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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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and growth factors, indicating that the antibody is highly specific for the placental peptide.

The antibody was used in the following studies: (a) to investigate the localization of the peptide in placenta and (b) to test A431 and other human carcinomas for their ability to biosynthesize this peptide. The results demonstrate that the placental growth factor is a synctial membrane protein, originating from cytotrophoblasts, that is expressed at a high level during the first trimester of pregnancy, but at a significantly lower level near term. The A431 vulvar carcinoma cell line and a number of other human carcinomas biosynthesize a 34-kDa peptide that is antigenically related to the placental protein. These results suggest an association of the 34-kDa growth factor with cell types that are intensely involved in proliferation and invasion.

Materials and Methods

Materials. Mouse EGF,[1] human PDGF, porcine insulin, human somatomedin C, and human α-thrombin (M_r ~30000) were obtained as described (see the preceding paper in this issue). Goat anti-rabbit IgG antibody and protein A were from Sigma Chemical Co., St. Louis, MO. ^{125}I-labeled protein A was prepared as follows. Protein A (2 μg) was radioiodinated with carrier-free Na^{125}I (2 mCi; Amersham Corp.) by using chloramine-T (5 mg/mL) in a total volume of 20 μL of 0.1 M potassium phosphate buffer, pH 7.5. After incubation at 20 °C for 1 min, the reaction was terminated by the addition of sodium metabisulfite and KI, and the labeled protein was separated from unreacted Na^{125}I by gel filtration through Sephadex G-15.

Purification of the 34-kDa Growth Factor from Human Placenta and Radioiodination of the Peptide. These were done as described earlier (see the preceding paper in this issue).

Cellular Binding of ^{125}I-Labeled 34-kDa Peptide and Assay of Mitogenic Activity of the Peptide. These were done as described previously (see the preceding paper in this issue).

Generation of a Specific Antiserum to the 34-kDa Peptide. Antibodies to the pure 34-kDa placental peptide were raised in rabbits as follows. About 100 μg of a homogeneous preparation of the peptide (see the preceding paper in this issue), in 200 μL of phosphate-buffered saline (PBS), was emulsified with an equal volume of Freund's complete adjuvant and then injected intradermally at multiple sites into rabbits (New Zealand, albino, 3–4-month-old females). The rabbits were reinjected twice, at 3-week intervals, with the antigen emulsified in Freund's incomplete adjuvant. Eight days after the last injection, the rabbits were bled. The IgG fraction of the antiserum was purified by protein A–Sepharose column chromatography using 0.1 M glycine hydrochloride, pH 3.0, as the eluting buffer. All the antibody activity was bound to the column, and over 90% was eluted. The eluted IgG was neutralized immediately with Tris, dialyzed extensively against PBS, and stored at −70 °C.

Radioiodination of Placental Microvillous Membranes and Mouse Liver Membranes. Membranes were isolated as described (Smith et al., 1977; Bishayee et al., 1982). About 500 μg of membrane protein was labeled with carrier-free ^{125}I (2 mCi) in 100 μL of PBS at 4 °C for 30 min with 50 μg of iodogen (Pierce) as the oxidizing agent (Fraker & Speck, 1978). The radioactively labeled membranes were pelleted by high-speed centrifugation and washed with 20 mL of 20 mM Hepes and 0.15 M NaCl, pH 7.4.

Immunoprecipitation Technique. Appropriate amounts of IgG (immune or nonimmune) were incubated at 4 °C for 1 h with pure ^{125}I-labeled 34-kDa protein (6 mM) in the absence or presence of other unlabeled peptides in 20 μL of 20 mM Hepes, pH 7.4, 0.15 M NaCl, and 1 mg/mL bovine serum albumin (BSA). The immune complexes were isolated by incubating the mixture at 20 °C for 15 min with 1 mg of formaldehyde-fixed *Staphylococcus aureus*. The bacteria were washed free of unbound radioactivity with 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, and 0.1% Triton X-100. The bound radioactivity was eluted by boiling for 2 min in 1% sodium dodecyl sulfate (SDS) containing 0.1 M β-mercaptoethanol in 0.15 M Tris-HCl, pH 6.8. It was subjected to electrophoresis on SDS–polyacrylamide gradient (5–20%) gels and subsequently to autoradiography using Kodak X-Omat AR films and Du Pont Cronex Lightning Plus intensifying screens.

Immunoblotting Method. The test samples were subjected to SDS–polyacrylamide gel electrophoresis under reducing conditions. The electrophoretically separated proteins were electrophoretically transferred to nitrocellulose sheets as described by Towbin et al. (1979), using a Bio-Rad apparatus. The electrode buffer contained 25 mM Tris–192 mM glycine–20% (v/v) methanol at pH 8.3. The run time was 5 h (at 250 mA, 20 V). After transfer, the nitrocellulose blots were soaked in 3% BSA, 0.15 M NaCl, and 10 mM Tris-HCl, pH 7.4, for 5 h at 20 °C to saturate additional protein binding sites. They were then rinsed in saline and incubated at 20 °C for 2 h with anti-34-kDa peptide rabbit IgG (100 μg of IgG/mL) in 3% BSA–0.15 M NaCl–10 mM Tris-HCl, pH 7.4. The antibody-treated sheets were washed extensively in saline, then incubated with goat anti-rabbit IgG antibody (100 μg/mL) in saline–BSA at 20 °C for 2 h, again washed, and finally incubated at 20 °C for 2 h with ^{125}I-labeled protein A (10^8 cpm, 500 ng) in saline–BSA. The papers were then washed extensively in saline, dried with a hair dryer, and then subjected to autoradiography.

Preparation of Preadsorbed Antiserum. Following the manufacturer's instructions (Das et al., 1984), 34-kDa protein–agarose columns were prepared by using Affi-Gel 10 (Bio-Rad Labs). The washed agarose (1 mL) containing 0.4 mg of covalently bound 34-kDa protein was stirred at 4 °C for 12 h with 1 mL of PBS–1 mg/mL BSA containing 0.5 mg of the IgG fraction of the antiserum (see Results). The supernatant obtained after centrifugation was used as a control in immunoperoxidase staining experiments.

Imunoperoxidase Staining of Fixed Placental Tissue. Tissues obtained from the hospital of the University of Pennsylvania were processed for staining as described (Hsu et al., 1981a,b). The specimens were first fixed in Bouin's fixative, dehydrated in graded alcohols, cleared in xylene, embedded in paraffin, and then cut into 4–5-μm sections. The tissue sections were deparaffinized in xylene and absolute ethanol and then incubated in methanol containing 0.3% hydrogen peroxide to quench endogenous peroxidase activity. The sections were washed with water, incubated at 20 °C for 20 min in a normal goat serum solution (1:20) to block nonspecific binding sites, washed with PBS–1 mg/mL BSA, and then incubated at 20 °C for 30 min with the test rabbit antibody in PBS–BSA. The treated slides were washed with PBS–BSA, incubated at 20 °C for 30 min with biotinylated

[1] Abbreviations: EGF, epidermal growth factor; PDGF, platelet-derived growth factor; HCG, human chorionic gonadotropin; PBS, phosphate-buffered saline; BSA, bovine serum albumin; DME medium, Dulbecco's modified Eagle's medium; SDS, sodium dodecyl sulfate; EBSS, Earle's balanced salt solution; IgG, immunoglobulin G; Tris, tris(hydroxymethyl)aminomethane; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride.

goat anti-rabbit IgG (1:600, from Vector Laboratories, Burlingame, CA), again washed, and then incubated at 20 °C for 45 min with avidin–biotin–peroxidase complex (1:50, Vector Laboratories, Burlingame, CA). After a final wash with PBS–BSA, the slides were incubated with a solution of PBS containing 0.06% 3,3′-diaminobenzidine and 0.0001% hydrogen peroxide to yield a reddish brown precipitate. The sections were counterstained with hematoxylin, dehydrated, mounted, and then examined by conventional light microscopy.

Immunoperoxidase Staining of Cultured Carcinoma Cells. Cells were removed from cultures by using 0.02% trypsin–EDTA, washed twice with PBS–1 mg/mL BSA, and spun onto slides by using a cytospin (Shandon). Cells were then fixed with 95% ethanol at 20 °C for 10 min (Keebler & Regan, 1977) and then washed with PBS. These were subjected to treatment with test antibody and immunoperoxidase staining as described in the previous paragraph for placental tissue sections.

Biosynthetic Labeling of Cultured Carcinoma Cells with [^{35}S]Methionine. The cells were seeded into 35-mm dishes (2.5×10^5 cells per dish) in Dulbecco's modified Eagle's (DME) medium containing 10% fetal bovine serum (FBS). After 2 days the cells were washed with methionine-free DME medium containing 5% dialyzed FBS and kept in the same medium for 3 h at 37 °C. The cells were then incubated at 37 °C for 4 h with [^{35}S]methionine (250 μCi/mL; 3.5 mCi/μmol) in 1 mL of DME–5% FBS medium. Next, the cells were washed and lysed with 100 μL of PBS containing 1% Triton X-100, 2 mM PMSF, 50 mM benzamidine, and 25 μg/mL aprotinin. The supernatant obtained after repeated freezing–thawing and centrifugation was first treated with 250 μg of rabbit nonimmune IgG at 4 °C for 1 h and then with 10 mg of washed *S. aureus* at 4 °C for 30 min. The resultant supernatant was subjected to immunoprecipitation using 25 μg of anti-34-kDa peptide immune IgG and 10 μg of washed *S. aureus* as described in a previous section. The immunoprecipitates were subjected to SDS gel electrophoresis, then prepared for fluorography by immersion in dimethyl sulfoxide containing 2,5-diphenyloxazole (Laskey & Mills, 1975), finally dried, and exposed to X-ray films.

Results

Peptide Specificity of the Antiserum. The reactivity of the anti-34-kDa peptide immune IgG was examined by immunoprecipitation analysis. Figure 1 demonstrates that the immune IgG can specifically immunoprecipitate the ^{125}I-labeled pure 34-kDa peptide. Unlabeled 34-kDa protein competed effectively with the labeled protein for the immune IgG (Figure 1). However, other unlabeled peptides—EGF, insulin, PDGF, somatomedin C, and α-thrombin (Table I)—were unable to compete even at micromolar concentrations in these radioimmunoassays. Note that these peptides were also noncompetitive in radioreceptor assays for the 34-kDa peptide (see the preceding paper in this issue).

Antibody Specificity Studied by Immunoprecipitation. The specificity of the anti-34-kDa peptide antiserum was further examined by using Triton X-100 solubilized extracts of human placental crude membranes. As shown in Figure 2, the antiserum immunoprecipitates only one radioiodinated peptide, a 34-kDa peptide, and this reaction was blocked by pretreatment of the antiserum with unlabeled pure 34-kDa peptide.

Similar experiments with radioiodinated extracts of mouse liver membranes also showed immunoprecipitation of a 34-kDa peptide by the antibody (Figure 3). Thus a murine homologue of the human placental peptide is present in liver membranes.

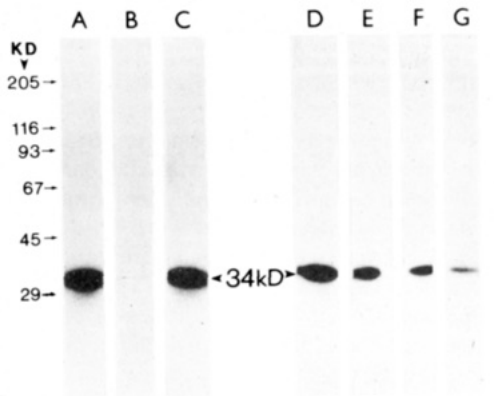

FIGURE 1: Electrophoretic/autoradiographic visualization of immunoprecipitated ^{125}I-labeled pure 34-kDa peptide. (Lane A) Input ^{125}I-labeled 34-kDa peptide (100 fmol, 200 000 cpm), no immunoprecipitation. (Lane B) ^{125}I-Labeled 34-kDa peptide (100 fmol) precipitated with 10 μg of rabbit nonimmune IgG. (Lanes C and D) ^{125}I-labeled 34-kDa peptide (60 fmol) precipitated with 10 (lane C) or 1 μg (lane D) of rabbit immune IgG. (Lanes E–G) ^{125}I-Labeled 34-kDa peptide precipitated with 1 μg of immune IgG in the presence of the following amounts of unlabeled 34-kDa peptide: lane E, 200 fmol; lane F, 500 fmol; lane G, 2 pmol. The conditions used for antigen–antibody reactions (in a total volume of 20 μL), the methods used for isolation of immune complexes with *S. aureus*, and electrophoretic/autoradiographic analysis are described under Materials and Methods.

Table I: Antigen Recognition Specificity of Rabbit Immune IgG[a]

unlabeled peptide added	immunoprecipitation of ^{125}I-labeled 34-kDa protein (% of control)
none	100
34-kDa protein (10 nM)	36
34-kDa protein (100 nM)	7
EGF (2 μM)	107
insulin (2 μM)	112
somatomedin C (0.5 μM)	103
PDGF (100 units/mL)	102
α-thrombin (2 μM)	103

[a] Anti-34-kDa peptide immune IgG (1 μg) was tested for binding to ^{125}I-labeled 34-kDa protein (100 fmol, 200 000 cpm) in the presence and absence of the indicated peptides in a 20-μL final volume, as described under Materials and Methods. The immune complexes were isolated by using *S. aureus*, subjected to SDS gel electrophoresis, and then analzyed by counting the gel slice containing the 34-kDa band. One hundred percent immunoprecipitation represents 106 000 cpm of bound ^{125}I-labeled 34-kDa protein. The blank value, i.e., the radioactivity bound by using identical amounts (1 μg) of rabbit nonimmune IgG, was about 1100 cpm.

These membranes were shown earlier to be mitogenic for cultured fibroblasts (Bishayee & Das, 1982; Bishayee et al., 1982).

Antibody Specificity Studied by Immunoblotting. Immunoblot analysis of crude placental membranes with the anti-34-kDa peptide antibody showed that the antibody binds only to a 34-kDa polypeptide and to no other membrane component (Figure 4). Thus the antibody is a highly specific probe for the 34-kDa peptide.

The immunoblot method was used to investigate the presence of this peptide in serum. Murine and human sera were found to contain the antigen, but bovine sera, including those of fetal origin, were devoid of it (not shown).

Effect of Antibody on the Biological Activity of the 34-kDa Peptide. The 34-kDa peptide is mitogenic for mouse 3T3 fibroblasts, and it interacts with these cells through specific receptors (see the preceding paper in this issue). We tested whether the antibody can block this interaction. The results

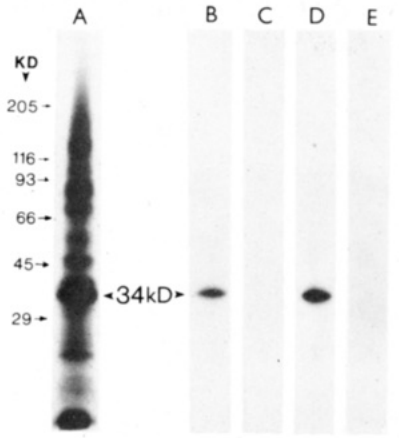

FIGURE 2: Immunoprecipitation of 34-kDa peptide from detergent extracts of placental brush border membranes. (Lane A) Input ^{125}I-labeled placental extract (10^6 cpm), no immunoprecipitation. (Lanes B and C) ^{125}I-Labeled placental extract (10^6 cpm) precipitated with 20 μg of rabbit immune IgG in the absence (lane B) and presence (lane C) of 20 pmol of unlabeled 34-kDa peptide. (Lane D) ^{125}I-Labeled placental extract (10^6 cpm) precipitated with 100 μg of rabbit immune IgG. (Lane E) ^{125}I-Labeled placental extract reacted with 100 μg of rabbit nonimmune IgG. Radioiodinated placental membranes (500 μg of protein) were stirred at 4 °C for 2 h with 100 μL of 2% Triton X-100 in 20 mM Hepes and 0.15 M NaCl, pH 7.4. After centrifugation at 10000*g* for 60 min, the supernatant was collected. The conditions used for immunoprecipitation (using 5 μL of supernatant), for immune complex isolation (using 3 mg of *S. aureus*), and for electrophoresis/autoradiography are described under Materials and Methods.

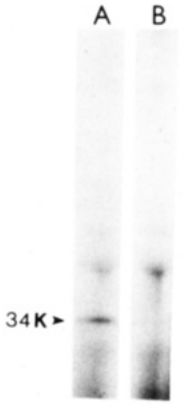

FIGURE 3: Immunoprecipitation of a 34-kDa peptide from detergent extracts of mouse liver membranes. ^{125}I-Labeled membrane extract (10^6 cpm) was reacted with either 20 μg of anti-34-kDa peptide immune IgG (lane A) or 20 μg of nonimmune IgG (lane B) and then subjected to precipitation with *S. aureus* and electrophoresis/autoradiography as described in the Figure 2 legend and under Materials and Methods.

in Figure 5 show that the immune IgG blocks the specific binding of ^{125}I-labeled 34-kDa peptide to 3T3 fibroblasts. It also blocks the mitogenic response to the 34-kDa peptide in these cells (Figure 6). However, these effects are seen only at a high antibody to antigen ratio. This indicates that most of the antibodies in the polyclonal population are directed to nonfunctional epitopes of the 34-kDa peptide and that antibodies directed to the active site constitute a minor fraction of the total.

Immunoperoxidase Localization of the 34-kDa Peptide in Human Placenta. Immunoperoxidase staining of histologic tissue sections of first trimester placenta revealed the presence of antigen in the cytotrophoblast cytoplasm, as well as in the brush border of syncytiotrophoblasts (Figure 7, panel I). The antigen was not present in any other placental structure. Preadsorption of the antiserum on columns of agarose con-

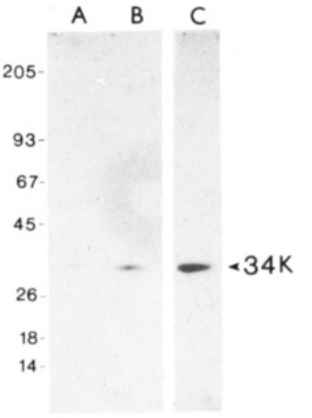

FIGURE 4: Immunoblot analysis of crude placental membranes with anti-34-kDa peptide antibody, second antibody, and radioiodinated protein A. Crude placental membranes (lane A, 0.1 μg of total protein; lane B, 0.3 μg of protein) and pure 34-kDa protein (lane C, 30 ng of protein) were subjected to SDS gel electrophoresis and immunoblotting as described under Materials and Methods. In experiments with nonimmune rabbit IgG, no labeled band was seen at 34 kDa or elsewhere.

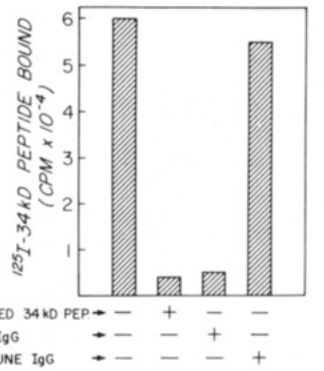

FIGURE 5: Effect of immune IgG on ^{125}I-labeled 34-kDa peptide binding to mouse 3T3 fibroblasts. Cell monolayers in 16-mm dishes were incubated at 4 °C for 1 h with 6 nM radioiodinated 34-kDa protein (50000 cpm/ng) in the presence and absence of rabbit immune IgG (100 μg), rabbit nonimmune IgG (100 μg), or unlabeled 34-kDa protein (1 μM) in a total volume of 250 μL, and then cell-bound radioactivity was determined as described earlier (see the preceding paper in this issue).

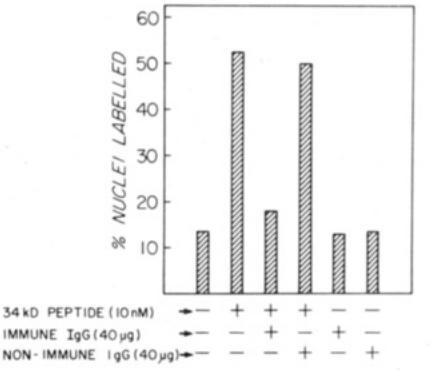

FIGURE 6: Effect of immune IgG on mitogenic activity of the 34-kDa peptide in mouse 3T3 fibroblasts. Mitogenic activity was assayed by nuclear autoradiography (see the preceding paper in this issue). Cell monolayers in 2.5-mm dishes were incubated at 37 °C for 24 h with [^{3}H]thymidine and the indicated proteins in 100 μL of DME medium containing 5% platelet poor human plasma and then processed for autoradiography as described earlier (see the preceding paper in this issue). The values shown are the average of quadruplicate assays. For each assay at least 400 nuclei were counted.

taining covalently bound pure 34-kDa protein abolished all staining reaction (Figure 7, panel III). Analysis of placenta at term revealed an identical pattern of staining (Figure 7,

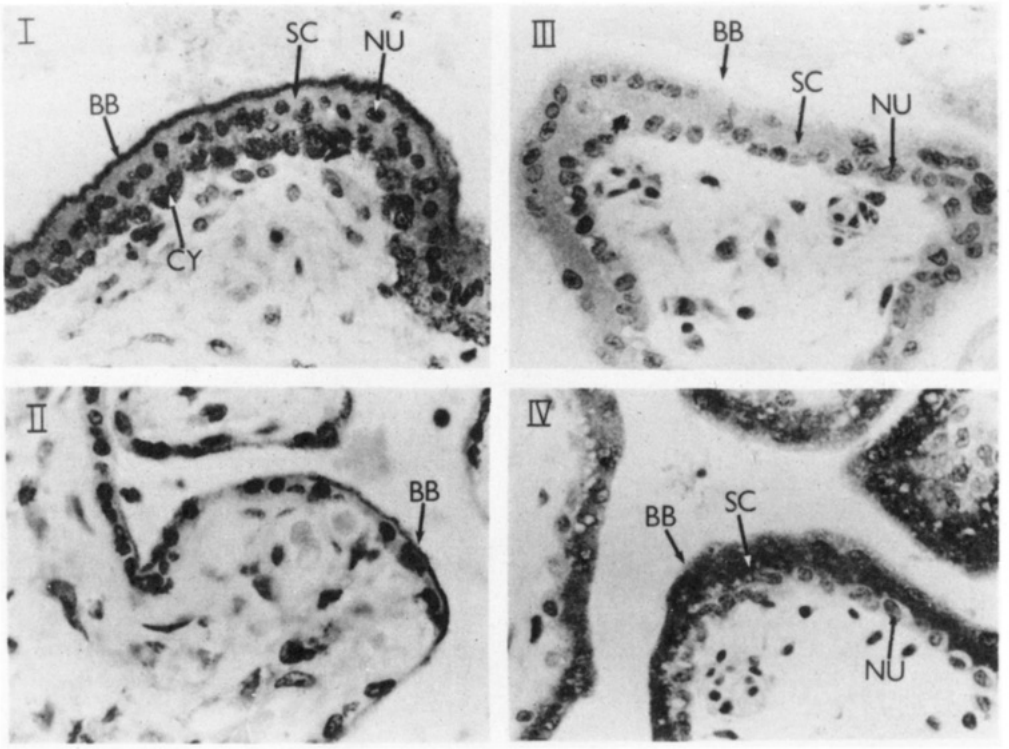

FIGURE 7: Immunoperoxidase staining of histologic tissue sections of human placenta. (I) Staining of a typical first trimester placenta with a 1:1000 dilution of anti-34-kDa peptide antiserum. (II) Staining of a typical term placenta with a 1:1000 dilution of anti-34-kDa antiserum (the magnification is the same as that for first trimester placenta). (III) Lack of staining of first trimester placenta with a 1:1000 dilution of preadsorbed anti-34-kDa antiserum (preadsorption was done on columns of agarose containing covalently bound pure 34-kDa protein). (IV) Staining of first trimester placenta with mouse anti-HCG monoclonal antibody (from Cappel Lab, West Chester, PA; the second antibody used here was biontinylated horse anti-mouse IgG form Vector Laboratories, Burlingame, CA). Details of techniques used are described under Materials and Methods. No staining was seen with nonimmune rabbit (or mouse) serum. The organelles marked with black- or white-tipped arrows are as follows: BB, brush border membrane of syncytiotrophoblasts; SC, syncytiotrophoblast cytoplasm; CY, cytotrophoblast cytoplasm; NU, nuclei of syncytiotrophoblasts, cytotrophoblasts, and other cells. Staining of BB and CY seen with the anti-34-kDa antibody and of SC seen with the anti-HCG antibody was reddish brown, the expected color of immunoperoxidase stain. The dark appearance of nuclei (NU) seen in the figure is not due to immunoperoxidase (reddish brown) staining, but to blue counterstaining of these organelles with hematoxylin. The results shown are typical and have been consistently seen in all placenta specimens examined. The numbers of first trimester placenta and term placenta examined were six and five, respectively.

panel II). However, in term placenta the size of villi is very greatly reduced (panel III in Figure 7 is at the same magnification as panels I, II, and IV), and there is a significant decrease in the amount of 34-kDa protein in both the syncytiotrophoblast brush border and cytotrophoblast cytoplasm.

Antibodies to two other placental proteins were run as positive controls. One of these proteins is the EGF receptor, a transmembrane protein (Das & Fox, 1978; Cohen et al., 1982), which was found to be located in the brush border membranes of syncytiotrophoblasts, but not in its cytoplasm or in the cytoplasm of cytotrophoblasts (not shown). The other protein is human chorionic gonadotropin (HCG), a soluble hormone, which was located in the cytoplasm of syncytiotrophoblasts, but not in the brush border of those cells or in the cytoplasm of cytotrophoblasts (Figure 7, panel IV). It is significant that the staining pattern for HCG, a secretory protein, is an almost perfect negative image of that seen for the 34-kDa mitogen.

The results of staining shown in Figure 7 are very typical. We have tested placenta specimens from more than 10 women. All the first trimester placenta tested (more than five) stained strongly for the 34-kDa antigen especially in the brush border region. In contrast all the five term placenta tested showed reduced villi and weak staining with the anti-34-kDa peptide antibody. The pattern of staining in all cases was the same as that seen in Figure 7.

Expression of the 34-kDa Peptide in Human Carcinomas. We tested whether any antigenically cross-reactive substance is present in A431, a human vulval epidermoid carcinoma cell line. In previous work we had reported the identification (although not the purification) of a mitogenic activity of 34 kDa associated with plasma membranes from these cells (Bishayee et al., 1984). Figure 8 reveals an immune serum specific staining of these cells that is abolished by preadsorption of the antiserum with pure placental 34-kDa protein. Interestingly, three other human carcinoma cell lines tested—SKBR-5 and MCF-7 of breast origin and T-24 of bladder origin—were also reactive with the anti-34-kDa peptide antiserum (not shown).

To test whether the observed immunoreactivity in carcinoma cells is due to cellular synthesis of the 34-kDa protein, we biosynthetically labeled these cells with [^{35}S]methionine and immunoprecipitated the lysates from labeled cells with the anti-34-kDa peptide antibody. The results in Figure 9 show that A431 cells biosynthesize a 34-kDa peptide that is specifically recognized by the antibody. The binding of this peptide to the antibody was totally abolished in the presence of 100 nM unlabeled 34-kDa peptide (not shown). Other labeled proteins seen in the antibody lane (lane Ab in Figure 9) are nonspecifically bound proteins which are also seen in experiments with nonimmune IgG (lane Ni, Figure 9) and in experiments with competing unlabeled 34-kDa protein (not shown). Thus A431 cells biosynthesized a 34-kDa peptide that is antigenically related to the placental 34-kDa growth factor. Biosynthesis of an immunoreactive 34-kDa peptide was also detected in SKBR-5 cells (not shown).

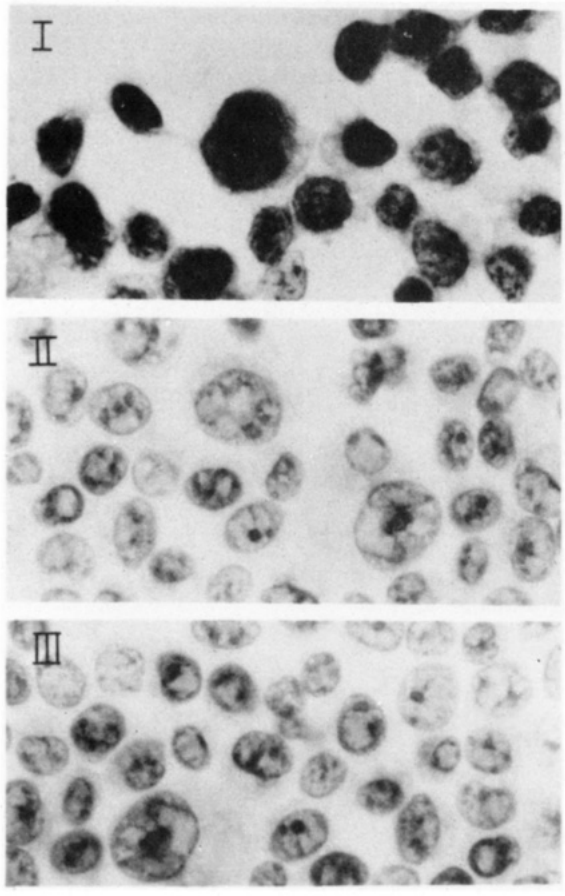

FIGURE 8: Immunoperoxidase staining of human A431 epidermoid carcinoma cells. (I) Staining with a 1:1000 dilution of the anti-34-kDa peptide antiserum. (II) Staining with a 1:1000 dilution of preadsorbed antiserum. (III) Staining with a 1:1000 dilution of normal rabbit serum. See Materials and Methods for experimental details.

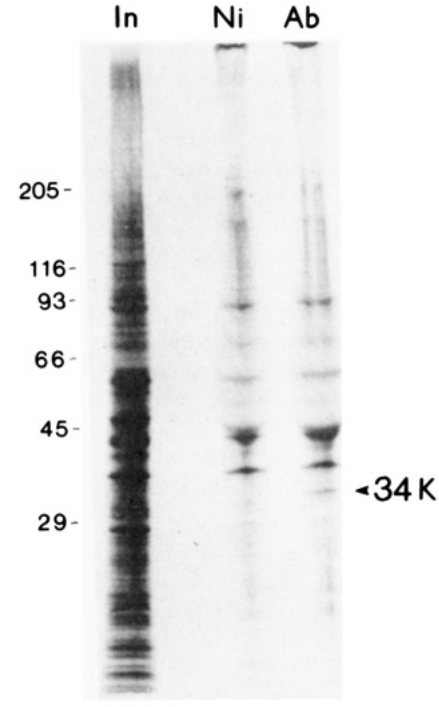

FIGURE 9: Immunoprecipitation of lysates from biosynthetically labeled human A431 carcinoma cells. Cells were labeled with [^{35}S]methionine, and the lysates (In) were subjected to immunoprecipitation using anti-34-kDa peptide immune IgG (Ab) or nonimmune rabbit IgG (Ni) as described under Materials and Methods. In experiments where unlabeled 34-kDa protein (100 nM) was included with the antibody, the results were the same as those shown for the nonimmune (Ni) IgG experiment; i.e., binding of the ^{35}S-labeled 34-kDa peptide was abolished, but certain other labeled proteins continued to adhere nonspecifically.

Discussion

In a previous paper we described the purification to homogeneity of a new peptide growth factor of 34 kDa from human placenta and showed that it acts through receptors that are not shared by a number of other growth factors and hormones (see the preceding paper in this issue). In this paper, a specific antibody was used to examine the antigenic relationship of this growth factor to other circulating peptides. The antibody meets the following criteria: (a) in immunoblot experiments with crude placental membranes the antibody recognizes only the 34-kDa peptide and no other membrane component; (b) in immunoprecipitation experiments with membranes from placenta and liver, it specifically binds to the 34-kDa peptide and to no other material; (c) in radioimmunoassays, only the 34-kDa peptide, and no other growth factor or hormone tested, competed with the radiolabeled antigen for binding to the antibody. These results demonstrate that the antibody is a highly specific probe for both the native and denatured 34-kDa peptide. The epitopes recognized by this antibody are not carried by other membrane proteins and a host of circulating hormones and growth factors.

We have used this antibody to analyze the genesis and location of the 34-kDa membranous mitogen. Antigenically active substances, similar in molecular weight to the placental peptide, have been detected in human A431 carcinoma cells (Figures 8 and 9) and in mouse liver (Figure 3). A431 cells and other human carcinomas of diverse tissue origin biosynthesize the peptide (Figure 9); in mouse liver the peptide is restricted to the ductal regions (A. Sen-Majumdar, K. Richardson, J. Brown, D. Chianese, U. Murthy, and M. Das, unpublished results). Plasma membranes from these different sources were shown earlier to contain a 34-kDa mitogen (Bishayee & Das, 1982; Bishayee et al., 1982). It now appears that these membranous mitogens are structurally related to the placental peptide.

In placenta, the peptide is found only in cells of embryonal origin, namely, cytotrophoblasts and syncytiotrophoblasts; the cells of maternal origin are devoid of any immunoreactive material. In cytotrophoblasts, which are the precursors of syncytiotrophoblasts, the growth factor is present in cytoplasmic organelles. Fusion and differentiation into multinucleated syncytiotrophoblasts are associated with the disappearance of the 34-kDa peptide from cytoplasm and its concomitant appearance in brush border membranes. These results, and our preliminary metabolic labeling studies with cultured cytotrophoblasts (A. Sen-Majumdar, J. E. Nestler, H. Kliman, U. Murthy, J. F. Strauss, and M. Das, unpublished results), indicate that these cells are the biosynthetic sources of the 34-kDa growth factor in placenta.

The antigen described here is a plasma membrane associated protein (Figures 2, 3, and 7; Sen-Majumdar et al., 1986; Bishayee et al., 1984). Although its association with membranes is tight, it does not involve any intercalation into the lipid bilayer [see salt-dependent release of the peptide from membranes in the preceding paper in this issue and in Bishayee et al. (1984)]. Regarding the nature of this association, it should be noted that placental trophoblasts are known to synthesize growth factors as well as their receptors (Goustin et al., 1985) and may therefore be unusually prone to autocrine stimulation by endogenous growth factors. On the basis of these characteristics, we hypothesize that the membrane association of the 34-kDa peptide represents a ligand–receptor

type of interaction; i.e., the 34-kDa growth factor made by cytotrophoblasts (and carcinomas) binds to an endogenous membrane receptor, which may lead to an autocrine stimulation of growth.

The consistently stronger immunochemical staining seen with trophoblasts from first trimester placenta than with those from term placenta suggests a nontrivial role for the 34-kDa peptide in early prenatal development. The high level of 34-kDa antigenicity seen in the brush border during first trimester very likely reflects a high degree of expression of this protein during early pregnancy; it could equally reflect the presence of a greater number of receptors for the peptide early in ontogeny. Some of the questions that can be asked regarding the role of the 34-kDa peptide in placenta are as follows: (a) whether the 34-kDa peptide is a mitogenic factor or a differentiation-inducing factor for cytotrophoblasts either in culture or in vivo; (b) whether the peptide is needed to maintain the differentiated secretory characteristics of syncytiotrophoblasts (such as synthesis and secretion of HCG and placental lactogen); (c) whether the actions of this peptide on cytotrophoblasts/syncytiotrophoblasts are mediated through binding to a 50-kDa membrane receptor, similar to that seen in fibroblasts (see the preceding paper in this issue). Lastly, it should be noted that this peptide is expressed by A431, a human vulval epidermoid carcinoma cell line, and also by other human carcinomas of breast and bladder origin. This finding is of interest in the context of the view that the trophoblast has been considered a "pseudomalignant tissue" because it grows invasively into the uterine epithelium or any surrounding tissue (Kirby, 1965). Thus it is of interest to test the role of this peptide in the genesis/maintenance of the transformed phenotype, as well as in early trophoblast development.

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