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Placental Keratinocyte Growth Factor: Partial Purification and Comparison with Epidermal Growth Factor

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A water-soluble extract of term human placenta, which was previously shown to promote proliferative growth of human keratinocytes in defined medium, enhanced both cellular attachment and proliferative growth. We have partially purified the activity which enhanced cell growth and examined its action in keratinocytes. Activity was precipitated from the crude extract by $(NH_4)_2SO_4$ between 33 and 60% saturation and chromatographed by gel filtration. The activity did not bind to heparin-Sepharose at low ionic strength but was adsorbed to DEAE-cellulose from which it was eluted with NaCl and then passed over phenyl-HPLC to remove bovine serum albumin previously added to protect the activity. The active fraction was applied to gel exclusion HPLC in the presence of 0.02% octyl-β-D-glucopyranoside, which yielded an apparent M_r 35,000 for the factor. Purification was ~200-fold with ~4% recovery. The factor appears to be a protein, since activity is destroyed by trypsin. Autoradiography of cultures treated with the placental factor or epidermal growth factor (EGF) revealed that approximately 50% of cells were labeled after treatment with either growth factor compared to 9% in control cultures after a [^{3}H]thymidine pulse. Protein synthesis was increased by about 50% 42 h after treatment with either agent, consistent with a 50% increase in nuclear labeling. Cell number was increased fivefold after 6 days in the presence of the partially purified factor, whereas EGF increased cell number eightfold. Stimulation of [^{3}H]-thymidine incorporation by the partially purified factor, in contrast, was about twice that produced by EGF, indicating that thymidine incorporation is preferentially stimulated by the placental factor and does not correlate well with other parameters of proliferative growth. The placental keratinocyte growth factor is a unique factor with a novel effect on incorporation of thymidine into DNA.

We have reported previously (1) that term human placenta contains a water-soluble activity which is a potent promoter of proliferative growth of human epidermal keratinocytes. Preliminary studies indicated that the active fraction was distinct from bovine hypothalamic activity which stimulated growth of keratinocytes (2) in its high molecular weight and in its sensitivity to temperature. It was also distinct from epidermal growth factor (EGF),[2] which produced an easily recognized change in colonial morphology at 1 ng/ml or higher and which was synergistic with the extract at low cell density and from some other agents known to affect keratinocyte growth.

We subsequently found that this potent activity was composed of an activity stim-

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[2] Abbreviations used: EGF, epidermal growth factor; PBS, phosphate-buffered saline; TCA, trichloroacetic acid; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; DTT, dithiothreitol; BSS, buffered saline solution; ppPX, partially purified placental ex-

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ulating cell attachment in addition to an activity stimulating cell proliferation. In this paper we describe partial purification of the growth-promoting activity and describe the effect of the activity on human keratinocytes whose growth has been arrested by being plated in incomplete medium. Growth arrest of keratinocytes as described in this study may provide a useful model for characterization of control of growth in keratinocytes, and possibly other epithelial cells, by specific growth factors, such as has been possible in density-arrested Balb/c-3T3 cells, a model for the study of growth control in fibroblasts (3). We also describe a novel effect of crude or partially purified placental extract on keratinocytes not produced by other growth factors studied. The activity increased proliferative growth, the percentage of nuclei labeled with [^{3}H]thymidine, and protein synthesis, and these increases were similar to the increases produced by EGF. The increase in incorporation of thymidine, however, was substantially greater with the placental factor than with EGF, suggesting that the placental factor has a different mechanism of action.

MATERIALS AND METHODS

Cell culture and assay for growth using cell counts (1) were performed as described previously. Placentas were refrigerated after delivery and were used fresh within 5–10 h. Briefly, placenta was cut with scissors in PBS to remove the bulk of clotted blood and the tissue was then washed at 4°C for 6 h in Dulbecco's phosphate-buffered saline lacking Ca^{2+} and Mg^{2+}. The wash was centrifuged to remove cells and debris and the supernatant dialyzed against distilled water for 16 h at 4°C and lyophilized. The lyophilized extract was stored at −20°C until needed, when it was dissolved in Hanks' buffered salt solution lacking Ca^{2+} for incubation with cells or else processed as described below.

Thymidine Incorporation Assay

Assay for incorporation of labeled thymidine was performed as described previously (4) with some modifications. MCDB 153 medium containing 0.1 mM $CaCl_2$ and supplemented with insulin (5 μg/ml), hydrocortisone (0.4 μg/ml), transferrin (5 μg/ml), ethanolamine (0.1 mM), and phosphoethanolamine (0.1 mM) ("incomplete medium") would not support proliferative growth of keratinocytes without addition of EGF, placental extract, or bovine pituitary extract, and was used to assay partially purified placental extract during purification. Cells (1.0–1.5 × 10^4) were added to each well (2 cm^2) of a 24-well tray in 0.5 ml of incomplete medium and incubated for 7 h at 37°C in a humidified incubator with 5% CO_2. Control wells then received in addition 0.5 ml of incomplete medium only, and experimental wells received 0.5 ml of incomplete medium supplemented with fractions to be tested, and the incubation was continued for another 16 h. One to 2 μCi of [*methyl*-^{3}H]thymidine (sp act 10 Ci/mmol, ICN, Irvine, CA) was then added to each well, and the incubation was continued for another 6 h. At the end of the incubation period, medium was removed by aspiration, and 1 ml of ice-cold PBS (0.15 M NaCl, 10 mM sodium phosphate, pH 7.4) was added to each well and aspirated. The same procedure was repeated with 2 ml PBS. One and one-half milliliters of ice-cold 5% trichloroacetic acid (TCA) was added to each well and aspirated after 10 min on ice. [^{3}H]Thymidine incorporated into DNA was extracted with 1 ml of 0.1 N NaOH containing 1% SDS for 10 min at room temperature and measured as described (4).

Methionine Incorporation Assay

Cells (1.0–1.5 × 10^4) were added to each well in 0.5 ml incomplete medium as in the thymidine incorporation assay and incubated for 7 h. Control wells then received an additional 0.5 ml incomplete medium, while experimental wells received 0.5 ml containing additions, and both sets of wells were then incubated for 42 h before addition of 0.6 μCi of [^{35}S]methionine (800 Ci/mM, New England Nuclear, Boston, MA). After 4 to 6 h, medium was aspirated and the cells were washed with PBS, extracted with 5% TCA, and solubilized with 0.1 N NaOH/1% SDS as described above. TCA-insoluble radioactivity was counted.

Autoradiography

Autoradiography was performed as described previously (4). Briefly, experiments were performed in parallel with thymidine incorporation under the same conditions; parallel wells were processed either for measurement of incorporated activity by scintillation counting of TCA-insoluble residue (see below) or by processing for autoradiography. Cultures to be autoradiographed were incubated in 24-well trays with [^{3}H]thymidine (1 μCi/ml) for 6 h, washed twice with PBS, fixed by two 10-min incubations in absolute methanol, and coated with photographic emulsion. The film was developed and fixed cells were scored by

tract; PX, placental extract; TGF-β, transforming growth factor β; FGF, fibroblast growth factor.

counting the percentage of nuclei with or without grains in 300 cells under phase contrast microscopy as described previously (4).

Ammonium Sulfate Precipitation

Crude placental extract (0.56 g) was suspended in 20 ml ice-cold acetone for 2 h and centrifuged. The precipitate was air-dried at room temperature and dissolved in 13 ml of PBS containing 1 mM ethylenediaminetetraacetic acid (EDTA). Phenylmethylsulfonyl fluoride, *N*-ethylmaleimide, iodoacetic acid, and *p*-chloromercuribenzoic acid (all at 0.2 M) were then added to achieve final concentrations of 2 mM each, and the sample was left on ice for 30 min. Active carbon (0.3 g wet weight) was added, and after 10 min on ice with occasional gentle stirring, the sample was centrifuged for 10 min at 37,000*g* at 4°C in a Sorvall SS34 rotor and the precipitates were discarded. To the supernatant, saturated $(NH_4)_2SO_4$ containing 1 mM EDTA and 10 mM sodium phosphate, pH 7.0, was added dropwise until the desired final concentration was reached. After 2 h on ice the sample was centrifuged at 37,000*g* for 10 min.

Gel Filtration Chromatography

The precipitate from $(NH_4)_2SO_4$ fractionation was dissolved in 8 ml PBS containing 1 mM EDTA, and 220 mg of bovine serum albumin (BSA) previously purified by precipitation between 65 and 85% $(NH_4)_2SO_4$ was added. The sample was applied to a Fractogel (Fractogel TSK, HW-55, MCB Reagents, Gibbstown, NJ) column (2.5 × 84 cm) equilibrated and developed with the same buffer at the rate of 14 ml/h at 4°C. Four-milliliter fractions were collected, and protein peaks were determined by absorption at 280 nm.

Heparin-Sepharose Column Chromatography

Heparin-Sepharose was prepared by coupling heparin (Sigma Chemical Co., St. Louis, MO) to Sepharose 4B-CL at 10 mg/ml of solid support as described (5). The active fractions from the Fractogel column (determined by thymidine incorporation assay) were pooled and dialyzed against 10 mM sodium phosphate containing 1 mM dithiothreitol (DTT), pH 7.1, for 2 h at 4°C. The dialysate was applied to a heparin-Sepharose column (2.5 × 5 cm) equilibrated with the same buffer at 4°C.

DEAE-Cellulose Column Chromatography

The active fraction from the heparin-Sepharose column was dialyzed against 0.05 M imidazole containing 1 mM DTT, pH 7.1, for 2 h and applied to a DEAE-cellulose column (DE52, Whatman, Inc., Clifton, NJ) (2.5 × 5 cm) equilibrated with the same buffer and was then washed with the same buffer at 4°C.

High-Pressure Liquid Chromatography

Phenyl-HPLC. Pooled active fractions, as assessed by thymidine incorporation, eluted from the DEAE-cellulose column, were collected into a dialysis bag, dried with Aquacide III (Calbiochem, San Diego, CA), and dissolved in approximately 20 ml of 0.8 M $(NH_4)_2SO_4$ in 20 mM sodium phosphate buffer containing 1 mM DTT and 1 mM EDTA, pH 7.2. The sample was applied to a phenyl-HPLC column (TSK phenyl-5PW, 7.5 × 75 mm, Pharmacia LKB, Piscataway, NJ) equilibrated with the same buffer.

Gel filtration HPLC. A gel filtration HPLC column (TSK 3000SW, 75 × 600 mm, Pharmacia LKB) was equilibrated with 10 mM sodium phosphate, pH 7.1, containing 0.2 M Na_2SO_4, 1 mM EDTA, 0.5 mM DTT, and 0.02% octyl-β-D-glucopyranoside (octyl glucoside). The sample to be chromatographed was dissolved in the same buffer but containing 5 mM DTT and concentrated by Centricon 10 microconcentration (Amicon, Danvers, MA) and was filtered (filter type HV, pore size 0.45 μm, Millipore, Bedford, MA). A 250-μl sample was applied to the HPLC column and eluted with equilibrating buffer at a flow rate of 0.25 ml/min, 0.5 ml per fraction. One milligram of BSA was added to each eluted fraction and the fractions were dialyzed against deionized water and dried with Aquacide III, and activity was determined by bioassay.

^{125}I-EGF Binding

EGF purified according to Savage and Cohen (6) was iodinated with ^{125}I-NaI (Amersham, Arlington Heights, IL) with chloramine-T as previously described to a specific activity of 1000 Ci/mmol (7). Competition for binding to placenta membrane fractions was performed as described (7) using 25 μg of protein. Binding to cells was measured by incubating ^{125}I-EGF (1.1×10^{-9} M final concentration) with intact keratinocyte monolayers in 2-cm^2 wells at 4°C for 4 h as described (8). Cells were washed three times with ice-cold buffer containing 0.1% BSA, solubilized with 1.0 N NaOH, and counted in a gamma counter. Nonspecific binding in the presence of 0.5 μg/ml of native EGF was subtracted from experimental values.

Protein Determination

The amount of protein was initially measured by absorption at 280 nm assuming 1 unit of absorbance as 1 mg/ml. In the partially purified fractions, protein was determined by the microprotein assay described by Bradford (9).

TABLE I

ATTACHMENT OF KERATINOCYTES IN THE PRESENCE OF PLACENTAL EXTRACTS DETERMINED BY CELL NUMBER AND [^{3}H]THYMIDINE INCORPORATION

Method of determination	Times of addition	Addition	Number of cells harvested[b]	Attachment efficiency (%)
Cell number[a]	At plating	None	15,280 ± 2740	19.1 ± 3.4
		PX, 200 μg/ml[c]	40,050 ± 3940	50.1 ± 4.9
		ppPX, 39 μg/ml[d]	16,670 ± 2900	20.8 ± 3.6
			cpm[f] incorporated	Fold stimulation
[^{3}H]Thymidine incorporation[e]	At plating	None	470 ± 40	1.0 ± 0.1
		PX, 400 μg	16,830 ± 1280	35.8 ± 2.7
		PX, 200 μg	14,240 ± 730	30.3 ± 1.6
		ppPX, 30 μg	2,540 ± 50	5.4 ± 0.1
		ppPX, 15 μg	1,550 ± 90	3.3 ± 0.2
		ppPX, 6 μg	1,090 ± 70	2.3 ± 0.1
	24 h after plating	None	1,410 ± 40	1.0 ± 0.0
		PX, 400 μg	8,850 ± 910	6.3 ± 0.6
		PX, 200 μg	5,920 ± 710	4.2 ± 0.5
		ppPX, 30 μg	6,530 ± 180	4.6 ± 0.1
		ppPX, 15 μg	4,270 ± 50	3.0 ± 0.0
		ppPX, 6 μg	2,700 ± 120	1.9 ± 0.1

[a] Cells were plated at a density of 8×10^4 cells per 35-mm dish in 2 ml of incomplete MCDB 153 medium with or without additions. After incubation for 17 h at 37°C, the cultures were washed with 3 ml of Hanks buffered saline solution and the remaining cells were suspended with trypsin and counted in a Coulter counter.

[b] Mean ± standard deviation, $N = 3$.

[c] PX, placental extract.

[d] ppPX, DEAE eluate, which has been corrected for BSA.

[e] [^{3}H]Thymidine was added 17 h after the addition, as described under Results.

[f] Average ± range of duplicates.

RESULTS

During purification studies, crude placental extract was found to stimulate attachment of keratinocytes in incomplete medium if it was added at the time of plating of the cells. Attachment was increased from 20% in incomplete medium to 50% in the presence of placental extract (Table I). Since stimulation of attachment increased the apparent thymidine incorporation independently of activity specifically stimulating proliferative growth, we modified the bioassay to eliminate the effect of the attachment-promoting activity of the extract. Since attachment of the keratinocytes was not stimulated when the placenta extract was added 6.5 h or more after the cells were plated (data not shown), we added fractions to be tested to the culture 7 h or more after the cells were plated. We added thymidine 16 h later and continued the incubation for another 6 h to determine incorporation of thymidine.

Partial Purification of Growth-Promoting Activity

Most of the growth-stimulating activity in the crude extract, as assessed by thymidine incorporation, was precipitated between 33 and 50% of saturation of $(NH_4)_2SO_4$. Once the activity was separated from crude placental extract, which

probably contains protease inhibitors present in plasma, it was very susceptible to proteases, but protease inhibitors alone did not preserve biological activity. Immediate addition of BSA after $(NH_4)_2SO_4$ fractionation was required to preserve activity. Since repeated freezing and thawing also resulted in loss of activity, lyophilization was not useful for concentration of samples. Similar high losses were encountered in attempts to concentrate fractions by stepwise elution from small DEAE-cellulose columns. Pooled samples were therefore concentrated in dialysis bags by drying with high-molecular-weight polyethylene glycol (Aquacide III).

The $(NH_4)_2SO_4$ precipitates obtained between 33 and 60% of saturation and containing an added equal amount of BSA were separated into two peaks by gel filtration. Bioassay revealed activity in the second peak, which also contained the bulk of the BSA. This second peak was pooled and applied to heparin-Sepharose in 10 mM sodium phosphate buffer as described under Materials and Methods, and activity was found in the unabsorbed fraction, which also contained BSA. This active fraction was absorbed to DEAE-cellulose in 0.05 M imidazole at neutral pH and was eluted, accompanied by part of the BSA, with 0.05 M imidazole containing 0.06 M NaCl, pH 7.2. This DEAE eluate was tested for attachment activity by counting cells released by trypsin and by incorporation of [^{3}H]thymidine (Table I). Unlike the crude extract, the DEAE eluate did not contain attachment activity, so that its action reflected a separate growth-promoting activity. Most of the BSA was removed by phenyl-HPLC in 0.8 M $(NH_4)_2SO_4$, since the active fraction was absorbed with only minor amounts of BSA at this concentration of salt. Activity was eluted from phenyl-HPLC with 5% isopropanol–20 mM sodium phosphate, pH 7.1, containing 1 mM EDTA and 1 mM DTT. Activity was preserved without BSA at this stage, possibly because proteases were removed.

The activity eluted from phenyl-HPLC was concentrated by Centricon 10 microconcentration and applied to gel filtration HPLC as described under Materials and Methods. The activity migrated as a globular protein with M_r 35,000 on gel filtration HPLC (Fig. 1). If the detergent, octyl-β-D-glucopyranoside, was omitted from the eluting buffer, however, the activity comigrated with BSA (M_r 67,000), suggesting that the activity may migrate as a dimer or complexed to another protein in the absence of detergent. The active fractions from gel filtration HPLC were combined, BSA was added (50 mg of BSA per 500 mg crude starting material), and the material was dialyzed briefly against distilled water and dried with Aquacide III. Dried material was dissolved in buffered saline solution (BSS), dried again with Aquacide III, redissolved in BSS (5 ml of BSS per 500 mg starting material), and stored at −70°C in 0.1-ml aliquots. Recovery of activity was determined by [^{3}H]thymidine incorporation produced by the fractions tested. A unit of activity was defined as the amount of protein required to increase [^{3}H]-thymidine incorporation half-maximally in the standard assay. Recovery of activity was 3.7% after the last step (Table II).

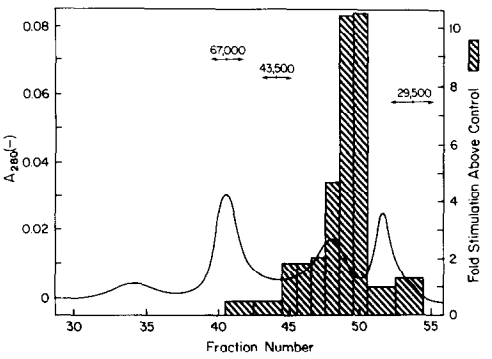

FIG. 1. Gel exclusion HPLC of phenyl-HPLC eluate. Partially purified placental extract eluted from a phenyl-HPLC column and containing less than 0.5 mg protein in 0.25 ml was applied, eluted, and assayed as described under Materials and Methods. Markers used were bovine serum albumin (67,000), ovalbumin (43,500), and carbonic anhydrase (29,500).

The effect of various conditions on the activity was examined. Activity was abolished at pH 3 or pH 11, as reported previously with crude placental extract (1). The activity was not altered by exposure of the lyophilized crude extract to cold ace-

TABLE II

RECOVERY OF BIOLOGICAL ACTIVITY AFTER PARTIAL PURIFICATION

Purification step	Protein (mg)	Specific activity[a] (unit/μg)	Total activity (units)	Recovery (%)	Fold purification
Crude extract	560	0.005	2800	100	1
$(NH_4)_2SO_4$ precipitation	180	0.009	1624	58	1.8
Gel filtration	122	0.012	1512	54	2.48
Heparin-Sepharose	43	0.035	1512	54	7
DEAE-cellulose	—[b]	—	644	23	—
Phenyl-HPLC	—[c]	—	148	5.3	—
Gel filtration HPLC	0.1	0.99	104	3.7	198

[a] One unit of activity gives 50% maximal stimulation of [^{3}H]thymidine incorporation in 1 ml of medium in which 15,000 cells per 2 cm^2 were plated.

[b] Not determined because of added BSA.

[c] Not determined because of contamination by BSA.

tone, since it could be quantitatively recovered from the precipitate in physiologic buffers, but acetone diluted in aqueous buffers reduced activity severely. Treatment with protease inhibitors including phenylmethylsulfonyl fluoride, *N*-ethylmaleimide, diisopropyl fluorophosphate, *p*-chloromercuribenzoic acid, iodoacetic acid, iodoacetamide, ethylenediaminetetraacetic acid, or DTT did not affect activity, but reduction with 50 mM DTT (30 min, 4°C) followed by five-fold dilution and alkylation by 50 mM iodoacetamide (2 h, 4°C) destroyed activity, suggesting that derivatization of reduced sulfhydryl groups, but not reduction alone, destroys activity. The activity of partially purified placental extract (ppPX) was readily destroyed by trypsin (0.15 mg/ml, 1 h, 24°C). Heating for 10 min at 63°C resulted in a 50% loss of activity, and more extensive heating destroyed activity completely (data not shown).

Restoration of Proliferative Growth of Arrested Cells and Stimulation of Thymidine and Methionine Incorporation by Partially Purified Placental Extract

The partially purified active fraction (ppPX), purified using incorporation of thymidine as a bioassay, stimulated proliferative growth of keratinocytes (Table III) as assessed by counting of cells. The stimulation of proliferative growth of the cells (Table III), nuclear labeling, and thymidine incorporation (Table IV) by ppPX were concentration dependent, and the degree of stimulation of cell growth and nuclear labeling were similar to that produced by EGF. Stimulation of thymidine incorporation by placental extract (both PX and ppPX) was substantially higher

TABLE III

STIMULATION OF GROWTH OF KERATINOCYTES BY PARTIALLY PURIFIED PLACENTAL EXTRACT OR EGF[a]

Addition	Cells harvested[b]	Fold stimulation
None	3,920 ± 100	1 ± 0.0
ppPX,[c] 4.5 μg	19,800 ± 1060	5.1 ± 0.3
ppPX, 3 μg	15,900 ± 2000	4.1 ± 0.5
PX, 300 μg	83,280 ± 7320	21.2 ± 1.9
EGF, 15 ng	31,920 ± 2060	8.1 ± 0.5

[a] Keratinocytes were cultured at a density of 1×10^4 cells in 1.5 ml incomplete MCDB 153 medium in 35-mm plastic dishes. Additions in 1.5 ml of the same medium were added 7.5 h later. Cells were harvested with trypsin 6 days later without refeeding and counted in a Coulter counter.

[b] Mean ± standard deviation.

[c] ppPX, partially purified placental extract.

TABLE IV

COMPARISON OF PERCENTAGE LABELED NUCLEI WITH INCORPORATION OF THYMIDINE IN RESPONSE TO ppPX OR EGF[a]

Addition	% Nuclei labeled[b]	Fold stimulation	[^{3}H]Thymidine incorporation[c]	Fold stimulation
None	9.0 ± 0.0	1.0 ± 0	800 ± 70	1.0 ± 0.1
ppPX, 1.8 μg	42 ± 0.5	4.7 ± 0.1	12,380 ± 430	15.5 ± 0.5
ppPX, 1.4 μg	36 ± 5	4.0 ± 0.6	9,370 ± 140	11.7 ± 0.2
ppPX, 0.7 μg	28 ± 3	3.1 ± 0.3	4,730 ± 210	5.9 ± 0.3
ppPX, 0.35 μg	22 ± 3	2.4 ± 0.3	2,080 ± 100	2.6 ± 0.1
PX, 200 μg	50 ± 1	5.6 ± 0.1	24,990 ± 5720	31.3 ± 7.7
PX, 100 μg	48 ± 0	5.3 ± 0	13,640 ± 310	17.1 ± 0.4
EGF, 5 ng	50 ± 0.1	5.6 ± 0.0	6,300 ± 300	7.9 ± 0.4
EGF, 1 ng	50 ± 0.15	5.6 ± 0.0	6,890 ± 130	8.6 ± 0.2

[a] Keratinocytes were plated in incomplete medium in 24-well trays and ppPX, PX, or EGF was added after 7.5 h. [^{3}H]Thymidine was added to each well 16 h after addition of ppPX, PX, or EGF, the incubation was continued for 6 h more, and assays were terminated. Autoradiography and thymidine incorporation were performed in parallel wells in the same experiment. Three hundred nuclei were counted in each well, and the results were from duplicate wells.

[b] Average ± ½ range of duplicate determinations.

[c] cpm, average ± ½ range of duplicate determinations.

than by EGF, however (Tables IV and V), even though the concentration of ppPX employed yielded slightly lower nuclear labeling than that produced by EGF in the same experiments (Table IV). Furthermore, deposition of silver grains over nuclei was considerably more intense in the cells treated with either partially purified or crude placental extract than in cells treated with EGF, although the percentage of labeled nuclei was similar. To determine whether the differences in intensity of nuclear labelling were secondary to different time courses of incorporation of label in response to EGF or placental extract, we determined thymidine incorporation at various time intervals after the addition of EGF or placental extract. As shown in Fig. 2, the time intervals required for the cells to enter S phase after the exposure to EGF and placental extract were similar, but incorporation of thymidine remained substantially higher with placental extract than with EGF. Since thymidine incorporation by scintillation counting and autoradiography were performed in parallel, and since ppPX did not increase the percentage of nuclei labeled, the incorporation studies provide a quantitative estimate of the increased labeling per nucleus found on autoradiography in cultures treated with ppPX.

In order to compare another feature of cells stimulated by EGF or placental extract in addition to nuclear labeling, we measured protein synthesis 20 h after the peak in thymidine incorporation, or S-phase. Incorporation of [^{35}S]methionine increased 40 to 80% in response to either placental extract (including PX, DEAE eluate, and ppPX) or EGF at this time, in agreement with similar increases in nuclear labeling in response to either agent (Table V). Thymidine incorporation, therefore, appeared to be preferentially stimulated by the crude or partially purified placental extract; the fold stimulation of thymidine incorporation did not correlate with the percentage of nuclei labeled or with protein synthesis by postmitotic cells.

Placental extract, unlike EGF, was inactivated by heating or by exposure to acid and produced a colony morphology different from that produced by EGF (1). In order to test directly whether placental ex-

TABLE V

COMPARISON OF THYMIDINE INCORPORATION AND PROTEIN SYNTHESIS IN RESPONSE TO ppPX, PX, OR EGF[a]

Additions	[^{3}H]Thymidine incorporation[b] (cpm)	Fold stimulation	[^{35}S]Methionine incorporation[c] (cpm)	Fold stimulation
		Experiment 1		
None	60 ± 4	1.0 ± 0.10	1660 ± 10	1 + 0.0
ppPX, 2.2 μg	2,180 ± 120	36.4 ± 2.0	2320 ± 160	1.4 ± 0.1
PX, 200 μg	4,900 ± 120	81.7 ± 2.0	3130 ± 90	1.9 ± 0.1
PX, 100 μg	2,620 ± 470	43.7 ± 7.8	2960 ± 130	1.8 ± 0.1
EGF, 5 ng	1,390 ± 10	23.2 ± 0.2	2850 ± 110	1.7 ± 0.1
EGF, 1 ng	1,040 ± 10	17.3 ± 0.2	2630 ± 60	1.6 ± 0.0
		Experiment 2		
None	300 ± 50	1.0 ± 0.2	2120 ± 200	1.0 ± 0.1
ppPX, 12 μl[d]	11,280 ± 170	37.6 ± 0.6	3480 ± 60	1.6 ± 0.0
ppPX, 9.5 μl[d]	10,520 ± 110	35.1 ± 0.4	3340 ± 130	1.6 ± 0.1
PX, 200 μg	12,680 ± 380	42.3 ± 1.3	3420 ± 20	1.6 ± 0.0
PX, 100 μg	7,720 ± 0	25.7 ± 0	3920 ± 20	1.8 ± 0.0
EGF, 5 ng	5,180 ± 320	17.3 ± 1.1	3230 ± 90	1.5 ± 0.0

[a] Cultured keratinocytes (10,000 cells/0.5 ml) were plated in incomplete medium in 24-well trays and EGF, ppPX, or PX in 0.5 ml medium was added after 8 h.

[b] [^{3}H]Thymidine (2 μCi) was added to each well after 16 h and the incubation was continued for another 6 h. Residue remaining after extraction with cold 5% TCA was counted as described under Materials and Methods. Results are cpm ± ½ range of duplicates.

[c] [^{35}S]Methionine (0.625 μCi for Experiment 1 and 0.3 μCi for Experiment 2) was added to each well of a parallel set of keratinocyte cultures 42 h later for 6 h and the fraction insoluble in cold 5% TCA was extracted and counted as described. Results are cpm ± ½ range of duplicates.

[d] DEAE eluate.

tract acted similarly to EGF, we measured EGF binding to receptors in a placental membrane fraction, which is rich in EGF receptors (7). As shown in Table VI, binding of ^{125}I-labeled EGF was not reduced in the presence of PX, indicating that the active material in placental extract did not bind to EGF receptors. In other experiments, the ability of placental extract to down-regulate EGF receptors on keratinocytes was studied by incubating keratinocytes with placental extract or EGF for 16 h at 37°C and then measuring EGF binding for 4 h at 4°C. These experiments showed that placental extract did not alter EGF binding by viable keratinocytes (data not shown), and is therefore not able to alter either binding affinity or receptor number of EGF receptors. This material is also distinct from transforming growth factor β (TGF-β), which has also been purified from placenta (10), since TGF-β inhibited [^{3}H]thymidine and [^{35}S]methionine incorporation in keratinocytes (Table VII), in keeping with its reported growth inhibitory activity in keratinocytes (11). A synergistic or paradoxical action of TGF-β was not found in experiments combining TGF-β and ppPX (data not shown).

DISCUSSION

We have previously demonstrated that crude extract of human placenta was able to stimulate proliferative growth of human keratinocytes. In attempting to purify the activity, we found that the crude extract was able to enhance apparent growth by stimulating attachment to plastic and in addition contained an activity stimulat-

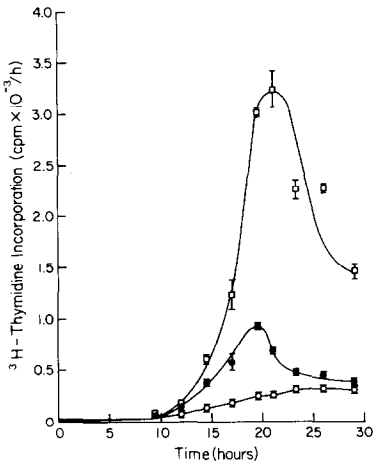

FIG. 2. Effect of time of exposure of cells to EGF or placental extract on stimulation of thymidine incorporation. Cells were plated in duplicate at 1×10^4 cells per well in 0.5 ml incomplete medium. EGF (5 ng) or placental extract (200 μg) in 0.5 ml of the same medium was added to experimental wells after 14 h (time zero) and medium alone was added to parallel control wells. One hour before indicated times, 2 μCi of [^{3}H]thymidine was added to each well, and incorporation was determined after 2 h as described. Points shown are the averages ± range of duplicates. (○) Control; (●) EGF; (□) placental extract.

ing thymidine incorporation (Table I). We modified our assay in order not to detect attachment-stimulating activity and used thymidine incorporation to monitor purification. An activity responsible for stimulation of growth was purified about 200-fold (Table II), was able to stimulate proliferative growth of keratinocytes (Table III), and did not compete for binding to EGF receptors (Table VI). The material appears to be a protein, since it was destroyed by trypsin or by reduction and alkylation. The partially purified material migrates on gel filtration with a Stokes radius of a globular protein of M_r 35,000. After the DEAE-cellulose step, the partially purified material failed to stimulate attachment of cells (Table I), suggesting that attachment and thymidine-incorporating activities differ.

Cultured keratinocytes suspended with trypsin and replated under the conditions of our assay appeared to be arrested at the G_0/G_1 boundary, since they did not incorporate thymidine or proliferate in the absence of an added growth factor, and there was a lag of 15–17 h after addition of EGF or placental extract before the cells entered S phase (Fig. 2). When the cells were plated and then treated with partially purified activity from human placenta, thymidine incorporation, methionine incorporation, nuclear labeling, and cell growth were stimulated, establishing the fact that this material is able to restore progression to S phase and to support proliferative growth. EGF has been reported to be required for the first 8 h during traverse of G_0/G_1 in Balb/c-3T3 cells, whereas insulin or insulin-like growth factor I (somatomedin C) was required for the traverse of late G_1 (12). The use of the model presented here, in which keratinocyte growth is arrested at the G_0/G_1 boundary, for the study of growth control in keratinocytes may yield this type of information about cell cycle-specific control of growth.

In addition to EGF, heparin-binding growth factors related to basic fibroblast growth factor (FGF) can promote growth

TABLE VI

COMPETITION OF PARTIALLY PURIFIED PLACENTAL EXTRACT FOR BINDING TO EGF RECEPTOR IN PLACENTA MEMBRANES[a]

Addition	^{125}I-labeled EGF bound[b]
None	15,200 ± 1100
EGF	
100 ng/ml	2,110 ± 180
30 ng/ml	6,500 ± 810
10 ng/ml	11,100 ± 940
3 ng/ml	13,100 ± 600
ppPX[c]	
15 μl[d]	15,750 ± 1750
7 μl	16,570 ± 670

[a] Placental microsomal membranes (25 μg/tube) were incubated with 18,000 cpm ^{125}I-labeled EGF in the presence or absence of additions in a final volume of 0.22 ml for 2 h at 24°C and filtered as described under Materials and Methods.

[b] cpm ± ½ range of duplicates.

[c] Heparin-Sepharose eluate.

[d] Containing 3 half-maximally stimulating units of activity in the [^{3}H]thymidine assay.

TABLE VII

EFFECTS OF INCORPORATION OF THYMIDINE AND PROTEIN SYNTHESIS BY TGF-β[a]

Addition	[^{3}H]Thymidine incorporation[b] (cpm)	Fold stimulation	[^{35}S]Methionine incorporation[b] (cpm)	Fold stimulation
None	280 ± 10	1.0 ± 0.0	3860 ± 200	1.0 ± 0.1
PX, 200 μg	7270 ± 1010	26.0 ± 3.6	7340 ± 290	1.9 ± 0.1
PX, 100 μg	3610 ± 100	12.9 ± 0.4	6330 ± 320	1.6 ± 0.1
TGF-β, 10 ng	50 ± 0	0.2 ± 0.0	3230 ± 170	0.8 ± 0.0
TGF-β, 4 ng	40 ± 0	0.1 ± 0.0	3400 ± 280	0.9 ± 0.1
TGF-β, 2 ng	50 ± 0	0.2 ± 0.0	3420 ± 50	0.9 ± 0.0
TGF-β, 1 ng	40 ± 0	0.1 ± 0.0	3320 ± 10	0.9 ± 0.0

[a] Cultured keratinocytes (15,000 cells/0.5 ml) were plated in incomplete medium in 24-well trays and PX or TGF-β (Collaborative Research, Bedford, MA) in 0.5 ml medium was added after 8 h.

[b] [^{3}H]Thymidine (2 μCi) or [^{35}S]methionine (0.79 Ci) was added and counted as described in Table V.

of epithelial cells, and we have recently found that basic FGF can promote keratinocyte proliferation (13). Although placental extract would be expected to contain some FGF, the activity we have purified, unlike FGF and related factors, does not bind to heparin-Sepharose columns (Table II). The material is also distinct from insulin-like growth factors I and II, since it is active in the presence of micromolar concentrations of insulin added to incomplete medium used in our assays; furthermore, IGF-1 is positively charged at neutral pH (14) and would not be expected to bind to anion-exchange resins under these conditions, unlike the placental factor. These findings suggest that at least part of the activity of the crude extract is a unique growth factor which has not been previously described.

The degree of stimulation of proliferative growth (Table III), the percentage of nuclei labeled (Table IV), and protein synthesis (Table V) were similar in the presence of EGF or ppPX, but stimulation of thymidine incorporation was several-fold higher in response to PX. Keratinocytes treated with bFGF (13) also demonstrated comparable increases in labeling of nuclei (3.7 times control) and thymidine incorporation (3.9 times control). In contrast, keratinocytes treated with ppPX increased nuclear labeling 4.6 times control, whereas thymidine incorporation increased by 15.5 times. Stimulation of thymidine incorporation by ppPX, therefore, while indicative of a stimulus to proliferative growth (4), is not accompanied by a proportionate stimulation of proliferative growth. In agreement with the finding of increased TCA-precipitable radioactivity in PX-treated cells, the density of grains of autoradiographically labeled nuclei was also considerably higher and produced very intense labeling of these nuclei in comparison with those labeled in response to EGF or FGF.

The reason for the disproportionate increase in thymidine incorporation per nucleus in response to PX or ppPX over that produced by EGF or bFGF is not known. One possibility is that increased thymidine incorporation could result from reduced catabolism of thymidine in the presence of the placental extract, since catabolism of thymidine was recently described in cultured keratinocytes (15). It is unlikely, however, that catabolism of thymidine contributed to the observed differences in thymidine incorporation, since significant catabolism of thymidine probably does not occur under the conditions of our assays. Thymidine catabolism was observed by Schwartz *et al.* (15) in confluent cultures in the presence of 0.2 μM thymidine only after at least 2 h and increased markedly thereafter; in our study very subconfluent cells were incubated in 3 μM thymidine, but the

difference between EGF and placental extract was very marked after a 2-h interval (Fig. 2).

Other possibilities are that disproportionately high stimulation of thymidine incorporation by the placental factor may result from increased usage of the salvage pathway in preference to *de novo* synthesis, increased transport of thymidine, or increased phosphorylation of thymidine as a result of increased activity or synthesis of thymidine kinase. Whatever the mechanism of this novel effect, it is important to note that although determination of thymidine incorporation is a useful tool to assess stimulation of growth, for example by chromatographic fractions during purification, it is not applicable for the quantitative assessment of proliferative growth. A similar conclusion was reached by other workers; it was reported previously that both salvage and *de novo* pathways were available for pyrimidine nucleotide biosynthesis in keratinocytes (16) and that thymidine incorporation into keratinocytes did not correlate consistently with other parameters of proliferation (17).

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