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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)

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