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Swiss 3T3 Mouse Embryo Fibroblasts Transfected With a Human Prepro-GRP Gene Synthesize and Secrete Pro-GRP Rather Than GRP

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A prepro-gastrin-releasing peptide (GRP) gene was introduced into Swiss 3T3 mouse embryo fibroblasts by DNA transfection in an attempt to establish autocrine growth stimulation. Clonal transfectants expressed varying amounts of GRP encoding mRNA. They synthesized and secreted a ~15-kd pro-GRP hormone but not fully processed 2.8-kd GRP. Accordingly, no changes in growth properties were associated with GRP gene expression. We postulate that Swiss 3T3 fibroblasts lack the enzymes necessary to process significantly pro-GRP into biologically active peptides and that this deficiency may be responsible for the failure to establish autocrine growth stimulation in the transfected cells.

Key words: autocrine growth, transfection, prohormone processing, bombesin

The gastrin-releasing peptide (GRP) is a 27-amino-acid neuropeptide homologous to the amphibian tetradecapeptide bombesin [1,2]. Both peptides bind to cell-surface receptors through their identical carboxyl heptapeptide [3], which triggers a physiological response in various central and peripheral neurons, in smooth muscle cells, and in secretory cells [4]. In addition, bombesin and GRP are potent mitogens for a number of cultured cells including Swiss 3T3 mouse embryo fibroblasts [5], human bronchial epithelial cells [6], and small-cell lung cancer cells [7,8]. The latter cells both contain and secrete a bombesinlike immunoreactive peptide [9–12] that was shown by cDNA cloning to be the 27-amino-acid GRP [13]. When a monoclonal antibody reacting with the carboxyl heptapeptide of GRP prevented this ligand from binding to its cell-surface receptors, the growth of some small-cell lung cancer cell lines was significantly impaired, both in soft agar and in nude mice [14]. Taken together, these data suggested that GRP could stimulate the growth of the cells that

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synthesized and secreted it. Such autocrine growth stimulation is thought to contribute to the acquisition of a malignant phenotype [15], and indeed both autocrine growth stimulation and tumorigenicity were induced in a factor-dependent hemopoietic cell line given a constitutively expressed GM-CSF growth factor gene [16], as well as in rat fibroblasts transfected with a TGF-α gene [17]. We tried to reconstitute an autocrine growth stimulation system by giving Swiss 3T3 mouse embyro fibroblasts a constitutively expressed prepro-GRP gene. These cells have a well-characterized mitogenic response to GRP [18], mediated by high-affinity cell-surface receptors [19], but do not normally synthesize or secrete this hormone. For DNA transfection, we used a cDNA clone of the human prepro-GRP gene [13,20] that encodes a 23-amino-acid signal sequence, the 27-amino-acid GRP, and a novel 95-amino-acid GRP-gene-associated peptide hereafter called GGAP. The present work shows that prepro-GRP transfected Swiss 3T3 cell lines synthesize and secrete large amounts of 15-kd pro-GRP but fail to process significantly this prohormone into biologically active GRP (2.8 kd).

MATERIALS AND METHODS

Prepro-GRP Expression Vectors

Two 8.1-kb GRP expression constructs were assembled (Fig. 1). Each construct contained the prepro-GRP and neomycin coding regions transcribed from opposite strands, with both genes being driven by the SV40 early region promoter (0.3-kb PvuII-HindIII fragment) and followed by the SV40 t antigen splice and polyadenylation site. The two constructs differ in that one transcribes the GRP coding region in a sense orientation with respect to the promoter, while the other should transcribe antisense GRP. To build these vectors, the pJB327 plasmid [21] (pBR322, with

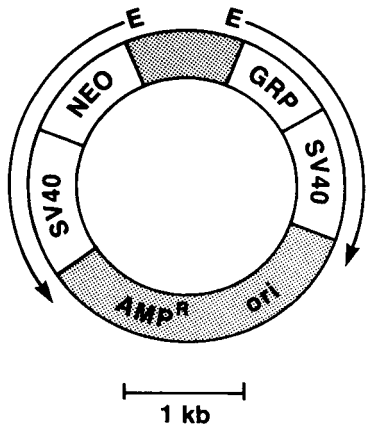

Fig. 1. Prepro-GRP sense and antisense expression vectors. Arrows indicate that the GRP and the neomycin genes are driven on opposite strands by the SV40 early promoter and enhancer (E). The only difference between the sense and antisense vectors is that they transcribe the prepro-GRP coding sequences in either the sense (5′–3′) or antisense (3′–5′) orientation. GRP = 0.7-kb EcoRI-DraI fragment from prepro-GRP cDNA form I [13]. NEO = 1.4-kb BamHI fragment from pmyc-neo [23]. SV40 = SV40 sequences, including the splice (0.6 kb) and polyadenylation (0.22 kb) sites from pSV2-gpt [22]. Stippling = pJB327 sequences, including the ampicillin resistance gene (AMP^R) and the origin of replication (ori) [21].

nucleotides 1,427–2,516 deleted, and the EcoRI-HindIII fragment replaced by the mp11 EcoRI-HindIII polylinker region) was reduced in size by deleting 737 bp in the tetracycline gene, eliminating the unique HindIII and EcoRV sites. This plamid, pJBΔ, was digested with SmaI and EcoRI, and a 2.9-kb PvuII-EcoRI fragment from pSV2-gpt [22] (which contained the Ecogpt coding region under control of the SV40 early promoter, followed by SV40 t antigen splice and polyadenylation site) was ligated into these sites. This plasmid, pJBΔ-gpt, was digested with EcoRV, and a HindIII linker was added by ligation. Subsequent digestion with HindIII released a 0.6-kb fragment containing the 5′ portion of the gpt coding sequence. The prepro-GRP coding region was then substituted for the gpt coding region, by (1) preparing a 0.7-kb EcoRI-DraI fragment encoding the prepro-GRP cDNA form I [13], (2) filling in the ends with Klenow polymerase, (3) adding HindIII linkers by ligation, and (4) ligating this fragment into pJBΔ-gpt missing the 0.6-kb HindIII-EcoRV gpt encoding fragment. Plasmids were selected which contained the prepro-GRP in either sense (5′–3′) or antisense (3′–5′) orientation. These two plasmids were linearized with NruI, and a 2.6-kb BamHI fragment from pmyc-neo [23] encoding the neomycin gene was ligated into the NruI site after filling in the staggered termini with Klenow polymerase. Plasmids carrying the prepro-GRP gene and the neomycin gene in opposite orientations were grown in *E. coli* LE392 and purified by CsCl density centrifugation [24] for transfection.

GGAP and GRP/GGAP Expression Vectors

The human GGAPI and GGAPIII cDNA sequences [13] were cloned in frame into the pUR290 expression vector [25], in order to produce β-galactosidase/GGAP fusion proteins (Fig. 2). This was done (1) by digesting pUR290 with BamHI and filling in the ends with Klenow polymerase; (2) by digesting further the linearized pUR290 with HindIII; and (3) by cloning a 0.5-kb RsaI-HindIII cDNA fragment encoding all but the first 21 amino acids of human GGAPI and GGAPIII (95 and 85 amino acids, respectively) into the modified BamHI and HindIII sites of pUR290. A 0.8-kb SmaI-HindIII cDNA fragment encoding the last amino acids of the signal sequence, GRP_{1-27} and $GGAPI_{1-95}$, was cloned into the same vector. The DNA regions containing the β-galactosidase junctions were sequenced by the dideoxy method [24], which showed that the three fragments had been cloned in frame. *E. coli* RR1Δm15 cells were transformed with the pUR290, pUR290-GGAP, and pUR290-GRP-GGAP plasmids. For expression, 20-ml bacterial cultures were grown at 37°C to an OD_{600} of 0.3 and induced for 30 min at the same temperature with 40 μl of 0.1 M IPTG. The bacterial pellets were washed once in 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and 5 mM EDTA, boiled for 5 min in SDS sample buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 5% beta-mercaptoethanol, 10% glycerol), and cleared by centrifugation. The protein concentration of the lysates was determined by the Biorad colorimetric assay, using lysozyme as a standard. For Western blot analysis, 10 μg of total proteins were electrophoresed on a 7.5% SDS-polyacrylamide gel [26], transferred to nitrocellulose [27], and incubated with 1:200,000 anti-GGAPI antiserum or 1:5,000 anti-N-GRP antiserum followed by ^{125}I-protein A (380,000 cpm/ml), as described below.

Transfected Cell Lines

The Swiss 3T3 mouse embryo fibroblast cell line was obtained from the American Type Culture Collection (CCL 92) and grown in Dulbecco's Modified

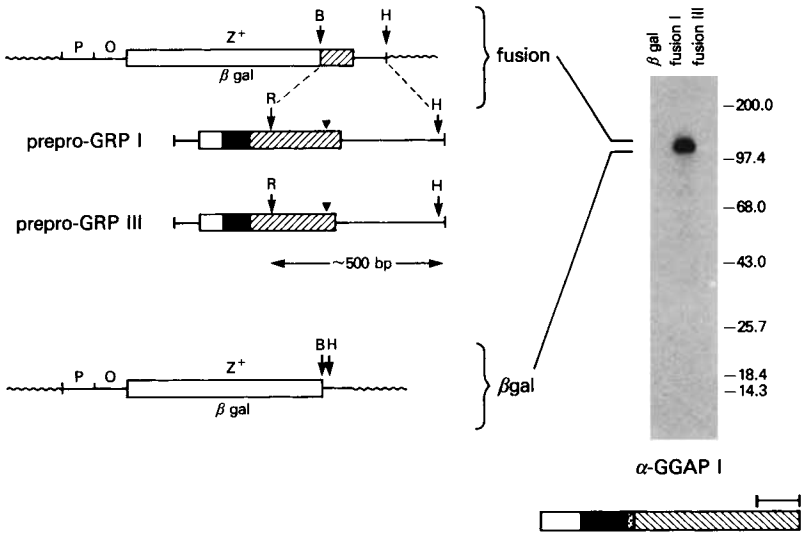

Fig. 2. The anti-GGAPI antiserum binds specifically to near full-length recombinant GGAPI in Western blot analysis. **Left:** prepro-GRP I and III: cDNA clones encoding the human prepro-GRP hormones I and III [13], ⊢⊣ = untranslated sequences; nucleotides encoding the signal sequence (□), GRP_{1-27} (■), and the GRP-gene-associated peptide GGAP (hatched bar); the arrowhead indicates the point from which the carboxyl-termini from GGAPI and GGAPIII differ; R = RsaI site; H = Hind III site; B = BamHI site. A 500-bp fragment encoding most of GGAPI or of GGAPIII was fused to the 3′ end of the β-galactosidase gene (Z^+) in pUR290 [25], thus generating two fusion protein expression vectors (fusion); the unmodified pUR290 vector expresses β-galactosidase (βgal); p = promotor; o = operon. **Right:** Western blot analysis (10 μg/lane) with the anti-GGAPI antiserum that was raised against the synthetic peptide identified by the horizontal bar above the prepro-GRP cartoon; βgal, fusion I, and fusion III: lysates from bacteria transformed with the βgal, GGAPI, and GGAPIII vectors, respectively; molecular weight markers, in kilodaltons, are shown on the right; the bars on the left indicate the positions of the ~ 130-kd fusion proteins and of the 115-kd unfused βgal.

Eagle's Medium with 10% fetal bovine serum at 37°C in a humidified atmosphere with 5% CO_2. One million cells in 100-mm tissue culture dishes were transfected with 25 μg of purified DNA in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), using the calcium phosphate DNA transfection method [24]. Mock transfections were done with TE buffer alone. Twenty-four hours after transfection, cells were plated for neomycin selection in 24-well microtiter plates, using 10^4 cells/well and 400 μg/ml of the biologically active aminoglycoside G-418 (Geneticin, Gibco). Preliminary experiments had established that in these conditions, all parent Swiss 3T3 cells were killed within 10–14 days of selection. Accordingly, after 2 wk of selection, none of the 84 wells containing mock-transfected cells showed evidence of growth, whereas 33% of the wells with sense or antisense transfected cells (104/312) contained at least one clone of growing cells, thus indicating an approximate transfection efficiency of 1:30,000. The contents of 24 wells were cloned by limiting dilution, generating 15 independent sense and four independent antisense transfected cell lines.

Northern Blot Analysis

Methods were essentially as described [24]. Total cellular RNA was prepared by the guanidine-isothiocyanate method, electrophoresed through a formaldehyde-

containing 1% agarose gel, and transferred to nitrocellulose. The filters were hybridized to DNA fragments purified by gel electrophoresis and nick-translated to a specific activity of 100–500 cpm/pg of DNA. The GRP probe was a 0.9-kb full-length prepro-GRP cDNA [13] that had been excised with HindIII from pBR322; the neomycin probe was a 1-kb HindIII-SmaI fragment of pSV2-neo [28] that contained exclusively neomycin coding sequences.

In Vitro Transcription and In Vitro Translation of the Human Prepro-GRP Gene

A 0.7-kb EcoRI-DraI fragment of the human prepro-GRP cDNA form I [13] was cloned into the EcoRI and SmaI sites of the expression vectors pSP64 and pSP65 (Promega). In these vectors, the bacterial SP6 promoter directs the transcription of RNA either identical (sense RNA) or complementary (antisense RNA) to the prepro-GRP mRNA. The plasmids were grown in *E. coli* HB101 and purified by CsCl density centrifugation; restriction mapping confirmed that they contained a single insert in the correct orientation; 5 μg of linearized and gel-purified plasmid DNA was capped and transcribed in vitro (Promega catalogue, 1987). Ten percent of the synthesized RNA (1 μl) was electrophoresed on a formaldehyde-containing 1% agarose gel. A single 0.7-kb band was visible after ethidium bromide staining and shown by Northern blot analysis to hybridize to the nick-translated GRP probe; 2 μl of prepro-GRP RNA was translated in an RNAase-treated rabbit reticulocyte lysate (Promega), using 75 μCi of ^{35}S-methionine (Amersham) and methionine-free amino acids in a total volume of 50 μl.

Antisera and Iodinated Protein A

The antisera used in this paper are described in detail elsewhere (F. Cuttitta et al., submitted for publication). Briefly, synthetic peptides corresponding to the 15 amino-terminal amino acids of human GRP (N-GRP$_{1-15}$) and to the 23 carboxyl-terminal amino acids of human GGAPI (GGAPI$_{73-95}$) were conjugated in equal weight ratios to keyhole limpet hemocyanin in the presence of 0.125% glutaraldehyde and injected subcutaneously into New Zealand White rabbits; the resulting antisera bound specifically to the immunizing peptides in a solid-phase radioimmunoassay; their specificity is being further characterized in this paper. Purified protein A (Pharmacia) was labeled with ^{125}I (Amersham) by the chloramine T method [29] to a specific activity of 25 μCi/μg (1 Ci = 3.7×10^{10} becquerels).

Cell Lysates and Western Blot Analysis

Monolayers of Swiss 3T3 transfected cell lines and cells from the GRP-producing human small-cell lung cancer cell line NCI-H209 [30] were lysed in SDS-sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol). The lysates were cleared by ultracentrifugation and their protein concentration was determined by the Biorad colorimetric assay. Equal amounts of proteins were electrophoresed on a 15% SDS-polyacrylamide gel [26] and transferred electrophoretically to nitrocellulose [27]. The filters were coated with 5% skimmed milk in Tris buffer (50 mM Tris-HCl pH 7.5, 5 mM EDTA, 150 mM NaCl, 0.02% NaN_3) for 1 hr at 40°C and further incubated at room temperature with rabbit antisera followed by iodinated protein A (380,000 cpm/ml) diluted in the same buffer. After extensive

washes, including one final 10-min wash in 0.5% NP40, filters were processed for autoradiography.

Metabolic Labeling and Immune Precipitation

Half-confluent monolayers of Swiss 3T3 transfected cell lines in T150 culture flasks ($\pm$ 5 $\times$ 10^6 cells/flask) were labeled for 4 hr at 37°C with 500 μCi of ^{35}S-methionine (Amersham) in 10 ml of methionine-free medium containing 2% dialyzed fetal bovine serum. Culture supernatants were centrifuged, made 250 μM PMSF, and fractionated on Sep-Pak C18 cartridges (Waters Associates). A fraction was eluted with 50% acetonitrile in 0.1 N NH_4OH after equilibrating the column with 15% acetonitrile in water, and the eluted material was lyophilized and resuspended in 1 ml of precipitation buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 1% Na deoxycholate). This procedure concentrated proteins smaller than 20 kd and allowed recovery of more than 90% of the ^{125}I-porcine GRP_{1-27} (Amersham) added to Swiss 3T3 culture supernatant. For immune precipation, 90,000 cpm of ^{125}I-porcine GRP_{1-27}, 150,000 TCA-insoluble cpm of reticulocyte lysate, and 350,000 TCA-insoluble cpm of culture supernatant in 450 μl of precipitation buffer were precleared twice for 1 hr in the cold with 50 μl of normal rabbit serum followed by 50 μl of protein A-Sepharose 4B CL (Pharmacia). After centrifugation, the supernatants were immune precipitated with 1:10 anti-N-GRP antiserum, followed by protein A-Sepharose 4B CL. After extensive washes [31], the immunoadsorbents were boiled for 10 min in SDS-sample buffer; the supernatants were electrophoresed on a 15% SDS-polyacrylamide gel that was eventually processed for fluorography.

RESULTS

Transfected cell lines were first analyzed at the nucleic acid level. Total RNA from 19 independent Swiss 3T3 transfected cell lines and from the GRP-producing small-cell lung cancer line NCI-H209 was assayed by Northern blot analysis (Fig. 3). As previously described, a major 0.9-kb prepro-GRP transcript was observed in the NCI-H209 cell line [13]. In the transfected cell lines, the prepro-GRP transcripts were expected to include the SV40 early promoter (0.34 kb), the prepro-GRP cDNA fragment (0.7 kb), the 3′ untranslated end of the gpt gene (0.5 kb), and the SV40 t antigen splice and polyadenylation signals (0.6 kb and 0.2 kb, respectively). Accordingly, $\pm$ 2.3-kb transcripts were detected in 13/15 sense and 4/4 antisense transfected cell lines. Minor 1.8-kb and 4.8-kb transcripts were also observed in these lines; they were possibly generated by the use of alternative SV40 polyadenylation sites. The levels of prepro-GRP mRNA varied from barely detectable to as high as those observed in a good GRP-producing small-cell lung cancer cell line. Hybridization of the same blot to the neomycin probe confirmed that all the transfected lines expressed the expected 2.6-kb neomycin transcript, as well as some other minor transcripts (not shown). Southern blot analysis of genomic DNA prepared from four of these lines showed that multiple copies of both unaltered and rearranged prepro-GRP genes had been integrated (not shown). Finally, the identity of the sense and of the antisense transfected lines was confirmed in an S1 nuclease protection assay, where total cellular RNA protected the appropriate single-stranded DNA probes [13] from S1 nuclease digestion (not shown).

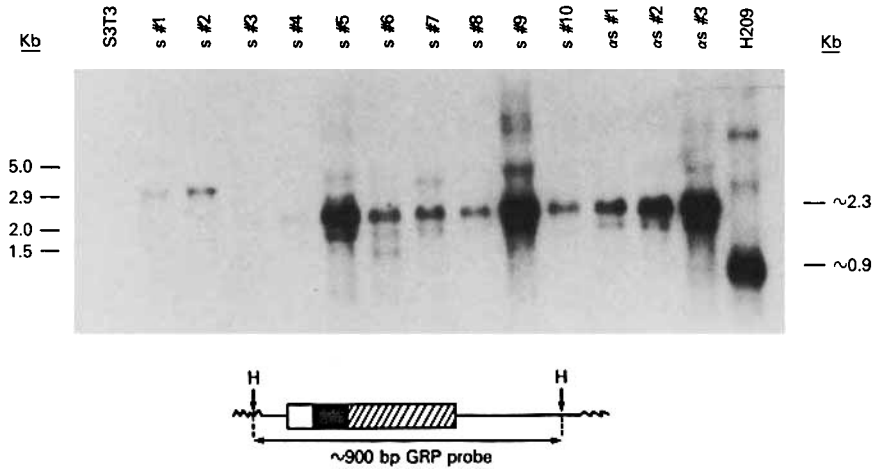

Fig. 3. Transfected Swiss 3T3 cell lines express varying amounts of prepro-GRP-encoding mRNA. Northern blot analysis of total cellular RNA (10 μg/lane) prepared from Swiss 3T3 mouse embryo fibroblast cell line (S3T3), from human small-cell lung cancer cell line NCI-H209 (H209), as well as from cloned Swiss 3T3 transfected cell lines; s# and αs# refer to cell lines transfected with sense and antisense prepro-GRP expression vectors, respectively. The probe is a 900-bp prepro-GRP cDNA, excised with HindIII (H) from pBR322 [13]. Molecular weight standards are indicated on the left; the approximate size of the transfected (2.3 kb) and endogenous (0.9 kb) GRP mRNAs are shown on the right.

Four transfected cell lines were further evaluated for protein expression. In a preliminary Western blot analysis, the anti-GGAPI antiserum bound specifically to the ± 130-kd β-galactosidase/GGAPI fusion protein, as well as to some minor degradation products of the latter (Fig. 2). It failed to react with bacterial lysates that contained either the unfused β-galactosidase (115 kd) or the β-galactosidase/GGAPIII fusion protein. This experiment confirmed that the anti-GGAPI antiserum bound specifically to near-full-length recombinant GGAPI. When the transfected cell lysates were analyzed in the same way, the anti-GGAPI antiserum bound specifically to a single 15-kd GRP precursor present in the NCI-H209 cell line, as well as in the two sense, but not in the two antisense, transfected cell lines (Fig. 4). Strikingly, the transfected cell lines contained at least ten times more GRP precursor than the NCI-H209 line. This precursor could formally be either prepro-GRP or pro-GRP. The former would have retained its signal sequence while the latter would have lost it (calculated molecular weights of 16 kd and 13.5 kd, respectively). Since in mammalian cells, the removal of signal sequences is usually a cotranslational procedure [32], it was assumed that the 15-kd GRP precursor was pro-GRP. In order to detect in cell lysates not only the 15-kd putative pro-GRP, but also processed GRP_{1-27} (calculated molecular weight of 2.8 kd), we used the anti-N-GRP antiserum in Western blot analysis. However, the latter antiserum failed to react with any of the cell lysates previously tested with the anti-GGAPI antiserum (not shown). This was probably due to the low affinity of this antiserum, since at high concentrations it failed to react in Western blot analysis with the ± 135-kd β-galactosidase/GRP-GGAPI fusion protein unless the latter was visible by Coomassie blue staining (at least 0.5 μg/lane).

Since cell-surface receptor-mediated autocrine growth requires the presence of processed GRP_{1-27} not only in the cells but also in the culture medium, the latter was

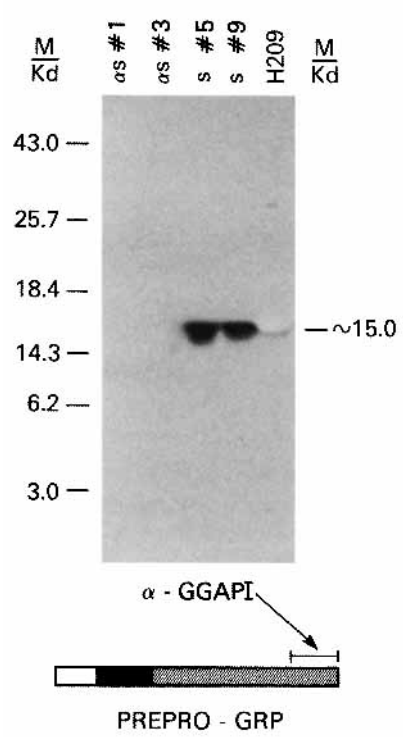

Fig. 4. Cell lysates from Swiss 3T3 cells transfected with the sense (s#5, s#9) but not with the antisense (αs#1, αs#3) prepro-GRP expression vector contain at least ten times more pro-GRP than a small-cell lung cancer cell line lysate (H209). Western blot analysis (500 μg/lane) with the anti-GGAPI antiserum (see legend Fig. 2); the filter was exposed for 24 hr at −70°C to Kodak XAR film in the presence of an intensifying screen; molecular weight markers, in kilodaltons, are shown on the left; the apparent molecular weight of the pro-GRP hormone is shown on the right.

immune precipitated with the anti-N-GRP antiserum. In a preliminary experiment, we tested reticulocyte lysates that contained the in vitro labeled and in vitro translated products of the sense and of the antisense prepro-GRP mRNAs (Fig. 5). The anti-N-GRP antiserum immune precipitated a single 17.0 kd prepro-GRP hormone, which further suggested that the 15-kd GRP precursor (Fig. 3) was pro-GRP. In another experiment, 50 fM of iodinated porcine GRP_{1-27} was mixed with Swiss 3T3 culture medium and immune precipitated by the anti-N-GRP antiserum as a 2.8-kd molecular species (Fig. 5). However, when culture supernatants of two Swiss 3T3 transfected cell lines were immune precipitated with the anti-N-GRP antiserum, a single ± 15-kd pro-GRP hormone was recovered from the medium surrounding the sense but not the antisense transfected cell line (Fig. 5). When the same experiment was done on NCI-H209 culture supernatants, no GRP-containing peptides of any size were detected, which suggested that only very small amounts of such unbound and undegraded peptides were available for immune precipitation (not shown).

In accord with the immune precipitation data, none of the sense transfected cell lines displayed any obvious growth advantage over the antisense transfected lines. In medium with 10% fetal bovine serum, sense transfectants displayed a wide range of growth rates that could always be matched by an antisense counterpart; in medium with 2% or 0% fetal bovine serum, some of the sense as well as some of the antisense

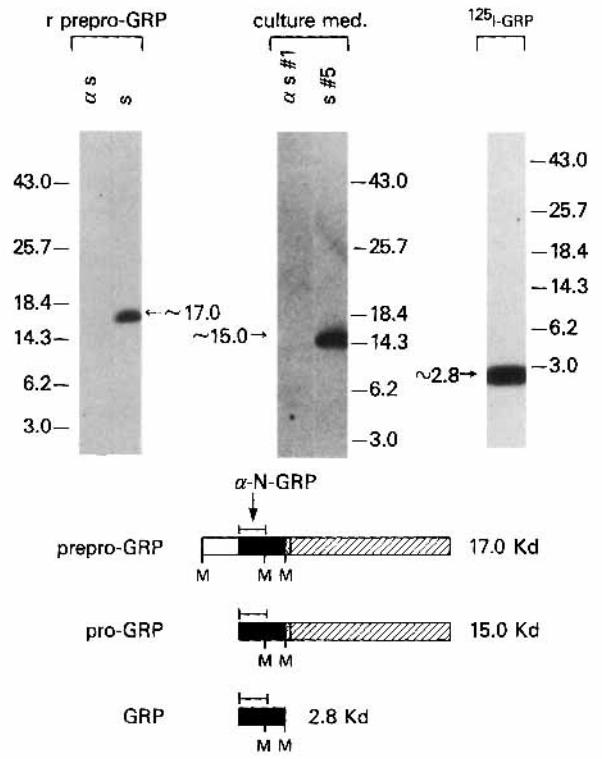

Fig. 5. **Left:** The anti-N-GRP antiserum immune precipitates a ~17.0 kd recombinant prepro-GRP hormone (r prepro-GRP); the synthetic peptide used to raise this antiserum is identified by the horizontal bar above the cartoons; s and α s: in vitro translation products of in vitro-transcribed sense and antisense prepro-GRP mRNA; fluorography exposed for 24 hr at −70°C to Kodak XRP film. **Right:** The anti-N-GRP antiserum does immune precipitate a ~2.8-kd iodinated GRP_{1-27} (^{125}I-GRP) added to Swiss 3T3 culture medium; autoradiography exposed for 4 days at −70°C to Kodak XAR film. **Middle:** Swiss 3T3 cells transfected with the sense (s#5) but not with the antisense (αs#1) prepro-GRP expression vector synthesize and secrete the pro-GRP hormone rather than fully processed GRP_{1-27}; immune precipitation with anti-N-GRP antiserum of culture medium (culture med) from in vitro-labeled cells; fluorography exposed for 5 days at −70°C to Kodak XAR film; molecular weight markers are shown in kilodaltons; the apparent molecular weights of prepro-GRP, pro-GRP, and GRP_{1-27} are indicated on the gels and on the corresponding cartoons; M = position of ^{35}S-methionine.

transfected cell lines managed to grow. However, none of these growth variations could be related to the expression of the prepro-GRP gene.

DISCUSSION

In this paper, we show that Swiss 3T3 mouse embryo fibroblasts express a transfected human prepro-GRP gene but that they secrete the 15-kd prohormone rather than processed GRP. Concomitantly, prepro-GRP transfected cell lines fail to show any obvious growth advantage over control transfected cell lines. We also provide the first data available on human GRP precursor proteins. Thus, the immune precipitation of recombinant human prepro-GRP establishes its apparent molecular weight as 17.0 kd. This strongly suggests that the 15-kd GRP precursor is the pro-GRP hormone, which was formally demonstrated in later experiments (A.M. Lebacq-

Verheyden et al., submitted for publication). However, nothing is known about the processing intermediates that separate the prohormone from the free hormones. The latter include at least GRP_{1-27} and GP_{18-27}, which were identified by radioimmunoassay in acid-boiled and size-fractionated extracts of human fetal lung [33,34], and of small-cell lung cancers [35]. Whether in all these tissues free peptides were being generated by a physiological process or by the experimental conditions remains unclear. Our inability to immune precipitate GRP_{1-27} from the supernatant of a small-cell lung cancer cell line that was shown in the past both to contain [10,30] and to secrete [36] bombesinlike immunoreactive material suggests that only very small amounts of free GRP are presently available in the culture medium. We are currently investigating the processing of prepro-GRP in several small-cell lung cancer cell lines in order to identify the GRP-related peptides that can be involved in autocrine growth stimulation [15].

In order to achieve autocrine growth stimulation, small-cell lung cancer cells and prepro-GRP transfected Swiss 3T3 cells need (1) to synthesize, process, and secrete biologically active GRP; (2) to bind this hormone to high-affinity cell-surface receptors [19]; and (3) to signal this binding through second messengers that include inositol 1,4,5-trisphosphate breakdown and calcium flux [18,37]. Failure at any of these three levels could account for the observed lack of autocrine growth stimulation. The data presented here suggest that Swiss 3T3 fibroblasts lack the enzymes required for processing pro-GRP into biologically active peptides. In common with many other peptide hormones [38], GRP needs to be α-carboxyl-amidated in order to bind to its receptor and exert its biological activities [39]. From processing data published about other prohormones [32,40,41], it can be inferred that the processing of pro-GRP should at least include (1) the trypsinlike cleavage of the 15-kd prohormone at a dibasic lys-lys in the sequence Met-Gly-Lys-Lys-Ser, where Met is the carboxyl-terminal amino acid of GRP_{1-27} [20]; (2) the removal of the two basic residues; and (3) the cleavage of the glycine—that is, the amide donor—with amidation of the methionine. For pro-insulin, proparathyroid hormone and pro-opiomelanocortin, it has been demonstrated that most if not all of the processing occurred in secretory granules [reviewed in 40]. Hence, it may not be surprising that Swiss 3T3 fibroblasts, devoid of secretory granules, do not process efficiently some prohormones. Indeed, monkey-fibroblastlike cell lines (namely, AGMK, CV1, and COS) transfected with the rat or with the human prepro-insulin gene synthesize and presumably secrete pro-insulin rather than insulin [42,43]. Similarly, COS cells transfected with the porcine pro-opiomelanocortin gene synthesize and secrete authentic POMC, which is subsequently degraded into smaller immunoreactive peptides, distinct from the biologically active peptides, by nonspecific proteases present in the culture medium [44]. In contrast, some mature somatostatin-14 was secreted by COS cells transfected with angler fish preprosomatostatin, thus showing that at least some simple processing, involving only a trypsinlike cleavage of a single pair of basic amino acids, could occur in fibroblastlike cells [45].

In conclusion, the immune precipitation data and the absence of autocrine growth stimulation support the view that Swiss 3T3 cells transfected with the human GRP gene synthesize and secrete readily detectable amounts of the 15-kd prohormone but fail to process significantly this precursor into GRP_{1-27}. From these experiments, it is clear that GRP may function as an autocrine growth factor only in certain specific cell types which possess the necessary enzymatic activities to process biologically active GRP from its prohormone precursor.

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