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 database search. The granulin/epithelin precursor is a little known growth factor, secreted by a variety of epithelial and hematopoietic 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) 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 database 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...
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 × 10^5/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-Protein II cell system (Bio-Rad) unless otherwise noted. 4–20% linear gradient Tris-glycine gels were used for the 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. 3B, whereas 4–15% linear gradient Tris-glycine gels were used for Fig. 3A 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. 3B) 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 out 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) and Ishii et al. (21).

Preparation of Protein for Peptide Analysis—To obtain a homogeneous protein preparation for peptide analysis, the active factor was purified by Sep-Pak C18. 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 mass spectrometry (MALDI-TOF MS). The bulk of the peptide mixture (90%) was subjected to reverse phase capillary chromatography (Applied Biosystems 173A cLC Micro blotter 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 software2 running on an Apple Power Macintosh 7600/120. IKS-1 and MAP Kinase Phosphorylation—Cells were treated as described for DNA synthesis (see above), and lysates were prepared from them for IKS-1 and MAP kinase phosphorylation following techniques previously described (10). For IKS-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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**Procedure for the Purification of BRL-GF**

1. **BRL-3A cell cultures**
2. **Production of BRL-3A CM**
3. **Collection of the CM**
4. **Ultrafiltration by YM10 & YM100 membranes, generating fraction 10-100 kDa**
5. **Chromatography using FPLC instruments**
6. **Anion exchange by DEAE column generating fraction at 0.3 M NaCl**
7. **Hydrophobic interaction by phenyl-Sepharose column generating active fraction in 20 mM Tris-HCl (pH 8.0)**
8. **RESOURCE™ RPC column resulting in homogeneity**

**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-1 (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

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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).
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.

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 non-denatured 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 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...
IGF-IR shares with other growth factor receptors the main epithelins may also be involved in transformation. 3) The medium. This raises the question whether the expression of expressed by transformed cell lines that can grow in serum-free medium. 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 (TGFε), and this growth factor is partially homologous to, but distinct from, the granulin/epithelins (33, 38). This TGFε-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). 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 b-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 R² 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 tissue (34) purified the precursor protein (34) made available. A 170–175-kDa protein has been reported (37) that binds the epithelial-type transforming growth factor (TGFε), and this growth factor is partially homologous to, but distinct from, the granulin/epithelins (33, 38). This TGFε-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 small band of about 25–28 kDa by silver stain (data not shown). Whether it is a TGFε 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 R² 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

![Graph](image_url)
for the optimal growth of cells and, as mentioned in the Introduction, is necessary applied point of view (see above). The IGF-IR is found in many growth factor that bypasses the requirement for an activated precursor. One we have purified and identified is the granulin/epithelin. The identification of the granulin/epithelin precursor as a growth factor that bypasses the requirement for an activated precursor.

is likely that there are other growth factors in the BRL-3A conditioned medium that can stimulate growth of R² cells. The one we have purified and identified is the granulin/epithelin precursor.

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 may be involved in granulin/epithelin signaling. The connection between c-src and MAP kinases 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 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).

Summary of Identities

| Polypeptide | Identity | Summary of Identities |
|------------|----------|-----------------------|
| BRL-GF/P1  | 100      |                       |
| rGrn/Epn   | 87       |                       |
| mGrn/Epn   | 72       |                       |
| hGrn/Epn   | 72       |                       |

TABLE I

Sequence analysis of polypeptides from BRL-GF

| Partial amino acid sequence compared with granulin/epithelin precursor | Identities |
|-----------------------------------------------------------------------|------------|
| BRL-GF/P1                                                             | 100        |
| rGrn/Epn                                                              | 87         |
| mGrn/Epn                                                              | 72         |
| hGrn/Epn                                                              | 72         |

**Fig. 5. IRS-1 and MAP kinase activation by the granulin precursor.** Stimulation of R² 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.

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 R² 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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Epithelin and IGF-1

20083