Nuclear Targeting Is Required for Hepatoma-derived Growth Factor-stimulated Mitogenesis in Vascular Smooth Muscle Cells*

Allen D. Everett¶‡, Tamara Stoops‡, and Coleen A. McNamara§

From the ¶Departments of Pediatrics and Internal Medicine, ‡Cardiovascular Division, and §Cardiovascular Research Center, University of Virginia Health Sciences Center, Charlottesville, Virginia 22908

Received for publication, June 4, 2001, and in revised form, July 26, 2001
Published, JBC Papers in Press, July 31, 2001, DOI 10.1074/jbc.M105109200

We recently identified hepatoma-derived growth factor (HDGF) as a nuclear targeted vascular smooth muscle cell (VSM) mitogen that is expressed in developing vascular lesions. In the present study, VSM in culture express endogenous HDGF only in the nucleus and target a green fluorescent protein (GFP)-HDGF fusion to the nucleus. To define the features of the HDGF molecule that are essential for nuclear localization and mitogenic function, deletion and site-directed mutagenesis were performed. Deletion analysis identified the carboxyl-terminal half of HDGF to be responsible for nuclear targeting in VSM. Overexpression of tagged HDGF proteins with point mutations in the putative bipartite nuclear localization sequence in the carboxyl terminus demonstrated that single Lys → Asn mutations randomized HDGF expression to both the nucleus and cytoplasm similar to the empty vector. Importantly, the Lys → Asn mutation of all three lysines blocked nuclear entry. Point mutation of a pS4ed2 kinase consensus motif within the nuclear localization sequence had no effect on nuclear targeting. Moreover, nuclear entry was essential for the HDGF mitogenic effect, as transfection with the triple Lys → Asn mutant HA-HDGF significantly attenuated bromodeoxyuridine uptake when compared with transfection with wild type HA-HDGF. We conclude that HDGF contains a true bipartite nuclear localization sequence with all three lysines necessary for nuclear targeting. Nuclear targeting of HDGF is required for HDGF stimulation of DNA replication in VSM.

Growth factors play a key role in vascular injury-induced smooth muscle cell (VSM) proliferation leading to neointimal formation and narrowing of the vascular lumen (1–4). However, the mechanisms of growth factor-stimulated VSM proliferation are still not fully understood. After binding and receptor activation, most receptor-growth factor complexes are thought to be internalized, transported to lysosomes, and degraded (5). However examples have emerged where growth factors exert their effect directly on the nucleus. For example, exogenously applied PDGF and bFGF accumulate in the nucleus of endothelial and mesenchymal cells (6, 7), and bFGF can regulate gene transcription in a cell-free system (8). These findings suggest that growth factors may function within the nucleus (9).

Nuclear targeting has a structural basis as exemplified by the well characterized basic cluster of seven amino acids that comprise the nuclear localization sequence (NLS) of the SV40 large T antigen. The SV40 sequence has served as a NLS consensus and has allowed the identification of a more complex NLS in vertebrate nuclear targeted proteins (reviewed in Ref. 10). Studies of nucleoplasmin, a major Xenopus nuclear protein, revealed that the NLS was bipartite, containing two required basic amino acid clusters of lysines separated by a variable spacer of 8–10 amino acids (11). Mutation and deletion studies of nucleoplasmin have demonstrated that the basic amino acid cluster is necessary for nuclear targeting whereas the composition and spacer length appeared unimportant. This bipartite NLS structure is common to many nuclear targeted proteins including the growth factors PDGF and bFGF, glucocorticoid receptors, the transcription factors Jun and Fos, and the cell cycle control protein p53 (10, 11). The importance of nuclear targeting in the regulation of growth factor function is supported by the evidence that deletion of a putative NLS in bFGF or schwannoma-derived growth factor was no longer mitogenic (12, 13) but continued to demonstrate cell membrane-associated signaling.

HDGF is a heparin-binding growth factor originally purified from media conditioned by the human hepatoma-derived cell line, HuH-7 (14). HDGF fits the definition of a true growth factor as endogenous HDGF is mitogenic for fibroblasts, HuH-7 cells (14), aortic endothelial cells (15), and VSM (16). HDGF represents a new family of growth factors as the nearest sequence homology (32%) is to high mobility group-1 (HMG-1), a DNA-binding protein (14). Importantly HDGF lacks many of the specific features characteristic of an HMG protein, especially an “HMG box,” responsible for DNA binding of these proteins. HDGF appears to be a secreted protein present in the conditioned media of HuH-7 cells, mesenchymal kidney cells, and COS-7 cells expressing recombinant HDGF; however, its sequence lacks a secretory leader sequence and contains a putative bipartite nuclear localization cassette (14). HDGF is the first member of a new family of proteins (17–19). All identified members to date contain a putative bipartite nuclear localization sequence (14, 17–19). Although the specific function of HDGF is unknown, recent studies suggest a role for HDGF in renal vascular development (15) and in vascular lesion formation (16). In the present study, we have mapped the sequence motif responsible for HDGF nuclear targeting and...
HDGF Nuclear Targeting

MATERIALS AND METHODS

Plasmids and Mutagenesis—A full-length rat HDGF cDNA (16) was subcloned into the eukaryotic expression vectors pK7-GFP or pHK3 (generous gifts of Ian Macara, University of Virginia) to express HDGF as an amino GFP or triple hemagglutinin fusion (HA), respectively (16). All mutated forms of GFP- or HA-HDGF were generated using the Stratagene Quick Change mutagenesis kit according to the manufacturer’s protocol. GFP-HDGF-(1–110) and HDGF-(111–237) deletions were generated by restriction digest.

Cell Culture and Transfection—Low passage (8–12) primary rat aortic VSM (a generous gift from Gary Owens, University of Virginia) were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) with 10% fetal calf serum (Life Technologies, Inc.) as previously described (16). A10 cells derived from fetal rat aortic smooth muscle cells (American Type Tissue Culture) were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc., Cells). Cells were grown to 50–60% confluence on glass coverslips and transfected for 20 h using FuGene 6 (Roche Diagnostics) according to the manufacturer’s instructions. For DNA synthesis analysis cells were pulsed with 10 μM BrdUrd (Sigma) for 12 h.

Immunocytochemistry—Transfected VSM on coverslips were washed with phosphate-buffered saline and fixed in 4% buffered paraformaldehyde for 20 min at room temperature. Coverslips were blocked for 1 h in 3% bovine serum albumin/phosphate-buffered saline (Roche Diagnostics) and incubated with a monoclonal anti-BrdUrd alkaline phosphatase-conjugated antibody (1:500) (Roche Diagnostics) and a polyclonal anti-HA epitope tag antibody (1:200) (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. A fluorescein isothiocyanate-conjugated anti-rabbit secondary antibody (1:200) (Amersham Pharmacia Biotech) and a red alkaline phosphatase substrate (Vector Laboratories, Burlingame, CA) were used for visualization. For endogenous HDGF non-transfected VSM were stained with a polyclonal anti-HDGF antibody (1:1000) (16) using the ABC staining method for alkaline phosphatase (Vector). The coverslips were subsequently rinsed with tap water and inverted onto microscope slides containing a drop of mounting media ( Vectashield, Vector) for visual inspection with an Olympus BX50WI Fluoview confocal microscope.

RESULTS

HDGF Is Nuclear Targeted in Cultured VSM—Endogenous HDGF is expressed in the nucleus of fetal aortic VSM and by VSM in the neointima after carotid injury (16). To examine the expression of endogenous HDGF in cultured rat VSM, VSM were grown to 75% confluency on glass coverslips, fixed, and stained for HDGF using a carboxyl-terminal affinity-purified rabbit polyclonal antibody (16). In these subconfluent cells, HDGF was expressed by less than 10% of cells in an exclusive nuclear pattern (Fig. 1A). Similarly, when wild type rat HDGF was expressed as an amino GFP fusion, HDGF localized exclusively to the nucleus using double labeling with the DNA stain H33342 (blue) and in the merged images. Bar = 10 μm.

A p34cdc2 Kinase Motif in the NLS Spacer Region Does Not Affect Nuclear Targeting—The HDGF spacer region between the two basic domains of the NLS contains a consensus sequence (Thr/Ser-Pro/Arg/Arg) that is phosphorylated by p34cdc2 kinase (11, 20). As phosphorylation of this consensus motif increases the rate of nuclear targeting for p53 and SV40 large T antigen (21) we examined the importance of this motif on HDGF nuclear targeting. The target serine in the p34cdc2 kinase consensus motif was point-mutated to result in a S165A substitution (cdc2-HDGF). As shown in Fig. 4, cdc2-HDGF continues to exclusively target the nucleus. The rate of nuclear targeting was also not affected as cdc2-HDGF was never observed in the

FIG. 1. Endogenous (A) or expressed (B) HDGF is localized to the nucleus of VSM. A, immunocytochemical localization of endogenous HDGF (red) to the nucleus of rat aortic VSM in culture. Bar = 10 μm. B, expression of GFP-HDGF in VSM. The GFP-HDGF fusion (green) localizes to the nucleus. Nuclear localization is confirmed using double staining with the DNA stain H33342 (blue) and in the merged images. Bar = 10 μm.

The “hath” region for homologous at the amino terminus of HDGF (17). The carboxyl-half of the protein is not homologous among family members and by sequence similarities contains a bipartite domain homologous to the NLS found in Xenopus nucleoplasmin (11). To determine whether the conserved hath region or carboxyl-half of HDGF containing a putative NLS is responsible for HDGF nuclear targeting, two HDGF deletions were produced: HDGF-(1–110) containing the intact hath region without the putative NLS and HDGF-(111–237) containing the NLS and carboxyl-terminal portion of the protein. As shown in Fig. 2B, when these HDGF deletions are expressed as GFP fusions in VSM nuclear targeting is altered. Like wild type HDGF (Fig. 2B), HDGF-(111–237) containing the putative NLS is only expressed in the nucleus (Fig. 2B). However, HDGF-(1–110) containing the hath domain only is expressed diffusely within the cytoplasm of the cell and is excluded from the nucleus (Fig. 2B).

HDGF contains a true bipartite NLS—As the carboxyl-terminal portion of HDGF containing the putative bipartite NLS alone can direct HDGF to the nucleus of VSM, we determined if the putative bipartite NLS domain was responsible for HDGF nuclear targeting. In Xenopus nucleoplasmin it was found that two basic domains containing lysines flanking a variable length spacer were important to NLS function. Using the scheme shown in Fig. 3A, we subsequently performed single-residue point mutations in the basic domains that resulted in a substitution of lysine for asparagine (Lys -> Asn) to test the effect on HDGF nuclear targeting. As shown in Fig. 3B, transfection of cells with the empty GFP vector (pK7-GFP) resulted in a diffuse distribution of GFP throughout the cytoplasm and nucleus in both VSM and A10 cells with wild type HDGF localized only to the nucleus. However when lysine 156 in the first basic domain is substituted for asparagine (K155N), HDGF is now expressed in a diffuse pattern similar to the empty vector (Fig. 3B). Similarly when lysines 167 and 170 in the second basic domain were substituted for asparagines (K167N/K170N) HDGF was again diffusely redistributed in both the nuclear and cytoplasmic compartments (Fig. 3B).

When all three lysines in both basic domains were substituted for asparagines (K155N/K167N/K170N) HDGF was diffusely distributed within the cytoplasm and excluded from the nucleus in both VSM and A10 cells.

A p34cdc2 Kinase Motif in the NLS Spacer Region Does Not Affect Nuclear Targeting—The HDGF spacer region between the two basic domains of the NLS contains a consensus sequence (Thr/Ser-Pro/Arg/Arg) that is phosphorylated by p34cdc2 kinase (11, 20). As phosphorylation of this consensus motif increases the rate of nuclear targeting for p53 and SV40 large T antigen (21) we examined the importance of this motif on HDGF nuclear targeting. The target serine in the p34cdc2 kinase consensus motif was point-mutated to result in a S165A substitution (cdc2-HDGF). As shown in Fig. 4, cdc2-HDGF continues to exclusively target the nucleus. The rate of nuclear targeting was also not affected as cdc2-HDGF was never observed in the
cytoplasm even at early time points (16, 20, and 24 h after transfection).

Nuclear Targeting Is Necessary for HDGF Mitogenic Function—VSM in the neointima after carotid injury (16) or in culture express HDGF exclusively in the nucleus. To determine whether HDGF nuclear targeting is essential for the mitogenic effect of HDGF, VSM were transfected with empty vector as a control, wild type HDGF, or triple NLS mutant HDGF (K155N/K167N/K170N, 3N-HDGF) containing a HA epitope tag. The cells were assayed for BrdUrd incorporation as a marker of DNA synthesis. As shown Fig. 5A, expression of wild type HDGF in VSM and A10 cells stimulated BrdUrd incorporation...
indicating increased DNA synthesis, whereas cells expressing the NLS mutant HDGF demonstrated a cytoplasmic expression pattern of HDGF without a stimulation of BrdUrd incorporation. When cells were counted, >90% (p < 0.05) of cells transfected with wild type HDGF were positive for BrdUrd, whereas 7% of cells transfected with NLS mutant HDGF were BrdUrd-positive (Fig. 5B). Mutation of the p34\(^{cd2}\) kinase consensus (S166A, cdc2-HDGF) did not affect BrdUrd uptake with 93% of transfected cells BrdUrd-positive (Fig. 5B).

DISCUSSION

Growth factors produced by the vascular wall after balloon injury play an important role in the pathologic proliferation of VSM. The paradigm of growth factor function involves interaction of a growth factor with a surface receptor, triggering an intracellular signaling event leading to increased proliferation of VSM. This paradigm has been challenged recently by the identification of growth factors within the nucleus of cells (6, 9, 12, 13). At least in the circumstances of bFGF, nuclear targeting and not cell surface receptor signaling appears responsible for mediating bFGF cell proliferation (12).

Although HDGF was originally identified from conditioned media, HDGF when observed in cells in vitro or in cells in intact tissues is only found in the nucleus. Nuclear targeting is likely to be an important mechanism of function for the HDGF family of proteins as all identified members of the HDGF family of related proteins (HRP1–HRP3) and p52/p75 contain a highly conserved bipartite NLS domain (17–19). Furthermore, like HDGF, p52/p75, HRP-1, and HRP-3 all traffic to the nucleus (18, 19, 22).

HDGF is a growth factor for VSM as overexpression results in DNA synthesis and increased cell number (16). In the present study, we demonstrate that the NLS triple lysine mutant of HDGF (3N-HDGF) that does not enter the nucleus did not stimulate BrdUrd incorporation. Therefore the bipartite NLS is not only required for the delivery of HDGF to the nucleus but is also necessary for HDGF-induced mitogenesis. Similarly, bFGF, which has been thought to function exclusively by signaling through a cell surface receptor, also requires nuclear entry to stimulate mitogenesis (12). Mutation of a nuclear localization signal in bFGF did not interfere with receptor binding or activation of early growth response genes but did significantly prevent cell proliferation. Therefore nuclear targeting of growth factors may represent a conserved pathway of cell growth regulation.

The efficiency of nuclear targeting may not always be related only to the presence of a NLS. Modulation of nuclear targeting can also be accomplished by phosphorylation of flanking sequences (10). For example, SV40 large T antigen contains a p34\(^{cd2}\) kinase substrate motif (S/T)P\(^\times\)(K/R) flanking the basic clusters of the NLS (11, 20), and p34\(^{cd2}\) kinase phosphorylation of the SV40 large T antigen regulates the rate of nuclear entry (21). The p34\(^{cd2}\) kinase motif is not necessary for nuclear targeting or mitogenic function of HDGF in VSM. Overexpression of HDGF with substitution of the serine for an alanine in the consensus motif did not block nuclear targeting or DNA
synthesis. Therefore in HDGF the overall function of this motif is unclear.

Presently the nuclear function of HDGF or any growth factor is unknown. It is intriguing that one member of the HDGF family of proteins, p52, is a transcription coactivator protein (19). p52 has been shown to modulate the transcriptional activation of class II genes in vitro and serves as a general transcription coactivator enhancing activated transcription through direct interactions with the general transcription factor TFIIF and RNA polymerase II (23). Interestingly p52 also specifically interacts with the binding domains of the splicing factor alternative/essential splicing factor 2 to increase pre-mRNA splicing activity (23). Whether the nuclear function of HDGF and the other related proteins is the specific regulation of target gene transcription by enhancing/inhibiting the general transcription apparatus is yet to be explored.

REFERENCES
1. Clowes, A. W., Reidy, M. A., and Clowes, M. M. (1983) Lab. Invest. 49, 327–333
2. Raines, E. W, and Ross, R. (1993) Br. Heart J. 69, S30–S37
3. Yoshida, Y., Mitsumata, M., Yamane, T., Temikawa, M., and Nishida K. (1998) Arch. Pathol. Lab. Med. 122, 987–996
4. Walsenberger, J. (1997) Circulation 96, 4083–4094
5. Honegger, A. M., Dull, T. J., Felder, S., Van Obberghen, E., Bellot, F., Szapary, D., Schmidt, A., Ulrich, A., and Schlessinger, J. (1987) Cell 51, 199–209
6. Rabozew-Szulczynska, E. M., Rodeck, U., Herlyn, M., and Koprowski, H. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 3728–3732
7. Baldin, V., Roman, A. M., Bose-Bierne, I., Alamri, F., and Bouche, G. (1990) EMBO J. 9, 1511–1517
8. Nakanishi, Y., Kihara, K., Mizuno, K., Masamune, Y., Yoshitake, Y., and Nishikawa, K. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 5216–5220
9. Jans, D. A. (1994) PASEF J. 8, 841–847
10. Dingwall, C., and Laskey, R. A. (1991) Trends Biochem. Sci 16, 478–481
11. Robbins, J., Dilworth, S. M., Laskey, R. A., and Dingwall, C. (1991) Cell 64, 615–623
12. Isamura, T., Engleka, K., Zhan, X., Tokita, Y., Forough, R., Roeder, D., Jackson, A., Maier, J. M., Hla, T., and Maciag, T. (1990) Science 248, 1567–1570
13. Kimura, H. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 2165–2169
14. Nakamura, H., Izumoto, Y., Kuroda, T., Mori, T., Kawamura, K., Yamamoto, H., and Kishimoto, T. (1994) J. Biol. Chem. 269, 25143–25149
15. Oliver, J. A., and Al-Awqati, Q. (1998) J. Clin. Invest. 102, 1208–1219
16. Everett, A. D., Lobe, D. R., Matsumura, M. E., Nakamura, H., and McNamara, C. A. (2000) J. Clin. Invest. 105, 567–575
17. Izumoto, Y., Kuroda, T., Harada, H., Kishimoto, T., Nakamura, H. (1997) Biochem. Biophys. Res. Commun. 238, 26–32
18. Ikegami, K., Yamamoto, M., Kishima, Y., Enomoto, H., Yoshida, K., Suemura, M., Kishimoto, T., and Nakamura, H. (1999) Biochem. Biophys. Res. Commun. 266, 61–87
19. Ge, H., Yuanzheng, S., and Wolfe, A. P. (1998) Mol. Cell 2, 751–759
20. Moreno, S., and Nurse, P. (1990) Cell 61, 549–551
21. McVey, D., Brizuela, L., Mohr, I., Marshak, D. R., Gluzman, Y., and Beach, D. (1989) Nature 341, 503–507
22. Toshifumi, K., Tanaka, H., Nakamura, H., Nishimune, Y., and Kishimoto, T. (1999) Biochem. Biophys. Res. Commun. 262, 433–437
23. Ge, H., Si, Y., and Wolfe, A. P. (1998) Mol. Cell 2, 751–759
