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A Heparin Sulfate-Regulated Human Keratinocyte Autocrine Factor Is Similar or Identical to Amphiregulin

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A novel human keratinocyte-derived autocrine factor (KAF) was purified from conditioned medium by using heparin affinity chromatography as the first step. Purified KAF stimulated the growth of normal human keratinocytes, mouse AKR-2B cells, and a mouse keratinocyte cell line (BALB/MK). Heparin sulfate inhibited KAF mitogenic activity on all cell types tested and inhibited the ability of KAF to compete with epidermal growth factor for cell surface binding. Interestingly, KAF stimulated the growth of BALB/MK cells at high cell density but failed to stimulate these cells at clonal density. Protein microsequencing of the first 20 NH_2-terminal amino acid residues of purified KAF revealed identity to the NH_2 terminus of human amphiregulin (AR). Northern (RNA) blot analysis with AR-specific cRNA demonstrated that human keratinocytes, as well as mammary epithelial cell cultures, expressed high levels of AR mRNA. In contrast, AR mRNA was not detected in normal human fibroblasts or melanocytes and was present at reduced levels in several mammary tumor cell lines. The mitogenic activity of purified AR was also shown to be inhibited by heparin sulfate, and an AR-specific enzyme-linked immunosorbent assay (ELISA) revealed that KAF and AR are antigenically related. We have previously shown that human keratinocytes can grow in an autocrine manner. Our present study demonstrates that one of the growth factors responsible for this autocrine growth (KAF) is similar or identical to AR and that KAF and AR bioactivity can be negatively regulated by heparin sulfate.

The integument of the human body includes an outermost multilayered group of epithelial cells called the epidermis. The epidermis lacks blood vessels and must derive its nutrients from the underlying dermis, which contains numerous small blood vessels. The major cell type in the epidermis is the keratinocyte. Keratinocytes from the proliferative layer in the epidermis (basal layer) can be isolated and grown in vitro, and numerous investigations into the properties of these cultured cells have led to the formulation of a specialized basal medium for the growth of these cells in the absence of undefined supplements (for a review, see reference 29). It has been demonstrated that epidermal growth factor (EGF), transforming growth factor type alpha (TGFα), and fibroblast growth factors (FGFs) are stimulatory for the growth of these cells under certain conditions (1, 18, 28, 29, 34). We have shown that human keratinocyte cultures synthesize TGFα peptide (4). These and subsequent investigations have also shown that TGFα levels increase when keratinocytes in defined medium are treated with EGF or TGFα, active phorbol esters, or serum (4, 6, 19). Our investigations to date have been unable to demonstrate whether mature TGFα is a mediator of autocrine growth in these cells.

Heparin sulfate is a sulfated glycosaminoglycan (GAG) which has been extensively used as an anticoagulent. Several members of the FGF family have been demonstrated to bind to heparin sulfate with various affinities, and heparin can positively regulate the potency of some members of this family, in particular acidic FGF (aFGF) (2). Interestingly, heparin sulfate has been shown to exert antiproliferative effects on several different cell types, in vitro and in vivo (3, 11, 12, 16, 24, 28, 35), and the mechanism by which heparin mediates its antiproliferative effect is not understood. To our knowledge, heparin sulfate has not been demonstrated to inhibit the mitogenic activity of any purified polypeptide growth factor. Heparan sulfate is closely related to heparin sulfate and has also been shown to exert growth-inhibitory effects in some cell types (11). These sulfated GAGs may be covalently linked to protein core molecules in the form of proteoglycans (PGs) and are produced and externalized by mammalian cells (11, 23). Underexpression of sulfated PGs has also been correlated with diminished density-dependent growth inhibition and neoplastic transformation (11). These observations suggest that sulfated GAGs or PGs could act as important negative regulators of growth in vivo.

We have shown that cultured human keratinocytes are capable of autonomous proliferation (growth in the absence of EGF, TGFα, or FGFs) when cell densities exceed 10^3 cells per cm^2 (7, 28). We have also demonstrated that the growth factor-independent proliferation of human keratinocyte cultures is inhibited by the addition of heparin sulfate to the culture medium (7, 28). Interestingly, serum-free conditioned medium derived from keratinocyte cultures was shown to contain mitogenic activity, which was inhibited by coincubation with heparin sulfate or anti-EGF receptor monoclonal antibody (7). Moreover, other investigators have demonstrated the existence of TGFα-like growth factors which interact with heparin sulfate (15). These observations led us to hypothesize that normal human keratinocytes may produce a novel heparin-binding, EGF-like autocrine growth factor whose mitogenic activity is inhibited by interaction with heparin sulfate.

In the work described here, we demonstrate for the first

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time that a normal epithelial cell type (human keratinocytes) produces a novel keratinocyte-derived growth factor (KAF) which acts to stimulate the growth of these cells in culture. We show that KAF mitogenic activity is inhibited by the addition of heparin sulfate to the culture medium and that heparin sulfate inhibits the ability of KAF to compete with EGF for binding to common cell surface binding sites. A variety of data, including protein and cDNA sequencing, Northern (RNA) blot analysis, biological assays, and immunological analysis, suggests that KAF is identical to amphiregulin (AR) (21, 30, 31).

MATERIALS AND METHODS

Cell culture. Modified basal medium MCDB 153 (29) was prepared in our laboratory or obtained from Clonetics Corp., San Diego, Calif., as keratinocyte basal medium (KBM). McCoy 5A medium, hydrocortisone (H-4001), and bovine insulin (1-5500) were purchased from Sigma Chemical Co., St. Louis, Mo. MCDB 402 (27), MCDB 170 (10), and MCDB 202a (10, 17) were also prepared in our laboratory. Human recombinant EGF and insulinlike growth factor type 1 were purchased from Amgen Corp., Thousand Oaks, Calif. Human recombinant TGFα was generously supplied by Rik Derynck, Genentech Inc. Human AR derived from phorbol ester-treated MCF 7 mammary carcinoma cells (30) was generously supplied by M. Shoyab, Oncogen, Seattle, Wash. Basic FGF (bFGF) was isolated from bovine pituitary glands and stored as previously described (28). Heparin sulfate (from porcine intestinal mucosa) was purchased from Hepar Industries, Franklin, Ohio (lot PM-19286), or Sigma Chemical Co. (H-3125; lot 78F-0633), dissolved in solution A (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES], 0.15 M NaCl, 3.3 μM phenol red [pH 7.4]) at 10 mg/ml and stored at −20°C. Methods for the preparation of culture additives have been published previously (28).

Primary cultures of normal foreskin keratinocytes were established from newborn foreskin tissue as previously described (34). Keratinocytes were routinely cultured in KBM supplemented with 0.2% bovine pituitary extract, 10 ng of EGF per ml, 5.0 μg of insulin per ml, 5×10^{-7} M hydrocortisone and 10 μg of gentamicin sulfate per ml (referred to in the text as complete medium) as previously described (28, 29). Complete medium without bovine pituitary extract, insulin, or EGF supplementation is referred to as standard medium. Normal human mammary epithelial cell strain 161 was a generously supplied by Martha Stampfer, LBL, Berkeley, Calif.

The BALB/MK-2 (BALB/MK) mouse keratinocyte cell line (33) was obtained from Bernard Weissman, University of Southern California. These cells were routinely grown in KBM supplemented with 5 μg of insulin per ml, 10 ng of EGF per ml, 0.5% fetal bovine serum, and 10 μg of gentamicin sulfate per ml. This medium is referred to as MK medium. The AKR-2B mouse cell line was cultured in McCoy 5A medium supplemented with 5% fetal bovine serum as previously described (26).

DNA synthesis assays. Incorporation of [^{3}H]methyl thymidine (50 to 80 Ci/mM; Dupont, NEN) into monolayers of AKR-2B cells in serum-free medium was performed as previously described (26). Experimental factors were added to each well in the presence of 500 ng of insulin per ml as described in the figure legends. For the data reported here, relative incorporation in each experimental well was determined and the background incorporation (insulin only) was subtracted.

BALB/MK-2 cells were plated in 24-well tissue culture plates at a density of 3×10^4 cells per cm^2 in MK medium. After 72 h of culture, each well was washed twice with solution A; 1 ml of MK medium without EGF was added to each well, and the cells were cultured for an additional 24 h. After this culture period, the medium was replaced with 0.25 ml of MK medium without EGF, and growth factors were added to the indicated cultures as described in the figure legends. [^{3}H]thymidine was added to each well at a final concentration of 8 μCi/ml, and the cells were cultured for an additional 24 h. After this culture period, incorporation of [^{3}H]thymidine in each experimental well was determined as previously described for AKR-2B cells (26).

Clonal growth assays. Clonal growth of human keratinocytes was performed by using six-well tissue culture plates. Briefly, cells were removed from stock culture flasks with 0.025% trypsin–0.01% EDTA and resuspended in cold solution A supplemented with 0.5% fetal bovine serum. The cells were centrifuged at 180 × *g*, and the pellet was resuspended in complete medium. The cells were inoculated into the six-well plates (600 cells per well; 9 cm^2), and the plates were incubated for 24 h. The medium was removed, and the cells were washed twice with solution A. After the washes, the medium was replaced with 2 ml of standard medium supplemented with insulin (5 μg/ml), and experimental factors were subsequently added as described in the legends to figures. The keratinocytes were grown without a medium change for 10 days. After the incubation, the colonies which developed were fixed in 3.7% formaldehyde and stained with 0.1% crystal violet for photography.

Clonal growth of BALB/MK-2 cells was carried out by using a method similar to that described for human keratinocytes. Briefly, BALB/MK cells were trypsinized from stock flasks, pelleted, resuspended in MK medium, and plated at 275 cells in 0.5 ml per well in 24-well plates. After 24 h of culture, each well was washed twice with solution A and 0.5 ml of MK medium without EGF was added to each well. Experimental factors were added as described in the figure legends. After a 10-day incubation, the colonies which developed were fixed in 3.7% formaldehyde and stained with 0.1% crystal violet for photography.

Purification and partial NH_2-terminal sequencing of KAF from keratinocyte conditioned medium. Keratinocytes were plated in 10-level cell factories (600 cm^2 per level; Nunc) at an initial density of 2.5×10^3 cells per cm^2 in 1.5 liters of complete medium. After 72 to 96 h of culture, the cells were washed three times with solution A and the medium was replaced with 1.5 liters of standard medium. At 24 h later, the cell culture medium was discarded and replaced with 1.5 liters of standard medium. Medium was collected at 48- to 72-h intervals until the cultures reached 70 to 90% confluency. Conditioned medium (CM) was stored at −20°C until use. Frozen keratinocyte conditioned medium was thawed in 9- to 18-liter batches, and protein was precipitated by the addition of 2 M zinc sulfate to a final concentration of 0.1 M, as previously described (5). After centrifugation, the pellets were resuspended in 0.5 M EDTA (pH 7.4), combined, and dialyzed against three 5-liter volumes of 1% acetic acid for 48 h at 4°C (Spectropor dialysis tubing; 3,500 molecular weight cutoff). The dialyzed material was concentrated to a small volume (150 to 300 ml) in several 15-ml Centriprep-10 (Amicon) centrifugal concentrators, lyophilized to dryness, and stored at −20°C until the first purification step.

Lyophilized batches of keratinocyte-derived CM protein were resuspended in 40 to 50 ml of solution A (pH 7.0), and the pH was adjusted to 7.4 with NaOH. The final molarity was

adjusted to approximately 0.1 M NaCl. Final volumes were 75 to 100 ml. Five separate batches of resuspended CM protein were passed twice over a 1-ml column of heparin-acrylic beads (no. H-5263; Sigma Chemical Co.) by using a Pharmacia fast protein liquid chromatography (FPLC) apparatus. The column was washed and proteins were eluted with a NaCl gradient in 20 mM HEPES (pH 7.0) as described in the legend to Fig. 1A. A small portion of each fraction was diluted in HEPES-buffered saline containing 50 μg of bovine serum albumin (BSA) per ml, and multiple volumes of the diluted material were tested for mitogenic activity in the AKR-2B DNA synthesis assay as described above. One unit of KAF activity was defined as the amount of active material required to obtain a half-maximal response in the DNA synthesis assay when compared with a maximal dose of EGF. A total of 31,053 U of KAF activity was present in the most active fractions from five heparin affinity purification runs representing 65.5 liters of keratinocyte CM. The most active fractions (fractions 15 to 17) from each of the five heparin affinity purifications were pooled and concentrated on a Centricon-10 centrifugal concentrator to a final volume of 2.5 ml in 20 mM HEPES–0.15 M NaCl (pH 7.0). The sample (25,939 U of KAF) was chromatographed, by using a NaCl gradient, on a Mono-S HR 5/5 cation-exchange column (Pharmacia) as described in the legend to Fig. 1C. The fractions were tested for mitogenic activity in the AKR-2B assay as described above. Fractions 10, 11, and 12 were pooled and brought to a final volume of 3 ml in 0.1% trifluoroacetic acid (TFA). The sample (18,593 U of KAF) was then chromatographed on a C1/C-8 ProRPC reversed-phase column (Pharmacia) as described in the legend to Fig. 1E. The most active reversed-phase fraction (fraction 13, Fig. 1E) was lyophilized, resuspended in 0.1% TFA (6,129 U of KAF) and rechromatographed on the same reversed-phase column as described in the legend to Fig. 1E. Fractions 21 to 23 from the second reversed-phase separation were combined into a single tube to a final volume of 1 ml and lyophilized. The sample was resuspended in 200 μl of 0.1% TFA, and 100 μl was used for NH_2-terminal protein microsequencing. The sample was subjected to 20 rounds of Edman degradation and analyzed by using an Applied Biosystems 470 A protein sequencer and a 120A phenylthiohydantoin analyzer. Protein microsequencing revealed that the preparation contained 42.5 pmol of polypeptide. The remainder of the resuspended material was frozen at −80°C and later tested in the AKR-2B DNA synthesis assay. Results from this assay indicated that the KAF preparation used for amino acid sequencing contained 3.05 U of KAF per μl. Therefore, 1.0 U of KAF activity was equivalent to 0.14 pmol of purified polypeptide. This value was used to determine the molar concentration of KAF in other purified preparations. KAF used in the AR-specific enzyme-linked immunosorbent assay (ELISA) (see Fig. 7C) and in the comparison AR/KAF heparin-sensitive mitogenic activity (see Fig. 7B) was purified by using a method identical to the heparin affinity, cation-exchange, and second reversed-phase chromatography steps described in this section.

^{125}I-EGF binding competition assays. AKR-2B cells were cultured in 24-well tissue culture plates as described above. After 48 h of culture in serum-free MCDB 402 medium, the medium from each well was aspirated and replaced with 0.2 ml of binding buffer (20 mM HEPES-buffered MCDB 402 medium [pH 7.4] containing 1 mg of BSA per ml). The cells were incubated at 4°C on ice for 1 to 2 h. After this incubation period, the binding buffer was removed from each well and replaced with 0.25 ml of cold binding buffer containing 50 pmol of ^{125}I-EGF (1,316 Ci/mmol, 0.1 mCi/ml; Amersham) and various unlabeled experimental factors as described in the figure legends. The cells were incubated with gentle rocking on ice for 3 h, washed three times with 1 ml of cold phosphate-buffered saline (PBS) per well, and subsequently lysed by the addition of 1 ml of lysis buffer (0.1% sodium dodecyl sulfate [SDS], 0.1 M NaOH) per well. After 15 min of rocking at room temperature, 0.9 ml of lysed material was removed from each well and the relative amount of bound ^{125}I-EGF was determined in a gamma counter. Nonspecific binding was determined in the presence of 30 nM unlabeled EGF and was equal to 10.5% of total labeled EGF bound in the absence of any competing unlabeled ligand. The mean value for nonspecific counts per minute was subtracted from all experimental values. These adjusted experimental values were then used to calculate a mean, which was subsequently converted to percent maximal competition by using the competition exerted by 30 nM unlabeled EGF as 100% (maximal) competition.

Electrophoretic separation and detection of proteins. Fractions obtained from different purification steps were subjected to electrophoretic separation in 15% polyacrylamide–SDS gels under both reducing (plus dithiothreitol) and nonreducing conditions as previously described (14, 20). Protein in each gel was detected by silver staining with a rapid Ag staining kit (ICN, Costa Mesa, Calif.). Molecular weight markers were purchased from Sigma Chemical Co.

Molecular cloning of AR cDNA from human keratinocytes. Human keratinocytes were cultured essentially as described for the preparation of concentrated keratinocyte-derived CM. Poly(A)$^+$ mRNA was isolated (25, 32) from nearly confluent, proliferating keratinocytes cultured in standard medium. Single-stranded cDNA was synthesized [from 2 μg of keratinocyte-derived poly(A)$^+$ mRNA] by using mouse mammary leukemia virus reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, Md.). Oligonucleotide primers were synthesized corresponding to the sense, 5′-GC AC/(RI)GAATTC-153-CGTGTCCCAGAGACCGAGTTGC CCC-177-3′, and antisense, 3′-998-CCTCAGTGACGGTTC AGTATCGGT-1021-TCGAA/(HIII)CACG-5′, strands of the published cDNA sequence of human AR (21) on an Applied Biosystems PCRmate DNA synthesizer. Underlined bases correspond to AR-specific cDNA sequences, nonunderlined sequences correspond to linkers (slash marks indicate restriction enzyme cleavage sites for *Eco*RI and *Hin*dIII, respectively), and the numbers correspond to the numbering of the previously published AR cDNA sequence (21). These primers were used to amplify AR sequences present in keratinocyte-derived cDNA by using *Taq* polymerase (Bethesda Research Laboratories) in a polymerase chain reaction (40 cycles; 95°C, 60°C, 72°C). Amplified cDNA was cloned into the *Hin*dIII and *Eco*RI sites of M13mp19 and M13mp18 (36) vectors and sequenced by using a Sequenase DNA sequencing kit (U.S. Biochemical Corp., Cleveland, Ohio). Amplified cDNA was also cloned into the pTZ19U vector (U.S. Biochemical Corp.), and the construct was designated pTZ/AR-2. Restriction analysis revealed the expected insert fragment of approximately 0.9 kb.

Northern blot analysis. AR-specific, [α^{32}P]UTP-labeled antisense cRNA was synthesized from 200 ng of *Eco*RI-linearized pTZ/AR-2 as described previously (4). A Northern blot was generated, as previously described (32), by using poly(A)$^+$ mRNA (5 μg per lane) derived from a variety of normal and abnormal human cell types cultured as described in the legend to Fig. 6. The Northern blot was hybridized with 5 × 10^6 cpm of the pTZ/AR-2 probe per ml for 16 h (60°C, 5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 50% formamide, 0.5% SDS, 10× Denhardt solution,

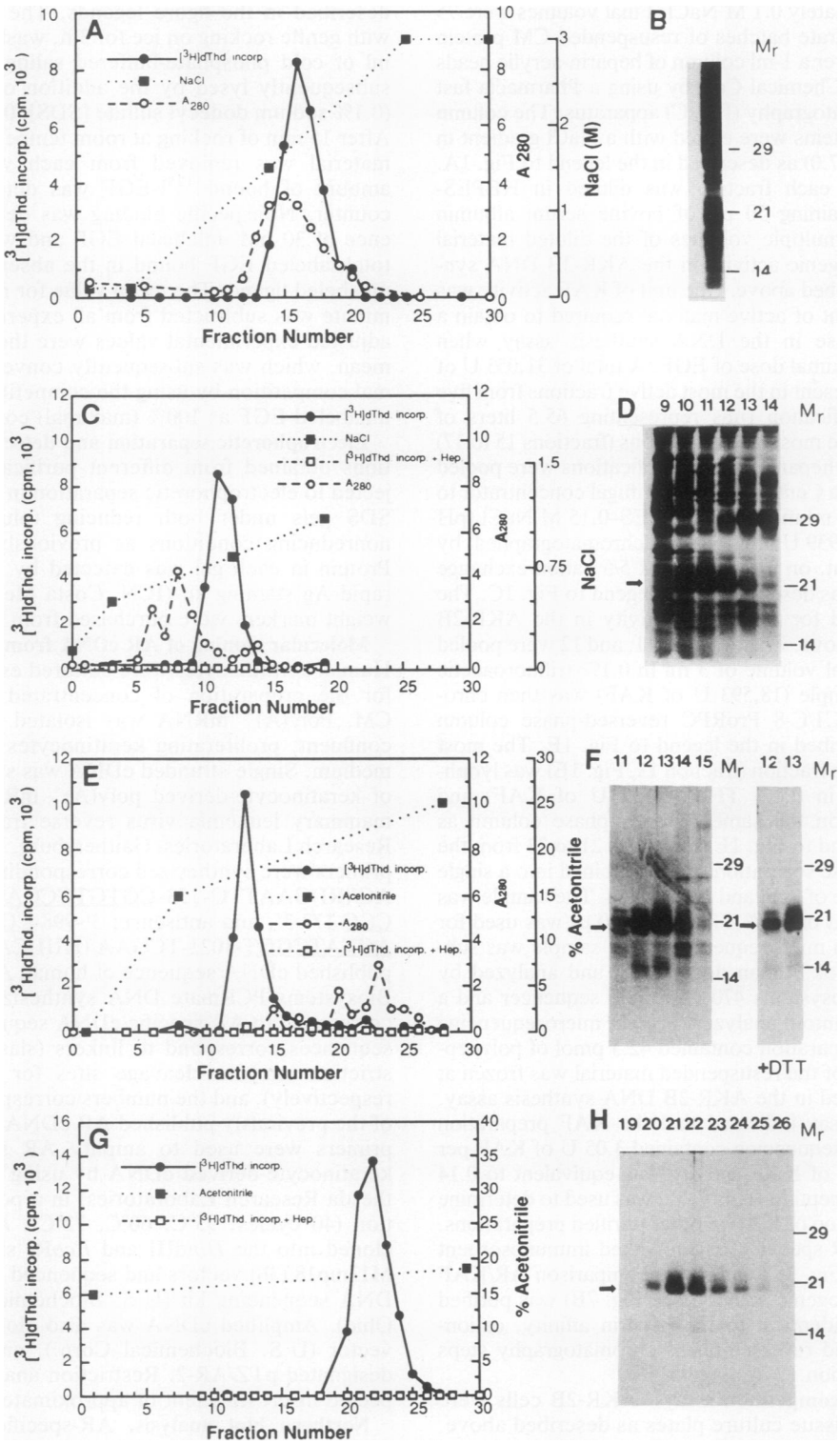

FIG. 1. Purification of KAF. (A) Concentrated keratinocyte-derived CM was chromatographed on a heparin-acrylic column. Proteins bound to the column were eluted with the indicated NaCl gradient, and 0.5-ml fractions were collected every minute. The relative protein concentration was monitored by measuring the A_{280}. Each of the indicated fractions were tested at a final dilution of 1:3,000 in the AKR-2B cell thymidine incorporation assay. (B) The peak three fractions from five separate heparin affinity purification steps (panel A) were combined and concentrated. A volume equivalent to 300 U of KAF was subjected to SDS-PAGE under nonreducing conditions and visualized by silver staining. Molecular mass protein markers used were carbonic anhydrase (29 kDa), trypsin inhibitor (21 kDa), and α-lactalbumin (14 kDa). (C) The pooled heparin affinity-purified KAF was subjected to cation-exchange chromatography (MonoS). The sample was passed through the cation-exchange column, and the column was washed. The column was then eluted with the indicated NaCl gradient, and 0.5-ml fractions were collected every minute for 20 min. Each of the indicated fractions was tested (in the presence or absence of 30 μg of heparin sulfate per

100 μg of herring sperm DNA per ml, 50 μg of polyadenosine per ml). The blot was rinsed briefly twice with washing buffer (0.1× SSC, 0.1% SDS, 5 mM EDTA) and three times (45 min each) with 400 ml of washing buffer at 65°C. Transcripts hybridizing to the probe were visualized by exposure of the blot to film (Kodak X-Omat A/R) for 20 min. An identical method was used to probe the same blot for the expression of the constitutively expressed cyclophillin (1B15) gene product (8, 32).

ELISA for the detection of AR-specific peptide sequences. Mouse monoclonal antibody AR1 was raised against a peptide (amino acids 144 to 184) spanning the EGF-like domain of mature AR (21) and used as uncloned hybridoma culture supernatant 6R1C2.8. Samples containing 0.5 pg to 1.0 ng of mammary tumor cell-derived AR (30) were bound to the bottom of a 96-well Immulon 2 microtiter plate in 0.1 ml of coating buffer (0.1 M sodium carbonate [pH 9.6]) and incubated at 37°C for 2 h or at 4°C overnight. Excess liquid was removed, 0.2 ml of diluting buffer (0.25% BSA plus 0.05% Tween 20 in PBS) was added per well, and the wells were incubated at 25°C for 30 min. Excess liquid was removed, 0.05 ml of mouse hybridoma supernatant 6R1C2.8 was added per well, and the wells were incubated at 25°C for 1 h. The wells were washed three times with PBS; then 0.1 ml of affinity-purified F $(ab')_2$ goat anti-mouse immunoglobulin G plus immunoglobulin M conjugated with horseradish peroxidase (Pel-freeze; 1:1,000 in diluting buffer) was added per well, and the wells were incubated at 37°C for 30 min. The wells were washed four times with PBS; then 0.1 ml of chromagen reagent (1 mg of 3',3'5',5'-tetramethylbenzidine per ml in dimethyl sulfoxide) diluted 1:100 in buffered substrate (0.01% hydrogen peroxide in 0.1 M sodium acetate [pH 6.0]) was added per well, and the wells were incubated at room temperature until a blue color appeared (10 to 60 min). The reaction was stopped by adding 0.1 ml of 1 N H_2SO_4 per well, and the A_{450} was determined on a microplate reader.

RESULTS

Purification of KAF from human keratinocyte conditioned medium. Previous experimental results from our laboratories suggested that keratinocytes growing in the absence of growth factors produced an autocrine-acting factor (KAF) which bound to and was negatively regulated by heparin (7, 28). Prior to large-scale purification, we determined that partially purified (heparin-affinity) KAF stimulated [^{3}H]thymidine incorporation in quiescent, serum-free AKR-2B cells (26) and that this stimulation was sensitive to inhibition by heparin. Heparin had no significant inhibitory effect on the mitogenic activity of bFGF, aFGF, EGF, or TGFα in these cells (data not shown). As a result of these observations, we used an AKR-2B cell DNA synthesis assay to detect KAF activity during the course of large-scale purification. Concentrated protein from medium conditioned by human keratinocytes was chromatographed on a heparin-acrylic column. Figure 1A illustrates the elution profile of one representative heparin affinity purification step. A major protein peak eluted in fractions 12 to 20 (approximately 1.7 M NaCl), whereas KAF activity eluted within fractions 14 to 20 at approximately 1.75 M NaCl. The peak fractions from five separate heparin affinity purification steps were combined and concentrated. A volume of this sample corresponding to 300 U of KAF was analyzed on a nonreducing SDS-polyacrylamide gel. Figure 1B displays the total fractionated proteins present in the KAF preparation as visualized by silver staining of the gel. Visual analysis indicated

	1	2	3	4	5	6	7	8	9	10
KAF-1	Ser	Val	Arg	Val	Glu	Gln	Val	Val	Lys	Pro
KAF-2	.....						Val	Val	Lys	Pro
AR	Ser	Val	Arg	Val	Glu	Gln	Val	Val	Lys	Pro
AR'	.....						Val	Val	Lys	Pro

	11	12	13	14	15	16	17	18	19	20
KAF-1	Pro	Gln	Asn	Lys	Thr	Glu	Ser	Glu	Asn	Thr
KAF-2	Pro	Gln	Xxx	Lys	Thr	Glu	Ser	Glu	Asn	Thr
AR	Pro	Gln	Asp	Lys	Thr	Glu	Ser	Glu	Asn	Thr
AR'	Pro	Gln	Asp	Lys	Thr	Glu	Ser	Glu	Asn	Thr

	21	22	23	24	25	26
KAF-1	.....					
KAF-2	Ser	Asp	Lys	Pro	Lys	Arg
AR	Ser	Asp	Lys	Pro	Lys	Arg
AR'	Ser	Asp	Lys	Pro	Lys	Arg

FIG. 2. NH_2-terminal protein microsequencing of reversed phase purified KAF. Fractions 21 to 23 from the second reversed-phase purification step (Fig. 1G) were combined, lyophilized, and resuspended in 200 μl of 0.1% TFA, and 100 μl was subjected to protein microsequencing as described in Materials and Methods. The results represent amino acid residues detected in 20 rounds of Edman degradation. Analysis demonstrated that two similar polypeptides, KAF-1 (full-length KAF) and KAF-2 (truncated KAF), were present in the purified preparation. The figure depicts the alignment of the deduced amino acid sequences for KAF-1 and KAF-2 with the reported amino acid sequence of AR and the AR' (30, 31). Xxx represents residues which were not determined.

ml) at a final dilution of 1:10,000 in the AKR-2B cell thymidine incorporation assay. (D) An equal volume (equal to 300 U of KAF in fraction 11) of fractions 9 to 14 from the cation-exchange purification step (panel C) was subjected to electrophoretic analysis under nonreducing conditions. The arrow indicates the location of stained protein bands correlating with heparin-sensitive mitogenic activity eluted from the cation-exchange column. (E) The three peak fractions from the cation-exchange purification step (panel C) were pooled and subjected to reversed-phase column chromatography. The sample was passed over a C-1/C-8 ProRPC reversed-phase column, and the column was washed. Bound material was eluted in the indicated acetonitrile gradient, and 0.5-ml fractions were collected every minute for 20 min. Each of the indicated fractions was tested (in the presence or absence of 30 μg of heparin sulfate per ml) at a final dilution of 1:5,000 in the AKR-2B cell thymidine incorporation assay. (F) An equal volume (equal to 300 U of KAF in fraction 13) of fractions 11 to 15 from the reversed-phase purification step shown in panel D was lyophilized and subjected to electrophoretic analysis under nonreducing conditions. Samples from fractions 12 and 13 were also analyzed under reducing (with dithiothreitol) conditions. Arrows indicate the location of stained protein bands correlating with heparin-sensitive mitogenic activity eluted from the reversed-phase column purification step. (G) Fraction 13 from the previous reversed-phase purification step (panel D) was subjected to a second reversed-phase purification step as described in Materials and Methods. The KAF preparation was introduced onto the same reversed-phase column, washed, and eluted with a different gradient as indicated in the figure. Fractions (0.25 ml) were collected every minute. Each of the indicated fractions were tested (in the presence or absence of 30 μg of heparin sulfate per ml) at a final dilution of 1:6,000 in the AKR-2B cell thymidine incorporation. (H) An equal volume (equal to 300 U of KAF in fraction 22) of fractions 19 to 26 from the second reversed-phase purification step (panel G) was lyophilized and subjected to SDS electrophoretic analysis under reducing (with dithiothreitol) conditions. The arrow indicates the location of stained protein bands correlating with heparin-sensitive mitogenic activity eluted from the second reversed-phase column purification step.

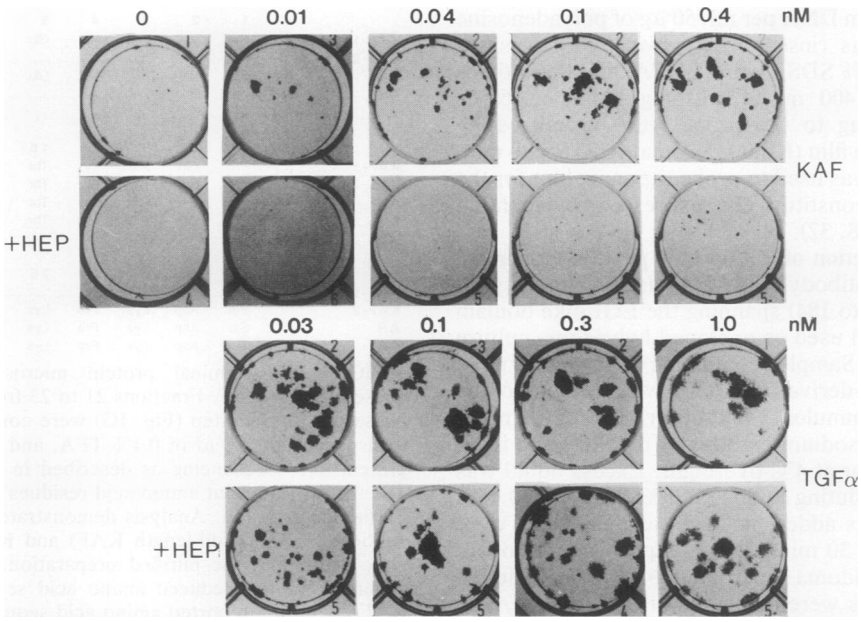

FIG. 3. Effect of purified KAF on the clonal growth of human keratinocytes. Fractions 20 and 24 from the second reversed-phase purification step (Fig. 1G) were pooled, lyophilized, resuspended in solution A-BSA, and tested for colony-stimulating activity in the human keratinocyte clonal growth assay, as described in the legend to fig. 1 and in Materials and Methods. The concentration of KAF was determined by using the conversion factor described in Materials and Methods. As positive controls, clonal cultures were treated with TGFα (0.03 to 1.0 nM). All experiments were performed in the absence or presence (+HEP) of 30 μg of heparin sulfate per ml. The results shown are representative samples of triplicate determinations.

that heparin affinity-purified KAF contained many different proteins of different molecular masses.

The pooled, heparin affinity-purified KAF preparation (25,939 U) was introduced onto a MonoS cation-exchange column at pH 7.0 and eluted with an NaCl gradient. The elution profile of this purification step is shown in Fig. 1C. Most protein eluted from the column within fractions 5 to 12 (0.55 to 0.8 M NaCl), whereas all of the heparin-sensitive KAF mitogenic activity eluted in fractions 10 to 13 at approximately 0.8 M NaCl. An equal volume (equivalent to 300 U in peak fraction 11) was removed from fractions 9 to 14 and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) under nonreducing conditions and subsequently silver stained. Analysis of the gel (Fig. 1D) revealed partial purification of a 21-kDa protein (arrow) in fractions 10 to 13 which correlated with the levels of heparin-sensitive KAF mitogenic activity. Fractions 10 to 12 were pooled for the next purification step.

The cation-exchange purified KAF preparation (18,593 U) was introduced onto a C-1/C-8 reversed-phase column in the presence of 0.1% TFA. Proteins which bound to the column were eluted with an acetonitrile gradient. Several protein peaks eluted within fractions 19 to 26 (21 to 23% acetonitrile), whereas heparin-sensitive KAF mitogenic activity eluted from the column in fractions 12 to 15 at approximately 18% acetonitrile (Fig. 1E). An equal volume (equivalent to 300 U in peak fraction 13) was removed from fractions 11 to 17 and subjected to SDS-PAGE under nonreducing or reducing (fractions 12 and 13 only) conditions. Visualization of the gels by silver staining (Fig. 1F) revealed significant purification; the nonreducing gel demonstrated the presence of a 21-kDa protein (Fig. 1F, left arrow) which correlated with the profile of KAF mitogenic activity. Electrophoretic analysis of fractions 12 and 13 under reducing conditions (with dithiothreitol) demonstrated that the migration of the 21-kDa protein (right arrow) in SDS-gels was not altered by reduction. However, reduction did alter the migration of the molecular mass standards and indicated that KAF had a molecular mass of 18.5 kDa under these conditions.

Fraction 13 from the separation shown in Fig. 1E was lyophilized, resuspended in 0.1% TFA (6129 U), and reintroduced onto the same reversed-phase column. Protein bound to the column was eluted with a different acetonitrile gradient. Figure 1G represents the elution profile of KAF activity over the second acetonitrile gradient. Most of the heparin-sensitive KAF mitogenic activity eluted in fractions 20 to 24 at approximately 17% acetonitrile. An equal volume (equivalent to 300 KAF U in peak fraction 22) was removed from fractions 19 to 26, subjected to SDS-PAGE under reducing conditions, and subsequently silver stained (Fig. 1H). Analysis of the proteins present in the most active fractions demonstrated the presence of a broad 18.5-kDa band that we believed to be the KAF polypeptide(s) (arrow) in the most active reversed-phase fractions. We also observed small amounts of a 15-kDa polypeptide which appeared to copurify with the 18.5-kDa polypeptide.

Fractions 21 to 23 were pooled, lyophilized, and resuspended in 200 μl of 0.1% TFA. One half of this sample (305 U of KAF, 100 μl) was subjected to 20 rounds of Edman degradation on a gas phase microsequencing apparatus. The results indicated that 42.5 pmol of two related polypeptides was present in the sample (Fig. 2); one of the polypeptides (KAF-2) was a truncated (minus 6 residues) version of the other (KAF-1). A comparison with known sequences revealed that KAF-1 and KAF-2 were nearly identical at the first 20 amino acid residues to the two forms of human AR, AR and AR′ (30, 31), and identical to the corresponding amino acid sequence predicted from human AR cDNA (21). The deduced NH_2-terminal amino acid sequences of the

KAF polypeptides and their alignment with the reported sequences for AR are shown in Fig. 2.

Purified KAF mitogenically stimulates both normal human keratinocytes and a mouse keratinocyte cell line. Highly purified KAF preparations stimulated the formation of human keratinocyte colonies in the absence of EGF and TGFα (Fig. 3). Half-maximal stimulation of keratinocyte clonal growth occurred at approximately 0.04 nM, and addition of heparin sulfate to the cultures significantly inhibited the activity of KAF at all concentrations tested. In contrast to these results, the ability of TGFα to stimulate clonal growth was insensitive to inhibition by heparin. Thus, KAF purified from medium conditioned by keratinocytes at high density stimulated human keratinocyte clonal growth, and the mitogenic activity of KAF on these cells was negatively regulated by heparin.

We also tested purified KAF for its ability to stimulate the clonal growth of a mouse keratinocyte cell line (BALB/MK). Surprisingly, KAF did not stimulate BALB/MK clonal growth, whereas both EGF and TGFα demonstrated significant activity (Fig. 4A). However, the same KAF preparation stimulated [^{3}H]thymidine incorporation in higher-density quiescent (without EGF) monolayers of BALB/MK cells (Fig. 4B). Purified KAF caused a heparin-sensitive morphological change in high-density cell cultures which was not observed in BALB/MK cells treated with KAF at clonal cell density (data not shown). Unlike EGF or TGFα, the mitogenic response to KAF by BALB/MK cells appears to be dependent on cell density and is inhibited by heparin.

KAF-mediated competition of cell surface EGF receptor occupancy is inhibited by heparin. AR has been demonstrated to belong to the EGF family of growth-regulatory polypeptides and possesses the ability to compete with EGF for binding to EGF cell surface receptors (31). Because our data indicated that KAF was similar or identical to AR, we tested purified KAF for its ability to compete with EGF for cell surface binding. Unlabeled EGF, TGFα, and KAF were able to compete with labeled EGF for cell surface binding (Fig. 5A). Heparin blocked the ability of unlabeled KAF to compete with radiolabeled EGF for cell surface binding sites, but had no effect on the ability of unlabeled TGFα to compete for these sites (Fig. 5B). Competition by unlabeled EGF was also not affected by the addition of heparin (data not shown). Collectively, these experiments indicated that heparin sulfate inhibited the ability of KAF to compete with ^{125}I-EGF for cell surface binding, whereas the competitive activities of TGFα and EGF were essentially unaffected by treatment with heparin sulfate.

Molecular cloning of AR cDNA from human keratinocyte-derived mRNA. Information obtained from amino acid sequencing of KAF, ^{125}I-EGF-binding competition assays, and molecular mass determination by SDS-PAGE indicated that KAF was similar or identical to AR. To further investigate this possibility, we used the published cDNA sequence of AR (21) to synthesize homologous oligonucleotide primers which were used for enzymatic amplification of putative AR-specific cDNAs from human keratinocyte-derived cDNA. Amplified sequences from keratinocyte cDNA were cloned into vectors, and both the minus and plus strands were sequenced. The results demonstrated that the cloned insert possessed identity to nucleotides 153 to 1021 of the reported sequence for human AR cDNA (21) and encompassed the region corresponding to the entire 252-amino-acid primary translation product (21). Amplified AR-specific sequences were also cloned into an in vitro transcription vector for the generation of radiolabeled antisense cRNA.

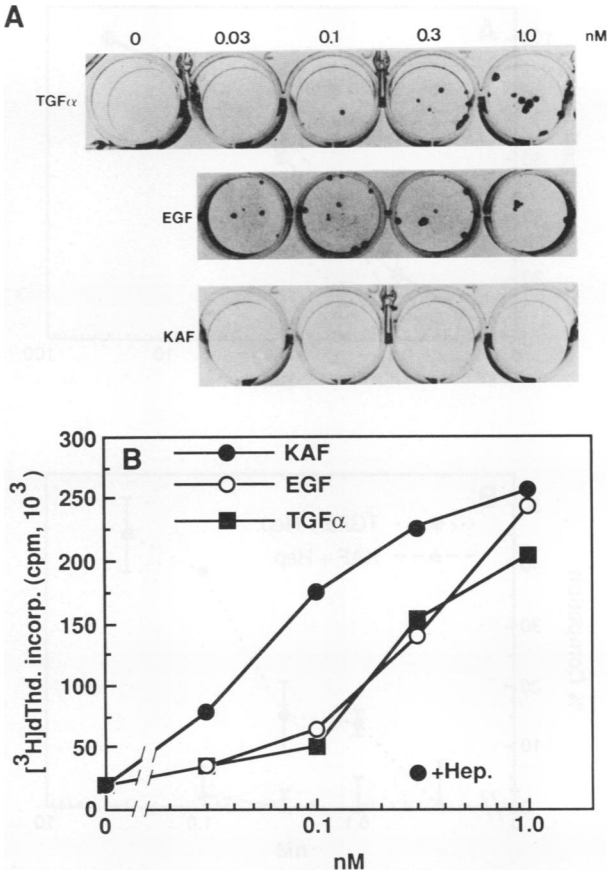

FIG. 4. Effect of purified KAF on the growth of BALB/MK cells cultured at clonal and high cell density. (A) BALB/MK cells were plated at clonal cell density, washed, and subsequently placed in medium lacking EGF, as described in Materials and Methods. The clonal cultures were then treated with the indicated concentrations of KAF (pooled fractions 21 to 23 from the second reversed-phase purification step [Fig. 1G]), TGFα, or EGF. The cells were cultured for 10 days and processed as described in Materials and Methods. The results shown are representative samples of triplicate determinations. (B) High-density BALB/MK cells were cultured in the absence of EGF for 24 h and treated with the indicated concentrations of KAF (same KAF preparation used in panel A), TGFα, or EGF in the presence of [^{3}H]thymidine as described in Materials and Methods. The cells were then cultured for an additional 24 h, and incorporation of [^{3}H]thymidine was determined. Heparin sulfate (30 μg/ml) was added to identical wells containing 0.3 nM KAF (+Hep.) or EGF (data not shown). All data points represent the average of duplicate determinations.

Expression of AR-specific mRNA in normal and abnormal human cells. Radiolabeled antisense AR-specific cRNA was hybridized to a Northern blot containing fractionated poly(A)$^+$ mRNAs derived from a variety of cell types including normal keratinocytes, fibroblasts, and melanocytes as well as normal and abnormal human mammary-derived cell lines. The AR-specific cRNA probe hybridized to a prominent 1.7-kb transcript in three different strains of keratinocytes (Fig. 6, lanes C to I) and normal mammary epithelial cells (lane J). No hybridization to this 1.7-kb transcript was detected in mRNA derived from cultured normal fibroblasts (lane A), normal melanocytes (lane B), or the Hs578T mammary tumor cell line (lane L), even after longer exposures. However, longer exposures did reveal the

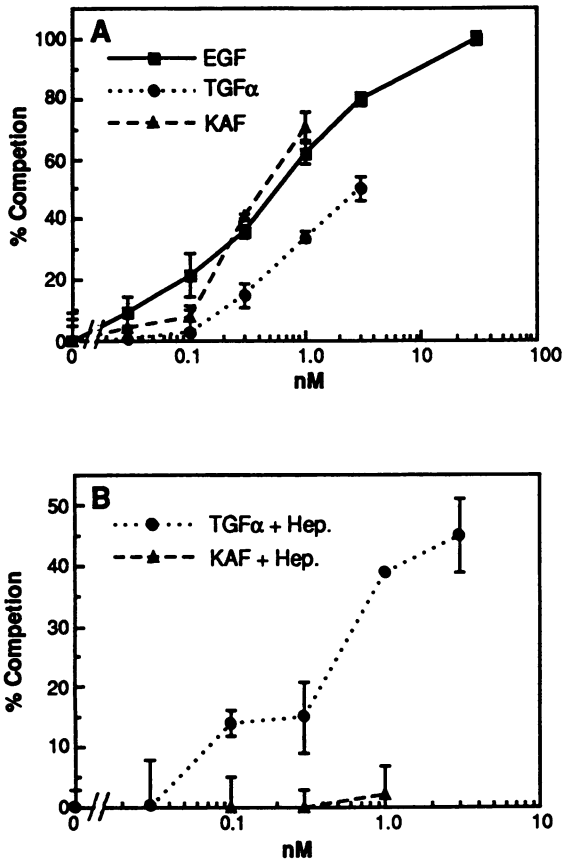

FIG. 5. Effect of heparin on the ability of KAF to compete with ^{125}I-EGF for cell surface-binding sites on AKR-2B cells. (A) Reversed-phase purified KAF (same KAF preparation used for Fig. 3) was tested for its ability to compete with ^{125}I-EGF for cell surface-binding sites on AKR-2B cells, as described in Materials and Methods. As positive controls, some AKR-2B cultures were treated with TGFα or EGF at the indicated concentrations. The percent maximal competition for each experimental point was determined as described in Materials and Methods. The results represent the mean of triplicate determinations; bars indicate the standard error of the mean. (B) An identical experiment (EGF competition data not shown) to that presented in panel A was performed in the presence of heparin (30 μg/ml).

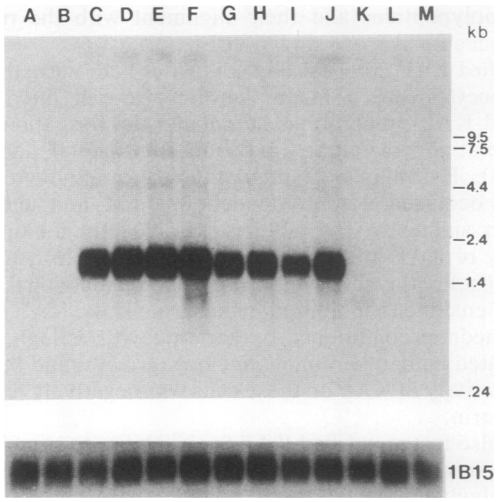

FIG. 6. Expression of AR-specific mRNAs in normal human skin-derived cell types, normal human mammary epithelial cells, and human mammary-derived cell lines. Northern blot analysis of poly(A)$^+$ mRNA was performed as described in Materials and Methods. Poly(A)$^+$ mRNA (5 μg per lane) was isolated from the following sources: lane A, normal human fibroblasts cultured in MCDB 202a plus bFGF (2 ng/ml) and insulin (1 μg/ml); lane B, normal human melanocytes cultured in standard medium plus phorbol-12-myristate 13-acetate (10 ng/ml), bFGF (2 ng/ml), and insulin (5 μg/ml); lane C, normal human keratinocytes (strain 8) cultured in standard medium; lane D, normal human keratinocytes (strain 8) cultured in standard medium plus insulinlike growth factor type 1 (10 ng/ml); lane E, normal human keratinocytes (strain 8) cultured in standard medium plus insulinlike growth factor type 1 (10 ng/ml) and EGF (10 ng/ml); lane F, normal human keratinocytes (strain 775) cultured in standard medium plus insulin (5 μg/ml); lane G, normal human keratinocytes (strain 11) cultured in standard medium plus insulin (5 μg/ml); lane H, normal human keratinocytes (strain 11) cultured in standard medium plus insulin (5 μg/ml) and EGF (10 ng/ml); lane I, normal human keratinocytes (strain 8) cultured in standard medium plus insulin (5 μg/ml); lane J, normal human mammary epithelial cells (strain 161) cultured in MCDB 170 plus KDS supplements (KDS supplements are 0.1 mM phosphoethanolamine, 0.1 mM ethanolamine, 0.4% bovine pituitary extract 1.4 × 10^{-7} M hydrocortisone, 10 ng of EGF per ml, and 5 μg of insulin per ml); lane K, HBL-100 human mammary cell line cultured in MCDB 170 plus KDS supplements (without EGF); lane L, Hs578T human mammary tumor cell line cultured in MCDB 202a plus 5% calf serum; lane M, BT474 human mammary tumor cell line cultured in MCDB 202a plus 5% fetal bovine serum. mRNA was fractionated against RNA standards of known size, as indicated at the right of the figure. A ^{32}P-labeled antisense cRNA probe corresponding to the constitutively expressed cyclophillin (1B15) gene product (0.9 kb) was also used for hybridization analysis of this blot (see lower panel).

presence of the 1.7-kb AR mRNA in the immortal HBL-100 mammary cell line (lane K) and in the BT474 mammary tumor cell line (lane M). Longer exposures also revealed relatively minor hybridization to several other higher-molecular-weight transcripts in all the cell types tested.

KAF and AR are both immunologically related and are inhibited by interaction with heparin sulfate. The present study clearly demonstrated that heparin sulfate acts as an inhibitor of KAF mitogenic activity. Even though our results suggested that KAF and AR were identical molecules, AR has not been reported to interact with heparin sulfate. To more clearly test the assumption that KAF and AR are indeed identical, we determined whether AR derived from phorbol ester-treated mammary tumor cells (30) could also be negatively regulated by heparin sulfate. AR and KAF stimulated DNA synthesis in quiescent AKR-2B cells with similar potency (Fig. 7B). Moreover, heparin sulfate inhibited the mitogenic activity of both KAF and AR, but had little effect on the growth-stimulating activity of EGF (Fig. 7A).

To further test the assumption that KAF and AR are identical molecules, we used an AR-specific ELISA to measure AR immunoreactivity in a purified preparation of KAF. Figure 7C demonstrates the results obtained when mammary tumor cell-derived AR (30), KAF, and EGF were analyzed in an AR-specific ELISA. This assay uses a monoclonal antibody raised against the carboxy-terminal EGF-like region (amino acid residues 144 to 184) of mature AR. The results show that mammary tumor cell-derived AR and KAF share antigenic similarity in this assay, whereas EGF and TGFα (not shown) fail to show this characteristic.

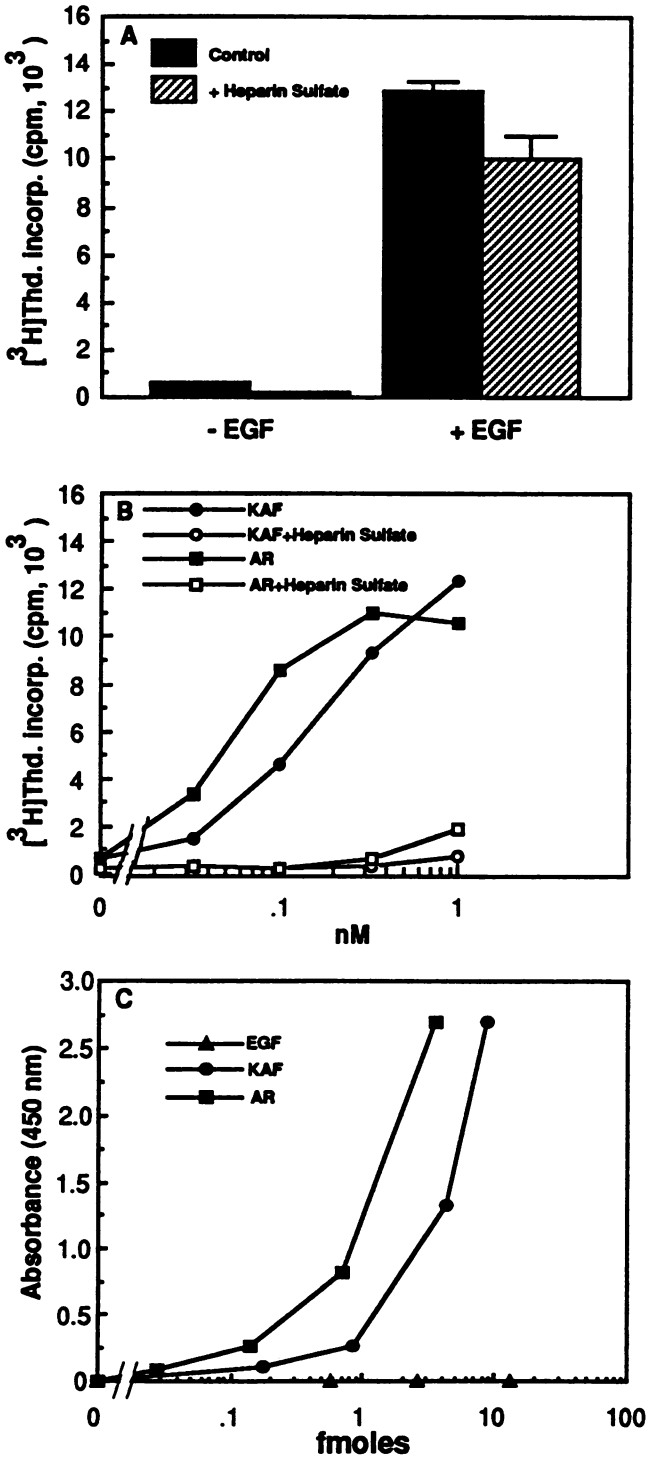

FIG. 7. AR and KAF are functionally and antigenically related. (A) An AKR-2B cell thymidine incorporation assay was performed as described in the legend to Fig. 1 and in Materials and Methods. AKR-2B cells were treated with (+EGF) or without (−EGF) 0.33 nM EGF in the presence or absence of 30 μg of heparin sulfate per ml. All values represent the mean of three separate determinations; bars indicate the standard error of the mean. (B) KAF was purified as described in the legend to Fig. 1 and in Materials and Methods, and human mammary tumor cell-derived AR was purified as previously described (30). KAF and AR were tested for DNA synthesis-stimulating activity in quiescent AKR-2B cells at the concentrations indicated in the figure, in the presence or absence of heparin sulfate (30 μg/ml). All values represent the average of duplicate determinations. (C) EGF and the same KAF and AR preparations as in panel B were tested at the indicated levels in an AR-specific ELISA, as described in Materials and Methods. Values are expressed as A_{450} and represent the average of triplicate determinations.

DISCUSSION

The human epidermis is a continually self-renewing organ that can be separated into regions based on proliferative capacity, expression of differentiation-specific gene products, and cell death. The basal and suprabasal layers of the epidermis are characterized by cell proliferation, and the factors responsible for regulating growth in these regions are not clearly understood. Although the results from some previous inquiries have led investigators to conclude that mitogens such as EGF, TGFα, and FGFs were required for sustained proliferation of normal human keratinocytes in vitro (1, 18, 29, 34), we have recently shown that these growth factors are required for initiation of growth only at clonal densities (unpublished observation) and that proliferation of these cells at densities above 10^3 cells/cm^2 can occur in the absence of these factors (7, 28). Moreover, the growth factor-independent proliferation of human keratinocytes was inhibited by heparin sulfate (7, 28). The activity of mitogens present in the medium conditioned by these keratinocytes was inhibited by heparin sulfate as well as by an anti-EGF receptor monoclonal antibody (7). The results of these studies led us to hypothesize that normal human keratinocytes produce an EGF-like, heparin-binding growth factor which acts in an autocrine manner to stimulate the proliferation of these cells. We called this activity KAF. In the results presented here, we demonstrate the purification of KAF and show that it is similar or identical to a growth factor previously purified from cultured human mammary tumor cells, AR (21, 30, 31). We show that the mitogenic activity of KAF/AR, unlike other heparin-binding growth factors, is negatively regulated by its interaction with heparin sulfate.

AR, a member of the EGF family of polypeptide growth factors (31), was isolated from medium conditioned by phorbol ester-treated human mammary carcinoma cells and was so named because of its ability to stimulate the growth of some cell lines and inhibit the growth of others (30). AR competes with EGF for cell surface binding and may exert at least some of its biological effects through the EGF receptor (31). It has not been clearly demonstrated whether AR uses other growth factor receptors to mediate its actions. Cloning and sequencing of the AR gene revealed that AR is synthesized as a 252-amino-acid membrane-bound precursor which is processed to non-membrane bound mature forms of 84 and 76 amino acids (21). AR appears to be unique among the EGF-like polypeptides in that its primary structure possesses a highly positively charged, Lys-Arg-rich, N-terminal hydrophilic region adjacent to a C-terminal EGF-like domain (21, 31). It seems likely that this hydrophilic domain could confer heparin regulation of biological activity to this molecule. Our results demonstrate that KAF/AR is a heparin-binding member of the EGF family.

Because KAF/AR is produced by keratinocytes and stimulates the clonal growth of these cells, it fits the classic definition of an autocrine acting factor. Further evidence for the autocrine nature of KAF/AR activity in human keratinocyte cultures is provided by the ability of heparin sulfate to inhibit the growth of these cells at high cell densities in the

absence of exogenously added factors such as EGF, aFGF, bFGF, or TGF-α (7, 28). KAF/AR is the only purified growth factor that we are aware of whose mitogenic activity is completely blocked by heparin sulfate. Neither aFGF, bFGF, TGF-α, nor EGF exhibited this property in any of the assays used in this study. In addition, purified KAF/AR competed with ^{125}I-EGF for cell surface binding, and the addition of heparin to the binding medium prevented this interaction. Thus, heparin sulfate may inhibit the mitogenic activity of KAF/AR via a mechanism in which it binds KAF/AR and thereby prevents interaction of KAF/AR with its cognate cell surface receptor (EGF or EGF-like receptors). It is not clear whether the growth inhibition exerted by heparin in other cell types (3, 11, 12, 16, 24, 35) is mediated by blocking the mitogenic activity of endogenous KAF/AR. Our observations suggest that sulfated GAGs or PGs produced in vivo could regulate the activity of KAF/AR. Moreover, these molecules could also be used as agents to treat diseases (neoplasia, psoriasis) caused by the inappropriate expression of, or responsiveness to, KAF/AR. KAF/AR may associate with sulfated GAGs or PGs present in extracellular matrix, thus creating mechanisms for delivery, storage, and/or regulation of this growth factor. Alternatively, artificial matrices composed of sulfated GAGs or PGs could theoretically be used as solid supports for the delivery of KAF/AR.

We have shown in this study that KAF/AR, as well as EGF and TGFα, stimulates DNA synthesis in nearly confluent BALB/MK keratinocyte cultures. At clonal cell density these cells did not respond to the same preparation of KAF/AR, but retained responsiveness to both EGF and TGFα. Although we cannot rule out the possibility that the mitogenic activity of KAF/AR is specifically abrogated by factors associated with the clonal assay culture conditions, these results could indicate that the biological response to KAF/AR may in some cases be dependent on the cell density of the target cell. In reference to this observation, it has been shown that AR is unable to stimulate the low-density anchorage-independent growth of NRK-SA6 cells (31). Moreover, the fact that KAF/AR does not supplant the requirement for EGF under clonal culture conditions suggests that it may mediate its biological response through receptors (other than the EGF-R) which are not expressed by BALB/MK cells at clonal cell density. TGFα has also been proposed as an autocrine growth factor for human keratinocytes (4). Although it has not been demonstrated that TGFα mediates the growth factor-independent proliferation of human keratinocyte cultures, TGFα was detected in medium conditioned by keratinocytes growing logarithmically in standard medium without added polypeptide growth factors. Thus, at least two EGF-like growth factors are present in the medium of these cultures, and these factors could be acting in concert to stimulate the autocrine growth of these cells. It has been suggested that AR may elicit some of its biological activity via a receptor separate from the EGF-R, perhaps by binding to the newly discovered HER-3 (ERBB3) protein (13, 22). Kraus et al. (13) have shown that ERBB3 mRNA is expressed in human keratinocytes. Thus, it is tempting to speculate, on the basis of our earlier studies (7, 28) and the results shown here, that the autonomous growth of human keratinocytes in vitro requires the cooperation of at least two growth factors (TGFα and KAF/AR) and the occupancy of at least two types of receptors.

Our studies have broad implications in the analysis of the transforming ability of autocrine-acting growth factors. The general assumption that normal cells do not produce growth factors to which they themselves respond, whereas many tumor cells coexpress growth factors and their receptors, has been based in large part on studies which compared the properties of normal fibroblasts (or fibroblastlike cell lines) with tumor cells that are often of epithelial origin. The ability to culture normal human keratinocytes (epithelial cells) under highly defined conditions has clearly revealed that normal epithelial cells in culture can proliferate in an autocrine fashion. In addition, we have reported here for the first time that normal human mammary epithelial cells express high levels of AR mRNA, whereas the immortal and tumor-derived mammary cell lines expressed significantly smaller amounts of this growth factor mRNA. Other investigators have demonstrated that AR may act as a growth inhibitor for some human tumor cell lines (30). It may be the case that these tumor-derived cell lines do not express elevated levels of AR by virtue of the fact that AR, acting as a growth inhibitor, would impede the proliferation of these cells in vivo or in vitro. Collectively, our observations suggest that generalized theories which claim that only transformed cells express autocrine growth stimulators are not applicable to some epithelial cell types. In addition, these generalized theories are further complicated by the fact that normal and transformed epithelial cells may respond differently to the same growth factor (30, 31).

Our present investigation has demonstrated that human keratinocyte and mammary epithelial cell cultures express human AR mRNA. In contrast, AR-specific mRNA was not detected in normal human epidermis (21). Thus, rapidly proliferating subconfluent human keratinocyte cultures may express higher levels of AR mRNA than the same cells in vivo. These keratinocyte cultures may mimic a wound-healing response in which growth factor gene expression is induced. In support of this notion, it is known that the level of TGFα mRNA expression in cultured keratinocytes decreases dramatically when these cultures become confluent (unpublished observation). The low level of TGFα expression observed in the human epidermis in vivo (9) may be analogous to the decreased TGFα expression observed in confluent versus subconfluent keratinocyte cultures. We have yet to determine whether AR expression is also reduced when keratinocyte cultures become confluent.

In summary, we have demonstrated for the first time that normal human keratinocyte cultures produce a heparin-binding autocrine growth factor (KAF) which is similar or identical to the human AR gene product (21, 30, 31). We have described several new biological properties (heparin affinity, heparin inhibition, density-dependent target cell responsiveness) of KAF/AR and have shown that it is an autocrine factor for normal human keratinocytes. Using an AR-specific cDNA, we have demonstrated new cellular sources for AR expression (normal keratinocytes and normal mammary epithelial cells). Future investigations will be necessary to ascertain the role of KAF/AR in normal growth and development, wound healing, and the development of pathological (cancer, psoriasis, hyperproliferative disease) states.

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REFERENCES

1. **Barrandon, Y., and H. Green.** 1987. Cell migration is essential for sustained growth of keratinocyte colonies: the role of transforming growth factor-α and epidermal growth factor. Cell **50**:1131–1137.
2. **Burgess, W. H., and T. Maciag.** 1989. The heparin-binding (fibroblast) growth factor family of proteins. Annu. Rev. Biochem. **58**:575–606.
3. **Clowes, A. W., and M. J. Karnovsky.** 1977. Suppression by heparin of smooth muscle proliferation in injured arteries. Nature (London) **265**:625–626.
4. **Coffey, R. J., Jr., R. Derynck, J. N. Wilcox, T. S. Bringman, A. S. Goustin, H. L. Moses, and M. R. Pittelkow.** 1987. Production and autoinduction of transforming growth factor-α in human keratinocytes. Nature (London) **328**:817–820.
5. **Cook, P. W., C. P. Edwards, T. Haraguchi, and G. L. Firestone.** 1989. Partial characterization of a glucocorticoid suppressible mitogenic activity secreted from a rat hepatoma cell line hypersensitive to the antiproliferative effects of glucocorticoids. J. Biol. Chem. **264**:14151–14158.
6. **Cook, P. W., B. E. Magun, M. R. Pittelkow, R. J. Coffey, Jr., and G. D. Shipley.** 1990. Expression and regulation of mRNA coding for heparin binding growth factors and transforming growth factor type-alpha in human skin-derived cells. Mol. Endocrinol. **4**:1377–1385.
7. **Cook, P. W., M. R. Pittelkow, and G. D. Shipley.** J. Cell. Physiol., in press.
8. **Danielson, P. E., S. Forss-Petter, M. A. Brow, L. Calavetta, J. Douglass, R. J. Milner, and J. G. Sutcliffe.** 1988. p1B15A cDNA clone of the rat mRNA encoding cyclophilin. DNA **7**:261–267.
9. **Elder, J. T., G. J. Fisher, P. B. Lindquist, G. L. Bennett, M. R. Pittelkow, R. J. Coffey, Jr., L. Ellingsworth, R. Derynck, and J. J. Voorhees.** 1989. Overexpression of transforming growth factor α in psoriatic epidermis. Science **243**:811–814.
10. **Hammond, S. L., R. G. Ham, and M. R. Stampfer.** 1984. Serum-free growth of human mammary epithelial cells: rapid clonal growth in defined medium and extended serial passage with pituitary extract. Proc. Natl. Acad. Sci. USA **81**:5435–5439.
11. **Hook, M., L. Kjellen, and S. Johansson.** 1984. Cell surface glycosaminoglycan. Annu. Rev. Biochem. **53**:847–869.
12. **Imamura, T., and Y. Mitsui.** 1987. Heparan sulfate and heparin as a potentiator or a suppressor of growth of normal and transformed vascular endothelial cells. Exp. Cell Res. **172**:92–100.
13. **Kraus, M. H., I. Wolfgang, M. Toru, N. C. Popescu, and S. A. Aaronson.** 1989. Isolation and characterization of *ERBB3*, a third member of the *ERBB*/epidermal growth factor receptor family: evidence for overexpression in a subset of human mammary tumors. Proc. Natl. Acad. Sci. USA **86**:9193–9197.
14. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
15. **Madtes, D. K., E. W. Raines, K. S. Sakariassen, R. K. Assoian, M. B. Sporn, G. I. Bell, and R. Ross.** 1988. Induction of transforming growth factor-α in activated human alveolar macrophages. Cell **53**:285–293.
16. **Majesky, M. W., S. M. Schwartz, M. M. Clowes, and A. W. Clowes.** 1987. Heparin regulates smooth muscle S phase entry in the injured rat carotid artery. Circ. Res. **61**:296–300.
17. **McKeehan, W. L., and R. G. Ham.** 1977. Methods for reducing the serum requirement for growth in vitro of nontransformed diploid fibroblasts. Dev. Biol. Stand. **37**:97–108.
18. **O'Keefe, E. J., M. L. Chiu, and R. E. Payne.** 1988. Stimulation of growth of keratinocytes by basic fibroblast growth factor. J. Invest. Dermatol. **90**:767–769.
19. **Pittelkow, M. R., P. B. Lindquist, R. T. Abraham, R. Graves-Deal, R. Derynck, and R. J. Coffey, Jr.** 1989. Induction of transforming growth factor-α expression in human keratinocytes by phorbol esters. J. Biol. Chem. **264**:5164–5171.
20. **Platt, E. J., K. Karlsen, A. Lopez-Valdivieso, P. W. Cook, and G. L. Firestone.** 1986. Highly sensitive immunoadsorption procedure for detection of low-abundance proteins. Anal. Biochem. **156**:126–135.
21. **Plowman, G. D., J. M. Green, V. L. McDonald, M. G. Neubauer, C. M. Disteche, G. J. Todaro, and M. Shoyab.** 1990. The amphiregulin gene encodes a novel epidermal growth factor-related protein with tumor-inhibitory activity. Mol. Cell. Biol. **10**:1969–1981.
22. **Plowman, G. D., G. S. Whitney, M. G. Neubauer, C. M. Green, V. L. McDonald, G. J. Todaro, and M. Shoyab.** 1990. Molecular cloning and expression of an additional epidermal growth factor receptor-related gene. Proc. Natl. Acad. Sci. USA **87**:4905–4909.
23. **Rahemtulla, F., C. Moorer, and J. J. Wille, Jr.** 1989. Biosynthesis of proteoglycans by proliferating and differentiating normal human keratinocytes cultured in serum-free medium. J. Cell. Physiol. **140**:98–106.
24. **Robertson, P. L., and G. W. Goldstein.** 1988. Heparin inhibits the growth of astrocytes in vitro. Brain Res. **447**:341–345.
25. **Schwab, M., K. Alitalo, H. E. Varmus, and J. M. Bishop.** 1983. A cellular oncogene (c-Ki-ras) is amplified, overexpressed, and located within karyotypic abnormalities in mouse adrenocortical tumour cells. Nature (London) **303**:497–501.
26. **Shipley, G. D., C. B. Childs, M. E. Volkenant, and H. L. Moses.** 1984. Differential effects of epidermal growth factor, transforming growth factor, and insulin on DNA and protein synthesis and morphology in serum-free cultures of AKR-2B cells. Cancer Res. **44**:710–716.
27. **Shipley, G. D., and R. G. Ham.** 1981. Improved medium and culture conditions for clonal growth with minimal serum protein and for enhanced serum-free survival of swiss 3T3 cells. In Vitro (Rockville) **17**:656–670.
28. **Shipley, G. D., W. W. Keeble, J. E. Hendrickson, R. J. Coffey, Jr., and M. R. Pittelkow.** 1989. Growth of normal human keratinocytes and fibroblasts in serum-free medium is stimulated by acidic and basic fibroblast growth factor. J. Cell. Physiol. **138**:511–518.
29. **Shipley, G. D., and M. R. Pittelkow.** 1987. Control of growth and differentiation in vitro of human prokeratinocytes cultured in serum-free medium. Arch. Dermatol. **123**:1541a–1544a.
30. **Shoyab, M., V. M. McDonald, J. G. Bradley, and G. J. Todaro.** 1988. Amphiregulin: a bifunctional growth-modulating glycoprotein produced by the phorbol 12-myristate 13-acetate-treated human breast adenocarcinoma cell line MCF-7. Proc. Natl. Acad. Sci. USA **85**:6528–6532.
31. **Shoyab, M., G. D. Plowman, V. M. McDonald, J. G. Bradley, and G. J. Todaro.** 1989. Structure and function of human amphiregulin: a member of the epidermal growth factor family. Science **243**:1074–1076.
32. **Sternfeld, M. D., J. E. Hendrickson, W. W. Keeble, J. T. Rosenbaum, J. E. Robertson, M. R. Pittelkow, and G. D. Shipley.** 1988. Differential expression of mRNA coding for heparin-binding growth factor type-2 in human cells. J. Cell. Physiol. **136**:297–304.
33. **Weismann, B. E., and S. A. Aaronson.** 1983. Balb and Kirsten murine sarcoma viruses alter growth and differentiation of EGF-dependent Balb/c mouse epidermal keratinocyte lines. Cell **32**:599–606.
34. **Wille, J. J., Jr., M. R. Pittelkow, G. D. Shipley, and R. E. Scott.** 1984. Integrated control of growth and differentiation of normal human prokeratinocytes cultured in serum-free medium: clonal analysis, growth kinetics, and cell cycle studies. J. Cell. Physiol. **121**:31–44.
35. **Wright, T. C., Jr., J. J. Castellot, Jr., M. Petitou, J.-C. Lormeau, J. Choay, and M. J. Karnovsky.** 1989. Structural determinants of heparins growth inhibitory activity. J. Biol. Chem. **264**:1534–1542.
36. **Yanisch-Peron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of M13mp18 and pUC19 vectors. Gene **33**:103–119.