Insulin Receptor Substrate-4 Enhances Insulin-like Growth Factor-I-induced Cell Proliferation*

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The insulin receptor substrates (IRSs)-1–4 play important roles in signal transduction emanating from the insulin and insulin-like growth factor (IGF)-I receptors. IRS-4 is the most recently characterized member, which has been found primarily in human cells and tissues. It interacts with SH2-containing proteins such as phosphatidylinositol 3-kinase (PI3K), Grb2, Crk-II, and CrKL. In this study, we transfected IRS-4 in mouse NIH-3T3 cells that overexpress IGF-I receptors. Clones expressing IRS-4 showed enhanced cellular proliferation when cells were cultured in 1% fetal bovine serum without added IGF-I. Addition of IGF-I enhanced cellular proliferation in cells overexpressing the IGF-I receptor alone but had an even greater proliferative effect in cells overexpressing both the IGF-I receptors and IRS-4. When etoposide and methylmethane sulfonate (MMS), both DNA damaging agents, were added to the cells, they uniformly induced cell cycle arrest. Fluorescence-activated cell sorter analysis demonstrated that the arrest of the cell cycle occurred at the G1 checkpoint, and furthermore no significant degree of apoptosis was demonstrated with the use of either agent. In cells, overexpressing IGF-I receptors alone, IGF-I addition enhanced cellular proliferation, even in the presence of etoposide and MMS. In cells overexpressing IGF-I receptors and IRS-4, the effect of IGF-I in overcoming the cell cycle arrest was even more pronounced. These results suggest that IRS-4 is implicated in the IGF-I receptor mitogenic signaling pathway.

Insulin and insulin-like growth factor-I (IGF-I)1 receptors stimulate a number of common intracellular events. Following ligand binding to their respective receptors, each hormone induces autophosphorylation of the receptor followed by activation of the tyrosine kinase activity inherent to these receptors. Receptor tyrosine kinase then phosphorylates a number of proteins including the insulin receptor substrate (IRS) family of proteins (1–4), Shc (5), pp120 (6), Grb2-associated binder-1 (7). Phosphorylated tyrosine residues in these molecules bind to the SH2 domains in other molecules, resulting in the activation of downstream signaling cascades. There are four members in the IRS family of proteins (IRS 1–4). IRS-1 contains about 20 tyrosine residues that form binding sites for molecules such as phosphatidylinositol 3-kinase (PI3K), Grb2, Nck and SH-PTP2, when phosphorylated (1). IRS-2, -3, and -4 have similar binding sites. This apparent redundancy in the IRS family of proteins is off-set by specificity in tissue and cellular distribution as well as unique features in each molecule that affect the interaction with upstream and downstream proteins. For example, there are differences in the domain that interacts with the insulin receptor in IRS-1 and IRS-2 (1, 2), whereas IRS-3 is much smaller than the other members, with fewer phosphorylation sites and therefore fewer SH2-interacting consensuses sites in its C-terminal region (8).

Deletion of the IRS-1 gene using homologous recombination technology in mice leads primarily to growth retardation (9), whereas deletion of the IRS-2 gene leads to a diabetic state (10). In contrast, deletion of both IRS-3 and IRS-4 genes had no discernible phenotype (11). The lack of phenotype in the IRS-3 gene-deleted mice may be due to redundancy by the expression of IRS-1 and IRS-2. In the case of IRS-4, the absence of a phenotype is not too surprising since its expression in mice is so low (12). In vitro expression of the IRS family of proteins in adipocytes, however, demonstrates that all IRS molecules can mediate insulin-induced glucose transport protein-4 translocation (13–15), an important step in insulin-induced glucose uptake.

Interestingly, the most recently characterized member, IRS-4, is expressed in human cells and tissues, whereas its expression in mouse tissues and cells is below the level of detection (4). IRS-4 does interact with SH2-containing proteins such as PI3K, Grb2 (16), Crk-II, and CrKL (17), but the functional characteristics of IRS-4 have not yet been ascertained.

To further characterize the role of IRS-4 in IGF-I receptor signaling and biological responses, we expressed IRS-4 in mouse NIH-3T3 cells that had previously been stably transfected with and overexpressed IGF-I receptors (18). Using this system, we studied the effect of the expression of IRS-4 on IGF-I-induced cellular proliferation and the ability of IGF-I to overcome cell cycle arrest.

EXPERIMENTAL PROCEDURES

Construction of IRS-4 Expression Vector—hIRS-4 cDNA was cloned by reverse transcriptase polymerase chain reaction using total RNA from HEK 293 cells as a template. Strategy and primers are described elsewhere.2 Full-length IRS-4 cDNA was sub-cloned into the pCEFL hemagglutinin eukaryotic expression vector kindly provided by S. Gutkind (National Institutes of Health, Bethesda, MD). The construct was sequenced in full to ensure in-frame ligation. In addition, the sub-cloned IRS-4 cDNA was completely identical to the previously published IRS-4 sequence (4).

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Establishment of Stable Cell Lines Expressing IRS4—The IRS-4 expression vector pCDNA3.1-IRS4 that contains the entire IRS-4 cDNA sequence was transfected into the NWTB3 cell line (18) using LipofectAMINE Plus transfection reagent (Life Technologies). The NWTB3 cell line established in this laboratory overexpresses the IGF-I receptor (~ 4 x 10^5 receptors per cell). The IRS-4 expression vector was cotransfected with the pIREShyg vector (CLONTECH) for colony selection. The ratio of pCDNA3.1-IRS4 to pIREShyg was 20:1, and the total DNA used per transfection was 6.3 μg. The pIREShyg vector alone was transfected into NWTB3 cells to generate the control cell lines. After 48 h of transfection, cells were digested with 0.05% Trypsin, 0.02% EDTA solution and transferred to 150-mm tissue culture plates containing Dulbecco's modified Eagle's medium (DMEM) plus 10% FBS, 0.5% g/liter G418 and 0.2 μl/g hemoglycin B. After selection for 2–3 weeks, the colonies were picked and cultured for several weeks.

The stable cell lines were screened by Western blot using a polyclonal anti-IRS-4 antibody (C-16) developed against 125I/DARRGNGQFDSP-KRG38 of the IRS-4 molecule. Cells were grown on 100-mm plates in the medium described above. Confluent cells were lysed in 0.6 ml of lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 10 mM NaF, 2 mM Na3VO4, 10 μg/ml aprotinin, 5 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). The cell lysate was incubated on ice for 30 min, and sonicated for three times for 5 s each, then centrifuged at 16,000 × g, 4°C for 10 min. The supernatant was separated, and the total protein concentration was measured using the BCA protein assay kit (Pierce). IRS-4 and IGF-I receptor levels were detected in 20 μg of cell lysate by Western blot using an antibody against the IGF-I receptor β-subunit (IGF-IRB 1-20, Santa Cruz Biotechnology) and an IRS-4 antibody.

Cell Proliferation—The cell proliferation assay was performed using the CyQUANT Cell Proliferation kit (Molecular Probes, Eugene, OR) and HTS 7000 Bio Assay reader (Perkin Elmer, Norwalk, CT). The NBS4 (cells overexpressing IGF-I receptors and IRS-4) and control cell lines (overexpressing only IGF-I receptors) were cultured in 75-cm² flasks. When flasks became confluent, cells were digested and counted using a Hemacytometer. Cells were seeded on 96-well Costar Cluster plates (Corning Inc., Corning, NY) in 200 μl of DMEM medium with 10% FBS, 48 h of transfection, cells were digested with 0.05% Trypsin, 0.02% EDTA solution and transferred to 150-mm tissue culture plates containing Dulbecco's modified Eagle's medium (DMEM) plus 10% FBS, 0.5% g/liter G418 and 0.2 μl/g hemoglycin B. After selection for 2–3 weeks, the colonies were picked and cultured for several weeks.

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RESULTS

Overexpression of IRS-4 in NBS4 Cell Line—The IRS-4 expression vector, pCDNA3.1-IRS4, containing the full-length of IRS-4 cDNA was transfected into the NWTB3 cell line (18) overexpressing human IGF-I receptors. After G418 and hygromycin B antibiotic selection, stable cell lines were established and designated as NBS4 (IGFIR plus IRS-4). To check protein expression level, lysates were prepared from NBS4 and control cells (IGFIR only) and were separated by SDS-polyacrylamide gel electrophoresis and immunoblotted with either anti-IGF-I receptor β-subunit antibody or anti-IRS-4 antibody. Fig. 1 shows the overexpression of both IRS-4 and IGF-I receptor in NBS4 cell lines. The IGF-I receptor typically appears as two bands, 90 and 200 kDa, which represent the β-subunit and its precursor, respectively (Fig. 1, panel A). IRS-4 runs as a 160-kDa band (Fig. 1, panel B). The control cells studied overexpress the IGF-I receptor but express very little endogenous IRS-4.

IRS-4 Enhances IGF-I-stimulated Cell Growth—To examine the effects of IRS-4 on cell growth, cell proliferation assays were carried out in the stable cell lines. Cells were cultured in the absence or presence of rhIGF-I. Two NBS-4 cell lines were tested (clones 1 and 2). Only one example of the control cells studied is shown. The results are shown in Fig. 2 (panel A).

Fic. 1. Overexpression of IRS-4 and IGF-I receptors in NBS4 cell lines. Shown is a Western blot analysis of the whole cell lysates of the stable cell lines. 20 μg of total protein was loaded in each lane of 8% polyacrylamide gels. Panel A, immunoblotting using a polyclonal anti-human IGF-I receptor β-subunit antibody (C-20). Panel B, immunoblotting using a polyclonal anti-human IRS-4 antibody (C-16). Lane C, a single band of constitutive control cell line. Lanes 1 and 2, two clones of the NBS4 cell lines (labeled 1 and 2). The arrows indicate the molecular mass markers showed in kDa.

Under basal conditions, i.e. using 1% FBS, both NBS-4 cell lines grew faster than the control cell line. For example, by 120 h of incubation, the number of control cells (C) increased 2.6 times, from a basal of 2,700 to 7,000. Cell number for NBS-4 clone 1 increased 3.6-fold, from a basal of 3,800 to 13,800, and clone 2 increased 7.1-fold, from a basal value of 3,600 to 25,500. Following 50 μg IGF-I stimulation, NBS-4 cells grew even faster than control cells. At 120 h of incubation, the number of control cells increased 5.7-fold, from a basal of 2,700 to 15,500. The NBS-4 clone 1 grew 10.9-fold, from a basal of 3,800 to 41,600, and clone 2 increased 13.7-fold, from a basal of 3,600 to 49,300. When the results (-fold increase of 120 h over basal level) of three independent experiments were combined and expressed as mean ± S.D. (panel B), IRS-4-expressing cells enhanced the increase in cell number significantly when compared with control cells both in the absence and presence of IGF-I (p < 0.05).

IRS-4 Inhibits MMS- and ES-induced Cell Cycle Arrest—Both MMS and ES are chemical reagents that induce cell cycle arrest and apoptosis. Cell growth inhibitory effects of 0.3 mM MMS and 0.25 μM etoposide were not accompanied by apoptotic cell death as was evaluated by the absence of DNA fragmentation in treated cells (Fig. 3) but caused cell cycle arrest in G1 (data not shown). In contrast, strong apoptosis was demonstrated when both control and IRS-4-overexpressing cells were treated with high doses of 1 mM MMS and 10 μM etoposide. Cell proliferation assays were carried out in the presence of MMS (0.3 mM) and etoposide (0.25 μM) to examine the effect of IRS-4. Fig. 4 shows that cells grew more slowly in the presence of MMS and ES than under normal conditions (compare results to Fig. 2). Again, both NBS-4 cell lines grew faster than the control cell line. At 120 h of incubation with MMS (Fig. 4, left panel, A) in the absence of IGF-I stimulation, the control cells grew 1.8-fold from 2,800 to 5,000. The NBS-4 clone 1 cells grew 2.6-fold, from 3,900 to 10,300, and clone 2 increased 3.7-fold,
The effects of IRS-4 on IGF-I-stimulated cell growth.

The cell proliferation assay was carried out using the CyQUANT Cell Proliferation kit. The NBS4 and control cells were seeded on 96-well Costar Cluster plates in 200 μl of DMEM medium with 10% FBS. After 16 h of incubation, medium was changed to DMEM in the absence or presence of 50 nM human IGF-I (rhIGF-I). The cells were incubated for the indicated time and then lysed with the lysis buffer. The fluorescence intensity of each sample was measured using an HTS 7000 microplate reader with excitation at 485 nm and emission at 535 nm. Panel A shows a representative experiment. ○, control cells, with added IGF-I; ●, control cells, without added IGF-I; ▽, clone 1 with added IGF-I; ▽, clone 1 without added IGF-I; □, clone 2 with added IGF-I; ▼, clone 2 without added IGF-I. Panel B, the fold increase (above basal) in cell number at 120 h from three experiments were combined and are represented as mean ± S.D. The black bars represent cell numbers in the absence of IGF-I, and gray bars represent the results in the presence of IGF-I.

from 3,600 to 13,400. Following 50 nM IGF-I stimulation, the control cells grew 2.8-fold from 2,800 to 7,700. The NBS-4 clone 1 cells grew 6.7-fold, from 3,900 to 26,000, and clone 2 cells grew 6.7-fold, from 3,600 to 24,100. The results for ES (right panel, A) are similar to MMS. When the results (-fold increase of 120 h over basal level) of three independent experiments were combined and expressed as mean ± S.D. (panel B), IRS-4-expressing cells enhanced the increase in cell number significantly when compared with control cells both in the absence and presence of IGF-I (p < 0.05). These results indicate that IRS-4 enhances the ability of the cell to overcome cell cycle arrest induced by MMS and ES, in the presence or absence of IGF-I.

Both control and IRS-4-overexpressing cells demonstrated low level of basal proliferation in the presence of MMS, as indicated by the low percentage of cells in S phase. IGF-I treatment was able to rescue this cell growth arrest in clone 1 as shown by an increase in S phase from 4 to 11% (Fig. 5). Similar results were obtained for ES treatment (data not shown). These findings further support the results from the cell proliferation experiments with MMS and ES.

DISCUSSION

IRS-1 was the first endogenous substrate of the insulin receptor to be identified and characterized. Subsequently numerous substrates of the insulin and IGF-I receptor have been identified, and the family of IRS molecules have enlarged. These protein substrates play key roles in signal transduction from the insulin and IGF-I receptors. In addition, IRS-1 and IRS-2 have been shown, at least in cell culture systems, to also play a role in signal transduction from many cytokine receptors including receptors for growth hormone and some interleukins (2, 19). IRS-1 and IRS-2 interact with the Npx(p)y motif in the juxtamembrane domain of the insulin and IGF-I receptors and potentially with the tyrosine kinase region (20). All four IRS molecules have conserved pleckstrin homology (PH) and phosphotyrosine binding (PTB) domains at their amino-terminal ends (1–4). The PH domain is apparently necessary for efficient tyrosine phosphorylation by the receptors even though this region of the IRS molecules do not appear to interact directly with the receptors. The PTB domains of IRS-1, IRS-2, and IRS-3, on the other hand, interact with the NPx(p)y motif of both receptors. IRS-4 has conserved PH and PTB domains similar to those present in the other IRS molecules although their functions have not yet been evaluated. C-terminal to the PH and PTB domains are the SAIN domains (21), one in IRS-1 and two in IRS-2. The SAIN domains are non-PTB domains, which are also important for the interaction with the receptors. There is little conservation between the SAIN domains of the various IRS molecules.

The C-terminal region of the IRS molecules contain multiple potential tyrosine phosphorylation motifs. The tyrosine resi- dues are in motifs that are expected to bind to proteins containing SH2 domains including PI3K, Grb2, and the tyrosine phosphatase SH-PTP2 and phospholipase Cγ. While the C-terminal regions of the IRS molecules are poorly conserved, many of the tyrosine phosphorylation motifs in the C-terminal region are conserved. Indeed there are 20 in both IRS-1 and IRS-2, and about 12 in IRS-4. Many of these potential tyrosine phosphorylation sites are in XXXM motifs that bind to the SH2 domains of PI3-kinase. Other sites in IRS-1, IRS-2, and IRS-4 are expected to bind Grb2, SH-PTP2, and phospholipase Cγ. Thus studies have shown that IRS-1 binds PI3K, Grb2, and SH-PTP2, as well as Nck and Fyn, whereas IRS-2 associates with PI3K and Grb2. IRS-3 is a much smaller molecule than the others and binds PI3K, SH-PTP2 and only slightly with Grb2 (8).

Current research continues to identify the roles of individual IRS molecules in the signaling cascades of these receptors. The biological responses mediated by IRS-1 and IRS-2 have been extensively studied (22–25). Experiments in cultured cells suggested that IRS-1 regulates gene expression and stimulates mitogenesis, and appears to mediate insulin-stimulated glucose transport (13). However, IRS-1 gene deletion in mice resulted in growth retardation with only mild resistance to insulin (9). Interestingly, IRS-2 has similar in vitro effects as for IRS-1, but IRS-2 gene-deleted mice showed insulin resistance and a diabetic phenotype (10). These results were interpreted as suggesting that in vivo IRS-1 may be responsible for IGF-I receptor signaling and IRS-2 responsible for insulin receptor signaling. Gene deletion experiments for IRS-3 and IRS-4 failed to result in a phenotype (11), suggesting that there may be redundancy in the system. Furthermore, the presence of multiple IRS molecules raises the question of whether there are distinct functions for IRS-3 and IRS-4 as has been shown for IRS-1 and IRS-2. One group of investigators has shown that in rat adipose cells in culture, all four IRS molecules can substitute and mediate insulin-responsive glucose transport protein 4 translocation to the cell surface (13–15), an important step in insulin-induced glucose uptake. While the IRS molecules are highly conserved both phylogenetically as well as between themselves, there are a number of differences in structure, tissue and cellular distribution, developmental expression, and subcellular localization. Thus they may have distinct functions.

IRS-4 is unique in that it was discovered in human cells and has not been found to any significant level in rodent cells or
tissues. It was first identified as a 160-kDa protein which underwent tyrosine phosphorylation following insulin and IGF-I stimulation of HEK 293 cells (4) but was immunologically distinct from IRS-1 which also migrates at 160 kDa (26). IRS-4 differs from IRS-2 (190 kDa) (27) and IRS-3 (60 kDa) (3) that migrate differently. Characterization of the gene, revealed a molecule with PH, PTB domains, and a C-terminal region with many potential tyrosine phosphorylation sites in motifs that could bind proteins with SH2 domains. IRS-4 binds PI3K, Grb2, CrkII, and CrkL which are all SH2-containing proteins.

FIG. 3. MMS and etoposide treatment cause cell cycle arrest at low concentrations and cell death at very high concentrations. Cells were plated in 100-mm dishes in DMEM supplemented with 10% FBS. After 16 h of incubation, medium was changed to DMEM with 1% FBS, and cells were treated as indicated. After 3 days of treatment, cells were collected, fixed, and analyzed by flow cytometry as described under "Materials and Methods."

FIG. 4. The effects of IRS-4 on MMS- and ES-induced cell cycle arrest. The experiments were performed as described in Fig. 2 except the addition of 0.3 mM MMS (left panel) and 0.25 μM of ES (right panel) to the medium, respectively, in the absence or presence of 50 nM IGF-I. ○, control cells, with added IGF-I; ◇, control cells, without added IGF-I; ◊, clone 1 with added IGF-I; ▽, clone 1 without added IGF-I; □, clone 2 with added IGF-I; ▲, clone 2 without added IGF-I.

| ES 0.25 μM | MMS 0.3 mM | ES 10 μM | MMS 1 mM |
|------------|------------|----------|----------|
| ![Graph](image1) | ![Graph](image2) | ![Graph](image3) | ![Graph](image4) |

C

| ES 0.25 μM | MMS 0.3 mM | ES 10 μM | MMS 1 mM |
|------------|------------|----------|----------|
| ![Graph](image5) | ![Graph](image6) | ![Graph](image7) | ![Graph](image8) |

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however, its role in IGF-I receptor signaling has not been studied. Interestingly, transfection of IRS-1 or IRS-2 into 32D cells expressing insulin receptors enhanced the insulin-mediated cellular responses, while IRS-4 failed to do so (28). In the present study, we wanted to determine the role of IRS-4 in IGF-I receptor signaling. To this end, we utilized mouse NIH-3T3 cells which had been stably transfected with a cDNA expressing IGF-I receptors and were responsive to IGF-I-induced cellular proliferation (18). IRS-4 expression in mouse cells and tissues is extremely low. Thus, these cells serve as a useful system for expressing IRS-4. Clones overexpressing IRS-4 showed enhanced cellular proliferation both when cells were cultured in 1% FBS in the absence of added IGF-I and in response to added IGF-I.

Etoposide and MMS are capable of arresting the cell cycle and inducing apoptosis (29, 30). When added to the cells used in this study, they uniformly induced cell cycle arrest. FACS analysis demonstrated that the arrest of the cell cycle occurred at G1 phase of the cell cycle (data not shown), and furthermore, no significant degree of apoptosis was demonstrated with the use of either agent. In control cells, overexpressing IGF-I receptors, IGF-I addition enhanced cellular proliferation, i.e. overcame the cell cycle arrest induced by etoposide and MMS. In cells overexpressing IGF-I receptors and IRS-4, the effect of IGF-I in overcoming the cell cycle arrest was even more pronounced.

We conclude from these results that IRS-4 is implicated in IGF-I receptor signaling pathways involved in mitogenesis. Future studies will be carried out to determine which pathways downstream of IRS-4 are involved. Since the p85 subunit of PI3K, Grb2, and the Crk family of adapter proteins bind IRS-4 in intact cells, the PI3K pathway, MAP kinase pathway, and pathways emanating from Crk-binding proteins may be involved.

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Fig. 5. Cell cycle arrest by 0.3 mM of MMS and rescue by IGF-I. Cell were plated in 60-mm dishes in DMEM supplemented with 10% FBS. After 16 h of incubation, medium was changed to DMEM with 1% FBS, and cells were treated with 0.3 mM MMS in the presence or absence of 50 nM IGF-I. After 3 days of treatment, cells were collected, fixed, and analyzed by flow cytometry as described under "Materials and Methods." C, control cells; I, clone 1.
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