Identification of a Heparin-binding Growth Factor-1 Nuclear Translocation Sequence by Deletion Mutation Analysis*

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We have shown previously that a deletion mutant of human heparin-binding growth factor (HBGF)-1, HBGF-1U, lacking the sequence Asn-Tyr-Lys-Lys-Pro-Lys-Leu is capable of initiating c-fos mRNA expression and polypeptide phosphorylation on tyrosine residues at concentrations that do not induce either DNA synthesis or cell proliferation (1). The fact that addition of the nuclear translocation signal from the yeast histone 2B protein to the HBGF-1U mutant caused reconstitution of the biological activity of HBGF-1 indicated that nuclear translocation may be an important component of the mitogenic signal induced by HBGF-1. In order to examine the nuclear translocation potential of HBGF-1α, the deletion mutant HBGF-1U, and the yeast histone 2B-HBGF-1 chimeric, HBGF-1U2, we expressed these forms of HBGF-1 in murine endothelial cells. Western blot and two-dimensional Western blot analysis of cytosol and nuclei demonstrated that although the three forms of HBGF-1 are readily detectable in the cytosol of the individual transfectants, HBGF-1α and HBGF-1U2 but not HBGF-1U was detected in the nucleus. Furthermore, murine endothelial cells expressing HBGF-1α and HBGF-1U2 exhibited an atypical cellular phenotype in vitro that was absent in the HBGF-1U transfectants. These data suggest that HBGF-1 contains a functional nuclear translocation sequence that may be responsible for the initiation of DNA synthesis, and these data further correlate the presence of the nuclear translocation sequence with an abnormal endothelial cell phenotype in vitro.

Heparin-binding growth factor (HBGF)-1 is a potent polypeptide mitogen for mesenchymal- and neuroectoderm-derived cells (2). HBGF-1 is the precursor for biologically active NH2-terminal truncated derivatives isolated previously as acidic fibroblast growth factor (3) and HBGF-1α (4). Interestingly, HBGF-1 (5) and HBGF-2 (6) are the prototypes for a larger family of structurally related polypeptides (7-11, 22), many of which contain functional signal sequences (7-10). In contrast, the HBGF prototypes, unlike the majority of HBGF family members, do not contain recognizable signal sequences for secretion (2, 5, 6). Because the HBGF prototypes induce cell proliferation through a high affinity receptor (12-17), the extracellular transport of the HBGF prototypes has been the subject of considerable interest. We have previously used deletion mutagenesis and prokaryotic expression to identify a putative nuclear translocation signal at the NH2 terminus of HBGF-1α (1). Prokaryotic expression of the deletion mutant, HBGF-1U, lacking the sequence Asn-Tyr-Lys-Lys-Pro-Lys-Leu yields a polypeptide with the ability to bind to immobilized heparin, induce c-fos transcript expression, and enhance tyrosine-specific polypeptide phosphorylation at concentrations where HBGF-1U fails to induce either DNA synthesis or cell proliferation in vitro (1). It also was possible to reconstitute the mitogenic activity of HBGF-1U by ligation of the nuclear translocation signal from the yeast histone 2B gene (18) at the NH2 terminus of HBGF-1U; prokaryotic expression of this chimeric polypeptide HBGF-1U2 yields a functional endothelial cell mitogen (1), including the property of heparin potentiation, a feature that discriminates between the HBGF-1 prototypes (1, 19-21). However, it was not possible to follow the intracellular fate of exogenous HBGF-1U, because unlike HBGF-1α, HBGF-1U proved difficult to radiolabel due to deletion of the tyrosine near the NH2 terminus of HBGF-1α (1). Therefore, to study the nuclear translocation potential of HBGF-1α, HBGF-1U, and HBGF-1U2, we obtained stable murine endothelial cell transfectants expressing the wild-type and mutant forms of HBGF-1 using the pMEXneo vector in which the expression of the individual forms of HBGF-1 are driven by the RSV-LTR promoter independent of the selectable marker, the neomycin-resistance gene, which is regulated by the early SV40 promoter. This strategy enables us to examine the subcellular localization of HBGF-1 and its mutants irrespective of their receptor affinity which may alter their potential intracellular concentration as a result of receptor-mediated endocytosis (1). Our data suggest that deletion of the sequence Asn-Tyr-Lys-Lys-Pro-Lys-Leu from HBGF-1 not only impairs nuclear translocation but also fails to induce an atypical endothelial cell phenotype observed in the HBGF-1 transfectants that may present a growth advantage to the endothelial cell in vitro. These data argue that HBGF-1 may ultimately act as an intracellular nuclear-targeted polypeptide mitogen, a feature that would not require a signal sequence or interaction with an extracellular high affinity receptor.

MATERIALS AND METHODS

Cells and Vectors—Murine lung endothelial (LEII) cells were routinely cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (HyClone) (10% (v/v) FBS/DMEM). Complementary DNAs encoding HBGF-1α, HBGF-1U, and HBGF-1U2 were generated by the polymerase chain reaction as described elsewhere (1), except that the oligonucleotides primers used were 5'-A AGT CGA CGT CGA  CGA  CCC  ACC ATG GCT AAT

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§ The abbreviations used are: HBGF, heparin-binding growth factor; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.
**Nuclear Translocation Sequence of a Growth Factor**

TAC AAG AAG CCC AAA CTC CTC-3' for HBGF-la sense, 5'-AAG TCG ACG TCG ACC CAC CAT GCT CTA TAG CAA CCG G-3' for HBGF-lU sense, 5'-AAG TCG ACG TCG ACC CAC CAT G AAG AAG AAG AAG GCC ATG CTC TAC TG TGC AAC GGG GGC-3' for HBGF-lU2 sense, and 5'-CCG AAT TCG AAT TCT TTA ATG AGA GAC TGG-3' as control sequences to make convenient linkers on the 5'- (SfiI) and 3'-ends (EcoRI), as well as to introduce the Kozak sequence (CCAC-CATG) at the translation initiation sites (23). The amplified cDNAs were digested with Sall and EcoRI, followed by purification by agarose gel electrophoresis, and ligated to the cohesive cloning sites in the plasmid pMEXneo. Plasmids containing correct inserts were obtained, and the sequence was verified by using a Sequenase kit (U. S. Biochemical Corp.).

**Methods of Transfection**—Exponentially growing LEI1 cells were trypsinized, washed twice in phosphate-buffered saline, pH 7.4 (PBS), and suspended in 50 mM glucose in FBS to a density of 1 X 10⁶ cells/ml. To 250 μl of the cell suspension, the plasmids were added to a final concentration of 100 μg/ml. The cells were placed in an electrophoration chamber and kept on ice for 10 min. The cells received three electric pulses of 2000 V/cm and kept on ice for an additional 5 min. The cells were mixed with ice-cold 10% (v/v) FBS/DMEM and then left at room temperature for 5 min. The cells were seeded into four T75 flasks, with 10% (v/v) FBS/DMEM, and incubated in an atmosphere containing 5% CO₂ at 37°C. The following day, the medium was replaced with a fresh 10% (v/v) FBS/DMEM containing 400 μg/ml G418 and selected for G418 resistance. The medium was changed every 4 days, and transfected colonies appeared after 2 weeks. The transfected cells were trypsin-treated and subcultured in 10% (v/v) FBS/DMEM containing 200 μg/ml G418.

**Detection of Cytosolic HBGF-1**—The transfected cells were trypsin-treated, and 5 X 10⁶ cells were washed twice with PBS and solubilized with 200 μl of 1% (v/v) Triton X-100 containing 0.5 mM NaCl, 10 mM Tris-Cl, pH 7.4, for 4°C for 20 min. The solubilized cells were centrifuged at 15,000 X g for 10 min, the supernatant transferred to a fresh microcentrifuge tube, and 1 ml of PBS containing 0.5 mM NaCl and 100 μl of heparin-Sepharose (40% (v/v) slurry in distilled H₂O; Pharmacia) was added. The cap was sealed, the tube rotated at 4°C for 2 h, and the heparin-Sepharose beads washed twice with 1 ml of PBS. The beads were boiled in SDS-PAGE sample buffer and the samples subjected to SDS-PAGE (15% gel), electrotransferred to a nitrocellulose membrane, followed by visualization of HBGF-1 with affinity-purified rabbit anti-HBGF-1 antibody (1). As demonstrated by the appearance of elongated fibroblast-like cells and an apparent loss of contact inhibition. In addition, it was observed that the proliferative potential of the U-LEI1 and p-LEI1 cell transfectants was similar but significantly slower than the a-LEI1 and U2-LEI1 cell transfectants in the presence of 5% FBS. The ability of the individual LEI1 cell transfectants to translocate the various forms of HBGF-1 to the nucleus was studied by fractionation of the individual transfectants into cytosol and nuclear fractions. As shown in Fig. 2, it was possible to readily identify by Western blot analysis the presence of HBGF-1α, HBGF-1U, and HBGF-1U2 in the cytosol derived from their respective cellular transfectants (arrowheads). Interestingly, LEI1 cells contained endogenous proteins irrespective of the transfected constructs which bind to heparin-Sepharose in the presence of 0.5 M NaCl and are recognized by the affinity-purified anti-HBGF-1 antibody constantly (Fig. 2). The feature of these proteins remains to be clarified.
Two-dimensional Western blot analysis of nuclear HBGF-1. Nuclear HBGF-1 of each transfectant cells were analyzed by two-dimensional Western blotting using anti-HBGF-1 antibody as described under "Materials and Methods." A, p-LEI1; B, α-LEI1; C, U-LEI1; D, U2-LEI1. First dimensions of isoelectric focusing were run from left to right. Second dimensions of SDS-PAGE from top to bottom. The arrows indicate the positions of HBGF-1s as determined by running recombinant proteins on separate gels. The arrowheads indicate the edges of the gels, where isoelectric points were 6.6 for the left and 4.4 for the right edge as determined by a parallel isoelectric focusing gel. Four separate experiments yielded similar results.

One-dimensional Western blot analysis of the nuclei obtained from the individual transfectants did not yield similar results as shown in Fig. 2. Interestingly, the abundance of nuclear-derived heparin-binding proteins that co-eluted with HBGF-1 significantly reduced the resolution of this method (data not shown). These proteins also interfered with the detection of nuclear HBGF-1 by immunohistochemical staining. Therefore, we employed two-dimensional electrophoresis to separate HBGF-1 (Isoelectric point, pI, of about 6) from nuclei-derived polypeptides possessing more acidic pI. As shown in Fig. 3, B and D, it was possible to identify by one-dimensional Western blot analysis of nuclei derived from α-LEI and U2-LEI1 cell transfectants both HBGF-1α and HBGF-1U2. However, nuclei derived from p-LEI1 and U-LEI1 cell transfectants did not contain detectable levels of endogenous HBGF-1 or HBGF-1U (Fig. 3, A and C, respectively). Similar data were obtained in three separate experiments. Thus, we suggest that the absence of the sequence Asn-Tyr-Lys-Lys-Pro-Lys-Leu in the HBGF-1U polypeptide prevents nuclear translocation.

The failure to detect HBGF-1U in the nucleus of LEI1 cell transfectants correlates with the inability of HBGF-1U to act as an exogenous (1) and endogenous mitogen for these cells (described above). Although the significance of the altered phenotype assumed by the α-LEI1 (25) and U2-LEI1 cell transfectants is difficult to interpret, these transfectants do attain an increased proliferative potential in response to serum, and this response is attenuated in the p-LEI1 and U-LEI1 transfectants. Interestingly, nuclear HBGF-2 has also been found to be present in endothelial cells (26), an event that occurs in the late G1 phase of the cell cycle (27). In addition, int-2, a member of the HBGF family, contains an alternative CUG translation start site that yields an int-2 polypeptide with a functional nuclear translocation signal (28). This contrasts with the translation product obtained from translation of the int-2 polypeptide at the AUG start site that enables int-2 polypeptide to undergo secretion (28). Furthermore, nuclear localization of the HBGF prototypes has been observed in rheumatoid arthritic tissue (29) and in the heart (30).

The ability to delete and reconstitute the mitogenic activity of HBGF-1 (1) by manipulation of a nuclear translocation signal indicates that HBGF-1 may ultimately function as an intracellular polypeptide that may utilize nuclear translocation as a component of this pathway. This feature may not necessitate the interaction between extracellular HBGF-1 and a high affinity plasma-membrane receptor, and this suggestion is consistent with the absence of a signal peptide sequence in the structure of the HBGF-1 precursor (5), However, our data do not eliminate the potential interaction between cytosolic and/or nuclear HBGF-1 and an intracellular high affinity binding site(s) responsible for the regulation of cell proliferation. More detailed study on the state of nuclear localization of HBGF-1 will help us understanding the role of nuclear growth factors and their roles. It is also interesting to note that with the possible exception of HBGF-5 (9), all members of the HBGF family contain a sequence motif resembling a nuclear translocation signal prior to the Leu-Tyr-Cys sequence that is conserved among all HBGF family members (2).
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