Insulin-like Growth Factor-I is an Autocrine Regulator of Chromogranin A Secretion and Growth in Human Neuroendocrine Tumor Cells

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ABSTRACT

Carcinoid tumors are predominantly found in the gastrointestinal tract and are characterized by hypersecretion of various substances, including bioamines and neuropeptides, leading to functional tumor disease. Here, we demonstrate that human BON carcinoid tumor cells express functionally active insulin-like growth factor-I (IGF-I) receptors and secrete IGF-I, suggesting an autocrine action of this growth factor. The IGF-I receptor was functionally active. IGF-I stimulated phosphatidylinositol 3-kinase (PI3-kinase), mammalian target of rapamycin/p70s6k, and extracellular signal-regulated kinase 2 activity in BON cells. Furthermore, immunoneutralization of endogenously released IGF-I markedly reduced the high basal activity of p70s6k and extracellular signal-regulated kinase 2 in serum-starved BON cells. Exogenously added IGF-I induced a marked increase in chromogranin A secretion, a marker protein for neuroendocrine secretion, by a process that was largely dependent on PI3-kinase activity. In addition, immunoneutralization of endogenously released IGF-I markedly reduced basal chromogranin A release by BON cells. Thus, the autocrine IGF-I loop regulates basal neuroendocrine secretion in BON cells. Next, we investigated the role of IGF-I as a growth promoting agent for BON cells. Our data demonstrate that IGF-I stimulates anchorage-dependent and anchorage-independent growth of BON cells by a pathway that involves PI3-kinase, mammalian target of rapamycin/p70s6k, and mitogen-activated protein kinase kinase 1 activity. Interestingly, mitogen-activated protein kinase kinase 1 activity was less important for anchorage-independent growth of BON cells. Endogenously released IGF-I was found to be largely responsible for autonomous growth of BON cells in serum-free medium and for the constitutive expression of cyclin D1 in these cells. In conclusion, IGF-I is a major autocrine regulator of neuroendocrine secretion and growth of human BON neuroendocrine tumor cells. Because our data also demonstrate that a significant proportion of neuroendocrine tumors express the IGF-I receptor and its ligand, interference with this pathway could be useful in the treatment of hypersecretion syndromes and growth of human neuroendocrine tumors.

INTRODUCTION

Carcinoid tumors are neuroendocrine neoplasms that are derived from neuroectodermal cells of the neural crest (1). These tumors are predominantly found in the gastrointestinal tract, although they may arise in various organs throughout the body. Carcinoids are characterized by hypersecretion of various substances, including bioamines and neuropeptides, leading to functional tumor disease. BON cells have been established from a human pancreatic carcinoid tumor and are a useful model to study the biology of neuroendocrine tumors in vitro (2).

Neuropetides (3) and polypeptide growth factors, such as nerve growth factor and fibroblast growth factor (4, 5), have been implicated in the regulation of neuroendocrine tumor cell growth. In addition, the presence of IGF-I and the IGF-I receptor has been reported in neuroendocrine tumors, such as midgut carcinoids (6, 7). However, the signaling pathways induced by IGF-I and its precise role for secretion and/or growth in human neuroendocrine tumors are largely unknown. IGF-I is a 70-amino acid peptide closely related to insulin that binds to distinct high affinity receptors with intrinsic tyrosine kinase activity. Upon binding to the IGF-I receptor, IGF-I stimulates cell cycle progression and growth in various cell lines (8–10). In addition, IGF-I and the IGF-I receptor have been implicated in multistage carcinogenesis: overexpression of the IGF-I receptor or its ligand IGF-I causes abnormal growth, cellular transformation, inhibition of apoptosis, and spontaneous tumor formation in transgenic mice (11, 12). Furthermore, expression of the IGF-I receptor is crucial for tumorigenesis in athymic mice (10, 13).

Here, we report that human BON carcinoid cells express functionally active IGF-I receptors and secrete IGF-I. Exogenously added IGF-I stimulated PI3-kinase, p70s6k, and ERK activity in BON cells. The endogenously released IGF-I was found to be largely responsible for the high basal activity of p70s6k and ERK2 in serum-starved BON cells. Exogenously added IGF-I markedly increased chromogranin A secretion by BON cells by a PI3-kinase-dependent pathway. This kinase also mediated autocrine IGF-I secretion. Immunoneutralization of endogenously released IGF-I substantially reduced basal chromogranin A release. These data demonstrate, for the first time, the existence of an autocrine IGF-I loop regulating neuroendocrine secretion in a carcinoid tumor cell line. In addition, both exogenously added and endogenously released IGF-I stimulated growth of BON cells by a PI3-kinase, p70s6k, and MEK-1-dependent signaling pathway. At the level of the cell cycle, endogenously released IGF-I was found to selectively regulate the expression of cyclin D1 and p27kip1. Thus, neuroendocrine secretion and autonomous growth of human BON tumor cells are largely regulated by endogenously released IGF-I. Because the presence of IGF-I and the IGF-I receptor can be demonstrated in various neuroendocrine tumors, the IGF-I signaling pathway is a potential novel target for the treatment of hypersecretion syndromes and growth of these tumors.

MATERIALS AND METHODS

Cell Culture. Human BON carcinoid tumor cells were maintained in DMEM medium supplemented with 10% (v/v) fetal bovine serum in a humidified atmosphere of 5% CO2; 95% air at 37°C and passed every 4 days. MiaPaCa-2, COS, LCC18, and HEK 293 cells were maintained in DMEM supplemented with 10% (v/v) fetal bovine serum in a humidified atmosphere of 5% CO2, 95% air at 37°C and passed every 3 days. 125I-IGF-I Binding Assays. IGF-I equilibrium-competition-inhibition binding studies were performed as described previously (14). Briefly, BON...
cells (1–2 × 10^6/dish) were washed twice with PBS and once with binding buffer and then incubated for 1 h at room temperature with 1^25I-IGF-I (10 pm), and different concentrations of unlabeled IGF-I, r3-IGF-I, and insulin (each at 10 pm to 0.1 μM). To assess whether binding of IGF-I changes during cell proliferation, binding assays with 125I-IGF-I or 125I-rIGF-I were performed in BON cells cultured for 24, 36, 48, and 72 h. At the end of each experiment, cells were washed three times with PBS containing 0.1% BSA. In all experiments, degradation of tracer was less than 10%, thus excluding differences in binding caused by hormone degradation. Cell bound and free intact activity were counted in an automatic gamma counter (Berthold, Munich, Germany) with 70% efficiency. Specific binding was determined by subtracting the amount of 125I-IGF-I or 125I-rIGF-I unspecifically bound (<0.5%) in the presence of 0.1 μM IGF-I and ranged between 5 and 10% in all experiments. A computer-assisted protein kinase fitting program was used in the presence of various concentrations of unlabeled peptide yielding a 50% inhibition (IC50) of 125I-tracer binding (15). As described by Scatchard (16), the ratio of bound to free hormone was plotted as a function of total hormone, and the number of binding sites per cell and binding affinity (Kd values) were calculated.

**Western Blotting.** BON cells were washed twice in serum-free DNM and incubated in fresh serum-free medium for further 24 h. Cells were then treated with factors as indicated in the legends to Figs. 3 and 6 and lysed in 50 mM Tris-HCl, 5 mM EDTA, 100 mM NaCl, 20 mM β-glycerophosphate, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, pH 7.6 (lysis buffer). For detection of the IGF-I receptor in HEK 293, BON, COS, MiaPaCa-2, and LCC18 cells, serum-starved cells were lysed in lysis buffer, and protein content was determined. Proteins were subsequently extracted in 5× SDS-PAGE sample buffer. Equal amounts of proteins were further analyzed by SDS-PAGE and Western blotting with a polyclonal anti-IGF-I receptor antibody, followed by ECL detection. For p70^s6k mobility shift assays and detection of cyclin D1, cyclin E, and p27^Kip1, cells were treated as indicated in the legends to Figs. 3 and 6 and lysed in SDS-PAGE sample buffer, and samples were further analyzed by SDS-PAGE and Western blotting using specific antisera to these proteins.

**P13-Kinase Assays.** Serum-starved BON cells were incubated with IGF-I for 10 min as indicated, subsequently lysed in lysis buffer, and incubated with an antiphosphotyrosine antibody for 2 h with antimouse IgG agarose added for the second h. Immunoprecipitates were subsequently washed three times with lysis buffer, twice with Buffer A (0.5 M LiCl, 0.1 M Tris, pH 7.4) and once with Buffer B (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA). Immunoprecipitates were subsequently incubated in the kinase reaction mix containing Buffer B, 5 mM MgCl2, 50 μM ATP, 5 μCi/ml [γ-32P]ATP, and 20 μg of phosphatidylinositol 5 in 500 μM HEPES, pH 7.4, per condition for 25 min at 25°C. Reactions were stopped by adding 100 μl of 1 M HCl and 200 μl of a mixture of CHCl3 and methanol (1:1, v/v). Samples were mixed by vortexing and subsequently briefly centrifuged. The CHCl3 phase was further purified by mixing with HCl/methanol (1:1, v/v). Finally the CHCl3 phase was transferred into a new tube, dried under N2, and subsequently run on TLC plates in a mixture containing H2O, CHCl3, methanol, aceton, and glacial acetic acid (16:30:26:30:24, v/v). TLC plates were then developed by autoradiography. Autoradiographs were scanned using the UMAX Vistascan program (version 3.1).

**p70^S6K and ERK2 Immune Complex Kinase Assays.** Serum-starved BON cells were incubated with IGF-I in the presence or absence of rapamycin or PD 098059 as indicated in the legend to Fig. 3. Controls received an equivalent amount of solvent. Cells were then lysed at 4°C in 1 ml of lysis buffer. Immunoprecipitations were performed at 4°C using an anti-p70^S6K or anti-ERK2 antibody for 2 h, with protein A-agarose added for the second h. Immunocomplexes were washed three times in lysis buffer and once with p70^S6K kinase buffer (20 μM HEPES, pH 7.4, 10 mM MgCl2, 1 mM DTT, and 10 mM β-glycerophosphate) or ERK kinase buffer (15 mM MgCl2, 15 mM Tris-HCl, pH 7.4). Kinase reactions were performed by resuspending the protein A-Sepharose pellets in 25 μl of kinase assay mixture containing the appropriate kinase buffer with 0.2 mM S6 peptide (RRRLSS-LRA) or myelin basic protein, 20 μM ATP, 5 μCi/ml [γ-32P]ATP, 2 μM cAMP-dependent protein kinase inhibitor peptide, and 100 μM microcystin LR. Incubations were performed under linear assay conditions at 30°C for 20 min and terminated by spotting 25 μl of the supernatant onto Whatman p81 chromatography paper. Papers were washed four times for 5 min in 0.5% o-phosphoric acid, immersed in acetone, and dried before scintillation counting. The average radioactivity of two blank samples containing no immune complex was subtracted from the result of each sample.

**Measurement of Chromogranin A.** To determine chromogranin A secretion, BON cells were incubated in Krebs-Ringer-HEPES buffer for 2 h at 37°C. Cells were subsequently treated with IGF-I for 25 min at 37°C in the absence or presence of various inhibitors as indicated in the legend to Fig. 4. The supernatant was then aspirated and stored at −80°C until assayed. Chromogranin A was subsequently determined in the supernatants using a specific chromogranin A ELISA. Release is expressed as fold stimulation above untreated controls.

**Determination of IGF-I Secretion.** To characterize the autocrine release of IGF-I, cells were washed three times in serum-free medium and incubated in conditioned medium from the presence of various compounds as indicated. Medium was aspirated on specific days as indicated in the legend to Figs. 2 and 4 and stored at −80°C until assayed. IGF-I concentration was determined using a specific RIA. Values are expressed as ng of IGF-I/ml of supernatant.

**Analysis of IGFBP-2 Expression in BON Cell Conditioned Media.** Conditioned media were analyzed by Western ligand blot analysis according to the method of Hossenlopp (17). Briefly, media were concentrated as described previously (18), diluted 1:5 with sample buffer [50 mM NaHPO4, pH 7.0, 1% (w/v) SDS, 50% (w/v) glycerin] and boiled (5 min), and proteins were separated by SDS-PAGE. Separated proteins were transferred to a nitrocellulose membrane. The blots were blocked with 1% fish gelatin and incubated with 125I-IGF-II (10^6 cpm per blot). Binding proteins were visualized on a PhosphorImager Storm (Molecular Dynamics, Krefeld, Germany). All hybridization and washing steps were performed at 4°C. IGFBP-2 in the conditioned media was identified by Western immunoblot analysis using antisera to human IGFBP-2 (kindly provided by Dr. M. Eilinger, Universität-Kinderklinik Tübingen, Tübingen, Germany). Membranes were prepared as described above and incubated with human IGFBP-2 antiserum (1/1000) for 1 h, followed by peroxidase-coupled antirabbit IgG antibody. Signals were generated using dianimobenzidine.

**Immunostaining of Cells and Cryostat Sections.** Serum-starved cultures of BON cells were washed with PBS and fixed in 4% formaldehyde at room temperature for 20 min. Cells were then permeabilized with 0.2% Triton X-100 and stained with specific anti-IGF-receptor or anti-IGF-I antibodies for 1 h followed by detection with an Alexa-labeled secondary antibody. Samples were further analyzed by confocal microscopy. Cryostat sections of neuroendocrine tumor specimens obtained endoscopically or surgically were fixed in 4% ethanol-formaldehyde at room temperature for 30 min. Tissue sections were then permeabilized with 0.1% Triton X-100 for 10 min and preblocked with 2% BSA (w/v) for 2 h at room temperature. Sections were subsequently incubated overnight in 2% BSA (w/v) with a monoclonal anti-IGF-I antibody or a polyclonal anti-IGF-I receptor antibody. Tissues were then incubated with Alexa-labeled antirabbit or antimouse antibodies and further analyzed by immunofluorescence microscopy.

**Growth Assay.** BON cells, 3 days postpassage, were washed in serum-free DNM, trypsinized, and resuspended in serum-free DNM. Cells were plated at a density of 1 × 10^3 cells in 1 ml of serum-free DNM in the presence or absence of 20 ng/ml rapamycin in duplicate. At the times indicated in the figure legends, cell number was determined using a cell counting chamber.

**Clonogenic Assay.** BON cells were washed, trypsinized, and resuspended in DNM. Cell number was determined using a cell counting chamber. Cells (3 × 10^4) were mixed with serum-free DNM containing 0.3% agarose in the presence or absence of rapamycin at the concentrations indicated and layered over a solid base of 0.5% agarose in serum-free DNM in the presence or absence of rapamycin at the same concentrations in 33-mm dishes. The cultures were incubated in humidified 5% CO2/95% air at 37°C for 14 days and then stained with the vital stain nitroblue tetrazolium. Colonies of >120 μm in diameter (20 cells) were counted using a microscope.

**Statistical Analysis.** Cerebrovolumes of the ERK and p70^S6K immune complex kinase assays (n = 6 for each condition) were tested for normal distribution and extreme values. The levels of significance were determined by Pearson correlation.

**Materials.** The monoclonal antibody against Tyr(P) (clone 4G10), the monoclonal anti-IGF-I antibody used for the immunoneutralization experiments, and the polyclonal antibodies against IRS-2, the p85 subunit of PI3-
kinase, and the N-terminally directed anti-p70S6K polyclonal antibody used to determine p70S6K activity were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). The polyclonal antibodies directed against the IGF-I receptor, IRS-1, p70S6K, p27kip1, cyclin D1, and cyclin E were from Santa Cruz Biotechnology (Santa Cruz, CA). Peroxidase-coupled antirabbit IgG antibody was from Dianova (Hamburg, Germany). The polyclonal anti-ERK2 antibody was the kind gift of Dr. Jo van Lint (Katholieke Universiteit Leuven, Leuven, Belgium). Rapamycin, Ly 294002, and GF 109203X were from Calbiochem-Novabiochem (Schwalbach, Germany). PD 098059 was from New England Biolabs (Schwalbach, Germany). Protein A-Sepharose was obtained from Roche Molecular Biochemicals (Mannheim, Germany). Human IGF-I, ECL reagent, and [γ-32P]ATP were obtained from Amersham Pharmacia Biotech (Freiburg, Germany). The Alexa red-labeled and Alexa green-labeled antirabbit and antirabbit IgG antibodies were from Molecular Probes (Leiden, the Netherlands). The ELISA kit to determine chromogranin A in the supernatants was from DAKO Diagnostica GmbH (Hamburg, Germany). The RIA to determine IGF-I in the supernatants was from Nichols Institute Diagnostics (San Juan, CA). Nitrocellulose membranes and polyvinylidene difluoride membranes for Western blotting were obtained from Millipore (Eschborn, Germany). All other reagents were of the purest grade available.

RESULTS

Human BON Carcinoid Tumor Cells Express Functional IGF-I Receptors and Secrete IGFBP-2. The expression of the IGF-I receptors in BON cells was examined by Western blotting. A specific anti-IGF-I receptor antibody detected a single band migrating with a Mr of 90,000 in BON cells corresponding to the β chain of the IGF-I receptor. Comparison of IGF-I receptor expression in BON cells with other human (HEK 293, MiaPaCa-2, and LCC18) or monkey (COS) cell lines by Western blotting revealed that IGF-I receptor expression in BON cells was comparable to cell lines that exhibit high levels of IGF-I receptor expression (Fig. 1A, top panel). IGF-I receptor immuno-reactivity was mainly detected at the plasma membrane and around the nucleus (Fig. 1A, bottom panels).

Binding of 125I-IGF-I to the IGF-I receptor was assessed using increasing concentrations of IGF-I, r3-IGF-I, and insulin as competitive ligands. Consistent with typical IGF-I receptors (Fig. 1B, top panel), IGF-I competed for 125I-IGF-I binding with approximately 100-fold higher affinity than insulin.

Secretion and identification of IGFBP-2 in the conditioned media of BON cells by Western ligand analysis (top panel) and immunoblot analysis (bottom panel). Cells were kept up to 6 days under serum-free conditions before IGFBP expression was analyzed as described in “Materials and Methods” using rabbit anti-hIGFBP-2 antiserum.
lower affinity (IC$_{50}$ 71 ± 3 nM). Scatchard analysis (Fig. 1B, bottom panel) revealed a single class of IGF-I receptors (72700 ± 2000 sites/cell, $K_d = 0.77 ± 0.1$). As shown in Fig. 1B, bottom panel, the number of IGF-I receptors markedly increased during BON cell proliferation, reaching a maximum after 48 h.

Interestingly, r3-IGF-I, which does not cross-bind with IGFBPs, showed a slightly higher affinity (IC$_{50}$ 0.4 ± 0.1 nM) than IGF-I (Fig. 1B, top panel), suggesting the existence of IGFBPs in the supernatant of BON cells. As demonstrated by ligand blotting, only a single class of IGFBPs could be detected in BON cell supernatants (Fig. 1C, top panel). This band was identified as IGFBP-2 by Western blotting (Fig. 1C, bottom panel). The amount of IGFBP-2 increased in the supernatant of serum-starved cells with time, reaching a maximum 5 days after plating of the cells.

To examine whether the increase in $^{125}$I-IGF-I binding with time could be attributable to cross-binding of $^{125}$I-IGF-I to IGFBPs, we performed binding assays using $^{125}$I-r3-IGF-I, which acts as a ligand for the IGF-I receptor but not for IGFBPs. These experiments revealed binding data very similar to those obtained with $^{125}$I-IGF-I: using $^{125}$I-r3-IGF-I, a 1.7-fold increase in IGF-I receptor number/cell was detected after 48 h of incubation (data not shown). Thus, the increase in $^{125}$I-IGF-I binding was not caused by increased binding to IGFBPs but was indeed the result of an increase in receptor numbers.

BON Cells Secrete IGF-I. It has been speculated that IGF-I could act on neuroendocrine tumors by an autocrine mechanism (6). Indeed, IGF-I could be demonstrated in the cytoplasm of human BON cells by immunocytochemistry using a specific anti-IGF-I antibody (Fig. 2, left and middle panels). To examine whether IGF-I was also secreted by BON cells, we determined the amount of IGF-I in the supernatants of serum-starved BON cells at various times. As shown in Fig. 2, right panel, IGF-I concentrations in the supernatant of serum-starved BON cells increased in a time-dependent manner: a maximum concentration of 15 ng IGF-I/ml of conditioned medium was obtained 7 days after plating of the cells. These data suggest the existence of an autocrine loop involving IGF-I in human BON cells. To substantiate these findings, we examined a panel of various human neuroendocrine tumors by immunohistochemistry using specific antibodies against IGF-I and the IGF-I receptor. As shown in Table 1, all but one neuroendocrine tumor exhibited positive staining for the IGF-I receptor. IGF-I immunoactivity was also detected in a large proportion of tumor specimens either in the tumor itself and/or in the surrounding connective tissue, suggesting an autocrine and/or paracrine mechanism of action.

IGF-I Activates PI3-kinase, p70s6k, and ERKs in BON Cells. The IGF-I receptors in human BON carcinoid cell lines were functionally active: incubation of BON cells with IGF-I led to a marked increase in tyrosine phosphorylation of the IGF-I receptor and of two major IGF-I receptor substrates, IRS-1 and IRS-2 (data not shown). IGF-I markedly stimulated the activation of PI3-kinase, which could be prevented by treatment of cells with the selective PI3-kinase inhibitor LY 294002 (Fig. 3A, left panel). Similar results were obtained with a different PI3-kinase inhibitor, wortmannin (data not shown). The effect of LY 294002 on PI3-kinase activation was selective, as treatment of cells with the MEK-1 inhibitor PD 098059 did not block IGF-I-induced PI3-kinase activation (data not shown). PI3-kinase is composed of two subunits, a $M_r$ 110,000 catalytic subunit and a $M_r$ 85,000 regulatory subunit, which possesses two Src-homology 2 domains. Both of these Src-homology 2 domains specifically associate with tyrosine-phosphorylated IRS-1 and IRS-2 (19). To examine which IRS would mediate PI3-kinase activation in response to IGF-I, we determined the IRS-1- and IRS-2-associated PI3-kinase activity in BON cells stimulated with IGF-I. Cells were treated with 100 ng/ml IGF-I for 10 min and lysed. The lysates were immunoprecipitated with specific anti-IRS-1 or anti-IRS-2 antibodies and further analyzed by PI3-kinase assays. A 2- and 1.5-fold increase in PI3-kinase activity could be detected in IRS-1 and IRS-2 immunoprecipitates, respectively (Fig. 3A, middle and right panels). Thus, the IGF-I and the IGF-I receptor are expressed in a variety of neuroendocrine tumors

<table>
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<tr>
<th>Tumor type (total n = 11)</th>
<th>IGF-I</th>
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<tr>
<td></td>
<td>Tumor</td>
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<tr>
<td>Carcinoid (n = 6)</td>
<td>3/6</td>
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<td>Phaeochromocytoma (n = 2)</td>
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<tr>
<td>Gastrinoma (n = 2)</td>
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<tr>
<td>Insulinoma (n = 1)</td>
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Cryostat sections of six carcinoid tumors, two pheochromocytomas, two gastrinomas, and one insulinoma were stained as described in "Materials and Methods" using antibodies directed against chromogranin A, IGF-I, and the IGF-I receptor. All tumors exhibited positive chromogranin A staining. About 60% of the tumors (7 of 11) stained positive for IGF-I. Interestingly, 10 of 11 tumors exhibited IGF-I immunoreactivity within the tumor and/or in the surrounding connective tissue (ct). Ninety percent (10 of 11) of the tumors examined stained positive for the IGF-I receptor. The tissue sections of the pheochromocytomas did not contain any connective tissue (indicated by @).
**IGF-I is an Autocrine Regulator of Chromogranin A**

PI3-kinase regulates several downstream targets, one of which is p706k (20). To determine whether IGF-I-induced PI3-kinase activation was sufficient to trigger the activation of downstream targets, we examined p706k activation in response to IGF-I. As shown in Fig. 3B, stimulation of cells with IGF-I induced a moderate but significant increase in p706k activity in immune complex kinase assays (P < 0.02). IGF-I-stimulated p706k activity could be prevented by treatment of cells with the PI3-kinase inhibitor LY 294002, as well as the specific inhibitor of p706k activation, rapamycin (21). Rapamycin and LY 294002 also strikingly inhibited basal p706k activity in BON cells, suggesting that this kinase was constitutively active in BON cells. In contrast, the MEK-1 inhibitor PD 098059 had no effect on basal and IGF-I-induced p706k activity (Fig. 3B, inset).

IGF-I also stimulated activation of ERK2 in human BON cells: as shown in Fig. 3C, 100 ng/ml IGF-I induced a moderate but significant increase in ERK2 activity (P < 0.01). Similar results were obtained in ERK1 immune complex kinase assays (data not shown). IGF-I-induced ERK2 activation could be prevented by treatment of cells with the selective MEK-1 inhibitor PD 098059 (Ref. 22; Fig. 3C). PD 098059 also reduced basal ERK2 activation by 40%, suggesting that ERK2 is also constitutively active in these cells. Because IGF-I could activate PI3-kinase in BON cells, we examined whether this kinase could be an upstream regulator of ERK activation in BON cells. Treatment of cells with the selective PI3-kinase inhibitor LY 294002 reduced basal and IGF-I-stimulated ERK2 activation. Thus, IGF-I-stimulated ERK activation requires the activation of MEK-1 and PI3-kinase (Fig. 3C).

**Constitutive Activation of ERK2 and p706k in Human BON Cells Is Caused by Autocrine IGF-I Secretion.** As shown in Fig. 3, A and B, both p706k and ERK2 can be further activated by exogenously added IGF-I but exhibit a high basal activity in serum-starved BON cells (Fig. 3, B and C). Therefore, we examined whether basal activation of these kinases in BON cells could be attributable to stimulation by the endogenously released IGF-I. Serum-starved BON cells were incubated with the immunoneutralizing antibody directed against IGF-I, and subsequently, p706k and ERK2 activities were determined. Activation of p706k by mitogens can be determined by the appearance of slower migrating forms in SDS-PAGE attributable to phosphorylation of p706k on Thr229, Thr389, and Ser404, which are not basally phosphorylated in quiescent cells (23). As shown in Fig. 3D, left panel, incubation of cells with the IGF-I-blocking antibody reduced basal phosphorylation of p706k in BON cells as demonstrated by the increased electrophoretic mobility of the protein in SDS-PAGE. Incubation with the IGF-I-blocking antibody was almost as efficient in inducing dephosphorylation of p706k as treatment with the selective inhibitor of p706k activation, rapamycin. Upon treatment of cells with the IGF-I-blocking antibody basal kinase activity of ERK2 was also decreased by 50% in immune complex kinase assays. Indeed, inhibition of basal ERK2 activation by the IGF-I-blocking antibody was comparable to the maximal inhibitory effect of the MEK-1 inhibitor PD 098059 in BON cells (Fig. 3D, right panel). Thus, endogenously released IGF-I is largely responsible for constitutive activation of both the ERK cascade and p706k in human BON cells.

**Exogenously Added and Endogenously Released IGF-I Stimulate Chromogranin A Release in Human BON Carcinoid Tumor Cells.** Carcinoid tumors are characterized by hypersecretion of various neuroendocrine marker proteins such as chromogranin A (24). There are few reports demonstrating that members of the IGF family can regulate exocytosis in cultured cells (25, 26). Having established a functionally active IGF-I signaling pathway in BON cells, we examined whether IGF-I could stimulate neuroendocrine secretion in these cells as judged by the secretion of chromogranin A into the...
supernatant of cells. As expected, chromogranin A could be detected in the cytoplasm of BON tumor cells by immunocytochemistry (data not shown). Incubation of BON cells with 100 ng/ml IGF-I for 25 min led to a 5-fold increase in chromogranin A release into the supernatant. Chromogranin A release in response to IGF-I reached about 60% of the maximal secretory response induced by PDB, a potent secretagogue for BON cells (Ref. 27; Fig. 4A, left panel).

If IGF-I plays a major role in the regulation of neuroendocrine secretion in BON cells, endogenously released IGF-I should contribute to basal chromogranin A secretion. Therefore, IGF-I in the supernatants of cells was immunoneutralized by adding an IGF-I-blocking antibody. As shown in Fig. 4A, right panel, this treatment led to a marked, 45% inhibition of basal chromogranin A secretion. Thus, endogenously released IGF-I indeed regulates basal chromogranin A secretion in human BON neuroendocrine tumor cells.

Chromogranin A Secretion by IGF-I Is Mediated by PI3-kinase. Next, we examined which of the signaling pathways induced by IGF-I could mediate chromogranin A secretion in BON cells. Activation of PI3-kinase has been implicated in the regulation of exocytosis in mast cells, neutrophils, and chromaffin cells (28–30). Observation of cells with the selective PI3-kinase inhibitor LY 294002 completely blocked chromogranin A secretion in response to IGF-I (Fig. 4B). Activation of ERKs has also been implicated in the regulation of secretion by some receptors (31). Treatment of cells with the selective MEK-1 inhibitor PD 098059 reduced IGF-I-induced chromogranin A release by about 30% (Fig. 4B). Inhibition of p70s6k activation using rapamycin reduced chromogranin A secretion in response to IGF-I also by only 35%. IGF-I has been demonstrated to activate phospholipase-sensitive members of the PKC family of serine threonine kinases (32). However, GF 109203X treatment completely abolished chromogranin A secretion in response to IGF-I only by about 35% (Fig. 4B). In contrast, chromogranin A release by PDB treatment was completely abolished when cells were treated with the selective PKC inhibitor GF 109203X but was only reduced by about 20% in the presence of the PI3-kinase inhibitor LY 294002 (Fig. 4C). These data demonstrate that IGF-I and phorbol esters induce chromogranin A secretion by distinct signaling pathways in BON cells.

Autocrine IGF-I Secretion Is Regulated by PI3-kinase. To examine whether PI3-kinase could also mediate IGF-I secretion, serum-starved BON cells were incubated in the presence or absence of LY 294002 for 80 h, and IGF-I concentrations in the supernatants were determined. As shown in Fig. 4D, endogenous IGF-I secretion was completely blocked in the presence of the PI3-kinase inhibitor LY 294002. Thus, PI3-kinase regulates both autocrine IGF-I secretion and neuroendocrine chromogranin A release in BON cells.

IGF-I Stimulates Anchorage-dependent and Anchorage-independent Proliferation of Human BON Carcinoid Tumor Cells. IGF-I and its receptor play an important role in the growth of various cells, including tumor cells (10, 33). However, its role for anchorage-dependent and anchorage-independent growth of neuroendocrine tumor cells is unknown. To examine whether IGF-I could induce proliferation of BON cells, serum-starved cells were incubated with 100 ng/ml IGF-I for various times, and cell numbers were determined at the days indicated. IGF-I stimulated proliferation of BON cells in a time-dependent manner: a maximum, 1.6-fold stimulation of cell proliferation was obtained after 7 days of incubation with IGF-I (Fig. 5, A and B, top panels). The effect of IGF-I on cell proliferation was concentration-dependent: half-maximum and maximum effects were achieved at 50 and 100 ng/ml IGF-I (data not shown).

Role of PI3-kinase, ERK, and p70s6k Activity for Autonomous and IGF-I-stimulated Anchorage-dependent Growth of BON Carcinoid Tumor Cells. Next, we examined the role of PI3-kinase, p70s6k, and ERK activation for basal and IGF-I-induced anchorage-dependent growth of serum-starved human BON cells. Incubation of cells with rapamycin or PD 098059 markedly inhibited basal (Fig. 5A) and IGF-I-stimulated (Fig. 5B) anchorage-dependent proliferation of BON cells, by about 70 and 65%, respectively. Incubation of cells with the PI3-kinase inhibitor LY 294002 led to an even more pronounced inhibition of basal and IGF-I-stimulated proliferation of serum-starved BON cells; they were inhibited by 80 and 75%, respectively (Fig. 5, A and B). Thus, activation of PI3-kinase, p70s6k, and the ERK cascade are important mediators of autonomous and IGF-I-stimulated anchorage-dependent growth of BON cells.

IGF-I Stimulates Anchorage-independent Growth of Human BON Cells. Tumors and transformed cells are able to grow in an anchorage-independent manner by forming colonies in agarose me-

Fig. 4. A, left panel. IGF-I stimulates chromogranin A secretion in BON cells. Serum-starved BON cells were incubated with 400 ng PDB or 100 ng/ml IGF-I for 30 min, and chromogranin A concentrations in the supernatant of cultured cells were determined using a specific ELISA. A, right panel. Immunoneutralization of IGF-I inhibits basal chromogranin A release by BON cells. Serum-starved BON cells were incubated with 25 µg/ml IGF-I neutralizing antibody for 24 h. The concentration of chromogranin A in the supernatant was subsequently determined using a specific ELISA. B, IGF-I-stimulated chromogranin A secretion is largely dependent on PI3-kinase activity. Serum-starved BON cells were incubated with 20 ng/ml rapamycin (Rapa, +), 20 µM PD 098059 (PD, +), 3.5 µM GF 109203X (GF, +), or 20 µM LY 294002 (Ly, +) for 40 min. Control cells received an equivalent amount of solvent (+). Cells were then incubated with 100 ng/ml IGF-I (IGF-I) for 25 min. The amount of chromogranin A in the supernatant of treated cells was determined as described in "Materials and Methods." C, PDB-stimulated chromogranin A release is dependent on PKC activity but independent of PI3-kinase. Serum-starved BON cells were incubated with 3.5 µM GF 109203X (GF, +), or 20 µM LY 294002 for 40 min. Control cells received an equivalent amount of solvent. Cells were then incubated with 400 ng/ml PDB (PDB, +) for 25 min. The amount of chromogranin A in the supernatants of cells was determined as described in "Materials and Methods." D, autocrine IGF-I secretion is mediated by PI3-kinase in BON cells. Serum-starved BON cells were incubated with 20 µM LY 294002 for 24 h. Control cells received an equivalent amount of solvent. The IGF-I concentration in the supernatant of treated and untreated cells was subsequently analyzed using a specific ELISA.

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The Autocrine IGF-I Loop Regulates Constitutive Expression of Cyclin D1 in Human BON Cells. Growth factors regulate progression from G1 to S. This process requires the expression of cyclins D and E, which modulate the activities of the cyclin-dependent kinases (36), and their respective inhibitors, such as p27kip1. Cyclins and cyclin-related genes are amplified and/or overexpressed in a major fraction of human tumors at a relatively early stage in the carcinogenic process (37, 38). To further elucidate the role of the autocrine IGF-I loop for BON cell cycle progression, cyclin D1, cyclin E, and p27kip1 expression was examined in serum-starved BON cells in the presence or absence of the IGF-I neutralizing antibody. Cyclin D1, cyclin E, and p27kip1 were found to be constitutively expressed in BON cells (Fig. 6B). Upon incubation of cells with the IGF-I-blocking antibody, expression of cyclin E was unchanged in Western blots. In contrast, expression of cyclin D1 was markedly reduced, and p27kip1 expression slightly increased with time. Thus, the endogenously released IGF-I is indeed a major mediator of autonomous proliferation of human BON neuroendocrine tumor cells.

DISCUSSION

In this paper, we demonstrate, for the first time, the presence of a functionally active autocrine IGF-I loop in human neuroendocrine BON tumor cells. This loop is regulated by a positive feedback mechanism: both the number of IGF-I receptors as determined by IGF-I or rIGF-I and the amount of IGF-I secreted into the supernatant increase with time. Our data further show that BON cells secrete a single class of IGFBPs, IGFBP-2. Because expression of IGFBP-2 correlates with proliferation of some tumor cell lines (39) and has been associated with increased malignancy of certain tumors (40, 41), IGFBP-2 could facilitate autocrine action of IGF-I by binding to IGF-I and thereby increasing its half-life.

Exogenously added IGF-I activated distinct signaling cascades in BON cells, such as PI3-kinase, p70s6k, and ERKs. Both IRS-1 and IRS-2 mediated IGF-I-stimulated PI3-kinase activation in these cells, in contrast to breast cancer cells, in which PI3-kinase activation in response to IGF-I is largely mediated by IRS-1 (42). p70s6k and the ERKs exhibited a high level of basal activity in serum-starved BON cells that could be prevented by treatment of the cells with their respective selective inhibitors. The constitutive activation of certain kinases under serum-free conditions is increasingly appreciated as a

mental conditions (35). Thus, in contrast to anchorage-dependent proliferation, autonomous and IGF-I-stimulated anchorage-independent growth are largely dependent on PI3-kinase/p70s6k activity and less dependent on the activation of the ERK cascade.

Endogenously Released IGF-I Regulates Autonomous Growth of BON Cells. Our data demonstrate that endogenously released IGF-I regulates constitutive activation of the ERK cascade and p70s6k (Fig. 3D) and that autonomous proliferation of BON cells requires the activity of both kinases (Fig. 5A). Thus, the interruption of the autocrine IGF-I loop should inhibit the autonomous growth of BON cells in serum-free medium. As shown in Fig. 6A, treatment of BON cells with the IGF-I-blocking antibody markedly inhibited proliferation of serum-starved BON cells by about 50%. The inhibitory effect of the antibody treatment was comparable to the effect of a maximal efficient concentration of rapamycin and was concentration dependent: a maximum effect was achieved at an antibody concentration of 25 μg/ml (data not shown). The effect of the IGF-I-blocking antibody on cell growth was selective. Incubation of cells with a distinct monoclonal antibody directed against tyrosine-phosphorylated proteins at the same concentration had no effect on cell growth (Fig. 6B). Thus, endogenously released IGF-I is indeed a major mediator of autonomous proliferation of human BON neuroendocrine tumor cells.
were counted at the days (B,d) indicated. Control cells received an equivalent amount of solvent (−). Cells were lysed and further analyzed by Western blotting with antibodies directed against cyclin E, cyclin D1, p70s6k and ERK2 that was comparable to the effect of their respective selective kinase inhibitors. Thus, endogenously released IGF-I led to a marked reduction in basal kinase activity of both p70s6k and ERK2 that was comparable to the effect of their respective selective kinase inhibitors. Therefore, endogenously released IGF-I is largely responsible for the constitutive activation of p70s6k and the ERK cascade in serum-starved BON cells. Despite the fact that receptors for other growth factors have been reported in BON cells (2), these data stress the importance of the autocrine IGF-I loop for the basal activity of certain signaling pathways in these human neuroendocrine tumor cells.

Human carcinoid tumor cells are characterized by strong secretory properties that are often debilitating for patients. Exogenously added IGF-I markedly increased secretion of chromogranin A, a marker protein for neuroendocrine secretion, in human BON cells. This is the first report demonstrating that the IGF-I receptor tyrosine kinase stimulates secretion in any tumor cell line. Interestingly, immunoneutralization of endogenously released IGF-I led to a slight increase in the expression of cyclin D1 and p27kip1 in human BON cells. Subconfluent serum-starved cultures of BON cells were treated with 25 μg/ml anti-IGF-I immunoneutralizing antibody for the times indicated. Control cells received an equivalent amount of solvent (−). Cells were lysed and further analyzed by Western blotting with antibodies directed against cyclin E, cyclin D1, or p27kip1 as indicated. The results shown in each case are representative of at least three independent experiments.

Fig. 6. Endogenously released IGF-I is responsible for autonomous growth of BON cells. A, BON cells were incubated at a density of 1 × 10^6 cells in 1 ml of serum-free DNM in the presence of 20 ng/ml rapamycin (Rapa) or 25 μg/ml IGF-I immunoneutralizing antibody (Ab) for various days. Control cells received an equivalent amount of solvent (−). Cells were counted at the days (d) indicated. B, BON cells were incubated at a density of 1 × 10^6 cells in 1 ml of serum-free DNM in the presence of 25 μg/ml IGF-I immunoneutralizing antibody (anti-IGF-I) or 25 μg/ml anti-Tyr(P) antibody (anti-Tyr(P)) for 3 days. Control cells received an equivalent amount of solvent (−). Cells were subsequently counted. Each data point represents the mean of three determinations ± SE and is representative of at least two independent experiments. C, the autocrine IGF-I loop regulates the constitutive expression of cyclin D1 and p27kip1 in human BON cells. Subconfluent serum-starved cultures of BON cells were treated with 25 μg/ml anti-IGF-I immunoneutralizing antibody for the times indicated. Control cells received an equivalent amount of solvent (−). Cells were lysed and further analyzed by Western blotting with antibodies directed against cyclin E, cyclin D1, or p27kip1 as indicated. The results shown in each case are representative of at least three independent experiments.
of human BON neuroendocrine tumor cells. We also demonstrate the presence of IGF-I and its receptor in various human neuroendocrine tumors. IGF-I could hence be one of the major mediators of the so called hypersecretion syndromes leading to functional (“hormone-active”) neuroendocrine tumor disease (24). Treatment of patients with antibodies against the Her2/neu receptor tyrosine kinase is appreciated as a novel strategy for breast cancer therapy (50). Thus, targeting IGF-I or the IGF-I receptor tyrosine kinase may constitute a novel therapeutic strategy for patients suffering from carcinoid tumors.

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Insulin-like Growth Factor-I Is an Autocrine Regulator of Chromogranin A Secretion and Growth in Human Neuroendocrine Tumor Cells

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