Hepatoma-derived Growth Factor Stimulates Cell Growth after Translocation to The Nucleus by Nuclear Localization Signals

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Summary

Hepatoma-derived growth factor (HDGF) is the original member of the HDGF family of proteins, which contain a well-conserved N-terminal amino acid sequence (homologous to the amino terminus of HDGF; hath) and nuclear localization signals (NLSs) in gene-specific regions other than the hath region. In addition to a bipartite NLS in a gene-specific region, an NLS-like sequence is also found in the hath region. In cells expressing green fluorescence protein (GFP)-HDGF, green fluorescence was observed in the nucleus, whereas it was detected in the cytoplasm of cells expressing GFP-HDGF with both NLSs mutated or deleted. GFP-hath protein (GFP-HATH) was distributed mainly in the nucleus although some was present in the cytoplasm, while GFP-HDGF with a deleted hath region (HDGFnonHATH) was found only in the nucleus. Exogenously supplied GFP-HDGF was internalized and translocated to the nucleus. GFP-HATH was internalized, whereas GFP-HDGFnonHATH was not. Overexpression of HDGF stimulated DNA synthesis and cellular proliferation, although HDGF with both NLSs deleted did not. Overexpression of HDGFnonHATH caused a significant stimulation of DNA synthesis, while that of hath protein did not. HDGF containing the NLS sequence of p53 instead of the bipartite NLS did not stimulate DNA
synthesis, and also truncated forms without the C-terminal or N-terminal side of NLS2 did not.

These findings suggest that the gene-specific region, at least the bipartite NLS sequence and the N-terminal and C-terminal neighboring portions, is essential for the mitogenic activity of HDGF after nuclear translocation.
Hepatoma-derived growth factor (HDGF) is the first member of the HDGF family of proteins that contain a well-conserved N-terminal amino acid sequence which we call the *hath* (homologous to amino terminus of HDGF) region (1,2). HDGF was purified from the conditioned medium of human hepatoma-derived HuH-7 cells, which proliferate autonomously in a serum-free chemically defined medium, and its complementary DNA was cloned from a HuH-7 cell cDNA library (1,3). HDGF has growth-stimulating activity in fibroblasts, hepatoma cells, vascular smooth muscle cells and endothelial cells (1,3-5). Its deduced amino acid sequence demonstrated that it lacked a signal peptide-like hydrophobic sequence essential for classical secretion, however, the conditioned medium of Cos7 cells transfected with cDNA expressing copious amounts of human HDGF showed growth-stimulating activity (1,4). Furthermore, HDGF family proteins have a basic motif homologous to the reported consensus sequences for bipartite nuclear localization signals (NLSs) in gene-specific amino acid sequences other than the *hath* region of the molecule (1,2,6,7). Additionally, an NSL-like basic amino acid-rich region is found in the *hath* region. Indeed, one of the HDGF family proteins, HDGF-related protein (HRP)-1 is detected
immunohistochemically in the nucleus of germ cells, and another member, HRP-3, can translocate to the nucleus of its target cells (6,7). Recently, HDGF was detected in the nucleus using anti-C terminus HDGF antibody, and found to co-localize with the proliferating cell nuclear antigen in vascular smooth muscle cells (5). Among the growth factors, cytokines and hormones identified previously, the following polypeptide factors have been reported to contain NLS(s) and undergo nuclear translocation for mitogenesis: acidic or basic fibroblast growth factor (FGF), FGF-3, schwannoma-derived growth factor (SDGF), ciliary neurotrophic factor (CNTF), amphiregulin, angiogenin and lens epithelium-derived growth factor (LEDGF) (8-23). These findings prompted us to study whether HDGF translocates to the nucleus via these NLSs, and whether its nuclear translocation is essential for induction of cell growth activity.

In this study, we investigate the functional roles of the putative NLSs by use of polymerase chain reaction (PCR)-directed mutagenesis, and the relationship between its nuclear translocation ability and growth-stimulating activity in the HDGF molecule. Further, we analyze which part of the HDGF molecule is essential for internalization and the mitogenic activity.
MATERIALS AND METHODS

Plasmid Construction of truncated and mutant forms of HDGF: We have named an NLS-like basic amino acid-rich region in the hath region and the basic region homologous to a bipartite NLS in a gene-specific part as NLS1 and NLS2, respectively. We constructed chimera proteins of wild HDGF or 12 truncated or mutant forms. The truncated or mutant forms of HDGF were named as follows: hath region from S\textsuperscript{2} to S\textsuperscript{98} (HATH), HDGF with deleted hath region (HDGFnonHATH), truncated form consisting of peptide from S\textsuperscript{2} to K\textsuperscript{170} (HDGF-LA), truncated form consisting of peptide from S\textsuperscript{2} to L\textsuperscript{154} (HDGF-SA), truncated form consisting of peptide from K\textsuperscript{155} to C-terminal L\textsuperscript{240} (HDGF-LP), truncated form consisting of peptide from E\textsuperscript{171} to C-terminal L\textsuperscript{240} (HDGF-SP), NLS1-mutated HDGF (HDGFmN1), NLS2-mutated HDGF (HDGFmN2), both NLS1- and NLS2-mutated HDGF (HDGFmN1mN2), both NLS1- and NLS2-deleted HDGF (HDGFdN1dN2), hath region with mutated NLS1 (HATHmN1), and hath region with deleted NLS1 (HATHdN1) (Figure 1).

First, we constructed four mutants of two putative NLSs in the HDGF molecule. The four HDGF mutants were HDGFmN1, HDGFmN2, HDGF with deleted NLS1 (HDGFdN1) and HDGF with deleted NLS2 (HDGFdN2). Deletions and mutations were introduced into the
putative NLSs of human HDGF by PCR-directed mutagenesis. PCR was performed using appropriate synthetic oligonucleotides as primers and full-length HDGF cDNA as template. The point mutations introduced into NLS1 were K-75(AAG) to N(AAC), K-78(AAG) to N(AAT), R-79(AGG) to Q(CAG), and K-80(AAA) to N(AAT). A BstEII site was created by changing the codon of G-74 from GGC to GGT and used to make a PCR-generated fragment spanning the mutated NLS1. The same strategy was used in the construction of NLS2 point mutations. The amino acid changes were K-155(AAG) to N(AAT), R-156(AGG) to Q(CAG), R-157(AGA) to Q(CAA), K-167(AAA) to N(AAT), and K-170(AAG) to N(AAT). A XbaI site was introduced by changing the codons of L-161 from TTG to CTT and L-162 from CTG to CTA. The junction point of the in-frame deletion mutants of NLS1 was made by NruI linker, which made a change of G-79 (GGG) to A (GCC). Deletion mutants of NLS1 also include a XhoI restriction site at the junction point with no amino acid substitution. Briefly, for HDGFDN1, two PCR products amplified using HF-S1 and dN1-S described below as sense primers, and dN1-R and HF-R1 as antisense primers, which contained only sequences upstream of NLS1 and downstream of NLS1, were ligated at the NarI site. For HDGFmN1, two PCR products amplified using HF-S1 and mN1-S described below as sense primers and
mN1-R and HF-R1 as antisense primers, which contained NLS1 with mutations K75N, K78N, R79Q and K80N, were ligated at the BstEII site. These HDGF cDNAs were inserted into the SpeI and XbaI sites of pBluescriptII vectors. The cDNA for HDGFdN2 was constructed by ligation at the XhoI site after amplification by PCR using HF-S2 and dN2-S as sense primers, and dN2-R and HF-R2 as antisense primers. For HDGFmN2, two PCR products amplified using HF-S2 and mN2-S as sense primers and mN2-R and HF-R2 as antisense primers, which contained an upstream sequence of NLS2 with mutations K155N, R156Q and R157Q and downstream sequence of NLS2 with mutations K167N and K170N, were ligated at the XbaI site. The cDNA for HDGFdN1dN2 and HDGFmN1mN2 were constructed after amplification by PCR using HDGFdN1 and HDGFmN1 cDNA as templates, respectively, as described above for HDGFdN2 and HDGFmN2. These cDNAs were inserted into the HindIII and SpeI sites of pRc/RSV vector. The sequences of primers were as follows: HF-S1: 5’-GCACATAGTCGATCCAACCGGCAGAAGGA-3’,

HF-R1: 5’-GCTCTAGACAGGCTCTCATGATCTCTGA-3’,

mN1-R: 5’-GCTCTAGACAGGCTCTCATGATCTCTGA-3’,

HF-S2: 5’-AATGGCGCCAAACTTCTCCTTGATT-3’,

dN1-R: 5’-AATGGCGCCAAACTTCTCCTTGATT-3’,

dN1-S: 5’-AATGGCGCCTTCAGCGAGGGGCTGTGGGA-3’,

dN2-R: 5’-AATGGCGCCTTCAGCGAGGGGCTGTGGGA-3’,

HF-R2: 5’-GCTCTAGACAGGCTCTCATGATCTCTGA-3’,

mN2-R: 5’-GCTCTAGACAGGCTCTCATGATCTCTGA-3’,

mN2-S: 5’-GCTCTAGACAGGCTCTCATGATCTCTGA-3’,
GGGCTTGCCAAACTTCTCCTTGATTC-3', mN1-S: 5'-

TTTGGTAAACCAAACAATCAGAATGGGTTCAGCGAGGGGC-3', HF-S2: 5'-

GCAAGCTTAGCCCCGCAATGTCGCGATC-3', dN2-R: 5'-

CGCTCGAGCGCTCTTTCTGTTCTTCTCCTT-3', dN2-S: 5'-

CGCTCGAGGAGAAAACCCTGAAGGAGAG-3', HF-R2: 5'-

GCACTAGTCAGGCTCTCTAGATCTCTGA-3', mN2-R: 5'-

CCTCTAGAAAGTCCCTATTGCTGATCAACGCTCCTT-3', mN2-S: 5'-

GCTCTAGAGGACTCTCTCTAATCGTCCAATGAGGC-3'.

For construction of green fluorescence protein (GFP)-chimera proteins, we amplified the wild HDGF and its 6 truncated forms by PCR using appropriate synthetic oligonucleotides as primers and full-length HDGF cDNA as template (Figure 1). Further, six mutant forms of HDGF were amplified by PCR using appropriate synthetic oligonucleotides as primers and the corresponding HDGF mutant cDNA as template. Primer 1: 5'-

GCCCCAGATCTTCGCAGATCCAACCGGCAGAAGGAG-3', primer 2: 5'-

AACATGTCGACCAGGCTCTCATGATCTCGATGAC-3', primer 3: 5'-

AGTCAGTCGAGGAAGCTTGGACTAGTAGGGTTGTC-3', primer 4: 5'-
ACGTTGTCGACCTACTTGGGACGTTTAGGAGAGTCCTC-3’, primer 5: 5’-
CCTCGTACCTACAACGCTCTTTCGTTCTTTTC-3’, primer 6: 5’-
AAGGAAGACCTAAGGAGAGGAGGACATTGCTG-3’, primer 7: 5’-
ACGTAAGTCTGAGGCAGAAAACCTGAAGGAGAG-3’, primer 8: 5’-
TCAAGAGATCTGGCTATTCAGTCTCCAGAAAGAGA-3’, primer 9: 5’-
ACGAGAGATCTAAGGAGAGTTTGGC-3’, primer 10: 5’-
CACAGGTCGACTCAGAAACCCTTCTCTCTT-3’. For GFP-wHDGF and its mutant forms
including GFP-HDGFn1, GFP-HDGFn2, GFP-HDGFn1mN2 and GFP-
HDGFdN1dN2, primer 1 was used as the sense primer and primer 2 as the antisense primer.
For GFP-HATH, GFP-HATHmN1 and GFP-HATHdN1, primer 1 and primer 3 were used as
the sense and antisense primers, respectively. For GFP-HDGFnonHATH, primer 8 and primer
2 were used. For GFP-HDGF-LA and GFP-HDGF-SA, primer 1 and primer 4 and 5 were
used as the sense and antisense primers, respectively. For GFP-HDGF-LP and GFP-HDGF-SP,
primer 2 and primer 6 and 7 were used as the antisense and sense primers, respectively. For
GFP-NLS1 (GFP-fusion peptide consisting of 13 amino acids (K70~F82) including NLS1),
primer 9 was used as the sense and primer 10 as the antisense primer. For GFP-NLS2 (GFP-
NLS2 peptide (K155~K170), primer 4 and primer 6 were used as the antisense and sense primers, respectively. These 15 amplified cDNAs were inserted into the BglII and SalI sites of pEGFP-C1 vector (CLONTECH).

We constructed a mutant form of HDGF containing the NLS sequence of p53 (K305~K321) instead of NLS2 (GFP-HDGF-p53NLS). The cDNA for the NLS of p53 was amplified by PCR using appropriate synthetic oligonucleotides: 5'-ATACGCTCGAGAAGCGAGCACTGCCCAACA-3' as the sense primer and 5'-TACGTCTCGAGTTTCTTCTTTTGCTGGA-3' as the anti-sense primer, and 5'-CTCGAGAAGCGAGCACTGCCCAACAACCAGCTCTCTCCAGCCAAAGAAGAAACTCGAG-3' corresponding to the NLS sequence of p53 as a template (24). The amplified cDNA was inserted into the Xho I site of HDGFdN1dN2 cDNA in pEGFP-C1.

We constructed myc-tagged proteins of wild HDGF and HDGFdN1dN2 for stable transformants. We amplified these cDNAs by PCR using 5'-GAGCCGGATCCGCTCGAGATCCAAACCGGCAGAAGG-3’ as the sense primer and 5’-AACATTCTAGACTACAGGCTCTCATGATCTCTGATG-3’ as the anti-sense primer and the cDNA of HDGF and HDGFdN1dN2 as templates for myc-wHDGF and myc-HDGFdN1dN2,
respectively. These amplified cDNAs were inserted into the BamHI and XbaI sites of pEF-BOS vector (25). All PCR products were analyzed by restriction mapping and dideoxynucleotide sequencing.

**Gene transfection;** A human renal epithelial cell line (293 cells), a human hepatoblastoma cell line (HepG2 cells) and NIH3T3 cells were cultured in Dulbecco’s modified Eagle’s medium (D-MEM) supplemented with 10% FCS and antibiotics. Using a Mammalian transfection kit (STRATAGENE), 293 cells were transiently transfected with 6 μg of pEGFP-C1 containing cDNA of wild type or mutant forms of HDGF. HepG2 cells and NIH3T3 cells were co-transfected with 18 μg of pEF-BOS containing myc-tagged proteins, and 2 μg of pST vector with the neomycin-resistance gene. Transfected cells were cultured in D-MEM with 10% FCS in 3% CO₂ at 37°C, and 24 hours later the D-MEM was replaced with fresh medium and cultured for three days in 5% CO₂ at 37°C. After cultivation with 0.9 mg/ml of neomycin for about one month, stable transformants of HepG2 cells and NIH3T3 cells were selected and cloned.

**Visualization of GFP-chimera proteins;** Living 293 cells transfected with pEGFP-C1 were evaluated on day 1, 2, 3 or 4 using con-focal microscopy equipped for GFP visualization
(488nm excitation and FITC filter set). The conditioned media of 293 cells expressing GFP-chimera proteins were concentrated about 20-fold with Centricon 10 (Amicon), and then supplied to parent 293 cells. GFP-HDGF was examined after being purified from the conditioned medium of 293 cells expressing GFP-HDGF by heparin-Sepharose column chromatography, too. Three days later, we evaluated the intracellular fluorescence of these cells by con-focal microscopy equipped for GFP visualization.

**DNA synthesis and cell proliferation assay:** For DNA synthesis assay, HepG2 cells and NIH3T3 cells expressing myc-tagged proteins were plated onto 96-well plates at a density of \(2 \times 10^3\) cells/well in D-MEM supplemented with 10% FCS. Twenty-four hours later, HepG2 cells and NIH3T3 cells were given fresh medium supplemented with 1% and 5% FCS, respectively. Two or four days later, these cells were harvested and \(^{3}\text{H}\)-thymidine (Thd) incorporation into DNA was measured. 293 cells expressing GFP-chimera proteins were plated onto 96-well plates at a density of \(1 \times 10^3\) cells/well in D-MEM supplemented with 10% FCS. Twenty-four hours later, these cells were given fresh medium supplemented with 1% FCS and the cells were harvested four days later.

For cell proliferation assay, HepG2 cells expressing myc-tagged proteins were plated onto
96-well plates at a density of $3 \times 10^3$ cells/well in D-MEM supplemented with 10% FCS.

After 24 hours, HepG2 cells were given fresh medium supplemented with 10% FCS. Cell numbers were measured 2 and 4 days later using MTS colorimetric assay (Promega, Madison).

**Western blot analysis:** HepG2 cells and NIH3T3 cells expressing myc-tagged proteins were cultured in 6-well plates to semi-confluence. Then cells were collected in 1.5ml tubes and washed twice with PBS. They were resuspended with 50 μl of 2X sample buffer (containing 0.5 M Tris(hydroxymethyl)aminomethane hydrochloride (pH 6.8), 10% sodium dodecyl sulphate (SDS), 20% glycerol and 0.01% bromphenol blue) and boiled at 99.9°C for 5 minutes. After SDS-polyacrylamide gel electrophoresis (PAGE), each sample was transferred to an Immobilon membrane (Millipore, Bedford, Tx). To detect myc-tagged proteins, membranes were incubated with anti-c-myc monoclonal antibody (Ab-1 monoclonal) (CALBIOCHEM, Cambridge, UK) and visualized with horseradish peroxidase-linked anti-mouse IgG using the enhanced chemiluminescence (ECL) system (Amersham, UK). For detection of HDGF, we used rabbit polyclonal anti-C terminus human HDGF IgG, and visualized the samples with horseradish peroxidase-linked anti-rabbit IgG using the ECL.
system.
Results

**HDGF translocates to the nucleus in living cells via nuclear localization signals.**

HDGF contains a basic motif, $\text{K}^{155}\text{RRAGLLEDSPKRPK}^{170}$ (basic residues underlined) homologous to the consensus sequences of bipartite NLS (NLS2), in a gene-specific area other than the *hath* region that consists of two clusters of basic residues separated by 10-12 amino acids including proline residues (26, 27). Additionally, another NLS-like basic amino acid-rich region, $\text{K}^{75}\text{PNKRK}^{90}$ (basic residues underlined) (NLS1), was found in the *hath* region (29). To clarify whether HDGF can translocate to the nucleus, and which NLS is the functional sequence in the HDGF molecule, 293 cells were transfected with an expression vector encoding a GFP-tagged human HDGF cDNA and mutant HDGF cDNAs. As shown in Figure 2, transfected cells expressing GFP-wHDGF showed green fluorescence only in the nucleus, however cells expressing GFP-HDGFdN1dN2 or GFP-HDGFmN1mN2 showed fluorescence in the cytoplasm, not in the nucleus. In the transfected cells expressing GFP-HDGFmN2 or the truncated form without NLS2 (GFP-HDGF-SA), green fluorescence was detected in the nucleus, with some amount also found in the cytoplasm. HDGFmN1 translocated to the nucleus, as did the truncated forms with NLS2 (HDGF-LP and
HDGFnonHATH), and neither was found in the cytoplasm. Most of the hath proteins with an intact NLS1 were transported to the nucleus, but variable amounts of hath protein remained in the cytoplasm. HATHmN1 and HATHdN1 remained in the cytoplasm. On the other hand, only NLS2 peptide could translocate green fluorescence protein to the nucleus. Most of the GFP-NLS1 was transported to the nucleus, showing a similar pattern of fluorescence to hath protein. NLS1 also has nuclear translocational potential, but its activity is weaker than that of NLS2. The two NLSs can together undoubtedly translocate HDGF protein to the nucleus.

**Exogenously added GFP-HDGF is internalized and accumulated in the nucleus.**

Exogenous HDGF stimulated the growth of fibroblasts and endothelial cells, vascular smooth muscle cells and some hepatoma cells. We investigated whether exogenously added GFP-HDGF was transported to the nucleus. After the addition of the concentrated conditioned medium of GFP-wHDGF, GFP-HDGFdN1dN2, GFP-HATH or GFP-HDGFnonHATH-expressing cells, 293 cells were observed 24, 48, 72 and 96hr later by fluorescence detection. The purified fraction of GFP-wHDGF protein from the conditioned medium of 293 cells expressing GFP-wHDGF was examined, too (data not shown). In 293 cells given GFP-wHDGF extracellularly, green fluorescence in the nucleus was observed from 72hr (Figure 3).
Nuclear fluorescence was also observed at 72hr in NIH3T3 cells given GFP-wHDGF. Whereas, green fluorescence was observed only in the cytoplasm, not nucleus, in 293 cells given GFP-HDGFDN1dN2. Interestingly, in cells treated with GFP-HATH extracellularly, green fluorescence was observed in both the cytoplasm and nucleus, similar to GFP-HATH-expressing cells. Exogenously supplied wild HDGF, HDGFdN1dN2 and hath protein show similar patterns of intracellular localization to their overexpressing cells. On the other hand, green fluorescence was not observed in wild 293 cells given GFP-HDGFnHATH and GFP-HDGFLP extracellularly, suggesting that HDGF protein with a deleted hath region could not be internalized into the cells.

**Nuclear translocation of HDGF is essential for its growth stimulating activity.**

We investigated the role of nuclear translocation in the cell growth activity of HDGF. Since the effect of HDGF on cell proliferation is more prominent in cells overexpressing HDGF, we investigated the cell growth activity of truncated or mutant forms of HDGF by use of overexpressing cells. HDGF stimulated the growth of some hepatoma cells. First, we constructed myc-tagged HDGF expressing vector, and produced stable transformants. HepG2 cells have a low level of intrinsic HDGF (Figure 4a). Native HDGF is detected as about a
40kDa band on SDS-PAGE analysis, and a smaller form of 35kDa is supposed to be a degraded product or post-translationally modified form. After selection with neomycin, we cloned stable transformants of HepG2 cells that expressed myc-wHDGF and myc-HDGFdN1dN2. The expression of these myc-tagged proteins was confirmed by Western blotting using anti-myc antibody (Figure 4c). The faint band of about 40kDa detected by anti-myc antibody in each lane is a non-specific band, since it is also detected in parent and mock cells. As shown in Figure 5a, myc-wHDGF-expressing HepG2 cells showed a significantly stimulated DNA synthesis that was about 2 times greater than the level in neomycin-resistance gene-transfected (neo)-cells in culture medium supplemented with 1% FCS. On the other hand, myc-HDGFdN1dN2-expressing HepG2 cells did not show significantly stimulated DNA synthesis greater than neo-cells. We produced stable myc-wHDGF- and myc-HDGFdN1dN2-expressing NIH3T3 cells, too (Figure 4b,d). Myc-wHDGF-expressing NIH3T3 cells showed significant stimulation of DNA synthesis 2.5 to 3 times greater than that in neo-cells in culture medium supplemented with 5% FCS (Figure 5b). However, myc-HDGFdN1dN2-expressing NIH3T3 cells showed no stimulated DNA synthesis. Additionally, the number of myc-wHDGF-expressing HepG2 cells increased significantly to 1.5 to 2-fold.
that of the parent cells or neo cells after a 4-day-culture in medium supplemented with 10% FCS (Figure 6). However, the increase in the number of myc-HDGFdN1dN2-expressing HepG2 cells was significantly lower than that of myc-wHDGF-expressing cells. Thus, the nuclear translocation potential seems to be essential to the growth stimulating activity of HDGF.

Next, we examined DNA synthesis in the bulk of cells expressing GFP-chimera proteins of HDGF after neomycin selection for about 1 week. The GFP-protein-expressing 293 cells were microscopically examined for green fluorescence. As shown in Figure 7, GFP-wHDGF-expressing 293 cells showed significantly stimulated DNA synthesis, about 85% greater than that of 293 cells transfected with vector only. However, GFP-HDGFdN1dN2-expressing cells and GFP-HDGFmN1mN2-expressing cells showed significantly diminished [3H]-Thd incorporation to a level almost equal to that of cells transfected with vector only. GFP-HDGFmN1-expressing cells showed significantly stimulated DNA synthesis, whereas GFP-HDGFmN2-expressing cells did not. GFP-HATH-expressing cells did not show significantly stimulated DNA synthesis although most of hath protein was translocated to the nucleus (Figure 7a). Further, we investigated which part other than the hath region functioned for
growth stimulating activity, by use of cells expressing GFP-chimera proteins. Interestingly, GFP-HDG\textit{non}HATH-expressing cells showed significantly stimulated DNA synthesis, about 120\% greater than that in neo-cells, the level of which was almost equal to that of GFP-wHDGF-expressing cells (Figure 7). However, neither GFP-HDG\textit{F}-LA-expressing cells nor GFP-HDG\textit{F}-LP-expressing cells showed significantly stimulated DNA synthesis, although both protein forms were transported to the nucleus (Figure 7b). Furthermore, GFP-HDG\textit{F}-p53NLS, which was translocated to the nucleus, did not stimulate DNA synthesis (Figure 8).

These findings suggest that \textit{hath} protein alone does not have growth-stimulating activity and gene-specific areas other than the \textit{hath} region contribute to the mitogenic activity of HDGF after translocation to the nucleus.
Discussion

Exogenously supplied HDGF stimulates the growth of fibroblasts, endothelial cells, smooth muscle cells and some hepatoma cells, and is considered to function in these cells as an autocrine or paracrine factor (1,3-5). HDGF contains not only a basic amino acid-rich region (NLS1) in the *hath* region, but also a putative bipartite NLS (NLS2) in a gene-specific area other than the *hath* region. These experiments using GFP-chimera proteins demonstrated that HDGF could be translocated into the nucleus via these two NLSs. NLS2 translocated the truncated forms as well as whole HDGF protein to the nucleus, in the presence or absence of NLS1. On the other hand, variable amounts of the *hath* proteins and NLS2-mutated HDGF proteins remained in the cytoplasm. Furthermore, NLS2 peptide translocated GFP to the nucleus completely, and NLS1 peptide translocated most of the GFP to the nucleus although some amount remains in the cytoplasm. Thus, NLS2 functions as a major NLS signal in the HDGF molecule, and NLS1 may have the ability of NLS but functions more weakly as an assistant for nuclear translocation. A mutated form without basic amino acids in the NLS sequences (HDGFmN1mN2) as well as NLS-deleted forms (HDGFdN1dN2, HATHdN1) could not be translocated to the nucleus. Thus, basic residues in NLSs are essential for the
nuclear translocational potential of NLSs, as reported previously (26-28).

HDGF was detected in the nucleus of endothelial cells and SMC by immunohistochemistry using an anti-C terminus HDGF antibody (5). We previously reported the cytoplasmic localization of HDGF in HuH-7 cells, using an anti-N terminus antibody generated in rabbit (1). Oliver et al. also showed a cytoplasmic localization in rat metanephrergic mesenchymal cells with no signal in the nucleus, using immunoaffinity-purified anti-peptide antibody (the peptide used as antigen had the amino acid sequence of the N-terminal region) (4). One possible explanation for the difference in these findings is that antibodies used in the previous reports were generated against the N-terminus of HDGF or the peptide in the N-terminal region and the antibodies in this study were generated against the C-terminus of HDGF. Some amount of N-terminal protein was also detected in the cytoplasm in the present study. These antibodies reacted with the HDGF molecule and with N-terminal protein cleaved from HDGF, probably abundant in the cytoplasm of any cell type. However, in these previous studies, nuclear staining was not found. Therefore, either a specific cell type or stage of the cell cycle might influence the fate of the intracellular localization of HDGF. Conversely, some amount of imported HDGF in the target cells might accumulate and remain in the
cytoplasm in any cell type, or it might shuttle between the cytoplasm and the nucleus during cell differentiation or at different stages of the cell cycle. The high mobility group (HMG)-1 protein was reported to be present in both the cytoplasm and nucleus, and most of it was found in the cytoplasm in confluent cells whereas it was found in the nucleus in dividing cells (29). HDGF was co-localized with proliferating cellular nuclear antigen (PCNA) in the nucleus of proliferating vascular smooth muscle cells (4). It is reported that another member of the HDGF family, named LEDGF, changed its intracellular localization from the nucleus to the cytoplasm with culture temperature (30). Thus, HDGF may shuttle between the cytoplasm and nucleus as do LEDGF and HMG-1, depending on the phase of the cell cycle, the state of differentiation or the environmental conditions.

In this study, we showed that the nuclear translocation of HDGF was essential for its mitogenic activity. Overexpression of the gene-specific region (HDGFnonHATH) did significantly stimulate DNA synthesis, almost equal to the effect of wild HDGF, while hath protein could not. HDGFmN1 with intact NLS2 stimulated the DNA synthesis, suggesting that NLS1 was not essential for the mitogenic activity. Further, a HDGF mutant containing the NLS sequence of p53 instead of NLS2, which had nuclear translocation ability, did not
stimulate DNA synthesis, and also truncated forms without the C-terminal or N-terminal side of NLS2 (HDGF-LA and HDGF-LP) did not. We consider that the mitogenic activity disappeared because a conformational change occurred on replacement of the p53 NLS sequence of the NLS2 locus, or that the amino acid sequence itself including NLS2 may be necessary for the activity, in addition to the ability for nuclear translocation. These findings suggest that, the whole sequence of the gene-specific region of HDGF, or at least NLS2 and the N-terminal and C-terminal neighboring portions, is essential for the mitogenic activity.

Recently, it has become evident that some polypeptide factors, subsequent to receptor-ligand internalization, may translocate to the nucleus, and that nuclear translocation may be essential for mitogenic activity. Induction of mitogenesis by FGF is directly dependent on nuclear localization, whereby the deletion of its NLS abolishes the mitogenic activity with no effect on other functions (11-13,31-34). Additionally, nuclear localization has been reported to be essential for the mitogenic activity of SDGF, CNTF, amphiregulin and angiogenin (18-23). The mitogenic activity of HDGF is dependent on nuclear translocation. Thus, HDGF may belong to a group of these growth factors that require nuclear translocation to induce mitogenic activity, at least as one signal pathway. Basic FGF stimulated cell proliferation and
angiogenesis after internalization and translocation to the nucleus in a receptor-mediated manner (35). Exogenous acidic FGF was associated with the nucleus in a receptor-dependent manner and some cell surface FGF receptors translocated to a perinuclear location as a functional tyrosine kinase (36, 37), suggesting that both the tyrosine kinase signal from the activated FGF receptor and growth factor internalization were required for the induction of cell proliferation (38). On the other hand, another group asserted that receptor-mediated endocytosis of acidic FGF does not result in its translocation to the nucleus, since acidic FGF, microinjected into the cytoplasm of BHK cells, enters the cell nucleus by free diffusion and becomes trapped on binding to nuclear structures (39). Since the receptor of HDGF has not yet been identified, it is not clear whether nuclear translocation is essential for the receptor-mediated signal transduction as seen in basic FGF and SDGF. However, it is notable that green fluorescence was detected in the nucleus 72 hr after the extracellular addition of GFP-HDGF, suggesting that exogenously supplied HDGF was truly internalized. Further, GFP-HATH protein was internalized, but GFP-HDGF-LP or GFP-HDGfnonHATH was not. Although the time till visualization, 72 hours seems to be longer for DNA synthesis, we consider that the accumulation of sufficient amounts of GFP-proteins must be necessary to
detect the fluorescence. These findings suggest that exogenously supplied HDGF is internalized into the cytoplasm, probably after binding to the cell membrane (possible receptor) via the **hath** region, and then is transported to the nucleus via NLS in the HDGF molecule.

The growth stimulating behavior of the stable transformants overexpressing the growth factor lacking a signal peptide may be explained by the prediction that HDGF is largely sequestered in a cellular compartment different from that of its receptor. Non-secreted acidic FGF lacking the secretion signal functions in cell motility and invasive potential compared to the secreted form of acidic FGF encoding cDNA coupled to a signal peptide sequence, indicating its ability for intracellular signaling in a so-called intracrine manner (40). Our current findings demonstrating that the HDGF-producing cells have growth stimulating activity after nuclear translocation may support the functional intracrine signaling of HDGF. HRP-1 and HRP-3 were detected in the nucleus by immunohistochemistry and GFP-tagged fluorescence microscopy (6,7). Another member of the HDGF family, HRP-2, had a higher molecular weight than other HDGF proteins, and was a more basic protein containing many K and R amino acids in a gene-specific region, strongly suggesting that it might be a
transcriptional regulatory protein (2). P52/p75 were cloned first as transcriptional coactivators, however they contain the hath region in the N-terminal sequence, and therefore must belong to the HDGF family (41). Interestingly, P75 (which we would like to call HRP-5) was reported to be a survival and growth factor for lens epithelial cells, fibroblasts and keratinocytes (23). Synthetic peptides containing the NLS of acidic FGF were able to stimulate DNA synthesis in an FGF receptor-independent manner after delivery into living NIH3T3 cells by a cell permeable peptide import technique, suggesting that exogenous FGF was internalized and directly involved in the nuclear transport (31). Further, basic FGF stimulated gene transcription in a cell-free system, suggesting a direct function in nuclear events (16). FGF-2 activated ribosomal DNA transcription and showed a strong affinity for histone H1. FGF-2 regulated gene expression through modulation of chromatin structure in the nucleus (42). In our study, HDGFnonHATH-overexpressing cells showed increased DNA synthesis, suggesting that HDGFnonHATH was transported to the nucleus directly and stimulated DNA synthesis. It may be postulated that HDGF translocates to the nucleus directly and regulates gene expression. Therefore, HDGF may function in an intracrine fashion for cell proliferation as well as having autocrine or paracrine functions, or might
function as a transcriptional regulatory protein after direct translocation to the nucleus in any cell type.

HDGF has no signal peptide, but some amount of HDGF was detected in the conditioned medium of HuH-7 cells, HepG2 cells and Cos cells overexpressing HDGF (1). Some growth factors were reported to have no signal peptides, such as FGF-1, 2, 9, 11~14 and IL-1 (43-46). It is reasonable to consider that the HDGF protein in the conditioned medium is released from lysed or dead cells, although we cannot exclude the possibility that HDGF is secreted via an internal hydrophobic sequence as an internal signal sequence (1) or by a non-classical secretory pathway. Most of the HDGF present extracellularly must be a consequence of cell lysis and would not be physiological. However, we consider these factors to have possible roles under pathogenic conditions. For example, when the tissue was damaged and cells were destroyed by mechanical stress or inflammation, growth factors such as HDGF in the cytoplasm and/or nucleus were released, and may act on adjacent progenitor cells and stimulate cell proliferation to repair the tissue. In any cell type, these non-receptor-mediated signals may induce cell proliferation or survival after direct translocation to the nucleus. These pathways might work for some factors as a primitive signal pathway in biological
functions for cell survival and repair.

In summary, HDGF translocates to the nucleus via nuclear localization signals, mainly NLS2 in a gene-specific region as the major potential NLS, and NLS1 in the hath region may be an assistant. The nuclear translocation is essential for the mitogenic activity of HDGF. Exogenously supplied GFP-HDGF is undoubtedly internalized and translocated to the nucleus, although its receptor has not yet been identified. HDGF is supposed to be internalized into the cytoplasm after binding to a possible membrane receptor in the hath region and then translocated to the nucleus via mainly NLS in a gene-specific part other than the hath region. The gene-specific region, or at least NLS2 and the N-terminal and C-terminal neighboring portions, may contribute to mitogenic activity in the HDGF molecule. HDGF (and other members of the HDGF family) may function as nuclear/growth factors in an autocrine, paracrine and/or intracrine manner.
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LEGENDS TO FIGURES

Figure 1. Plasmid construction of truncated and mutant forms of HDGF.

The shaded boxes show the NLS1 or NLS2 with mutated amino acids, and the mark shows the deletions in NLSs, as described in Materials and Methods.

Figure 2. Two nuclear localization signals function in the HDGF molecule.

293 cells were transiently transfected with pEGFP-C1 containing cDNA of wild type, truncated or mutant forms of HDGF. The living 293 cells transfected with pEGFP-C1 were evaluated on day 3 using confocal microscopy equipped for GFP visualization (488nm excitation and FITC filter set). The photographs show 293 cells expressing a) Mock, transfected with pEGF-C1 vector, b) GFP-wHDGF, c) GFP-HATH, d) GFP-HDGFnonHATH, e) GFP-HDGFmN1, f) GFP-HDGFmN2, g) GFP-HDGFmN1mN2, h) GFP-HDGFdN1dN2, i) GFP-HDGF-LA, j) GFP-HDGF-SA, k) GFP-HDGF-LP, l) GFP-HDGF-SP, m) GFP-HATHmN1, n) GFP-HATHdN1, o) GFP-NLS1+, p) GFP-NLS2.

Figure 3. Exogenously added GFP-HDGF accumulates in the nucleus of target cells.
The conditioned media of 293 cells expressing GFP-chimera proteins of HDGF, HDGF<sub>dN1dN2</sub> and HATH were concentrated about 20-fold by Centricon10, and then added to parent 293 cells extracellularly. Three days later, we evaluated intracellular fluorescence by microscopy equipped for GFP visualization. The photographs show the 293 cells supplied exogenously a) GFP-wHDGF, b) GFP-HDGF<sub>dN1dN2</sub>, c) GFP-HATH, and d) GFP-HDGF<sub>nonHATH</sub>.

Figure 4. Overexpression of myc-tagged HDGF proteins in stable transformants.

The protein levels of wild type and mutant forms of HDGF expressed in stable transformants were evaluated by Western blotting. Myc-tagged wild HDGF was highly expressed in HepG2 cells and NIH3T3 cells relative to intrinsic HDGF in their neomycin-selected and parent cells, using an anti-C terminus HDGF polyclonal antibody. Myc-tagged HDGF and its mutant forms in stable transformants of HepG2 cells (a, b) and NIH3T3 cells (c, d) were detected with an anti-C terminus HDGF polyclonal antibody at a dilution of 1000-fold (a, c) or anti-myc monoclonal antibody at a dilution of 10-fold (b, d). a) and b): lane 1; parent HepG2, lanes 2 and 3; Mock clones 1 and 2, lanes 4 and 5; clones 1 and 2.
expressing myc-HDGFdN1dN2, lanes 6 and 7; clones 1 and 2 expressing myc-wHDGF. c) and d): lane 1; parent NIH3T3, lanes 2 and 3; Mock clones 1 and 2, lanes 4 and 5; clones 1 and 2 expressing myc-HDGFdN1dN2, lanes 6 and 7; clones 1 and 2 expressing myc-wHDGF. Each arrow shows a protein band corresponding to intrinsic HDGF (I), myc-tagged wild type or mutant HDGF (M).

Figure 5. HepG2 and NIH3T3 cells overexpressing wild type HDGF stimulate DNA synthesis, but cells overexpressing mutant forms of HDGF without NLSs do not.

HepG2 cells were cultured in D-MEM supplemented with 1% FCS for 96 hrs and then harvested for DNA synthesis. NIH3T3 cells were cultured in D-MEM supplemented with 5% FCS for 48 hrs and then harvested. a) HepG2 cells. Lanes 1 and 2; Mock clones 1 and 2, lanes 3 and 4; clones 1 and 2 expressing myc-HDGFdN1dN2, lanes 5 and 6; clones 1 and 2 expressing myc-wHDGF. b) NIH3T3 cells. Lanes 1 and 2; Mock clones 1 and 2, lanes 3 and 4; clones 1 and 2 expressing myc-HDGFdN1dN2, lanes 5 and 6; clones 1 and 2 expressing myc-wHDGF. *: p<0.05 vs mock clones and myc-HDGFdN1dN2-expressing cells.
Figure 6. HepG2 cells overexpressing wild type HDGF stimulate cell proliferation, but cells overexpressing mutant forms of HDGF without NLSs do not.

HepG2 cells were cultured in D-MEM supplemented with 10% FCS, and 2 and 4 days later cell numbers were determined by the MTS method. Open and closed square; Mock clones 1 and 2, open and closed triangle; clones 1 and 2 expressing myc-HDGFdN1dN2, open and closed circle; clones 1 and 2 expressing myc-wHDGF. *: p<0.05 vs mock clones and myc-HDGFdN1dN2-expressing cells.

Figure 7. Increased DNA synthesis in 293 cells expressing GFP-wHDGF or GFP-HDGFnonHATH, but not in cells expressing GFP-HDGF without NLSs or GFP-HATH.

293 cells expressing GFP-chimera proteins of wild type HDGF, its mutant or truncated forms were cultured in D-MEM supplemented with 1% FCS, and were harvested 4 days later. a) Lane 1; mock, transfected with pEGFP-C1 vector, lane 2; GFP-HDGFmN1mN2, lane 3; GFP-HDGFdN1dN2, lane 4; GFP-HDFGmN1, lane 5; GFP-HDGFmN2, lane 6; GFP-HATH, lane 7; GFP-HDGFnonHATH, lane 8; GFP-wHDGF. b) Lane 1; mock, lane 2; GFP-HATH, lane 3; GFP-HDGFnonHATH, lane 4; GFP-HDGF-SA, lane 5; GFP-HDGF-LA, lane
6; GFP-HDGF-LP. *: p<0.05 vs mock, GFP-HDGFmN1mN2, GFP-HDGFdN1dN2, GFP-HDGFmN2 and GFP-HATH, **: p<0.001 vs mock, GFP-HATH, GFP-HDGF-SA, GFP-HDGF-LA and GFP-HDGF-LP.

Figure 8. HDGF containing the NLS sequence of p53 instead of NLS2 cannot stimulate DNA synthesis.

a) Confocal microscopy of 293 cells expressing GFP-HDGF-p53NLS. The living 293 cells transfected with pEGFP-C1 containing HDGF-p53NLS were evaluated on day 3 using confocal microscopy equipped for GFP visualization (488nm excitation and FITC filter set).

b) DNA synthesis in 293 cells. 293 cells expressing GFP-HDGF-p53NLS were cultured in D-MEM supplemented with 1% FCS, and were harvested 4 days later. Lane 1; mock, lane 2; GFP-HDGFdN1dN2, lane 3; GFP-HDGF-p53NLS, lane 4; GFP-wHDGF. *: p<0.01 vs mock, GFP-HDGFdN1dN2 and GFP-HDGF-p53NLS.
Fig. 5

Fig. 6
Fig. 8

a) NLS of p53

KRALPNNTSSSPQPKKK

NLS of p53

b) [3H]-Thd uptake (×10^3 cpm)

![Graph showing [3H]-Thd uptake](image)
Hepatoma-derived growth factor stimulates cell growth after translocation to the nucleus by nuclear localization signals
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