The APS adapter protein couples the insulin receptor to the phosphorylation of c-Cbl and facilitates ligand-stimulated ubiquitination of the insulin receptor

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Abstract **The APS adapter protein is rapidly tyrosine-phosphorylated following insulin stimulation. In insulin-stimulated 3T3-L1 adipocytes, APS co-precipitated with phosphorylated c-Cbl. In CHO.T-APS cells overexpressing the insulin receptor and APS, APS co-precipitated with c-Cbl but not in CHO.T cells which do not express APS. APS-mediated recruitment of c-Cbl to the insulin receptor led to rapid ubiquitination of the insulin receptor β-subunit in CHO.T-APS but not in parental CHO.T cells. These results suggest that the function of APS is to facilitate coupling of the insulin receptor to c-Cbl in order to catalyse the ubiquitination of the receptor and initiation of internalisation or degradation.** © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Insulin receptor; Tyrosine kinase; c-Cbl; Adapter protein containing a PH and SH2 domain; Adapter protein; Ubiquitination

1. Introduction

Tyrosine kinase receptor catalytic domains are activated following the binding of cognate ligands to the extracellular domains. The binding of insulin to the α-subunit of the receptor triggers autophosphorylation of the β-subunit of the receptor on a conserved set of tyrosine residues (Tyr 1158, 1162 and 1163) in the activation loop of the kinase domain [1,2]. This results in maximal activation of the receptor tyrosine kinase and subsequent tyrosine phosphorylation of a number of intracellular substrates, including the insulin receptor substrate (IRS) family (IRS1,2,3,4) and Shc. Phosphorylation of these proteins then facilitates the activation of the phosphatidylinositol 3-kinase and Ras-mitogen-activated protein kinase pathways, respectively [3].

Although, it was previously thought that the insulin receptor utilised intermediates such as IRS and Shc to signal, recent evidence indicates that the insulin receptor, by virtue of autophosphorylation in the activation loop, can recruit signalling adapters directly. For example, the family of adapter proteins that include the Grb10/14 family bind directly to the activa-

tion loop of the insulin receptor [4]. More recently, we and others identified Src homology 2 (SH2)-B and an adapter protein containing a PH and SH2 domain (APS) as insulin receptor binding proteins [5–9]. The APS protein [10] is the newest distinct member of this family of adapter proteins that include SH2-B splice variants, α, β and γ [8] and the lymphocyte specific adapter protein, Lnk [11,12]. These are characterised by the presence of a PH domain, proline rich regions and an SH2 domain. APS was first identified as a c-kit interacting protein [10] but was subsequently found to also interact with the insulin receptor activation loop and found to be a substrate for insulin-stimulated tyrosine phosphorylation in intact cells. The expression of APS is restricted in comparison with SH2-B [7]. APS appears to be highly expressed in adipose tissue, skeletal muscle and heart which are target tissues for insulin [6,7]. Since the role of APS in insulin signalling is not understood, we stably expressed myc-tagged APS in Chinese hamster ovary (CHO) cells expressing the insulin receptor and studied initial signalling events in these cells.

2. Materials and methods

2.1. Materials, buffers and antibodies

9E10 Myc antibody was prepared from 9E10 hybridoma. Antiphosphotyrosine antibody RC20 conjugated to horseradish peroxidase (HRP) and anti-c-Cbl antibodies were purchased from Transduction Labs (Lexington, KY, USA). Anti-ubiquitin antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-insulin receptor antibody 83-14 was previously obtained from Ken Siddle. Antipeptide insulin receptor peptide antibody was obtained from Dr. J. Olefsky (UC San Diego, CA, USA). The N-terminus of rat APS (amino acids 117–465) [13] was subcloned into a pGEX and expressed as a glutathione *S*-transferase fusion protein in DH5α bacteria. The expressed protein was purified by affinity chromatography on glutathione agarose and used to immunise rabbits as described [6]. The resulting sera (anti-APS 6113-40) were screened using cell lysates derived from CHO.T-APS cells and used to probe anti-Cbl immunoprecipitates.

2.2. Expression vectors and cell lines

CHO.T cells were previously obtained from Dr. Gus Gustafson. Myc-tagged rat APS [13] was kindly provided by Dr. David Ginty (Johns Hopkins University, Baltimore, MD, USA) and subcloned into pIRES Hygro (Clontech) and transfected into CHO.T cells using Superfect (Qiagen). Forty-eight hours after transfection, the cells were selected in 800 μg/ml hygromycin and clones were isolated by limiting dilution and screened using anti-myc 9E10 monoclonal antibody. Clones were maintained in 400 μg/ml hygromycin.

2.3. Lysate preparation, immunoprecipitation and Western blotting

CHO.T cells were cultured and lysates prepared as previously described [6,9]. Proteins were immunoprecipitated using 5–10 μg of anti-

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body. Antibodies were immunoprecipitated using antimouse agarose (monoclonal) or protein A Sepharose. Immunoprecipitates were then analysed by immunoblotting and visualisation using HRP-conjugated secondary antibodies and luminol-based substrates (Supersignal, Pierce Chemical Company, Rockford, IL, USA).

3. Results and discussion

3.1. Insulin stimulates tyrosine phosphorylation of c-Cbl in cells transfected with APS

The stimulation of some cell types with platelet-derived growth factor (PDGF), erythropoietin or insulin results in the tyrosine phosphorylation of APS on tyrosine residue 618 [7,10,14,15]. This phosphorylated tyrosine has been shown to interact with the variant SH2 domain of Cbl [15]. In 3T3-L1 adipocytes, insulin stimulates the phosphorylation of c-Cbl [16]. This does not occur in other cell types, including CHO cells expressing high levels of insulin receptor, suggesting that an accessory protein is required to facilitate phosphorylation of Cbl in 3T3-L1 adipocytes. We performed a similar experiment in differentiated 3T3-L1 adipocytes. Insulin-stimulated c-Cbl phosphorylation was determined by anti-Cbl immunoprecipitation and antiphosphotyrosine immunoblotting. In addition, anti-c-Cbl immunoprecipitates revealed the presence of a phosphoprotein of M_r 90–95 kDa (Fig. 1). Probing the membranes with anti-APS antibody showed that this phosphoprotein was detected with an anti-APS antibody (Fig. 1). We next examined c-Cbl phosphorylation in CHO.T-APS cells (Fig. 2). Both CHO.T cells and CHO.T-APS cells were stimulated with insulin and c-Cbl was immunoprecipitated and its tyrosine phosphorylation state was examined by antiphosphotyrosine immunoblotting. In CHO.T-APS cells, a tyrosine phosphoprotein corresponding to the mobility of APS

Fig. 2. Overexpression of APS allows insulin-stimulated c-Cbl tyrosine phosphorylation. CHO.T cells stably transfected with myc-tagged APS (CHO.T-APS) were serum-starved and stimulated with insulin for 5 min. c-Cbl was then immunoprecipitated from these cells. The immunoprecipitates were analysed by antiphosphotyrosine immunoblotting (a). b: The membrane shown in (a) was then stripped and reprobed for c-Cbl using polyclonal anti-Cbl. A sample of the whole cell lysate is shown alongside for comparison. w.c.l., whole cell lysate; IP, immunoprecipitating antibody.

was observed in whole cell lysates indicating that APS was being phosphorylated to a high stoichiometry in these cells. In CHO.T-APS cells (Fig. 1) insulin-stimulated rapid and robust tyrosine phosphorylation of c-Cbl but no phosphorylation of c-Cbl was observed in CHO.T cells which do not express endogenous APS. This indicated that APS is sufficient to facilitate c-Cbl phosphorylation in response to insulin. Interestingly, as we have previously shown, CHO.T cells express en-
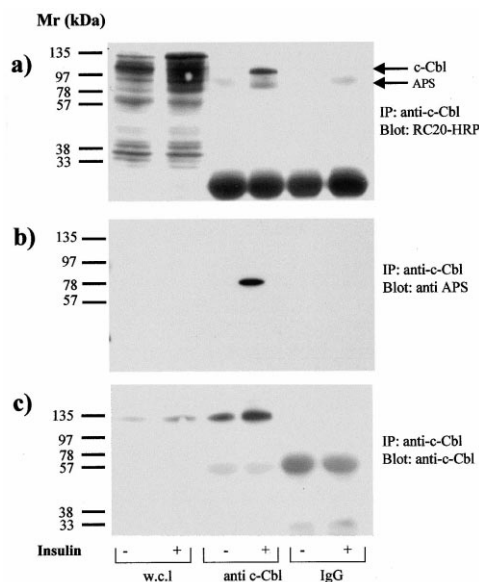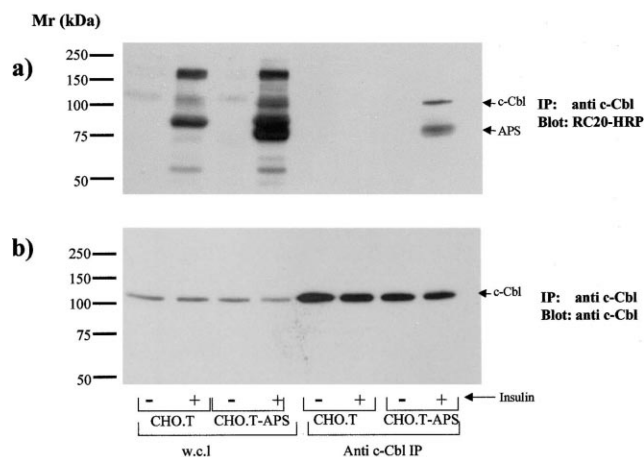

Fig. 1. Insulin-stimulated phosphorylation in 3T3-L1 adipocytes. Differentiated 3T3L1 adipocytes were serum-starved and then stimulated with 100 ng/ml insulin for 5 min. The cells were lysed and c-Cbl was immunoprecipitated using monoclonal anti-Cbl. a: Immunoprecipitates were analysed using anti-phosphotyrosine conjugated to HRP (RC-20HRP). The blot shown in (a) was then stripped and analysed using polyclonal anti-APS (b) and anti-c-Cbl (c). w.c.l., whole cell lysates; IP, immunoprecipitating antibody; Blot, immunoblotting antibody.
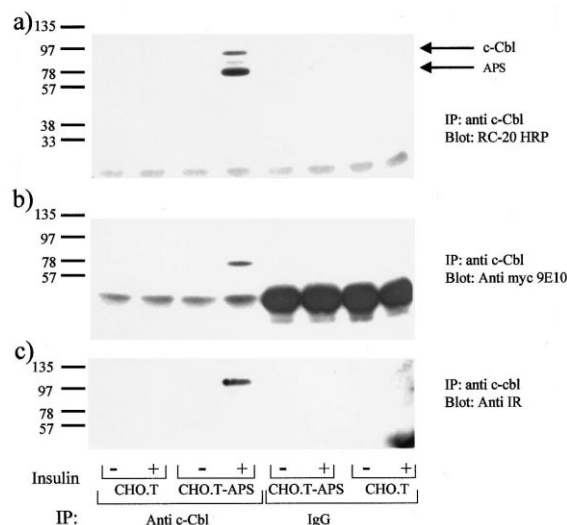

Fig. 3. APS-mediated interaction of c-Cbl with the insulin receptor. The phosphorylation of c-Cbl was compared in CHO.T cells and CHO.T cells stably transfected with pIRES Hygro APS (CHO.T-APS). Cells were serum-starved overnight and stimulated with 100 ng/ml insulin for 5 min and solubilised. Cell lysates (200 mg) were used for immunoprecipitation with monoclonal anti-Cbl antibody or purified mouse IgG as a control. Proteins were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and blotted with (a) anti-phosphotyrosine antibody RC20H, (b) anti-myc 9E10 and (c) anti-IR peptide polyclonal antibody. IP, immunoprecipitating antibody; Blot, immunoblotting antibody.

dogenous SH2-B [6] which is weakly phosphorylated by insulin but does not appear to be sufficient to facilitate insulin-stimulated phosphorylation of c-Cbl. In addition, in the c-Cbl immunoprecipitates, we observed a phosphoprotein that was similar in mobility to APS or the insulin receptor β-subunit suggesting that one of these proteins was possibly present in the c-Cbl immunoprecipitates (Fig. 2).

3.2. Co-precipitation of c-Cbl, insulin receptor and APS

We next determined whether insulin receptor could be detected in the c-Cbl immunoprecipitates (Fig. 3). The immunoprecipitates were also analysed by antiphosphotyrosine, anti-insulin receptor and anti-myc immunoblotting (Fig. 3). Again, in c-Cbl immunoprecipitates, we observed the co-precipitation of a major phosphoprotein with c-Cbl as well as a weaker phosphoprotein migrating between the position of c-Cbl and APS. The membranes were then stripped and reprobed for insulin receptor and APS. Insulin receptor is clearly detectable in c-Cbl immunoprecipitates from insulin-stimulated CHO.T APS cells (Fig. 3c). Similarly, using anti-myc 9E10 to detect the presence of epitope-tagged APS, reveals that APS is present in the c-Cbl immunoprecipitates from insulin-stimulated CHO.T APS cells (Fig. 3b). Thus, these results indicate that insulin stimulates the formation of a complex comprised of the insulin receptor, c-Cbl and APS.

3.3. Insulin stimulates ubiquitination of the insulin receptor in cells transfected with APS

c-Cbl has been shown to promote the ubiquitination of tyrosine kinase receptors by functioning as a RING-type E2-dependent ubiquitin protein ligase [17–25]. The association of c-Cbl with tyrosine kinase receptors catalyses the ubiquitination of these receptors and targets them for degradation and this is dependent on the variant SH2 domain and RING finger region of c-Cbl [17,23–25]. We next addressed the question of whether expression of APS facilitated or enhanced ubiquitination of the insulin receptor (Fig. 4). CHO.T cells and CHO.T APS cells were stimulated with insulin and the insulin receptor was immunoprecipitated with anti-insulin receptor antibody 83-14. The immunoprecipitates were analysed using anti-ubiquitin antibody. Anti-ubiquitin blotting reveals the
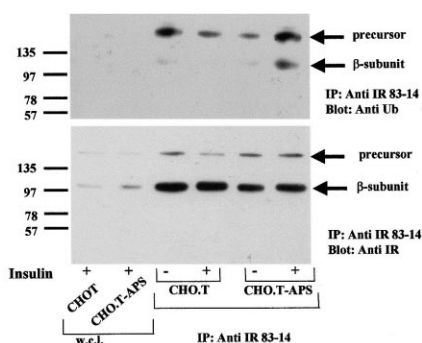

Fig. 4. Expression of APS enhances ligand-stimulated ubiquitination. CHO.T cells stably transfected with insulin receptor and/or APS (CHO.T-APS) were serum-starved, then stimulated with 100 ng/ml insulin for 5 min and lysed. Insulin receptor was then immunoprecipitated using anti-insulin receptor monoclonal 83-14. The immunoprecipitates were then analysed by immunoblotting using anti-ubiquitin antibody (top panel). The membrane shown in the top panel was then stripped and probed for using anti-insulin receptor peptide polyclonal antibody (bottom panel). Ub, ubiquitin.

presence of two major bands corresponding to the precursor and the mature β-subunit. In CHO.T cells, we could only observe ubiquitination of the precursor form of the insulin receptor indicating that a substantial amount of unprocessed precursor is ubiquitinated and targeted for degradation and this does not alter with ligand stimulation. In contrast, expression of APS resulted in increased ubiquitination of the mature β-subunit following ligand stimulation (Fig. 4).

The proto-oncogene c-Cbl is a substrate for a number of tyrosine kinase receptors, including epidermal growth factor, PDGF and CSF-1 [22,26]. These receptors appear to associate directly with c-Cbl through its variant SH2 domain resulting in the phosphorylation of c-Cbl. The increased association of c-Cbl with these receptors facilitates ubiquitination and targets the receptors for degradation. In comparison, insulin stimulation cannot result in c-Cbl phosphorylation even in cells overexpressing insulin receptor [16]. An exception to this is the 3T3-L1 adipocyte which is widely used as a model cell line for the study of insulin signalling. In these cells, insulin stimulates phosphorylation of c-Cbl (Fig. 1 and [16]) but not in the 3T3-L1 pre-adipocyte. c-Cbl does not associate directly with the insulin receptor and the variant SH2 domain of c-Cbl does not bind to phosphorylation sites on the insulin receptor. This indicates that an accessory adapter or docking protein is required to facilitate the phosphorylation of c-Cbl following insulin stimulation. This correlates with the expression of APS in the differentiated adipocyte. In view of this correlation, we asked the question whether the expression of APS is sufficient to allow phosphorylation of c-Cbl. As we have demonstrated (Fig. 3) and as has been previously shown by Saltiel et al. [16], in CHO.T cells which do not express APS, insulin does not stimulate c-Cbl tyrosine phosphorylation. On the contrary, the overexpression of APS is sufficient to allow insulin-stimulated tyrosine phosphorylation of c-Cbl. Thus, in contrast to other tyrosine kinase receptors, c-Cbl phosphorylation in response to insulin can only occur in cells that co-express insulin receptor and APS indicating the tissue or cell-type specific nature of insulin-stimulated c-Cbl phosphorylation.

What is the physiological role of this insulin-stimulated c-Cbl phosphorylation? We determined whether the co-expression of APS was associated with increased receptor ubiquitination. We found that overexpression of APS enhanced ligand-stimulated ubiquitination of the insulin receptor. Thus, the role of APS in insulin signalling would be to facilitate coupling of c-Cbl to the insulin receptor in order to facilitate ubiquitination of the insulin receptor. Therefore target tissues for insulin such as adipose and skeletal muscle which would coexpress insulin receptor and APS are likely to have the unique ability to negatively regulate insulin receptor signalling by virtue of increased coupling to the c-Cbl, a RING-type E2-dependent ubiquitin protein ligase [17]. In addition, mono-ubiquitination may carry a novel internalisation signal that is appended to activated receptors [27]. We have only observed a single form of the ubiquitinated receptor suggesting that receptor is mono-ubiquitinated rather than polyubiquitinated. While we have shown that APS is sufficient to couple to c-Cbl, we do not know if it is necessary and whether this is the only mechanism. The c-Cbl-associated protein CAPS [28] which is also preferentially expressed in the differentiated adipocyte also couples the insulin receptor to c-Cbl. This insulin receptor-CAPS-c-Cbl pathway may play a role in enhancing

sensitivity to insulin [29], in contrast to the downregulation that will arise from receptor ubiquitination mediated by APS and c-Cbl. Thus, these two mechanisms may be mutually exclusive or redundant. Future studies will be required to address the relative role of each. It will also be of interest to determine whether other signalling molecules that interact with the insulin receptor also undergo increased ubiquitination as a result of increased coupling to c-Cbl.

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