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Review

Role of Sam68 as an adaptor protein in signal transduction

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Abstract. Sam68, the substrate of Src in mitosis, belongs to the family of RNA binding proteins. Sam68 contains consensus sequences to interact with other proteins via specific domains. Thus, Sam68 has various proline-rich sequences to interact with SH3 domain-containing proteins. Moreover, Sam68 also has a C-terminal domain rich in tyrosine residues that is a substrate for tyrosine kinases. Tyrosine phosphorylation of Sam68 promotes its interaction with SH2 containing proteins. The association of Sam68 with SH3 domain-containing proteins, and its tyrosine phosphorylation may negatively regulate its RNA binding activity. The presence of these consensus sequences to interact with different domains allows this protein to participate in signal transduction pathways triggered by tyrosine kinases. Thus, Sam68 participates in the signaling of T cell receptors, leptin and insulin receptors. In these systems Sam68 is tyrosine phosphorylated and recruited to specific signaling complexes. The participation of Sam68 in signaling suggests that it may function as an adaptor molecule, working as a dock to recruit other signaling molecules. Finally, the connection between this role of Sam68 in protein-protein interaction with RNA binding activity may connect signal transduction of tyrosine kinases with the regulation of RNA metabolism.

Key words. Sam68; signal transduction; tyrosine kinases; tyrosine kinases substrates; RNA binding proteins; SH2 domain; SH3 domain; proline-rich motif.

Introduction

Sam68 was first identified as p62, a tyrosine-phosphorylated GTPase activating protein (GAP)-associated protein in in cells transformed with oncogenes of the tyrosine kinase family, including Src [1]. In 1992, a p62 protein was purified by affinity chromatography from NIH-3T3 cells transformed with Src, using anti-phosphotyrosine antibodies [2]. In 1994, five groups identified p62 as an SH3 domain binding protein [3–7]. P62 GAP-associated protein cloned in 1992, turned out to be Sam68, a substrate of Src in mitosis with RNA binding activity that migrates in SDS-polyacrylamide gel electrophoresis (PAGE) with an apparent molecular weight of 68 kDa [3,5]. Sam68 has been cloned from different species, including human, mouse, rat, chicken, fish and fly with highly conserved sequences within the putative functional domains [4, 5, 8, 9].

Sam68 belongs to the family of RNA binding proteins that contain a domain with homology to ribonucleoprotein heteronuclear K (KH domain). The RNA binding activity and the protein interaction domains of Sam68 and related proteins led to the term STAR (signal transducers and activators of RNA). In addition to Sam68, proteins of the STAR family include GRP33 from *Artemia salina* [10], GLD-1 from *Caenorhabditis elegans* [11], QK1 [12], SLM-1 and SLM-2 [13], How from *Drosophila* [14], KEP1 [8] and the splicing factor 1 (SF1) [15].

There is growing evidence of the role of Sam68 and STAR proteins in signaling. In a recent review [16] a complete picture of the function of Sam68 was provided, including the possible role of Sam68 in tumor biology,

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cell cycle and viral pathogenesis. In the present review we focus on the published data regarding the function of Sam68 in signal transduction of different cellular communication systems.

Sam68 Structure

Human Sam68 is located in chromosome 1p32 with nine exons similar in size to those of mouse Sam68 [16]. The primary structure of the polypeptide contains specific sequences conserved in other STAR proteins. In addition, the amino acid sequence in these putative functional domains of Sam68 is highly conserved through evolution, from *Drosophila* [8] and Torpedo [9].

Sam68 and other STAR proteins contain a KH domain located within a larger domain of 200 amino acids, with RNA binding activity named GSG, after the three proteins GRP33, Sam68 and GLD-1. Thus, the KH domain is flanked by an 80-amino acid N-terminal region and a 30-amino acid C-terminal region called NK and CK, respectively. In addition, GSG regions in STAR proteins such as Sam68, GLD-1, GRP33 and QKI can form homomultimers through interaction with the GSG domains [13, 17– 20].

Sam68 also contains proline-rich regions which allow interaction with SH3 and WW domains containing proteins. Thus, Sam68 has six proline-rich domains (P0 through P5) out of the GSG domain, three N-terminal (P0–P2) and three C-terminal (P3–P5). The interaction of Sam68 with the SH3 domains of the Src family of kinases is required for its tyrosine phosphorylation.

The WW and SH3 domains have affinity for similar proline-rich sequences, and therefore they may compete for the same ligands [21, 22]. However, Sam68 methylation in arginine residues may prevent interaction with SH3 but not WW domains.

The C-terminal domain of Sam68 and other STAR proteins (SLM-1, SLM-2, QK1) has various tyrosine residues which are potential phosphorylation sites [2, 12, 13]. In fact, Sam68 can be tyrosine phosphorylated by soluble tyrosine kinases, including Src [3, 5], p59fyn [4], p56lck [6], ZAP-70 [23] and Sik/BRK [24]. Moreover, binding of the ligands to CD16, CD32, TCR, leptin receptor and insulin receptor increases the tyrosine phosphorylation level of Sam68 [25–29].

Another important feature of the structure of Sam68 and STAR proteins is the presence of methylation sites, arginine-glycine-rich sequences or RGG box [30]. Arginine methylation is a post-translational modification known to modulate various cellular processes, including protein-protein interactions, transcription and intracellular localization [31–33]. The interaction of Sam68 with protein arginine methyltransferase 1 (PRMT1) and its arginine methylation have been found in vivo [34]. Hypomethylated Sam68 is located in the cytoplasm, whereas methylated Sam68 is located in the nucleus. It has been shown that methylation is an irreversible process. Sam68 contains a nuclear localization signal (NLS) within the last 24 amino acids in the C-terminal part of the protein [35]. Thus, two motifs of nuclear localization are present in Sam68, PPXXR [35] and RXHPYQ/GR [19].

Protein acetylation is another known post-translational modification. Acetylation of lysine residues in Sam68 has been demonstrated in vivo, positively correlating with the RNA binding activity of the protein [36]. Thus, the acetyltransferase CBP has been shown to acetylate Sam68 and enhance its binding to poly(U) RNA. The highest level of acetylation was found in tumorigenic breast cancer cell lines.

Sam68 and protein-protein interactions

The amino acid sequence of Sam68 presents different domains that mediate the interaction with different signaling proteins. In fact, the modular structure of Sam68 containing protein-protein interaction motifs led to the concept that Sam68 is an adaptor protein with a putative role in signal transduction.

Since Sam68 has six proline-rich motifs, it can interact with the SH3 domains of Src [3, 5, 7], and other kinases of this family, such as Sik or BRK [24], p59fyn [4] or Itk/Tec/BTK [37, 38]. P0, P3, P4 and P5 are the motifs in Sam68 known to interact with SH3 domains of tyrosine kinases of the Src family. This interaction of Sam68 with SH3 domains enables these kinases to tyrosine-phosphorylate the substrate [4, 7].

Sam68 can also interact with SH3 domains of various signaling enzymes and adaptor proteins. Thus, Sam68 has been found to bind the SH3 domains of p85 phosphatidylinositol 3-kinase (PI3K) [39], phospholipase C gamma-1 (PLC-γ-1) [4, 40], protein arginine methyltransferase (PRMT) [22], Grb-2, Grap [41] and Nck [42]. P1, P3 and P4 are the preferential motifs for interaction with the SH3 domains of p85 PI3K and PLCγ-1, whereas the C-terminal SH3 domain of Grb-2 binds Sam68 with higher affinity than the N-terminal SH3 domain [43]. The binding of Sam68 with SH3 domains has been found to negatively regulate RNA binding activity. On the other hand, arginine methylation of Sam68 may prevent the interaction with SH3 domain-containing proteins.

The proline-rich motifs in Sam68 structure also allow interaction with proteins containing WW domains. Thus, Sam68 has been found to interact with WW domains of the formin binding proteins FBP21 and FBP30, especially through the P3 and P4 motifs in Sam68 [44]. This interaction of Sam68 with WW domains probably occurs in the nucleus.

Tyrosine phosphorylation of Sam68 leads to its association with different SH2 domain-containing proteins [45], including kinases of the Src family [3–7], Sik/BRK [24], as well as the kinases of the Itk/Tec family [37, 38]. Moreover, tyrosine-phosphorylated Sam68 can also interact with SH2-containing adaptor proteins and signaling enzymes such as Grb2 [4, 41], Grap [41], Nck [42], PLCγ-1 [4], Ras-GAP [4, 46, 63] and p85a-PI3K [29, 39]. The association of Sam68 with these SH2 domains exhibits different affinities. Thus, it has been shown that tyrosine-phosphorylated Sam68 preferentially associates with the N-terminal SH2 domain of p85 compared to the C-terminal [29], whereas Sam68 binds the C-terminal SH2 domain of GAP with higher affinity than the N-terminal SH2 domain [46].

All these interactions are good candidates for playing some role in signal transduction and are consistent with the hypothesis that Sam68 is an adaptor molecule that can be recruited by several signaling systems triggered by tyrosine kinases.

Role of Sam68 in signal transduction

The modular structure of Sam68 with protein-protein interaction domains has provided a multifunctional element that may have a role as an adaptor protein in signal transduction. In this line, Sam68 has been implicated in the signaling of T cell receptor (TCR), leptin receptor (Ob-R) and insulin receptor.

Sam68 and TCR signaling

Stimulation of TCR leads to sequential activation of kinases of the Src family p56lck and p59fyn, which initiate TCR signaling. This signal is amplified by the ZAP-70/Syk kinases, finally leading to activation of the mitogen-activated protein kinase (MAPK) cascade [47]. Accordingly, Sam68 has been found to be tyrosine phosphorylated after TCR activation and to interact with the SH2 domains of PLCγ-1 and p120GAP [48, 49]. Moreover, Sam68 can interact with the SH2 and SH3 domains of Fyn and Lck in lymphocytes and can be tyrosine phosphorylated by Fyn, promoting the association of Sam68 with various signaling proteins, such as PLCγ-1, p85 PI3K, Grb2, SHP-1, Cbl and JAK3 [25]. ZAP-70 has also been found to tyrosine phosphorylate Sam68 in vitro and in vivo in Jurkat cells [23].

Another tyrosine kinase required for signaling through TCR, Itk (a member of the Tec family of non-receptor tyrosine kinases), interacts with Sam68 by means of the SH3 domains of the kinase and the proline-rich motifs in Sam68, and thus allowing the tyrosine phosphorylation of Sam68 [37, 50].

In summary, these data reported in the literature suggest that Sam68 participates in the signal transduction pathway downstream of TCR, via tyrosine phosphorylation by Fyn, Lck, ZAP-70 and Itk, promoting the formation of signaling complexes with different adaptor proteins and enzymes that may mediate the TCR signal (fig. 1).

Sam68 and leptin receptor signaling in immune cells

Sam68 has also been implicated as a signaling molecule in the Ob-R system in human monocytes and lymphocytes [51]. Similar to other receptors of the cytokine receptor superfamily, Ob-Rs lack intrinsic tyrosine kinase activity but requires activation of receptor-associated kinases of the Janus family (JAKs) [52], which initiate downstream signaling, including members of the STAT (signal transducers and activators of transcription) family of transcription factors [53]. After ligand binding, JAKs autophosphorylate and tyrosine-phosphorylate various STATs. Activated STATs by leptin stimulation in the hypothalamus dimerize and translocate to the nucleus, where specific gene responses are elicited [54]. In this context, we have found constitutive association of JAK2 and JAK3 with Ob-R (the leptin receptor) in human peripheral blood mononuclear cells, and subsequent activation by tyrosine phosphorylation of STAT3, which then translocates from the cytoplasm to the nucleus in human peripheral blood mononuclear cells [55]. We have observed that human leptin time and dose dependently stimulates Tyr phosphorylation of Sam68, promoting the association with STAT3, probably by interacting with the SH2 domains. Whether leptin-stimulated tyrosine phosphorylation of Sam68 is mediated by JAK or another kinase activity remains to be studied. On the other hand, we cannot rule out the possible involvement of STATs other than STAT-3. In any case, STAT-3 is the only STAT that has been shown to be activated by leptin in the hypothalamus [56]. Moreover, activation of STAT-3 by leptin stimulation has recently been confirmed in a murine macrophage cell line [57]. Even though we do not know the function of tyrosine-phosphorylated Sam68 associated with activated STAT3, the translocation to the nucleus and the known RNA binding activity of Sam68 suggest that Sam68 may play some role in the transduction of leptin messages from the plasma membrane to the nucleus (fig. 1).

Different pathways in addition to STATs are known to be involved in Ob-R signaling, in a similar way to other members of the cytokine family. Thus, leptin has been shown to activate MAPK, and PI3K in peripheral blood mononuclear cells [28]. We have found that human leptin stimulates the association of tyrosine-phosphorylated Sam68 with p85 PI3K, leading to increased PI3K activity associated with tyrosine-phosphorylated proteins. These p85 PI3K-associated tyrosine-phosphorylated proteins in response to leptin include Sam68 and the insulin receptor substrate-1 (IRS-1), which mediate PI3K activation by

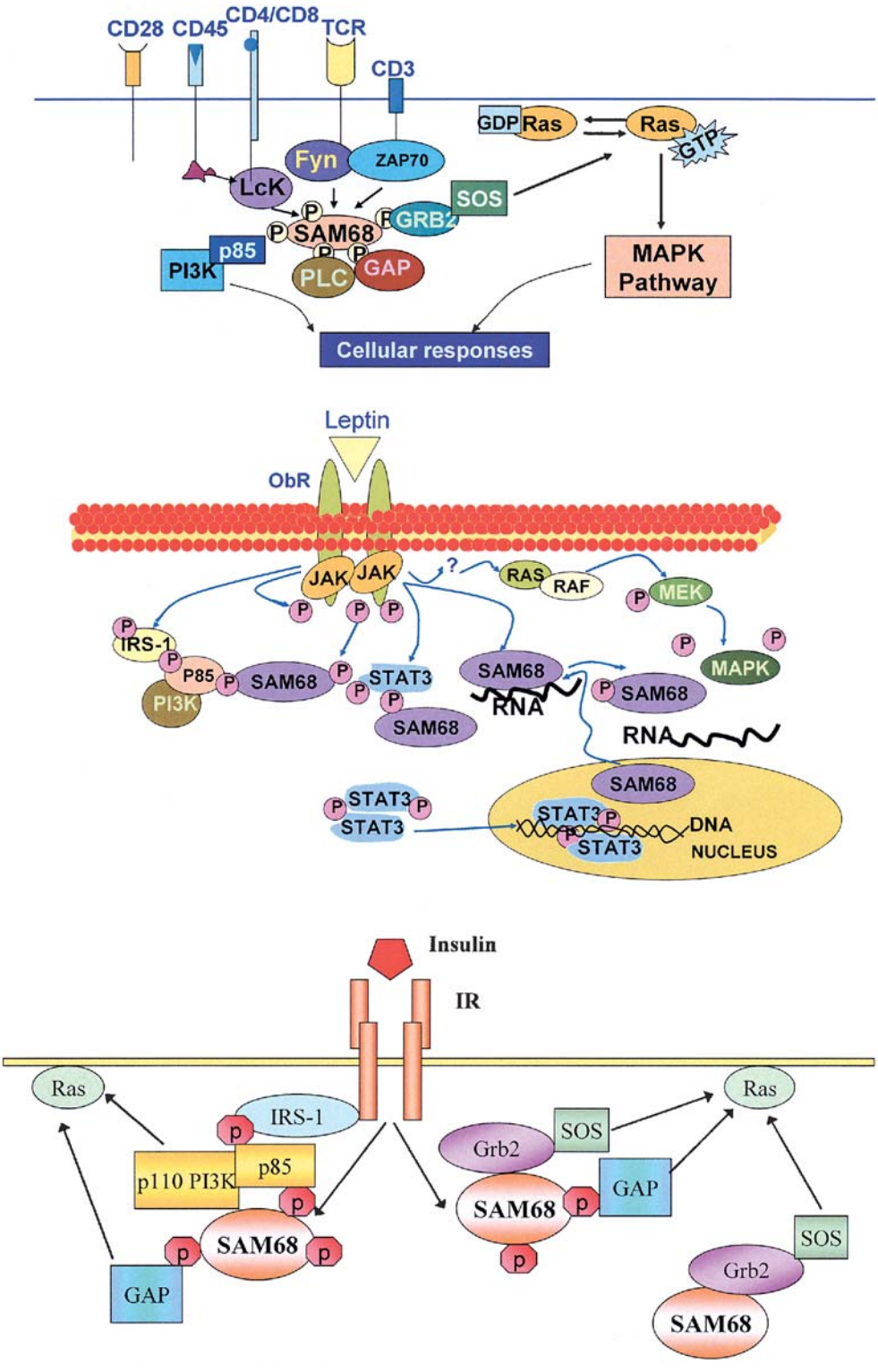

Figure 1. Role of Sam68 in signal transduction. Schematic representation of the proposed role of Sam68 in signal transduction of T cell receptor (TCR) (upper), leptin receptor (Ob-R) (middle) and insulin receptor (IR) (lower).

association with the SH2 domains of p85, the regulatory subunit of PI3K (fig. 1).

Tyrosine phosphorylation of Sam68 by the Src family kinase p59fyn has been previously shown to negatively regulate its association with RNA [58] and poly(U) [59]. Leptin stimulation of peripheral blood mononuclear cells dose dependently increased the tyrosine phosphorylation level of Sam68 [28], inhibiting the binding efficiency of Sam68 to poly(U) [28]. This effect of leptin regulating the RNA binding capacity of Sam68 may be involved in the post-transcriptional modulation of RNA (fig. 2). In this way, Sam68 may facilitate a rapid pathway for regulating protein expression by modifying the messenger RNA (mRNA) stability and/or mRNA translation. Moreover, Sam68 has been shown to interact with the splicing-associated factor YT521-B in nuclear dots, and this interaction is regulated by Tyr-phosphorylation [60]. Thus, Tyr-phosphorylation of Sam68 by leptin stimulation could modulate its association with the splicing machinery in a similar way to that described for p59fyn, and in this way it could influence splice site selection. In any case, these hypotheses remain speculative until investigated.

Sam68 and insulin receptor signaling

Sam68 can also be a substrate of the insulin receptor tyrosine kinase, and therefore a role for Sam68 in insulin receptor signaling has been proposed [29] (fig. 1).

Insulin stimulation of HTCs (rat hepatoma cells) transfected with the human insulin receptor (HTC-IR) promotes the tyrosine phosphorylation of Sam68. The effect is dependent on the dose of insulin, and it is rapid, within 3–5 min, and reversible, turning back to basal levels after 30 min [29]. These results were then confirmed in vitro with recombinant Sam68 and purified insulin receptor. Tyrosine phosphorylation of Sam68 in response to insulin in vivo was also assessed in CHO (Chinese hamster ovary) cells overexpressing the insulin receptor (CHO-IR) and in a more physiologically relevant system, such as rat adipocytes, with similar results [61]. Moreover, we have found that Sam68 tyrosine phosphorylated by the activated insulin receptor can be recruited to different insulin signaling complexes. Thus, we have observed in adipocytes, HTC-IR and CHO-IR cells that one of the insulin receptor substrates that associates with PI3K via the SH2 domains of the p85 subunit is Sam68, along with IRS-1. We also studied the differential affinity of the two SH2 domains of p85 with tyrosine-phosphorylated Sam68 in response to insulin in vitro, by using the recombinant Sam68 and fusion proteins separately containing the SH2 domains of p85. Sam68 has 10 times higher affinity for the N-terminal SH2 domain of p85 compared with the C-terminal SH2 domain [29]. The higher affinity of the N-terminal SH2 domain of p85 for Sam68 is consistent with the higher affinity of the C-terminal SH2 domain of p85 for IRS-1 [62], supporting the hypothesis of the formation of ternary complexes containing p85 and IRS-1 along with Sam68 in response to insulin. Moreover, it has been shown that insulin stimulation increases PI3K activity in Sam68 immunoprecipitates, suggesting that the association of tyrosine-phosphorylated Sam68 with the regulatory subunit p85 also correlates with an increase in PI3K activity.

Sam68 was previously described to associate with Ras-GAP in mitosis by interacting with the SH2 domains [39, 63]. Sam68 tyrosine phosphorylated by insulin receptor also associates with Ras-GAP, via the SH2 domains, recruiting this signaling protein to the PI3K pathway. In this way, Sam68 may provide a link between the PI3K and Ras pathways. Moreover, in vitro studies with recombinant and fusion proteins have shown that Sam68 preferentially binds the C-terminal domain of GAP, therefore leaving the N-terminal SH2 domain available for other GAP-associated proteins.

On the other hand, Sam68 is constitutively associated with the SH3 domains of Grb2 in vivo [43]. In vitro experiments with recombinant Sam68 and fusion proteins containing the SH3 domains of Grb2 have shown that Sam68 preferentially associated with the C-terminal SH3 domain, and in vivo experiments have shown that this association does not change upon insulin stimulation [43]. These results are consistent with the preferential binding of the N-terminal SH3 domain of Grb2 with SOS (son-of-sevenless) [64]. 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. On the other hand, since Tyr phosphorylation of Sam68 promotes the association with GAP, this direct interaction provides a dock for the recruitment of GAP to the Grb2-SOS-Ras signaling pathway [43]. 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, it has been found predominantly in the nucleus because of the non-conventional nuclear localization signal in the C-terminal of the polypeptide [35]. However, we have demonstrated the preferential cytoplasmic expression of this protein in HTC-IR [46]. Actually, we have also reported that Sam68 further relocalizes from the nucleus to the cytoplasm when cells overexpress insulin receptors, and in response to insulin [46, 61]. Moreover, we have found that Sam68

localizes in the cytoplasm in a typical insulin target cell such as the adipocyte [61]. These data regarding cytoplasmic localization of Sam68 and traffic from the nucleus to the cytoplasm are consistent with previously reported results from other groups [65]. Therefore, Sam68 may be part of the signaling machinery of the insulin receptor in the cytoplasm, functioning as a docking protein. On the other hand, the presence of Sam68 in the nucleus does not rule out the putative function of Sam68 as a substrate of the insulin receptor, since the insulin receptor itself can be found in the nucleus [66], as well as other substrates such as IRS-1 [67] and signaling molecules, i.e. PI3K [68], PDK [69] and Ras [70]. In any case, the physiological relevance of insulin receptor signaling in the nucleus is not yet well understood, although Sam68 could still play its role as a substrate and docking protein for the insulin receptor in the nucleus.

Another important feature for most insulin receptor substrates is regulation of expression by insulin stimulation. In this regard, we have found that overexpression of insulin receptor in CHO cells as well as insulin stimulation in CHO-IR and adipocytes increase the expression of Sam68. These data further support the importance of Sam68 for insulin action.

Conclusion

Sam68 can be tyrosine phosphorylated by activation of different receptor systems, and recruited for their signal transduction cascades. Thus, Sam68 can be used by TCRs, leptin and insulin receptors, in a similar way to other tyrosine kinase substrates that may function as adaptor proteins in different signaling pathways. IRS-1 can be tyrosine phosphorylated by insulin receptor, Ob-R activation and by activation of different cytokine receptors [8, 71, 72].

On the other hand, Sam68 is a putative regulator of the RNA metabolism, and it may provide a rapid pathway for regulating protein expression by modifying mRNA stability and/or mRNA translation [73]. Moreover, Sam68 may serve to target the mRNA in the cytoplasm to deliver specific proteins to specific structures for mediating interactions. Sam68 may thus be a substrate for tyrosine phosphorylation in different signaling systems, functioning as an adaptor protein, but eventually providing a way to regulate the metabolism of RNA. This mechanism could allow the cell to respond much faster than protein expression from de novo transcription, when stimulated by extracellular signals. However, we do not know the precise biological role of Sam68 in the signaling systems where it is recruited. Studies of the RNA targets of Sam68 and their regulation in response to activation of different receptors may provide some clues to finally elucidate the biological role of Sam68 in signal transduction.

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