JOURNAL OF VIROLOGY, Jan. 1992, p. 448–457
0022-538X/92/010448-10$02.00/0

Vol. 66, No. 1

The Fibroblast Growth Factor Receptor Is Not Required for Herpes Simplex Virus Type 1 Infection

DANIEL P. MIRDA,[1,2,3] DAVID NAVARRO,[4] PEDRO PAZ,[4] PAULINE L. LEE,[5]†
LENORE PEREIRA,[4] AND LEWIS T. WILLIAMS[2,3,5]*

Cancer Research Institute,[1] Cardiovascular Research Institute,[2] Program of Excellence in Molecular Biology,[3] Department of Stomatology,[4] and Howard Hughes Medical Institute,[5] University of California, San Francisco, California 94143-0724

Received 22 August 1991/Accepted 10 October 1991

The early events mediating herpes simplex virus type 1 (HSV-1) infection include virion attachment to cell surface heparan sulfates and subsequent penetration. Recent evidence has suggested that the high-affinity fibroblast growth factor (FGF) receptor mediates HSV-1 entry. This report presents three lines of experimental evidence showing that the high-affinity FGF receptor is not required for HSV-1 infection. First, rat L6 myoblasts lacking FGF receptors were as susceptible to HSV-1 infection as L6 cells genetically engineered to express the FGF receptor. Second, a soluble FGF receptor fragment that inhibited FGF binding and receptor activation did not inhibit HSV-1 infection. Finally, basic FGF (but not acidic FGF) inhibited HSV-1 infection in L6 cells lacking FGF receptors, presumably by blocking cell surface heparan sulfates also required for HSV-1 infection. These results show that the high-affinity FGF receptor is not required for HSV-1 infection but instead that specific low-affinity basic FGF binding sites are used for HSV-1 infection.

Herpes simplex virus type 1 (HSV-1) is a common human pathogen that infects a wide spectrum of cell types, including those of mesodermal and neuroectodermal lineages. The initial steps in viral infection include virion attachment to the cell surface followed by penetration through fusion of the virion envelope with the cell membrane (20, 29, 43, 51). Virion attachment is mediated by binding to heparan sulfate proteoglycans (64), cell surface molecules important for cell-cell and cell-substratum interactions (21). The interaction of the viral glycoproteins (gC and potentially other viral glycoproteins) with the cell surface heparan sulfate proteoglycans has been proposed to be a mechanism of viral attachment (9, 23, 57). The HSV-1 glycoproteins gB, gD, and gH mediate viral penetration and are required for infection of cells in culture (7, 8, 14, 19, 28, 38, 39, 56). A putative receptor for gD which is not a heparan sulfate proteoglycan has been identified, and it has been proposed that this putative receptor may mediate viral penetration and infection (27).

It was recently reported that the high-affinity fibroblast growth factor (FGF) receptor mediates HSV-1 entry into cells (32) and that basic FGF (bFGF) associates with the HSV-1 virion (3). It has been proposed that the virion-associated bFGF mediates viral attachment and cellular entry through the FGF receptor. In support of this hypothesis, it was shown that bFGF and a related peptide inhibited HSV-1 attachment to or infection of vascular cells and that attachment and internalization of HSV-1 virions by Chinese hamster ovary (CHO) cells was enhanced by genetically engineering the cells to express the high-affinity FGF receptor (FGFR1 or *flg*) (32). However, this latter finding has not been reproduced by other investigators (58).

The FGFs are a family of related polypeptides that are potent mitogens for cells of mesodermal and neuroectodermal origin (1, 13, 26, 41, 55, 66), cell types susceptible to HSV-1 infection. The best described of these growth factors are bFGF and acidic FGF (aFGF), which are identical in 55% of their amino acids and have similar biochemical and biological properties (6). Like other growth factors, bFGF and aFGF mediate their cellular effects through binding to high-affinity cell surface receptors (30). Recent evidence has shown that the cell surface heparan sulfates are required for the high-affinity interaction between bFGF and the receptor (54, 65). The FGF receptor reported to mediate HSV-1 entry, also known as FGF receptor 1 (FGFR1) or *flg*, was the first of the four known FGF receptors for which a full-length cDNA clone was obtained (15, 35, 36, 52). The FGF receptors are transmembrane proteins that share structural features: they have two or three extracellular immunoglobulin (Ig)-like domains and a split intracellular tyrosine kinase domain. FGF binds to the extracellular portion of the receptor which activates the tyrosine kinase, resulting in receptor autophosphorylation and phosphorylation of certain cellular proteins (5, 11, 15, 24). The FGF receptor thus activates intracellular signalling pathways ultimately leading to cell division.

In this study, we performed experiments to clarify the role of the high-affinity FGF receptor in HSV-1 infection. We present evidence showing that the high-affinity FGF receptor is not required for HSV-1 infection.

MATERIALS AND METHODS

Materials, cells, and virus. bFGF and aFGF were the gift of L. Coussens (Chiron). The bFGF-related peptide (3, 4) was prepared by C. Turck by conventional peptide synthesis on a model 430A peptide synthesizer (Applied Biosystems, Foster City, Calif.). Vero cells, rat L6 myoblasts, and *Spodoptera frugiperda* (SF9) cells were obtained from the American Type Culture Collection. Vero and L6 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and antibiotics. SF-9 cells were grown in TNM-FH (63) supplemented with

* Corresponding author.
† Present address: Department of Molecular and Experimental Medicine, Division of Biochemistry, Scripps Research Institute, La Jolla, CA 92037.

10% fetal calf serum and antibiotics. *Autographa californica* nuclear polyhedrosis virus was a gift from M. D. Summers. The isolation and properties of HSV-1 strain F are reported elsewhere (16). HSV-1 virions were purified in dextran T-10 gradients as described by Spear and Roizman (60). Virus preparations were titrated in Vero cells.

Radiolabelling and immunoprecipitation of viral proteins from herpesvirus-infected L6 cells. Confluent L6 myoblasts were infected with HSV-1 strain F at a multiplicity of infection (MOI) of 10. Infected cells were radiolabelled with 50 μCi of [^{35}S]methionine per ml in methionine-deficient DMEM from 5 to 24 h after infection. Cells were lysed in phosphate-buffered saline (PBS), pH 7.4, containing 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and protease inhibitors. The cell lysates were aliquoted and immunoprecipitated as described by Pereira et al. (53) with the following antibodies: H640-28 (specific for ICP4) (2), H126 (specific for gB), and HD-1 (specific for gD). Samples were analyzed by SDS–9% polyacrylamide gel electrophoresis (PAGE) and autoradiography.

Expression of the 2-Ig and 3-Ig FGF receptors in rat L6 myoblasts. The cDNAs for the FGF receptors were isolated from cDNA libraries from human placenta (2-Ig FR) and from chicken embryos (3-Ig FR) (30, 36). The 2-Ig and 3-Ig FGF receptor forms were cloned into an expression vector, pSV7d, in which the cDNA was under the transcriptional control of the simian virus 40 early promoter. Rat L6 myoblasts were cotransfected with the expression plasmid and with pSV2*neo* (vector) by the Lipofectin method (Bethesda Research Laboratories-GIBCO). Stable transfectants were selected in DMEM containing G418 (Bethesda Research Laboratories-GIBCO) and screened by immunoblotting with antibody to the receptor (44). Eleven L6 cell lines were isolated as individual clones from transfections of the same parental L6 cells. Unless indicated, subsequent experiments used the following clones of transfected L6 cell lines (nomenclature same as used in Fig. 2 and 3): cell line V1 (vector), cell line 2 (2-Ig FR), and cell line 4 (3-Ig FR). These cell lines were representative of other clones isolated from the same transfection.

Covalent cross-linking of ^{125}I-bFGF to the L6 cell lines. bFGF was radioiodinated as described by Lee et al. (36). L6 cells were incubated overnight at 4°C with ^{125}I-bFGF (30 pM) in the presence or absence of a 100-fold excess of unlabelled bFGF in six-well plates. The cell monolayers were washed with PBS and PBS–2 M NaCl at 4°C and then cross-linked with 1 mM 3,3′-bis(sulfosuccinimidyl)suberate (Pierce). Cells were lysed in 0.5% Nonidet P-40 (Sigma) lysis buffer (10 mM Tris [pH 8.0], 100 mM NaCl, 2 mM EDTA, 10% glycerol, protease inhibitors). Cell lysates were analyzed by SDS–5% PAGE and autoradiography.

Viral plaque assays of herpesvirus-infected L6 cell lines. Vero cells and all L6 cell lines were grown to confluence in six-well plates. Purified virions (500 to 2,000 PFU per well) were adsorbed for 1 h at 37°C. Cells were then washed, and DMEM containing 0.1% pooled human gamma globulin (overlay medium) was added. At 72 h postinfection, cells were fixed with methanol and were stained with crystal violet for counting plaques.

Indirect immunofluorescence of ICP4 expression in herpesvirus-infected L6 cells. L6 cell lines were seeded onto chamber slides and were infected with purified HSV-1 virions at a high MOI (1 to 100). At 16 h postinfection, cells were fixed in acetone and were stained for ICP4 expression with monoclonal antibody H640-28 as described by Ackermann et al. (2). A minimum of 200 cells was scored in several different fields for specific nuclear staining.

Construction of a recombinant baculovirus for expression of a soluble FGF receptor in insect cells. The cDNA fragment (*Bam*HI-*Aat*II; 1,153 bp) encoding the extracellular region of the 3-Ig FGF receptor was cloned into the baculovirus expression plasmid pVL1393 (*Bam*HI-*Eco*RI) (63) by using an *Aat*II-*Eco*RI linker containing an in-frame stop codon at position 372 with codon assignment according to that of Lee et al. (36). A recombinant baculovirus encoding a soluble form of the 3-Ig FGF receptor (sxFR) was isolated and purified as described by Webb and Summers (63). Insect cells (SF9) were infected (MOI of 10) with the recombinant baculovirus encoding sxFR or with a nonrecombinant baculovirus (control). Control medium or medium containing recombinant protein (sxFR) was purified by wheat-germ agglutinin chromatography (36). Subsequent experiments used the same partially purified protein (approximately 20 ng/μl) for direct cross-linking to ^{125}I-bFGF, to inhibit bFGF binding and activation of FGF receptors, and for viral inhibition studies.

Assay for binding of ^{125}I-bFGF to the soluble FGF receptor. Control medium or medium containing sxFR (1 μl) was incubated with 30 pM ^{125}I-bFGF in the presence or absence of a 100-fold excess of unlabelled bFGF. The ^{125}I-bFGF-protein mixture was cross-linked with 1 mM 3,3′-bis(sulfosuccinimidyl)suberate for 1 h at 4°C and analyzed by SDS-PAGE (7 to 12% gradient) and autoradiography.

Assay of the soluble FGF receptor fragment as an inhibitor of bFGF binding to whole cells. ^{125}I-bFGF (30 pM) was preincubated with 10 μl of sxFR (10-fold more than cross-linked above) or control medium for 1 h at 4°C in a total volume of 0.5 ml of binding medium (DMEM with 0.2% bovine serum albumin, 15 U of heparin per ml, 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES], pH 7.4). Where indicated, L6 cells expressing the 3-Ig FGF receptor were then incubated for 12 h at 4°C with ^{125}I-bFGF alone or with ^{125}I-bFGF preincubated with control medium or sxFR. After binding, the cells were lysed in a 0.5% Nonidet P-40 lysis buffer and specific ^{125}I-bFGF binding was measured with a gamma counter (Beckman).

Assay of the soluble FGF receptor fragment as an inhibitor of bFGF-stimulated tyrosine phosphorylation in whole cells. Increasing amounts of bFGF (2, 20, and 50 ng/ml) were preincubated with 10 μl of sxFR or control medium at 4°C for 1 h in a total volume of 0.5 ml of binding medium. Then quiescent L6 cells expressing the 3-Ig FGF receptor were incubated in duplicate with the bFGF-protein mixture for 15 min at 37°C. Cell lysates were analyzed by SDS-PAGE (7.5% polyacrylamide) and immunoblotted with phosphotyrosine antibody 4G10, as described by Morrison et al. (44).

Assay of the soluble FGF receptor fragment as an inhibitor of HSV-1 infection of cells. Purified HSV-1 virions were preincubated with 100 μl of sxFR or control medium for 1 h at 4°C in 1 ml of DMEM. Then the virion-protein mixture was plated onto Vero cells or L6 cells expressing the 3-Ig FGF receptor in six-well plates (Vero, 250 PFU per well; 3-Ig FR, 10^3 PFU per well) and incubated for 1 h at 37°C. An additional 100 μl of sxFR or control medium was added during viral adsorption (1 h at 37°C). Cells were then washed, and overlay medium was added. Plaques were counted at 72 h postinfection.

Assay for inhibition of HSV-1 infection by bFGF, aFGF, and bFGF-related peptide. L6 cell lines in six-well plates were incubated with increasing concentrations (5 to 100 nM) of bFGF, aFGF, or (5 to 10 μM) a bFGF-related peptide for

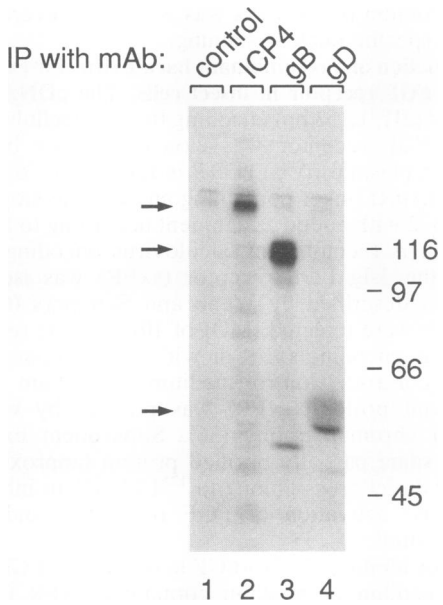


FIG. 1. Expression of viral proteins in herpesvirus-infected rat L6 myoblasts. Herpesvirus-infected L6 cells were radiolabelled with [^{35}S]methionine, and cell lysates were immunoprecipitated (IP) with monoclonal antibodies (mAb) recognizing the viral proteins ICP4, gB, and gD (indicated by arrows for lanes 2 to 4). The control lysate (lane 1) was treated identically except for the presence of antibody. Immunoprecipitates and control were analyzed by SDS-PAGE (9%) and autoradiography. Molecular mass markers (in kilodaltons) are indicated to the right.

2 h at 37°C. Purified HSV-1 virions (0.5×10^3 to 1.0×10^3 PFU per well) were then added, and the pretreated cell monolayers were further incubated for 1 h at 37°C. Cells were then washed, and overlay medium was added. Plaques were counted at 72 h postinfection.

RESULTS

Rat L6 cells which lack FGF receptors are susceptible to HSV-1 infection. To determine whether the FGF receptor is required for HSV-1 infection, we analyzed viral infectivity of rat L6 myoblasts, a cell line reported to lack high-affinity FGF binding sites (18, 49, 50). We confirmed that the parental L6 cell line did not express high-affinity FGF receptors as assessed by binding of ^{125}I-bFGF (36), covalent cross-linking of ^{125}I-bFGF (see Fig. 2B), and bFGF-stimulated tyrosine phosphorylation (data not shown). In L6 myoblasts infected with HSV-1, the expression of the immediate-early viral protein ICP4 as well as the late viral proteins (envelope glycoproteins gB and gD) was detected by radioimmunoprecipitation with specific monoclonal antibodies (Fig. 1). HSV-1 was able to infect and fully replicate in L6 myoblasts even though these cells lack high-affinity FGF receptors.

Then the susceptibility of L6 cells to HSV-1 infection was quantitated by plaque assay and compared with that of Vero cells, a cell line commonly used to propagate HSV-1 in vitro. Cells were infected at different MOIs, and plaques were allowed to develop. L6 cells were susceptible to HSV-1 infection, but with an efficiency 10 to 20% of that of Vero cells (data not shown). These results clearly demonstrated that the FGF receptor was not absolutely required for HSV-1 infection.

L6 cells were genetically engineered to express the high-affinity FGF receptor (FGFR1). To determine whether susceptibility to HSV-1 infection could be enhanced by the FGF receptor, L6 cells were genetically engineered to express two forms of the FGF receptor. For these experiments, the cDNA encoding two variant forms of the FGF receptor (FGFR1) were used. These FGF receptor forms, produced by alternative splicing of mRNA (31), differ only in the extracellular region; one form has three extracellular Ig-like domains (3-Ig FR), while the other form has only two of these domains (2-Ig FR), lacking the first Ig domain (Fig. 2A). Both forms are known to bind aFGF and bFGF with comparable affinities (30).

From the same parental L6 cell line, several stable cell lines that expressed functional FGF receptors were established. bFGF bound with high affinity (K_d, 0.1 nM) to either form of the receptor expressed in L6 cells (30). Covalent cross-linking of ^{125}I-bFGF bound to L6 cell lines confirmed cell surface expression of the 2-Ig and 3-Ig forms of the receptor (Fig. 2B). In this experiment, the lower-molecular-mass bands observed in L6 cells expressing the FGF receptors (below the expected positions of the receptors) likely represented receptor precursors or degradation products. The receptor immunoblot similarly demonstrated immunoreactive bands corresponding to the 2-Ig and 3-Ig forms of the receptor, with apparent molecular masses of 125 and 145 kDa, respectively (Fig. 2C). bFGF treatment of different L6 cell lines expressing FGF receptors resulted in receptor autophosphorylation on tyrosine (37) and tyrosine phosphorylation of cellular proteins at 45 and 42 kDa (Fig. 3). This pattern of bFGF-stimulated tyrosine phosphorylation was similar to that observed in fibroblasts, which express native FGF receptors (11, 24). In contrast, vector-transfected L6 cells, like the parental L6 cell line, had no detectable high-affinity FGF receptors as shown by binding of ^{125}I-bFGF (30), by covalent cross-linking to ^{125}I-bFGF (Fig. 2B), by immunoblot (Fig. 2C), and by bFGF-stimulated tyrosine phosphorylation (Fig. 3). These studies confirmed the activity of the receptor expressed in L6 cells and the absence of detectable high-affinity FGF receptors in parental and vector-transfected L6 cells.

Susceptibility to HSV-1 infection is not enhanced by expression of FGF receptors in L6 cells. We then compared HSV-1 infectivity of L6 cells with or without FGF receptors by performing plaque assays and by quantitating the expression of the immediate-early viral protein ICP4 after virus infection. For these experiments, L6 cell lines were infected over a range of MOIs, and the number of plaques that developed were counted (Fig. 4). The results of these infectivity studies showed a range in the level of infectivity of the L6 cell lines; however, this variability was comparable between L6 cells expressing the 2-Ig or 3-Ig FGF receptor and L6 cells lacking FGF receptors (parental or vector-transfected L6 cells) (Fig. 4). Among the L6 cell lines genetically engineered to express the FGF receptor, susceptibility to HSV-1 infection did not correlate with the ability to respond to bFGF stimulation (Fig. 3). Therefore, L6 cells lacking high-affinity bFGF receptors were as susceptible to HSV-1 infection as L6 cells expressing either form of the FGF receptor.

To confirm these differences in plaque-forming ability among the various cell lines, ICP4 expression in L6 cell lines after viral infection was quantitated. Representative results of these experiments are shown in Fig. 5. Overall, the level of ICP4 expression correlated with the plaque assay results, indicating that FGF receptor expression does not enhance HSV-1 infectivity of L6 cells.

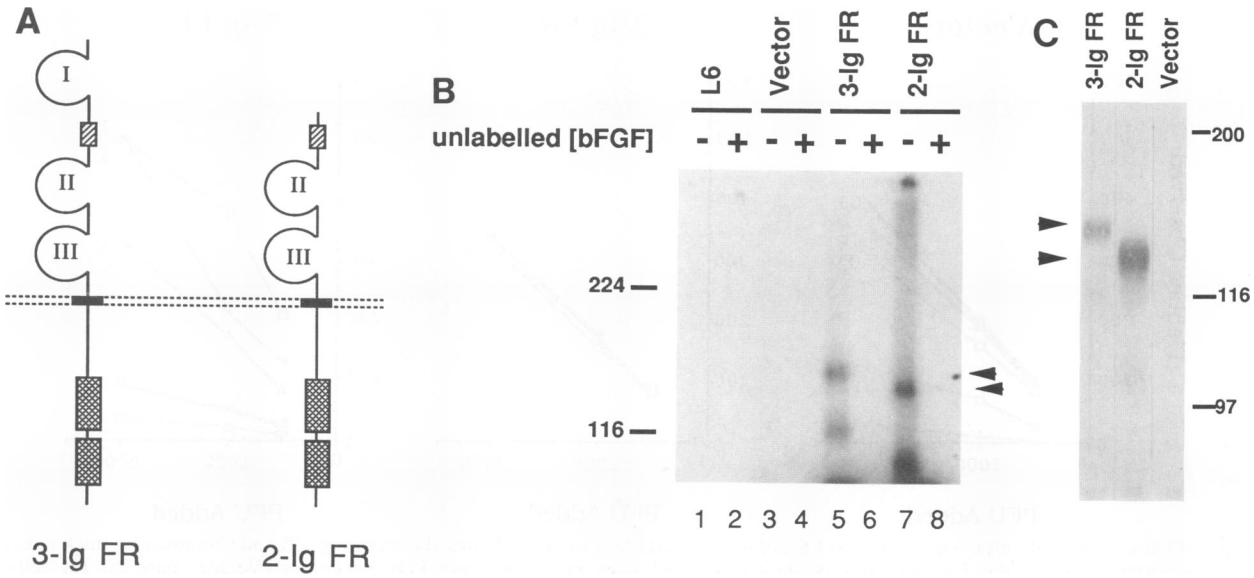


FIG. 2. FGF receptor expression in L6 cell lines. (A) The schematic diagram of the FGF receptor variant forms (3-Ig FR and 2-Ig FR) shows the following predicted structural features: the Ig-like domains (I, II, and III), the highly acidic region (hatched box), the transmembrane region (solid box), and the intracellular tyrosine kinase region (cross-hatched boxes). (B) Parental L6 cell (L6) and L6 cell transfectants without (Vector) or with FGF receptors (2-Ig FR and 3-Ig FR) were covalently cross-linked to ^{125}I-bFGF in the absence (lanes 1, 3, 5, and 7) or presence (lanes 2, 4, 6, and 8) of a 100-fold excess of unlabelled bFGF. Cell lysates were analyzed by SDS-PAGE (5%) and autoradiography. (C) Lysates of L6 cell transfectants (not cross-linked to ^{125}I-bFGF) were purified by lectin affinity and analyzed by SDS-PAGE (7.5%) followed by immunoblotting with antibody to the receptor. The arrowheads identify the positions of the 2-Ig and 3-Ig FGF receptors. Molecular mass markers (in kilodaltons) are indicated to the right.

A soluble form of the FGF receptor blocks bFGF binding and receptor activation yet did not inhibit HSV-1 infection. Soluble extracellular regions of virus receptors have been shown to be potent inhibitors of viral infection, e.g., soluble CD4 and human immunodeficiency virus (10, 12, 17, 25, 40, 62), soluble poliovirus receptor and poliovirus (33, 34, 42), and soluble CR2 and Epstein-Barr virus (47, 61). It has been proposed that bFGF, or a bFGF-like protein, associated with the HSV-1 virion interacts with the FGF receptor, leading to viral entry (3). We hypothesized that if the receptor plays a significant role in viral entry, a soluble FGF receptor should inhibit HSV-1 infection.

We produced a soluble FGF receptor fragment (3-Ig form) that binds bFGF directly and specifically inhibits bFGF-mediated receptor activation. The cDNA encoding the entire extracellular region of the 3-Ig FGF receptor was introduced into the genome of *Autographa californica* nuclear polyhedrosis virus (baculovirus) through homologous recombina-

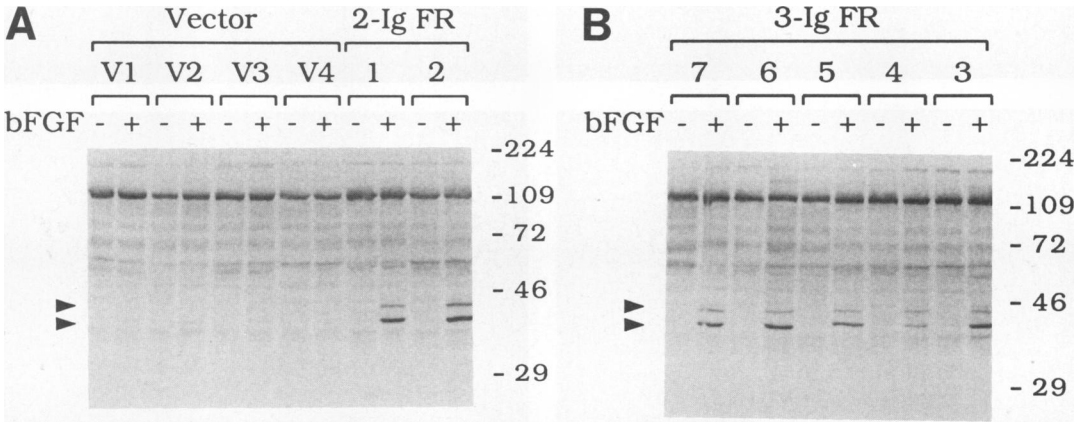


FIG. 3. bFGF-stimulated tyrosine phosphorylation in L6 cell lines expressing FGF receptors. In addition to the cell lines for which results are shown in Fig. 2B and C, other L6 cell transfectants were independently isolated from the same cDNA transfection experiments. (A) Four L6 cell lines transfected with pSV*neo* only (V1 to V4) and two L6 cell lines expressing the 2-Ig FGF receptor (2-Ig FR, 1 and 2). (B) Five L6 cell lines expressing the 3-Ig FGF receptor (3-Ig FR, 3 to 7). These cell lines were incubated in the presence or absence of bFGF (50 ng/ml) for 15 min at 37°C. Cells were lysed and then analyzed by SDS–7.5% PAGE and immunoblot with phosphotyrosine antibody (4G10). The arrowheads indicate the tyrosine-phosphorylated proteins induced by bFGF treatment. The identity of these proteins is unknown. Molecular mass markers (in kilodaltons) are indicated to the right.

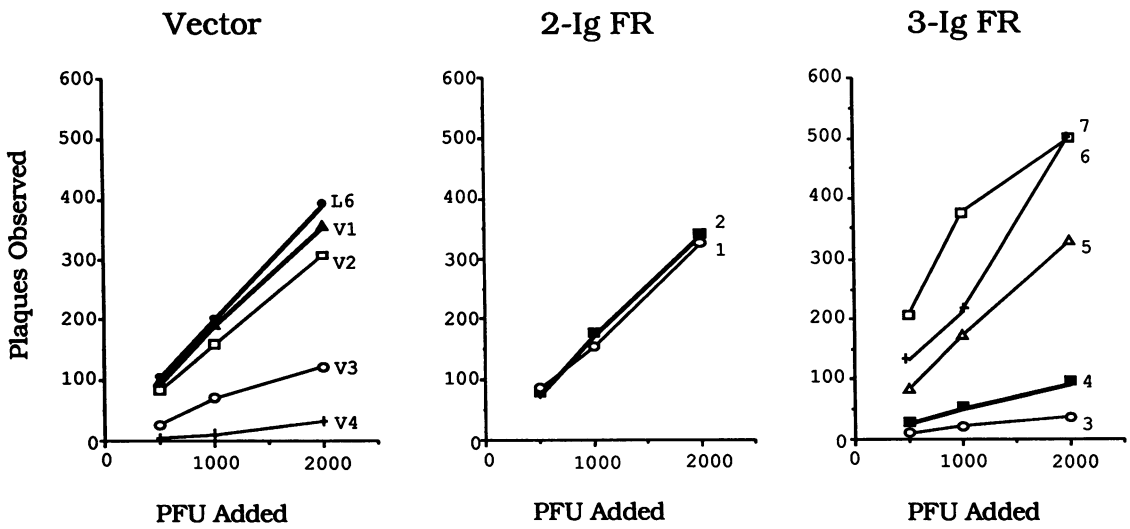


FIG. 4. Plaque assays of herpesvirus-infected L6 cell lines. In addition to the cell lines shown in Fig. 2B and C (shown by thick lines), the HSV-1 susceptibility of all the L6 cell lines is shown. L6 cell lines not expressing FGF receptors (Vector; parental L6 cells and vector-transfected L6 cell lines V1 to V4) and L6 cell lines expressing FGF receptors (2-Ig FR, lines 1 and 2; 3-Ig FR, lines 3 to 7) were tested for susceptibility to HSV-1 infection by using conventional plaque assays. Values are mean numbers of plaques observed (six determinations for each cell line; error bars showing standard errors are within the symbol outlines) for a given amount of HSV-1 virions added (PFU added).

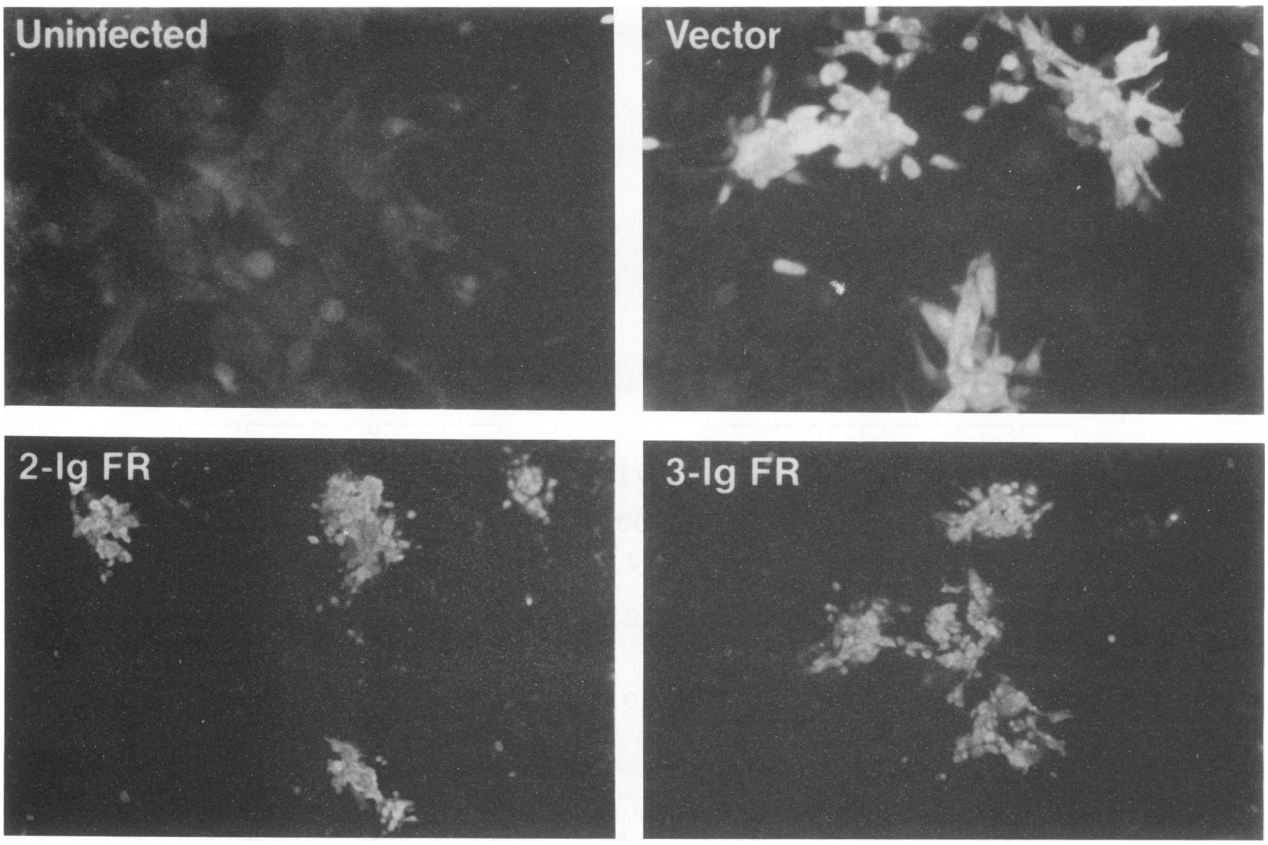
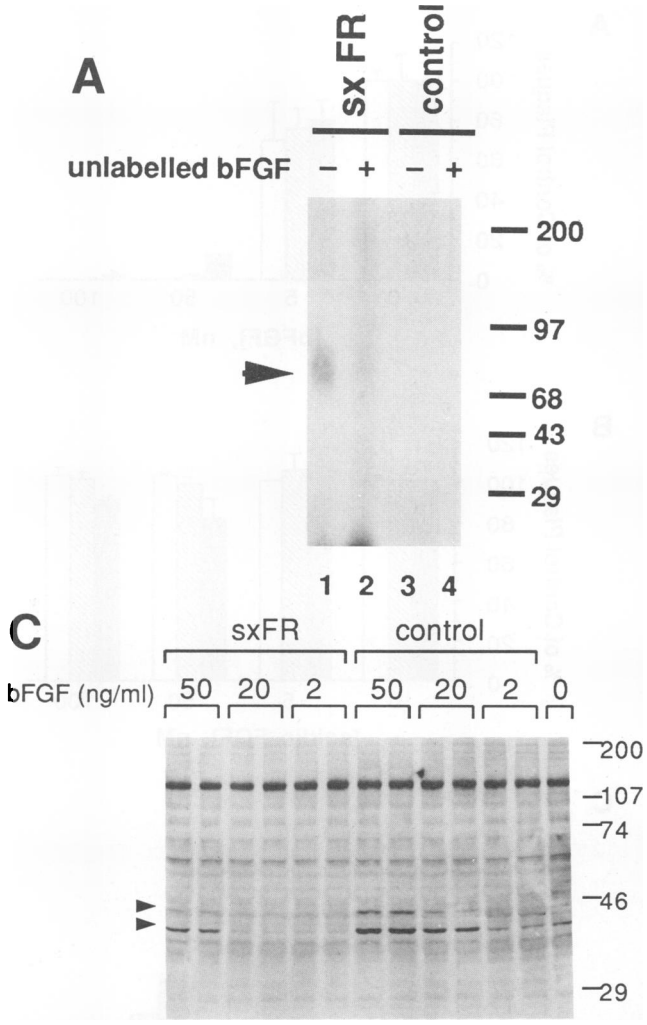


FIG. 5. Indirect immunofluorescence of ICP4 expression in herpesvirus-infected L6 cells. The panels show ICP4 expression after HSV-1 infection (MOI of 1) in L6 cells lacking FGF receptors (Vector) and L6 cells expressing the 2-Ig (2-Ig FR) or 3-Ig FGF receptor (3-Ig FR). Nonspecific fluorescence is shown for uninfected vector-transfected L6 cells (Uninfected).



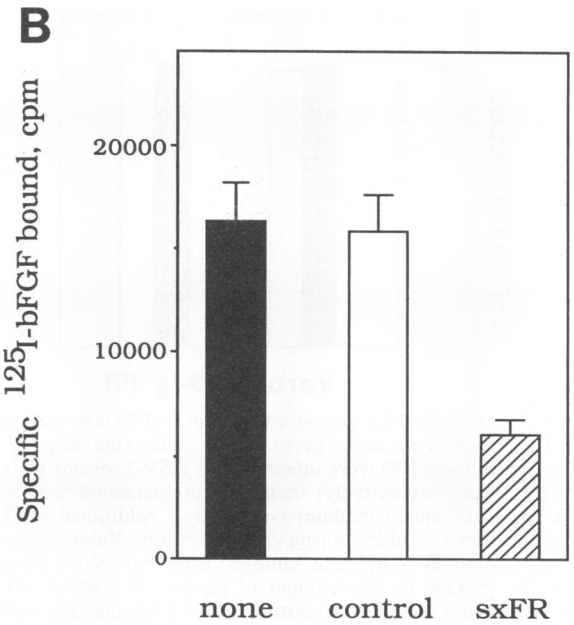


FIG. 6. A soluble FGF receptor fragment, sxFR, binds bFGF and blocks bFGF binding and stimulation of cells. (A) Autoradiogram of ^{125}I-bFGF cross-linked to a soluble FGF receptor fragment. Control medium or medium containing sxFR (1 μl) was covalently cross-linked to ^{125}I-bFGF in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of a 100-fold excess of unlabelled bFGF. Samples were analyzed by SDS-PAGE (12%) and autoradiography. The arrowhead indicates the position of sxFR cross-linked to ^{125}I-bFGF. Molecular mass markers (in kilodaltons) are indicated to the right. (B) Inhibition of ^{125}I-bFGF binding to cells. The soluble FGF receptor fragment (sxFR; hatched bar) or control medium (control; open bar) was preincubated with ^{125}I-bFGF and was then added to L6 cells expressing the 3-Ig FGF receptor. The counts per minute (cpm) of specific ^{125}I-bFGF binding in the absence of sxFR or control is shown (none; solid bar). Samples were performed in triplicate; error bars show standard deviations. (C) Inhibition of bFGF-stimulated tyrosine phosphorylation. Increasing amounts of bFGF (2, 20, and 50 ng/ml) were preincubated with sxFR or control medium and then added to quiescent L6 cells expressing the 3-Ig FGF receptor. The bFGF-stimulated tyrosine phosphorylation pattern was analyzed by SDS–7.5% PAGE and immunoblot by using phosphotyrosine antibody (4G10). Arrows identify cellular proteins with enhanced tyrosine phosphorylation in response to bFGF stimulation (apparent molecular masses of 45 and 42 kDa). Molecular mass markers (in kilodaltons) are indicated to the right.

tion. A recombinant baculovirus encoding the extracellular region of the 3-Ig FGF receptor was then used to express a soluble FGF receptor fragment (sxFR) in insect cells (63). The recombinant protein was partially purified by lectin affinity chromatography (36) and then tested for activity. The soluble FGF receptor fragment was able to bind bFGF directly, as shown by covalent cross-linking to ^{125}I-bFGF (Fig. 6A). Further, in L6 cells expressing the 3-Ig FGF receptor, sxFR inhibited 65% of specific bFGF binding to whole cells (Fig. 6B) and also blocked bFGF-stimulated tyrosine phosphorylation of cellular proteins with apparent molecular masses of 45 and 42 kDa (Fig. 6C). Similar results were obtained with Swiss/3T3 cells; sxFR inhibited 85% of ^{125}I-bFGF binding to whole cells and blocked bFGF-stimulated tyrosine phosphorylation of cellular proteins with apparent molecular masses of 145, 90, 45, and 42 kDa (11, 48) (data not shown). The identities of these proteins, except for the tyrosine-phosphorylated protein at 145 kDa (phospholipase C-γ) (5), are not known. These experiments establish for the first time that a soluble FGF receptor fragment can act as an FGF receptor antagonist.

The soluble FGF receptor fragment was then tested as an inhibitor of HSV-1 infection of Vero cells and L6 cells expressing the 3-Ig FGF receptor. sxFR was used in significantly higher concentrations for these antiviral studies (20-fold over that required to inhibit 65% of bFGF binding or receptor activation) to enhance any antiviral activity. In addition, sxFR was preincubated with relatively low numbers of virions and additional soluble receptor was added during viral adsorption. Despite these conditions, the soluble FGF receptor fragment did not inhibit herpesvirus infection of Vero cells or L6 cells expressing the 3-Ig FGF receptor (Fig. 7). These results do not support the proposal that bFGF associated with HSV-1 virions interacts with cell surface FGF receptors, thus leading to viral infection.

bFGF but not aFGF blocks HSV-1 infection. Given the reported observation that bFGF inhibited HSV-1 infection (32), we tested the inhibitory effect of bFGF and a closely related member of the FGF family, aFGF, on viral infection of various L6 cell lines. bFGF and aFGF bind to cell surface heparan sulfates with low affinity (K_d, ~10 nM), whereas binding of either ligand to the FGF receptor (FGFR1) occurs

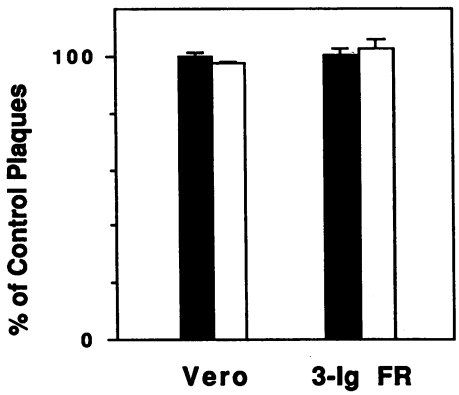


FIG. 7. A soluble FGF receptor fragment (sxFR) does not inhibit HSV-1 infection. Vero cells (Vero) and L6 cells expressing the 3-Ig FGF receptor (3-Ig FR) were infected with HSV-1 virions (250 and 1,000 PFU/well, respectively) that were preincubated with sxFR (open bars) or control medium (solid bars). Additional sxFR or control medium was added during viral adsorption. Values represent mean plaque counts (triplicate samples; error bars show standard deviations) divided by the number of plaques in control cultures (without addition of sxFR or control; 122 ± 2 plaques per well for Vero cells, and 178 ± 4 plaques per well for L6 cells expressing 3-Ig FR).

with high affinity (K_d, 0.1 to 0.01 nM) (6, 30). In L6 cells lacking high-affinity FGF receptors (parental or vector-transfected L6 cells), bFGF inhibited HSV-1 infection in a dose-dependent manner, whereas aFGF had no effect on HSV-1 infection (Fig. 8A and B, solid and hatched bars). A bFGF-related peptide representing the binding domain of bFGF (3, 4) similarly inhibited HSV-1 infection in L6 cells lacking FGF receptors (Fig. 8C, solid and hatched bars). In L6 cells expressing the 3-Ig FGF receptor, bFGF and the bFGF-related peptide also inhibited HSV-1 infection, while aFGF again had no effect (Fig. 8, open bars). It should be noted that half-maximal inhibition of infection required concentrations of bFGF higher than 5 nM, which is much higher than the concentration required to occupy half of the high-affinity receptors (K_d, 0.1 to 0.01 nM) (30) or to elicit a half-maximal response (Fig. 6C). We suggest that the likely target of bFGF inhibition of HSV-1 infection is the cell surface heparan sulfates that bind bFGF with low affinity (K_d, ~10 nM) (45, 54, 65) and that are required for HSV-1 attachment and infection (64).

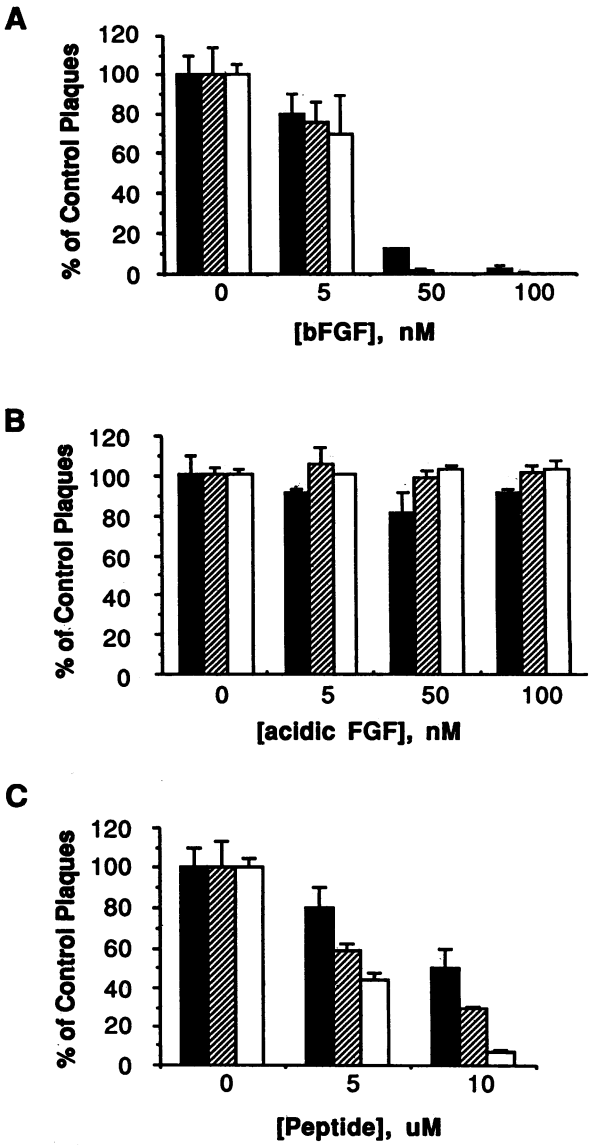


FIG. 8. Inhibition of HSV-1 infection by bFGF, aFGF, and a bFGF-related peptide. L6 cells without FGF receptors (parental L6 cells, solid bars; vector-transfected, hatched bars) and L6 cells with 3-Ig FGF receptors (open bars) were incubated with increasing concentrations (5 to 100 nM) of (A) bFGF, (B) aFGF, or (C) bFGF-related peptide. Purified HSV-1 virions were then added to cells (approximately 10^3 PFU per well), and conventional plaque assays were performed. Values represent mean numbers of plaques observed (duplicate samples; error bars show standard deviations) divided by the number of plaques in control cultures that were not incubated with bFGF or aFGF.

DISCUSSION

It has been proposed that bFGF and the FGF receptor mediate HSV-1 infection on the basis of findings that bFGF and a related peptide inhibited HSV-1 infection and that CHO cells expressing the FGF receptor enhanced HSV-1 attachment and internalization (32). However, the latter finding has not been reproduced by other investigators (58). Using several different experimental approaches, we attempted to clarify the role of bFGF and the FGF receptor in HSV-1 infection.

Our results show that cells lacking high-affinity FGF receptors (assessed by binding of ^{125}I-bFGF, cross-linking to ^{125}I-bFGF, immunoblot with receptor antibodies, and bFGF-stimulated tyrosine phosphorylation) are nevertheless susceptible to HSV-1 infection, suggesting that the FGF receptor is not absolutely required for HSV-1 infection. It remains possible that these cells may have extremely low levels of high-affinity FGF receptors, below the limits of detection by these methods, and that these low levels of receptor expression are sufficient for HSV-1 infection. However, when exogenous FGF receptors were expressed at high levels in L6 cells, there was no enhancement of their susceptibility to HSV-1 infection, nor was the attachment of radiolabelled virions enhanced by the expression of FGF receptors in L6 cells (data not shown). These data suggest that the FGF receptor does not have a significant role in HSV-1 infection of L6 cells.

A soluble FGF receptor fragment was tested as an inhibitor of HSV-1 infection. Soluble derivatives of viral receptors have been shown to act as specific antiviral agents by interfering with the initial events of viral infection, as exemplified by soluble CD4 for human immunodeficiency virus infection (10, 12, 17, 25, 40, 62). It has been proposed that bFGF associates with the virion and thereby mediates the interaction between HSV-1 and the FGF receptor leading to viral entry (3). In this case, we found that a soluble FGF receptor fragment was able to bind bFGF directly and inhibited bFGF binding and receptor activation; however, the soluble receptor did not inhibit HSV-1 infection of either Vero cells or L6 cells expressing the FGF receptor. It can be argued that the quantity of soluble FGF receptor used to inhibit HSV-1 infection was not sufficient to interfere with the virus-receptor interaction. However, antibodies that inhibited bFGF binding to cells also did not inhibit HSV-1 infection of Vero cells or the L6 cell lines (3, 22) (data not shown). The results of the viral infectivity studies and the lack of antiviral activity by the soluble FGF receptor do not support a significant role for bFGF or the FGF receptor in HSV-1 infection.

HSV-1 attachment to cell surface heparan sulfates is known to be required for infection (46, 59, 64). Cell surface heparan sulfates also serve as low-affinity binding sites for bFGF (45) and are required for high-affinity binding to the receptor (54, 65). We suggest that HSV-1 and bFGF both bind to a related site on the cell surface heparan sulfates. In support of this is our finding that bFGF inhibits HSV-1 infection of cells lacking detectable high-affinity bFGF receptors. In addition, HSV-1 interacts with specific cell surface heparan sulfates since only bFGF, and not aFGF (a closely related heparin-binding growth factor), inhibits HSV-1 infection. This is the first report to suggest that bFGF and aFGF may bind to different low-affinity sites.

The results of these experiments suggest that bFGF inhibits HSV-1 infection, presumably by interfering with HSV-1 attachment to cell surface heparan sulfates and not to the high-affinity FGF receptor. Therefore, antagonists that block bFGF binding to the FGF receptor, as shown here (Fig. 6), are unlikely to be efficacious agents in preventing herpesvirus infection. Instead, the future development of antiviral therapeutics should focus on inhibitors of bFGF binding to cell surface heparan sulfates as potential specific antagonists of HSV-1 infection.

ACKNOWLEDGMENTS

We thank E. Leash, D. Ganem, W. VanVoorhis, and members of our laboratory for critical reading of the manuscript and for insightful suggestions. We thank D. R. Duan for assistance in the purification of the soluble FGF receptor.

This work was supported by the National Institutes of Health Program of Excellence in Molecular Biology (HL-43821) and by Public Health Service grants AI-30873 and AI-23592 from the National Institute of Allergy and Infectious Diseases. D.P.M. was supported by American Heart Association fellowship 89-22. D.N. was supported by a fellowship from the Spanish Ministry of Education and Science.

REFERENCES

1. **Abraham, J. A., A. Mergia, J. L. Whang, A. Tumolo, J. Friedman, K. A. Hjerrild, D. Gospodarowicz, and J. C. Fiddes.** 1986. Nucleotide sequence of a bovine clone encoding the angiogenic protein, basic fibroblast growth factor. Science **233:** 545–548.
2. **Ackermann, M., D. K. Braun, L. Pereira, and B. Roizman.** 1984. Characterization of herpes simplex virus 1 α proteins 0, 4, and 27 with monoclonal antibodies. J. Virol. **52:**108–118.
3. **Baird, A., R. Z. Florkiewicz, P. A. Maher, R. J. Kaner, and D. P. Hajjar.** 1990. Mediation of virion penetration into vascular cells by association of basic fibroblast growth factor with herpes simplex virus type 1. Nature (London) **348:**344–346.
4. **Baird, A., D. Schubert, N. Ling, and R. Guillemin.** 1988. Receptor- and heparin-binding domains of basic fibroblast growth factor. Proc. Natl. Acad. Sci. USA **85:**2324–2328.
5. **Burgess, W. H., C. A. Dionne, J. Kaplow, R. Mudd, R. Friesel, A. Zilberstein, J. Schlessinger, and M. Jaye.** 1990. Characterization and cDNA cloning of phospholipase C-γ, a major substrate for heparin-binding growth factor 1 (acidic fibroblast growth factor)-activated tyrosine kinase. Mol. Cell. Biol. **10:** 4770–4777.
6. **Burgess, W. H., and T. Maciag.** 1989. The heparin-binding (fibroblast) growth factor family of proteins. Annu. Rev. Biochem. **58:**575–606.
7. **Cai, W., B. Gu, and S. Person.** 1988. Role of glycoprotein B of herpes simplex virus type 1 in viral entry and cell fusion. J. Virol. **62:**2596–2604.
8. **Cai, W., S. Person, S. C. Warner, J. Zhou, and N. A. DeLuca.** 1987. Linker-insertion nonsense and restriction-site deletion mutations of the gB glycoprotein gene of herpes simplex virus type 1. J. Virol. **61:**714–721.
9. **Campadelli-Fiume, G., D. Stirpe, A. Boscaro, E. Avitabile, L. Foa-Tomasi, D. Barker, and B. Roizman.** 1990. Glycoprotein C-dependent attachment of herpes simplex virus to susceptible cells leading to productive infection. Virology **178:**213–220.
10. **Clapham, P. R., J. N. Weber, D. Whitby, K. McIntosh, A. G. Dalgleish, P. J. Maddon, K. C. Deen, R. W. Sweet, and R. A. Weiss.** 1989. Soluble CD4 blocks the infectivity of diverse strains of HIV and SIV for T cells and monocytes but not for brain and muscle cells. Nature (London) **337:**368–370.
11. **Coughlin, S. R., P. J. Barr, L. S. Cousens, L. J. Fretto, and L. T. Williams.** 1988. Acidic and basic fibroblast growth factors stimulate tyrosine kinase activity in vivo. J. Biol. Chem. **263:** 988–993.
12. **Deen, K. C., J. S. McDougal, R. Inacker, W. G. Folena, J. Arthos, J. Rosenberg, P. J. Maddon, R. Axel, and R. W. Sweet.** 1988. A soluble form of CD4 (T4) protein inhibits AIDS virus infection. Nature (London) **331:**82–84.
13. **Delli-Bovi, P., and C. Basilico.** 1987. Isolation of a rearranged human transforming gene following transfection of Kaposi sarcoma DNA. Proc. Natl. Acad. Sci. USA **84:**5660–5664.
14. **Desai, P. J., P. A. Schaffer, and A. C. Minson.** 1988. Excretion of non-infectious virus particles lacking glycoprotein H by a temperature-sensitive mutant of herpes simplex virus type 1: evidence that gH is essential for virion infectivity. J. Gen. Virol. **69:**1147–1156.
15. **Dionne, C. A., G. Crumley, F. Bellot, J. M. Kaplow, G. Searfoss, M. Ruta, W. H. Burgess, M. Jaye, and J. Schlessinger.** 1990. Cloning and expression of two distinct high-affinity receptors cross-reacting with acidic and basic fibroblast growth factors. EMBO J. **9:**2685–2692.
16. **Ejercito, P. M., E. D. Kieff, and B. Roizman.** 1968. Characterization of herpes simplex virus strains differing in their effect on social behavior of infected cells. J. Gen. Virol. **3:**357–364.
17. **Fisher, R. A., J. M. Bertonis, W. Meier, V. A. Johnson, D. S. Costopoulos, T. Liu, R. Tizard, B. D. Walker, M. S. Hirsch, and R. T. Schooley.** 1988. HIV infection is blocked in vitro by recombinant soluble CD4. Nature (London) **331:**76–78.
18. **Florini, J. R., and K. A. Magri.** 1989. Effects of growth factors on myogenic differentiation. Am. J. Physiol. **256:**c701–c711.
19. **Fuller, A. O., R. E. Santos, and P. G. Spear.** 1989. Neutralizing antibodies specific for glycoprotein H of herpes simplex virus permit viral attachment to cells but prevent penetration. J. Virol. **63:**3435–3443.
20. **Fuller, A. O., and P. G. Spear.** 1987. Anti-glycoprotein D antibodies that permit adsorption but block infection by herpes simplex virus 1 prevent virion-cell fusion at the cell surface. Proc. Natl. Acad. Sci. USA **84:**5454–5458.

21. **Gallagher, J. T., M. Lyon, and W. P. Steward.** 1986. Structure and function of heparan sulfate proteoglycans. Biochem. J. **236**:313–315.
22. **Gonzalez, A. M., M. Buscaglia, M. Ong, and A. Baird.** 1990. Distribution of basic fibroblast growth factor in the 18-day rat fetus: localization in the basement membranes of diverse tissues. J. Cell Biol. **110**:753–765.
23. **Herold, B. C., D. WuDunn, N. Soltys, and P. G. Spear.** 1991. Glycoprotein C of herpes simplex virus type 1 plays a principal role in the adsorption of virus to cells and in infectivity. J. Virol. **65**:1090–1098.
24. **Huang, S. S., and J. S. Huang.** 1986. Association of bovine brain-derived growth factor receptor with tyrosine kinase activity. J. Biol. Chem. **261**:9568–9571.
25. **Hussey, R. E., N. E. Richardson, M. Kowalski, N. R. Brown, H. C. Chang, R. F. Siliciano, T. Dorfman, B. Walker, J. Sodroski, and E. L. Reinherz.** 1988. A soluble CD4 protein selectively inhibits HIV replication and syncytium formation. Nature (London) **331**:78–81.
26. **Jaye, M., R. Howk, W. H. Burgess, G. A. Ricca, I. M. Chiu, M. W. Ravera, J. S. O'Brien, W. S. Modi, T. Maciag, and W. Drohan.** 1986. Human endothelial cell growth factor: cloning, nucleotide sequence, and chromosomal localization. Science **233**:541–545.
27. **Johnson, D. C., R. L. Burke, and T. Gregory.** 1990. Soluble forms of herpes simplex virus glycoprotein D bind to a limited number of cell surface receptors and inhibit virus entry into cells. J. Virol. **64**:2569–2576.
28. **Johnson, D. C., and M. W. Ligas.** 1988. Herpes simplex viruses lacking glycoprotein D are unable to inhibit virus penetration: quantitative evidence for virus-specific cell surface receptors. J. Virol. **62**:1486–1494.
29. **Johnson, D. C., M. Wittels, and P. G. Spear.** 1984. Binding to cells of virosomes containing herpes simplex virus type 1 glycoproteins and evidence for fusion. J. Virol. **52**:238–247.
30. **Johnson, D. E., P. L. Lee, J. Lu, and L. T. Williams.** 1990. Diverse forms of a receptor for acidic and basic fibroblast growth factors. Mol. Cell. Biol. **10**:4728–4736.
31. **Johnson, D. E., J. Lu, H. Chen, S. Werner, and L. T. Williams.** 1991. The human fibroblast growth factor receptor genes: a common structural arrangement underlies the mechanisms for generating receptor forms that differ in their third immunoglobulin domain. Mol. Cell. Biol. **11**:4627–4634.
32. **Kaner, R. J., A. Baird, A. Mansukhani, C. Basilico, B. D. Summers, R. Z. Florkiewicz, P. A. Maher, and D. P. Hajjar.** 1990. Fibroblast growth factor receptor is a portal of cellular entry for herpes simplex virus-1. Science **248**:1410–1413.
33. **Kaplan, G., M. S. Freistadt, and V. R. Racaniello.** 1990. Neutralization of poliovirus by cell receptors expressed in insect cells. J. Virol. **64**:4697–4702.
34. **Kaplan, G., D. Peters, and V. R. Racaniello.** 1990. Poliovirus mutants resistant to neutralization with soluble cell receptors. Science **250**:1596–1599.
35. **Keegan, K., D. E. Johnson, L. T. Williams, and M. J. Hayman.** 1991. Isolation of an additional member of the fibroblast growth factor receptor family, FGFR-3. Proc. Natl. Acad. Sci. USA **88**:1095–1099.
36. **Lee, P. L., D. E. Johnson, L. S. Coussens, V. A. Fried, and L. T. Williams.** 1989. Purification and complementary DNA cloning of a receptor for basic fibroblast growth factor. Science **245**:57–60.
37. **Lee, P. L., and L. T. Williams.** Unpublished data.
38. **Ligas, M. W., and D. C. Johnson.** 1988. A herpes simplex virus mutant in which glycoprotein D sequences are replaced by β-galactosidase sequences binds to but is unable to penetrate into cells. J. Virol. **62**:1486–1494.
39. **Little, S. P., J. T. Jofre, R. J. Courtney, and P. A. Schaffer.** 1981. A virion-associated glycoprotein essential for infectivity of herpes simplex virus type 1. Virology **115**:149–160.
40. **Maddon, P. J., A. G. Dalgleish, J. S. McDougal, P. R. Clapham, R. A. Weiss, and R. Axel.** 1986. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. Cell **47**:333–348.
41. **Marics, I., J. Adelaide, F. Rayband, M. G. Mattei, F. Coulier, J. Planche, O. de Lapeyuere, and D. Birnbaum.** 1989. Characterization of the HST-related FGF.6 gene, a new member of the fibroblast growth factor gene family. Oncogene **4**:335–340.
42. **Mendelsohn, C. L., E. Wimmer, and V. R. Racaniello.** 1989. Cellular receptor for poliovirus: molecular cloning, nucleotide sequence, and expression of a new member of the immunoglobulin superfamily. Cell **56**:855–865.
43. **Morgan, C., H. M. Rose, and B. Mednis.** 1968. Electron microscopy of herpes simplex virus. I. Entry. J. Virol. **2**:507–516.
44. **Morrison, D. K., D. R. Kaplan, J. A. Escobedo, U. R. Rapp, T. M. Roberts, and L. T. Williams.** 1989. Direct activation of the serine/threonine kinase activity of Raf-1 through tyrosine phosphorylation by the PDGF beta-receptor. Cell **58**:649–657.
45. **Moscatelli, D.** 1987. High and low affinity binding sites for basic fibroblast growth factor on cultured cells: absence of a role for low affinity binding in the stimulation of plasminogen activator production by bovine capillary endothelial cells. J. Cell. Physiol. **131**:123–130.
46. **Namhias, A. J., and S. Kibrick.** 1964. Inhibitory effect of heparin on herpes simplex virus. J. Bacteriol. **87**:1060–1066.
47. **Nemerow, G. R., J. J. Mullen III, P. W. Dickson, and N. R. Cooper.** 1990. Soluble recombinant CR2 (CD21) inhibits Epstein-Barr virus infection. J. Virol. **64**:1348–1352.
48. **Olwin, B. B., and S. D. Hauschka.** 1986. Identification of the fibroblast growth factor receptor of Swiss 3T3 cells and mouse skeletal muscle myoblasts. Biochemistry **25**:3487–3492.
49. **Olwin, B. B., and S. D. Hauschka.** 1988. Cell surface fibroblast growth factor and epidermal growth factor receptors are permanently lost during skeletal muscle terminal differentiation in culture. J. Cell Biol. **107**:761–769.
50. **Olwin, B. B., and S. D. Hauschka.** 1989. Cell type and tissue distribution of the fibroblast growth factor receptor. J. Cell. Biochem. **39**:443–454.
51. **Para, M. F., R. B. Baucke, and P. G. Spear.** 1980. Immunoglobulin G (Fc)-binding receptors on virions of herpes simplex virus type 1 and transfer of these receptors to the cell surface by infection. J. Virol. **34**:512–520.
52. **Partanen, J., T. P. Makela, E. Eerola, J. Korhonen, H. Hirvonen, W. L. Claesson, and K. Alitalo.** 1991. FGFR-4, a novel acidic fibroblast growth factor receptor with a distinct expression pattern. EMBO J. **10**:1347–1354.
53. **Pereira, L., T. Klassen, and J. R. Baringer.** 1980. Type-common and type-specific monoclonal antibody to herpes simplex virus type 1. Infect. Immun. **29**:724–732.
54. **Rapraeger, A. C., A. Krufka, and B. B. Olwin.** 1991. Requirement of heparan sulfate for bFGF-mediated fibroblast growth and myoblast differentiation. Science **252**:1705–1708.
55. **Rubin, J. S., H. Osada, P. W. Finch, W. G. Taylor, S. Rudihoff, and S. A. Aaronson.** 1989. Purification and characterization of a newly identified growth factor specific for epithelial cells. Proc. Natl. Acad. Sci. USA **86**:802–806.
56. **Sarmiento, M., M. Haffey, and P. G. Spear.** 1979. Membrane proteins specified by herpes simplex viruses. III. Role of glycoprotein VP7(B_2) in virion infectivity. J. Virol. **29**:1149–1158.
57. **Sears, A. E., B. S. McGwire, and B. Roizman.** 1991. Infection of polarized MDCK cells with herpes simplex virus 1: two asymmetrically distributed cell receptors interact with different viral proteins. Proc. Natl. Acad. Sci. USA **88**:5087–5091.
58. **Shieh, M. T., and P. G. Spear.** 1991. Fibroblast growth factor receptor: does it have a role in the binding of herpes simplex virus? Science **253**:208–210.
59. **Shieh, M.-T., D. WuDunn, R. Montgomery, J. D. Esko, and P. G. Spear.** Cell surface receptors for herpes simplex virus are heparan sulfate proteoglycans. J. Cell Biol., in press.
60. **Spear, P. G., and B. Roizman.** 1972. Proteins specified by herpes simplex virus. V. Purification and structural proteins of the herpesvirion. J. Virol. **9**:143–159.
61. **Tanner, J., J. Weis, D. Fearon, Y. Whang, and E. Kieff.** 1987. Epstein-Barr virus gp350/220 binding to the B lymphocyte C3d receptor mediates adsorption, capping, and endocytosis. Cell **50**:203–13.

CD4 molecules neutralize human immunodeficiency virus type 1. Nature (London) **331**:84–86.
63. **Webb, N. R., and M. D. Summers.** 1990. Expression of proteins using recombinant baculovirus. Technique **2**:173–188.
64. **WuDunn, D., and P. G. Spear.** 1989. Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. J. Virol. **63**:52–58.
65. **Yayon, A., M. Klagsbrun, J. D. Esko, P. Leder, and D. M. Ornitz.** 1991. Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. Cell **64**:841–848.
66. **Zhan, X., B. Bates, X. Hu, and M. Goldfarb.** 1988. The human FGF-5 oncogene encodes a novel protein related to fibroblast growth factors. Mol. Cell. Biol. **8**:3487–3495.