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Structure-Function Studies of Heparin-Binding (Acidic Fibroblast) Growth Factor-1 Using Site-Directed Mutagenesis

Wilson H. Burgess, Anne M. Shaheen, Brian Hampton, Patrick J. Donohue, and Jeffrey A. Winkles

American Red Cross, Laboratory of Molecular Biology, Jerome H. Holland Laboratory for the Biomedical Sciences, 15601 Crabbs Branch Way, Rockville, Maryland 20855

Abstract The heparin-binding or fibroblast growth factors (HBGFs) modulate cell growth and migration, angiogenesis, wound repair, neurite extension, and mesoderm induction. Relatively little is known regarding the precise mechanism of action of these growth factors or the structural basis for their action. A better understanding of the structural basis for the different activities of these proteins should lead to the development of agonists and antagonists of specific HBGF activities. In this report, we summarize evidence that indicates that the heparin-binding and mitogenic activities of HBGF-1 can be dissociated from the receptor-binding activities of the growth factor by site-directed mutagenesis of a single lysine residue. Thus, the mutant HBGF-1 has normal receptor-binding activity and is capable of stimulating tyrosine kinase activity and proto-oncogene expression but is not able to elicit a mitogenic response. A similar dissociation of early events such as proto-oncogene expression from the mitogenic response is observed when the human wild-type HBGF-1 is used in the absence of added heparin. These results indicate that intracellular sites of action by the growth factor may be required to complete the mitogenic response. Further evidence for this idea is provided by transfection experiments where NIH 3T3 cells are engineered to produce large quantities of wild-type or mutant HBGF-1. Production of wild-type induces a transformed phenotype, whereas over-production of the mutant does not. The majority of both forms of the protein is found in the nuclear fraction of the transfected cells. Additional site-directed mutagenesis of putative nuclear translocation sequences in the wild-type protein do not affect mitogenic activity. Thus, the role of nuclear translocation in the mechanisms of action of HBGF-1 remains unclear.

Key words: proto-oncogene expression, nuclear translocation, mitogenesis, tyrosine kinase, angiogenesis

The heparin-binding growth factor (HBGF) family presently consists of seven structurally related polypeptides [1]. Two of the proteins, HBGF-1 and HBGF-2, have been characterized under many different names, most often as acidic and basic fibroblast growth factor, respectively. Functions associated with HBGF-1 and HBGF-2 include stimulation of mitogenesis, chemotaxis, neurite extension, plasminogen activator activity, and mesoderm induction. They are also capable of inducing angiogenesis and wound repair in vivo (see [1,2] for reviews). The precise mechanisms by which the two HBGFs are able to elicit these pleiotropic responses remain largely unknown. A family of high-affinity HBGF receptors with intrinsic tyrosine kinase activities has been identified recently [3–5], as have low-affinity receptors that consist of heparan sulfate proteoglycans [6,7]. Binding of HBGFs to the high-affinity receptors leads to stimulation of protein tyrosine kinase activity [8,9], phosphorylation of phospholipase C-γ [10], and activation of immediate-early gene transcription [11,12]. However, the relationship of these events to HBGF-stimulated mitogenesis remains unclear.

The glycosaminoglycan heparin can potentiate HBGF-1-stimulated mitogenesis [13], neurite outgrowth [14], and neovascularization [15]. The mechanism(s) by which potentiation occurs is not completely understood. It has been demonstrated that heparin increases the biological half-life of HBGF-1 in tissue culture medium [14] and can protect HBGF-1 from proteolytic degradation [16]. The three responses described above may require biologically active HBGF-1 to be present for a relatively long time; indeed, Da-

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Address reprint requests to Wilson H. Burgess, American Red Cross, Laboratory of Molecular Biology, Jerome H. Holland Laboratory for the Biomedical Sciences, 15601 Crabbs Branch Way, Rockville, MD 20855.

mon et al. [14] reported that HBGF-1 must be present for 25 h in order to observe neurite extension in PC12 cells. To date, there is only one report investigating the effect of heparin on HBGF-1 function using a short-term biological assay. Schreiber et al. [17] demonstrated that bovine HBGF-1 can bind cell surface receptors in the absence of heparin; however, heparin increased the apparent affinity of HBGF-1 for its receptor by two to threefold. It is important to determine whether heparin can potentiate the early cellular responses that occur following HBGF-1 receptor binding.

Both HBGF-1 and HBGF-2 are relatively resistant to degradation following internalization by receptor-mediated endocytosis [18,19]. Intact growth factor persists intracellularly for several hours and large fragments (M_r 15,000 and 10,000 for HBGF-1; M_r 16,000 for HBGF-2) are detectable after as many as 24 h. These observations suggest a possible intracellular target may be important to the mechanism of HBGF signal transduction. Further support for this idea is provided by the direct demonstration of nuclear or nucleolar localization of HBGF-2 [20,21] and indirect evidence for nuclear localization of HBGF-1 [22].

Recently, a consensus for the structural basis of nuclear translocation has developed. Chelsky et al. [23] examined the ability of 18 synthetic peptides to target chicken serum albumin to the nucleus following microinjection into HeLa cells. They arrived at a consensus sequence where all the peptides that induced full or partial nuclear localization fit the sequence $K\text{-}^{K}/_{R}\text{-}X\text{-}^{K}/_{R}$, where X could be K, R, P, V, or A; further, X could not be N. Examination of the amino acid sequences of HBGF-1 and HBGF-2 reveals that both proteins contain amino-terminal sequences that fit the consensus described above.

In addition, the methylation of arginine residues may be important in directing the nuclear localization of certain proteins, such as histones [24]. It has been demonstrated that the "high" molecular weight forms of HBGF-2 contain methylated arginines [25]. Also, HBGF-1 contains a region (surrounding lysine 132) which is similar to the sequence of adenovirus protein E1a that has been shown to direct nuclear localization [26]. Thus, HBGF-1 and HBGF-2 contain both common and unique sequences that may provide the signals necessary for nuclear translocation of these proteins. Clearly, it is important to determine the structural basis for nuclear localization of these growth factors and more importantly to determine what role, if any, nuclear localization plays in mediating the actions of the HBGFs.

It is established that deletion of the first 20 residues of HBGF-1 is without significant effect on the mitogenic activity of the growth factor [27]. Recently, Imamura et al. [22] reported that deletion of an additional seven residues reduces significantly the mitogenic activities of the protein without affecting its heparin-binding activity. Further, they reported that mitogenic activity was not observed at concentrations of the growth factor that were sufficient to stimulate intracellular kinase activity and induce c-*fos* gene expression. Interestingly, this deletion involves the consensus nuclear translocation sequence described above. Finally, they reported that construction of a chimeric protein containing the nuclear translocation sequence of yeast histone 2B adjacent to the des 1-27 form of HBGF-1 resulted in restoration of full mitogenic activity to the chimeric protein. In this report we 1) summarize recent work [12] using site-directed mutagenesis to dissociate the receptor-binding from the mitogenic properties of HBGF-1; 2) provide evidence that heparin is not required for the induction of proto-oncogene expression by wild-type HBGF-1; 3) demonstrate nuclear localization of wild-type HBGF-1 and a mutant that is deficient in its mitogenic activities; and 4) present evidence that "classical" nuclear translocation sequences found in HBGF-1 can be disrupted without affecting its mitogenic activity.

MATERIALS AND METHODS

Construction of Prokaryotic Expression Plasmids and Purification of Recombinant Proteins

The plasmid expressing wild-type HBGF-1 (corresponding to the α-form of endothelial cell growth factor [27]) was kindly provided by R. Forough, American Red Cross. The plasmids expressing HBGF-1 mutants containing single amino acid substitutions, designated pG23 (lys 23 → gly), pG24 (lys 24 → gly), pG26 (lys 26 → gly), and pE132 (lys 132 → glu), were kindly provided by M. Ravera and M. Jaye, Rorer Biotechnology, Inc. *E. coli* cultures bearing the recombinant plasmids were grown, and wild-type and mutant forms of HBGF-1 were purified using heparin-Sepharose chromatography and reversed-phase HPLC [12]. All preparations were >95% pure as analyzed by SDS-PAGE. Protein

concentrations were determined by amino acid analysis.

Mitogenic Assays

Balb/c 3T3 clone A31 cells obtained from the American Type Culture Collection (ATCC) were grown to confluence at 37°C in Falcon 96-well plates with DME (Irvine Scientific) containing 10% calf serum (Inovar). The media was then replaced with DME, 0.5% calf serum, and the cells were incubated for 48 h. They were then either left unstimulated or stimulated with wild-type or mutant HBGF-1. Heparin (Upjohn) was also added to all wells to a final concentration of 5 units/ml. After 16 h of incubation, the cells were pulsed for 4 h with 3 μCi/ml of [^{3}H]methyl-thymidine (25 Ci/mmol, Amersham). The media was removed, and the cells were washed with phosphate-buffered saline and treated with ice cold 10% trichloroacetic acid (TCA) for 30 min at 4°C. The TCA was removed, 1 M NaOH was added, and the plates were incubated at 37°C for 30 min. The amount of [^{3}H]methyl-thymidine incorporated into DNA was determined by scintillation counting.

RNA Gel Blot Analysis

NIH 3T3 cells (ATCC) were grown to confluence at 37°C in DMEM (Mediatech) containing 10% calf serum (Hyclone). They were incubated for 48 h in DMEM/0.5% FCS and then either left unstimulated or stimulated with 10 ng/ml wild-type HBGF-1 or 10 ng/ml mutant HBGF-1 (lys 132 → glu) for the indicated times. In some cases, 5 units/ml heparin (Upjohn) or 10 μg/ml cycloheximide (Sigma) were also added. Cells were harvested, total RNA was prepared as previously described [11], and 10 μg of each sample was denatured and subjected to electrophoresis in 1.2% agarose gels containing 2.2 M formaldehyde. The gels were stained with ethidium bromide to verify that each lane contained similar amounts of undegraded ribosomal RNA. RNA was electroblotted onto Zetabind nylon membranes (AMF Cuno) and cross-linked by UV irradiation using a Stratalinker (Stratagene). The restriction fragments used and source of the DNA probes were as follows: (a) c-*fos*, 2.8 kb *Nco*I/*Xho*I fragment of pc-*fos*-1; ATCC; (b) c-*jun*, 1.5 kb *Hin*dIII/*Bam*HI fragment of ph-cJ-1; gift of P. Angel, University of California, La Jolla, CA; (c) c-*myc*, 1.4 kb *Sst*I fragment of pHSR-1; ATCC; (d) glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 0.8 kb *Pst*I/*Xba*I fragment of pHcGAP; ATCC. The probes were labeled to high-specific activity with [^{32}P]dCTP (3,000 Ci/mmol, Amersham) using a random primer labeling kit (Boehringer Mannheim). Hybridization and membrane washing conditions were as described [11]. Blots were exposed to film (XAR-5, Kodak) with an intensifying screen at −80°C.

Transfection of NIH 3T3 Cells With HBGF-1 Expression Vectors

NIH 3T3 cells were transfected with pSV2neo or co-transfected with pSV2neo and either p267 (wild-type HBGF-1 expression vector) or p268 (mutant HBGF-1 [lys 132 → glu] expression vector) as previously described [12]. G418-resistant cells were isolated and maintained in selective media.

Subcellular Fractionation and Western Blot Analysis

Two 10-cm dishes containing NIH 3T3 cells transfected with expression vectors for wild-type or the lys 132 → glu mutant of HBGF-1 were grown to confluence. The cells were harvested by gentle scraping in 0.25 M sucrose containing 0.001 M EDTA, 0.01 M Tris-HCl pH 7.1. The cells were ruptured with ten strokes of a loose-fitting dounce homogenizer. The homogenate was centrifuged at 1,000*g* for 10 min to obtain a supernatant and a crude nuclear pellet. The pellet was washed two times by resuspension in the same buffer and centrifugation as above. An equivalent percentage of the supernatant and nuclear fractions were mixed with Laemmli sample buffer, subjected to polyacrylamide gel electrophoresis in the presence of SDS, and transferred to nitrocellulose membranes for Western blotting as described [12]. The relative amounts of HBGF-1 in the samples was determined by probing the blots with an HBGF-1-specific polyclonal antibody followed by incubation with ^{125}I-labeled Protein A and autoradiography.

RESULTS AND DISCUSSION

We determined previously that the presence of heparin is not necessary for two early events that occur following the addition of HBGF-1 to quiescent cells: stimulation of tyrosine kinase activity [9] and phosphorylation of phospholipase C-γ [10]. Another early event associated with HBGF-1-stimulated mitogenesis is the increased expression of mRNAs encoding the c-*fos*,

c-*jun*, and c-*myc* polypeptides. This has been observed in HBGF-1-stimulated human endothelial cells [11] and murine NIH 3T3 fibroblasts [12]. These experiments were performed in the presence of heparin. To determine whether heparin was necessary for HBGF-1-induced gene expression, quiescent NIH 3T3 cells were either left unstimulated or stimulated with 10 ng/ml recombinant human HBGF-1 in the absence of heparin. Cells were collected at various times post-stimulation, RNA was prepared, and levels of c-*fos*, c-*jun*, c-*myc*, and GAPDH (as a control for RNA loading) mRNA were assayed by RNA gel blot analysis. The proto-oncogene mRNAs were transiently expressed after HBGF-1 addition (Fig. 1). The kinetics of induction were identical to those previously observed in cells treated with both HBGF-1 and heparin [12]. Induction also occurred in the presence of cycloheximide, an inhibitor of protein synthesis. In fact, the presence of cycloheximide "superinduced" proto-oncogene mRNA levels, presumably by preventing the synthesis of transcriptional repressors and/or RNAses involved in mRNA degradation.

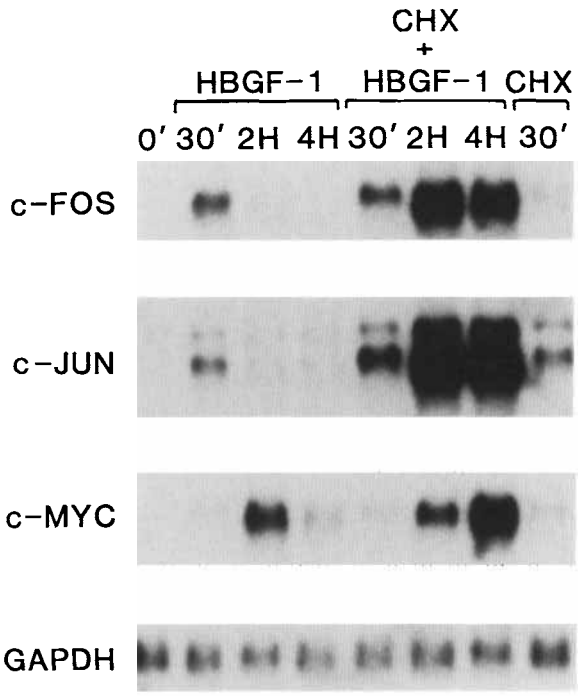

Fig. 1. Expression of proto-oncogene mRNAs in HBGF-1-stimulated NIH 3T3 cells. Serum-starved NIH 3T3 cells were *either left untreated or treated with* HBGF-1, HBGF-1 and cycloheximide (CHX), or cycloheximide alone for the indicated time periods. RNA was prepared, and equivalent amounts of each sample were used for RNA gel blot analysis. The cDNA probes used are indicated on the left side.

Human recombinant HBGF-1 is highly dependent on the presence of heparin for optimal mitogenic activity [12]. In contrast, recombinant HBGF-1 that has a glutamic acid residue instead of a lysine residue at position 132 is a relatively poor mitogen in both the presence and absence of heparin [12]. This polypeptide also 1) has a reduced apparent affinity for heparin, 2) has a normal affinity for HBGF cell surface receptors, and 3) can stimulate tyrosine kinase activity, phospholipase-Cγ phosphorylation, and proto-oncogene mRNA expression [12]. Thus, although this mutant polypeptide is a poor mitogen, it can activate early events associated with HBGF-1 stimulation of cell growth. Our previous experiments comparing the early responses induced by wild-type and mutant HBGF-1 were performed in the presence of heparin. We determined whether the wild-type and mutant forms of HBGF-1 could also induce c-*fos* gene expression to a similar degree in the absence of heparin. NIH 3T3 cells were serum-starved and either left unstimulated or stimulated for different lengths of time with either wild-type HBGF-1 in the presence and absence of heparin or mutant (lys 132 $\rightarrow$ glu) HBGF-1 in the absence of heparin. Cells were collected, RNA was prepared, and mRNA levels were analyzed by RNA gel blot analysis. Transient expression of c-*fos* mRNA was detected after each of the three treatments and the level of induction was similar (Fig. 2).

The results presented above, when considered with our previous studies as well as those of other investigators, suggest two general conclusions. First, heparin is not required for HBGF binding to cell surface receptors or for at least three early responses that occur following HBGF-1 stimulation of quiescent cells. If heparin is present, it does not potentiate significantly the early responses. In contrast, heparin potentiation of HBGF-1 activity is evident when late responses such as cellular division or neurite extension are assayed and probably reflects the requirement for relatively long exposures to biologically active growth factor. Second, although various studies have implicated proto-oncogene proteins, in particular c-*fos* [28], in the control of cell proliferation, HBGF-1-inducible proto-oncogene expression is not sufficient to initiate a mitogenic response. This proposal is supported by the ability of wild-type HBGF-1 to

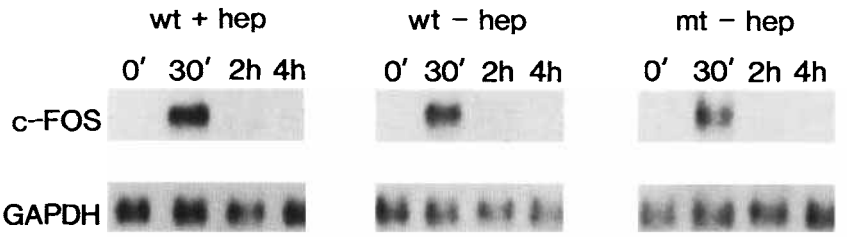

Fig. 2. Expression of *c-fos* mRNA in NIH 3T3 cells treated with wild-type or mutant (lys 132 → glu) HBGF-1. Serum-starved NIH 3T3 cells were either left untreated or treated with wild-type (wt) HBGF-1 or mutant (mt; lys 132 → glu) HBGF-1 for the indicated time periods. In some cases, heparin (+hep) was also added to the treated cells. RNA was prepared, and equivalent amounts of each sample were used for RNA gel blot analysis. The cDNA probes used are indicated on the left side.

induce proto-oncogene expression, but not mitogenesis, when heparin is absent. Similarly, when added alone or in the presence of heparin, mutant HBGF-1 can induce gene expression, but is a relatively poor mitogen. Our observation that proto-oncogene induction can occur without subsequent DNA synthesis agrees with a study by Severinsson et al. [29], who reported that the addition of PDGF to cells expressing mutant PDGF receptors induces *c-fos* expression, but not mitogenesis. Also, proto-oncogene mRNA expression is induced by a variety of agents, some of which promote cell differentiation but not division (see [30] for review).

Thus, one of the important questions regarding the mechanism of action of HBGF-1 relates to identifying the "late events" or other "early events" that are required to complete the mitogenic response. Clearly, "high-affinity" receptor-binding, activation of tyrosine kinase activity, and induction of proto-oncogene expression may be necessary but are not, by themselves, sufficient to initiate cell division. A second, and related, question is whether an intracellular event mediated by HBGF-1 or one of its fragments is required for completion of the signal transduction pathway following receptor-mediated internalization of the growth factor.

Based on the data described above and in [12], it appears that the mutant (lys 132 → glu) HBGF-1 is capable of performing all of the known functions of the wild-type protein that are thought to involve binding to cell surface receptors without initiating a mitogenic response. It is known that high levels of wild-type HBGF-1 or HBGF-2 expression in transfected cells leads to an altered, transformed phenotype, loss of contact inhibition of growth, and the ability of the cells to form colonies in soft agar (reviewed in [1,2]). The results shown in Figure 3 are consistent with these observations, but, more importantly, demonstrate that high level expression of the mutant HBGF-1 is basically without affect on the morphology of NIH 3T3 fibroblasts or their ability to overcome contact inhibition of growth. The transfected cells selected for this comparison expressed similar levels of wild-type or mutant HBGF-1 as judged by Western blot analysis of whole cell lysates. It is presently unclear from our own work or that of others whether the induction of the transformed phenotype by high-level expression of HBGF-1 or HBGF-2 requires release of the growth factor from cells. It should be noted that suramin, a compound which acts in part by dissociating ligands from cell surface receptors, is able to reverse the transformed phenotype of cells overexpressing HBGF-2 [31] and HBGF-1 (data not shown).

Reports of nuclear localization of HBGF-1 and HBGF-2 and the structures that may direct nuclear localization of these proteins were summarized above. We examined the distribution of HBGF-1 in the wild-type and mutant transfected cells by subcellular fractionation and Western blot analysis. One advantage to the expression vectors used in these studies is that they encode for the biologically active des 1-20 form of HBGF-1 (referred to originally as αECGF [27]). Thus, the difference in apparent molecular weight (~ 2,000 daltons) of the endogenously produced full-length HBGF-1 and that of the plasmid-derived protein can be detected following SDS-PAGE and Western blotting. The immunoreactive HBGF-1 proteins found in the cytosolic or nuclear subcellular fractions of 1) untransfected NIH 3T3 cells, 2) neo-only transfected, 3) wild-type HBGF-1 transfected, and 4) lys 132 → glu mutant HBGF-1 transfected cells are shown in Figure 4. It can be seen from this

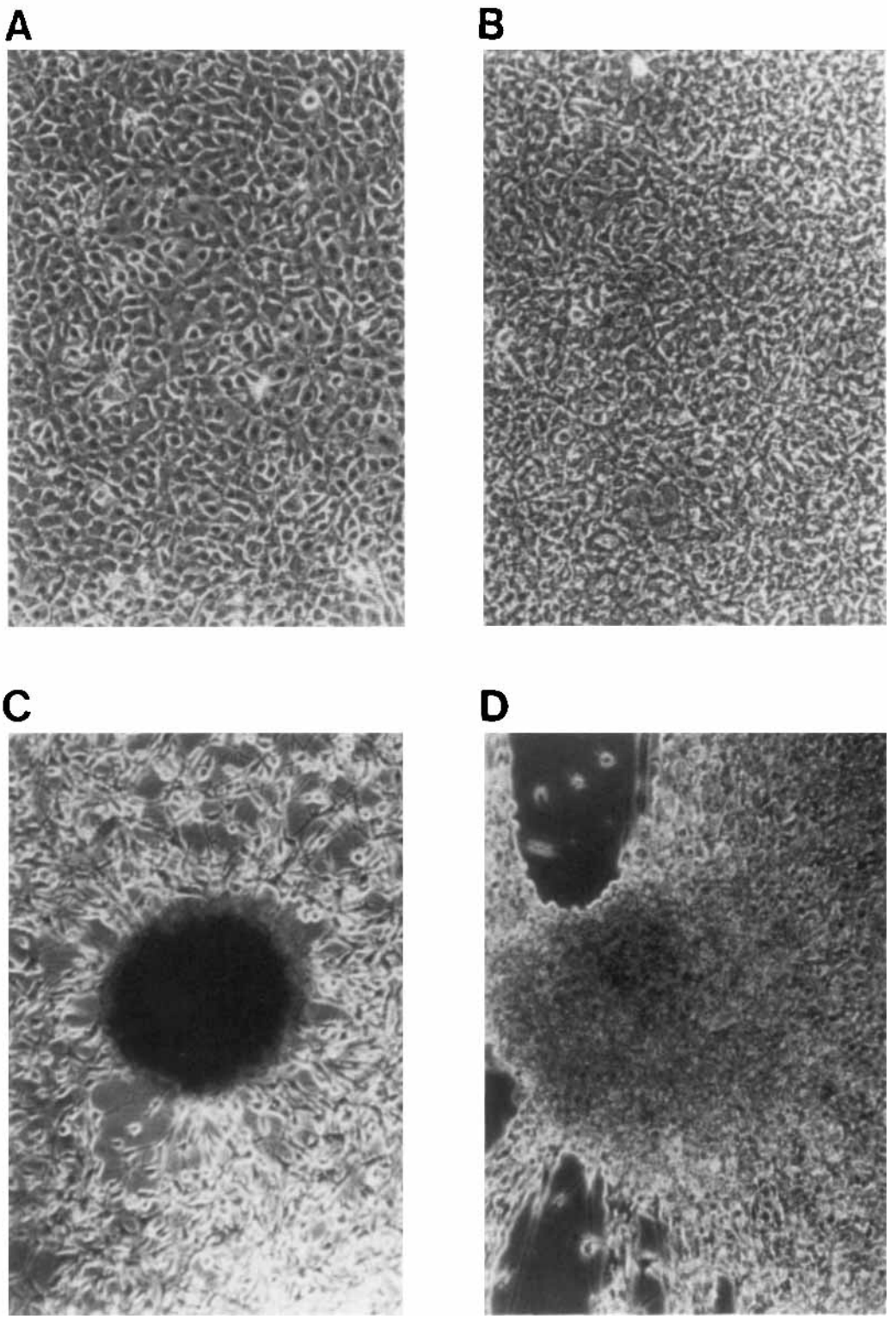

Fig. 3. Morphology of NIH 3T3 cells transfected with wild-type or mutant HBGF-1 expression plasmids. Cells were plated at low density in identical growth media and photographed at confluence. **A:** Untransfected NIH 3T3 cells; **B:** transfected NIH 3T3 cells expressing mutant HBGF-1 (lys 132 → glu); **C,D:** transfected NIH 3T3 cells expressing wild-type HBGF-1. Magnification 100×.

figure that all detectable endogenous HBGF-1 immunoreactivity is associated with the nuclear fraction. In addition, the majority of the plasmid-derived HBGF-1 (either wild-type or mutant) has been directed from the cytosolic to the nuclear fraction. These results demonstrate that the lack of mitogenic or transformation-like activity of this mutant does not result from any gross change in intracellular stability or compartmentalization. They do not address whether the mutant has an altered affinity for any nuclear macromolecule that may be involved in completing the mitogenic response.

As described above, the sequence of residues 23-26 (K-K-P-K) in HBGF-1 is in good agreement with a consensus sequence for nuclear translocation. Imamura et al. [22] demonstrated that amino-terminal deletion mutants of HBGF-1 lacking this region and several adjacent residues reduced the mitogenic activity of the growth factor to a greater extent than receptor-binding or other activities. They suggested that nuclear translocation of HBGF-1 may be important for mitogenic activity based on the observation that mitogenic activity was restored when an unrelated nuclear translocation sequence was added to the amino terminus of this mutant. We utilized site-directed mutagenesis to generate three-point mutations that change each of the lysine residues individually to a glycine. Each of these mutations disrupt the consensus sequence for nuclear translocation found in HBGF-1. Although the subcellular distribution of these mutants has not yet been

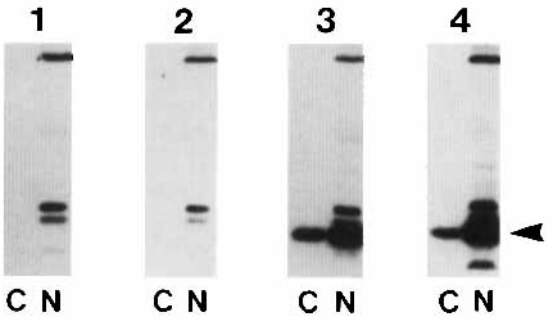

Fig. 4. Subcellular distribution of HBGF-1 immunoreactivity. NIH 3T3 cells were fractionated into nuclear (N) and cytosolic (C) fractions as described in Materials and Methods. The fractions from untransfected **(Panel 1)**; Neo transfected **(Panel 2)**; wild-type HBGF-1 transfected **(Panel 3)**, or mutant HBGF-1 transfected cells **(Panel 4)** were subjected to SDS-PAGE and Western blotting. The arrowhead indicates the position of HBGF-1 expressed from the transfected plasmids. The loads of each fraction represents an equivalent percent of the total volume of each fraction.

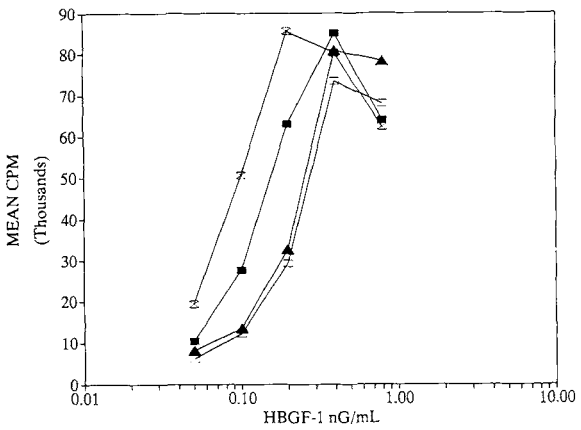

Fig. 5. Stimulation of DNA synthesis in Balb/c 3T3 cells by wild-type and mutant HBGF-1. Stimulation of DNA synthesis was assessed by measuring the amount of [^{3}H]thymidine incorporated into DNA. Incorporation obtained with the indicated concentrations of wild-type (■), pG23 (□), pG24 (▲), or pG26 (⊠) HBGF-1 is shown; values represent the mean of triplicate cultures.

determined, their heparin-binding, receptor-binding, mitogenic, and neurite-promoting activities are similar to those of the wild-type protein. Figure 5 compares the abilities of wild-type and these three mutant polypeptides to stimulate DNA synthesis in Balb/c 3T3 cells. The maximum stimulation and the dose response by each of the four proteins are very similar. We have not examined the biological properties of double or triple mutants in this region. A mutant that would be of interest to study would be one that contained the sequence K-K-N-K in that Lanford et al. [32] showed that in the consensus sequence K-$^{K}/_{R}$-X-$^{K}/_{R}$, X could not be an asparagine residue. It should be noted that other sequences capable of directing nuclear localization of growth factors have been identified. Finally, it is possible that the importance of this region of HBGF-1 to its mitogenic capacity may relate to the fact that it represents a rare basic cluster in an acidic protein.

The question as to the significance of nuclear localization of HBGF-1, HBGF-2, and other growth factors (see [33] for review) should be the focus of intense effort over the coming years. It clearly relates to the dissection of the "early" and the "late" events involved in growth factor action. Site-directed mutagenesis of these proteins and continued analysis of the events that may be necessary and those that are sufficient for eliciting a particular response should provide important reagents for understanding the mechanisms of action of the HBGF proteins.

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