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Biochemistry

Characterization of the murine BEK fibroblast growth factor (FGF) receptor: Activation by three members of the FGF family and requirement for heparin

(receptor binding/32D cells/growth factors)

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ABSTRACT **The *bek* gene encodes a member of the high-affinity fibroblast growth factor receptor family. The BEK/FGFR-2 receptor is a membrane-spanning tyrosine kinase with the typical features of FGF receptors. We have cloned a murine *bek* cDNA and expressed it in receptor-negative Chinese hamster ovary cells and in 32D myeloid cells. The BEK receptor expressed in Chinese hamster ovary cells binds acidic FGF, basic FGF, and Kaposi FGF equally well but does not bind keratinocyte growth factor or FGF-5 appreciably. Upon treatment with basic FGF or Kaposi FGF, the BEK receptor is phosphorylated and a mitogenic response is achieved. Heparan sulfate proteoglycans have been shown to play an obligate role in basic FGF binding to the high-affinity FLG receptor. Unlike the BEK-expressing Chinese hamster ovary cells, 32D cells expressing the BEK receptor require the addition of exogenous heparin in order to grow in the presence of basic FGF or Kaposi FGF. We show that the addition of heparin greatly enhances the binding of radio-labeled basic FGF to the receptor. Thus the BEK receptor, like FLG, also requires an interaction with heparan sulfate proteoglycans to facilitate binding to its ligands.**

Fibroblast growth factors (FGFs) are a family of seven heparin-binding polypeptides that share 30–40% homology (1). Like other growth factors, FGFs act by binding and activating specific cell-surface receptors, and the FGF receptors also represent a family of related, but distinct, membrane-spanning tyrosine kinases. Four such FGF receptors have thus far been cloned. These include the FLG receptor or FGFR-1 (2–5), the BEK receptor or FGFR-2 (6–10), FGFR-3 (10, 11), and, recently, a human FGFR-4 (12). Related receptors capable of binding bFGF have also been described in *Xenopus* and *Drosophila* (13, 14).

Each of these receptors consists of an extracellular ligand binding region containing three immunoglobulin-like domains (15) and an intracellular split tyrosine kinase domain. The FLG and BEK receptors are most closely related and bind acidic FGF (aFGF) and basic FGF (bFGF) with equal affinities (7, 16). FGFR-3 has also been shown to bind both growth factors (11), whereas FGFR-4 binds aFGF but not bFGF (12), thus providing one example of strict ligand specificity in this receptor family. The FLG receptor binds Kaposi FGF (K-FGF) with ≈15× lower affinity than aFGF or bFGF (5).

In addition to multiple receptors for the FGF family, variant forms of FLG and BEK have been described that probably arise from alternative RNA splicing, thereby increasing the complexity of this receptor family (4, 16, 17). A shorter form of FLG, which is missing the N-terminal, immunoglobulin-like domain, binds with high affinity to aFGF, bFGF, and K-FGF, indicating that the first immunoglobulin-like domain of the receptor is dispensable for ligand binding (5, 16). An isoform of BEK, which results from alternative splicing, was cloned from a keratinocyte expression library as a receptor for keratinocyte growth factor (KGF) (17). It binds aFGF and KGF with high affinity but exhibits very low affinity for bFGF (17). Adding another dimension to FGF–receptor interaction are the low-affinity FGF receptors or heparan sulfate proteoglycans (HSPGs), which have been shown to play an essential role in the binding of bFGF to FLG (18) and in bFGF-mediated fibroblast growth and myoblast differentiation (19).

Since the FGFs seem to interact with only partial specificity with a family of receptor tyrosine kinases, which are often coexpressed in the same cells, the question arises of why such a degree of redundancy exists in this system. A first step necessary to answer this question is the precise characterization of each of the FGF receptors, its binding specificity, and the factors required for its activation. In this report we describe the cloning and characterization of a three-immunoglobulin-domain form of murine BEK. This receptor, when expressed in Chinese hamster ovary (CHO) cells, is capable of binding aFGF, bFGF, and K-FGF with equal affinity but does not bind KGF or FGF-5 appreciably. The BEK receptor is phosphorylated in response to the three former factors, and a proliferative response is elicited. When BEK is introduced in the 32D myeloid cell line, which is normally interleukin 3 (IL-3) dependent (20) and does not appear to express high- or low-affinity FGF receptors, the cells can respond to FGFs instead of IL-3, but only in the presence of exogenous heparin. In the presence of heparin, the binding of ^{125}I-labeled bFGF (^{125}I-bFGF) to the BEK receptor is dramatically enhanced, indicating that this receptor also requires HSPGs to facilitate ligand binding.¶

MATERIALS AND METHODS

Library Screening and Sequencing. A λgt10 cDNA library from A-15 cells was screened using an 800-base-pair (bp) fragment from the tyrosine kinase portion of the partial *bek* cDNA as a probe (6). Filters were hybridized as described (5). The cDNAs were subcloned into pBluescript (Stratagene) and sequenced (21).

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Abbreviations: FGF, fibroblast growth factor; bFGF, basic FGF; aFGF, acidic FGF; K-FGF, Kaposi FGF; KGF, keratinocyte growth factor; CHO, Chinese hamster ovary; HSPG, heparan sulfate proteoglycan; IL-3, interleukin 3.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. M86441).

Cell Lines and Transfections. CHO-DG44 cells were maintained as described (5). Cells were transfected by calcium phosphate precipitation with a 3.7-kilobase (kb) *bek* cDNA in p91023B vector (22). Positive clones were selected in medium lacking hypoxanthine and thymidine (5). The stably transfected 32D cells were isolated after electroporation with the *bek* cDNA expressed in the pZip-NeoSV(X)1 vector (23). For electroporation and selection, 5×10^6 cells were suspended in 1 ml of cold phosphate-buffered saline (PBS) with 50 μg of plasmid DNA in a Gene Pulser cuvette. The cuvette was exposed to a 650-V impulse, 25 μF (Gene Pulser, Bio-Rad), and kept on ice for 15 min. The cells were diluted in Iscove's medium with 10% heat-inactivated fetal calf serum and IL-3 (10 units/ml). After 24 hr the cells were put in medium containing geneticin (625 μg/ml).

Binding Assays. Recombinant bFGF was iodinated as described (24) (specific activity = 552 cpm/fmol). Recombinant K-FGF was iodinated with ^{125}I-labeled Bolton–Hunter reagent. The radioactive K-FGF was repurified on heparin-Sepharose with 0.15% gelatin added to all buffers and had a specific activity of 63 cpm/fmol. For Scatchard analysis, clone CHO bek 3.7-5 was incubated with various concentrations of ^{125}I-bFGF from 0.25 to 10 ng/ml. Radioactive bFGF bound to high-affinity receptors was quantitated as described (5). Competition assays were performed as described (5). Heparin (10 μg/ml) was included in all assays (24).

Growth Curves. On day 0, 32D cells were washed with serum-free medium and seeded at 1.5×10^5 cells per ml in Iscove's medium with 10% heat-inactivated fetal calf serum, and various growth factors were added as indicated. Cells were counted in duplicate on days 2, 4, and 6.

Crosslinking to 32D Cells. ^{125}I-bFGF was prepared by mild chloramine-T treatment and purified over a Sephadex G-25 column. The specific activity of ^{125}I-bFGF was 4×10^4 cpm/ng (800 cpm/fmol). Cells (5×10^6) were washed twice in ice-cold PBS and resuspended in 0.5 ml of binding medium (Iscove's medium/0.15% gelatin) and ^{125}I-bFGF at 10 ng/ml. Unlabeled bFGF (1 μg/ml) and heparin (10 μg/ml) were included as indicated. The binding was performed for 2 hr at 4°C with gentle agitation of samples. The cells were washed twice in ice-cold PBS and crosslinked with disuccinimidyl suberate (Pierce) (3 mM in PBS) for 30 min at room temperature. Samples were loaded on an 8% polyacrylamide/SDS gel.

RESULTS

Cloning of the Murine *bek* cDNA Encoding a FGF Receptor. The mouse BEK protein was originally identified as a tyrosine kinase in an expression cloning system using antiphosphotyrosine antibodies to screen a liver cDNA library (6). The partial sequence of BEK was 91% similar to the intracellular catalytic domain of the chicken and human FLG FGF receptor (2, 3). We used an 800-bp *Eco*RI fragment of *bek* as a probe to isolate a full-length *bek* cDNA. Two clones that had a 240-bp extension in the 5′ direction were isolated and the 240-bp *Eco*RI fragment (corresponding to amino acids 399–475 in Fig. 1) was used to screen several cDNA libraries. A mouse brain cDNA library yielded a 2-kb *Eco*RI fragment that overlapped perfectly with the entire probe and encoded the amino terminus of the BEK protein.

The complete deduced protein sequence of BEK is shown in Fig. 1. Based on the homology to the FLG sequence, the initiator methionine is located at position 607 of the cDNA and is followed by a stretch of 20 hydrophobic amino acids that could serve as a signal sequence. The 600 bp of 5′ untranslated leader sequence has four ATG codons that could play a role in the regulation of BEK translation. The BEK open reading frame of 821 amino acids is flanked by a 1.3-kb 3′ untranslated region. The predicted protein shares 71% homology with murine FLG and has all of the features of a membrane-spanning tyrosine kinase receptor (25). In the

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  1 MVSWGRFICL VLVTMATLSL ARPSFSLVED TTLEPEEPPT KYQISQPEAY
 51 VVAPGESLEL QCMLKDAAVI SWTKDGVHLG PNNRTVLIGE YLQIKGATPR
101 DSGLYACTAA RTVDSETWIF MVNVTDAISS GDDEDDTDSS EDVVSENRSN
151 QRAPYWTNTE KMEKRLHACP AANTVKFRCP AGGNPTSTMR WLKNGKEFKQ
201 EHRIGGYKVR NQHWSLIMES VVPSDKGNYT CLVENEYGSI NHTYHLDVVE
251 RSPHRPILQA GLPANASTVV GGDVEFVCKV YSDAQPHIQW IKHVEKNGSK
301 YGPDGLPYLK VLKAAGVNTT DKEIEVLYIR NVTFEDAGEY TCLAGNSIGI
351 SFHSAWLTVL PAPVREKEIT ASPDYLEIAI YCIGVFLIAC MVVTVIFCRM
401 KTTTKKPDFS SQPAVHKLTK RIPLRRQVTV SAESSSSMNS NTPLVRITTR
451 LSSTADTPML AGVSEYELPE DPKWEFPRDK LTLGKPLGEG CFGQVVMAEA
501 VGIDKDKPKE AVTVAVKMLK DDATEKDLSD LVSEMEMMKM IGKHKNIINL
551 LGACTQDGPL YVIVEYASKG NLREYLRARR PPGM[EYSYDI NRVPEEQM]TF
601 KDLVSCTYQL ARGMEYLASQ KCIHRDLAAR NVLVTENNVM KIADFGLARD
651 INNIDYYKKT TNGRLPVKWM APEALFDRVY THQSDVWSFG VLMWEIFTLG
701 GSPYPGIPVE ELFKLLKEGH RMDKPTNCTN ELYMMMRDCW HAVPSQRPTF
751 KQLVEDLDRI LTLTTNEEYL DLTQPLEQYS PSYPDTSSSC SSGDDSVFSP
801 DPMPYEPCLP QYPHINGSVK T 821
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FIG. 1. Deduced amino acid sequence of murine BEK protein. The heavy underline is the transmembrane region. Cysteine residues are marked by black squares and N-linked glycosylation sites are marked by shaded ovals. The acidic region and conserved tyrosine kinase motifs are highlighted. The kinase insert region is demarcated by brackets.

356-amino acid extracellular portion, it has three immunoglobulin-like loops similar to those of human BEK (7). Like FLG, BEK has an acidic box (DDEDDTD) between the first and second immunoglobulin loops, a characteristic motif of the FGF receptor family. Although the overall homology between BEK and FLG is high, it is lower (45%) in the highly variable first immunoglobulin-like loop, which is dispensable for FLG binding to aFGF, bFGF, or K-FGF (5, 16). Like the other FGF receptors, BEK has a short (14 amino acid) kinase insert region in the tyrosine kinase domain.

BEK Expression in Transfected CHO Cells. The *bek* cDNA (3.7 kb) was cloned into the p91023B vector (22) and transfected into dihydrofolate reductase-negative CHO cells (clone DG44). These cells have very low numbers of FGF receptors and do not express any RNA that hybridizes to bek (data not shown). Dihydrofolate reductase-positive clones were selected and tested for their ability to bind ^{125}I-bFGF. Clone CHO bek 3.7-5 was used for further characterization of the receptor. When CHO bek 3.7-5 cells were exposed to ^{125}I-bFGF or ^{125}I-labeled K-FGF (^{125}I-K-FGF) and a crosslinking agent, a ligand–receptor complex of ≈160 kDa was obtained (Fig. 2). The estimated size of the receptor (≈140 kDa) is probably due to glycosylation. Higher molecular mass forms were also observed that could represent receptor oligomerization.

The dissociation constant of BEK for bFGF was determined by saturation binding analysis of clone CHO bek 3.7-5 with iodinated bFGF. Scatchard analysis of binding gives a straight line, indicating a single class of binding sites on these cells. From these data, bek 3.7-5 was calculated to have 40,000 receptors per cell with a K_d of 21 pM (Fig. 3). The K_d was equal to that for our previously described CHO clone 3-4, a CHO line expressing a similar number of two-immunoglobulin-domain FLG receptors with a K_d of 17 pM for bFGF (5) (Fig. 3).

To determine the relative affinities of bFGF, K-FGF, and aFGF for the BEK receptor, competition curves were generated by measuring the ability of the unlabeled ligands to displace labeled K-FGF. We had previously shown that about 15 times more K-FGF than bFGF is needed to com-

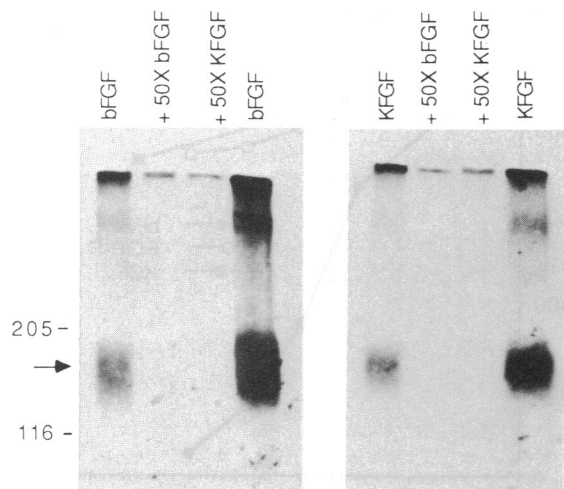

FIG. 2. Crosslinking of ^{125}I-bFGF and ^{125}I-K-FGF to CHO cells expressing the BEK receptor. CHO bek 3.7-5 cells were incubated with the labeled ligand (10 ng/ml) in DMEM with 0.15% gelatin and 25 mM Hepes (pH 7.5). In the lanes marked + 50× bFGF and + 50× KFGF, 500 ng of unlabeled ligand per ml was included. Molecular masses are indicated in kDa. The rightmost lane in each panel was loaded with three times more lysate than the left lane.

petitively inhibit the binding of ^{125}I-bFGF to FLG-expressing CHO cells (5), whereas aFGF is as efficient as bFGF (data not shown). The curves in Fig. 4 show that on CHO bek 3.7-5 cells, K-FGF is as competent as bFGF or aFGF at displacing the ^{125}I-K-FGF. Similar results were obtained in the reverse experiment using ^{125}I-bFGF (data not shown).

In similar experiments we determined the ability of two other members of the FGF family, FGF-5 and KGF, to displace ^{125}I-bFGF binding from clone CHO 3-4 (FLG-expressing) cells and clone CHO bek 3.7-5 (BEK-expressing) cells. The mitogenic activity of FGF-5 was tested on NIH 3T3 and BALB 3T3 cells to ensure that there was no loss of activity of the growth factor. The mitogenic activity of KGF was tested on human cervical epithelial cells 183ATLL1 and 183BTLLI. Neither KGF nor FGF-5 can displace ^{125}I-bFGF from CHO 3-4 (FLG) cells (Fig. 5*A*). On CHO bek 3.7-5 cells, KGF and FGF-5 are unable to displace the labeled bFGF (Fig. 5*B*). At a concentration of 100 ng/ml bFGF displaced >90% of the ^{125}I-bFGF bound to these cells, whereas KGF and FGF-5

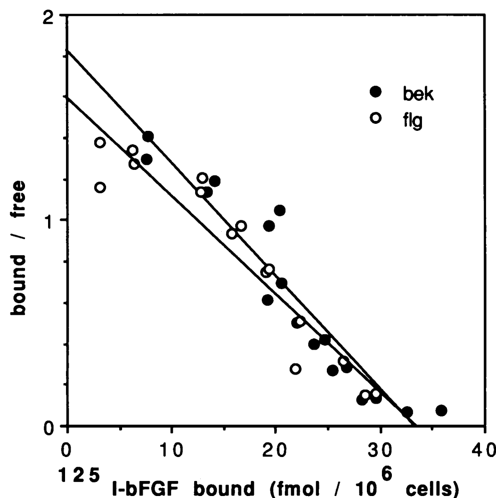

FIG. 3. Saturation binding analysis of ^{125}I-bFGF on CHO 3-4 (flg) and on CHO bek 3.7-5 cells. Cells were plated at 2×10^5 cells per 35-mm dish and incubated for 2 hr at 4°C with ^{125}I-bFGF. Scatchard analysis of the high-affinity binding is presented.

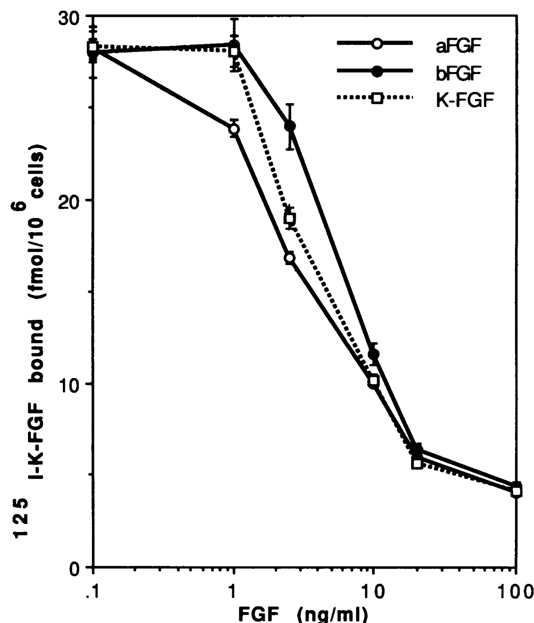

FIG. 4. Competition assay. Competition for binding of ^{125}I-K-FGF (10 ng/ml) by bFGF, K-FGF, and aFGF on CHO bek 3.7-5 cells. All assays were performed in the presence of heparin (10 μg/ml).

displace, at best, 15% of the labeled ligand, indicating an affinity at least 100-fold lower than that of bFGF.

Receptor Activation. To ensure that our cDNA clone encoded a functional receptor, we analyzed ligand-dependent receptor tyrosine phosphorylation in CHO bek 3.7-5 cells. Clone CHO bek 3.7-5 and CHO parental cells stimulated with bFGF and K-FGF were extracted and tested by Western analysis with antiphosphotyrosine antibodies. A band of ≈140 kDa was seen in BEK clones treated with bFGF and K-FGF at 10 ng/ml but not in those treated with platelet-derived growth factor or in untreated cells (data not shown). Thus FGF stimulation of CHO cells expressing the FGF bek receptor leads to the phosphorylation of a protein whose molecular mass corresponds to the receptor that is crosslinked to ^{125}I-bFGF and ^{125}I-K-FGF. The CHO bek 3.7-5 cells also show an increase in the rate of DNA synthesis in response to K-FGF or bFGF in a similar fashion to CHO 3-4 (flg) cells (data not shown). Additionally, CHO bek 3.7-5 cells exhibit an increase in cell number in response to bFGF and K-FGF at 10 ng/ml in defined medium (data not shown). Although the response is weak, with a cell doubling time up to 40 hr, it is reproducible. The addition of heparin at 10 μg/ml to the growth assay made no noticeable difference in the growth curves.

***bek* Expression in Transfected 32D Cells.** The *bek* cDNA was cloned into the pZip-NeoSV(X)1 vector (23) and introduced into 32D cells. The 32D cells are murine myeloid cells that are negative for *bek* mRNA by Northern analysis and express no detectable FGF receptors as determined by their inability to bind ^{125}I-bFGF (unpublished observations). These cells are dependent on IL-3 for their proliferation and survival (20). Clones expressing *bek* mRNA were tested for their ability to bind ^{125}I-bFGF. Two clones, 32D bek B3 and 32D bek B5, expressed about 3000 receptors per cell (data not shown) and were used for further studies.

32D cells grow extremely poorly when the IL-3 concentration is reduced to about 10% of the optimal concentration for growth, and FGFs with or without heparin are not mitogenic (see Fig. 7 and data not shown). When we tested the ability of BEK-expressing 32D cells to grow in the presence of bFGF or K-FGF in lowered IL-3 conditions, no

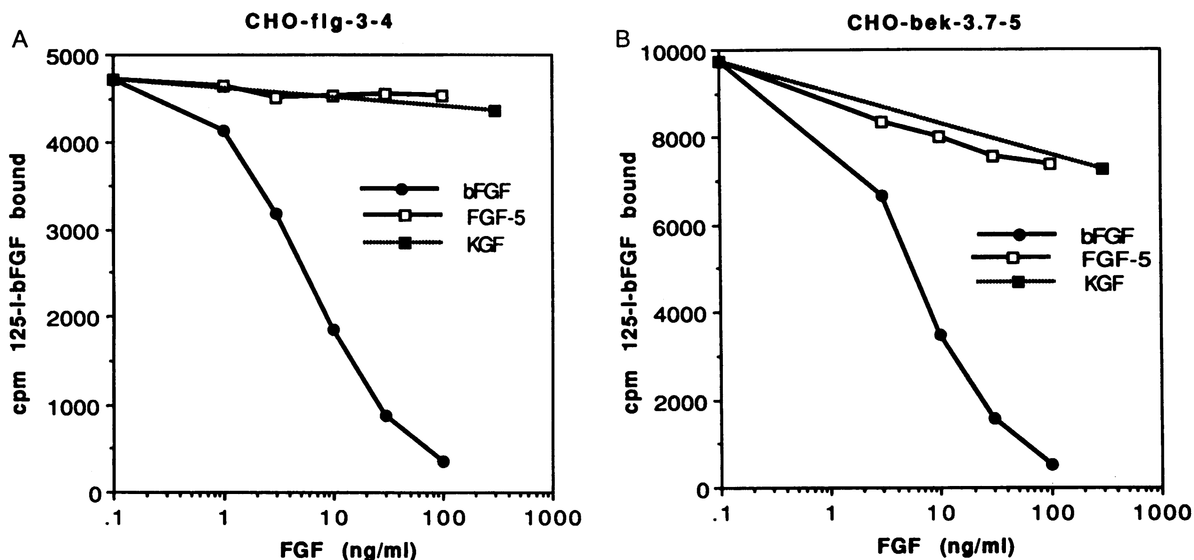

FIG. 5. Competition for binding of ^{125}I-bFGF by bFGF, FGF-5, and KGF on CHO flg 3-4 cells (*A*) and on CHO bek 3.7-5 cells (*B*).

proliferation was observed even in the presence of very high concentrations (1 μg/ml) of the growth factors. However, the addition of heparin (50 μg/ml) made a dramatic difference and the cells grew in the presence of K-FGF (50 ng/ml) plus heparin as readily as in the presence of 10 units of IL-3 per ml (Fig. 6). A similar effect was seen in the presence of bFGF plus heparin, although the growth was somewhat slower than in the presence of 10 units of IL-3 per ml (Fig. 6). We have been unable to sustain prolonged growth of these cells with FGFs and heparin in the complete absence of IL-3. The reasons for this situation are not clear but could result from the existence of a specific IL-3 signal transduction pathway that is necessary for cell survival.

To determine whether the heparin-dependent growth of the 32D bek cells was due to heparin-dependent binding of the ligand to the BEK receptor, we performed crosslinking experiments. ^{125}I-bFGF was crosslinked to 32D bek B3 cells in the presence and absence of heparin. A crosslinked band of ≈160 kDa corresponding to the size of the ligand–receptor complex was seen in the presence of heparin. Although a faint band was visible in the absence of heparin, this complex was dramatically increased upon addition of heparin and was competively inhibited by excess unlabeled bFGF (Fig. 7). This band was absent in 32D parental cells that were similarly treated (data not shown). 32D cells expressing the FLG receptor showed identical results—i.e., they only grew in the presence of FGFs and heparin—and the binding of iodinated bFGF was almost completely heparin dependent (data not shown). We did not detect any difference in the growth responses of 32D flg and 32D bek cells to K-FGF. This result is somewhat surprising given the lower affinity K-FGF has for the FLG receptor. However, FLG-expressing CHO cells also do not differ in their mitogenic response to K-FGF or bFGF in spite of the difference in affinity for the two growth factors (5).

DISCUSSION

We have demonstrated that the murine BEK receptor expressed in CHO cells is activated by three growth factors of the FGF family, bFGF, aFGF, and K-FGF, whereas it does not bind FGF-5 or KGF to any appreciable extent. This last result is not surprising in the case of KGF, since a distinct receptor has been identified for KGF from keratinocytes that does not bind bFGF (17). In addition, KGF is known to act specifically on epithelial cells and to have no effect on fibroblast-like NIH 3T3 cells (26), which express BEK and FLG. The lack of competition by FGF-5 is somewhat harder to explain given that FGF-5 is mitogenic for NIH 3T3 and BALB 3T3 cells. One possible explanation may be that FGF-5 interacts with a BEK/FLG heterodimer and the CHO cells used express only one or the other receptor. Alternatively, FGF-5 may interact with another FGF receptor on NIH 3T3 cells or be unable to interact with the endogenous low-affinity receptors present in CHO cells. The affinity of K-FGF for BEK is the same as bFGF, whereas the affinity

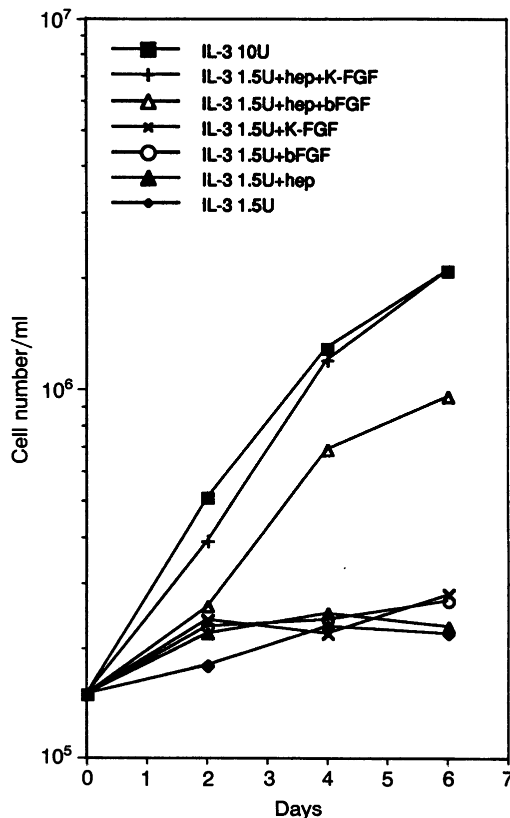

FIG. 6. Growth curves of 32D bek B5 cells. Growth factors were added at the following concentrations: K-FGF, 50 ng/ml; bFGF, 30 ng/ml; heparin (hep), 50 μg/ml. IL-3 units are units/ml. The same results were obtained using 32D bek B3 cells.

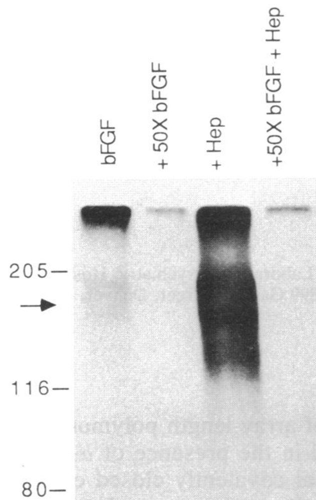

FIG. 7. Crosslinking of ^{125}I-bFGF to 32D bek B3 cells. In each lane ^{125}I-bFGF was added at 10 ng/ml; + 50× bFGF = 500 ng of unlabeled bFGF per ml; + Hep = 10 μg of heparin per ml. Molecular masses are indicated in kDa.

of K-FGF for FLG is ≈15× lower than that of bFGF. Thus, at present, BEK is the highest-affinity receptor known for K-FGF.

Yayon *et al.* (18) have shown that the binding of bFGF to FLG requires an obligatory interaction with HSPGs or low-affinity receptors on the cell surface. Although heparan sulfate has been shown to bind the FGFs and play indirect roles in FGF function, such as stabilization and storage of the growth factor (reviewed in ref. 1), its physiological significance was probably underestimated until this direct demonstration of its obligatory role in bFGF binding to the FLG receptor.

Since 32D cells grow in suspension and lack an extracellular matrix, they are likely not to have the HSPGs that are usually cell surface-matrix associated. In fact, these cells have no detectable mRNA for syndecan, a low-affinity FGF receptor that is a HSPG (27, 28) and is a potential candidate molecule for the endogenous HSPG that cooperates with FLG. The murine FLG receptor, expressed in another IL-3-dependent myeloid cell line, FDC-P1, showed an absolute requirement for heparin for receptor binding and growth (29). Our studies with 32D cells demonstrate that the murine BEK receptor, like FLG, requires the presence of HSPGs in order to activate signal transduction and cell growth.

Thus, when the *bek* cDNA is introduced in CHO cells that express HSPGs, it is capable of binding and of being activated by FGFs, in the absence of exogenous heparin. In 32D cells that do not express low-affinity receptors (HSPG), BEK cannot bind FGF and the cells do not proliferate unless heparin is added to the medium. Presumably, heparin-bound FGFs undergo a conformational change that is essential for "presenting" the growth factor to the receptor. It should be noted that a low level of binding to the BEK receptor can be detected even in the absence of heparin (Fig. 7). The significance of this low-level binding, which is competitively inhibited by unlabeled bFGF, is unclear, given the strict growth dependence of these cells on added heparin. It may be due to other low-affinity receptors present on 32D cells that can partially substitute for the HSPGs and allow a weak interaction between bFGF and the BEK receptor. Alternatively, it is possible that FGFs can bind the BEK receptor with low efficiency even without the conformational changes induced by heparin or HSPGs but that this type of ligand–receptor interaction is unstable. In any case, binding in the absence of heparin is insufficient to activate the BEK mitogenic response in BEK-expressing 32D cells. Although the precise nature of the interaction between these three species remains to be elucidated, it is evident that the biological effects of the FGFs may be regulated at several levels—by the presence of the growth factor, its affinity for the type of tyrosine kinase receptor, and its interaction with the HSPG components that are required to present the growth factor to its receptors.

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