Insulin receptor substrates-5 and -6 are poor substrates for the insulin receptor

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Received September 30, 2009; Accepted November 13, 2009

DOI: 10.3892/mmr_00000239

Abstract. Insulin receptor substrates (IRS)-5 and -6 are two recently identified members of the IRS family. We investigated their roles as insulin receptor substrates and compared them with Src-homology-2-containing (Shc) protein, a well-established substrate. Bioluminescence resonance energy transfer (BRET) experiments showed no interaction between the receptor and IRS-5, while interaction with IRS-6 was not enhanced by insulin. By contrast, Shc showed an insulin-induced BRET response, as did a truncated form of IRS-1 (1-262). While Shc was heavily phosphorylated after stimulation of the insulin receptor, IRS-5 and -6 showed very weak phosphorylation levels. These results suggest that, although these two adaptors have previously been proposed as substrates for the insulin receptor, they are poor substrates for the insulin receptor. This calls into question their relevance to insulin signalling.

Introduction

Insulin is crucial for the regulation of metabolism, growth and development. Binding of insulin to the insulin receptor (IR) leads to the activation of receptor tyrosine kinase and receptor phosphorylation, which enables the binding of docking proteins such as insulin receptor substrates (IRS)-1, -2, -3 and -4 and Src-homology-2-containing protein (Shc). This in turn leads to their phosphorylation and, thereby, intracellular signalling (1).

Until recently, the IRS family included IRS-1, -2, -3 and -4 (2) and three downstream of kinases, Dok-1, -2 and -3. These seven proteins have similar amino-terminal pleckstrin homology (PH) and similar phosphotyrosine binding (PTB) and carboxyl-terminal phosphorylation domains which, when tyrosine-phosphorylated, dock Src-homology-2 (SH2) domain proteins. Despite their similar domain architecture, the Dok proteins can be distinguished from the IRS family based on sequence homology and functional interactions. Recently, two new members of the family were identified: IRS-5 and -6 (also called Dok-4 and -5, respectively). Based on PH and PTB domain sequence identity, IRS-5 and -6 have been shown to be more closely related to each other than to either the IRS or the Dok family (3,4). Dok-6, defined as a novel member of the Dok-4/5 'subclass' of the Dok family (5), and Dok-7 have also recently been identified (6).

The physiological processes regulated by the Dok family are poorly understood. IRS family members and other receptor tyrosine kinase-associated adaptor molecules are generally involved in signal amplification. Dok-1, -2 and -3 function primarily as inhibitors of tyrosine kinase signalling (7-9). Dok-4 and -5, however, have been shown to be positively involved in tyrosine kinase signalling (3,10-12), although Dok-4 was suggested to be an inhibitor of tyrosine kinase signalling in epithelial cells (13,14). It has been suggested that Dok-4 and -5 are involved in insulin and related insulin-like growth factor (IGF)-I signalling, and play a role as substrates for the insulin and the IGF-I receptor (3). However, contradictory results have been obtained in other studies (13).

Shc is a well-established and -characterized substrate of the IR and plays an important role, mainly in insulin-induced mitogenesis (15,16).

In order to examine the function of IRS-5 and -6 in insulin signalling, we compared the roles of these adapters as IR substrates with the role played by Shc. Contrary to studies

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Abbreviations: BRET, bioluminescence resonance energy transfer; Dok, downstream of kinase; GFP, green fluorescent protein; HEK, human embryonic kidney; IGF, insulin-like growth factor; IR, insulin receptor; IRS, IR substrate; mBU, milliBRET Unit; PH, pleckstrin homology; PTB, phosphotyrosine binding; Rluc, Renilla luciferase; Shc, Src-homology-2-containing protein; SH2, Src-homology-2; YFP, yellow fluorescent protein

Key words: insulin signalling, insulin receptor substrate protein, bioluminescence resonance energy transfer, phosphorylation

suggesting that these two proteins are substrates of the IR (3), our results indicate their weakness as IR substrates.

Materials and methods

Materials. The 4G10 anti-phosphotyrosine antibody was from Upstate Biotechnology (Lake Placid, NY, USA), and the anti-Green Fluorescent Protein (GFP) monoclonal antibody (clones 7.1 and 13.1) was from Roche Applied Science (Indianapolis, IN, USA). All other materials have been described previously (17,18).

Expression vectors. The IR-Renilla luciferase (Rluc) plasmid, coding for the entire IR sequence (19), has been described previously (20).

The yellow fluorescent protein (YFP)-Shc fusion construct was prepared by PCR on pcDNA3 p52Shc (coding for rat p52Shc) with forward primer 5'GGGAAGATCTAACAAGC TGAGTGGAGGCGGCGG3' and reverse primer 5'CCGGAA TTCCTCACACTTTTCGATCCACAGGT3'. After digestion by Bgl II and EcoR I (restriction sites present in the primers), the insert was cloned into appropriately digested pEYFP-C1 (Clontech, Mountain View, CA, USA).

The IRS-5-YFP and IRS-6-YFP fusion constructs were prepared by PCR on cDNAs (coding for human IRS-5 and -6) using the following primers: IRS-5 forward, 5'CGGAAT TCAAATGGCGACCAATTTCAGTGAC3'; IRS-5 reverse, 5'CCGGGATCCTTCTGGGATGGGGTCTTGGCCTC3'; IRS-6 forward, 5'CTTCGAATTCAAATGGCTTCCAAT TTTAATGACATAG3'; IRS-6 reverse, 5'TACCGTCGACTT GTGCTCAGATCTGTAGGCTGG3'. The PCR products were cloned into pCR2.1 using a TOPO-TA cloning kit (Invitrogen, Denmark). After digestion by EcoR I/BamH I (IRS-5) or EcoR I/Sal I (IRS-6) (restriction sites present in the primers), the insert was shuttled into appropriately digested pEYFP-N1 (Clontech).

The IRS-1(1-262)-YFP fusion construct codes for the first 262 amino acids (and thus for the PH and the PTB domain of the protein) of human IRS-1 coupled to YFP at the C-terminal. The construct was prepared as described by Jacobs *et al* (21), but with the use of pEYFP-N1 (Clontech) instead of pEGFP-N2/3.

The constructs were transfected into human embryonic kidney (HEK)-293 cells using FUGENE6 (Roche Diagnostics, Indianapolis, IN, USA) and visualised by fluorescence microscopy.

Bioluminescence resonance energy transfer (BRET) experiments. HEK-293 cells were transfected as described previously (18). To study the interaction between the IR and Shc, 300 ng IR-Rluc cDNA and 300 ng YFP-Shc cDNA or 300 ng empty vector per 35-mm dish were used. For BRET measurements between the IR and IRS-5 or -6, 300 ng IR-Rluc cDNA and 600 ng IRS-5-YFP cDNA, IRS-6-YFP cDNA or empty vector, or 25 ng YFP cDNA and 575 ng empty vector were used. For BRET measurements between the IR and IRS-1(1-262), 300 ng IR-Rluc cDNA and 150 ng IRS-1(1-262)-YFP cDNA or 150 ng empty vector was used. One day after transfection, cells were transferred into 96-well microplates, in which all BRET measurements were carried out on the following day. Results were expressed in milliBRET Units (mBU) as described previously (18).

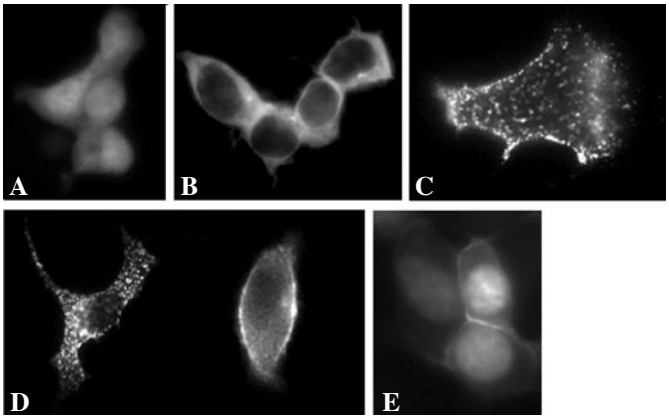

Figure 1. Localization of Shc, IRS-5, IRS-6 and IRS-1(1-262) YFP fusion constructs. HEK-293 cells were transfected with cDNAs encoding YFP alone or YFP-tagged Shc, IRS-5, IRS-6 or IRS-1(1-262). Localization of the proteins was observed using fluorescence microscopy. (A) Expression of YFP alone resulted in a fluorescent signal distributed uniformly throughout the cell. (B) Expression of YFP-Shc resulted in a cytoplasmic distribution of the fluorescence. (C) IRS-5-YFP exhibited a punctuate cytoplasmic fluorescence, while in (D) IRS-6-YFP a fluorescent signal was observed at the plasma membrane and the cytoplasm or in a punctuate cytoplasmic manner. (E) IRS-1(1-262)-YFP exhibited a fluorescent signal partially localized at the plasma membrane.

Phosphorylation measurements. HEK-293 cells were transfected as described previously (18). IR-Rluc cDNA (600 ng) and 300 ng of YFP-Shc, IRS-5-YFP or IRS-6-YFP cDNA or empty vector per 35-mm dish were used. Forty-eight hours after transfection, the cells were incubated with or without 100 nM insulin for 5 min in Dulbecco's modified Eagle's medium at 37˚C. Proteins were then extracted as previously described (17), subjected to Western blotting (22) and detected using chemiluminescence.

Results

Localization of Shc, IRS-5, IRS-6 and IRS-1(1-262)-YFP fusion constructs. In HEK-293 cells, YFP-p52Shc localized to the cytoplasmic regions (Fig. 1B). This is in agreement with the findings of Clark *et al* (23) and Lotti *et al* (24).

IRS-5-YFP showed a punctuate cytoplasmic localization (Fig. 1C). In fractionation studies, a large quantity of IRS-5-YFP fusion construct was found to be localized in the mitochondria of HEK-293 cells (results not shown). The punctuate cytoplasmic localization was also found by Bedirian *et al* (13) and Itoh *et al* (25). Itoh *et al* also identified these compartments by cell fractionation studies, and confirmed them to be mitochondrial.

In separate cells, but in the same experiment, IRS-6-YFP was either detected at the plasma membrane and in the cytoplasm or showed a punctuate cytoplasmic localization (Fig. 1D). In cell fractionation studies, a small quantity of IRS-6-YFP was found to be present in the mitochondria of HEK-293 cells (results not shown). The localization patterns found were also presented by Shi *et al* (11). Fu *et al* (26) also detected IRS-6-YFP at the plasma membrane.

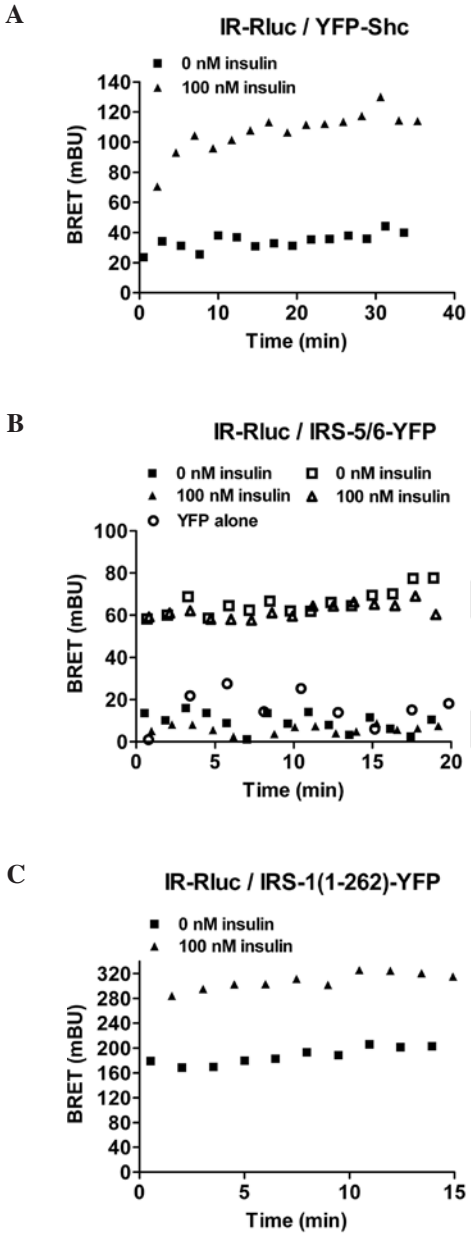

Figure 2. Real-time interaction of the insulin receptor with Shc, IRS-5, IRS-6 and IRS-1(1-262). HEK-293 cells were transfected with IR-Rluc + YFP-Shc, IR-Rluc + IRS-5-YFP, IR-Rluc + IRS-6-YFP, IR-Rluc + YFP or IR-Rluc + IRS-1(1-262)-YFP. The BRET signal was measured in real-time in living cells after stimulation with 0 (■,□) or 100 nM (▲,△) insulin. (A) Dynamics of the interaction between IR-Rluc and YFP-Shc before and after stimulation. Results are representative of three independent experiments. (B) Dynamics of the interaction between IR-Rluc and IRS-5-YFP or IRS-6-YFP before and after stimulation. In order to indicate the specificity of the interaction between IR and IRS-6, the BRET signal after transfection with IR-Rluc and YFP alone at a similar YFP/Rluc ratio is shown (○). ■ and ▲, IRS-5; □ and △, IRS-6. Results are representative of two independent experiments. (C) Dynamics of the interaction between IR-Rluc and IRS-1(1-262)-YFP before and after stimulation. Results are representative of two independent experiments.

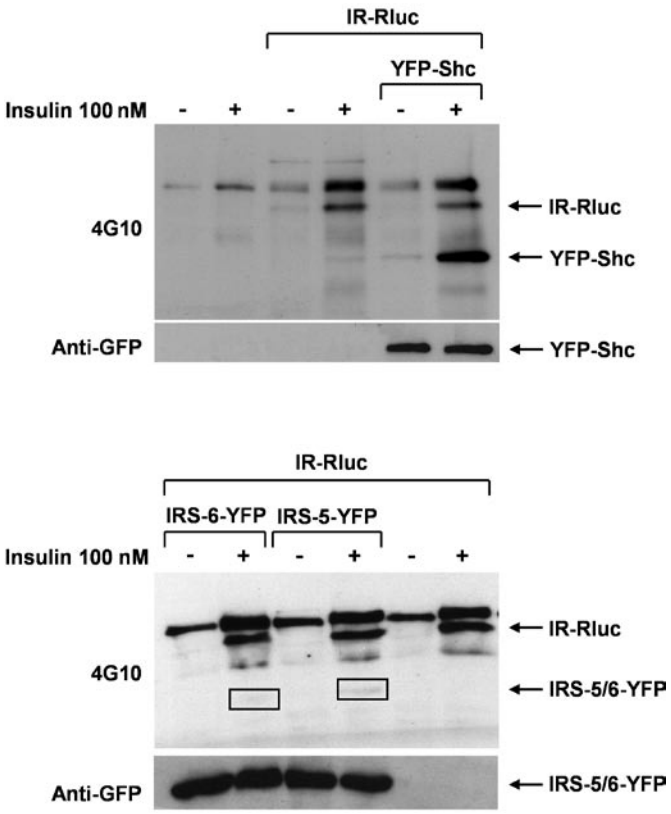

Figure 3. Phosphorylation of Shc, IRS-5 and IRS-6 in response to insulin. HEK-293 cells were transfected with empty vector, IR-Rluc, IR-Rluc + YFP-Shc, IR-Rluc + IRS-5-YFP or IR-Rluc + IRS-6-YFP. Forty-eight hours after transfection, the cells were incubated in the absence or presence of 100 nM insulin for 5 min. Proteins were extracted and tyrosine phosphorylation was determined by immunoblotting using an anti-phosphotyrosine antibody (4G10). Results are representative of two independent experiments.

IRS-1(1-262)-YFP localized partially to the plasma membrane (Fig. 1E). This localization pattern is in accordance with that presented by Jacobs *et al* (21) for IRS-1(1-262)-GFP.

These results show that the fusion constructs used in this study are correctly expressed according to the currently available knowledge.

Real-time interaction of the insulin receptor with Shc, IRS-5, IRS-6 and IRS-1(1-262). Real time interactions between the IR and Shc, IRS-5, IRS-6 and, as a positive control, IRS-1(1-262) were measured in living cells by BRET. HEK-293 cells were transfected with IR-Rluc and YFP-Shc, IRS-5-YFP, IRS-6-YFP or IRS-1(1-262)-YFP. In the BRET assay, Rluc is excited by the addition of its substrate, coelenterazine. If the potential interaction partners bring Rluc and YFP <100 Å apart, an energy transfer occurs between luciferase and YFP, and a signal emitted by YFP can be detected.

Shc interacted with the IR in an insulin-dependent manner (Fig. 2A). In cells transfected with IRS-5, virtually no BRET signal could be detected (Fig. 2B). With IRS-6, a substantial and specific basal BRET signal was detected, but insulin had no effect on this signal (Fig. 2B). As a positive control within the IRS family, the BRET signal observed between IR-Rluc and IRS-1(1-262)-YFP was enhanced by insulin stimulation (Fig. 2C).

Phosphorylation of Shc, IRS-5 and IRS-6 in response to insulin. We decided to evaluate whether the insulin-induced BRET response between the IR and Shc, IRS-5 and IRS-6 correlated with their phosphorylation levels after the stimulation of the cells with insulin. Insulin strongly induced the tyrosine phosphorylation of Shc (Fig. 3). Shc was substantially

phosphorylated in comparison to the phosphorylation levels of the IR. In contrast, insulin induced only a very poor phosphorylation of IRS-5 and -6 (Fig. 3). In the same experiment, the IR itself was heavily phosphorylated after stimulation.

Discussion

The physiological roles of IRS-5 (Dok-4) and -6 (Dok-5), two recently discovered members of the IRS/Dok family of adaptor proteins, are still very poorly understood. We compared the roles of these two adaptors as substrates of the IR with Shc, a well-established and -characterized insulin receptor substrate.

By BRET measurements, Shc was demonstrated to interact with the IR in an insulin-dependent manner, while IRS-5 did not show any BRET response. IRS-6 produced a solid and specific basal BRET response; however, this signal was not enhanced by stimulation with insulin. By contrast, a truncated form of the major insulin receptor substrate IRS-1 (amino acids 1-262) did, just like Shc, show a solid basal BRET signal that was enhanced by stimulation with insulin (a truncated form of IRS-1 was used since full length IRS-1-YFP shows an anomalous cell localization) (21). Just like IRS-1(1-262), IRS-5 and -6 were fused to YFP at their C-terminal, leaving the N-terminal PH domain and the PTB domain free for interaction with the cell membrane and the receptor. However, the lack of BRET response between IRS-5 and the IR does not completely rule out an interaction between these two proteins. Since the energy transfer (leading to a BRET signal) between Rluc and YFP depends not only on the distance but also on the relative orientation of the two partners, the lack of BRET response with IRS-5 could be due to an unfavourable orientation of the Rluc-YFP BRET pair for energy transfer to occur. The difference in basal BRET levels between IRS-5 and -6 might also be explained by their different cellular localizations. IRS-5 was shown in the present and previous studies (25) to be localized in the mitochondria. This could mean that the protein is not as readily available for a BRET interaction. IRS-6 partially localized to the cell membrane and the cytoplasm, possibly providing the opportunity for an easier interaction and a higher basal BRET.

The lack of an insulin-inducible BRET signal for IRS-6 could indicate that IRS-6 is constitutively bound to the IR and does not get recruited to the receptor after stimulation. Although it is unclear whether IRS-6 binds to the same locus on the IR as its two major substrates, IRS-1 and -2, our data suggest that it may act as a competitive inhibitor of IRS-1 and -2. This warrants further investigation, including whether the interaction of IRS-6 and the IR can be regulated by factors other than insulin.

In addition, we showed that, while Shc phosphorylation is heavily induced by insulin stimulation, both IRS-5 and -6 show very weak phosphorylation levels after IR activation in HEK-293 cells. To be categorized as genuine 'IRS', an adaptor must be tyrosine-phosphorylated by the IR in addition to having the appropriate PH-PTB domain architecture. Our data therefore suggest the weakness of IRS-5 and -6 as insulin receptor substrates. In contrast with our results, Cai *et al* found IRS-5 and -6 to be phosphorylated after stimulation of the insulin and the related IGF-I receptor in HEK-293 and Chinese hamster ovary cells (3). However, Bedirian *et al* showed that, in COS cells, overexpressed IGF-I receptor was able to phosphorylate itself, but unable to phosphorylate IRS-5, even after stimulation with IGF-I (13). This group was also unable to detect IRS-5 phosphorylation in epithelial cells or in IRS-5-overexpressing 293 cells after treatment with IGF-I.

Although these contradictory results are possibly due to differences in experimental conditions, it is clear that further studies are needed to elucidate which role these adaptors play as substrates for the insulin and IGF-I receptor and in insulin and IGF-I receptor signalling. Animal knockouts of these adaptors would provide valuable information and further clarify the physiological roles of IRS-5 and -6.

Acknowledgements

Soetkin Versteyhe was the recipient of an Industrial PhD scholarship from the Danish Ministry of Science, Technology and Innovation. Steven Gray was the recipient of a BIO+IT postdoctoral fellowship from the Oeresund IT Academy. This study was supported by the Association pour la Recherche sur le Cancer (Grant no. 3781) and by the Ligue contre le Cancer, Comité de Paris (Grant no. R04/75/75). The Hagedorn Research Institute and the Receptor Systems Biology Laboratory are independent basic research components of Novo Nordisk A/S.

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