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Sam68 mediates leptin-stimulated growth by modulating leptin receptor signaling in human trophoblastic JEG-3 cells

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BACKGROUND: Sam68, a member of the signal transduction and activation of RNA metabolism (STAR) family of RNA-binding proteins, has been previously implicated as an adaptor molecule in different signaling systems, including leptin receptor (LEPR) signaling. LEPR activation is known to stimulate JAK-STAT, MAPK and PI3K signaling pathways, thus mediating the biological effects of leptin in different cell types, including trophoblastic cells. We have recently found that leptin stimulation also promotes the overexpression and tyrosine phosphorylation of Sam68 in human trophoblastic JEG-3 cells, suggesting a role for Sam68 in leptin signaling and action in these cells. In the present work, we have studied the participation of Sam68 in the main signaling pathways activated by LEPR to increase growth and proliferation in trophoblastic JEG-3 cells.

METHODS: We used an antisense strategy to down-regulate *Sam68* expression in these cells, and we studied LEPR signaling by immunoprecipitation and poly-U affinity precipitation and by analyzing phosphorylation levels of signaling proteins by immunoblot. The effect of leptin on protein synthesis and proliferation was studied by ^{3}[H]-leucine and ^{3}[H]-thymidine incorporation.

RESULTS: Sam68 knockdown impaired leptin activation of JAK-STAT, PI3K and MAPK signaling pathways in JEG-3 cells. We have also found that leptin-stimulated Sam68 tyrosine phosphorylation is dependent on JAK-2 activity, since the pharmacological inhibitor AG490 prevents the phosphorylation of Sam68 in JEG-3 cells. Finally, the trophic and proliferative effect of leptin in trophoblastic cells is dependent on *Sam68* expression, since its down-regulation impaired the leptin-stimulated DNA and protein synthesis.

CONCLUSIONS: These data demonstrate that Sam68 participates in the main signaling pathways of LEPR to mediate the trophic and proliferative effect of leptin in human trophoblastic cells.

Key words: Sam68 / leptin / LEPR signaling / placenta / trophoblast

Introduction

Sam68 (Src associated in mitosis; 68 kDa) belongs to the signal transduction and activation of RNA metabolism (STAR) family of RNA-binding proteins, which are characterized by a single heterogeneous nuclear ribonucleoprotein particle K (hnRNP K) homology (KH) domain for their RNA-binding activity. Moreover, Sam68 contains a variety of protein domains that allow protein–protein interaction and modification by multiple signaling pathways. It has proline-rich domains, which enable the interaction with signaling molecules through SH3 and WW domains (Richard *et al.*, 1995), and Sam68 is also a target of several tyrosine kinases (Fusaki *et al.*, 1997; Lang et al., 1997; Sanchez-Margalet and Najib, 1999; Derry *et al.*, 2000). The Tyr phosphorylation of Sam68 by these kinases modulates its SH2-binding activity. Sam68 interactions and its post-translational modifications affect Sam68 intracellular localization and RNA-binding activity (Cote *et al.*, 2003; Lukong *et al.*, 2005; Rho *et al.*, 2007).

Sam68 has been implicated in different cellular processes (Matter *et al.*, 2002; Babic *et al.*, 2006; Busa *et al.*, 2007; Paronetto *et al.*, 2007; Paronetto *et al.*, 2010). Regarding its biological functions, one of the physiological systems where Sam68 is known to participate in is the reproductive system. Thus, in mice, targeted deletion of the *Sam68* gene seems to cause male infertility (Richard *et al.*, 2005; Paronetto *et al.*, 2009) and subfertility in female mice with several defects

in sexual organs at puberty (Richard *et al.*, 2008). Recently, other authors have proposed a role for Sam68 in the proper expression of the gonadotrophin receptor transcripts in pre-ovulatory follicles in the adult ovary (Bianchi *et al.*, 2010). In addition, it has been observed that Sam68 shuttles from nucleus to cytoplasm and associates with polysomes during meiosis in mice spermatocytes (Paronetto *et al.*, 2006). Finally, Sam68 has been related to early signaling events triggered by fertilization (Paronetto *et al.*, 2008).

Leptin, the 16-kDa multifunctional hormone, is another factor that has been also clearly implicated in reproduction exerting roles at different levels, including the placenta (Masuzaki *et al.*, 1997; Caprio *et al.*, 2001; Henson and Castracane, 2006). Leptin exerts an autocrine and trophic effect in the placenta (Magarinos *et al.*, 2007), which is the most important source of this hormone during pregnancy (Masuzaki *et al.*, 1997; Dotsch *et al.*, 1999). In fact, placental leptin expression has been shown to be up-regulated by different pregnancy hormones such as chorionic gonadotrophin (Maymo *et al.*, 2009) and 17beta-estradiol (Gambino *et al.*, 2010), and also by second messengers such as cyclic adenosine 5′-monophosphate (Maymo *et al.*, 2010).

In trophoblastic JEG-3 cells and also in human placental explants, leptin has been shown to activate JAK-STAT, PI3K and MAPK pathways through the long form of leptin receptor ($LEPR_L$), which mediate some of the biological effects of leptin, such as the prevention of apoptotic death (Perez-Perez *et al.*, 2008), and a trophic effect enhancing cell growth and proliferation. Thus, in human trophoblastic BeWo and JEG-3 cells, leptin was shown to increase DNA synthesis and to displace cells toward the G2/M phase, promoting an increase in cyclin D1 (Magarinos *et al.*, 2007). Moreover, MAPK and PI3K pathways have been shown to be necessary for leptin stimulation of protein synthesis in trophoblastic cells, which seems to be mediated by the activation of the translation machinery, promoting the phosphorylation of RPS6KB1, EIF4E and EIF4EBP1 (Perez-Perez *et al.*, 2008, 2009, 2010). Regarding the mechanisms of action of leptin in placenta, we have recently found that LEPR and Sam68 are functionally linked in human trophoblast cells (Sanchez-Jimenez *et al.*, 2011), suggesting that Sam68 could play a role in LEPR signaling.

The RNA-binding capacity of Sam68, and also its protein–protein interaction domains suggest that Sam68 may have an important function as a putative link for transducing information from signaling systems to mRNA metabolism (Najib *et al.*, 2005). In this sense, Sam68 has been previously implicated in signal transduction as an adaptor protein in the signaling of T cells receptor (TCR) (Fusaki *et al.*, 1997; Lang *et al.*, 1997), insulin receptor (IR) (Sanchez-Margalet and Najib, 1999, 2001; Najib and Sanchez-Margalet, 2002; Sanchez-Margalet *et al.*, 2003a,b) and LEPR (Sanchez-Margalet *et al.*, 2003a,b), where we have also demonstrated the leptin-dependent association of Sam68 with STAT-3 (Martin-Romero and Sanchez-Margalet, 2001). Furthermore, Sam68 has been found to be tyrosine phosphorylated after leptin stimulation in human peripheral blood mononuclear cells (Martin-Romero and Sanchez-Margalet, 2001) and in C2C12 muscle cells (Maroni *et al.*, 2009), exerting a negative effect on Sam68 RNA-binding capacity. More importantly, a direct association of Sam68 with LEPR mRNA has been previously found (Maroni *et al.*, 2009), suggesting a possible mechanism underlying this effect. Recently, leptin-dependent Sam68 Tyr phosphorylation has also been found in trophoblastic JEG-3 cells (Sanchez-Jimenez *et al.*, 2011). In this system, *Sam68* expression has been shown to

be positively regulated by leptin stimulation in a dose-dependent manner. We have also demonstrated that LEPR activity is decreased as a result of Sam68 down-regulation using an antisense strategy (Sanchez-Jimenez *et al.*, 2011). Nonetheless, the relative importance of Sam68 in LEPR signaling in trophoblastic cells is not known, and this question was the major aim of the present work. We have found that Sam68 down-regulation not only inhibits full signal transduction of LEPR, but also prevents the trophic and proliferative effects of leptin in JEG-3 trophoblastic cells.

Materials and Methods

Antibodies and reagents

The recombinant human leptin was provided by Sigma (Sigma Chemical); agarose immobilized poly(U) and protein A-sepharose for affinity and immunoprecipitation were purchased from Amersham Pharmacia Biotech. AG-490 was obtained from Calbiochem. Antibodies against Sam68 (C-20), Sam68 N-term (N-15), STAT-3 (C-20) and anti-tubulin were from Santa Cruz Biotechnology; anti-P-ERK1/2 (pT202-Y204/pT185-Y187) and anti-P-MEK1/2 (pS217-pS221) were from Sigma-Aldrich, anti-P-PKB (pS473), anti-P-STAT3 (pY705), anti-P-RPS6KB1 (pT389), anti-P-EIF4E (pS209) and anti-P-EIF4EBP (pT37-46) were from Cell signaling Technology; anti-P-GSK3αβ (pY216) was from BD BioSciences and anti-P-JAK2 (pY1007-1008) was from Calbiochem. Horseradish peroxidase-linked anti-rabbit/anti-mouse immunoglobulins, L-[4,5-³H]-leucine (162 Ci/mmol) and [5′-³H]-thymidine (11.9 Ci/mmol) were purchased from Amersham Pharmacia. Oligonucleotide antisense of *Sam68* sequence 5′-cagTGGC aCCTCtGGTgatg-3′ and standard control sequence 5′-catcACCaGA GGtGCCActg-3′ were purchased from Biomedal S.L. (Seville, Spain).

Cell culture and treatments

Human choriocarcinoma cell line JEG-3 was grown in Dulbecco's modified Eagle's medium (DMEM)-F12 medium (Invitrogen) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 g/ml streptomycin at 37°C in 5% CO_2. Cells were transfected 24 h prior to the leptin stimulus in Sam68 down-regulation experiments, or treated with 50 μM of AG490 for 5 min before leptin addition, in JAK-2 inhibition experiments. A total 24 h period of starvation with a medium supplemented with 1% FCS was also maintained before adding leptin. Cells were treated in the absence or presence of leptin 1 nM, corresponding to the physiological dose of leptin at which we had previously achieved maximal response in trophoblastic cells (Perez-Perez *et al.*, 2008, 2009).

The cells were washed with cold PBS and the lysates were 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 $CaCl_2$, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride and 0.4 mM sodium orthovanadate. After centrifugation, the soluble cell lysates were used for immunoprecipitation or western blot analysis. Total protein levels were determined by the bicinchoninic acid method (Smith *et al.*, 1985) using bovine serum albumin as the standard.

Transfection experiments

For transfection experiments, JEG-3 cells were plated at a density of 2.5×10^5 cells/ml onto six-well dishes containing 2 ml of DMEM-F12 plus 10% FCS. Cells were incubated for 24 h. The medium was replaced, and transfection of cells was performed according to the standard liposome-mediated method optimized for JEG-3 cells (Zhang *et al.*, 2007). Typically, 1.3 μg of the Sam68 antisense oligonucleotide were transfected using 5 μl of LipofectAMINE (Life Technologies). Control cells were transfected

using the same quantity of Sam68 sense oligonucleotide and LipofectAMINE reagent. The medium was replaced after 8–12 h with DMEM-F12 with 1% FCS, and maintained for 24 h. Transfection experiments were performed by duplicate in each of at least three independent experiments.

Immunoprecipitation and poly(U)-binding assay

Soluble cellular lysates (0.5 mg of protein) were precleared with 50 μl of protein A-sepharose for 2 h at 4°C by end-over-end rotation. The precleared cellular lysates were incubated with appropriate antibodies for 3 h at 4°C. Next, 50 μl of protein A-sepharose was added to immune complexes and incubation was continued for 2 h at 4°C. For the poly(U)-binding assay, 60 μl of a 50% solution of agarose-poly(U) beads were added to 0.5 mg of proteins of cellular lysates. The mixture was rotated for 2 h at 4°C.

Western blot analysis

The immunoprecipitates and poly(U)-affinity precipitates were washed three times with lysis buffer. We added 40 μl of SDS-stop buffer containing 100 mM dithiothreitol to the immunoprecipitates, followed by boiling for 5 min. Soluble supernatants were then resolved by 7 or 10% SDS–PAGE and electrophoretically transferred onto nitrocellulose membranes. The membranes were blocked with buffered saline–0.05% Tween 20 (PBST) containing 3% albumin, 1 h at 23°C. The blots were then incubated with primary antibody for 1 h, washed in PBST and further incubated with secondary antibodies using horseradish peroxidase-linked anti-rabbit/anti-mouse immunoglobulins. Bound horseradish peroxidase was visualized by a highly sensitive chemiluminescence system (SuperSignal from Pierce). The bands obtained in the blots were scanned and analyzed by the PCBAS 2.0 program. The relative optical density of the different bands was normalized by the corresponding intensity of the tubulin immunoblot in each individual experiment.

[^{3}H]-thymidine and [^{3}H]-leucine incorporation

After 9 h of Sam68 antisense or sense oligonucleotide transfection, JEG-3 cells were starved in a medium supplemented with 1% FCS to lower the basal growth rate before leptin treatment. Twenty-four hours after transfection, the leptin stimulus was added for another 16 h in DNA synthesis experiments. Then JEG-3 cells were incubated with 1 μCi/ml [^{3}H]-thymidine for 4 h. In protein synthesis experiments, leptin was added for 4 h and cells were further incubated with 1 μCi/ml [^{3}H]-leucine for 2 h. The same protocol was then followed for both experiments. Cells were washed three times with cold PBS, harvested in 0.03% SDS and centrifuged at 5000g for 5 min. The cellular pellet was lysed with 20% trichloroacetic acid, centrifuged and washed twice with ethanol 95%. The pellet was resuspended in 150 μl of 1 M NaOH + 0.1% SDS for 1 h at room temperature. The incorporated radioactivity was quantified by scintillation counting and DNA synthesis and leucine synthesis were estimated as a percent of effect according to its basal rates. Transfection was performed by triplicate in each of at least three independent experiments.

Data analysis

Experiments were repeated separately at least three times to assure reproducible results. Results are expressed as means ± standard deviation (SD) in arbitrary units (AU). AU were calculated as a normalized band intensity in western blot analyses. Statistical analysis was performed using the Graph Pad Prism computer program (GraphPad Software).

Statistical significance was assessed by analysis of variance followed by Bonferroni's *post hoc* tests. *P*-value of <0.05 was considered to be statistically significant.

Results

Sam68 down-regulation prevents the activation of MAPK and PI3K pathways, but not JAK-2 activation upon leptin stimulation in trophoblastic JEG-3 cells

Cells were transfected with Sam68 antisense, or sense oligonucleotide as a control, and stimulated with leptin 1 nM for 5 min at 37°C, which are the conditions previously found to achieve maximal response and a previously reported 60% reduction in Sam68 expression (Sanchez-Jimenez et al., 2011). Total extracts of transfected and lysed cells were analyzed with specific antibodies that recognize the Sam68 protein as a control of Sam68 down-regulation, and anti-tubulin antibodies were used as loading control in each experiment. A representative experiment is shown in Fig. 1.

We measured the activation of the central kinase of PI3K pathway, i.e. PKB, studying the effects of Sam68 down-regulation on the PKB phosphorylation, previously shown to be increased under leptin stimulation in trophoblastic cells (Perez-Perez et al., 2008, 2010). Sam68 knockdown significantly diminished leptin-stimulated PKB phosphorylation, as assessed by using specific antibodies against PKB-phosphorylated form (Fig. 1). GSK3 is the target of PKB, and is inhibited by the phosphorylation on Ser-21 (the α-isoform) and Ser-9 (the β-isoform) (Cross et al., 1995). Figure 1 also shows similar effects on leptin-mediated phosphorylation of GSK3-α and β, where the Sam68 down-regulation in JEG-3 cells impaired the leptin effect on the phosphorylation–inhibition of GSK3. The total amount of protein in every sample was controlled using anti-tubulin antibodies.

Since Sam68 has been found to be also recruited to the Ras-MAPK pathway in different signaling systems including IR (Najib and Sanchez-Margalet, 2002) and LEPR (Martin-Romero and Sanchez-Margalet, 2001; Sanchez-Margalet et al., 2003a,b; Maroni et al., 2009), we wanted to test the possible role of Sam68 in the leptin-dependent activation of MAPK pathway in JEG-3 trophoblastic cells, by using the antisense strategy to down-regulate Sam68 expression. We employed antibodies that specifically recognize the phosphorylated forms of the kinases. As shown in Fig. 1, the leptin-mediated MEK1−2 Ser phosphorylation was significantly reduced in cells where Sam68 was down-regulated. A similar effect was observed in the leptin activation of ERK1−2 proteins by Thr/Tyr phosphorylation, which was impaired by decreasing the expression of Sam68.

We have previously found that leptin dose-dependently stimulates the signaling pathway known to regulate protein synthesis, promoting the phosphorylation and activation of the protein RPS6KB1 (also known as p70S6K) (Perez-Perez et al., 2008), the phosphorylation of the translation initiation factor EIF4E, as well as the phosphorylation of the EIF4E-binding protein EIF4EBP1 (PHAS-I) (Perez-Perez et al., 2009, 2010). As shown in Fig. 1, RPS6KB1 phosphorylation by leptin was impaired in cells that were treated with Sam68 antisense oligonucleotide to down-regulate Sam68 expression. Leptin-stimulated EIF4E and EIF4EBP1 phosphorylation were also decreased in Sam68 knockdown cells, as shown in Fig. 1.
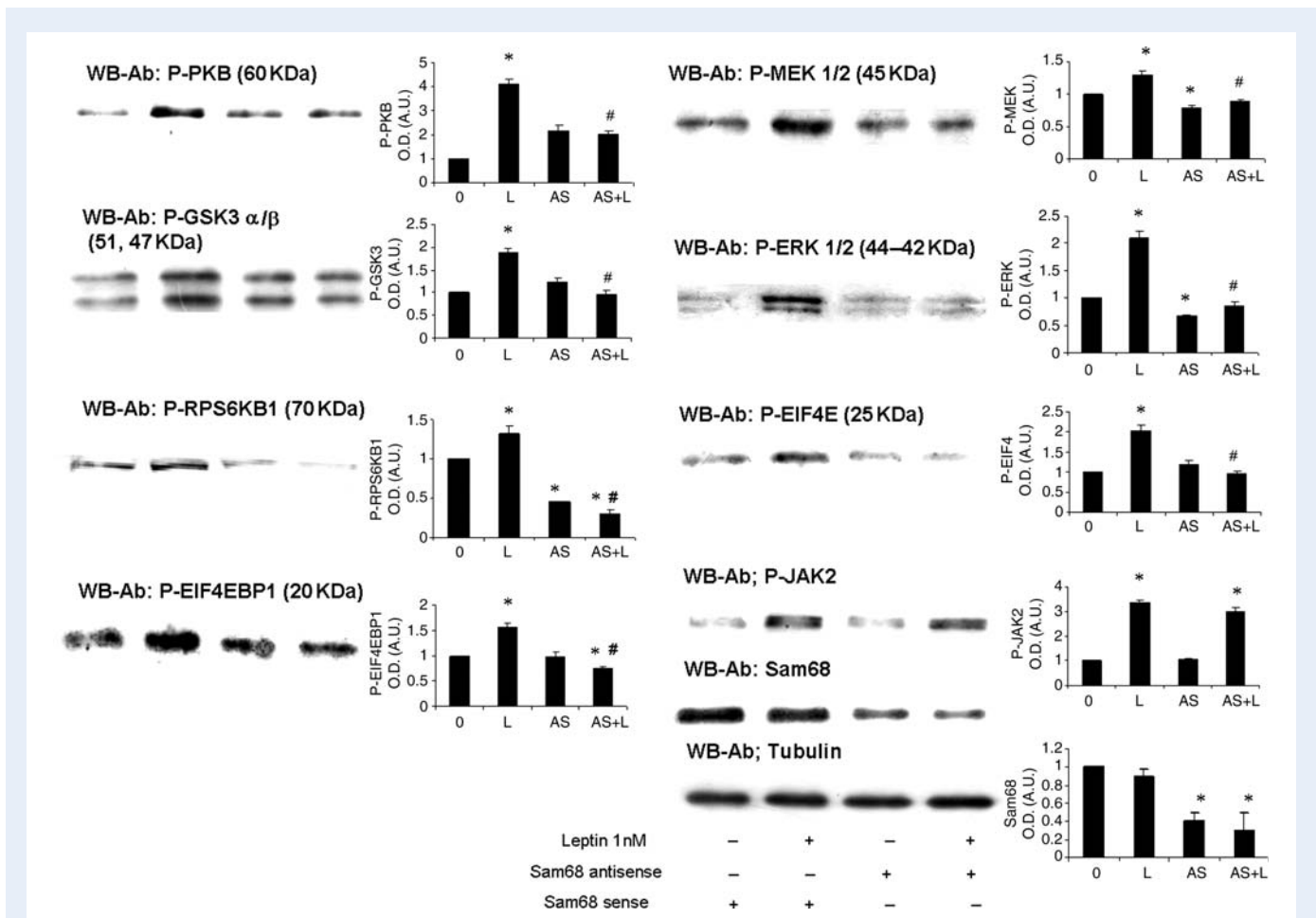
Figure 1 Sam68 down-regulation by Sam68 antisense oligonucleotide prevents the leptin-dependent activation of PI3K and MAPK pathways, but does not prevent JAK2 activation with leptin. JEG-3 cells were treated with Sam68 antisense oligonucleotide or sense oligonucleotide as control, for 24 h prior to stimulation with 1 nM leptin for 5 min. Cells were lysed and soluble clarified cell lysates were separated by SDS−PAGE. A western blot analysis was performed by using anti-P-PKB and anti-P-GSK3-α and β antibodies to study leptin activation of the PI3K signaling pathway. Anti-P-MEK1/2 and anti-P-ERK1/2 antibodies were also used to study the leptin-dependent activation of the MAPK pathway. Anti-P-RSP6KB1, anti-P-EIF4E and anti-P-EIF4EBP1 antibodies were used to test PI3K- and MAPK-dependent activation of these proteins by leptin. A western blot analysis was performed by using anti-Sam68 antibodies to control Sam68 down-regulation. Anti-P-JAK2 antibodies were used to assess the effect of Sam68 down-regulation on leptin-dependent JAK-2 phosphorylation. Sample protein loading was controlled by using anti-tubulin antibodies. We show the corresponding densitometric analysis of three independent experiments as means ± SD, * $P < 0.05$ versus control, # $P < 0.05$ versus leptin-stimulated. 0, Sam68 sense oligonucleotide-treated and non-stimulated cells; L, Sam68 sense oligonucleotide-treated and leptin-stimulated cells; AS, Sam68 antisense-treated non-stimulated cells; AS + L, Sam68 oligonucleotide-treated and leptin-stimulated cells.

Even though down-regulation of Sam68 expression prevented the activation of MAPK and PI3K signaling pathways, the activation of JAK-2 by leptin was not abolished, as assessed by the immunoblot that recognizes the tyrosine-phosphorylated form of JAK-2 (Fig. 1).

Sam68 interacts with STAT-3 and Sam68 down-regulation diminishes STAT-3 phosphorylation in leptin-stimulated trophoblastic JEG-3 cells

STAT-3 is the major effector of the JAK signaling pathway known to be activated by LEPR activation in different systems (Schwartz *et al.*, 1996; Sanchez-Margalet and Martin-Romero, 2001; O'Rourke and Shepherd, 2002), including trophoblastic cells (Perez-Perez *et al.*, 2008).

Thus, we studied the possible role of Sam68 in STAT-3 activation upon leptin stimulation by using the antisense strategy. As shown in Fig. 2A, down-regulation of Sam68 caused a decrease in the leptin-stimulated Tyr phosphorylation level of STAT-3, even though the activation of JAK-2 was not prevented, as shown in Fig. 1. The total amount of protein in cell lysates was checked by immunoblot using anti-tubulin antibodies.

Since the down-regulation of Sam68 expression in JEG-3 cells prevented STAT-3 phosphorylation but maintained JAK-2 activation in response to leptin, a possible mechanism could be the physical association of Sam68 with STAT-3. To study this possibility, we immunoprecipitated Sam68 and searched for the presence of STAT-3 in the immunoprecipitates. As shown in Fig. 2B, higher levels of STAT-3 are found in immunoprecipitates from cells stimulated by leptin,
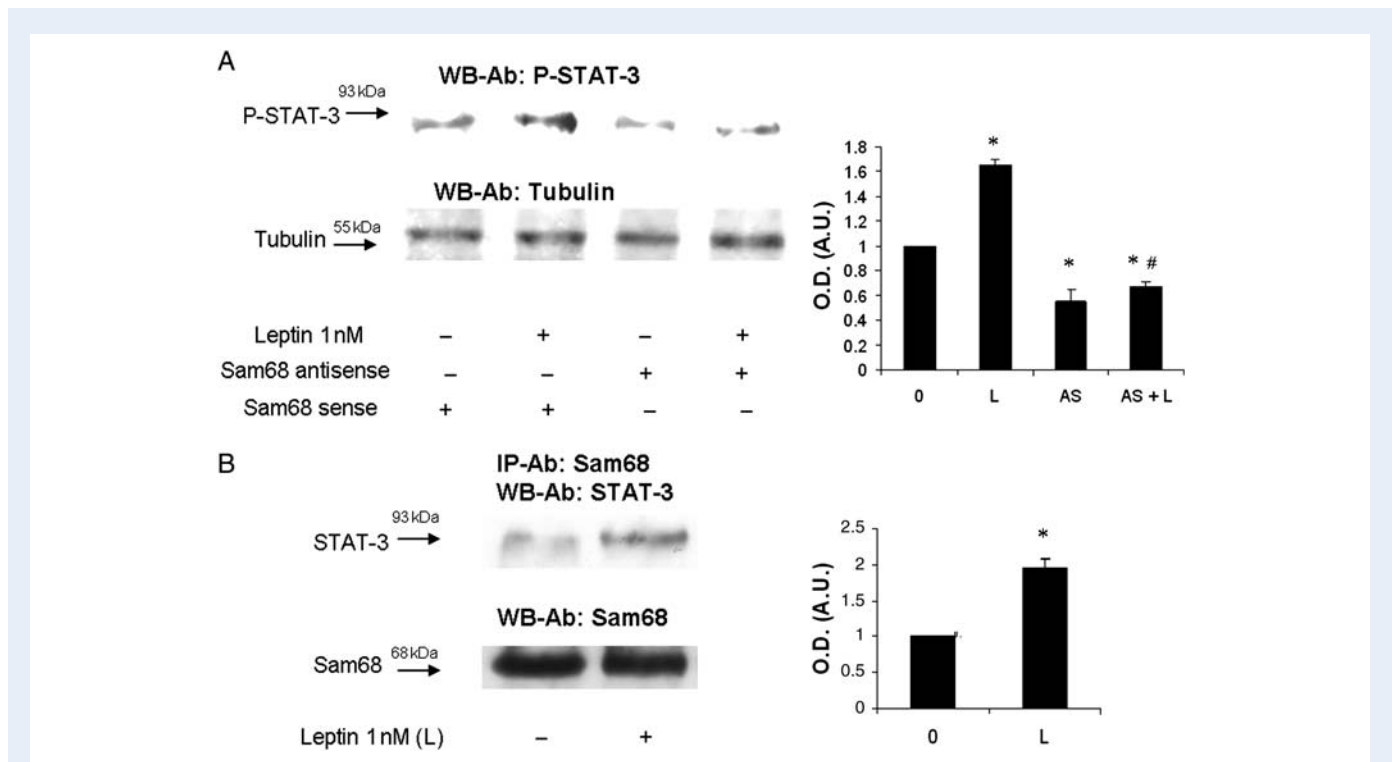
Figure 2 Sam68 down-regulation decreased leptin-stimulated STAT-3 phosphorylation and both proteins associate in response to leptin. JEG-3 cells were treated with Sam68 sense or antisense oligonucleotide for 24 h prior to the stimulation with leptin 1 nM for 5 min. Cells were then lysed and proteins were separated by SDS−PAGE. STAT-3 phosphorylation was tested by western blot analysis using specific anti-P-STAT-3 antibodies. Anti-tubulin immunoblot was performed as loading controls (**A**). Cells were incubated at 37°C for 5 min with 1 nM leptin. Cells were lysed and immunoprecipitated with antibodies to Sam68, and the immunoprecipitates were analyzed by immunoblot with anti-STAT-3 antibodies to assess the association between both proteins (**B**). Each experiment is a western blot representative of three. Densitograms with means ± SD are shown, *$P < 0.05$ versus control, #$P < 0.05$ versus leptin-stimulated. 0, control, Sam68 sense oligonucleotide treated and non-stimulated cells; L, Sam68 sense oligonucleotide-treated and leptin-stimulated cells; AS, Sam68 antisense-treated non-stimulated cells; AS + L, Sam68 oligonucleotide-treated and leptin-stimulated cells.

whereas the amount of Sam68 precipitated was the same, suggesting that leptin stimulation promotes the association of these proteins in these cells.

Sam68 tyrosine phosphorylation by leptin stimulation in trophoblastic JEG-3 cells is dependent on JAK-2 activity

Since we had found that leptin stimulation promotes the Tyr phosphorylation of Sam68 (Sanchez-Jimenez et al., 2011), and JAK-2 is activated (Fig. 1), our aim was to further assess whether JAK-2 activity was mediating this effect. Thus, we used the pharmacological inhibitor AG490, a member of the tyrphostin family of tyrosine kinase inhibitors, known to prevent leptin-mediated specific JAK-2 activation at 50 μM (Gao et al., 2009). As shown in Fig. 3A, AG490 (50 μM) prevented Sam68 Tyr phosphorylation after the leptin stimulus in cells previously treated with the JAK-2 inhibitor. Basal Tyr phosphorylation of Sam68 was decreased in the presence of AG490 and no change in the phosphorylation level was observed upon leptin stimulation. Since the Tyr phosphorylation of Sam68 has been demonstrated to negatively regulate its RNA-binding activity (Wang et al., 1995; Martin-Romero and Sanchez-Margalet, 2001), we used immobilized poly(U) polymer to test Sam68 RNA-binding capacity in the presence of the JAK-2 inhibitor AG490. As shown in Fig. 3B, AG490 (50 μM)

prevented the leptin-stimulated inhibition of Sam68 binding to poly(U) polymer. Consistent with the effect of AG490 decreasing basal Tyr phosphorylation of Sam68, more Sam68 bound to poly(U) is found under non-stimulated conditions. We further controlled the AG490 inhibition of leptin signaling by checking the activation of MAPK and PI3K signaling pathways, which depends on JAK activation. As shown in Fig. 3C, a representative immunoblot shows that both ERK and PKB activation were prevented in the presence of the JAK-2 inhibitor AG490, which should be expected since ERK and PI3K signaling pathways depend on JAK activation, as previously described by other authors (Myers, 2004; Gao et al., 2009).

Sam68 down-regulation prevents leptin stimulation of growth and proliferation in trophoblastic JEG-3 cells

Leptin has been shown to play a role in promoting growth and proliferation in trophoblastic cells (Magarinos et al., 2007; Perez-Perez et al., 2009), which is dependent on MAPK and PI3 pathways (Perez-Perez et al., 2010). Cells were incubated in the absence or presence of leptin (1 nM) for 16 h after 24 h treatments with Sam68 antisense oligonucleotide or Sam68 sense oligonucleotide as control. As shown in Fig. 4A, when *Sam68* expression was decreased, the effect of leptin on [³H]-thymidine incorporation in DNA was prevented. Similarly,
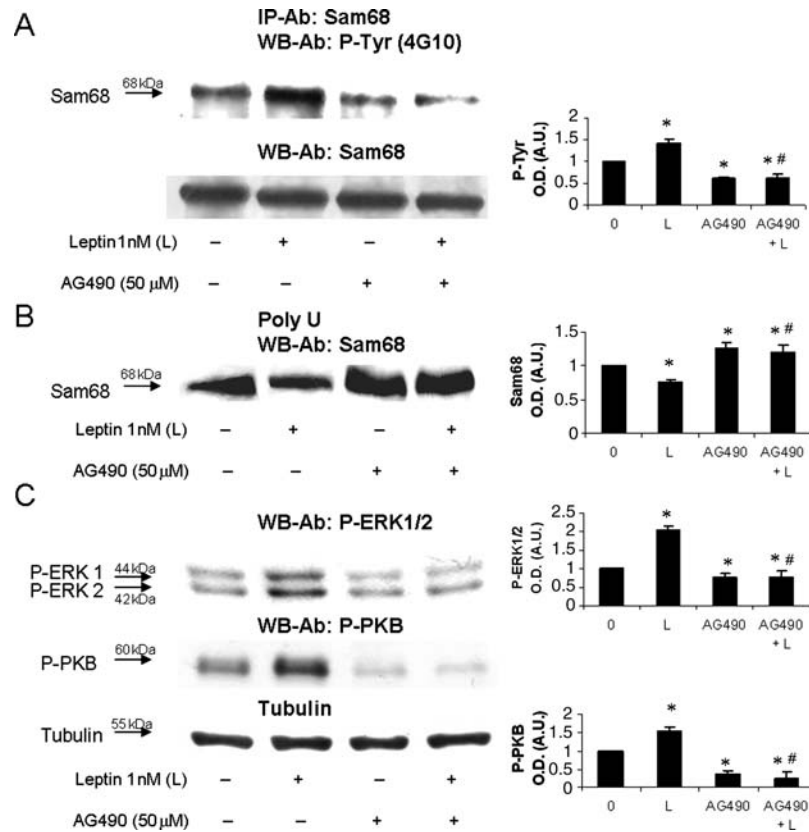
Figure 3 The JAK-2 inhibitor, AG490, prevents both leptin-stimulated Sam68 phosphorylation and the consequent inhibition of Sam68 binding to poly(U). JEG-3 cells were incubated in the presence of leptin (1 nM) for 5 min with or without a previous treatment with AG490 (50 μM) for 5 min. (**A**) Cells were lysed, and soluble clarified cell lysates were subjected to immunoprecipitation with anti-Sam68 antibodies. Immunoprecipitates were resolved by SDS–PAGE and analyzed by western blot with anti-phosphotyrosine antibodies. The same lysates were analyzed by immunoblot using anti-Sam68 antibodies to control the amount of Sam68 immunoprecipitated in each lane. (**B**) Cells precleared lysates were also incubated with Sepharose-coupled poly(U) beads for Sam68 affinity precipitation. Samples were then washed and analyzed by immunoblot with anti-Sam68 antibodies. (**C**) Cells lysates were analyzed by immunoblot to test the AG490 effect on leptin-mediated activation of MAPK and PI3K pathways by using, respectively, anti-P-ERK1/2 and anti-P-PKB antibodies. An experiment representative of three independent experiments is shown and densitograms are expressed as means $\pm$ SD, $*P < 0.05$ versus control, $\#P < 0.05$ versus leptin stimulated. 0, control, non-stimulated cells; L, leptin-stimulated cells; AG490, JAK-2 inhibitor-treated and non-stimulated cells; AG490 + L, JAK-2 inhibitor-treated and leptin-stimulated cells.

previously transfected JEG-3 cells were also incubated in the absence or presence of leptin (1 nM) for 4 h and further treated with [³H]-leucine for two more hours. As shown in Fig. 4B, a decrease in leptin-stimulated leucine incorporation to protein synthesis was observed in Sam68 down-regulated cells.

Discussion

The RNA-binding protein Sam68 has multifunctional domains that enable protein–protein interactions, pointing to a possible role in signal transduction. In fact, Sam68 has been previously shown to be involved in cell signaling in different biological systems (Najib *et al.*, 2005; Maroni *et al.*, 2009) that could also be connected to different roles of Sam68 in RNA metabolism (Wang *et al.*, 1995; Matter *et al.*, 2002; Lukong *et al.*, 2005; Paronetto *et al.*, 2007). In addition, a function for Sam68 in reproduction and fertility has been previously suggested (Bianchi *et al.*, 2010; Sette *et al.*, 2010). Along this line, we

have also linked leptin, one of the most important hormones having a role in reproduction, to Sam68 in trophoblastic JEG-3 cells (Sanchez-Jimenez *et al.*, 2011).

Sam68 has been found to be Tyr phosphorylated by Src and other members of the Src family Tyr kinases (Richard *et al.*, 1995) in systems like the TCR (Fusaki *et al.*, 1997; Lang *et al.*, 1997) or the IR (Sanchez-Margalet and Najib, 2001). Sam68 has also been found to be Tyr phosphorylated in response to leptin in LEPR-dependent signaling systems (Sanchez-Margalet and Martin-Romero, 2001; Maroni *et al.*, 2009), including that in human trophoblastic JEG-3 cells, where we have found that leptin dose-dependently promotes the tyrosine phosphorylation of Sam68 and its overexpression. Moreover, Sam68 seems to be necessary for LEPR expression, suggesting a possible role of Sam68 in leptin signaling in trophoblastic JEG-3 cells (Sanchez-Jimenez *et al.*, 2011).

In these trophoblastic cells and also in placental explants, leptin has been previously shown to stimulate tyrosine phosphorylation of JAK-2
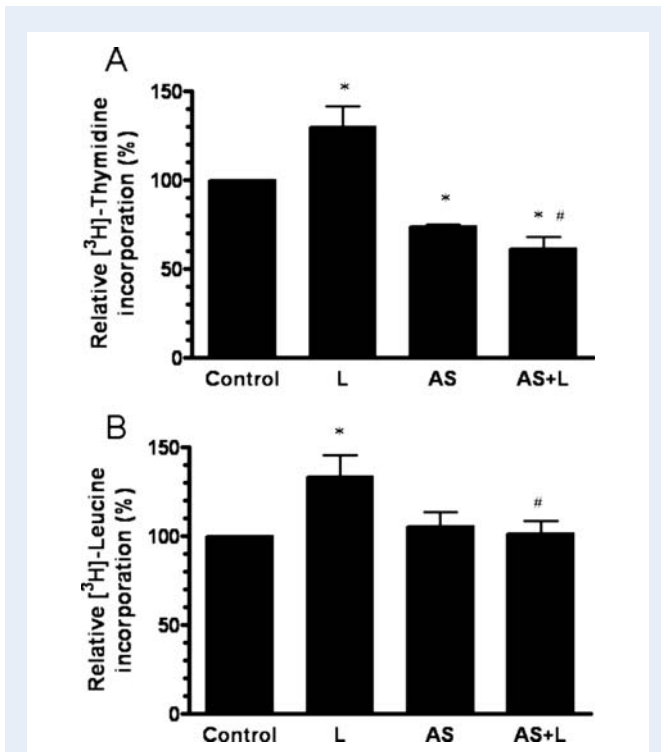
Figure 4 Leptin effect on cellular growth and proliferation is impaired in Sam68 down-regulated trophoblastic JEG-3 cells. JEG-3 cells were treated with Sam68 antisense or Sam68 sense oligonucleotides. After 12 h, medium was replaced by DMEM-F12 supplemented with 1% FCS. Cells were cultured for another 12 h in the presence or absence of 1 nM leptin prior to the [³H]-thymidine addition (**A**) or stimulated with 1 nM leptin for 4 h prior to the [³H]-leucine addition (**B**). The radioactive incorporation was determined as indicated in Materials and Methods section. Control, Sam68 sense oligonucleotide-treated cells; L, Sam68 sense oligonucleotide-treated and leptin-stimulated cells; AS, Sam68 antisense-treated non-stimulated cells; AS + L, Sam68 oligonucleotide-treated and leptin-stimulated cells. Data are expressed as means $\pm$ SD from four independent experiments, *$P < 0.05$ versus control, #$P < 0.05$ versus leptin stimulated.

(Janus kinase-2) (Perez-Perez et al., 2008), which is the most important JAK isoform to mediate the physiological effect of leptin through its kinase activity (Ghilardi and Skoda, 1997; Banks et al., 2000; Kloek et al., 2002). In the present work, we have pointed out that JAK-2 may be the kinase responsible for the leptin-dependent phosphorylation of Sam68 in JEG-3 trophoblastic cells, also mediating the negative effect on Sam68 RNA-binding capacity, as assessed by the experiments using the pharmacological JAK-2 inhibitor AG490, which has been previously employed at 30–100 μM (Chakravarti et al., 2006; Gao et al., 2009; Mauvoisin et al., 2010). Basal Tyr phosphorylation of Sam68 is also decreased in the presence of the inhibitor, probably preventing the autocrine effect of the leptin released by these cells. However, AG490 may inhibit not only JAK-2 but also JAK-3 phosphorylation (Wang et al., 1999). Therefore, we cannot rule out the possible role of other JAKs that could participate in leptin-mediated Sam68 phosphorylation in JEG-3 cells. Moreover, whether this observed role of JAK-2 on Sam68 Tyr phosphorylation is

exerted directly or indirectly through another downstream kinase remains to be investigated. In this sense, LEPR has previously been shown to activate other Tyr kinases, such as c-Src or members of the family, which could also mediate leptin signaling (Jaffe and Schwartz, 2008; Jiang et al., 2008). In any case, we have found that the leptin-mediated Sam68 tyrosine phosphorylation is dependent on JAK activity and, therefore, the possible activation of any other kinase should be taking place downstream of this point. Likewise, we have found that the leptin activation of MAPK and PI3K signaling pathways are also dependent on JAK activity in JEG-3 cells, similarly to what has been previously described in other systems (Myers, 2004).

In this context, LEPR signaling activates JAK-STAT, PI3K and MAPK signaling pathways in different systems (Sanchez-Margalet et al., 2003a,b; Fruhbeck, 2006; Cirillo et al., 2008; Myers et al., 2008). Along this line, we have previously demonstrated that leptin stimulation promotes the activation of the major signaling pathways of LEPR in trophoblastic cells (Perez-Perez et al., 2008). To further investigate the role of Sam68 on leptin-stimulated signaling pathways, we have used the antisense strategy to down-regulate Sam68 expression. We have found that the leptin-dependent Tyr phosphorylation of STAT-3 was dependent on the expression of Sam68. However, this effect on STAT-3 phosphorylation was not a consequence of the modulation of JAK-2 activity, since leptin-stimulated Tyr phosphorylation of JAK-2 was not changed by the down-regulation of Sam68, as assessed by the specific immunoblot. Even though we have previously found that the knockdown of Sam68 expression decreases the LEPR activity (Sanchez-Jimenez et al., 2011), this effect is not sufficient to down-regulate the JAK-2 activation by the stimulated LEPR. Therefore, this effect on LEPR activity may not account for the inhibition of LEPR signaling, which we have observed in the present work by down-regulation of Sam68 expression. In a similar way, the relationship between the possible apoptotic event of the JEG-3 cells and the inhibition of leptin signaling as a result of Sam68 down-regulation may be ruled out, since the leptin-mediated activation of JAK-2 is preserved.

We have previously shown that Sam68 and STAT-3 interact upon leptin stimulation in human peripheral blood mononuclear cells (Martin-Romero and Sanchez-Margalet, 2001) and now, this effect has been corroborated in trophoblastic JEG-3 cells. Thus, we have provided new evidence for the possible role of Sam68 in the JAK-STAT pathway, as its interaction with STAT-3 may be responsible for the activation of STAT-3 by leptin stimulation.

Previously, we have observed that Sam68 is constitutively associated with the SH3 domains of Grb2 and may interact with the SH2 domains of GAP after insulin stimulation in HTC-IR cells (Najib and Sanchez-Margalet, 2002), suggesting a role of Sam68 in the MAPK pathway. Moreover, the MAPK pathway has also been shown to be activated in trophoblastic JEG-3 cells and placental trophoblast explants upon leptin stimulation, as it has previously been assessed by studying leptin-mediated MEK and ERK phosphorylation (Perez-Perez et al., 2008). Now, we have found that Sam68 also plays a role in the leptin-activated MAPK pathway in trophoblastic cells, since we observed a significant decrease of leptin-dependent MEK1/2 and ERK1/2 phosphorylation in Sam68 down-regulated cells.

The PI3K pathway can also be recruited by LEPR in both JEG-3 cells and placental trophoblast explants, where we have previously found that leptin dose-dependently stimulates the phosphorylation of PKB

and its downstream targets, mediating the effect of leptin on cellular growth and protein synthesis (Perez-Perez *et al.*, 2008, 2009). In the present work, we have studied the leptin-dependent phosphorylation of PKB and GSK3α/β in Sam68 down-regulated cells, showing that Sam68 is necessary for the activation of the PI3K pathway upon leptin stimulation. Previous works have shown that Tyr-phosphorylated Sam68 associates with p85, the regulatory subunit of PI3K, via its SH2 domains in response to insulin in hepatocytes and adipocytes (Sanchez-Margalet and Najib, 1999). This association was found to be preferentially mediated by the N-terminal SH2 domain of p85, allowing the tertiary complex along with IRS-1, which binds with higher affinity to the C-terminal SH2 domain (Sanchez-Margalet *et al.*, 1995). Tyr-phosphorylated Sam68 also inter-acts with p85 after leptin stimulation in human peripheral blood mono-nuclear cells (Martin-Romero and Sanchez-Margalet, 2001). This interaction may enhance the activation of PI3K pathway, thus support-ing a role of Sam68 in the regulation of this pathway by leptin.

Leptin promotes cell survival, proliferation and protein synthesis in different cellular types, including JEG-3 trophoblastic cells (Magarinos *et al.*, 2007; Perez-Perez *et al.*, 2008, 2009). PI3K and MAPK pathways seem to be implicated in these biological effects of leptin (Perez-Perez *et al.*, 2010). Thus, downstream to these signaling pathways, RPS6KB1, EIF4E and EIF4EBP1 have been previously found to be phosphorylated in response to leptin, activating the initiation of translation and there-fore mediating the leptin stimulation of protein synthesis (Perez-Perez *et al.*, 2008, 2009). In the present work, we have demonstrated that Sam68 down-regulation diminishes the effect of leptin upon the phos-phorylation of these proteins involved in the control of protein syn-thesis in trophoblastic JEG-3 cells.

According to the aforementioned observations regarding the par-ticipation of Sam68 in signaling pathways that mediate the biological function of leptin, we next studied the effect of Sam68 down-regulation on leptin-dependent cell growth and proliferation, measured as [^{3}H]-thymidine incorporation to DNA synthesis and [^{3}H]-leucine incorporation to protein synthesis. We have found that the down-regulation of *Sam68* expression prevents both effects of leptin on trophoblastic JEG-3 cells. These results are consistent with the participation of Sam68 in the activation of the main signaling path-ways activated by leptin in these cells.

In conclusion, we have provided new evidence for the participation of Sam68 in LEPR signaling in trophoblastic JEG-3 cells. We have found that Sam68 Tyr phosphorylation is dependent on JAK-2 activation and that Sam68 also interacts with STAT-3 after leptin stimulation in these cells. Moreover, Sam68 participates in the main pathways activated by leptin in JEG-3 cells: JAK-STAT, MAPK and PI3K to mediate the effects of leptin. Thus, we have demonstrated by *in vitro* studies that Sam68 not only participates in LEPR signal transduction, but also has an important role mediating the trophic and proliferative effect of leptin in JEG-3 trophoblastic cells.

Authors' roles

F.S.-J. contributed toward the acquisition of data, analysis and interpretation of data, and drafting and final approval of the manu-script. A.P.-P. contributed toward the acquisition of data, analysis and interpretation of data, and drafting and final approval of the manu-script. C.G.-Y. made a substantial contribution to the conception and design, revision of the article for intellectual content and final approval. C.V. made a substantial contribution to the conception and design, revision of the article for intellectual content and final approval. V.S.-M. made substantial contributions to the conception and design of the article, analysis and interpretation of data, drafting and revision of the article, and final approval.

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