Externally Added aFGF Mutants Do Not Require Extensive Unfolding for Transport to the Cytosol and the Nucleus in NIH/3T3 Cells†

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ABSTRACT: Acidic fibroblast growth factor (aFGF) is transported to the cytosol and the nucleus when added to cells expressing FGF receptors, implying that aFGF must cross cellular membranes. Since protein translocation across membranes commonly requires extensive unfolding of the protein, we were interested in testing whether this is also necessary for membrane translocation of aFGF. We therefore constructed mutant growth factors with intramolecular disulfide bonds to prevent complete unfolding. Control experiments demonstrated that translocation of aFGF by the diphtheria toxin pathway, which requires extensive unfolding of the protein, was prevented by disulfide bond formation, indicating that the introduced disulfide bonds interfered with the unfolding of the growth factor. On the other hand, when the growth factor as such was added to cells expressing FGF receptors, the disulfide-bonded mutants were translocated to the cytosol and the nucleus equally well as wild-type aFGF. The possibility that the translocation of the mutants was due to reduction of the disulfide bonds prior to translocation was tested in experiments using an irreversibly cross-linked mutant. Also this mutant was transported to the cytosol and to the nucleus. The results suggest that extensive unfolding is not required for membrane translocation of aFGF.

Acidic fibroblast growth factor (aFGF[1] or FGF-1) belongs to a large family of growth factors that are involved in angiogenesis, differentiation, stimulation of proliferation, and several other processes (*1*). The fibroblast growth factors bind to four closely related transmembrane receptors, the fibroblast growth factor receptors 1−4 (FGFR-1−4). The receptors contain a split cytoplasmic tyrosine kinase domain, and they are autophosphorylated upon binding of the ligand. An intracellular phosphorylation cascade is then initiated, culminating in the activation of immediate early genes.

There is now evidence that upon binding to the specific FGFR, aFGF can be translocated to the cytosol and to the nucleus. Evidence for this was obtained by fractionation of cells where extracellularly added aFGF was recovered from the nuclear fraction. An aFGF mutant lacking the N-terminal nuclear localization signal (NLS) found in wild-type aFGF was not found in the nuclear fraction and failed to induce DNA synthesis in the cells (*2*, *3*). The ability to stimulate DNA synthesis was restored when a yeast NLS was added to the aFGF mutant (*4*).

It is not trivial to demonstrate that an extracellular protein is translocated to the cytosol and nucleus rather than being present in intracellular membrane-bounded organelles. Upon binding of the growth factor to the specific receptors, it is endocytosed and transported to a juxtanuclear organelle that appears to be the recycling endosome compartment (*5*). Part of the FGFR appears to be anchored to cytoskeletal elements that are associated with the nuclei and therefore appear in the nuclear fraction upon cell fractionation (*5*). To demonstrate that the growth factor has really crossed cellular membranes and reached the cytosol and nucleus in vivo, it was therefore necessary to employ additional methods.

Evidence for membrane translocation of aFGF was obtained in studies where aFGF was modified to contain a C-terminal farnesylation signal, a CAAX-box (*6*). When aFGF with a CAAX-box was added to cells containing FGF receptors, the growth factor was farnesylated. Since the farnesyl transferase is only found in the cytosol and possibly in the nucleus, this can be taken as evidence that the externally added aFGF had indeed crossed cellular membranes. Part of the farnesylated growth factor was found in the nuclear fraction.

Another method used for the same purpose takes advantage of the fact that aFGF can be phosphorylated on Ser^{132} by protein kinase C. When recombinant aFGF was added to cells expressing FGFR, it was phosphorylated at this site (*7*). aFGF lacking the consensus site for protein kinase C was not phosphorylated upon incubation with living cells, although it did penetrate into the cells as demonstrated by farnesylation experiments. Since protein kinase C is a

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[1] Abbreviations: aFGF, acidic fibroblast growth factor; FGFR, fibroblast growth factor receptor; SLO, streptolysin O; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; MESNA, β-mercaptoethane sulfonate; PMSF, phenylmethylsulfonyl fluoride; DT, diphtheria toxin; NLS, nuclear localization sequence; DBB, dibromobimane.

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cytosolic enzyme, it appears that aFGF must have been translocated to the cytosol to be phosphorylated.

Further evidence that this is the case was obtained in experiments where the plasma membrane was permeabilized with streptolysin O under conditions where intracellular organelles remained intact. Under these conditions, the phosphorylated growth factor was released into the buffer together with soluble cytosolic proteins (*8*).

Translocation of the growth factor to the cytosol and nucleus as measured by these methods requires that the growth factor binds to FGFR. Thus, although the growth factor can bind extensively to surface heparan sulfate proteoglycans in cells lacking FGFR, this binding does not result in translocation of the growth factor to the cytosol (*9*). The cytoplasmic part of one such receptor, i.e., FGFR-4, is important for this translocation, since when most of the cytoplasmic domain was removed, the growth factor was not translocated although it was still endocytosed (*10*). Also, it appears that the very C-terminal end is required, but not sufficient for translocation. It is so far not clear which other parts of the cytoplasmic domain are required, but the tyrosine kinase activity is not necessary for translocation of the growth factor (*10*).

Translocation of aFGF into cells and transport to the nucleus appear to be involved in the stimulation of DNA synthesis. Thus, fusion proteins of aFGF that are unable to translocate into cells were found not to stimulate DNA synthesis in U2OSR4 cells despite their ability to bind to the FGFR-4 expressed in these cells and to activate its tyrosine kinase (*9*). On the other hand, when aFGF was fused to diphtheria toxin A-fragment and reconstituted with diphtheria toxin B-fragment, the fusion protein was translocated to the cytosol, and, upon further incubation, it accumulated in the nucleus. Under these conditions, the fusion protein stimulated DNA synthesis in serum-starved cells lacking functional FGF receptors (*3*).

To reach the cytosol and the nucleus, the growth factor must translocate across a cellular membrane, either at the cell surface or across the limiting membrane of an intracellular organelle. The same challenge of crossing a membrane barrier is shared by many protein toxins that bind to cell-surface receptors and exert their effect in the cytosol. Diphtheria toxin binds to the uncleaved precursor of HB-EGF at the cell surface (*11*) and is subsequently endocytosed. Upon exposure to the low pH obtained in endosomes, the A-fragment of the toxin is translocated to the cytosol where it enzymatically modifies elongation factor 2 and thereby inhibits protein synthesis. Falnes et al. have shown that introduction of artificial disulfide bonds in the diphtheria toxin A-fragment blocks the translocation step, apparently due to the inability of the protein to unfold sufficiently (*12*). Also, the toxicity of a mutant of the plant toxin ricin containing an internal disulfide bridge was lower than that of wild-type ricin (*13*). Experiments with a fusion protein of anthrax toxin and diphtheria toxin A-fragment containing an internal disulfide bond demonstrated that unfolding was also necessary for translocation of anthrax toxin (*14*).

To investigate in more detail the molecular mechanisms involved in membrane translocation of aFGF, we introduced intramolecular disulfide bonds at three different locations in the growth factor. Control experiments where the mutants were fused to diphtheria toxin A-fragment indicated that the internal disulfide bonds prevent the unfolding of the mutant growth factors. We therefore used these mutants to test if aFGF as such must unfold in order to translocate to the cytosol and appear in the nucleus.

EXPERIMENTAL PROCEDURES

Materials, Media, and Buffers. [^{35}S]Methionine (1000 Ci/mmol) and [*methyl*-^{3}H]thymidine (25 Ci/mmol) were obtained from Amersham. DBB was from Calbiochem. Other chemicals were from Sigma. HEPES medium: bicarbonate- and serum-free Eagle's minimal essential medium buffered with HEPES to pH 7.4. Dialysis buffer: 140 mM NaCl, 20 mM HEPES, and 2 mM $CaCl_2$, adjusted to pH 7.0 with NaOH. Lysis buffer: 0.1 M NaCl, 20 mM NaH_2PO_4, 10 mM EDTA, 1% Triton X-100, 1 mM PMSF, and 1 mM NEM, pH 7.4. PBS: 140 mM NaCl and 10 mM NaH_2PO_4, pH 7.4. MES-gluconate buffer: 140 mM NaCl, 5 mM sodium gluconate, and 20 mM MES, adjusted with Tris to pH 4.8 or pH 7.0.

Site-Directed Mutagenesis and Plasmid Construction. E. coli strain TG-1 was used as a host for M13mp18 during mutagenesis, and *E. coli* strain DH5α was used in the cloning procedures. A cysteine-free mutant of aFGF was obtained from the plasmid pFT1 (a kind gift from Dr. T. Maciag) by PCR using the following primers: GGCCATGGCTAATTACAAGAAGCCCAAA and CCGGATGCATCATTACATGATTACGCAATCAGAAGAGACTGGCAGGGG. The primers were used to introduce a *Nco*I site at the 5′-end, and a *Nsi*I site at the 3′-end. The fragment was cloned into M13 mp18. Site-directed mutagenesis was performed using the Sculptor kit from Amersham. In the first round of mutagenesis, the *Nco*I site in the middle of the fragment was eliminated, and a *Xho*I site near the end was added using the following primers: CGGTGTCCATTGCCAAGTAC and GATTACGCAATCACTCGAGACTGGCAGG. To generate the five different double cysteine mutants, the following complementary mutant primers were used: P11C, AGAGGAGTTTGCACTTCTTGTAA; T59C, GGCCAGTCTCGCAACTCTTTATA; I25C, CATCCGGAAGGCACCTCAGGAAG; S58C, CAGTCTCGGTACACTTTATATAC; G75C, GTCTGTGAGCAGTATAAAAGC; Q43C, CACTGAGCTGCAGACAAATGTGCTGGTC; V137C, CAATCACTCGAGCATGGCAGGGGG; T123C, GGCCATAGTGACACCGAGGACCG; I130C, GGAGAAACAAGCATGCTTTCTGGC. The sequences of the mutated fragments were verified by dideoxy sequencing and cloned into a vector derived from pBS (Stratagene) for expression in rabbit reticulocyte lysate and into the pTrc-99A (Amersham Pharmacia Biotech) vector for expression in *E. coli*.

Cell Cultures. NIH/3T3 and Vero cells were propagated as earlier described (*3*). Cells were seeded into Costar (Cambridge, MA) tissue culture plates the day preceding the experiments.

In Vitro Transcription and Translation. Plasmid DNA was linearized downstream of the encoding gene and transcribed with T3 RNA polymerase as described (*15*). The mRNA was precipitated with ethanol and dissolved in H_2O containing 10 mM DTT and 0.1 unit/μL RNasin. The translation was performed for 1 h at 30 °C in micrococcal nuclease treated rabbit reticulocyte lysate (Promega, Madison, WI). Radioactive proteins were prepared in lysates containing 1 μM [^{35}S]-

methionine and 25 μM aliquots of the other 19 amino acids. Labeled methionine was replaced by 25 μM unlabeled methionine when nonradioactive proteins were synthesized. The amount of protein in the nonlabeled lysates was estimated as earlier described (*16*) by translating in parallel a small aliquot of the lysate in the presence of 5 μM [^{35}S]-methionine. The lysates were finally dialyzed against PBS to remove free [^{35}S]methionine and reducing agents, and to allow the formation of disulfide bonds.

Expression and Purification of Recombinant Proteins. Expression of recombinant protein from the plasmids pTrc-aFGF, pTrc-CC1, pTrc-CC3, pTrc-CC4, or pTrc-CF in *E. coli* BL21DE was induced with 1 mM isopropyl-β-D-thiogalactopyranoside. The bacterial pellet was resuspended in 20 mM Na_2HPO_4, pH 7.5, 0.5 M NaCl, 1mM dithiothreitol, 1 mM EDTA and sonicated. The supernatant was applied to a heparin cartridge (BioRad) or, alternatively, to a heparin−Sepharose column (Pharmacia), and the bound material was eluted with a NaCl gradient (0.5−2 M) in 20 mM Na_2HPO_4, pH 7.5, 1 mM dithiothreitol, 1 mM EDTA.

SDS−PAGE. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was carried out in 13.5% gels as described by Laemmli (*17*). After electrophoresis, the gel was fixed for 30 min in 27% methanol/4% acetic acid and then incubated for 30 min in 1 M sodium salicylate/2% glycerol, pH 5.8. Kodak XAR-5 film was exposed to the dried gel at −80 °C.

Cell Binding and Translocation Assay. The experiments were performed as earlier described (*3*, *9*). To measure binding, dialyzed translated proteins were added to NIH/3T3 cells and incubated for 2 h at 4 °C. Five millimolar NEM was added during the last 10 min of the incubation, and the cells were then washed 3 times in HEPES medium and lysed in lysis buffer. Proteins were adsorbed to heparin−Sepharose and analyzed by nonreducing SDS−PAGE and fluorography. To fractionate the cells, NIH/3T3 cells were incubated with radiolabeled proteins for 6 h at 37 °C, washed, and treated with Pronase. After additional washing of the cells, they were lysed in lysis buffer and centrifuged. The supernatant was designated cytosolic/membrane fraction. The pellet was washed 3 times in lysis buffer containing 0.4 M sucrose and then sedimented through a cushion of 0.7 M sucrose in lysis buffer by centrifuging at 720*g* for 15 min. After sonicating 2 times for 10 s on ice, the lysate was centrifuged for 5 min at 15800*g*, and the supernatant was designated the nuclear fraction. The different fractions were adsorbed to heparin−Sepharose and subsequently washed with PBS containing 0.7 M NaCl. The adsorbed material was analyzed by reducing SDS−PAGE and fluorography.

To analyze translocation to the cytosol, the cells were incubated with radiolabeled proteins as above and then treated with Pronase. The cells were then incubated at 0 °C with 2 μg/mL streptolysin O (SLO) preactivated with 10 mM MESNA in HEPES medium. After 10 min, the cells were washed briefly to remove unbound toxin and incubated for 10 min at 37 °C for permeabilization to occur. Subsequently, the cells were kept on ice for additional 30 min to allow components of the cytosol to diffuse into the buffer. Finally, the cells were centrifuged, and the supernatant was designated the cytosolic fraction. The cellular pellet was lysed with 1% Triton X-100. The nuclei were sedimented, and the soluble fraction was taken as the membrane fraction. The nuclei were then sonicated in lysis buffer containing 0.5 M NaCl, and the soluble fraction thus obtained was designated the nuclear fraction. All fractions were incubated with heparin−Sepharose, and the adsorbed material was washed with 0.7 M NaCl in PBS and then subjected to reducing SDS−PAGE.

Translocation of Diphtheria Toxin Fusion Proteins. Dialyzed translation mixtures were added to Vero cells and kept for 20 min at 24 °C in the presence of 1 mM methionine, 10 μM monensin, and 10 μg/mL aFGF. Then the cells were washed 3 times with HEPES medium and exposed to pH 4.5 at 37 °C, treated with Pronase, lysed, and analyzed by SDS−PAGE and fluorography as described (*18*).

Cross-Linking with DBB. The growth factor mutants were expressed in a rabbit reticulocyte lysate and purified by adsorption to heparin−Sepharose. Acetylated BSA (1 mg/mL) was added, and the proteins were incubated with 100 μM dibromobimane (DBB) for 24 h at 4 °C. The reaction was terminated by adding 1 mM DTT. The cross-linked protein was dialyzed to remove excess DBB and DTT, and analyzed by reducing SDS−PAGE.

Measurement of DNA Synthesis. Cells growing in 24-well tissue culture plates (10^5 cells per well) were preincubated for 48 h in serum-free medium at 37 °C. Then the cells were treated with increasing concentrations of aFGF or aFGF mutants, and the incubation was continued for 24 h at 37 °C. During the last 6 h, the cells were incubated with 1 μCi/mL [*methyl*-^{3}H]thymidine as described (*4*), and the incorporated radioactivity was measured.

Phosphorylation Assay in NIH/3T3 Cells. NIH/3T3 cells growing in 6-well tissue culture plates (2×10^5 cells per well) were incubated for 24 h in serum-free medium at 37 °C. The cells were then incubated for 8 h with $Na_3{}^{32}PO_4$ and unlabeled aFGF or growth factor mutants. The cells were then washed in PBS (pH 7.4) containing phosphatase inhibitors (100 μM sodium orthovanadate, 50 mM sodium fluoride, and 30 mM sodium pyrophosphate), and incubated with SLO as described above. The cells were lysed in P-lysis buffer (10 mM Tris, 50 mM NaCl, 5 mM EDTA, 1% Triton X-100, 100 μM sodium orthovanadate, 50 mM sodium fluoride, 30 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 200 units/mL aprotinin, pH 7.4). The nuclei and membrane fraction was sonicated and incubated with heparin−Sepharose. To reduce the background of phosphorylated proteins, the heparin−Sepharose with adsorbed material was treated with 0.1 mg/mL trypsin for 15 min on ice. The digestion was terminated by washing 2 times with PBS containing aprotinin. Proteins were eluted from the heparin−Sepharose with 2 M NaCl and incubated with antibodies against aFGF coupled to protein A−Sepharose. The immunoprecipitated material was analyzed by SDS−PAGE and fluorography.

RESULTS

Characterization of aFGF Mutants Containing Internal Disulfide Bonds. aFGF contains three cysteine residues which are not necessary for biological activity and do not form intramolecular disulfide bonds. A mutant aFGF where the three cysteines were mutated to serines did bind to heparin and to FGFR, and had the same mitogenic potential as wild-type aFGF (*19*, *20*). We constructed five different double

A

Mutant	Mutations introduced
CC1	P11C/T59C
CC2	I25C/S58C
CC3	I25C/G75C
CC4	Q43C/V137C
CC5	T123C/I130C

B

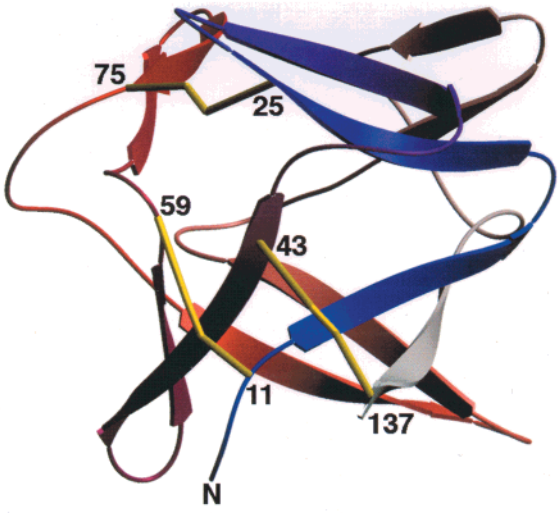

FIGURE 1: Schematic representation of constructs used. (A) List of the constructed mutants. CC1, CC2, CC3, CC4, and CC5 were made using the cysteine-free (CF) mutant as a template. The wild-type cysteines have been mutated to serines in addition to the introduced cysteines. (B) Representation of the location of the intramolecular disulfide bonds in the aFGF mutants. For simplicity, the disulfide bonds in the three different mutants CC1, CC3, and CC4 are here drawn into the same molecule. The mutants CC2 and CC5 did not form intramolecular disulfide bonds and are therefore not represented in this figure.

cysteine mutants using the cysteine-free mutant as a basis (Figure 1A). The three-dimensional structure of aFGF (*21*) was used to identify potential sites for disulfide formation.

Due to their smaller Stoke's radius, proteins with internal disulfide bonds migrate faster in SDS−PAGE than their reduced counterparts. [^{35}S]Methionine-labeled mutant growth factors were produced in vitro using rabbit reticulocyte lysate and purified using heparin−Sepharose columns to remove the reducing agents. The mutants were run on SDS−PAGE in the presence or absence of the reducing agent dithiothreitol (DTT). As shown in Figure 2, the oxidized forms (lanes 2, 4, 6) of the double cysteine mutants (CC1, CC3, and CC4) migrated clearly faster than the reduced forms (lanes 1, 3, 5). The cysteine-free mutant (CF) migrated as the reduced form of the disulfide-bonded mutants whether reducing agent was added or not (lanes 7, 8). We therefore conclude that the double cysteine mutants, CC1, CC3, and CC4, form intramolecular disulfide bonds. The engineered disulfide

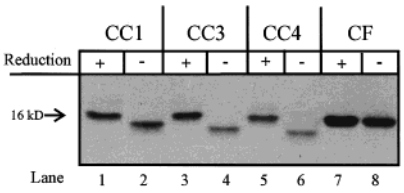

FIGURE 2: Comparison of migration rates of the mutant proteins in SDS−PAGE. The mutant proteins were translated in vitro in a rabbit reticulocyte lysate and purified with heparin−Sepharose to allow disulfide bond formation. The proteins were analyzed by nonreducing SDS−PAGE. Where indicated, the samples were reduced with 70 mM DTT, which was subsequently quenched by NEM (100 mM) before the samples were loaded onto the gel. The arrow at 16 kD corresponds to the migration rate of wild-type aFGF.

bonds are represented schematically in Figure 1B. The two double cysteine mutants CC2 and CC5 did not form intramolecular disulfide bonds efficiently (data not shown) and were not used in further experiments.

Translocation of Fusion Proteins Consisting of the Mutant Growth Factors and Diphtheria Toxin. We first decided to test if the introduced disulfide bonds were capable of blocking the translocation in a transport system which is known to require unfolding. Falnes et al. have shown that the introduction of internal disulfide bonds in the diphtheria toxin A-fragment (dtA) inhibits its translocation (*12*). In addition, a fusion protein between wild-type aFGF and dtA was translocated to the cytosol when reconstituted with diphtheria toxin B-fragment (dtB), but the translocation was inhibited in the presence of heparin, which inhibits unfolding of the growth factor (*22*). These studies demonstrated that extensive unfolding is required for translocation by the diphtheria toxin pathway.

We fused the different mutant growth factors to dtA, expressed the fusion proteins in a reticulocyte lysate, and dialyzed them together with dtB to form a disulfide bond between dtA and dtB, as well as an internal disulfide in the aFGF part of the fusion proteins (see Figure 3A). The formation of disulfide bonds was verified by SDS−PAGE analysis (data not shown). After binding to Vero cells, translocation of dtA can be induced by treating the cells with low-pH buffer, thus mimicking at the level of the cell surface the conditions in the endosomes. The translocated protein is shielded against subsequent treatment of the cells with Pronase. As shown in Figure 3B, the fusion between wild-type aFGF and dtA was translocated to the cytosol when the cells were treated with low-pH buffer (lane 2), but no translocation was observed when the experiment was performed at neutral pH (lane 1). Heparin, which interferes with the unfolding of the growth factor, prevented the translocation (lane 3). This was also the case for the fusion between the cysteine-free aFGF mutant and dtA (CF-dtA, lanes 4−6). On the other hand, the fusion proteins containing the three mutants with internal disulfide bonds (CC1-dtA, CC3-dtA, and CC4-dtA) were not translocated (lanes 7, 11, and 12).

The disulfide bond in the mutant CC1 can be reduced by 10 mM DTT, while reduction of the disulfide bond between the diphtheria toxin A- and B-fragments requires 50 mM DTT. It is therefore possible to reduce the intramolecular disulfide bond in the fusion protein without reducing the intermolecular bond between the two toxin chains. As seen in Figure 3B, the fusion protein CC1-dtA was translocated

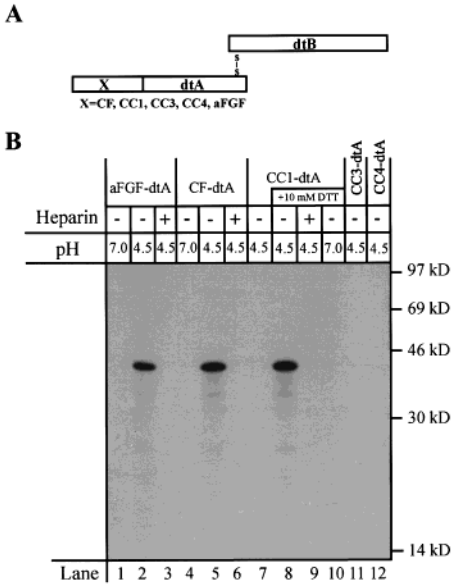

FIGURE 3: Translocation of diphtheria toxin fusion proteins. (A) Linear representation of the aFGF-dtA fusion proteins reconstituted with diphtheria toxin B-fragment. (B) Radiolabeled fusion proteins were bound to cells, where indicated, in the presence of 10 units/mL heparin and/or 10 mM DTT. Then the cells were exposed to pH 4.5 or pH 7.0, treated with Pronase to remove untranslocated protein, and analyzed by reducing SDS−PAGE and fluorography. In all cases, 10 μg/mL unlabeled aFGF was present to avoid binding of the fusion protein to FGFR and heparan sulfate proteoglycans at the cell surface.

to the cytosol when 10 mM DTT was added to cleave the internal disufide bond (lane 8). No translocation was observed when heparin was added (lane 9) or when the treatment with low-pH buffer was omitted (lane 10). The mutants CC3 and CC4 required higher concentrations of DTT (40 and 50 mM, respectively) to be reduced, and these amounts of DTT affect the intermolecular disulfide bond as well. These mutants were therefore not suitable for this experiment.

Altogether, the results demonstrate that the engineered disulfide bonds in the aFGF mutants are stable and capable of blocking the unfolding of the growth factor that is required for translocation by the diphtheria toxin pathway.

Binding to FGF Receptors and Stimulation of DNA Synthesis. The stimulation of DNA synthesis by aFGF and the translocation of aFGF to the cytosol and the nucleus are mediated by specific FGF receptors (*9*). To test the binding of the mutant growth factors to FGF receptors, [^{35}S]-methionine-labeled proteins were added to NIH/3T3 cells which express FGFR-1. The cells were incubated with the growth factor mutants for 2 h at 4 °C and washed, and cellular proteins were analyzed by nonreducing SDS−PAGE. The results showed that similar amounts of CC1, CC3, CC4, and CF were bound to NIH/3T3 cells (Figure 4). When heparin was added to inhibit binding to heparan sulfate proteoglycans, less labeled growth factor was bound to the cells. An excess of unlabeled aFGF competed out this binding, indicating that the binding in the presence of heparin was to the specific FGF receptors (Figure 4). The mutants CC1, CC3, and CC4 migrated faster in SDS−PAGE than CF, demonstrating that the disulfide bonds were not reduced after binding to the cells. The experiment demonstrates that all the mutants had retained the ability to bind to specific

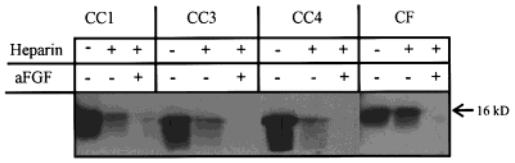

FIGURE 4: Binding of mutant growth factor to cells. NIH/3T3 cells were incubated with radiolabeled growth factor mutants for 2 h at 4 °C in the absence or presence of 50 units/mL heparin and in some cases 10 μg/mL unlabeled aFGF as well. NEM (5 mM) was added during the last 10 min of incubation. The cells were then washed 4 times in HEPES medium, lysed, and analyzed by nonreducing SDS−PAGE and fluorography.

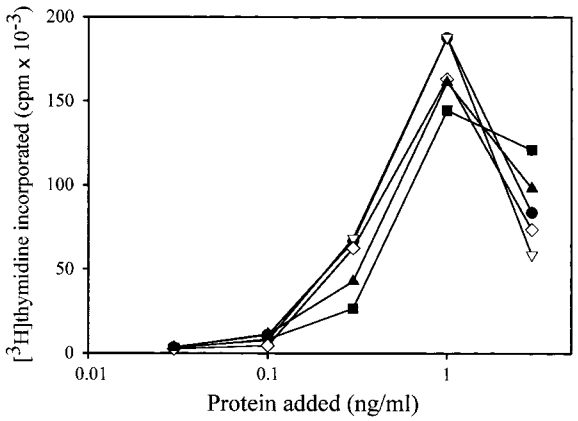

FIGURE 5: Ability of aFGF and the different mutants to stimulate DNA synthesis. NIH/3T3 cells growing in 24-well tissue culture plates (10^5 cells/well) were preincubated for 48 h in serum-free medium. Then the cells were treated with increasing amounts of aFGF or mutant growth factors, and the incubation was continued for 24 h more. During the last 6 h, the cells were labeled with [^{3}H]thymidine, and the incorporated radioactivity was measured. CC1, closed circles; CC3, open triangles; CC4, closed squares; CF, open diamonds; aFGF, closed triangles.

receptors and that the disulfide bonds in CC1, CC3, and CC4 remain intact after binding.

aFGF stimulates the synthesis of DNA when given to serum-starved NIH/3T3 cells. We compared the abilities of CC1, CC3, CC4, CF, and aFGF to stimulate DNA synthesis as measured by the capacity of treated cells to incorporate [^{3}H]thymidine. As seen in Figure 5, all the mutants stimulated DNA synthesis with about the same potency as wild-type aFGF. This demonstrates that the introduced cysteines and the formation of intramolecular disulfide bonds at three different locations in the growth factor do not impair the mitogenic activity of the growth factor.

Transport to the Nuclear Fraction. To test if the disulfide-bonded mutants were transported to the nuclear fraction of cells such as wild-type aFGF, we incubated NIH/3T3 cells with the radiolabeled growth factors and fractionated the cells into nuclear and membrane/cytosolic fractions. Although most material was usually found in the cytosol/membrane fraction (Figure 6A, lanes 1−7), all three mutants, CC1, CC3, and CC4, as well as aFGF and CF were partially recovered from the nuclear fraction, indicating transport to the nucleus (Figure 6A, lanes 8−12). When analyzing the samples by nonreducing SDS−PAGE, we observed that the disulfide-bonded growth factor mutants in the nuclear fraction were reduced (data not shown). The disulfide bonds are probably cleaved in the reducing environment of the cytosol or the nucleus. Therefore, to get sharper bands in SDS−PAGE, we

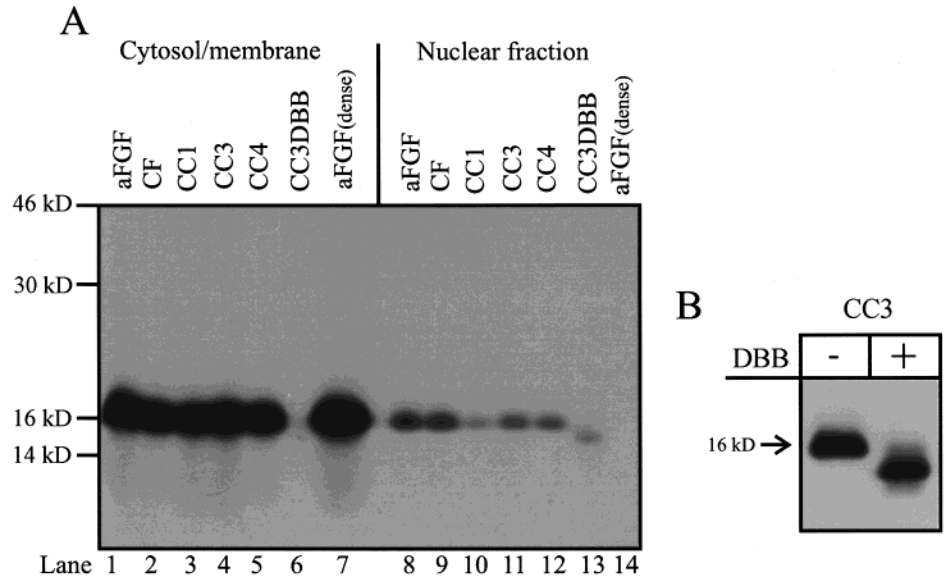

FIGURE 6: Transport of aFGF and the mutant growth factors to the nuclear fraction of NIH/3T3 cells. (A) Radiolabeled proteins were added to serum-starved NIH/3T3 cells in the presence of 10 units/mL heparin. The cells were incubated for 8 h and then washed and treated with Pronase to remove growth factor bound to the cell surface or to the plastic surface of the tissue culture plate. The cells were washed once more and then lysed in lysis buffer. The lysate was centrifuged, and the supernatant was designated the cytosol/membrane fraction. The pellet containing the nuclei was washed 3 times in PBS containing 0.4 M sucrose and spun through a cushion of 0.7 M sucrose. The nuclei were then sonicated. The cytosol/membrane fraction and the solubilized nuclear fraction were incubated with heparin−Sepharose, and the adsorbed material was analyzed by reducing SDS−PAGE and fluorography. (B) Formation of an irreversible cross-link in CC3. The mutant CC3 was expressed in reticulocyte lysate and purified with heparin−Sepharose. The protein was incubated for 24 h at 4 °C in the absence or presence of 100 μM DBB, as indicated. The reaction was terminated by adding 1 mM DTT, and then the mixture was dialyzed against dialysis buffer and analyzed by reducing SDS−PAGE.

used reducing conditions during the gel electrophoresis of the presented experiments.

A possible pitfall when performing fractionation studies is that the nuclear fraction may be contaminated with material from the membrane/cytosolic fraction. Additionally, the growth factor can be imported into or bound to the nucleus after lysis of the cells. To rule out these possibilities, we took advantage of the observation that aFGF is not transported to the nuclear fraction when NIH/3T3 cells are grown at high density (*23*). Using the same fractionation protocol as in the previous experiments, aFGF was not present in the nuclear fraction when dense cells were incubated with radiolabeled aFGF (Figure 6A, lanes 7, 14). Furthermore, it was recently shown that the phosphatidylinositol 3-kinase (PI 3-kinase) inhibitor LY294002 inhibits the transport of aFGF to the nuclear fraction (*24*). In accordance with this, aFGF was not found in the nuclear fraction when the experiment was performed in the presence of the PI 3-kinase inhibitor (data not shown).

The results of the previous experiment, demonstrating that the introduced disulfide bonds do not block the transport of the growth factor mutants to the nuclear fraction, indicate that unfolding is not necessary for aFGF translocation. However, the possibility also existed that the disulfide bonds were cleaved by a reducing power at the membrane or in an internal organelle before membrane translocation. To test the latter possibility, we introduced a nonreducible thioether bond between the sulfhydryl groups in the aFGF mutant CC3. The bifunctional cross-linker dibromobimane (DBB) reacts with sulfhydryl groups. Purified CC3 was incubated with DBB and then analyzed by reducing SDS−PAGE. As seen in Figure 6B, DBB-treated CC3 migrated faster in reducing SDS−PAGE than CC3 alone. This indicates that DBB cross-links the two cysteines present in the molecule, resulting in an internal nonreducible bond. The two other mutants, CC1 and CC4, as well as CF, did not migrate faster when incubated with DBB, suggesting that these mutants were not cross-linked (data not shown). When incubated with NIH/3T3 cells, the irreversibly cross-linked mutant was also transported to the nuclear fraction (Figure 6A, lane 13). For unknown reasons, very little of the cross-linked mutant was found in the membrane/cytosolic fraction in several experiments.

Membrane Translocation. To monitor membrane translocation of the mutant growth factors, we used the toxin streptolysin O (SLO), which under defined conditions makes large pores in the plasma membrane, but not in intracellular organelles, and therefore allows soluble cytosolic proteins to selectively leak out into the medium. First, we tested the time-dependent transport of wild-type aFGF. NIH/3T3 cells were incubated with radiolabeled aFGF. The cells were subsequently treated with Pronase to remove surface-bound ligand and then treated with SLO. The medium containing cytosolic proteins was designated the cytosolic fraction, whereas the remaining cell structures were lysed and fractionated into a membrane fraction and a nuclear fraction. The material present in the different fractions was incubated with heparin−Sepharose, and the adsorbed material was analyzed by reducing SDS−PAGE.

As seen in Figure 7A, lanes 8−13, aFGF appeared in the cytosol after 4 h and reached a maximum after 6 h. Interestingly, the amount of aFGF in the cytosol decreased after 6 h, while the amount of aFGF in the nuclear fraction (lanes 15−20) increased. This could mean that aFGF is first translocated to the cytosol and then transported into the nucleus, most likely by the N-terminal NLS present in aFGF.

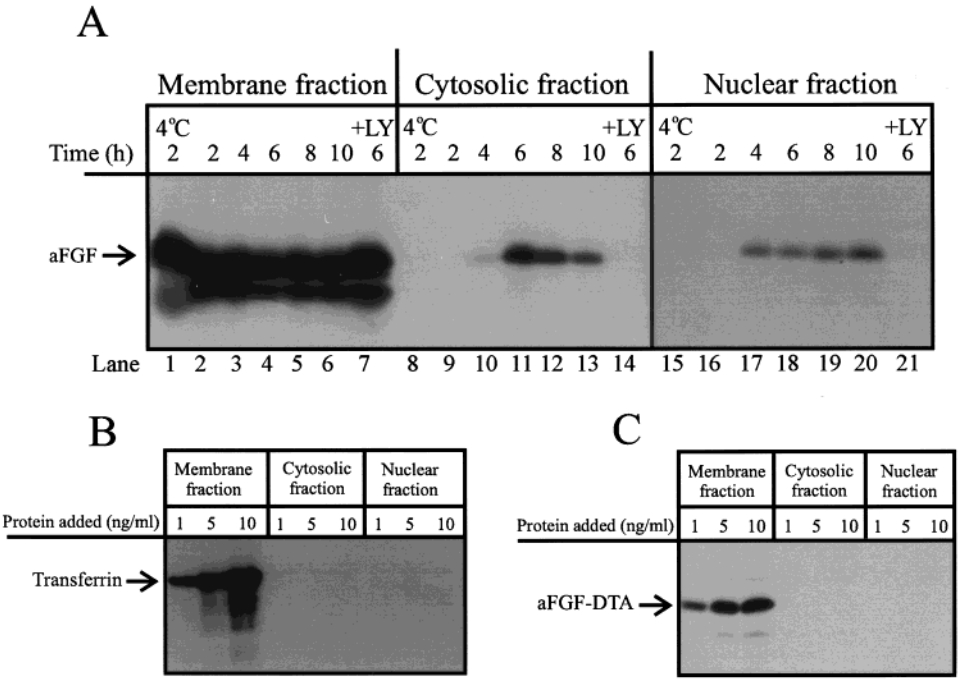

FIGURE 7: Transport of aFGF to the cytosol and nuclear fraction of NIH/3T3 cells. (A) Radiolabeled aFGF was added to serum-starved NIH/3T3 cells in the presence of 10 units/mL heparin, and incubated for different periods of time. To measure binding, cells were incubated for 2 h at 4 °C (lanes 1, 8, and 15), and the cells were then washed and lysed. To measure transport, cells were incubated at 37 °C for the indicated period of time. In one case, the inhibitor LY294002 (50 μM) was present (lanes 7, 15, and 21). The cells were then treated with Pronase, washed, and incubated with SLO at 4 °C. The cells were then transferred to 37 °C to allow permeabilization to occur and subsequently kept at 4 °C to let the cytosol leak out from the cells. The remaining cell components were fractionated into a membrane and a nuclear fraction as in Figure 6A. The different fractions were incubated with heparin−Sepharose and analyzed by reducing SDS−PAGE and fluorography. (B and C) Radiolabeled transferrin (B) or aFGF-dtA (C) was added to the cells and incubated for 6 h. The experiments were then performed as in (A).

The inhibitor of PI 3-kinase LY294002, completely blocked the translocation of aFGF to the cytosol and the nuclear fraction (lanes 14, 21).

A smaller, faster migrating band was observed in the membrane fraction (lanes 2−7), and represents most likely a degradation product of aFGF. This band was not seen when the cells were incubated at 4 °C (lane 1), which is consistent with the band being a degradation product. The growth factor seems to be stable after translocation, since the faster migrating degradation product is not seen in the cytosolic or nuclear fraction.

In a control experiment, the cells were incubated with radiolabeled transferrin, which binds to transferrin receptors and is subsequently endocytosed and transported to the recycling compartment. In this case, all the material was found in the membrane fraction, which includes intracellular vesicles (Figure 7B). In addition, a fusion protein between aFGF and diphtheria toxin A-fragment (aFGF-dtA) which binds to FGF receptors, but is not translocated into cells, was also only found in the membrane fraction (Figure 7C). Thus, aFGF found in the cytosolic fraction is unlikely to originate from leakage of proteins from endosomes or other membranous structures in the SLO-treated cells.

NIH/3T3 cells were then incubated for 6 h with the different growth factor mutants. After SLO treatment of the cells, CC1, CC3, CC4, CF, and aFGF were all recovered from the cytosolic fraction, indicating transport of the mutants to the cytosol (Figure 8, lanes 8−12). Part of the ligands was also found in the nuclear fraction (lanes 15−19), consistent with the previous fractionation studies.

In the same experiment, we incubated the cells with the chemically cross-linked mutant CC3-DBB with and without the inhibitor LY294002. CC3-DBB was recovered from the cytosolic (Figure 8, lanes 13, 14) and nuclear fractions (lanes 20, 21) in the absence, but not in the presence, of the inhibitor. Thus, even the irreversibly cross-linked mutant CC3-DBB appears to be transported from the cell surface to the cytosol, and it can partly be recovered from the nuclear fraction.

Phosphorylation of the Cross-Linked Growth Factors. To further test if the mutants are transported across the membrane and gain access to the cytosol, we took advantage of the observation that aFGF is phosphorylated by protein kinase C after translocation to the cytosol (*7*). NIH/3T3 cells were incubated with radiolabeled phosphate and unlabeled wild-type or mutant aFGF. After 8 h incubation, the cells were treated with SLO to analyze the cytosol for phosphorylated growth factor mutants. The rest of the cells (which includes membranes and nuclei) was lysed and analyzed for phosphorylated growth factor (see Experimental Procedures). As seen in Figure 9, phosphorylated aFGF was recovered from the cytosolic fraction (lane 6), but not from the membrane/nuclei fraction (lane 1). An aFGF mutant where the protein kinase C site has been abrogated (aFGF K132E) gave no phosphorylated band (lanes 2 and 7). The Cys-free mutant was also phosphorylated in this assay (lane 8). This mutant has the cysteine located within the protein kinase C site mutated to serine, but this does not seem to change the ability of the growth factor mutant to be phosphorylated. Interestingly, even the mutant with an irreversible, internal

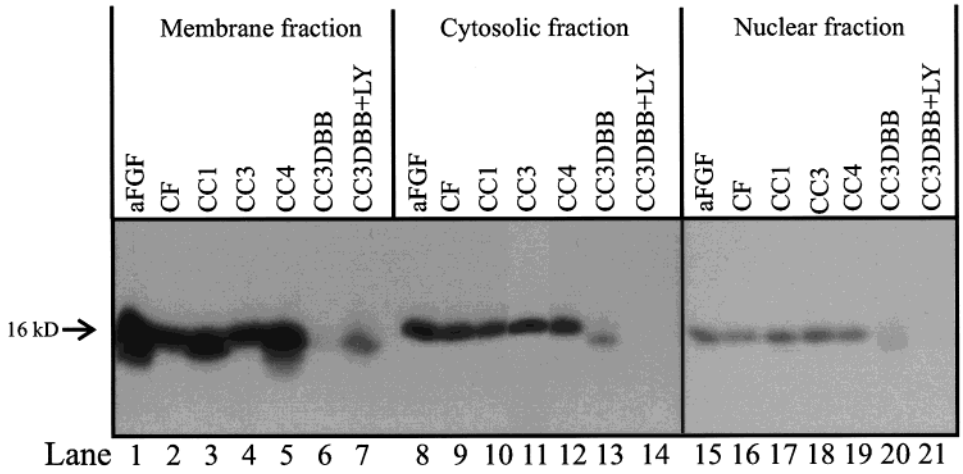

FIGURE 8: Translocation of mutant growth factors to the cytosol and the nuclear fraction. The experiments were performed as in Figure 7. In some cases, the PI 3-kinase inhibitor LY294002 (50 μM) was added (lanes 7, 14, and 21).

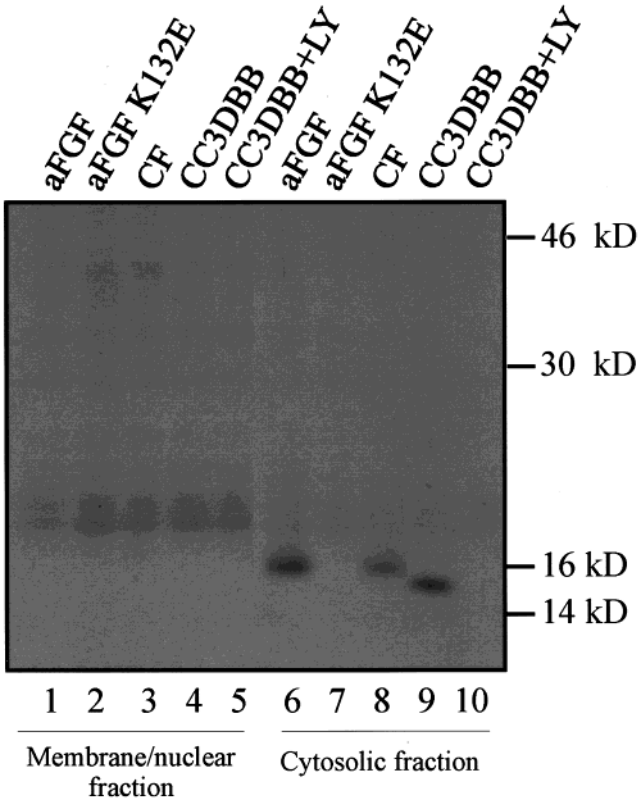

FIGURE 9: Phosphorylation of aFGF and mutant growth factors in NIH/3T3 cells. Serum-starved NIH/3T3 cells were preincubated with $Na_3{}^{32}PO_4$, and then 100 ng/mL of aFGF or growth factor mutants was added. In addition, 10 units/mL heparin was added. After 8 h incubation, the cells were washed and incubated with SLO. The cytosol which leaked out into the medium was collected and incubated with heparin−Sepharose (lanes 6−10). The remainder of the cells was lysed in lysis buffer, sonicated, and incubated with heparin−Sepharose (lanes 1−5). The heparin−Sepharose with adsorbed material was washed with PBS containing 0.5 M NaCl. Proteins were eluted from the heparin−Sepharose with 2 M NaCl and then incubated with antibodies against aFGF coupled to protein A−Sepharose. The immunoprecipitated material was analyzed by reducing SDS−PAGE and autoradiography.

cross-link (CC3DBB) was phosphorylated in the absence (lane 9), but not in the presence, of the PI 3-kinase inhibitor LY294002 (lane 10).

Also the mutants CC1, CC3, and CC4 were phosphorylated when added to NIH/3T3 cells (data not shown). Treatment of the cells with 10% serum, or with aFGF-dtA that is not translocated, gave no phosphorylated band (data not shown). These results further indicate that an internal cross-link does not inhibit the translocation of aFGF mutants.

DISCUSSION

The results presented here indicate that aFGF does not have the same requirement for unfolding during translocation across cellular membranes as diphtheria toxin, where the presence of disulfide bonds in the A-fragment prevented its translocation to the cytosol. We here demonstrate that the same disulfide bonds that allowed translocation of aFGF mutants, as such, prevented translocation of a fusion protein between the same aFGF mutants and the diphtheria toxin A-fragment. Three different aFGF mutants were engineered to contain an internal disulfide bond to prevent unfolding of the growth factor. The three mutants were translocated into NIH/3T3 cells in the same manner as wild-type aFGF. Our concern that the membrane translocation of mutant aFGF became possible after reduction of the disulfide bond in intracellular membrane-bounded compartments, was ruled out by experiments with an irreversibly cross-linked aFGF mutant. Also in this case, the internal cross-link did not block translocation of the protein. It is therefore clear that translocation of the growth factor as such does not require the same extensive unfolding as when the growth factor is translocated by the diphtheria toxin pathway. Furthermore, in our membrane translocation assays, heparin is present. As opposed to translocation of aFGF by the diphtheria toxin pathway where heparin inhibits the translocation, the presence of heparin does not prevent the transport of aFGF to the cytosol and the nucleus in NIH/3T3 cells.

aFGF lacks a classical signal sequence for secretion and is transported out of the cells by an unknown mechanism (*25*). Export of aFGF out of the cells also requires that the protein translocates across a membrane, but in the opposite direction of what we have studied here. It was shown that Cys^{30} was necessary for export (*19*). Thus, a cysteine-free mutant was not exported. Evidence presented here indicates that the two systems are fundamentally different, since the cysteine-free mutant was translocated across the membrane and to the cytosol and the nucleus. Whether extensive unfolding is necessary for export of aFGF remains to be elucidated.

Membrane translocation of folded proteins is not without precedence. For instance, peroxisomal protein import is believed to progress without unfolding of the proteins. Walton et al. showed that human serum albumin stabilized with disulfide bonds and chemical cross-links was imported into peroxisomes (*26*). Surprisingly, even 9 nm gold particles covered with peptides representing a peroxisomal targeting signal were translocated. Another example of a membrane translocation system where unfolding seems not to be required is the Sec-independent transport system in the plant chloroplast thylakoid membrane. It was recently shown that a small molecule with an internal cross-link was translocated across the thylakoid membrane (*27*). Also, dihydrofolate reductase with the appropriate targeting signal was translocated in the presence of methotrexate which keeps the protein tightly folded (*28*). The twin-arginine translocation system for export of proteins in bacteria is closely related to the Sec-independent transport system of the thylakoid membrane in chloroplasts, and also in this case unfolding of the exported proteins seems not to be necessary (*29*).

Very little is known about the mechanisms involved in translocation of aFGF to the cytosol and nucleus. We have previously shown that binding to the high-affinity receptors is necessary for translocation (*9*). FGF receptors have been reported to traffic to the nucleus in response to ligand stimulation (*30*). The possibility therefore exists that aFGF is transported to the nuclear fraction together with its receptor. However, how the ligand, or alternatively the ligand−receptor complex, is translocated across the membrane remains to be explained.

Certain polypeptides, e.g., the antennapedia homeodomain (*31*), TAT-peptide fusion proteins (*32*), and VP22 (*33*), have been reported to transduce directly through the lipid bilayer apparently without interacting with any specific membrane protein. In the case of aFGF translocation, this is different, since binding of the growth factor to specific FGF receptors is necessary for translocation to occur. Additionally, aFGF is a soluble, hydrophilic protein, and it is difficult to see how it could penetrate the lipid bilayer on its own. Therefore, it is likely that aFGF interacts with proteins in the membrane which facilitate the translocation, conceivably by forming a proteinaceous pore. Considering the relatively small size of aFGF, ∼40 Å, translocation through a pore is not conceptually impossible even without prior unfolding of the protein. Hamman et al. estimated the diameter of the Sec61p translocon aqueous pore to be between 40 and 60 Å during cotranslational translocation (*34*). A compactly folded aFGF molecule could then theoretically translocate through a similar pore. An upper size exclusion limit of a hypothetical pore could explain why the aFGF-dtA fusion protein is not translocated into cells by the aFGF pathway, as the dtA moiety has a larger size.

In cells lacking specific FGF receptors, aFGF can bind to the cell surface by binding to heparan sulfate proteoglycans. This association to the cell surface does not lead to membrane translocation (*9*). It has been reported that aFGF can assume a "molten globule"-like state, which interacts with lipid bilayers (*35*). However, since the specific FGFR is necessary for translocation, the molten globule state cannot account for membrane translocation alone. Nevertheless, our results showing that internal disulfide bonds do not inhibit membrane translocation do not exclude that a "molten globule"-like state of aFGF may be involved in this process. Even if the introduced disulfide bonds inhibit extensive unfolding, different conformations might be achieved leading to partially structured states of aFGF. The flexibility endowed in a "molten globule"-like state might facilitate the translocation through a pore or across the lipid bilayer with the help of cofactors.

It is an interesting possibility that a specific translocation apparatus is involved in the transport of aFGF and possibly other growth factors and cytokines across cellular membranes.

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