Integrins play a role in fibroblast growth factor (FGF) signaling through cross-talk with FGF receptors (FGFRs), but the mechanism underlying the cross-talk is unknown. We discovered that FGF1 directly bound to soluble and cell-surface integrin αβ3 (Kd about 1 µM). Antagonists to αβ3 (monoclonal antibody 7E3 and cyclic RGDfV) blocked this interaction. αβ3 was the predominant, if not the only, integrin that bound to FGF1, because FGF1 bound only weakly to several β1 integrins tested. We presented evidence that the CYDMKTTC sequence (the specificity loop) within the ligand-binding site of FGF1 overlaps with the heparin-binding site but is distinct from the FGFR-binding site using docking simulation and mutagenesis. We identified an FGF1 mutant (R50E) that was defective in integrin binding but still bound to heparin and FGFR. R50E was defective in inducing DNA synthesis, cell proliferation, cell migration, and chemotaxis, suggesting that the direct integrin binding to FGF1 is critical for FGF signaling. Nevertheless, R50E induced phosphorylation of FGFR1 and FRS2α and activation of AKT and ERK1/2. These results suggest that the defect in R50E in FGF signaling is not in the initial activation of FGF signaling pathway components, but in the later steps in FGF signaling. We propose that R50E is a useful tool to identify the role of integrins in FGF signaling.

Fibroblast growth factors (FGFs) constitute a family of heparin-binding polypeptides involved in the regulation of biological responses such as growth, differentiation, and angiogenesis (1–4). The FGF family currently consists of 22 members with FGF1 (acidic FGF) and FGF2 (basic FGF) the most extensively studied. The biological effects of FGFs are mediated by four structurally related receptor tyrosine kinases designated FGFR1–4. The binding of FGF to its receptor results in receptor dimerization and subsequent autophosphorylation of specific tyrosine residues within the cytoplasmic domain (1–4). Activation of the receptor allows proteins containing Src homology 2 or phosphotyrosine binding domains to bind to sequence recognition motifs in the FGFR, resulting in phosphorylation and activation of these proteins (5). This leads to the activation of intracellular signaling cascades. The main signaling cascade activated through the stimulation of FGF1 is the Ras/MAPK pathway.

FGF signaling enhances multiple biological processes that promote tumor progression (6). Therapies targeting FGF receptors and/or FGF signaling not only affect the growth of the tumor cells but also modulate tumor angiogenesis (7). FGF1 and FGF2 are responsible for resistance to chemotherapeutic agents in cancer (8–11) and are also pro-inflammatory growth factors that play a role in pathological angiogenesis in chronic inflammatory diseases (12). Thus FGF signaling is a potential therapeutic target for cancer and pathological angiogenesis in chronic inflammatory diseases.

It has been proposed that cross-talk between integrins and growth factor receptors plays a critical role in growth factor signaling (13), but the specifics of the cross-talk are unclear. Integrins are a family of cell adhesion receptors that recognize extracellular (ECM) ligands and cell-surface ligands (14). Integrins are transmembrane α-β heterodimers, and at least 18 α and 8 β subunits are known (15). Integrins play an important role in anchorage-dependent cell survival and proliferation (16). Integrin-stimulated pathways are very similar to those mediated by growth factor receptors and are intimately coupled with them. Many cellular responses to soluble growth factors, such as epidermal growth factor, platelet-derived growth factor, and thrombin, are dependent upon the adherence of the cell to ECM ligands via integrins. FGF2-induced angiogenesis requires integrin signaling from the ECM (cross-talk between integrins and FGFs). Indeed mAb against integrin αβ3 blocks FGF2-induced angiogenesis (17, 18).

It has been reported that substrate-bound FGF2 promotes endothelial cell adhesion by directly interacting with integrin αβ3 (19) and induces endothelial cell proliferation, motility, and the recruitment of FGFR1 in the cell substrate contact (20). However, because heat-denatured FGF2 still supports integrin binding (20), it is unclear whether this interaction is biologically
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The human FGF1 cDNA was amplified using PCR with human placenta library as a template. A BglII restriction site was introduced at the 5’ end and an EcoRI site at the 3’ end of the cDNA fragment with following primers: 5’-CCGAGATCTTCTGCTACAGAGGATCT-3’ and 5’-GCGGAATTCTTAAATCGAGGAGCTCCAGGAAT-3’. The resulting fragments were digested with BglII and EcoRI and then subcloned into the BamHI/EcoRI sites of the vector pGEX-2T (Amersham Biosciences) as a template. The refolded protein and refolded as described (29). The refolded protein was purified by affinity chromatography on heparin-Sepharose to enrich the properly folded protein. Bound protein was eluted with 1 M NaCl.

Surface Plasmon Resonance (SPR) Analysis

Heparin–FGF Interaction—Biotinylated heparin (Sigma) in Biacore HBS-EP buffer (0.01 M Hepes, pH 7.4, 0.15 M NaCl, 3 mM EDTA, and 0.0005% of surfactant P20) was immobilized by a streptavidin sensor chip (Biacore SA Chip) by injecting at 2 μl/min for 15 min. One response unit difference represents about 1 pg/mm² of the analytes on the surface matrix of the sensor chip. 5-Fold serially diluted WT, R50E, and 3xA ranging from 800 nM to 51.2 pm and 2-fold serially diluted 4xE ranging from 1.6 μM to 50 nM in HBS-EP were injected at 50 μl/min for 3 min.

Integrin–FGF Interaction—Soluble αβ3 was immobilized on the CM5 sensor chip using a standard amine coupling procedure (30). The WT and R50E FGF1 were individually 2-fold serially diluted from 8 μM to 125 μM in HBS-P buffer (0.01 M Hepes, pH 7.4, 0.15 M NaCl, and 0.0005% of surfactant P20) with 1 mM of Mn²⁺, and the 3xA FGF1 was 2-fold serially diluted from 6 μM to 93.75 μM in the same buffer. Samples were injected at 50 μl/min for 1.8 min. The HBS-P buffer with 1 mM of Mn²⁺ was then injected at 50 μl/min for 3 min to allow the bound FGF1s to dissociate from the integrin.

FGF1–FGFR1 D2D3 Interaction—About 1500 response units of FGF1–FGFR1 D2D3 was immobilized to a sensor chip CM5 by using a standard amine coupling method. WT or mutant FGF1 was 2-fold serially diluted from 800 to 50 μM in HBS-P buffer containing 1 mM of Mn²⁺ and injected at a flow rate 30 μl/min for 3 min. The same buffer was injected at the same flow rate for 3 min to measure the dissociation of the bound FGF1 from FGFR1 D2D3.

Docking Simulation

In the AutoDock 3.05 program, the ligand is presently compiled to a maximum size of 1024 atoms. The solvent-exposed Mg²⁺ octahedral vertex was left empty in the model during docking calculations. Atomic solvation parameters and frac-
tional volumes were assigned to the protein atoms by using the AddSol utility, and grid maps were calculated by using AutoGrid utility in AutoDock 3.05. A grid map with $127 \times 127 \times 127$ points and a grid point spacing of $0.603 \AA$ included the whole metal ion-dependent adhesive site-containing face of the I-like domain of $\beta$ and the $\beta$-propeller domain containing repeats 2–4, which are large enough to accommodate the FGF1 structure. Kollman “united-atom” charges were used. AutoDock 3.05 uses a Lamarckian genetic algorithm (LGA) that couples a typical Darwinian genetic algorithm for global searching with the Solis and Wets algorithm for local searching. The LGA parameters were defined as follows: the initial population of random individuals had a size of 50 individuals; each docking was terminated with a maximum number of $1 \times 10^6$ energy evaluations or a maximum number of 27,000 generations, whichever came first; mutation and crossover rates were set at 0.02 and 0.80, respectively. An elitism value of 1 was applied, which ensured that the top ranked individual in the population always survived into the next generation. A maximum of 300 iterations per local search was used. The probability of performing a local search on an individual was 0.06, whereas the maximum number of consecutive successes or failures before doubling or halving the search step size was 4. This set of parameters was used for all dockings.

**Integrin Binding Assays**

Cell adhesion and soluble integrin binding assays were performed as described previously (24). Native or heat-denatured (70 °C for 10 min) FGF in 0.1 M carbonate buffer, pH 9.4, was incubated in a polystyrene 96-well non-tissue culture plate overnight at 4 °C. Unbound FGF was removed, and 200 μl of 0.1% BSA in PBS was added and incubated for 60 min at room temperature. The wells were washed with PBS, and soluble integrin αβ3 in 50 μl in Hepes-Tyrode’s buffer supplemented with 1 mM Mn$^{2+}$ was added to the wells and incubated at room temperature for 60 min. Then non-bound soluble integrin was removed by rinsing the wells with the same buffer. Horseradish peroxidase (HRP)-conjugated anti-His tag mouse IgG was added to the wells and incubated for 60 min. Non-bound antibodies were removed by rinsing the wells with the same buffer, and bound integrins were quantified by measuring the absorbance at 450 nm developed from adding the substrate 3,3′,5,5′-tetramethylbenzidine of HRP.

**DNA Synthesis**

DNA synthesis was measured by BrdUrd incorporation. Balb 3T3 cells were plated on sterile coverslips in 6-well culture plates and serum-starved in DMEM supplemented with 0.4% FCS for 48 h. They were stimulated with 5 ng/ml WT FGF1 and its mutants for 24 h in the presence of 5 μg/ml heparin. BrdUrd (10 μg/ml) was added to the medium for the last 6 h of the incubation. Cells were then fixed with 70% ethanol and incubated at 37 °C for 15 min at 37 °C. Afterward, cells were washed twice with ice-cold PBS, and lysed with the lysis buffer (20 mM Tris-HCl, pH 8.0, 120 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 10 mM NaF, 1 mM Na$_2$VO$_4$, 10 μg/ml aprotinin). Protein concentrations in the cell lysates were determined using the Bradford protein assay (Bio-Rad). Equal amounts of cell proteins were analyzed by SDS-PAGE in a 10% polyacrylamide gel and Western blotting. The first antibodies were anti-FGFR1, anti-phospho-FGFR1 (Tyr-653/Tyr-654, BIOSOURCE), anti-FRS2α, anti-phospho-FRS2α (Tyr-196), anti-p44/42 ERK1/2 MAPK, anti-phospho-p44/42 ERK1/2 MAPK (Thr-202/Tyr-204), anti-AKT, or anti-phospho-AKT antibody (Cell Signaling Technology, Inc, Danvers, MA). Bound antibodies were detected with HRP-conjugated anti-mouse or anti-rabbit IgG, and ECL Western blotting detection reagents (Pierce).

**In Vitro Wound Healing Assay**

Balb 3T3 cells were plated into 6-well cell culture plates. Cells were allowed to grow in DMEM containing 10% FCS overnight, and then cells were washed with serum-free medium and starved for 24 h. A scratch was made across the cell layer using a pipette tip. After washing with serum-free medium twice, DMEM containing 10 ng/ml WT or mutant FGF1 together with 5 μg/ml heparin was added to the cells. The wounded areas were quantified by using ImageJ.

**Chemotaxis**

A polycarbonate filter of 8 μm pore size of the Transwell insert was coated with 10 μg/ml fibronectin (Sigma) overnight at 4 °C. After washing, the insert was placed into a 24-well cell culture plate, and the lower portion of the plate was filled with 600 μl of serum-free DMEM containing 5 ng/ml WT or mutant FGF1. Balb 3T3 cells (10$^5$ cells/filter) were plated on the filter and incubated at 37 °C for 24 h, and cells were visualized by crystal violet staining (0.5% crystal violet in 50 mM borate, pH 9.0, and 2% ethanol). The uncoated side of each filter was wiped with a cotton swab to remove cells that had not migrated through the filter. Chemotaxed cells were counted from the digital images of the stained cells, determining the mean num-
**FIGURE 1. Direct binding of FGF1 to integrin αvβ3.**

- **a,** recombinant soluble αvβ3 bound to WT FGF1 but not to heat-denatured FGF1. Wells of 96-well microtiter plates were coated with WT or heat-denatured FGF1 at the indicated concentrations, and the remaining protein-binding sites were blocked with BSA. Recombinant soluble αvβ3 was added to the wells and incubated for 1 h at room temperature. Bound αvβ3 was determined using HRP-conjugated anti-His6 antibody and peroxidase substrate at A_{450}. Data are shown as means ± S.E. of triplicate experiments.

- **b,** effects of antibodies and EDTA on the binding of soluble αvβ3 to FGF1. The ELISA-type integrin-binding assay was performed as described above. Soluble αvβ3 was incubated with 10 µg/ml 7E3 or control mouse IgG for 10 min on ice prior to adding to the wells. Concentrations of MgCl2 and EDTA are 10 and 5 mM, respectively. BSA was used as a negative control. Data are shown as means ± S.E. of triplicate experiments.

- **c,** adhesion of K562 cells that express different integrins to FGF1. Wells of 96-well microtiter plates were coated with FGF1, and the remaining protein-binding sites were blocked with BSA. Cells were added to the wells and incubated for 1 h at 37 °C in Hepes-Tyrode's buffer containing 1 mM MnCl2, and bound cells were quantified after removing unbound cells by using phosphatase assays. Data are shown as means ± S.E. of triplicate experiments.

- **d,** inhibition of cell adhesion to FGF1 by anti-integrin antibodies. Adhesion assay was performed as described in **c.** Cells were incubated with 10 µg/ml control mouse IgG or mAb AIIB2 (anti-β1, function blocking), or KH72 (anti-α5) for 30 min on ice prior to adding to the wells. Data are shown as means ± S.E. of triplicate experiments. *p < 0.05 to control IgG, n = 3 by t test.

- **e,** inhibition of αvβ3-FGF1 interaction by cyclic RGDfV, a specific antagonist to αvβ3. Adhesion assay was performed as described in **c.** Cyclic RGDfV was used at 10 µM, and DMSO, in which stock cyclic RGDfV was solubilized, was used as a control. *p = 0.0024 between αvβ3-K562 (αvβ3-K)/DMSO and αvβ3-K562/cyclic RGDfV.

- **f,** binding of the β1–3–1 mutant to FGF1. Adhesion assay was performed as described in **c.** Cells were incubated with 10 µg/ml control mouse IgG or mAb AIIB2 (anti-β1, function blocking). Data are shown as means ± S.E. of triplicate experiments. *p < 0.05 to DMSO, n = 3 by t test.

- **g,** effect of FCS to αvβ3-FGF1 interaction. Adhesion assay was performed as described in **c.** Assays were performed in the absence and presence of FCS (10%). Data are shown as means ± S.E. of triplicate experiments.
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RESULTS

FGF1 Specifically Binds to Integrin αβ3—We tested whether FGF1 directly interacts with integrins in vitro. We found that recombinant soluble αβ3 bound to immobilized WT FGF1 in a dose-dependent manner but did not bind to heat-denatured FGF1 in ELISA-type integrin binding assays (Fig. 1a). These results suggest that FGF1 binds to integrin αβ3, and this interaction requires an intact three-dimensional structure of FGF1. EDTA and mAb 7E3 (anti-β3) blocked the binding of soluble αβ3 to FGF1, suggesting that binding to FGF1 is cation-dependent and specific to αβ3 (Fig. 1b). An SPR analysis of the FGF1-αβ3 interaction showed that FGF1 binds to αβ3 at a high affinity (see below).

We next tested whether cell-surface αβ3 binds to FGF1. We found that K562 erythroleukemic cells (α5β1+) expressing exogenous αβ3 (αβ3-K562, α5β1+/αβ3+) cells adhered to FGF1, but mock-transfected K562 cells showed only weak adhesion to FGF1 (Fig. 1c). This suggests that FGF1 binds to αβ3, but not to α5β1, on the cell surface under the current assay conditions. mAb 7E3 blocked, but mAb KH72 (anti-α5) did not significantly block, the adhesion of αβ3-K562 cells to FGF1 (Fig. 1d). Consistent with this finding, cyclic RGDfV, which is an antagonist specific to αβ3 (32), blocked the adhesion of αβ3-K562 cells to FGF1 (Fig. 1e). K562 cells that express several β1 integrins only weakly adhered to FGF1 (Fig. 1c). These results suggest that αβ3 is a predominant, if not the only, integrin that binds to FGF1 in our assay conditions.

Because FGF1-αβ3 interaction is blocked by mAb 7E3 that has been mapped in the ligand-binding site of the β3 subunit (33, 34), and blocked by cyclic RGDfV, it is suggested that FGF1 binds to the ligand-binding site of αβ3. We previously reported that a small disulfide-linked loop in the I-like domain of β3 plays a critical role in determining ligand specificity of αβ3 (27). To test if FGF1 binding requires the specificity loop of αβ3, we used a β1 mutant in which a disulfide-linked loop of β1 (the specificity loop, residues 187–193) was swapped with the corresponding amino acid residues of β3 (the CYDMKTTC sequence) (the β1–3–1 mutation) (27). αβ1–3–1 has been shown to bind to several αβ3 ligands, including vitronectin, fibrinogen, von Willebrand factor (27, 35), and a viral surface protein (36). Nevertheless function-blocking anti-β1 mAbs such as AIIB2 blocked ligand binding to αβ1–3–1 (27). We found that CHO cells that express β1–3–1 (β1–3–1-CHO) strongly adhered to FGF1, whereas those expressing WT β1 (β1-CHO) did not (Fig. 1f). Furthermore, anti-β1 mAb AIIB2

1AXM was used for docking. The headpiece of 1LSG was used as a receptor. The pose in the cluster 1 with the lowest docking energy (–26.3 kcal/mol) is shown. This pose represents the most stable pose of FGF1 when FGF1 interacts with integrin αβ3. b, positions of amino acid residues that are selected for mutagenesis at the predicted interace between FGF1 and αβ3. Several amino acid residues within the predicted integrin-binding site in FGF1 were selected for mutagenesis. c, positions of the amino acid residues (E102A, Y109A, and N110A) at the FGR-binding site selected for mutagenesis. Note that the predicted integrin-binding site is distinct from the FGR-binding site.
**TABLE 1**
Amino acid residues at the predicted interface between FGF1 and integrin αvβ3
Amino acid residues of FGF1 or αvβ3 that are within a 6-Å distance from αvβ3 or FGF1, respectively, were selected using Swiss-pdb viewer (version 3.7). Amino acid residues of FGF1 (bold) were selected for mutagenesis.

| FGF1 | αv | β3 |
|------|----|----|
| Asn-33, Gly-34, Gly-35 | Met-118 | Tyr-122, Ser-123, Met-124, Lys-125, Asp-126, Asp-127, Trp-129 |
| His-36, Arg-39, Leu-41 | Ser-144, Gln-145, Asp-146, Ile-147, Asp-148, Ala-149, Asp-150, Gly-151 | Asp-179, Met-180, Lys-181, Thr-182, Arg-214, Asn-215, Arg-216, Ala-218, Asp-251, Ala-252, Lys-253 |
| Asp-43, Thr-45, Val-46 | Tyr-178 | Ser-131, Cys-132, Lys-133, Arg-134, Arg-137, Thr-138, Gly-141, Gln-142, Lys-143, Ala-144 |
| Lys-127, Lys-128, Asn-129, Gly-130, Ser-131, Cys-132, Lys-133, Arg-134, Arg-137, Thr-138, Gly-141, Gln-142, Lys-143, Ala-144 |
| Asp-47, Gly-48, Thr-49 | Thr-212, Gln-214, Ala-215, Ile-216, Asp-218, Asp-219 | Threo-212, Gln-214, Ala-215, Ile-216, Asp-218, Asp-219 |
| Arg-50, Asp-51, Arg-52 | Arg-248 | Arg-248 |
| Ser-53, Asp-54 | |

**FIGURE 3. Effect of FGF mutations on the binding to integrin αvβ3.**

(a) R50E mutation of FGF1 reduced its binding to integrin αvβ3. Amino acid residues in the integrin-binding site or in the FGFR-binding site were mutated individually or in groups. WT FGF1 and the R50E mutant were coated to a plastic plate at various concentrations as indicated, and the binding of soluble αvβ3 was determined as described in Fig. 1a. The results show that the R50E mutation reduced αvβ3 binding. The summary of the effects of FGF1 mutations on integrin binding. The results suggest that mutations in the predicted integrin-binding site of FGF1 reduced the binding of soluble αvβ3 to FGF1, but mutations in the FGFR-binding site did not. (b) Binding of WT FGF1 (c) and R50E FGF1 (d) to αvβ3 in SPR. Soluble αvβ3 was immobilized on a CMS sensor chip. WT and R50E FGF1 were individually 2-fold serially diluted from 80 μM to 125 nM in HBS-P buffer with 1 mM of Mn2⁺, and the 3X A FGF1 was 2-fold serially diluted from 6 μM to 93.75 nM in the same buffer. 4X did not show any binding to integrin (data not shown).

This suggests that the specificity loop is critical for FGF1 binding to αvβ3 and that FGF1 interacts with αvβ3 in a manner similar to those of known αvβ3 ligands (e.g., vitronectin). These results also suggest that αvβ3 and αvβ3 that are expressed in poses clustered well with the lowest docking energy, 26.1 kcal/mol (cluster 1). These results predict that the docking pose of cluster 1 (Fig. 2a) may represent the most probable stable FGF1 pose upon binding to αvβ3. This model predicts that the integrin-binding interface of FGF1 with integrin αvβ3 is parent CHO and β1-CHO cells do not appear to be important for FGF1 binding.

It is possible that integrin αvβ3 may not interact with FGF1 in the presence of plasma adhesive proteins, because they may compete with FGF for binding to αvβ3. We found that the presence of 10% fetal bovine serum did not suppress αvβ3-specific cell adhesion to FGF1 (Fig. 1g). This consistent with the previous reports that integrin-binding sites in serum-adhesive proteins are cryptic (see “Discussion”).

**Generation of Integrin-binding Defective FGF1 Mutants**—To localize the integrin-binding site in FGF1, we used a docking simulation program and site-directed mutagenesis. AutoDock is a set of docking tools widely used for predicting the pose of small ligands bound to receptors (37), and the methods are being extended to predict protein-protein complex poses (38). We performed 50 dockings of the FGF1-integrin αvβ3 interaction, each one starting with a random initial position and orientation of FGF1 (PDB code 1AXM) with respect to the integrin αvβ3 headpiece (PDB code 1L5G). The results were clustered together by positional root mean square distance into families of similar poses (data not shown). Many of the docking
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distinct from the FGFR-binding site (39) but is close to or overlapping with the heparin-binding site (Fig. 2, b and c).

We introduced several mutations within the predicted integrin-binding site (Fig. 2b and Table 1) to localize the integrin-binding site in FGF1. We tested the ability of the mutants to bind to αvβ3 in ELISA-type binding assays. The Arg-50 to Glu (R50E) mutation effectively reduced the binding of soluble αvβ3 to FGF1 (Fig. 3a). Mutating positively charged residues (Lys-127, Lys-128, Lys-133, and Arg-134) in the predicted integrin-binding site to Glu in combination diminished the binding of soluble αvβ3 to FGF1 (Fig. 2b and Fig. 3b). Mutating all these residues simultaneously to Glu (designated the 4xE mutation) effectively diminished integrin binding. We predicted that Glu-102, Tyr-109, and Asn-110 are located in the FGFR-binding site from the FGF1-FGFR-2 complex structure (39) and mutagenesis data of FGFR2 (40). Mutating these residues to Ala individually or simultaneously (designated 3xA mutant) did not affect integrin binding (Fig. 2c and Fig. 3b), whereas the 3xA mutation effectively suppressed the binding to FGFR1 (below).

We assessed the ability of the FGF1 mutants to bind to αvβ3 in surface plasmon resonance (SPR) using a sensor chip immobilized with soluble αvβ3. WT FGF1 bound to αvβ3 at a high affinity (K_D 1.1 μM) (Fig. 3c). The R50E mutant showed much lower R_max value and enhanced off-rate to αvβ3, whereas it has a K_D value to αvβ3 (1.31 μM) similar to WT FGF1 (Fig. 3d). The ability of the 3xA mutant to bind to αvβ3 was intact (K_D 0.18 μM), but the 4xE mutant did not show binding affinity to αvβ3 (data not shown). These results are consistent with those obtained by ELISA-type binding assays.

We assessed the ability of these FGF1 mutants to interact with the FGF1 fragment (domains 2 and 3, or FGF1 D2D3) in SPR using a sensor chip immobilized with FGF1 D2D3. WT FGF1 (K_D 30 pM) and R50E FGF1 (K_D 14 pM) had similar affinity to FGF1 (Fig. 4, a and b). The 4xE mutant showed low affinity to FGF1 (K_D 5.5 μM; data not shown). The 3xA mutant has a much lower affinity (K_D 0.41 nM) (Fig. 4c).

We assessed the ability of the mutants to bind to heparin-Sepharose. WT FGF1, and the R50E and 3xA mutants, bound strongly to heparin-Sepharose and required 1.2 M NaCl for elution, but the 4xE mutant did not show affinity to heparin (Fig. 4d). In SPR R50E (Fig. 4e) had slightly lower affinity to heparin (K_D 66 nM) than WT FGF1 (K_D 16.6 nM) (Fig. 4f), whereas the slightly reduced affinity of R50E to heparin did not affect its ability to induce FGFR1 activation and ERK1/2 activation (see below). The 3xA mutant (data not shown) showed affinity to heparin (K_D 11.5 nM) similar to that of WT FGF1, but the 4xE did not show any binding to heparin (data not shown).

These results are consistent with the prediction that in mutations in the predicted integrin-binding site in FGF1 compromised integrin binding and heparin binding, but those in the FGFR-binding site affected FGF binding but did not affect integrin binding. The R50E mutation was unique in that it reduced integrin binding and induced minimal effect on heparin or FGF binding. Thus we propose that the R50E mutant is useful to determine the potential role of integrin αvβ3 in FGF binding.
Effect of the Mutations on FGF-induced DNA Synthesis and Cell Migration—We examined the effect of the FGF1 mutations on FGF1-induced DNA synthesis in Balb 3T3 cells. The R50E, 4xE, and 3xA mutants induced much lower BrdUrd incorporation than WT FGF1 (Fig. 5a), suggesting that these mutants are defective in inducing DNA synthesis. To test the ability of the FGF1 mutants to induce cell proliferation, we used BaF3 mouse pro-B cells that express human FGFR1c (designated BaF3-FGFR1c) (41), which have been used to sensitively detect cell proliferation that is dependent on exogenous FGF1. We found that WT FGF1 robustly induced proliferation of BaF3-FGFR1c cells, whereas the R50E, 4xE, and 3xA mutants did not induce detectable cell proliferation (Fig. 5b). This is consistent with the results that these mutants were defective in inducing DNA synthesis.

Because FGF1 is a potent inducer of cell migration and chemotaxis (42, 43), we tested the ability of the mutants to induce cell migration. Using scratch wound healing assays with Balb 3T3 cells, we found that although WT FGF1 closed the wound by 59% in 24 h, the R50E, 4xE, and 3xA mutants did not close the wound in this time frame (Fig. 5c). We found that the R50E, 4xE, and 3xA mutants induced much lower chemotaxis than WT FGF1 (Fig. 5d). These results suggest that these mutants are defective in inducing cell migration.

Effect of the Integrin-binding Defective Mutations on FGF1 Signaling—Next we studied whether these FGF1 mutants induce FGF signaling inside the cells. The binding of FGF to FGFR leads to tyrosine phosphorylation of the docking protein FRS2α, which functions as a major mediator of signaling via FGFR (1–4). This results in recruitment of multiple Grb2-Sos complexes leading to activaton of the Ras/MAPK signaling pathway (44). The mitogenic action of the FGFs is mediated in part by the activation of ERK1/2 (45). WT FGF1 induced phosphorylation of FGR1, FRS2α, AKT (protein kinase B), and ERK1/2. The 4xE and 3xA

signaling. The 4xE mutation was defective in integrin, heparin, and FGFR1 binding and could be used as a control together with the 3xA mutant. Positions of these mutations in FGF1 are shown in Fig. 2, B and C.
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mutants showed little or no ability to induce FGFR1, FRS2α, or ERK1/2 activation as compared with WT FGF1 and the R50E mutant (Fig. 6), which is consistent with the finding that the 4xE and 3xA mutants are defective in binding to FGFR1. Notably, the R50E mutant induced FGFR1, FRS2α, AKT, and ERK1/2 activation to the similar extent of WT FGF1 (Fig. 6). These results suggest that the integrin-binding defective R50E FGF1 mutant can induce the initial FGFR activation and subsequent FGF signaling and that the slightly reduced heparin binding to this mutant did not affect it.

DISCUSSION

Although it has been reported that integrins play a critical role in FGF signaling (13), the specifics governing their interaction in this process are still unclear. In this study, we showed that integrin αvβ3 directly bound to FGF1 at an affinity comparable with those of known integrin ligands. Heat denaturation of FGF1 destroyed integrin binding, and the amino acid residues of FGF1 that are critical for integrin-binding were discontinuous, indicating that proper folding of FGF1 is required for integrin binding. Because mAb 7E3 and cyclic RGDFV blocked the FGF1-αvβ3 interaction, it is suggested that FGF1 binds to the ligand-binding site of αvβ3. We showed that the specificity loop plays a role in the binding of FGF1 to αvβ3, suggesting that FGF1 binds to αvβ3 in a manner similar to that of other αvβ3 ligands tested (27, 35, 36). We showed that 10% fetal bovine serum did not suppress αvβ3-mediated cell adhesion to FGF1. This is consistent with the reports that integrin-binding sites are cryptic in fibrinogen (46), fibronectin (47), and vitronectin (48). These findings suggest that the observed FGF1 binding to αvβ3 may happen in biological body fluid. It has been proposed that productive integrin-ligand interactions require ligand “activation” (e.g. by immobilization and proteolysis) in addition to activation of integrins (46). We recently showed that αvβ3 binds to the C-terminal fragment of the fibrinogen γ-chain but not to whole fibrinogen in solution (49).

We localized critical amino acid residues of FGF1 for binding to αvβ3 using docking simulation and mutagenesis. We found that the integrin-binding site overlaps with the heparin-binding site. These findings suggest that docking simulation and mutagenesis are useful tools for studying how integrins bind to protein ligands. The R50E mutation affected integrin binding, although its effect on heparin and FGFR binding was minimal, suggesting that this mutation primarily affected integrin binding. The R50E mutant did not induce cell proliferation and migration, whereas R50E-induced FGF1 signaling (FGFR1 phosphorylation, FRS2α phosphorylation, and ERK1/2). This suggests that the integrin binding may not be required for the early activation of ERK1/2 but is required for the later steps in FGF signaling that leads to cell proliferation and migration. Indeed it has been reported that that transient ERK1/2 activation is not integrin-dependent and not related to cell proliferation, but that sustained ERK1/2 activation is integrin-dependent and directly related to cell cycle entry (50, 51). The R50E mutant of FGF1 may be a useful tool to study the role of integrins in FGF signaling. Further studies in this direction are under way.

We showed that WT FGF1 free in solution had a K_D of 1.1 μM to αvβ3 and a K_D of around 1 pm to FGFR1. It is thus expected that FGF1 will bind preferentially to FGFR on the cell surface. We expect that the binding of FGF1 to FGFR will facilitate the binding of FGF1 to αvβ3 because FGF1 is concentrated on the cell surface through binding to FGFR. Our results predict that the integrin-binding site of FGF1 would be still exposed upon binding to FGFR. The results in this study suggest that integrin αvβ3 is a major, if not the only, integrin that binds to FGF1. It has been reported that αvβ3 is detectable in NIH3T3 cells (52) and that αvβ3 is markedly induced when the Ras-ERK1/2 pathway is activated by Raf oncogene in NIH3T3 cells (53). The R50E mutation is close to the FGF1-FGFR1 domain D2 interface as shown in the crystal structure of the FGF1-FGFR1 complex (PDB code 1EVT). We showed that the R50E mutation did not affect the binding of FGF1 to FGFR1 D2D3. Also we showed that this mutant induced phosphorylation of FGFR1 and FRS2α and activation of ERK1/2 and AKT. It is thus unlikely that the R50E mutation suppressed FGFR binding. We propose that the primary defect in the R50E mutant is in its ability to bind to integrins.

In current models of the role of integrins in growth factor signaling, integrins interact with ECM ligands, and the resulting integrin-mediated signaling merges with growth factor-mediated signaling (54). The present results suggest that the direct binding of FGF to integrin αvβ3 is critical for proper FGF signaling and that FGF1 itself acts as an integrin ligand. If this is the case, it is likely that integrin-FGFR cross-talk may be mediated by direct binding of FGF to integrins and FGFR. We
showed that FGF1 binds to integrins in the presence of 10% serum, consistent with the previous reports that integrin-binding sites in major serum adhesive proteins (e.g. fibrinogen) are cryptic in solution as discussed above. We propose that FGF-integrin interaction occurs in body fluids. Also, we propose that FGF may be concentrated on the cell surface through high affinity binding to FGFR. This would facilitate FGF-integrin interaction on the cell surface. Because FGFR and integrins bind to distinct sites in FGF1, FGFR binding to FGF would not block FGF-integrin interaction. Thus we expect that FGF may bind to distinct sites in FGF1, FGFR binding to FGF would not interfere with interactions in FGF signaling. We propose that FGF-integrin interaction on the cell surface is biologically relevant. The present results, however, do not rule out the possibility that ECM-integrin interaction is required for FGF signaling. Integrin antagonists or down-regulation of integrins block both integrin-ECM and integrin-FGF interactions, and do not distinguish the two interactions in FGF signaling. The R50E mutant of FGF1 is thus an important tool that selectively blocks FGF1-integrin interaction in FGF signaling.

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