The Carboxyl Terminal Extension of the Drosophila Insulin Receptor Homologue Binds IRS-1 and Influences Cell Survival*

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The Drosophila insulin receptor (INR) homolog includes an extension of approximately 400 amino acids at the carboxyl-terminal end of its β subunit containing several tyrosine-based motifs known to mediate interactions with signaling proteins. In order to explore the role of this extension in INR function, mammalian expression vectors encoding either the complete INR β subunit (β-Myc) or the INR β subunit without the carboxyl-terminal extension (βΔ) were constructed, and the membrane-bound β subunits were expressed in 293 and Madin-Darby canine kidney cells in the absence of the ligand-binding α subunits. β-Myc and βΔ proteins were constitutively active tyrosine kinases of 180 and 102 kDa, respectively. INR β-Myc co-immunoprecipitated a phosphoprotein of 170 kDa identified as insulin receptor substrate-1 (IRS-1), whereas INR βΔ did not, suggesting that the site of interaction was within the carboxyl-terminal extension. IRS-1 was phosphorylated on tyrosine to a much greater extent in cells expressing INR β-Myc than in parental or INR βΔ cells. Despite this, a variety of PTB or SH2 domain-containing signaling proteins, including IRS-2, mSos-1, Shc, p55 subunit of phosphatidylinositol 3-kinase, SHP-2, Raf-1, and JAK2, were not associated with the INR β-Myc-IRS-1 complex. Overexpression of INR β-Myc and βΔ kinases conferred an equivalent increase in cell proliferation in both 293 and Madin-Darby canine kidney cells, indicating that this growth response is independent of the carboxyl-terminal extension. However, INR β-Myc-expressing cells exhibited enhanced survival relative to parental and βΔ cells, suggesting that the carboxyl-terminal extension, through its interaction with IRS-1, plays a role in the regulation of cell death.

Like the mammalian insulin receptor, the Drosophila insulin receptor (INR) is a tetramer formed by two α subunits and two β subunits. INR α and β subunits are synthesized together as a proreceptor precursor, proteolytically processed, and linked together by disulfide bonds (1, 2). The α subunits, with a molecular mass of 110–120 kDa (1), are extracellular and contain the ligand binding domains that are capable of binding mammalian insulin with a Kd of 15 nM (3). The β subunits traverse the plasma membrane and have an insulin-stimulated tyrosine kinase in the cytoplasmic portion (1, 2), DNA sequence analysis (2) and expression of the INR β subunit in mammalian (4) and Drosophila cells (5) indicate that the INR β subunit is larger than its mammalian homolog and exhibits an apparent molecular mass of ~180 kDa. The increased mass is due to the presence of a 400-amino acid carboxyl-terminal extension (2). However, the majority of INR β subunits are processed to 92/102-kDa forms in Drosophila embryos and some cell lines, the difference being due to proteolytic cleavage of the carboxyl-terminal extension (5, 6). Both truncated and full-length β subunits are autophosphorylated on tyrosine residues in response to insulin binding (1, 6).

The 400-amino acid carboxyl-terminal extension of the β INR contains clusters of motifs known to be involved in the interaction with SH2 and PTB domain-containing proteins (2), suggesting a role for this domain in signaling through interaction with other signaling molecules. Interestingly, four tyrosines are found in “hybrid” amino acid motifs in which residues amino-terminal to each tyrosine form the motif NPXY, resembling known PTB domain binding sites, and residues carboxyl-terminal to the same tyrosines form the motifs YXXM, YMXM, or YXXLD, known to be involved in binding to SH2 domains (7). Thus, tyrosines 1993 and 2030 appear in the motif SYN-PYXXYM, tyrosine 2009 is part of SXPYXMMX, and tyrosine 1969 appears in the sequence SDNPYRYLD (2). Whether these motifs serve to bind SH2 or PTB domain-containing proteins upon tyrosine phosphorylation and whether one is preferred over the other is not clear. The cytoplasmic domain of the INR expressed in cells lacking IRS-1 has been shown to bind PI3-kinase (8). However, a similar construct expressed in Chinese hamster ovary cells that contain IRS-1 failed to do so (4). Since a significant percentage of the INR β subunit undergoes tissue- or stage-specific proteolytic processing in Drosophila embryos to remove the carboxyl-terminal extension (6) and once it is removed it appears not to be phosphorylated (5), its role in signal transduction by the INR is not clear. Therefore, the signaling capacity conferred by the β INR carboxyl-terminal extension was explored in the following studies by expressing either full-length or truncated INR β subunit forms in mammalian cells and determining the effect on protein-protein interactions and cell growth.

EXPERIMENTAL PROCEDURES

Cell Culture

Human embryonic kidney cells (293 cells) (9) were obtained from ATCC and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, at 37 °C and 5% CO2. Madin-Darby canine kidney (MDCK) cells (10) were obtained from Dr. George Ojakian (State University of New York Health Science Center at Brooklyn) grown in DMEM supplemented with 5% fetal bovine serum.
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serum, at 37 °C and 5% CO2. Culture conditions for the stably transfected cell lines 293 β-Myc, 293 β-MyC, MDCK β-Myc, and MDCK ßA were identical to the parental cell lines except that the transfected cells were kept under selection with 100 μg/ml geneticin (Life Technologies, Inc.).

Antibodies

Synthesis and characterization of Ab dp1040 against the peptide corresponding to amino acids 1702–1720 in the β subunit of the INR (numbering according to Ref. 2) have been described previously (6). Synthesis and characterization of antibodies to peptide P5 (AbP5), from the carboxyl terminus of the human insulin receptor (amino acids 1328–1343, numbering according to Ref. 11), have been described previously (12). Monoclonal Ab 9E10 directed against the c-Myc epitope tag (numbering according to Ref. 2) have been described previously (6). Synthesis and characterization of antibodies to peptide P5 (AbP5), from the carboxyl terminus of the human insulin receptor (amino acids 1328–1343, numbering according to Ref. 11), have been described previously (12). Monoclonal Ab 9E10 directed against the c-Myc epitope tag (numbering according to Ref. 2) have been described previously (6).

Receptor Autophosphorylation and Immunoprecipitation

For in vitro autophosphorylation, 293 or MDCK cells and transiently or stably transfected 293 or MDCK β-Myc and 293 β-A cells were washed three times with phosphate-buffered saline and lysed with 0.3 ml of lysis buffer (20 mM HEPES, pH 7.6; 150 mM NaCl; 1 mM EDTA; 2.5 mM MgCl2; 2.5 mM phenylmethylsulfonyl fluoride; 25 μg/ml each of leupeptin, aprotinin, and soybean trypsin inhibitor; and 1 mM sodium orthovanadate) per 100-mm dish for 1 h at 4 °C and clarified by ultracentrifugation at 100,000 g for 40 min at 4 °C. Solubilized membranes, prepared as described previously (14), or total cell lysates were immunoprecipitated with the indicated antibodies (1.100 dilution) and incubated in the presence or absence of 100 μM insulin in autophosphorylation buffer (50 mM HEPES, pH 7.8; 2.5 mM MnCl2) for 1 h at 4 °C. Autophosphorylation was carried out in the immunocomplex by the addition of [γ-32P]ATP (20 μM final, 20 μCi/nmol). Reactions were terminated as described previously (14), except that the concentration of sodium orthovanadate was increased to 100 μM in the autophosphorylation reaction and 800 μM in the stop mix. Autophosphorylated receptors were analyzed by electrophoresis on 6.0–6.5% SDS-polyacrylamide gels (PAGE) (15) and detected by autoradiography. For whole cell experiments, cells were incubated in serum-free DMEM containing 1 mM sodium orthovananade at 37 °C and 5% CO2 for 4–6 h, followed by incubation in HEPES/saline (50 mM HEPES, pH 7.8; 150 mM NaCl; 1 mM sodium orthovanadate) with or without 100 μM insulin for 10 min at 37 °C and 5% CO2. Cells were lysed as described above, and after immunoprecipitation with the indicated Abs, proteins were separated on 6.0–6.5% SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting (see below).

Immunoblotting

After transfer of proteins to nitrocellulose (16), membranes were incubated with the primary Ab for 1 h at 22 °C followed by horseradish peroxidase–protein A (Amersham Pharmacia Biotech) for polyclonal primary Abs and goat anti-mouse Ab (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) in the case of monoclonal Abs. When blots were probed with the horseradish peroxidase–PY20 Ab, the use of a secondary Ab was not necessary. Enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech) was utilized for detection.

Construction of cDNAs Encoding Inr β Subunits

pc3-β-myc—As a first step in the construction of a plasmid to express the β subunit of INR, a signal sequence was added to the β subunit amino terminus to direct it to the membrane. Two partially overlapping oligonucleotides with the sequences 5′-CTCTGAATTCCACATGTTCA-AGATCTGGTCTCTGCTCCGGCGGCTGGTGGCCGCCAAC-9′ and 5′-CTCTGAATTCCACATGTTCA-AGATCTGGTCTCTGCTCCGGCGGCTGGTGGCCGCCAAC-3′ were annealed and a fill-in reaction with Klenow fragment of DNA polymerase was used to synthesize the double-stranded, 124-base pair-long DNA fragment. This was subcloned into the plasmid p19-p121, after EcoRI and XhoI digestions. p19-p121 contains the cDNA sequence encoding the β subunit of the INR (nucleotides 3277–6447, according to Ref. 2) cloned into the pUC19 plasmid. The new construct termed p19-β-myc, was digested with EcoRI and PstI, and the resulting DNA fragment containing the complete INR β subunit and its new signal sequence was cloned into the vector pcDNA3 at the EcoRI and EcoRV sites to form pc3-β-myc.

pc3-β—To construct a plasmid that lacked the 1143 carboxy-terminal nucleotides of the INR β subunit, the plasmid p19-β-myc was digested with EcoRI and SphI. The 2151-base pair-long DNA fragment (nucleotides 3277–5304, according to Ref. 2) was subcloned into the vector pcDNA3 at the EcoRI and EcoRV restriction sites to form the resulting vector called pc3-β.

Transient and Stable Transformations

Transient transfections were performed with LipofectACE reagent (Roche Molecular Biochemicals), and stable transformants with Lipofectin reagent (Roche Molecular Biochemicals) according to the manufacturer’s instructions. Cells were grown in 100-mm dishes and transfected with 14 μg of DNA in 34 μl of the liposome reagent. For transient transfection, cells were grown for 60–65 h after DNA addition. Total cell lysates or crude membranes were prepared, and the presence of endogenous insulin receptors and transfected β-INR was demonstrated by autophosphorylation, in the presence or absence of insulin, following immunoprecipitation of receptors with Abs P5, dp1040, and 9E10. Stable transfectants were grown for 65 h after transfection before adding selective agent. Neomycin-resistant colonies were selected using 200 μg/ml geneticin (G418) (Life Technologies, Inc.) in DMEM. Total cell death was achieved in 7 days in nontransfected cells used as controls. Single cell-derived clones were picked, grown, and tested for the expression of β INR by autophosphorylation reactions.

Cell Growth and Survival Assays

5 × 104 cells were plated in 96-well tissue culture dishes in their respective growth medium. Cell number was measured at the indicated times from 0 to 4 weeks after seeding, using the Cell Titer96AQueous kit (Promega, Madison, WI) according to the manufacturer’s instructions. The absorbance at 490 nm was read in a microtiter plate reader (Bio-Rad).

RESULTS

Expression of β INR in Mammalian Cells—Deletion of the α subunit of mammalian insulin receptors leads to the constitutive activation of the tyrosine kinase activity of the β subunit (19, 20). The structural homology between the mammalian and Drosophila insulin receptors suggested that the INR would be similarly activated by removal of its α subunit. Therefore, in order to express constitutively activated receptors to explore the role of the 400-amino acid carboxy-terminal extension in INR function, expression vectors encoding either the complete β subunit (amino acids 1083–2148, pc3-β-myc) or the β subunit without the carboxy-terminal extension (amino acids 1083–1768, pc3-β) were constructed (see “Experimental Procedures”). pc3-β-myc encodes the complete INR β subunit preceded by a Drosophila signal sequence and an epitope tag recognized by a c-Myc antibody (9E-10) (Fig. 1). pc3-β is derived from pc3-β-myc by deletion of the sequence encoding 380 amino acids of the carboxy-terminal extension (1769–2148) (Fig. 1). 293 and MDCK cells were transiently and stably transfected with these recombinants giving rise to 293 β-Myc and 293 β-Δ cells, MDCK β-Myc and MDCK β-Δ cells, respectively.

Consistent with the finding that the INR β subunit is longer than that of the HIR (1058 versus 619 amino acids) (2), in autophosphorylation experiments Ab dp1040 and monoclonal Ab 9E10 immunoprecipitated phosphoproteins of 170 and 180 kDa from total cell lysates of 293 β-Myc cells (Fig. 2 lanes 5 and 7, arrowheads). The same Abs immunoprecipitated a phosphoprotein of 102 kDa from lysates of the 293 β-Δ cells (lanes 8–10, arrow). Immunoprecipitation of β-Myc and β-Δ with Ab dp1040 was blocked by an excess of peptide dp1040 (lanes 6 and 9). From 293 cell lysates Ab P-5 immunoprecipitated the 97-
when MDCK β-Myc and MDCK βA cells were tested for the presence of the INR proteins (data not shown).

The 170-kDa Protein Is a Distinct Protein Co-immunoprecipitated with β INR from 293 β-Myc Cells—In order to determine if the 170- and 180-kDa bands in Ab dp1040 immunoprecipitates from 293 β-Myc cells represent different forms of β INR or distinct proteins, immunoblotting assays of proteins immunoprecipitated with Abs dp1040 and 9E10 from total lysates of 293, 293 pc3, 293 β-Myc, and 293 βA cells were performed (Fig. 3A). In contrast to the results obtained in autophosphorylation experiments (Fig. 2), Ab dp1040 directly recognized only one protein of 180 kDa in immunoprecipitates from 293 β-Myc lysates (lanes 5, 6, and 8, upper arrow). Immunoprecipitation of this protein was blocked when carried out in the presence of an excess of peptide dp1040 (lane 7), indicating that it corresponds to the INR β subunit. In samples from the 293 βA cells, Ab dp1040 recognized the 102-kDa βA INR (lanes 9, 10, and 12, lower arrow), and immunoprecipitation of this protein was also blocked by an excess of peptide dp1040 (lane 11). As expected, Ab dp1040 did not recognize any protein in 293 (lanes 1–4) and 293 pc3 cell lysates (data not shown). Thus, Abs dp1040 and 9E10 immunoprecipitate two phosphoproteins of 170- and 180-kDa from 293 β-Myc lysates (Fig. 2), yet only one, the 180-kDa protein, is directly recognized by Ab dp1040 (Fig. 3A). Furthermore, when autophosphorylation and immunoprecipitation with Abs dp1040 and 9E10 were carried out utilizing solubilized membrane proteins rather than total cell lysates of 293 β-Myc cells, only one protein of 180 kDa was detected (Fig. 3B, arrow). This suggests that the 170-kDa protein that co-immunoprecipitates with the INR β subunit from total cell lysates is a cytoplasmic or peripheral membrane protein, and its interaction with the INR β subunit is not stable enough to persist during the membrane preparation process.

The 170-kDa Protein Contains Phosphotyrosine—The above experiments indicate that the 170-kDa protein is immunologically distinct from β INR, co-immunoprecipitates with it, and is phosphorylated in vitro. To examine if the 170-kDa protein is tyrosine-phosphorylated by the INR kinase in intact cells, 293 β-Myc cells were lysed and proteins immunoprecipitated with Ab dp1040 without in vitro autophosphorylation. The samples were analyzed by immunoblotting (Fig. 4) with Abs PY20 (lane 1) and dp1040 (lane 2). Anti-phosphotyrosine Ab PY20 reacted with both 170- and 180-kDa proteins in immunoblots (Fig. 4A, lanes 1, arrowheads), whereas Ab dp1040 recognized only the β subunit of the INR (lane 2, arrowhead). The same results were obtained when the protein phosphorylation reaction was performed in vitro (Fig. 4B). In this case, proteins from 293 β-Myc total cell lysates were first immunoprecipitated with Ab dp1040, and then protein phosphorylation was performed in the presence of unlabeled ATP for 10 min.

Taken together, these results demonstrate that in 293 β-Myc cells a cytoplasmic protein of 170 kDa, distinct from the INR β subunit, associates with the β subunit and is phosphorylated on tyrosine. The inability of this interaction to withstand the process of membrane preparation suggests that it is not covalent.

Identification of the 170-kDa Protein as IRS-1—IRS-1, a major substrate of HIR, is a cytoplasmic protein that migrates in SDS-PAGE with an apparent molecular mass of 160–190 kDa (21). If the 170-kDa phosphoprotein that binds directly to the INR β subunit is IRS-1, then INR β subunits should be recovered in samples immunoprecipitated with aIRS-1 Abs. aIRS-1 immunoprecipitates from total cell lysates of 293, 293 pc3, and 293 β-Myc cells were probed with the aIRS-1 Ab (Fig. 5A), and similar amounts of 170-kDa IRS-1 were recovered in all cases (Fig. 5A, arrow). The same nitrocellulose membrane
treated 293 cells (Fig. 5B, lane 2, arrow), but the phosphorylated endogenous receptors were not observed, indicating that they do not co-immunoprecipitate with IRS-1. The same Ab recognized a broad band from 293 β-Myc cells that contains two phosphoproteins, one co-migrating with IRS-1, and the single 180-kDa INR β subunit recognized by Ab dp1040 (A and B, lane 2). Proteins were detected using the ECL method as described. Molecular mass standards are indicated by the bars on the left and correspond to 205, 116, and 80 kDa from top to bottom, respectively.

β-INR Does Not Co-precipitate with IRS-1—A 170-kDa protein is not detected in Ab dp1040 immunoprecipitates from β3 cell lysates (Fig. 2, lanes 8–10). This result was confirmed by anti-phosphotyrosine immunoblotting of total cell lysates immunoprecipitated with Abs dp1040 and 9E10 and phosphorylated in vitro (Fig. 6A). No phosphoprotein of 170 kDa was recovered along with the 102-kDa β3 INR (Fig. 6A, lanes 1, 2, and 4) whether or not insulin was present. Similarly, when phosphoproteins in Ab dp1040 immunoprecipitates from intact cells were examined, only the β3 INR was observed in immunoblots probed with Ab PY20 (Fig. 6B, lane 3).

In order to determine the phosphorylation state of IRS-1 in 293 β3 cells, total cell lysates from 293 β-Myc and 293 βΔ cells were immunoprecipitated with αIRS-1 Ab. Immunoblotting with αIRS-1 and PY20 Abs was used to assess the amount of IRS-1 and its state of phosphorylation, respectively (Fig. 7). A similar amount of IRS-1 was present in both 293 β-Myc and 293 βΔ cell lysates (Fig. 7B). However, IRS-1 in 293 βΔ cells was phosphorylated to a much lower extent than in 293 β-Myc cells (Fig. 7A). Thus, despite the presence of constitutively activated INR β subunit kinases in 293 β-Myc and 293 βΔ cell lines, increased phosphorylation of IRS-1 and association with the INR β subunit was only observed in 293 β-Myc cells. This suggests that the carboxyl-terminal extension in the full-length INR β subunit is the site of interaction with IRS-1.

Inr β Subunits Do Not Associate with Other Signaling Mol-

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**Fig. 4.** Immunoblot (IB) analysis of phosphorylated INR β subunit from 293 β-Myc cells. Ab dp1040 immunoprecipitates of total cell lysates from 293 β-Myc cells were separated by SDS-PAGE, transferred to nitrocellulose, and reacted with Affinity purified Ab dp1040. (A) either directly (A) or following autophosphorylation in vitro in the presence of unlabeled ATP (B). Arrowheads indicate the 180- and 170-kDa phosphoproteins recognized by Ab PY20 (A and B, lane 1) and the single 180-kDa INR β subunit recognized by Ab dp1040 (A and B, lane 2). Proteins were detected using the ECL method as described. Molecular mass standards are indicated by the bars on the left and correspond to 205, 116, and 80 kDa from top to bottom, respectively.

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**Fig. 3.** INR β subunits in 293-β-Myc, and 293-βΔ cell lines. A, immunoblot (IB) analysis. Untransfected 293 cells (lanes 1–4), stably transfected 293 β-Myc (lanes 5–8), or 293 βΔ cells (lanes 9–12) were incubated in the absence (lanes 1, 5, 7, 8, 9, 11, and 12) or presence (lanes 2, 3, 4, 6, and 10) of insulin. Cells were lysed and INR β subunits immunoprecipitated from total cell lysates with the indicated Abs in the absence (lanes 1–6, 8–10, and 12) or presence (lanes 7 and 11) of excess peptide dp1040 (40 μg/ml), separated by SDS-PAGE, transferred to nitrocellulose, and reacted with affinity purified Ab dp1040. Upper and lower arrows indicate the 180- and 102-kDa INR β subunits, respectively. Proteins were detected using the enhanced chemiluminescence (ECL) method as described under “Experimental Procedures.” Molecular mass standards are indicated by the bars on the left and correspond to 205, 116, 80, and 49.5 kDa from top to bottom, respectively. B, INR β subunit autophosphorylation in solubilized membranes prepared from stably transfected 293-β-Myc cell lines. Proteins from membrane preparations of untreated 293 cells (lanes 1 and 2) or stably transfected 293 β-Myc cells (lanes 3 and 4) were incubated in the presence (lane 2) or absence (lanes 1, 3, and 4) of insulin. Autophosphorylation reactions were initiated by addition of [γ-32P]ATP, and samples were then immunoprecipitated with the indicated Abs. The arrowhead indicates the single 180-kDa INR β subunit from 293 β-Myc cells recognized by Ab dp1040 and 9E10. Molecular mass standards are indicated by the bars on the left and correspond to 205, 116, 97, and 66 kDa from top to bottom, respectively.

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**Fig. 2.** Immunoblot (IB) analysis of phosphorylated INR β subunit from 293 β-Myc cells. Ab dp1040 immunoprecipitates of total cell lysates from 293 β-Myc cells were separated by SDS-PAGE, transferred to nitrocellulose, and probed with Abs PY20 (lane 1) and dp1040 (lane 2) either directly (A) or following autophosphorylation in vitro in the presence of unlabeled ATP (B). Arrowheads indicate the 180- and 170-kDa phosphoproteins recognized by Ab PY20 (A and B, lane 1) and the single 180-kDa INR β subunit recognized by Ab dp1040 (A and B, lane 2). Proteins were detected using the ECL method as described. Molecular mass standards are indicated by the bars on the left and correspond to 205, 116, and 80 kDa from top to bottom, respectively.

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In order to determine if other signaling proteins were co-immunoprecipitated with the full-length or truncated INR β subunits, total cell lysates of 293, 293 pc-3, 293 β-Myc, and 293 βD cells were immunoprecipitated with antibodies against IRS-2, mSos-1, Shc, p85 subunit of PI3'-kinase, SHP-2, Raf-1, and JAK2 and probed with Ab dp1040. These experiments failed to demonstrate co-immunoprecipitation of β INR with any of these proteins (data not shown).

The opposite experiments were also performed, in which the total cell lysates were immunoprecipitated with Ab dp1040 and probed with...
antibodies against each protein. Likewise, in these experiments no co-immunoprecipitation of any of these proteins with the INR β subunits was detected (data not shown). All of the signaling proteins examined were present in all cell lines in similar amounts as shown by immunoprecipitation and immunoblotting with Abs against the respective proteins (data not shown). Thus, neither direct interaction between these proteins and the constitutively active INR β subunits nor alteration in the content of these proteins in the different cell lines was found.

The Carboxyl-terminal Extension of β INR Promotes Cell Survival—One of the physiological functions of insulin receptors is to regulate mitogenesis and cell proliferation. In fact, a defect in regulation of cell growth or proliferation may underlie the growth deficiency phenotype observed in flies harboring ins mutations, as evidenced by the decrease in the number of imaginal disc cells observed (22). Therefore, it was of interest to test if the constitutively active β-Myc or βΔ receptors had an effect on cell growth and if there was a difference in the regulation of this biological response between these two receptors.

These experiments were carried out on both 293 and MDCK stably transfected cell lines. 293 β-Myc and 293 βΔ cell lines both exhibited similar increases in the rate of cell proliferation relative to the parental 293 cells (Fig. 8A). Between days 5 and 10, the transfected cells grew more rapidly and to a higher cell density. Maximum cell number was reached on day 10, as compared with day 14 for the untransfected 293 cells (Fig. 8A). The maximum cell number was 37 and 26% higher than that of the parental 293 cells for 293 β-Myc, and 293 βΔ cells, respectively. In contrast, the behavior of the β-Myc and the βΔ cell lines was remarkably different upon prolonged incubation (up to 4 weeks). β-Myc cells were able to survive significantly longer than the parental cell line, whereas βΔ cells died at a faster rate (Fig. 8A). For example, on day 17, 293-β-Myc cell density was 2.7-fold higher than the parental cell line which, in turn, was 2.4-fold higher than that of 293-βΔ cells. Cell number declined to 50% of the maximum on day 13 for the 293-βΔ cells, day 16.5 for the 293 cells, and day 24 for the 293-β-Myc cells (Fig. 8A). Thus, although both β-Myc and βΔ receptors appear similar in their ability to promote cell proliferation, they have very distinct effects on long term survival of the cells.

The increase in rate of growth induced by both β-Myc and βΔ receptors and the inability of the βΔ receptors to support long term survival were confirmed when similar experiments were performed using stably transfected MDCK cell lines (Fig. 8B). In these experiments, the transfected MDCK β-Myc and βΔ cells reached maximum cell number by day 2, as compared with day 7 for untransfected MDCK cells (Fig. 8B). The maximum cell number was 25% higher for β-Myc and βΔ cells as compared with parental MDCK cells. Overexpression of INR β-Myc in MDCK cells conferred prolonged survival capacity, although the difference with untransfected MDCK cells was not as pronounced as that observed for 293 cells. Cell number declined to 50% of maximum on day 12 in MDCK cells versus day 14 in the β-Myc cells (p < 0.05 for days 10 and 14). However, the rapid decline in cell number in βΔ expressing cells was more pronounced in the MDCK cell background. This contrasted significantly with the much more gradual decline observed in the β-Myc-expressing MDCK cells. Cell number was 50% of maximum on day 5.5 in βΔ cell lines and declined to 0 by 10 days, when greater than 50% of MDCK and MDCK β-Myc cells was still present. The time to 50% survival differed by 8.5 days between βΔ and β-Myc MDCK cells (p < 0.05), similar to the 11 days difference noted in 293 cell lines (Fig. 8A). Thus, a pronounced difference in cell survival due to β-Myc and βΔ INR expression was consistently observed in two different cell backgrounds. This dramatic difference may be due to functional properties of the carboxyl-terminal extension.

These experiments strongly suggest the following: (a) overexpression of constitutively activated β-Myc and βΔ receptors in the 293 and MDCK cells increases the rate of growth of these cell lines, and (b) the carboxyl-terminal extension of the INR plays a role in promoting cell survival.

DISCUSSION

Two forms of recombinant, membrane-bound INR β subunits, either the complete INR β subunit (β-Myc) or a β subunit lacking the carboxyl-terminal extension (βΔ), were expressed in mammalian cells in the absence of their α subunit and found to be constitutively active as protein tyrosine kinases. The full-length INR β subunit in 293 β-Myc cells interacted more strongly with IRS-1 than the truncated form in 293 βΔ cells or the endogenous HIR in 293 cells. IRS-1 co-immunoprecipitated with the β INR from 293 β-Myc cells, whereas it was not recovered in anti-receptor immunoprecipitates from 293 and 293 βΔ cell lysates. Since the only difference between the βΔ...
and β-Myc proteins is the lack of the carboxyl-terminal extension in βΔ, it is likely that the strong interaction with IRS-1 occurred within this domain. In the INR carboxyl-terminal extension, tyrosines 1969, 2030, and 2009 reside within NPXY motifs that, after phosphorylation, would represent binding sites for the PTB domain of IRS-1 (23–25). Tyrosines 1969 and 2030, especially, have residues at the −5 position (Ser) and −8 position (Met or Val, respectively) relative to the phosphotyrosines that are important for high affinity binding to the IRS-1 PTB domain (25). These motifs, despite the NPXY sequence, do not represent ideal Shc-binding sites (26, 27), which may explain why Shc was not found to co-immunoprecipitate with the β-Myc receptors from 293 cells. Indeed, the sequence NPNY, in particular, which is present in the four INR NPXY motifs (2), has been shown to be a binding site for IRS-1 but not SHC (27). Stable association of IRS-1 molecules with the carboxyl-terminal extension of the β INR is consistent with the higher level of IRS-1 phosphorylation observed in 293 β-Myc cells, as compared with 293 βΔ and 293 cells. Notably, IRS-2 did not associate with the INR, suggesting it has distinct requirements for binding. The potential interaction of IRS-1, recently shown to be the predominant IRS protein in 293 cells (28), with INR is currently under study. However, the complete loss of the INR-associated phosphoprotein following membrane preparation (Fig. 3A) suggests that the interacting protein is primarily cytoplasmic, as is IRS-1, whereas at least 50% of IRS-4 appears membrane-associated (28).

The tyrosine residues present in the NPXY motifs of the carboxyl-terminal extension also form the motifs YXXM, YMXM, and YXXLD with their carboxyl-terminal residues. The motifs YXXM and YMXM are known to be binding sites for the SH2 domains of the p85α regulatory subunit of PI3-kinase (29–31), and the motif YXXLD is similar to the sequence YIDLD that interacts with the SH2 domain of SHP-2 (30). Thus, it was expected that PI3-kinase and possibly SHP-2 would bind to the INR carboxyl-terminal extension. However, this was not observed in these studies. These sites appear capable of binding PI3-kinase in the absence of IRS-1. Chimeric receptors composed of human α subunits and INR β subunits expressed in 32D cells which lack IRS-1 have been shown to associate with PI3-kinase (8). However, similar chimeric receptors expressed in Chinese hamster ovary cells, which contain IRS-1, failed to bind PI3-kinase (4). No association of IRS-1 with the INR β subunit was noted by Yamaguchi et al. (4); however, receptor phosphorylation was acutely stimulated by insulin in those studies rather than constitutive as in the studies described here. The constitutive kinase activity of β-Myc may lead to constitutive association with IRS-1, accounting for the co-precipitation observed in these studies. However, a functional interaction between hormone-activated INR and IRS-1 is suggested by the IRS-1 requirement for an INR-induced proliferative response (8). Thus, it would appear that both PTB domain- and SH2 domain-containing proteins are capable of binding to the phosphotyrosines within the hybrid NPNYMP, NPNYQP, or NPNYRLLD motifs in the carboxyl-terminal extension. However, when both are present, the binding of IRS-1 to these residues seems dominant over the the binding of SH2-containing proteins and may block the SH2 sites located immediately after the phosphotyrosine residue.

Overexpression of constitutively active INR β and βΔ receptors in 293 and MDCK cells promoted cell proliferation, indicating that the INR can engage the mammalian proliferation pathways. The equivalent proliferative responses induced by INR β-Myc and βΔ kinases suggests that the growth-promoting function of the INR in these cells is independent of the carboxyl-terminal extension. In contrast, cells expressing the full-length INR β subunit exhibit significantly enhanced survival as compared with cells expressing the βΔ INR. Relative to the parental 293 and MDCK cells, the INR β-Myc and βΔ proteins conferred somewhat different behavior; β-Myc clearly promoted survival in 293 cells, whereas βΔ more dramatically accelerated cell death in MDCK cells. Nonetheless, a clear difference in the behavior of cells expressing the full-length or truncated INR β subunits is evident in both backgrounds. Despite the presence of a juxtapathemembrane NPXY motif predicted to interact with IRS-1 in both β-Myc and βΔ proteins, IRS-1 is not highly phosphorylated in βΔ cells (Fig. 7A). This suggests that the carboxyl-terminal extension of the INR β subunit is required for sustained association and phosphorylation of IRS-1. This persistent IRS-1 phosphorylation distinguishes β-Myc from βΔ cells and may be of primary importance in promoting cell survival. Without this sustained interaction, cell death may actually be accelerated, as observed in MDCK cells transfected with the INR βΔ kinase.

IRS-1 that was bound to the INR β subunit was phosphorylated on tyrosine; however, no evidence was found for increased association of PI3-kinase or other candidate signaling molecules with this complex. Therefore, the mechanism whereby this association led to increased cell survival is unclear at present. Interestingly, a recent report demonstrates that expression of a truncated IRS-1 containing only the pleckstrin homology and phosphotyrosine binding domains, without any tyrosine phosphorylation sites, mediates PI3-kinase and phosphotyrosine-independent signals that contribute to the regulation of cell survival and apoptosis (32). IRS-1 that was bound to the carboxyl-terminal extension of INR in 293 and MDCK cells may have similarly activated pathways that promote cell survival in the absence of PI3-kinase activation.

Thus, two isoforms of an activated INR β subunit have been expressed in mammalian cells, and a functional difference between them has been demonstrated. The data presented here indicate that the stimulation of cell proliferation by INR was mediated by the kinase domain independent of the carboxyl-terminal extension. In contrast, the carboxyl-terminal extension mediated an interaction with IRS-1 and influenced cell survival. Since an IRS homolog is present in Drosophila (33), this may reflect an inherent function of the INR which, in flies, is modulated by tissue- or stage-specific processing of the receptor. Importantly, these data also suggest that in mammalian cells persistent localization of IRS-1 to membranes via its interaction with receptors and/or persistent tyrosine phosphorylation generates signals independent of association with PI3-kinase which modulate cell survival.

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