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Nerve Growth Factor Binds to Normal Human Keratinocytes through High and Low Affinity Receptors and Stimulates Their Growth by a Novel Autocrine Loop*

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Eddi Di Marco, Monica Mathor‡, Sergio Bondanza, Nunzio Cutuli, Pier Carlo Marchisio§, Ranieri Cancedda, and Michele De Luca¶

From the Laboratorio di Differenziamento Cellulare, Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy and the §Dipartimento di Scienze Biomediche ed Oncologica Umana, Università di Torino, Torino, Italy

Normal human keratinocytes synthesize and secrete biologically active nerve growth factor (NGF) in a growth regulated fashion (Di Marco, E., Marchisio, P. C., Bondanza, S., Franzi, A. T., Cancedda, R., and De Luca, M. (1991) *J. Biol. Chem.* 266, 21718–21722). Here we show that the same human keratinocytes bind NGF via low and high affinity receptors. In parallel with the course of NGF synthesis, the expression of low affinity NGF receptor ($p75^{NGFr}$) decreases when a confluent, differentiated, and fully stratified epithelium is obtained. In skin sections, $p75^{NGFr}$ is present in basal keratinocytes and absent from suprabasal, terminally differentiated cells. The *trkA* protooncogene product ($p140^{trkA}$), a component of the NGF receptor, is not expressed by keratinocytes. Instead, keratinocytes express a new member of the *trk* family (that we termed *trkE*), which generates 3.9-kilobase transcripts. Keratinocyte-derived NGF plays a key role in the autocrine epidermal cell proliferation. This has been proven by (i) direct effect of NGF on [^{3}H]thymidine incorporation, (ii) inhibition of autocrine keratinocyte growth by monoclonal antibodies (αD11) inhibiting human NGF biological activity, and (iii) inhibition of autocrine keratinocyte proliferation by a *trk*-specific inhibitor, the natural alkaloid K252a. These data provide evidence that NGF, in addition to its effect as a survival and differentiation factor, is a potent regulator of cell proliferation, at least in human epithelial cells.

Nerve growth factor (NGF)[1] (Levi-Montalcini and Angeletti, 1968) is a polypeptide hormone member of a family of related molecules called neurotrophins (reviewed by Thoenen (1991)), responsible for the maintenance, development, and differentiation of several neural crest-derived cell types (Thoenen and Barde, 1980, Levi-Montalcini, 1987) and central cholinergic neurons (Korshing *et al.*, 1985). NGF, synthesized by and secreted from target tissues of sympathetic, sensory, and cholinergic basal forebrain neurons (Thoenen and Barde, 1980; Levi-Montalcini, 1987), binds to both low and high affinity receptors (formed by $p75^{NGFr}$ and $p140^{NGFr}$/*trkA* protooncogene product) (Johnson *et al.*, 1986; Klein *et al.*, 1991; Kaplan *et al.*, 1991; Hempstead *et al.*, 1991), transduces appropriate signals in nerve terminals (Berg *et al.*, 1991; Cordon-Cardo *et al.*, 1991; Hempstead *et al.*, 1991; Kaplan *et al.*, 1991), is internalized in membrane-bound vesicles of peripheral nerves and transported along microtubules up the axon to the cell body, where it exerts its biological activity (Thoenen and Barde, 1980). In agreement with a target derived neurotrophic role for NGF, a good correlation exists between levels of NGF in target tissues and degree of their innervation by NGF-sensitive fibers (Levi-Montalcini (1987), but see Davies *et al.* (1987)). However, in male mouse submandibular glands, guinea pig and rabbit prostate, the expression of NGF does not correlate with the level of innervation, suggesting that NGF exerts non-neurotrophic activities in these tissues, and that it evokes biological responses also in non-neuronal cells (for review, see Levi-Montalcini (1987)). Indeed, NGF affects growth and histamine release from mast cells (Levi-Montalcini, 1987), modulates human B lymphocyte differentiated functions (Otten *et al.*, 1989), and regulates the onset of meiosis in rat seminiferous epithelium through Sertoli cells (Parvinen *et al.*, 1992).

Human keratinocytes and lining epithelial cells can be serially cultivated (Rheinwald and Green, 1975; De Luca *et al.*, 1990a; Romagnoli *et al.*, 1990). Under the appropriate culture conditions (Green *et al.*, 1979), keratinocytes reconstitute *in vitro* transplantable sheets of epithelium and maintain virtually the same differentiation features and gene expression pattern of their *in vivo* counterparts (for reviews, see Green (1980) and De Luca and Cancedda (1992)), such as to represent an ideal system to investigate epidermal physiology. We and others have shown that epidermal keratinocytes regulate melanocyte growth and differentiation through the paracrine secretion of several polypeptides (De Luca, 1988a, 1988b; Gordon *et al.*, 1989; Halaban *et al.*, 1988; De Luca *et al.*, 1993). In particular, basal keratinocytes synthesize and secrete biologically active NGF (Di Marco *et al.*, 1991; Yaar *et al.*, 1991), which plays a pivotal role in regulating melanocyte migration and differentiation in epidermal morphogenesis (Yaar *et al.*, 1991; De Luca *et al.*; 1993), and

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‡ On leave of absence from the Department of Application of Nuclear Techniques in Biological Sciences, National Nuclear Energy Commission (IPEN-CNEN), São Paulo, Brazil.

¶ To whom correspondence and reprint requests should be addressed: Laboratorio di Differenziamento Cellulare, IST, Istituto Nazionale per la Ricerca sul Cancro, Viale Benedetto XV 10, 16132, Genova, Italy. Tel.: 39-10-355504/3534248; Fax: 39-10-352999.

[1] The abbreviations used are: NGF, nerve growth factor; EGF, epidermal growth factor; NT, neurotrophin; DMEM, Dulbecco's modified Eagle's medium; mAb, monoclonal antibody; PCR, polymerase chain reaction.

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induces the widespread sprouting of sensory nerve fibers during wound healing.[2]

In this paper we show that (i) normal human keratinocytes themselves bind NGF through both low and high affinity receptors, (ii) keratinocytes express the $p75^{NGFr}$ and a new member of the *trk* family (that we termed *trkE*), but not the *trkA* protooncogene, (iii) NGF, in addition to its well known effects as a survival and differentiation factor, is a potent autocrine stimulator of keratinocyte proliferation, (iv) K252a, a selective inhibitor of the tyrosine protein kinase activity of the *trk* family of protooncogenes (Tapley *et al.*, 1992; Berg *et al.*, 1992; Knusel and Hefti, 1992), is sufficient to nearly abolish the autocrine keratinocyte growth stimulation.

EXPERIMENTAL PROCEDURES

Cell Culture—Hybridoma cells producing the anti-mouse NGF mAb (αD11, Cattaneo *et al.* (1988)), able to inhibit human NGF biological activity (Di Marco *et al.*, 1991), were a gift from Drs. Antonio Cattaneo and Pietro Calissano, University of Rome, Rome, Italy. αD11 does not recognize other neurotrophins, such as brain-derived neurotrophic factor or NT-3.[3] 3T3-J2 cells were a gift from Dr. Howard Green, Harvard Medical School, Boston, MA.

Human epidermal keratinocytes were obtained from skin biopsies of healthy volunteers and cultivated on a feeder-layer of lethally irradiated 3T3-J2 cells as described (Green *et al.*, 1979; Di Marco *et al.*, 1991). In brief, skin biopsies were minced and trypsinized (0.05% trypsin, 0.01% EDTA) at 37 °C for 3 h. Cells were collected every 30 min, plated ($2.5 \times 10^4/cm^2$) on lethally irradiated 3T3-J2 cells ($2.4 \times 10^4/cm^2$), and cultured in 5% CO_2 and humidified atmosphere in keratinocyte growth medium: Dulbecco-Vogt Eagle's (DMEM) and Ham's F-12 media (3:1 mixture) containing fetal calf serum (10%), insulin (5 μg/ml), transferrin (5 μg/ml), adenine (0.18 mM), hydrocortisone (0.4 μg/ml), cholera toxin (0.1 nM), triiodothyronine (2 nM), epidermal growth factor (EGF, 10 ng/ml), glutamine (4 mM), penicillin-streptomycin (50 IU/ml). Subconfluent primary cultures were passaged in secondary cultures as described (De Luca *et al.*, 1988b). 3T3-J2 cells were cultured in DMEM containing calf serum (10%), glutamine (4 mM), and penicillin-streptomycin (50 IU/ml).

Hybridoma cells (αD11) were subcloned and cultured in DMEM containing 10% fetal calf serum, glutamine (4 mM), and penicillin-streptomycin (50 IU/ml). Clones used in these experiments were selected by their capacity of inhibiting NGF induced neurites in chick embryo dorsal root ganglia (Di Marco *et al.*, 1991). Supernatants were prepared by conditioning hybridoma cell medium for 72 h in DMEM, glutamine (4 mM), and penicillin-streptomycin (50 IU/ml) and tested on chick embryo dorsal root ganglia before use.

Binding Assays—(3-[^{125}I]Iodotyrosyl)-murine-2.5S-NGF (1560 Ci/mmol) was purchased from Amersham International, Bucks, United Kingdom. Purified murine 2.5S βNGF was a gift from Dr. Delio Mercanti, Consiglio Nazionale delle Ricerche, Istituto di Neurobiologia, Rome, Italy. Purified murine 2.5S βNGF was also purchased from Sigma. Human recombinant NGF was purchased from Austral Biologicals (San Ramon, CA).

For equilibrium binding studies, two ranges of ^{125}I-NGF concentrations (4–100 pM and 1–15 nM) were used to explore high and low affinity sites. Subconfluent primary or secondary cultures of normal human keratinocytes were trypsinized as described above. Isolated keratinocytes were plated (1×10^5 cells/well) in 24-well plates in keratinocyte growth medium, in the absence of fetal calf serum and feeder-layer. After 16 h, cells were washed twice in binding buffer (ice-cold DMEM containing 0.1% bovine serum albumin). ^{125}I-NGF solutions were prepared in binding buffer and added (150 μl, final volume) to keratinocyte cultures. After 2 h at 4 °C under gentle agitation, cells were rinsed five times in binding buffer and solubilized in 0.5 ml of 1 N NaOH for 4 h at 37 °C. The solutions were then γ-counted (Gamma 5500, Beckman Instruments, Inc.). Nonspecific binding was measured in the presence of 100 μg/ml nonradioactive NGF. Scatchard analysis was performed following the EBDA/LIGAND program (McPherson, 1985). For nonequilibrium binding studies (aimed to specifically detect high affinity binding sites), cells were incubated in the presence of ^{125}I-NGF (4–100 pM) for 5 min at 4 °C under gentle agitation. Nonspecific binding was measured in the presence of 100 μg/ml nonradioactive NGF. Samples were then processed as above.

RNA Blotting—The *Eco*RI 0.8-kilobase fragment of human $p75^{NGFr}$ cDNA was a gift from Dr. Moses Chao, Cornell University Medical College, New York. The *Bam*HI-*Eco*RI fragment of the plasmid pDM-17, corresponding to the entire cytoplasmic portion of the human $p140^{NGFr}$/*trkA* protooncogene ($p140^{trkA}$), was a gift from Dr. Mariano Barbacid, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ.

Total cellular RNA was isolated by lysing cultured keratinocytes in primary or secondary culture with 4.2 M guanidine thiocyanate followed by cesium chloride gradient centrifugation as described (Di Marco *et al.*, 1991). Poly(A^+) RNA was prepared by an oligo(dT)-cellulose column (Pharmacia) chromatography. Two mg of total cellular RNA, dissolved in 1 ml of 20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 0.1% SDS, were denatured in boiling water for 2 min followed by addition of an equal volume of 1 M sodium chloride. The sample was then applied to the column and the poly(A^-) fraction was eluted with 50 ml of 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5 M sodium chloride, 0.1% SDS. The poly(A^+) fraction was eluted using 2 ml of H_2O kept at 37 °C and precipited with 300 mM sodium acetate, pH 5, and 2.5 volumes of absolute ethanol. Twenty μg of total RNA or 5–10 μg of poly(A^+) RNA were size fractionated through a 1% agarose gel and transferred to a nylon membrane (GeneScreen Plus, Du Pont-New England Nuclear) in 1.5 M sodium chloride, 0.15 M sodium citrate. After immobilization by shortwave UV exposure, blots were prehybridized at 42 °C for 3 h in 50% deionized formamide, 0.75 M sodium chloride, 25 mM sodium phosphate, 5 mM EDTA, 0.2 mg/ml salmon sperm DNA, 0.5% SDS. Hybridization buffer was identical to the above buffer with the addition of the indicated (see "Results") ^{32}P-labeled probes (2×10^6 cpm/ml) and 10% dextran sulfate. A final wash was done at 65 °C for 30 min in 15 mM sodium chloride, 1 mM sodium phosphate, 1 mM EDTA, 0.1% SDS. All filters were autoradiographed on x-ray films (hyperfilm-MP Amersham) with intensifying screens at −70 °C.

Polymerase Chain Reaction (PCR)—Reagents were purchased from Perkin-Elmer. PCR was performed using two distinct oligonucleotide primers, *i.e.* 5′-GTCTTCCTTGCTGAGTGC-3′ (3α) and 5′-CTTGGCATCGGGTCCATG-3′ (4α). One μg of total RNA obtained from K562 cells and normal human keratinocytes was reverse transcribed using 2.5 μM random hexamers or 0.75 μM 4α downstream from oligonucleotide in 5 mM MgCl, 5 mM KCl, 10 mM Tris-HCl, pH 8.3, dNTPs, 1 mM each, 1 unit of RNase inhibitor, and 2.5 units of reverse transcriptase in a final volume of 20 μl. The reverse transcription reaction was performed in a Perkin-Elmer Cetus DNA thermal Cycler at 42 °C for 15 min, 99 °C for 5 min, and then soaked at 4 °C for 5 min. For the PCR reaction, downstream and upstream primers were used at a concentration of 0.15 mM in the presence of 2 mM MgCl, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, and 2.5 units of AmpliTag DNA polymerase. The downstream primer (4α) was omitted in the PCR reaction when used in the reverse transcription reaction. The mixture was subjected to PCR amplification in the Perkin-Elmer thermal Cycler apparatus for 35 cycles. The temperatures were: 2 min at 95 °C and 1 min at 60 °C for 35 cycles, 7 min at 60 °C for 1 cycle. Ethidium bromide-stained 1.5% agarose gel was used to visualize PCR fragments.

Immunocytochemistry—Anti-human $p75^{NGFr}$ mAb (ME20.4) was purchased from Amersham. Rabbit polyclonal antibodies elicited against a synthetic peptide corresponding to the 14 carboxyl-terminal residues of the *trk* protooncogene products (Martin-Zanca *et al.*, 1989) were a gift from Dr. Mariano Barbacid, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ.

Keratinocytes from subconfluent primary cultures (1×10^4 cells/cm^2) were plated onto 24-well Costar plates containing 1.4-cm^2 round glass coverslips previously coated with feeder-layer and cultured as described above. When keratinocyte colonies were evident in phase-contrast microscopy (3–5 days after plating), colonies were treated for immunofluorescence. Coverslip-attached keratinocyte colonies were fixed in 3% formaldehyde (from paraformaldehyde) in phosphate-buffered saline, pH 7.6, containing 2% sucrose for 5–15 min at room temperature. After rinsing in phosphate-buffered saline, cells were permeabilized by soaking coverslips for 3–5 min at 0 °C in HEPES-Triton X-100 buffer (20 mM HEPES, pH 7.4, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, and 0.5% Triton X-100).

Indirect immunofluorescence on cell cultures was performed as previously reported (De Luca *et al.*, 1990b; Marchisio *et al.*, 1991). Briefly, the primary antibody (10–30 μg/ml) was layered on fixed and permeabilized cells and incubated in a humid chamber for 30 min.

[2] M. De Luca and R. Cancedda, unpublished data.

[3] Dr. A. Cattaneo, personal communication.

After rinsing in PBS, 0.2% bovine serum albumin, coverslips were incubated in the appropriate rhodamine-tagged secondary antibody (Dakopatts, Copenhagen, Denmark) for 30 min at 37 °C in the presence of 2 μg/ml fluorescein-labeled phalloidin (Sigma). Coverslips were mounted in Mowiol (Hoechst AG, Frankfurt/Main, Germany) and observed in a Zeiss Axiophot photomicroscope equipped with epifluorescence lamp and usually with planapochromatic oil immersion lenses. Fluorescence images were recorded on Kodak T-Max 400 films exposed at 1000 ISO and developed in T-Max Developer for 10 min at 20 °C.

For immunoperoxidase staining, punch biopsies from normal skin of healthy volunteers or from the leading edge of healing wounds of otherwise healthy volunteers, were snap frozen in liquid nitrogen and sectioned (4–6 μm) in a cryostat; sections were air dried and fixed in a mixture of chloroform/acetone (1:1) at 4 °C for 10 min. Immunoperoxidase staining was performed following a procedure previously described (Pellegrini *et al.*, 1992).

Growth Assay—The natural alkaloid K252a was purchased from Calbiochem. EGF was from Austral Biologicals. αD11 mAb was used as nonconcentrated conditioned medium of subcloned hybridoma cells. ME20.4 mAbs were purified immunoglobulins.

Assays were performed in defined medium. Keratinocytes from subconfluent primary cultures were plated in basal medium (keratinocyte growth medium depleted of fetal calf serum, EGF, cholera toxin, and containing 10 ng/ml insulin) onto 96-well plates (1×10^4 cells/well). Indicated factors (see "Results") were added 12 h after plating. After an additional 12 h, 5 μCi/ml [^{3}H]thymidine (Amersham, 90 Ci/mmol) were added. Cells were trypsinized 12 h later, collected with the aid of a cell harvester, and incorporated radioactivity was β counted. The NGF-dependent autocrine keratinocyte proliferation was also evaluated by plating keratinocytes (4×10^5 cells/well) onto 6 multiwell plates (precoated for 24 h with hybridoma supernatants) in basal medium, in the presence of indicated factors (see "Results"). After 4 days, cells were stained with rhodamine B (De Luca *et al.*, 1988a, 1988b) and photographed, or trypsinized and counted.

RESULTS

NGF Binding Assays—Saturation binding assays were performed in normal human keratinocytes as described under "Experimental Procedures." As shown in Fig. 1, two ranges of ^{125}I-NGF concentration (4–100 pM and 1–15 nM) were used to explore high (*upper panels*) and low (*lower panels*) affinity binding sites. Scatchard analysis (*right panels*) pointed to two classes of binding sites with a dissociation constant (K_d) of 1.086 (±0.108) × 10^{-11} (*upper panel*) and 8.698 (±0.693) × 10^{-9} (*lower panel*), respectively. Calculation of the B_{max} indicated the presence of 934 ± 38 high affinity and 223,200 ± 12,173 low affinity receptors per cell. Nonequilibrium binding conditions are particularly suitable for demonstrating high affinity binding sites (Eveleth and Bradshaw, 1992). As shown in Fig. 2, binding assays performed in nonequilibrium conditions (reaction stopped 5 min after ^{125}I-NGF addition), emphasized the presence of high affinity receptors on normal human keratinocytes. Values obtained from the Scatchard analysis of nonequilibrium binding assays cannot be considered accurate estimation of the binding constants of the NGF sites (Green *et al.*, 1986; Eveleth and Bradshaw, 1992). Comparable data, both in terms of dissociation constants and binding sites, were obtained from three different keratinocyte strains (not shown).

NGF Receptors in Normal Human Keratinocytes—The expression of p75^{NGFr} and p140^{trkA} was investigated in skin sections and in cultured normal human keratinocytes. Equal amounts of poly(A$^+$) RNA were isolated from keratinocytes either in the exponential phase of growth or at different time after confluence (reconstitution of multilayered sheets of cohesive epithelium), and hybridized with the human specific p75^{NGFr} probe. As shown in Fig. 3, a 3.8-kilobase transcript, consistent in size with p75^{NGFr} mRNA detected in the human melanoma cell line Hs-294T (*lane 1*), was present in growing keratinocyte colonies (*lane 2*) and in reconstituted epithelium

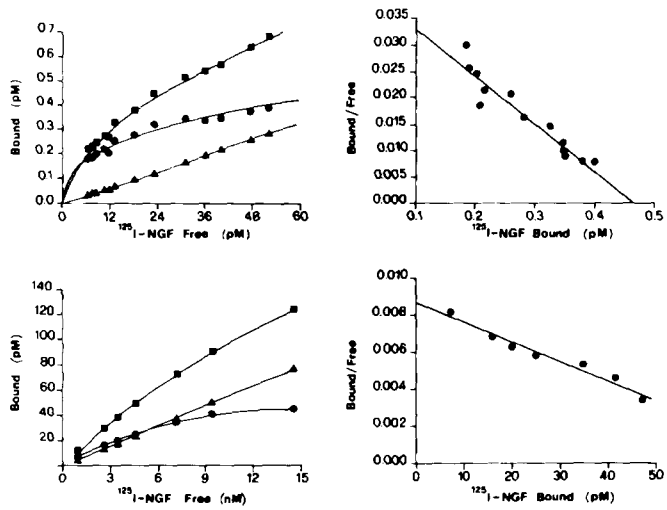

FIG. 1. **Saturation binding assay.** Saturation binding assays were performed on normal human keratinocytes (strain 200) obtained from primary or secondary cultures as described under "Experimental Procedures." Two ranges of ^{125}I-NGF concentration (4–100 pM and 1–15 nM) were used to explore high (*upper panels*) and low (*lower panels*) affinity binding sites. *Left panels*, total (*squares*), nonspecific (*triangles*), and specific (*close circles*) binding curves are shown. Scatchard analysis (*right panels*) pointed to two classes of binding sites with a dissociation constant (K_d) of 1.086 (±0.108) × 10^{-11} (*upper panel*) and 8.698 (±0.693) × 10^{-9} (*lower panel*), respectively. Calculation of the B_{max} indicated the presence of 934 ± 38 high affinity and 223,200 ± 12,173 low affinity receptors per cell. Each point was averaged from triplicates. Variation among triplicates was <6%. Comparable data, both in terms of dissociation constants and binding sites, were obtained from three different keratinocyte strains.

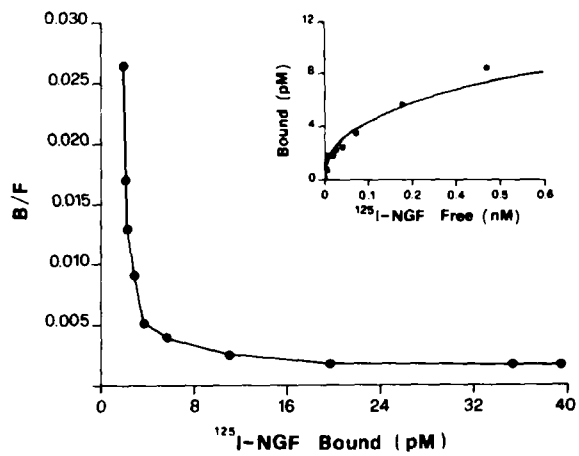

FIG. 2. **Saturation binding assay.** Saturation binding assays in nonequilibrium conditions were performed on normal human keratinocytes (strain 200) as described under "Experimental Procedures." Nonequilibrium binding studies specifically detect high affinity binding sites. Each point was averaged from triplicates. Variation among triplicates was <3%. Comparable data were obtained from three different keratinocyte strains.

1 and 6 days after confluence (*lanes 3 and 4*, respectively). The level of expression of p75^{NGFr} mRNA strongly decreased when a confluent differentiated and fully stratified epithelium was obtained (*lanes 2–4*). The expression of p75^{NGFr} was also determined by immunocytochemistry using the mAb ME20.4 (Ross *et al.*, 1984). Fig. 4 (*panel b*) shows expression of p75^{NGFr} in a colony of growing keratinocytes plated on lethally irradiated 3T3-J2 cells. Positivity was limited to keratinocytes (compare the F-actin staining in *panel a*) and was evenly diffused over their surface, with no obvious receptor cluster-

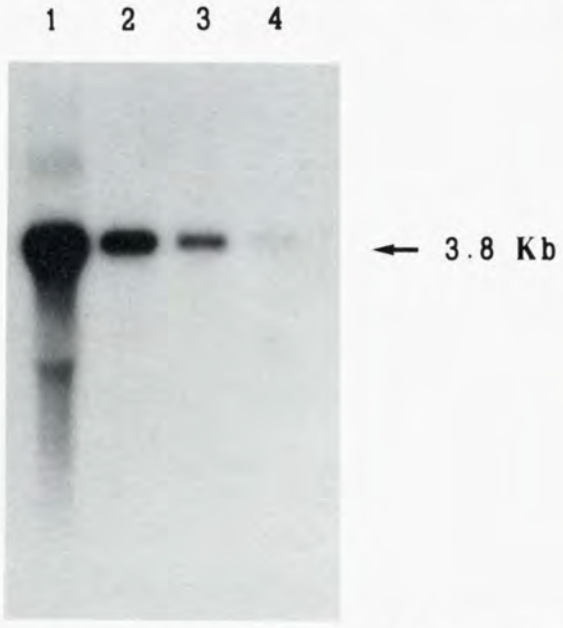

FIG. 3. **Northern blot analysis.** Five μg of poly(A$^+$) RNA from human melanoma cells (Hs 294T, *lane 1*), growing keratinocyte colonies (*lane 2*), and reconstituted epithelium 1 and 6 days after confluence (*lanes 3* and *4*, respectively) were hybridized with the human specific p75^{NGFr} cDNA probe. Equal amounts of poly(A$^+$) RNA were loaded in each lane as assessed by ethidium bromide staining and β-actin hybridization (not shown). *B/F*, bound/free.

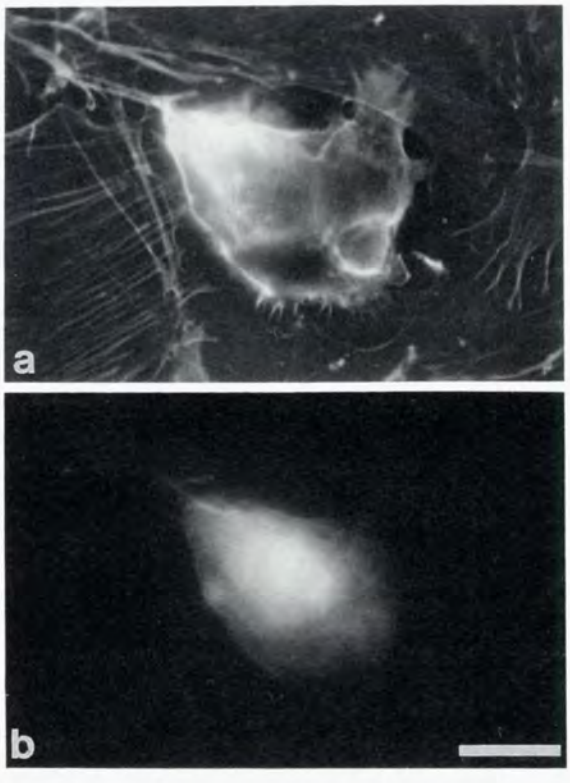

FIG. 4. **Immunofluorescence.** Immunofluorescence detection of p75^{NGFr} in a small colony of human keratinocytes growing on a feeder-layer of lethally irradiated 3T3-J2 fibroblasts. The colony was stained for F-actin with fluorescein-tagged phalloidin (*panel a*) and simultaneously for p75^{NGFr} using the mAb ME20.4 (*panel b*). The positivity for p75^{NGFr} was limited to keratinocytes and evenly diffused over their surface. No evidence for the formation of receptor aggregates was obtained. *Bar* denotes 5 μm. *kb*, kilobase.

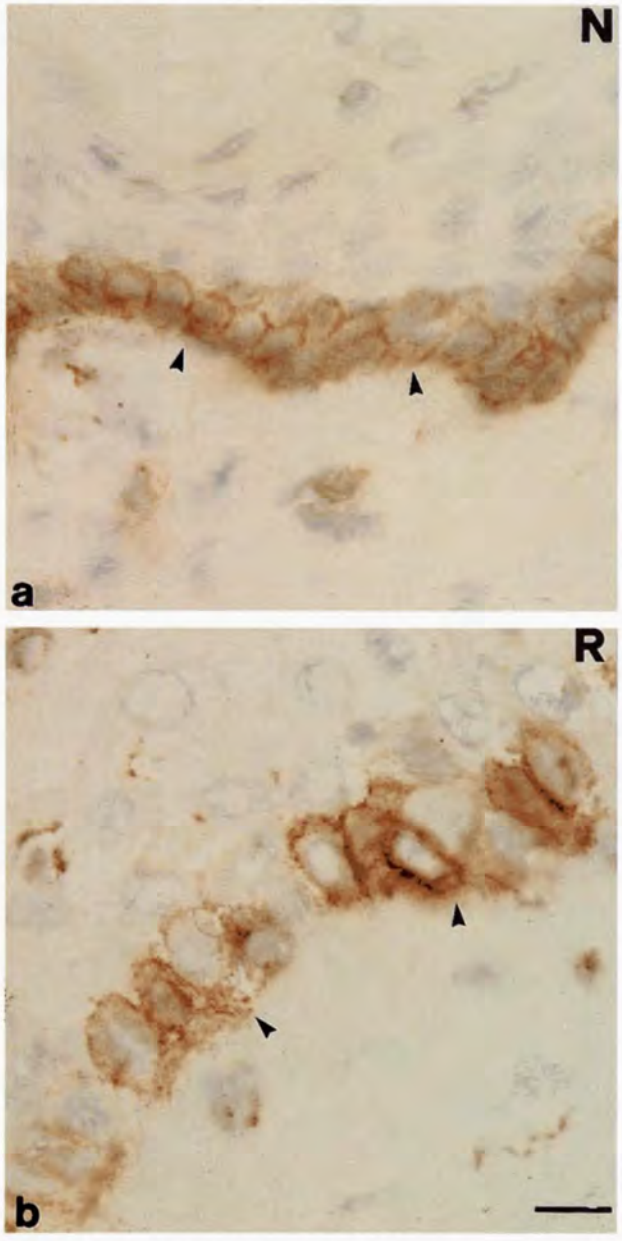

FIG. 5. **Immunoperoxidase staining.** Immunoperoxidase staining of a normal (*N*) and regenerating (*R*) skin section. In the skin sections of normal donors (*panel a*), positivity to p75^{NGFr}, detected with the mAb ME20.4, was evenly distributed over the surface of basal keratinocytes and absent from suprabasal cells. In sections of small punch biopsies taken at the regenerating boundary of burnt human skin (*panel b*), p75^{NGFr} positivity was also detected at the surface of rather swollen basal cells; its positivity, however, was less homogeneous than in normal epidermis with strongly positive cells alternating with cells provided with weaker surface positivity. *Arrowheads* indicate the position of the basement membrane. Some punctate irregular positivity to p75^{NGFr} was detected both in the underlying dermis and within epidermal suprabasal layers, presumably associated to thin nerve fibers. *Bar* denotes 3 μm.

ing. In skin sections of normal donors (Fig. 5, *panel a*), p75^{NGFr} was evenly distributed over the surface of basal keratinocytes and absent from suprabasal terminally differentiated cells (see also Bothwell (1991a), Wheeler and Bothwell (1992), and Fantini and Johansson (1992)). Interestingly, the expression of biologically active NGF is limited to basal keratinocytes and decreases after full epithelial maturation as well (Di Marco *et al.*, 1991), suggesting a role for NGF during the exponential phase of keratinocyte growth. In sections of small

punch biopsies taken at the regenerating boundary of burnt human skin (Fig. 5, *panel b*), $p75^{NGFr}$ was also detected at the surface of rather swollen basal cells; its positivity, however, was less homogeneous than in normal epidermis, with strongly positive cells alternating with cells showing weaker surface staining.

The high affinity binding properties of NGF have been associated with the expression of the protooncogene *trkA* (Klein *et al.*, 1991; Kaplan *et al.*, 1991). To determine the level of *trkA* expression in keratinocytes, 10 μg of poly(A^+) RNA were isolated from keratinocytes in primary or secondary culture in their exponential phase of growth, and hybridized with a human $p140^{trkA}$ cDNA probe. The 3.2-kilobase *trkA* transcript, present in human K562 leukemia cells (Fig. 6, *lane 1, arrows*), was undetectable in keratinocytes (Fig. 6, *lanes 3–6*). Instead, the probe gave multiple but weak bands ranging from 1.8 (*lower arrowhead*) to 3.9 kilobases (*upper arrowhead*). The expression of $p140^{trkA}$ was also investigated by PCR (Fig. 7). K562 (*lanes 2* and *3*) and keratinocyte (*lanes 4 and 5*) mRNA were reverse transcribed to cDNA using either random hexamers (*lanes 3* and *5*) or 4α oligonucleotide primer (*lanes 2* and *4*) and subsequently used as template in PCR with oligonucleotide 3α and 4α as primers. The predicted band of 258 base pairs was present in K562 mRNA (*lanes 2* and *3, arrow*), but was absent in normal human keratinocytes (*lanes 4* and *5*). One ng of PDM-17 plasmid DNA gave the expected 258-base pair fragment after PCR amplification using the 3α and 4α oligonucleotide primers (not shown). Futhermore, antisera against the 14 carboxyl-terminal residues of $p140^{trkA}$ did not immunoprecipitate the receptor from metabolically labeled keratinocytes, and did not stain normal human skin section or cultured keratinocytes (not shown), further demonstrating the absence of *trkA* in human epidermal cells.

Given the apparent absence of *trkA* in keratinocytes, the presence of specific high affinity NGF binding sites, and the sequence homology between the different members of the *trk* family, we therefore decided to further analyze the multiple bands recognized (in low stringency conditions) by the specific *trkA* probe. By screening normal human keratinocyte cDNA libraries with the $p140^{trkA}$ probe, we have identified, by sequence comparison, a new member of the *trk* protooncogene

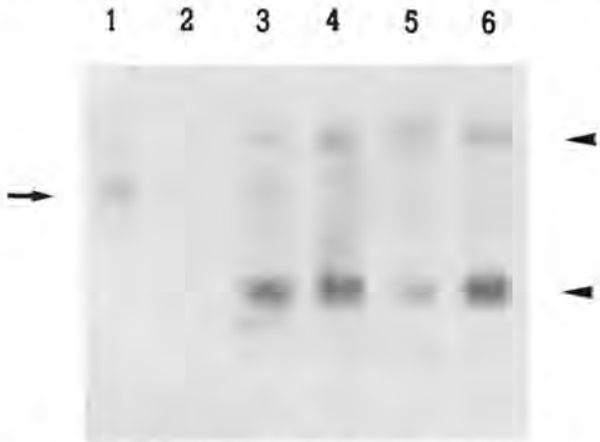

FIG. 6. **Northern blot analysis.** Twenty μg of total RNA from K562 cells (*lane 1*) and 10 μg of poly(A^+) RNA from 3T3-J2 cells (*lane 2*) and from four different keratinocyte strains (*lanes 3–6*) were hybridized (in low stringency conditions) with the human $p140^{trkA}$ cDNA probe. *Arrow* indicates the 3.2-kilobase *trkA* transcript. *Arrowheads* indicates the 1.8- and 3.9-kilobase transcripts detected in keratinocytes. Equal amounts of poly(A^+) RNA were loaded *lanes 2–6* as assessed by ethidium bromide staining and β-actin hybridization (not shown). The film has been exposed for 15 days.

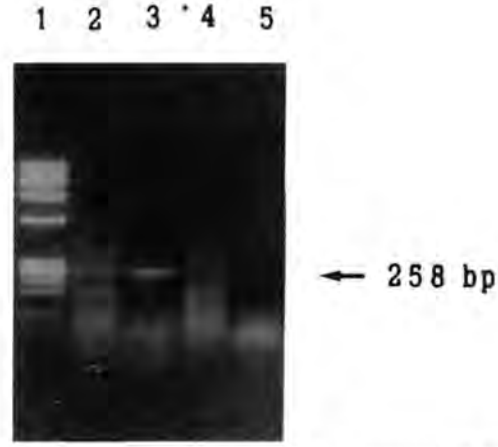

FIG. 7. **Polymerase chain reaction.** Expression of $p140^{trkA}$ by PCR. Ethidium bromide-stained PCR products of reverse transcribed K562 cell line mRNA (*lanes 2* and *3*) and normal human keratinocytes mRNA (*lanes 4* and *5*). The DNA amplification has been performed using 3α and 4α oligonucleotide primers (see "Experimental Procedures"). The predicted band of 258 base pairs (*bp*) was present in K562 mRNA transcribed with random hexamers (*lane 3, arrow*) and 4α primer (*lane 2, arrow*), but was absent in keratinocytes mRNA transcribed with random hexamers (*lane 5*) and 4α (*lane 4*) oligonucleotide primer. Molecular markers are in *lane 1*.

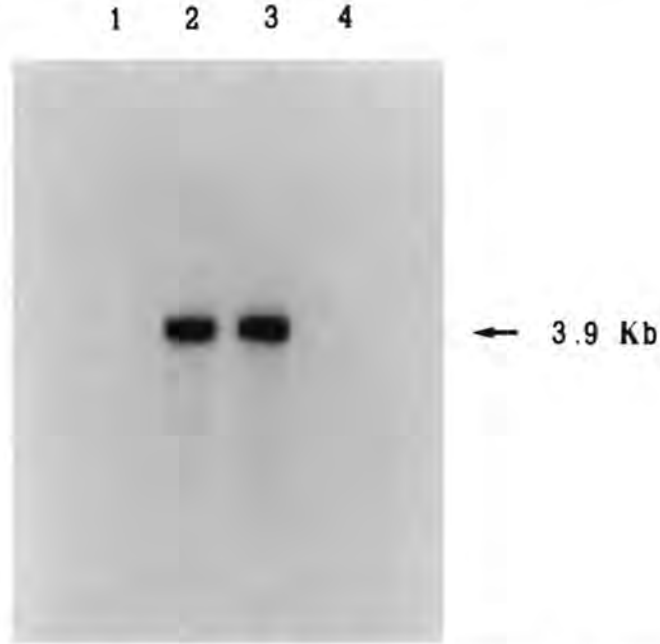

FIG. 8. **Northern blot analysis.** Five μg of poly(A^+) RNA from K562 cells (*lane 1*), growing keratinocyte colonies from two different strains (*lanes 2* and *3*), and 3T3-J2 cells (*lane 4*), were hybridized with a cDNA probe corresponding to the entire cytoplasmic domain of *trkE*. Equal amounts of poly(A^+) RNA were loaded in each lane as assessed by ethidium bromide staining and β-actin hybridization (not shown). The film has been exposed for 16 h.

family (which we have termed *trkE*), which is likely to provide the high affinity NGF binding in keratinocytes.[4] As shown in Fig. 8, when 5 μg of poly(A^+) RNA obtained from growing keratinocyte colonies, were hybridized (in high stringency conditions) with a ^{32}P-labeled cDNA probe corresponding to the entire cytoplasmic domain of the new *trk* (*trkE*), a mRNA of 3.9 kilobases was shown to be expressed by normal human

[4] E. Di Marco, N. Cutuli, L. Guerra, R. Cancedda, and M. De Luca, submitted for publication.

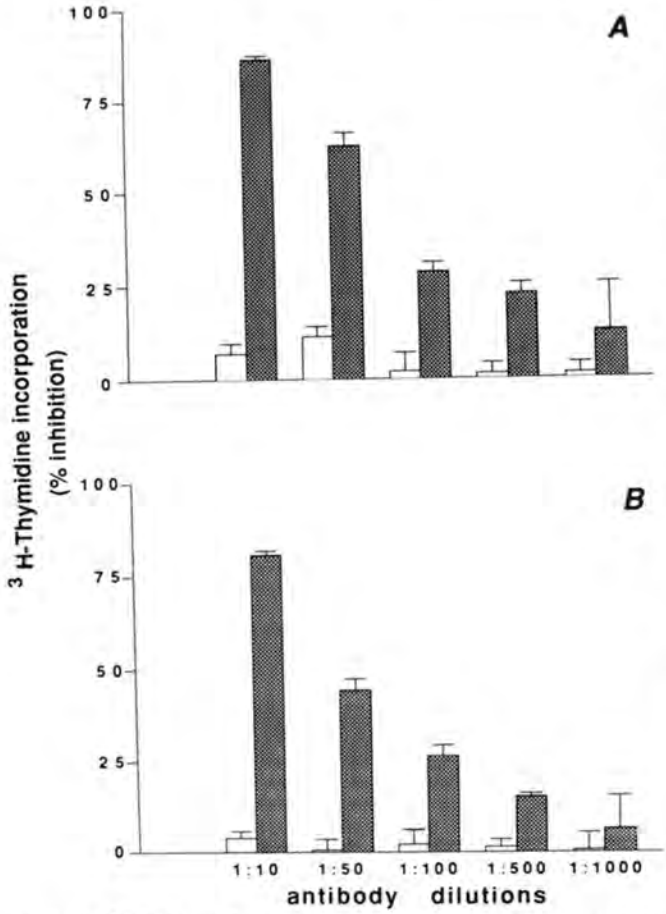

FIG. 9. **[^{3}H]Thymidine incorporation assay.** Dose-dependent inhibition of [^{3}H]thymidine incorporation obtained with mAbs to NGF (αD11) (*close bars*). Keratinocytes obtained from subconfluent primary cultures were plated in basal medium onto 96-well plates (1×10^4 cells/well). Assays were performed as described under "Experimental Procedures" in the absence of exogeneously added NGF, in the absence (*panel A*) or in the presence (*panel B*) of the simultaneous addition of other growth stimuli such as EGF, cholera toxin, and insulin. Unrelated mAbs were ineffective (*open bars*). Each point is the average of triplicates from three experiments. These data were reproduced in four different keratinocyte strains, and using three different mAbs as negative controls.

keratinocytes (*lanes 2* and *3*) but not by K562 cells (*lane 1*) or 3T3-J2 cells (*lanes 4*). Its size was identical to the 3.9-kilobase transcript detected in low stringency conditions by the p140^{trkA} probe (Fig. 6, *upper arrowhead*).

NGF-dependent Autocrine Stimulation of Keratinocyte Growth—Normal human keratinocytes control their growth in an autocrine fashion (Coffey *et al.*, 1987; Eisinger *et al.*, 1988; Cook *et al.*, 1991a, 1991b).[5] However, the underlying molecular mechanisms of these autocrine loops are poorly characterized. The potential role for NGF in stimulating keratinocyte growth in an autocrine fashion was thus investigated. Fig. 9 shows a dose-dependent inhibition of [^{3}H] thymidine incorporation obtained with mAbs to NGF (αD11) (*close bars*). Assays were performed in defined medium, in the absence of exogeneously added NGF, in the absence (*panel A*) or in the presence (*panel B*) of the simultaneous addition of other growth stimuli such as EGF, cholera toxin, and high insulin concentrations (5 μg/ml, able to stimulate the type-1 insulin-like growth factor receptor; Barreca *et al.* (1992)). Interestingly, in both conditions, there was a strong inhibition of [^{3}H]thymidine incorporation that reached approximately 80% with a 1:10 dilution of the hybridoma supernatant. Unrelated mAbs, whose hybridoma cells were conditioned in the same way as to αD11, were ineffective (*open bars*). These data were reproduced at least three times in four different keratinocyte strains, and using three different mAbs as negative controls. Growth inhibition was also evaluated by plating keratinocytes (4×10^5 cells/well) onto 6 multiwell plates in the same conditions as in Fig. 9 (*panel B*) in the presence or in the absence of αD11 (1:10 dilution). As shown in Fig. 10 (*panel B*), αD11 gave an almost complete inhibition of keratinocyte growth compared to an unrelated antibody (*panel A*). This experiment was repeated with four different keratinocyte strains; cells were counted after 4 days and the αD11-dependent growth inhibition ranged between 63 and 85% (not shown).

The growth-regulated synthesis and secretion of biologically active NGF from basal keratinocytes (Di Marco *et al.*, 1991), the expression of high and low affinity NGF binding sites (Fig. 1), the potent inhibition of [^{3}H]thymidine incorporation and cell number by anti-NGF mAbs, in defined-medium and in the absence of exogeneously added NGF (Figs.

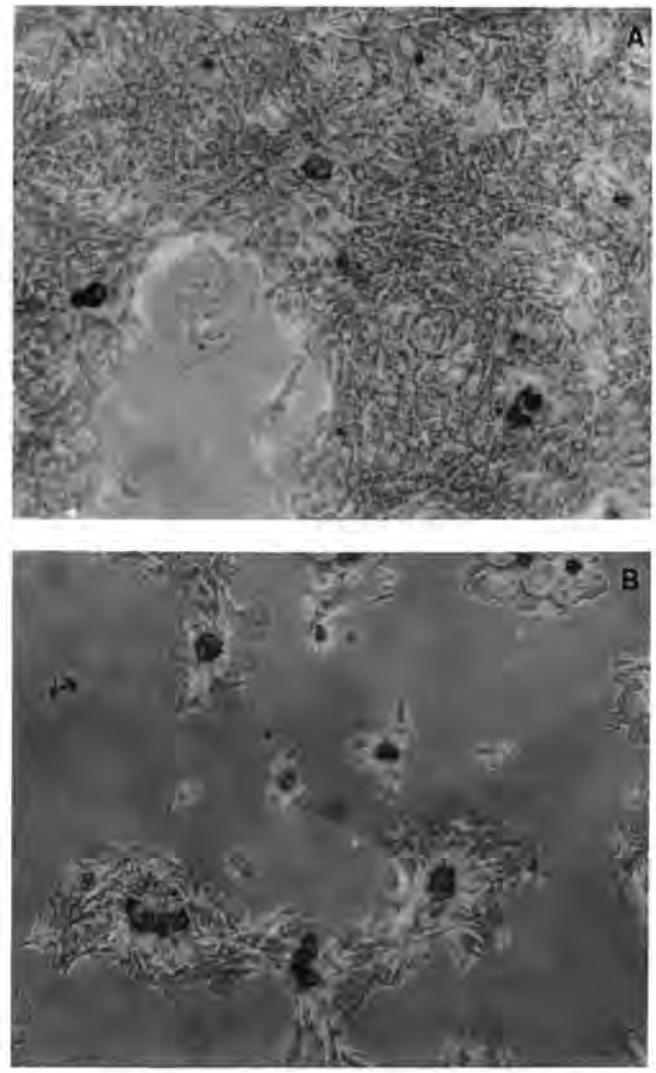

FIG. 10. **Growth assay.** Keratinocytes obtained from subconfluent primary cultures were plated (4×10^5 cells/well) onto 6 multiwell plates and assay was performed in the same conditions as in Fig. 9*B* in the presence of unrelated mAbs (*panel A*), or aD11 (1:10 dilution, *panel B*). Cells were fixed 4 days later, stained with rhodamine B, and photographed.

[5] M. De Luca, unpublished data.

9 and 10), demonstrate the presence of a novel autocrine loop in normal human keratinocytes. The strong inhibition of αD11 in the presence of other growth stimuli suggests that this autocrine loop is crucial for keratinocyte growth. However, this complicates the demonstration of a direct effect of exogenous NGF on keratinocyte growth. Cultured keratinocytes form colonies, each colony being the progeny of a single basal keratinocyte (Rheinwald and Green, 1975). If exogenous NGF is added to cells (in the absence of feeder-layer, and in defined medium) only a few hours after plating, when the concentration of endogenous NGF is presumably still very low (Fig. 11, *close bars*), a significative increase in [^{3}H]thymidine incorporation is observed compared to the control (Fig. 11, *open bars*). When keratinocyte colonies become larger, the exogenous NGF effect is no longer visible (not shown).

Roles of p75^{NGFr} and trk—Several reports indicate that the stimulation of the p140^{trkA} in cell lines responsive to NGF is necessary and/or sufficient to elicit a biological response (Cordon-Cardo *et al.*, 1991; Loeb *et al.*, 1991; Ibanez *et al.*, 1992). However, other reports highlight the crucial role of the association of p140^{trkA} and p75^{NGFr} in regulating biological activities of NGF (Hempstead *et al.*, 1990, 1991; Lee *et al.*, 1992; for recent reviews, see Meakin and Shooter (1992) and Barker and Murphy (1992)). In intact cells, the natural alkaloid K252a (at nanomolar concentrations) inhibits the action of NGF and other neurotrophins without affecting other growth factors acting through stimulation of their tyrosine kinase receptors (Berg *et al.*, 1992; Tapley *et al.*, 1992; Knusel and Hefti, 1992). K252a acts by selectively inhibiting the tyrosine kinase domain of the *trk* family of protooncogenes (Berg *et al.*, 1992; Tapley *et al.*, 1992). ME20.4 is a mAb which recognizes human (but not rat) p75^{NGFr} and inhibits NGF binding to the low affinity receptor (Ross *et al.*, 1984), also in normal human keratinocytes (not shown).

We investigated the effect of K252a and ME20.4 on the NGF-mediated autocrine growth stimulation of normal human keratinocytes. Assays were performed in the same conditions as in Fig. 9 (*panel B*). As shown in Fig. 12 (*closed circles*) there was a dose-dependent inhibition of [^{3}H]thymidine incorporation by nanomolar concentrations of K252a. The alkaloid gave a 50% inhibition at a concentration as low as 5 nM. Matching the effect of the anti-NGF mAb (see Fig. 9), K252a gave a maximal inhibition of the autocrine loop of approximately 80%. Anti-p75^{NGFr} mAb (ME20.4) (*open squares*) did not inhibit [^{3}H]thymidine incorporation even at a concentration of 500 ng/ml. These data indicate that the *trk* tyrosine kinase stimulation in these cells is necessary and sufficient to induce the NGF-dependent autocrine loop. The EGF-dependent enlargement of colonies and thinning of their interior (Barrandon and Green, 1987b) and the insulin-like growth factor-1 dependent stimulation of keratinocyte growth (Barreca *et al.*, 1992), were not affected by the simultaneous addition of EGF or insulin-like growth factor-1 and K252a (50 nM) (not shown).

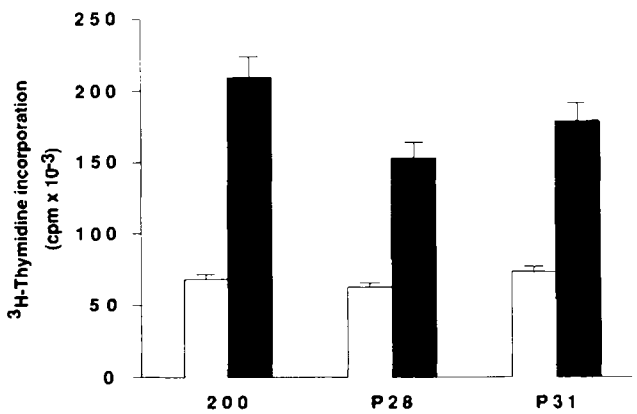

FIG. 11. **[^{3}H]Thymidine incorporation assay.** Keratinocytes obtained from subconfluent primary cultures (strains 200, P28, and P31) were plated in basal medium onto 96-well plates (1 × 10^4 cells/well). [^{3}H]Thymidine incorporation was performed as described under "Experimental Procedures" in the presence of EGF (10 ng/ml) (*open bars*) or EGF + NGF (200 ng/ml) (*closed bars*).

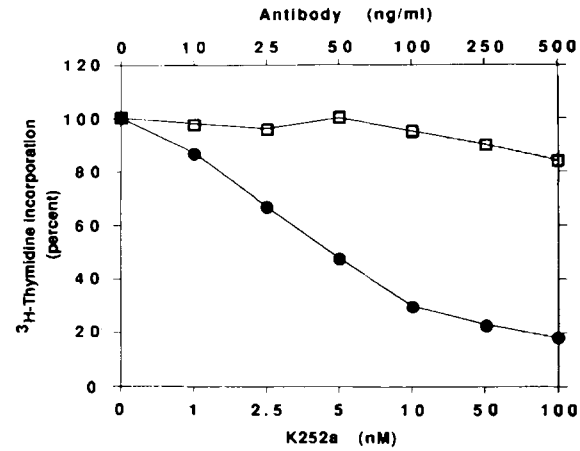

FIG. 12. **[^{3}H]Thymidine incorporation assay.** Keratinocytes obtained from subconfluent primary cultures were plated onto 96-well plates (1 × 10^4 cells/well) and assay was performed in the same conditions as in Fig. 8*B*. Dose-dependent inhibition of [^{3}H]thymidine incorporation by K252a (*close circles*). Increasing concentrations (10–500 ng/ml) of anti-p75^{NGFr} mAbs (ME20.4) (*open squares*) were ineffective.

DISCUSSION

Epidermis, the outermost layer of skin, consists of stratified squamous epithelium accounting for only one-fortieth of skin thickness. Yet, its function is essential in maintaining the stability of the interior milieu of the entire organism and in protecting the body against environmental hazards. Epidermis survives physical and chemical traumas by a continuous self-renewal. To do this, small progenitor cells (Barrandon and Green, 1985, 1987a), forming the innermost epidermal basal layer, undergo mitosis to replace terminally differentiated cornified cells (see Watt (1989), for review). Within a growing keratinocyte colony, migrating and proliferating cells are located at the narrow peripheral rim of the colony, whereas internal cells are destined for terminal differentiation (Barrandon and Green, 1987b). In particular situations, such as healing of large wounds, basal keratinocytes have to strongly increase their migration and growth rates.

The regulation of normal human keratinocyte growth is a complex phenomenon involving both nutritive support from dermal blood vessel and a network of paracrine and autocrine loops which act mainly within the local skin environment. Fibroblasts are a source of several polypeptides binding to receptors endowed with tyrosine kinase activity and stimulating keratinocyte functions. For example, hepatocyte growth factor (scatter factor) is a potent modulator of epithelial cell motility (Stoker *et al.*, 1987; Matsumoto *et al.*, 1991), while fibroblast-derived keratinocyte growth factor and insulin-like growth factor-1 stimulate keratinocyte proliferation (Finch *et al.*, 1989; Barreca *et al.*, 1992). Indeed, optimal keratinocyte clonal growth *in vitro* is obtained when cells are plated on a feeder-layer of lethally irradiated fibroblasts (Rheinwald and Green, 1975) in the presence of factors which cooperate in

sustaining their growth (see Green (1980)). However, when cultured human keratinocytes reach a critical density, they become capable of autonomous growth (Cook *et al.*, 1991a, 1991b).[5] These autocrine-paracrine loops have been related to the synthesis and secretion of transforming growth factor α (which modulates keratinocyte migration and consequent growth) (Barrandon and Green, 1987a; Coffey *et al.*, 1987), basic fibroblast growth factor (Halaban *et al.*, 1988; Shipley *et al.*, 1989), and amphiregulin (Cook *et al.*, 1991b), which, however, acts through the EGF/transforming growth factor-α receptor (Johnson *et al.*, 1993).

In this report we demonstrate that keratinocyte-derived NGF (Di Marco *et al.*, 1991), in addition to its fundamental role in regulating migration and dendritic arborization of surrounding melanocytes (Yaar *et al.*, 1991; De Luca *et al.*, 1993), plays a key role in the autocrine keratinocyte growth stimulation. This has been proven by (i) presence of high affinity receptors on keratinocyte plasma-membrane, (ii) direct effect of NGF on [^{3}H]thymidine incorporation, (iii) potent inhibition of autocrine keratinocyte growth by αD11 mAbs (able to inhibit human NGF biological activity), and by a *trk*-specific inhibitor, the natural alkaloid K252a. It is worth noting that both αD11 and K252a gave an 80% inhibition of keratinocyte growth in defined medium, and in the presence of other growth stimuli such as EGF, cholera toxin, and a high concentration of insulin, suggesting that the NGF-dependent autocrine loop is crucial for the optimal keratinocyte growth, and that the absence of NGF make other growth stimuli unfit for eliciting their activity. Keratinocyte-derived NGF is also responsible for the widespread sprouting of sensory nerve fibers observed during the healing of wounds.[2] This further contributes to keratinocyte proliferation by the paracrine secretion of nerve fiber-derived vasoactive-intestinal polypeptide, which activates keratinocyte adenylate cyclase and stimulates its growth (Haegerstrand *et al.*, 1989). Thus, NGF cooperates in defining the epidermal cytokine network, which regulates, within the local epidermal environment, growth and differentiation of different epidermal cell types as well as epidermal immunological functions (see Luger and Schwartz (1990), for review).

The most important NGF biological effect has always been related to survival and maintenance of differentiated functions of vertebrate neurons (see Levi-Montalcini and Angeletti (1968), Levi-Montalcini (1987), and Thoenen (1991), for reviews). Indeed, NGF promotes the survival of ganglionic cells (neurotrophism) by preventing their physiological death, and it induces neurite elongation from peripheral ganglia and their proper orientation (neurotrophism). These data clearly demonstrate a growth promoting activity of NGF outside the nervous system (Cattaneo and McKay, 1990). NGF induces normal human epithelial cells, in primary culture, to enter the S-phase of the cell cycle and sustains their proliferation.

NGF is the prototype of a family of related molecules, called neurotrophins (see Thoenen (1991)). NGF, brain-derived neurotrophic factor, and neurotrophins (NT-3, -4, and 5) are structurally related molecules which bind to a common low affinity receptor, the p75^{NGFr} (Meakin and Shooter, 1992; Barker and Murphy, 1992). Based on some reports, high affinity binding and specificity appear to be conferred by a heterodimeric complex of p75^{NGFr} and the products of the *trk* family of protooncogenes (*trk A*, *B*, and *C*) (Klein *et al.*, 1991; Squinto *et al.*, 1991; Soppet *et al.*, 1991; Lamballe *et al.*, 1991; Bothwell, 1991b; Hempstead *et al.*, 1991). Alternatively, *trk* monomers might bind NGF with low affinity (Kaplan *et al.*, 1991), while *trk* dimerization (Jing *et al.*, 1992) or p75^{NGFr}-mediated NGF endocytosis (Eveleth and Bradshaw, 1988, 1992; Kahle and Hertel, 1992) could confer high affinity binding properties. Normal human keratinocytes have approximately 1000 high affinity receptors and more than 200,000 low affinity binding sites per cell in conditions where NGF internalization is blocked. Whether the low affinity binding capacity is conferred only by p75^{NGFr} or also by *trk* monomers, remains to be determined. Moreover, contrasting data exist in the literature concerning the role of p75^{NGFr} and *trk* in mediating signal transduction and biological activity of NGF in target cells. The stimulation of the p140^{trkA} in cell lines responsive to NGF is necessary and sufficient to elicit a biological response (Cordon-Cardo *et al.*, 1991; Loeb *et al.*, 1991; Ibanez *et al.*, 1992). In a recent paper, even the physical association between p140^{trkA} and p75^{NGFr} has been refuted (Jing *et al.*, 1992). Other reports, instead, highlight the crucial role of the association of p140^{trkA} and p75^{NGFr} in regulating biological activities of NGF (Hempstead *et al.* (1990, 1991), Lee *et al.* (1992), for recent reviews, see Meakin and Shooter (1992) and Barker and Murphy (1992)). Of particular interest are data obtained in transgenic mice, showing that targeted mutation of the gene encoding p75^{NGFr} leads to deficits in the peripheral sensory nervous system (Lee *et al.*, 1992). Eveleth and Bradshaw (1992) linked signaling dysfunction to altered NGF receptor mediated endocytosis and degradation.

Our data, obtained with the *trk* specific inhibitor K252a and the mAb ME20.4 against the p75^{NGFr}, clearly demonstrate that, at least in normal human keratinocytes, (i) NGF-mediated autocrine growth stimulation requires the activation of the *trk* tyrosine kinase receptor, (ii) such a stimulation is necessary and sufficient to induce keratinocyte proliferation, and (iii) ME20.4 alone does not interfere with NGF biological activity. However, p75^{NGFr} is up-regulated in the exponential phase of keratinocyte growth (Fig. 3) as well as in injured Schwann cells (Taniuchi *et al.*, 1986), it is abundantly expressed during the very early stages of embryonic development, in skin as well as in other organs (Ernfors *et al.*, 1988), and it is associated with several members of the *trk* family (Meakin and Shooter, 1992). This suggests a regulatory role for p75^{NGFr} which awaits experimental confirmation.

The *trkA* protooncogene product has been considered as the high affinity NGF receptor (Klein *et al.*, 1991). Interestingly, although NGF elicits biological activities in peripheral tissues, *in vivo trkA* expression studies in mice has shown that the expression of *trkA* mRNA is restricted to sensory cranial and spinal ganglia of neural crest origin, becoming a specific marker of neural crest derived sensory neurons (Martin-Zanca *et al.*, 1990). Thus, the absence of *trkA* in normal human keratinocytes, even after PCR amplification, is not surprising. However, the presence of specific and high affinity NGF binding sites in human keratinocytes, and the sequence homology between the different members of the *trk* family, led us to the discovery of a new member of the *trk* family (*trkE*), which generates a 3.9-kilobase transcript, present in keratinocytes and absent in K562 cells. To our knowledge, no member of the *trk* family of protooncogenes has yet been described in normal human cells. These observation raise interesting questions on the different roles of *trkA* and *trkE* in humans and suggest that NGF activity in different human tissues might be mediated by different members of the *trk* family of protooncogenes.

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