Characterization of the nuclear translocation of acidic fibroblast growth factor

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SUMMARY

The subcellular localization of human acidic FGF (aFGF; FGF-1) expressed to high levels by using a bacteriophage T7 RNA polymerase-driven vaccinia virus expression system was studied in BHK21 and HeLa cells. Acidic FGF was detected by immunoblotting or immunofluorescence using an affinity-purified rabbit polyclonal antibody. The nuclei of most transfected cells, but not nuclei of control cells, were strongly immunoreactive. The nuclear accumulation of aFGF was confirmed by subcellular fractionation and immunoblotting, indicating that about 50% of the expressed protein was located in the nuclei at 12 h after transfection. It has previously been reported that a putative N-terminal nuclear localization sequence (NLS) in aFGF is required for full mitogenic activity (Imamura et al., Science 249, 1567-1570, 1990). We found that deletion of the first 27 residues including the putative NLS did not prevent the nuclear translocation of aFGF in either cell type. This observation suggests that the putative NLS sequence is not essential for targeting aFGF to the cell nucleus. To analyze further the mechanism of nuclear import, purified aFGF was microinjected into the cytoplasm of growing BHK21 cells under various conditions. In chilled (4°C) or ATP-depleted cells, the injected aFGF entered the nucleus with similar efficiency to that in control cells at 37°C. This suggests that aFGF, which has a molecular mass of only 16,500, enters the cell nucleus by free diffusion, and possibly becomes trapped by binding to some nuclear structures. When added exogenously to growing BHK21 cells, aFGF was not localized to the nucleus. Instead, a punctate staining pattern in the cytosol was observed, reminiscent of that in the endosomal-lysosomal compartments. In addition, a diffuse extracellular surface-staining was evident. This result demonstrates that receptor-mediated endocytosis of aFGF does not result in its translocation to the nucleus, as has been reported for basic FGF.

Key words: acidic fibroblast growth factor, vaccinia virus expression system, nuclear localization

INTRODUCTION

The fibroblast growth factor (FGF) family comprises seven structurally and functionally related polypeptides including the two prototype members acidic and basic FGF (aFGF and bFGF). These factors are also called FGF-1 and FGF-2, respectively (Baird and Klagsbrun, 1991). FGFs are potent inducers of proliferation and differentiation of a variety of cell types of mesodermal and neuroectodermal origin (Gospodarowicz et al., 1987; Goldfarb, 1990). Since they bind strongly to heparin and heparin-like molecules, such as heparan sulfate proteoglycans, FGFs are also called heparin-binding growth factors (HBGFs) (Shing et al., 1984; Burgess and Maciag, 1989; Rifkin and Moscatelli, 1989; Klagsbrun, 1990). Because of their strong mitogenic and chemotactic activities on endothelial cells, these growth factors are classified as angiogenic growth factors (Folkman and Klagsbrun, 1987; Folkman and Shing, 1992). Five of the members (int-2 (FGF-3), hst/K-FGF(FGF-4), FGF-5, FGF-6, and KGF (FGF-7)) are synthesized with an N-terminal signal sequence that targets the proteins onto the normal secretory pathway (Goldfarb, 1990). In contrast, aFGF and bFGF lack such a signal sequence (Abraham et al., 1986; Jaye et al., 1986), but yet have to be released from cells to interact with high-affinity receptors on the cell surface (see e.g. Lee et al., 1989; Partanen et al., 1991; Werner et al., 1992). As a consequence, aFGF and bFGF remain sequestered intracellularly when they are expressed in tissue culture cells (Cao and Pettersson, 1990; D’Amore, 1990). To date, the mechanism for release of the signal sequence-negative FGF prototypes remains unknown.

Although the mitogenic signals of FGFs are clearly mediated by various high-affinity receptors located on the cell surface, the finding that aFGF and bFGF are inefficiently released into the extracellular space points to the possibility that they might also play an important intracellular role. Immunocytochemical and subcellular fractionation studies have shown that bFGF is localized both to the cytoplasm...
and to the nucleus in a variety of cell types (Renko et al., 1990; Tessler and Neufeld, 1990; Florkiewicz et al., 1991). Only N-terminally extended forms initiated at upstream CUG codons are translocated to the nucleus, while the normal AUG-initiated form is confined to the cytoplasm (Bugler et al., 1991; Florkiewicz et al., 1991). Exogenously added bFGF has been found to be translocated to the nucleolus in a cell cycle-dependent fashion (Baldin et al., 1990). An N-terminally extended CUG-initiated form of int-2 has also been localized to the nucleus (Acland et al., 1990). Finally, it has been suggested that exogenously added aFGF has to enter the nucleus in order to exert its full mitogenic activity (Imamura et al., 1990). Unlike bFGF and int-2, aFGF has an in-frame translation termination codon just upstream from the AUG codon (Jaye et al., 1986), precluding the possibility of N-terminally extended forms. Instead, the sequence -NYKKPKL- close to the N terminus of aFGF has been suggested to serve as a nuclear localization signal (NLS) (Imamura et al., 1990, 1992).

These findings, prompted us to analyze this nuclear targeting in more detail. Using a T7 RNA polymerase-driven expression system and an affinity-purified rabbit anti-aFGF antibody (Cao and Pettersson, 1990; Elde et al., 1991), we localized aFGF and an N-terminal deletion mutant to the cytoplasm and nucleus of both BHK21 and HeLa cells. We also studied the fate of aFGF microinjected into the cytoplasm, and aFGF added exogenously. Our results suggest that aFGF is likely to enter the nucleus by free diffusion followed by accumulation in the nucleus due to trapping. We failed to observe nuclear translocation of exogenously added aFGF.

MATERIALS AND METHODS

Cells and viruses
BHK21, clone 13, and HeLa cells were grown in plastic dishes or bottles (Nunc) or on coverslips in Eagle’s minimum essential medium (MEM) (Gibco Ltd., Middlesex, England), supplemented with 10% fetal calf serum (FCS), glutamine and antibiotics. The recombinant vaccinia virus (vTF7-3) expressing bacteriophage T7 RNA polymerase (Fuerst et al., 1986; Elroy-Stein et al., 1989) was kindly provided by Dr. Bernard Moss. Virus stocks were prepared from infected HeLa cell homogenates and the titer of virus was determined by plaque assay on HeLa cells according to standard methods (Mackett et al., 1985).

Construction of aFGF expression plasmid
The construction of the recombinant plasmid pT7EMC-aFGF containing the complete cDNA coding sequence for human aFGF will be described in detail elsewhere (Cao and Pettersson, unpublished). In this plasmid, aFGF mRNA is expressed from the T7 promoter and translation initiation is enhanced by the introduction of an internal ribosome entry site (IRES) derived from encephalomyocarditis virus (EMC) virus (Elroy-Stein et al., 1989) (Fig. 1). To generate the deletion mutant of aFGF, a PCR-based method was used. The 5’ sense oligonucleotide primer (CCGTACCATGGGACTCCTCTACTGTAGCAACG) corresponds to the region coding for the initiation ATG and part of the aFGF N terminus starting from residue 28. The 5’ antisense primer (CTCTATTGGTTGCTCTGTGACCC) is complementary to the sequence coding for residues 89-94 of aFGF, and lies downstream of an NcoI cleavage site. Using pT7EMC-aFGF as a template, the PCR amplification was carried out for 30 cycles in a 100 µl reaction volume, with each cycle consisting of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C. The amplified DNA fragment was digested with NcoI and the resulting 223 bp fragment was isolated and used to replace the NcoI-NcoI fragment in pT7EMC-aFGF. The recombinant plasmid was named as pT7EMC-ΔaFGF (Fig. 1). The intactness of the inserts in pT7EMC-aFGF and pT7EMC-ΔaFGF was confirmed by restriction mapping and DNA dideoxynucleotide-sequencing using Sequenase® (United States Biochemical Corp., Ohio). The recombinant plasmids contain, downstream from the T7 promoter, the internal ribosomal entry site (IRES) from encephalomyocarditis virus (EMC), the aFGF cDNA insert, and the T7 transcription termination region (Fig. 1). The AUG initiation codon of aFGFs is placed in the optimal position (in the NcoI site) relative to the IRES sequence.

Infection and transfection
Monolayers of BHK or HeLa cells were usually grown to 70-80% confluency in MEM supplemented with 10% FCS in 25 cm² flasks prior to infection. The medium was removed and replaced with serum-free MEM. The cells were infected with the recombinant vaccinia virus vTF7-3 at a multiplicity of infection (MOI) of about 10-20 PFU/cell and incubated at 37°C for 45 min. The virus inoculum was then removed and replaced by 2 ml of fresh Opti-MEM medium (Gibco BRL) followed by transfection with plasmid DNA (2-5 µg) using 20-30 µg Lipofectin® according to the protocol recommended by manufacturer (Gibco BRL).

Immunoblot analysis
Proteins from transfected cell lysate and nuclear fraction were separated on a 10% to 15% SDS-PAGE gradient gel and transferred onto a nitrocellulose membrane (Towbin et al., 1979). The membrane was soaked for 30 min at room temperature in a blocking buffer (1% Tween-20, 5% skim milk, 150 mM NaCl and 20 mM Tris-HCl, pH 7.5) followed by incubation with rabbit anti-aFGF (1:500 dilution) for 2 h at room temperature in the same blocking buffer. Incubation with the secondary antibody, alkaline phosphatase-conjugated goat anti-rabbit IgG (Promega), and development of the phosphatase reaction was carried out as described before (Cao and Pettersson, 1990).

Subcellular fractionation
Isolation of nuclei from BHK21 and HeLa cells was essentially

![Fig. 1. Schematic representation of the aFGF cDNA inserts in the expression plasmids.](image-url)
as described by Renko et al. (1990) and Tessler and Neufeld (1990). Cells from three 25 cm² bottles, infected and transfected as described above, were harvested after removing the medium. After washing twice with PBS, the cells were collected by trypsinization and resuspended in MEM containing 10% FCS. The cells were then washed twice with MEM-FCS, and once with PBS containing 100 units aprotinin, and resuspended in 0.6 ml of TECK buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 3 mM CaCl₂, and 10 mM KCl) containing a mixture of protease inhibitors (100 units aprotinin, 10 µg phenylmethylsulfonyl fluoride (PMSF), 10 µg antipain, 10 µg dryomstatin, 10 µg leupeptin, 10 µg pepstatin per ml) followed by incubation for 15 min on ice. The cells were then homogenized with 30 strokes in a tight-fitting Dounce homogenizer followed by centrifugation at 10000 g for 10 min at 4°C. The supernatant was used as the cytoplasmic fraction. The pellet containing nuclei was resuspended in TECK buffer, washed three times with TECK, and suspended in 0.5 ml of sample buffer, incubated for 3 min at 95°C, and analyzed on a 10% to 15% SDS-gel.

Indirect immunofluorescence
Monolayers of BHK21 and HeLa cells grown on coverslips to 70-80% confluence were infected with recombinant vaccinia virus, vTF7-3, and transfected with 2 µg of plasmid as described above. After 4-6 h post-transfection, cells were washed three times with PBS, and fixed with 3% paraformaldehyde in phosphate buffer (pH 7.2) for 30 min at room temperature. After rinsing three times with PBS containing 0.2% bovine serum albumin (PBS-BSA), cells were permeabilized with 0.1% Triton X-100 for 30 min. The cells were then washed three times with PBS-BSA, and incubated with the affinity-purified antibody to aFGF (2 µg/ml) for 2 h at room temperature, or overnight at 4°C. The cells were then rinsed with PBS and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (1:80 dilution; Boehringer-Mannheim) for 1 h at room temperature, washed with PBS three times, and mounted in a mixture of glycerol and PBS (3:1, v/v) containing 0.1% p-phenylenediamine.

Microinjection
BHK21 cells were grown on coverslips to 70-80% confluence in MEM supplemented with 10% FCS. Microinjection of recombinant aFGF, purified from insect cells (Cao and Pettersson, 1990), into the cytoplasm of growing cells was carried out with a Zeiss automated injection system using glass capillaries from Eppendorf. The concentration of injected protein was 1 µg/µl in TNE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 100 mM NaCl). Before injection, the samples were centrifuged at 10,000 g for 10 min to remove aggregates. In the low temperature experiments, cells were maintained in ice-cold medium during the injection. The temperature was kept constant by a circulating pump. After injection, cells were incubated in MEM-FCS medium at 4°C for an additional hour. In the control experiments, cells were injected at room temperature and incubated at 37°C for 1 h after injection. Sodium azide (Na₃N₃) treatment of BHK cells was carried out by preincubating cells with 10 mM Na₃N₃ at 37°C for 30 min. It has been shown previously that this treatment reduces the ATP level to less than 5% of control cells (Persson et al., 1988). Na₃N₃ was present in the medium during injection and during the following hour of incubation at 37°C. Following incubation, the cells were fixed and analyzed by indirect immunofluorescence as described above.

Incubation of BHK cells with exogenous recombinant aFGF
Recombinant aFGF, purified from insect cells (Cao and Pettersson, 1990) by chromatography on a heparin-Sepharose column, was added to dividing, approximately 80% confluent, BHK21 cells. aFGF was adjusted to final concentrations of 0.1 µg, 1 µg and 10 µg per ml with MEM-FCS and incubated with the cells at 37°C for 2 h. The cells were then washed 5 times with PBS, fixed, permeabilized and stained with the affinity-purified anti-aFGF antibody as described above.

RESULTS
Intracellular localization of aFGF expressed in HeLa and BHK21 cells
The plasmid pT7EMC-aFGF containing the coding sequence of the human aFGF downstream from the T7 promoter and the internal ribosome entry site (IRES) from encephalomyocarditis (EMC) virus (Fig. 1) was transfected into HeLa and BHK21 cells that had been infected 45 min earlier with the T7 RNA polymerase-encoding recombinant vaccinia virus vTF7-3 (Elroy-Stein et al., 1989). aFGF was localized 4 h after transfection by indirect immunofluorescence using an affinity-purified rabbit antibody produced against native aFGF (Cao and Pettersson, 1990). As shown in Fig. 2A and B (BHK21 cells) and C and D (HeLa cells), immunoreactivity was observed in both the cytoplasm and the nuclei. The relative intensity between these two compartments varied from one cell to another. Nuclear staining was more prominent in HeLa cells than in BHK cells. At early time points, as shown in Fig. 2A, it appeared that the more aFGF was produced the stronger the staining was. At later time points (8 to 12 h post transfection), an increase in the cytoplasmic staining was seen (data not shown). In particular in BHK cells, pronounced immunoreactive cytoplasmic inclusions were evident (Fig. 2A) in some, but not all experiments (see e.g. Fig. 3A). These were in general absent from HeLa cells. As they were also absent from BHK21 cells microinjected with aFGF, these inclusion bodies were most likely related to the vaccinia virus infection. Cells that apparently had not taken up the recombinant plasmid showed no immunoreactivity and served as internal specificity controls. Cells transfected with the control plasmid pTM1 lacking an insert (Fig. 2E) and transfected cells stained with the flow-through fraction from the affinity purification column (Fig. 2F) displayed no immunoreactivity. From these results we conclude that aFGF expressed at a high level accumulates in the nucleus of both cell types.

Deletion of the N-terminal region does not prevent nuclear localization of aFGF
In two previous reports, it was speculated that nuclear translocation of exogenously added (Imamura et al., 1990), or endogenously synthesized (Imamura et al., 1992), aFGF could be mediated by an N-terminally located potential nuclear localization signal (NLS), -GNYKKPK-. To test this hypothesis, we removed this sequence by deleting the first 27 amino acids of aFGF (Fig. 1) and expressed the truncated protein in BHK21 and HeLa cells. The deleted form had the expected mobility on an SDS-gel, migrating clearly faster than wild-type aFGF (Fig. 4). The subcellu-
lar localization of the truncated aFGF was similar to that of the wild-type protein in both BHK21 cells (Fig. 3A and B) and HeLa cells (Fig. 3C and D), although the relative amount of protein found in nuclei as compared to the cytoplasm was somewhat decreased. Thus, we conclude that the N-terminal 27 residues are not essential for nuclear localization, but deletion of the sequence results in decreased nuclear accumulation.

To get an independent confirmation of the above results, we subjected \[^{35}S\]methionine-labeled cells expressing either the wild-type or the mutant form of aFGF to subcellular fractionation. Cells were harvested and disrupted 12 h after transfection, and the cytoplasm and nuclei were separated by centrifugation and equal amounts of material were analyzed by immunoblotting using the aFGF antibody. In two separate experiments the same results were obtained.

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Fig. 2. Immunolocalization of aFGF in BHK and HeLa cells. Cells were infected with vTF7-3 and transfected with pT7EMC-aFGF (A-D) or with the control plasmid pTM1 (E). Four hours later, the cells were fixed with paraformaldehyde, permeabilized with Triton X-100, and indirectly stained with the affinity-purified rabbit anti-aFGF antibody (A, C and E), or the flow through fraction from the affinity-purification column (F). (A, B, E and F) represent transfected BHK21 cells, while (C and D) are transfected HeLa cells. (B and D) are phase-contrast micrographs corresponding to A and C.
Nuclear localization of aFGF (Fig. 4). In cells expressing the wild-type protein, about 40-50% of aFGF was recovered in the nuclear fraction (lanes 2 and 6), while in mutant-transfected cells 25-30% was found in the nuclear fraction. These results were in conformity with the distribution observed by immunolocalization carried out at 12 h post-transfection (data not shown).

Low temperature and ATP depletion do not prevent nuclear localization of microinjected aFGF. Active transport into the nucleus requires energy and does not take place at low temperature, while free diffusion can readily take place under such conditions. We therefore introduced aFGF into the cytoplasm of BHK21 cells by microinjection and analyzed the fate of the protein 60 min later after incubating either at 37°C (Fig. 5A and B), or at 4°C (Fig. 5C and D). In both cases, microinjected aFGF was found in both the cytoplasm and the nuclei in about the same ratio. Thus, aFGF entered the nucleus in a temperature-independent manner.

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We next studied the effect on nuclear translocation of energy-depletion by first pretreating the cells with 10 mM sodium azide for 20 min at 37°C before microinjecting aFGF. Sodium azide was also present during the microinjection period and for 1 h prior to immunostaining. This treatment has been shown to lower the ATP level to less than 5% and to inhibit intracellular transport (Persson et al., 1988). As shown in Fig. 6, the subcellular distribution of aFGF in BHK21 cells was the same in control cells (A and

Fig. 3. Immunolocalization of the N-terminally deleted aFGF in HeLa and BHK cells. BHK21 cells (A and B) and HeLa cells (C and D) were transfected with pT7EMC-ΔaFGF and the cells were fixed and permeabilized at 4 h post-transfection. (A and C) show fluorescence micrographs, (B and D) are the corresponding phase-contrast micrographs.
Fig. 4. Immunoblotting of aFGF and ΔaFGF in nuclear and cytoplasmic fractions. BHK cells were infected with vTF-3 and transfected with plasmids pT7EMC-aFGF (lanes 1, 2, 5 and 6) or pT7EMC-ΔaFGF (lanes 3, 4, 7 and 8). After 12 h, the cells were homogenized and the nuclei were separated from cytoplasm as described in Materials and methods. Samples from each fraction (representing about 100,000 cells) were analyzed by SDS-PAGE and immunoblotting with the anti-aFGF antibody. Two independent experiments were performed as indicated. Lanes marked by odd numbers represent the nuclear fractions and lanes marked by even numbers are the cytoplasmic fractions. The positions of aFGF and mutant aFGF are indicated on the right. Molecular mass markers in kilodaltons are shown on the left.

**Exogenously added aFGF is not translocated to the nucleus**

Since a previous report (Imamura et al., 1990) has suggested that exogenously added aFGF might enter the nucleus, we analyzed the fate of exogenously added purified aFGF. BHK21 cells, known to express high levels of FGF-receptors (Gospodarowicz et al., 1987), were treated with various concentrations of aFGF (0.1–10 μg/ml). The permeabilized cells were then immunostained 2 h later to localize aFGF. With a concentration of 0.1 μg/ml, only weak immunofluorescence was observed, while with 1 μg/ml or 10 μg/ml clear positive immunoreactivity was obtained. Both the latter concentrations gave similar results. As shown in Fig. 7A, using 1 μg/ml, a punctate immunoreactivity was observed in a perinuclear region throughout the cytoplasm, while untreated cells were negative (Fig. 7C). This pattern is reminiscent of the endosomal-lysosomal compartments. Notably, immunoreactivity was absent from nuclei, indicating that internalized aFGF had not reached the nucleus during the 2 h incubation.

**DISCUSSION**

The development of a suitable antibody to aFGF and an efficient vaccinia virus-based expression system has allowed us to study the intracellular localization of aFGF in both BHK21 and HeLa cells. Our results can be summarized as follows. Immunofluorescence and subcellular fractionation experiments showed that endogenously expressed aFGF localized to the cell nucleus in addition to the cytoplasm. A mutant aFGF lacking the first 27 N-terminal amino acids, previously suggested to harbor a nuclear localization signal (NLS) (Imamura et al., 1990), was also localized to the nucleus, albeit to a slightly lesser extent. Since nuclear accumulation of aFGF injected into the cytosol was independent of ATP and occurred at low temperature, we conclude that nuclear translocation is not due to active transport, but is likely to occur by free diffusion. Finally, we found that exogenously added aFGF localized to cytoplasmic vesicular structures probably representing endosomes and lysosomes. No indication of nuclear uptake was obtained. Taken together, we interpret these results to mean that nuclear localization of aFGF is due to free (or facilitated) diffusion and may not require active transport mediated by a specific NLS.

The present studies were initially carried out to study the process of release of a FGF expressed to high levels. The mechanism by which aFGF and bFGF, both lacking an N-terminal signal sequence, are released from the cells has been enigmatic. To date, release by cell damage and leakage seems to be the only plausible suggestion (McNeil et al., 1989). In conformity with this notion, our studies showed that aFGF is released slowly and inefficiently into the cell culture medium, and that heparin increases the recovery of extracellular aFGF (Cao and Pettersson, unpublished). During the course of these studies, we found that endogenously expressed aFGF accumulated also in the cell nucleus. This finding, together with those by Imamura et al. (1990), indirectly suggesting that nuclear entry of exogenously added aFGF is required for full mitogenic activity, prompted us to analyze the nuclear translocation in more detail. During the course of this work a report by Imamura et al. (1992) appeared showing that endogenously expressed aFGF accumulated also in the cell nucleus. This finding, together with those by Imamura et al. (1990), indirectly suggesting that nuclear entry of exogenously added aFGF is required for full mitogenic activity, prompted us to analyze the nuclear translocation in more detail. During the course of this work a report by Imamura et al. (1992) appeared showing that endogenously expressed aFGF accumulated also in the cell nucleus. Thus, the issue of nuclear entry of aFGF is at present quite unclear.

Nuclear entry of endogenously expressed and exogenously added aFGF must in all likelihood take place by two very different mechanisms. Exogenously added aFGF must first be taken up via the endocytic pathway and then become translocated across the endocytic membrane in order to reach the cytoplasm where a NLS could become functional. Alternatively, aFGF could cross directly through the plasma membrane. Neither mechanism has as yet been shown to exist experimentally. In contrast, endogenously expressed protein may enter the nucleus directly from the cytoplasm.
by either passive or facilitated diffusion, if the size of the protein is small enough (less than about 40-60 kDa (see Peters, 1986) to allow entry through the nuclear pore, or by an active NLS-mediated import (Silver, 1991; Nigg et al., 1991). The size of aFGF is only 16.5 kDa and therefore it is small enough to be able to diffuse freely into the nucleus. We further suggest that aFGF, which strongly binds to negatively charged heparin-like molecules (Shing et al., 1984), could become trapped inside the nucleus by binding to, e.g., DNA or RNA, or some other negatively charged macromolecules. Deletion of the N-terminal 27 residues, which are exposed on the surface of the molecule (Zhu et al., 1991), removes four lysines and therefore would be expected to bind less tightly to negatively charged targets.

Our results are at variance with those reported by Imamura et al. (1992), in that we found no or only a minor role for the N-terminal 27 amino acid residues. Furthermore, in contrast to the observations by Imamura et al. (1990) we found no evidence for nuclear translocation of exogenously

**Fig. 5.** Immunolocalization of microinjected aFGF in BHK21 cells after incubation at 4°C and 37°C. The cytoplasm of dividing BHK21 cells were injected with purified recombinant human aFGF at a concentration of 1 µg/ml and the injected cells were fixed, permeabilized and stained with the antibody to aFGF 1 h later. (A and B) represent cells injected at room temperature followed by incubation at 37°C for 1 h. In (C and D) the cells were injected in ice-cold medium and incubated at 4°C for an additional hour. (A and B) are immunofluorescent micrographs, and (C and D) the corresponding phase-contrast micrographs. The non-injected cells served as negative controls.
added aFGF even after a 2 h incubation. Instead, aFGF probably localized to the endosomal/lysosomal compartment. We find it likely that internalized aFGF is ultimately transferred to the lysosomes where it is finally degraded. We wish to emphasize that, since we have analyzed the fate of internalized aFGF only by immunofluorescence, it is possible that we were unable to detect small amounts of aFGF taken up by the nuclei. Nuclear uptake of minute amounts of aFGF could be sufficient to elicit the full mitogenic activity as described by Imamura et al. (1990). In their study no attempts to localize internalized aFGF were made.

One important question is whether nuclear localization of endogenously expressed aFGF plays any physiological role, e.g. by mediating an intracellular autocrine effect. There is no clear answer to this. Although deletion of the N-terminal 27 residues resulted in an altered cell morphol-

Fig. 6. Immunolocalization of microinjected aFGF in ATP-depleted BHK21 cells. Dividing BHK21 cells were either left untreated (A and B) or treated with 10 mM sodium azide at 37°C for 20 min prior to injection into the cytoplasm of purified aFGF (1 µg/ml). The injected cells were incubated at 37°C for 1 h in either the absence (A and B) or presence (C and D) of sodium azide, fixed, permeabilized and stained with the specific antibody to aFGF.
ogy as compared to wild-type transfected cells (Imamura et al., 1992), this effect could be due to deficient function (Imamura et al., 1990) of the released mutant aFGF rather than a defective nuclear translocation. Since aFGF, according to our results, freely diffuses into the nucleus, an unambiguous answer to this question will be very difficult to obtain.

**Fig. 7.** Immunolocalization of aFGF added exogenously to BHK21 cells. Dividing BHK cells were incubated with purified aFGF at a final concentration of 1 µg/ml in FCS-MEM for 2 h at 37°C (A and B). The cells were fixed, permeabilized and indirectly stained with the affinity-purified antibody to aFGF. Inset in A shows a larger magnification of a treated cell. The punctate pattern of immunoreactivity is evident. (C and D) show untreated control cells.

It should be noted that bFGF, a close relative to aFGF, has also been shown to enter the nucleus, both when added exogenously (Bouche et al., 1987; Baldin et al., 1990), and when expressed endogenously (Renko et al., 1990; Tessler and Neufeld, 1990; Florkiewicz et al., 1991). In the former case, nuclear uptake is cell cycle-dependent, occurring only during G1 to S transition (Baldin et al., 1990). In the latter
case, only three larger forms (22-25 kDa) of bFGF initiated at CUG codons upstream from the regular AUG codon are found in the nucleus, while the AUG-initiated 155-residue form remains cytoplasmic (Bugler et al., 1991; Tessier and Neufeld, 1990; Renko et al., 1990; Florkiewicz et al., 1991). The NLS of the extended forms has been identified as methylated arginine residues in a repeated RGRG sequence (Burgess et al., 1991). There is to date no evidence that the various forms of bFGF have different biological functions. Since there is an in frame stop-codon immediately upstream from the initiating AUG in the aFGF mRNA (Jaye et al., 1986), no extended forms can be generated.

From a large body of work it is clear that the mitogenic, chemotactic and differentiation-inducing effects of FGFs are mediated by specific cell-surface tyrosine kinase receptors (see review by Goldfarb, 1990). To find out whether aFGF and bFGF have, in addition, intracellular modes of action, e.g. on transcription, remains a tough challenge for the future. The possibility of such alternative modes of action is supported by the fact that many other growth factors (or variant forms of them) known to interact with specific cell surface receptors are also found in the cell nucleus, either when synthesized endogenously or when added exogenously. Examples include IL-1β (Grenfell et al., 1991) and an N-terminally extended CUG-initiated form of Int-2 (FGF-3), another member of the FGF family (Acland et al., 1990).

The authors thank Robert Persson and Erich A. Nigg for helpful advice and stimulating discussions. Thanks are also due to Anita Bergström for excellent technical assistance.

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