APS, an adapter protein with a PH and SH2 domain, is a substrate for the insulin receptor kinase

Zamal AHMED*†, Beverley J. SMITH*, Kei KOTANI†, Peter WILDEN‡ and Tahir S. PILLAY*†¹

*Molecular Endocrinology Group, Institute of Cell Signalling and School of Biomedical Sciences, University of Nottingham Medical School, Queen's Medical Centre, Nottingham NG7 2UH, U.K., †Cell Signalling Laboratory, Department of Metabolic Medicine, Imperial College School of Medicine, Hammersmith Campus, 150 Du Cane Road, London W12 0NN, U.K., and ‡Department of Pharmacology and the Molecular Biology Program, University of Missouri-Columbia, School of Medicine, One Hospital Drive, Columbia, MO 65212, U.S.A.

APS (adapter protein with a PH and SH2 domain) is the newest member of a family of tyrosine kinase adapter proteins including SH2-B and Lnk. We previously identified SH2-B as an insulin-receptor-binding protein and substrate [Kotani, Wilden and Pillay (1998) *Biochem J.* 335, 103–109]. Here we show that APS interacts with the insulin receptor kinase activation loop through its SH2 domain and insulin stimulates the tyrosine-

phosphorylation of APS. Furthermore, the phosphorylation of activation-loop tyrosine residues 1158 and 1162 are required for this interaction.

Key words: tyrosine kinase, tyrosine phosphorylation, yeast two-hybrid system.

INTRODUCTION

After the activation of the insulin receptor tyrosine kinase by insulin, several proteins are phosphorylated on tyrosine residues [1–4]. These include the well-characterized substrates insulin receptor substrate (IRS) proteins and SHC, which can activate the Ras pathway by recruiting growth-factor-receptor-bound protein 2 (Grb2) through its SH2 domain. The IRS family can also recruit and activate phosphoinositol 3-kinase (PI3-K) by binding to the p85 subunit [5]. The Ras pathway leads to mitogenesis, whereas PI3-K activation is required for the stimulation of glucose transport, glycogen synthesis and suppression of apoptosis by insulin [5]. However, the precise basis of signalling specificity is not clearly understood because a number of other hormones and cytokines activate similar pathways in a variety of cell types [2]. This has led to a search for additional substrates and adapter proteins that could interact with the insulin receptor cytoplasmic domain [6–9]. These adapter proteins might initiate signalling pathways unique to the insulin receptor tyrosine kinase or might initiate inhibitory pathways that modulate the Ras and PI3-K kinase pathways, for example [10,11].

By using the yeast two-hybrid system, a number of groups have successfully identified new potential interactors for the insulin receptor cytoplasmic domain [6–8,12,13]. These include growth-factor-receptor-binding protein (insulin receptor) (Grb-IR)/Grb10, which is an SH2-domain-containing adapter protein [7,8,12]. Recently, we and others identified SH2-B as an insulin-receptor-binding protein [14–16]. In addition, we found that SH2-B interacted with the activation loop via its SH2 domain and underwent tyrosine phosphorylation in response to insulin [14]. Database sequence similarity searches have revealed that SH2-B [17] belongs to a family of related proteins including adapter protein with a PH and SH2 domain (APS) [18] and Lnk [19]. The APS and SH2-B structures are similar [18]. Both have a central PH domain with a C-terminal SH2 domain and N-terminal proline-rich regions, along with several conserved tyrosine phosphorylation sites. In addition, APS has a second

proline-rich region C-terminal to the SH2 domain. On the basis of its sequence similarity with SH2-B, particularly in the SH2 domain, it was possible that APS would also interact with the insulin receptor. We tested this hypothesis directly by using the yeast two-hybrid system and mammalian cell transfection. Our results indicate that APS, which is expressed in insulin-sensitive tissues, displays a strong interaction with the insulin receptor and is phosphorylated in response to insulin in cells expressing both low and high levels of insulin receptor.

EXPERIMENTAL

Antibodies

Anti-APS, AntiLexA, polyclonal anti-Myc and anti-phosphotyrosine PY99 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). RC20 was purchased from Transduction Labs (Lexington, KY, U.S.A.). Peroxidase conjugates of anti-goat, anti-mouse and anti-rabbit antibodies were purchased from Sigma (Poole, Dorset, U.K.).

DNA constructs

Myc-tagged rat APS cDNA [20] was provided by Dr. David Ginty (Johns Hopkins University, Baltimore, MD, U.S.A.) and subcloned into pGAD424 derivatives [21]. Insulin receptor cytoplasmic domain and tyrosine phosphorylation point mutants were cloned into pBTM116 [22] as LexA fusions. Insulin receptor activation-loop mutants were generated by PCR or reverse-transcriptase-mediated PCR with the use of mutant plasmids or mRNA from Chinese hamster ovary (CHO) cell lines overexpressing activation-loop mutants. Residues are numbered as in [23]. These include Y1158F, Y1162F and Y1163F. YE3 contains glutamate substitutions of Tyr¹¹⁵⁸, Tyr¹¹⁶² and Tyr¹¹⁶³. These were generated by using the Quikchange mutagenesis kit (Stratagene). Insulin receptor in pSG5 and insulin receptor YF2 [24] mutant constructs were obtained from Dr. N.

Abbreviations used: APS, adapter protein with a PH and SH2 domain; CHO, Chinese hamster ovary; Grb2, growth-factor-receptor-bound protein 2; Grb-IR, growth-factor-receptor-binding protein (insulin receptor); GST, glutathione S-transferase; IGF, insulin-like growth factor; IRS, insulin receptor substrate; PI3-K, phosphoinositol 3-kinase.

¹ To whom correspondence should be addressed (e-mail tpillay@nottingham.ac.uk).

Webster (University of California, San Diego, CA, U.S.A.). C-terminal deletions of rat APS in pGAD424 were generated by digestion with restriction enzymes. The APS deletion mutants are designated as follows: APS (1–532) and APS (1–415). The integrity of constructs was confirmed by automated dideoxy DNA sequencing. The expression and tyrosine phosphorylation of insulin receptor yeast constructs were verified by Western blotting with anti-phosphotyrosine antibodies and anti-LexA antibodies.

Transfections

Cells (293 and CHO.T) were transfected with Effectene or Superfect reagents (Qiagen). Cells were transfected in 6 cm dishes and experiments were performed 48 h later. After stimulation with insulin, the cells were lysed in Hepes buffer containing 50 mM Hepes, pH 7.5, 1% (v/v) Nonidet P40, 10% (v/v) glycerol, 1 mg/ml Bacitracin, 1 mM EDTA, 10 mM NaF and 1 mM Na_3VO_4 . APS was then immunoprecipitated with polyclonal anti-Myc or anti-APS antibody and probed with anti-phosphotyrosine and horseradish peroxidase-conjugated secondary antibodies as indicated. All blots were developed with luminol-based substrates (Supersignal; Pierce).

Yeast two-hybrid interaction assays

These were performed as described previously [14] after the freeze–thaw permeabilization of yeast colonies lifted to circular filters. Insulin receptor cytoplasmic domain fusions to Lex A in pBTM116 [22] were co-transformed with rat APS fused to GAL DNA activation domain in pGAD424 derivatives [21] and selected for tryptophan and leucine prototrophy. Transformants were then lifted to filter papers and tested for expression of the β -galactosidase reporter gene with 5-bromo-4-chloroindol-3-yl β -D-galactopyranoside (X-Gal) as a substrate.

RESULTS AND DISCUSSION

Interaction of wild-type and mutant APS with wild-type and mutant insulin receptors in the yeast two-hybrid system

We have previously shown that a related protein SH2-B interacts with the insulin receptor activation loop and undergoes tyrosine phosphorylation and an electrophoretic mobility shift in response to insulin [14]. Because the overall structure of SH2-B is very similar to that of APS and there is marked sequence similarity in the SH2 domains, we tested the interaction of APS with the insulin receptor cytoplasmic domain. As measured by standard filter assay for β -galactosidase reporter expression, the kinase-active LexA insulin receptor cytoplasmic domain fusion was found to display a strong interaction with rat APS cloned into a GAL4 activation domain vector (Figure 1, upper panels). In contrast, there was no interaction with an insulin receptor ATP-binding site (Lys¹⁰³⁰) mutant, which is kinase-dead. This indicated that kinase activity and/or tyrosine phosphorylation was required for the interaction. We then tested the interaction of full-length APS with various insulin receptor phosphorylation-site point mutants to map the site of interaction in the insulin receptor cytoplasmic domain (Figure 1, upper panels). Mutation of the C-terminal tyrosine phosphorylation sites, Tyr¹³²⁷ and Tyr¹³³⁴, to phenylalanine (YF2 mutant) did not abolish interaction with APS. Similarly, the mutation of Tyr¹¹⁶³ to phenylalanine did not abolish the interaction. However, the mutation of Tyr¹¹⁵⁸ or Tyr¹¹⁶² abolished the interaction of the insulin receptor cytoplasmic domain with rat APS (Figure 1, upper panels). We and others have previously shown that activation-loop mutants

Figure 1 Interaction of wild-type and mutant APS with the wild-type and mutant insulin receptor cytoplasmic domain in the yeast two-hybrid system

A β -galactosidase filter assay of yeast colonies is shown. Upper panels: a GAL4 activation domain fusion of full-length APS was tested for interaction with a LexA fusion of the insulin receptor cytoplasmic domain in the L40 yeast strain with the use of a filter assay for β -galactosidase as described in the Experimental section. Abbreviations: IR, insulin receptor; wt, wild-type insulin receptor; 1158F, 1162F, 1163F, single mutants Y1158F, Y1162F and Y1163F; YE3, triple mutant containing glutamic substitutions of Tyr¹¹⁵⁸, Tyr¹¹⁶² and Tyr¹¹⁶³; YF2, double mutant with phenylalanine substitutions of Tyr¹³²⁷ and Tyr¹³³⁴; KD, insulin receptor ATP-binding-site mutant (AK1030). Lamin-BTM116 was used as a DNA-binding domain control. A diagram of the tyrosine phosphorylation sites and mutants is also shown, in which TM stands for transmembrane domain. Lower panels: deletions of the APS C-terminus (APS 1–532) and APS C-terminus and SH2 domain (APS 1–415) fused to the GAL4 activation domain were tested against a LexA fusion of wild-type insulin receptor kinase domain as described in the Experimental section.

with single substitutions retain detectable autophosphorylation [14,25,26]. Similarly, mutation of the activation-loop tyrosine residues 1158, 1162 and 1163 to glutamic residues abolished interaction, even though this mutant has detectable kinase activity and autophosphorylates [27]. Our results therefore indicate that, in the yeast two-hybrid system, interaction with APS occurs through the insulin receptor kinase activation loop with a requirement for Tyr¹¹⁵⁸ and Tyr¹¹⁶², whereas Tyr¹¹⁶³ is not required.

We then attempted to determine the region of APS that was required for the interaction (Figure 1, lower panels). Deletions in the full-length APS cDNA were generated by digestion with restriction enzymes. Deletion of the SH2 domain and the C-terminus of APS (residues 415–620) of APS abolished the interaction of the insulin receptor cytoplasmic domain with APS, whereas deletion of the C-terminus alone (residues 532–620) did not abolish the interaction. This confirmed that the SH2 domain of APS was required for interaction with the insulin receptor cytoplasmic domain.

Interaction of the isolated SH2 domain with insulin receptor derived from intact cells

We used a glutathione S-transferase (GST) fusion protein of the human APS SH2 domain to precipitate insulin receptors derived from insulin-stimulated CHO cells expressing either wild-type

Figure 2 Precipitation of wild-type and mutated insulin receptors with the APS-GST SH2 fusion protein

Serum-starved CHO.T cells (IR^{wt}) or cells expressing activation-loop mutants (IR^{Y1158F}, IR^{Y1162F} and IR^{Y1158F/Y1162F}) were stimulated with insulin; whole cell lysates were then prepared. These were incubated with an immobilized GST fusion protein of the human APS SH2 domain. The GST fusion proteins were then precipitated and bound proteins were analysed with anti-phosphotyrosine immunoblotting. Abbreviations: APS SH2 ppt, precipitates with the APS-GST SH2 domain; w.c.l., whole cell lysate.

insulin receptor or activation-loop mutants. As controls, we used comparable amounts of SH2B SH2 domain, Grb2 SH2 domain and Grb10 SH2 domain. We found that the APS SH2 domain precipitated the Y1163F mutant, indicating that this residue was not required for the interaction, whereas both the Y1158F and Y1162F mutants were poorly precipitated by the APS-GST SH2 domain. These results with the intact insulin receptor and the isolated APS SH2 domain (Figure 2) confirmed the results obtained in the yeast two-hybrid system (Figure 1).

Insulin stimulates tyrosine phosphorylation of APS

Next we investigated whether APS would undergo insulin-stimulated tyrosine phosphorylation as an indication of an interaction in intact cells, *in situ*. In contrast with SH2-B [14], many cultured cell lines do not express APS or express very low levels. For this reason it was necessary to transfect epitope-tagged APS. Myc-tagged rat APS was transfected into 293 cells or CHO.T cells. Expression was detected by using anti-APS antibody or anti-Myc antibody. Although the predicted size of APS is 69 kDa, on SDS/PAGE APS migrates at approx. 84 kDa. A similar discrepancy between predicted size and migration on SDS/PAGE has been observed with SH2-B [14]. After serum starvation, the cells were stimulated with insulin and lysed. The cells were then immunoblotted with anti-phosphotyrosine or anti-Myc antibodies.

In 293 cells transfected with APS, insulin stimulated the tyrosine phosphorylation of an 84 kDa protein, which corresponded to the mobility of APS. This tyrosine phosphorylation was further enhanced by the transfection of exogenous insulin receptor (Figures 3A and 3B). The anti-APS antibody that we used was relatively inefficient at precipitating APS, possibly because it bound to the distal C-terminus, which contains a tyrosine-phosphorylation site [28].

We then attempted to determine whether higher levels of tyrosine phosphorylation of APS could be observed in CHO.T cells, which stably express high levels of human insulin receptor. These cells were transiently transfected with exogenous Myc-tagged APS. APS protein was then immunoprecipitated with anti-Myc antibody and the immunoprecipitates were probed with anti-phosphotyrosine antibody. The results show clearly that APS is tyrosine-phosphorylated in response to insulin (Figures 3C and 3D). To confirm that the phosphoprotein observed was APS, the membrane shown in Figure 3(C) was

Figure 3 Insulin-stimulated tyrosine phosphorylation of APS

293 cells (A, B) or CHO.T cells (C, D) were transfected with Myc-APS (APS) or insulin receptor (IR). After 48 h, the cells were serum-starved for 3 h before stimulation with 100 ng/ml insulin and lysis. (A, B) APS from lysates of transfected 293 cells was immunoprecipitated with anti-APS and probed with monoclonal PY99 (A). Whole cell lysates were also immunoblotted with monoclonal anti-Myc tag (9E10) (B) to confirm expression of APS protein. (C, D) CHO.T cells were transfected with Myc-tagged APS. APS in CHO.T lysates was precipitated with anti-Myc and then probed with anti-phosphotyrosine RC20-horseradish peroxidase conjugate as indicated. The membrane shown in (C) was then reprobed with anti-APS antibody (D). Abbreviations: mock, mock-transfected cells; IP, immunoprecipitates; ins, insulin (100 ng/ml).

reprobed with anti-APS (Figure 3D). The APS protein in Figure 3(D) co-migrated with that shown in Figure 3(C).

Conclusion

In conclusion, we have provided evidence that APS functions as an insulin-receptor-interacting protein. First, with the use of the yeast two-hybrid system, we found evidence of a strong interaction of full-length APS with the insulin receptor cytoplasmic domain; this was dependent on a functional tyrosine kinase and two of the three activation-loop tyrosine phosphorylation sites, namely Tyr¹¹⁵⁸ and Tyr¹¹⁶². These results were confirmed with the use of an immobilized GST fusion protein *in vitro*. The mechanism of interaction of APS with the insulin receptor therefore seems similar to that of SH2-B [14]. This is in contrast

with Grb10, which requires Tyr¹¹⁶² and/or Tyr¹¹⁶³ for interaction [26]. Furthermore, Grb10 is not a substrate for the insulin receptor kinase.

Secondly, we have found that insulin stimulates the tyrosine phosphorylation of transfected APS in 293 cells and CHO.T cells. This was not dependent on the level of expression of insulin receptor and was observed with both low (293 cells) and high (CHO.T cells) levels of insulin receptor expression, although as expected this was technically easier to detect in CHO.T cells. Under the current conditions of the immunoprecipitation, we could not detect insulin receptor co-precipitation, which might indicate that the interaction *in vivo* is transient. Similar observations have been made with insulin receptor–IRS protein interactions, in which IRS does not seem to co-precipitate even though it interacts with the insulin receptor [29].

What are the potential functions of APS? in contrast with SH2-B, APS is not ubiquitously expressed but is present in cell types considered to be physiologically insulin-sensitive, including skeletal muscle and adipose tissue [18]. APS has been shown to interact with Grb2 and c-Cbl in lymphocytes in response to stimulation with platelet-derived growth factor [28]. Furthermore, Grb2 is constitutively associated with APS or SH2-B in neurons [20]. In neurons, SH2-B is required for neuronal differentiation, and both APS and SH2B are substrates for the Trk family of receptors [20]. Recently, it has also been observed in SAOS-2 cells that insulin-like growth factor 1 (IGF-1) stimulates the tyrosine phosphorylation of endogenous APS [28]. Therefore APS is a substrate for both the insulin and IGF-1 receptors and joins the list of tyrosine kinase activation-loop-interacting SH2 domain proteins including Grb-IR/Grb10, SH2-B and JAB [30]. Proteins with SH2 domains that interact with activation-loop sites seem to act as inhibitors [8]. Examples include Grb10, which binds to the activation loop of the insulin receptor, and JAB, which binds to the activation loop of JAK2 [30]. Thus activation-loop phosphorylation sites might also serve to recruit signalling proteins as well as activating the tyrosine kinase. The recruitment of APS and subsequent tyrosine phosphorylation in response to IGF-1, platelet-derived growth factor [28] and insulin (the present study) would allow it to recruit secondary signalling molecules that interact with tyrosine phosphorylation sites or with the PH and proline-rich regions. Insulin has been found to stimulate the phosphorylation of c-Cbl in 3T3L1 adipocytes but not in CHO.T cells; the insulin receptor does not seem to interact directly with Cbl [31]. c-Cbl seems to have a role in insulin action in adipocytes [32]. It is possible that APS might recruit c-Cbl after insulin-stimulated phosphorylation. It will therefore be important to identify the downstream signalling partners for APS involved in insulin/IGF-1 signalling.

Note added in proof (received 18 June 1999)

Following submission of this paper, Moodie et al. [33] reported the identification of APS as a novel insulin receptor substrate.

We thank Dr. David Ginty and Dr. Xiaozong Qian for providing us with the Myc-tagged APS DNA, Dr. Michael Schweizer for providing the modified pGAD plasmids, Dr. Gus Gustafson for the Grb10 SH2 GST construct, and Dr. N. Webster for the pSG5IR and YF2 constructs. Z.A. was the recipient of a Wellcome Prize studentship; T.S.P. is a Wellcome Senior Fellow in Clinical Science.

REFERENCES

- White, M. F. (1998) *Mol. Cell. Biochem.* **182**, 3–11
- Yenush, L. and White, M. F. (1997) *Bioessays* **19**, 491–500
- White, M. F. (1997) *Diabetologia* **40** (Suppl. 2), S2–S17
- Holman, G. D. and Kasuga, M. (1997) *Diabetologia* **40**, 991–1003
- Shepherd, P. R., Withers, D. J. and Siddle, K. (1998) *Biochem. J.* **333**, 471–490
- O'Neill, T. J., Zhu, Y. and Gustafson, T. A. (1997) *J. Biol. Chem.* **272**, 10035–10040
- O'Neill, T. J., Rose, D. W., Pillay, T. S., Hotta, K., Olefsky, J. M. and Gustafson, T. A. (1996) *J. Biol. Chem.* **271**, 22506–22513
- Liu, F. and Roth, R. A. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 10287–10291
- Hansen, H., Svensson, U., Zhu, J., Laviola, L., Giorgino, F., Wolf, G., Smith, R. J. and Riedel, H. (1996) *J. Biol. Chem.* **271**, 8882–8886
- Nantel, A., Mohammad-Ali, K., Sherk, J., Posner, B. I. and Thomas, D. Y. (1998) *J. Biol. Chem.* **273**, 10475–10484
- Liu, F. and Roth, R. A. (1998) *Mol. Cell. Biochem.* **182**, 73–78
- Frantz, J. D., Giorgetti-Peraldi, S., Ottinger, E. A. and Shoelson, S. E. (1997) *J. Biol. Chem.* **272**, 2659–2667
- Laviola, L., Giorgino, F., Chow, J. C., Baquero, J. A., Hansen, H., Ooi, J., Zhu, J., Riedel, H. and Smith, R. J. (1997) *J. Clin. Invest.* **99**, 830–837
- Kotani, K., Wilden, P. and Pillay, T. S. (1998) *Biochem. J.* **335**, 103–109
- Riedel, H., Wang, J., Hansen, H. and Yousaf, N. (1997) *J. Biochem. (Tokyo)* **122**, 1105–1113
- Wang, J. and Riedel, H. (1998) *J. Biol. Chem.* **273**, 3136–3139
- Osborne, M. A., Dalton, S. and Kochan, J. P. (1995) *Biotechnology (N. Y.)* **13**, 1474–1478
- Yokouchi, M., Suzuki, R., Masuhara, M., Komiya, S., Inoue, A. and Yoshimura, A. (1997) *Oncogene* **15**, 7–15
- Huang, X., Li, Y., Tanaka, K., Moore, K. G. and Hayashi, J. I. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 11618–11622
- Qian, X., Riccio, A., Zhang, Y. and Ginty, D. D. (1998) *Neuron* **21**, 1017–1029
- Roder, K. H., Wolf, S. S. and Schweizer, M. (1996) *Anal. Biochem.* **241**, 260–262
- Vojtek, A. B., Hollenberg, S. M. and Cooper, J. A. (1993) *Cell* **74**, 205–214
- Ebina, Y., Ellis, L., Jarnagin, K., Eder, M., Graf, L., Clauser, E., Ou, J. H., Masiarz, F., Kan, Y. W., Goldfine, I. D. et al. (1985) *Cell* **40**, 747–758
- Takata, Y., Webster, N. J. and Olefsky, J. M. (1991) *J. Biol. Chem.* **266**, 9135–9139
- Wilden, P. A. and Kahn, C. R. (1994) *Mol. Endocrinol.* **8**, 558–567
- Dong, L. Q., Farris, S., Christal, J. and Liu, F. (1997) *Mol. Endocrinol.* **11**, 1757–1765
- Zhang, B., Tavaré, J. M., Ellis, L. and Roth, R. A. (1991) *J. Biol. Chem.* **266**, 990–996
- Yokouchi, M., Wakioka, T., Sakamoto, H., Yasukawa, H., Ohtsuka, S., Sasaki, A., Ohtsubo, M., Valius, M., Inoue, A., Komiya, S. and Yoshimura, A. (1999) *Oncogene* **18**, 759–767
- Gustafson, T. A., He, W., Craparo, A., Schaub, C. D. and O'Neill, T. J. (1995) *Mol. Cell. Biol.* **15**, 2500–2508
- Yasukawa, H., Misawa, H., Sakamoto, H., Masuhara, M., Sasaki, A., Wakioka, T., Ohtsuka, S., Imaizumi, T., Matsuda, T., Ihle, J. N. and Yoshimura, A. (1999) *EMBO J.* **18**, 1309–1320
- Ribon, V. and Saltiel, A. R. (1997) *Biochem. J.* **324**, 839–845
- Ribon, V., Printen, J. A., Hoffman, N. G., Kay, B. K. and Saltiel, A. R. (1998) *Mol. Cell. Biol.* **18**, 872–879
- Moodie, S. A., Alleman-Sposeto, J. and Gustafson, T. A. (1999) *J. Biol. Chem.* **274**, 11186–11193

Received 6 April 1999/27 April 1999; accepted 27 May 1999