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The Role of Heparin-Binding Growth-Associated Molecule (HB-GAM) in the Postsynaptic Induction in Cultured Muscle Cells

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The heparan sulfate proteoglycan (HSPGs) is a components of the extracellular matrix of skeletal muscle that is concentrated at the neuromuscular junction (NMJ). Recent studies have suggested that HSPG, together with its bound peptide growth factors, plays important roles in autocrine or paracrine types of regulation of cell growth and differentiation. Heparin-binding growth-associated molecule (HB-GAM; also known as pleiotrophin, or p18) is a newly discovered HSPG-bound factor that is expressed at high levels in the developing CNS and PNS. In this study, we examined the role of this factor in NMJ development by examining its relationship to the formation of ACh receptor (AChR) clusters. Using an antibody against recombinant rat brain HB-GAM, we found that this protein is present prominently on the surface of cultured *Xenopus* myotomal muscle cells by immunocytochemistry. It is associated with HSPGs as evidenced by the fact that heparin and heparinase treatment greatly diminished the antibody labeling. HB-GAM is concentrated at preexisting AChR hot spots as well as at those induced by polystyrene beads. In addition, this molecule is also concentrated at AChR clusters induced by spinal cord neurons in nerve–muscle cocultures. To assess its function in synaptic induction, we applied recombinant HB-GAM-coated beads to cultured muscle cells to effect its focal presentation. Over 70% of these beads induced the formation of AChR clusters as shown by fluorescent α-bungarotoxin labeling. Furthermore, bath application of HB-GAM inhibited the nerve-induced formation of AChR clusters. Thus, HB-GAM is an endogenous muscle-derived factor that may be a component of the molecular mechanism in postsynaptic induction.

[Key words: heparin-binding growth-associated molecule (HB-GAM), pleiotrophin, neuromuscular junction (NMJ), synaptogenesis,* Xenopus, *heparan sulfate proteoglycan (HSPG)]

Synaptic development at the neuromuscular junction (NMJ) is induced by the contact between the growth cone of the motor axon and the skeletal muscle cell. This contact presents a highly localized signal to both the nerve and the muscle to initiate the formation of pre- and postsynaptic specializations that are precisely registered in space (Hall and Sanes, 1993). Clustering of nicotinic ACh receptors (AChRs) is the hallmark of vertebrate postsynaptic development. In response to innervation, AChRs are clustered discretely at the nerve–muscle (N-M) contact. This spatial restriction in AChR clustering necessitates a signaling system that has a limited diffusion radius.

Previous studies have shown that the heparan-sulfate proteoglycan (HSPG) is an important component of the extracellular matrix (ECM) of skeletal muscle (Anderson and Fambrough, 1983; Anderson et al., 1984; Bayne et al., 1984; Anderson, 1986; Swenarchuk et al., 1990). They are concentrated at the NMJ *in vivo* and *in vitro* as well as at aneural ACh receptor (AChR) clusters ("hot spots") in cultured muscle cells. Recent studies have demonstrated that this proteoglycan can harbor heparin-binding growth factors (HBGFs) at the cell surface (Risau and Ekblom, 1986; Rifkin and Moscatelli, 1989; Klagsbrun and Baird, 1991; Bernfield et al., 1992; Salmivirta et al., 1992; Higashiyama et al., 1993). These factors can be released or presented to their receptors during cell–cell interaction to signal cell growth or differentiation (Saksela and Rifkin, 1990; Brunner et al., 1991). An important feature of this class of molecules is that they are spatially restricted when bound to HSPG (Flaumenhaft et al., 1990). The role of HBGFs in NMJ development is suggested by our recent studies that local presentation of basic fibroblast growth factor (bFGF), which binds to HSPG with high affinity, via latex beads stimulates the formation of AChR clusters in cultured *Xenopus* muscle cells (Peng et al., 1991a). However, immunocytochemical study has not revealed an abundance of bFGF at the surface of *Xenopus* skeletal muscle cells, nor at the NMJ (Baker et al., 1992). In addition, the primary sequence of this factor lacks a classical signal peptide (Esch et al., 1985; Mignatti et al., 1992). Thus, it is not clear how this molecule can be externalized to effect synaptic induction.

To further explore the role of HSPG and its associated molecules in NMJ development, we examined the expression and function of a newly discovered HBGF, the heparin-binding growth-associated molecule (HB-GAM; also known as pleiotrophin, or p18). As its name implies, it binds to heparin and HSPG with high affinity (Rauvala, 1989; Li et al., 1990; Merenmies and Rauvala, 1990; Hampton et al., 1992; Raulo et al., 1992). The cDNA for this factor encodes a protein with 136 amino acids with an N-terminal signal sequence. The amino acid se-

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quence of this molecule is highly conserved across species. Two features of this sequence are particularly striking. First, it shows a high content of basic amino acids which are clustered at its N- and C-terminals. Second, it possesses 10 cysteines, all of which have been shown to form disulfide bonds. These features, as well as the overall sequence, are shared by members of a family of retinoid acid–induced heparin-binding factors, such as midkine (Kadomatsu et al., 1988; Matsubara et al., 1990; Kretschmer et al., 1991). Previous studies have shown the expression of HB-GAM in a variety of tissues, including brain, skeletal muscle, and kidney, in a developmentally regulated manner (Rauvala, 1989; Li et al., 1990; Hampton et al., 1992). While its expression is downregulated in other tissues during development, it persists in the brain beyond neonatal stages (Rauvala, 1989; Li et al., 1990). *In vitro* assays have shown that it exhibits neurite-promoting activity when applied on the substratum (Rauvala, 1989; Kuo et al., 1990; Kretschmer et al., 1991; Hampton et al., 1992) and may also be a mitogen to certain fibroblast cell lines (Li et al., 1990; Fang et al., 1992).

In this study, we examined the distribution of HB-GAM in cultured muscle cells and its relationship to NMJ development. Here we report that HB-GAM is present prominently at the surface of cultured *Xenopus* myotomal muscle cells, where it is associated with HSPG. It becomes concentrated at AChR clusters induced by innervation as well as by other stimuli. Local presentation of this factor by beads induces the formation of AChR clusters in these muscle cells. On the other hand, bath application interferes with the nerve-induced AChR clustering. These data suggest that HB-GAM is an endogenous muscle-derived growth factor that may play an important role in the signal transduction process leading to postsynaptic differentiation at the developing NMJ.

Materials and Methods

Production of recombinant proteins and antibodies. Recombinant HB-GAM was produced with the aid of a baculovirus vector and the secreted protein was purified to apparent homogeneity from the culture medium of the SF9 cells as described previously (Raulo et al., 1992). For comparison, amphoterin, another heparin-binding factor was also used in this study (Rauvala et al., 1988; Merenmies et al., 1991). Recombinant amphoterin was also expressed in SF9 cells using a baculovirus vector and purified in a similar manner as HB-GAM (Parkkinen et al., 1993). Immune sera against the purified recombinant HB-GAM were produced in rabbits as described previously (Raulo et al., 1992). IgG was isolated from the immune sera using protein A–Sepharose (Pharmacia, Sweden) as recommended by the manufacturer. For affinity purification of the antibodies, 5 mg of recombinant HB-GAM was coupled to a 1 ml cynogen bromide-activated Sepharose 4B column (Pharmacia, Sweden). Antibodies bound to the affinity column from the immune sera in the presence of 0.6 M NaCl were purified as described previously (Rauvala et al., 1988).

Cell cultures. Muscle cells were isolated from *Xenopus* embryos according to a previously published method (Peng et al., 1991b). They were plated on 35 mm tissue culture dishes for Western blot experiments or on 12 mm coverglass circles for immunofluorescence experiments. The cultures were maintained in Steinberg's solution [60 mM NaCl, 0.7 mM KCl, 0.4 mM $Ca(NO_3)_2$, 0.8 mM $MgSO_4$, 10 mM HEPES, pH 7.4] supplemented with 10% L-15 medium, 1% fetal bovine serum, and 100 μg/ml gentamycin at 22°C (Peng et al., 1991b). To study NMJs in culture, neural tubes from *Xenopus* embryos were separated from the myotomes after collagenase treatment (1 mg/ml for 30 min) and dissociated into single neurons in Ca^{2+}, Mg^{2+}-free Steinberg's solution. They were then plated onto 3–4 d muscle cultures. A ratio of five neural tubes per muscle culture was found to be satisfactory in the establishment of easily identifiable nerve–muscle pairs. Functional NMJs can be observed within the first day of N-M coculture.

Xenopus kidney epithelial cell line A6 (Rafferty, 1969) was obtained from Dr. Howard Fried (Department of Biochemistry, University of North Carolina at Chapel Hill) and from American Type Culture Collection (Rockville, MD). It was cultured in Waymouth's culture medium with 10% fetal calf serum and karamycin/gentamycin at 22°C in tissue culture flasks.

Western blots. Muscle and epithelial cultures were washed three times with phosphate-buffered saline (PBS) and then lysed by the addition of lysis buffer (25 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.5% Na-deoxycholate, 2% NP-40, 0.2% SDS, 10 μg/ml aprotinin, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, and 0.5 mM PMSF). Cells were scraped off from the dishes and briefly homogenized with a Brinkmann (Westbury, NY) PT-1200 Polytron-type homogenizer. The protein concentration of the lysate was determined with the Bio-Rad (Richmond, CA) method using a kit supplied by the company. Samples were prepared according to Laemmli (Laemmli, 1970) and were resolved on a 12% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membrane (Bio-Rad) with a semi-dry electrophoretic transfer device (Pharmacia-LKB, Piscataway, NJ). The membrane was treated with the blocking buffer [10 mM Tris, pH 8, 50 mM NaCl, 0.05% Tween-20, 2% BSA, and 1% cold fish gelatin (Sigma, St. Louis, MO)] for 1 hr and then incubated with antibody against HB-GAM at 2 μg/ml reconstituted in Tris buffer (same as the blocking buffer except for the omission of BSA) at room temperature. After washing with the Tris buffer, the membrane was incubated with HRP-conjugated secondary antibody (Sigma) and the protein bands were visualized with the enhanced chemiluminescence (ECL) method with a kit supplied by Du Pont (Boston, MA).

Immunofluorescent labeling. Cultures were immunolabeled with antibody against HB-GAM in the living state. Cultures were incubated with antibodies reconstituted with culture medium to a concentration of 50–66 nM for 45 min, rinsed and labeled with a FITC or rhodamine-conjugated goat anti-rabbit secondary antibody (Organon Teknika, Durham, NC) for 30 min. The labeling was carried out either at room temperature or at 4°C. They were either observed alive or fixed with 95% ethanol at −20°C followed by mounting in a polyvinyl alcohol based mounting medium which contained 5% *n*-propyl gallate as antifading agent (Giloh and Sedat, 1982). To visualize AChR clusters, muscle cultures were also double labeled with tetramethylrhodamine-conjugated α-bungarotoxin (R-BTX) at a concentration of 0.3 μM for 30 min. As a control, we labeled cultures with the secondary antibody alone without the primary antibody. These cultures were completely devoid of fluorescent staining. The competition between HB-GAM and bFGF in binding to HSPG was studied by treating cultures with recombinant bFGF (Synergen, Boulder, CO) at a concentration of 1 μg/ml for 30 min, followed by labeling with a mouse monoclonal antibody against this protein (Type I from UBI, Lake Placid, NY) and the rabbit polyclonal HB-GAM antibody to visualize these two proteins at the cell surface.

A monoclonal antibody against heparan sulfate (HepSS-1 from Seikagaku) was used to detect the HSPG on the surface of muscle cells (Kure and Yoshie, 1986). It was used at 1:200 dilution to label live cultures. The staining pattern of this antibody was similar to that obtained through the use of mAb 2AC2, an anti-HSPG core protein antibody kindly provided by Dr. M. J. Anderson (University of Calgary).

Heparin and heparinase treatment. Low molecular-weight heparin (Sigma, M.W. 4000–6000) was used to displace HB-GAM from the cell surface. Cultures were treated with heparin at a concentration of 100–500 μg/ml for 1 hr, followed by immunolabeling. To remove the HSPG from the cell surface, we treated cultures at room temperature with heparinase (Type I from Sigma) at a concentration of 50 U/ml for 4 hr in culture medium for immunolabeling or in PBS for Western blot. This was followed by antibody labeling. For Western blot, the conditioned medium after heparinase treatment was collected and centrifuged at 13,000 rpm. The supernatant was freeze dried and desalted with spin columns (Bio-Rad) and prepared for PAGE and Western blots. After the heparinase treatment, the cells were also harvested by scraping them off the substrate, pelleted by centrifugation, washed and lysed with lysis buffer, and prepared for PAGE.

Induction of AChR clustering by beads coated with HB-GAM. Ten micrometer polystyrene latex beads (Polysciences) were washed with 95% ethanol, rinsed, and incubated with recombinant HB-GAM in the range of 1–100 μg/ml in PBS for 1–3 hr. The beads were then washed with PBS and the unoccupied area on the bead surface was blocked with culture medium containing 1% fetal bovine serum or with PBS containing 1 mg/ml BSA. The beads were then applied to muscle cultures. After an overnight incubation, the cultures were labeled with R-BTX or HB-GAM antibody. To visualize the adsorption of HB-GAM

on beads, we labeled the beads with anti-HB-GAM antibody followed by fluorescently conjugated secondary antibody. The size of bead-induced clusters was quantified by digital video imaging methods. The images were acquired with a SIT camera connected to a computer running IMAGE-1 image processing software (Universal Imaging, Chester, PA). The clusters were then individually quantified with the analysis program in the same software package.

Suramin and tyrphostin application. Suramin was obtained from FBA Pharmaceuticals (New York, NY). Cultures were treated with this drug at a concentration ranging from 1–100 μM for 15 min before the addition of HB-GAM–coated beads. The drug concentration was maintained through the duration of the 24 hr bead–muscle coculture. Tyrphostins, RG-50864 (active) and RG-50862 (inactive), were kind gifts of Rhone-Poulenc Rorer Central Research (Horsham, PA). They were dissolved in dimethyl sulfoxide (DMSO) to make a 40 mM stock that was stored at −80°C. Cultures were pretreated with tyrphostin in the range of 20–100 μM for 3 hr before the beads were added. Thereafter, they were maintained in the tyrphostin-containing medium for 24–48 hr before R-BTX labeling.

Bath application of HB-GAM and amphoterin. Stock solution of recombinant HB-GAM or amphoterin was dialyzed against Steinberg solution through a membrane with 3000 M.W. cutoff. The dialyzed solution was then diluted with culture medium. To each muscle culture on 12 mm round coverglass circle placed in an 8 well dish whose bottom was lined with parafilm, 50 μl of medium with HB-GAM or amphoterin was added. Neurons, suspended in minimum amount of medium after dissociation, were seeded into each culture. The 8 well dish was then sealed, placed in a moisture chamber and incubated at 22°C for 18–24 hr. The cultures were then labeled with R-BTX and examined live under a fluorescence microscope. The presence of AChR clusters along each unambiguous N-M contact visualized with phase-contrast optics was scored. The percentage of contacts that were positive in AChR clusters was calculated for each sample.

Immunofluorescent labeling of embryos. The dorsal parts of embryos at stage 22–32, containing the myotomes, the neural tube and the notochord, were excised. They were briefly treated with Ca^{2+}, Mg^{2+}-free Steinberg's solution to remove the skin. The pieces were then labeled in the living state with HB-GAM antibody followed by rhodamine-conjugated secondary antibody. In some experiments, pieces were double labeled with FITC-conjugated α-bungarotoxin. The embryo pieces, either in the living state or after fixation with 95% ethanol at −20°C, were then mounted by sandwiching them between a slide and a coverslip, which caused their flattening. This made the myotomes clearly visible by their characteristic segmented appearance. The edge of the coverslip was sealed with nail enamel, and the specimen was observed under a fluorescence microscope.

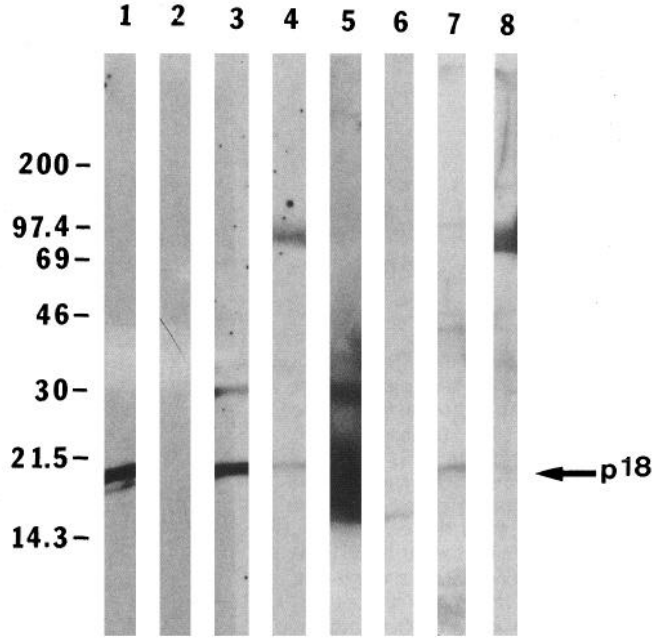

Figure 1. Western blot of HB-GAM. *Lane 1,* Recombinant HB-GAM, 0.25 μg; *lane 2,* recombinant bFGF, 0.25 μg; *lane 3,* A6 cell lysate; *lane 4,* muscle cell lysate; *lane 5,* conditioned medium from A6 cultures after heparinase treatment; *lane 6,* A6 cell lysate after heparinase treatment; *lane 7,* conditioned medium from muscle cultures after heparinase treatment; *lane 8,* muscle cell lysate after heparinase treatment. In *lanes 1, 3, 4, 7,* a band at 18 kDa (*p18*) corresponding to HB-GAM can be seen. The 100 kDa band seen in *lanes 4* and *8* is due to yolk protein in these muscle cells, which is very sticky and tends to give nonspecific antibody binding. The 30 kDa minor band in *lanes 3* and *5* is routinely seen in the A6 preparation. Since it is eluted from the cell surface after heparinase treatment (*lane 5*) and is no longer seen in the cell lysate (*lane 6*), this protein is probably also bound to the HSPG. It may represent another protein that shares structural homology with HB-GAM. The broadening of the bands in *lane 5* is due to protein overloading. Affinity-purified HB-GAM antibody was used in this experiment.

Results

Immunoblot analysis of HB-GAM in Xenopus *cells*

To examine the presence of HB-GAM in *Xenopus* muscle cells, we first conducted Western blot analyses on these cells. As shown in Figure 1 (lane 1), the polyclonal antibody made against baculovirus-derived recombinant HB-GAM recognized the recombinant protein as a major 18 kDa band. The minor band at a slightly lower molecular mass in this lane is probably a proteolytic breakdown product. In contrast, the same amount of recombinant bFGF, which also has a molecular mass of 18 kDa, was not recognized by this antibody (Fig. 1, lane 2). When the lysate of cultured *Xenopus* muscle cells was electrophoresed, blotted, and probed with this antibody, a band at 18 kDa was clearly seen (Fig. 1, lane 4). In this and other muscle samples, a nonspecific band at 100 kDa was also observed. This band is due to the yolk protein that is present in large amount inside these cells in the form of yolk platelets. Since the yolk protein is very sticky, it tends to adsorb antibodies in a nonspecific manner, resulting in nonspecific bands in Western blots (Evans and Kay, 1991). A previous study has shown that HB-GAM is also found in embryonic rat kidney (Merenmies and Rauvala, 1990). Thus, we also studied the expression of this protein in the A6 cell line derived from *Xenopus* kidney epithelial cells (Rafferty, 1969) as a way to further confirm the specificity of the antibody to the *Xenopus* protein. As shown in Figure 1 (lane 3), the A6 cell lysate also exhibited a prominent band at 18 kDa when the Western blot was probed with the anti-HB-GAM antibody.

Since HB-GAM is a heparin-binding molecule, it may be bound to the HSPG at the cell surface. To test this, we treated cultures with heparinase, collected and concentrated the conditioned medium, and probed it with the HB-GAM antibody. We found that most of the HB-GAM was released into the medium in both A6 (Fig. 1, lane 5) and muscle (Fig. 1, lane 7) cultures by heparinase. Only a small amount of this protein was left on cells after enzymatic treatment (Fig. 1, lane 6 for A6 and lane 8 for muscle). These studies suggest that the bulk of HB-GAM is present on the cell surface in a heparinase-sensitive manner.

Immunolocalization of HB-GAM

Immunofluorescence studies were conducted on live cultures to determine the cellular localization of this molecule. As shown in Figure 2, *A* and *B,* HB-GAM antibody labeled the cell surface in a punctate, streaky or filamentous pattern in noninnervated muscle cells. The confocal image in Figure 2*A* shows that the entire cell surface was labeled. Most, but not all, of the labeling was removed by treating cells with heparinase (Fig. 2*B*) or with

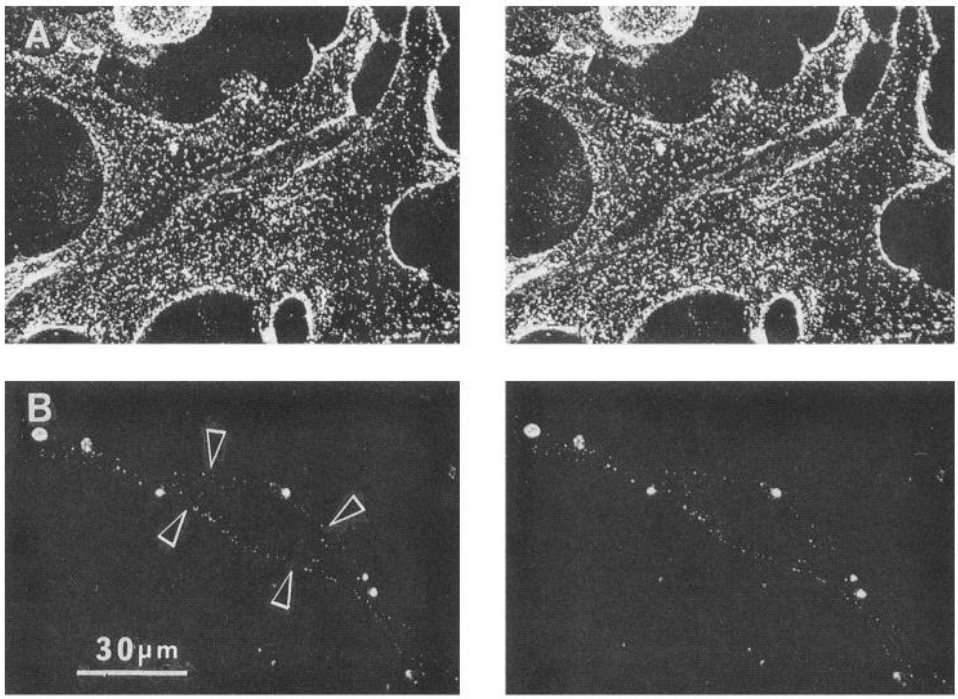

Figure 2. Confocal stereo image pairs of HB-GAM distribution on the surface of muscle cells before (*A*) and after (*B*) heparinase treatment. HB-GAM is present on the entire cell surface. Heparinase removes a significant amount of this protein from the cell surface. The *arrowheads* in *B* point to residual HB-GAM staining after the enzymatic treatment. The larger fluorescent objects around the cell periphery are autofluorescent yolk granules released from damaged cells during dissociation.

heparin (data not shown). Heparinase partially removes heparan-sulfate glycosaminoglycan (GAG) side chains from the core protein of HSPG, whereas heparin presumably solubilizes factors that are bound to HSPG at the cell surface. These results thus indicate that HB-GAM is associated with HSPG at the surface of *Xenopus* muscle cells, in consistency with Western blot data presented above. A punctate labeling pattern of HB-GAM was also observed on the surface of A6 cells and heparinase also abolished the bulk of the labeling on these cells (data not shown). In addition to the diffusely distributed pattern, patches of the cell surface where HB-GAM was concentrated were also observed (Figs. 3*B,D*; 4*B,D,F*). These HB-GAM hot spots were, in fact, often colocalized with AChR clusters (Fig. 4) as described below. No difference in the appearance of HB-GAM labeling at 20°C versus 4°C was observed. This rules out the possibility that the labeling pattern is due to a redistribution of the antigen.

To demonstrate further the association of HB-GAM with HSPG, we double-labeled muscle cells with the rabbit polyclonal HB-GAM antibody and a mouse monoclonal antibody against heparan sulfate (mAb HepSS-1). The patterns of HB-GAM and HSPG hot spots were then compared. As shown in Figure 3, *A* and *B*, and *C* and *D*, a good colocalization of these two molecules was often observed at these patches. However, this is not a constant feature as some of the HSPG patches were clearly under populated by HB-GAM as shown in Figure 3, *E* and *F*. This suggests that HB-GAM only occupies a fraction of the available binding sites on the GAG chains of the HSPG molecules. By double labeling muscle cells with mAb HepSS-1 and a rabbit polyclonal antibody against perlecan (kindly provided by Dr. J. R. Hassell, University of Pittsburgh), the major basement-membrane HSPG (Noonan et al., 1991), we recently found that the HSPG that HB-GAM binds to is indeed perlecan (D.F.D. and H.B.P., unpublished results).

In our previous study, we showed that exogenously applied bFGF binds to HSPG at the surface of *Xenopus* muscle cells (Baker et al., 1992). To determine whether the site on HSPG occupied by HB-GAM is also recognized by bFGF, we incubated cells with a 1 μg/ml solution of bFGF for 30 min and then doubly labeled them with the HB-GAM antibody and a mouse monoclonal antibody against bFGF. Our results showed that the application of exogenous bFGF nearly abolished HB-GAM staining (Fig. 3*G,H*). This observation shows that exogenous bFGF was able to displace the endogenous HB-GAM from its binding sites on the HSPG. To determine whether this displacement was simply due to the basic nature of bFGF, we also examined the effect of another heparin-binding protein amphoterin (p30), which is highly basic in its N-terminal amino-acid composition (Merenmies et al., 1991), and two polycations, polyornithine and polylysine. We found that amphoterin at 1 μg/ml was much less effective and polyornithine and polylysine at similar concentration were ineffective in displacing HB-GAM. Thus, bFGF and HB-GAM may compete specifically for the same GAG sequences of HSPG.

Colocalization of HB-GAM with AChR clusters and the NMJ

To understand whether HB-GAM is involved in the formation of AChR clusters in cultured muscle cells, we compared the localization of these two molecules at sites where they were concentrated. Double labeling cultures with R-BTX and HB-GAM and FITC-conjugated secondary antibody showed a general colocalization of HB-GAM antibody and AChR hot spots in uninnervated muscle cells (Fig. 4*A–D*). However, when examined at high magnification as in Figure 4*A–D*, it became clear that these two types of specializations were not identical despite their colocalization. The HB-GAM clusters were in general slightly larger than the AChR clusters (Fig. 4*C,D*). Quantitation of this relationship showed that 61% of AChR hot spots were substantially overlapped by HB-GAM (as shown in Fig. 4*A–D*), 31% of them showed partial overlap and 8% were not correlated with HB-GAM at all (n = 144). We also examined the relationship between these two molecules at bead-induced AChR clus-

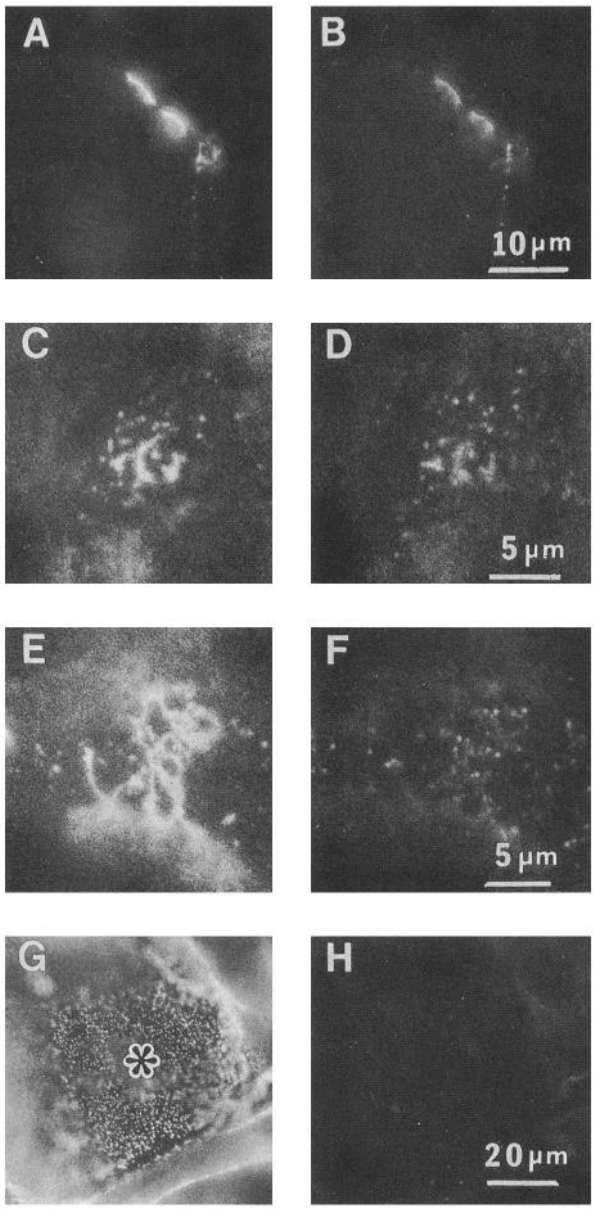

Figure 3. *A–F,* Correlation between HSPG and HB-GAM in muscle cells. Cultures were double labeled with a heparan-sulfate antibody (*A, C, E*) and HB-GAM antibody (*B, D, F*). The top two examples (*A–B* and *C–D*) show good spatial correlation of HSPG and HB-GAM hot spots. In the third example (*E–F*), only a small amount of HB-GAM is associated with this HSPG hot spot. (*G–H*) Applying recombinant bFGF (1 μg/ml for 30 min) to the cells resulted in bright bFGF labeling (*asterisk* in *G*) at the expense of HB-GAM (*H*). This shows that bFGF can compete with HB-GAM for binding sites on HSPG.

ters. In this study, uncoated polystyrene beads were used to induce receptor clustering according to previous methods (Baker et al., 1992). As shown in Figure 4, *E* and *F,* HB-GAM was also concentrated at bead-induced AChR clusters, although there was a discrepancy both in morphology and in size.

To study the influence of innervation on HB-GAM distribution, we cocultured muscle cells with neurons dissociated from neural tubes of *Xenopus* embryos. R-BTX labeling revealed the formation of NMJ at 50%–90% of the nerve–muscle contacts as evidenced by the clustering of AChRs. Double labeling with both R-BTX and HB-GAM antibody showed that this molecule was concentrated at AChR clusters at the NMJ. Two examples of this colocalization are shown in Figure 5. These nerve-induced AChR clusters were generally associated with a relatively precise colocalization of HB-GAM. Thus, innervation induces both AChRs and HB-GAM to cluster at the nerve–muscle contact. It should be noted, however, that the HB-GAM was still present diffusely at extrasynaptic membrane even after innervation (Fig. 5*B,E*). Labeling live, cultured *Xenopus* spinal cord neurons not in contact with muscle cells showed no HB-GAM at their surface (H.B.P., unpublished results).

HB-GAM was also detected at AChR clusters in C2C12 myotubes by immunostaining with the anti-recombinant HB-GAM antibody and with an affinity-purified anti-HB-GAM peptide antibody that binds to the rodent sequence (Rauvala, 1989), whereas polyclonal antibodies to the mouse midkine protein did not stain these myotubes (data not shown). This indicates that the immunostaining of AChR clusters by the HB-GAM antibodies is not due to midkine, another member of this protein family. Thus, the colocalization of HB-GAM with AChR clusters is a conserved phenomenon from *Xenopus* to mouse.

Induction of AChR clustering by local presentation of HB-GAM via beads

To examine whether HB-GAM can function as an inducer for postsynaptic development in skeletal muscle, we locally applied this molecule to cultured *Xenopus* muscle cells via beads. Polystyrene 10 μm latex beads were coated with recombinant rat HB-GAM by incubating them in a solution containing HB-GAM at a concentration of 100 μg/ml for several hours. Beads coated with HB-GAM showed intense fluorescence after labeling with HB-GAM antibody and fluorescent secondary antibody, indicating the adsorption of HB-GAM to the beads. Control beads coated with bovine serum albumin (BSA), fetal bovine serum, or other factors, such as EGF, NGF, and PDGF, were not labeled by the antibody (data not shown). These beads were then applied to muscle cultures. After an incubation period ranging from 6 to 24 hr, the cultures were labeled with R-BTX. As shown in Figure 6, a high percentage (between 60% and 90%) of bead–muscle contacts were associated with AChR clusters. These clusters were discretely localized to the contact area. In contrast, control beads coated with BSA, serum proteins, EGF, PDGF, NGF, immunoglobulins, laminin, or tenascin were ineffective in inducing AChR clustering even though they also adhered to the cells (Peng et al., 1991a). The efficacy of the beads in inducing AChR clustering depends on the surface HB-GAM concentration. Beads incubated in a solution containing 10 μg/ml or less of HB-GAM were ineffective in inducing AChR clustering and there was an abrupt increase in the cellular response at 25 μg/ml incubation concentration.

In addition to inducing AChR clustering at sites of contact, these HB-GAM-coated beads were also effective in causing a dispersal of preexisting AChR hot spots at sites away from the beads (data not shown). Thus, these beads can mimic two effects of nerve innervation on the skeletal muscle: the local induction of AChR clustering at nerve–muscle contacts and the global dispersal of AChR hot spots at extrasynaptic sites.

The role of tyrosine kinase activation in the bead-induced AChR clustering

To test whether the effect of HB-GAM in inducing AChR clustering is mediated by a cell surface receptor, we studied the effect of suramin on the bead-induced AChR clustering. Suramin

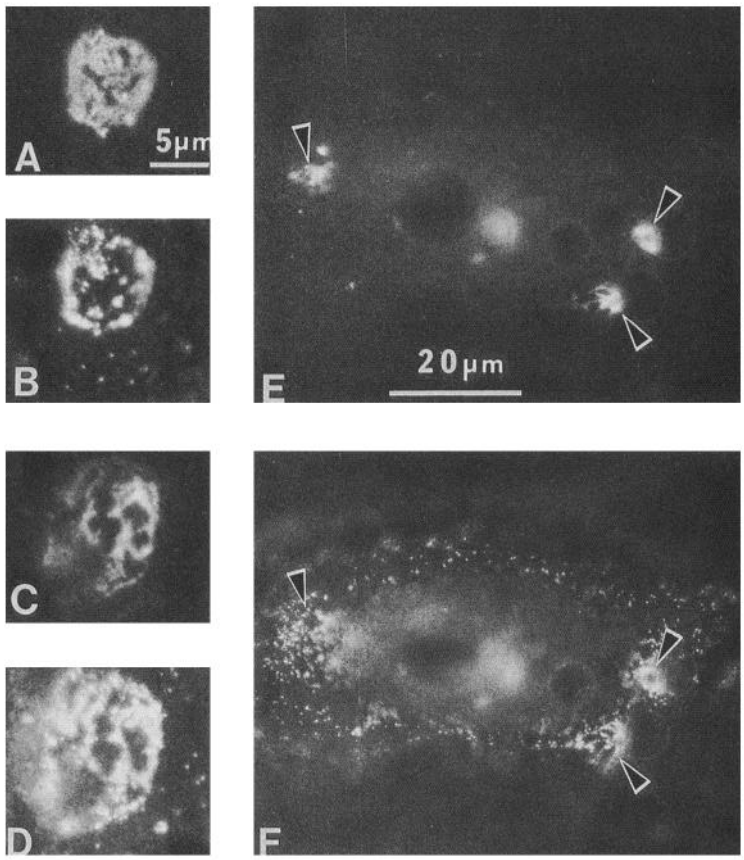

Figure 4. Association of HB-GAM with AChR clusters. *A, C, E,* R-BTX labeling; *B, D, F,* HB-GAM labeling. HB-GAM is closely associated with preexisting AChR hot spots (*left column*) or bead-induced clusters (*right column, arrowheads*), although they are not congruent in their fine patterns.

is a small (MW 1500), polyanionic molecule that has been widely used to interfere with the ligand–receptor interaction involved in peptide growth factor signaling (Betsholtz et al., 1986; Coffey et al., 1987; Huang and Huang, 1988; Fleming et al., 1989; Behrendt et al., 1993). At a concentration of 100 μM, suramin reduced the percentage of HB-GAM bead–muscle contacts that were positive in AChR clusters to 30% of the control value. The clusters that did form in the presence of suramin were much smaller in size. The median cluster size was about 10.3 μm^2 in the control ($n = 100$) and was reduced to 2.7 μm^2 in 50 μM suramin ($n = 100$).

Although the receptor for HB-GAM has not been elucidated, we suspected that the activation of a tyrosine kinase–mediated signaling pathway may be involved in the induction of AChR clustering by this molecule as suggested by previous studies (Qu et al., 1990; Wallace et al., 1991; Baker and Peng, 1993). This was tested by the use of the tyrosine kinase inhibitor tyrphostin RG-50864 (Lyall et al., 1989; Levitzki and Gilon, 1991; Seckl and Rozengurt, 1993). As shown in Figure 7, the efficacy of beads in inducing AChR clustering was blocked by this compound with a half-inhibitory concentration of 50 μM. Cells remain viable at these concentrations of tyrphostin as shown in previous studies (Peng et al., 1991a; Baker and Peng, 1993). In addition to blocking the formation of AChR clusters, tyrphostin also abolished the effect of beads in causing the dispersal of preexisting AChR hot spots (data not shown). Thus, this tyrosine kinase inhibitor reversed both the local, cluster-inducing and the global, cluster-dispersing effects of the beads. An inactive form of tyrphostin, RG-50862, was used as a control in these studies and was found to have no effect on bead-induced AChR clustering.

Effect of bath HB-GAM application on NMJ formation

To further understand the role of HB-GAM in NMJ formation, we investigated the effect of interfering with the local HB-GAM signaling in the formation of AChR clusters induced by N-M contact. Since a good neutralizing antibody against HB-GAM had not been generated, we tested the effect of exogenous HB-GAM in AChR clustering. Muscle cultures were treated with recombinant HB-GAM and then seeded with spinal cord neurons. After an 18–24 hr coculture period, the cultures were labeled with R-BTX and AChR clustering at N-M contacts was assessed. The percentage of contacts that exhibited AChR clustering was determined by scoring all of the clearly identifiable contacts in each culture. In control cultures not treated with HB-GAM, more than 90% of contacts exhibited AChR clustering in this series of experiments. HB-GAM at a concentration of 25–50 μg/ml (1.4–2.8 μM) significantly suppressed the nerve-induced clustering process and essentially abolished it at 75–100 μg/ml (4.2–5.6 μM) (Fig. 8). An example of this suppression is shown in Figure 9 (compare *A* with *B* and *C*). Clusters that did develop at these high HB-GAM concentrations appeared to be more diffuse and less intense than the control ones (Fig. 9*D*). Bath application of HB-GAM, however, did not result in an increase in the formation of AChR clusters in extrasynaptic area of nerve-contacted muscle cells or in noninnervated muscle cells. Nor did we observe a decrease in the number of AChR hot spots at these concentrations. This suggests that the bath-

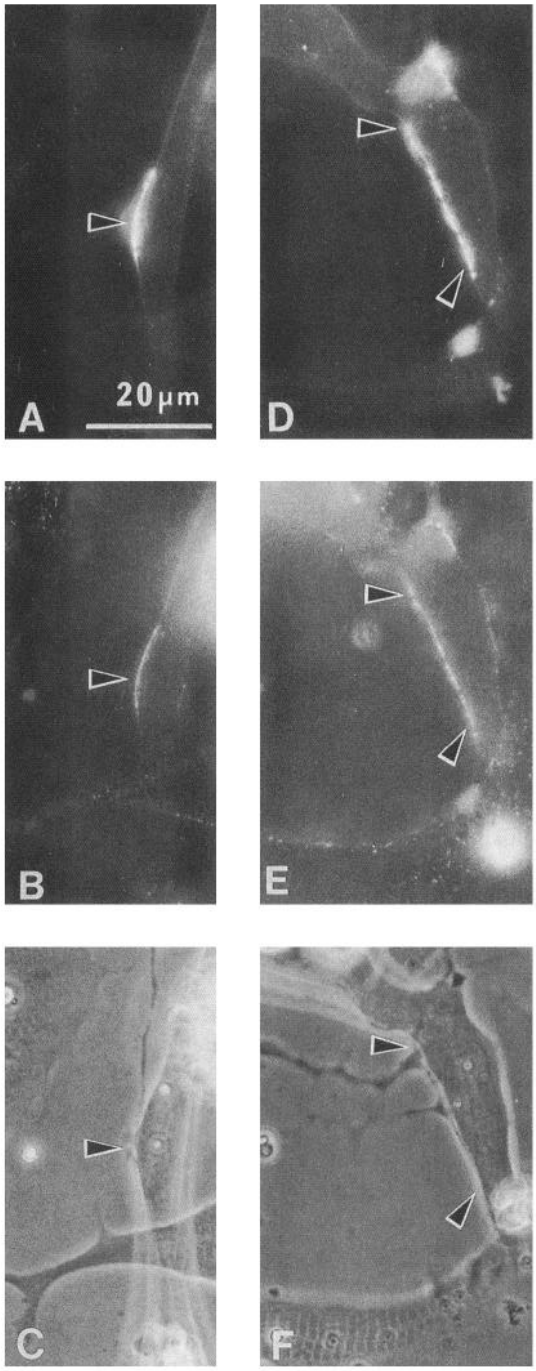

Figure 5. Association of HB-GAM with the NMJ in culture. The *left* and *right columns* are two different examples. *A, D,* R-BTX labeling; *B, E,* HB-GAM labeling; *C, F,* phase contrast. HB-GAM is concentrated at nerve-induced AChR clusters (*arrowheads*), although it is also present in extrajunctional areas (*B, E*). The bright object at the lower right-hand side of *E* is a fluorescent cell debris.

applied HB-GAM did not cause deleterious effect on the muscle cell. When the HB-GAM concentration was raised to 200 μg/ml, hot spots were indeed dispersed over a 24 hr period. The cells remained viable even at this concentration.

To determine the specificity of the HB-GAM inhibition in nerve-induced AChR clustering, we also treated cultures with amphoterin (Atn), another heparin-binding protein that contains approximately the same proportion (28%) of cationic, mainly

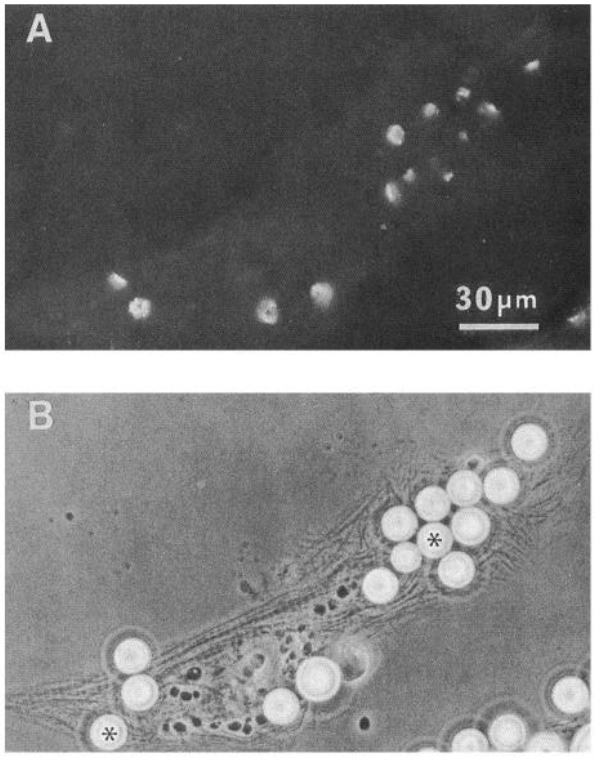

Figure 6. Induction of AChR clustering by HB-GAM-coated beads. R-BTX labeling (*A*) revealed the high efficacy of these beads in inducing this specialization: only 2 out of a total of 15 bead-muscle contacts (*B, asterisks*) failed to induce clusters.

lysine, residues as in HB-GAM (Merenmies et al., 1991). As shown in Figures 8 and 9, *E* and *F,* this protein had no effect on nerve-induced AChR clustering at concentrations as high as 100 μg/ml.

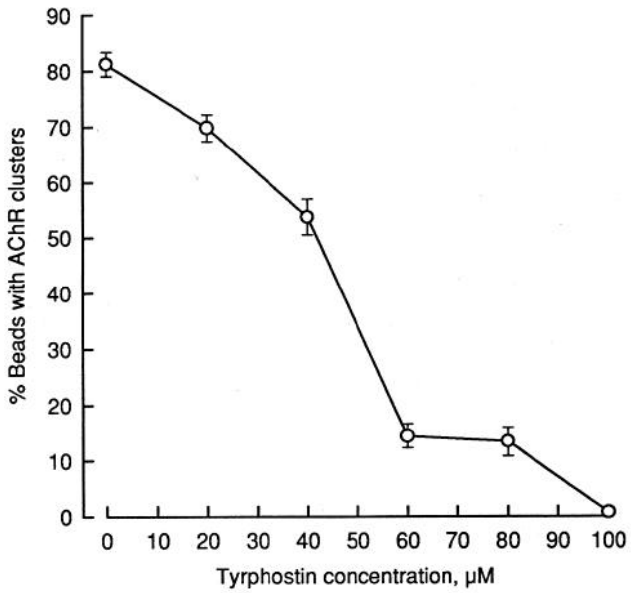

Figure 7. Inhibition of AChR clustering induced by HB-GAM-coated beads by the tyrosine kinase inhibitor tyrphostin RG50864.

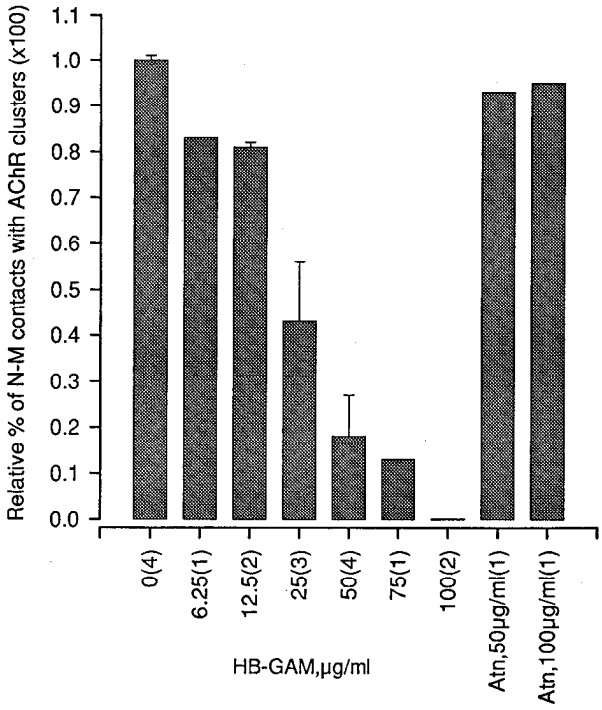

Figure 8. Effect of bath HB-GAM application on NMJ formation. The percentage of N-M contacts with AChR clustering was calculated from each N-M coculture and is expressed as a relative value to the control culture. The value for each concentration is the mean of several experiments. The number of experiments for each concentration is indicated in the parentheses. The error bars are SEs. HB-GAM, at concentrations above 25 μg/ml, significantly suppressed AChR clustering induced by the nerve. In contrast, another heparin-binding protein amphoterin (*Atn*) was ineffective.

Distribution of HB-GAM in vivo

To begin to understand the role of HB-GAM *in vivo,* we conducted preliminary experiments to examine its distribution in *Xenopus* embryos. Dorsal embryo pieces were labeled with HB-GAM antibody followed by fluorescent secondary antibody in living state without fixation, similar to the method used in labeling cell cultures described above. As shown in Figure 10, *A* and *C* (arrowheads), HB-GAM labeling was observed within the space between adjacent myotomes in embryos at stage 22–24. This intermyotomal area, filled with various ECM molecules, is traversed by processes from motoneurons to establish NMJs at the ends of the myotomal muscle fibers which abut this space. However, at this early stage, NMJs had not formed as shown by the absence of AChR clusters (Fig. 10*B*). When older embryos (after stage 30) were examined, more prominent HB-GAM labeling was detected in this region (Fig. 10*D*). At these later stages, the HB-GAM localization was comparable to the sites of NMJs as shown by the location of AChR clusters (Fig. 10*E*). These data suggest that HB-GAM is first concentrated at future sites of NMJ formation prior to the arrival of the nerve and later becomes colocalized with the junctions. HB-GAM was absent from the notochord with the live-labeling method employed in this study.

Discussion

In this study, we have shown that HB-GAM is an endogenous muscle-derived factor normally stored on the HSPG at the surface of *Xenopus* muscle cells. Despite its concentration at AChR hot spots, this molecule is generally distributed in a diffuse manner on the cell surface in a pattern analogous to the HSPG distribution. Innervation results in concentration of HB-GAM at the NMJ. The induction of AChR clustering by HB-GAM–coated beads suggests a role for this molecule in signaling postsynaptic development. The activation of a tyrosine kinase–mediated pathway is probably involved in the signal transduction process activated by HB-GAM, since it is blocked by an inhibitor to tyrosine kinase. In addition, bath application of HB-GAM antagonizes the nerve-induced AChR clustering process. To our knowledge, this is the first demonstration of an endogenous peptide growth factor that colocalizes with a synapse and can act as its inducer.

Previous works have demonstrated two other roles for HB-GAM. This molecule has been shown to be a neurite-promoting and guidance factor when it is bound to the substratum (Rauvala, 1989; Kuo et al., 1990; Li et al., 1990; Hampton et al., 1992; Raulo et al., 1992; Rauvala et al., 1994). In fact, neurons in the CNS are rich sources for HB-GAM (Rauvala et al., 1994). Immunocytochemical studies using fixed and permeabilized neurons have shown its presence in the soma and the neurite (Rauvala, 1989). Its absence from the surface of live neurites as shown in this study may be correlated with the lack of an extensive HSPG-containing ECM at the neuronal surface. HB-GAM may also be a mitogen for certain cell lines (Li et al., 1990; Fang et al., 1992), although this effect is controversial (Merenmies, 1992; Raulo et al., 1992). This molecule shares high amino acid homology with two other heparin-binding factors, midkine (Matsubara et al., 1990; Muramatsu et al., 1993) and retinoic acid–inducible heparin-binding factor, which may be the avian homolog of midkine (Vigny et al., 1989; Cockshutt et al., 1993). Thus, HB-GAM is a member of an emerging family of new HBGFs which may play regulatory roles in development.

Although the receptor for HB-GAM has not yet been elucidated, a recent study showed that this factor can be crosslinked to 127 and 155 kDa cell surface proteins on NIH 3T3 cells (Kuo et al., 1992) and can stimulate tyrosine phosphorylation of a 200 kDa protein in two fibroblast cell lines (Li and Deuel, 1993). Thus, a kinase-dependent signaling pathway is likely involved in the interaction of HB-GAM with cells. This is also supported by our current result that a tyrosine kinase inhibitor blocks AChR clustering induced by HB-GAM beads.

The role of HSPG and its associated factors in synaptogenesis

HSPGs are ubiquitous components of the ECM in a variety of cell types, including skeletal muscle and neurons (Ruoslahti, 1989; Bernfield et al., 1992; Yanagishita and Hascall, 1992; Halfter, 1993). Recent studies have elucidated two roles for this class of molecules. First, they serve as storage sites for HBGFs, such as bFGF, heparin-binding EGF, and HB-GAM (Rauvala, 1989; Rifkin and Moscatelli, 1989; Higashiyama et al., 1992). This provides a mechanism for localizing these factors to the cell surface, a strategic location for intercellular signaling. In addition, a second role for HSPG as an active participant in the signaling process has recently been elucidated (Klagsbrun and Baird, 1991; Ruoslahti and Yamaguchi, 1991). As shown in the case of bFGF receptor, the formation of a ternary complex, involving the receptor, bFGF and HSPG, is essential for cell stimulation (Rapraeger et al., 1991; Yayon et al., 1991; Kan et al., 1993). Whether this paradigm also applies to the action of HB-GAM awaits further studies.

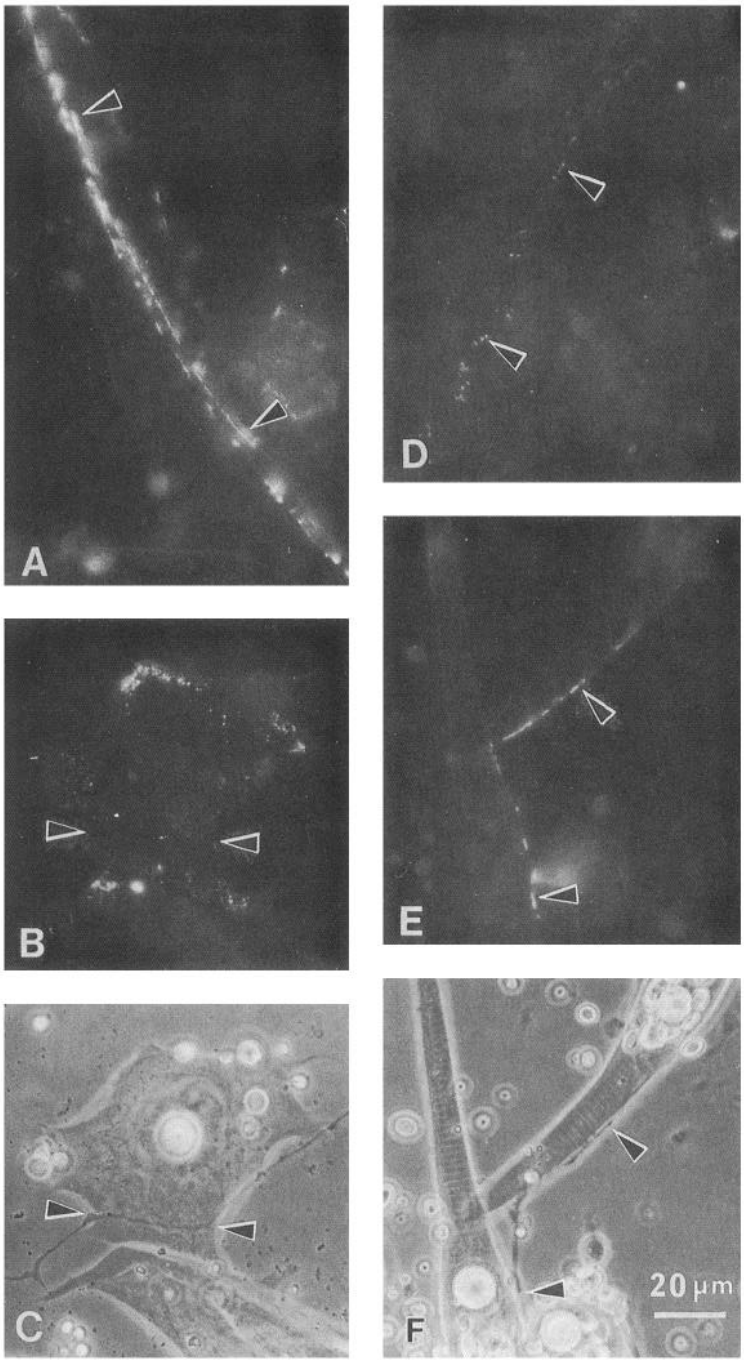

Figure 9. Effect of bath HB-GAM application on NMJ formation. *A,* NMJs in a control N-M coculture. The R-BTX labeling shows extensive clustering of AChRs (*arrowheads*) on two adjacent muscle cells innervated by a common nerve. *B, C,* In the presence of 50 μg/ml HB-GAM in the bath, the nerve-induced AChR clustering was substantially suppressed as shown in this example. The N-M contact shown in *C* (between *arrowheads*) was devoid of AChR clustering (*B*), although hot spots were present on this muscle cell. *D,* At higher HB-GAM concentration (75 μg/ml), the few remaining nerve-induced clusters showed a diffuse appearance and were generally much dimmer than control ones as shown by R-BTX fluorescence. *E, F,* In contrast to HB-GAM, amphoterin at 100 μg/ml had no effect on AChR clustering induced by the nerve.

In the case of NMJ development, the involvement of HSPG in the induction of AChR clustering is suggested by several lines of evidence. First, HSPG is concentrated at the NMJ both *in vivo* and *in vitro* and is coclustered with AChRs during innervation of cultured muscle cells (Anderson and Fambrough, 1983; Anderson et al., 1984; Bayne et al., 1984). This property is also shared by HB-GAM as shown in this study. Second, culturing nerve and muscle cells in the presence of heparin and heparan sulfate, but not condroitin sulfate, suppresses the formation of AChR clustering induced by the nerve in culture (Hirano and Kidokoro, 1989; see also Baker et al., 1992). In genetic variants of the mouse C2 myotubes that are defective in GAG synthesis, either in its elongation or sulfation, spontaneous clustering of AChRs into hot spots fails to occur (Gordon and Hall, 1989; Gordon et al., 1993). Recent studies have shown that heterokaryons of these defective cells that have restored GAG synthesis exhibit normal AChR clustering (Jung et al., 1993). This further illustrates the necessity of HSPGs in AChR clustering.

Our results suggest that the importance of HSPG in AChR clustering may lie in its association with HBGFs and HB-GAM is probably one such factor. The strong heparin-binding property of this class of proteins enables them to be localized in the ECM. When these molecules are bound to the HSPG, they exist in a kind of latent form. Upon cell–cell contact, these factors are locally presented to interacting cells and become active in signaling (Saksela and Rifkin, 1990). This presentation is presumably carried out by the nerve during innervation. The capability to present these molecules may be specific to the neuron, since

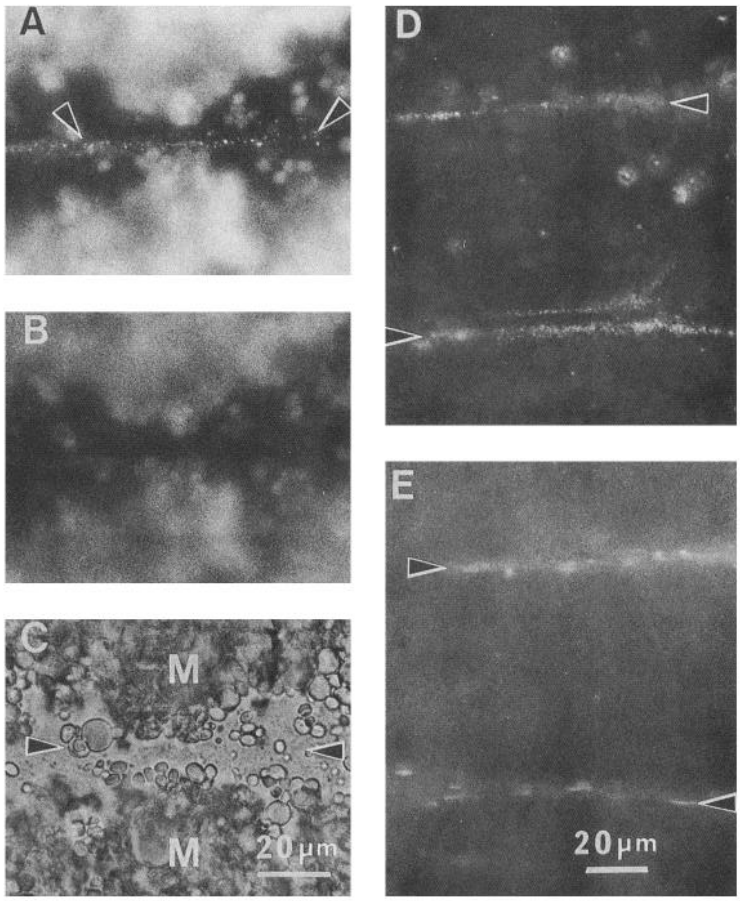

Figure 10. HB-GAM localization in the myotomes of the *Xenopus* embryo. *A–C,* Stage 24 embryo. HB-GAM was concentrated within the inter-myotomal region (*arrowheads* in *A*), which can be seen in bright field in *C*. At this stage, NMJs had not formed as shown by the absence of fluorescent BTX labeling in this region (*B*). The myotomes (*M*) contained a lot of yolk which yielded autofluorescent background as shown in *A* and *B*. *D–E,* Stage 30–32 embryos. The HB-GAM labeling (*arrowheads* in *D*) can be seen more prominently in the intermyotomal region in these older embryos. This labeling pattern was comparable to the distribution of AChR clusters as shown by fluorescent BTX labeling (*arrowheads* in *E*).

AChR clustering does not occur at muscle–muscle contact in *Xenopus* cultures. The nature of the presentation mechanism is unknown. Previous studies suggest that the release of proteases by the growth cone may modulate the HSPG at N-M contact to effect the presentation of its bound factors (Anderson, 1986; Seeds et al., 1992). Alternatively, the nerve may secrete specific molecules, such as neuronal HSPG (Halfter, 1993), that can competitively displace factors bound to the muscle HSPG to effect stimulation at the contact site. The stimulation probably involves the activation of a receptor tyrosine kinase by the locally displaced HB-GAM as our inhibitor study shows. In any event, our study demonstrates that beads can effectively mimic the nerve in presenting these factors to the muscle cell to effect synaptic induction. On the other hand, bath application of exogenous HB-GAM suppresses the cluster formation. This can be explained by the fact that muscle cells usually respond only to local signals presented by the nerve during synaptogenesis. When they are globally stimulated by exogenous ligand, the cell is most likely overwhelmed so that it can no longer discern the positional information provided by the nerve via its local presentation of endogenous HB-GAM, thus resulting in failure to cluster AChRs.

In agreement with the suggested role as a muscle-derived synaptogenic factor, HB-GAM is expressed in developing muscle *in vivo*. This was previously shown by Northern and Western blots (Li et al., 1990; Merenmies and Rauvala, 1990) and by *in situ* hybridization (Vanderwinden et al., 1992). Immunostaining of the rat limb buds shows that after the midgestation period (on embryonic days 15–17) HB-GAM is mainly found in desmin-positive muscle cells, in which it partially colocalizes with the AChR clusters (E. Szabat and H. Rauvala, unpublished results). The localization of this molecule at the intermyotomal junction where NMJs develop as revealed in this study is also consistent with its role *in vivo*. Significantly, the HB-HAM promoter contains several consensus (CANNTG) sequences for MyoD binding (Li et al., 1992; Milner et al., 1992). This suggests that the expression of HB-GAM is linked to muscle differentiation, which is known to be controlled by transcription factors of the MyoD family. The presence of MyoD binding sites within the HB-GAM promoter suggests a coordinate expression of HB-GAM and AChR in muscle cells, since the expression of AChR subunits is also known to be controlled by MyoD-type transcription factors (Salpeter et al., 1992).

Extensive studies have established the central role of agrin, an ECM-bound molecule enriched at the NMJ, in directing postsynaptic differentiation (McMahan et al., 1992; Cohen et al., 1994; Bowe and Fallon, 1995). The results of this study suggest that agrin and HB-GAM may both be involved in postsynaptic induction. Whether these represent independent or overlapping pathways awaits further investigation.

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