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Research Article

The expression of Sam68, a protein involved in insulin signal transduction, is enhanced by insulin stimulation

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Abstract. The role of Sam68, an RNA binding protein and putative substrate of the insulin receptor (IR) in insulin signaling was studied using CHO wild type (WT) cells, CHO cells overexpressing IR, and rat white adipocytes as a physiological system. In CHO-IR cells and adipocytes, Sam68 was tyrosine phosphorylated in response to insulin, and then associated with p85 phosphatidylinositol-3 kinase along with IRS-1. Sam68 was localized mainly in the nucleus of CHO-WT, and both in the nucleus and cytoplasm of CHO-IR cells, but only in the cytoplasm of rat white adipocytes. Insulin stimulation for 16 h enhanced the expression of Sam68 in rat adipocytes and CHO-IR cells. Moreover, CHO-IR cells expressed more Sam68 than CHO-WT, suggesting that overexpression of the IR is enough to induce the expression of Sam68. In summary, these results demonstrate that Sam68 works as a cytoplasmic docking protein which is recruited by IR signaling and whose expression is induced by insulin stimulation, suggesting a putative role for Sam68 in insulin signal transduction.

Key words. Sam68; insulin receptor; insulin signaling; insulin receptor substrate; insulin action; expression.

Sam68, the 68-kDa Src substrate associated during mitosis [1, 2] contains an RNA-binding motif named KH for its homology to the heterogeneous nuclear ribonucleoprotein (hnRNP) K [3, 4]. The Sam68 KH domain is embedded in a larger conserved domain of ~200 amino acids called the GSG domain [5, 6] that can affect protein localization [7, 8] and cell cycle progression [9]. This domain is also called STAR, for signal transduction and activator of RNA [10]. The GSG domain (GRP33, Sam68, GLD-1) is present in functionally diverse proteins such as the *Caenorhabditis elegans* germ line-specific tumor suppressor protein GLD-1 [5], the fragile X mental retardation gene product FMR1 [11] and the mouse quaking gene product Qk1 [12]. Sam68 also contains a region similar to an RGG box (a domain containing several Arg-Gly-Gly motifs) [13, 14]. Both the KH and the RGG box enable Sam68 to bind RNA [13, 14]. Sam68 has, furthermore, been shown to bind to single-stranded nucleic acids and homopolymeric RNA in vitro [15, 16]. A splice variant within the KH domain of Sam68 has decreased RNA-binding properties and can antagonize cell cycle progression [9]. Tyrosine phosphorylation of Sam68 by $p59^{fyn}$ [15] and binding to SH3 domains [17] can negatively regulate its nucleic acid-binding properties. The function of Sam68 remains, however, unknown.

Sam68 is tyrosine phosphorylated in mitotic cells and it forms a complex with Src by interactions with both SH2 and SH3 domains of Src [1, 2]. In addition, because it is Tyr phosphorylated and has proline-rich domains, Sam68 interacts with signaling molecules both through SH2 and SH3 domains [18, 19]. Thus, a role for Sam68 in signal transduction of the T cell receptor and leptin receptor in

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lymphocytes has been proposed [20–23]. Sam68 has therefore been envisioned as an adaptor protein in signaling systems.

After insulin stimulation of cells, phosphatidylinositol-3 kinase (PI3K) forms various signaling complexes with downstream proteins [24, 25]. They include the insulin receptor (IR), IRS-1 and p60–70-kDa phosphoproteins [26–28]. Recently, we have found in hepatoma cells transfected with insulin receptor (HTC-IR) that one of these substrates that associates with PI3K via the SH2 domains of the p85 subunit is Sam68 [29]. Thus, a role for Sam68 in signal transduction of the IR has been proposed [29]. In this regard, we have also found in HTC-IR that Sam68 associates with the SH2 domains of GAP (preferentially at the C terminal) when it is tyrosine phosphorylated by the IR [30]. Furthermore, Sam68 is constitutively associated with the SH3 domains of Grb2 (preferentially at the C-terminal SH3 domain) and this association does not change upon insulin stimulation. The Tyr phosphorylation of Sam68 promotes the association with GAP, and this direct interaction provides a dock for the recruitment of GAP to the Grb2-SOS-Ras signaling pathway [31].

Sam68 has been observed to localize in novel nuclear bodies called Sam68-SLM nuclear bodies in cancer cell lines, due to a nuclear localization signal [8]. However, Sam68 has also been found to translocate out of the nucleus, because of a nuclear export signal [32]. In fact, Sam68 has been shown to functionally substitute for the HIV-1 Rev protein, which plays an essential role in the transport of unspliced HIV virus into the cytoplasm [32–35]. In HTC hepatoma cells, we demonstrated the presence of Sam68 in the nucleus, but the preferential cytosolic localization of Sam68, especially in cells overexpressing IRs (HTC-IR) [32]. Insulin stimulation of HTC-IR for 5 min produced further translocation of Sam68 from the nucleus to the cytosolic fraction [31].

In the present work, we sought to confirm the possible role of Sam68 as a substrate and signaling molecule of the IR in another cell line overexpressing insulin receptors (CHO-IR), and, more importantly, to check the recruitment of Sam68 to IR signaling in physiologically relevant systems such as adipocytes. We also checked the localization of Sam68 in CHO cells and rat adipocytes. Finally, we studied the expression of Sam68 in response to insulin stimulation.

Materials and methods

Antibodies and reagents

Monoclonal antibodies anti-Sam68 (α-Sam68) were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.). Antiserum to the p85α regulatory subunit of PI3K (α-p85) was from Upstate Biotechnology (Lake Placid, N. Y.). Monoclonal antibodies to phosphotyrosine (α-PY) were purchased from Transduction Laboratories (Lexington, Ken.). Dexamethasone, IBMX, insulin and general reagents were from Sigma (Alcobendas, Madrid, Spain).

Cells and preparation of soluble cell lysates

Wild-type Chinese hamster ovary cells (CHO-WT) and CHO cells overexpressing human IR (CHO-IR) were kindly provided by Dr. M. Ouwens (University of Leyden, The Netherlands). Cells were prepared and maintained in Dulbecco's modified Eagle's medium (DMEM) as previously described [36]. For experiments, cells were grown in 100 mm dishes to 90% confluency and serum starved for 24 h. They were treated at 37 °C with 100 nM insulin 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 and 0.4 mM sodium orthovanadate [27]. After centrifugation, the soluble cell lysates were used for the study. Protein concentration was determined with a Bio-Rad (Richmond, Calif.) kit, using bovine serum albumin (BSA) as standard.

Adipocyte isolation, incubation and differentiation

Adipocytes were prepared from the epididymal fat pads of ad libitum-fed, 120- to 180-g male Wistar rats according to the method described by Rodbell [37], with minor modifications [38].

Adipocytes were incubated in Krebs Ringer bicarbonate (KRB) with 20 mM Hepes, supplemented with 0.1% BSA, and 6 mM glucose at 37 °C [38]. When cells were treated with insulin, we used 10 nM for 5 min. Then the incubation was stopped by washing with iced incubation buffer and cells were then solubilized for 30 min in lysis buffer [38].

Preadipocytes were obtained from the infranatant of the adipocyte isolation [39]. Cells were plated in six-well plates and cultured in DMEM with 10% fetal calf serum (FCS) as previously described [40]. After 4 days in culture at 37 °C in an atmosphere of 5% CO_2, differentiation was induced by the addition of medium supplemented with isobutyl methylxanthine (0.5 mM), and dexamethasone (0.25 µM). After 48 h, the induction medium was removed and replaced with medium containing 10% FCS supplemented with insulin (10 µg/ml). This medium was changed every 2 days [40]. Immunohistochemical studies were performed after 4 days in the same plates. The medium was changed to serum free in the absence of insulin for 16 h.

Immunoprecipitation

Soluble cell lysates (2 mg protein) were first precleared with 50 µl protein A-sepharose for 2 h at 4 °C and incubated with appropriate antibodies for 2 h at 4 °C. Fifty microliters of protein A-sepharose were then added to the an-

tibody-containing sample and incubation proceeded for a further 1 h at 4 °C [27]. The immunoprecipitates were washed three times with lysis buffer. Fifty microliters of SDS-stop buffer containing 100 mM DTT were 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% nonfat dry milk for 1 h and washed in TBST. The membranes were then incubated with primary antibodies, 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; Pierce, Rockfold, Ill.) [29]. The bands obtained were scanned and analyzed by the PCBAS 2.0 program.

Sam68 localization

Cells were fixed in 4% paraformadehyde/PBS at room temperature (RT) for 30 min. Endogenous peroxidase activity was inhibited by incubating in 3% hydrogen peroxide for 20 min at RT. In the case of CHO cells, endogenous biotin was blocked by sequential incubation with avidin (Biomeda, Hayward, Calif.) for 15 min at RT and biotin (Biomeda) for 15 min at RT, in a humidity chamber. Plates were rinsed with PBS for 10 min and then permeabilized in 0.1% Triton X-100 (Sigma. St Louis, Mo.). The samples were incubated in 2.5% normal horse serum (Vector, Burlingame, Calif.) and subsequently incubated with the primary anti-Sam68 antibody (1 µg/ml) for 60 min at RT. After incubation, samples were rinsed with PBS for 10 min at RT, and then incubated with the biotinylated secondary antibody (Vector) for 30 min at RT. Subsequently, samples were rinsed with PBS for 10 min, incubated with the streptavidin-peroxidase complex (Vector) for 30 min at RT, and rinsed with PBS for 30 min. A positive reaction was visualized by incubating the samples with 3,3′-diaminobenzidine under microscope control until color development. Finally, samples were rinsed with distilled water. In the case of adipocyte culture, samples were slightly counterstained with Harris' hematoxylin and mounted in a mixture of glycerol-gelatin (Sigma).

mRNA detection by RT-PCR

CHO cells and primary cultured adipocytes were incubated at 37 °C in a 5% CO_2 atmosphere for 16 h in the absence or presence of insulin. Total RNA (1×10^6 cells) was extracted with the QuickPrep Total RNA extraction kit (AmershamPharmacia Biotech, Barcelona, Spain). First-strand cDNA synthesis was performed using an oligo-dT primer (kit from Roche Molecular Biochemicals, Barcelona, Spain) and used for detection of the Sam68 mRNA by RT-PCR as previously described [9]. The sequences of primers and hybridization probes for Sam68, encompassing nucleotides 511–534 and 1101–1125 of Sam68 cDNA located within the KH domain, have been previously used for the detection of Sam68 expression [41]. β-Actin mRNA expression was used as an internal control. The PCR products were analyzed by 2% agarose gel with ethidium bromide staining and Southern hybridization. The hybridization probe was labeled by tailing the oligonucleotide with digoxigenin-dUTP (kit from Roche Molecular Biochemicals). Specific hybridization was developed with anti-digoxigenin antibodies (Roche Molecular Biochemicals) labeled with peroxidase [23]. The bands were visualized by a high-sensitive chemiluminescence system (SuperSignal from Pierce). Values are expressed as means ± SE. Student's t test was used for comparisons, with differences being considered significant at $p < 0.05$.

Results

Tyrosine phosphorylation of Sam68 and its association with p85 PI3K in CHO-IR cells

Using CHO-IR cells, we studied the in vivo tyrosine phosphorylation of Sam68 in response to insulin stimulation. Cells were stimulated with or without 100 nM insulin for 5 min, and lysed. Cell lysates were then used for precipitation with anti-Sam68 and precipitates were analyzed for the presence of Tyr-phosphorylated proteins. As shown in figure 1, immunoprecipitated Sam68 was tyrosine phos-

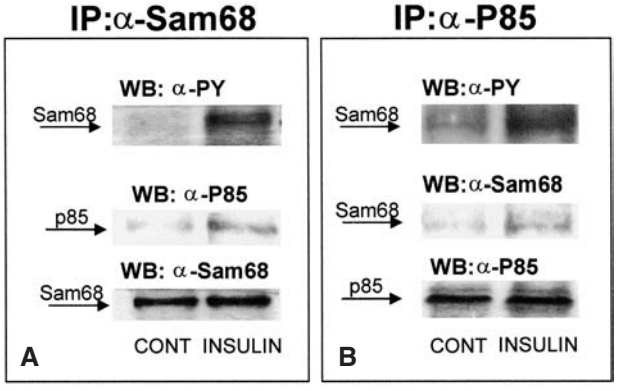

Figure 1. Illustration of Sam68 phosphorylation and its association with the p85 subunit of PI3K in CHO-IR cells without (CONT) or with (INSULIN) insulin stimulation. The pictures are representative of four experiments. (*A*) Western blot analysis of proteins immunoprecipitated with anti-Sam68 antibodies from CHO-IR cell lysate after 5 min incubation without or with 100 nM insulin. Individual protein bands were visualized either with anti-phosphotyrosine (α-PY), anti-p85 subunit (α-P85) or anti-Sam68 antibodies. (*B*) Western blot analysis of proteins immunoprecipitated with anti-p85 antibodies from CHO-IR cell lysate after 5 min incubation without or with 100 nM insulin. Individual protein bands were visualized either with anti-phosphotyrosine (α-PY), anti-Sam68 (α-Sam68) antibodies, or anti-p85 subunit (α-P85).

phorylated when the cells were incubated with 100 nM insulin for 5 min. The immunoprecipitate with anti-p85 also showed p60–70 tyrosine-phosphorylated proteins after 5 min insulin stimulation (fig. 1). To check whether Sam68 was one of these tyrosine-phosphorylated proteins, we probed the samples of the anti-p85 immunoprecipitates with anti-Sam68. Some Sam68 seems to be associated with p85 in basal conditions, but this association was increased about four times after 5 min of insulin stimulation. To confirm the in vivo association of Sam68 with p85, we looked at the co-immunoprecipitation of p85 in anti-Sam68 immunoprecipitates. We found that the association of p85 with Sam68 was increased by insulin stimulation. The amount of protein immunoprecipitated in every lane was assessed by immunoblot with the same immunoprecipitating antibody to control that the differences were not due to technical problems.

Tyrosine phosphorylation of Sam68 and its association with p85 PI3K in rat adipocytes

To confirm the role of Sam68 as a signaling molecule for the IR in a more physiological system, we employed rat white adipocytes. As shown in figure 2, an anti-phosphotyrosine immunoblot of anti-Sam68 immunoprecipitates showed that Sam68 is tyrosine phosphorylated in response to 10 nM insulin, after 5 min stimulation. Similar to the results observed in CHO-IR cells, Sam68 associated with p85 PI3K in rat adipocytes when the cells were stimulated with 10 nM insulin for 5 min. Thus, anti-Sam68 immunoprecipitates showed the co-immunoprecipitation of p85 in insulin-stimulated cells, although some co-immunoprecipitation was observed even in basal conditions. Consistently, anti-p85 immunoprecipitates showed some Sam68 in the specific immunoblot, and the co-immunoprecipitation was significantly increased after 5 min incubation with 10 nM insulin.

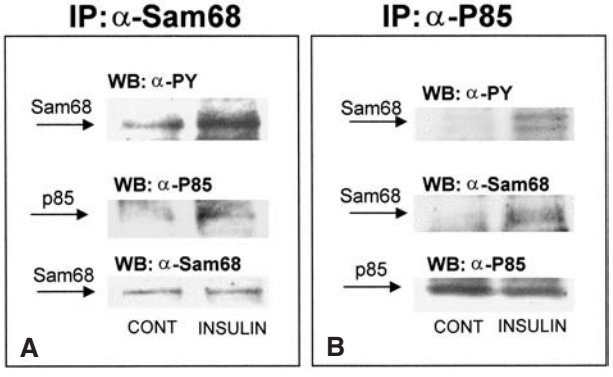

Figure 2. Illustration of Sam68 phosphorylation and its association with the p85 subunit of PI3K in adipocytes without (CONT) or with (INSULIN) insulin stimulation. The pictures are representative of four experiments. (*A*) Western blot analysis of proteins immunoprecipitated with anti-Sam68 antibodies from adipocyte lysate after 5 min incubation without or with 10 nM insulin. Individual protein bands were visualized either with anti-phosphotyrosine (α-PY), or anti-p85 subunit (α-P85) or anti-Sam68 antibodies. (*B*) Western blot analysis of proteins immunoprecipitated with anti-p85 antibodies from adipocyte lysate after 5 min incubation without or with 10 nM insulin. Individual protein bands were visualized either with anti-phosphotyrosine (α-PY), or anti-Sam68 (α-Sam68) antibodies, or anti-p85 subunit (α-P85).

Sam68 localization in CHO-WT, CHO-IR and adipocytes

We have previously found that Sam68 localizes in both the nucleus and the cytosolic fractions of HTC cells, using immunoblots of subcellular fractions, and that insulin stimulation of HTC-IR promotes the translocation from the nucleus to the cytoplasm [30, 31]. To study the subcellular localization of Sam68 in CHO cells and rat adipocytes we performed immunohistochemical studies in cultured cells.

As shown in figure 3A, both CHO-WT and CHO-IR showed marked expression of Sam68 but displayed a different immunolocalization pattern for this protein. A consistent nuclear-positive signal in nuclei with low cytoplasmic staining was visible in all CHO-WT cells, whereas CHO-IR cells showed the intranuclear pattern, and also a strong cytoplasmic and perinuclear immunostaining for Sam68. No changes in the staining pattern were observed in insulin-stimulated CHO-IR cells (data not shown). Immunohistochemistry was controlled without anti-Sam68 primary antibody.

Immunoperoxidase staining of in vitro-differentiated rat adipocytes from epididymal white adipose tissue using anti-Sam68 and hematoxylin nuclear counterstaining is shown in figure 3B. Specific immunostaining for Sam68 was localized to adipocyte perinuclear areas and lipid droplet-surrounding cytoplasm, while nuclei and lipid droplets (arrowheads) remained negative. The same pattern was observed in the presence or absence of insulin.

Sam68 expression in response to insulin stimulation

Next, we sought to assess the modulation of Sam68 expression by insulin receptor stimulation in CHO and rat adipocytes. As shown in figure 4A, overexpression of IR in CHO cells increased the expression of Sam68. Thus, CHO-IR express significantly more Sam68 than CHO-WT (about 50% increase). Furthermore, CHO-IR cells showed an increase in Sam68 expression more than twice the basal levels when the cells were stimulated with insulin for 16 h (fig. 4B). Similarly, insulin stimulation for 16 h increased Sam68 expression about three times the basal levels in rat adipocytes (fig. 4C). Southern blot showed further evidence of the amplified bands.

To check the regulation of Sam68 expression by insulin stimulation in adipose tissue, rat adipocytes were incubated with 10 nM insulin for 8–24 h. As shown in figure 5, the insulin effect was time dependent and a significant increase was observed at 8 h stimulation (about

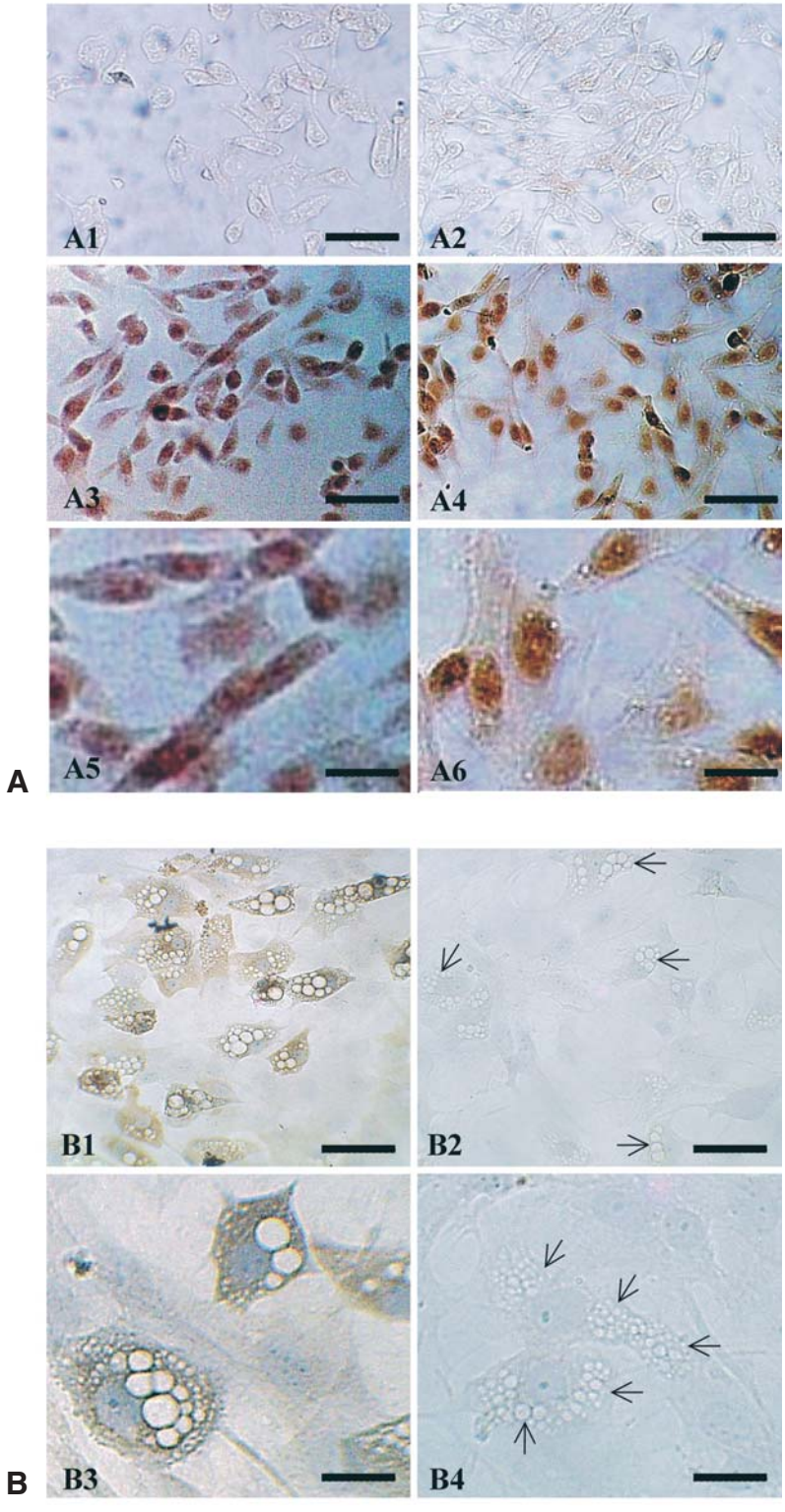

Figure 3. Sam68 is localized in the cytoplasm of CHO cells and in rat adipocytes. (*A*) Immunoperoxidase staining of CHO-IR (1, 3, 5) and CHO-WT (2, 4, 6) monolayers using a monoclonal antibody against Sam68, with no counterstaining. No specific signal and a low background were seen in controls without anti-Sam68 primary antibody (1, 2). Both CHO-WT (4, 6) and CHO-IR (3, 5) cells showed marked expression of Sam68 but displayed a different immunolocalization pattern for this protein. 1–4 bar = 30 μm; 5, 6, bar = 10 μm (*B*) Immunoperoxidase staining of in vitro-differentiated rat adipocytes from epididymal white adipose tissue using a monoclonal antibody against Sam68, with Harris' hematoxilin nuclear counterstain. No specific signal was observed in control adipocytes without anti-Sam68 primary antibody (2, 4). Specific immunostaining for Sam68 was localized to adipocyte perinuclear areas and lipid droplet-surrounding cytoplasm, with nuclei and lipid droplets (arrowheads) remaining negative (1, 3). 1, 2, bar = 40 μm; 3, 4, bar = 10 μm.

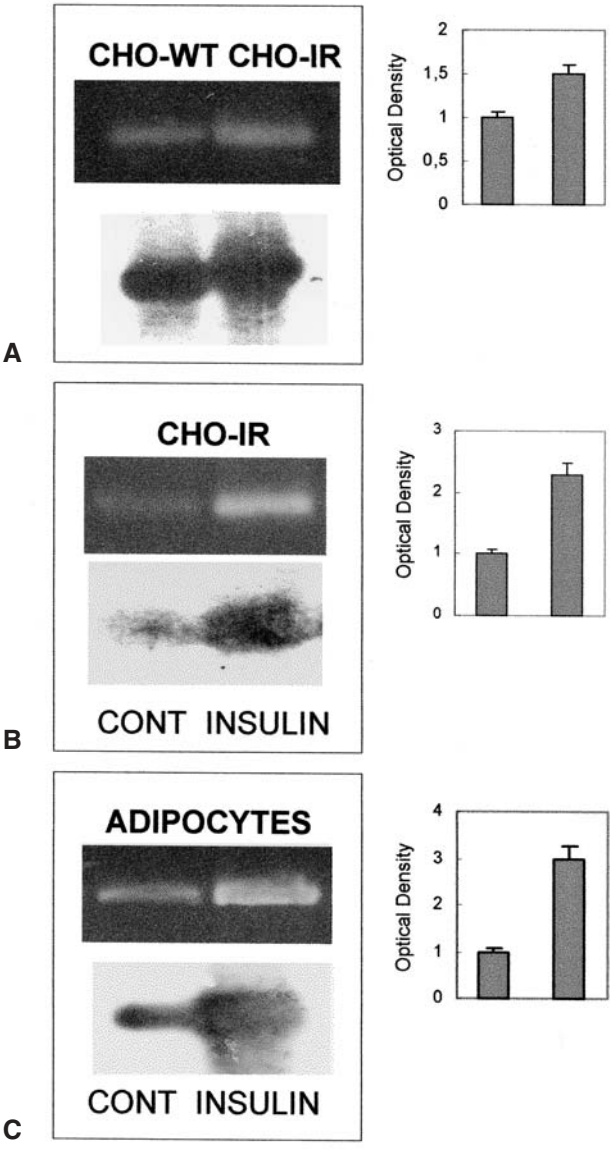

Figure 4. Sam68 expression in CHO-WT, CHO-IR and rat adipocytes. The pictures are representative of four experiments. Bands correspond to the PCR product of the KH domain of Sam68. Sam68 bands were quantified and normalized with the β-actin bands. Densitograms with SE are shown. (*A*) Basal expression of Sam68 in CHO-WT and CHO-IR was assessed by RT-PCR amplification from total RNA. (*B*) CHO-IR cells were cultured in serum-free medium for 16 h in the presence or absence of 100 nM insulin. Total RNA extracted from CHO-IR cells was reverse transcribed and Sam68 expression determined by RT- PCR amplification. (*C*) Adipocytes were cultured in serum-free medium for 16 h in the presence or absence of 10 nM insulin. Total RNA was reverse transcribed and Sam68 expression determined by RT-PCR.

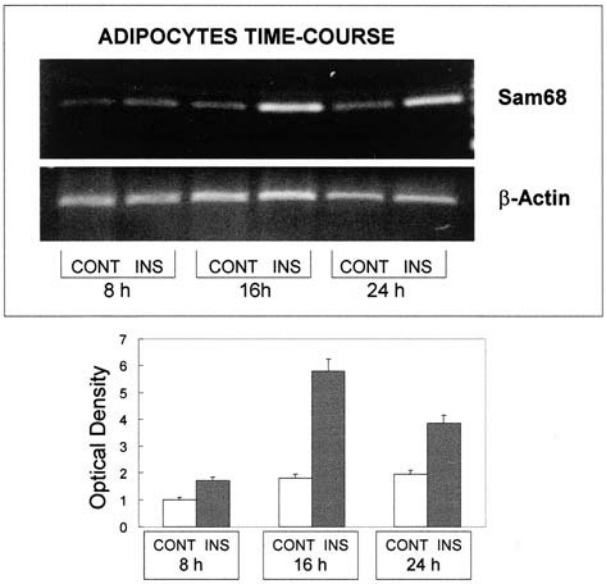

Figure 5. Time-course of insulin stimulation of Sam68 expression. The pictures are representative of four independent experiments. Sam68 bands were quantified and normalized with the β-actin bands. Densitograms with SE are shown. Adipocytes were cultured for different times in serum-free medium in the presence or absence of 10 nM insulin. Total RNA was reverse transcribed and Sam68 expression determined by RT-PCR.

50% increase of Sam68 expression). Insulin stimulation increased the expression of Sam68 about three times the basal levels after 16 h incubation. The insulin effect could be observed after 24 h incubation. After 48 h incubation, the stimulatory effect of insulin on Sam68 expression was no longer observed (not shown).

Discussion

IR activation triggers signaling that begins with the stimulation of tyrosine kinase activity [42], resulting in autophosphorylation of the β subunit and tyrosine phosphorylation of cellular proteins [43, 44]. The major IR substrates belong to the IRS family [45]. During insulin-stimulated tyrosine phosphorylation, the IRS proteins mediate a broad biological response by binding and activating various enzymes or adapter molecules, such as PI3K [46]. Thus, after insulin stimulation of cells, PI3K forms various signaling complexes with downstream proteins, including p60–70-kDa phosphoproteins [27, 28]. We have recently found that one of these substrates of the IR that associates with PI3K via the SH2 domains of the p85 subunit is Sam68 [29, 30]. In fact, we have previously demonstrated that Sam68 preferentially associates with the N-terminal domain of p85.

Our results in CHO-IR cells and rat adipocytes show the tyrosine phosphorylation of Sam68 and the association with PI3K in response to insulin, confirming a role for Sam68 as a signaling molecule in the transduction of the IR. Even though similar expression of Sam68 was observed in CHO-IR cells and adipocytes, higher association with p85 was found in CHO-IR cells in response to insulin, due to greater tyrosine phosphorylation. This result is not striking since CHO-IR cells express more IRs than adipocytes. That is why we employed the supraphysiological dose of 100 nM insulin for maximal stimulation of

CHO-IR cells, whereas 10 nM insulin is enough to reach maximal IR activation in rat adipocytes, as previously described [38]. Nevertheless, similar affinities have previously been demonstrated for IRs from CHO-IR and rat adipocytes [47, 48]. Therefore, even though the participation of Sam68 in IR signaling is magnified by overexpression of the IR, it can also be observed in a physiological system.

Sam68 has been described mainly as a nuclear protein [8], although it can translocate from the nucleus to the cytoplasm [32, 49]. The fact that Sam68 is present in the cytoplasm of CHO cells, especially the CHO-IR cells, and adipocytes (physiological target cells for insulin) is consistent with the hypothesis that considers Sam68 as a signaling molecule for the IR. In fact, insulin stimulation does not induce further cytoplasmic localization in CHO-IR cells, suggesting that there is enough Sam68 present in the cytoplasm to play its role in insulin signaling. In any case, other important substrates of IR such as IRS-1 have been found to translocate to the nucleus and to be tyrosine phosphorylated in response to insulin-like growth factor (IGF-1) [50]. Sam68 has also been demonstrated to be phosphorylated by other tyrosine kinases, such as Sik [48] in the nucleus, negatively regulating its RNA-binding activity [51].

The regulation of Sam68 RNA-binding activity by phosphorylation has been demonstrated in different systems [15, 23], suggesting a link between tyrosine kinase signaling and RNA metabolism. Nevertheless, we have not found any regulation of Sam68 capacity to bind RNA by IR tyrosine phosphorylation in vitro or in vivo, either in HTC cells, CHO or rat adipocytes (data not shown), suggesting a different regulation of Sam68 function depending on the kinase that recruits this protein as a substrate.

Sam68 is a ubiquitously expressed protein [1, 2], and this might count against it playing a specific role as a signaling molecule of IR. Nevertheless, insulin stimulation of target cells seems to regulate the expression of Sam68, and to recruit it to the signaling complexes, in a similar way to other known substrates of the IR [52]. Therefore, Sam68 may be a common signaling molecule to different receptors, including the IR, but also IGF-1, leptin or T cell receptors. In this context, even the substrates of the IRS family have been found to be shared by insulin, IGF-1 and cytokine receptors [53].

In conclusion, the role of Sam68 as a signalling molecule for the IR is confirmed in CHO-IR cells, and it has been demonstrated for the first time in a physiological target cell for insulin, i.e. the adipocyte. Regulation of Sam68 expression by insulin stimulation further supports the physiological role of Sam68 as a signaling molecule for the IR.

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