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Research Article

Insulin increase in MAP kinase phosphorylation is shifted to early time-points by overexpressing APS, while Akt phosphorylation is not influenced

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ABSTRACT

Upon insulin stimulation, the adaptor protein APS is recruited to the insulin receptor and tyrosine phosphorylated. APS initiates the insulin-induced TC10 cascade which participates to GLUT4 translocation to the plasma membrane. Nevertheless, the molecular mechanism that governs APS and its SH2 and PH domains action on the insulin transduction cascade is not yet fully understood. Here, we show that APS co-immunoprecipitates with the class I PI 3-kinase regulatory subunit p85, through its SH2 domain but that APS does not modulate neither PtdIns(3,4,5)P3 levels nor Akt phosphorylation provoked by insulin. We have confirmed a previously described positive effect of APS overexpression on insulin-induced MAPK phosphorylation upregulation. Consequently, we analyzed the role of SH2 and PH domains of APS in the APS increased MAPK phosphorylation observed upon insulin stimulation and correlated this with the membrane localization of the protein. The effect observed on MAPK phosphorylation requires the intact PH binding domain of APS as well as its SH2 domain.

Introduction

APS, Lnk and SH2-B, are members of a family of adaptor proteins that share proline-rich regions, PH and SH2 domains and a conserved C-terminal tyrosine phosphorylation site [1,2]. It has been previously reported that Lnk is involved in the regulation of T-cell receptor [1], whereas SH2-B and APS are important signalling molecules implicated in growth factor receptor signalling pathways [2–7].

Following insulin stimulation, the insulin receptor is autophosphorylated; this provides docking sites for SH2 or PTB domain of proteins including the insulin receptor substrate (IRS) [8], Shc [9] and APS [4,10]. These adaptor proteins are essential for transducing the insulin pathway. Indeed, recruitment of IRS and APS to the insulin receptor activates two complementary and correlated pathways that lead to the translocation of the glucose transporter GLUT4 to the cell membrane [8,11]. One involves activation of PI 3-kinase and its downstream target Akt [12], and the other involves the activation of the small G protein, TC10 [11]. A role of APS in GLUT4 translocation has also been reported through its interaction with a protein associated with the actin cytoskeleton, Enigma [13]. On the other hand, phosphorylation of IRS and Shc by the receptor provokes the activation of the mitogenic effects of insulin [9]. IRS and Shc tyrosine phosphorylation provides docking sites for the

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Abbreviations: APS, Adaptor protein with a PH and SH2 domain; CAP, c-Cbl associated protein; CHO-IR, Chinese hamster ovary cells-overexpressing the insulin receptor; IRS, insulin receptor substrate; MAPK, Mitogen activated protein kinase; PH, Pleckstrin Homology; PtdIns (3,4,5)P3, phosphatidylinositol 3,4,5 triphosphate; SH2, Src homology 2; SHIP2, SH2-containing inositol phosphatase 2; wt, wild type

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SH2 domain of Grb2 which is then recruited, together with the Ras guanine nucleotide exchange factor son of sevenless (Sos), from the cytoplasm to the plasma membrane. Membrane localization of Sos enables Ras activation through guanine exchange and consequently initiates MAPK pathway [9].

Although the implication of the adaptor protein APS in the insulin pathway has been approached in different studies, the molecular mechanism that governs APS action on this cascade is not fully understood. In this report, we observed that, upon insulin stimulation, APS could be associated with the p85 regulatory subunit of the classI PI 3-kinase. This interaction was in part mediated by APS SH2 domain. However, overexpression of APS in CHO-IR cells has no effect on insulin-induced-PtdIns(3,4,5)P_3 levels nor on Akt activation. On the contrary, APS specifically increases insulin-induced MAPK phosphorylation at short time of continuous insulin stimulation. Expression of APS-induced enhancement of MAPK activation requires both its PH domain and SH2 domain. These domains are also crucial for APS insulin-induced membrane localization.

Materials and methods

Plasmids and antibodies

Single point mutations within the PH domain (W290L), the SH2 domain (R437K) and on the tyrosine phosphorylation site (Y618F) of APS were generated by Quick Change (Stratagene, Amsterdam, The Netherlands). Anti-MYC (9E10) from Sigma (Bornem, Belgium), anti-Akt, anti-phospho-Akt (Ser473), anti-phospho-Akt (Thr308), anti-phospho p44/p42 MAPK and anti-p44/p42 MAPK from Cell Signalling (Leiden, The Nertherlands), anti-APS V19 from Santa Cruz Biotechnology (Santa Cruz, CA), anti-p85 and 4G10 anti-phosphotyrosine from Upstate Biotech (NY, USA) and IRDye 800CW, IRDye 680 secondary antibodies from Li-Cor Biosciences (Westburg, The Netherlands). Complete protease inhibitors cocktail and Fugene 6 were purchased from Roche Diagnostics (Vilvoorde, Belgium), ECL kit from Amersham Pharmacia Biotech (AT Roosendaal, The Nertherlands), and Ham's F12, streptomycin/penicillin, fungizone and fetal bovine serum from Gibco (Paisley, UK). Wortmaninn, LY294002 were purchased from Sigma (Bornem, Belgium) and PD98059 from Merck (Darmstadt, Germany).

Cell lines

CHO-IR cells were maintained in Ham's F12 supplemented with fetal bovine serum 10%, 100 U/ml penicillin and 100 mg/ml streptomycin, fungizone 2.5 μg/ml and G-418 0.5% in 5% CO_2/humidified atmosphere at 37 °C.

Immunoblotting

CHO-IR cells were transfected using Fugene 6 with HIS-tagged SHIP2 and/or with MYC-tagged APS in pcDNA3. The percentage of transfected cells varies between 20 and 30%. 24 h after transfection, cells were deprived of serum for 18 h prior to stimulation with insulin (100 nM) for 5 min. Cells were then harvested in cell lysis buffer [14]. 16 μg of cell extract were dissolved in sample buffer (Laemmli buffer). Proteins were separated by SDS/PAGE, transferred to nitrocellulose membranes, detected with the specified antibodies and revealed either by horseradish peroxidase-linked secondary antibodies and developed using an ECL kit (Perkin Elmer, Massachusetts, USA) or by fluorescent secondary antibodies using the Odyssey infrared imaging system.

Immunofluorescence

Immunofluorescence and confocal imaging of MYC-tagged APS proteins in CHO-IR cells were performed as described previously [14] using mouse anti-MYC and Texas Red anti-mouse antibodies.

PtdIns(3,4,5)P_3 measurements

Cells were cultured in 10% serum overnight. Cells were washed twice in medium without serum and twice in medium without either phosphate or serum. They were labeled for 4 h in medium with [^{32}P] orthophosphate (250 μCi/ml) and without serum. Cells were stimulated or not with 100 nM insulin for 5 min. Lipids were extracted as previously described [15]. Radioactivity was estimated with an online detector from Raytest (Straubenhardt, Germany).

Quantification and statistical analysis

The amount of phosphorylated MAPK normalized by the total amount loaded was quantified by the software program as specified the Odyssey software manual. Data are expressed as means ± SD. Data were analyzed by Anova plus *t*-test for determination of the significance of differences. A p value < 0.05 was considered statistically significant.

Results

APS and p85 subunit of PI 3-kinase co-precipitate in CHO-IR cells

Experiments performed in CHO-IR cells transfected with APS interestingly revealed that p85 subunit of the PI 3-kinase was present in the APS immunoprecipitate. As shown in Fig. 1, this association was only seen in cells treated with 100 nM of insulin for 5 min. By the use of two punctual mutants of APS respectively in the PH domain (W290L) and in the SH2 domain (R437K), we showed that this interaction was dependent on a functional SH2 domain of APS as the interaction with the R437K mutant is largely impaired.

This data is of particular interest considering that it was previously described that stable overexpression of APS enhances Akt phosphorylation [16]. We were thus tempted to unravel in more details the effect of APS on the PI 3-kinase pathway by measuring the levels of PtdIns(3,4,5)P3 and insulin-induced Akt phosphorylations.

APS overexpression does not influence PtdIns(3,4,5)P_3 levels

As PtdIns(3,4,5)P_3 level is the product of PI 3-kinase in the control of insulin pathway, we next measured the influence of APS overexpression on PtdIns(3,4,5)P_3 levels. Serum-starved CHO-IR cells were labeled with [^{32}P] orthophosphate. Lipids extraction was performed after stimulation of the cells with insulin for 5 min. The