A growth factor from the conditioned medium of NIH-Sape-4, an embryonic cell line of the flesh fly, was purified to homogeneity. This growth factor, termed IDGF, stimulated the proliferation of NIH-Sape-4 cells in an autocrine manner; it was a homodimer of a protein with a molecular mass of 52 kDa, and its specific activity was comparable with those of mammalian growth factors. Immunoblotting experiments revealed that unfertilized mature eggs of the flesh fly contained this growth factor, a certain level of which was maintained throughout embryonic development. Analysis of cDNA for this growth factor showed that this factor is a novel protein consisting of 553 amino acid residues. No significant sequence similarity was found between this factor and other proteins except atrial gland granule-specific antigen of Aplysia californica.

During the development of holometabolous insects such as the flesh fly, undifferentiated cells proliferate as diploid cells, but at a certain stage of development, most cells cease proliferation and become polyploid. Therefore, the increases in the size of a larval body reflect increases in the sizes of the polyploid cells, not their numbers, which is a characteristic of insect development. Therefore, there appear to be two important mechanisms that regulate insect development: diploid cell growth and polyploid cell extension.

Little information about insect growth factors was available until recently, when insect homologs of mammalian growth factors and their receptors were genetically identified in Dro sophila and suggested to play roles in oogenesis (1–4), embryogenesis (5–10), and morphogenesis of specified organs (11–14). However, until now, no insect growth factor had been purified and characterized at the molecular level.

In this paper, we report the purification of an insect growth factor from the culture medium of an embryonic cell line, NIH-Sape-4, of Sarcophaga peregrina (flesh fly). Like many other cell lines established from various insects, NIH-Sape-4 cells inoculated at high density proliferated in the absence of fetal calf serum and known growth factors (15). This finding suggested that NIH-Sape-4 cells may produce a growth factor that stimulates their proliferation in an autocrine manner. We purified a growth factor from the conditioned medium of NIH-Sape-4 cells, and the results of our experiments with it suggest that it participates in the embryogenesis of this insect. The cDNA for this growth factor encoded a novel protein. The predicted amino acid sequence showed a significant identity to AGSA2 of Aplysia californica (16), but its biological relevance is unclear.

**MATERIALS AND METHODS**

Cells and the Conditioned Medium—NIH-Sape-4 cells were cultured in M-M medium (17) at 25 °C, as described previously (18). Briefly, cells were inoculated at a density of about 5 × 10^5 cells/ml into 4 liters of M-M medium by adding 500 ml of preculture (about 4 × 10^6 cells/ml) and a spinner flask with constant stirring for 7 days. When the cell density reached 4 × 10^6/ml, the cells were removed by centrifugation, and the resulting conditioned medium was stored at −20 °C until used as a source of the growth factor.

Assay of Growth Factor Activity—This was carried out by assessing the stimulation of cell proliferation. NIH-Sape-4 cells were suspended at a density of 2.5 × 10^5 cells/ml in M-M medium containing streptomycin (0.5 mg/ml) and penicillin G (120 units/ml), and 100 µl of cell suspension was poured into each well of a 96-well microtiter plate. The cell numbers did not increase appreciably, even when cultured for 7 days at 25 °C under these conditions, because the initial cell density was low. However, a significant increase in cell proliferation was detected when 20 µl of conditioned medium was added to the wells and the cells were cultured as described above.

Cell proliferation was determined by one of two methods. The first method was measurement of DNA synthesis. The cells were incubated for 1 day at 25 °C in the presence or absence of test sample. Then, 10 µl of [3H]thymidine ([100 µCi/ml, Amersham Corp., 25 Ci/mmol) was added and incubation was continued for 1 more day. The medium was discarded, and the cells were washed thoroughly and solubilized with 150 µl of 0.5 N NaOH solution, and 150 µl of N,N,N-trichloroacetic acid was added to the cell lysate. The acid-insoluble radioactive material was trapped on a glass fiber filter (Whatman, GF/C), and the radioactivity was measured (19). The second method was measurement of the enhancement of cellular metabolism by the Alamar Blue assay (20, 21). The cells were incubated for 2 days at 25 °C in the presence or absence of test sample. Then, 15 µl of Alamar Blue (Alamar Biosciences, Inc.) was added, and the change in the color of the culture medium was monitored by measuring A570 and A610. In this assay, the reducing activity generated by the proliferating cells changes the color of Alamar Blue. Throughout purification of the growth factor, the latter assay was used to assess the growth factor activity. One unit of growth factor was defined as the amount giving half-maximal stimulation of cell proliferation induced by 20 µl of conditioned medium.

Purification of the Growth Factor from the Conditioned Medium of NIH-Sape-4—About 30 liters of conditioned medium was concentrated to 1 liter by ultrafiltration (Millipore, PTKK membrane). The concentrated solution was dialyzed against 3-fold with buffer I (10 mM phosphate buffer (pH 6.8)) and applied to a DEAE-cellulose column (9.0 × 19 cm) equilibrated with buffer I, and the adsorbed material was eluted with this buffer containing 150 mM NaCl. About 34 ml of saturated (NH₄)₂SO₄ solution to give a final saturation of 30% (v/v) (NH₄)₂SO₄ was added to.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank/EBI Data Bank with accession number(s) D83125.

The abbreviations used are: AGSA, atrial gland granule-specific antigen; FPLC, fast protein liquid chromatography; HPLC, high performance liquid chromatography; IDGF, insect-derived growth factor.
about 80 ml of fraction from the DEAE-cellulose column. This mixture was applied to a butyl-Toyopearl 650M column (1.5 x 11.5 cm) equilibrated with buffer I containing 30% (NH₄)₂SO₄, and the column was washed well with this buffer and the adsorbed material was eluted with a decreasing linear gradient of 30–0% (NH₄)₂SO₄ in buffer I. The growth factor activity was eluted from the column as a single peak. The fractions containing activity were pooled (about 40 ml), diluted 50-fold with buffer I, and concentrated to about 10 ml by ultrafiltration (Diaflo, UF PM10 membrane). This solution was subjected to FPLC on a Mono Q column (Pharmacia Biotech Inc.) equilibrated with buffer I. The column was developed with an linear gradient of 100–400 mM NaCl, and the growth factor activity was eluted as a single peak. Fractions containing activity were pooled (about 10 ml) and concentrated to about 1 ml (Amicon, Centricon 10 membrane), and the concentrated solution was subjected to HPLC on a hydroxylapatite column (Pentax) equilibrated with buffer I. The growth factor activity was recovered in the flow-through fraction (about 2 ml), which was concentrated to about 0.2 ml (Amicon, Centricon 10 membrane), and subjected to HPLC on a molecular sieve column (G3000SWXL, Tosoh) equilibrated with buffer I containing 100 mM NaCl. The growth factor activity was eluted from the column as a single peak. At this stage, the growth factor was almost pure, giving a single band of 52 kDa when subjected to SDS-polyacrylamide gel electrophoresis. The protein concentration was measured by the method of Lowry et al. (22) using bovine serum albumin as a standard.

Antibody Against the Growth Factor—An antibody against the growth factor was raised by injecting 5 µg of purified protein into a male albino rabbit with complete Freund's adjuvant followed by a booster injection of the same amount of protein 14 days later (23). Affinity purification of the resulting antibody was performed using the purified growth factor as follows. First, the purified protein (100 µg) was electrophoresed on a 12.5% (w/v) polyacrylamide gel containing 0.2% (v/v) SDS, and then the proteins were blotted onto a polyvinylidene difluoride membrane filter. The small region of the filter on which the growth factor had been concentrated was excised and treated with 5% (w/v) skim milk solution. Then, the filter paper strip was incubated in 15 ml of an antiserum solution at 4°C for 12 h with gentle shaking, rinsed well, and cut into pieces, and the antibody specifically bound to the growth factor was extracted with 0.2 M glycine/HCl buffer (pH 2.8). The resulting extract was neutralized with 1 M KOH, and bovine serum albumin to a final concentration of 0.1% (w/v) was added.

Electrophoresis and Immunoblotting—Electrophoresis on SDS-polyacrylamide slab gels was carried out by the method of Laemmli (24). The protein concentration was measured by the method of Lowry et al. (25). The electrophoretically separated proteins were transferred electrophoretically from the gel onto polyvinylidene difluoride membrane filters, which were immersed in a 5% skim milk solution for 1 h at room temperature and then transferred to rinsing solution (10 ml Tris/HCl buffer, pH 7.9, containing 150 mM NaCl, 1 mM EDTA, 0.1% (w/v) Triton X-100, and 0.25% skim milk) containing affinity-purified antibody (IgG) against the growth factor (3 ng/ml) and kept at 4°C for 12 h. Then they were washed well with rinsing solution, transferred to a 5-ml rinsing solution containing radiiodinated anti-rabbit IgG (2 x 10⁶ cpm), and kept for 2 h at room temperature. Finally, they were washed well with rinsing solution, dried, and subjected to autoradiography using Kodak XAR film.

Cloning Procedure and Sequencing of cDNA—The purified growth factor was digested with lysylendopeptidase, and the resulting peptides were applied to a reverse-phase HPLC column of Synchropak RP-P (C₁₈) connected to a Waters HPLC system. The fractions containing each peptide were lyophilized and subjected to automated sequence analysis. By this procedure, the sequences of four peptides were determined. We also determined the sequence of 15 amino acid residues from the amino-terminal end of the growth factor. These sequences were: peptide 1, TREVAGLATAP; peptide 2, YQPLVCADLQG; peptide 3, FIPQLLGMPFMDFYFAGH; peptide 4, ATPLSHDFYIAFLGISAH; and peptide 5, ANHMYQHAPFEOQA. The oligodeoxyribonucleotide probe designed to correspond to PDFVAGFD in peptide 2 was synthesized as 5'-CCIGACTC5'GTGCIGCTCAG5'TTCG-3' and 5'-end-labeled with ³²P by the method of Sgaramella and Khorana (26). The construction of the cDNA library for NIH-Sape-4 cells and the hybridization procedure were essentially the same as described before (27, 28). About 100,000 colonies of Escherichia coli SOLRT strain carrying recombinant plBluescript (Stratagene) were transferred to duplicate sets of nylon filters. One hybridization-positive colony was cloned. For nucleotide sequencing of cDNA, various deletion derivatives of the DNA fragment were prepared using exonuclease III and mung bean nuclease (29). Each deletion derivative was sequenced by the

### Table 1

| Purification step | Protein | Activity | Specific activity | Yield | Purification |
|------------------|---------|----------|-------------------|-------|-------------|
| Culture medium   | 4800    | 1400     | 0.29              | 100   | 1.0         |
| Concentrated solution | 1900 | 1000     | 0.55              | 72    | 1.9         |
| DEAE-cellulose   | 330     | 930      | 2.8               | 65    | 9.4         |
| Butyl-Toyopearl  | 7.6     | 1900     | 250               | 130   | 830         |
| Mono Q           | 1.2     | 930      | 760               | 65    | 2500        |
| Hydroxylapatite  | 0.15    | 310      | 2100              | 22    | 6900        |
| G3000SWXL        | 0.017   | 110      | 6300              | 7.6   | 21000       |

FIG. 1. Effect of the conditioned medium on NIH-Sape-4 cell growth. Increasing concentrations of conditioned medium (20-µl aliquots) were added to the cells, inoculated at a density of 2.5 x 10⁴ cells/ml, and cultured for 1 day, after which cell proliferation was determined by measuring [³²P]thymidine incorporation (A) and the Alamar Blue assay (B). Each point represents the mean of duplicate measurements, and the standard deviations are within the symbols. (A) conditioned medium; (B) fresh medium (without dilution).
dideoxy chain termination method of Sanger et al. (30) using a Taq dye primer cycle sequencing kit (Applied Biosystems). The nucleotide sequences of both strands were determined.

RESULTS

Purification of a Growth Factor from the Conditioned Medium of NIH-Sape-4—We found that the conditioned medium of NIH-Sape-4 cells contained a factor that promoted growth of these cells. As shown in Fig. 1, cell proliferation was stimulated markedly in the presence of the conditioned medium, as revealed by the $[^3]$H thymidine incorporation and Alamar Blue assays, whereas no growth factor activity was detected when the same amount of fresh M-M medium was added to the cell culture. We purified this insect-derived growth factor (IDGF) in the conditioned medium to homogeneity by five successive column chromatographies. The results of a typical purification procedure are summarized in Table I. About 17 μg of pure growth factor was routinely obtained from 9,000 ml of conditioned medium, and its specific activity was about 20,000-fold.

Fig. 2. Elution profile and electrophoretic analysis of IDGF from the molecular sieve column. A, at the final step of purification, IDGF was eluted from a G3000SWXL column. The growth factor activity in each fraction was assayed, and the amounts of protein recovered in each fraction relative to that in the original sample were calculated. ●, growth factor activity; □, relative amount of protein. B, the fractions containing growth factor activity were pooled, and samples (0.5 μg of protein) were subjected to SDS-polyacrylamide gel electrophoresis. The gel was calibrated with the following molecular mass markers: a, phosphorylase b (97 kDa); b, bovine serum albumin (66 kDa); c, ovalbumin (43 kDa); and d, α-chymotrypsinogen (26 kDa).

Fig. 3. Molecular mass estimation of the intact IDGF. Purified IDGF (18 μg) was subjected to HPLC on a molecular sieve column (G3000SWXL), which was calibrated with β-galactosidase (116 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), and α-chymotrypsinogen (26 kDa), the molecular masses of which (open symbols) are plotted against their elution volumes. The closed symbol represents IDGF.

Fig. 4. Dose-response curve for the stimulation of NIH-Sape-4 cell growth by IDGF. NIH-Sape-4 cell proliferation in the presence of increasing concentrations of IDGF was examined. Each point represents the mean of duplicate measurements, and the standard deviations are within the symbols.

Fig. 5. Immunoblotting of IDGF. A, the purified IDGF (4.5 ng) and conditioned medium (70 μg of protein) were subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblotting with an affinity-purified antibody against IDGF. Lane 1, purified IDGF; lane 2, conditioned medium. B, immunoblotting analysis of mature eggs and embryos at various developmental stages. Unfertilized eggs were collected from 6-day-old virgin female flies, and embryos were collected from 6–10-day-old female flies mated when they were 6 days old. Embryos from 10-day-old flies correspond to first instar larvae. Protein (70 μg) sources: lane 1, purified IDGF (4.5 ng); lane 2, unfertilized eggs; lanes 3–7, embryos from flies of 6-, 7-, 8-, 9- and 10-day flies, respectively.
that of the initial conditioned medium.

At the final step of purification, the growth factor activity was eluted by HPLC on a molecular sieve column as a single peak, which coincided with a protein peak, as shown in Fig. 2A. A single band with a molecular mass of 52 kDa was detected when the final growth factor fractions were combined and subjected to SDS-polyacrylamide gel electrophoresis (Fig. 2B). In order to confirm that this major protein peak was the growth factor, we subjected this final pooled fraction to FPLC on a chromatofocusing column of Mono P. The growth factor activity and major protein were eluted again from the column simultaneously (data not shown), indicating that the purified protein with a molecular mass of 52 kDa was the growth factor.

Characterization of IDGF—

The molecular mass of the intact IDGF, estimated from the elution volume from the molecular sieve column, was 98 kDa, as shown in Fig. 3, which indicated that the intact IDGF was a homodimer of the 52-kDa subunit.

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The dose-response curve is comparable with those obtained with various mammalian growth factors, indicating that the specific activity of IDGF is almost in the same range as those of mammalian growth factors (31–33).

IDGF and Sarcophaga Embryogenesis—In order to characterize IDGF further, we raised an antibody against it and affinity-purified this antibody. We used the affinity-purified antibody to perform immunoblotting analysis of extracts of embryos at various developmental stages (34), as NIH-Sape-4 cells were established from Sarcophaga embryos. As is evident from Fig. 5A, the conditioned medium contained the 52-kDa protein that cross-reacted immunologically with this antibody, indicating that the purified IDGF was secreted by the cells in this form and was not a processed or degraded product of a larger precursor molecule.

As shown in Fig. 5B, all the embryonic extracts prepared from the embryos at various developmental stages tested so far, including an extract of first instar larvae, contained significant amounts of IDGF, indicating that it is needed for proliferation of Sarcophaga cells at various developmental stages. Unexpectedly, even unfertilized eggs contained IDGF, which may, therefore, be stored in unfertilized eggs as a maternal protein and stimulate cell proliferation as soon as embryonic cells are formed at the cellular blastodermal stage.

Isolation of a cDNA for IDGF—To determine the complete amino acid sequence of IDGF, we isolated a cDNA clone for IDGF. For this we determined the partial amino acid sequences of four peptides derived from IDGF by digestion with lysylendopeptidase and synthesized an oligodeoxyribo nucleotide probe according to the partial sequence of one of these peptides. By using this probe, we screened about 100,000 transformants of a cDNA library for NIH-Sape-4 poly(A) RNA and isolated a hybridization-positive clone. This cloned plasmid contained a cDNA insert of about 2.3 kilobase pairs.

The nucleotide sequence and deduced amino acid sequence of the insert are shown in Fig. 6. This insert contains an open reading frame of 1659 nucleotides corresponding to 553 amino acid residues. This amino acid sequence contained the sequences of four peptides obtained by digesting IDGF with lysylendopeptidase.

We determined the sequence of 15 amino acid residues from the amino-terminal end of IDGF and assigned the amino-terminal residue to Ala at position 29. Thus, IDGF consists of 553 amino acid residues. This amino acid sequence contained the sequences of four peptides obtained by digesting IDGF with lysylendopeptidase.

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No significant sequence identity was found between IDGF and other known growth factors. However, a significant identity was found between IDGF and AGSA of A. californica. The percentage of identity was about 25% when gaps were introduced to optimize the identity (Fig. 7).

Aplysia AGSA was reported to be an antigen restricted to the cortex of granules in the atrial gland, but its biological function is unknown.

DISCUSSION

Like many established insect cell lines, NIH-Sape-4 cells have been maintained as a mixture of heterogenous embryonic cells (15). When the cells were inoculated at a high density (1 × 10^6 cells/mL), a significant effect on the proliferation of NIH-Sape-4 cells, which was detected with 0.7 ng/ml and reached saturation above 5 ng/ml (Fig. 4). This dose-response curve is comparable with those obtained with various mammalian growth factors, indicating that the specific activity of IDGF is almost in the same range as those of mammalian growth factors (31–33).

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10^6 cells/ml) into M-M medium, they proliferated in the absence of fetal calf serum and any other growth factors, but when inoculated at a low density (2.5 × 10^5 cells/ml), little proliferation was detected. Therefore, cooperative effects of multiple cells are needed to maintain them in culture, and the production of a growth factor(s) seemed indispensable for their growth.

We purified a growth factor from the conditioned medium of NIH-Sape-4 cells to homogeneity. This is, to our knowledge, the first report of the purification of a growth factor from insect cells. During this purification procedure, growth factor activity was recovered from each column chromatography as a major single peak, suggesting that the conditioned medium contained predominantly a single growth factor, which alone is sufficient for the cooperative growth of NIH-Sape-4 cells. Actually, under our assay conditions, the number of cells increased 2–3-fold in the presence of saturation levels of the purified IDGF for 2 days, whereas no appreciable change was detected in the cell population before this 2-day incubation nor any further change thereafter when the cultures were examined under a microscope.

The specific activity of IDGF was of the same order as those of various mammalian growth factors (31–33). As specific receptors for mammalian growth factors have been identified, we assume that a specific receptor for IDGF is present on the surfaces of NIH-Sape-4 cells.

Homologues of mammalian growth factors and their receptors have been suggested to be present in insects (1–14). For instance, a member of the Drosophila transforming growth factor-β family, decapentaplegic (dpp), was shown to act as a morphogen for dorsal-ventral pattern organization in Drosophila (2). The growth factor we purified was a homodimer of the 52-kDa subunit. As the molecular masses of most mammalian growth factors and their receptors form mammalian growth factors have been identified, we assume that a specific receptor for IDGF is present on the surfaces of NIH-Sape-4 cells.

cDNA cloning of IDGF confirmed that it is a novel growth factor, since no significant sequence identity was found between IDGF and other growth factors. Overall sequence identity between IDGF and AGSA was about 25%, but there are several partial sequences showing much higher identity. If these sequences are needed for growth factor activity, AGSA is likely to be an Aplysia growth factor.

Immunoblotting revealed that IDGF is present in mature unfertilized eggs as a maternal protein, suggesting that IDGF is needed at the very early stages of embryonic development, such as the cellular blastodermal stage of Sarcophaga. Clearly, IDGF can stimulate NIH-Sape-4 cell proliferation, but it does not appear to induce polyploid cellular extension, as the chromosomes of the cells do not polyteneize during culture in the presence of IDGF.

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