSM may negatively regulate insulin action by counterbalancing IRS-1, IRS-2, Shc-mediated, or other signals. There are few precedents for such a function which may, however, provide important regulatory controls for many aspects of the insulin signal in analogy to the opposing actions of Tyr kinases and phosphatases (*3*). A negative regulatory role in insulin action has recently been proposed for the IR target Grb-IR as a first example for such a mechanism (*15*). Since relatively little is known about the role of the insulin-stimulated and IR-dependent Ser kinase (*71*) PSM may be a mediator of these signals compatible with the identified putative target site for this kinase in its primary structure. Future experiments will begin to address these alternative mechanisms.

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