Specific Receptor Detection by a Functional Keratinocyte Growth Factor–Immunoglobulin Chimera

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Abstract. Fibroblast growth factor receptors (FGFRs) are encoded by at least four distinct highly conserved genes, and alternative splicing generates multiple gene products. The close relationship among different FGFRs has greatly increased the difficulty in generating specific immunochemical probes. As an alternative strategy, we constructed a fusion protein comprising keratinocyte growth factor (KGF) and an IgG_1 Fc domain (HFc). The chimeric molecule was efficiently secreted from transfectants as a disulfide-linked dimer that bound KGFRs with high affinity. Moreover, the KGF-HFc, like native KGF, induced DNA synthesis by epithelial cells implying normal functional receptor activation. Because it retained the convenient detection properties of an immunoglobulin, it was possible to use the KGF-HFc in ligand-mediated histochemical analysis of KGFRs. Flow cytometry revealed KGF-HFc chimera detection of the KGFR, an alternative FGFR2 product, but not FGFR1 (flg) or FGFR2 (bek). Histochemical analysis of normal skin demonstrated the specific localization of KGFRs within the spinous layer, a zone of epithelial cell differentiation. KGFRs were also localized to epithelial cells within a specific region of the hair follicle, and they were not detectable in cells of the sweat gland. Tissue sections of soft palate and tonsil, two examples of nonkeratinizing epithelium, revealed staining of stratum spinosum and some staining of the basal cell layer as well. Neither salivary gland epithelium nor lymphoid cells were positive. The ciliated epithelium of the trachea exhibited KGFR expression in intermediate and basal cell layers. In striking contrast to the normal pattern of staining in the adjacent epithelium, a squamous cell carcinoma of skin lacked detectable KGFRs. Our present findings suggest that growth factor–Ig fusion proteins may be generally applicable in ligand-mediated histochemical detection and localization of growth factor receptors.

KERATINOCYTE growth factor (KGF)[1] is a member of the FGF family. Originally isolated from human embryonic fibroblast conditioned medium, KGF is expressed by stromal fibroblasts of many epithelial tissues, and it is unique among FGF family members in its epithelial cell target specificity (Rubin et al., 1989; Finch et al., 1989). During wound healing, KGF mRNA expression is dramatically increased in comparison to that of the other FGFs (Werner et al., 1992*a*). Additionally, KGF appears to play an important role as a paracrine effector in the development and differentiation of a variety of epithelial tissues (Werner et al., 1992*b*; Marchese et al., 1990; Alarid et al., 1994).

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1. *Abbreviations used in this paper*: DAB, diamino benzidine tetrachloride; FGFR, fibroblast growth factor receptor; KGF, keratinocyte growth factor; KGFR, KGF receptor.

KGF binds and activates the KGF receptor (KGFR), a membrane-spanning tyrosine kinase generated by alternative splicing of FGFR2 (one of four members of the FGF receptor [FGFR] family). The FGFR extracellular domain is comprised of Ig-like domains. By analysis of a series of naturally occurring FGFR alternative products, Ig-like domain I appears dispensable for ligand binding (Miki et al., 1991). Additional gene products arise from the alternative splicing of the Ig-like domain III–encoded exons, which confer very different ligand binding properties (Miki et al., 1991; Givol and Yayon, 1992; Fantl et al., 1993). In particular, the FGFR2 gene product binds aFGF and bFGF, but not KGF, while the alternative KGFR product binds aFGF and KGF at high affinity, but bFGF at much lower affinity (Bottaro et al., 1990; Yayon et al., 1992).

The nearly identical nature of the KGFR and FGFR2 isoforms has made it difficult to develop specific immunochemical probes with which to elucidate patterns of receptor distribution and expression. To overcome these obstacles, we engineered a chimeric molecule between cDNAs encoding KGF and the immunoglobulin G Fc domain. Our results

demonstrate that this strategy may be generally applicable to the development of highly specific probes for cell surface receptors and in targeting molecules to intracellular sites of specific cell populations.

Materials and Methods

Construction of Recombinants

The region encoding the hinge, CH2, and CH3 domains (HFc) of a cDNA (LaRochelle, W. J., unpublished observation) for the mAb sis 1 (LaRochelle et al., 1989) heavy chain was amplified by the PCR (Cetus Corp., Norwalk, CT) so as to contain an XhoI-cloning site 5′ and in frame with the HFc PCR product, as well as BamHI sites at both termini. BamHI restriction endonuclease digestion of the HFc PCR product and ligation into the BamHI site of the pUC18 plasmid cloning vector created pUC18-HFc. The BamHI excision sites of pUC 18-HFc allowed insertion of the HFc cDNA into the BglII site of the MMTneo plasmid (LaRochelle et al., 1990) resulting in the MMTneo-HFc expression vector (Fig. 1). A second vector, MMTneo-spHFc, was generated by adding the PDGF A signal peptide (sp) upstream and in frame with the XhoI-cloning site of MMTneo-HFc. The KGF-encoded cDNA (Finch et al., 1989) minus the stop codon was amplified with XhoI- or SalI-compatible ends by PCR, digested with the appropriate restriction enzyme and cloned in frame with the HFc cDNA of MMTneo-HFc.

Transfection and Analysis of KGF-HFc Expression

Plasmid DNA from the KGF-HFc MMTneo construct was transfected into NIH 3T3 cells by the calcium phosphate precipitation technique (Wigler et al., 1977). Colony formation after selection in G418-containing medium was used as an internal marker of transfection efficiency.

KGF-HFc transfectants were washed and incubated for 30 min in methionine- and cysteine-free DME containing 25 μM zinc chloride followed by metabolic labeling with [^{35}S]methionine (125 μCi/ml) and [^{35}S]cysteine (125 μCi/ml) for 3.5 h. After collection, conditioned medium was immunoprecipitated with a KGF monoclonal antibody 1G4 (Alarid et al., 1994) or anti-mouse Fc (Pierce Chemical Co., Rockford, IL) followed by *Staphylococcus aureus* protein A-Sepharose CL-4B. The immunoprecipitated proteins were then resolved by SDS-PAGE and visualized after fluorography.

Ligand-binding Assay

32D cells are an IL-3–dependent hematopoietic cell line that do not normally express KGFRs. An expression vector containing the KGFR was introduced into naive 32D cells by electroporation and marker selected (Pierce, J. H., unpublished observation). 32D cell KGFR transfectants were harvested by centrifugation, washed in DME, and gently resuspended in binding buffer (DME, 25 mM Hepes, pH 7.4, 1 mg/ml BSA, and 1 μg/ml heparin). To this cell suspension was added increasing concentrations of unlabeled KGF-HFc, enriched by protein A chromatography, as well as a saturating amount of ^{125}I-KGF (2 ng) prepared by our standard iodination protocol (Bottaro et al., 1990; Ron et al., 1993) in binding buffer. After 1 h at 16°C, the cell suspension was layered onto 300 μl of a chilled oil mix (*n*-butyl phthalate [Fischer Scientific, Pittsburgh, PA]/*bis* (2-ethylhexyl) phthalate 1.5:1 (Eastman Kodak Co., Rochester, NY). Cells were centrifuged in an Eppendorf microfuge at 10K for 10 min at 4°C. The cell pellet was removed and counted in a gamma counter (model 5500; Beckman Instruments, Inc., Fullerton, CA).

Mitogenic Assay

Thymidine incorporation into BALB/MK cells was performed as described (Rubin et al., 1989; Ron et al., 1993). Briefly, varying concentrations of recombinant KGF (Ron et al., 1993) or KGF-HFc protein were added to quiescent cultures. Cells were incubated at 37°C for 16 h, followed by [^{3}H]thymidine addition for 5 h. Cells were washed, harvested, and [^{3}H]thymidine uptake was measured by liquid scintillation counting. In some cases, a KGF-neutralizing monoclonal antibody or heparin (Sigma Chemical Co., St. Louis, MO) was added to mitogenically equivalent amounts of KGF or KGF-HFc.

Flow Cytometric Analysis

NIH 3T3 cells were transfected with either the KGFR, FGFR2 (bek), or FGFR1 (flg) by standard calcium phosphate precipitation technique (Wigler et al., 1977). Similar levels of each receptor were expressed by each marker-selected cell population (Bottaro, D. P., unpublished observation). B5-589 cells or NIH 3T3 cells transfected with either the KGFR, FGFR2 (bek), or FGFR1 (flg) were removed from 10-cm culture dishes with 1% EDTA, PBS, pH 7.4, pelleted, and incubated for 10 min at room temperature in DME, 10% fetal calf serum, 0.5 % milk, 0.01% Tween 20, and 0.3 M NaCl to block nonspecific binding. The cells were again pelleted and resuspended in conditioned medium from KGF-HFc or control HFc transfectants adjusted to 0.5% milk, 0.01% Tween 20, 0.3 M NaCl, and 10 mM

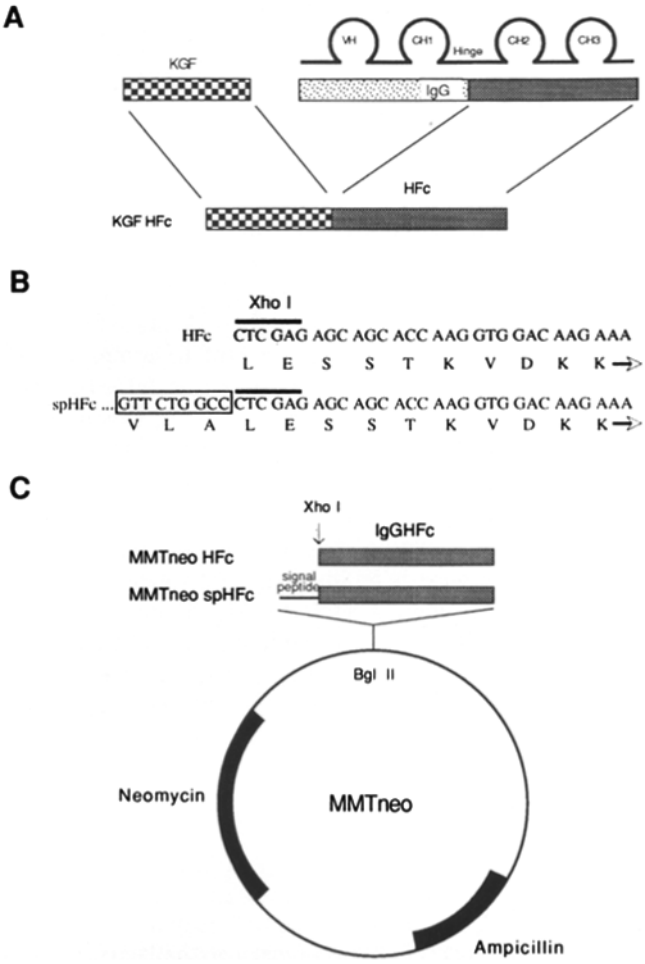


Figure 1. Engineering a KGF-HFc chimera. (*A*) The KGF-encoded cDNA minus its stop codon was recombined with the HFc portion of the mouse immunoglobulin IgG_1 heavy chain cDNA at the hinge region as described in Materials and Methods. (*B*) cDNA sequence and encoded amino acid residues adjacent to the XhoI-cloning site of MMTneo HFc are shown. (*C*) The HFc portion of the immunoglobulin heavy chain gene was subjected to the polymerase chain reaction to generate BamHI-compatible ends and cloned into pUC 18. The HFc PCR product also contained an XhoI-cloning site introduced by PCR 5′ and in frame with the Fc domain, but downstream of the 5′ BamHI site. The HFc fragment was removed from pUC18 by BamHI restriction endonuclease digestion, and it was cloned into the BglII site of the MMTneo vector. The KGF cDNA was amplified by PCR with SalI-compatible ends, digested with restriction enzyme, and ligated into the XhoI site of the MMTneo HFc vector in frame with the Fc domain.

sodium benzoate for 10 min at room temperature. Cells were washed twice with PBS, pH 7.4, 0.01% Tween 20. Bound primary antibody was detected after incubation for 10 min with FITC-conjugated goat anti-mouse IgG (Biosource International, Camarillo, CA) diluted 1/100 in DME, 0.5% milk, 0.01% Tween 20, and 10 mM sodium benzoate. Cells were washed twice with PBS, pH 7.4, 0.01% Tween 20, resuspended in 600 μl of Haemaline and processed using a FACSCAN™ analyzer (Becton Dickinson Immunocytometry Systems, Mountain View, CA) (Ashmun et al., 1989).

Ligand-mediated Histochemistry

Ligand-mediated histochemistry was performed on frozen sections of human skin fixed in 1% acetone/1.0 mM trichloroacetic acid/ethanol for 2 min, then transferred to 100% ethanol, followed by 50% ethanol, and then PBS, pH 7.4. Slides were treated in PBS, pH 7.4, 1.5 M NaCl for 30 min, then washed in PBS, pH 7.4. The sections were blocked for 1 h at room temperature with 5.0% Milk, 0.1% Tween 20, and PBS, pH 7.4, which was previously cleared by centrifugation at 20,000 *g* for 20 min. The slides were then incubated with KGF-HFc or control HFc-conditioned medium adjusted to 5.0% milk, 0.01% Tween 20, 0.3 M NaCl, and 10 mM sodium benzoate for 1 h at room temperature followed by three washes with PBS, pH 7.4, 0.01% Tween 20. The sections were then treated with rabbit anti-mouse IgG_1 conjugated to horseradish peroxidase (Boehringer Mannheim Biochemicals, Indianapolis, IN) diluted 1/100 in DME, 5.0% milk, 0.01% Tween 20, and 10 mM sodium benzoate for 1 h. The slides were then washed three times in PBS, pH 7.4, 0.01% Tween 20 and three times in PBS, pH 7.4. The color reaction was performed as described (Harlow and Lane, 1988) using diamino benzidine tetrachloride (DAB) according to the manufacturer's protocol (Pierce). In some experiments, the ImmunoPure DAB substrate kit (Pierce) was used according to the manufacturer's protocol. Similar protein levels of KGF-HFc or control HFc were used in ligand-mediated histochemical experiments. However, the control HFc showed no staining of tissue sections, even at a 10-fold higher concentration than KGF-HFc.

In Situ Hybridization

In situ hybridization was performed on deparaffinized tissue sections essentially as described (Wilkinson et al., 1987), using ^{33}P-labeled probes at a concentration of 0.2 ng/ml per kb length of cloned fragment in hybridization buffer (50% deionized formamide, 0.3 M NaCl, 20 mM Tris HCl, pH 8.0, 5 mM EDTA, 10% Dextran sulfate, 1× Denhardt's buffer, and 0.5 mg/ml yeast RNA). Tissue sections were hybridized at 55°C for 16–18 h and then washed under high stringency conditions (2× SSC and 50% formamide at 65°C) for 30 min. Nonhybridized probe was digested with 20 mg/ml RNase A for 30 min at 37°C. After further washing under high stringency conditions, slides were dehydrated through graded alcohols containing 0.3 M ammonium acetate. Sections were dipped in NTB-2 emulsion (Kodak), diluted 1:1 in water, air dried, and stored desiccated at 4°C. After appropriate exposure times, slides were developed in Kodak D-19 developer and counterstained with 0.02% toluidine blue.

Results

Strategy for KGF-HFc Chimera Construction

In an effort to generate a high affinity KGFR-specific probe with the well-characterized detection properties of an immunoglobulin, we designed a chimera in which the KGF cDNA was recombined with the HFc portion of the mouse immunoglobulin IgG_1 heavy chain cDNA at the hinge region (Fig. 1). Inherent difficulties with this approach included poor secretion and rapid degradation of the heavy chain in the absence of the partner light chain, respectively (Capon et al., 1989). However, Cogne and co-workers reported that structurally abnormal heavy chains, containing only CH2 and CH3 domains, were efficiently secreted apparently because of the deletion of the CH1 domain (Cogne et al., 1992). Additionally, the flexibility and adaptability of the hinge region of the heavy chain was well documented (Burton, 1985). Thus, we reasoned that deletion of the variable and CH1 domains from the heavy chain would facilitate dimer secretion and that grafting KGF to the random structure of the hinge would allow maximal adaptability consistent with presentation of growth factor biologic function.

To engineer the HFc expression construct, we exploited the polymerase chain reaction to generate BamHI-compatible ends on the HFc portion of the immunoglobulin heavy chain cDNA that was subsequently cloned into the BamHI site of pUC 18. The HFc cDNA insert was also engineered to contain an XhoI-cloning site in frame and 5′ to the HFc region, but within the BamHI sites (Fig. 1). The HFc fragment was removed from pUC18-HFc by BamHI digestion and cloned into the BglII site of the MMTneo vector. The KGF coding sequence was then amplified by PCR with either XhoI- or SalI-compatible ends, restriction enzyme digested, and subcloned into the MMTneo HFc vector in frame with the IgG HFc domain (Fig. 1).

KGF-HFc Chimeric Gene Product Possesses Both KGF and IgG_1 Fc Domain Determinants

The KGF-HFc construct was transfected into NIH 3T3 cells to investigate the immunochemical and biochemical properties of the encoded product. Marker selected mass cultures were radiolabeled with [^{35}S]methionine and [^{35}S]cysteine. Although protein A alone detected the chimeric gene product in culture fluids, the signal was ~15- to 20-fold greater when protein A was used with either KGF monoclonal antibody or anti-mouse Fc (data not shown). To establish that KGF-HFc possessed the immunochemical determinants of KGF, culture fluids were collected and incubated with a KGF

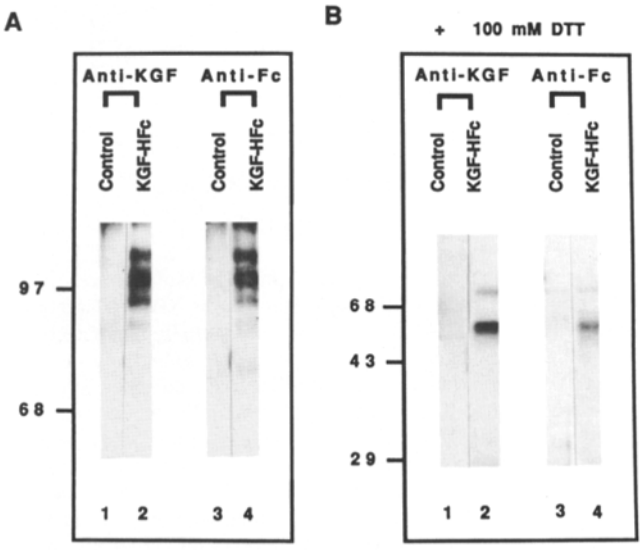
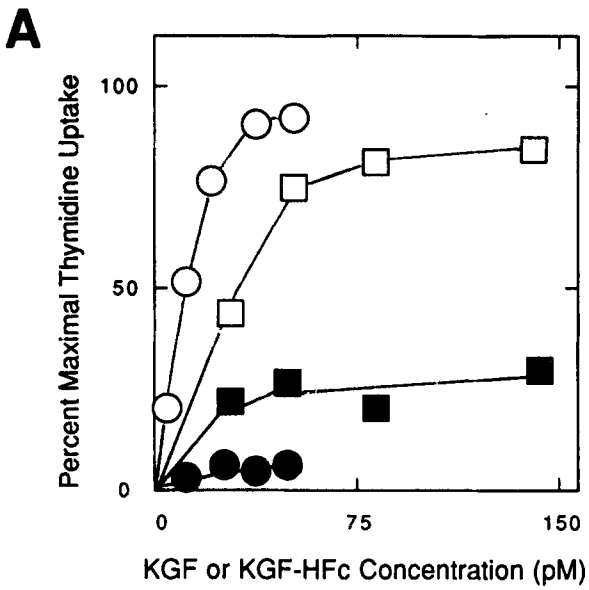


Figure 2. Immunochemical analysis of KGF-HFc chimera. Metabolically labeled conditioned medium from mock (lanes *1* and *3*) or KGF-HFc MMTneo (lanes *2* and *4*) transfectants was immunoprecipitated with anti-KGF monoclonal antibody (lanes *1* and *2*) or anti-mouse Fc antibody (lanes *3* and *4*), as described in Materials and Methods. Immunoprecipitates were recovered with protein A–Sepharose, subjected to SDS-PAGE, and the immunoreactive species were visualized after fluorography.



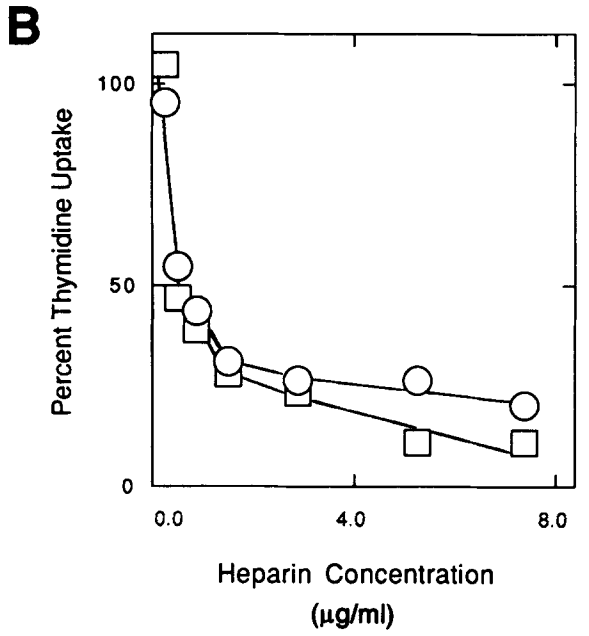


Figure 3. Comparison of KGF-HFc and KGF biologic activity. (*A*) The biologic activity of the KGF-HFc (□) or KGF (○) was measured on quiescent BALB/MK cells. Cells were first incubated with the indicated concentration of chimeric gene product or growth factor. After 16 h at 37°C, [^{3}H]thymidine was added for 5 h. Cells were then washed, harvested, and [^{3}H]thymidine uptake was measured by liquid scintillation counting. In some cases, a KGF-neutralizing monoclonal antibody (closed symbols) was added to confirm the specificity of either the KGF-HFc (■) or KGF (●). (*B*) Quiescent BALB/MK cells were incubated with the indicated concentration of heparin followed by addition of mitogenically equivalent amounts of either KGF-HFc (□) or KGF (○), as described in Materials and Methods.

monoclonal antibody followed by immunoprecipitation with protein A–Sepharose. As shown in Fig. 2, three distinct p94-98 immunoreactive species were observed. When the conditioned medium was incubated instead with anti-mouse IgG Fc, the same p94-98 species were observed (Fig. 2). In contrast, these species were not found in immunoprecipitates of conditioned medium from control MMTneo transfectants.

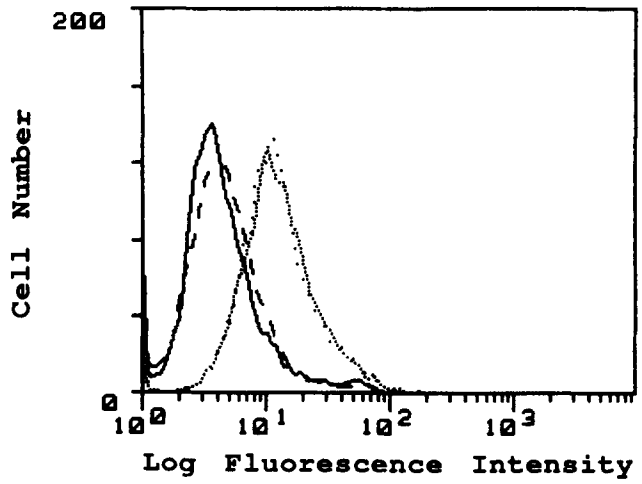


Figure 4. Flow cytometric analysis of KGF-HFc binding to members of the FGFR superfamily. KGF-HFc was incubated with B5-589 cells (· · ·) or NIH 3T3 cells transfected with the KGFR (·····), bek (- - -), or flg (-----), and it was subjected to flow cytometric analysis, as described in Materials and Methods. As an additional control, untransfected NIH 3T3 cells showed background staining comparable to bek and flg. Furthermore, the control HFc domain of the parental IgG did not recognize B5-589 or NIH 3T3 KGFR transfectants (data not shown). Bound KGF-HFc was detected with FITC-conjugated rabbit anti-mouse IgG. Cells were processed as described (LaRochelle et al., 1993) using a FACSCAN™ analyzer.

Since KGF-HFc possessed the IgG_1 heavy chain hinge region known to cause IgG dimer formation, we sought to determine whether KGF-HFc was a disulfide-linked dimer. Addition of 100 mM DTT before SDS-PAGE was associated with increased mobility of an apparent 48-kD species after immunoprecipitation with either the KGF mAb or anti-mouse HFc (Fig. 2). Thus, this efficiently secreted molecule possessed structural determinants of both KGF and the immunoglobulin Fc domain.

Functional Properties of the KGF-HFc Chimeric Gene Product

To determine whether the KGF-HFc exhibited KGF biologic properties, we investigated its ability to induce [^{3}H]thymidine uptake in KGF responsive BALB/MK cells. As shown in Fig. 3 *A*, the KGF-HFc readily stimulated [^{3}H]thymidine uptake. In comparison with recombinant bacterially expressed KGF, which induced half-maximal stimulation at ~10 pM, the mammalian expressed KGF-HFc half-maximally stimulated DNA synthesis at ~45 pM. Thus KGF-HFc was comparable to KGF purified from mammalian cells which is also fivefold lower in mitogenic activity than the bacterial recombinant ligand (Ron et al., 1993). DNA synthesis induced by the chimera was inhibited by >75% by a KGF-neutralizing monoclonal antibody, demonstrating the KGF specificity of the mitogenic activity. As additional controls, neither the secreted HFc portion of IgG_1 nor control IgG_1 monoclonal antibody induced detectable [^{3}H]thymidine uptake (data not shown).

Functional properties of KGF include its ability to interact with heparin, which dramatically inhibited KGF induced

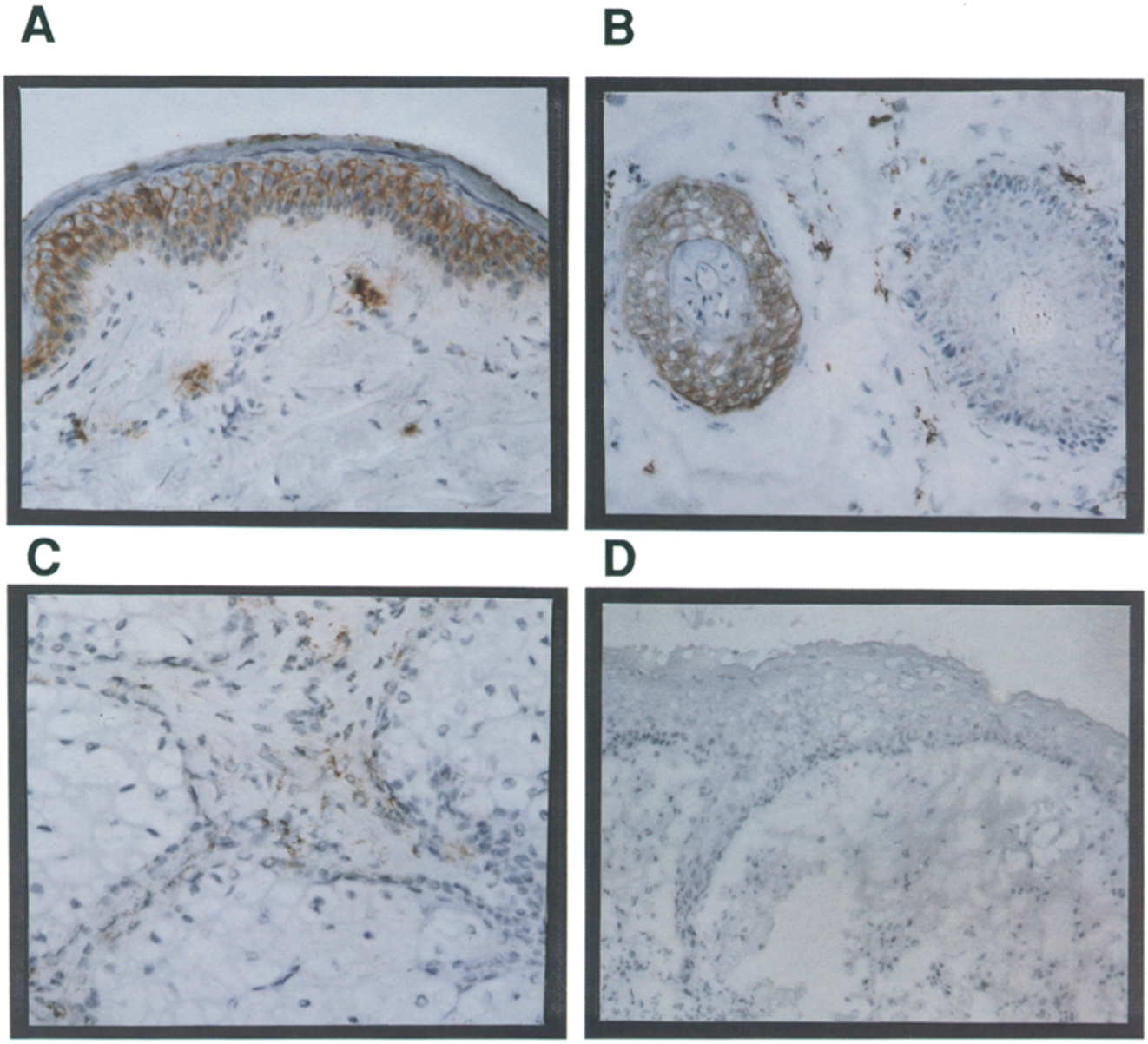


Figure 5. Ligand-mediated histochemical analysis of KGFR expression in human epidermis. KGFR expression was probed with KGF-HFc (*A–C*) or control HFc (*D*) on tissue sections from normal human skin. KGF-HFc was detected with rabbit anti-mouse IgG_1 conjugated to horseradish peroxidase, as described in Materials and Methods. KGFR was localized to the stratum spinosum of normal epidermis (*A*) and the bulb region of the hair follicle (*B*). The stratum basale also stained to a lesser extent. No staining was observed in the upper portion of hair follicles (*B*) or in sweat glands (*C*). Control HFc showed no staining of the epidermis (*D*). Bar, 10 μm.

proliferation (Bottaro et al., 1990; Ron et al., 1993). As shown in Fig. 3 *B*, heparin in the range of 1–5 μg/ml inhibited both KGF-HFc and recombinant KGF to equivalent extents of >80%. In other studies, heparin Sepharose, but not Sepharose, precipitated both KGF-HFc and KGF, but had no effect on the secreted HFc portion of IgG_1 (data not shown). These findings confirmed that heparin interacted specifically with the KGF portion of the chimeric molecule.

We next compared the ability of KGF-HFc and KGF to bind 32D cell transfectants expressing the KGFR by Scatchard analysis (data not shown). KGF-HFc bound to 32D KGFR transfectants with a K_d of ~1.4 nM, while recombinant KGF possessed a dissociation constant of 0.13 nM. Under these conditions, neither molecule detectably bound parental 32D cells nor control HFc failed to compete with iodinated growth factor binding (data not shown). All of the above results indicated that the KGF-HFc possessed the KGFR-binding properties and mitogenic signaling functions of native KGF.

KGFR-specific Detection by KGF-HFc

To demonstrate the specificity of KGF-HFc in KGFR immunodetection assays, we used flow cytometry. The chimeric molecule was incubated with NIH 3T3 cell transfectants expressing the KGFR, FGFR2 (bek), or FGFR1 (flg), as well as B5-589 human breast epithelial cells. As shown in Fig. 4, flow cytometry revealed that the KGF-HFc readily

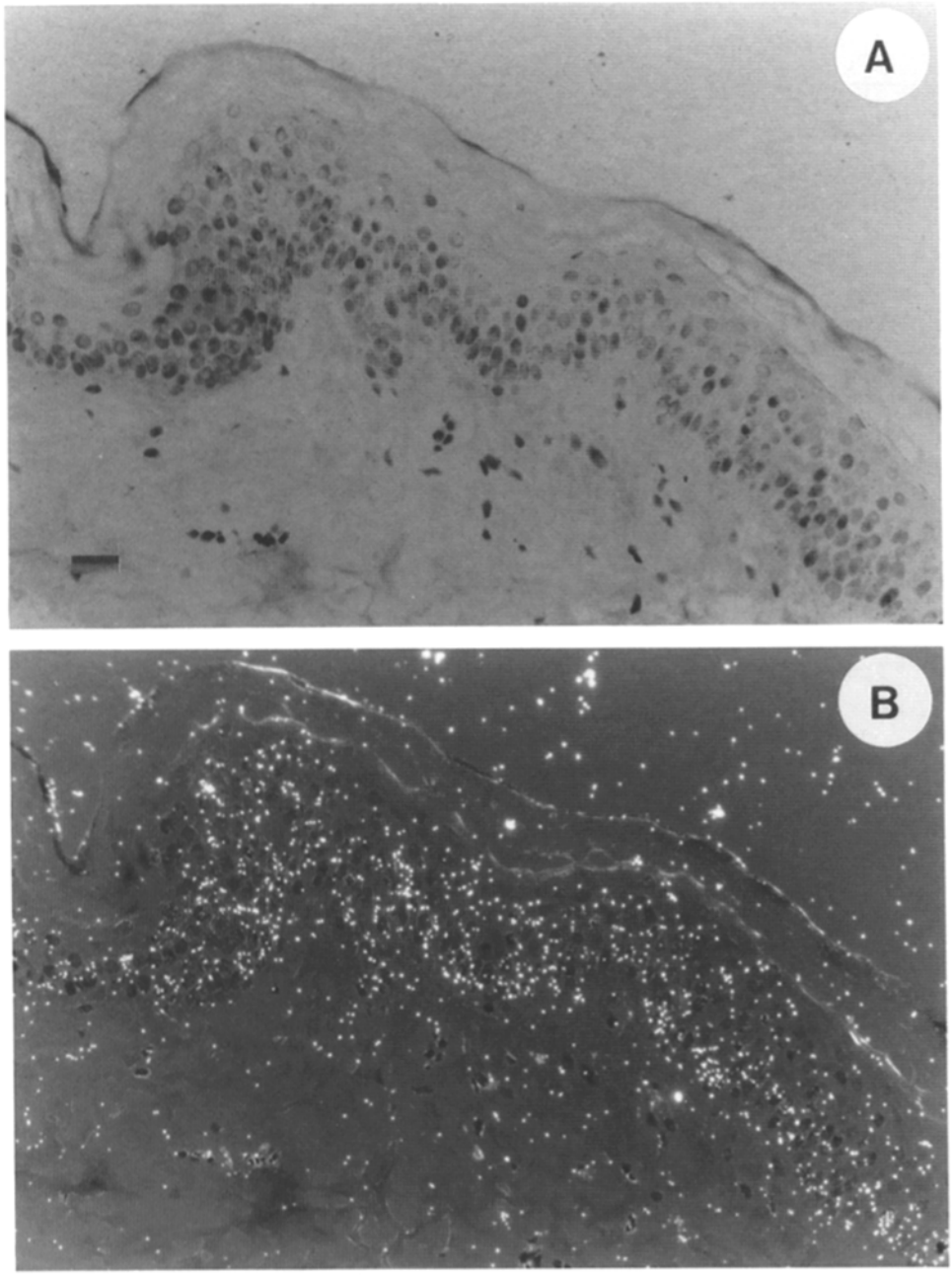


Figure 6. Localization of KGFR mRNA in human epidermis by in situ hybridization. (*A*) Bright-field micrograph of a toluidine blue–stained tissue section of normal human epidermis. (*B*) Corresponding dark-field micrograph demonstrating hybridization signal for KGFR mRNA in the stratum spinosum and basale of the epidermis. Bar, 10 μm.

recognized the NIH 3T3 KGFR transfectants as indicated by the 5- to 10-fold increase in fluorescent intensity compared to untransfected NIH 3T3 cells. In contrast, NIH 3T3 transfectants expressing similar receptor numbers of the alternatively spliced FGFR2 or FGFR1 showed no increase in staining over that of untransfected NIH 3T3 cells. The chimera also readily detected B5-589 cells that express $\sim 5 \times 10^4$ KGFRs (Bottaro et al., 1990). Under the same conditions, the control HFc failed to recognize either B5-589 or the NIH 3T3 KGFR transfectants (data not shown). All of these findings demonstrated the exquisite specificity of KGF-HFc in the recognition of the KGFR.

Ligand-mediated Histochemical Localization of KGFRs in Human Epithelium

The highly specific nature of KGFR recognition by the KGF-HFc chimera led us to investigate its application in the localization of KGFRs in human epithelial tissues. Thus, cryopreserved sections of human epithelium were incubated with the KGF-HFc or control HFc followed by detection with anti-mouse Fc conjugated to horseradish peroxidase. As shown in Fig. 5 *A*, specific staining with KGF-HFc was observed within the stratum spinosum of the epithelium and to a lesser extent, the stratum basale. Staining was uniform around the spinous cell surface with no evidence of polarity. The stratum corneum and granulosum all lacked detectable reactivity under the same conditions. Fig. 5 *B* demonstrates that the bulb region of the hair follicle (Fig. 5 *B*, *left side*) stained intensely with the KGF-HFc, while the upper portion of a hair follicle (Fig. 5 *B*, *right side*) did not stain. A typical sweat gland, as shown in Fig. 5 *C*, showed little or no staining under these conditions. As a specificity control, HFc alone showed no staining (Fig. 5 *D*).

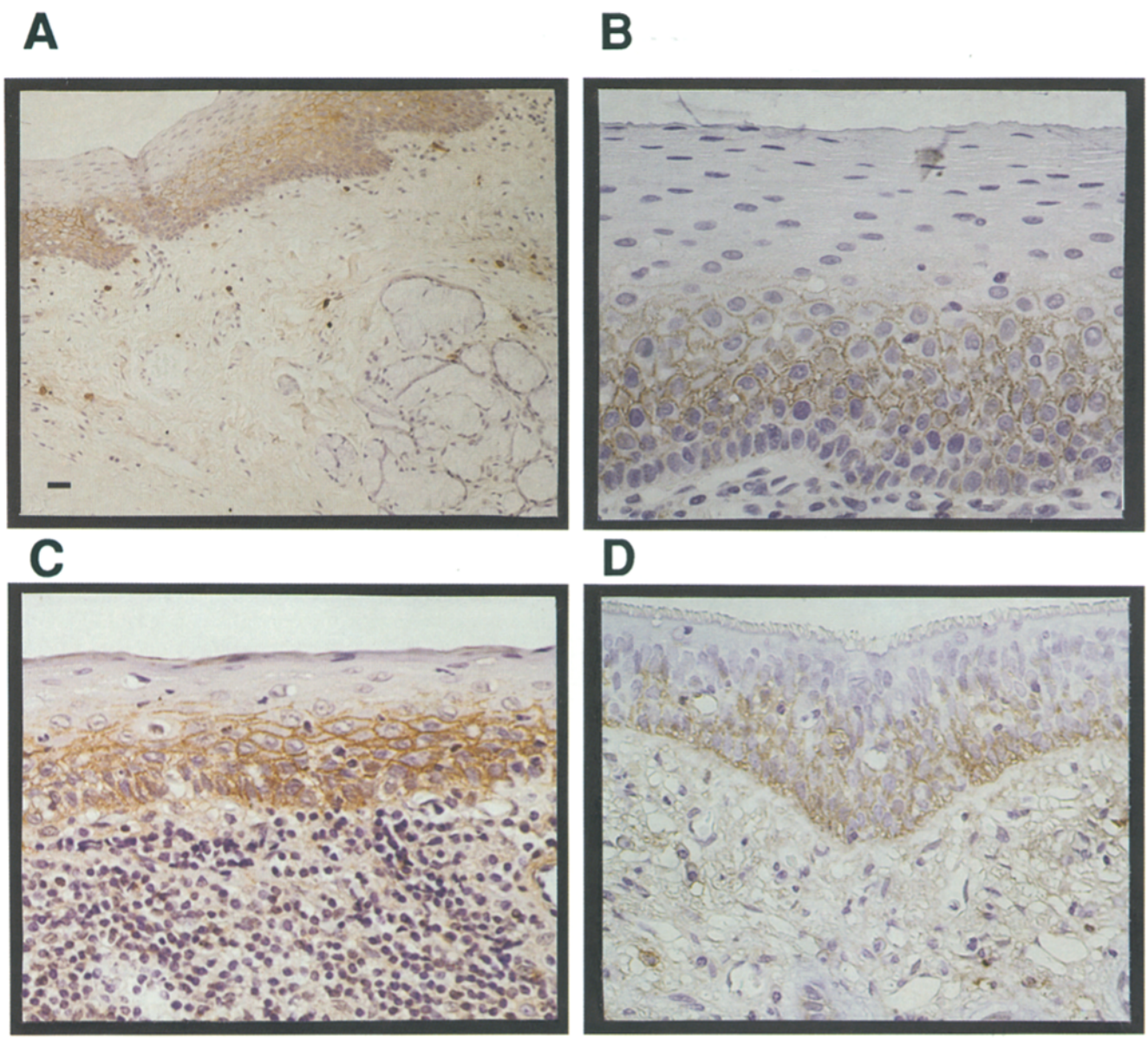


Figure 7. Ligand-mediated histochemical analysis of KGFR expression in normal human tissues of the oral cavity. KGFR expression was probed with KGF-HFc on tissue sections from normal human soft palate (*A* and *B*), tonsil (*C*), or trachea (*D*), as described in Materials and Methods. (*B*) KGF-HFc staining of KGFRs of soft palate was detected with DAB-enhanced metal staining. No KGFR staining was observed in salivary glands (*A*), lymphoid tissue (*C*), or mesenchyme. In each case, control HFc at similar protein concentrations showed no staining of tissue sections (data not shown). Bar, 10 μm.

To provide further evidence for the specificity of the staining pattern obtained with the KGF-HFc chimera, we examined the spatial expression of the KGFR transcript in paraffin-embedded sections of normal human skin using in situ hybridization. As shown in Fig. 6, KGFR mRNA was expressed in both the stratum spinosum and basale of the epidermis, but not in the stratum corneum or granulosum. Additionally, KGFR mRNA was also detected in hair follicles (data not shown), in agreement with the pattern of staining seen with the KGF-HFc chimera. Control sections hybridized with the corresponding sense strand transcript showed no signal (data not shown). These experiments further demonstrate the specificity of KGF-HFc staining in tissues that express the KGFR transcript.

We next investigated the presence of the KGFR in the soft palate, an example of nonkeratinizing stratified epithelial tissue. As shown in Fig. 7 *A*, the stratum spinosum stained most intensely for the KGFR. Of note, KGF-HFc staining of salivary glands was not detectable in the same sections. A metal-enhanced DAB reaction showed intense staining throughout the stratum spinosum with weaker staining adjacent to the stratum granulosum and in the basal cell layer (Fig. 7 *B*). Another nonkeratinizing epithelial tissue, the tonsil (Fig. 7 *C*) demonstrated KGF-HFc staining of both stratum spinosum and basale with little or no KGFR-specific staining of the lymphoid cells. KGFR expression was also studied in the trachea, an example of ciliated cylindrical epithelial tissue. KGF-HFc was immunoreactive with both the intermediate and more basale layers (Fig. 7 *D*). The salivary glands and mesenchymal cells of the trachea showed no

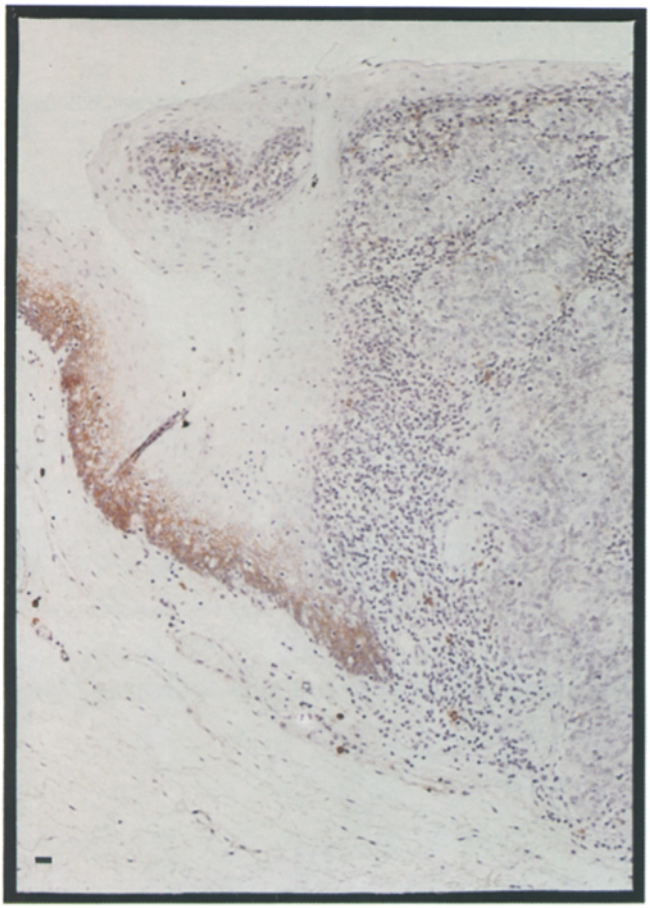


Figure 8. Ligand-mediated histochemical analysis of KGFR expression in a human squamous cell carcinoma of the oral cavity. KGFR expression was probed with KGF-HFc on tissue from a squamous cell carcinoma, as described in Materials and Methods. Note the striking absence of tumor cell KGFR staining, while the adjacent epithelium exhibited the normal pattern of KGFR expression. Control HFc did not stain (data not shown). Bar, 10 μm.

KGF-HFc staining. The control HFc did not stain any of these epithelial tissues under identical conditions (data not shown).

Finally, we investigated KGFR expression in a squamous cell carcinoma of the oral cavity. The carcinoma cells were entirely devoid of KGFRs, while the normal pattern of KGFR expression was observed in the adjacent epithelium (Fig. 8). Whether the lack of detectable KGFRs represents a general pattern for squamous cell carcinoma or provides a marker for a particular tumor phenotype remains to be determined. In any case, all of the above findings demonstrate the highly specific patterns of KGFR expression observed in the different epithelial tissues examined.

Discussion

Our present studies describe the application of a growth factor Ig fusion protein to the specific immunodetection of cell surface receptors. This approach confers particular advantages where highly related receptors make their discrimination very difficult. To our knowledge, there are as yet no polyclonal or monoclonal antibodies that can even discriminate between the external domains of different members of the FGFR gene family. We demonstrated that the KGF-HFc chimera readily detected its cell surface receptor, the KGFR, an alternative product of FGFR2, yet did not detect the FGFR2 despite the fact that these receptors differ only within a small stretch of their respective external domains (Miki et al., 1991; Givol and Yayon, 1992; Fantl et al., 1993). The ability to generate such specific immunochemical probes in a systematic manner also obviates the time-consuming process of generating and screening monoclonal antibodies. We demonstrated further that KGF-HFc exhibited high receptor affinity and the ability to induce DNA synthesis in target cells that express KGFRs. The ability to transduce a mitogenic signal contrasts with many monoclonal antibodies directed against receptors such as those to the α-PDGFR or CSF-1R, which bind avidly but do not activate receptors (Ashmun et al., 1989; LaRochelle et al., 1993).

The secretory properties of the KGF-HFc chimera were such that with the metallothionein expression construct used, it was possible to generate quantities only a few fold less than that produced from cultured hybridoma cell lines. Efficient secretion in part was related to the choice of promoter in the construct. However, it was likely also caused by the deletion of the CH1 domain from the heavy chain. There is evidence that the normal heavy chain is poorly secreted in the absence of light chain (Capon et al., 1989; LaRochelle, W. J., unpublished observations), and there are reports that individual heavy chains efficiently secreted during immunoproliferative disorders lack the CH1 domain (Cogne et al., 1992). Functional heavy chain molecules that lack the CH1 domain have also been found to be secreted in at least one nonhuman mammalian species (Hamers-Casterman et al., 1993). Thus, deletion of the CH1 domain may circumvent the need for the light chain partner in heavy chain secretion (Capon et al., 1989). KGF-HFc secretion was sufficiently robust that it was possible to use culture fluids harvested in the absence of further purification for KGFR immunodetection.

Ligand-mediated histochemical staining of skin with the KGF-HFc provided the first observations of KGFR expression and distribution in an intact tissue. We observed KGFRs most intensely in the spinous cell layer directly adjacent to the basal cell layer. Most importantly, correlation of the histochemical localization of KGFRs in normal human skin by KGF-HFc with the spatial pattern of KGFR mRNA expression detected by in situ hybridization demonstrates mechanistically that the cellular sites of KGFR transcription correspond to the sites of its gene product expression.

It has been reported that keratinocyte proliferation is confined to and defines the basal cell layer (Fuchs, 1990). The spinous cells ultimately differentiate into the granular cell layer and finally into the stratum corneum. Thus, KGFR staining appears to coincide with cells undergoing differentiation in addition to proliferation. We showed that KGF-HFc, like KGF itself (Bottaro et al., 1990; Ron et al., 1993) directly interacts with heparin. However, several lines of evidence suggest that heparan sulfate–like proteoglycans were not bound under the conditions that were used. First, KGF-HFc immunodetection performed in the presence of 0.5 M NaCl eliminated a broad and weakly diffuse staining pattern presumably attributable to the extracellular matrix heparan sulfate–like proteoglycans. Furthermore, the

low concentration of KGF-HFc that is required to achieve maximum saturable staining implies the presence of high affinity rather than low affinity binding sites. Finally, the pattern of staining makes it unlikely that extracellular matrix components were recognized, since regions of epidermis known to contain these components, as well as control NIH 3T3 cell transfectants that secrete heparan sulfate–like proteoglycans, were not stained.

Recent reports have demonstrated the functionality of chimeric receptor proteins fused to immunoglobulins. The use of bifunctional molecules engineered as IgG heavy chain chimeras was first reported for the CD4 immunoadherin (Capon et al., 1989). Since then, receptor–IgG chimeras have been used for structural and functional studies of the Ig-like superfamily of receptors (Williams and Barclay, 1988), including the T cell antigen receptor (Eilat et al., 1992), flt3/flk-2 (Lyman et al., 1993), c-kit (Liu et al., 1993), the β PDGFR (Heidaran et al., 1995), and the KGFR (Cheon et al., 1994). In the case of the KGFR, this approach provided a framework for dissection of its ligand-binding domains and made it possible to demonstrate that high affinity binding sites for two high affinity ligands, aFGF and KGF, reside within different receptor Ig-like domains (Cheon et al., 1994). The ligand binding properties of other non–Ig-like receptors, including the natriuretic peptide receptors (Bennett et al., 1991), tumor necrosis factor receptor (Howard et al., 1993), and hepatocyte growth factor receptor (Mark et al., 1992) have also been analyzed by means of immunoglobulin fusion proteins.

Growth factor immunoglobulin chimeras have been much less actively investigated. In one report, IGF-1 was grafted to the carboxyl terminus of the IgG heavy chain (Shin and Morrison, 1990). This resulted in a poorly secreted molecule with markedly impaired receptor-binding properties. In another study, IL-2 fused to the immunoglobulin CH1 domain demonstrated high affinity receptor binding and mitogenic activity, but it was not tested in ligand-mediated histochemical assays (Landolfi, 1991). Our present results suggest that growth factor–Ig fusion proteins generated as described here can be used to define the expression and distribution of specific receptors in histologic specimens. It is known that certain growth factor receptors can be abnormally upregulated in tumor cells (Aaronson, 1991). Thus, such probes may prove useful in diagnosis and prognosis of malignancies and other pathologic states. This approach has the potential of identifying novel target cells and novel receptors for known growth factors. Finally, the ability of growth factor Ig fusion proteins to cause functional receptor activation suggests normal receptor processing associated with downregulation and internalization. If so, such chimeric ligands may provide ideal vehicles for delivering toxins and other therapeutic modalities to intracellular sites in specific target cell populations.

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