Identification of the APS Protein as a Novel Insulin Receptor Substrate*

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Insulin binding to its receptor results in tyrosine autophosphorylation of the β-subunit. This results in an active insulin receptor that is able to phosphorylate several cytoplasmic proteins on tyrosine residues. The tyrosine-phosphorylated insulin receptor interacts with downstream docking proteins that appear to be essential for insulin signaling. Phosphorylation of these adapter proteins induces their association with proteins containing SH2 domains, resulting in activation of a variety of downstream signaling pathways. Examples of such adapter proteins include the insulin receptor substrates (IRS) 1, 2, 3, and 4 and the SHC proto-oncogene product. These adapter proteins appear to link the insulin receptor to at least two signaling pathways, the phosphatidylinositol 3-kinase (PI3K) pathway and the Ras-mitogen-activated protein kinase pathway. It remains unclear whether these adapters constitute the full complement of signaling intermediates utilized by the insulin receptor. In fact, much recent evidence has begun to accumulate to suggest that the known signaling pathways that emanate from the IR may be insufficient to fully explain insulin-mediated metabolic regulation, and in particular the regulation of GLUT4 translocation.

In order to identify novel substrates involved in insulin receptor signaling, a yeast two-hybrid 3T3-L1 adipocyte cDNA library was screened with the cytoplasmic domain of the human insulin receptor as bait. Here we describe the isolation and characterization of an interacting protein, APS, which contains pleckstrin homology and Src homology 2 domains and several potential tyrosine phosphorylation sites. APS mRNA and protein are expressed primarily in skeletal muscle, heart, and adipose tissue, and in differentiated 3T3-L1 adipocytes. We show that APS associates with phosphotyrosines situated within the activation loop of the insulin receptor via the APS Src homology 2 domain. Insulin stimulation of 3T3-L1 adipocytes resulted in rapid tyrosine phosphorylation of endogenous APS on tyrosine 618, whereas platelet-derived growth factor treatment resulted in no APS phosphorylation. In summary, we have identified a new insulin receptor substrate that is primarily expressed in insulin-responsive tissues and in 3T3-L1 adipocytes whose phosphorylation shows insulin receptor specificity. These findings suggest a potential role for APS in insulin-regulated metabolic signaling pathways.

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1 The abbreviations used are: SH2, Src homology domain 2; IR, insulin receptor; APS, adapter protein containing a PH and SH2 domain; PH, pleckstrin homology; IRS, insulin receptor substrate; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PIP2, phosphatidylinositol 3,4,5-triphosphate; PDK1, PIP2-dependent protein kinase 1; CHO, Chinese hamster ovary; PDGF, platelet-derived growth factor; GST, glutathione S-transferase; PVDF, polyvinylidene difluoride; kbp, kilobase pair(s); PBS, phosphate-buffered saline.
tively activated form of p110 or a form of p110 that was targeted to GLUT4 vesicles in 3T3-L1 adipocytes was analyzed. These manipulations resulted in PI3K activities that exceeded insulin-stimulated levels and increased GLUT4 translocation and glucose transport, yet these effects were significantly reduced when compared with insulin (19, 20). In other experiments, PI3K was activated in 3T3-L1 adipocytes using thio-phosphorylated peptides corresponding to the binding motif of PI3K within IRS1; again, only a modest increase in GLUT4 translocation was observed (21). Other studies have shown that, although growth factors such as PDGF can activate PI3K activity in 3T3-L1 adipocytes, this is not sufficient to stimulate significant GLUT4 translocation to the plasma membrane (22, 23). In other experiments, interleukin-4 stimulation of L6 myoblasts overexpressing the interleukin-4 receptor induced IRS1 tyrosine phosphorylation and increased PI3K activity to the same degree as insulin but had no effect upon glucose uptake (22). More recently, a mutant PKB protein in which the regulatory phosphorylation sites were substituted with alanine was shown to exert a dominant negative effect on endogenous PKB activity stimulated by insulin. Expression of this dominant negative PKB in 3T3-L1 adipocytes had no effect on insulin-induced glucose uptake or GLUT4 translocation but was found to decrease the rate of protein synthesis and inhibit the ability of insulin to activate p70S6 kinase (24). These data strongly suggest that PKB may not be necessary for insulin-dependent GLUT4 translocation. More recently it was found that introduction of membrane-permeant esters of PIP3 into 3T3-L1 adipocytes by themselves did not stimulate glucose uptake or GLUT4 translocation to the plasma membrane. However, these esters were able to reverse the wortmannin blockade of insulin-stimulated GLUT4 translocation (25). This suggests that PIP3 is necessary but not sufficient and raises the possibility that additional signaling pathways may originate from the insulin receptor to mediate the full response. In other experiments, microinjection of competitive inhibitory IRS1 proteins, which effectively blocked the interaction of the IR with endogenous IRS1, had no effect upon GLUT4 translocation or glucose uptake in response to insulin (8). These same proteins blocked insulin-dependent membrane ruffling and mitogenesis, suggesting that IRS-1 was critical for some insulin actions but not for GLUT4 translocation. In summary, the current data strongly support an essential role for PI3K in the regulation of glucose uptake by insulin. However, the data from a variety of studies outlined above suggest the possibility that other signaling pathways may exist that emanate specifically from the insulin receptor that are responsible for the full metabolic response of a muscle or adipose cell to insulin.

In this paper we report the cloning of mouse APS, which was shown to interact strongly with the insulin receptor. APS mRNA and protein were expressed predominantly in skeletal muscle, heart, fat, and 3T3-L1 adipocytes. The SH2 domain of APS interacted with the activation loop of the kinase domain of the insulin receptor. Endogenous APS becomes robustly tyrosine-phosphorylated on tyrosine 618 after insulin treatment of 3T3-L1 adipocytes. These results are suggestive of a physiological role for APS in insulin signaling in the insulin-responsive tissues muscle and fat.

**EXPERIMENTAL PROCEDURES**

**Cell Lines—**CHO-IR cells overexpressing the human insulin receptor (26) were grown in F12 nutrient medium containing 10% fetal bovine serum and antibiotics. 3T3-L1 fibroblasts were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics. Differentiation to adipocytes was induced as described previously (9). The cells were then cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics for 8–12 days. Before growth factor stimulation the cells were serum-starved for 2–18 h. Unless otherwise indicated, 100 nM insulin was added directly to the medium and the incubation was continued at 37 °C for the indicated times.

**Plasmid Constructions—**Full-length and subdomains of APS were subcloned into either the two-hybrid plasmid pBluescript II KS+ or the mammalian expression plasmid, pcDNA3.1 His (Invitrogen), pcDNA5 (American Pharmacia Biotech), or pET28 (Novagen) using standard methods. All site-directed mutants were generated by QuikChange kit (Stratagene).

**Yeast Strains and Plasmids—**S. cerevisiae EGY48 (a-trp1, ura3-52, his3, leu2) and all expression plasmids were provided by the laboratory of Roger Brent and have been described previously (9, 27–31). All procedures for routine growth and maintenance of yeast strains have been described previously. Plasmid transformation of yeast was by the lithium acetate method (32). The insulin receptor and insulin-like growth factor 1 receptor cDNA fusions have been reported previously (30, 31). The colony color β-galactosidase assay was performed as described (30). A 3T3-L1 adipocyte cDNA was generated with the ZAP cDNA synthesis kit (Stratagene) and subcloned in the pG4–5library vector.

**Northern Blots—**Northern blot analysis was performed on commercial human and rat multiple tissue poly(A)⁺ RNA blots (CLONTECH), or, purified poly(A)⁺ RNA prepared from either 3T3-L1 fibroblasts or adipocytes. For the Northern blots shown in Fig. 2, probes were generated by polymerase chain reaction and labeled by nick translation. Hybridization was performed overnight at 60 °C in 5% formamide, 1× sodium acetate, 0.5 M sodium phosphate (pH 7.2), 7% SDS, 100 μ M EDTA, and 50 μg ml⁻¹ denatured salmon sperm DNA with high stringency washes.

**In Vitro Interaction Studies—**A GST fusion protein was generated by introducing the APS cDNA fragment corresponding to the SH2 domain (amino acids 408–507) into the pGEX5X expression plasmid. After transformation of DH5α, induction with 1× isopropyl-l-thio-β-D-galactopyranoside, cell collection, and lysis by sonication, the proteins were purified using immobilized glutathione-agarose beads. Serum-starved cultured CHO-IR cells were stimulated with 100 nM insulin for 0, 5, 15, or 30 min at 37 °C, washed twice with ice-cold 1× PBS, and solubilized with lysis buffer (1× PBS supplemented with 1% Nonidet P-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml each of aprotinin, leupeptin, and pepstatin, 1 mM sodium vanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate). The samples were homogenized and clarified by centrifugation, and incubated (500 μg of total protein/reaction) for 2 h at 4 °C with 3–5 μg of immobilized GST fusion protein. After extensive washing with ice-cold HNTG buffer (10 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol), the proteins co-associating with the GST fusion protein were separated by SDS-polyacrylamide gel electrophoresis, transferred to PVDF membrane (Amersham Pharmacia Biotech), and immunoblotted as described below with either anti-phosphotyrosine antibody 4G10 or antibodies against the β subunit of the insulin receptor.

**Transient Transfection of CHO-IR Cells—**CHO-IR cells were transiently transfected with 5 μg of the various APS constructs subcloned in the mammalian expression plasmid pcDNA3.1 His (Invitrogen) by LipofectAMINE according to manufacturer’s instructions (Life Technologies, Inc.). After 48 h, the cells were serum-starved overnight and then stimulated by the addition of 100 nM insulin for 5 min. Lysates were prepared as described under “Immunoprecipitation and Immunoblots.”

**Immunoprecipitation and Immunoblots—**Cells treated as described under “Results and Discussion” were washed twice with ice-cold 1× PBS and then lysed in 1 ml of lysis buffer. After a 30-min centrifugation at 4 °C to remove the insoluble material, proteins of interest were immunoprecipitated with either mouse monoclonal antibodies or rabbit polyclonal antibodies coupled to protein A/G-Sepharose (Santa Cruz Biotechnology, Santa Cruz, CA) during a 2–3-h incubation at 4 °C with constant mixing. The immune complexes were washed three times with ice-cold HNTG buffer. Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membrane (Amersham Pharmacia Biotech). Membranes were incubated for 1 h at room temperature in TBST buffer (Tris-HCl, pH 7.4, NaCl, 0.01% Tween 20) containing 5% nonfat dry milk and blotted with specific antibodies in TBST containing 5% bovine serum albumin for 2 to 3 h. Immunoreactive bands were detected by ECL immunodetection system (Pierce).

**Antibodies—**Anti-phosphotyrosine antibody 4G10 was purchased from Upstate Biotechnology (Lake Placid, NY), anti-insulin receptor antibody was purchased from Santa Cruz Biotechnology, and anti-XPRESS mouse monoclonal was purchased from Invitrogen.

Fusion protein was produced in Escherichia coli from a pGEX5X (Amersham Pharmacia Biotech) expression plasmid containing the COOH domain (amino acids 508–591) of APS. The GST fusion protein was...
was purified by standard procedures. Immunizing rabbits with the purified GST fusion raised anti-APS antisera.

**Protein Determination and Gel Analysis**—Protein assays were performed by modified Lowry method (33). SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli (34).

**RESULTS AND DISCUSSION**

Identification of APS as an Insulin Receptor Interactor Using the Yeast Two-hybrid System—To be able to identify new substrates of the insulin receptor that may be important components in metabolic signaling pathways, we carried out a yeast two-hybrid screen with the cytoplasmic domain of the human insulin receptor as bait and searched for interacting proteins within a 3T3-L1 adipocyte cDNA library. Of $4 \times 10^6$ transformants, 90 colonies showed specific interaction with the insulin receptor. Sequence analysis of the rescued plasmids revealed that 17 encoded a recently identified adapter protein termed APS (adapter protein containing PH and SH2 domain). The sequence of the longest clone contained an insert of 2832 base pairs. Analysis of potential coding sequences suggested a protein of 621 amino acids with a predicted molecular weight of 66,553. Analysis of the predicted amino acid sequence revealed a PH domain encoded by amino acids 197–299 and an SH2 domain between amino acids 408 and 507. Two potential tyrosine phosphorylation sites were located at amino acids 46 (NPX) and 618 (ENQY). Alignment of the mouse APS amino acid sequence with the published human and a partial rat cDNA (that we also cloned in an insulin receptor two-hybrid screen with a skeletal muscle library) revealed that there was significant conservation between species (Fig. 1). The only marked difference between these proteins was within the amino-terminal domain where two short stretches are missing from the mouse: amino acids 12-16 (APVPV), amino acids 84–89 (GPTTRG) of the human APS, and one short stretch missing from the human: amino acids 161–164 (PASE) of mouse APS.

APS is a recently described protein whose function is unknown. APS was first described to interact with an oncogenic mutant of the tyrosine kinase receptor, c-Kit (35).
immunoreceptor signaling induced tyrosine phosphorylation of APS in response to a variety of cytokines including stem cell factor. As reported previously, APS is similar to but not identical to SH2B and LNK (35). The PH domains of SH2B and LNK are 58% and 40% identical to APS, whereas the SH2 domains of SH2B and LNK are more similar with 80% and 72% identity, respectively. The LNK protein that has been reported may not represent the complete cDNA or may be a result of alternative splicing or start codon usage. Comparison of the remaining sequences of the three proteins outside either the PH or SH2 domain showed little sequence similarity. However, one of the potential tyrosine phosphorylation sites at the carboxyl terminus of APS, tyrosine 618, is conserved in both SH2B (amino acid 753) and LNK (amino acid 297). SH2B was originally cloned from mast cells because of its ability to bind in a yeast tribrid system to the tyrosyl-phosphorylated γ subunit of the high affinity immunoglobulin E (IgE) receptor (36). SH2B has also been reported to bind to tyrosine-phosphorylated JAK2 upon growth hormone stimulation of 3T3-F442A adipocytes (37). These data suggest that growth hormone activation of JAK2 recruits SH2B, thereby initiating an as-yet-unidentified signal transduction pathway. The second family member, LNK, is tyrosine-phosphorylated upon T-lymphocyte activation by antibody-mediated cross-linking of the T cell receptor and CD4. LNK has been suggested to provide a link between the activated T cell receptor and phosphatidylinositol 3-kinase, phospholipase Cγ1, and Ras signaling pathways through a multifunctional tyrosine phosphorylation site within the protein (38, 39). Thus, APS, SH2B, and LNK appear to be a family of adapter molecules involved in tyrosine kinase signaling in hematopoietic cells.

Expression of APS in Tissues and Cell Lines—To determine the tissue distribution of APS mRNA, both human and rat multiple tissue Northern blots were hybridized with a probe generated against the SH2 domain of APS. Two APS mRNA transcripts were detected with tissue specificity and variation in their relative abundance (Fig. 2A). The transcripts were approximately 1.9 and 2.9 kbp in size. Both were expressed with equal intensity in poly(A)+ mRNA prepared from either rat or human skeletal muscle. However, the lower RNA band of approximate 1.9 kbp was absent in other tissues examined. The significance of the multiple mRNA bands remains to be determined.

Since the full-length APS cDNA was isolated from a 3T3-L1 adipocyte cDNA library, we examined the expression of APS mRNA in 3T3-L1 cells. Northern blot analysis of poly(A)+ RNA isolated from fibroblasts and fully differentiated adipocytes was performed with a cDNA probe encoding the SH2 domain of APS. As shown in Fig. 2B, a transcript of 2.9 kbp was observed in RNA prepared from both fibroblasts and adipocytes. This corresponds well to the size of the full-length cDNAs that we identified in the two-hybrid assay, the longest of which was 2833 base pairs. APS mRNA expression was highest in the adipocytes. The blot was stripped and rehybridized with a GLUT4 probe. In agreement with published data, GLUT4 expression was highest in the adipocytes.

In Vitro Interaction of the APS SH2 Domain with the Insulin Receptor—To further examine the interaction of APS and the insulin receptor, we produced a GST fusion protein that contained the SH2 domain of APS (amino acids 408–507). We examined the ability of this GST fusion protein to interact with proteins in lysates derived from unstimulated or insulin-stimulated CHO cells that overexpress the insulin receptor. After incubation of the immobilized GST protein with cellular extracts, the samples were extensively washed and the coprecipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotted with an anti-insulin receptor antibody (Fig. 3A, upper panel). The blots were then stripped and rebotted with an anti-phosphotyrosine antibody (Fig. 3A, lower panel). GST-SH2 APS fusion protein precipitated the insulin receptor from these lysates in a time-dependent manner. The coprecipitation was dependent upon prior stimulation of the insulin receptor, as shown by the lack of association between these two proteins in unstimulated lysates.

Recent work has suggested that the SH2 domain of SH2B, an APS-related protein, was able to interact with the insulin receptor (40, 41). We therefore produced GST fusion proteins that contained the SH2 domains of APS, SH2B, or LNK and directly compared the ability of these GST fusion proteins to interact with the cytoplasmic domain of the insulin receptor in lysates derived from unstimulated or insulin-stimulated 3T3-L1 adipocytes (Fig. 3B). As shown previously, the GST-SH2 APS fusion protein was found to bind the insulin receptor in an insulin-dependent manner. Using equivalent amounts of GST fusion (5 µg), neither the GST-SH2 SH2B or GST-SH2 LNK was able to interact with the receptor to the same degree as the SH2 domain of APS. This difference between our results and the previous studies may be due to the higher amounts of GST fusion proteins were used (10 µg), which revealed a weak interaction between both SH2B and LNK with the insulin receptor (data not shown).

Characterization of the Site of Interaction between APS and the Insulin Receptor—To further analyze insulin receptor binding to APS, we mapped the site of interaction between these two proteins using a detailed two-hybrid analysis with a variety of insulin and the insulin-like growth factor receptor mutants. We detected strong interaction between APS and wild-type insulin receptor and insulin-like growth factor receptor as
Insulin Receptor Phosphorylation of APS

**Fig. 3.** In vitro interaction of the APS SH2 domain with insulin receptor. GST fusion proteins corresponding to the SH2 domain of APS, SH2B, and LNK were assayed for the ability to interact in vitro with the IR expressed in CHO-IR cells or 3T3-L1 adipocytes. A, cellular extracts (500 μg) were prepared from CHO-IR cells, stimulated with or without insulin (100 nM) for the times indicated and incubated with 3 μg of GST-SH2 fusion protein of APS. Samples were resolved by 8% SDS-polyacrylamide gel electrophoresis, transferred to PVDF and immunoblotted (IB) with anti-insulin receptor antibodies (IR), and then stripped and reprobed with anti-phosphotyrosine antibodies (PY). An arrow on the right denotes the SH2 domain plasmid pJG4–5 and assayed for interaction with the wild-type insulin receptor (WTIR) in the yeast two-hybrid system. Transformants were assayed by colony color, and the data represent an average from a minimum of three independent colonies. ++++ indicates strong interaction (dark blue), + indicates weak interaction (faint blue), and – refers to no interaction (white).

**Fig. 4.** Two-hybrid insulin receptor-APS interaction. Full-length and various domains of APS were cloned in the activation domain plasmid pJG4–5 and assayed for interaction with the wild-type insulin receptor (WTIR) in the yeast two-hybrid system. Transformants were assayed by colony color, and the data represent an average from a minimum of three independent colonies. ++++ indicates strong interaction (dark blue), + indicates weak interaction (faint blue), and – refers to no interaction (white).

The fact that the interaction of APS with the insulin receptor required tyrosine phosphorylation of the insulin receptor suggested that the SH2 domain within APS was the domain that bound the autoactivation loop of the insulin receptor. Although this interaction was probably direct, it remained possible that additional region(s) of APS were also important. To investigate whether the SH2 domain of APS was critical, we substituted this motif has been shown previously to lead to the loss in the ability of SH2 domains to bind to phosphotyrosine. As shown in Fig. 4, the wild-type full-length APS interacted strongly with the insulin receptor as determined by colony color studies. We found that the point mutation within the SH2 domain (R437K) of the full-length APS almost completely abrogated the binding of APS to insulin receptor. Point mutations within either the PH domain (W290L), which would be predicted to destroy lipid binding, or the putative tyrosine phosphorylation site (Y618F) had no effect on the interaction. Furthermore, a series of APS hybrids were generated and tested for insulin receptor interaction using the two-hybrid assay: the amino-terminal domain (amino acids 1–196), the PH domain (197–299), the SH2 domain (408–507), the carboxy-terminal domain (508–621), the SH2 domain plus the carboxy-terminal domain lacking the tyrosine phosphorylation site (408–591), and the SH2 domain plus the full carboxy terminus (408–621). Only the proteins that contained the intact SH2 domain were able to interact efficiently with the insulin receptor (Fig. 4). These results suggest that the SH2 domain alone is sufficient for interaction.

**Antibodies and Tissue Expression**—In order to be able to characterize the endogenous APS protein, rabbit polyclonal antibodies were raised against the carboxy-terminal (amino acids 507–591) domains of APS. Lysates prepared from 3T3-L1 adipocytes were immunoprecipitated with the anti-APS antibodies. The immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis and immunoblotted. Proteins with a molecular mass of between 60 and 70 kDa were detected in 3T3-L1 adipocyte lysates with a major band at approximately 70 kDa (Fig. 5A). These protein bands were not observed in immunoprecipitates with any of the preimmune sera tested (data not shown). The multiple bands may represent degraded protein or alternatively spliced variants of APS expressed in 3T3-L1 adipocytes. In agreement with the Northern blot data, which showed induction of APS mRNA levels in differentiated 3T3-L1 adipocytes (Fig. 2B), APS protein was only detected in adipocytes (Fig. 5A). The fact that we observed no protein in fibroblasts despite expression of detectable mRNA may be due to different sensitivity of the blotting procedures or, alternatively, may be indicative of post-transcriptional regulation. We next performed immunoblot analysis of several human and rat tissues. APS protein was specifically detected in human skeletal muscle and heart (Fig. 5B). No APS protein was detected in extracts prepared from either human spleen or liver.
Arrows

Samples were immunoblotted with anti-APS antibodies.

between lanes 2 and 7 denote APS with standard molecular size markers (kDa) shown right.

...immunoprecipitated with anti-APS antibodies and immunoblotted (IB) with anti-phosphotyrosine antibodies (PY) or with anti-APS antibodies (APS). B, 50 μg of total cellular extracts prepared from unstimulated (lane 1), 5 min (lane 2) or 15 min (lane 3) insulin-stimulated or 5 min (lane 4) or 15 min (lane 5) PDGFββ-stimulated 3T3-L1 adipocytes were immunoblotted (IB) with anti-phosphotyrosine antibodies (PY).

Arrows on the right denote the position of tyrosine phosphorylated insulin receptor (IR) and the PDGF receptor (PDGFR) with standard molecular size markers (kDa) shown on the left.

Interestingly, the human APS was slightly reduced in molecular mass with an approximate molecular mass between 60 and 65 kDa compared to the protein detected in the mouse 3T3-L1 adipocyte cell line. The origin of these proteins of distinct mobilities remains unclear. Immunoblot analysis of adult rat tissues clearly demonstrated that the APS protein was expressed in a variety of insulin responsive tissues including adipose, heart, and pancreas (Fig. 5C). APS protein was also detected in rat skeletal muscle with a molecular mass similar to that found for human APS (data not shown). The apparently high level of APS protein in pancreas and the low level observed in spleen would not be predicted by the Northern blot data, which is otherwise consistent. Nevertheless, the demonstration that APS is expressed primarily in insulin-responsive tissues and in 3T3-L1 adipocytes supports a potential physiological role of this protein in insulin signaling.

Endogenous APS Is Tyrosine-phosphorylated by Insulin Stimulation of 3T3-L1 Adipocytes—It is clear from the GST pull-down experiments that the SH2 domain of APS interacts with the insulin receptor in a phosphotyrosine-dependent manner. Furthermore, the results from the yeast two-hybrid experiments indicate that APS possesses the ability to interact directly with insulin receptor. To gain further insight regarding the functional importance of the insulin receptor and APS association, we examined the effects of insulin or PDGF treatment on tyrosine phosphorylation on endogenous APS in 3T3-L1 adipocytes.

Fig. 6. Tyrosine phosphorylation of endogenous APS in 3T3-L1 cells. A, 500 μg of total cellular extract prepared from 3T3-L1 adipocytes, stimulated with or without insulin (100 nm, lanes 1–3) or PDGF-ββ (50 ng/ml, lanes 4–6) for the times indicated. The samples were immunoprecipitated with anti-APS antibodies and immunoblotted (IB) with anti-phosphotyrosine antibodies (PY) or with anti-APS antibodies (APS). B, 50 μg of total cellular extracts prepared from unstimulated (lane 1), 5 min (lane 2) or 15 min (lane 3) insulin-stimulated or 5 min (lane 4) or 15 min (lane 5) PDGFββ-stimulated 3T3-L1 adipocytes were immunoblotted (IB) with anti-phosphotyrosine antibodies (PY). Arrows on the right denote the position of tyrosine phosphorylated insulin receptor (IR) and the PDGF receptor (PDGFR) with standard molecular size markers (kDa) shown on the left.

Fig. 5. Expression of APS protein in human and mouse tissues. A, total cellular extracts were prepared from either 3T3-L1 fibroblasts (lane 1) or fully differentiated adipocytes (lane 2). 2 μg of total protein were immunoprecipitated with anti-APS antibodies as described under “Experimental Procedures.” Samples were resolved on an 8% SDS-polyacrylamide gel, transferred to PVDF, and immunoblotted. APS is denoted by arrows on the right, and standard molecular mass markers (kDa) are shown on left. B, human tissue extracts were purchased from CLONTECH. 5 μg of total protein from human skeletal muscle (lane 2), human heart (lane 3), human liver (lane 4), and human spleen (lane 5) or 500 μg of total protein from mouse 3T3-L1 adipocytes (lane 1) were immunoprecipitated with anti-APS antibodies and immunoblotted with anti-APS antibodies. C, rat tissue extracts were prepared from either adipose (lanes 1 and 2), heart (lanes 3 and 4), pancreas (lanes 5 and 6) or spleen (lanes 7 and 8). 10 μg of total protein were immunoprecipitated with either pre-immune (lanes 1, 3, 5, and 7) or anti-APS (lanes 2, 4, 6, and 8) antibodies as described under “Experimental Procedures.” Samples were immunoblotted with anti-APS antibodies. Arrows on the right denote APS with standard molecular size markers (kDa) shown between lanes 2 and 3.
results that APS is an endogenous substrate of the insulin receptor. Immunoblotting with the anti-APS antibodies indicated that equivalent amounts of APS had been immunoprecipitated under all conditions examined. APS was not tyrosine-phosphorylated in response to PDGFββ stimulation of 3T3-L1 adipocytes, even though the receptor was clearly shown to be tyrosine-phosphorylated, suggesting some degree of receptor specificity (Fig. 6B). Despite our demonstration that APS is a substrate of the IR, we have thus far been unable to demonstrate a stable interaction between the IR and APS by co-immunoprecipitation (data not shown). In this regard, it should be noted that co-immunoprecipitation of other substrates of the IR, including SHC and IRS proteins, has also been difficult to demonstrate. Our data do not allow us to compare the relative levels of APS to other known substrates of the IR such as IRS-1, IRS-2, or SHC since we do not know the relative efficiencies of immunoprecipitation or immunoblotting of the various antibodies. The fact that we cannot easily see a tyrosine-phosphorylated band in total cell lysates from 3T3-L1 cells or tissues suggests that that APS may not be as abundant as IRS-1. Alternatively, this may be partly due to the finding that APS is phosphorylated upon only a single site, whereas IRS-1 is multiply phosphorylated. In this regard, the SHC protein, which is a known substrate of the IR that is phosphorylated upon a single tyrosine, is also not easily observed in insulin-stimulated lysates without prior immunoprecipitation.

Unlike activation of PI3K and mitogen-activated protein kinase, which can both become activated to varying degrees by insulin and PDGF, APS appears to be a specific substrate of the insulin receptor. In agreement with our studies showing limited in vitro interaction between the insulin receptor and the APS-related protein SH2B, no tyrosine phosphorylation of SH2B has been reported in response to insulin stimulation either in CHO cells overexpressing the insulin receptor (40, 41) or in 3T3-F442A adipocytes (44). More recently SH2Bβ has been shown to be recruited and tyrosine-phosphorylated via a direct interaction to the PDGF receptor. Co-immunoprecipitation of endogenous SH2Bβ and the PDGF receptor was detected after ligand stimulation in both NIH3T3 and 3T3-F442A adipocytes (44). We have been unable so far to detect either a stable association between APS and the PDGF receptor (data not shown) or to detect tyrosine phosphorylation of APS by PDGFββ in 3T3-L1 adipocytes. This appears to be one more difference between the members of this family of adapter proteins.

The SH2 Domain and Tyrosine 618 Are Essential for APS Phosphorylation—In order to be able to characterize the relative importance of the SH2 domain, the PH domain and the carboxyl-terminal tyrosine residue in the tyrosine phosphorylation of APS by the insulin receptor, plasmids containing the full-length wild-type and mutant forms of APS with an XPRESS epitope tag at the amino terminus were transiently expressed in several insulin-responsive tissues. Endogenous APS was identified through a yeast two-hybrid screen using a known substrate of the insulin receptor that is necessary for the full activation of glucose transport by insulin. These unidentified signaling proteins could interact specifically with the insulin receptor in yeast. PH domains are found in a large variety of proteins and are likely to be involved in the localization of proteins to the proximity of the membrane. Transient transfection of CHO cells overexpressing the insulin receptor with APS containing a mutation of the conserved tryptophan greatly reduced the phosphotyrosyl content of APS in response to insulin. Although detailed analysis of the binding affinity of phospholipids has not been undertaken, the PH domain may be important in regulating the intracellular localization of APS and its recruitment to the receptor after insulin stimulation. Identification of the phosphorylation site within APS was clearly demonstrated to be the carboxyl-terminal tyrosine (position 618) since mutation of this residue abolished all tyrosine phosphorylation of APS in response to insulin. Immunoblotting with the epitope tag anti-XPRESS antibody indicated equivalent amounts of transiently expressed APS had been immunoprecipitated under all conditions. Transfection of CHO cells with these plasmid cDNAs produced two proteins, of which only the higher molecular weight form was able to be tyrosine-phosphorylated.

As discussed earlier, many recent studies have suggested the existence of additional signaling pathways which emanate from the insulin receptor that are necessary for the full activation of glucose transport by insulin. These unidentified signaling proteins could interact specifically with the insulin receptor and in collaboration with PI3K produce the full metabolic response within a muscle or adipose cell. This report identifies a new substrate of the insulin receptor that may play such a role. APS was identified through a yeast two-hybrid screen using the insulin receptor as bait. The APS protein is highly expressed in several insulin-responsive tissues. Endogenous APS was found to be a direct substrate of the insulin receptor and underwent tyrosine phosphorylation in response to insulin in 3T3-L1 adipocytes. We have also demonstrated a clear difference in APS tyrosine phosphorylation by insulin and PDGF.
The function of tyrosine phosphorylation of APS in mediating the effects of insulin remains to be determined. Taken together, the characteristics of APS suggest that this protein may be an important adapter involved in insulin receptor signaling in metabolically responsive tissues. The identification of signaling molecules that are downstream of APS and the physiological role(s) of APS in insulin-mediated signal transduction is the focus of further work.

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