The Granulin/Epithelin Precursor Abrogates the Requirement for the Insulin-like Growth Factor 1 Receptor for Growth *in Vitro**

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3T3 cells null for the type 1 insulin-like growth factor receptor are refractory to stimulation by a variety of purified growth factors that are known to be required for the stimulation of other 3T3 cells. However, these cells, known as R⁻ cells, grow in serum-supplemented medium and also in media conditioned by certain cell lines. We report here the purification of a growth factor that stimulates DNA synthesis (and growth) of R⁻ cells. The growth factor, purified to homogeneity by SDS-polyacrylamide gel electrophoresis, was identified as the granulin/epithelin precursor by an accurate determination of the masses of endoproteinase Lys-C peptides using matrix-assisted laser desorption ionization mass spectrometry, followed by a data base search. The granulin/epithelin precursor is a little known growth factor, secreted by a variety of epithelial and hemopoietic cells. It is at present the only purified growth factor that can stimulate the growth of mouse embryo fibroblasts null for the type 1 insulin-like growth factor receptor.

It has been known for several years that the insulin-like growth factors (IGF-I and -II)[1] play a central role in the growth of cells in culture (reviewed in Ref. 1). Most cells in the animal body have type 1 IGF receptors (IGF-IR) and require the activation of this receptor by its ligands for optimal growth, both *in vivo* (2, 3) and *in vitro* (reviewed in Ref. 4). The requirement for IGF-I is especially evident with mouse and human fibroblasts, where it combines with other growth factors (for instance, platelet-derived growth factor) in stimulating growth of cells under defined conditions (serum-free medium). Singly, these growth factors cannot stimulate the growth of normal fibroblasts, like 3T3 cells (5). R⁻ cells (6, 7) are 3T3 fibroblasts that originated from mouse embryos with a targeted disruption of the IGF-IR genes (2, 3). R⁻ cells have been extensively used in the past years to study the role of the IGF-IR in mitogenesis, transformation, and apoptosis (see the editorial in Ref. 8). R⁻ cells do not grow in serum-free medium supplemented with the growth factors that are known to sustain the growth of other 3T3 cells, with a physiological number of IGF-IRs (1). Singly or in combination, the following growth factors failed to stimulate the growth of R⁻ cells: platelet-derived growth factor, epidermal growth factor, IGF-I and II, insulin, basic and acidic fibroblast growth factor, TGFα, TGFβ, and hepatocyte growth factor (7, 9, 10). In fact, even R⁻ cells overexpressing either the epidermal growth factor receptor (11) or the platelet-derived growth factor β receptor (12) are unresponsive to their respective growth factors, indicating a central role of the IGF-IR in fibroblast growth. Reintroduction of a wild type IGF-IR promptly restores the growth deficits of R⁻ cells (6, 7). However, the fact that R⁻ cells grow in 10% serum clearly indicates that serum contains one or more growth factors that bypass the requirement for IGFs. R⁻ cells also grow in medium conditioned by certain (but not all) cell lines (9). In previous papers (9, 13), we reported that the conditioned medium of BRL-3A cells stimulated the growth of R⁻ cells. The partial fractionation of a growth-stimulating polypeptide derived from BRL-3A cells was reported by Xu *et al.* (9). The growth factor has now been identified as the granulin/epithelin precursor (14–16) by high accuracy peptide mass mapping with matrix-assisted laser desorption ionization (MALDI) mass spectrometry (MS), followed by data base searching with a set of measured peptide masses. Further evidence for its identity was provided by sequence analysis of a peptide by Edman degradation after purification by reverse phase chromatography. Given the central role of the activated IGF-IR in the growth of cells *in vivo* and *in vitro* (see above), the identification of the granulin/epithelin precursor as a growth factor that bypasses the requirement for IGFs can be of considerable interest to the many investigators in the field of IGFs and IGF-binding proteins and of cell proliferation in general.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—All experiments used as the test cell line R⁻ cells (6, 7), which are 3T3-like cells derived from mouse embryos with a targeted disruption of the IGF-IR genes (2, 3). These cells have been repeatedly described and characterized in previous papers from this laboratory (11). They grow in 10% fetal bovine serum, but they do not grow at all in serum-free medium supplemented with purified growth factors. R⁻ cells were maintained routinely in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum.

For the production of conditioned medium, BRL-3A cell line was chosen after a primary screening of several different cell lines for their ability to secrete a growth stimulating activity into serum-free medium (9). BRL-3A is a cell line originated as a clone from a 5-week-old female Buffalo rat liver (Life Technologies, Inc.). Serum-free medium conditioned by the BRL-3A cells produces a family of polypeptides, including a multiplication-stimulating activity that was reported to be related to somatomedins and stimulate [³H]thymidine incorporation into DNA in chick embryo fibroblasts and human skin fibroblasts (17). BRL-3A cells were maintained in F-10 medium plus 10% fetal bovine serum.

Preparation of Conditioned Medium—Conditioned medium from

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[1] The abbreviations used are: IGF, insulin-like growth factor; IGF-IR, type 1 IGF receptor; TGF, transforming growth factor; TGFe, epithelial-type TGF; MALDI, matrix-assisted laser desorption ionization; TOF, time-of-flight; MS, mass spectrometry; PAGE, polyacrylamide gel electorphoresis; MAP, mitogen-activated protein.

BRL-3A cells was prepared as described in detail by Xu *et al.* (9). Collected medium was sterilized using a 0.2-µm filter and stored frozen at −20 °C until needed.

DNA Synthesis—R⁻ cells were seeded at a density of $5 \times 10^3/\text{cm}^2$ on coverslips in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and allowed to attach for 24 h. The cultures were then made quiescent for 96 h in serum-free medium prior to the addition of growth factors or conditioned medium. Tritiated thymidine (0.5 µCi/ml) was added at the same time as growth factors or conditioned medium, and the incubations were continued for 24 h. The cells were then fixed in cold methanol and autoradiographed by standard procedures. The percentage of labeled cells was determined by scoring a total of 1,000 cells. DNA synthesis stimulating activity was expressed as the percentage of labeled nuclei.

Chromatography Procedures—The purification of the growth factor is schematically represented in Fig. 1.

Polyacrylamide Electrophoresis—Polyacrylamide electrophoresis of proteins in any sample was performed using Precast Ready Gels and Mini-Protean II cell system (Bio-Rad) unless otherwise noted. 4–20% linear gradient Tris-glycine gels were used for the *inset* of Figs. 2 and for Fig. 3*B*, whereas 4–15% linear gradient Tris-glycine gels were used for Fig. 3*A* and the *inset* of Fig. 4.

Recovery of Protein Resolved under Nonreducing Condition—200 µl of fraction 38 (about 3 µg of protein) from the reverse phase chromatography procedure was lyophilized by Speed-Vac concentrator and resolved by polyacrylamide gel electrophoresis under nonreducing condition. One-sixth of the sample was run onto a separate lane for silver stain as reference. Slices above, below, and corresponding to the desired band detected by silver stain (Fig. 3*B*) were cut out from the gel and were incubated in 1 ml of phosphate-buffered saline at 4 °C for 12 h. The supernatant was dialyzed by Slide-A-Lyzer 10K Dialysis Cassettes (Pierce) twice against saline for 6 h and twice against distilled water for 6 h (18). The product was concentrated by Centricon-10 (Amicon, molecular weight cut-off of 10,000) unit; four-fifths were tested for the ability to stimulate DNA synthesis, and one-fifth was analyzed by SDS-PAGE under reducing conditions (Fig. 4).

Renaturation of Protein Reduced by SDS-PAGE—Target protein (3 µg) in 200 µl of fraction 38 from reverse phase chromatography procedure was also studied by cutting the desired band from the gel after resolution by SDS-PAGE. The protein was eluted from the gel band, renatured, and characterized, basically according to the method of Hager and co-workers (19, 20) and Ishii *et al.* (21).

Preparation of Protein for Peptide Analysis—To obtain a homogenous protein preparation for peptide analysis, the active factor was purified by SDS-PAGE. The preparation was lyophilized in a vacuum concentrator, and the protein was subjected to reduction by dithiothreitol and cysteine alkylation by 4-vinylpyridine in Laemmli SDS-PAGE sample buffer. The mixture was applied to a single lane of a 14% Novex precast Tris-glycine gel and subjected to electrophoresis under normal conditions. The protein was visualized by Coomassie Blue stain, the major component was excised, and the gel slice was destained in preparation for endoproteinase digestion *in situ* (22).

Protein Digestion, Peptide Isolation, and Sequence Analysis—The protein present in the gel slice was subjected to endoproteinase Lys-C (*Achromobacter lyticus* from Wako) digestion for 16 h. Peptides were extracted from the gel slice in organic solvents, and 10% of the mixture was subjected to analysis by MALDI reflectron time-of-flight (MALDI-TOF) MS. The bulk of the peptide mixture (90%) was subjected to reverse phase capillary chromatography (Applied Biosystems 173A cLC Microblotter system) with deposition of the peptides on a polyvinylidene difluoride membrane after passage through the detector cell. One peak in the chromatogram was subjected to Edman degradation using an Applied Biosystems Procise 494 protein sequencer through direct introduction of the polyvinylidene difluoride membrane and use of "blot" cycles.

Protein Data Base Search with Peptide Mass Data—Peptide monoisotopic masses were used for protein data base searching using PeptideSearch software[2] running on an Apple Power Macintosh 7600/120.

IRS-1 and MAP Kinase Phosphorylation—Cells were treated as for DNA synthesis (see above), and lysates were prepared from them for IRS-1 and MAP kinase phosphorylation following techniques previously described (10). For IRS-1, 300 µg of protein lysate were immunoprecipitated overnight at 4 °C with anti-IRS-1 and protein A-agarose (Onco-

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[2] M. Mann (1996) peptide search software for Apple Macintosh computers (available via anonymous ftp at ftp://mac-mannb.embl-heidelberg.de/saturn/pub/software).

Procedure for the Purification of BRL-GF

BRL-3A cell cultures

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Production of BRL-3A CM

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Collection of the CM

↓

Ultrafiltration by YM10 & YM100 membranes, generating fraction 10~100 kDa

↓

Chromatography using FPLC instruments

Anion exchange by DEAE column generating fraction at 0.3 M NaCl

↓

Hydrophobic interaction by phenyl-sepharose column generating active fraction in 20 mM Tris-HCl (pH 8.0)

↓
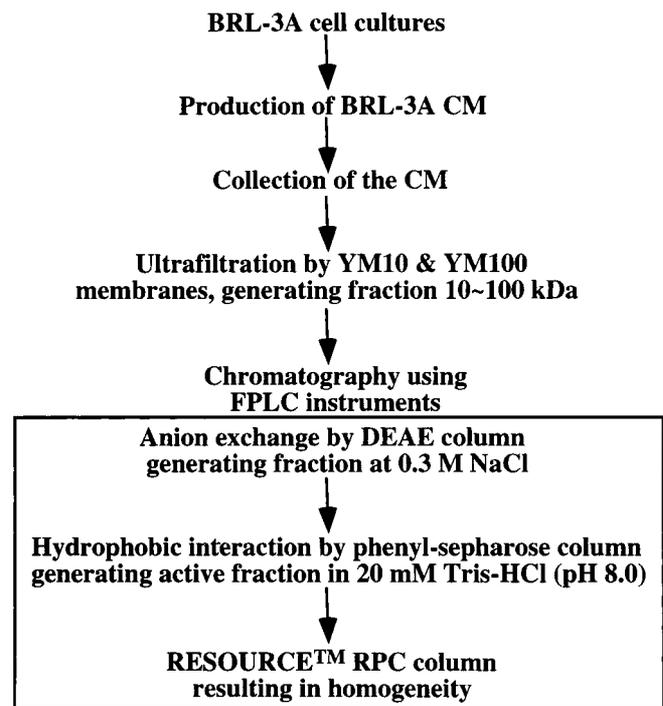
RESOURCE™ RPC column resulting in homogeneity

FIG. 1. **Diagram of the purification procedure.** The growth-promoting activity was originally designated as BRL-GF, for growth factor from BRL-3A cells. It will be referred to, in the rest of the paper, as granulin/epithelin.

gene Science). After resolution on a 4–15% SDS-PAGE (Bio-Rad) and transfer to a nitrocellulose filter, the membrane was probed with anti-phosphotyrosine antibody (Transduction Laboratories). Detection was carried out with ECL (Amersham Pharmacia Biotech). After membrane incubation with stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 7.6) at 50 °C for 30 m, membrane was washed twice with TBS-T buffer (0.1 M Tris, 1.5 M NaCl, 0.5% Triton Y-100) blocked overnight in 5% nonfat milk in TBS-T buffer and probed with anti IRS-1 antibody (Oncogene Science). MAP kinase phosphorylation was determined following the instructions of the antibody's manufacturer (Promega).

RESULTS

Purification of the Growth Factor—In a previous paper (9), we showed that R⁻ cells could grow in media conditioned by three different cell lines, BRL-3A, T98G (a human glioblastoma cell line), and T24H (a 3T3 cell line transformed by an activated *ras*). Other cell lines were also tested and found to be negative, *i.e.* their conditioned media did not elicit a response in R⁻ cells. Among the other cell lines tested were two normal cell lines, WI-38 human diploid fibroblasts and Balb/c 3T3 cells, and a human breast cancer cell line, MCF-7. In general, the cells whose conditioned media stimulated R⁻ cells were cell lines capable of growing in serum-free medium, whereas the negative cell lines required IGF-I (as well as other growth factors) for growth. We concentrated on BRL-3A conditioned medium, because it seemed more potent than other conditioned media in stimulating the growth of R⁻ cells. The partial purification of the unidentified growth factor has been described by Xu *et al.* (9). A diagram of the purification procedure is given in Fig. 1. At every step, the various fractions were used to stimulate R⁻ cells. Although the activity was originally identified as capable of causing an increase in cell number in R⁻ cells (9), in later experiments, the usual procedure was to monitor each fraction for the ability to stimulate DNA synthesis. This procedure is easier and more sensitive than cell number, because even when

Epithelin and IGF-1

FIG. 2. **Last step in the purification procedure.** The active fractions from the phenyl-Sepharose column were pooled and fractionated by reverse phase chromatography. *Solid line* with *crossed circle*: protein amounts. *Dashed line* with *open circle*, activity in stimulating DNA synthesis in R⁻ cells. *Inset*, gel of the most active fractions, showing a strongly visible single band in fractions 38, 39, and 40.
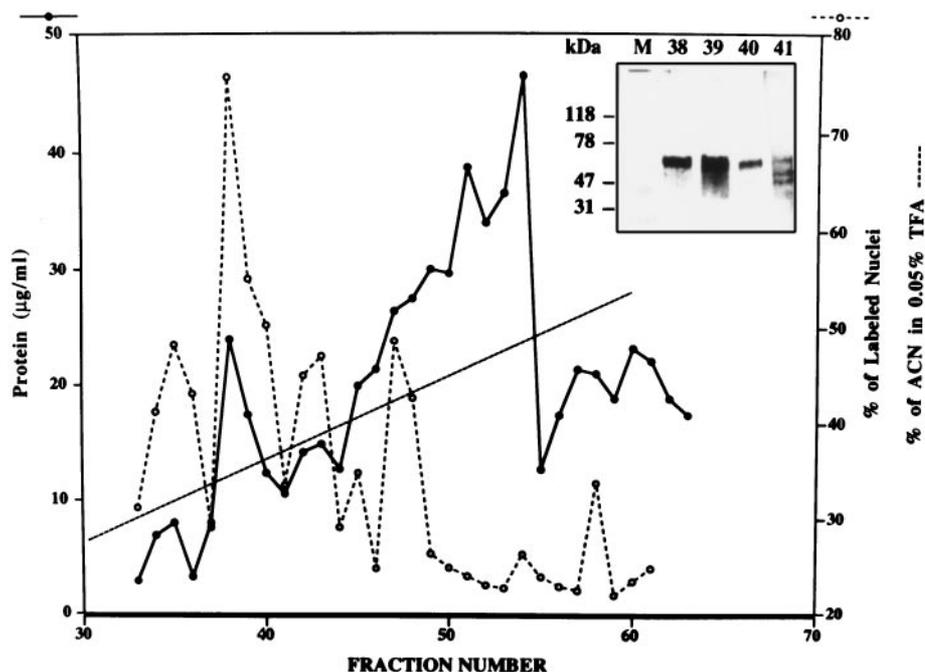
close to 100% of cells enter S phase, the increase in cell number is only a doubling. However, the purified growth factor can also stimulate cell growth, in addition to stimulating DNA synthesis. A pool of fractions from the phenyl-Sepharose column that was active in stimulating the growth of R⁻ cells was further fractionated by reverse phase chromatography (Fig. 2). The highest stimulating activity was found in fractions 38–40. When these fractions were run on a gel, a single band was found at approximately 60–65 kDa (*inset* of Fig. 2). This band was eluted (see "Experimental Procedures") and rerun again on a gel (Fig. 3), both under denaturing and nondenaturing conditions. Again, only one band was detectable on silver-stained gels.

These bands were again eluted and tested for their ability to stimulate DNA synthesis in R⁻ cells in serum-free medium and for peptide analysis. Fig. 4 shows that the protein from the nondenatured gel of Fig. 3, was capable of inducing DNA synthesis in R⁻ cells, to an extent (about 70% of cells) only a little inferior to 10% serum (80%). The band eluted from the denaturing gel was essentially inactive, as were the eluates from regions above or below the active band. The remaining material was again loaded on gels, and the *inset* of Fig. 4 shows the bands (much decreased in intensity) at the same location as in the previous figures.

Identification of the Growth Factor as the Granulin/Epithelin Precursor—The active fraction was subjected to a number of analyses, as described under "Experimental Procedures." An aliquot of the protein preparation was analyzed by direct Edman degradation, because analysis by SDS-PAGE suggested a purity level of 95% for the growth factor; however, no sequence information was obtained (an indication of protein blockage). A new and rapid method for the identification of proteins involved high accuracy peptide mass mapping using delayed ion extraction MALDI time-of-flight-TOF MS, followed by protein data base searching with the peptide masses (24). The protein band shown in Fig. 3 was digested with endoproteinase Lys-C, and an aliquot of the peptide mixture was used to determine the accurate molecular masses (generally <30 parts/million) by delayed ion extraction MALDI-TOF MS. The molecular masses of the peptides were used to search an in-house nondegenerate protein data base, and the protein was identified as the rat granulin/epithelin precursor. Table I compares the regions of
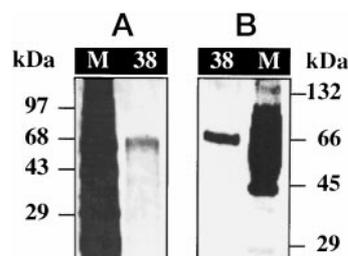
FIG. 3. **Purity of the growth factor.** Only fraction 38 of the previous figure was used. By silver staining, a single band was visible on gels, whether under denaturing (*A*) or native conditions (*B*). *M*, markers.

the epithelin/granulin precursor identified by delayed ion extraction MALDI reflectron TOF MS with the mouse protein and human protein, both predicted from their respective cDNA. The sequences obtained by us showed 100% homology with the rat granulin/epithelin, 87% homology to the mouse protein, and 72% homology to the human protein. The growth factor, it should be noted, was purified from BRL-3A cells that are of rat origin. The sequences obtained from our band cover 179 of 589 amino acids or 30.4% of the rat granulin precursor. It seems reasonable to conclude that at least one of the growth factors in the BRL-3A conditioned medium that stimulates the growth of R⁻ cells is the granulin/epithelin precursor.

IRS-1 and MAP Kinase Activation—We have conducted preliminary experiments on the possible pathways used by the granulin/epithelin precursor to induce DNA synthesis in R⁻ cells. To circumscribe the problem, we have investigated the activation (tyrosyl phosphorylation) of IRS-1 as well as MAP kinase activation by the granulin/epithelin precursor, following the same procedures outlined above. These experiments are shown in Fig. 5 (*A*, *B*, and *C*). IRS-1 tyrosyl phosphorylation is not increased by stimulation with the granulin/epithelin precursor (Fig. 5A). There is a faint band of phosphorylated IRS-1, but this is present also in unstimulated cells. We have already noticed before a slight activation of IRS-1 in cells attached to the substratum (10, 11), probably because of the known interaction of IRS-1 with other cellular components. Fig. 5B shows the levels of IRS-1 protein in the immunoprecipitates. We conclude that stimulation with the granulin/epithelin precursor
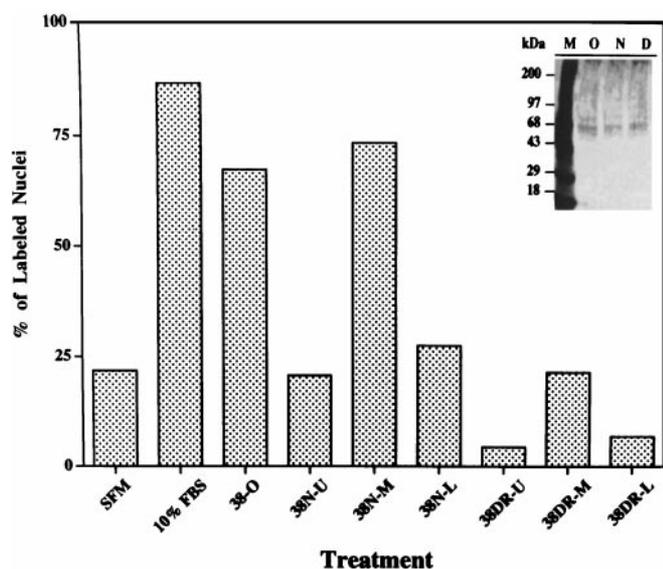
FIG. 4. **Activity of the bands of Fig. 3.** The bands of Fig. 3 were eluted and used for two purposes: 1) stimulation of DNA synthesis in R⁻ cells and 2) sequencing. DNA synthesis was determined as described under "Experimental Procedures." The sequences are given in Table I. *O*, original sample; *N*, nondenatured sample; *DR*, denatured sample. *U* and *M* are slices of gels immediately above or below the silver-stained bands. The *ordinate* gives the percentage of labeled cells. *Inset*, gels of the material left over. *SFM*, serum-free medium.

has no effect on tyrosyl phosphorylation levels of IRS-1. This is not the case with MAP kinases, which are sharply activated by the granulin/epithelin precursor (Fig. 5*C*), indicating that this growth factor eventually connects with the MAP kinase pathway in stimulating DNA synthesis.

DISCUSSION

We have purified, partially sequenced, and identified a polypeptide from BRL-3A conditioned medium that stimulates DNA synthesis (and growth in number) of R⁻ cells that are null for the IGF-IR (6, 7). This growth factor has been identified as the granulin/epithelin precursor (14–16). The identification of the granulin/epithelin precursor as a growth factor that bypasses the requirement for a functional IGF-IR is of considerable interest for several reasons: 1) The IGF-IR plays a major role in the growth of cells, both *in vivo* and *in vitro* (see Introduction), but clearly its requirement is not an absolute one, because it can be circumvented by a single purified growth factor. 2) Although epithelins are produced also by normal tissues (see below), in cells in culture they are predominantly expressed by transformed cell lines that can grow in serum-free medium. This raises the question whether the expression of epithelins may also be involved in transformation. 3) The IGF-IR shares with other growth factor receptors the main mitogenic pathway (commonly referred to as the *ras* pathway). However, the discovery of a purified growth factor that bypasses this pathway opens the possibility of identifying a new mitogenic pathway that is presumably *ras*-independent (7). 4) One can also predict the usefulness of the granulin/epithelin precursor in studies dealing with the inhibitory action of IGF-binding proteins, because stimulation of cell proliferation could be achieved in the absence of IGFs.

Epithelins were originally purified from rat kidney as two small single chain peptides (approximately 6 kDa) containing about 20% cysteine (14). Epithelin1 stimulated the proliferation of murine keratinocytes, whereas epithelin2 had inhibitory potential (14, 25). At about the same time, the same peptides were isolated from leukocytes and termed granulins (26). Subsequently, Bhandari *et al.* (15) cloned the granulin precursor

cDNA and reported that the prepropeptide of the human granulins is a 593-residue glycoprotein containing seven tandem repeats of the 12-cysteine granulin domain. The human granulin/epithelin gene codes for at least four small epithelin peptides (27). In addition to granulin A and B, an additional peptide, granulin F, which had never been isolated before but whose primary structure is known on the basis of the cDNA sequence, was isolated from human urine (28). The rat granulin precursor codes for 589 amino acid residues (BRL-3A cells are from rat liver) and is 72% homologous to the human granulin precursor and 87% homologous to the mouse granulin precursor (16, 25). The primary structure of the epithelins has been reported by Belcourt *et al.* (29). It has also been reported that the granulin/epithelin protein motif has an unusual structure consisting of a parallel stack of β-hairpins stapled together by six disulfide bonds (30). The granulin gene has an unusual genomic structure containing 12 exons interrupted by 11 introns mapped to chromosome 17 in human and 13 exons interrupted by 12 introns mapped to chromosome 11 in mouse, respectively (15, 31, 32). A putative promoter has also been characterized at the 5′ end of the granulin gene (33). The granulin gene is conserved widely in species, suggesting a possibly widespread growth regulatory function (30).

Although granulin/epithelins have been usually tested as the small peptides, Zhou *et al.* (34) purified the precursor protein and showed that it was, by itself, a mitogen for cells in culture. We are now showing that the granulin/epithelin precursor is mitogenic per se, in fact that it can stimulate DNA synthesis (and growth, see Ref. 9) in serum-free medium in R⁻ cells that are known to be refractory to stimulation by other purified growth factors, singly or in combination. Interestingly, Zhou *et al.* (34) purified the granulin/epithelin precursor from an "insulin-independent" cell line. Because insulin in tissue cultures is generally used at concentrations that activate the IGF-IR (35), their observation is an indirect confirmation of our observation that the granulin/epithelin precursor bypasses the requirement for the IGF-IR. The stimulatory activity of the precursor, in respect to the small epithelins, may be due to the fact that the small epithelins are a mixture of stimulatory and inhibitory peptides (14, 25).

Our active polypeptide was inactivated by a denaturing gel. This was not surprising, given the large number of cysteine residues in the sequence (see above); furthermore, structural evidence indicated the importance of proper formation of intrachain disulfide bonds of the granulin/epithelin (30). Finally, epithelin-binding sites have been found in human breast carcinoma cells (36), but to our knowledge, no further information on a putative receptor has been made available. A 170–175-kDa protein has been reported (37) that binds the epithelial-type transforming growth factor (TGFe), and this growth factor is partially homologous to, but distinct from, the granulin/epithelins (33, 38). This TGFe-specific receptor with a size of approximately 170–175 kDa (37) is presumably different from the putative receptor for granulin/epithelin, a membrane protein of 140–145 kDa (36).

It is worth mentioning that SDS-PAGE of proteins in fractions 34 and 35 corresponding to the first active peak in Fig. 2 showed a single band of about 25–28 kDa by silver stain (data not shown). Whether it is a TGFe or a processed granulin/epithelin or another factor remains to be established. BRL-3A conditioned medium is rich in growth-stimulating activities (39–41), including IGF-II, originally identified as multiplication stimulating activity (17). IGF-II, however, cannot stimulate DNA synthesis in R⁻ cells, even at concentrations up to 200 ng/ml (10), and, in addition, the size of IGF-II is much smaller than the size of the growth factor we have purified. It

T A B L E I
Sequence analysis of polypeptides from BRL-GF

r, rat; m, mouse; h, human; Grn, granulin; Epn, epithelin.

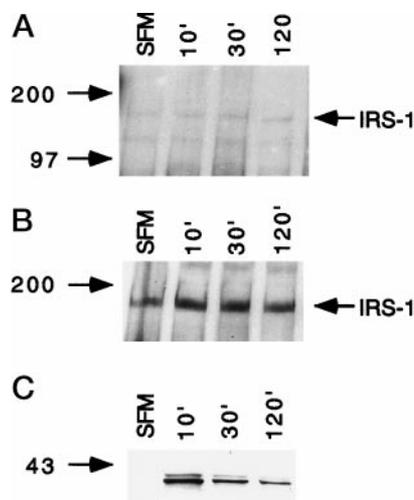| Partial amino acid sequence compared with granulin/epithelin precursor	Identities
	%
BRL-GF/P1 [1]KFPAQRTNRAVAFPFSVVCPDAKTQCPDDSTCCELPTGK[39]	
rGrn/Epn [189]KFPAQRTNRAVAFPFSVVCPDAKTQCPDDSTCCELPTGK[227]	100
mGrn/Epn [189]KFPAQKTNRAVSLPFSVVCPDAKTQCPDDSTCCELPTGK[227]	92
hGrn/Epn [190]KLPAQRTNRAVALSSSVMCPDARSRCPDGSTCCELPSGK[228]	74
BRL-GF/P2 [1]LPGYPVNEVKCDLEVSCPDGYTCCRLNTGAWGCCPFTK[38]	
rGrn/Epn [272]LPGYPVNEVKCDLEVSCPDGYTCCRLNTGAWGCCPFTK[309]	100
mGrn/Epn [272]LPGYPVKEVKCDMEVSCPEGYTCCRLNTGAWGCCPFAK[309]	89
hGrn/Epn [274]LPAHTVGDVKCDMEVSCPDGYTCCRLQSGAWGCCPFTQ[311]	76
BRL-GF/P3 [1]CMDEGYCQKGDRMVAGLEKMPVRQTTLLQHGDIGCDQHTSCPVGQTCCPSLK[52]	
rGrn/Epn [408]CMDEGYCQKGDRMVAGLEKMPVRQTTLLQHGDIGCDQHTSCPVGQTCCPSLK[459]	100
mGrn/Epn [408]CLAQGYCQKGDTMVAGLEKIPARQTTPLQIGDIGCDQHTSCPVGQTCCPSLK[459]	84
hGrn/Epn [410]CVAEGQCQRGSEIVAGLEKMPARRASLSHPRDIGCDQHTSCPVGQTCCPSLQ[460]	70
BRL-GF/P4 [1]DSQGGWACCPYVKGVCCRDGRHCCPIGFHCSAK[33]	
rGrn/Epn [532]DSQGGWACCPYVKGVCCRDGRHCCPIGFHCSAK[564]	100
mGrn/Epn [532]DSAGVWACCPYLKGVCCRDGRHCCPGGFHCSAR[564]	84
hGrn/Epn [536]DNRQGWACCPYRQGVCCADRRHCCPAGFRCAAR[568]	66
BRL-GF/P5 [1]TPRWDILLRDPAPRPLL[17]	
rGrn/Epn [574]TPRWDILLRDPAPRPLL[589]	100
mGrn/Epn [574]IPRWDMFLRDPVPRPLL[589]	76
hGrn/Epn [578]APRWDAPLRDPALRQLL[593]	71
Summary of Identities	
BRL-GF/P1~5 (cover 179 out of 589 amino acids (30.39%) of rat Granulin precursor)	
rGrn/Epn	100
mGrn/Epn	87
hGrn/Epn	72

FIG. 5. **IRS-1 and MAP kinase activation by the granulin precursor.** Stimulation of R⁻ cells and determination of IRS-1 phosphorylation and MAP kinase activation are described under "Experimental Procedures." *A*, IRS-1 tyrosyl phosphorylation at various times after stimulation with the granulin precursor. The times are indicated above the respective lanes. *B*, the same blot, stripped and stained for IRS-1. *C*, MAP kinase activation under the same conditions. *SFM*, serum-free medium.

is likely that there are other growth factors in the BRL-3A conditioned medium that can stimulate growth of R⁻ cells. The one we have purified and identified is the granulin/epithelin precursor.

The identification of the granulin/epithelin precursor as a growth factor that bypasses the requirement for an activated IGF-IR is, in our opinion, important, both from a basic and an applied point of view (see above). The IGF-IR is found in many cell types and, as mentioned in the Introduction, is necessary for the optimal growth of cells *in vivo* and *in vitro*. Furthermore, down-regulation of the IGF-IR causes massive apoptosis

of tumor cells *in vivo*, whereas overexpression causes transformation and protection from apoptosis (reviewed in Ref. 4). The granulin/epithelin family could then become a target in those tumor cells that have escaped regulation by the IGF-IR. At a more basic level, our finding raises some interesting questions, for instance, whether the granulin/epithelin peptides use and, so to speak, usurp the IGF-IR signaling pathway(s), or whether they induce mitogenicity (and/or transformation) by a totally different pathway. This problem is being actively investigated, but our preliminary results shown in Fig. 5 clearly indicate that MAP kinases are activated, but IRS-1 is not. Thus, the pathway stimulated by the granulin/epithelin precursor does not seem to be dependent on IRS-1 and is therefore different from the IGF-IR mitogenic pathway, which is heavily dependent on IRS-1 (reviewed in Ref. 4). On the other hand, this pathway connects again with one of the main pathways for stimulation of DNA synthesis, which passes through the MAP kinases. A considerable amount of evidence has accumulated, indicating a crucial role of MAP kinases in the stimulation of DNA synthesis by either growth factors or integrins or simply attachment to the substratum (23, 42–45). Indeed, MAP kinase activation has been reported to increase transcription from the serum response element (45), which is usually correlated with stimulation of DNA synthesis. Thus it seems that the granulin/epithelin precursor bypasses the IRS-1 mitogenic pathway but eventually joins the mitogenic pathway that is common to many growth factors and mitogenic stimuli (23, 42–45). The question now is where the granulin/epithelin pathway reinserts itself into the main mitogenic pathway, downstream from IRS-1. At present, the only clue we have is that a plasmid expressing the human granulin/epithelin precursor (courtesy of Dr. Bateman) can make R⁻ cells grow in serum-free medium, a property that, so far, is shared only by v-*src* (13). This finding suggests that *src* may be involved in granulin/epithelin signaling. The connection between c-*src* and MAP kinases has been elucidated recently by Schlaepfer *et al.* (23), and the *src* pathway may therefore be a prime candidate for further studies.

However, the complexities of the mitogenic signaling pathways is such that several options must be kept open, especially in view of the fact that no receptor for the granulin/epithelin precursor has yet been cloned. The R⁻ cells expressing the granulin/epithelin precursor are now being investigated in detail for their growth phenotype, including transformation and ability to protect cells from apoptosis and the ability to process the precursor and their signaling potentials.

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The Granulin/Epithelin Precursor Abrogates the Requirement for the Insulin-like Growth Factor 1 Receptor for Growth *in Vitro*

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Additions and Corrections

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Page 20082: Table I is incorrect. The corrected table is shown below.

TABLE I

Measured and calculated peptide masses for the Achromobacter Lys-C peptides from the rat epithelin/granulin precursor (PIR2:B38128)

The table provides a list of the experimental molecular masses of the peptides found in the Lys-C digest of the growth factor in comparison with the theoretical masses of the corresponding peptides of the granulin/epithelin precursor found in the data base file. The mass spectrum was acquired on a Bruker Reflex instrument equipped with a delayed-extraction ion source. Calibrations were established with the use of an internal peptide calibrant [ACTH-(18–39)] and a matrix ion (α-cyano-4-hydroxycinnamic acid). The sequence of one peptide (272–281) was established by Edman degradation after isolation by capillary chromatography. MH^+ refers to the monoisotopic mass of the protonated molecular ion.

Measured MH^+	Calculated MH^+	ppm[a]	Residue span	Amino acid sequence from data base file
1075.609	1075.556	48	417–426	(K)GDRMVAGLEK(M)
1115.653	1115.609	39	272–281	(K)LPGYPVNEVK(C)[b]
1286.503	1286.500	1.5	408–416	(K)CMDEGYCQK(G)
1481.866	1481.857	6	346–359	(K)VTASLSLPDPQILK(N)
1623.656	1623.708	32	532–544	(K)DSQGGWACCPYVK(G)
1639.691	1639.763	43	496–511	(K)DAGSVQPSMDLTFGSK(V)
2012.806	2012.855	24	212–227	(K)TQCPDDSTCCELPTGK(Y)
2029.108	2029.170	30	573–589	(K)TPRWDILLRDPAPRPLL( )
2526.335	2526.307	10	190–211	(K)FPAQRTNRAVAFPFSVVCPDAK(T)
2654.390	2654.402	4	189–211	(K)KFPAQRTNRAVAFPFSVVCPDAK(T)
2674.259	2674.202	21	545–564	(K)GVCCRDGRHCCPIGFHCSAK(G)
3672.537	3672.585	13	282–309	(K)CDLEVSCPDGYTCCRLNTGAWGCCPFTK(A)
3973.792	3973.879	21	427–459	(K)MPVRQTTLLQHGDIGCDQHTSCPVGQTCCPSLK(G)

[a] Mass accuracy in parts per million (ppm).
[b] Identity confirmed by Edman degradation after isolation by capillary chromatography. Residues in parentheses are those which precede and follow the Lys-C peptide in the precursor sequence.

The homology comparisons of the original Table I arise from the alignment of the data base sequences of the precursors from the three species of organisms and do not relate to empirical sequence determinations. The peptide mass search of the data base resulted in the identification of the growth factor as rat epithelin/granulin precursor, the sequence of which is 87% homologous to the mouse precursor and 72% homologous to the human precursor. The growth factor was purified from BRL-3A cells of rat origin. The peptide matches represent a sequence coverage of 30.4%, or 179 of the 589 amino acids in the precursor sequence, and encompass the sequence from residues 189 to 589 (the C terminus).