tain other mutations that are responsible for the loop repair defect. We examined loop repair in 13 additional cell extracts (*18*), including several made from tumor cell lines that exhibit microsatellite instability. All extracts efficiently repaired a heteroduplex containing a five-base loop. One extract was from an endometrial tumor cell line (HEC59) known to be defective in mismatch repair and to contain a mutation in one *hMSH2* allele (*4*). Thus, whether hMSH2 participates in loop repair remains to be resolved.

Candidate gene products that may be required for repair of DNA with loops include putative mismatch repair proteins already identified, such as other MSH or MLH homologs, or proteins yet to be discovered. The latter could include a human homolog of a yeast protein that specifically binds to DNA substrates containing loops of three to nine bases, a protein found even in yeast *msh2* and *msh3* mutants (*19*). The possible existence of mutant cell lines defective in some but not all forms of heteroduplex repair is suggested by reports indicating both qualitative and quantitative differences in the stability of various microsatellite alleles in tumor cells and tumor cell lines (*5*, *6*). Identification of extracts defective in repair of loops but not mismatches would reinforce the suggestion that mismatch and loop repair activities have one or more distinct requirements.

Note added in proof: The recent demonstration that purified human MSH2 protein binds to DNA containing loops of up to 14 nucleotides (*20*) is consistent with a possible role for MSH2 protein in loop repair activity.

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14. Even with unrepaired DNA heteroduplexes, plaques that are exclusively blue or colorless are obtained upon electroporation of an *E. coli* strain that is defective in mismatch repair. This is believed to reflect selective strand loss in vivo.
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16. Two explanations for repair of looped heteroduplexes can be considered other than repair of DNA containing several consecutive unpaired bases. One is that the sequences of the extra bases allow formation of multiple, adjacent shorter loops that are recognized by the same system that recognizes mismatches. However, except for the substrate containing four extra bases (Fig. 1), the sequences of the looped heteroduplexes used here make this possibility unlikely. A second explanation is that these substrates inadvertently contain an unknown mismatch elsewhere that signals concomitant repair of the loop. This possibility is also unlikely because these same heteroduplexes are efficiently repaired in an extract that is defective in mismatch repair.
17. That these extracts are not generally inactive for all DNA transactions is indicated by the fact that they are competent for simian virus 40 (SV40) origin-dependent DNA replication. The relative replication efficiency of the extracts was: HeLa, 100%; TK6, 120%; HCT116, 90%; LoVo, 41% (*4*).
18. Additional cell lines examined include the transformed lymphoblast line MT1 [V. S. Goldmacher, R. A. Cuzick, W. G. Thilly, *J. Biol. Chem.* **261**, 12462 (1986)]; the colon tumor cell lines SW48 and SW480 [A. Leibovitz *et al.*, *Cancer Res.* **36**, 4562 (1976)], DLD1 and HCT15 [D. L. Dexter, J. A. Barbosa, P. Calabresi, *Cancer Res.* **39**, 1020 (1979)], LS 180 and LS174T [B. H. Tom *et al.*, *In Vitro* **12**, 180 (1976)]; the endometrial tumor cell lines HEC59 [T. Morisawa, *J. Jpn. Soc. Clin. Cytol.* **26**, 433 (1987)] and AN_3CA [C. J. Dawe, W. G. Banfield, W. D. Morgan, M. S. Slat- ick, H. O. Curth, *J. Natl. Cancer Inst.* **33**, 441 (1964)]; the pancreatic cancer cell line HPAF-II [R. S. Metzgar *et al.*, *Cancer Res.* **42**, 601 (1982)]; the breast cancer cell lines T47D [H. C. Freake, C. Marcocci, J. Iwasaki, I. MacIntyre, *Biochem. Biophys. Res. Commun.* **101**, 1131 (1981)] and ZR-75-1 [L. W. Engel *et al.*, *Cancer Res.* **38**, 3352 (1978)]; and the ovarian teratocarcinoma cell line PA-1 [J. Zeuthen *et al.*, *Int. J. Cancer* **25**, 19 (1980)].
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Ligands for EPH-Related Receptor Tyrosine Kinases That Require Membrane Attachment or Clustering for Activity

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The EPH-related transmembrane tyrosine kinases constitute the largest known family of receptor-like tyrosine kinases, with many members displaying specific patterns of expression in the developing and adult nervous system. A family of cell surface–bound ligands exhibiting distinct, but overlapping, specificities for these EPH-related kinases was identified. These ligands were unable to act as conventional soluble factors. However, they did function when presented in membrane-bound form, suggesting that they require direct cell-to-cell contact to activate their receptors. Membrane attachment may serve to facilitate ligand dimerization or aggregation, because antibody-mediated clustering activated previously inactive soluble forms of these ligands.

Intercellular communication is often mediated by protein factors produced in one cell and recognized by receptors on the surface of other cells. Many of these factors, such as insulin and nerve growth factor, bind to and activate cell surface receptors with intrinsic protein tyrosine kinase activity (*1*). Ligand-mediated activation of these receptor tyrosine kinases regulates cell growth, survival, and differentiation in various cell types (*1*). There remain numerous receptor-like tyrosine kinases whose ligands have yet to be identified, and many of these orphan receptors are specifically expressed in the nervous system (*2*). The EPH-related kinases constitute the largest known family of orphan receptor-like tyrosine kinases, with several members of this family displaying specific expression in the developing and adult nervous system (*2–17*). In the adult a number of EPH-related kinases, such as EHK1 (*16*), EHK2 (*16*), and ELK (*7*), are restricted in their expression to discrete neuronal populations, including locus coeruleus neurons and the dopaminergic neurons in the substantia nigra. To identify ligands that might func-

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tion in the nervous system, we used an expression cloning strategy involving two kinases related to EPH that exhibit neural-specific expression—EHK1 and ELK. We identified three members of a family of cell surface–bound ligands that display distinct, but overlapping, specificities for EPH-related kinases. These ligands did not act as conventional soluble ligands, but instead seemed to require cell-to-cell contact to activate their receptors.

Fusion proteins consisting of the ectodomains of either EHK1 or ELK linked to the Fc portion of human immunoglobulin G1 (IgG1) [designated EHK1-Fc and ELK-Fc, respectively (*18*)] were used as probes in binding assays on various cell lines. Two neural cell lines with reciprocal patterns of surface binding to these receptor-Fc fusion proteins were found: The human neuroblastoma cell line SH-SY5Y bound EHK1-Fc but not ELK-Fc, whereas the human neuroepithelioma cell line CHP100 bound ELK-Fc but not EHK1-Fc (Fig. 1A). We constructed complementary DNA (cDNA) libraries from these cell lines and used them to identify, by expression cloning in COS cells, ligands that bound to EHK1 or ELK. Two different ligands specific for EHK1-Fc binding were isolated from the SH-SY5Y library (Fig. 1B). One of these was identical with B61 (*19*), a protein previously identified as a tumor necrosis factor–inducible sequence in endothelial cells, and the other was a related molecule, which we termed EHK1-L (Fig. 2). Cells expressing either of these ligands on their surface bound EHK1-Fc with a dissociation constant (K_d) of approximately 200 to 500 pM (Fig. 1C). A single ELK-specific ligand (termed ELK-L) was isolated from the CHP100 library (Fig. 1B); this ligand was also related to both B61 and EHK1-L (Fig. 2). Cells expressing surface-bound ELK-L had an affinity for ELK-Fc similar to that of cells expressing EHK1 ligands for EHK1-Fc (Fig. 1C). A soluble form of B61, released from tumor cells, has been purified on the basis of its ability to bind the EPH-related receptor ECK (*20*). An ECK-Fc fusion protein, like EHK1-Fc, recognized surface-associated forms of both B61 and EHK1-L but not ELK-L (Fig. 1B). Thus, the pattern of receptor interactions for known ligands to EPH-related receptors can, as presently understood, be schematically summarized as in Fig. 1D.

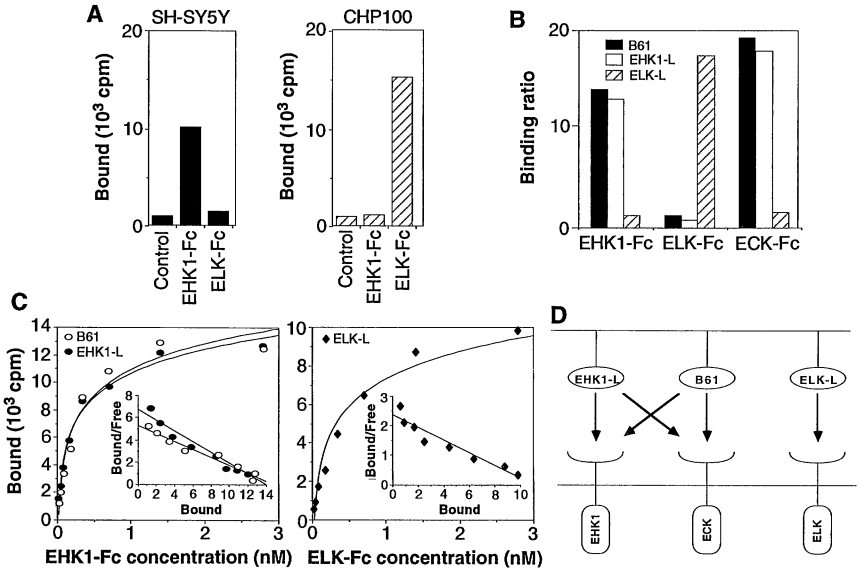

Fig. 1. Identification, expression cloning, and characterization of ligands for the EHK1 and ELK receptors. (**A**) Identification of cell lines expressing membrane-bound ligands for EHK1 and ELK. Indicated cell lines (SH-SY5Y and CHP100) were assayed for binding to EHK1-Fc and ELK-Fc (*32*). (**B**) Binding specificity of EHK1-Fc and ELK-Fc for COS cells expressing membrane-bound B61, EHK1-L, and ELK-L. COS cells transfected with B61, EHK-1L, or ELK-L cDNA expression constructs were assayed for binding to indicated receptor-Fc proteins (*32*). (**C**) Binding analysis of EHK1-Fc and ELK-Fc fusion proteins to COS cells expressing membrane-bound B61, EHK1-L, and ELK-L. Insets show data plotted in Scatchard format; Bound is given in 10^3 cpm, and Bound/Free is in 10^4 cpm/nM. (**D**) Schematic summary of interactions between ligands and receptors of this family.

The amino acid sequences of B61, EHK1-L, and ELK-L (Fig. 2) display both conserved and variable regions, and all share four conserved cysteine residues. Pairwise comparisons reveal that B61 and EHK1-L are more closely related to each other (~50% identity within the main conserved region) than either is to ELK-L (~37% and ~31% identity, respectively, within the same region), perhaps reflecting the similar binding specificities of B61 and EHK1-L. Whereas the ELK ligand is evidently a transmembrane protein with a small intracytoplasmic domain, the COOH-terminal sequences of both B61 and EHK1-L contain hydrophobic regions that instead strongly resemble recognition sequences for generating glycophosphatidylinositol (GPI) linkages to the membrane. Consistent with this possibility, both B61 and EHK1-L were cleaved from the cell surface with phosphatidylinositol-specific phospholipase C, whereas the ELK ligand was not (*21*).

Northern (RNA) blot analysis (Fig. 3) revealed restricted and reciprocal patterns of expression for EHK1-L and B61, in contrast

Fig. 2. Sequence comparison of ligands for EHK1 and ELK receptors. Aligned sequences of B61, EHK1-L, and ELK-L are displayed. Residues shared by all three sequences are boxed, and residues shared by at least two sequences are shown in the Consensus line; bold dots indicate conserved cysteines, and asterisks demarcate residues bordering the main conserved region. Lowercase letters show the presumed NH_2-terminal signal sequences as well as the transmembrane domain of ELK-L and the COOH-terminal hydrophobic GPI-recognition tails of B61 and EHK1-L. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

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B61                          meflwapl lglccslaaa DRHTVFWNSS NPKF-RNEDY   37
EHK1-L      maaaplllll llvpvpllpl laqgpggalg NRHAVFWNSS NQHL-RREGY   49
ELK-L       marpgqrwlg kwlvamvvwa lcrlatplaK NLEPVSWSSL NPKFLSGKGL   50
Consensus                                   V.FWNSS NPKF.R.EGY      50

B61         TIHVQLNDYV DIICPHYEDH SV---ADAAM FQYMLYLVEH EEYQLCQPQS   84
EHK1-L      TVQVNVNDYL DIMCPHYNSS GAGPGPGGGA FQYMLYMVSR NGYRTCNA-S   98
ELK-L       VIYPKIGDKL DIICPRAE-- -----AGRPY FMYKLYLVRP EQAAACSTVL   93
Consensus   TI.V..NDYL DIICPHYE.. .....AG... FQY.LYLV.. E.Y..C...S   100

B61         KDQVRWQCNR PSAKHGPEKL SEKFQRFTPF TLGKEFKEGH SYYYISK---   131
EHK1-L      QGFKRWECNR PHAPHSPIKF SEKFQRYSAF SLGMEFHAGH EYYYIST---   145
ELK-L       DPNVLVTCNR PEQE---IRF TIKFQEFSPN YMGLEFKKHH QYYITSTSNG   140
Consensus   ...VRW.CNR P.A.H.PIKF SEKFQRFSPF .LG.EFK.GH .YYYIST...   150

B61         PIHQHEDR-- --C-LR-LKV TV-SGKITHS PQAHVNPQEK RLAADDPEVR   174
EHK1-L      PTHNLHWK-- --C-LR-MKV FVCCASTSHS GEKPVPTLPQ FTMGPNVKIN   189
ELK-L       SLEGLENREG GVCRTRTMKI IMKVGQDPNA VTPEQLTTSR PSKEADNTVK   190
Consensus   P.H.LE.R.. ..C.LR.MK                                     200

B61         VLHSIGHSAA PRlfplawtv lllpllllqtp                       206
EHK1-L      VLEDFEGENP QVPKLEKSIS GTSPKREHlp lavgiafflm tflas       234
ELK-L       MATQAPGSRG SLGDSDGKHE TVNQEEKSGP GASGGSSGDP DGFFNSKval  237

ELK-L       faavgagcvi flliiifltv lllKLRKRHR KHTQQRAAAL SLSTLASPKG  297

ELK-L       GSGTAGTEPS DIIIPLRTTE NNYCPHYEKV SGDYGHPVYI VQEMPPQSPA  347

ELK-L       NIYYKV                                                  353
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to broad distribution of ELK-L. Like EHK1 (*16*), EHK1-L is expressed almost exclusively in the central nervous system, with the notable exception of high EHK1-L expression in skin, where EHK1 expression is almost undetectable (*16*). In contrast, B61 is expressed primarily in nonneuronal tissues (Fig. 3A; longer exposures reveal small amounts of B61 mRNA in most neural structures). Unlike the ELK receptor, which is expressed only in the brain and testes (*7*), ELK-L is widely expressed in both neuronal and nonneuronal tissues. In brain, mRNAs for both B61 and ELK-L, but not EHK1-L, were preferentially expressed early in development (Fig. 3B). As with B61 and ELK-L, the ligands for other nervous system–specific receptor tyrosine kinases (such as the neurotrophin ligands for the Trk receptors) are also expressed in nonneuronal tissues (*22*, *23*), apparently because they serve as target-derived factors for axonal processes innervating these tissues (*24*). The nonneuronal expression of B61 and ELK-L also raises the possibility that they serve as ligands for members of the EPH receptor family, such as ECK, that are expressed by nonneural cells.

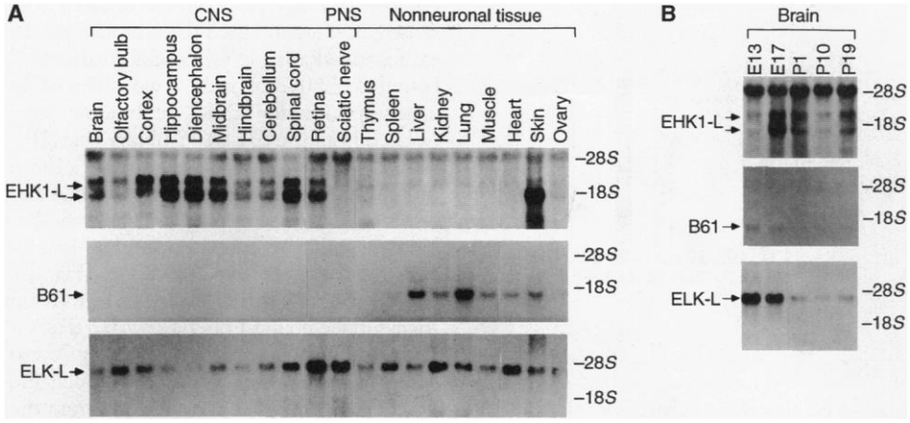

Fig. 3. Northern blot analysis of B61, EHK-1L, and ELK-L expression in adult rat tissues (**A**) and in developing brain (**B**). Total RNA (20 μg per lane) was isolated from the indicated tissues, fractionated on 1% formaldehyde-agarose gels, and transferred to nylon membranes. Blots were hybridized to ^{32}P-labeled probes derived from restriction fragments internal to the coding regions of each cDNA.

Although receptor activation, as judged by phosphorylation, was markedly induced by stimulation of receptor-expressing reporter cells with COS cells overexpressing membrane-bound forms of these ligands (Fig. 4A), we were unable to show ligand-induced receptor phosphorylation with soluble forms of B61, EHK1-L, or ELK-L (Fig. 4A). This discrepancy might be explained if membrane attachment facilitates ligand dimerization or clustering, or both, which in turn might promote receptor multimerization and activation. If this were true, then the soluble forms might also become active if they were clustered. To test this possibility, we constructed secreted forms of the ligands with epitope tags added at their COOH-termini; antibodies to the tags were then used to aggregate the ligands. This type of clustering enabled previously inactive soluble ligands to strongly induce receptor tyrosine phosphorylation in reporter cells expressing ELK and EHK1 receptors (Fig. 4A), as well as proliferation in reporter cells expressing an ECK receptor chimera (Fig. 4B). Dose-response studies demonstrated that clustered ligands had at least 100 times greater potency than unclustered ligands, and responses were saturable with low concentrations of clustered ligand in both the phosphorylation assay [tested for ELK-L (*25*)] and in the proliferation assay (tested for EHK1-L and B61; Fig. 4B). High concentrations of soluble B61 reportedly induced tyrosine phosphorylation of ECK receptors (*20*), but, consistent with our proliferation results (Fig. 4B), saturation was not apparent even at concentrations of soluble B61 of 1 to 2 μg/ml (*20*). These minimal effects might be attributed to a weak ability of the soluble ligand itself to dimerize or aggregate, either spontaneously or as a result of purification.

Our results define a class of ligands that function only as membrane-bound ligands. The Boss ligand for the sevenless receptor, in *Drosophila*, is a ligand for a receptor tyrosine kinase that is thought to function only in membrane-bound form (*26*). Ligands for EPH-related receptors, like Boss, may require cell-to-cell contact for receptor activation. This requirement appears consistent with

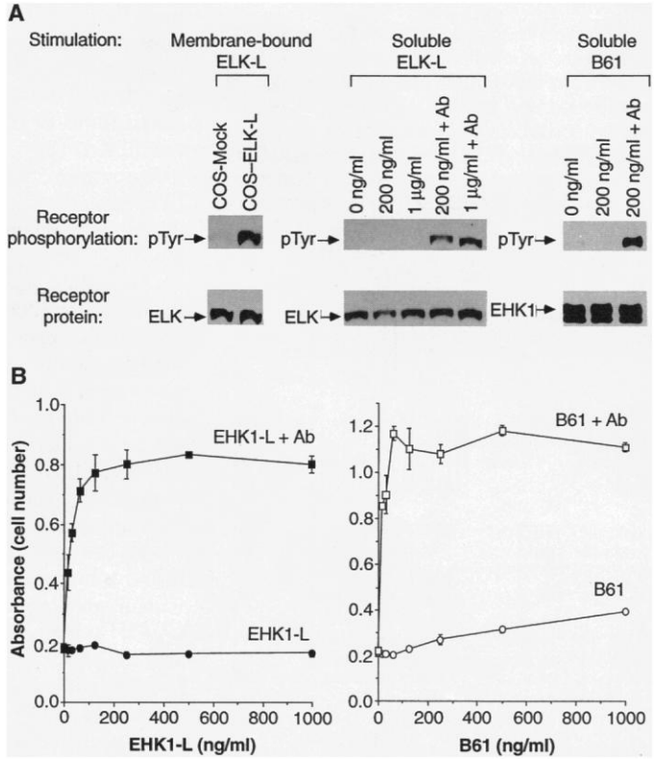

Fig. 4. Activation of EPH-family receptors by membrane-bound or clustered ligands. (**A**) Induction of ELK receptor tyrosine phosphorylation by membrane-bound ELK-L (**left panel**) and by clustered but not unclustered soluble ELK-L (**center panel**), and induction of EHK1 receptor tyrosine phosphorylation by clustered but not unclustered soluble B61 (**right panel**). Membrane-bound ELK-L was expressed on the surface of COS cells; "+Ab" following the concentration of soluble ligands denotes clustering with antibody (*33*). Reporter cell lines were NIH 3T3 fibroblasts transfected with ELK (left and center panels) or C2C12 cells transfected with EHK1 (right panel). After immunoprecipitation of receptors, total receptor protein was visualized by immunoblotting with antibodies to the receptors (lower panels), while tyrosine-phosphorylated receptor protein was revealed by immunoblotting with antibodies to phosphotyrosine (upper panels) (*33*). (**B**) Clustered, but not unclustered, forms of soluble EHK1-L and B61 potently stimulate growth in BAF cells expressing an ECK receptor chimera; "+Ab" denotes soluble ligands clustered with antibody. An ECK receptor chimera (in which the ectodomain of the ECK receptor was fused to the cytoplasmic domain of the fibroblast growth factor receptor) was used because the catalytic domain of the ECK receptor does not normally mediate growth responses in BAF cells. Two days after exposure to ligand, cell number was assessed by absorbance after addition of a vital dye as described (*34*).

evidence that EPH-related receptors transiently concentrate in areas of cell-to-cell contact (*17*) and that they participate in refining intersegmental boundaries during hindbrain development (*27*). Although the mechanism by which membrane attachment participates in receptor activation has not been established, the activity of clustered soluble forms suggests that membrane attachment somehow facilitates dimerization or aggregation of ligands.

There is overlap in the binding specificities of these ligands for different EPH-related receptors. Because these cross-specificities preclude assigning a particular ligand solely to an individual EPH-related receptor, we suggest that this family of ligands be termed the EFLs (for EPH-family ligands) and that each factor be numbered sequentially; thus, B61 would be designated as EFL-1, EHK1-L as EFL-2, and ELK-L as EFL-3. For the currently known EFLs, membrane linkage seems to provide a specialized mechanism for coupling receptor activation to direct cell-to-cell contact. In addition to potential roles in hindbrain development, the particular distributions of EPH-related receptors in the developing and adult nervous system make them likely candidates for mediators of various neuronal processes that depend on cell-to-cell interactions (*28*, *29*).

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32. To assay for binding to receptor-Fc proteins, we incubated cells plated confluently on 24-well dishes for 30 min with the indicated receptor-Fc proteins (used at either an approximate concentration of 1 μg/ml or at the indicated concentrations; the receptor-Fc proteins were produced as supernatants from COS cells transiently transfected with appropriate expression constructs, and concentrations estimated by a two-site enzyme-linked immunosorbent assay, with a polyclonal antibody to human (anti-human) IgG as a capturing antibody and an alkaline phosphatase–conjugated anti-human IgG as a secondary antibody), washed twice with phosphate-buffered saline (PBS), and then incubated for an additional 30 min with radio-iodinated goat anti-human IgG (NEN/Dupont; 1 μCi/ml in PBS containing 10% calf serum). After two additional washes, cells were solubilized and bound radioactivity was determined. Total cpm bound (Fig. 1A), a ratio of cpm bound in the presence versus absence of receptor-Fc protein (Binding Ratio; Fig. 1B), or cpm bound after nonspecific binding was subtracted (Fig. 1C; nonspecific binding was assessed as binding in the absence of receptor-Fc protein) are presented. Expression cloning was largely performed as previously described (*30*) with the following modifications: cDNA libraries were constructed in the JFE14 vector, and single-cell transfectants expressing ligands were detected by incubation with receptor-Fc fusion proteins, followed by methanol fixation, incubation with an alkaline phosphatase–conjugated second antibody and finally with alkaline phosphatase substrate. Positive cells were scraped from the tissue culture dish, and plasmid DNA was purified from these cells and rescued by electroporation. Plasmid DNA was then used for subsequent rounds of enrichment until single clones were isolated.
33. To assay membrane-bound ligand, we transfected COS cells with either vector alone (COS-Mock) or ELK-L expression vector (COS–ELK-L), detached from dishes with PBS + 1 mM EDTA, pelleted, resuspended in PBS, and layered on top of reporter cells. Soluble ligands were designed by replacement of the COOH-terminal GPI-recognition sequences of EHK1-L (residues 211 to 234) or B61 (residues 182 to 205), or the transmembrane and cytoplasmic domains of ELK-L (residues 235 to 353), with a myc-epitope tag (*31*). These soluble ligands were produced in COS cell supernatants and used as unclustered ligands, or clustered by incubation with both a mouse monoclonal antibody recognizing the myc epitope as well as anti-mouse immunoglobulin polyclonal antiserum.
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The Function of KGF in Morphogenesis of Epithelium and Reepithelialization of Wounds

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The function of keratinocyte growth factor (KGF) in normal and wounded skin was assessed by expression of a dominant-negative KGF receptor transgene in basal keratinocytes. The skin of transgenic mice was characterized by epidermal atrophy, abnormalities in the hair follicles, and dermal hyperthickening. Upon skin injury, inhibition of KGF receptor signaling reduced the proliferation rate of epidermal keratinocytes at the wound edge, resulting in substantially delayed reepithelialization of the wound.

Cutaneous wound repair is a complex process that involves formation of granulation tissue, reepithelialization, and tissue remodeling (*1*). These processes are mediated by a large number of growth factors and cytokines that have been only partially identified (*1*). Recently we demonstrated a large induction of KGF expression in fibroblasts below the wound and at the wound edge (*2*). Because KGF is a highly specific and potent mitogen for keratinocytes (*3*), these findings suggest that dermally derived KGF stimulates wound reepithelialization in a paracrine manner. To address this possibility, we selectively blocked KGF receptor signaling by targeted expression of a dominant-negative KGF receptor mutant in the undifferentiated basal keratinocytes of transgenic mice.

We have previously demonstrated that mutated fibroblast growth factor receptors (FGFRs) that lack kinase activity block

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