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Insulin receptor substrate-1 (IRS-1) forms a ribonucleoprotein complex associated with polysomes

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ABSTRACT

Insulin receptor substrates (IRSs) are known to play important roles in mediating intracellular insulin-like growth factors (IGFs)/insulin signaling. In this study, we identified components of messenger ribonucleoprotein (mRNP) as IRS-1-associated proteins. IRS-1 complex formation analysis revealed that IRS-1 is incorporated into the complexes of molecular mass more than 1000 kDa, which were disrupted by treatment with RNase. Furthermore, oligo(dT) beads precipitated IRS-1 from cell lysates, showing that the IRS-1 complexes contained messenger RNA. Taken together with the data that IRS-1 was fractionated into the polysome-containing high-density fractions, we concluded that IRS-1 forms the novel complexes with mRNPs.

Structured summary of protein interactions:
IRS1 physically interacts with **PABPC1** by anti bait coimmunoprecipitation (View Interaction: 1, 2)
IRS1 physically interacts with **PABPC1** by anti tag coimmunoprecipitation (View interaction)
IRS1 physically interacts with **PABPC1** by anti bait coimmunoprecipitation (View interaction)
IRS1 physically interacts with **EIF4F** and **PABPC1** by anti bait coimmunoprecipitation (View interaction)

1. Introduction

Insulin-like growth factors (IGFs) and insulin induce fundamental bioactivities, supporting embryonic development and growth, and postnatal somatic growth and regulation of glucose, lipid and protein metabolism [1]. The intracellular processes are accomplished by a variety of molecules of the IGF/insulin signaling pathway, including insulin receptor substrates IRS-1 and -2 [2–4]. When IGFs or insulin bind to their specific receptors, receptor-intrinsic tyrosine kinases are activated and phosphorylate IRSs. Phosphotyrosyl IRSs are then recognized by Src homology region 2 (SH2) domain-containing proteins, leading to the activation of phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways. The activated PI3K transmits the signal to up-regulate growth and metabolism through the Akt signaling pathway [5]. In particular, the downstream mammalian target of rapamycin (mTOR) is important in promoting growth-related intracellular activities such as protein synthesis and ribosome biogenesis [6]. The signaling pathway triggered by tyrosine phosphorylation of IRSs plays important roles in controlling translational processes required for IGF/insulin-induced protein synthesis resulting in cell proliferation, differentiation and survival.

Among the IRS isoforms, it was well established that IRS-1 and IRS-2 are the main isoforms that mediate signal transduction essential for IGF/insulin bioactivities. We found that IRS-1/-2 form high-molecular-mass complexes (we named these complexes IRSomes) with various proteins even in a phosphotyrosine-independent manner, and the components of IRSome vary in different tissues/cell-types and under conditions of hormone/cytokine

Abbreviations: IRSs, insulin receptor substrates; IGFs, insulin-like growth factors; PABPC1, poly(A) binding protein cytoplasmic 1; MALDI-TOF-MS, matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry; BN-PAGE, blue native-polyacrylamide gel electrophoresis; mRNA, messenger RNA; mRNP, messenger ribonucleoprotein; eIF, eukaryotic initiation factor; PI3K, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; EJC, exon junction complex; 4EBPs, 4E binding proteins; rpS6, ribosomal subunit protein S6; S6Ks, S6 protein kinases; PIC, translation preinitiation complex; HEK293, human embryonic kidney 293; DMEM, Dulbecco's modified Eagle's medium; RNase A, ribonuclease A; FBS, fetal bovine serum

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stimulation [7]. We and others have identified phosphotyrosine-independent binding partners of IRSs and these IRS-associated proteins are shown to positively/negatively modulate IGF/insulin signaling through altering the availability of IRSs to their receptors and regulating the intracellular quantity of IRSs [8–15]. While much has been studied about the phosphotyrosine-dependent signaling pathway of IRSs, it is largely unclear how the phosphotyrosine-independent association of proteins with IRS modulates IGF/insulin bioactivities. Thus, we have been investigating the components of IRS-1 complexes using proteomic approaches, and as shown herein, our studies unexpectedly identified an interaction of IRS-1 with poly(A) binding protein cytoplasmic 1 (PABPC1).

Newly transcribed pre-messenger RNA (mRNA) is capped at the 5′ end and bound by a nuclear cap-binding heterodimer CBP80/20 [16,17]. Pre-mRNA undergoes RNA processing including 3′ poly(A) tail addition and splicing, in which introns are removed and a large-protein complex called the exon junction complex (EJC) is deposited upstream of each exon–exon junction [18,19]. These proteins remain bound to mRNA while the messenger ribonucleoprotein (mRNP) complex is exported to the cytoplasm [20–22] and are subsequently replaced by cytoplasmic translation initiation factors during the first ribosomal passage along the mRNA in the cytoplasm; the EJC is removed [23,24] and CBP80/20 is replaced by eukaryotic initiation factor (eIF) 4E [25] and poly(A)-tail is protected by PABPC1 [26]. Passing through the pioneer round of translation, eIF4E-bound mRNAs undergo steady-state translation, which is regulated by mTORC1 mainly through phosphorylation of downstream effectors eIF4E inhibitory proteins, 4E binding proteins (4EBPs) and the 40S ribosomal subunit protein S6 (rpS6) protein kinases (S6Ks) in response to various cellular conditions, including the presence of growth factors [27]. eIF4E promotes translation initiation by recruiting eIF4G, eIF4A, eIF3 and the 40S ribosomal subunit to the 5′ end of mRNA, and the ternary complex (eIF2/Met-tRNA/GTP) is also recruited to the cap, resulting in the assembly of the translation preinitiation complex (PIC) [28]. Poly(A)-associated PABPC1 interacts with eIF4G in the PIC, leading to circularization of the mRNA and translation enhancement [29].

In this study, we show that IRS-1 forms RNA-dependent high molecular mass complexes including PABPC1 and other mRNP components and IRS-1 was distributed into high-density fractions containing polysomes in proliferating cells.

2. Materials and methods

Experimental materials and cell cultures and transfection are described in Supplementary manuscripts.

2.1. Identification of PABPC1 as an IRS-1-associated protein

IRS-1-associated proteins were immunopurified using anti-IRS-1 antibody and separated by SDS–PAGE, followed by silver staining as described previously [7]. Excised protein bands were digested and subjected to matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF-MS) as described previously [30]. Peptide mass fingerprinting analysis were performed with the Mascot search engine (http://www.matrixscience.com/search_form_select.html).

2.2. Immunoprecipitation followed by immunoblotting

Immunoprecipitation and immunoblotting were performed as described in Supplementary methods.

2.3. Oligo(dT) pull down assay

mRNP capturing was performed using Dynabeads oligo$(dT)_{25}$ magnetic beads (Dynal). Extracts from MCF-7 cells (1×10^7 cells) were adjusted to 5 ml in Binding buffer [20 mM HEPES (pH 7.6), 150 mM NaCl, 1 mM EDTA, 0.5% (v/v) NP40, 15 μg/ml calpain inhibitor, 10 μg/ml leupeptin, 5 μg/ml pepstatin, 20 μg/ml PMSF, 100 KIU/ml aprotinin, 10 mg/ml PNPP], and incubated with 20 μl of beads for 60 min at 4 °C. As controls, extracts were treated with 1 mg/ml ribonuclease A (RNase A) for 10 min at room temperature, prior to binding to the oligo(dT) beads. After six washes in the same buffer, the mRNPs were eluted by boiling in1× Laemmli′s buffer.

2.4. Blue native-polyacrylamide gel electrophoresis (BN-PAGE)

To separate protein complexes containing IRSs, BN-PAGE was performed as described previously [7].

2.5. Sucrose gradient density centrifugation analysis

The sucrose gradient density centrifugation was performed according to the previous report [7], except for modifications as described in supplementary methods.

2.6. Polysome fractionation

Polysome fractionation was performed as described in supplementary methods

3. Results

3.1. PABPC1 is a component of IRS-1 complex

We have previously set up immunoprecipitation assays using FLAG-tagged IRS-1 and -2 proteins and found many proteins that are candidates for IRSome components [7]. To further characterize proteins that associate with IRS independently of its tyrosine phosphorylation, L6 myotubes were cultured under serum-free conditions and cell lysates were immunoprecipitated with anti-IRS-1 antibody. Immunoprecipitated proteins were detected by silver staining, and the protein profile of the precipitated fraction was quite similar to that of FLAG-IRS-1, which we have previously reported [7] (data not shown). Those proteins were subjected to MALDI-TOF-MS and peptide mass finger printing analysis, and as a result, we identified poly(A) binding protein cytoplasmic 1 (PABPC1) as one of the IRS-associated proteins. We confirmed the association in serum-starved L6 cells by immunoprecipitation using anti-IRS-1 antibody followed by immunoblotting with anti-PABPC1 antibody (Fig. 1A). To address the question whether IRS-2 also interacts with PABPC1, we overexpressed FLAG-IRS-1 or FLAG-IRS-2 and immunoprecipitated with anti-FLAG antibody, followed by immunoblotting with anti-PABPC1 antibody. The specific interaction of PABPC1 with IRS-1 was observed but not in the case of IRS-2 (Fig. 1B). In addition, we used MCF-7 cells since this cell line abundantly expresses both IRS-1 and IRS-2 proteins to check endogenous association between PABPC1 and IRS-1 or IRS-2. Co-immunoprecipitation analysis also showed that PABPC1 specifically associated with IRS-1 but not with IRS-2 (Fig. 1C).

3.2. IRS-1 forms a ribonucleoprotein complex

Since it is well established that PABPC1 interacts with the poly(A)-tail of mRNA [31], we next investigated the possibility that IRS-1 complexes contain polyadenylated mRNAs. To this end, we performed oligo(dT) pull-down assays using serum-starved MCF-7 whole cell lysates. Poly$(A)^+$ RNAs were isolated by incubating cell lysates with oligo(dT)-conjugated beads, and proteins associated with the RNA were separated by SDS–PAGE and analyzed by immunoblotting. IRS-1, but not IRS-2, was isolated by oligo(dT)

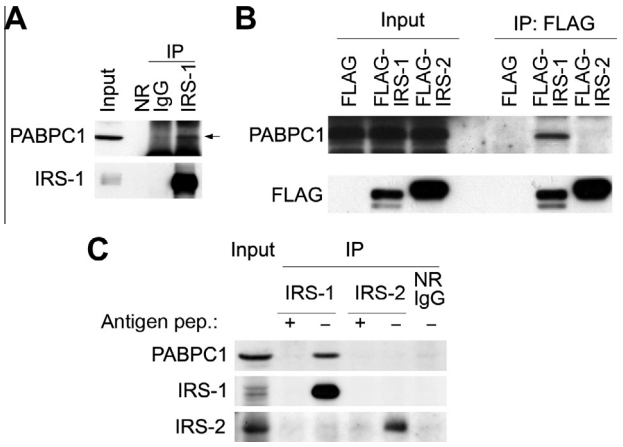

Fig. 1. IRS-1 forms a complex with PABPC1. (A) L6 myoblasts were cultured under serum-free conditions, and cell lysates were subjected to immunoprecipitation with anti-IRS-1 antibody, followed by immunoblotting with anti-PABPC1 antibody. Immunoprecipitates with normal rabbit IgG (NR IgG) served as negative controls. (B) HEK293 cells transiently expressing FLAG-IRS-1 or FLAG-IRS-2 were cultured under serum-free conditions. Cell lysates were subjected to immunoprecipitation with anti-FLAG antibody conjugated-agarose, followed by immunoblotting with the indicated antibodies. (C) MCF-7 cells were cultured under serum-free conditions, and cell lysates were subjected to immunoprecipitation with anti-IRS-1 antibody or anti-IRS-2 antibody, followed by immunoblotting with anti-PABPC1 antibody. Immunoprecipitates with normal rabbit IgG (NR IgG) or with anti-IRS-1 and anti-IRS-2 antibody in the presence of peptide used to raise the anti-IRS-1 or IRS-2 antibody (Antigen pep.) served as negative controls.

affinity extraction, whereas this interaction was impaired in the presence of RNase A (Fig. 2A). This strongly suggests that IRS-1 forms complexes with poly(A)$^+$ RNA.

3.3. Ribonuclease treatment dissociates IRS-1 complexes

Given the association of IRS-1 with poly(A)$^+$ RNA, we considered the possibility that the formation of IRSomes is dependent on RNAs. Thus, the effects of ribonuclease A (RNase A) treatment on the IRSomes were investigated. IRSomes were immunopurified from serum-starved human embryonic kidney 293 (HEK293) cells expressing FLAG-IRS-1 using anti-FLAG antibody. They were then treated with RNase A, and separated under the native conditions by BN-PAGE, followed by immunoblotting with anti-FLAG antibody (Fig. 2B). The results showed that IRS-1 was contained in complexes of about 400 kDa and over 1000 kDa, which is consistent with our previous report [7]. RNase A treatment clearly decreased the amount of complexes over 1000 kDa, and increased the amount of the 400-kDa complexes. We also investigated endogenous IRSomes by treating MCF-7 cell lysates with RNase and found that endogenous IRS-1 showed a similar trend (Fig. 2C). On the other hand, the amounts of the high-molecular mass complexes of IRS-2 were not as much reduced after RNase A treatment (Fig. 2C). Consistently, sucrose gradient fractionation analysis showed that although IRS-1 and IRS-2 were detected in relatively high-molecular-mass fractions as well as in low-molecular-mass fractions, RNase A treatment of lysates greatly decreased high-molecular-mass complexes containing IRS-1 but not IRS-2, and increased low-molecular-mass complexes (Fig. 2D). Taken together, we concluded that the formation of 1000 kDa-complex containing IRS-1 is dependent on RNAs.

3.4. IRS-1 associates with protein components of mRNP

Since PABPC1 is a major component of mRNP and is involved in multiple key steps of translation initiation, such as cap recognition by eIF4E [31], and translation enhancement by the circularization of the mRNA through eIF4G–PABPC1 interaction [29], we next examined whether components of cytoplasmic cap-binding protein also interact with IRS-1 (Fig. 3). We found that IRS-1 interacts with eIF4G and eIF4E in both serum-starved and IGF-I-stimulated MCF-7 cells. In contrast, we could not detect the association between IRS-1 and 4EBP1, which sequesters eIF4E in the eIF4E–4EBP1 complexes from mRNPs [32,33], indicating that IRS-1

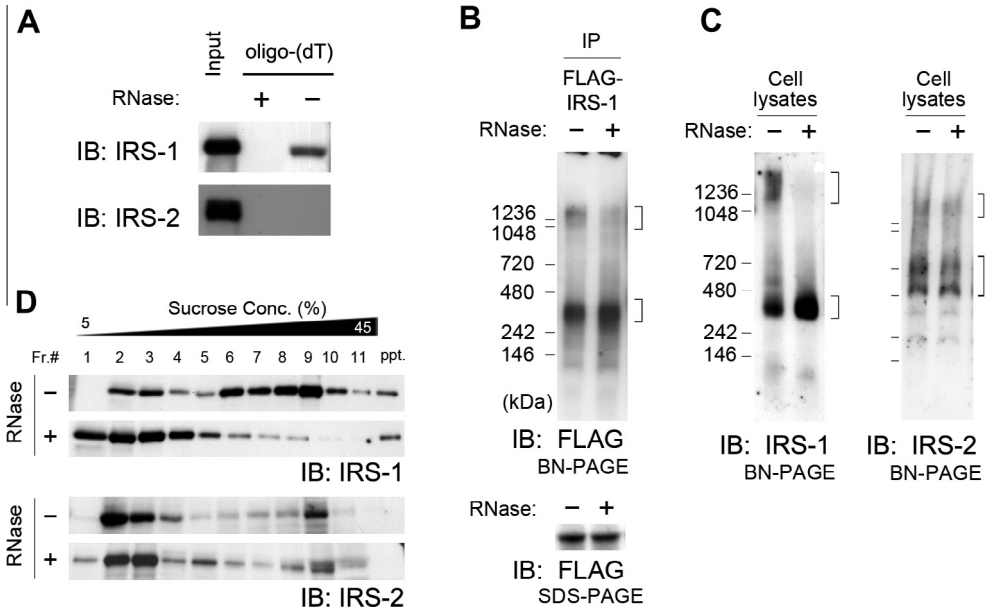

Fig. 2. High-molecular-mass complexes of IRS-1 contain mRNAs. (A) mRNPs in MCF-7 cell extracts were captured using oligo(dT) beads in the presence or absence of RNase A. Proteins bound to the beads were analyzed by immunoblotting with anti-IRS-1 or IRS-2 antibody. (B) HEK293 cells transiently expressing FLAG-IRS-1 were cultured under serum-free conditions. Cell lysates were subjected to immunoprecipitation with anti-FLAG antibody conjugated-agarose in the absence or presence of RNase A, and then FLAG-IRS-1 was eluted with FLAG peptides. Nine-tenths of the precipitate was subjected to BN-PAGE (top panel), and the remaining to SDS–PAGE (bottom), followed by immunoblotting. (C) MCF-7 cells were serum-starved, and the lysates were treated with or without RNase A, and subjected to BN-PAGE and immunoblotting with anti-IRS-1 or IRS-2 antibody. (D) Cell lysates of MCF-7 cells were treated with or without RNase. Each lysate was fractionated by sucrose density gradient centrifugation, and the aliquots of each fraction were analyzed by immunoblotting with anti-IRS-1 or IRS-2 antibody. Fraction number (Fr. #) and sucrose concentration (%) are indicated above the panels. Ppt: precipitates.

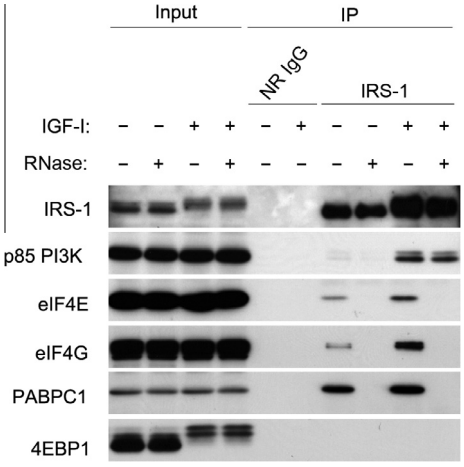

Fig. 3. IRS-1 forms complexes with the components of mRNPs. MCF-7 cells were serum-starved, then either left untreated or stimulated with IGF-I (100 ng/ml) for 2 h. The cell lysates were subjected to immunoprecipitation with anti-IRS-1 antibody in the presence or absence of RNase A. Precipitated IRS-1 complexes were eluted with the IRS-1 immunizing peptides and analyzed by immunoblotting with the indicated antibodies.

certainly interacts with RNA-bound eIF4E rather than the eIF4E–4EBP1 complexes. Furthermore, our results revealed that the interactions of IRS-1 with both PABPC1 and eIFs were abolished by RNase A treatment, whereas the protein–protein interaction of IRS-1 with a p85 regulatory subunit of PI3K was not affected. These results suggest that IRS-1 is incorporated into mRNP complexes.

3.5. IRS-1 associates with polysomes

To investigate whether IRS-1-associated mRNAs are translationally activated under the growth conditions, we compared the sedimentation profile of IRS-1 with that of polysomes in a 10–45% sucrose gradient. Under serum-starved conditions, IRS-1 was primarily detected in monosome fractions (Fig. 4A, before fraction #13) as well as non-ribosomal fractions (Fig. 4A, fraction #4–6). IRS-2 was mainly detected in non-ribosomal fractions (Fig. 4A, before fraction #6). In the case of MCF-7 cells cultured in the presence of serum, we observed IRS-1 in the polysome-containing fractions (Fig. 4B, after fraction #13) as well as 40S and 60/80S fractions (Fig. 4B, before fraction #13). The shift of PABPC1 distribution to the polysome-containing fractions was also observed. On the contrary, IRS-2 distribution remained unchanged (Fig. 4B). Consistent with the findings that IRS-1, but not IRS-2, forms complexes with mRNAs, these results suggested that IRS-1 specifically interacts with mRNPs, and that IRS-1-associated mRNPs, forming polysomes in response to growth factor stimuli, are active to be translated.

4. Discussion

IRSs are phosphorylated by the activated tyrosine kinases in IGF-I receptor and insulin receptor, and phosphotyrosyl IRSs associate with various effecter proteins containing the SH2 domain, resulting in the activation of the downstream signaling pathways. In contrast to this canonical phosphotyrosine-dependent association between IRSs and signaling molecules, we have recently shown that IRSs associate with various proteins in a phosphotyrosine-independent manner, and that those IRS-associated proteins play important roles in modulating IGF/insulin bioactivities [8–10]. In this study, we have identified several RNA-binding proteins as novel binding partners with IRS-1 and have shown that the IRS-1 complexes contained mRNA. These IRS-1 complexes containing mRNAs are likely to have some functions in translation.

IRS-1 is widely expressed in various IGF/insulin target tissues [34]. Previously, we have reported that the formation of high-molecular mass complexes containing IRS-1 is observed in various tissues/cell-types [7]. In this study, the interaction of IRS-1 with RNA-binding proteins as well as mRNA by themselves was detected in all cell-types that we have tested, suggesting that complex formation of IRS-1 with mRNAs or RNA-binding proteins generally occurs in various tissues/cell-types.

BN-PAGE and sucrose gradient fractionation analyses revealed that IRS-1 forms two types of complexes, relatively high- and low-molecular mass complexes, and in both analyses, the high-molecular mass complexes were disrupted by RNase treatment and disappeared whereas the amount of the low-molecular mass complexes increased instead (Fig. 2B–D). These data suggested that

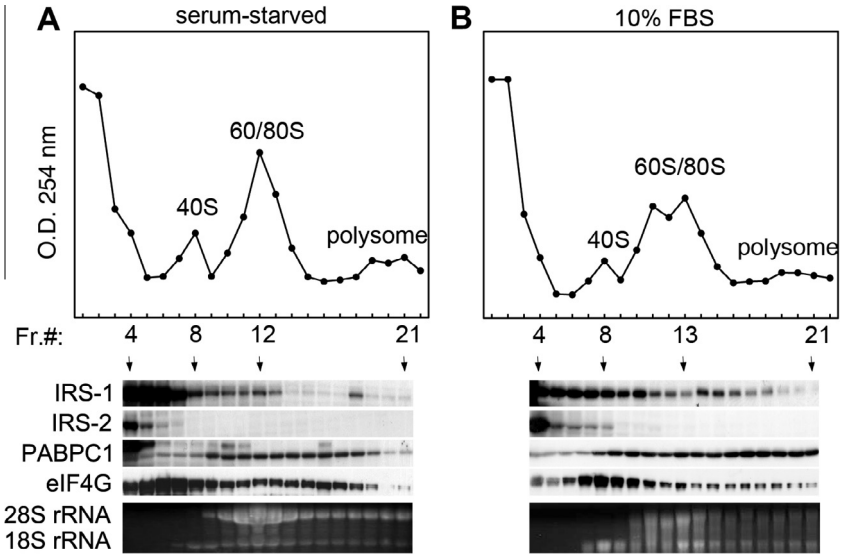

Fig. 4. IRS-1 associates with polysomes. Polysomes were fractionated using extracts prepared from MCF-7 cells cultured in the (A) serum-starved or (B) growth condition (10% fetal bovine serum (FBS)/Dulbecco's modified Eagle's medium (DMEM)). Proteins in each fraction were subjected to immunoblotting with the indicated antibodies. The curve denotes the O.D. 254 of each fraction, and the positions of 40S, 60S, and 80S ribosomal particles and polysomes are indicated. Fraction numbers (Fr. #) are indicated.

RNAs are contained only in the high-molecular mass complexes and that the low-molecular complexes are formed by different mechanisms independent of RNAs. In addition, this complex formation with RNAs was specific for IRS-1, since IRS-2 could not interact with PABPC1 (Fig. 1B and C) and high-molecular mass complexes containing IRS-2 were less sensitive to RNase treatment (Fig. 2C and D). Even though we could not rule out the possibility that IRS-2 also forms high-molecular mass complexes with RNAs and PABPC1 in small amounts, it remains unclear how only IRS-1 can associate with mRNPs, but specific interaction of IRS-1 with RNA complex suggests specific function of IRS-1 in RNA metabolism at the post-transcriptional level.

How does IRS-1 form complexes with RNAs? One possibility is that some proteins, directly interacting with both RNA and IRS-1, recruit RNAs around IRS-1. In this study we newly discovered that PABPC1, eIF4E and eIF4G were co-immunoprecipitated with IRS-1 (Fig. 3). But all these co-immunoprecipitation were disrupted by RNase treatment, indicating that these associations are not direct but RNA-mediated. Since none of the RNA binding proteins we tested showed RNase-resistant binding to IRS-1, we could not identify proteins that recruit RNAs around IRS-1 by directly binding to IRS-1. Comprehensive analysis of proteins in the IRS-1 ribonucleoprotein complexes is required to identify protein candidates for bridging RNA and IRS-1 in the complexes. Another possibility is that IRS-1 directly interacts with RNAs. Although IRS-1 bears no known conserved RNA binding motifs as far as we could determine, we cannot rule out the possibility that IRS-1 binds to RNAs in a direct manner through a novel RNA-binding motif. Understanding of the structural nature of the IRS-1 complexes will help identify mRNAs packaged and translationally regulated within them.

Recently, IRSs are shown to function as an integrator of signaling information rather than as a canonical adaptor protein [35]. We and others reported that IRSs are associated with various proteins to modulate the downstream signaling through IRS as well as control its intracellular localization [8–15,36]. This study indicating the identification of mRNA in complex with IRS-1 adds a novel function of IRS-1 as a scaffold protein to control mRNA metabolism. It is well known that tyrosine phosphorylation of IRSs by IGF-I receptor/insulin receptor kinase activated by IGFs/insulin playing important roles in controlling global translational processes through the downstream mTOR complex [6]. Taken together, specific mRNAs in high-molecular mass complexes with IRS-1 are possibly translated in response to IGFs/insulin, mediating their bioactivities in novel manners.

Our study demonstrated that IRS-1 interacts with several translation-related proteins and forms high-molecular mass complexes with mRNAs in a phosphotyrosine-independent manner. These interactions suggest that IRS-1 may play novel role(s) in controlling post-transcriptional RNA programs in addition to the canonical phosphotyrosine-dependent roles of IRSs in IR and IGFR signaling pathways. The components of IRS-1-associated mRNPs might be remodeled into polysomes for translational activation using IRS-1 as a scaffold. Further study is required to evaluate the role of IRS-1 complex formation with mRNP in translational activation.

Author contributions

Conceived and designed the experiments: A.O., M.S., T.F., N.K., K.C., A.T., F.H., S.-I.T. Performed the experiments: A.O., M.S., T.F. Analyzed the data: A.O., M.S., T.F., N.K., K.C., F.H., S.-I.T. Contributed reagents/materials/analysis tools: A.O., M.S., T.F., N.K., F.H., S.-I.T. Wrote the paper: A.O., N.K., F.H., S.-I.T. Primary responsibility for final content: S.-I.T. Read and approved the final manuscript: A.O., M.S., T.F., N.K., K.C., A.T., F.H., S.-I.T.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.05.066.

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