p68 Sam is a substrate of the insulin receptor and associates with the SH2 domains of p85 PI3K

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Received 18 May 1999; received in revised form 21 June 1999

Abstract The 68 kDa Src substrate associated during mitosis is an RNA binding protein with Src homology 2 and 3 domain binding sites. A role for Src associated in mitosis 68 as an adaptor protein in signaling transduction has been proposed in different systems such as T-cell receptors. In the present work, we have sought to assess the possible role of Src associated in mitosis 68 in insulin receptor signaling. We performed in vivo studies in HTC-IR cells and in vitro studies using recombinant Src associated in mitosis 68, purified insulin receptor and fusion proteins containing either the N-terminal or the C-terminal Src homology 2 domain of p85 phosphatidylinositol-3-kinase. We have found that Src associated in mitosis 68 is a substrate of the insulin receptor both in vivo and in vitro. Moreover, tyrosine-phosphorylated Src associated in mitosis 68 was found to associate with p85 phosphatidylinositol-3-kinase in response to insulin, as assessed by co-immunoprecipitation studies. Therefore, Src associated in mitosis 68 may be part of the signaling complexes of insulin receptor along with p85. In vitro studies demonstrate that Src associated in mitosis 68 associates with the Src homology 2 domains of p85 after tyrosine phosphorylation by the activated insulin receptor. Moreover, tyr-phosphorylated Src associated in mitosis 68 binds with a higher affinity to the N-terminal Src homology 2 domain of p85 compared to the C-terminal Src homology 2 domain of p85, suggesting a preferential association of Src associated in mitosis 68 with the N-terminal Src homology 2 domain of p85. This association may be important for the link of the signaling with RNA metabolism.

Key words: Insulin receptor; Insulin receptor substrate; Src associated in mitosis 68; Src homology 2 domain; p85 phosphatidylinositol-3-kinase

1. Introduction

Insulin binding to the extracellular domain of insulin receptors (IRs) on the surface of the cell induces a signal transduction cascade, which then propagates inside the cell to mediate the uptake and storage of energy and to control gene expression and proliferation [1–3]. IRs are heterotetrameric transmembrane glycoproteins (two α- and two β-subunits) [4]. The α-subunit is completely extracellular and mediates insulin binding, whereas the β-subunit has an intracellular domain containing a tyrosine-specific kinase similar to that of the proto-oncogene product Src [5]. When insulin binds to the α-subunit, intrinsic tyrosine kinase of the β-subunit is activated, autophosphorylates [6] and tyrosine phosphorylates cellular proteins [7]. IR substrate 1 (IRS-1) is a major cellular substrate for the IR [8]. Multiple tyrosine phosphorylations of IRS-1 in specific motifs renders IRS-1 to interact with various Src homology (SH) 2 containing proteins including the p85 regulatory subunit of phosphatidylinositol-3-kinase (PI3K) [9,10]. The IR itself, via its C-terminal Tyr-1322-Thr-His-Met motif following tyrosine phosphorylation, is also capable of interacting with a SH2 domain of a PI3K p85 subunit [11,12]. PI3K is a heterodimer enzyme that consists of a p110 catalytic subunit [13] and a p85 regulatory subunit [14]. A P85 subunit contains two SH2 domains and one SH3 domain that interact with phosphorylated tyrosines in specific motifs [15] and proline-rich domains, respectively [16]. PI3K activity has been implicated in insulin-stimulated glucose transport and glycogen synthesis [17–19]. Following insulin stimulation, PI3K forms various signaling complexes with downstream IR proteins [20]. They include the IR, IRS-1 and p60–p70 kDa phosphoproteins. One of these proteins has been previously identified as the p62 GTPase activating protein (GAP)-associated protein [21,22]. A protein first thought to be p62 GAP-associated protein [23] is Src associated with mitosis (Sam) 68 which is known to be tyrosine-phosphorylated in mitotic cells and to form a complex with Src by interactions with both SH2 and SH3 domains of Src [24,25]. Sam68 is able to bind RNA because it contains a KH domain and a region similar to a RGG box [26,27] that are predictive for RNA binding proteins. Sam68 has been shown to bind to single-stranded nucleic acid [28]. A splice variant within the KH domain of Sam68 has decreased RNA binding properties and can antagonize cell cycle progression [29]. Because Sam68 is tyrosine-phosphorylated in mitotic cells and has proline-rich domains, Sam68 interacts with signaling molecules both through SH2 and SH3 domains [30,31]. Besides, tyrosine phosphorylation of Sam68 negatively regulates its nucleic acid binding properties [28,32]. These data suggested that Sam68 might have a role in signaling events. In fact, Sam68 participates in the signal transduction pathway downstream of the T-cell receptor in lymphocytes [33,34]. Our hypothesis was that Sam68 might be part of the IR signaling.

2. Materials and methods

2.1. Antibodies and recombinant proteins

Recombinant Sam68, monoclonal antibodies anti-Sam68 (α-Sam68) and anti-IR (α-IR) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antiserum to the p85α regulatory subunit of PI3K (α-p85) and glutathione *S*-transferas (GST) fusion proteins containing either the N- or C-SH2 domain were from Upstate Biotech-

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Abbreviations: Sam, Src associated in mitosis; PI3K, phosphatidylinositol-3-kinase; IR, insulin receptor; IRS-1, insulin receptor substrate-1; SH, Src homology; GST, glutathione *S*-transferase

PII: S0014-5793(99)00887-X

nology (Lake Placid, NY, USA). Monoclonal antibodies to phosphotyrosine (α-PY) were purchased from Transduction Laboratories (Lexington, KY, USA).

2.2. Cells and preparation of soluble cell lysates

Rat HTC hepatoma cells overexpressing human IR (HTC-IR) were kindly provided by Dr. Ira D. Goldfine (UCSF, San Francisco, CA, USA). Cells were prepared and maintained in Dulbecco's modified Eagle's medium as previously described [21]. For experiments, cells were grown in 100 mm dishes to 90% confluency and serum-starved for 24 h. They were treated for different times at 37°C with 100 nM insulin or different insulin concentrations for 5 min and solubilized for 30 min at 4°C in lysis buffer containing 20 mM Tris, pH 8, 1% nonidet P-40, 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM dithiothreitol (DTT), 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.4 mM sodium orthovanadate [21]. After centrifugation, the soluble cell lysates were used for the study. The protein concentration was determined by a kit from Bio-Rad (Richmond, CA, USA), using bovine serum albumin as standard.

2.3. Immunoprecipitation

Soluble cell lysates (2 mg protein) were first pre-cleared with 50 μl protein A-Sepharose for 2 h at 4°C and incubated with appropriate antibodies for 2 h at 4°C. 50 μl of protein A-Sepharose was then added and the incubation was further proceeded for 1 h at 4°C. The immunoprecipitates were washed three times with lysis buffer. 50 μl of SDS-stop buffer containing 100 mM DTT was added to immunoprecipitates and boiled for 5 min. These were then analyzed by Western blotting. Samples were resolved by SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes. The membranes were blocked with Tris-buffered saline-0.05% Tween 20 (TBST) containing 5% non-fat dry milk for 1 h, washed in TBST and further incubated with secondary antibodies linked to horseradish peroxidase. Bound horseradish peroxidase was visualized by a high sensitive chemiluminescence system (SuperSignal from Pierce, Rockfold, IL, USA).

2.4. IR purification

IRs were semi-purified by wheat germ agglutinin (WGA) chromatography from HTC-IR cells as previously described [12]. Cells were solubilized in 1% Triton X-100 containing 50 mM HEPES, pH 7.6, 1 mM PMSF, 1 mM sodium orthovanadate and 0.1 mg/ml aprotinin. Next, the solubilized cells were centrifuged at 50 000 × *g* for 1 h. The soluble extract was purified on a 2 ml column containing WGA coupled to agarose and eluted with 0.3 M *N*-acetyl-β-glucosamine as previously described [12]. Semi-purified (WGA) material was further purified by immunoaffinity with anti-IR antibodies coupled to protein A-Sepharose. IRs were immunoprecipitated as previously described [35]. IR immunoprecipitates were employed for the in vitro tyrosine phosphorylation reaction.

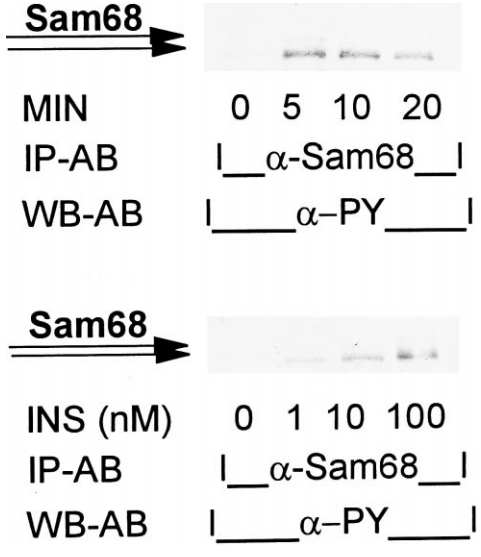

Fig. 1. Time-course and dose-response of insulin-induced tyrosine phosphorylation of Sam68. Cells were incubated with 100 nM insulin for different times (upper panel) or for 5 min at different concentrations (lower panel) and solubilized. Cell lysates were then immunoprecipitated with α-Sam68 and analyzed by Western blotting with α-PY. One experiment representative of three for each panel is shown.

2.5. In vitro phosphorylation of Sam68

Affinity-purified IR from HTC-IR, immobilized with protein A-Sepharose, was incubated with or without 100 nM insulin for 1 h in 50 mM HEPES (pH 7.6), 150 mM NaCl, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 0.1% Triton X-100, 1 mg/ml bacitracin and 1 mM PMSF. ATP (for 10 μM final concentration) was then added and incubation continued for 1 h. Next, 100 fmol recombinant Sam68 (Santa Cruz Biotechnology) was added to the reaction mixture and further incubated for 1 h. The mixture was centrifuged and supernatant containing Sam68 was employed for in vitro interaction with GST fusion proteins.

2.6. Affinity precipitation with GST fusion proteins

Reaction mixture containing in vitro phosphorylated or basal recombinant Sam68 (diluted 10-fold with reaction buffer minus Mg/Mn and ATP) was incubated for 2 h at 4°C with the GST-(PI3K) p85 N- or C-SH2 domain fusion protein (2 μg) conjugated with glutathione agarose (GST-p85 (N) and GST-p85 (C), respectively). After centrifugation, affinity precipitates were washed three times with lysis reaction mixture (minus Mg/Mn and ATP) and boiled in 50 μl SDS-stop buffer. Samples were then analyzed by Western blotting as described above.

3. Results

3.1. Sam68 is tyrosine-phosphorylated in HTC-IR cells upon insulin stimulation and associates with p85 PI3K

Using HTC-IR cells, we studied the in vivo tyrosine phosphorylation of Sam68 by insulin stimulation. Sam68 is phosphorylated in the presence of serum (not shown), but after 24 h of serum starving, the phosphorylation level is almost undetectable. As shown in the immunoprecipitate with α-Sam68 (Fig. 1, upper panel), Sam68 was slightly tyrosine-phosphorylated in basal conditions. Nevertheless, when cells were incubated with 100 nM insulin, the tyrosine phosphorylation level of Sam68 was transiently increased. The time-course of this effect of insulin showed that the Sam68 phosphorylation level was maximal at 5 min and slowly decreased after 10 min, although the level of phosphorylation after 20 min incubation was still significantly increased. Scanning of the bands and analysis with the PCBAS2.0 program showed that phosphorylation levels at 10 and 20 min were 76 ± 15% and 47 ± 9% (means ± S.D.) of that observed at 5 min. To further demonstrate the physiological importance and the specificity of this effect of insulin on tyrosine phosphorylation of Sam68, we performed dose-response experiments (Fig. 1, lower panel) at the 5 min time-point. This effect of insulin was dependent on the concentration, it was significantly observed at 1 nM (27 ± 4% of maximum) and was maximal at 100 nM insulin. At 10 nM insulin, phosphorylation of Sam68 reached 48 ± 5% of the maximum.

The immunoprecipitate with α-p85 also showed p60–70 tyrosine-phosphorylated proteins after 5 min insulin stimulation (Fig. 2A) as previously reported [12,21]. To check whether Sam68 was one of these tyrosine-phosphorylated proteins, we probed the samples from Fig. 2A with α-Sam68 after stripping the membrane (Fig. 2C). Some amount of Sam68 seems to be associated with p85 in basal conditions (6 ± 2% of the immunoprecipitable Sam68), but this association was increased about four times after 5 min of insulin stimulation (up to 26 ± 5%). Therefore, Sam68 is one of the proteins associ-

ated with p85 after insulin stimulation. To confirm the in vivo association of Sam68 with p85, we used the opposite approach (Fig. 2B). Thus, we look for the co-immunoprecipitation of p85 with α-Sam68. Similar results to those of α-Sam68 were obtained. Thus, some amount of p85 is associated with Sam68 (4 ± 1% of the immunoprecipitable p85), but this association is significantly increased (about four times) upon insulin stimulation (up to 21 ± 6%) (Fig. 2B). Sam68 recognizes two bands of 62 and 68 kDa as described in other cell lines [34] and both are tyrosine-phosphorylated after insulin stimulation (Figs. 1 and 2A). To quantify the percentage of Sam68, we calculated the average of the two bands.

3.2. Sam68 is tyrosine-phosphorylated by the activated IR in vitro and associates with the SH2 domains of p85

Affinity-purified IR immobilized with protein A-Sepharose was incubated with or without 100 nM insulin for 1 h and further incubated with 10 μM ATP in the presence of divalent cations. Recombinant Sam68 (100 fmol) was then added and the incubation continued for 1 h. The reaction mixture (supernatant containing Sam68) was then incubated with immobilized GST-p85 fusion proteins and the precipitates were analyzed by Western blotting with α-PY (Fig. 3A). Analysis of the total reaction mixture revealed that Sam68 was tyrosine-phosphorylated by the activated IR. Both GST-p85 (N) and GST-p85 (C) precipitated Sam68. Besides, GST-p85 (N) more effectively precipitated Sam68 compared to GST-p85 (C). To confirm this differential affinities, we repeated the experiment with different amounts of fusion proteins (Fig. 3B and C). GST-p85 (N) at 0.02 μg (500 fmol) interacted significantly with phosphorylated Sam68 (100 fmol), but it did not interact with GST-p85 (C) until 0.1 μg (2.5 pmol) of this fusion protein was employed. Maximal binding of Sam68 was obtained with 1 μg (25 pmol) of GST-p85 (N) (Fig. 3B) whereas 5 μg (125 pmol) of GST-p85 (C) was needed to bind the same amount of Sam68 (Fig. 3C).

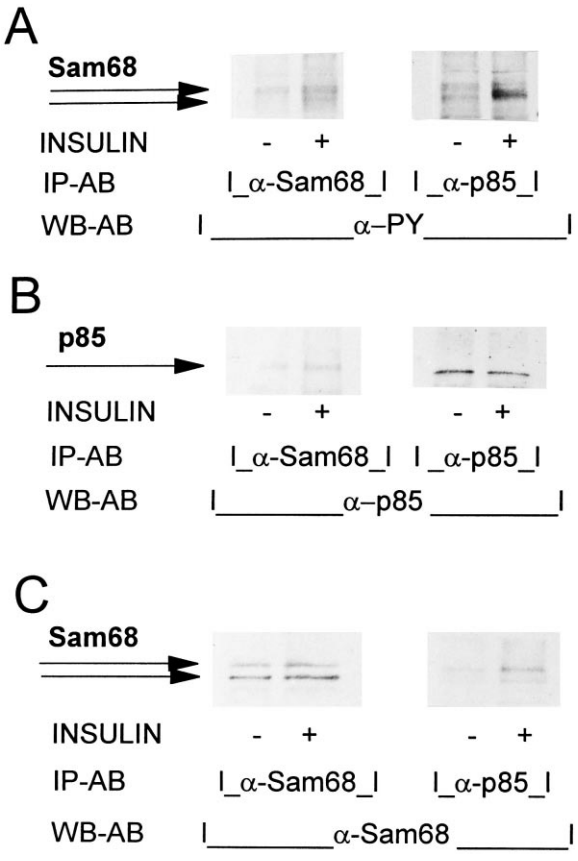

Fig. 2. Insulin-induced tyrosine phosphorylation of Sam68 and its association with p85 in vivo. Cells were incubated for 5 min with 100 nM insulin and solubilized. Cell lysates were then immunoprecipitated with α-Sam68 and α-p85 and analyzed by Western blotting with α-PY (A). The same immunoprecipitates were then analyzed by Western blotting with α-p85 (B) and α-Sam68 (C). One representative experiment of four is shown.

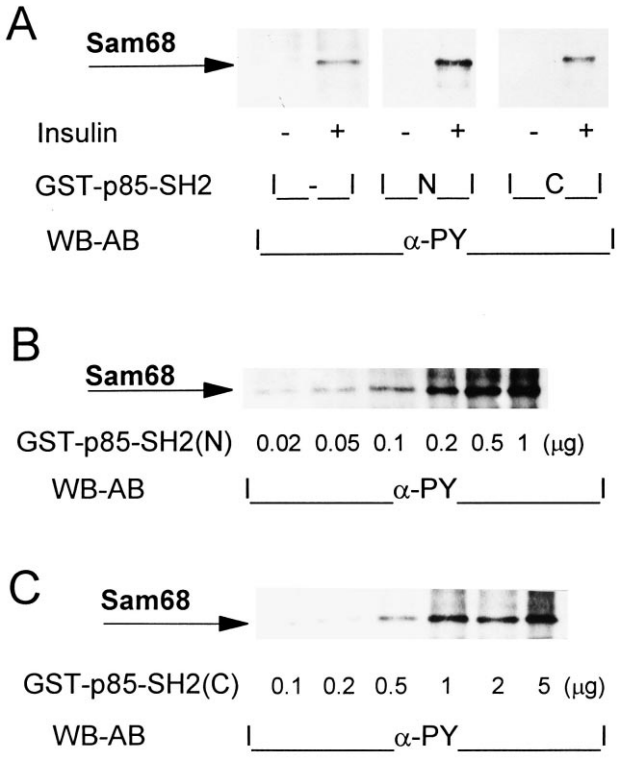

Fig. 3. Insulin-induced tyrosine phosphorylation of Sam68 in vitro and association with the SH2 domains of p85. (A) Affinity-purified IR (bound to protein A-Sepharose) was incubated with recombinant Sam68 (100 fmol) in a tyrosine phosphorylation reaction with or without insulin, in the presence of 10 μM ATP and divalent cations as described in Section 2. Samples were then centrifuged and supernatant containing Sam68 was taken and diluted in reaction buffer (minus divalent cations and ATP), incubated for 1 h with the GST-p85-SH2 (N or C) conjugated with glutathione agarose and then precipitated. Samples were then denatured and analyzed by Western blotting with α-PY. One experiment representative of five is shown. (B) Differential affinities of phosphorylated Sam68 with GST-p85 (N) and GST-p85 (C) fusion proteins. Sam68 was tyrosine-phosphorylated in vitro as described in A. The reaction mixture containing phosphorylated Sam68 was then diluted in reaction buffer (minus divalent cations and ATP) and incubated for 2 h with different amounts of either GST-p85 (N) or GST-p85 (C) fusion proteins conjugated to glutathione agarose and precipitated. These precipitates were then washed and analyzed by Western blotting with α-PY. One representative experiment of three is shown.

4. Discussion

The present study indicates that Sam68, the Src-associated protein in mitosis, is tyrosine-phosphorylated by the IR and is part of the signaling complexes elicited by insulin stimulation. Previous work has shown that Sam68 is a substrate of the

tyrosine kinase c-Src and the threonine kinase Cdc2 during mitosis [24,25,36]. We have studied tyrosine phosphorylation of Sam68 in resting cells, which were serum-starved for 24 h. The time-course of Sam68 tyrosine phosphorylation showed a rapid and transient effect after insulin stimulation, even at a nanomolar concentration, suggesting a direct action of the IR kinase. We cannot exclude the participation of other kinases in this effect of insulin in vivo, however. Nevertheless, in vitro studies with purified IR and recombinant Sam68 have demonstrated that Sam68 may be a direct substrate of IR. Even though we have employed a cell line overexpressing IRs, the dose-response effect of insulin suggests that this effect may be also relevant in a more physiological model with a normal number of IRs. The effect of insulin at a nanomolar concentration also demonstrates the specificity of the action through IRs rather than IGF-1 receptors.

Sam68 is able to bind RNA and contains a KH domain and a region similar to a RGG box [26,27], characteristic for a distinct family of RNA binding proteins [37]. Sam68 is therefore a putative regulator of the RNA metabolism and it could give means for a rapid pathway to regulate protein expression by modifying the mRNA stability and/or mRNA translation [38,39]. Besides, p68 may serve to target the mRNA in the cytoplasm [40] to deliver specific proteins to specific structures, i.e. the cytoskeletal proteins that usually contain SH3 domains, and therefore, to provide means to mediate the interactions. We propose that p68 Sam may be a substrate for the IR and by tyrosine phosphorylation of p68, the metabolism of RNA might be regulated as it has been suggested in other systems [28,32]. This mechanism could allow the cell to respond much faster than protein expression from the novo transcription, when stimulated by insulin.

Sam68 is known to associate with the SH2 and SH3 domains of Src and other signaling molecules such as Grb2, PLC-γ-1 or p85α with different affinities [24,35,30–32]. Sam68 has therefore been envisioned as an adaptor protein in signaling systems such as the T-cell receptor [33,34]. In the present study, we have found an in vivo association of Sam68 with p85α even in basal conditions, but this association is increased upon insulin stimulation about four times, probably due to tyrosine phosphorylation. In vitro studies seem to support this hypothesis, since tyrosine phosphorylation of Sam68 by IR increases the association with the SH2 domains of p85. Moreover, the in vitro association studies with phosphorylated Sam68 and p85 (N) or p85 (C) demonstrated that the binding affinity of Sam68-p85 (N) was higher than that of Sam68-p85 (C). These data strongly suggest that Sam68 is part of the signaling complexes of the IR and that it may function as an adaptor molecule to link the IR signaling cascade with the regulation of ARN metabolism.

Acknowledgements: This work was supported by a Grant from the Ministry of Education, Spain (DGCYT PB94/1437). We are grateful to Dr. I.D. Goldfine (UCSF, Mt Zion Hospital, San Francisco) for providing the HTC-IR cells.

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