Inhibition of Endosomal Insulin-like Growth Factor-I Processing by Cysteine Proteinase Inhibitors Blocks Receptor-mediated Functions*

The receptor for the type 1 insulin-like growth factor (IGF-I) has been implicated in cellular transformation and the acquisition of an invasive/metastatic phenotype in various tumors. Following ligand binding, the IGF-I receptor is internalized, and the receptor-ligand complex dissociates as the ligand is degraded by endosomal proteinases. In the present study, we show that the inhibition of endosomal IGF-I-degrading enzymes in human breast and murine lung carcinoma cells by the cysteine proteinase inhibitors, E-64 and CA074-methyl ester, profoundly altered receptor trafficking and signaling. In treated cells, intracellular ligand degradation was blocked, and although the receptor and two substrates, Shc and Insulin receptor substrate, were hyperphosphorylated on tyrosine, IGF-I-induced DNA synthesis, anchorage-independent growth, and matrix metalloproteinase synthesis were inhibited. The results suggest that ligand processing by endosomal proteinases is a key step in receptor signaling and function and a potential target for therapy.

Previously one of our laboratories reported that treatment of murine Lewis lung carcinoma subline H-59 cells with the cysteine proteinase inhibitor E-64 blocked their invasiveness in vitro and inhibited liver metastases formation in vivo (12). In the present work, we investigated further the molecular mechanism underlying the E-64 effects using H-59 and MCF-7 cells. We show here that E-64 and a second cathepsin B inhibitor, CA074-methyl ester (ME), inhibited endosomal proteolysis of IGF-I, and this led to an alteration of IGF-I receptor signaling. Under these conditions, the IGF-I receptor, as well as the signaling molecules Shc and IRS-1, were highly tyrosine-phosphorylated. On the other hand, IGF-I-induced DNA synthesis, anchorage-independent growth, and MMP-2 synthesis were all inhibited. Selective intervention at the internalization/degradation level may be of therapeutic relevance in abrogating IGF-I-mediated tumor formation and metastasis.

EXPERIMENTAL PROCEDURES

Cell Lines and Tissues—H-59 is a highly metastatic subline of the Lewis lung carcinoma with metastatic predilection for the liver, developed by one of our laboratories (13). Human breast carcinoma cell line MCF-7 (14) was a gift from Dr. Mader (Dept of Biochemistry, University of Montreal, Quebec, Canada). Endosomal fractions were prepared from livers of male Harlan Sprague-Dawley rats after an 18-h period of fasting. The livers were homogenized, and the endosomal fractions were isolated by discontinuous sucrose gradient centrifugation and collected at the 0.25 to 1.0 M sucrose interface (9, 10, 15). The soluble extract

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The abbreviations used are: IGF-IR, receptor for the type 1 insulin-like growth factor; IRS-1, insulin receptor substrate-1; ME, methyl ester; kb, kilobase; bp, base pair; mAb, monoclonal antibody; MMP-2, matrix metalloproteinase-2; HRP, horseradish peroxidase; HPLC, high pressure liquid chromatography.
(ENs) from the endosomal fractions was isolated by freeze/thawing in 5 mM sodium phosphate, pH 7.4, and disrupted in the same hypotonic medium using a small Dounce homogenizer (15 strokes with the tight Type A pestle) followed by centrifugation at 300,000 g for 30 min as described previously (9, 10, 15).

**Reagents and Antibodies**—E-64 (trans-epoxysuccinyl-l-leucylamido (4-guanidino)-butane), protein A-Sepharose beads, and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; thiazolyl blue) were purchased from Sigma. CA074-ME (N-(L-3-trans-propylcarbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline), a pro-inhibitor of intracellular cathepsin B (16), was from Peptides International (Louisville, KY). [3H]thymidine (2.0 Ci/mmol) was from DuPont. Recombinant Human IGF-I used for all the biological assays was purchased from InterGen (Purchase, NY). [125I]thymidine (2000 Ci/mmol) used for the ligand binding assay was obtained from Amersham Pharmacia Biotech. Human recombinant IGF-I used for the IGF-I proteolysis assay was radioiodinated by the lactoperoxidase method as described previously for insulin (15) to specific activities of 350–500 Ci/mmol and purified by gel filtration on Sephadex G-50. A 1.1-kb type IV collagenase cDNA fragment was kindly provided by Dr. W. Stetler-Stevenson (NIH, Bethesda, MD). A 700-bp IGF-IR cDNA fragment was a kind gift from Dr. M. Pollak (Lady Davis Research Institute, Montreal, PQ, Canada). The following antibodies were used: rabbit antiserum to MMP-2 (Ab-45), a kind gift from Dr. William Stetler-Stevenson (NIH); rabbit antiserum against IRS-1 were either obtained as a kind gift from Dr. Barry I. Posner (McGill University) or purchased from Calbiochem; anti-phosphotyrosine mAb PT-66 was from Sigma; horseradish peroxidase (HRP)-conjugated mAb RC20-H was from Transduction Laboratories (Mississauga, Ontario, Canada); mAb a IR3 to human IGF-IR was from Calbiochem; HRP-conjugated goat anti-mouse and goat anti-rabbit IgG antibodies were from Bio-Rad.

**Fig. 1.** IGF-IR functions are blocked in tumor cells treated with the cysteine proteinase inhibitor E-64. A, reduced anchorage-independent growth. H-59 and MCF-7 cells were cultured in semi-solid agar for 12 days in the absence (a and c) or presence (b and d) of 10 μg/ml E-64. Colonies exceeding 250 μm in diameter were scored. Shown are the results of one of two experiments performed in triplicate. A light microscopic view of the agar colonies (× 250) is shown on the right (a and b, H-59; c and d, MCF-7). The number of colonies/plate expressed as means and S.D. of three plates is shown on the left. B, inhibition of IGF-I-induced [3H]thymidine incorporation. Serum-starved, E-64-treated, or untreated H-59 (left) and MCF-7 (right) cells were seeded in 96-well microtiter plates and incubated for 72 h in serum-free medium containing IGF-I with or without E-64. Shown are the results of a representative experiment of three performed. They are expressed as the increase in [3H]thymidine incorporation relative to cells incubated without IGF-I and represent means ± S.D. of triplicates. C, loss of MMP-2 synthesis. Top, Northern blot analysis was performed on total RNA extracted from H-59 cells cultured for 48 h in the presence of serum, with or without 10 μg/ml E-64. The blots were probed consecutively with [32P]-labeled MMP-2 and cyclophilin cDNA probes, and the bands were analyzed by laser densitometry. Results of the densitometry are shown in the bar graph and are expressed as MMP-2: cyclophilin ratios. Bottom, proteins in the concentrated media (× 60) collected from H-59 cells cultivated for 48 h with or without 10 μg/ml E-64 and in the presence or absence of 10 ng/ml IGF-I were analyzed by immunoblotting using mAb Ab-45 to MMP-2 (I) and by gelatin zymography (II). a, control (untreated); b, E-64-treated; c, untreated, IGF-I stimulated; and d, E-64 treated, IGF-I stimulated H-59 cells.
Functional Assays for IGF-IR—Thymidine incorporation and soft agar cloning assays were performed as follows: semiconfluent cultures of H-59 or MCF-7 were cultured in serum free-medium for 24 h with or without E-64, dispersed, seeded onto 96-well polystyrene plates (Falcon), and incubated with different concentrations of IGF-I with or without E-64 for 54 h prior to pulsing with 0.1 mCi/ml of \([3H]\)thymidine for 18 h. For soft agar cloning (17) the tumor cells were mixed with a solution of 0.8% agar added to an equal volume of a 2× concentrated RPMI-fetal calf serum medium with or without 10 μg/ml of E-64, plated on solidified 2% agar at a concentration of 10^4 cells/plate, and supplemented with 1 ml of RPMI-fetal calf serum containing or not 10 μg/ml of E-64. This medium was replenished on alternate days for 12 days. MMP-2 synthesis was analyzed by Northern and Western blotting and by gelatin zymography, which were performed as previously described (18). For Northern blotting, 32P-labeled 1.1-kb human MMP-2 and 800-bp rat cyclophilin (19) cDNA fragments were used as hybridization probes. Western blotting and gelatin zymography were performed on concentrated (60×) serum-free media conditioned by H-59 cells for 48 h in the presence or absence of IGF-I with or without 10 μg/ml E-64. Blots were probed with a 1:500 dilution of mAb Ab-45 to MMP-2 and an alkaline phosphatase-conjugated affinity purified goat anti-rabbit IgG, diluted 1:2000.

Measurement of Cell Surface IGF-I Receptors—The ligand binding assay and fluorocytometry were used to measure cell surface IGF-I receptors on the murine H-59 and human MCF-7 cells, respectively. 2-day-old H-59 cultures were replenished with fresh medium containing or not 10 μg/ml E-64. Blots were probed with a 1:500 dilution of mAb Ab-45 to MMP-2 and an alkaline phosphatase-conjugated affinity purified goat anti-rabbit IgG, diluted 1:2000.

FIG. 2. The cysteine proteinase inhibitor E-64 blocks endosomal IGF-I degradation. The effect of E-64 on IGF-I degradation was tested using lysates of E-64-treated cells (A and B) and endosomal fractions isolated from whole rat livers (C and D). H-59 and MCF-7 cells were cultured for 24 h with or without 10 μg/ml E-64. The cells were lysed, and cell lysates were immediately incubated with 10^{-7} M IGF-I and analyzed by reverse-phase HPLC. Shown are profiles of eluates monitored on-line for absorbance at 214 nm. Typically, two peaks corresponding to intact (arrowhead) and degraded (arrow) IGF-I were observed in untreated cells. E-64-treated cells produced essentially one peak corresponding to the intact IGF-I. Loss of IGF-I degradation was confirmed by assessing the integrity of the ligand in 10% trichloroacetic acid precipitates following incubation of the cell lysates for up to 1 h at 37 °C with \([125I]\)-labeled IGF-I (B). Similar patterns were observed when soluble endosomal extracts prepared from rat liver parenchyma were incubated with unlabeled (C) or \([125I]\)-labeled (D) IGF-I in the presence or absence of 10^{-7} M E-64. EN, endosome.
FIG. 3. E-64 treatment causes a reduction in post-ligand binding cell surface receptor expression without affecting IGF-IR synthesis. A, analysis of cell surface IGF-IR by 125I-IGF-I binding. H-59 cultures were incubated with fresh medium containing or not 10 μg/ml E-64. 24 h later, cultures were incubated with 8–1500 pm 125I-labeled IGF-I (triplicate wells were used for each concentration), with or without graded concentrations of unlabeled IGF-I as described under “Experimental Procedures.” Counts were analyzed using the Ligand program and are presented as a Scatchard plot. B/F, bound/free. B, analysis of cell surface IGF-IR by flow cytometry. Serum-starved, MCF-7 cells incubated for 24 h with or without 10 μg/ml E-64 were incubated for 10 min with or without 10 ng/ml IGF-I and then allowed an additional 30 min of incubation at 37°C prior to labeling with mAb RC-20 (Transduction Laboratories) conjugated to fluorescein isothiocyanate and a fluorescein isothiocyanate-conjugated goat anti-mouse IgG (diluted 1:50). Prior to labeling, the cells were cultured for 24 h in serum free-RPMI with or without 10 ng/ml E-64 then dispersed, reseeded at a density of 10^5 cells/well into 96-well plates, stimulated with 10 ng/ml of IGF-I for 10 min, and incubated for an additional 30 min at 37°C. Labeled cells were fixed in phosphate-buffered saline containing 1% formalin and analyzed using a FACScalibur system (Becton-Dickinson, San Jose, CA).

Ligand Proteolysis Assays—Proteolysis of IGF-I was measured using the soluble endosomal extract prepared from rat liver parenchyma (1 ng), and cell lysates (3–15 ng) were derived from H-59 and MCF-7 cells cultured for 24 h with or without 10 μg/ml E-64, lysed by incubation in 50 mM phosphate buffer, pH 7.4, containing 0.5% Triton X-100, 0.5% deoxycholate, and 0.2 M NaCl for 30 min at 4°C and then clarified by centrifugation at 30,000 × g for 30 min. These preparations were incubated for various lengths of time at 37°C with 10^-4 M unlabeled or 50,000 cpm [125I]-labeled IGF-I in 200 or 400 μl of 50 mM citrate-phosphate, pH 5, respectively. The integrity of the radiolabeled ligand was assessed by precipitation with 10% trichloroacetic acid (9, 10, 15).

To measure proteolysis of the unlabeled IGF-I, the samples were acidified with acetic acid (15%) and immediately loaded onto a reverse-phase HPLC column. Reverse-phase HPLC was performed on a Waters 2765 HPLC system (Becton-Dickinson, San Jose, CA). Samples were chromatographed using an eluent mixture of 0.1% trifluoroacetic acid in water (Solvent A) and 0.1% trifluoroacetic acid in acetonitrile (Solvent B) with a flow rate of 1 ml/min. Elution was carried out using two sequential linear gradients followed by the following isocratic elution: an initial gradient of 0–20% Solvent B (30 min); a second gradient of 20–39% Solvent B (15 min); and a third isocratic elution of 39% Solvent B (15 min). Eluates were monitored on-line for absorbance at 214 nm with a liquid chromatography spectrophotometer.

Immunoprecipitation and Western Blot Analysis—MCF-7 and H-59 cells were treated with 10 ng of IGF-I for 5 min following or not pretreatment with E-64 or CA074-ME as described above. Cells were then washed with phosphate-buffered saline, solubilized in 30 ml Hepes, pH 7.4, 150 mM NaCl, 1% Triton X-100 and spun at maximal speed in a microfuge for 15 min at 4°C. Cell lysates (1 to 3 mg) were then immunoprecipitated, respectively, with anti-Shc, anti-IRS-1 (from B. I. Posner), or anti-IGF-II overnight at 4°C. Immunoprecipitates were collected by addition of protein A-Sepharose beads, washed three times with lysis buffer, and resuspended in Laemmli sample buffer (22).

Immunoprecipitates were resolved by SDS polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes followed by either immunoblotting with anti-phosphotyrosine antibodies (Sigma) or RC-20 (Transduction Laboratories) conjugated to HRP or with anti-IRS-1 (Calbiochem), anti-Shc, or anti-IGF-II antibodies. The blots were revealed by enhanced chemiluminescence followed by radioautography on Kodak X-Omat AR films.

RESULTS

Abrogation of IGF-IR Functions by the Cysteine Proteinase Inhibitor E-64—DNA synthesis, anchorage-independent growth, and production of the matrix metalloproteinase MMP-2 are three IGF-I-regulated cellular functions that are critical for the expression of the malignant phenotype (4, 18). Treatment of MCF-7 and H-59 cells with the cysteine protease inhibitor E-64 at the non-toxic concentration of 10 μg/ml (12) reduced by factors of 7 and 10, respectively, the cloning efficiency of these cells in semi-solid agar (Fig. 1A) and abrogated [3H]thymidine incorporation in response to IGF-I in both cell lines (Fig. 1B).

Furthermore, MMP-2 mRNA synthesis in serum-containing medium, which was previously shown to depend on IGF-II expression in these cells (23), was reduced by a factor of 2, and this was reflected in reduced levels of MMP-2 protein levels in H-59 conditioned medium analyzed by immunoblotting and gelatin zymography (Fig. 1C). IGF-I-induced MMP-2 production and activity measured following addition of 10 ng/ml IGF-I to serum-starved cells were likewise reduced (Fig. 1C).
dopeptidases such as cathepsin B and L are inhibited by E-64 and have been implicated in the processing of receptor-ligand complexes (9, 10). We postulated that IGF-I receptor-mediated cellular functions in E-64-treated cells were altered as a consequence of perturbed endosomal processing of the internalized receptor-bound IGF-I. Changes in IGF-I proteolysis were therefore investigated in lysates of E-64-treated tumor cells, as well as in isolated liver parenchymal endosomal fractions, which were incubated with exogenous IGF-I at acidic pH values. Reverse-phase HPLC analysis revealed that IGF-I degradation products, which were detectable in the untreated preparations, were absent following E-64 treatment (Fig. 2, A and C). This was subsequently confirmed when cell lysates and endosomal fractions were incubated with radiodinated IGF-I for 1 h, and trichloroacetic acid precipitation was used to monitor ligand integrity. An increase in trichloroacetic acid-soluble radioactivity over time was evident in the untreated preparation, but this was completely abolished by E-64 pretreatment (Fig. 2, B and D) indicating that IGF-I proteolysis was dramatically reduced.

Reduced Cell Surface Levels of IGF-IR in E-64-treated Cells—One possible consequence of ligand proteolysis blockade is the endosomal trapping of receptor-ligand complexes leading to a decreased availability of free receptor for recycling at the cell surface. We measured the effect of E-64 treatment on cell surface IGF-IR receptor levels on H-59 and MCF-7 cells. Ligand binding analysis revealed that the number of IGF-I binding sites as measured after the addition of 125I-IGF-I to H-59 cells was reduced by more than 2-fold, from 3.9 × 10^4 sites/cell on untreated to 1.8 × 10^4 sites/cell on E-64-treated cells (Fig. 3A). Flow cytometric analysis with a monoclonal antibody (mAb α IR3) to the α subunit of the human IGF-I receptor revealed that 40 min after the addition of ligand to serum-starved MCF-7 cells, there was a reduction of 45% in the number of immunolabeled cells with the mean intensity of fluorescence declining from 255 to 82 (Fig. 3B). In neither of these cell types did E-64 treatment cause a reduction in IGF-IR mRNA levels (Fig. 3C) or in the total level of immunoprecipitable receptor (Fig. 3D). These experiments suggested that the reduction of IGF-IR expression at the cell surface was not because of a change in receptor transcription or translation.

Increased Levels of Tyrosine-phosphorylated IGF-IR and Substrates in Cells Treated with Cysteine Protease Inhibitors—One of the earliest molecular events in IGF-IR ligand-induced signaling is the autophosphorylation of tyrosine residues on the receptor β subunit and the subsequent phosphorylation of downstream substrates such as IRS-1 and Shc. We first measured ligand-induced tyrosine phosphorylation of the receptor in E-64- and ME-treated cells by immunoprecipitation with anti-IGF-IR antibodies followed by immunoblotting with either anti-phosphotyrosine antibodies or anti-IGF-IR antibodies. Three experiments were performed for each condition. In all the experiments, we found an increase in tyrosine-phosphorylated receptor β subunit in inhibitor-treated cells. For ME-treated cells the means of the increases were 3.75 (p < 0.0007, n = 3) and 2.08 (p < 0.001, n = 3)-fold, and for ME-treated cells they were 2.1 (p < 0.002, n = 3)- and 1.33 (p < 0.005, n = 3)-fold compared with IGF-I-treated H-59 and MCF-7 cells, respectively (Fig. 4, A and B). The different responses to the two inhibitors may reflect a higher efficiency of intracellular uptake of ME because of its increased membrane permeability. Two major substrates of the IGF-IR are Shc and IRS-1. Their interactions with the activated IGF-IR have been mapped to the Tyr^950 residue suggesting that they could potentially compete for binding (24). In MCF-7 cells, a 1.7-fold increase in tyrosine-phosphorylated IRS-1 was noted in ME-treated cells stimulated with IGF-I as compared with cells stimulated with IGF-I only (Fig. 4D, n = 3, p < 0.0008), but no significant change in p52^shc tyrosine phosphorylation was noted (data not shown). In H-59 cells, on the other hand, p52^shc tyrosine phosphorylation increased by 1.9-fold (n = 2) in response to ligand binding in E-64- or ME-pretreated cells (Fig. 4C, upper and middle panels), whereas Shc expression per se did not change (Fig. 4C, bottom panel, n = 3). In these cells, no detectable levels of IRS-1 were observed by Western blotting or immunoprecipitation using experimental conditions that identified this protein in MCF-7 cells. It appears therefore that in these cells, differential expression levels of the adaptor proteins Shc and IRS-1 may determine their preferential IGF-IR binding and phosphorylation in response to IGF-I.

**DISCUSSION**

Our results show that inhibition of cysteine protease activity by E-64 resulted in reduced cell surface IGF-IR expression levels and abrogation of cellular responses to IGF-I. In an apparent paradox, however, treatment with this or a second cathepsin B inhibitor, CA074-ME, also caused an increase in tyrosine phosphorylation of the IGF-IR β subunit, IRS-1, and Shc.

When taken together with our findings that IGF-I proteolysis was blocked in E-64-treated liver parenchymal endosomes and in tumor cell lysates, our results indicate that inhibition of processing of the IGF-IR/IGF-I complex has the following two
major consequences: (i) receptor recycling to the plasma membrane is decreased, and (ii) the IGF-IR β subunit and the IRS-1/Shc substrates remain hyperphosphorylated, and this attenuates rather than activates IGF-IR-mediated biological functions such as the induction of DNA synthesis and MMP-2 production. A direct effect of cysteine proteinase inhibitors on functions such as the induction of DNA synthesis and MMP-2 production. Receptor phospho-

It has been clearly shown that ligand degradation is a key event in receptor recycling (11, 15) and signaling (27). Recently it was reported that anti-p185/HER2 antibody-mediated targeting of a cysteine proteinase inhibitor to a cathepsin B-containing intracellular compartment resulted in growth inhibition of two breast carcinoma cell lines including MCF-7 (32). Our model offers mechanistic insight into these observations, suggesting that growth impairment in these cells was related to defective ligand processing in the endosomes. Collectively, the results identify the endocytic apparatus as a critical component of growth factor receptor signaling that can be accessible and sensitive to specific proteinase inhibitors.

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Fig. 5. Schematic representation of the postulated effects of the proteinase inhibitors E-64/ME on IGF-I-induced DNA synthesis. En, endosomes; E-64 Comp, E-64/ME-dependent compartment.