Structural Requirements of FGF-1 for Receptor Binding and Translocation into Cells†

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ABSTRACT: FGF-1 binds to and activates specific transmembrane receptors (FGFRs) and is subsequently internalized and translocated to the interior of the cell. To elucidate the role of the receptor in the translocation process, we studied the effects of the elimination of distinct sites of the ligand−receptor interaction. On the basis of the structure of the FGF-1−FGFR1 complex, we substituted four key amino acid residues of FGF-1 from the FGF-receptor binding site with alanines, constructing four point mutants and one double mutant. We determined by *in vivo* assays in NIH 3T3 cells the ability of the mutants to bind to specific FGF receptors, to stimulate DNA synthesis, and to activate downstream signaling pathways. We found that correct binding to the receptor is necessary for optimal stimulation of DNA synthesis. All four single mutants became phosphorylated to different extents, indicating that they were translocated to the cytosol/nucleus with varying efficiency. This indicates that despite a low affinity for FGFR, translocation to the cytosol/nucleus can still occur. However, simultaneous substitution in two of the positions led to a total loss of biological activity of the growth factor and prevented its internalization, implying that there is only one strongly receptor-dependent, productive way of translocating FGF-1. We also found that the process of translocation did not correlate with the thermal stability of the protein. Additionally, we observed a clear negative correlation between the stability of the FGF-1 mutants and the efficiency of their phosphorylation, which strongly suggests that protein kinases prefer the unfolded state of the protein substrate.

Human acidic fibroblast growth factor (FGF-1[1]) is a member of a large family of growth factors that exhibit a wide variety of biological activities and activate several signaling pathways (*1*, *2*). FGF-1 is a potent mitogen, stimulating DNA synthesis and proliferation in many cell types, and plays important roles in angiogenesis, tissue regeneration, and inflammation (*3*).

FGF-1 is synthesized as a cytosolic protein without an *N*-terminal signal sequence and is exported from the cell by a non-classical mechanism independent of the endoplasmic reticulum and Golgi apparatus (*4*). FGF-1 acts by activating specific transmembrane phosphotyrosine kinase receptors, of which there are four closely related isoforms, FGFR1−4 (*1*, *2*, *5*). FGFRs consist of an extracellular ligand binding part composed of three immunoglobulin-like domains (D1, D2, and D3), a single transmembrane helix, and a cytoplasmic split tyrosine kinase domain (*5*, *6*). Ligand binding and specificity depend on D2, D3, and the linker that connects them.

Upon binding to the receptor, the growth factor induces receptor dimerization and phosphorylation of specific cytoplasmic tyrosine residues (*7*). The phosphorylation of FGFR triggers the activation of cytoplasmic signal tranduction pathways, such as Ras-mitogen-activated protein kinase (MAPK), phospholipase C gamma (PLC-γ), phosphatidylinositol-3 kinase (PI3K)/Akt kinase, and p38 kinase cascades (*1*, *8*−*10*).

FGF-1 also binds specifically to extracellular heparan sulfate proteoglycans (HSPGs) and interacts with heparin (*1*). These polysaccharides play an essential role in FGF signaling by direct association with FGF and FGFR in a ternary complex at the cell surface (*11*). This observation is supported by crystallographic studies of FGF−FGFR−heparin complexes (*12*−*16*), which have provided a wealth of information about the interaction between FGF-1 and its partners and enabled us to identify amino acids in the growth factor that are important for receptor binding.

Externally added FGF-1 is able to enter the cytosol and nucleus, and it is likely that this protein has an intracellular function in addition to receptor activation (*17*−*22*). Evidence for FGF-1 translocation was obtained in experiments showing that externally added growth factor was modified *in vivo* by enzymes that are located in the cytosol or in the nucleoplasm

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[1] Abbreviations: FGF-1, fibroblast growth factor 1; FGFR, fibroblast growth factor receptor; WT, wild type; CD, circular dichroism; GdmCl, guanidinium chloride; MAPK, mitogen-activated protein kinase; PLC-γ, phospholipase Cγ; SDS−PAGE, SDS−polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride.

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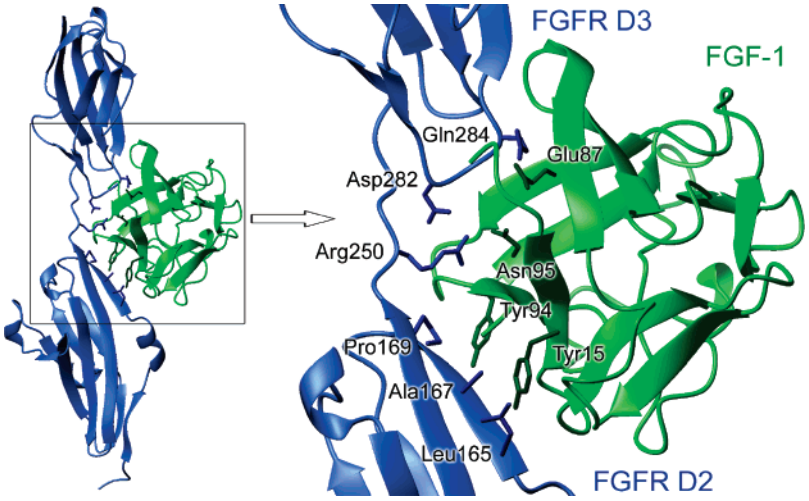

FIGURE 1: Fragment of the 3D structure of the FGF-1−FGFR1 complex (pdb code: 1evt) indicating the location of the mutated residues in FGF-1 and the residues in FGFR1 interacting with them.

and not at the cell surface or in vesicular compartments.

FGF-1, extended to contain a *C*-terminal farnesylation signal, a CAAX box, is farnesylated *in vivo* (*23*). Because farnesyl transferase is located exclusively in the cytosol and nucleus, farnesylation of exogenously added FGF-1 is possible only if the growth factor has crossed cellular membranes. The phosphorylation of FGF-1 *in vivo* is another assay that enables the verification of the presence of the growth factor in the interior of the cell. Although FGF-1 can be phosphorylated *in vitro* by casein kinase II (CK2) (*24*), the major kinase carrying out the phosphorylation of the growth factor *in vivo* is protein kinase C (PKC) (*25*). This enzyme is present in the cytosol and nucleus but not at the cell surface or inside vesicular compartments (*25*, *26*). Therefore, *in vivo* phosphorylation indicates that FGF-1 must be translocated into the cell (*25*). Wiedlocha et al. (*27*) found that the phosphorylation of exogenous FGF-1 occurs in the nucleus by the δ isoform of PKC and suggested that phosphorylation is involved in the regulation of the localization of internalized FGF-1. Another indication that FGF-1 plays a role inside cells is its interactions with intracellular proteins, such as FIBP, p34, and CK2 (*24*, *28*, *29*). It seems that the localization of FGF-1 to the nucleus is determined by the presence of two nuclear localization sequences (NLSs) (*17*, *30*).

The translocation of FGF-1 to the cytosol/nucleus has been observed in several cell types naturally carrying FGF receptors, such as NIH 3T3, CPAE, and HUVEC cells (*31*), and a growing number of studies suggest that exogenous FGF-1 is transported into a cell in a receptor-dependent manner (*32*). Translocation of FGF-1 to the cytosol occurs from the lumen of intracellular vesicles possessing vacuolar proton pumps and requires vesicular membrane potential (*31*, *33*). The major proportion of FGF-1 in the cytosol is translocated to the nucleus and phosphorylated, apparently by PKCδ, and then rapidly exported back to the cytosol where it is dephosphorylated and degraded (*27*). Entry of the growth factor into the nucleus does not require phosphorylation. The detailed mechanism by which FGF-1, or alternatively the FGF-1−FGFR complex, is transported through the membrane requires further investigation.

In contrast to its binding to FGFRs, the binding of FGF-1 to heparans on the cell surface does not result in its translocation. In cells lacking specific FGF receptors but expressing HSPGs, the growth factor is transported to the lysosome, where it is degraded (*21*, *34*).

Here, we describe studies where we have mutated selected amino acid residues in the receptor-binding region of FGF-1. We found that despite a considerably reduced affinity of the mutated FGF-1 for FGFR, single mutations in the receptor-binding region still allowed translocation to the cytosol/nucleus to take place. However, the simultaneous substitution in two positions involved in the interaction with the receptor resulted in a total loss of the growth factor activity and blocked its translocation. Thus, the results show that the internalization and translocation of FGF-1 are strongly receptor-dependent, and there appears to be no alternative productive way to translocate the extracellular growth factor. Furthermore, we found that the translocation of FGF-1 did not depend on the stability of the protein. Our results also imply that phosphorylation is favored for the less stable mutants in which the specific sequence recognized by the kinase is unfolded.

EXPERIMENTAL PROCEDURES

Design of FGF-1 Mutants Defective in Receptor Binding. The residues of FGF-1 interacting with the receptor were selected for site-directed mutagenesis on the basis of an analysis of the FGF-1−FGFR1 complex crystal structure (*35*) with the use of NACCESS (http://wolf.bms.umist.ac.uk/naccess) and Ligplot (*36*) software. On the basis of the expected amino acid contribution in the binding to the receptor, four singly and one doubly alanine-substituted FGF-1 variants were designed: Y15A, E87A, Y94A, N95A, and Y94A/N95A. Figure 1 illustrates the location of the mutated residues in the 3D structure of FGF-1. We mutated two tyrosine residues of FGF-1 (at position 15 and 94) from the hydrophobic interface between the growth factor and the D2 domain of FGFR1, asparagine 95 interacting mainly with the D2−D3 linker in the receptor, and the glutamic acid residue at position 87 taking part in the binding to the βB′−βC loop of the D3 domain of the receptor.

Tyr15 and Tyr94 make hydrophobic contacts with Ala167 and Pro169 in FGFR, respectively. Additionally, the side chain of Tyr15 forms two hydrogen bonds with the main

chains of Leu165 and Ala167 receptor residues, and Tyr94 makes a water-mediated hydrogen bond with the backbone of Ala167. The highly conserved asparagine in the FGF family (at position 95 in FGF-1) involved in the FGF−linker interface forms a hydrogen bond with the Arg250 side chain of FGFR. The conserved interactions between FGF-1 and the βB′−βC loop of the D3 domain of the receptor are mediated mainly by a hydrogen bond between Glu87 of FGF-1 and the Gln284 residue in the receptor. This interface seems also to be supported by a water-mediated hydrogen bond between the side chain of Asn95 in FGF-1 and the backbone carbonyl oxygen of Asp282.

Mutagenesis, Protein Expression and Purification. A construct comprising a truncated form (residues 21−154) of human FGF-1 in the pET-3c vector was used. Mutagenesis, protein expression, and purification followed previously published procedures (*37*, *38*). Briefly, we used a site-directed mutagenesis protocol and mutagenic oligonucleotides of 28−36 bases in length to introduce mutations. All constructs were confirmed by DNA sequencing. Recombinant proteins were expressed at 37 °C in an *E. coli* Bl21(DE3)pLysS strain and purified on a heparin−sepharose CL-6B column (*21*, *37*, *38*). Protein purity was confirmed by silver-stained SDS−PAGE, and molecular masses were verified by electrospray ionization mass spectrometry. The concentrations of wild type FGF-1 and its mutants were determined spectrophotometrically using molar extinction coefficients calculated as described by Pace et al. (*39*). The numbering system used to identify amino acids in FGF-1 has been reported previously (*37*).

Spectroscopic Studies. For all proteins, the high-salt elution buffer was changed to phosphate buffer (25 mM H_3PO_4, pH 7.3) before spectroscopic measurements using a HiTrap desalting column and an ÄKTA Explorer chromatography system (Amersham Biosciences).

To analyze recombinant proteins in context to the differences in the secondary and tertiary structure, we acquired circular dichroism (CD) and fluorescence spectra at 21 °C. The CD spectra were collected in the 195−340 nm wavelength range on a Jasco J-715 spectropolarimeter with a slit width set to 2 nm and a response time of 1 s. Fluorescence measurements were performed using an FP-750 spectrofluorimeter (Jasco) with an excitation wavelength of 280 nm and emission spectra with a range from 300 to 450 nm. Circular dichroism and fluorescence were also used to study thermal denaturation. Measurements were performed in the presence of 0.7 M guanidinium chloride (GdmCl) in the phosphate buffer. The temperature-induced ellipticity changes at 227 nm were followed in cuvettes of 10 mm path length using a slit width of 2 nm and an 8 s response time. Denaturation performed in the spectrofluorimeter was monitored by changes in the emission signal at 353 nm of the single tryptophan residue excited at 280 nm (*40*). Both excitation and emission slits were 5 nm wide.

All denaturation data, irrespective of the method, were collected using a scan rate of 0.25 °C/min. The temperature of the sample was monitored directly using a probe immersed in the cuvette and controlled by an automatic Peltier accessory. The measurements were carried out at a protein concentration of 2×10^{-6} M. The data were analyzed using PeakFit software (Jandel Scientific Software) assuming a two-state reversible equilibrium transition as described previously (*37*, *38*).

Protease Digestion Assay. Digestion experiments were carried out at 37 °C by incubation of the wild type and mutants of FGF-1 with trypsin in a 1:400 ratio in 100 mM Tris and 20 mM calcium chloride at pH 8.3. The products of the protease reaction were visualized by SDS−PAGE and Coomassie staining.

Receptor Binding Assay. Binding assays were performed in manner essentially the same as those published previously (*21*, *37*, *38*). Wild type FGF-1 in high-salt buffer was dialyzed against PBS and iodinated using the Iodogen method (*41*). Labeled growth factor was then purified on a disposable PD-10 desalting column (Amersham Biosciences). Confluent NIH 3T3 cells were washed with an ice-cold binding buffer (DMEM containing 50 mM HEPES and 10 U/mL heparin at pH 7.4) and incubated with 6 ng/mL of ^{125}I-labeled FGF-1 and increasing concentrations of unlabeled FGF-1 or its mutants for 3 h at 4 °C. Subsequently, the cells were washed twice with ice-cold binding buffer, once with ice-cold PBS, followed by the addition of ice-cold 1 M NaCl in PBS to dissociate the growth factor from heparans at the cell surface. After washing, the cells were lysed in 0.1 M KOH, and the solubilized radioactivity, representing the cell-surface-bound ^{125}I-FGF-1, was measured with a γ-counter. Nonspecific binding was determined by the incubation of the cells in the presence of a 100-fold excess of unlabeled growth factor. To calculate IC_{50} values, the data were fitted to a single binding site curve.

Measurements of DNA Synthesis. The mitogenic properties of FGF-1 mutants were assessed by monitoring [^{3}H]thymidine incorporation in response to the stimulation of NIH 3T3 cells (*25*, *37*, *38*). The cells were starved in serum-free DMEM containing 2.5 μg/mL insulin and 2.5 μg/mL transferrin for 24 h at 37 °C. Then, increasing amounts (0.1 to 100 ng/mL) of FGF-1 or its mutants were added, and the incubation was continued for 24 h at 37 °C in the presence of 10 U/mL heparin. For the last 6 h, 1 μCi/mL [^{3}H]-thymidine was added to the cells. Finally, the cells were treated with 5% trichloroacetic acid and then dissolved in 0.1 M NaOH, and the level of trichloacetic acid-insoluble radioactivity was measured using a liquid scintillation β-counter. The relationship between the [^{3}H]thymidine incorporation and log concentration of the added growth factor was fitted to a sigmoidal function. Specific mitogenic activity was expressed as the concentration giving 50% of maximal stimulation (EC_{50}).

Western Blot Analysis. To detect the phosphorylated forms of downstream signaling proteins, serum-starved NIH 3T3 cells were stimulated for 10 min with 2 ng/mL of the growth factor in the presence of heparin (10 U/mL). After washing with PBS, the cells were lysed with SDS sample buffer, scraped, and sonicated. Total cell lysates were separated by SDS−PAGE, transferred to a nitrocellulose membrane, and subjected to immunoblot analysis (*38*). The blot was incubated with the following primary anti-phospho antibodies: phospho-p44/42 MAP kinase antibody, phospho-Akt antibody (Cell Signaling), phospho-p38 antibody (BD Transduction Laboratories), and phospho-PLC-γ1 antibody (Santa Cruz Biotechnology). The primary antibodies were detected with the corresponding horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoReasearch Laboratories) and then visualized using a chemiluminescent sub-

strate. After stripping, the membrane was re-probed with antibodies against total signaling proteins: p44/42 MAP kinase antibody, Akt antibody, p38 antibody (Cell Signaling), and PLC-γ1 antibody (Santa Cruz Biotechnology) to ensure equal loading.

Phosphorylation in Vitro. Phosphorylation experiments *in vitro* were performed with protein kinase C (PKC) from rat brain (Sigma) and casein kinase II (CK2) (Sigma). Wild type FGF-1 and FGF-1 mutants (500 ng) were incubated with purified kinase and 40 μCi/mL [γ-^{32}P]ATP in reaction buffer (25 mM HEPES at pH 7.5, 10 mM $MgCl_2$, 100 μM Na_2MO_3, 100 μM Na_3VO_4, 10 mM sodium β-glycerophosphate, 1 mM DTT, and 10 mM NaF) at 30 °C for 30 min. Additionally, 1 mM $CaCl_2$ or 150 mM NaCl was added to the reaction buffer for CK2 or PKC, respectively. The reaction mixtures were resolved by SDS−PAGE, stained with Coomassie Blue dye to ensure equal loading, and subjected to autoradiography. The intensity of the bands corresponding to the phosphorylated FGF-1 variants was analyzed with Quantity One 1-D Analysis Software (Bio-Rad).

Phosphorylation in Vivo. Phosphorylation *in vivo* was performed essentially as described by Klingerberg et al. (*42*) and Wiedlocha et al. (*27*). NIH 3T3 cells were starved for 24 h in DMEM with 1% fetal bovine serum (FBS). For the last 8−12 h, starvation was continued in phosphate-free DMEM with 1% FBS and 20 μCi/mL [^{33}P]-phosphate. Then the cells were treated with 20−100 ng/mL of growth factor in the presence of 10 U/mL heparin, and the incubation was continued for an additional 6 h. Subsequently, the cells were washed once with PBS containing a phosphatase inhibitor cocktail, lysed in lysis buffer (10 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, 1% Triton X-100, and both protease- and phosphatase-inhibitor cocktails at pH 7.4), and sonicated. Then, the lysates were adsorbed onto heparin−sepharose. To reduce the background of phosphorylated proteins, the beads were washed with 0.6 M NaCl and subjected to trypsin digestion (2 μg/mL) for 30 min at room temperature. Finally, samples were washed twice with PBS containing 1 mM PMSF and analyzed by SDS−PAGE and fluorography.

RESULTS

Production and Physicochemical Characterization of FGF-1 Mutants. On the basis of the FGF-1−FGFR1 complex crystal structure (*35*), we selected four positions in the FGF-1 molecule for alanine mutations (Figure 1), constructing four singly and one doubly substituted FGF-1 variants. All of the expressed mutants were obtained with a yield of about 20 mg from 1 L of bacterial culture. A major fraction of each recombinant protein was found in the soluble fraction, and therefore, all mutants could be easily purified on a heparin−sepharose column. None of the mutated residues appeared to be involved in the interaction of the growth factor with cell surface HSPGs, and as expected, all variants equally bound to immobilized heparin as well as the wild type and exhibited the same elution profile during purification. Each mutant protein preparation was analyzed for purity by SDS−PAGE gels, and no contaminant protein bands were seen after silver staining. Conformation of the variants was studied by circular dichroism and fluorescence in phosphate buffer at 21 °C. No significant differences between the spectra of the mutants and the wild type were observed, suggesting that a native-like architecture and conformation was preserved in all variants. The fluorescence spectra of all mutants were dominated by multiple tyrosine fluorescence at approximately 305 nm (data not shown). There was no signal from the single Trp (position 107) at 353 nm because the fluorescence of this residue is completely quenched in the properly folded protein (*43*). Similarly, all CD spectra showed a minimum at around 206 nm, typical of β-sheet-rich proteins of the β-II type (*44*, *45*), and a positive peak between 220 and 240 nm, with the maximum centered near 228 nm (data not shown).

Table 1: Thermodynamic Parameters for Thermal Denaturation of the Wild Type and Alanine Mutants of FGF-1[a]

	circular dichroism			fluorescence		
mutant	T_{den} (°C)	ΔT_{den} (°C)	ΔH_{den} (kcal mol^{-1})	T_{den} (°C)	ΔT_{den} (°C)	ΔH_{den} (kcal mol^{-1})
WT	40.3[b]		64.9[b]	39.8[b]		66.2[b]
Y15A	30.1	−10.2	39.2	28.9	−10.9	43.0
E87A	37.0	−3.3	61.0	37.0	−2.8	60.7
Y94A	31.4	−8.9	44.3	29.2	−10.6	46.6
N95A	41.7	1.4	64.7	40.9	1.1	65.9
Y94A/N95A	33.9	−6.4	47.4	32.0	−7.8	49.9

[a] The data shown are mean values of three independent denaturation experiments. The standard errors in T_{den} and ΔH_{den} were estimated to be ±0.1 °C and ±5.0 kcal/mol, respectively. [b] The values are from Zakrzewska et al. (*38*).

Stability Studies. Because our previous study indicated that the stability of FGF-1 could correlate with its biological action (*38*), we determined the stability parameters for the FGF-1 mutants substituted at receptor binding sites. To measure accurately the differences in conformational stability, we employed thermal denaturation monitored by two independent spectroscopic techniques, fluorescence and circular dichroism. The fluorescence measurements enabled us to assess the disruption of the tertiary structure upon temperature increase by following changes in the emission signal around 353 nm. During unfolding, the protein structure becomes less rigid and the quenching effect of the pyrrole and imidazole groups surrounding the single Trp residue in FGF-1 is eliminated, resulting in a strong fluorescence signal, characteristic of the indole side group (*43*, *46*). To ensure that the increase in fluorescence was caused by the global unfolding of FGF-1 and not by the local relaxation of the structure, we also monitored the denaturation by ellipticity. Changes in the secondary structure of FGF-1 result in a replacement of the positive signal at 228 nm associated with the β-structure by a negative one (*46*). The unfolding profiles of wild type FGF-1 and its mutants obtained by circular dichroism superimposed the unfolding curves derived by the fluorescence method very well. The denaturation experiments were performed in the presence of 0.7 M GdmCl because such an addition of the denaturant successfully prevented both protein aggregation and the accumulation of folding intermediates during heating, thus leading to a two-state reversible process (*47*). The agreement between the thermodynamic parameters obtained by the two different measurement methods also confirms that all analyzed proteins undergo a two-state transition. Table 1 summarizes the denaturation temperatures (T_{den}), denaturation temperature changes ($\Delta T_{den} = T_{den}$(mutant) − T_{den}(WT)), and the van't Hoff enthalpy data (ΔH_{den}) from the denaturation experiments. The effects of mutations on the conformational

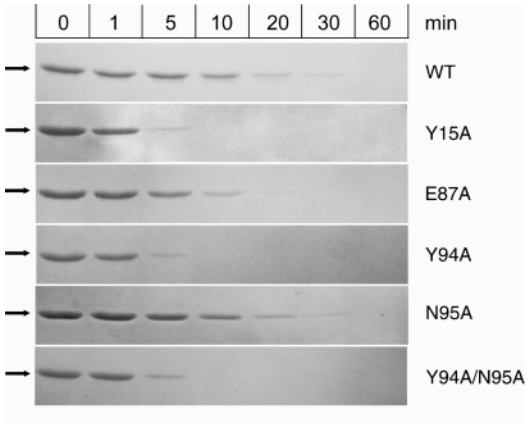

FIGURE 2: Proteolytic degradation of the wild type and alanine variants of FGF-1 by trypsin at 37 °C. The arrow indicates the position of intact FGF-1.

stability of FGF-1 can be easily assessed by comparing the T_{den} values of respective variants with the T_{den} value of the wild type. All but one mutant (N95A) exhibited a thermal stability lower than that of the wild type. Introduction of the alanine substitution at position 15 (variant Y15A) caused the most significant decrease in the denaturation temperature, over 10 °C. Similarly, the replacement of the tyrosine residue at position 94 (mutant Y94A) resulted in a strong reduction of the thermal stability of FGF-1. A less noticeable effect on the growth factor stability was observed in the case of the E87A variant. Its denaturation temperature was about 3 °C lower than that of the wild type.

The only alanine mutation that increased the stability of FGF-1 (by over 1 °C) was the substitution of asparagine at position 95. The double mutant, Y94A/N95A, exhibited lowered stability compared to that of the wild type, but because of the contribution of the N95A mutation, it showed a higher one than that of the single alanine mutant Y94A. The effects of these two point mutations accumulate in the double mutant, however, they are not fully additive. The reason for such behavior lies in the close vicinity of these two neighboring residues.

Susceptibility of FGF-1 Mutants to Proteolysis. Using spectroscopic methods, we demonstrated that the overall structure of FGF-1 remained unchanged upon the introduction of the alanine substitutions. However, mutations that do not influence the global conformation can result in local relaxation of the protein fold, thus effectively promoting proteolytic digestion. But it is well known that unfolded proteins are much more susceptible to protease attack (*48*−*50*). Because we observed differences in the conformational stability of the FGF-1 mutants, we decided to examine their susceptibility to proteases. We treated the growth factors with trypsin at 37 °C to be able to assess the possible correlation between proteolytic degradation and biological activities in NIH 3T3 cells studied at the same temperature. To digest wild type FGF-1 completely, a period of 1 h was required (Figure 2). All proteolytic results obtained for the FGF-1 mutants were in excellent agreement with the stability parameters reported in Table 1. As shown in Figure 2, the mutant most susceptible to trypsin digestion was the Y15A variant, which also exhibited the lowest denaturation temperature. As suggested by thermodynamic results, the Y94A and Y94A/N95A mutants were next in the proteolytic rank order. Mutant E87A, with T_{den} equal to 37.0 °C, was somewhat more sensitive to trypsin than the wild type. The most stable among the alanine variants, the N95A mutant, was similarly resistant to proteolysis as the wild type. The substitution of Asn95 with alanine improved the stability of the growth factor only slightly; therefore, it was not expected to strongly influence proteolytic processing. The results demonstrate a clear correlation between the conformational stability and resistance to protease action, suggesting that the degradation of FGF-1 variants is facilitated by global unfolding and not by local structural fluctuations.

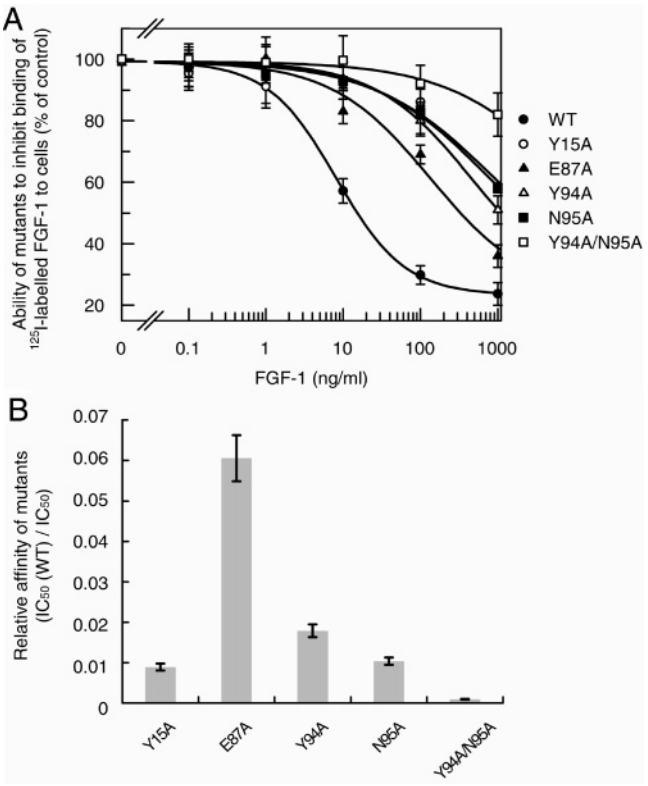

FIGURE 3: Ability of the wild type and alanine mutants of FGF-1 to bind to NIH 3T3 cells, measured as the ability to prevent the binding of ^{125}I-labeled wild type FGF-1. (A) Results expressed as a percentage of the control (cells treated with labeled FGF-1 only) and are the means of three experiments ± SD. The symbols denote wild type FGF-1 (●) and the Y15A (○), E87A (▲), Y94A (△), N95A (■), and Y94A/N95A (□) mutants. (B) Relative affinity for the receptor of each alanine mutant expressed as IC_{50}(WT)/IC_{50}.

Receptor-Binding Affinity of the FGF-1 Alanine Mutants. To experimentally determine how much the selected sites are involved in FGFR binding, we tested the affinity of the FGF-1 variants for the receptor by a competition assay in which we measured the ability of increasing concentrations of each mutant to prevent the binding of the radiolabeled wild type growth factor to the NIH 3T3 cells. Each of the single amino acid mutations strongly reduced the binding of FGF-1 to FGFR (Figure 3). The smallest decrease in affinity was observed for the E87A mutant, which bound to the cell surface receptors present on NIH 3T3 cells ~16-fold less strongly than the wild type FGF-1. In contrast, the Y15A mutation reduced the affinity for FGF-1 to FGFR by a factor of ~112. The single substitutions of Tyr94 or Asn95 with alanine reduced the receptor-binding affinity about 56- and 96-fold, respectively. In the case of the Y94A/N95A double mutant, a more than 1000-fold decrease in binding was observed, indicating that there is a cumulative effect of

each single-site replacement on the affinity of the growth factor for the receptor.

The strong reduction in the receptor affinity of the FGF-1 variants confirms a direct involvement of the selected residues of the growth factor in the interactions with the high-affinity receptor. The reduced binding can only be due to a lower affinity for the receptor because the affinity of the mutants for heparin was in each case like that of the wild type FGF-1. Moreover, spectroscopic analysis of the mutants did not indicate any conformational changes, thus providing additional evidence that the different abilities of the mutants to compete with the ^{125}I-labeled wild type FGF-1 depend only on the direct interactions of the FGF-1 molecule with the receptor.

Ability of the Mutants to Stimulate DNA Synthesis. The binding of FGF-1 to quiescent cells induces a strong stimulation of DNA synthesis and cell proliferation. When the growth factor binds to the cell surface receptor, it induces phosphorylation of the receptor tyrosine kinase domain that causes the activation of downstream signaling cascades, which in turn induce the expression of immediate early genes followed by mitogenesis (*1*, *32*). In addition, the growth factor is internalized by receptor-mediated endocytosis and then translocated into the cytosol and nucleus (*27*, *32*). We, therefore, used the FGF-1 mutants to study how the affinity of the growth factor for the receptor correlated with its mitogenic activity.

To test the ability of the mutants to stimulate DNA synthesis, we performed [^{3}H]thymidine incorporation experiments in NIH 3T3 cells in the presence of heparin, which facilitates the formation of the growth factor−receptor complex. As shown in Figure 4, none of the FGF-1 mutants were able to stimulate DNA synthesis at equally low concentrations as the wild type. The strongest mitogenic activity among the FGF-1 variants was found for the E87A mutant, which exhibited a 2.5-fold lower DNA synthesis stimulation than that of the wild type (EC_{50} 2.5-fold higher than that of the wild type). Both the Y94A and the N95A mutants were about five times less active than the wild type in this assay. The Y15A mutant exhibited a 7-fold reduction in mitogenic activity compared to that of the wild type. The double mutant Y94A/N95A was practically inactive, showing an over 500-fold lower mitogenic activity than that of the wild type. The results demonstrate that the reduction of FGF-1 affinity for specific cell surface receptors caused by one point mutation is strong enough to significantly reduce the mitogenic effect of the growth factor. The double substitution in the binding region of FGF-1 resulted in an almost total loss of the ability to stimulate DNA synthesis.

Activation of Downstream Signaling Pathways. To characterize the mutants further, we tested their ability to activate downstream signaling pathways induced by FGF-1. We used antibodies specific for the phosphorylated forms of Akt, MAPK, p38, and PLC-γ (Figure 5, upper panels) because these proteins are involved in the main signaling cascades initiated by FGF-1. These pathways are activated rapidly (within a few minutes) in response to mitogen stimulation, and we, therefore, measured phosphorylation after a 10-min incubation. In serum-starved cells with no added growth factor, there was only weak basal phosphorylation of the signaling proteins tested (Figure 5, upper panels; lane 1). After wild type FGF-1 stimulation, significant activation of

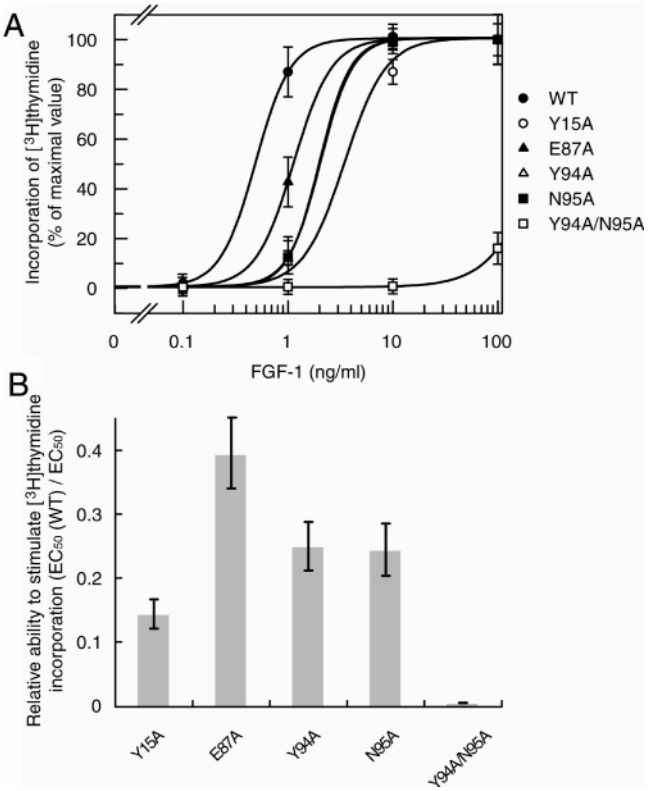

FIGURE 4: Normalized mitogenic activity curves of the wild type and mutants of FGF-1 in the presence of heparin from a [^{3}H]-thymidine incorporation assay in NIH 3T3 cells. (A) Data shown are mean values of at least five independent experiments. The error bars indicate standard deviations of the measurements. The identities of the curves are as follows: wild type FGF-1 (●) and the Y15A (○), E87A (▲), Y94A (△), N95A (■), and Y94A/N95A (□) mutants. (B) Relative mitogenic activity of each mutant expressed as EC_{50}(WT)/EC_{50}.

Akt, MAPK, p38, and PLC-γ was observed (Figure 5, upper panels; lane 2). Treatment with single mutants of FGF-1 induced only a very small increase in the level of phosphorylated and, therefore, active signaling molecules (Figure 5, upper panels; lanes 3−6). The double mutant Y94A/N95A had no influence on the level of phosphorylated signaling proteins, indicating that it was unable to activate the signaling cascades (Figure 5, upper panels; lane 7). The Western blots were stripped and reprobed with antibodies against total signaling proteins to ensure equal loading of the gels (Figure 5, lower panels).

As expected, single substitutions significantly altered the extent of activation of the downstream signaling proteins, which clearly correlated with the results obtained in the mitogenic and binding experiments. Also, after longer exposure to FGF-1 variants (3 and 6 h), the phosphorylation pattern was very similar to that obtained after the 10-min treatment (data not shown). However, in this case, all phosphorylated bands were much weaker.

Phosphorylation of FGF-1 Variants in Vitro. To test whether FGF-1 can be transported into the cell despite disturbed binding to the receptor, we used FGF-1 phosphorylation as a marker of its translocation. We first checked if all of the FGF-1 variants were substrates for the two kinases, PKC and CK2, known to phosphorylate the growth factor *in vitro*. In the *in vitro* phosphorylation experiments, we used both enzymes, although the main kinase phosphorylating FGF-1 *in vivo* is PKC (*25*, *27*).

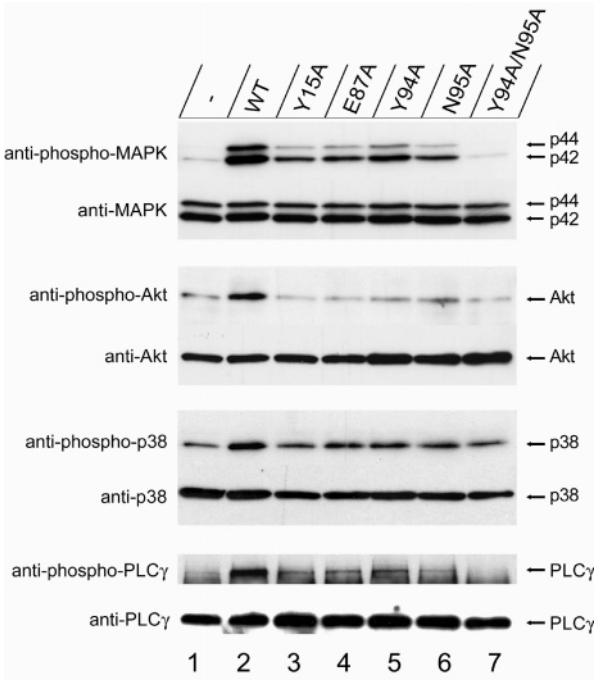

FIGURE 5: Ability of the wild type and alanine variants of FGF-1 to activate p44/42 MAP kinases, Akt kinase, p38 MAP kinase, and the PLC-γ signaling pathway in NIH 3T3 cells. To assay the activation of the downstream proteins, anti-phosphoprotein antibodies were used (upper panels). To verify equal loading, stripped membranes were blotted with antibodies that recognized the total amount of each signaling protein (lower panels).

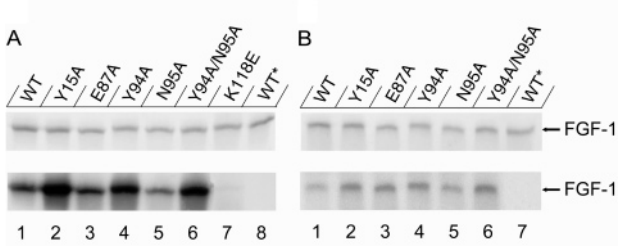

FIGURE 6: Phosphorylation of the wild type and alanine variants of FGF-1 *in vitro* by (A) PKC and (B) CK2 kinases. The upper panels represent Coomassie Blue-stained SDS−polyacrylamide gels and the lower panels their autoradiographs. (*) Control experiment with wild type FGF-1 without kinase.

Because the FGF-1 phosphorylation site by PKC is well known (*25*, *42*) and all of the alanine mutations introduced were located far away from the consensus sequence for the kinase, we did not expect to find large differences in phosphorylation between the variants and wild type FGF-1. As shown in Figure 6 A, all mutants were effectively phosphorylated in contrast to a control mutant, K118E (in the other numbering system, K132E) (lane 7), in which the phosphorylation site for PKC had been destroyed (*25*, *51*). However, unexpectedly, we found strong differences between the mutants in the intensity of *in vitro* phosphorylation. The most effectively phoshorylated mutants were Y15A, Y94A, and Y94A/N95A, which were the least thermodynamically stable ones. The most stable mutant, N95A, was slightly less phosphorylated than the wild type (Figure 6 A; lane 5). We observed a clear correlation between the stability of the FGF-1 variants and the extent of their phosphorylation. The results suggest that the unfolded state of the protein is preferred by the kinase because the *in vitro* experiments were performed at 30 °C. At this temperature, the less stable variants were denaturated to almost 50%. To confirm the correlation between the unfolding and the effectiveness of FGF-1 phosphorylation, we carried out an additional experiment with the chemically denatured wild type. The unfolded FGF-1 was several times more strongly labeled than the control (native) protein (data not shown).

Most studies regarding protein phosphorylation emphasize the importance of the amino acid sequence of a protein to be phosphorylated and not its conformation. We were, therefore, surprised that the phosphorylation of unfolded FGF-1 occurs much more efficiently. However, a recent article by Iakoucheva et al. (*52*) suggests a preference of phosphorylation sites for disordered regions. Even when the consensus sequence is ordered, there is a strong possibility of local unfolding of the region surrounding the phosphorylation site just prior to the phosphate attachment. These observations are in very good agreement with our results, which point to a significant increase in phosphorylation efficiency when the protein is less stable.

CK2 has been shown to bind FGF-1 via the catalytic α-subunit as well as the regulatory β-subunit (*24*). Moreover, it was shown that the growth factor is phosphorylated by CK2 *in vitro* (*24*). It is highly possible that the same phosphorylation event can take place *in vivo*. Therefore, we tested whether the alanine mutants of FGF-1 were phosphorylated not only by PKC but also by CK2. It should be mentioned that the phosphorylation sites for CK2 in the FGF-1 sequence have not yet been identified. In an *in vitro* experiment, CK2 generated phosphorylation patterns of the mutants that were very similar to the ones obtained in the PKC reaction, but the signals in this case were much weaker (Figure 6 B). All of the mutants were labeled, indicating that they were proper substrates for the kinase. However, again, we observed differences in the intensities of the bands, in agreement with the thermodynamic data and the phosphorylation results for PKC.

We conclude that the phosphorylation sites of the mutants were functional *in vitro*, and we proceeded to study the phosphorylation of the FGF-1 mutants in living cells.

Phosphorylation in Vivo as a Measure of Translocation. Because we were interested in the intracellular fate of the FGF-1 mutants, we employed the *in vivo* phosphorylation assay to monitor their translocation into the cell. This experimental method takes advantage of the fact that PKC, the enzyme known to be responsible for phosphorylation inside the cell, is a cytosolic and nuclear enzyme and that cells are only able to phosphorylate the growth factor that has crossed the cell membrane. Because it is believed that intracellular interactions of FGF-1 are important for its biological action (*32*, *53*), we investigated whether the observed decreased mitogenic potential of the mutants was caused not only by their lower affinity for the receptor but also by a reduced ability of the receptor-bound growth factors to be translocated to the interior of the cell.

The wild type and mutants of FGF-1 were added to serum-starved NIH 3T3 cells in the presence of radioactive phosphate and incubated for 6 h. We first employed 20 ng/mL of FGF-1. In this case, only the wild type FGF-1 and the E87A mutant exhibited visible labeling (Figure 7 A, lanes 1 and 3). At a higher concentration (50 ng/mL), all of the single mutants were labeled but to varying extents (Figure

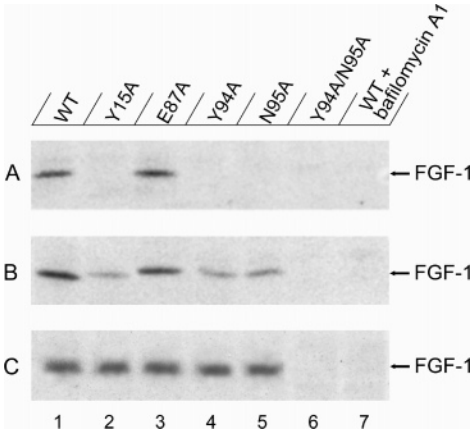

FIGURE 7: Phosphorylation *in vivo* of the wild type and alanine variants of FGF-1 in NIH 3T3. Different concentrations of the growth factor were applied: (A) 20 ng/mL; (B) 50 ng/mL; (C) 100 ng/mL. The control experiment was performed in the presence of 10 nM bafilomycin A1 preventing the internalization of FGF-1 (lane 7).

7 B). This indicates that all single alanine mutants of FGF-1 are translocated into the cytosol/nucleus and phosphorylated, although with differing efficiencies. At a concentration of 100 ng/mL, all of the single mutants and the wild type growth factor were labeled to the same extent (Figure 7 C).

The data indicate that despite the significant weakening of receptor binding, the single amino acid mutants were still able to enter the cell provided a sufficiently high concentration of the growth factor was present in the medium to compensate for the reduced affinity. The double mutant, which hardly interacted with the receptor (its affinity being more than 1000-fold lower than that of the wild type), was not phosphorylated even at a concentration of 100 ng/mL (Figure 7 C; lane 6). The result supports the contention that the internalization and translocation of FGF-1 is strongly receptor-dependent and that there is no alternative productive way of translocating extracellular FGF-1.

In the presence of a translocation inhibitor, bafilomycin A1, an inhibitor of the vesicular proton pump (*31*), no labeling of FGF-1 was observed (Figure 7, lane 7). This control experiment confirmed that externally added single FGF-1 mutants are indeed phosphorylated inside the cell.

Additionally, we verified the process of FGF-1 internalization in NIH 3T3 cells using Western blotting analysis (data not shown). As expected, the wild type of FGF-1 as well as the single alanine mutants were effectively internalized, in contrast to the double mutant (Y94A/N95A), which did not bind to the cell surface receptor and, therefore, was not internalized.

DISCUSSION

The main finding in this article is that four single amino acid mutations of FGF-1 in the site of interaction with the growth factor receptor, reducing the affinity for the receptor, do not prevent the translocation of FGF-1 to the cytosol/nucleus. We also found that in our translocation assay, the individual mutants were labeled to different extents. This might reflect differences in transport or the phosphorylation of the internalized growth factor mutants. The former explanation appears to be the most likely one because the mutant that was the most strongly labeled growth factor *in vitro* was not the most strongly labeled *in vivo* (compare Figures 6 and 7). Furthermore, the mutant most strongly labeled *in vivo* (E87A) was the one with the least reduced affinity for the receptor.

We constructed and characterized five mutants of FGF-1 (four single and one double) affecting the FGF-1−FGFR interface. The amino acids chosen for substitutions were selected on the basis of the structural information for the FGF-1−FGFR1 complex (*35*). To evaluate the contribution of individual FGF-1 residues in high-affinity receptor binding, we replaced each of them with alanine and determined the receptor-binding affinities of the resulting mutants. Moreover, we tested their conformational stability, susceptibility to protease digestion, and biological activities, such as activation of the downstream signaling pathways and stimulation of DNA synthesis as well as their ability to translocate to the cytosol/nucleus.

All cell culture experiments were performed in NIH 3T3 cells because this cell line expresses predominantly FGFR1 (*25*). Our results from competition assays with ^{125}I-labeled-FGF-1 demonstrate that all individual alanine mutations reduced the ability of the growth factor to bind to its receptor. Individual replacement of any of the four selected amino acids with alanine lowered the FGFR binding affinity 16- to 110-fold with no alteration in heparin affinity. An over 1000-fold decrease in FGFR binding was observed for the Y94A/N95A double mutant, suggesting the additivity of the effects of the single-site substitutions on binding affinity. The dramatic decrease in FGFR binding observed for the mutant proteins confirms the direct involvement in FGFR binding interactions.

The FGF−FGFR interactions involve two highly conserved interfaces between FGF and the receptor D2 domain and between FGF and the receptor linker (connecting D2 and D3), whose combination defines the overall binding site (*13*, *35*). Specificity is achieved through interactions between the *N*-terminal and central regions of FGFs and the two loop regions in D3 that may vary between receptors because of alternative splicing. In FGF-1, the side chains of Tyr15, Tyr94, Leu133, and Leu135 form a hydrophobic patch interacting with D2. Additionally, Tyr15 and Tyr94 form hydrogen bonds that further strengthen the mainly hydrophobic FGF-1−D2 interface (*35*). The interactions with the growth factor in the receptor linker region are mediated mainly via hydrogen bonds. The side chain of Asn95 and the backbone carbonyl oxygen of His93 of FGF-1 are involved in this region. Conserved interactions between FGF-1 and the βB′−βC segment of D3 include Glu87, which makes a central hydrogen bond with a glutamine in this loop. A water-mediated hydrogen bond created by the side chain of Asn95 also contributes to this interface (*13*).

A comparison of the crystal structures of FGF-1−FGFR1 and FGF-2−FGFR1 (*13*, *35*) shows very similar interactions of these two growth factors with the receptor, especially in the D2 and D2−D3 linker region. Substitutions of residues Tyr24, Gln96, Tyr103, and Asn104 in FGF-2 with alanines (corresponding to the substitutions in FGF-1 analyzed in this study) gave mutants with a strong reduction in affinity for FGFR1 (*54*−*56*). However, it should be noted that the values reported for binding varied somewhat depending on the method of measurement. Our results for FGF-1 are in very

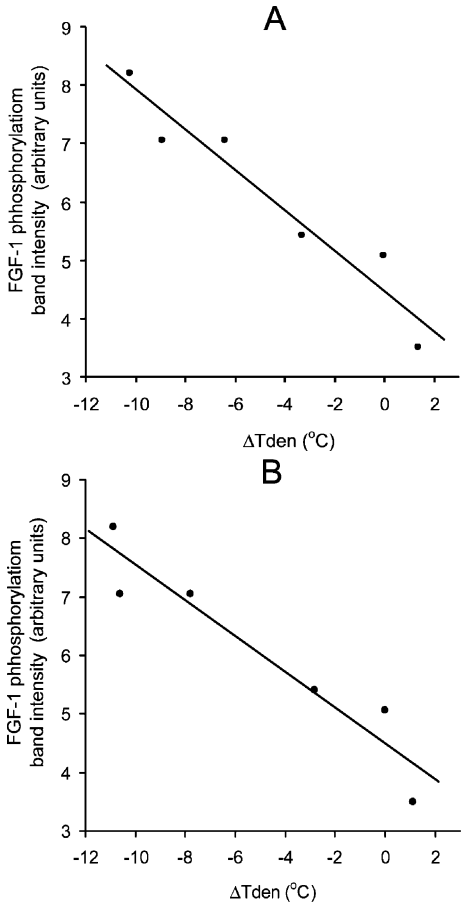

FIGURE 8: Correlation of the intensity of *in vitro* phosphorylation by PKC of the wild type and alanine variants of FGF-1 and their stability. The stability is expressed as the change in denaturation temperature (ΔT_{den}) obtained from denaturation experiments monitored by (A) CD and (B) fluorescence measurements.

good agreement with the study of Springer et al. (*54*), who assessed the binding affinity using a cell culture system.

Recently, we found that the stability of FGF-1 could correlate with its biological action (*38*). Therefore, we checked how the introduction of the alanine mutations affected the thermodynamical behavior of the growth factor. Three out of the four single substitutions significantly reduced the protein stability. Because all of the mutation sites were located within β-strands, we suggest that the differences in the stability of the FGF-1 variants reflect the propensities of the alanines at the mutated positions to form β-structure (*57*). Moreover, in the case of the E87A mutation, the decrease of T_{den} (in comparison to that of the wild type) can be caused by the disruption of the intramolecular hydrogen bonds upon alanine substitution. As a result of enhanced thermal unfolding, we observed an increased rate of trypsin digestion for the Y15A, E87A, and Y94A variants.

Because all but one mutant (N95A) exhibited lower stability than the wild type, we performed the mitogenic assay in the presence of heparin to prevent the extracellular degradation of the growth factor and to be able to directly observe the effect of the lower affinity for the receptor on the stimulation of DNA synthesis. The results confirm that proper binding to specific FGF receptors is required for FGF-1 mitogenic activity, as was proposed previously (*23*, *25*, *51*, *53*).

Because of the presence of heparin, a highly stabilizing compound for FGF-1, we did not notice any correlation between the stability of the FGF-1 mutants and their ability to activate the receptor. However, protein stability could influence the internalization and translocation of the FGF-1 variants. In our study, we checked how the lowering of the affinity for the receptor affects the internalization process. Surprisingly, all single alanine mutants were not only efficiently internalized but also effectively phosphorylated *in vivo*, confirming their translocation. The double mutant showed little or no binding to the specific FGF receptors (Figure 3), and in accordance with this, it did not translocate to the cytosol/nucleus, where the PKCδ was located. Even though the double mutant was a good substrate for the enzyme *in vitro*, it was not *in vivo*, where it remained separated from the enzyme by a membrane. All together, the data indicate that the conformational stability of FGF-1 does not influence its transport to the cytosol/nucleus.

To be able to study the translocation process of the mutants, we first performed *in vitro* phosphorylation experiments. All of the tested FGF-1 mutants subjected to the PKC and CK2 kinases were phosphorylated but to different extents. The level of radiolabeling inversely correlated with the thermal stability of the mutants, especially in the case of PKC (Figure 8). Bioinformatics studies indicate that phosphorylation commonly occurs within disordered protein regions and that unfolded regions are preferred by kinases (*52*). Our data are in agreement with this view. Thus, the less stable variants of FGF-1 were more efficiently phosphorylated *in vitro* than the more stable ones (Figure 8). Unfolded or disordered proteins are much more susceptible to protease action than tightly folded ones (*48*−*50*), and consequently, protein denaturation facilitates its digestion. It has been shown that folded regions of proteins cannot fit the active site of proteases without the disordering of several residues around the digestion site (*48*). An analogical model for substrate preferences for protein kinases has been suggested (*52*). In the case of FGF-1, a very well-ordered protein, the effectiveness of its phosphorylation seems to be dictated by the global unfolding of the molecule. The thermally induced denaturation process is much more efficient for the low-stability variants, and therefore, these are preferred in the phosphorylation reaction.

One of the main conclusions from the present work is that translocation of FGF-1 is a strongly receptor-dependent process and that there appears to be no alternative productive way of translocating the extracellular growth factor. Our data confirm the importance of the selected residues of FGF-1 for binding to the FGF receptor. However, we showed that despite the low affinity of FGF-1 single-site mutants for the receptor, their internalization and translocation to the cytosol/nucleus is still possible when the growth factor concentration is increased to compensate for the reduced affinity. We also demonstrated that translocation of the growth factor does not depend on its stability and that FGF-1 in an unfolded state is much more susceptible to phosphorylation.

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