Insulin-like growth factor-I-induced DNA synthesis in insulin-secreting cell line RINm5F is associated with phosphorylation of the insulin-like growth factor-I receptor and the insulin receptor substrate-2

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Abstract

A proliferative effect of insulin-like growth factor-I (IGF-I) was previously shown in pancreatic islets. However, the mechanism under which IGF-I actions are exerted in insulin-secreting cells is not clear. The rat insulinoma cell line, RINm5F, was shown to have both IGF-I receptors and IGF-II/mannose-6-phosphate receptors. IGF-I binding to cell surface receptors stimulated phosphorylation of 97 kDa and 93 kDa subunits of the IGF-I receptor and incorporation of $[^3H]thymidine$ into RINm5F cells. Both the IGF-I-induced protein phosphorylation and $[^3H]thymidine$ incorporation were abolished in the presence of the tyrosine kinase inhibitor, genistein. Under basal conditions, IGF-I did not induce insulin release or changes in cytosolic free $Ca^{2+}$ concentration. Immunoprecipitation of proteins from RINm5F cells, using phosphotyrosine antibodies, followed by western blotting using antibody against IRS-1 revealed no distinct band of phosphorylated insulin receptor substrate (IRS)-1. Instead, tyrosine-phosphorylated IRS-2 was detected and stimulated by IGF-I when western blotting was performed using antibody against IRS-2. These results indicate that IRS-1 is not likely to be involved in IGF-I signalling in RINm5F cells. Hence, IGF-I stimulated DNA synthesis in RINm5F cells was associated with phosphorylation of IGF-I receptors and IRS-2.

Introduction

The insulin-like growth factor-I (IGF-I) receptor, homologous with the insulin receptor, is a heterotetrameric protein, consisting of two $\alpha\beta$ heterodimers. The $\alpha$ subunit is located extracellularly and has a molecular mass of 120–130 kDa, whereas the $\beta$ subunit, which is mainly localized intracellularly, is a 93 kDa protein. IGF-I binds to the IGF-I receptor with high affinity. The cross-reactions of IGF-II and insulin with the IGF-I receptor are 5–10% and 0·1–1% respectively (Roth & Kiess 1994).

The IGF-I receptor belongs to the tyrosine kinase growth factor receptor family. Tyrosine kinase activity and tyrosine phosphorylation of the receptor and intracellular substrates are essential for all actions mediated by the IGF-I receptor (Kato et al. 1994, Li et al. 1994). Binding of IGF-I to its receptor induces autophosphorylation of the receptor and phosphorylation of intracellular substrates, catalysed by the intrinsic tyrosine kinase in the intracellular domain of the receptor $\beta$ subunits (Kato et al. 1994, Jones & Clemmons 1995). In certain cell types, IGF-I stimulates phosphorylation of two types of $\beta$ subunits of IGF-I receptors with molecular masses of about 97 kDa and 95 kDa (Moxham et al. 1989). The immunological properties and protein microsequencing of the two proteins have revealed the presence of IGF-I/insulin hybrid receptors (Siddle et al. 1994, Kasuya et al. 1993). The hybrid receptors can be formed in vitro using purified heterodimers from insulin receptors and IGF-I receptors (Treadway et al. 1989). Several intracellular proteins have been found to be phosphorylated after activation of IGF-I receptors (Oh et al. 1993, Yenush & White 1997). The major intracellular substrate for activated IGF-I receptor is insulin receptor substrate-1 (IRS-1), which is also involved in insulin-induced cell responses (Cantley et al. 1991, Sun et al. 1991, Tamemoto et al. 1994). IRS-1 cDNA has been cloned and sequenced (Sun et al. 1991). Phosphorylated IRS-1 is capable of interacting with and activating several SH2-domain-containing proteins, such as the regulatory subunit of phosphatidylinositol-3 kinase (PI3K), growth factor receptor-bound protein 2 (Grb2) (Cantley et al. 1991, LeRoith et al. 1995), protein tyrosine phosphatase (SHP2) (Rocchi et al. 1996, Yenush & White 1997).
and other intracellular proteins (Jones & Clemmons 1995). In mice carrying a disrupted IRS-1 gene, insulin and IGF-I stimulate phosphorylation of another docking protein, IRS-2, associated with activation of PI3K (Araki et al. 1994, Tobe et al. 1995). This pathway becomes an alternative, IRS-1-independent pathway in insulin or IGF-I signalling.

In comparison with the insulin receptor, the IGF-I receptor is more effective in mediating cell growth (LeRoith et al. 1995), as demonstrated by the use of antisense oligonucleotides to the IGF-I receptor RNA (Pietrzkowska et al. 1993) or applying cells with targeted disruption of the IGF-I receptor genes (Li et al. 1994). IGF-I receptors are essential during development (Liu et al. 1993) and in tumorigenesis (Baserga et al. 1995, Li et al. 1996). In addition, sufficient expression of the IGF-I receptor is required for protection of cell death from apoptosis (Steller 1995, O’Connor et al. 1997), through a signal transduction pathway similar to that involved in the metabolic and mitogenic actions of IGF-I (Parrizas et al. 1997).

IGF-I receptors are present in insulin-secreting cells (Van Schravendijk et al. 1987). Exogenous IGF-I stimulates proliferation of rat islets (Swenne et al. 1987, Hogg et al. 1993), in which IGF-I was shown to have a more potent effect than IGF-II on DNA synthesis (Hogg et al. 1993). A proliferative effect of IGF-I was also demonstrated in the rat insulin-secreting cell line, RINm5F (Dereli et al. 1988). In addition, IGF-I is capable of regulating insulin secretion in insulin-secreting cells. Both stimulatory (Dheen et al. 1996) and inhibitory effects (Van Schravendijk et al. 1990, Zhao et al. 1997) of IGF-I on insulin release have been reported. Therefore, the IGF-I receptor, through interacting with its ligands, has an important role in both growth and hormone secretion in insulin-secreting cells. These cells continuously secrete a large quantity of insulin, which not only binds to the insulin receptor, but also crossreacts with the IGF-I receptor. This raises the question of the nature of the cellular mechanism of the IGF-I receptor that mediates IGF-I actions in insulin-secreting cells. In the present study, we have investigated the role of the IGF-I receptor and underlying molecular mechanisms in the insulin-secreting cell line, RINm5F.

Materials and Methods

Materials

Recombinant IGF-I and II were generous gifts from Pharmacia, Stockholm, Sweden. Dupont New England Nuclear (NEN) Products provided [methyl-3H] thymidine. Enhanced chemiluminescence (ECL) reagents and [γ-32P]ATP were purchased from Amersham (Amersham, UK). Anti-IRS-1 antibody (C-terminal), anti-IRS-2 antibody and mouse 3T3 cell lysate were from Upstate Biotechnology Inc., NY, USA. Rabbit anti-phosphotyrosine IgG was from Zymed. Genistein and Fura-2/acetoxymethylester (fura-2/AM) were from Sigma, St Louis, USA. Bio-gel P-4 Gel (fine, 65 ± 20 μm, wet) and Bio-Rad protein assay reagent were obtained from Bio-Rad Laboratories (Richmond, CA, USA). Culture medium RPMI-1640 and fetal calf serum (FCS) were purchased from Life Technologies (Paisley, UK).

Cell culture

A rat insulinoma cell line, RINm5F, passage number ranging between 25 and 35, was maintained in RPMI-1640 tissue culture medium supplemented with 10% (v/v) FCS, 100 IU/ml penicillin and 100 μg/ml streptomycin.

[3H]Thymidine incorporation

[3H]Thymidine incorporation was performed according to the method of Kato et al. (1994). RINm5F cells were placed in 24 well tissue culture trays (5 × 10^4 cells/well) in RPMI-1640 medium supplemented with 10% FCS and grown to 80% confluence. Cells were washed three times with serum-free medium and kept in serum-free medium for 24 h. Thereafter, the medium was removed and replaced by fresh serum-free medium containing either vehicle or hormones with or without the tyrosine kinase inhibitor, genistein. After an incubation of 18 h, [3H]thymidine (0.5 μCi/well) was added and the incubation was continued for an additional 6 h. At the end of incubation, cells were washed three times in Hepes buffer and lysed with 500 μl 1 M NaOH; 400 μl of the lysate was transferred into vials containing scintillation fluid, and radioactivity was determined in a liquid scintillation counter. The remaining 100 μl was used for protein determination (Bio-Rad). Results are presented as c.p.m./mg protein.

Receptor binding

Trypsinized cells were washed three times with ice-cold Hepes buffer containing 10 mM Hepes, 135 mM NaCl, 4.8 mM KCl, 1.7 mM MgSO₄, 2.5 mM CaCl₂ and 1.0 mM NaH₂PO₄, pH 7.4 and resuspended in Hepes buffer–1% BSA. Cell suspension (100 μl, 5 × 10^5 cells, per tube) was incubated at 4°C overnight with iodine-labelled hormone in the presence or absence of unlabelled polypeptides at a final incubation volume of 300 μl. The incubation was terminated by aspirating 2 × 100 μl from each assay tube, followed by centrifugation through 250 μl of a mixture of phthalic acid esters. The supernatants were eliminated and the tips of the tubes containing cell pellets were excised and counted in a gamma counter. The 100 μl remaining in each assay tube was used as reference (Tally et al. 1984).
**Affinity crosslinking**

Affinity crosslinking was performed as described previously (Pilch & Czeck 1979). Trypsinized RINm5F cells were washed with ice-cold 50 mM phosphate buffer, pH 7-4. Incubation was carried out in 50 mM phosphate buffer—1% BSA, pH 7-4, with 125I-des(1–3)-IGF-I or 125I-IGF-II for 40 min at room temperature in the presence or absence of unlabelled des(1–3)IGF-I (100 ng/ml) or IGF-II (100 ng/ml). Affinity crosslinking was carried out on ice for 15 min using disuccinimidyl suberate (DSS). The reaction was quenched with 0·5M fluoride, 1µg/ml leupeptin, 1µg/ml pepstatin, 0·25% sodium deoxycholate and 1% Nonidet P-40. The lysate was boiled and proteins were separated on SDS–PAGE (10%) (Laemmli 1970) and visualized by autoradiography.

**Immunoprecipitation and western blotting**

Immunoprecipitation of proteins with phosphotyrosine antibody and western blotting using anti-IRS-1 or -2 was performed according to the manufacturer’s instructions. Cells were grown in culture dishes to a confluence of 80%. Before the experiment, cells were kept in serum-free RPMI-1640 medium for 24 h. Incubation of cells was carried out in serum-free medium in the presence or absence of 10 nM IGF-I (37 °C, 1 min). Cells were washed with ice-cold PBS and solubilized in a lysis buffer containing 50 mM Tris–HCl (pH 7-4), 150 mM NaCl, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 µg/ml aprotinin, 1 mM phenylmethylsulphonyl fluoride, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 0·25% sodium deoxycholate and 1% Nonidet P-40. The lystate was incubated with anti-phosphotyrosine antibody (1 µg/ml) overnight with gentle shaking. The immunocomplex was precipitated with protein A–Sepharose (4 °C, 2 h). The beads were collected by centrifugation and washed with lysis buffer. The immunoprecipitated proteins were solubilized in SDS–PAGE sample buffer. Equal amount of proteins was applied in each lane and separated on SDS–PAGE (7%) under reducing conditions. Mouse 3T3 C cell lysate and immunoprecipitated proteins from a 100 000 g supernatant from a rat liver were used as controls. The proteins in the gels were electrotransferred onto nitrocellulose sheets. The sheets were blocked in PBS–3% non-fat dry milk (PBS–MLK), followed by an overnight incubation with anti-IRS-1 (0·5 µg/ml) or anti-IRS-2 antibodies (1 µg/ml) at 4 °C. The nitrocellulose sheets were intensively washed and incubated with horseradish peroxidase-linked antibodies (1:3000) for 1·5 h in PBS–MLK at room temperature. The blots were washed and the immunoreactive proteins were detected with ECL.

**Preparation of IGF receptor proteins and protein phosphorylation**

Partial purification of IGF receptor proteins and protein phosphorylation were carried out using the methods described by Shemer et al. (1987). RINm5F cells were solubilized in 50 mM Hepes buffer, pH 7-4, with 1% Triton X-100, 100 KIE/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride, followed by centrifugation at 100 000 g for 20 min at 4 °C. The supernatant was allowed to bind to a wheat-germ agglutinin (WGA)-agarose in an affinity column (bed volume: 0·5 ml). The bound proteins were eluted in 0·3 M N-acetyl-d-glucosamine after the column had been washed with 10 bed volumes of 50 mM Hepes buffer–0·1% Triton X-100, pH 7-4. The partially purified receptor preparation was incubated for 40 min at room temperature, in the presence or absence of 10 nM IGF-I or 10 nM insulin or IGF-I plus insulin at the same concentrations. Phosphorylation reaction was initiated by the addition of [γ-32P]ATP (15 µM) to the buffer containing 8 mM MgCl2 and 4 mM MnCl2 (1 min, 37 °C) and terminated by addition of SDS–PAGE sample buffer. The labelled products were separated on SDS–PAGE under reducing conditions and visualized by autoradiography.

**Insulin release from RINm5F cells**

Insulin release from RINm5F cells was determined using a column perfusion system (Kanatsuna et al. 1981). After trypsinization, about 2 x 10^5 cells were carefully mixed with a small volume of pre-wetted Bio-Gel P-4 and placed on the top of each of two parallel microcolumns (bed volume: 0·5 ml), pre-packed with the same polyacrylamide beads. Perfusion was performed continuously at 37 °C with buffer A containing 125 mM NaCl, 5·9 mM KCl, 1·28 mM CaCl2, 1·2 mM MgCl2, 25 mM Hepes and 0·1% BSA, pH 7-4. The cells were exposed to 50 ng/ml IGF-I for 12 min and depolarized by 25 mM K^+ at the end of each experiment. The flow rate was 0·2 ml/min and fractions were collected every 2 min for insulin radioimmunoassay (Herbert et al. 1965).

**Measurement of cytoplasmic free Ca^{2+} concentration**

Cytoplasmic free Ca^{2+} concentration ([Ca^{2+}_{ci}]) was measured utilizing a microscope equipped with a photon-counting photometer and connected to a SPEX fluorolog-2 CM1T 111 system, allowing fluorimetry using two excitation wavelengths (Kindmark et al. 1992). RINm5F cells were placed on coverslips in RPMI-1640 medium supplemented with 10% FCS for 24 h. The coverslips with cells were incubated in buffer A containing 1·5 µM fura-2-AM for 30 min at 37 °C. During measurement, the cells were continuously superfused with the buffer (300 µl/min at 37 °C). Measurements were performed on a cell cluster (5–10 cells) at the excitation and emission wavelengths of 340 nm/380 nm and 510 nm, respectively. Cells were exposed to 50 ng/ml IGF-I for 4 min in buffer A containing 3 mM glucose.
The results are expressed as the 340/380 fluorescence ratios as described elsewhere (Kindmark et al. 1992).

Results

Effect of IGFs on[^H]thymidine incorporation, [Ca^{2+}], and insulin release

Both IGF-I and IGF-II stimulated thymidine incorporation into RINm5F cells (Fig. 1). At a concentration of 50 ng/ml IGF-I, the [^H]thymidine incorporation was doubled. A similar effect was obtained in the presence of 100 ng/ml IGF-II.

During continuous perifusion in the presence of 3 mM glucose, RINm5F cells failed to respond to IGF-I (50 ng/ml) with insulin release (Fig. 2). In addition, in the presence of 3 mM glucose, IGF-I did not cause any changes in [Ca^{2+}], (data not shown).

Presence of IGF receptors

The presence of cell surface receptors was investigated using[^251]I-des(1–3)IGF-I, human recombinant IGF-II or insulin (Fig. 3A–C). Des(1–3)IGF-I was applied instead of native IGF-I in order to minimize the effects of IGF binding proteins released from the cells; the receptor binding of des(1–3)IGF-I is indistinguishable from that of native IGF-I. The specific binding of[^251]I-des(1–3)IGF-I to RINm5F cells was 18%. Both des(1–3)IGF-I and native IGF-I were shown to be equipotent in the competitive inhibition of the labelled[^251]I-des(1–3)IGF-I binding, indicating that the binding was not attributed to IGF binding proteins. The affinity of IGF-II for the IGF-I receptor on these cells was approximately 6% in potency compared with IGF-I. Insulin crossreacted less than 0.2%.

Scatchard analysis of the data obtained from the competitive inhibition study using des(1–3)IGF-I revealed a K_d of 9.1 × 10^{-9} M, derived from a two-site model, with approximately 2.7 × 10^{4} high-affinity binding sites per cell. The specific binding for IGF-II was 24% in the assays using IGF-II as the labelled ligand. Des(1–3)IGF-I or insulin failed to compete with IGF-II for the IGF-II binding. Scatchard analysis of the data revealed a K_d of 2 × 10^{-10} M with 1.8 × 10^{4} IGF-II binding sites per cell.[^251]I-insulin did not display any specific binding to RINm5F cells.

Affinity crosslinking was performed using labelled des(1–3)IGF-I or IGF-II (Fig. 4). When[^251]I-des(1–3)IGF-I was used, a 135 kDa protein band was detected, and the labelled hormone in the gel was displaced by 100 ng/ml unlabelled des(1–3)IGF-I (A). Affinity crosslinking using[^251]I-IGF-II resulted in a 250 kDa protein (B), competed for by unlabelled IGF-II (100 ng/ml).
Effects of genistein on IGF-I-induced receptor phosphorylation and [3H]thymidine incorporation

In order to characterize the functional IGF-I receptor in RINm5F cells, protein phosphorylation was performed using a WGA-purified fraction of solubilized plasma membranes. As shown in Fig. 5A, two phospho-labelled protein bands with molecular masses of 97 kDa and 93 kDa were stimulated by IGF-I or insulin. Densitometric analysis of the protein bands, using a scanner connected to a computer, revealed a twofold increase in phosphorylation of the two proteins stimulated by IGF-I. Combination of IGF-I with insulin did not show any additional effect.

The IGF-I-induced protein phosphorylation was evaluated in the presence of the tyrosine kinase inhibitor, genistein (Fig. 5B). At a concentration of 50 ng/ml, IGF-I caused an increased phosphorylation of the 97 kDa protein, and, to a lesser extent, the 93 kDa protein. Densitometric analysis of the two protein bands revealed an increase of 174% for the 97 kDa protein and 148% for the 93 kDa protein. In the presence of 5 μM genistein, the
density of the two phosphorylated bands stimulated by IGF-I was suppressed by 25%. When the concentration of genistein was increased to 10 µM, the IGF-I stimulated phosphorylation of the two proteins was further suppressed and showed no statistically significant difference in density, compared with controls.

The involvement of tyrosine kinase in the IGF-I-induced DNA synthesis was investigated in the presence or absence of genistein. Genistein inhibited the IGF-I-induced [3H]thymidine incorporation into RINm5F cells in all concentrations used (Fig. 6). The viability of the cells, as determined by trypan blue exclusion, did not show a significant difference in the presence of genistein in the concentrations used (1–10 µM), compared with controls.

Discussion

The present study shows that both IGF-I and IGF-II/M6P receptors were present in RINm5F cells, with dissociation constants similar to those in other cell types (Steele-Perkins et al. 1988, Tong et al. 1988). IGF-I and IGF-II stimulated thymidine incorporation into RINm5F cells in a tyrosine kinase-dependent manner. The IGF-I-stimulated DNA synthesis in RINm5F cells was associated with phosphorylation of the IGF-I receptor and IRS-2.

In RINm5F cells, activation of the IGF-I receptor did not seem to be associated with the insulin stimulus–secretion machinery under basal conditions, as IGF-I had no effect on insulin release and [Ca²⁺]. However, in rat pancreatic islets, IGF-I has a stimulatory effect on insulin secretion (Dheen et al. 1996). The discrepancy in IGF-I response may be accounted for by a species difference.

IGF-I stimulated phosphorylation of two proteins with molecular masses of 97 kDa and 93 kDa in RINm5F cells, suggesting the presence of two types of β subunits of IGF-I receptors, as reported in other cell types (Alexandrides & Smith 1989, Garofalo & Rosen 1989, Moxham et al. 1989, Langlois et al. 1995). The two types of β subunits might be from the IGF-I receptor and the insulin receptor respectively, forming IGF-I/insulin hybrid receptors, or both types of β subunits may have been from the IGF-I receptor.
IRS-1 is the major substrate for IGF-I and insulin receptors responsible for interaction with cellular effectors, including PI3K (Sun et al. 1991, 1997, Kato et al. 1994). Dominant expression and function of IRS-1 in the cells are believed to contribute to a normal insulin response (Rondinone et al. 1997, Sun et al. 1997). Cells expressing IGF-I receptors but lacking IRS-1 have little or no response to insulin or IGF-I (Chuang et al. 1993), suggesting a key role of IRS-1 in IGF-I and insulin signalling. In RINm5F cells, however, activation of IGF-I receptors did not cause phosphorylation of IRS-1. The absence of phosphorylated IRS-1 in these cells cannot be explained by a rapid dephosphorylation of the protein, as phosphorylated IRS-1 remains in the cell longer than does phosphorylated IRS-2 before they are eventually dephosphorylated (Ogihara et al. 1997). In addition, phosphorylated IRS-1 was detected in the mouse 3T3 cells and the rat liver extracts in the same experiment, whereas it was absent in RINm5F cells. Hence, IRS-1 does not seem to have an important role in IGF-I signalling in RINm5F cells.

The presence of IRS-2 in RINm5F cells and an enhanced phosphorylation of the protein in response to IGF-I stimulation indicate the involvement of IRS-2 in IGF-I signalling in this cell type. IRS-2 shows both structural and functional similarities with IRS-1 (Sun et al. 1997, Yenush & White 1997) and is coexpressed with IRS-1 in many cells and tissues (Ogihara et al. 1997, Sun et al. 1997). However, IRS-2 tends to predominate in haematopoietic cells, whereas IRS-1 predominates in adipocytes and muscle cells (Sun et al. 1997). The cell-specific expression of IRS proteins may contribute to cell responses to different stimulators, such as insulin, IGF or cytokines (Sun et al. 1997, Yenush & White 1997). Although phosphorylated IRS-2 is capable of interacting with and activating PI3K, a greater concentration of insulin is required to achieve a response similar to that obtained through IRS-1 (Rondinone et al. 1997). It has been found that IRS-1 concentrations are reduced, and that IRS-2 is serving as the major docking protein for insulin receptors in pancreatic ß cells has been proposed (Bruning et al. 1997). Therefore, it is possible that insulin interacts with both insulin and IGF-I receptors in insulin-secreting cells.
cells. Signalling through IRS-2 in these cells may be important in decreasing the sensitivity of the cells to insulin, acting through either insulin or IGF-I receptors via autocrine and paracrine mechanisms.

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References


LeRoith D, Wener H, Beitner-Johnson D & Roberts CT Jr 1995 Molecular and cellular aspects of the insulin-like growth factor I receptor. Endocrine Reviews 16 143–163.


Yenush L & White MF 1997 The IRS-signalling system during insulin and cytokine action. Bioessays 19 491–500.


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