Import(ance) of Growth Factors in(to) the Nucleus

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POLYPEPTIDE growth factors (GFs) play a fundamental role during embryogenesis and regeneration (e.g., wound healing) by stimulating proliferation and differentiation of certain cell populations. Some GFs can be responsible also for malignant transformation and tumor growth (e.g., FGF-4; hstl oncogene). GF receptors (GFRs) are generally known as plasma membrane proteins which "send" signals to the nucleus principally via the MAPK and the JAK-STAT pathways (Karin and Hunte, 1995). However, in the past few years data were accumulating to suggest that, surprisingly, nuclear targeting and action of GFs and GF receptors could occur as well. This alternative or complementary signaling pathway appears to be involved in the induction of cell proliferation. In addition, nuclear GF–GFR complexes may participate in the activation of cell line–specific genes as well. Since by far the largest body of data has been published in relation to FGF-1 and -2 (aFGF and bFGF), we have focused here on the nuclear role of these GFs.

Mitogenic Effect and Developmental Appearance of Nucleus-associated GFs

Similar to steroid and thyroid hormones, vitamin D3, and retinoic acid, it appears that GFs may be present and function in cell nuclei. In different target cells, nuclear association was shown for FGF, EGF, NGF, PDGF, insulin, etc. (for reviews see Burwen and Jones, 1987; Jans, 1994; Prochiantz and Theodore, 1995; Jans and Hassan, 1998; on FGFs: Mason, 1994; M.K. Stachowiak et al., 1997). Although the idea of nuclear GFs is more or less accepted, the functional significance is generally debated based on some reports. For example, activation of the Raf-MAPK pathway was shown to be sufficient and necessary for transduction of the aFGF mitogenic signal in BaF3 hematopoietic cells (Hung et al., 1995). Although several data indicate that nuclear localization of GFs may be required for the mitogenic effect in certain conditions in different cell types.

The presence of radiolabeled, externally added aFGF in the nuclear fraction appeared to correlate with stimulation of DNA synthesis in a concentration-dependent manner in NIH 3T3 cells (with a submaximal [3H]thymidine incorporation rate at 10 ng/ml FGF-1). Correlation between nuclear association of aFGF and DNA synthesis was demonstrated also in diphtheria toxin–resistant U 2 Os Dr1 cells. Although these cells lack aFGF receptors, they were able to internalize aFGF via their cell surface toxin receptors, if the GF was fused to the diphtheria toxin fragments. After extracellular administration, the aFGF-toxin label was detected in the nuclear fraction. At the same time, DNA synthesis was found to increase fourfold (at a fairly low, 5 ng/ml aFGF-toxin). However, no significant increase in the number of cells was observed. Therefore, it appears that although nuclear action of aFGF seems to be sufficient for triggering DNA synthesis, FGF-R is indispensable for other processes of cell proliferation (Wiedlocha et al., 1994). Consistent with this idea, DNA synthesis was accompanied by cell proliferation only in cells which possess aFGF receptors or if the toxin-resistant cells were transfected with a FGF-R (Wiedlocha et al., 1996).

However, one has to keep in mind that cell lines, transfected cells, and tumor cells most probably do not behave and cannot be considered as normal cells. Nonetheless, cell proliferation rate and nuclear association of bFGF was reported to change in parallel not only in glioma cells for example, which express transfection-derived endogenous FGF-2, but also in primary cultures of human astrocytes stimulated with extracellular bFGF (concentration range: 0.09–2.5 nM) (Joy et al., 1997). These observations support the idea that nuclear translocation of FGFs could be related to mitogenesis in normal, nontransformed cells as well.

Uptake of extracellular bFGF to the nucleus and to the nucleolus was found to occur only in late G1 phase of the cell cycle in growing aortic endothelial (A B A E) cells, both by immunocytochemistry and by analysis of radioiodinated cell fractions (Baldin et al., 1990). Nuclear association of FGF-2 was also observed in mid-late G1 phase in proliferating epiphyseal plate chondrocytes (Kilkenny and Hill, 1996), suggesting a controlled nuclear entry of GFs around the restriction point of the cell cycle.

It is important to note that autocrine and intracrine FGF types can have different effects, which are related to their partially different sequence and to their characteristic site of action. From the four different forms of human FGF-2, the low molecular mass form (with 18 kD) is an autocrine/paracrine one. The three high molecular mass forms (with...
21–22, 22.5, and 24 kD, respectively) are the intracrine forms generated by alternative translation initiation at CUG codon, through an internal ribosome entry process regulated by a cis-acting mechanism (Vagner et al., 1996). These intracrine forms, which have a longer, arginine-rich NH₂-terminal with at least two possible short nuclear localization sequences (NLSs) (Gly-Arg-Gly-Arg-Gly-Arg) are preferentially targeted to the nucleus (Quarto et al., 1991). In contrast, the 18-kD form has only a weak or cryptic short NLS, and is found predominantly in the cytoplasm (Quarto et al., 1991; Davis et al., 1997). Only the short bFGF form can be released from the cell, and can, therefore, interact with the plasma membrane FGFR. Surprisingly, when intracrine bFGF types were expressed in NIH 3T3 cells, high proliferation rates and growth in soft agar were observed, even in the presence of mutant cell surface bFGF receptors lacking the Tyr kinase domain (Bikfalvi et al., 1995). This reflects a plasma membrane receptor-independent pathway, presumably via formation of complexes between intracrine GFs and intracellular receptors (see below). In vascular smooth muscle cell lines expressing different human bFGFs, the intracrine bFGF forms appeared to be significantly more effective in augmenting the rate of DNA synthesis than the autocrine one (Davis et al., 1997). Moreover, the continuous proliferation of two glioma cell lines is suggested to be related to the constitutive presence of endogenous FGF-2 in nuclei; these cells were nonresponsive to extracellular GFs (Joy et al., 1997). Synthesis of CUG-initiated forms could be induced also in primary human skin fibroblasts, producing normally the short bFGF form almost exclusively, by heat shock (45°C, 15–60 min) and by oxidative stress, which is probably due to translational activation (Vagner et al., 1996).

Developmental studies indicated that FGF-2, known as a maternal signal involved in mesoderm induction in amphibians, brings about mesoderm induction via Src-kinase (Bikfalvi et al., 1995). However, nuclear bFGF may be involved in other specific developmental phenomena, since nuclear association of bFGF becomes restricted to some cell populations during embryogenesis. In the mid-blastula stage, FGF-2 was demonstrated clearly in the nuclei of the animal hemisphere of Xenopus; in the prelarval embryo, nuclear bFGF was shown in most head regions (including the brain) and particularly in some muscle cells of the trunk region (immunocytochemical study by Song and Slack, 1994). This is consistent with the well-known stimulatory effects of bFGF on myoblast proliferation (Burgress and Macciag, 1989) and on proliferation plus differentiation of neuroblasts and glial precursor cells (M.K. Stachowiak et al., 1997). In early chicken embryos, nuclear FGF-2 isoforms were observed in most cells of the preblastodisc during hypoblast formation and mesoderm induction. Only the hypoblasts and the blastocoeic cells seemed to maintain their nuclear immunostaining during primitive streak formation and with the onset of gastrulation (Riese et al., 1995). In later phases, only a small proportion of limb bud cells, most likely migrating myoblasts, and differentiating kidney podocytes were shown to have considerable nuclear FGF-2 (immunohistochemical study by Dono and Zeller, 1994).

### Nuclear Targeting of GFs and GFRs

Recently, several data have accumulated which support the idea of GF receptor translocation to the nucleus. For example, three FGFR-1 variants (with 145, 118, and 103 kD, respectively) were detected in the nucleoplasmic and in the nuclear matrix fractions of human astrocytes and bovine adrenal medullary cells. In the majority of cells, the immunofluorescence signals of FGF-2 and FGFR-1 appeared to colocalize in the nuclei (Stachowiak et al., 1996a,b). So, how could the GFR gain access into the nucleus?

A according to the emerging view, NLS-bearing GFs like FGFs presumably facilitate the nuclear import of their receptors. Theoretically, GFs do not need NLS to enter the nucleus, since the molecular “sieves” of nuclear pores demand it only from compounds >40–45 kD. Possession of NLSs by low molecular mass GFs implies that this may be necessary for the nuclear import of their receptors which can be transported “piggyback” to the nucleus in association with NLS-bearing ligands (J ans, 1994; J ans and H assan, 1998). The concept that plasma membrane GFRs could enter the nucleus upon extracellular GF stimulation is supported by the accurate study of Mahe (1996), who demonstrated a dose- and time-dependent increase of nucleus-associated FGFR-1 immunoreactivity in Swiss 3T3 fibroblasts (onset: within 10 min, max: 1 h; concentration: 5–15–50 ng/ml). Moreover, the FGFR-1 in the nuclear fraction was shown to bear the impermeable biotin label of the cell surface proteins and was proven to be of full length, verifying its plasma membrane origin. Even intracrine GFs may enter the nucleus in complex with intracellular receptors. Consistent with this idea, several truncated forms of FGFR-1 and FGFR-2 have been described, which are devoid of the transmembrane region (Givol and Yayon, 1992). Furthermore, a truncated FGFR 3 variant missing the transmembrane part and half of the final Ig-like domain was shown to be characteristic with cell nuclei in breast epithelial cell lines by immunocytochemistry (J ohnston et al., 1995).

Considering the role of high-affinity GF receptors in nuclear targeting, they are probably prerequisite for the intracellular transport of GFs to the perinuclear region during receptor-mediated endocytosis. A according to the studies of Prudovsky et al. (1996) on transfected L6 myoblasts, the first Ig-like loop in type 1 FGFRs may facilitate the transport of exogenous FGF-1 to the perinuclear area, as mostly the α, 3-loop receptor isoforms possessing this domain (and not the β isoforms lacking it) were demonstrated in the nuclear/perinuclear fraction. N-glycosylation seems to be also important, as tunicamycin treatment significantly reduced the presence of the α receptor forms in the nuclear/perinuclear fraction. This can be interpreted on the basis of NLS-independent, but sugar-dependent nuclear import mechanism described by Duverger et al. (1995), as GFRs are glycoproteins (M.K. Stachowiak et al., 1997).

Regarding GFs, possible involvement of low-affinity saccharide receptors in nuclear translocation cannot be ruled out. Heparan sulfate proteoglycans (HSPGs) with highly O-sulfated oligosaccharide chains are well known to play a crucial role in the formation and in the mainta-
nance of the active FGFR-α/bFGF complexes at the plasma membrane (Luo et al., 1996). Perlecain, a basal lamina proteoglycan (A vizeer et al., 1994), syndicains, and glypican (Steinfeld et al., 1996) proved to be effective in stimulating FGF-FGFR interaction. It is thought that HSPG and extracellular bFGF bound to FGFR might be cotranslocated to the nucleus; HSPG could stabilize the complex and protect it from degradation in the endocytotic vesicles and in the lysosomes (R eland and R apaeg, 1993). Since glypican was observed in association with cell nuclei (in rat neurons and in glioma cells) and, moreover, it was shown to have functional NLS (L iang et al., 1997), this gives further credence to the mentioned idea.

In NIH 3T3 cells, the constitutively activated FGFR-3 mutant kinase domains in linkage with the plasma membrane appeared to be sufficient to trigger cell proliferation and transformation, in contrast to wild-type kinase domains, or to activated kinase domains targeted to the nucleus or to the cytoplasm (Web ster and D onoghue, 1997). However, in astrocyte and glioma cultures, cell proliferation appeared to correlate with the nuclear presence of FGFR-1. Continuously proliferating glioma cells, unresponsive to external FGF, displayed constitutive nuclear association of FGFR-1. In contrast, astrocytes had decreasing nuclear appearance of FGFR-1 in parallel to increasing cell density in cultures approaching confluency. Furthermore, enhanced cell proliferation rate could be achieved in glioma cells lacking FGFR-1 by transfecting them with the full-length receptor cDNA; thereafter, immunoreactivity of FGFR-1 was seen predominantly in association with the nucleus (E.K. Stachowiak et al., 1997). Both in astrocytes and in bovine adrenal medullary cells, the nuclear FGFR was shown to retain kinase activity. With this observation, we arrived at a basic, but currently unresolved question.

How could GFs and GFRs act in the nucleus? Nuclear FGFR kinase activity is thought to have no significant role in the induction of cell proliferation (Web ster and D onoghue, 1997). However, bFGF may be involved in induction of ribosomal gene transcription via stimulation of casein kinase-2, which is known to regulate nucleolin, a major component of the nucleolus implicated in ribosome biogenesis. Using nuclear extracts of FM3A cells and purified proteins, FGF-2 was shown to bind CK-2 and stimulate its activity, resulting in an increased phosphorylation of nucleolin (B onnet et al., 1996). (Enhancement of CK-2 activity reached its maximum at 10^{-7} M FGF-2 concentration, which was calculated to be a possible bFGF concentration in the nucleus.) Supporting these observations, rRNA was found to increase severalfold upon addition of bFGF (0.1–1 NM) to isolated nuclei from quiescent ABAE cells (B ouche et al., 1987). Furthermore, GF–GFR complexes may cotransport intranuclearly acting molecules to the nucleus, via binding to the receptor, as was suggested for IFN–γ–IFN receptor complex and STAT by J ohnson et al. (1998).

**Normal Cells: Anchorage-dependent GF Transport to the Nucleus?**

According to the classic view, extracellular GFs stimulate their receptor-mediated endocytosis, which leads to degradation (or to recycling) of GF receptors. However, it is plausible to suppose that a portion of GFs and GFRs can escape from the endosomes or lysosomes and may reach the nucleus. Growth hormone was demonstrated to undergo a receptor-dependent nuclear translocation via the endosomes in rat hepatocytes (L obie et al., 1994; electron microscopic autoradiography); its nuclear uptake could be significantly increased upon the addition of some lysosome inhibitors, indicating an escape route from the lysosomes. It is noteworthy that FGF-1 may be detected only in few regions at the nuclear envelope and displayed a patchy distribution within the nucleus of bovine adrenal medullary cells; this may reflect nuclear entry at determined membrane pores and controlled transport to special nuclear sites (immunoelectron microscopy by Stachowiak et al., 1996b).

It is intriguing to hypothesize that actin is involved not only in endocytosis and in the transport of endosomes to the perinuclear area (D urrbach et al., 1996), but also in the precise nuclear targeting of GF–GFR complexes from the perinuclear cytoplasm, since actin is known to be present in abundance in cell nuclei, both in the chromatin and in the nuclear matrix fraction (Capco et al., 1982). Furthermore, there is evidence that a GF receptor, the EGFR, is a (direct) actin-binding protein (den Hartigh et al., 1992); other GFRs may be linked to actin indirectly, via actin-binding proteins, during their nuclear translocation.

It should be noted in this context that extracellular matrix-dependent cytoskeletal organization supervises GF action on proliferation of normal cells, reflected in the well-known phenomenon of anchorage-dependent growth. Cell division is generally preceded by extensive cell spreading (A lberts et al., 1994). Spreading is probably necessary for nuclear translocation of GF–GFR complex in normal cells, since only astrocytes in subconfluent cultures (and not the ones in confluent cultures in short of extracellular surface) were observed to have nuclear-associated bFGF and FGFR-1. On the contrary, in continuously growing glioma cells, nuclear appearance of FGF-2 and FGFR-1 was constitutive and was largely independent of cell density (J oy et al., 1997; E.K. Stachowiak et al., 1997). Finally, bFGF gene is continuously activated in glioma cells irrespective of cell density, whereas in astrocytes bFGF transcription is induced by subconfluence (M offett et al., 1996).

All in all, it seems that a portion of internalized exogenous GFs plus their receptors may escape degradation and could be transported to the cell nucleus. Nuclear GF–GFR complexes appear to stimulate cell proliferation in certain conditions in several cell types; in addition, activation of cell line-specific genes may occur in some differentiating cells. Continuous proliferation of transformed cells could be partially due to the continuous nuclear presence of GFs and GFRs. Obviously, much work has to be done to elucidate details of the nuclear targeting of GF–GFR complexes and to be able to understand their nuclear action fully.
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