Biochemical Pharmacology 83 (2012) 78–87

Contents lists available at SciVerse ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Sam68 interacts with IRS1

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ARTICLE INFO

Article history:
Received 5 August 2011
Accepted 28 September 2011
Available online 10 October 2011

Keywords:
Sam68
IRS1
Insulin receptor
BRET

ABSTRACT

Sam68 (Src associated in mitosis) is a RNA binding protein that links cellular signaling to RNA processing. In previous studies we found that insulin promotes Sam68 relocalization in the cytoplasm allowing Sam68 to associate with p85PI3K, Grb2, GAP and probably the insulin receptor (IR), modulating insulin action positively. In the present work, we wanted to define the role of Sam68 in the first stages of IR signaling.

Both BRET and co-immunoprecipitation assays have been used for the study of Sam68 binding to IR, IRS1 and p85-PI3K.

BRET saturation experiments indicated, for the first time, that Sam68 associates with IRS1 in basal condition. To map the region of Sam68 implicated in the interaction with IRS1, different Sam68 mutants deleted in the proline-rich domains were used. The deletion of P0, P1 and P2 proline rich domains in N-terminus as well as P4 and P5 in C-terminus of Sam68 increased $BRET_{50}$, thus indicating that the affinity of Sam68 for IRS1 is lower when these domains are missing. Moreover, in IR-transfected HEK-293 cells, BRET saturation experiment indicated that insulin increases the affinity between Sam68-Rluc and IRS1-YFP.

In conclusion, our data indicate that Sam68 interacts with IRS-1 in basal conditions, and insulin increases the affinity between these two partners.

1. Introduction

Sam68 is the Src-associated in mitosis protein of 68 kDa, that belongs to the STAR signal transducer and activator of RNA family of RNA-binding proteins, which are implicated in signal transduction and RNA metabolism [1–3]. Sam68 (Fig. 1) contains a KH domain located within a larger domain of 200 amino acids, with RNA binding activity, named GSG, common to STAR proteins. This GSG domain is flanked by six proline rich sequences (from P0 to P5), located at its N-terminus (P0–P2) and C-terminus (P3–P5) and involved in Sam68 interaction with SH3 and WW domains containing proteins such as Src, Fyn, Sik, BRK [4,5], PI3K [6], PLC-γ-1 [7,8], PRMT [4], Grb2, Grap [9] and Nck [10]. The interaction of Sam68 with the SH3 domains of Src family kinases is required for its tyrosine phosphorylation [2,3]. In fact, Sam68 also contains a tyrosine rich region at the C-terminus of the protein. Tyrosine phosphorylation of Sam68 strongly affects its activity [11] and is necessary for its association with different proteins containing SH2 domains, that include kinases belonging to Src family [2,3,8,12], Sik/BRK [13], as well as those from Itk/Tec family [14,15]. Tyrosine-phosphorylated Sam68 also interacts with docking proteins and signaling enzymes containing SH2 domains such as Grb2 [8,9], Grap [9], Nck [10], PLC-γ-1 [8], Ras-GAP [8,16] and the p85 subunit of PI3K [6]. These numerous interactions suggest that Sam68 plays an adaptor role in signal transduction and is involved in several cellular processes.

We have previously shown the implication of Sam68 in IR signaling. Although Sam68 has been described as a nuclear protein [17,18], it can translocate from nucleus to cytoplasm in presence of methylase inhibitors, viral infections or during cell cycle transition [19–21]. Interestingly, we have demonstrated that Sam68 is exclusively located in the cytoplasm of rat adipocytes and its expression is enhanced upon insulin stimulation. In CHO cells, IR overexpression in itself also increases Sam68 expression, and insulin stimulation further increases its expression and targets Sam68 to the cytoplasm. Moreover, the IR activation *in vivo*, in both CHO-IR cells and rat adipocytes, stimulates Sam68 tyrosine phosphorylation, increasing its association with p85-PI3K [22,23]. In hepatoma cells that over-express IR (HTC-IR), Sam68 is associated with the SH2 domains of the p85 regulatory subunit of PI3K, forming a ternary complex with IRS-1 [24,25]. Furthermore, insulin stimulation promotes Sam68 association with the

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0006-2952/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2011.09.030

SH2 domains of GAP *in vivo* and *in vitro* [26,27]. These interactions with insulin signaling effectors suggest that Sam68 plays an adaptor role in IR signal transduction.

Insulin receptor substrates-1 (IRS-1) belong to a family of intracellular proteins with at least six members identified to date (IRS-1 to IRS-6) [28–32]. At first, this family was identified as insulin receptor substrates but now they are also known to be implicated in other signaling pathways. IRS-1 as well as other members of the family contain a Pleckstrin Homology (PH) domain at their N-terminus, that is used to keep the protein bound to membrane phosphoinositides in close proximity to the insulin receptor, and a Phospho-Tyrosine Binding (PTB) domain that recognizes and bind the phosphorylated NPXY motif in the IR [33,34]. IRS-1 also contains numerous tyrosine residues that, once phosphorylated, are recognized by proteins containing Src-homology 2 (SH2) domains such as the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K), Grb2, Nck, Crk, Fyn, and SHP-2 [35–37]. Upon insulin receptor activation, IRS-1 is phosphorylated on tyrosine residues, allowing downstream effectors such as PI3K to be recruited and activated. In addition to this canonical role of IRSs in IR and IGFR signaling pathways, IRS-1 and IRS-2 can also interact with many other signaling pathways in a non-canonical way [38] or translocate to the nucleus in response to IGF-I or certain oncogenes (although it does not bear a nuclear localization signal). IRS1 translocation into the nucleus is dependent on its PTB domain. Inside the nucleus, IRS-1 regulates transcription of several genes implicated in different stages of cancer progression. For instance, in the nucleus, IRS-1 associates to estrogen receptor α (ERα) and regulates the ERα gene transcription, inside the nucleus [39,40]. Nuclear IRS-1 also interacts with β-catenin, and regulates the cyclin D1 transcription [38,41].

In this work, our aim was to characterize the role of Sam68 in IR signaling pathway by BRET. The BRET technology is an important tool for the study of protein–protein interactions in living cells [42,43]. The use of this methodology has allowed us to describe, for the first time, the direct interaction between Sam68 and IRS1 as well as the regions of Sam68 involved and the implication of the said interaction in IR signaling.

2. Materials and methods

2.1. Reagents and materials

2-Amino-2-(hydroymethyl)-1,3-propanediol (Trizma base), sodium orthovanadate, pyrophosphate tetrasodium salt, sodium fluoride, Tween-20, Triton X-100, poly-L-lysine-hydrobromide and paraformaldehyde were from Sigma (St. Louis, MO, USA). phosphate buffer saline (PBS) was from BioWhittaker (Lonza, VerviersBelgium). Nonidet-P40 was from Fluka-Biochemika

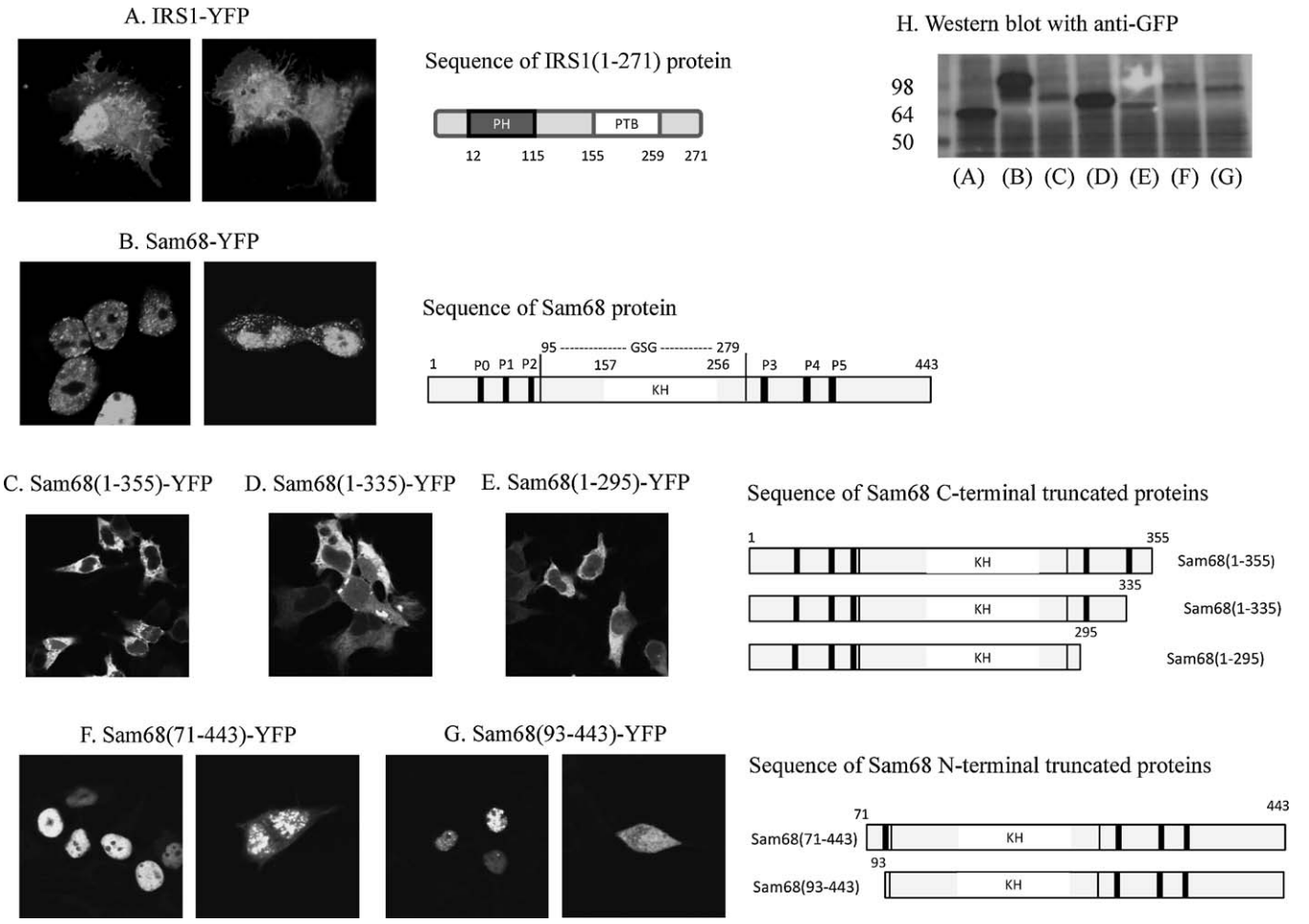

Fig. 1. Expression of yellow fluorescent protein (YFP) fusion constructions in human embryonic kidney (HEK)-293 cells. Cells were transfected with cDNAs encoding YFP-tagged proteins. The confocal images show the localization of each protein expressed. A schematic diagram of each protein is shown alongside the fluorescence image: (A) expression of IRS1(1–271)-YFP, (B) wild-type Sam68-YFP (C), Sam68(1–355)-YFP, (D) Sam68(1–335)-YFP and (E) Sam68(1–295)-YFP, (F) Sam68(71–443)-YFP and (G) Sam68(93–443)-YFP. The percentage of transfected cells showing a high fluorescence signal was around 25–30%. (H) Western blot showing the expression level of each construction. HEK293 cells were transfected with 600 ng of cDNAs, lanes (A) IRS1-YFP, (B) Sam68-YFP, (C) Sam68(1–335)-YFP, (D) Sam68(1–355)-YFP, (E) Sam68(1–295), (F) Sam68(71–443) and (G) Sam68(93–443). Two days after transfection, cells were lysed and immunoblotted with anti-GFP antibody.