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Cell Transformation by Fibroblast Growth Factors Can Be Suppressed by Truncated Fibroblast Growth Factor Receptors

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Ligand-induced dimerization and transphosphorylation are thought to be important events by which receptor tyrosine kinases generate cellular signals. We have investigated the ability of signalling-defective, truncated fibroblast growth factor (FGF) receptors (FGFR-1 and FGFR-2) to block the FGF response in cells that express both types of endogenous FGF receptors. When these dominant negative receptors are expressed in NIH 3T3 cells transformed by the secreted FGF-4, the transformed properties of the cells can be reverted to various degrees, with better reversion phenotype correlating with higher levels of truncated receptor expression. Furthermore, truncated FGFR-2 is significantly more efficient at producing reversion than FGFR-1, indicating that FGF-4 preferentially utilizes the FGFR-2 signalling pathway. NIH 3T3 clones expressing these truncated receptors are more resistant to FGF-induced mitogenesis and also exhibit reduced tyrosine phosphorylation upon treatment with FGF. The block in FGF-signalling, however, can be overcome by the addition of excess growth factor. The truncated receptors have binding affinities that are four- to eightfold lower than those of wild-type receptors, as measured by Scatchard analysis. We also observed a partial specificity in the responses of truncated-receptor-expressing clones to FGF-2 or FGF-4. Our results suggest that the block to signal transduction produced by kinase-negative FGF receptors is achieved through a combination of dominant negative effects and competition for growth factor binding with functional receptors.

Fibroblast growth factors (FGFs) are heparin-binding polypeptides which exert their biological action through binding and activating high-affinity cell surface receptors that have an intrinsic tyrosine kinase activity (2). Growth factor binding induces receptor dimerization, transphosphorylation, and subsequent association with cytoplasmic signalling molecules leading to DNA synthesis or differentiation (24). The FGF receptors are a family of related but distinct membrane-spanning tyrosine kinases. Four FGF receptors, flg or FGFR-1, bek or FGFR-2, FGFR-3, and FGFR-4, have been identified in different species (7, 10, 11, 18). The extracellular region of these receptors has two or three immunoglobulin (Ig)-like domains, and the intracellular region is composed of a split tyrosine kinase domain. FGFR-1 and FGFR-2 generate multiple mRNA transcripts via alternative splicing, thus giving rise to structurally diverse receptor molecules which can have different ligand binding properties (14, 25).

Both 3-Ig domain and 2-Ig domain FGFR-2 forms bind at least three members of the FGF family, FGF-1, FGF-2, and FGF-4, with nearly equal affinities (5, 12). Both 3-Ig domain and 2-Ig domain FGFR-1 forms bind FGF-1 and FGF-2 with equal affinities (8). FGFR-1 also binds FGF-4, but with a 10-fold-lower affinity compared with the binding of FGF-1 and FGF-2 (13). Thus, the first Ig domain is dispensable for high-affinity binding. The binding of FGFs to the FGF receptors is enhanced by heparan-sulfate proteoglycans, which are components of the extracellular matrix and constitute the low-affinity binding site for FGFs (17, 27). Furthermore, the frequent coexpression of FGFR-1 and FGFR-2 in the same cells raises the question of why such functional overlap exists in this system.

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FGF receptor activation is associated with phosphorylation of the receptor itself and of downstream substrates, not all of which been identified, culminating in a cascade of signal transduction which leads to various physiological responses (24). Studies using kinase-negative mutant receptors have helped to gain insights into FGF receptor signal transduction as well as the roles of FGFs in development. The tyrosine kinase activity of a *Xenopus* FGF receptor was eliminated by either a point mutation in the ATP-binding site or a deletion (truncation) of the cytoplasmic region (Δ). Such receptors have an inhibitory effect on FGF-mediated activities such as mesoderm formation when expressed in *Xenopus* embryos (1). Moreover, targeted expression of ΔFGFR-1 in the epidermis of transgenic mice disrupts the differentiation and morphogenesis of epidermal keratinocytes (26). When ΔFGFR-1 mRNA was coinjected with wild-type mRNA into *Xenopus* oocytes, the mutant receptor appeared to inhibit not only the function of wild-type FGFR-1 but also those of FGFR-2 and FGFR-3, as it abolished Ca^{2+} efflux upon FGF stimulation (23). Inhibition of wild-type receptor function by a mutant receptor is therefore termed a "dominant negative" effect, which is presumably mediated according to the receptor oligomerization model (24), via formation of a nonfunctional heterodimer between mutant and wild-type receptors. This has been shown in the case of the epidermal growth factor (EGF) receptor, with heterodimers between mutant and wild-type receptors not being phosphorylated (9). Autophosphorylation of FGF receptors, after treatment with FGFs, also seems to be mediated by an intermolecular mechanism, since a kinase-negative full-length mutant FGF receptor was found to be phosphorylated when coexpressed with a wild-type receptor (3). This transphosphorylation can also occur between two different FGF receptor species, FGFR-1 and FGFR-2.

To investigate the signalling pathways activated by FGFs, as

well as the specificity of FGF receptor activation by different members of the FGF family, we have introduced mutant forms of FGFR-1 and FGFR-2 into NIH 3T3 cells transformed by the k-fgf (FGF-4) oncogene. The mutant receptors are truncated after the transmembrane region and lack the entire tyrosine kinase domain. The FGF-4 gene encodes a secreted growth factor of the FGF family, and its expression results in transformation of NIH 3T3 cells, which express FGFR-1 and FGFR-2, by creating an autocrine growth loop (6, 22). We were able to show that the phenotype of FGF-4-transformed cells could revert as a result of expression of high levels of truncated FGFR-1 and FGFR-2 and that the truncated FGFR-2 was significantly more efficient at causing reversion of the transformed phenotype than FGFR-1. The same truncated receptor forms can make NIH 3T3 cells resistant to the mitogenic effects of FGF-4 and FGF-2. These and other results indicate that FGF-4 preferentially utilizes FGFR-2 and that truncated FGF receptors inhibit signal transduction through the formation of inactive heterodimers and by competition for growth factor binding with functional receptors.

MATERIALS AND METHODS

Cell lines. The 8bG and KDEL-2b cells have been previously described (22). Both were derived by transfecting NIH 3T3 murine fibroblasts with a cDNA encoding the FGF-4 growth factor and have transformed properties. The 8bG cell line secretes high levels of wild-type FGF-4, while the KDEL line secretes small amounts of FGF-4 since the cDNA was mutated to include the KDEL sequence which prevents efficient secretion of the growth factor. This short sequence at the C terminus is sufficient to cause retention of a normally secreted protein in the endoplasmic reticulum (16). CHO cell lines expressing FGFR-1 (CHO flg 4-1) or FGFR-2 (CHO bek 3-7) have also been previously described (12, 13).

Plasmids and transfections. The murine cDNAs for FGFR-1 (flg) and FGFR-2 (bek) have been previously described (12, 13). To construct the FGFR-1 truncated receptor, a stop codon was generated downstream of the transmembrane domain, resulting in a 340-amino-acid protein that encodes the entire extracellular 2-Ig domain form with 32 amino acids of the intracellular region. The 3-Ig domain form of the FGFR-2 receptor was truncated after 472 amino acids which include the entire 3-Ig domain extracellular portion and 74 amino acids of the intracellular region. Both truncated cDNAs were cloned into the *Bam*HI site of the pCEP4 expression vector (Invitrogen).

The transfections were performed by the calcium-phosphate precipitation method, and clones were selected in 400 μg of hygromycin B (Calbiochem) per ml.

DNA synthesis assays. About 1.5×10^4 cells per well were plated in 48-well dishes and starved the following day in Dulbecco modified Eagle medium–0.5% serum for 48 h. The indicated amounts of growth factor were added, and 12 h later cells were labelled with 0.5 μCi of [^{3}H]thymidine per ml (6.7 Ci/mmol) for 6 h. Medium was removed, and cells were gently washed once with Tris-buffered saline. Ice-cold 10% trichloroacetic acid was added for 10 min, and the cells were washed again in Tris-buffered saline. Sodium hydroxide (0.5 N; 0.127 ml) was added to each well at room temperature. The solution was neutralized with the addition of 0.127 ml of 0.5 N hydrochloric acid and transferred into scintillation vials. Scintillation counting fluid (ReadySafe; Beckman) was added to each vial (2.5 ml) and counted in a Beckman scintillation counter.

Growth in soft agar. Five thousand single cells were plated in 65-mm-diameter dishes in 1.5 ml of Dulbecco modified Eagle medium with 10% calf serum and 0.34% agar on top of 8 ml of the same medium containing 10% tryptose phosphate broth and 0.5% agar. The plates were incubated at 37°C, and the growth factor was added at 100 μl to the center of each plate. Colonies were counted 14 days later.

Cross-linking. ^{125}I-FGF-2 was prepared by mild chloramine T treatment and purified over a Sephadex G-25 column. The specific activity of ^{125}I-FGF-2 was 4×10^4 cpm/ng (800 cpm/fmol). Five million cells were washed twice in ice-cold phosphate-buffered saline (PBS) and resuspended in 0.5 ml of binding medium (Dulbecco modified Eagle medium–25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] [pH 7.4]–0.15% gelatin)–^{125}I-FGF-2 at 10 ng/ml. Unlabelled FGF-2 (1 μg/ml) was included at a 100-fold excess as a competitor in parallel experiments. The binding was performed for 2 h at 4°C with gentle agitation of samples. The cells were washed twice in ice-cold PBS and cross-linked with disuccinimidyl suberate (DSS; Pierce) (3 mM in PBS) for 30 min at room temperature. The cell pellet was resuspended in 1× sample buffer, sonicated, boiled, and loaded on a sodium dodecyl sulfate (SDS)–8% polyacrylamide gel. The gel was fixed for 30 min in 7% acetic acid, dried, and autoradiographed.

Scatchard analysis. Twenty thousand cells were plated in 65-mm-diameter dishes and incubated at 4°C in Dulbecco modified Eagle medium containing 0.15% gelatin, 25 mm HEPES (pH 7.4), 10 μg of heparin per ml, and various concentrations of iodinated FGF-2 from 0.06 to 10 ng/ml. After gentle shaking for 2 h, the medium was removed, cells were washed in ice-cold PBS, and radioactivity bound to the extracellular matrix was removed by washing twice with cold 2 M NaCl–25 mM HEPES (pH 7.4). Radioactive FGF-2 bound to high-affinity receptors was eluted with two washes with 2 M NaCl–25 mM sodium acetate (pH 4). Counts were quantitated on a γ scintillation counter (LKB). Cells in parallel cultures were counted by using a Coulter particle counter.

RESULTS

Expression of truncated FGF receptors causes phenotypic reversion of FGF-4-transformed NIH 3T3 cells. FGF-4 can transform NIH 3T3 cells via an autocrine loop mediated by endogenous FGF receptors, which can be interrupted by anti-FGF-4 neutralizing antibodies. In the presence of the antibody, FGF-4-transformed cells show phenotypic reversion, i.e., a flatter, contact-inhibited morphology, and are no longer serum independent for growth, indicating that the growth factor-receptor interaction occurs in the extracellular compartment (22). We wished to determine if the introduction of FGF receptors lacking the kinase domain could achieve a reversion of the transformed properties of these cells. A truncated 2-Ig domain form of murine FGFR-1, which has a stop codon after amino acid 340, and a truncated 3-Ig domain form of FGFR-2, which is truncated after amino acid 472, were cloned in the pCEP4 vector under the control of the cytomegalovirus promoter. The pCEP4 vector contains the hygromycin resistance gene as a selectable marker. Two FGF-4-transformed lines secreting high and low levels of FGF-4 were used as recipients for the kinase-negative mutant FGF receptors, ΔFGFR-1 and ΔFGFR-2. 8bG cells are FGF-4-transformed NIH 3T3 cells which express and release high levels of FGF-4 and exhibit a highly transformed phenotype, as assessed by their ability to grow in soft agar and in serum-free medium; KDEL-2b cells secrete lower levels of FGF-4 because they express a mutated FGF-4 which is inefficiently secreted (22).

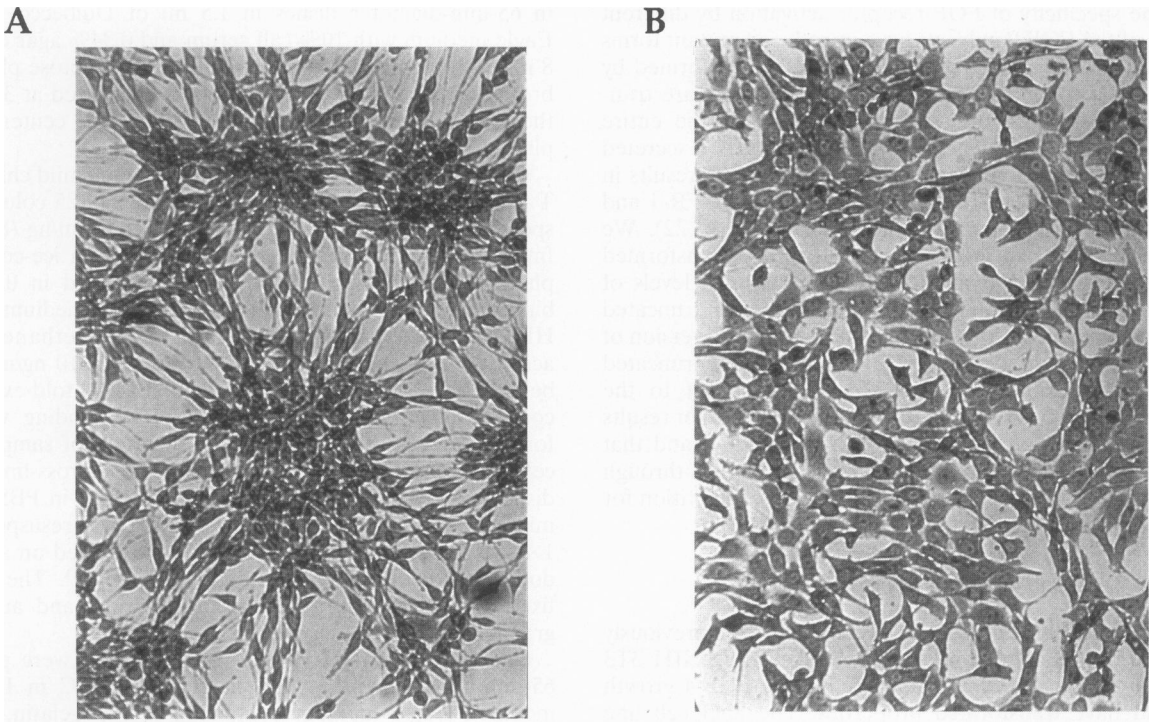

FIG. 1. Morphologies of FGF-4-transformed 8bG cells and one of its revertant derivatives. Cells were fixed in 10% formalin and stained with Giemsa. (A) 8bG parental cells are spindle-shaped and form foci. (B) 8bG-1rev is a typical revertant clone with a flat morphology. Magnification, ×6.

Each ΔFGFR construct was introduced into 8bG and KDEL cells, and the hygromycin-resistant clones from each transfection were observed and counted. Most colonies still had the spindle-shaped transformed phenotype of the parental cells. However, a subset of these showed a phenotypic reversion in that they appeared untransformed, flat, and contact inhibited as do NIH 3T3 cells (Fig. 1). The data in Table 1 show that, in both cell lines, ΔFGFR-1 and ΔFGFR-2 produced phenotypically revertant colonies. However, 8% of the colonies transfected with ΔFGFR-2 showed revertant morphology, while only 2% of the colonies transfected with ΔFGFR-1 appeared to have undergone phenotypic reversion, and this difference was reproducible in three different experiments. Thus, both receptors can produce morphological revertants in a subset of the transformed cells, but with a fourfold higher efficiency for ΔFGFR-2 than for ΔFGFR-1 (Table 1). Furthermore, the phenotypic reversion of the cells expressing FGFR-2 was more complete than that of cells expressing FGFR-1 (see below).

TABLE 1. Phenotypic reversion of FGF-4-transformed cells by truncated FGFR-1 and FGFR-2 receptors

Cell line	No. of revertant clones/total[a]		% Reversion[b]	
	ΔFGFR-1	ΔFGFR-2	ΔFGFR-1	ΔFGFR-2
KDEL-2b	22/1,011	73/862	2.18	8.47
8bG	32/1,147	101/1,380	2.80	7.32

[a] The indicated truncated (Δ) FGF receptor construct was used to transfect the recipient cells, and hygromycin-resistant clones were selected. Drug-resistant clones displaying a morphologically reverted phenotype were counted.

[b] Number of phenotypically revertant colonies divided by the total number of drug-resistant colonies.

To determine whether the morphological reversion of cells was correlated with expression of truncated receptors, Northern (RNA) analysis was performed on 18 revertant clones with cDNA probes for FGFR-1 and FGFR-2 as well as FGF-4. In 13 clones expressing the ΔFGFR-2 mRNA, higher expression of the truncated receptor mRNA was seen in the clones with the better revertant phenotype. Of five ΔFGFR-1 revertants, two had lost the ability to produce FGF-4 and the other three expressed the truncated receptor. In addition, full-length endogenous FGFR-1 and FGFR-2 receptor transcripts were detected in all the clones tested and could be distinguished from the truncated receptors on the basis of size differences. The ratios of mRNA levels of the truncated receptors to those of the endogenous receptors are shown in Table 2. To further confirm that the reversion of transformed morphology is mediated by expression of the truncated receptors and not merely by loss of the growth factor, we assayed FGF-4 activity in the conditioned medium from the revertant clones. Since FGF-4 causes an observable morphological transformation of NIH 3T3 cells, the presence of the growth factor can be easily detected in conditioned medium from the revertant clones. Conditioned medium was collected from comparable numbers of the clones and applied in serial dilutions onto NIH 3T3 cells that had been starved overnight. With two exceptions, all the clones tested were still secreting FGF-4 in amounts comparable to those for the parental clones (data not shown).

To assess the degree of reversion of the revertant clones more quantitatively and stringently, we tested the growth of the clones in soft agar. Table 3 shows the number of colonies obtained from each revertant clone. The parental cells 8bG and KDEL form large numbers of colonies in agar, indicating a high degree of transformation, although under these conditions KDEL cells grow less efficiently than 8bG cells. Rever-

TABLE 2. Cell lines used in this study

Cell line	Transfected FGF receptor	Transfected/endogenous receptor ratio[a]
8bG		
8bG-1rev	ΔFGFR-2	1:1
8bG-2rev	ΔFGFR-2	2:1
8bG-3rev	ΔFGFR-2	2:1
8bG-5rev	ΔFGFR-1	1:1
KDEL		
KDEL-1rev	ΔFGFR-2	1:1
KDEL-2rev	ΔFGFR-2	1:1
KDEL-3rev	ΔFGFR-2	1:1
KDEL-4rev	ΔFGFR-2	2:1
KDEL-5rev	ΔFGFR-2	2:1
KDEL-6rev	ΔFGFR-2	3:1
NIH 3T3		
3T3vec		
3T3flg12	ΔFGFR-1	1:1
3T3flg7	ΔFGFR-1	0.5:1
3T3bek8	ΔFGFR-2	1:1
3T3bek18	ΔFGFR-2	5:1
3T3bek21	ΔFGFR-2	3:1
CHO		
CHObek3.7	FGFR-2	
CHOflg4-1	FGFR-1	

[a] Ratio of the transfected-receptor mRNA level to the corresponding endogenous-receptor mRNA level, as assessed by Northern analysis.

TABLE 3. Growth of revertant 8bG and KDEL-2b clones in soft agar

Cells or clone	No. of colonies/plate (%)[a]
NIH 3T3	<1
8bG[b]	3,513 (100)
8bG-1rev[c]	2,915 (83)
8bG-2rev[c]	2,593 (73)
8bG-3rev[c]	2,610 (74)
8bG-5rev[d]	4,200 (119)
KDEL-2b[b]	2,086 (100)
KDEL-1rev[c]	35 (1.6)
KDEL-2rev[c]	41 (1.9)
KDEL-3rev[c]	35 (1.6)
KDEL-4rev[c]	766 (36)
KDEL-5rev[c]	<1
KDEL-6rev[c]	<1

[a] Ten thousand cells of each clone were plated in soft agar in 60-mm-diameter dishes. Colonies were counted after 14 days.
[b] Parental cells express FGFR-1 and FGFR-2.
[c] Expresses ΔFGFR-2.
[d] Expresses ΔFGFR-1.

tants derived from KDEL cells form fewer colonies than those from 8bG cells. The 8bG revertant clones 8bG-1rev, 8bG-2rev, and 8bG-3rev exhibit the normal, flatter shape of NIH 3T3 fibroblasts, show contact inhibition, and do not form foci in prolonged culture. However, they are only slightly inhibited (17 to 26%) in soft-agar growth compared with their parental cell line. In contrast, KDEL revertants exhibit a weakly transformed phenotype, as judged by the larger decrease (63 to 98%) in the number of colonies in soft agar. The simplest interpretation of the results is that the expression of truncated receptors is more efficient at producing reversion of transformation in the cells which secrete a smaller amount of the growth factor. This effect is evident when a stringent transformation parameter (growth in agar) is used, while it is almost undetectable when morphological changes are measured.

To further confirm this conclusion, we performed a DNA synthesis experiment with the revertant clones to assess their ability to respond to exogenously added FGF. 8bG revertants such as 8bG-1rev and 8bG-2rev are serum independent for growth and therefore did not become quiescent in medium containing a small amount of serum (0.5% calf serum). These cells showed no significant increase in [^{3}H]thymidine incorporation in response to exogenous FGF-2 and FGF-4. In contrast, KDEL revertants showed decreased [^{3}H]thymidine incorporation compared with parental cells under low-serum conditions. They also show an increase in DNA synthesis in response to exogenous FGFs but require a dose higher than that for NIH 3T3 cells to achieve a similar response. The results imply that reversion by truncated receptors can be overcome by an excess of growth factor (data not shown).

Thus, FGF-4-induced transformation can be reverted by truncated FGF receptors. Truncated FGFR-2 is more efficient than FGFR-1 at causing reversion, indicating that FGFR-2 is the receptor preferentially utilized by FGF-4. Complete reversion is obtained only in cells producing a limited amount of growth factor, suggesting that the dominant negative effect of truncated receptors is also caused by competition for growth factor binding with functional receptors.

Expression of ΔFGFR-2 and ΔFGFR-1 in NIH 3T3 cells inhibits the FGF response. To gain more insight into the mechanism by which mitogenic signalling by wild-type FGF receptors is blocked by truncated receptors, we transfected normal NIH 3T3 cells with the constructs expressing ΔFGFR-2 (bek clones) or ΔFGFR-1 (flg clones). Hygromycin-resistant clones were selected, and their resistance to morphological transformation by FGF-4 was measured. Clones 3T3bek18 and 3T3flg12 showed complete resistance to FGF-4 doses as high as 10 ng/ml. Several other clones showed partial resistance to FGF-4. Of these, 3T3bek8 and 3T3flg7 where chosen for further study.

Northern analysis revealed a close correlation between the level of expression of the truncated receptor and the ability of the clones to resist morphological transformation by FGF-4. For example, the level of truncated FGFR-2 transcripts in the 3T3bek18 clone is at least fivefold that of endogenous FGFR-2, compared with an approximately 1-to-1 ratio (truncated form/wild-type form) in the 3T3bek8 clone (Fig. 2). The resistance of the transfected clones to the mitogenic activity of FGFs was confirmed by measuring the incorporation of [^{3}H]thymidine into trichloroacetic acid-precipitable material after incubation of cells with FGF-4 (Fig. 3A). In NIH 3T3 cells, the optimal concentration of FGF-4 for maximal stimulation of DNA synthesis is generally on the order of 1 ng/ml and a higher dose of FGF-4 produces a partial inhibitory effect. Clones expressing ΔFGFR-2 or ΔFGFR-1 exhibit a considerably reduced response to FGF-4. In the 3T3flg7 clone, which has the lowest level of ΔFGFR-1, the maximal response is lower compared with that in NIH 3T3 cells, while in the 3T3flg12 and 3T3bek8 clones the dose required for maximal DNA synthesis is increased to 50 ng/ml. In the 3T3bek18 clone, which has the highest level of the truncated receptor, maximal DNA synthesis is achieved only with 250 ng of FGF-4 per ml. In addition, the doses of FGF-4 required to produce the inhibitory effects on DNA synthesis are also increased in the revertant clones. This high resistance to FGF-4 is not caused by secretion of the truncated form of FGFR-2 extracellularly,

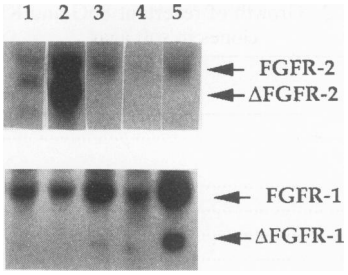

FIG. 2. Northern analysis of NIH 3T3 clones transfected with truncated FGF receptors. RNA was extracted by the guanidine isothiocyanate method, run on formaldehyde gels, and transferred to nitrocellulose. A 2-kb *Eco*RI fragment from FGFR-2 cDNA (upper panel) and a 1.2-kb *Eco*RI-*Bam*HI fragment from FGFR-1 cDNA (lower panel) were used. Lanes 1 and 2, clones transfected with ΔFGFR-2; lanes 3 and 5, clones transfected with ΔFGFR-1. The positions of the endogenous and transfected truncated receptors are indicated. Lane 1, 3T3bek8; lane 2, 3T3bek18; lane 3, 3T3flg7; lane 4, NIH 3T3 cells; lane 5, 3T3flg12. Differences in intensity among endogenous-receptor mRNA bands reflect the variation in the amount of RNA.

since the conditioned medium from 3T3bek18 fails to interfere with the mitogenic activity of FGF-4 on NIH 3T3 cells (data not shown). This suggests that, although truncated receptors interfere with mitogenic signalling through endogenous receptors in NIH 3T3 cells, a complete inhibition of mitogenesis can probably be achieved only when the truncated receptor is expressed in a large excess over endogenous forms.

The clones transfected with truncated ΔFGFR-2 or ΔFGFR-1 appear to exhibit different levels of resistance to FGF-4 and to FGF-2. As shown in Fig. 3B, ΔFGFR-2-transfected clones are somewhat more resistant to FGF-4 than to FGF-2, but ΔFGFR-1 transfectants are more resistant to FGF-2 than to FGF-4. In light of the fact that FGF-2 interacts with both receptor types with equal affinities while the preferential receptor for FGF-4 is FGFR-2, the different abilities of the truncated receptors to block FGF-2 and FGF-4 imply that FGFR-1 and FGFR-2 have a higher potential to form homodimers than to form heterodimers (see Discussion).

Further evidence of a block in FGF signalling in truncated-receptor-expressing clones is provided by soft-agar growth assays. 3T3bek18, 3T3flg12, and 3T3bek21 (which expresses a level of ΔFGFR-2 slightly lower than that expressed by 3T3bek18) as well as NIH 3T3 cells were plated in soft agar and treated with dilutions of FGF-4 or FGF-2 at 1, 10, and 50 ng/ml every 3 days. Colony counts after 2 weeks of growth are shown in Table 4. With control NIH 3T3 cells there was a significant response to all FGF-4 concentrations, with the highest concentration of FGF-4 (50 ng/ml) producing 800 to 1,000 colonies. On the other hand, 50 ng/ml of FGF-4 failed to stimulate soft-agar growth in 3T3bek18 cells, while clones 3T3flg12 and 3T3bek21 did not respond to 1 or 10 ng of FGF-4 per ml and produced only a reduced number of colonies at 50 ng/ml. Thus, clones expressing a higher level of the truncated receptor require a higher dose of growth factor to form agar colonies. A similar experiment with FGF-2 shows the same trend of resistance in these clones. Consistent with the thymidine incorporation data, the data also suggest that ΔFGFR-1- and ΔFGFR-2-expressing clones show different levels of resistance to FGF-2 and FGF-4. 3T3flg12 cells (expressing ΔFGFR-1) appear equally resistant to FGF-2 and FGF-4, while 3T3bek18 and 3T3bek21 (expressing ΔFGFR-2) are more resistant to FGF-4 than to FGF-2.

TABLE 4. Growth of mutant-receptor-expressing NIH 3T3 clones in soft agar with constant FGF treatment

Cells or clone	No. of colonies/plate[a]					
	FGF-4[b]			FGF-2[c]		
	1 ng/ml	10 ng/ml	50 ng/ml	1 ng/ml	10 ng/ml	50 ng/ml
NIH 3T3	117	233	818	77	619	994
3T3vec[d]	105	537	1,062	79	474	1,081
3T3flg12	<1	<1	350	<1	81	230
3T3bek21	<1	<1	163	38	74	182
3T3bek18	<1	<1	<1	<1	55	139

[a] Cells were plated in soft agar as described in Materials and Methods. Colonies were counted at the end of 2 weeks.
[b] Added every 4 days.
[c] Added every 3 days.
[d] Control clone transfected with the vector alone.

Truncated receptors ΔFGFR-2 and ΔFGFR-1 are expressed on the cell surface and inhibit receptor tyrosine phosphorylation. Clones 3T3bek18 and 3T3flg12 were incubated with radiolabelled FGF-2 and then treated with a chemical cross-linking agent, BS3. As shown in Fig. 4, there are two cross-linked bands around 148 and 160 kDa present in the clones which are also present in the control cells NIH 3T3 and 3T3vec (NIH 3T3 cells transfected with the PCEP4 vector alone), whose sizes correspond to the expected sizes of the cross-linked products of ^{125}I-labelled FGF-2 plus the endogenous 2-Ig domain and 3-Ig domain receptor forms of FGFR-1 or FGFR-2. Two faster-migrating bands around 115 and 90 kDa, the molecular sizes of which correspond to the estimated molecular size of ^{125}I-FGF-2 plus ΔFGFR-2 and ΔFGFR-1, appear in 3T3bek18 and 3T3flg12, respectively (Fig. 4). The level of ΔFGFR-2 is much higher than that of endogenous FGFR-2 in 3T3bek18 cells. In addition, a band of approximately 230 kDa appears in the 3T3bek18 clone, most probably representing a homodimer of ΔFGFR-2 and ^{125}I-FGF-2 (Fig. 4, lane 9). This band was not recognized by the anti-FGFR-2 antiserum (directed against the C-terminal tail of the receptor, which is absent in the truncated receptor), indicating that it does not contain full-length FGFR-2. Thus, cross-linking experiments reveal that the truncated receptors are expressed at the cell surface and that they are able to bind the growth factor, as well as to form dimers upon treatment with FGF. We did not observe the heterodimer between the endogenous receptor and the truncated form, a result which, on one hand, may be due to relatively low levels of endogenous receptor expression compared with that for the truncated receptor and/or, on the other hand, also may be due to the inefficiency of the cross-linking reagent.

Like other receptor tyrosine kinases, the FGF receptors are activated by phosphorylation on tyrosine residues upon exposure to a ligand. Activation of FGF receptors also leads to increased tyrosine phosphorylation of a number of cellular proteins including a 90-kDa protein unique to the FGF receptor signalling pathways (4, 13). In order to assess the blocking activity of the truncated FGF receptors on the endogenous wild-type receptors, we used a Western blot (immunoblot) with antiphosphotyrosine antibodies. Clones 3T3bek18 and 3T3flg12 and control cells were exposed to 5 ng of FGF-4 per ml for 10, 25, and 50 min, and phosphotyrosine-containing proteins were visualized. Tyrosine phosphorylation in FGF-4-treated control NIH 3T3 cells was obviously enhanced, especially in a 145-kDa band whose size corresponds to the expected size of the endogenous FGF receptors (Fig. 5).

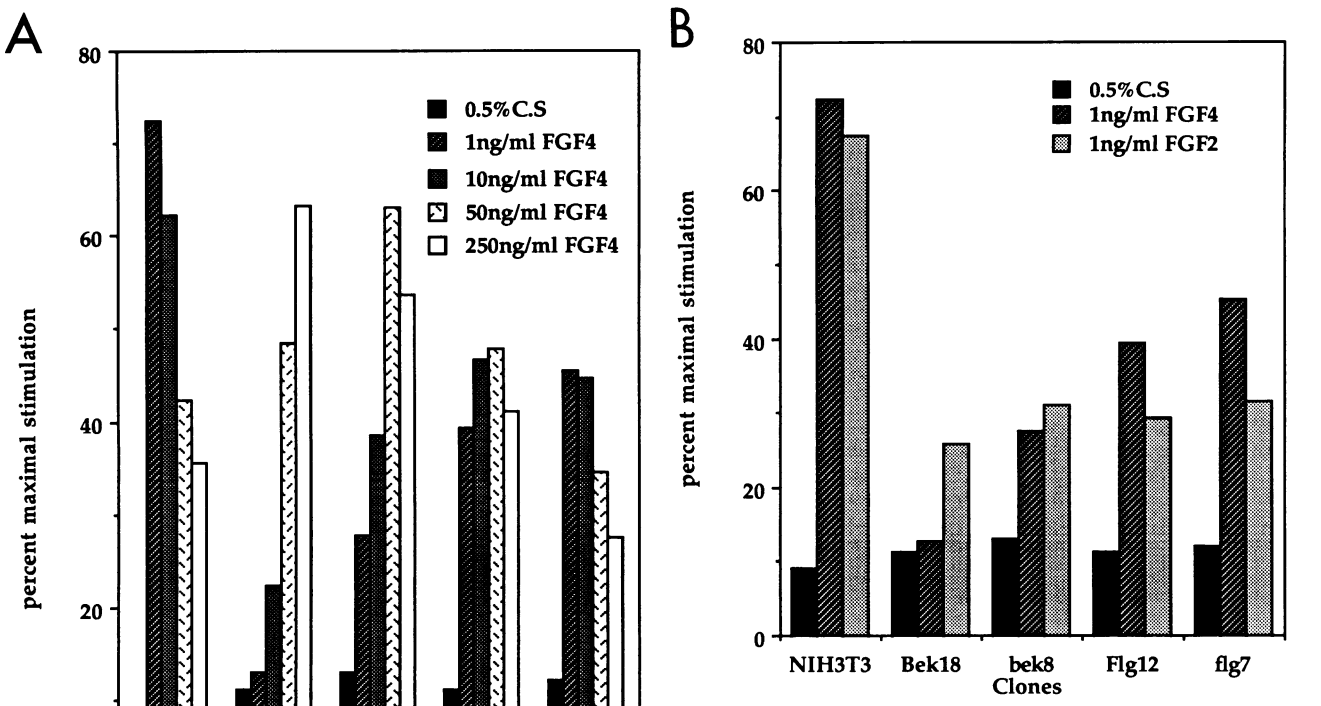

FIG. 3. DNA synthesis in response to FGF-4 or FGF-2 in NIH 3T3 cells expressing a truncated FGF receptor. (A) Response to FGF-4. NIH 3T3 cells were transfected with the same FGF mutant receptor constructs as KDEL and 8bG cells. Four clones expressing different levels of truncated-receptor mRNA relative to endogenous-receptor mRNA levels, as assessed by Northern blots, were subjected to thymidine incorporation assays. The incorporation induced by 10% calf serum (C.S.) was considered maximal (100%). Clone 3T3bek18 expressed a higher level of ΔFGFR-2 than clone 3T3bek8. Clone 3T3flg12 expressed more of ΔFGFR-1 than clone 3T3flg7. (B) Comparison of responses to FGF-4 with responses to FGF-2.

The ΔFGFR-1-expressing clone, 3T3flg12, showed enhanced phosphorylation of the 145-kDa band compared with control cells, while in the ΔFGFR-2-expressing 3T3bek18 cells FGF-4 treatment failed to induce tyrosine phosphorylation of the 145-kDa band. The simplest explanation of this result is that the ratio of truncated to endogenous receptors in 3T3flg12 cells is not high enough to completely prevent activation of the endogenous receptor by FGF-4. However, the phosphorylation of faster-migrating bands at 90, 60, and 44 kDa, which are likely to be downstream target proteins, is blocked in these cells. Thus, a partial inhibition of the FGF signalling pathway can be detected in these cells.

Ligand binding properties of truncated receptors. NIH 3T3 cells have ~20,000 FGF receptors per cell, with a dissociation value (K_d) of ~50 pM (2). The expression of ΔFGFR-2 and ΔFGFR-1 proteins on the cell surface was determined by Scatchard analyses of the transfected clones using iodinated FGF-2. In agreement with the Northern blot and cross-linking results, we found that, compared with NIH 3T3 cells, there are more high-affinity binding sites for FGF-2 on the cell surface in 3T3bek18 and 3T3bek21 (approximately eightfold), as well as in 3T3flg12 (approximately two- to fourfold). Surprisingly, the ΔFGFR-2- and ΔFGFR-1-expressing clones have notably higher dissociation constants, as calculated from the slope of the FGF-2 binding curves for these cells (Fig. 6A and B). This higher K_d value (200 to 400 pM) was reproducible in three experiments. This suggests that truncated receptors without a cytoplasmic domain have a lower binding affinity for growth factor. To further confirm this observation and eliminate the effects of the endogenous receptors present in NIH 3T3 cells, we transfected truncated ΔFGFR-2 and ΔFGFR-1 constructs

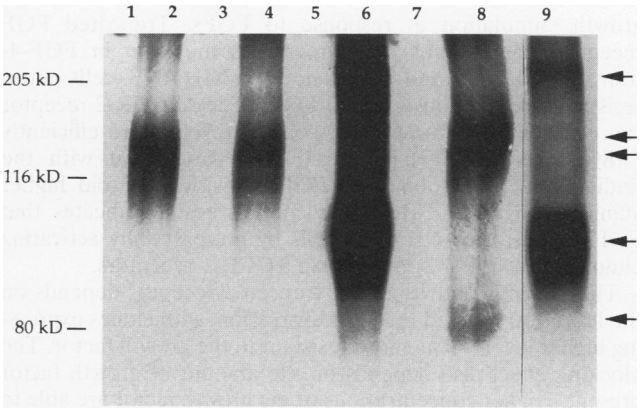

FIG. 4. Cross-linking of radiolabelled FGF-2 to receptors on the cell surface. The bands represent cross-linked products of ^{125}I-FGF-2 and FGF receptors on the cell surface. Arrows, major bands. The sizes of molecular weight markers are indicated on the left. For each cell line, the experiment was performed in parallel with a 50-fold excess of unlabelled FGF-2 (lanes 1, 3, 5, and 7). Lanes 1 and 2, NIH 3T3 cells; lanes 2 and 3, 3T3vec; lanes 5 and 6, 3T3bek18; lanes 7 and 8, 3T3flg12; lane 9, lower exposure of lane 6.

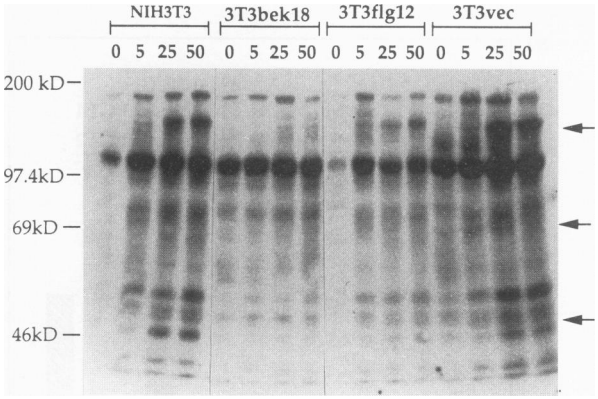

FIG. 5. Tyrosine phosphorylation in mutant-receptor-expressing clones. Cells were starved overnight in 0.5% calf serum and then stimulated with FGF-4 (5 ng/ml) for 5, 25, or 50 min. Cells were lysed, clarified, run on an SDS–8% polyacrylamide gel, and subjected to Western analysis using antiphosphotyrosine antiserum (Upstate Biotechnologies, Inc.). Arrows, major phosphorylated bands. The sizes of molecular weight markers are indicated on the left. The major phosphorylated band at 100 kDa is a nonspecific band.

into CHO cells, which have no detectable endogenous FGF receptor expression (13). Binding analysis was carried out on two pools of hygromycin-resistant clones from each transfection. CHO clones expressing full-length FGFR-1 or FGFR-2, which have been previously described, were used as controls. Scatchard analysis with the truncated-receptor-expressing CHO pools shows binding curves similar to those of transfected NIH 3T3 clones (Fig. 6C and D). In summary, both ΔFGFR-2 and ΔFGFR-1 show decreased (approximately four- to eightfold) binding affinities for FGF-2 in comparison with those of wild-type FGFR-1 and FGFR-2.

DISCUSSION

Truncated FGF receptors inhibit endogenous receptors and block FGF-induced mitogenesis. In the present study we have shown that the expression of truncated FGFR-1 and FGFR-2 in NIH 3T3 cells can inhibit FGF signalling, leading to reduced growth stimulation in response to FGFs. Truncated FGF receptors can disrupt the autocrine stimulation in FGF-4-transformed cells and make normal NIH 3T3 cells more resistant to exogenously added FGFs. The ΔFGFR-2 receptor can cause FGF-4-transformed cells to revert more efficiently (~8%) than ΔFGFR-1 does (~2%). Consistent with the finding that FGF-4 binds to FGFR-2 with a 10-fold higher affinity than to FGFR-1 (12, 13), this result indicates that FGF-4 transforms NIH 3T3 cells by preferentially activating endogenous FGFR-2 rather than FGFR-1 receptors.

The blocking activity of the truncated receptor depends on the level of truncated receptor expression, with clones expressing higher levels being more resistant to the growth factor. The blocking effect also depends on the amount of growth factor present. Higher concentrations of the growth factor are able to overcome the resistance to stimulation in truncated-receptor-expressing NIH 3T3 clones. Accordingly, it was easier to induce reversion of the transformed properties of KDEL cells than it was for the 8bG cells, which secrete a larger amount of FGF-4. The transformed properties of the 8bG cell line did not revert, as assessed by growth in soft agar, but we could observe morphological reversion in 8bG clones expressing the truncated FGFR-2. On the other hand, the reverted KDEL cells are reverted both morphologically and in their ability to grow in soft agar, but they still respond to exogenously added FGF with increased DNA synthesis.

Overall, these results show that growth in soft agar is a more stringent parameter of transformation than is morphology. They also indicate that the dominant negative effect of the truncated receptors in blocking mitogenic signalling can be overcome by treatment with high FGF concentrations. This was also confirmed by the experiments using normal NIH 3T3 cells, in which introduction of the truncated FGFR-1 and FGFR-2 receptors resulted in variable resistance to FGF-induced DNA synthesis, depending on the degree of expression of the truncated receptor and on the concentration of FGF applied to these cells. Thus, even in the most resistant cell line (3T3bek18), treatment with a very high dose of FGF was capable of inducing significant DNA synthesis.

Our results with truncated FGF receptors differ from those of the studies on the truncated EGF receptor. Kashles et al. (9) found that a mutant/endogenous receptor ratio as low as 0.1 to 2 was capable of blocking the EGF response by 25 to 50% over a range of EGF concentrations in DNA synthesis assays. In addition, they were unable to overcome the block by addition of up to 50 ng of EGF per ml.

The fact that, in the case of FGF receptors, the block can be overcome suggests that the effect of the truncated receptors occurs at least partially through competitive inhibition (i.e., competition with endogenous receptors for direct binding of the available growth factor) rather than only by a true dominant negative effect (i.e., formation of inactive heterodimers with endogenous receptors). If the action of truncated receptors is mediated entirely through heterodimerization, it should not be possible to overcome the effect of a signalling block with an increase in the amount of growth factor, because the number of functional homodimers formed should be minimal when the truncated receptor is overexpressed.

In vitro cross-linking experiments using ^{125}I-labeled FGF-2 have shown that different forms of the receptor (monomer, homodimer, and heterodimer) can be observed when wild-type and truncated receptors are coexpressed at high levels (23). However, in biological assays such as disruption of mesoderm induction or disruption of FGF-mediated calcium efflux in *Xenopus* oocytes, the dominant negative action of the truncated receptor can be achieved only with a 10- to 100-fold excess of the mutant receptor with respect to the wild type, implying that sufficient amounts of heterodimer can be formed only if the truncated receptor is overexpressed (1, 23). Even though a receptor oligomerization model can explain these effects, another possibility is that the mutant receptor functions simply as a competitive inhibitor to sequester binding of FGFs to wild-type FGF receptors. A mechanism of this type would predict that the block can be overcome by high doses of FGF, which, in fact, is what we find.

In conclusion, the properties of FGF-4-transformed and normal NIH 3T3 cells transfected with truncated FGF receptors suggest that their resistance to FGF signalling has two major components: competition for growth factor binding with functional receptors and formation of inactive heterodimers between truncated and wild-type receptors. In this respect, it is interesting that ΔFGFR-2-transfected clones tend to be more resistant to FGF-4 than to FGF-2 while ΔFGFR-1-expressing clones are more resistant to FGF-2 than to FGF-4. These results can be interpreted as suggesting that each receptor has a higher potential to form homodimers than to form heterodimers. The lower resistance of ΔFGFR-1-expressing clones to FGF-4, which binds to FGFR-2 with higher affinity than to FGFR-1, is probably expected, since FGF-4 would

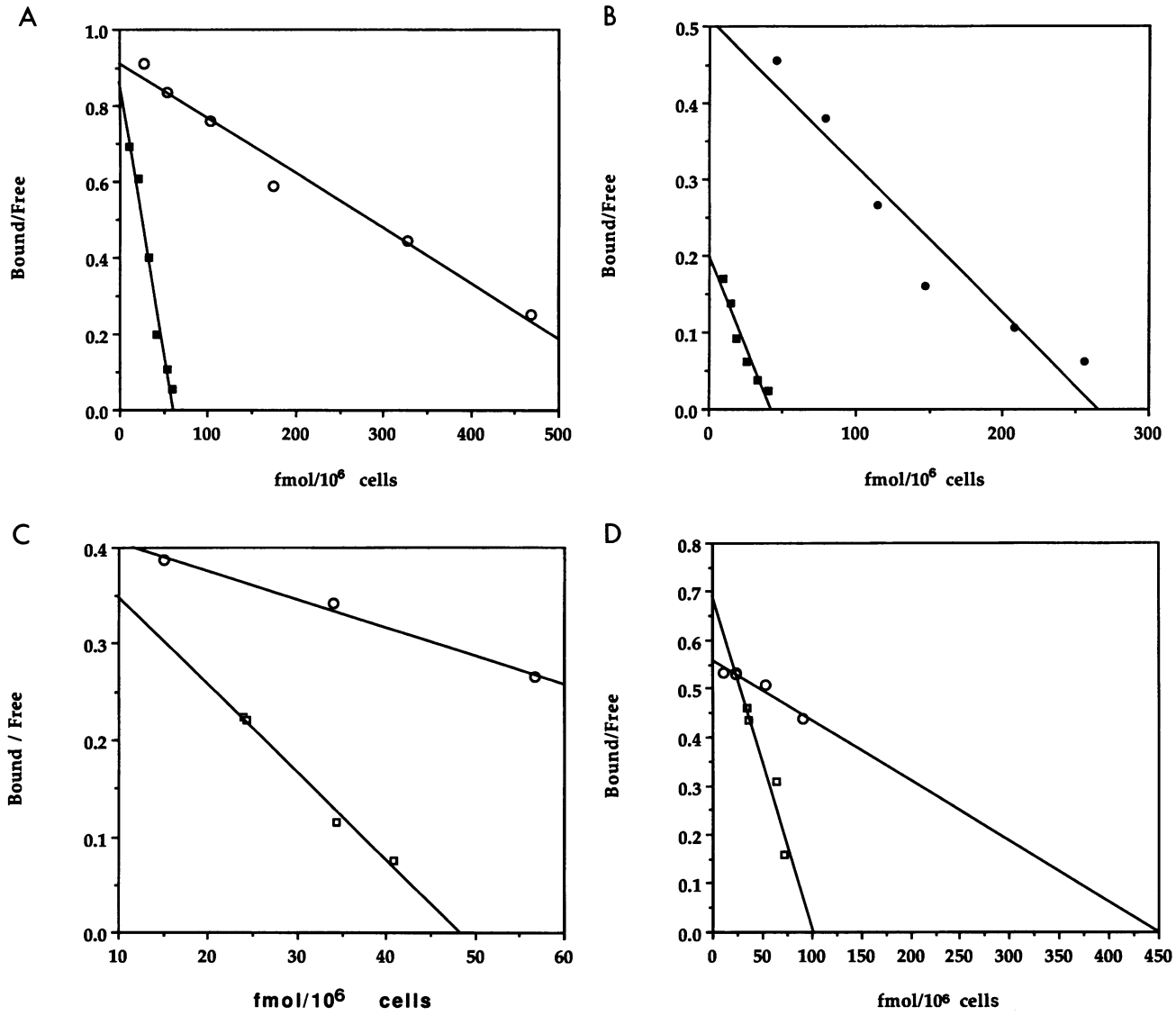

FIG. 6. Scatchard analysis, performed as described in Materials and Methods. Counts eluted by the acidic wash were used to calculate the high-affinity FGF-2-binding sites. (A) NIH 3T3 cells (■) and 3T3bek18 cells (○); (B) NIH3T3 cells (■) and 3T3flg12 cells (●); (C) CHObek3.7 cells (□) and a pool of CHO cells transfected with ΔFGFR-2 (○); (D) CHOflg4-1 cells (□) and a pool of CHO cells transfected with ΔFGFR-1 (○).

select FGFR-2 wild-type molecules during binding. However, in the case of ΔFGFR-2-expressing cells, while the high resistance to FGF-4 is again predicted for the reason stated above, the relative susceptibility to FGF-2 suggests a preference for receptor homodimerization. If FGF receptors are totally promiscuous in their dimerization pattern, FGF-2, which binds FGFR-1 and FGFR-2 equally, should not reveal any difference in susceptibility between cells expressing truncated receptors of either type. The interpretation of these experiments is unfortunately complicated by the fact that FGFR-1 is expressed much more abundantly in NIH 3T3 cells than FGFR-2 and by the somewhat lower affinities of truncated FGFR-1 and FGFR-2 for their ligands. Thus, further experiments will be necessary to confirm this conclusion.

Truncated FGF receptors have lower ligand binding affinities but inhibit phosphorylation of endogenous FGF receptors. Tyrosine kinase activity of the intracellular region is not necessary for proper synthesis, processing, glycosylation, and transport to the cell surface of FGF receptors. Experiments using kinase-defective mutant FGFR-1 and FGFR-2 with point mutations instead of truncation have shown no influence on the ligand binding ability (3). Truncated receptors without intracellular regions are still capable of binding the ligand and forming dimers after ligand stimulation; therefore, the tyrosine kinase domain is not obligatory for these functions. However, there is very little information on the influence of the intracellular region on the ligand binding property of the extracellular domain. A truncated EGF receptor was found to have lower affinity for EGF than the wild-type receptor, suggesting that cytoplasmic function or structure is required for high-affinity binding of EGF receptors (20). We found that the truncated FGF receptors had affinities for FGF-2 that were four- to eightfold lower than those of wild-type receptors. The Scatchard plot of ΔFGFR-2-expressing clone 3T3bek18 shows a single class of binding sites with an affinity lower than that of NIH 3T3 cells. This also indicates that the truncated receptors

can sequester FGF binding to wild-type endogenous receptors by functionally interfering or quantitatively overwhelming the latter. In contrast, 3T3bek8, which expresses approximately equal amounts of ΔFGFR-2 and wild-type receptors, exhibited a straight-line plot and a binding affinity only twofold higher than that of NIH 3T3 cells. Interestingly, ΔFGFR-1 has a binding curve different from that of ΔFGFR-2 and it actually resembles that reported for the truncated EGF receptor. Further investigations are needed to differentiate whether the decreased binding affinity in the truncated-receptor-expressing clones is a result of higher dissociation or lower association rates.

Individual phosphotyrosine residues of receptors appear to serve as highly selective binding sites that are specific for cytoplasmic signalling molecules. A mutant FGFR-1 in which the tyrosine residue at position 766 is removed no longer binds phospholipase C-γ, but the mutant-receptor-bearing cells are stimulated by FGF mitogenically to the same or a greater extent than the controls, strongly implying that phosphorylated Tyr-766 within the receptor is not required for FGF-induced mitogenesis (15, 19). Our studies of tyrosine phosphorylation of FGF receptors showed that receptor phosphorylation is strongly inhibited in the 3T3bek18 clone at physiological FGF concentrations. The use of a higher dose (>10 ng/ml) of FGF-4, however, produced significant receptor phosphorylation (data not shown). On the other hand, receptor phosphorylation was clearly detectable in 3T3flg12 cells even at lower FGF concentrations, but a number of putative receptor substrates did not appear to be phosphorylated. Receptor phosphorylation has always been considered a good parameter of receptor activation and signal transduction, but Shi et al. (21) have suggested that phosphorylation can be either intramolecular or intermolecular. Tyr-653 is stringently dependent on a *trans* intermolecular mechanism and appears obligatory for activation of the bound substrates, while phosphorylation of Tyr-766 may occur by a *cis* intramolecular mechanism within kinase-competent monomers either prior to dimerization or when complexed to either a wild-type or a kinase-defective monomer and is not correlated with mitogenic response. In our case, 3T3flg12 cells did not show a strong inhibition of receptor phosphorylation upon treatment with FGFs, but their mitogenic response was impaired to a significant extent, suggesting that the phosphorylated tyrosine residue(s) within endogenous receptors may not be coupling to mitogenic signalling molecules. The exact nature of the FGF signal transduction pathway is still unknown. These studies provide a basis to investigate the downstream targets of FGF-signalling and to study possible therapeutic uses of receptor blocking mechanisms in FGF-associated pathologies.

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