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A New Trophoblast-Derived Growth Factor from Human Placenta: Purification and Receptor Identification†

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ABSTRACT: This paper describes the identification and characterization of a new peptide growth factor. The peptide was isolated from trophoblastic brush border membranes of human placenta. The purified preparation was homogeneous and consisted of a single polypeptide of M_r 34 000 with a p*I* of about 6.0. This peptide stimulated DNA replication in cultured fibroblasts. The following association was seen between activity and protein: (a) During DEAE-cellulose chromatography, both the 34-kilodalton (kDa) protein and the mitogenic activity displayed identical binding and salt dependence of elution. (b) Nondenaturing electrophoresis at pH 8.3 revealed a comigration of the 34-kDa protein and the DNA replication stimulatory activity. (c) Identical electrophoretic mobilities were displayed for both activity and protein at pH 7.0. These results demonstrate that the preparation is homogeneous and show that growth factor activity is intrinsic to the 34-kDa polypeptide. Binding of the ^{125}I-labeled 34-kDa mitogen to target fibroblastic cells was specific; i.e., nanomolar concentrations of the unlabeled 34-kDa protein competed effectively with the labeled protein, whereas a variety of well-characterized growth factors and hormones were unable to compete even at micromolar levels. Thus the 34-kDa protein interacts with target cells through highly specific surface receptors. Chemical cross-linking techniques were used to investigate the identity of the receptor for the 34-kDa mitogen. Cross-linking of fibroblastic cells containing bound ^{125}I-labeled 34-kDa protein generated a radiolabeled complex of 86 kDa in all four different cell types examined. The amount of labeled complex formed decreased drastically in the presence of excess unlabeled 34-kDa protein but was unaffected in the presence of other growth factors. The results suggest that the binding of the placental mitogen to target cells is mediated through a 50-kDa surface protein which may represent the whole receptor or only the binding subunit of an oligomeric receptor. In its molecular weight and binding specificity, this "mitogen receptor" is different from the well-characterized receptors for epidermal growth factor, platelet-derived growth factor, insulin-like growth factor, or β-transforming growth factor. Implications of these findings and the teleological significance of the membranous growth factor are discussed.

Growth factors and their receptors play a central role in the control of cell proliferation. Demonstration in recent years of structural and functional homologies between these normal growth control molecules and those responsible for cell transformation adds considerable interest to studies on these proteins (Waterfield et al., 1983; Downward et al., 1984). Four important classes of polypeptide growth factors have been identified, and their interactions with cell-surface receptors have been well characterized. These are the following: (a) epidermal growth factor (EGF)[1] and EGF-like factors, a family of 6000–7000-dalton polypeptides that share a common receptor that shows homology with the *erb-B* oncogene product (Cohen, 1962; Das, 1982; Downward et al., 1984; Tam et al., 1984); (b) platelet-derived growth factor (PDGF), a 25 000–30 000-dalton protein that shows identity with the *sis* oncogene product (Waterfield et al., 1983; Doolittle et al., 1983); (c) insulin and insulin-like growth factors, a class of structurally related 6000–7000-dalton proteins that interact with multiple

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[1] Abbreviations: EGF, epidermal growth factor; PDGF, platelet-derived growth factor; IGF, insulin-like growth factor; β-TGF, β-transforming growth factor; Il-2, interleukin 2; LHRH, luteinizing hormone releasing hormone; BS^3, bis(sulfosuccinimido) suberate; DTSSP, bis(sulfosuccinimido) 3,3′-dithiobis(propionate); DME medium, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; EBSS, Earle's balanced salt solution; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; βME, β-mercaptoethanol; BSA, bovine serum albumin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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receptors (Blundell & Humbel, 1980; Czech & Massague, 1982); and (d) β-transforming growth factor (β-TGF), a 25000-dalton protein (Roberts et al., 1983). It is recognized that ectopic synthesis or overproduction of these growth factors or their receptors can lead to the expression of the transformed phenotype (Sporn & Todaro, 1980; Huang et al., 1984).

In this paper we describe the purification to homogeneity of a new peptide growth factor of 34000 daltons (Da) from human placenta. The peptide is different from the aforementioned growth factors and several other known mitogens in its polypeptide molecular weight, receptor binding specificity, and antigenic structure [also see the following paper in this issue (Sen-Majumdar et al., 1986)]. It originates from trophoblasts (placental cells of embryonal origin) and is associated with the plasma membrane fraction of these cells. Trophoblasts are known to express various hormones, growth factors, growth factor receptors, and oncogene products in a developmentally regulated pattern during pregnancy (Hertz, 1978; Muller et al., 1983; Goustin et al., 1985). The 34-kDa protein was found to be one of the most abundant peripheral proteins of trophoblastic brush border membranes, especially during the first trimester of pregnancy (see the following paper in this issue), suggesting that it may influence the growth of these cells in an autocrine manner.

Materials and Methods

Materials. The cross-linking reagents bis(sulfosuccinimido) suberate (BS^3) and bis(sulfosuccinimido) 3,3′-dithiobis(propionate) (DTSSP) were from Pierce Chemical Co. EGF was purified from mouse submaxillary glands (Savage & Cohen, 1972). Porcine insulin was a gift from Eli Lilly and Co. PDGF was purchased from Collaborative Research, Waltham, MA. Human somatomedin C was a kind gift from Dr. Richard Furlanetto of this institution. Human α-thrombin (M_r ~30,000) was from Sigma Chemical Co. Luteinizing hormone releasing hormone (LHRH) was a kind gift from Dr. Jerome Strauss of this institution.

Cell Culture. Monolayer cultures of Swiss mouse 3T3 cells, NR-6 cells (Das et al., 1977), and human foreskin fibroblasts were grown in Dulbecco's modified Eagle's (DME) medium containing 10% fetal bovine serum (FBS) and 10 μg/mL gentamycin (Bishayee et al., 1984). Rabbit skin fibroblasts, a gift from Dr. Roger Kennett of this institution, were grown in RPMI medium containing 10% FBS, 10 μg/mL gentamycin, and 25 mM Hepes.

Isolation of Placental Membrane. Microvillous membranes from human placenta were prepared according to the method of Smith et al. (1977). All procedures were performed at 4 °C. Fresh normal human placenta, obtained within 30 min of delivery, were placed on ice, trimmed of amnion and chorion, washed extensively with ice-cold Earle's balanced salt solution (EBSS) containing 20 mM Hepes, pH 7.4, and minced into small pieces. The pieces were washed again with fresh ice-cold EBSS–Hepes, pH 7.4, followed by three rinses with 100 mM $CaCl_2$, and transferred to approximately 2 volumes of isotonic phosphate-buffered saline (PBS). The suspension was stirred gently at 4 °C for 2 h and then filtered through cotton gauze. The filtrate was centrifuged at 800g for 10 min. The supernatant was carefully decanted and centrifuged at 10400g for 20 min. The supernatant was once again centrifuged at 100000g for 60 min. The resulting pellet was suspended in 20 volumes of isotonic PBS and centrifuged at 100000g for 60 min. The sedimented membranes were resuspended in 2 volumes of PBS and stored frozen at −70 °C. The typical yield of membranes from a single placenta is 150–200 mg of protein.

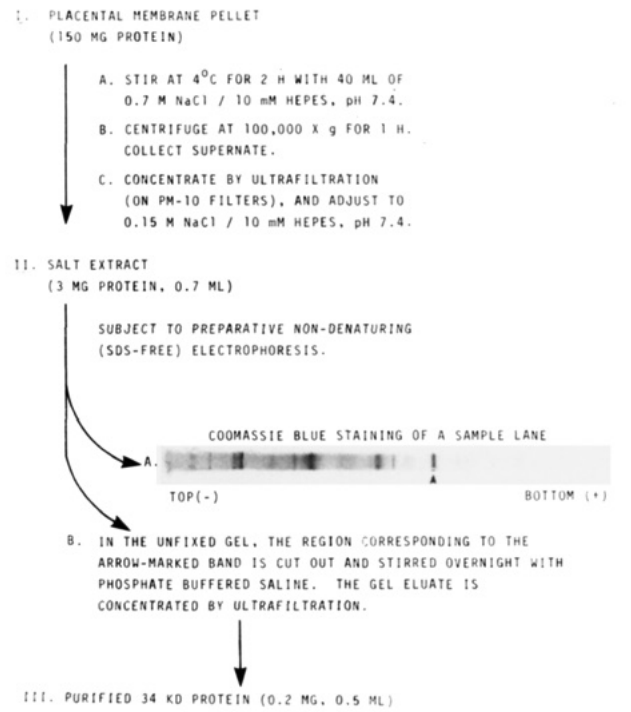

FIGURE 1: Purification of the 34-kDa mitogen from human placental membranes. See Materials and Methods for details on the preparation of placental membranes and nondenaturing gel electrophoresis at pH 8.3.

Nondenaturing Electrophoresis at pH 8.3. The gel and buffer systems were essentially the same as those described by Laemmli (1970), excepting that no sodium dodecyl sulfate (SDS) or β-mercaptoethanol (βME) was used. The electrophoresis was performed on 0.75 mm thick and 11 cm long slabs of 5–20% polyacrylamide gradient gels containing no SDS. The electrode buffer contained 0.025 M Tris and 0.192 M glycine, pH 8.3. The samples were run at 4 °C for 4 h (20 mA, 200–300 V). After the run, a sample lane was fixed and stained with Coomassie blue. The other lanes were sliced, and the slices were incubated with appropriate eluting solutions as described in Figure 1.

Nondenaturing Electrophoresis at pH 7.0. The electrophoresis was performed on 0.75 mm thick and 11 cm long slabs of 5–20% polyacrylamide gradient gels in 0.1 M sodium phosphate, pH 7.0, containing no SDS (Zahler, 1974). The electrode buffer contained 0.1 M sodium phosphate, pH 7.0. The samples were run at 4 °C for 20 h and then processed as above.

Purification of the 34-kDa Mitogen. Purification of the 34-kDa mitogen from placental membranes involved extraction of the membranes with high salt, and electrophoretic fractionation of the high-salt extract on nondenaturing gels at pH 8.3. Figure 1 depicts a typical purification protocol for a membrane batch derived from a single placenta. Placental membranes were pelleted by high-speed centrifugation prior to treatment with 0.7 M NaCl–10 mM Hepes, pH 7.4. Approximately 2% of the membrane proteins were extracted by high salt. The salt extract contained about 50% of membrane mitogenic activity. Nondenaturing gel electrophoresis of the high-salt extract revealed the presence of many protein bands. To determine the polypeptide molecular weight and mitogenic activity of these various fractionated proteins, we eluted the proteins from gel slices and tested them for the following properties: (a) DNA replication stimulatory activity in 3T3 cells and (b) their molecular weight, by electrophoresis in reducing and denaturing gels. One of the protein bands

(marked by arrow in Figure 1) was found to represent a mitogenically active 34-kDa polypeptide. During routine purification by nondenaturing electrophoresis, a sample lane was stained with Coomassie blue to yield a typical staining profile (Figure 1). A narrow region (0.2–0.3 cm wide) of the unfixed gel, corresponding to the arrow-marked band (representing the 34-kDa activity; mobility about 0.6), was cut out and eluted at 4 °C with PBS. The yield of eluted 34-kDa protein from a single placenta was about 200 μg. The protein purified in this way was found to be electrophoretically homogeneous (see Figures 2 and 3).

Denaturing Gel Electrophoresis and Autoradiography. The electrophoresis was performed on 5–20% gradient polyacryalmide gel slabs containing 0.1% SDS (Basu et al., 1984). The electrode buffer contained 0.1% SDS in 0.025 M Tris/0.192 M glycine, pH 8.3. The samples were dissolved in 1–3% SDS/0.15 M Tris-HCl, pH 6.8, under reducing (0.1 M βME) or nonreducing conditions as described in the figure legends. After completion of the electrophoresis, the gels were stained, destained, dried, and then subjected to autoradiography (for 12–48 h) using Kodak X-Omat AR films and Du Pont Cronex Lightning Plus intensifying screens.

Two-Dimensional Gel Electrophoresis. This was carried out as described (O'Farrell, 1975). The protein was desalted by gel filtration on a Sephadex G-10 column, lyophilized, and then subjected to isoelectric focusing in tube gels (5% polyacrylamide, 1.5-mm diameter) using an ampholyte gradient of pH 3–10 (Pharmacia). After an overnight run (~15 h) at 400 V, the gel was removed from the tube, equilibrated in SDS sample buffer, and then subjected to SDS gel electrophoresis on a 10% polyacrylamide gel slab of 0.75-mm width. The second-dimension run was at 20 mA for 4 h. The gel was stained for protein by using Coomassie brilliant blue.

DEAE-cellulose Chromatography. Purified 34-kDa protein (70 μg) in 300 μL of 10 mM Hepes, pH 7.4, was passed very slowly through a 100-μL DE-53 (Whatman) gel bed packed in an Eppendorf micropipet tip and equilibrated with the same buffer. The column was washed with 1 mL of the above buffer and then eluted sequentially with 400 μL of buffer containing 0.1, 0.3, 0.5, and 1 M NaCl. The washes and eluates were each diluted 10-fold with DME medium and concentrated by Amicon ultrafiltration (on PM-10 filters) to 300 μL. Aliquots of each fraction were tested for DNA replication stimulatory activity by a [^{3}H]thymidine incorporation assay and for the presence of the 34-kDa protein by SDS gel electrophoresis and Coomassie blue staining.

Nuclear Labeling and Autoradiography. Cells were plated into 2.5-mm dishes (in 96-well plates) at a density of 10^4 cells per well in 150 μL of DME medium containing 1% FBS. After 25 h at 37 °C the medium was replaced with 150 μL of DME medium containing 1% platelet poor human plasma (Scher et al., 1985). After 5 days at 37 °C, these cells were incubated with the appropriate mitogen and [^{3}H]thymidine (1 μCi/mL; 1.5 Ci/mmol) in 100 μL of DME medium containing 5% platelet poor human plasma. After 24 h at 37 °C, the cell monolayers were washed with PBS, fixed with methanol, and then treated with Kodak NTB2 emulsion (Das et al., 1984). After 5 days at 15 °C in the dark, the wells were developed and fixed. Labeled nuclei were visualized as heavy dark round spots (see Figure 4B). Unlabeled nuclei were stained with Giemsa. For each determination of percentage labeled nuclei, at least 400 nuclei were counted.

Incorporation of [^{3}H]Thymidine into Acid-Insoluble Material. Cells were plated into 16-mm dishes (in 24-well plates) at a density of 10^5 cells per well in 1 mL of DME medium containing 1% FBS. After 24 h at 37 °C the medium was replaced with 1 mL of DME medium containing 1% platelet poor plasma. After 5 days at 37 °C, these cells were incubated with the appropriate mitogen in 0.3 mL of DME medium containing 1% FBS. [^{3}H]Thymidine (1.5 Ci/mmol) was added 18 h after mitogen addition to a final concentration of 1 μCi/mL, and the incubation were continued at 37 °C for an additional 6 h. Trichloroacetic acid insoluble radioactivity was determined as described (Das, 1981).

Radioiodination of the 34-kDa Protein. Radioiodination was done by the chloramine-T method. Carrier-free Na^{125}I (5 mCi) and pure 34-kDa protein (25 μg) in 0.1 M potassium phosphate buffer, pH 7.5 (total volume, 60 μL), were mixed with 1 μL of chloramine-T (100 mg/mL) for 1 min at 24 °C. The reaction was terminated by addition of 1 μL of sodium metabisulfite (200 mg/mL) and 1 μL of KI (1 g/mL). The labeled protein was separated from unreacted Na^{125}I by gel filtration through Sephadex G-15. The buffer used for elution contained 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, and 1 mg/mL bovine serum albumin (BSA). The specific radioactivity of the preparation was 50 000–100 000 cpm/ng of 34-kDa protein.

Cellular Binding of ^{125}I-Labeled 34-kDa Protein. For binding experiments, cells were seeded into 16-mm dishes (in 24-well plates) at a density of 10^5 cells per well in 1 mL of DME medium containing 10% FBS. Then cells were ready for binding after 2–4 days at 37 °C. Prior to binding, the cell monolayers were washed 3 times at 4 °C with Earle's balanced salt solution (EBSS) containing 20 mM Hepes, pH 7.4, and 1 mg/mL BSA (EBSS–Hepes–1 mg/mL BSA), using 1 mL per wash. Binding was initiated by the addition of ^{125}I-labeled 34-kDa protein in 250 μL of EBSS–Hepes–1 mg/mL BSA. After a 2-h incubation at 4 °C, the monolayers were rapidly washed 4 times with EBSS–Hepes–1 mg/mL BSA, using 2 mL per wash. The washed monolayers were solubilized in 0.5 mL of 1 M NaOH and counted in a γ counter. Nonspecific binding was determined in the presence of 0.3–1 μM unlabeled 34-kDa protein.

Covalent Cross-Linking of ^{125}I-Labeled 34-kDa Protein to the Cellular Receptor. The two water-soluble cross-linking reagents used were (a) bis(sulfosuccinimido) suberate (BS^3), a noncleavable cross-linker, and (b) bis(sulfosuccinimido) 3,3′-dithiobis(propionate) (DTSSP), a cleavable cross-linker (Staros, 1982). For these experiments, cell monolayers were grown in 35-mm dishes as described above for the binding experiments. The monolayers were washed 3 times with EBSS–Hepes–0.1 mg/mL BSA and then incubated at 4 °C for 30 min with 6 nM ^{125}I-labeled 34-kDa protein in 0.5 mL of EBSS–Hepes–0.1 mg/mL BSA in the presence or absence of 0.3 μM unlabeled 34-kDa protein. The monolayers were then washed 4 times with cold EBSS–Hepes. Cross-linking was initiated by the addition of freshly prepared reagents (BS^3 or DTSSP) at appropriate concentrations in 0.5 mL of cold EBSS–Hepes. After a 10-min incubation at 4 °C, the monolayers were washed twice with EBSS–Hepes, solubilized with 50 μL of SDS sample buffer (with or without 0.1 M βME), and then subjected to SDS gel electrophoresis and autoradiography.

Protein Determination. Protein was determined according to the procedure of Lowry et al. (1951), using crystalline BSA as the standard.

Results

Purity and Intrinsic Mitogenic Activity of the 34-kDa Protein. Purity and activity of the protein preparation were investigated by various methods. A single polypeptide of 34

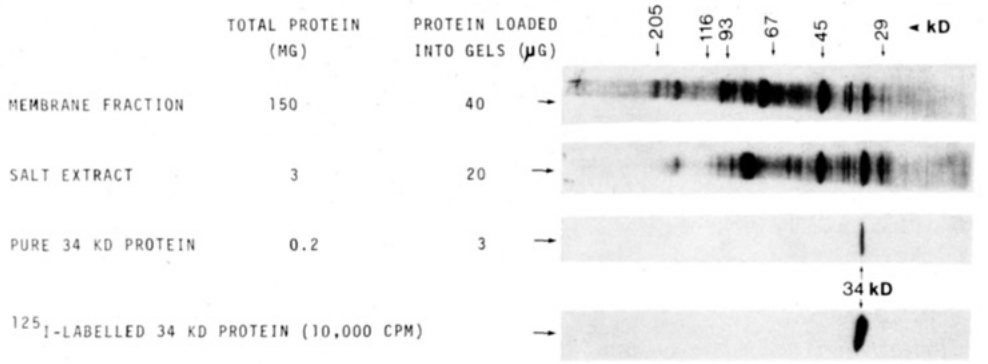

FIGURE 2: Visualization of the 34-kDa protein by Coomassie blue staining/autoradiography after reducing SDS gel electrophoresis.

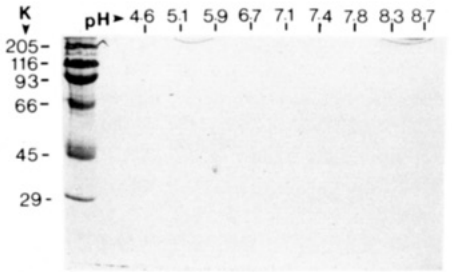

FIGURE 3: Visualization of the 34-kDa protein by Coomassie blue staining after two-dimensional gel electrophoresis involving isoelectric focusing in the first dimension and reducing SDS gel electrophoresis in the second dimension.

kDa was revealed after one-dimensional SDS gel electrophoresis by both Coomassie blue staining and autoradiography of the radioiodinated protein (Figure 2). In another experiment the protein was subjected to isoelectric focusing in the first dimension and reducing SDS gel electrophoresis in the second dimension. A single species with a p*I* of about 6.0 and M_r 34000 was seen in the gel electrophoretogram (Figure 3).

Figure 4 shows the effect of increasing amounts of pure 34-kDa protein on mouse 3T3 cells. A DNA replication stimulatory effect was seen at the lowest concentration of protein tested (3 nM). The replication stimulatory effect was completely abolished by boiling for 3 min at pH 7.4 (not shown).

To test the possibility that the observed mitogenic activity is due to a trace contaminant unrelated to the 34-kDa protein, we subjected the protein to various fractionation protocols. Chromatographic behavior of the protein on DEAE-cellulose was investigated (see Materials and Methods). The 34-kDa protein bound to a DE-53 column at pH 7.4 and did not elute with the equilibrating buffer or with buffer containing 0.1 M NaCl. However, 0.3 M NaCl was effective in eluting the protein and all the applied mitogenic activity (not shown). Electrophoretic examination of the other column fractions revealed an absence of any protein at 34 kDa or elsewhere. Thus the column binding affinity of the 34-kDa protein and the associated mitogenic activity display identical salt dependence.

Electrophoretic behavior of the protein at different pH values was investigated under nondenaturing conditions (see Materials and Methods). Reelectrophoresis at pH 8.3 for 4 h again revealed a comigration of the 34-kDa protein with DNA replication stimulatory activity (not shown). Identical electrophoretic mobilities for protein and activity were also displayed during electrophoresis at pH 7.0 for 20 h (not shown).

Overall, the above results demonstrate the homogeneity of the preparation and show that growth factor activity is intrinsic to the 34-kDa peptide.

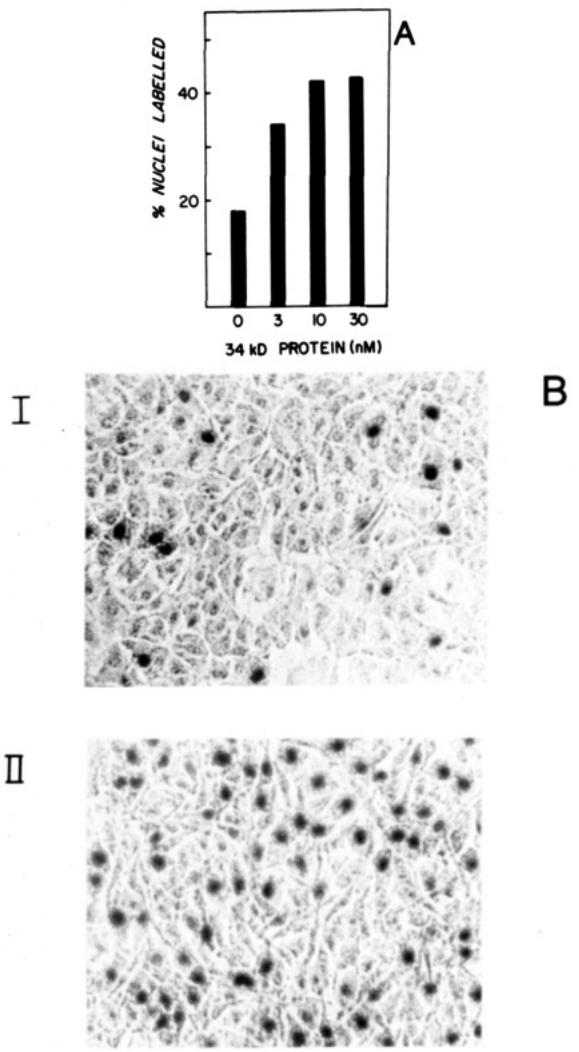

FIGURE 4: (A) Stimulation of nuclear labeling in mouse 3T3 fibroblastic cells by the 34-kDa protein. The values shown are the average of quadruplicates. No activity was seen with protein samples that were heat-treated (100 °C for 3 min) at pH 7.4. (B) Autoradiographic visualization of nuclear labeling in 3T3 cells with no 34-kDa protein (I) and with 30 nM 34-kDa protein (II).

Specific Binding of the 34-kDa Protein to Target Fibroblastic Cells. To test whether the 34-kDa protein interacts with target cells through specific and high-affinity sites, binding experiments were done with the radioiodinated protein (Figures 5 and 6, Table I). The radioiodinated preparation was found to be mitogenically active (not shown).

Incubation of 3T3 cells with ^{125}I-labeled 34-kDa protein resulted in cellular binding of the radiolabeled protein. As with mitogenic activity, the binding activity was totally abolished by boiling the protein for 3 min (Figure 5).

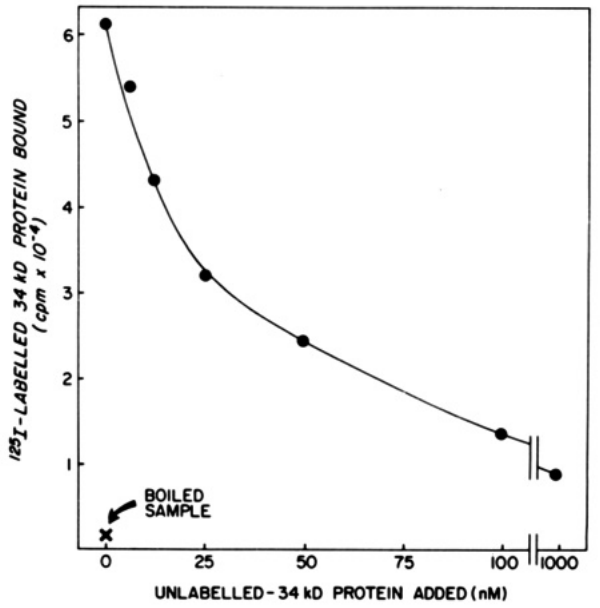

FIGURE 5: Binding of ^{125}I-labeled 34-kDa protein to mouse 3T3 fibroblastic cells. Cell monolayers in 16-mm dishes were incubated (at 4 °C for 2 h) with 6 nM radioiodinated 34-kDa protein (60 000 cpm/ng) in the presence and absence of the indicated amounts of unlabeled peptide. For preparing the boiled sample, the radioiodinated protein (40 μg/mL in 10 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl and 1 mg/mL BSA) was kept in a boiling water bath for 3 min. Then it was diluted with cold binding buffer (see Materials and Methods) to 6 nM concentration and tested for binding activity. Cell-bound radioactivity was determined as described under Materials and Methods.

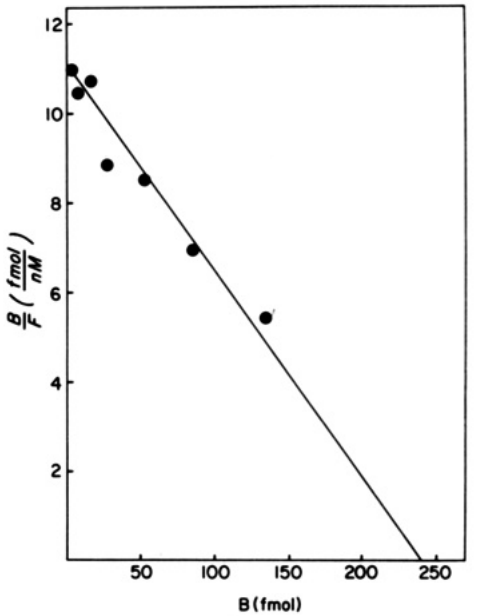

FIGURE 6: Scatchard analysis of the concentration-dependent binding of radioiodinated 34-kDa protein to mouse 3T3 cells. ^{125}I-Labeled 34-kDa protein (0.5–25 nM, 100 000 cpm/ng) was incubated with cell monolayers in 16-mm dishes at 4 °C for 2 h. Nonspecific binding, determined in the presence of 1 μM unlabeled protein, was 5–10% of total binding. For example, at 6 nM ^{125}I-labeled 34-kDa protein, the total binding was 190 000 cpm, and the nonspecific binding was 9000 cpm.

Table I: Specificity of Binding of the Radioiodinated 34-kDa Protein to 3T3 Cells[a]

unlabeled peptide added	specific binding (% of control)
none	100
34-kDa protein (100 nM)	9
EGF (2 μM)	105
insulin (2 μM)	97
somatomedin C (0.5 μM)	109
PDGF (100 units/mL)	95
α-thrombin (2 μM)	101
LHRH (2 μM)	98

[a] Cell monolayers in 16-mm dishes were tested for binding activity with 6 nM radioiodinated 34-kDa protein (60 000 cpm/ng) in the presence and absence of the indicated peptides, as described under Materials and Methods. One hundred percent binding activity represents 52 000 cpm specifically bound. Nonspecific binding (determined with 1 μM unlabeled 34-kDa protein) was about 9000 cpm.

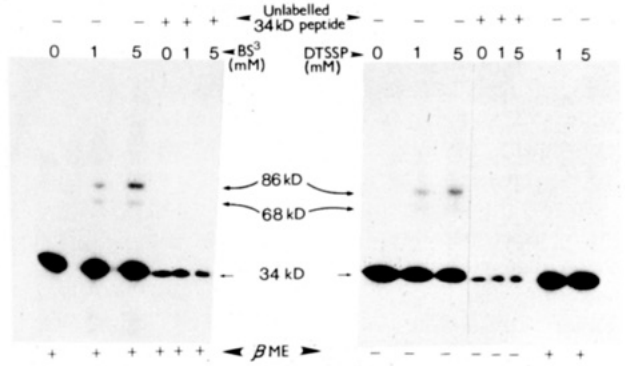

FIGURE 7: Identification of the receptor for the 34-kDa protein in NR-6 cells by chemical cross-linking. Washed NR-6 cell monolayers in 35-mm dishes were incubated at 4 °C for 30 min with 6 nM ^{125}I-labeled 34-kDa protein (100 000 cpm/ng) in the presence and absence of 0.3 μM unlabeled 34-kDa protein as described under Materials and Methods. The monolayers were washed free of unbound radioactivity and then incubated at 4 °C for 10 min with the indicated concentrations of cross-linking reagents (BS^3 and DTSSP) as described under Materials and Methods. After washing, the cells were solubilized with 50 μL of 0.1 M Tris-HCl, pH 6.8–3% SDS, containing either 0.1 M βME or no βME and then subjected to SDS gel electrophoresis and autoradiography as described under Materials and Methods.

Unlabeled 34-kDa protein competed effectively with the labeled protein for the cellular binding sites (Figure 5). However, other unlabeled peptides—EGF, insulin, PDGF, somatomedin C, and α-thrombin, which are known to interact with 3T3 cells—were unable to compete with the radiolabeled 34-kDa protein even at micromolar concentrations (Table I).

The concentration dependence of binding to cells was studied. A Scatchard analysis of the binding to mouse 3T3 cells (Figure 6) and mouse NR cells (not shown) revealed a relatively large number of binding sites (1–2 million sites per cell) and a K_d of about 20 nM.

Identification of the Cell-Surface Receptor for the 34-kDa Protein. The identity of the receptor in target cells was investigated by using a chemical cross-linking approach. We took the following precautions to avoid possible artifacts: (a) The experiments were done by using a low concentration of ^{125}I-labeled 34-kDa protein to minimize nonspecific binding. (b) All binding and cross-linking were done at 4 °C, a temperature at which internalization and degradation might be expected to be minimal. (c) The only cross-linking reagents used were hydrophilic membrane-impermeant *N*-hydroxysulfosuccinimido agents—BS^3 and DTSSP (Staros, 1982)—which would allow covalent linking of only cell-surface-located complexes.

Figure 7 shows the results of a cross-linking experiment done with mouse NR-6 cells. In these experiments cells containing bound ^{125}I-labeled 34-kDa protein were reacted with BS^3 (noncleavable agent) or DTSSP (cleavable agent), and the cross-links formed were visualized by electrophoresis and autoradiography. A major cross-linked complex of 86 kDa was seen, along with a minor complex of 68 kDa. The

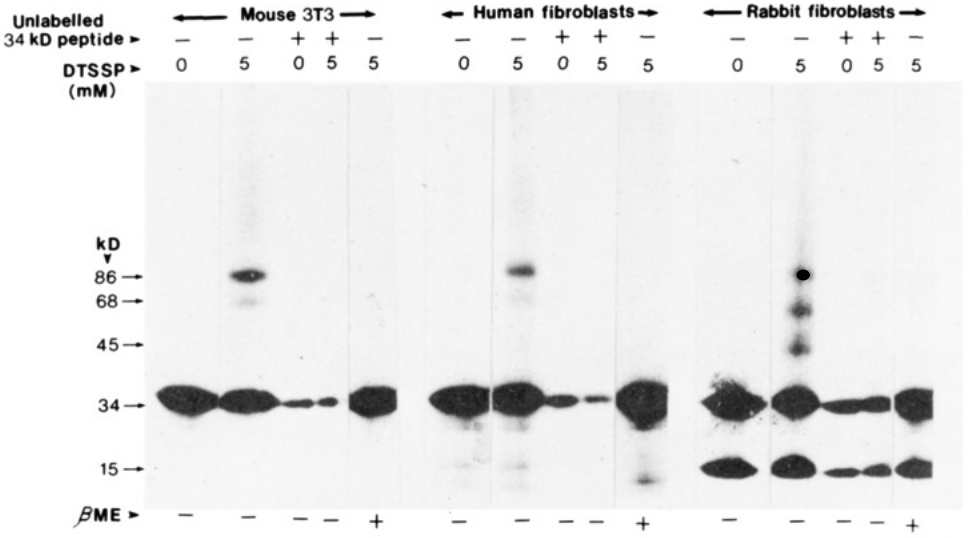

FIGURE 8: Visualization of putative receptors for the 34-kDa protein in different cell types. Chemical cross-linking studies with the cleavable cross-linker, DTSSP, were conducted as described in the Figure 7 legend and under Materials and Methods.

cross-linked complexes formed with the noncleavable agent (BS^3) were observed under both reducing and nonreducing conditions. However, with the cleavable agent (DTSSP), the cross-linked complexes were seen only under nonreducing conditions and disappeared after treatment with βME. In other cross-linking experiments where cells were treated with excess unlabeled 34-kDa protein, the amount of labeled cross-linked complex formed decreased in proportion to the decrease in ^{125}I-labeled 34-kDa protein bound (Figure 7). The presence of other growth factors, e.g., EGF, PDGF, insulin, and somatomedin C (an insulin-like growth factor), did not reduce the amount of radiolabel in the cross-linked complexes (not shown). In all these experiments the cell-bound 34-kDa protein remained in an intact undegraded form.

To test the generality of the phenomenon, similar cross-linking experiments were conducted with three additional cell types—mouse 3T3 cells, human foreskin fibroblasts, and rabbit skin fibroblasts (Figure 8). The autoradiograms showed a similar pattern of cross-linking in these cells from different species. In all cases a major cross-linked complex of 86 kDa was seen, accompanied by a minor complex of 68 kDa. In rabbit cells an additional labeled band of 45 kDa was also seen. In rabbit cells there was also a significant degradation of bound 34-kDa protein to a 15-kDa product. It should be noted that no cross-linked complex was formed in the absence of cells; i.e., the addition of cross-linking reagents to 6–20 nM solutions of ^{125}-labeled 34-kDa protein did not generate any intermolecular cross-links.

We tested whether the cross-linked complexes formed with the cleavable reagent (DTSSP) would generate, upon reduction, the 34-kDa protein (Figure 9). Both the 86- and 68-kDa bands (from both mouse NR-6 cells and rabbit skin fibroblasts) generated the 34-kDa band upon reduction. In addition, reduction of the rabbit cell-specific 45-kDa band also gave rise to the 34-kDa protein.

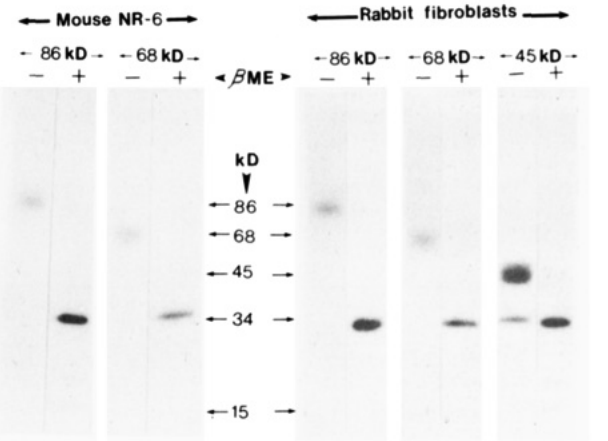

FIGURE 9: βME-induced generation of the 34-kDa protein from cross-linked species formed with DTSSP. Monolayers of mouse NR-6 cells and rabbit cells were allowed to bind ^{125}I-labeled 34-kDa protein and then were subjected to cross-linking with 5 mM DTSSP as described in the Figure 7 legend and under Materials and Methods. The cells were solubilized with SDS solution containing no βME and then subjected to nonreducing SDS gel electrophoresis. The regions of the unfixed wet gel corresponding to 86, 68, 45, and 34 kDa were cut out, stirred at 37 °C for 10 h with 100 μL of SDS sample buffer in the absence (−) or presence (+) of βME, and then subjected to electrophoresis and autoradiography. The higher visibility (radioactivity) of the +βME lanes compared with the −βME lanes (especially for the 86- and 68-kDa samples) may be due to effects of βME upon elution of proteins from gels.

Discussion

The growth factor described here is one of the peripherally attached membrane proteins of placental trophoblasts. The purified protein meets the following criteria: (a) in one-dimensional SDS gel electrophoretograms it shows only a single protein band of M_r 34000 (Figure 2); (b) only a single protein spot of M_r 34000 and with a pI of about 6 is seen after two-dimensional gel electrophoresis (Figure 3); (c) nondenaturing electrophoresis at pH 8.3 revealed only a single protein band that comigrated with the mitogenic activity; (d) comigration of mitogenic activity and the 34-kDa protein is also seen during electrophoresis at pH 7.0; (e) both mitogenic activity and protein bind to DEAE-cellulose at pH 7.4 and elute with 0.3 M NaCl but not with 0.1 M NaCl. Electrophoretic examination of DE-53 column fractions, other than the 0.3 M NaCl eluate, revealed an absence of protein at 34 kDa or elsewhere.

Overall, these results demonstrate the homogeneity of the preparation and show that growth factor activity is intrinsic to the 34-kDa protein. The protein is unique in many respects. (a) In its polypeptide molecular weight and antigenic structure it is unlike EGF, PDGF, insulin, insulin-like growth factors (IGFs), β-TGF, interleukin 2 (Il-2), and several other known growth factors (also see the following paper in this issue). (b)

Its binding to target cells is mediated through unique receptors that do not interact with a number of well-known growth factors and hormones (Table I). (c) Unlike a number of growth factors, e.g., EGF and PDGF, which are heat-stable, the 34-kDa mitogen is inactivated by boiling at neutral pH. (d) It is related (in its ion-exchange column binding characteristics) to our previously identified (although not purified) mitogenic polypeptide of 34 kDa, present in A431 carcinoma cell membranes (Bishayee et al., 1984), but it is unrelated to the reported 3T3 cell membrane associated mitogenic activity of 150 kDa (Lieberman, 1983).

The observed specificity of binding of the 34-kDa placental mitogen to target fibroblasts prompted us to investigate the identity of its receptor. In our experiments on receptor identification by chemical cross-linking, a major cross-linked complex of 86 kDa was seen in all the four cell types examined. A minor cross-linked band of 68 kDa was also seen; in addition, a 45-kDa complex was seen in the atypical rabbit cell, the only cell type that degraded bound 34-kDa protein at 4 °C. The cross-linked complexes in all four cell types meet the following criteria: (a) In the presence of excess unlabeled 34-kDa protein, the amount of labeled complexes formed decreased in proportion to the decrease in labeled 34-kDa protein bound. (b) The presence of other growth factors such as EGF, PDGF, insulin, or somatomedin C did not affect the amount of cross-linked complex formed. (c) The complexes formed with the cleavable cross-linking agent (DTSSP) were nonvisualizable after reduction. (d) The cross-linked complexes contained intact, undegraded 34-kDa protein; i.e., the DTSSP-linked complexes generated the 34-kDa protein upon reduction. (e) Cell lines from three different animal species generated cross-linked complexes of identical molecular weights. These results suggest that the observed cross-links are unlikely to be artifacts and may very well represent a ligand–receptor type of interaction. The major 86-kDa cross-linked complex may represent a cellular receptor of 50 kDa. This 50-kDa moiety could be the entire receptor or only a binding subunit of an oligomeric (non-disulfide-linked) receptor. It should be noted that in its molecular weight this putative receptor is unlike the receptors for EGF (Das et al., 1977; Cohen et al., 1982; Biswas et al., 1985), PDGF (Heldin et al., 1983), β-TGF (Massague, 1985), insulin, or insulin-like growth factors (Czech & Massague, 1982). However, the Il-2 receptor has been reported to have a similar molecular weight (Leonard et al., 1983).

The identities of the minor cross-linked species of 68 and 45 kDa are not clear at this time. It is possible that they represent receptor degradation products of 34 and 11 kDa (derived from 50-kDa receptor) that retain the ability to bind to the 34-kDa placental mitogen. It should be noted that these cross-linked species, especially the 45-kDa band, are prominent in the rabbit cell line that appears to display a markedly enhanced proteolytic activity (at least with respect to bound 34-kDa mitogen).

While the identity of the cellular receptor for the 34-kDa mitogen remains to be unequivocally established, it would be of interest to examine whether similar cross-linked species can be generated in donor placental cells or plasma membranes. We have demonstrated that this growth factor is biosynthesized by cultured cytotrophoblasts (A. Sen-Majumdar, J. E. Nestler, H. Kliman, U. Murthy, J. F. Strauss, and M. Das, unpublished results) as well as by A431 carcinoma cells and some other human carcinomas (see the following paper in this issue), and we have seen that the synthesized protein is tightly associated with the plasma membrane fraction of these cells, although it is not an integral membrane protein. The question is, how is it attached to the plasma membrane?. Studies with autocrine-stimulated cells that synthesize growth factors related to PDGF or EGF have shown that the effects of these growth factors are mediated through binding to endogenous membrane receptors (Sporn & Todaro, 1980; Huang et al., 1984; Goustin et al., 1985). Thus it is likely that the 34-kDa growth factor is bound to its membrane receptor in placental trophoblasts and other producer cells such as the A431 carcinoma line. Its possible role as an autocrine stimulant of cytotrophoblast growth remains to be tested.

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A Specific Antibody to a New Peptide Growth Factor from Human Placenta: Immunocytochemical Studies on Its Location and Biosynthesis†

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ABSTRACT: Recently, we isolated a new peptide growth factor of M_r 34 000 from synctial membranes of human placenta. In its polypeptide molecular weight and receptor binding specificity it is unlike several known growth factors. In this paper we describe immunocytochemical studies on its cellular location and biosynthesis. A rabbit antiserum was raised against a homogeneous preparation of the placental peptide. The specificity of the antibody was established by immunoprecipitation and immunoblot analyses. The antibody recognized both the native and denatured 34-kilodalton (kDa) peptide but showed no binding to a variety of other growth factors and hormones tested. The antibody was used to investigate the genesis and location of the 34-kDa membranous mitogen. Immunoperoxidase staining of placental tissue slices revealed a restricted localization of the antigen in the cytoplasmic organelles of cytotrophoblasts and in the brush border membranes of syncytiotrophoblasts. No other placental structures contained the antigen. A developmentally regulated appearance of the mitogen was suggested by the fact that first trimester placenta consistently stained far more strongly than term placenta. These studies show that the 34-kDa mitogenic protein originates in placenta from embryo-derived cellular structures and suggest that in its strategic location it may influence trophoblastic growth in an autocrine manner. In other studies we investigated the presence and biosynthesis of the 34-kDa peptide in the A431 vulval carcinoma cell line, which was shown earlier to contain a membrane-associated 34-kDa growth factor. The studies demonstrate that this cell line, as well as some other human carcinomas of breast and bladder origin, actively expresses this peptide. The tight, but peripheral, association of the growth factor with membranes of producer cells (cytotrophoblasts and carcinomas) suggests binding to a receptor and an autocrine mechanism of stimulation.

In placenta, various hormones, growth factors, receptors, and oncogene products appear in a developmentally regulated fashion during pregnancy (Hertz, 1978; Nexo et al., 1979; Stromberg et al., 1982; Muller et al., 1983). For example, hormones such as placental lactogen (Vilee, 1978) and receptors such as EGF receptor (O'Keefe et al., 1974; Lai & Guyda, 1984) increase in placenta throughout pregnancy in parallel with the appearance of syncytiotrophoblasts, which are multinucleated cells of embryonic origin (Kirby, 1965). Synctial microvillous membranes are also rich in receptors for insulin (Petruzelli et al., 1984), insulin-like growth factors (Jones & Harrison, 1980), and platelet-derived growth factor (Goustin et al., 1985). These various growth factor receptor systems may play a role in cytotrophoblast proliferation, and in the maintenance of the differentiated functions of syncytiotrophoblasts.

Recently, we isolated a new peptide growth factor of 34 000 dalton (Da) from syncytiotrophoblast membranes of human placenta [see the preceding paper in this issue (Sen-Majumdar et al., 1986)]. In its polypeptide molecular weight and receptor binding specificity it is unlike several known growth factors and hormones. However, it appeared to be related to a previously studied peptide growth factor associated with plasma membranes of human A431 carcinoma cells (Bishayee et al., 1984).

To further investigate the biology of this placental growth factor, we developed a specific immunologic probe. In this paper, we describe the characteristics of a specific antiserum raised against a homogeneous preparation of the 34-kDa growth factor. The specificity of the antiserum for the 34-kDa peptide was established by immunoprecipitation and immunoblot analyses. Binding of the 34-kDa peptide to this antibody was not competed for by a host of circulating hormones

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