Journal of Cellular Biochemistry 86:99–106 (2002)

Sam68 Associates With the SH3 Domains of Grb2 Recruiting GAP to the Grb2-SOS Complex in Insulin Receptor Signaling

Souad Najib and Víctor Sánchez-Margalet*

Department of Medical Biochemistry and Molecular Biology, Medical School, Investigation Unit, Virgen Macarena University Hospital, Seville, Spain

Abstract The 68 kDa Src substrate associated during mitosis (Sam68) is an RNA binding protein with Src homology (SH) 2 and 3 domain binding sites. We have recently found that Sam68 is a substrate of the insulin receptor (IR) that translocates from the nucleus to the cytoplasm and that Tyr-phosphorylated Sam68 associates with the SH2 domains of p85 PI3K and GAP, in vivo and in vitro. In the present work, we have further demonstrated the cytoplasmic localization of Sam68, which is increased in cells overexpressing IR. Besides, we sought to further study the association of Sam68 with the Ras-GAP pathway by assessing the interactions with SH3 domains of Grb2. We employed GST-fusion proteins containing the SH3 domains of Grb2 (N or C), and recombinant Sam68 for in vitro studies. In vivo studies of protein–protein interaction were assessed by co-immunoprecipitation experiments with specific antibodies against Sam68, GAP, Grb2, SOS, and phosphotyrosine; and by affinity precipitation with the fusion proteins (SH3-Grb2). Insulin stimulation of HTC-IR cells promotes phosphorylation of Sam68 and its association with the SH2 domains of GAP. Sam68 is constitutively associated with the SH3 domains of Grb2 and it does not change upon insulin stimulation, but Sam68 is Tyr-phosphorylated and promotes the association of GAP with the Grb2-SOS complex. In vitro studies with fusion proteins showed that Sam68 association with Grb2 is preferentially mediated by the C-terminal SH3 domains of Grb2. In conclusion, Sam68 is a substrate of the IR and may have a role as a docking protein in IR signaling, recruiting GAP to the Grb2-SOS complex, and in this way it may modulate Ras activity. J. Cell. Biochem. 86: 99–106, 2002.

Key words: RNA binding protein; Ras-GAP; signal transduction

Insulin binding to its cell surface receptor induces a signal transduction cascade that determines the insulin specific physiological response [Taha and Klip, 1999; Withers and White, 2000]. When insulin binds to the α-subunit of the receptor, it induces a conformational change in the β-subunit activating its intrinsic tyrosine kinase [White et al., 1988; White and Kahn, 1989] that results in autophosphorylation of the β-subunit [White et al., 1988; Lammers et al., 1990], and tyrosine phosphorylation of cellular proteins [Ullrich and Schlessinger, 1990; Cheatham and Kahn, 1995]. The tyrosine phosphorylation of the substrates then triggers the formation of different signaling complexes based on protein–protein interactions [Virkamaki et al., 1999]. Insulin receptor substrate 1 (IRS-1) is a major cellular substrate for the insulin receptor (IR) [White et al., 1985]. Multiple tyrosine phosphorylation 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) [Sun et al., 1992]. The IR itself, via its C-terminal Tyr-1322-Thr-His-Met motif, following tyrosine phosphorylation is also capable of interacting with an SH2 domain of PI3K p85 subunit [Levy-Toledano et al., 1994; Sánchez-Margalet et al., 1995]. PI3K has been proposed to be the key switch mechanism in insulin signaling [Shepherd et al., 1998]. Thus, PI3K activity has been implicated in insulin-stimulated

Grant sponsor: Consejería de Salud, Junta de Andalucía, Spain; Grant number: 07/00.

*Correspondence to: Víctor Sánchez-Margalet, Department of Medical Biochemistry and Molecular Biology, School of Medicine, Av. Sánchez Pizjuan 4, Seville-41009, Spain.
E-mail: margalet@us.es

Received 4 September 2001; Accepted 13 March 2002

DOI 10.1002/jcb.10198

activation of cell metabolism and proliferation [Cheatham et al., 1994; Sánchez-Margalet et al., 1994; Sánchez-Margalet, 2000].

After insulin stimulation of cells, PI3K forms various signaling complexes with downstream proteins [Kahn, 1994; Dorrestijn et al., 1998]. They include the IR, IRS-1, and p60-70 kDa phosphoproteins [Sung et al., 1994, 1998]. Recently, we have found that one of these substrates of IR that associates with PI3K via the SH2 domains of the p85 subunit is Sam68 (Src associated in mitosis) [Sánchez-Margalet and Najib, 1999]. Sam68 [Wong et al., 1992; Lock et al., 1996] is a protein tyrosine-phosphorylated in mitotic cells, and it forms a complex with Src [Fumagalli et al., 1994; Taylor and Shalloway, 1994] and other signaling molecules [Richard et al., 1995; Neet and Hunter, 1995] by interactions with both SH2 and SH3 domains. Thus, a role of Sam68 in signal transduction of IR has been proposed [Sánchez-Margalet and Najib, 1999]. In this regard, we have recently found that Sam68 associates with the SH2 domains of GAP when tyrosine phosphorylated by the IR [Sánchez-Margalet and Najib, 2001], and this seems to be primarily a function of the carboxy terminal SH2 domain of GAP.

Sam68 is able to bind RNA because it contains a KH (heterogeneous nuclear ribonucleoprotein K homology) domain and a region similar to a RGG box (a domain containing several Arg-Gly-Gly motifs) [Dreyfuss et al., 1993; Gibson et al., 1993]. Sam68 has been shown to bind to single-stranded nucleic acid [Wang et al., 1995]. A splice variant within the KH domain of Sam68 has decreased RNA binding properties and can antagonize cell cycle progression [Barlat et al., 1997]. The RNA binding capability of Sam68 can be negatively regulated by tyrosine phosphorylation [Wang et al., 1995] and binding to SH3 domains [Taylor et al., 1995].

Sam68 has been found to bind SH3 domains of different molecules in vitro: Src, p85, PLCγ, and Grb2 [Williger and Liscovitch, 1997; Shen et al., 1999]. Nevertheless, although the SH3-mediated interactions with p85 and PLC-γ has been confirmed [Richard et al., 1995; Taylor et al., 1995], no evidence of in vivo interaction of Sam68 with Grb2 in cells has been provided.

Grb2 is an adaptor protein with an SH2 domain that interacts with phosphorylated substrates of IR, such as IRS-1 and Shc [Skolnik et al., 1993; Downward, 1994; Songyang et al., 1994] and two SH3 domains that direct its association with the Ras guanyl nucleotide exchange factor SOS [Chardin et al., 1993; Egan et al., 1993]. The formation of the Shc-Grb2-SOS and IRS-1-Grb2-SOS complexes are thought to mediate the activation of Ras [Baltensperger et al., 1993; Ouwens et al., 1994].

To further investigate the role of Sam68 in IR signaling, we sought to assess the in vitro interaction of Sam68 with the SH3 domains of Grb2 and the in vivo consequences in the formation of IR signaling complexes. In the present study, we report that Sam68 associates with Grb2 in vivo and in vitro by interaction with the SH3 domains of Grb2 (preferentially the carboxy terminal SH3 domain), recruiting GAP to the Grb2-SOS complex when tyrosine phosphorylated by insulin stimulation.

MATERIALS AND METHODS

Antibodies and Reagents

Recombinant protein comprising residues 331-443 of Sam68 (containing potential SH3 binding sites and tyrosine phosphorylation sites), monoclonal antibodies anti-Sam68 (α-Sam68) and anti-IR (α-IR), anti-p120GAP (α-GAP), polyclonal anti-Grb2 (α-Grb2), anti-SOS (α-SOS), and glutathione S-transferase (GST) fusion proteins containing the N- or C-terminal SH3 domains of Grb2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibodies to phosphotyrosine (α-PY) were purchased from Transduction Laboratories (Lexington, KY).

Cells and Preparation of Soluble Cell Lysates

Wild type rat HTC hepatoma cells (HTC-WT) and overexpressing human IR (HTC-IR) were kindly provided by Dr. Ira D. Goldfine (UCSF, San Francisco, CA). Cells were prepared and maintained in Dulbecco's modified Eagle's medium (DMEM) as previously described [Sung et al., 1994]. 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 [Sung et al., 1994]. After centrifugation, the soluble cell lysates were used for the study. Protein concentration was determined by a kit

from Bio-Rad (Richmond, CA), using bovine serum albumin as standard.

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 antibody containing sample and incubation further proceeded for 1 h at 4°C (Sung et al., 1994). 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 analysed 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 from Pierce, Rockfold, IL) [Sánchez-Margalet and Najib, 1999].

Interaction of Cellular Proteins With GST Fused to SH3 Domains of Grb2 (GST-Grb2-SH3)

HTC-IR cell lysates were denatured with 1% SDS to disrupt endogenous protein–protein interactions [Sánchez-Margalet et al., 1995]. Then, cell lysates were diluted 25-fold in lysis buffer and incubated for 1 h at 4°C with 2 μg of GST-Grb2-SH3 fusion protein (N or C). After centrifugation, affinity precipitates were washed three times with lysis buffer and boiled in SDS-stop buffer.

Affinity Precipitation of Sam68 With GST Fusion Proteins

Reaction mixture containing recombinant Sam68 was incubated for 2 h at 4°C with the GST-Grb2 N- or C-SH3 domain fusion protein (2 μg) conjugated with glutathione agarose. 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.

Sam68 Localization

Serum-starved (24 h) HTC-IR and HTC-WT cells were harvested and homogenized in 10 mM Tris-ClH, pH 7.5 and protease inhibitors. Nuclei were obtained by centrifugation at 2,200*g* at 4°C for 5 min. Aliquots of the supernatants and precipitates were then denatured in SDS-stop buffer containing 100 mM DTT by boiling during 5 min. Samples were finally analyzed by SDS–PAGE and immunoblotting with anti-Sam68.

RESULTS

Sam68 Associates With Grb2 In Vitro by Interacting With the SH3 Domains of Grb2

To investigate the association of Sam68 with Grb2, we employed two fusion proteins containing either the N- or C-terminal SH3 domains of Grb2 (GST-Grb2-SH3 (N) or (C)). Recombinant Sam68 (100 fmol) was incubated for 1 h with immobilized GST-Grb2-SH3 fusion proteins, and the precipitates were analyzed by Western blotting with anti Sam68 (Fig. 1). Both GST-Grb2-SH3 (N) and (C) precipitated Sam68. Besides, GST-Grb2-SH3 (C) more effectively

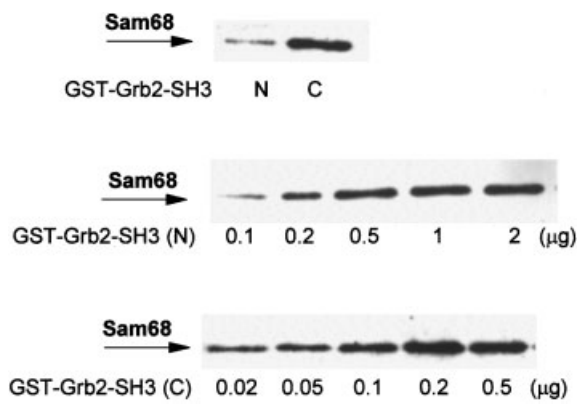

Fig. 1. Sam68 associates with Grb2 by direct interaction with the SH3 domains of Grb2. Affinity precipitation of Sam68 with GST-Grb2-SH3 fusion proteins in vitro. Recombinant Sam68 (100 fmol) was incubated with the GST-Grb2-SH3 (N or C) conjugated with glutathione agarose and then precipitated. Samples were then denatured and analyzed by Western blotting with anti-Sam68. One experiment representative of four is shown in upper panel. Differential affinities of Sam68 with the SH3 domains of Grb2 (N or C). The same amount of recombinant Sam68 (100 fmol) was incubated with different amounts of either GST-Grb2-SH3 (N) or GST-Grb2-SH3 (C) fusion proteins conjugated to glutathione agarose and precipitated. These precipitates were then washed and analyzed by Western blotting with anti-Sam68. One experiment representative of three is shown.

precipitated Sam68 compared to GST-Grb2-SH3 (N). Control experiments with Sepharose beads showed negative results (data not shown). To confirm these differential affinities, we employed different amounts of fusion proteins (Fig. 1). GST-Grb2-SH3 (C) at 0.02 μg (500 fmol) interacted significantly with Sam68 (100 fmol), but GST-Grb2 (N) did not interact with Sam68 (100 fmol) until 0.1 μg (3 pmol) of this fusion protein was employed.

Sam68 is Associated With Grb2 In Vivo and Recruits GAP to the Grb2-SOS Pathway

Although the SH3-mediated interactions of Sam68 with p85 and PLC-γ has been confirmed [Richard et al., 1995; Taylor et al., 1995], no evidence of in vivo interaction of Sam68 with Grb2 in cells has been provided. To test whether Sam68 was able to associate with Grb2-SOS complex in HTC-IR cells, we performed anti-Grb2 and anti-SOS immunoprecipitations in lysates from cells previously stimulated or not with insulin (Fig. 2) to look at the co-precipitation of Sam68. As shown in Figure 2, Sam68 was present in both anti-Grb2 and anti-SOS immunoprecipitates. Insulin stimulation did not induce further association of Sam68 with Grb2-SOS complex. However, Western blot analysis of the same immunoprecipitates with anti-PY showed strong Tyr-phosphorylation of Sam68 associated with Grb2 and SOS when cells are stimulated with insulin (Fig. 2), suggesting that the association is not dependent on tyrosine phosphorylation. Control experiments were performed with pre-immune serum with negative results in the immunoblots (data not shown).

We have previously shown that Sam68 associates with GAP when tyrosine phosphorylated by IR, through the interaction with the SH2 domains of GAP [Sánchez-Margalet and Najib, 2001]. Since Sam68 is associated with Grb2 with no change upon insulin stimulation, we tested whether Sam68 could recruit GAP to the Grb2-SOS complex when tyrosine phosphorylated by insulin stimulation. As shown in Figure 3, there is some amount of GAP associated with Grb2 and SOS, but there was a significant increase in the association when the cells were stimulated with insulin. Control experiments were performed using pre-immune serum (data not shown).

To further investigate the mechanism that mediates the association of Sam68 with Grb2,

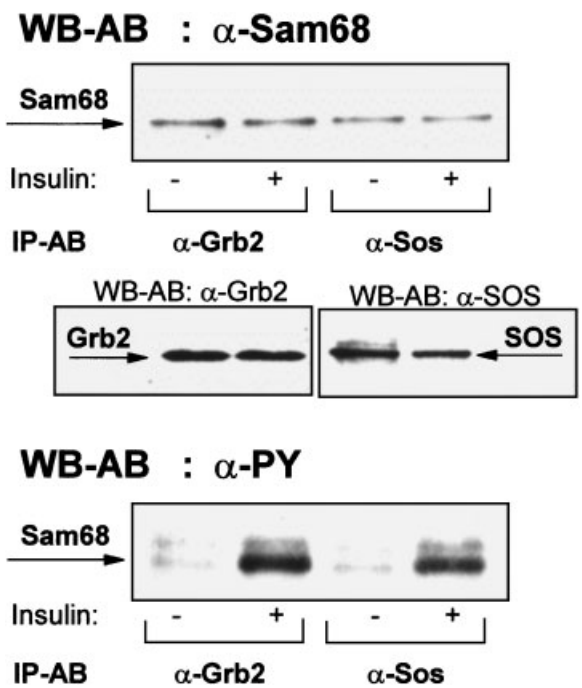

Fig. 2. Sam68 associates with Grb2-SOS complex in vivo. HTC-IR cells were serum-starved for 24 h. Then cells were incubated with or without 100 nM insulin and lysed. Cell lysates were then immunoprecipitated with α-Grb2, α-SOS, and α-Sam68. Samples were analyzed by Western blotting with α-Sam68, and α-PY. The immunoprecipitations were controlled by the specific immunoblots with α-Grb2 and α-SOS. An experiment representative of three is shown.

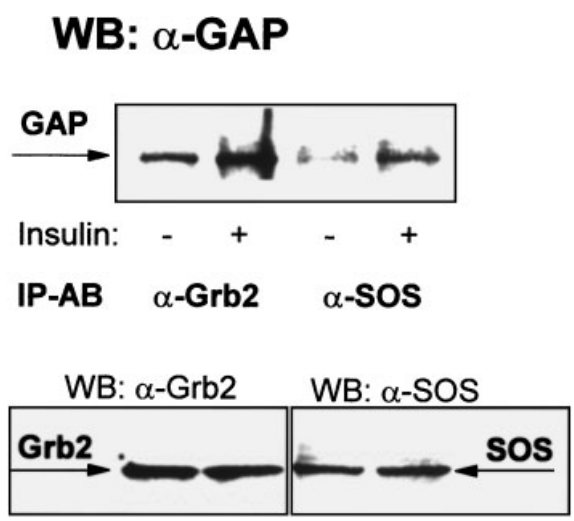

Fig. 3. Sam68 recruits GAP to Grb2-SOS complex in response to insulin. HTC-IR cells were serum-starved for 24 h. Then cells were incubated with or without 100 nM insulin and lysed. Cell lysates were then immunoprecipitated with α-Grb2 and α-SOS. Samples were analyzed by Western blotting with α-GAP. The immunoprecipitations were controlled by the specific immunoblots with α-Grb2 and α-SOS. An experiment representative of three is shown.

we employed two fusion proteins containing either the N- or C-terminal SH3 domains of Grb2. We employed the fusion proteins to affinity precipitate Sam68 in solubilized cell lysates from HTC-IR. Both the C- and N-terminal SH3 domains of Grb2 were able to affinity precipitate Sam68, although C-terminal SH3 domain was more effective compared to the N-terminal SH3 domain (data not shown). As shown in Figure 4A, Sam68 was associated with the Grb2-SH3 (C) fusion protein, and there was no further association when cells were stimulated with insulin. Besides, Sam68 associated with the SH3 domains of Grb2 was able to bind GAP after insulin stimulation (Fig. 4B). These data suggest that GAP can be recruited to the Grb2 complex by association with Sam68 after insulin stimulation.

Overexpression of the IR in HTC Hepatoma Cells Decreases the Presence of Sam68 in the Nucleus and Increases in Cytoplasm

We have recently found that Sam68 is preferentially present in the cytoplasm of HTC hepatoma cells overexpressing IRs [Sánchez-Margalet and Najib, 2001]. Besides, Sam68 present in the nucleus translocates to the cytoplasm in response to insulin in these cells [Sánchez-Margalet and Najib, 2001]. In order to check the effect of the IR overexpression on the localization of Sam68, we compare HTC-WT and HTC-IR by detecting the protein in nuclear and cytoplasmic extracts. As shown in Figure 5, HTC-IR cells preferentially express Sam68 in the cytoplasm as previously described [Sánchez-Margalet and Najib, 2001]. HTC-WT cells also express more Sam68 in the cytoplasm than the nucleus (Fig. 5). Comparing both cell lines, HTC-IR cells contain less Sam68 in the nucleus than HTC-WT, whereas the HTC-IR express higher amount of Sam68 in the cytoplasm than HTC-WT. These results suggest that overexpression of IRs correlates with some translocation of Sam68 from the nucleus to the cytoplasm.

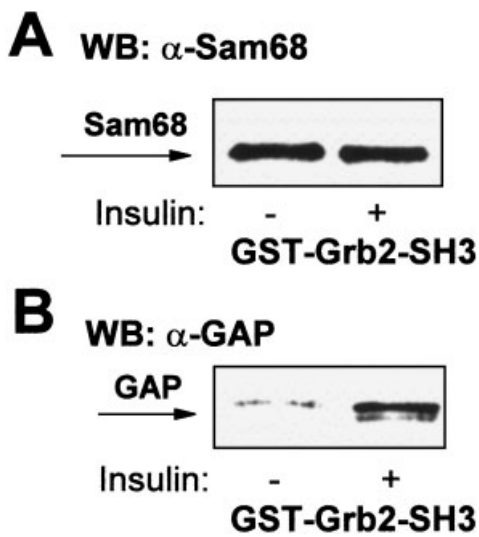

Fig. 4. The association of Sam68 and GAP with Grb2 in cell lysates is mediated by the interaction with the SH3 domains of Grb2. Affinity precipitation of Sam68 with GST-Grb2-SH3 fusion proteins in vivo. Serum-starved HTC-IR cells were incubated with or without 100 nM insulin and lysed. Cell lysates were denatured by boiling in 1% SDS to disrupt endogenous protein–protein interactions as described under Materials and Methods. These were diluted 25-fold in lysis buffer and incubated for 1 h with 2 μg fusion proteins bound to agarose beads. Cellular proteins associated with immobilized GST-Grb2-SH3 (C) fusion proteins were then precipitated and analyzed by Western blotting with α-Sam68 (**A**) and α-GAP (**B**).

DISCUSSION

Sam68 is an RNA binding protein with a putative role in signal transduction because of its capacity to interact with other proteins containing SH2 and SH3 domains. Both SH2- and SH3-binding domains are present in the C-terminal sequence of Sam68 [Richard et al., 1995], suggesting a role for protein–protein interactions [Taylor and Shalloway, 1994; Fumagalli et al., 1994]. Thus, Sam68 has been implicated in signal transduction of T-cell receptor [Fusaki et al., 1997; Jabado et al., 1998]. We have recently described the in vitro and in vivo association of Sam68 with the SH2 domains of p85 PI3K (preferentially the N-terminal) when it is Tyr-phosphorylated by the IR [Sánchez-Margalet and Najib, 1999]. Moreover,

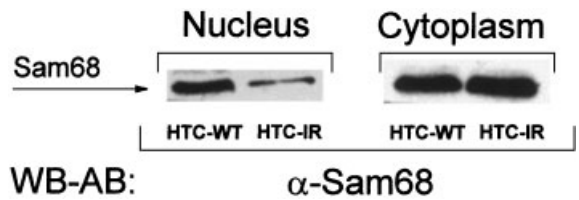

Fig. 5. Sam68 is preferentially localized in the cytoplasm in HTC-IR cells. HTC-IR and HTC-WT were homogenated as described under Materials and Methods. Cell lysates were centrifuged at 2,200*g* to precipitate nuclei. Precipitates (nuclei) and supernatants (cytoplasm and membranes) were then denatured and analyzed by immunoblot with anti-Sam68 antibody. WB-AB, Western blotting antibody. One immunoblot representative of three is shown.

we have found that Sam68 is tyrosine phosphorylated and associates with p85 PI3K after insulin stimulation in a complex that also contains IRS-1 and the IR itself. Tyr-phosphorylated Sam68 not only binds the p85 regulatory subunit but is also associated with an increased PI3K activity [Sánchez-Margalet and Najib, 2001].

Recently, we have also found that insulin-stimulated tyrosine phosphorylated Sam68 binds to the SH2 domains of p120GAP, both in vitro and in vivo (preferentially the C-terminal SH2 domain) [Sánchez-Margalet and Najib, 2001]. Thus, we have demonstrated the direct interaction of Tyr-phosphorylated Sam68 with the SH2 domains of GAP and p85 PI3K. The simultaneous association of Sam68 with p85 PI3K and GAP may help to bring together these two molecules, suggesting that Sam68 may link PI3K with the Ras pathway in IR signaling [Sánchez-Margalet and Najib, 2001]. In fact, in vivo interaction of p110 PI3K and Ras has been previously described, suggesting that PI3K is a target of Ras [Rodriguez-Viciana et al., 1994].

To further investigate the possible role of Sam68 in the Ras signaling pathway, in the present work we have studied the interaction between Sam68 and Grb2. Grb2 is an adaptor protein with central SH2 domain flanked by two SH3 domains that direct its association with the Ras guanyl nucleotide exchange factor SOS [Chardin et al., 1993; Egan et al., 1993].

Grb2 has previously been demonstrated to bind to Sam68 in an SH2 domain-dependent manner [Richard et al., 1995, Taylor et al., 1995]. Besides, in vitro association of Sam68 with the SH3 domains of Grb2 has also been suggested, although with no compelling evidence [Shen et al., 1999]. Our results in the present work indicate that Sam68 does interact with Grb2 SH3 domains in vitro and in vivo, providing the basis for a new signaling complex of the IR. Moreover, we have found that the C-terminal SH3 domain of Grb2 more effectively binds Sam68 compared to the N-terminal SH2 domain of p120GAP. These results are consistent with the preferential binding of the N-terminal SH3 domain of Grb2 with SOS [Egan et al., 1993]. Therefore, simultaneous association of Grb2 with SOS and Sam68 may be possible by interaction with the N- and C-terminal SH3 domains of Grb2, respectively.

We have not tested in vitro association of Sam68 with the SH2 domain of Grb2. However, the in vivo association between Sam68 and Grb2 that we have found in HTC-IR cells is not dependent on Tyr-phosphorylation by insulin stimulation. Therefore, if there is some SH2 mediated interaction it may not be the major mechanism for the in vivo association of Sam68 with Grb2 in HTC-IR cells.

Sam68 has been previously described to associate with GAP in mitosis by interacting with the SH2 domains [Taylor et al., 1995; Guitard et al., 1998]. We have also demonstrated that insulin-mediated Tyr-phosphorylated Sam68 associates with the SH2 domains of GAP [Sánchez-Margalet and Najib, 2001]. In the present study, we sought to assess whether Sam68 could recruit GAP to the Grb2-SOS complex upon insulin stimulation. We did find an increase in GAP amount associated with Grb2-SOS in vivo after insulin challenge. Since no change in Sam68 association with Grb2 was observed after Tyr-phosphorylation by IR in HTC-IR cells, a possible explanation could be that GAP associates indirectly with Grb2-SOS by interacting with Sam68 when it is Tyr-phosphorylated by insulin stimulation. On the other hand, some GAP was also present in the Grb2-SOS complex without insulin stimulation. In this context, a direct interaction between GAP and the SH3 domain of Src family kinases, mediated by a proline-rich N-terminal region of GAP, has been proposed [Briggs et al., 1995]. Therefore, further investigation would be needed to determine the possible contribution of the direct interaction between GAP and Grb2 via the SH3 domains. In any case, the Tyr-phosphorylation dependence of the GAP association with Grb2-SOS complex after insulin stimulation implies that the main mechanism may be through the association with Sam68, which is already bound to Grb2-SOS. To our knowledge, this is the first time that GAP has been proposed to be in the same complex of Grb2-SOS in any signaling pathway. This complex may provide increased GTPase activity in addition to GDP/GTP exchange activity to Ras.

Regarding the localization of Sam68, we have previously demonstrated the preferential cytoplasmic expression of this protein in HTC-IR [Sánchez-Margalet and Najib, 2001]. Actually, we have also reported that Sam68 further relocalizes from the nucleus to the cytoplasm in response to insulin [Sánchez-Margalet and

Najib, 2001]. In the present work, we are showing that the localization of Sam68 in the cytoplasm is also increased when the IR is overexpressed (Fig. 5). These data of cytoplasmic localization of Sam68 and traffic from the nucleus to the cytoplasm are consistent with previously reported results from other groups [McBride et al., 1996]. Therefore, Sam68 may be part of the signaling machinery of the IR in the cytoplasm, functioning as a docking protein.

In summary, our study demonstrates that Sam68 associates with Grb2 in vitro and in vivo by interacting with the SH3 domains of Grb2 (preferentially the C-terminal SH3 domain). This direct interaction provides a dock for the recruitment of GAP after Tyr-phosphorylation of Sam68 by IR activation, linking GAP to the Grb2-SOS-Ras signaling pathway. The possible consequences of these associations on GAP and Ras activity is an exciting question that remains to be investigated, but the present results suggest that they may play a role in IR signal transduction.

ACKNOWLEDGMENTS

We are grateful to Dr. I.D. Goldfine (UCSF, Mt. Zion Hospital, San Francisco, CA) for providing the HTC-WT and HTC-IR cells.

REFERENCES

Baltensperger K, Kozma LM, Cherniack AD, Klarlund JK, Chawla A, Banerjee U, Czech MP. 1993. Binding of the Ras activator son of sevenless to insulin receptor substrate-1 signaling complexes. Science 260:1950–1952.

Barlat I, Maurier F, Duchesne M, Guitard E, Tocque B, Schweighoffer F. 1997. A role for Sam68 in cell cycle progression antagonized by a spliced variant within the KH domain. J Biol Chem 272:3129–3132.

Briggs SD, Bryant SS, Jove R, Sanderson SD, Smithgall TE. 1995. The Ras GTPase-activating protein (GAP) is an SH3 domain-binding protein and substrate for the Src-related Tyrosine kinase, Hck. J Biol Chem 270:14718–14724.

Chardin P, Camonis JH, Gale NW, van Aelst L, Schlessinger J, Wigler MH, Bar-Sagi D. 1993. Human SOS1: a guanine nucleotide exchange factor for Ras that binds to GRB2. Science 260:1338–1343.

Cheatham B, Kahn CR. 1995. Insulin action and the insulin signaling network. Endocr Rev 16:117–142.

Cheatham B, Vlahos C, Cheatham L, Wang L, Blenis J, Kahn CR. 1994. Phosphatidylinositol 3-kinase activation is required for insulin stimulation of pp70 S6 kinase, DNA synthesis, and glucose transporter translocation. Mol Cell Biol 14:4902–4911.

Dorrestijn J, van Bussel FJ, Maassen JA, Gomes de Mesquita DS. 1998. Early steps in insulin action. Arch Physiol Biochem 106:269–289.

Downward J. 1994. The GRB2/Sem-5 adaptor protein. FEBS Lett 338:113–117.

Dreyfuss G, Matunis MJ, Pinol-Roma S, Burd CG. 1993. hnRP proteins and the biogenesis of mRNA. Annu Rev Biochem 62:289–321.

Egan SE, Giddings BW, Brooks MW, Buday L, Sizeland AM, Weinberg RA. 1993. Association of SOS Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. Nature 363:45–51.

Fumagalli S, Totti N, Hsuan JJ, Courtneidge SA. 1994. A target for Src in mitosis. Nature 368:871–874.

Fusaki N, Iwamatsu A, Iwashima M, Fujisawa JI. 1997. Interaction between Sam68 and Src family tyrosine kinases Fyn and Lck, in T cell receptor signalling. J Biol Chem 272:6214–6219.

Gibson TJ, Thompson JD, Heringa J. 1993. The KH domain occurs in a diverse set of RNA-binding proteins that include the antiterminator NusA and is probably involved in binding to nucleic acid. FEBS Lett 324:361–366.

Guitard E, Barlat I, Maurier F, Schweighoffer F, Tocque B. 1998. Sam68 is a Ras-GAP-associated protein in mitosis. Biochem Biophys Res Commun 245:562–566.

Jabado N, Jauliac S, Pallier A, Bernard F, Fischer A, Hivroz C. 1998. Sam68 association with p120GAP in CD4+ T cells is dependent on CD4 molecule expression. J Immunol 161:2798–2803.

Kahn CR. 1994. Insulin action, diabetogenes, and the cause of type II diabetes. Diabetes 43:1066–1084.

Lammers R, Van Obberghen E, Ballotti R, Schlessinger J, Ullrich A. 1990. Transphosphorylation as a possible mechanism for insulin and epidermal growth factor receptor activation. J Biol Chem 265:16886–16890.

Levy-Toledano R, Taouis M, Blaettler DH, Gorden P, Taylor SI. 1994. Insulin-induced activation of phosphatidylinositol 3-kinase: demonstration that the p85 subunit binds to the COOH terminus of the insulin receptor in intact cells. J Biol Chem 269:31178–31182.

Lock P, Fumagalli S, Polakis P, McCormick F, Courtneidge SA. 1996. The human p62 cDNA encodes Sam68 and not the Ras GAP-associated p62 protein. Cell 84:23–24.

McBride AE, Schlegel A, Kirkegaard K. 1996. Human protein Sam68 relocalization and interaction with poliovirus RNA polymerase in infected cells. Proc Natl Acad Sci USA 93:2296–2301.

Neet K, Hunter T. 1995. The nonreceptor protein-tyrosine kinase CSK complexes directly with the GTPase-activating protein-associated p62 protein in cells expressing v-Src or activated c-Src. Mol Cell Biol 15:4908–4920.

Ouwens DM, van der Zon GC, Pronk GJ, Bos JL, Moller W, Cheatham B, Kahn CR, Maassen JA. 1994. A mutant insulin receptor induces formation of a Shc-growth factor receptor bound protein 2 (Grb2) complex and p21ras-GTP without detectable interaction of insulin receptor substrate 1 (IRS1) with Grb2. Evidence for IRS1-independent p21ras-GTP formation. J Biol Chem 269:33116–33122.

Richard S, Yu D, Blumer KJ, Hausladen D, Olszowy MW, Connely PA, Shaw AS. 1995. Association of p62, a multifunctional SH2- and SH3-domain binding protein, with Src family tyrosine kinases, Grb2, and phospholipase Cγ1. Mol Cell Biol 15:186–197.

Rodriguez-Viciana P, Warne PH, Dhand R, Vanhaesebroek B, Gout I, Fry MJ, Waterfield MD, Downward J. 1994.

Phosphatidylinositol-3-OH kinase as a direct target of Ras. Nature 370:527–532.
Sánchez-Margalet V. 2000. Stimulation of glycogen synthesis by insulin requires S6 kinase and phosphatidylinositol-3-kinase in HTC-IR cells. J Cell Physiol 182:182–188.
Sánchez-Margalet V, Najib S. 1999. p68 Sam is a substrate of the insulin receptor and associates with the SH2 domains of p85 PI3K. FEBS Lett 455:307–310.
Sánchez-Margalet V, Najib S. 2001. Sam68 is a docking protein linking GAP and PI3K in insulin receptor signaling. Mol Cell Endocrinol 183:113–121.
Sánchez-Margalet V, Goldfine ID, Vlahos C, Sung CK. 1994. Role of phosphatidylinositol-3-kinase in insulin receptor signaling: studies with inhibitor LY294002. Biochem Biophys Res Commun 204:446–452.
Sánchez-Margalet V, Goldfine ID, Truitt K, Imboden J, Sung CK. 1995. Role of p85 subunit of phosphatidylinositol 3-kinase as an adaptor molecule linking the insulin receptor to insulin receptor substrate 1. Mol Endocrinol 9:435–442.
Shen Z, Batzer A, Koehler JA, Polakis P, Schlessinger J, Lydon NB, Moran M. 1999. Evidence for SH3 domain directed binding and phosphorylation of Sam68 by Src. Oncogene 18:4647–4653.
Shepherd PR, Withers DJ, Siddle K. 1998. Phosphoinositide 3-kinase: the key switch mechanism in insulin signalling. Biochem J 333:471–490.
Skolnik EY, Batzer A, Li N, Lee CH, Lowenstein E, Mohammadi M, Margolis B, Schlessinger J. 1993. The function of GRB2 in linking the insulin receptor to Ras signaling pathways. Science 260:1953–1955.
Songyang Z, Shoelson SE, McGlade J, Olivier P, Pawson T, Bustelo XR, Barbacid M, Sabe H, Hanafusa H, Yi T, Ren R, Baltimore D, Ratnofsky S, Feldman RA, Cantly LC. 1994. Specific motifs recognized by the SH2 domains of Csk, 3BP2, fps/fes, GRB-2, HCP, SHC, Syk, and Vav. Mol Cell Bio l4:2777–2785.
Sun X-J, Miralpeix M, Myers MG, Jr, Glasheen EM, Backer JM, Kahn CR, White MF. 1992. Expression and function of IRS-1 in insulin signal transmission. J Biol Chem 267: 22662–22672.
Sung CK, Sanchez-Margalet V, Goldfine ID. 1994. Role of p85 subunit of phosphatidylinositol-3-kinase as an adaptor molecule linking the insulin receptor, p62, and GTPase-activating protein. J Biol Chem 269:12503–12507.
Sung CK, Choi W, Sánchez-Margalet V. 1998. Guanosine triphosphate-activating protein-associated protein, but not Src-associated protein p68 in mitosis, is a part of insulin signaling complexes. Endocrinology 139:2392–2398.
Taha C, Klip A. 1999. The insulin signaling pathway. J Membr Biol 169:1–12.
Taylor SJ, Shalloway D. 1994. An RNA-binding protein associated with Src through its SH2 and SH3 domains in mitosis. Nature 368:867–871.
Taylor SJ, Anafi M, Pawson T, Shalloway D. 1995. Functional interaction between c-Src and its mitotic target, Sam68. J Biol Chem 270:10120–10124.
Ullrich A, Schlessinger J. 1990. Signal transduction by receptors with tyrosine activity. Cell 61:203–212.
Virkamaki A, Ueki K, Kahn CR. 1999. Protein–protein interaction in insulin signaling and the molecular mechanisms of insulin resistance. J Clin Invest 103:931–943.
Wang LL, Richard S, Shaw AS. 1995. p62 association with RNA is regulated by tyrosine phosphorylation. J Biol Chem 270:2010–2013.
White MF, Kahn CR. 1989. The cascade of autophosphorylation in the β-subunit of the insulin receptor. J Cell Biochem 39:429–441.
White MF, Maron R, Kahn CR. 1985. Insulin rapidly stimulates tyrosine phosphorylation of a Mr 185,000 protein in intact cells. Nature 318:183–186.
White MF, Shoelson SE, Kentmann N, Kahn CR. 1988. Cascade of tyrosine autophosphorylation in the β-subunit activates the insulin receptor. J Biol Chem 263:2969–2980.
Williger B-T, Liscovitch M. 1997. Ras GTPase-activating protein-associated p62 is a major v-Src-SH3-binding protein. FEBS Lett 403:35–39.
Withers DJ, White M. 2000. Perspective: the insulin signaling system–a common link in the pathogenesis of type 2 diabetes. Endocrinology 141:1917–1921.
Wong G, Müller O, Clark R, Conroy L, Moran MF, Polakis P, McCormick F. 1992. Molecular cloning and nucleic acid binding properties of the GAP-associated tyrosine phosphoprotein p62. Cell 69:551–558.