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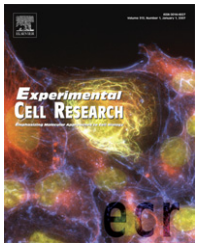

Research Article

Insulin receptor substrates 1 and 2 but not Shc can activate the insulin receptor independent of insulin and induce proliferation in CHO-IR cells

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ABSTRACT

Ligand-activated insulin receptor (IR) attracts and phosphorylates various substrates such as insulin receptor substrates 1–4 (IRS) and Shc. To investigate how binding affinity for substrate affects signalling we generated chimeric receptors with the β-chain of the insulin receptor containing NPXY motives with different affinities for receptor substrates. We found that the extent of receptor tyrosine phosphorylation positively correlates with binding affinity towards IRS1/2 but not towards Shc. Moreover, overexpression of IRS1 or IRS2 but not of Shc increased IR tyrosine phosphorylation in a dose-dependent manner, also independent of insulin. Molecular truncations of IRS1 revealed that neither the isolated PH and PTB domains nor the C-terminus with the tyrosine phosphorylation sites alone are sufficient for substrate-dependent receptor activation. Overexpression of IRS1 and IRS2 impaired insulin-induced internalization of the IR in a dose-dependent manner suggesting that IRS proteins prevent endosome-associated receptor dephosphorylation/inactivation. IRS1 and IRS2 could therefore target the activated IR to different cellular compartments. Overexpression of IRS1 and IRS2 inhibited insulin-stimulated activation of the MAP kinases Erk1/2 while it increased/induced activation of Akt/PKB. Finally, overexpression of IRS1 and IRS2 but not of Shc induced DNA synthesis in starved CHO-IR cells independent of exogenous growth factors. Our results demonstrate that variations in cellular IRS1 and IRS2 concentration affect insulin signalling both upstream and downstream and that IRS proteins could play instructive rather than just permissive roles in signal transmission.

Introduction

Insulin action (reviewed by [1]) depends on the activation of the insulin receptor (IR), a tetrameric transmembrane tyrosine kinase (RTK) consisting of two extracellular α- and two intracellular β-subunits with the RTK residing on the β-subunits. Binding of insulin to the α-subunits results in rapid autophosphorylation of several tyrosine residues in the

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intracellular part of the receptor [2,3]. The autophosphorylation sites are clustered in three regions. Two sites reside within the juxtamembrane region, three within the regulatory loop of the kinase and two within the COOH-terminal tail. The tyrosine clusters have different roles in the propagation of the insulin signal: First, phosphorylation of the regulatory loop is required for full activation of the RTK. The activated kinase then phosphorylates the tyrosine residue within the sequence NPEY located in the juxtamembrane region. This NPEY motif is a variation of the consensus binding site (NPXY) for phosphotyrosine binding (PTB) domain-containing proteins. Once phosphorylated it attracts receptor substrates which contain a PTB domain, like the insulin receptor substrate (IRS) proteins and Shc [4]. In addition to the recruitment of receptor substrates, phosphorylation of the NPEY motif in the juxtamembrane region of the IR is required for insulin-induced receptor endocytosis [5]. The role of the third tyrosine cluster in the COOH-terminal tail of the IR is not fully understood but it seems to be required for fine-tuning of the kinase activity [6], and eventually recruits signalling components with SH2 domains such as Grb10 [7].

Besides the IR, also the activated interleukin-4 (IL-4) receptor recruits IRS proteins via a NPXY (NPAY) motif. This raises the question of how these different receptors can transmit specific signals through the same receptor substrates. Previous work suggests that the NPAY motif found in the IL-4 receptor binds the PTB domain of IRS1 about 30 times stronger compared to the NPEY motif of the IR [8,9].

Two major pathways are activated downstream of insulin receptor substrates: Grb2 induces the MAP kinase cascade resulting in the activation of Erk1 and Erk2 (p42/p44) through phosphorylation of threonine 202 and tyrosine 204 [10,11]. The regulatory subunit (p85) of PI 3′ kinase (PI3K) stimulates the synthesis of PI3,4,5P3 and finally leads to activation of the serine–threonine kinase Akt/PKB. Akt activation requires phosphorylation of threonine 308 and serine 473 [12].

We hypothesized that the steady-state level of the receptor–substrate complex could influence signal propagation. In this study we show that modulating the affinity between receptor and substrates affects the phosphorylation state of both, the receptor and its substrate. In addition we present evidence that overexpression of IRS1 and IRS2, but not of Shc can mimic insulin action in CHO-IR cells which might in part depend on the inhibition of receptor-endocytosis.

Experimental procedures

Reagents and antibodies

Chemicals were purchased from SIGMA (Buchs, Switzerland) unless otherwise mentioned. Human recombinant M-CSF was obtained from Calbiochem (JURO, Lucerne, Switzerland), bovine insulin from SIGMA and [^{125}I]-insulin from Amersham Pharmacia (Dübendorf, Switzerland). Antibodies against phosphotyrosine (PY99), the insulin receptor β-subunit, IRS1, IRS2, Shc and Rabbit anti-Grb2 (C-23) were purchased from Santa Cruz Biotechnology Inc. Antibodies against phospho-Akt, phospho-Erk1/2 and p85 were obtained from Cell Signaling (Beverly, USA).

Cell culture and treatments

Cell culture media and other reagents were purchased from Gibco BRL. HEK 293 cells and CHO-K1 cells were a gift of the Department for Calcium Metabolism, Zurich. They were grown in DMEM supplemented with 10% FBS (HEK 293 cells) or HAM F12 supplemented with 10% FBS (CHO K1 cells). CHO-IR cells were a gift of Jeffery E. Pessin (Iowa City, USA) and were grown in HAM F12 supplemented with 10% FBS. All cells were grown at 37°C and 5% CO_2, and in the presence of 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL).

Cells were transfected with plasmid DNA in 6- or 24-well plates after they reached 80–90% confluence using Lipofectamine 2000 (Gibco BRL). CHO-K1 cells were harvested 24 h and HEK 293 cells 48 h post transfection for further analysis.

Plasmids and adenoviral vectors

We generated the CSF/IR chimeric receptor as described by Chaika et al. [13]. After subcloning into the expression vector pCMV, transfer mutations were introduced into the isolated *Bsu*36 I–*Xho* I fragment of the CSF/IR chimeric receptor by polymerase chain reactions. The cDNA of the human insulin receptor was subcloned into pcDNA3.1 (Invitrogen, Groningen, The Netherlands). The expression constructs encoding human Shc, human IRS1, and mouse IRS2 have been described previously [14].

Co-immunoprecipitation, Ni-NTA purification and Western blotting

HEK 293 cells were transfected in 6-well plates with a plasmid mixture containing 1.25 μg of expression vector coding for myc-tagged IRS1, IRS2 or Shc, respectively and additionally 1.25 μg of plasmid either encoding a respective mutant chimeric receptor or GFP. 44 h post transfection, cells were serum-deprived for 4 h in DMEM containing 0.5% BSA and subsequently stimulated for 10 min with 10 nM M-CSF. Thereafter, cells were lysed in *lysis buffer* (20 mM Tris, pH 7.5, 100 mM NaF, 1% NP-40, 5 mM EDTA) freshly supplemented with 1 mM PMSF, 3 mg/ml aprotinin, 3 mg/ml leupeptin, 1 mM $Na_2H_2P_2O_7$, and 1 mM Na_3O_4V. Precleared lysates were incubated with 0.5 μg of α-insulin receptor β-chain antibody for 1 h at 4°C under constant shaking. 20 μl of immobilized protein A on trisacryl (Pierce, Rockford, USA), equilibrated in *lysis buffer* were added to the cell lysates and equilibrated over night at 4°C on a rotating wheel. Thereafter beads were collected by a short centrifugation and washed 3 times with cold PBS. Finally, 20 μl of 1.5× SDS *gel loading buffer* (75 mM Tris/HCl, pH 6.8, 3% SDS, 15% glycerol, 0.15% bromophenol blue, 7.5% 2-mercaptoethanol) were added, samples were boiled for 3 min and beads were removed by short centrifugation. Supernatants were separated on SDS–PAGE.

For pull-down experiments with Ni-NTA CHO-IR cells were grown on 10 cm plates and infected with adenoviral vectors encoding myc-HIS-tagged IRS1, IRS2, Shc or GFP as a control. After 48 h, cells were washed with PBS and starved in appropriate medium as described for HEK293 cells. If required insulin was added for 4 min followed by 3 washes with ice-