Stimulation of cell motility by a viral fibroblast growth factor homolog: Proposal for a role in viral pathogenesis

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Abstract

The Autographa californica M nucleopolyhedrovirus (AcMNPV) encodes a gene (open reading frame 32) with homology to vertebrate and invertebrate fibroblast growth factors (fgfs), key regulators of developmental processes affecting the growth, differentiation, and migration of many cell types. We studied the temporal regulation of the AcMNPV vfgf, by Northern (RNA) blot hybridization; vfgf was transcribed as a 0.6-kb mRNA at early times but as part of a 1.4-kb bicistronic mRNA at late times. The product of vfgf, vFGF, exhibited a number of characteristics that have also been demonstrated for other FGF homologs. vFGF had strong affinity to heparin, a property important for FGF signaling via an FGF receptor. vFGF was secreted into the extracellular fluid when expressed in insect cells, suggesting that it acts as an extracellular ligand. Finally, vFGF was able to stimulate migration of several different types of insect cells. We discuss how this activity may be important for its function during virus infection.

Keywords: Fibroblast growth factor; Cell motility; Viral pathogenesis; Baculoviruses

Introduction

Fibroblast growth factors (FGFs) are a family of growth factors widespread in organisms ranging from nematodes to humans; however, they are not present in unicellular organisms (Ornitz and Itoh, 2001; Powers et al., 2000). In general, the structure of FGFs is conserved, and they share a core region of similarity throughout 140 amino acids in length, including 28 highly conserved amino acids, of which a subset interacts with the receptor (Ornitz and Itoh, 2001). Most, but not all, FGFs encode a terminal signal peptide and are secreted from cells. FGFs have an array of functions working as mitogens, motogens, and differentiation factors. In addition, they have crucial roles in many developmental processes that include neural cell differentiation, mesoderm induction, limb formation, osteogenesis, angiogenesis, and branching morphogenesis during the development of lungs, seminiferous tubules, and insect trachea. Inappropriate expression of some fgfs may lead to the pathogenesis of cancer. FGFs function by binding heparin or heparan sulfate proteoglycans to form oligomers, and this complex interacts specifically with FGF receptors (FGFRs), transmembrane tyrosine kinases that are activated upon FGF binding, leading to receptor dimerization and autophosphorylation. The activated FGFR then stimulates signal transduction pathways (reviewed in Powers et al., 2000).

The prototype baculovirus, Autographa california M nucleopolyhedrovirus (AcMNPV), contains a large DNA genome of 133,894 base pairs (bp) encoding about 150 genes (Ayers et al., 1994) and infects insects in the order Lepidoptera. AcMNPV produces two forms of the virus during its replication cycle with identical genetic makeup and nucleocapsid structure: budded virus (BV) and occluded derived virus (ODV). The BV is produced during the late phase of infection where viral DNA and proteins are packaged to form nucleocapsids that bud through the plasma membrane, thus acquiring an envelope (Adams et al., 1977). The BV spreads systemically within the insect...
and is also the form of the virus that is used in cell culture. The ODV is produced during the very late phase of infection where nucleocapsids remain in the nucleus, are enveloped, and then are embedded in a matrix that protects the virion in the environment. When the infected insect dies, it releases occlusion bodies that another insect ingests (O’Reilly et al., 1992).

Studies on baculovirus pathology concluded that after infection of midgut cells, tracheal epithelial cells become infected, and then BV appears in the hemolymph followed by the infection of other tissues (Keddie et al., 1989; Trudeau et al., 2001). The hemolymph may be the main route of BV distribution in some infected insects (Adams et al., 1977; Granados and Lawler, 1981). It has been proposed that the tracheal system of the insect serves as a conduit for systemic infection as the virus crosses the basal lamina into the hemocoel of the insect (Engelhard et al., 1994). Other studies suggest that the BV directly passes through the basal lamina into the hemolymph where the virus disseminates (reviewed in Federici, 1997).

Upon infection of insect cell lines with AcMNPV, three major classes of transcripts, early, late, and very late, are apparent (O’Reilly et al., 1992). Early transcripts encode factors necessary for DNA replication and transcription of late viral genes, as well as auxiliary genes that have roles in virus–host interactions. After DNA replication, late and very late genes are transcribed by a viral RNA polymerase activity (Glockner et al., 1993; Grula et al., 1981; Huh and Weaver, 1990). Translated late and very late products include, but are not limited to, structural proteins.

The AcMNPV open reading frame (ORF) 32 has homology to fibroblast growth factors (FGFs) (Ayers et al., 1994), and it is conserved in all baculoviruses that have been sequenced to date that infect insects in the order Lepidoptera (Garcia-Maruniak et al., 2004; Lauzon et al., 2004). However, fgf homologs are absent from baculoviruses that infect other orders than Lepidoptera: Culex nigripalpus NPV that infects insects in the order Diptera (mosquitoes) (Afonso et al., 2001), and Neodiprion sertifer NPV and Neodiprion lecontei NPV that infect insects in the order Hymenoptera (sawflies) (Garcia-Maruniak et al., 2004; Lauzon et al., 2004). The AcMNPV fgf, vfgf, was first discovered by sequence homology when the complete nucleotide sequence of AcMNPV was derived (Ayers et al., 1994); however, its function during infection is not known. A study of the BmNPV ffg indicated that it was expressed at early times post infection (p.i.) using reverse transcriptase-polymerase chain reaction analysis but the size of the transcripts or temporal expression in the presence of protein and DNA replication inhibitors was not presented (Katsuma et al., 2004). This report also concluded that the BmNPV FGF was secreted and glycosylated, but its functionality was not addressed (Katsuma et al., 2004). In this study, we characterize in detail the temporal expression and transcripts of vfgf, evaluate biochemical characteristics of its product that correlate with the function of other known FGFs, and assess its properties for chemoattraction of cells. Based on these characteristics, we propose a role for vFGF in viral pathogenesis, but emphasize that other roles are also possible.

Results

Baculovirus fgfs

vfgf is present in all baculoviruses sequenced to date that infect lepidopterans, but it is absent in the three baculoviruses that have been completely sequenced to date that infect other insects. Fig. 1 shows an alignment of the predicted polypeptide sequence of vfgfs in selected group I and II NPVs and granuloviruses, and compares them to the sequence of the Drosophila FGF branchless and a human FGF, highlighting a conserved signal sequence, and a heparin binding-growth factor/FGF family domain (Burgess and Macia, 1989; Thomas, 1988). Potential N-glycosylation sites in most, but not all, FGFs are indicated.

Northern blot analysis of vfgf transcripts

We determined the temporal regulation and overall size of vfgf mRNAs by Northern blot analysis. Inspection of sequences upstream of the vfgf ORF revealed the presence of a TATA box 29 bp upstream of the translational start codon. We infected cells with AcMNPV at a multiplicity of infection of 20 PFU per cell, purified total RNA at different times p.i., and performed Northern blot analysis using an RNA-specific probe derived from the vfgf ORF as described in Materials and methods (Fig. 2A).

A 0.6-kb transcript was first observed at 3 h p.i. and remained until 12 h p.i. when a probe complementary to the vfgf ORF was utilized (Fig. 2B). At 24 and 48 h p.i., transcription slightly decreased. Transcription was also observed in the presence of cycloheximide, a protein synthesis inhibitor, indicating that prior protein synthesis was not required for vfgf transcription. This is indicative of early genes, where synthesis is dependent on host proteins and/or viral proteins carried in the virus particle but not on viral gene products that need to be synthesized after virus infection. Similarly, vfgf was transcribed in the presence of aphidicolin, a DNA synthesis inhibitor. Late and very late viral genes require viral DNA replication for expression. Thus, since vfgf is transcribed at early times p.i., and in the presence of cycloheximide and aphidicolin, it can be designated an early gene. The early 0.6-kb RNA agrees with the predicted size for an RNA corresponding to the 546-nt vfgf ORF, given that the putative transcriptional start site and polyadenylation signals are fairly close the beginning and end of the ORF, respectively.

In addition, two prominent larger late RNAs of 1.4 and 3.1 kb were observed starting at 9–12 h p.i. (Fig. 2B). These late transcripts also hybridized to a probe complementary to ORF
33 (Fig. 2C). In agreement with late viral genes, these RNAs were not transcribed either in the presence of cycloheximide or aphidicolin. Interestingly, \textit{vfgf} may be transcribed at late times as part of a 1.4-kb bicistronic mRNA, since this mRNA hybridized to probes complementary to ORFs 32 and 33. The gene upstream of and in the same direction of \textit{vfgf}, \textit{orf 33} (550 nt in length), does not appear to contain a polyadenylation signal at its 3' end and its transcripts may coterminate with the \textit{vfgf} early mRNA using the polyadenylation signal downstream the \textit{vfgf} ORF (17 nt downstream of the translational stop codon for \textit{vfgf}). We do not know the identity of the larger 3.1-kb RNA and did not pursue it any further in this study.

Less abundant late mRNAs were also apparent (Figs. 2B and C, lanes 24). The first late transcriptional start sites upstream of the \textit{vfgf} ORF are present 202 and 15 nt upstream of the translational start codons of \textit{vfgf} and \textit{orf 33}, respectively. Whether these mRNAs utilize either of these late transcriptional start sites was not investigated. We also performed primer extension analysis and observed two early primer extension products that terminated 12 and 13 nt upstream of the translational ATG codon of the \textit{vfgf} ORF (results not shown). A short leader sequence starting at 10 nt upstream of the translational start codon was also observed in the BmNPV \textit{fgf} (Katsuma et al., 2004).

![Alignment of baculovirus FGFs, Drosophila FGF branchless (Bnl), and a human FGF. In the sequences shown, dark grey shading denotes identical residues, light grey shading shows similarities among proteins, bold-type residues highlight the putative heparin-binding growth factor/FGF family domain, italicized residues indicate putative cleavable signal sequence, underlined residues show putative N-glycosylation sites, numbers in parenthesis refer to amino acids of the corresponding polypeptide, and double slashes indicate the predicted cleavage sites. GenBank database accession numbers for the sequences shown are as follows: A. californica MNPV (AcMNPV) FGF (NC 001623), B. mori MNPV (BmNPV) FGF (NC 001962), Choristoneura fumiferana MNPV (CfMNPV) FGF (NC 004778), Orgyia pseudotsugata MNPV (OpMNPV) FGF (NC 001875), Lymantria dispar MNPV (LdNPV) FGF (NC 001973), Xestia c-nigrum GV (XecnGV) FGF (NC 2331), Plutella xylostella GV (PxGV) FGF (NC 002593), human FGF 16 (NM 003868), and Drosophila FGF Bnl (NM 169875).](image-url)

Fig. 1.
vFGF production and secretion

We constructed a plasmid, pHSFGFHA, using the Drosophila heat shock protein 70 (hsp70) promoter to express vfgf with an in-frame C-terminal influenza hemagglutinin (HA) epitope tag. SF-21 cells were transfected with pHSFGFHA and protein lysates from either the cell pellet or the culture supernatant were resolved by SDS–PAGE and immunoblotted with anti-HA monoclonal antibody. We found that a protein of about 20 kDa, in agreement with the predicted size for vFGF, was expressed from this plasmid. vFGF was found in the cell lysate but was also secreted into the culture medium (Fig. 3, lanes 1 and 2), consistent with its N-terminal predicted signal sequence. vFGF in the cell lysate may potentially contain vFGF bound to the extracellular matrix. A plasmid containing a viral RNA polymerase subunit (P47) tagged with HA that was not expected to be secreted was used as a control to monitor any contamination in the pellet and supernatant fractions (Fig. 3, lanes 3 and 4). The fgf from Bombyx mori NPV has also been shown to be secreted (Katsuma et al., 2004).

vFGF affinity to heparin

FGFs bind heparan sulfate proteoglycans in the extracellular matrix, and upon cleavage, complexes of heparin-bound FGFs bind and activate FGFRs. Previously characterized FGFs bind heparin-Sepharose and elute at high salt concentrations between 1.0 and 2.0 M NaCl (reviewed in Powers et al., 2000). To test whether vFGF had high affinity to heparin like other characterized FGFs, the supernatant was collected from SF-21 cells previously transfected with pHSFGFHA, applied to heparin-Sepharose, and bound proteins were eluted with increasing concentrations of NaCl. vFGF routinely started to elute between 1.0 and 1.2 M NaCl (Fig. 4A), indicating that vFGF conserved the property of binding heparin strongly that is critical for the function of other FGFs.

Fig. 2. Northern blot analysis of vfgf transcripts. (A) A schematic shows vfgf and adjacent open reading frames and the homologous region 2 (hr2) where the thick filled arrows indicate the direction of transcription. The transcripts that traverse the region (thin arrows) and their temporal expression (E, early; L, late) are shown above the open reading frames. The sizes of the transcripts in panels B and C below are indicated in parentheses and to the right of the thin arrows. The dotted arrow indicates that the 5’ and 3’ ends of the transcript were not defined. The asterisk (*) indicates the approximate location of a polyadenylation signal. Thin arrows below the open reading frames labeled “probe 1” and “probe 2” indicate RNA probes used in Northern blots in panels B and C, respectively. (B and C) Total RNA was extracted from SF-21 cells either mock-infected (mi) or infected with AcMNPV at the time indicated at the top in hours. RNA was extracted from cells treated with cycloheximide (12C) or aphidicolin (12A) at 12 h p.i. The size and position of predominant transcripts are indicated. M, molecular weight markers.

Fig. 3. Secretion of vFGF. SF-21 cells were transfected with either pHSFGFHA (lanes 1 and 2) or a plasmid expressing HA-tagged P47, pHSEpiHisp47, (lanes 3 and 4). Extracellular proteins present in the supernatant (sup; lanes 2 and 4) or intracellular proteins in the cell lysate (cell; lanes 1 and 3) were resolved by SDS–PAGE, proteins were transferred to a PVDF membrane, and reacted with anti-HA antibody. The migration of vFGF and P47 is indicated by arrows to the right. The asterisk (*) indicates a non-specific immunoreactive band in the supernatant.
Transfection of SF-21 cells with a plasmid expressing HA-tagged OpIAP, a baculovirus inhibitor of apoptosis (Birnbaum et al., 1994), a protein that we did not expect to have high affinity for heparin, eluted from heparin primarily in the flow through when a cell lysate was allowed to bind heparin-Sepharose (Fig. 4B).

Chemoattraction properties of vFGF for different cell types

One characteristic of FGFs is their ability to induce cell motility. We tested the ability of vFGF for chemoattraction of SF-21, TN-368, and hemocytes isolated from last instar Trichoplusia ni (Fig. 5). pHSFGFHA or pBluescript was transfected into SF-21 cells and the supernatant containing vFGF or proteins present in the supernatant of vector-transfected cells was partially purified by heparin-Sepharose and used in cell migration assays. Expression of vfgf before and after heparin purification was monitored by immunoblotting using anti-HA antibody (data not shown).

Since vFGF was not purified to homogeneity, we could not add a specific and known amount of vFGF in all experiments. Thus, we generated a dose-response curve for

Fig. 5. Chemoattractant activity of vFGF. (A) Different concentrations of heparin-purified vFGF or control proteins were tested for their ability to enhance motility using TN-368 cells in three independent assays. The relative CellTiter-Glo units of cells treated with vFGF compared to control-treated cells (assigned a value of 1.0) are presented on the ordinate axis. The numbers on the abscissa indicate the volume (µl) of heparin-purified and eluted proteins used. The optimal concentration of vFGF (2 µl) was used to perform transmigration experiments with TN-368 cells (C) below. Similar titration experiments were performed with SF-21 and T. ni hemocytes to determine the optimal concentration required for these cells. SF-21 (B), TN-368 (C), or last instar T. ni hemocytes (D) were plated in the upper chamber of a transwell and allowed to migrate to vFGF. vFGF and control proteins were obtained from SF-21 cells transfected with pHSFGFHA (vFGF) or pBluescript control (ctrl), respectively, and proteins in the extracellular fluid were partially purified by heparin-Sepharose. An optimal amount of heparin-purified vFGF, as determined by titration of vFGF in transmigration assays, was added to the lower compartment of the transwell. After 3.5 – 4 h, a measurement of viable cells that had migrated to the lower compartment was obtained by CellTiter-Glo. The bars above each column indicate the mean ± standard error of at least three independent experiments.
chemoattraction of insect cells for each batch of partially purified vFGF and used the optimal concentration of vFGF in transmigration assays. An example is presented in Fig. 5A. vFGF was not able to induce transmigration of insect cells when low concentrations of the protein (e.g., 0.2–1.0 μl, Fig. 5A) were assayed. Optimal concentrations were reached with 2.0 μl of vFGF, and addition of higher concentrations of vFGF (e.g., 10–20 μl, Fig. 5A, and results not shown) reduced activity, probably due to diffusion of vFGF, preventing the ability of a gradient of FGFs to be established.

We observed that vFGF was able to stimulate transmigration of cells derived from the ovarian cell-derived Lepidoptera cell lines permissive for AcMNPV infection, SF-21 and TN-368 cells, 2.3- and 1.8-fold, respectively, compared to transmigration of these cells to proteins purified from cells transfected with p Bluescript (Figs. 5B and C). Similar results have been reported for other better characterized FGFs (Lee et al., 2003; Ritch et al., 2003). Interestingly, vFGF was able to stimulate migration of hemocytes obtained from last instar T. ni larvae, consistent with the possibility that vFGF is able to attract hemocytes in the infected host (Fig. 5D). In addition, this vFGF-stimulated cell motility was verified by microscopic examination of cells in the lower transwell compartment (results not shown). In these experiments, the migration observed in the controls may be due in part to secreted cellular FGFs that were purified and to which target cells in the apical transwell insert may be responding.

Discussion

Most FGFs are secreted from cells where they act extracellularly. In addition, they have a high affinity to heparin. Finally, among their properties is their ability to function as chemoattractants. These three properties are conserved in the AcMNPV fgf in cell culture, since it has a functional signal peptide that allows secretion of the polypeptide (Fig. 3), it binds heparin (Fig. 4), and enhances cell migration (Fig. 5).

A few examples exist of viruses that encode growth factors other than fgfs or viruses that affect host fgfs. However, to our knowledge, the baculovirus fgfs are the only fgfs encoded by viruses. The vaccinia virus growth factor, VGF, has homology to epidermal growth factors (Bloomquist et al., 1984; Brown et al., 1985; Reisner, 1985) and is a virulence factor that stimulates cell growth (Buller et al., 1988). Another well-described example is the viral vascular endothelial growth factor, vVEGF, encoded by parapoxviruses (Lyttle et al., 1994; Ueda et al., 2003), that is involved in angiogenesis (Savory et al., 2000). In mice, retroviruses use either WNT or FGF as integration hot spots, activating the genes, and leading to carcinogenesis (Katoh, 2002). Respiratory syncytial virus (RSV) enhances the production of basic FGF, and this may lead to fibroblast activation involved in remodeling the airway epithelium due to chronic inflammatory changes associated with asthma (Dosanjh et al., 2003). Upregulation of basic FGF was suggested to explain prolonged wheezing after RSV infection (Dosanjh et al., 2003). More recently, Raf-induced VEGF augmented Kaposi’s sarcoma-associated herpesvirus infection of cells (Hamden et al., 2004).

Branching epithelial morphogenesis is central in vertebrate development of lungs, vasculature, kidneys, and most glands, involving cell migration, division, and attracting more cells to form complex structures apparent in mature organs. In insects, tracheal morphogenesis leads to a network of epithelial tubules that deliver oxygen to every tissue, and Drosophila has served as a model system to study the morphological, genetic, and molecular aspects of this process. The Drosophila FGF gene, branchless, is responsible for controlling tracheal cell migration and the pattern of branching (Sutherland et al., 1996). Its receptor, breathless, is expressed on the surface of developing tracheal cells (Glazer and Shilo, 1991; Klambt et al., 1992). Localized activation of the receptor guides the migration of tracheal cells forming primary and secondary branches aided by a number of factors (reviewed in Ghabrial et al., 2003). Terminal branching is regulated by oxygen requirements in cells signaled by branchless (Jarecki et al., 1999). Thus, branchless is an essential mitogen and chemoattractant in the development of the embryonic, larval, and differentiated adult tracheal system (Sato and Kornberg, 2002; Sutherland et al., 1996). The AcMNPV FGF is 42% identical to the FGF core sequences of branchless (amino acids 220–409 of branchless). These core sequences encode structural components composed of 12 antiparallel β-strands, heparin-, and receptor-binding regions.

Twenty-six baculovirus genomes are available to date and 23 encode vfgf. The viruses encoding vfgf infect Lepidoptera (moths), initiating infection in the midgut of the insect larvae. Infection then spreads systemically until the insect dies. Some granuloviruses encode more than one copy of vfgf (accession number NC004062; Hashimoto et al., 2000; Wormleaton et al., 2003). The advantage for more than one copy of vfgf with respect to pathogenesis or whether both copies are functional is not known. Furthermore, it would be interesting to find out if the diverse tissue tropisms exhibited by granuloviruses, ranging from midgut restrictions to virus distribution in several tissues (Federici, 1997), are related to vfgf function. The baculoviruses that do not encode fgf have a smaller genome and are phylogenetically considered to be more ancient and divergent from those that infect Lepidoptera (Garcia-Maruniak et al., 2004; Lauzon et al., 2004). These divergent viruses infect the Culex mosquito (Diptera) and Neodiprion sawflies (Hymenoptera). More interestingly, these baculoviruses only replicate at the primary site of infection, the epithelial cells of the midgut, and do not spread systemically (Federici, 1997; Moser et al., 2001). It is possible that vfgf is required...
for efficient spread of infection beyond the midgut or systemically. Thus, baculoviruses infecting Lepidoptera may have acquired the \( fgf \) homologs in order to efficiently invade and replicate in additional tissues.

Given the biological and biochemical characteristics of vFGF presented in this study, possible functions for \( vfgf \) in viral pathogenesis can be proposed, although alternative functions at other stages of the viral replication cycle are also possible. vFGF may attract uninfected hemocytes to trachea or other infected tissues by chemotaxis, allowing the virus to infect these cells and be spread through the open circulatory system of the insect. Hemocytes are motile cells that migrate towards wound sites or invading organisms for encapsulation. One possibility is that vFGF is used as a lure in which hemocytes are signaled by vFGF in a similar manner to that of chemokine signals that are sent by vertebrate cells upon virus infection to attract immune cells to sites of infection. vFGF may also induce cell proliferation of hemocytes or other cell types, thus augmenting its replication capacity by increasing the number of progeny virus. Cell proliferation was observed in response to the vaccinia virus virulence factor, VGF, yielding increased numbers of infectious progeny (Buller et al., 1988). Alternatively, vFGF may be serving as a signal for branching and migration of tracheal cells that allow virus accessibility to uninfected tissues.

This study presents the initial functional characterization of the Ac\( MNPV \) \( fgf \) and its implications in virus spread by modulating the host signal transduction pathways. We are in the process of characterizing a \( vfgf \) knockout virus in vitro and in vivo to discriminate among its possible roles during virus pathogenesis. Its function as a virulence factor may be useful in augmenting the applications of recombinant baculoviruses used for pest control, including baculoviruses that infect mosquitoes.

Materials and methods

Cells and virus

TN-368 cells (Hink, 1970) derived from the cabbage looper \( T. \ ni \) and IPLB-SF-21 cells (SF-21) derived from the fall armyworm \( Spodoptera frugiperda \) (Vaughn et al., 1977) were cultured at 27 °C in TC-100 medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 0.26% tryptose broth as described previously (O’Reilly et al., 1992). The Ac\( MNPV \) L1 strain (Lee and Miller, 1978) was propagated and titered in SF-21 cells, and used to infect cells at a multiplicity of infection of 20 plaque-forming units (PFU) per cell (O’Reilly et al., 1992).

Plasmid construction

The plasmid pHSFGFH\( A \) contained the \( vfgf \) ORF under control of the \( Drosophila \) hsp70 promoter, the HA epitope tag at the C terminus of FGF, and a polyadenylation signal derived from the \( Orygia pseudotsugata \) inhibitor of apoptosis gene (\( Opiap \) ) (Crouch and Passarelli, 2002). First, the \( vfgf \) ORF was amplified by PCR using a plasmid containing viral genomic DNA in this region. The amplified product expanded from the translational start ATG codon to the last codon excluding the translational stop codon and included an \( XhoI \) at the 5′ end and a \( NotI \) site at the 3′ end to facilitate cloning. Next, the amplified product was cloned into pBlue-script at the \( XhoI \) and \( NotI \) sites to generate pBSSF\( G \). The HA tag and a translational stop codon were introduced at the C terminus of FGF by annealing two complementary primers (5′-\( GGCCGCCTACCCATACGACGTCCCAGATTA-CGCTAACC\)-3′ and its complement) containing \( NotI \) and Sac\( II \) sites at the ends and cloned into the \( NotI \) and Sac\( II \) sites of p\( BSF \). The resulting plasmid containing a C-terminally tagged \( vfgf \), p\( BSSF\( GH \), was cut with \( XhoI \) and \( SacI \) to obtain a fragment with the HA-tagged \( vfgf \). Finally, the ends were repaired with T4 DNA polymerase and cloned into the Klenow-repaired \( Bsu 361 \)I sites of p\( HF \) (Crouch and Passarelli, 2002) to replace the green fluorescent gene for the HA-tagged FGF, and the resulting plasmid was named p\( HF \). The sequence of \( vfgf \) insert in p\( HF \) was verified by nucleotide sequencing.

The plasmids pH\( SEpiOpIAP \) and p\( HSEpiHisp47 \) have been previously described (Vucic et al., 1997). Briefly, the plasmid pH\( SEpiOpIAP \) contains the \( Op-\)iap\( 3 \) gene with an N-terminal HA epitope tag driven by the \( Drosophila \) hsp70 promoter. p\( HSEpiHisp47 \) contains the Ac\( MNPV \) p\( 47 \) gene with N-terminal HA and poly-histidine tags under control of the hsp70 promoter.

Transfection of cells

In experiments in which vFGF was detected in the cell lysate or supernatant of transfected cells, 2 \( \mu \)g of either p\( HF \) or p\( HSEpiHisp47 \) was introduced into 0.5 \( \times \) \( 10^6 \) SF-21 cells plated on 35-mm cell culture dishes by using 3 \( \mu \)l of a liposome preparation as previously described (Crouch and Passarelli, 2002). Cells were maintained for 4 h at 27 °C in the liposome-DNA mix. This mix was then replaced with TC-100 media containing 10% fetal bovine serum and incubated at 27 °C. Twenty-four hours after addition of DNA, cells were incubated at 42 °C for 30 min to induce expression of genes from the hsp70 promoter. Cells were allowed to express proteins for 4 h at 27 °C before cells and supernatant were harvested. In heparin purification experiments, 6 \( \mu \)g of either p\( HF \) or p\( Blue\)script II SK\( (+) \) (Stratagene) was transfected into 3.0 \( \times \) \( 10^6 \) SF-21 cells as described above, but proteins were collected 36 h post-transfection.

Insect rearing and hemocyte collection

\( T. \ ni \) eggs were obtained from Entopath Inc. (Easton, PA). Individual first instar caterpillars were transferred to 1-
oz cups containing cabbage looper diet (Southland Products, Inc.) and reared at 27 °C with a 12-h light/dark cycle. Caterpillars were allowed to grow to the last instar.

Last instar caterpillars were chilled at 4 °C for 30 min before hemolymph extraction. Hemolymph was collected by cutting an anal proleg and allowing the caterpillar to bleed onto a piece of parafilm. Hemolymph was transferred to ice-cold anti-coagulant buffer (4 mM NaCl, 40 mM KCl, 1.7 mM piperazine-\(N,N'\)-bis(2-ethanesulfonic acid) (PIPES), 146 mM sucrose, 0.1% polyvinylpyrrolidone, 8 mM EDTA, 9.5 mM citric acid, and 27 mM sodium citrate) at 1:1 vol/vol ratio and centrifuged at 300 \(\times\) g at 4 °C. Unseparated hemocyte cells were washed twice with ice-cold anti-coagulant buffer and then resuspended in the same buffer. Hemocytes were counted using a hemocytometer and 2–3 \(\times\) 10^4 cells were used in transmigration assays.

RNA isolation

SF-21 monolayers (2 \(\times\) 10^6 cells per 60-mm diameter dish) were infected with AcMNPV at a multiplicity of infection of 20 PFU per cell. The virus was allowed to adsorb for 1 h at room temperature, and time zero was defined as the time when the virus was removed and incubation at 27 °C commenced. The protein synthesis inhibitor cycloheximide was added to the cells at 100 \(\mu\)g/ml for 30 min before infection and maintained thereafter. The DNA synthesis inhibitor aphidicolin was added at 5 \(\mu\)g/ml following virus adsorption. Total RNA was isolated at selected hours p.i. with Trizol reagent (Invitrogen).

Northern blot analysis

Samples of total RNA (20 \(\mu\)g per lane) were electrophoresed on a formaldehyde–1% agarose gel, transferred to a nylon membrane, and hybridized to \(\alpha\)-32P-radiolabeled riboprobes. Two probes were generated by first PCR-amplifying vfgf from the translational start codon to the translational stop codon and ORF 33 from 27,727 to 28,284 nt of AcMNPV (Ayers et al., 1994) (from 6 nt downstream of the translational stop codon to 3 nt upstream of the translational start codon). PCR products were cloned by using the TA Cloning kit (Invitrogen) and the cRNA probes were synthesized by in vitro transcription using T7 RNA polymerase in the presence of \(\alpha\)-32P[UTP].

Immunoblotting

Cell culture supernatant from transfected cells was collected and spun down at 1000 \(\times\) g for 5 min to remove residual cells, and proteins in the supernatant were mixed with sodium dodecyl sulfate (SDS)-Laemmli buffer. Attached cells were washed twice with phosphate-buffered saline (PBS), pH 6.2 (Potter and Miller, 1980), and collected with SDS-Laemmli buffer. Proteins from cells and supernatant were resolved by SDS–15% polyacrylamide gel electrophoresis (PAGE), transferred to a PVDF membrane (Pierce), and immunodetected with 1:1000 dilution of HA.11 monoclonal antibody (Covance), 1:3000 dilution of goat anti-mouse IgG-horseradish peroxidase (Bio-Rad), and SuperSignal chemiluminescent substrate (Pierce).

Purification of vFGF through heparin-Sepharose

Supernatant from cells transfected with pHSFGFHA or pBluescript was harvested and spun down at 1000 \(\times\) g at 4 °C to remove residual cells. Proteins in the supernatant of transfected cells were then incubated with heparin-Sepharose 6 Fast Flow beads (Amersham Biosciences) previously washed with excess deionized water three times for 4 h at 4 °C and constant rotation. Subsequently, the mixture was loaded onto a 2-ml column (BD Bioscience Clontech) and washed with phosphate buffer (10 mM NaH2PO4) containing different concentrations of NaCl from 0.15 to 2.0 M as indicated. Each fraction was collected and proteins were detected by immunoblotting. Proteins eluted with phosphate buffer containing 30% glycerol and 1.4 M NaCl and were used in transmigration assays.

Transmigration assays

Cell migration was assessed using Costar transwells with polycarbonate membrane inserts. For migration of SF-21 and TN-368 cells, approximately 3–5 \(\times\) 10^4 cells were loaded onto 8 \(\mu\)M pore-size transwell inserts. Different amounts of heparin-purified vFGF or control proteins (pBluescript-transfected cells) as indicated were added to 24-well plates containing 600 \(\mu\)l of PBS, pH 6.2, or TC-100 lacking serum. For hemocyte migration, approximately 2–3 \(\times\) 10^4 cells were loaded onto 3 \(\mu\)M pore-size transwell inserts and different amounts of heparin-purified vFGF or control proteins were added to 24-well plates in which each well contained 600 \(\mu\)l of anti-coagulant buffer. The transwell inserts were then transferred to the 24-well plates containing heparin-purified proteins. After 3.5- to 4-h incubation at 27 °C, transwell inserts were removed and cells that migrated were quantified using CellTiter-Glo luminescent substrate to measure ATP present according to the protocol provided by the manufacturer (Promega) and viable cells determined with the Wallac Victor3 1420 Multilabel counter (Perkin-Elmer). ATP luminescence-based motility assays are considered more reliable and sensitive than other methods used to enumerate cells (de la Monte et al., 2002). All experiments were performed independently at least three times.

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