Interaction of Secreted Insulin-like Growth Factor-I (IGF-I) with Cell Surface Receptors Is the Dominant Mechanism of IGF-I’s Autocrine Actions*

In a prior report we presented evidence that insulin-like growth factor-I (IGF-I) can act in an autocrine fashion by demonstrating that FRTL-5 cells transfected with hIGF-IA fusion genes express and secrete biologically active IGF-I that renders the stimulation of DNA synthesis in FRTL-5 cells independent of their requirement for exogenous IGFs or insulin. To determine if IGF-I’s autocrine actions require secretion or can be mediated by interactions with intracellular receptors, we have created a new line of FRTL-5 cells that express a mutant IGF-IA precursor containing the endoplasmic reticulum retention amino acid sequence, Lys-Asp-Glu-Leu (KDEL), at its carboxyl terminus. The mutant IGF-IA/KDEL precursor expressed by stably transfected FRTL-5 cells was shown to be retained intracellularly and to have biological activity comparable to mature IGF-I, as judged by the activity of partially purified IGF-I/KADEL in wild type FRTL-5 cells. Expression of IGF-IA/KDEL in FRTL-5 cells, however, neither augmented TSH-stimulated DNA synthesis nor stimulated IGF-binding protein-5 expression, as does IGF-IA expression in transfecte...
(30 units/mg) for thymidine incorporation studies was a gift from the National Hormone and Pituitary Program (National Institute of Diabetes and Digestive and Kidney Diseases [NIDDK], Bethesda, MD). Recombinant human IGF-I (rhIGF-I) was obtained from Genentech (South San Francisco, CA). [methy1-3H]Thymidine (6.5 mCi/ml/mmol) and [32P]CTP (3,000 Ci/mmol) were obtained from Amersham Corp. (13,19) Cytochrome (113.9 Ci/ml/mmol) was purchased from Du Pont-New England Nuclear. FRTL-5 cells (ATCC CRL 8305) were kindly provided by Dr. Leonard Kohn (Section of Cell Regulation, NIDDK). Taus polymerase was purchased from New England Biolabs (Beverly, MA). Construction of an IGF-I Transgene Containing a KDEL Sequence—A polymerase chain reaction (PCR)-directed mutagenesis strategy was used to make a mutant human IGF-IA fusion gene containing the endoplasmic reticulum retention sequence KDEL (see Fig. 1 and its legend for details of the methods used). The hIGF-I expression vector pMIG (14,15) was used as a template for mutagenesis. PCR amplification was performed with Taus polymerase according to the specifications of the manufacturer (Perkin-Elmer Cetus) using a Perkin Elmer DNA thermal cycle. The resultant plasmid, called pNEO/IA/KDEL, was subjected to DNA sequencing (16) to confirm the appropriate insertion of the KDEL encoding sequence and to exclude unintended alterations in the coding sequence.

Transfection and Culture of FRTL-5 Cells—Transfection of FRTL-5 cells with pNEO/IA/KDEL was performed with the calcium phosphate precipitation method, as described previously (7,17). As with the other stably transfected FRTL-5 cell lines used in this study (7), cells were selected with G418, and single colonies isolated with cloning rings prior to amplification. After 4-5 passages, cells that had genomically integrated the KDEL mutant IGF-IA transgene were aliquoted and frozen as glycerol stocks in liquid N2. Similar procedures were used in preparing and preserving the other transfected cell lines used in this study (7). These include: pNEOMIG transfected FRTL-5 cells, which express a fusion of the rat somatostatin signal peptide and IGF-I, driven by the MT-I promoter; p5'II/IA-transfected cells, which express the same transgene driven by mIGF-II 5' genomic flanking region; and p5'II/NEO-transfected cells, which express the IGF-IA resistance gene under the regulation of the mIGF-II 5' genomic flanking region, thus serving as control-transfected cells that do not express IGF-I.

Unless specified otherwise, all wild type and transfected FRTL-5 cells used in this study were of similar passage number (passage 25-30) and were grown up in 100-mm dishes in Coon's F-12 containing 5% calf serum and three hormones (3H): TSH (1 milliunit/ml), insulin (10 /gm/ml), and transferrin (5 /gm/ml). Radioimmunoassay (RIA)—To obtain conditioned media and cell lysates for IGF-I RIA, quiescent cells were incubated in Coon's F-12 with 0.1% BSA for 24 h. After collecting the medium, cells were harvested in 80 mM sodium citrate, washed twice with PBS, and then lysed in a buffer consisting of 150 mM NaCl, 1% Nonidet P-40, and 50 mM Tris, pH 8.0 (lysis buffer). All cell lysates and conditioned media were incubated with equal volumes of 0.4 M acetic acid for 45 min and then neutralized prior to RIA (18,19).

Characterization of 32P-Labeled Transgene IGF-I—In vitro labeling of synthesized proteins with [35S]cytochrome, immunoprecipitation with a rabbit antiserum to hIGF-I (UBK 487; kindly donated by Dr. Louis E. Underwood), and SDS-PAGE analysis of transgenic IGF-I were performed, as described previously (7).

Partial Purification of IGF-I from Cell Lysates—Cells were collected from 3 near-confluent T-150 flasks, washed twice with PBS, resuspended in 300 ml of 1 M acetic acid, and lysed by repeated freezing and thawing. Lysates then were centrifuged for 15 min, and the supernatant collected and neutralized. An IGF-I polyclonal antibody immobilized on glass beads was then added to the supernatant, and the mixture was shaken gently for 1 h on ice, washed with PBS and then with distilled (d) H2O. Antibody-associated IGF-I was eluted with 1 M acetic acid and the eluate lyophilized. These IGF-I preparations were resuspended in 50 ml dH2O and stored at -80°C until they were evaluated. IGF-I was evaluated as 32P incorporation of 3 ml of each preparation to stimulate thymidine incorporation into wild type FRTL-5 cells.

Imethy1-3H]Thymidine Incorporation Assays—Incorporation of [methy1-3H]thymidine into FRTL-5 cells was performed as described previously (7,11).

Ligand Blot Analysis of IGF-Binding Proteins—Ligand blotting was performed as previously described (20). Conditioned media were collected from single 100-mm dishes of near-confluent cells incubated with Coon's F-12 for 24 h. After removing cellular debris by centrifuging at 3000 rpm for 15 min, media were dialyzed against 200 volumes of 0.1% acetic acid at 4°C using Spectropor-3 membrane (molecular weight cut-off 3,000) and concentrated 100-fold by using polyethylene glycol (M, = 8000). Concentrated media were electrophoresed through 12.5% discontinuous SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose filters (0.45 μm), and the resultant blot was incubated with a mixture of 125I-IGF-I and 125I-IGF-II. After several successive washes, blots were exposed to Kodak X-AR x-ray film at -70°C to determine the relative migration of proteins capable of binding radiolabeled IGFs. The relative intensity of autoradiographic signals was determined by densitometric scanning.

RNA Analysis—Total RNA was isolated from cells grown to confluence in single 100-mm dishes, and Northern hybridization analyses were performed as described previously (7, 21, 22), using a 32P-labeled 40 bp rat IGF-binding protein-5 (IGFBP-5; bp 588-1201; Ref. 20) cDNA probe generated from rat liver reverse transcribed total RNA by PCR. Autoradiographic bands were quantified by scanning densitometry, as with ligand blots.

Affinity Cross-linking—The cells were grown to confluence in 35-mm dishes in 3H Coon's F-12 culture medium with 5% calf serum and two PBS washes. After rinsing in PBS, the cells were incubated with Coon's F-12 with 0.1% BSA. After washing with HEPES buffer three times, cells were incubated with 125I-IGF-I (6 x 107 cpm/dish) in HEPES binding buffer with 0.5% BSA with or without 200 ng/ml unlabeled IGF-I or 100 μg/ml insulin for 18 h at 4°C. The cells were then washed with HEPES buffer three times, and 0.1 mM disuccinimidyl suberate in HEPES buffer was added for 15 min at room temperature (24). The reaction was terminated by the addition of 9 ml of 10 mM Tris-HCl, pH 7.4, with 1 mM EDTA for 15 min. 125I-IGF-I cross-linked cells were solubilized in 2% SDS, 0.05 M Tris-HCl, and 0.1 M DTT, and subjected to SDS-PAGE analysis and autoradiography.

IGF-I Binding Assay—Specific 125I-IGF-I binding to cell surface sites was measured on monolayers of wild type and transfected FRTL-5 cells as previously described (25), with minor modifications. Cells were grown to confluence in 24-well plates. After removing culture media (3H Coon's F-12 with 5% calf serum) and washing with PBS twice, cells were incubated with Coon's F-12, 0.1% BSA for 24 h, then washed three times with cold HEPES binding buffer containing 20 mM HEPES, pH 7.4, 120 mM NaCl, 5 mM KCl, 1.2 mM MgSO4, 10 mM NaHCO3, 1.3 mM CaCl2, 1.2 mM KH2PO4, with 0.5% BSA and incubated with 125I-IGF-I (26,000 cpm/ml, specific activity = 261 μCi/μg IGF-I) plus various concentrations of unlabeled IGF-I in a final volume of 0.5 ml at 4°C for 45 min. After removing the buffer, the monolayers were washed four times with cold PBS containing 0.5% BSA, then solubilized in 0.5 ml of 0.1% SDS, 0.2 M NaOH, and radioactivity determined in a Packard Auto-Gamma 6500.

RESULTS

The Expression of the pNEO/IA/KDEL Construct in FRTL-5 Cells Results in Intracellular Accumulation of the IGF-IA/KDEL Precursor—In a previous report we described the creation of autocrine models of IGF-I action in FRTL-5 cells (7). Two plasmids encoding a hIGF-I fusion gene, driven either by mouse MT-I or IGF-II 5' genomic regulatory regions (designated pNEOMIG and p5'II/IA, respectively), were transfected. With stable plaques characterizable for transfection, FRTL-5 cells secreted IGF-I into 24-h conditioned medium in concentrations ranging between 0.5 and 3 ng/ml. These lines exhibited high basal DNA synthesis and had a DNA synthetic activity that was indistinguishable from that induced by TSH and IGF-I in wild type or control-transfected cells (p5'II/NEO). In the present study a mutant hIGF-I expression vector containing a sequence encoding the KDEL endoplasmic reticulum retention signal, designated pNEO/IA/KDEL, was constructed and transfected into FRTL-5 cells (Fig. 1). After transfection and selection, single G418-resistant colonies were screened for IGF-I expression by RIA. As anticipated, immunoreactive IGF-I was detectable in cell lysates of transfectants, but not in their conditioned media. In the two colonies chosen for detailed characterization-
primers before the stop codon); oligonucleotide C pNEO/IA/KDEL. Initially, two DNA fragments were amplified from primers used were as follows: oligonucleotide A

AAG(K) GAC(D) GAG(E) CTT(L) TAG(stop) GAAGACC CTCCTG

complementary to the 3' end of the human growth hormone gene the 5' tail of a primer used to amplify the other fragment

TTCCTA AAG CTC GTC CTT CAT CCT GTA GTT CTT G

strand at the 3' end of the hIGF-IA cDNA, and containing sequences one in I sense strand regulatory sequence (37), i.e. this sequence

including the KDEL sequence) that hybridize, and thus it allowed amplification used a primer containing a 5' tail complementary to

sequence (C)
or its complement

(B)

and one of 474 bp using primers

ATTAGGAGTT GGAGACCAGC CT (sequence identical to the bases 478-504 of the sense strand, bases 2412-2433; Ref. 38). After purification of the first PCR products, the two fragments were combined, denatured and annealed, and then subjected to PCR with primers A and D to amplify a 956-bp fragment with the KDEL sequence inserted. This fragment was cut at internal BglII and SphI sites, and then subcloned into pMIG that was digested at the same sites. The resulting KDEL mutant precursor had biological activity comparable with that of the precursor without this sequence, the IGF-IA precursors from pNEOMIG and pNEO/IA/KDEL-transfected FRTL-5 cells were partially purified using an immunoadfinity method, and their capacity to stimulate thymidine incorporation was assessed in wild type FRTL-5 cells. As shown in Table I, the cell lysate IGF-IA precursor preparations from both pNEOMIG- and pNEO/IA/KDEL-transfected FRTL-5 cells exhibited the capacity to stimulate thymidine incorporation when incubated with wild type FRTL-5 cells. The pNEO/IA/KDEL cell lysate preparation had a greater immunoreactive IGF-1 content than the pNEOMIG preparation and exhibited greater bioactivity. To exclude the possibility that substances other than IGF-1 co-purified from FRTL-5 cell lysates (or contaminating substances derived from the purification process) might stimulate thymidine incorporation, cell lysates from FRTL-5 cells expressing a control plasmid (p5'II/NEO that encodes only the neomycin

tion, cell lysate-immunoreactive IGF-I was 320 and 700 pg/10⁶ cells and no immunoreactive IGF-1 was detectable in conditioned media. Northern analysis revealed a single 0.8-0.9-kilobase hIGF-IA transcript, the size expected for mRNA transcribed from this fusion gene (Ref. 7; data not shown). The intracellular accumulation of hIGF-I in pNEO/IA/KDEL-transfected cells was further confirmed by labeling cellular proteins with [³⁵S]cysteine and immunoprecipitation of hIGF-I using an IGF-I antibody. A radiolabeled 12-14-kDa protein, the predicted size of the the hIGF-1 precursor, was found in pNEO/IA/KDEL-transfected cells and appeared nearly identical in size to the predominant protein found in the cell lysates of p5'II/IA-transfected FRTL-5 cells (Fig. 2). A 7-8-kDa radiolabeled protein, representing mature hIGF-I, was found in the conditioned media of p5'II/IA-transfected cells, but not in that of pNEO/IA/KDEL-transfected cells. The addition of the KDEL coding sequence to the IGF-IA fusion gene, therefore, results in intracellular retention of the hIGF-IA transgene product.

The IGF-IA Precursor and IGF-IA/KDEL Mutant Are Biologically Active—To determine if the transgene IGF-IA/KDEL mutant precursor had biological activity comparable with that of the precursor without this sequence, the IGF-IA precursors from pNEOMIG and pNEO/IA/KDEL-transfected FRTL-5 cells were partially purified using an immunoadfinity method, and their capacity to stimulate thymidine incorporation was assessed in wild type FRTL-5 cells. As shown in Table I, the cell lysate IGF-IA precursor preparations from both pNEOMIG- and pNEO/IA/KDEL-transfected FRTL-5 cells exhibited the capacity to stimulate thymidine incorporation when incubated with wild type FRTL-5 cells. The pNEO/IA/KDEL cell lysate preparation had a greater immunoreactive IGF-1 content than the pNEOMIG preparation and exhibited greater bioactivity. To exclude the possibility that substances other than IGF-1 co-purified from FRTL-5 cell lysates (or contaminating substances derived from the purification process) might stimulate thymidine incorporation, cell lysates from FRTL-5 cells expressing a control plasmid (p5'II/NEO that encodes only the neomycin

Fig. 1. Construction of the hIGF-IA/KDEL expression vector, pNEO/IA/KDEL. Panel A, schematic of the strategy to create pNEO/IA/KDEL. Initially, two DNA fragments were amplified from the hIGF-IA expression vector, pMIG (14, 15), one of 482 bp using primers A and B and one of 474 bp using primers C and D. Each amplification used a primer containing a 5' tail complementary to the 5' tail of a primer used to amplify the other fragment (B and C). The 5' tails of these primers (B and C) contained the KDEL encoding sequence (C) or its complement (B), as well as additional complementary sequence. Consequently, the single-stranded DNA resulting after melting each PCR product contained complementary ends (including the KDEL sequence) that hybridize, and thus it allowed amplification of a single DNA fragment using the other primers (A and D) used to amplify the fragments. The four oligonucleotide primers used were as follows: oligonucleotide A = TACTCCCGTAG CTCCAGCTTC (identical to bases 41-60 of the mouse metallothionein-I sense strand regulatory sequence (37), i.e. this sequence hybridizes to the nonsense strand); oligonucleotide B = GGCTCA TTCCCTA AAG CTC GTC CTT CAT CCT G (sequence that is complementary to the bases 478-504 of the sense strand at the 3' end of the hIGF-IA cDNA, and containing sequences complementary to the KDEL encoding sequence inserted immediately before the stop codon); oligonucleotide C = AC TAC AGG ATG AAG(K) GAC(D) GAG(E) CTT(L) TAG(stop) GAAGACC CTCCTG (sequence identical to the 3' end of the hIGF-IA cDNA sense strand sequence, bases 483-509, and the inserted KDEL sequence); and oligonucleotide D = ATTAGGAGTT GGAGACCAGC CT (sequence complementary to the 3' end of the human growth hormone gene sense strand, bases 2412-2433; Ref. 38). After purification of the first PCR products, the two fragments were combined, denatured and annealed, and then subjected to PCR with primers A and D to amplify a 956-bp fragment with the KDEL sequence inserted. This fragment was cut at internal BglII and SphI sites, and then subcloned into pMIG that was digested at the same sites. The resulting KDEL containing IGF-IA fusion gene was digested with KpnI and EcoRI, and was subcloned into pNEONUT at the same restriction sites (7). Panel B, graphic of the pNEO/IA/KDEL, showing that the KDEL sequence is inserted immediately before the stop codon, as indicated in enlarged box. mMT-I, mouse metallothionein-I promoter; rSS, rat somatostatin signal sequence; 3'hoGH, 3' end of hGH gene containing polyadenylation signal sequence; Neo R, neomycin resistance gene.

Fig. 2. Characterization of [³⁵S]cysteine-labeled IGF-I from either conditioned media or cell lysates of pNEO/IA/KDEL-, p5'II/IA-, or p5'II/NEO-transfected FRTL-5 cells. Conditioned media or cell lysates were incubated with a polyclonal hIGF-I antiserum, and the immunocomplexes were harvested by further incubation with protein A-Sepharose, as described under "Materials and Methods." These immunocomplexes were analyzed by denaturing 15% SDS-PAGE. The arrows indicate the location of the bands representing the IGF-1 transgene precursors (~14-15 kDa) and mature (~7-8 kDa) peptides, respectively. Aliquots of conditioned media and/or cell lysates from pNEO/IA/KDEL- and p5'II/IA-transfected FRTL-5 cells also were incubated with 100 ng of unlabeled recombinant hIGF-I prior to immunoreaction (the lanes designated +IGF-I). The absence of a radiolabeled band in these lanes indicates that the antibody is reacting with authentic IGF-I.
resistance gene) were subjected to the same purification procedures. This preparation exhibited neither detectable immunoreactive IGF-I nor the capacity to stimulate thymidine incorporation in wild type cells. Compared with recombinant hIGF-I, both IGF-I precursor preparations exhibited greater bioactivity than would be predicted based upon their immunoreactive IGF-I, suggesting the possibility that the IGF-IA precursors are more potent mitogens than mature IGF-I.

**FRTL-5 Cells Expressing the IGF-IA/KDEL Mutant Do Not Exhibit the DNA Synthetic Activity of IGF-I-secreting Transfected FRTL-5 Cells**—To determine whether the intracellular IGF-IA expression created in pNEO/IA/KDEL-transfected FRTL-5 cells results in IGF-I autocrine actions, we compared TSH-stimulated DNA synthesis in p5'II/NEO (control-transfected cells), pNEOMIG, and pNEO/IA/KDEL-transfected FRTL-5 cells (Fig. 3, top panel). As previously demonstrated in IGF-I-secreting transfected FRTL-5 cells (pNEOMIG and p5'II/IA; Ref. 7), TSH stimulated thymidine incorporation with a peak response occurring at a concentration of 1 nM (see pNEOMIG in Fig. 3, top panel). In contrast, cells expressing the IGF-IA/KDEL mutant exhibited only a minimal increase in thymidine incorporation in response to TSH. This TSH response was comparable with that of control-transfected (p5'II/NEO) and wild type (not shown) FRTL-5 cells. Furthermore, pNEO/IA/KDEL-transfected cells responded to the addition of TSH with and without exogenous IGF-I in a fashion similar to control-transfected and wild type FRTL-5 cell (Fig. 3, bottom panel). The response of the pNEO/IA/KDEL cell lines to IGF-I alone, however, was blunted, consistently being about one-third of that observed in non-IGF-expressing cells. In the experiment shown in Fig. 3 (bottom panel), the IGF-I-secreting line, pNEOMIG, exhibited a modest increase in DNA synthesis in response to exogenous IGF-I and a marked increase in response to TSH, which is not appreciably augmented by exogenous IGF-I. Therefore, while the IGF-IA/KDEL mutant has the capacity to produce substances with substantial mitogenic activity (see above), its expression in transfected FRTL-5 cells does not replicate the autocrine actions of IGF-I observed in IGF-I-secreting transfected cell lines.

Because the IGF-I-secreting FRTL-5 cell lines pNEOMIG and p5'II/IA also express the IGF-IA precursor intracellularly, we used a monoclonal mouse-derived antibody raised against hIGF-I (Sm 1.2) to neutralize media IGF-I as a means of determining whether the IGF-I secreted by these cells was responsible for the increased TSH-stimulated DNA synthesis. Increasing concentrations of Sm 1.2 or a control preparation of mlgG were incubated with pNEOMIG (Fig. 4, top panel) and p5'II/IA (Fig. 4, bottom panel) transfected FRTL-5 cells, and TSH-stimulated thymidine incorporation was measured. In both cell lines, Sm 1.2 decreased TSH-stimulated thymidine incorporation in a dose-dependent fashion, with a 90% decrease occurring at 20 μg/ml and a near-complete abolition occurring at 100 μg/ml. Similar concentrations of mlgG had only a minimal effects on TSH-stimulated thymidine incorporation.

**IGF-I-secreting FRTL-5 Cells Exhibit Increased IGF-binding Protein-5 (IGFBP-5) Synthesis, an Activity That Is Not Replicated in IGF-IA/KDEL Mutant Expressing FRTL-5 Cells**—Because IGF-I is known to stimulate the synthesis of some IGFBPs (26, 27), we collected serum-free, conditioned media from IGF-I-expressing and control-transfected FRTL-5 cells and utilized Western ligand blots to determine if they contained IGFBPs. As shown in Fig. 5, a single band migrating at an apparent size of 30 kDa was seen in concentrated (125-fold) conditioned media from each cell line. This protein was identified as IGFBP-5 by demonstrating that the IGFBP-binding bands on the same ligand blot were immunostained with a specific IGFBP-5 antibody (the generous gift of Dr. D.R. Clemmons, Department of Medicine, University of North Carolina; Ref. 28) and by the identification of a specific IGFBP-5 transcript of the expected size (23) in total RNA extracted from FRTL-5 cells (see below). The abundance of IGFBP-5 was low in conditioned medium from control p5'II/NEO-transfected FRTL-5 cells, as it was in medium from

| TABLE I  
| Biological activity of cell lysate preparations |
|---|---|
| Cell lysate preparations | Immuneactive IGF-I | Thymidine incorporation* |
| | ng/30 μl | dpm/well |
| Coon's F-12 with 0.1% BSA | ND1 | 185 ± 41 |
| p5'II/NEO | ND | 177 ± 12 |
| pNEOMIG | 0.25 | 1,033 ± 69 |
| p5'II/IA/KDEL | 3.90 | 3,186 ± 267 |
| rhIGF-I2 | 10.00 | 1,684 ± 253 |

*Immunoreactive IGF-I concentrations are derived from the assay of each cell lysate preparation at multiple concentrations in duplicate. 1, 23 μl of each preparation was added to 1 ml of medium (Coon's F-12 with 0.1% BSA). The data represents the mean ± S.E. (n = 3).
1 ND, not detectable.
2 Recombinant hIGF-I.
ever, was comparable with that in control-transfected FRTL-5 cells. These findings indicate that IGF-I stimulates IGFBP-5 synthesis and that secreted IGF-I is primarily responsible for this IGF-I action, as it is for IGF-I stimulation of DNA synthesis.

Northern analysis of total RNA prepared from wild type and control, p5'II/NEO-transfected FRTL-5 cells demonstrated a low abundance 6.0-kilobase transcript that was increased about 10-fold when the cells were incubated with 100 ng/ml IGF-I (Fig. 6), findings that confirm IGF-I's capacity to stimulate IGFBP-5 synthesis in FRTL-5 cells. On the other hand, IGF-I-secreting FRTL-5 cells transfected with pNEOMIG and p5'II/IA exhibited an abundance of IGFBP-5 transcripts comparable with that observed in IGF-I-stimulated wild type and control-transfected FRTL-5 cells. Furthermore, incubation of these cells with Sm 1.2 (50 μg/ml) reduced IGFBP-5 transcript abundance to that found in wild type and control-transfected FRTL-5 cells not exposed to IGF-I. These findings indicate that secreted IGF-I is responsible for the stimulation of IGFBP-5 expression. Consistent with this interpretation is the finding that IGF-IA/KDEL expressing cells only exhibit appreciable IGFBP-5 mRNA when incubated with exogenous IGF-I. Their response to exogenous IGF-I, however, was only about one-third that of wild type or control-transfected cells. In addition, the finding that incubation of IGF-IA/KDEL-expressing cells with Sm 1.2 abolishes the low abundance IGFBP-5 transcript observed under basal conditions suggests that small amounts of IGF-IA/KDEL or mature IGF-I may be secreted.

The Influence of IGF-I and IGF-IA/KDEL Mutant Expression on Cell Surface IGF Type I Receptors—Affinity cross-linking experiments were performed with wild type and pNEO/IA/KDEL- and control (p5'II/NEO)-transfected FRTL-5 cells to characterize the cell surface receptors that interact with IGF-I (Fig. 7). Under reducing conditions, radiolabeled IGF-I was cross-linked to a 130–140-kDa protein that was nearly obliterated by competition with 200 ng/ml unlabeled IGF-I and reduced in intensity by a 500-fold higher concentration (100 μg/ml) of insulin. These findings are characteristic of the type I IGF receptor (29). The radiolabeled cross-linked bands migrating at the top of the gel could represent non-reduced type I receptor or the type II IGF/mannose-6-phosphate receptor. Because the apparent sizes of these bands are >270 kDa and their intensity is reduced by competition with insulin, they also likely represent type I

![Image](image_url)
FIG. 7. Affinity cross-linking of 125I-IGF-I to cell surface receptors. Wild type, p5'II/NEO-transfected, and pNEO/IA/KDEL-transfected FRTL-5 cells were incubated with 125I-IGF-I in presence or absence of IGF-I (200 ng/ml) or insulin (100 µg/ml), as indicated, and cross-linked with disuccinimidyl suberate, as described. Solubilized samples were reduced, analyzed by 7.5% SDS-PAGE, and autoradiographed. Molecular weight markers are shown in the right lane. The band migrating at about 130 kDa is characteristic of the α subunit of type I IGF receptor. This interpretation is confirmed by the dramatic reduction of the intensity of this band by 200 ng/ml IGF-I and its diminution by a 500-fold higher concentration of insulin (100 µg/ml).

FIG. 8. Scatchard plot of 125I-IGF-I binding to wild type (closed circles) and to pNEO/IA/KDEL (crosses), pNEOMIG (open squares), and p5'II/IA (closed squares) transfected FRTL-5 cells. All FRTL-5 cells were incubated with 125I-IGF-I in Coon's F-12, 0.1% BSA for 24 h, as described. Cell number was similar, as judged by protein content, in each cell line (range 191.4 ± 4 µg/well to 218.3 ± 20.3 µg/well; mean ± S.D.). Each data point represents the mean of duplicate experiments.

receptors (the type II receptor migrates at ~250 kDa under reducing conditions, ~220 kDa under non-reducing conditions, and does not bind insulin; Ref. 29).

To determine if expression of IGF-I or the IGF-I/KDEL mutant altered receptor affinity and/or number, 125I-IGF-I binding to each FRTL-5 cell line was determined in the presence of increasing concentrations of unlabeled IGF-I. Specific binding was greatest in wild type FRTL-5 cells (10.08 and 10.55% in two experiments), modestly lower in pNEO/IA/KDEL-transfected cells (8.36 and 8.49%), and much lower in IGF-I-secreting cells (5.81 and 6.21% in pNEOMIG and p5'II/IA cells, respectively). Scatchard plots of IGF-I binding in each cell line were linear (Fig. 8), and, therefore, IGF-I appears to interact with a single class of receptors, a finding that is in accord with the affinity cross-linking studies. The receptor concentrations (calculated from the x intercept of the Scatchard plot) were lower in IGF-I-secreting cells (29.5 and 28.3 pm in pNEOMIG and p5'II/IA-transfected FRTL-5, respectively) compared to wild type FRTL-5 cells (41.4 pm), suggesting that IGF-I secretion results in type I receptor down-regulation. A minimal reduction of type I receptor concentration was observed in pNEO/IA/KDEL-transfected cells (37.4 versus 41.4 pm in wild type cells). This reduction also may reflect receptor down-regulation by the secretion of small amounts of IGF-IA/KDEL or mature IGF-I. The affinities of IGF-I binding, however, were similar in each cell line (Kd = 0.35, 0.38, 0.43, and 0.39 nM for wild type, pNEO/IA/KDEL, pNEOMIG, and p5'II/IA FRTL-5 cells, respectively).

DISCUSSION

The results of this study provide strong evidence that the dominant mechanism of IGF-I autocrine actions are effected by secreted IGF-I and its subsequent interaction with cell surface receptors. Specifically, we have shown that IGF-I expression in FRTL-5 cells results in an autocrine phenotype when expression results in IGF-I secretion, while this phenotype does not occur when IGF-I expression is predominately intracellular. Furthermore, in FRTL-5 cell lines exhibiting IGF-I autocrine actions, exposure to an IGF-I antibody blocks IGF-I-stimulated activity, again indicating that the activities observed are exerted by the media-secreted IGF-I. Using in vitro radiolabeling, we found that mature IGF-I is the major form of secreted IGF-I, while the IGF-IA or IGF-IA/KDEL precursor forms predominate intracellularly. The molecular form of the IGF-I expressed, however, does not account for the differing phenotypes in these transfected FRTL-5 cell lines, because we have shown that each of the IGF-I precursors expressed has biological potency that is at least comparable with that of mature recombinant hIGF-I.

In this study we assessed IGF-I actions using two independent measures, thymidine incorporation and IGFBP-5 gene expression. IGF-I's stimulation of these activities probably involves differing mechanisms because IGF-I's major effects on DNA synthesis in FRTL-5 cells are exerted in concert with TSH, while its capacity to increase IGFBP-5 expression does not require additional exogenous regulatory factors. Nonetheless, our data show that both of these IGF-I actions is similarly dependent upon IGF-I secretion. Although each action may be exerted through differing ultimate pathways, IGF-I's signaling mechanism appears to be shared and to result from the interaction of secreted IGF-I and the cell surface type I IGF receptor. The affinity cross-linking studies confirm the expression of the type I receptor on the FRTL-5 cell surface. They also suggest that the type I receptor is the major, if not the only, FRTL-5 cell surface protein capable of binding IGF-I. This interpretation is supported by the Scatchard analyses of FRTL-5 cell IGF-I binding data, because the linear plots derived from these analyses indicate a single class of receptors. The Scatchard analyses also indicate that receptor concentration is reduced in IGF-I-secreting FRTL-5 cells, a finding that can be taken as further evidence of secreted IGF-I's interaction with the cell surface type I receptor.

As anticipated, expression of the IGF-IA/KDEL mutant in FRTL-5 cells resulted in the intracellular accumulation of the IGF-IA/KDEL precursor. The KDEL amino acid sequence is recognized by specific binding sites in the endoplasmic reticulum, and, thus, proteins containing this and similar sequences are retained intracellularly as they are cyclic through the endoplasmic reticulum and Golgi apparatus (12, 13). Such binding of KDEL-containing proteins, however, does not exclude protein processing or secretion. We were unable to detect secreted IGF-I in the IGF-IA/KDEL-expressing FRTL-5 cells. Two findings in IGF-IA/KDEL-expressing cell lines, however, suggest that small amounts of IGF-I were secreted. 1) The low abundance IGFBP-5 mRNA observed in
IGF-I/KDEL-expressing cells under basal conditions could not be visualized when cells were exposed to the antibody to IGF-I. 2) 125I-IGF-I specific binding and the calculated receptor concentration is modestly reduced in these cells. Of potential importance is the finding that IGF-I/KDEL-expressing cells exhibited a diminished response to exogenous IGF-I, being about one-third that of wild type and control-transfected cells for both thymidine incorporation and IGFBP-5 expression. The possible reduction in receptor number exhibited by the IGF-I/KDEL-expressing cells does not seem responsible for this blunted IGF-I response, because the IGF-I-secreting FRTL-5 cell lines have a greater reduction in responsiveness to IGF-I.

While our findings strongly argue that secreted IGF-I is a major effector of IGF-I's autocrine actions, they do not exclude actions of intracellular IGF-I or its precursors. The IGF-I-secreting FRTL-5 cell lines express the IGF-I precursor and mature IGF-I intracellularly. Either of these proteins could exert intracellular actions that make the effects of secreted IGF-I possible. In addition, our findings suggest a desensitization of IGF-I/KDEL-expressing cells to exogenous IGF-I also argue for intracellular IGF-I actions.

Evidence that growth factors can act intracellularly has been reported. For example, expression of interleukin-3 possesses a carboxyl-terminal KDEL signal been shown to make 32D cells independent of their requirement for this growth factor (32). Evidence for intracellular autocrine actions of the v-sis protein product, the viral homolog of the platelet-derived growth factor B chain, also have been reported (33, 34). Other reports, however, provide evidence that v-sis protein actions require interaction with the platelet-derived growth factor cell surface receptor (35, 36). Whether growth factors can exert their autocrine action intracellularly, therefore, remains controversial. It seems likely that the mechanisms of growth factor autocrine actions will prove to differ with different growth factors and to be dependent upon nature of the expressing cell.

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