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# Nuclear Translocation of Angiogenic Proteins in Endothelial Cells: An Essential Step in Angiogenesis†

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Eukaryotic cells continuously transport proteins and ribonucleoproteins between the nucleus and the cytoplasm. Proteins destined to function within the nucleus are synthesized in the cytoplasm and then transported posttranslationally across the nuclear envelope through nuclear pore complexes (Gerace & Burke, 1988). The latter are organelles composed of a unique set of proteins, termed nucleoporins, that play a specific role in protein translocation (Davis & Blobel, 1986). Active transport of a protein into the nucleus requires that it has a suitable nuclear localization signal (NLS),[1] typically, a specific, short sequence that contains a high proportion of basic amino acids. Such NLS's are both necessary and sufficient for nuclear targeting (Garcia-Bustos et al., 1991). They are recognized by NLS-binding proteins (NBP) in the cytosol.

The nuclear import of proteins can be separated into two steps that require different cytosolic factors: binding at the cytosolic face of the pore complex and translocation through the nuclear pore (Adam & Gerace, 1991; Moore & Blobel, 1992, 1994; Adam & Adam, 1994). Recently, it was reported that the GTP-binding protein Ran/TC4 is required for the import of the bound protein into the nucleus (Moore & Blobel, 1993, 1994; Melchior et al., 1993). Nuclear localization of many proteins is regulated since their presence in the nucleus is required only at specific times in the cell cycle or in response to short-acting stimuli. One of the main mechanisms to control such localization seems to be cytosolic anchoring (Hunt, 1989).

Cellular transport mechanisms also operate for proteins delivered, e.g., via the circulation, to the cell surface and brought into the cell by endocytotic pathways. These include processes in which (1) the ligand–receptor complex dissociates at the endosomal level, the receptor is recycled to the surface, and the ligand is degraded in lysosomes [i.e., LDL (Basu et al., 1981)]; (2) the ligand–receptor complex is recycled to the cell surface where it dissociates and the receptor is reused [i.e., transferrin (Hoppkins & Trowbridge, 1983)]; and (3) the ligand–receptor complex is delivered by transcytosis to the opposite surface of a polarized cell where the ligand is released intact [i.e., IgA (Mostov et al., 1986)].

Recently, another transport mechanism has been found that combines protein internalization and subsequent translocation into the nucleus. Exogenous angiogenic and other growth-inducing proteins are endocytosed by target cells and translocated from the cell surface to the nucleus where they accumulate in the nucleolus. We believe that this is a general nuclear transport pathway which should be considered an addition to the more classic endocytotic pathways. We here present possible mechanisms of nuclear translocation and action within the nucleus and discuss the physiological significance of nuclear localization of exogenous angiogenic factors for angiogenesis.

*Nuclear Translocation as a General Pathway for Angiogenic Proteins in Endothelial Cells.* Angiogenin is a 14-kDa basic protein and a potent inducer of angiogenesis (Fett et al., 1985). It is a member of the pancreatic ribonuclease superfamily (Strydom et al., 1985) with a unique ribonucleolytic activity that is critical for its angiogenic activity (Shapiro et al., 1986). It interacts specifically with both high- and

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[1] Abbreviations: ABAE, adult bovine aortic endothelial; CPAE, calf pulmonary artery endothelial; aFGF, acidic fibroblast growth factor; bFGF, basic FGF; NLS, nuclear localization signal; NBP, NLS-binding protein; TGF-$\alpha$, transforming growth factor $\alpha$; VEGF, vascular endothelial growth factor; EGF, epidermal growth factor; PDGF, platelet-derived growth factor.

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low-affinity binding sites on calf pulmonary artery endothelial (CPAE) cells (Badet et al., 1989) which express at least one membrane-associated angiogenin binding protein (AngBP) identified as a smooth muscle type of $\alpha$-actin (Hu et al., 1993; Moroianu et al., 1993). Immunofluorescence microscopy has demonstrated that human angiogenin is specifically endocytosed by subconfluent CPAE cells and is translocated from the cell surface to the nucleus where it accumulates in the nucleoli (Moroianu & Riordan, 1994). Two regions of the angiogenin molecule have been shown to be essential for its angiogenic activity: one is presumed to be involved in binding to endothelial cells and the other is a catalytic site capable of cleaving RNA (Shapiro et al., 1986; Hallahan et al., 1991, 1992; Shapiro & Vallee, 1992). Endocytosis, and hence nuclear localization, does not require the catalytic site of angiogenin but it does require the cell binding site.

Two other angiogenic proteins, basic fibroblast growth factor (bFGF) and acidic FGF (aFGF), promote the proliferation and differentiation of a wide range of cell types and in the case of endothelial cells stimulate them to migrate, form tubes, and increase production of proteases and plasminogen activator (Folkman & Klagsbrun, 1987; Folkman & Shing, 1992; Rifkin & Moscatelli, 1989). Immunofluorescence microscopy has revealed that when bFGF is provided exogenously to proliferating adult bovine aortic endothelial (ABAE) and CPAE cells, it is internalized and specifically translocated to the nucleus with accumulation in the nucleoli (Baldin et al., 1990; Bouche et al., 1987; Moroianu & Riordan, 1994). In addition, endogenous bFGF was found to be associated with the nuclei of bovine corneal, aortic arch, and adrenal cortex capillary endothelial cells in culture (Tessler & Neufeld, 1990). Analysis of the cellular distribution of the different forms of endogenous bFGF revealed that only the higher molecular mass forms (22.5 and 24 kDa) are found preferentially in the nuclear fraction, while the 18-kDa form is primarily cytosolic (Renko et al., 1990).

aFGF has been found to localize in the nucleus of mesenchymal cells as determined by immunohistochemical staining (Sano et al., 1990). It is also endocytosed by proliferating CPAE cells as shown by immunofluorescence microscopy and is specifically translocated to the nucleus and to the nucleoli (Moroianu & Riordan, 1994).

EGF is a 6-kDa polypeptide that stimulates the proliferation of endothelial cells in vitro and is considered a weak angiogenic factor (Folkman & Klagsbrun, 1987; Klagsbrun & D'Amore, 1991). It was detected in the nucleus of primary cultured bovine corneal endothelial cells by cell fractionation (Savion et al., 1981), and fluorescently labeled EGF was endocytosed and translocated to the nucleus in subconfluent CPAE cells, but not in confluent ones (Moroianu & Riordan, 1994). Nucleolar accumulation was not observed.

These findings suggest that other angiogenic factors, such as vascular endothelial growth factor (VEGF), transforming growth factor $\alpha$ (TGF-$\alpha$), or TGF-$\beta$, might also be translocated to the nucleus of endothelial cells. VEGF is a highly specific mitogen for endothelial cells and has 21–24% sequence identity with the A and B chains of platelet-derived growth factor (PDGF) (Klagsbrun & D'Amore, 1991). It has been shown that endocytosed PDGF accumulates in the nuclei of target cells (Rakowicz-Szulczynska et al., 1986), and PDGF-like proteins have been found in the nuclei of cells transformed by the simian sarcoma virus (Yeh et al., 1987). TGF-$\alpha$ has 40% sequence identity with EGF and binds to the EGF receptor (Klagsbrun & D'Amore, 1991). Both EGF and its receptor were found to be translocated to the nucleus of carcinoma cells (Rakowicz-Szulczynska et al., 1989).

Other growth factors and hormones, such as nerve growth factor, insulin, prolactin, somatostatin, and interferons have also been found to be translocated to the nucleus of cells bearing their respective receptors (Burwen & Jones, 1987). Thus, for all of these proteins many aspects of their functionality seem to be associated with nuclear targeting.

*Nuclear Localization Signals (NLS) of Angiogenic Factors.* Proteins are targeted to different locations in the cell via specific signal sequences (Blobel & Dobberstein, 1975). They are actively translocated to the nucleus by cytoplasmic and nuclear factors that recognize various NLS. These are typically short sequences of 5–10 amino acids that contain a high proportion of basic amino acids. NLS's have been identified as sequences that (1) when deleted or mutated, abolish the ability of the protein in which they reside to be taken up by the nucleus or (2) can by genetic or chemical fusion transform a normally cytoplasmic protein into a nuclear protein (Garcia-Bustos et al., 1991).

Although no strong consensus sequence has emerged from the identification of NLS's in a number of proteins, two classes of NLS may exist (Dingwall & Laskey, 1991). The first, typified by the SV-40 large T-antigen NLS, consists of a stretch of three to five basic residues conforming to the weak consensus Lys-Arg/Lys-X-Arg/Lys (Chelsky et al., 1989). The second is denoted by the *Xenopus* nucleoplasmin NLS which is bipartite and consists of two basic regions of three to four residues separated by a spacer of about ten amino acids (Robbins et al., 1991). Also, some nuclear proteins such as *Xenopus* histone $H_1$ bind to other proteins that in turn have an NLS for nuclear transport (Breeuwer & Goldfarb, 1990).

The $NH_2$-terminal extension of the high molecular weight forms of bFGF was identified as a NLS by constructing a chimeric protein in which this extension sequence was fused to $\beta$-galactosidase. After transfection in a transient expression system, the chimeric protein was found to accumulate in the nuclei of transfected cells, whereas wild-type $\beta$-galactosidase occurred predominantly in the cytosol (Quarto et al., 1991). The extra $NH_2$-terminal sequence is particularly rich in arginine residues present in Gly-Arg repeats. When chimeric proteins were constructed by fusion of one of the three forms of bFGF (18, 21, and 22.5 kDa) with chloramphenicol acetyltransferase and expressed in COS cells, the two higher molecular weight forms were nuclear but the 18-kDa form, which lacks the $NH_2$-terminal extension, was cytoplasmic (Bugler et al., 1991), indicating that cytoplasmic versus nuclear localization of the different endogenous bFGF forms is determined by the $NH_2$-terminal sequence. The 18-kDa form of bFGF does contain a sequence Lys-Asp-Pro-Lys-Arg-Leu that is homologous to the NLS of histone H2-B and is located between residues 27 and 35. However, since endogenous 18-kDa bFGF is primarily cytoplasmic, this putative NLS must be cryptic. As noted above, when 18-kDa bFGF is provided to endothelial cells exogenously, it is not only endocytosed but also translocated to the nucleus and concentrated in the nucleolus (Bouche et al., 1987; Baldin et al., 1990; Moroianu & Riordan, 1994). The putative NLS of 18-kDa bFGF is also similar to residues 21–27 of aFGF (Asn-Tyr-Lys-Lys-Pro-Lys-Leu), which have been demonstrated to be a NLS for this protein by deletion mutation analysis (Imamura et al., 1992). As in the case of 18-kDa bFGF, the NLS of aFGF is functionally active only when the protein is provided to the cells exogenously, suggesting that its nuclear translocation requires an endocytosis-dependent pathway (Zhan et al., 1992).

We have suggested that the positively charged sequence $Arg_{31}$-$Arg_{32}$-$Arg_{33}$ of human angiogenin (Strydom et al., 1985) is part of a NLS (Moroianu & Riordan, 1994). An angiogenin mutant, R33A, in which Arg-33 is changed to Ala, is not translocated to the nucleus of CPAE cells. The mutant has an intact cell binding site, exhibits 17% enzymatic activity toward tRNA, but is not angiogenic (Shapiro & Vallee, 1992). Thus a combination of ribonucleolytic activity and receptor binding capacity is not sufficient for angiogenic activity: nuclear translocation of angiogenin is required as well.

NLS's have also been identified in the two homodimeric forms of platelet-derived growth factor, PDGF-A and PDGF-B, near their C-terminus (Maher et al., 1989). It is interesting that the amino acid insertions of the high molecular weight forms of VEGF show strong conservation with the NLS of PDGF-A (Maher et al., 1989; Houck et al., 1991). Taken together, these data support the classic concepts of selective recognition and active translocation of angiogenic factors into the nucleus.

*Nuclear Translocation Mechanism.* The nuclear localization process for an exogenous angiogenic protein involves at least six distinct steps: (1) binding to the cell surface, (2) endocytosis, (3) transport of the endocytotic vesicle to the nucleus, (4) transport of the protein across the endosomal membrane, (5) entrance of the angiogenic factor into the nucleus, and (6) targeting to the nucleolus or some other specific nuclear site. Details of the molecular mechanisms of nuclear and nucleolar targeting of exogenous angiogenic factors or any other growth factor are as yet unknown. However, the absolute requirement of a NLS for nuclear translocation strongly supports the classic pore route. In this case, the angiogenic factor must first be released from the endocytotic vesicle in order to interact with a NBP and traverse the pore (Silver, 1991; Newmeyer, 1993). This implies dissociation of the internalized angiogenic molecule from, and accumulation in, the nucleus, without its membrane receptor, as was demonstrated for insulin by electron microscopic immunocytochemistry (Soler et al., 1989). When and how the protein is released from the endocytotic vesicle is not known.

Once inside the nucleus the angiogenic molecules are somehow targeted to the nucleolus. In the case of nucleolar protein no. 38, nuclear uptake depends on a typical NLS, while accumulation within the nucleolus requires oligomerization (Nigg et al., 1991). For several retroviral regulatory proteins, nucleolar targeting signals, which are modified NLS's, appear to direct the protein to the nucleolus (Garcia-Bustos et al., 1991; Cochrane et al., 1990; Adachi et al., 1993). Moreover, the nucleolar targeting signal of the Rex protein of human T-cell leukemia virus type I (HTLV-I) binds to the nucleolar protein B-23—a key participant in the shuttling of ribosomal components across the nuclear envelope—which suggested that B-23 mediates the import of HTLV-I Rex from the cytoplasm to the nucleolus. Similar mechanisms could operate to target angiogenic molecules to the nucleolus.

Another unanswered question is the role played by the actin cytoskeleton, microtubules, or intermediate filaments in the translocation of endocytotic vesicles to the nucleus and in the transport of angiogenic factors into the nucleus and to their final nucleolar destination. Nuclear translocation of karyophilic proteins injected into the terminal axoplasm of *Aplysia californica* neurons was inhibited by nocodazole (Ambron et al., 1992). Also, preliminary observations with specific inhibitors (colchicine and nocodazole) have indicated that microtubules may be involved in the nuclear translocation of angiogenin in endothelial cells (J. Moroianu and J. F. Riordan, unpublished experiments). It is not known whether and how microtubules are connected with the nuclear pore complex and whether any of the NBP's interact with microtubules. A NBP was found in the region of the microtubule organizing center, and treatment of cells with nocodazole perturbed this localization (Li et al., 1992). Further, immunoelectron microscopy has revealed that within the nucleus a nucleolar NBP, Nopp 140, shuttles between the nucleolus and the cytoplasm on tracks that extend from the dense fibrillar component of the nucleolus across the nucleoplasm to some nuclear pore complexes (Meier & Blobel, 1992).

*Mechanisms of Action of Angiogenic Molecules within the Nucleus in Relation to Angiogenesis.* Translocation of angiogenic factors into the nuclei of proliferating endothelial cells raises at least three important questions: (1) Is nuclear translocation essential to the regulation of angiogenesis? (2) What are the molecular mechanisms of action of angiogenic molecules within the nucleus? (3) What is the relation between the nuclear translocation pathway of angiogenic molecules and the classic signal transduction pathway through intracellular messengers.

Angiogenesis occurs by formation of new capillaries from established blood vessels under the action of a variety of angiogenic factors such as angiogenin, EGF, aFGF, bFGF, TGF-$\beta$, tumor necrosis factor $\alpha$, and VEGF (Folkman & Klagsbrun, 1987; Folkman & Shing, 1992; Klagsbrun & D'Amore, 1991). In involves activation of endothelial cells and degradation of the extracellular matrix followed by migration, proliferation, and differentiation of the endothelial cells into tubelike structures. Mitogenic and other physiological signals induced by angiogenic molecules may be transmitted to the nucleus by the action of intracellular messengers, and/or the molecules may act directly in the cell nucleus.

In aortic endothelial cells the nucleolar translocation of bFGF correlates with its stimulatory effect on the transcription of ribosomal genes during $G_0$-$G_1$ transition after it first induces a 10-fold increase in the nucleolar protein, nucleolin (Bouche et al., 1987). Moreover, bFGF acts directly on the transcription of rDNA in isolated nuclei from quiescent endothelial cells and increases RNA polymerase I transcriptional activity by almost 6-fold. Preliminary experiments have indicated that within the nucleolus bFGF activates a nucleolar cyclic AMP-independent protein kinase, NII, whose specific substrate is nucleolin. The endoproteolytic cleavage of phosphorylated nucleolin appears to be the event that triggers ribosomal gene transcription in vitro (Bouche et al., 1987). The proliferative state of a cell is closely related to ribosome biogenesis, which involves a series of coordinated nuclear events including transcription of ribosomal genes. However, it has not been established whether activation of this bFGF signaling pathway *in vivo* is either necessary or sufficient to trigger the full mitogenic response.

bFGF can also regulate gene transcription directly in a cell-free system in a promoter-specific manner (Nakanishi et al., 1992). This suggests an alternative mechanism for the signal transduction pathway provided by bFGF. Moreover, a mutant of bFGF lacking the putative NLS, residues 27–32, is at least 100 times less efficient than the natural protein in stimulating plasminogen activator gene expression in endothelial cells (Issacchi et al., 1991). Importantly, bFGF lacks a signal sequence for secretion (Rifkin & Moscatelli, 1989), and hence it was suggested that it might also act by an intracrine mechanism without being secreted, perhaps by direct nuclear translocation of the endogenous high molecular weight

forms of bFGF (Logan, 1990). Whether the endogenous versus exogenous pathways underlie different functions of different bFGF forms within the nucleus is still to be elucidated.

Nuclear translocation is an absolute requirement for the mitogenic activity of aFGF in endothelial cells (Imamura et al., 1990). A mutant of aFGF that lacks the putative NLS fails to induce DNA synthesis and cell proliferation. If the heterologous NLS sequence from histone H2-B is attached to the amino terminus of this aFGF mutant, however, mitogenic activity is restored completely. Moreover, the mutant retains its ability to bind heparin and can induce the tyrosine-specific phosphorylation of its membrane receptor and the expression of c-fos mRNA. Recently, it was found that exogenous aFGF contained in a fusion protein with the A fragment of diphtheria toxin is able to stimulate DNA synthesis in cells deficient in aFGF receptors without measurable increase in tyrosine phosphorylation. DNA synthesis is only stimulated if the fusion protein is translocated to the nucleus (Wiedlocha et al., 1994). The protein induced little increase in cell number, however, which suggests that the receptor-mediated signal transduction pathway is required to activate additional processes necessary for proliferation.

Angiogenin is unique among the known angiogenic molecules in that it has ribonucleolytic activity (albeit markedly different from that of RNase A) which in turn is essential for its angiogenic activity (Shapiro et al., 1986). Data obtained with angiogenin mutants indicate a direct correlation between nuclear translocation and induction of angiogenesis in the chorioallantoic membrane assay (Moroianu & Riordan, 1994). Moreover, accumulation of angiogenin in the nucleolus of proliferating endothelial cells, where rRNA synthesis and processing take place, seems to provide an important clue for the putative substrate(s) of angiogenin *in vivo* although the molecular mechanism of angiogenin action at the nucleolus has yet to be established. Angiogenin also stimulates endothelial cells to form diacylglycerol and to secrete prostacyclin by activating phospholipases C and $A_2$, respectively (Bicknell & Vallee, 1988, 1989). It will be interesting to determine how this signal transduction pathway correlates with nuclear translocation of angiogenin in endothelial cells.

*Conclusions and Prospects.* Nuclear translocation through a receptor-mediated endocytotic pathway appears to be a common feature of the mechanism of action of exogenous angiogenic molecules. Recognition of this pathway provides a new perspective on the process of angiogenesis. Many areas of this new field are certain to receive detailed attention by investigators in the next few years. Among these, clearly, molecular events occurring within the nucleus and at the nucleolus are most important. Similarly, the relationship between nuclear translocation and the various second messenger signal transduction pathways and their modes of regulation have fundamental significance. Moreover, the cytosolic and cytoskeletal constituents involved in translocating exogenous angiogenic proteins and other growth factors to the nucleus and nucleolus require exploration. How are the endocytotic vesicles that contain the angiogenic molecules transported to the nucleus? What is the carrier that mediates this movement and do actin cytoskeleton/microtubules have a role in this process? How many distinct signals and nuclear targeting pathways for exogenous factors are likely to pertain? The answers to these and related questions should add significantly to the present state of knowledge regarding the mechanism of angiogenesis.

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