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Hepatocyte Growth Factor Receptor Is a Coreceptor for Adeno-Associated Virus Type 2 Infection

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After the first attachment of virus to the cell surface through a primary receptor, efficient entry of virus requires the presence of a coreceptor. For adeno-associated virus type 2 (AAV2) infection, heparan sulfate proteoglycan is supposed as the primary receptor, and αvβ5 integrin and FGFR1 are reported to act as coreceptors. In this study, we were able to demonstrate that hepatocyte growth factor receptor, c-Met, is also a coreceptor for AAV2 infection. AAV2-mediated transgene analyses revealed that c-Met expression significantly up-regulated transgene expression without increasing AAV2 cell binding. Moreover, a viral overlay assay elucidated the physical interaction between AAV2 and the β subunit of c-Met. These data suggest that c-Met plays the role of coreceptor for AAV2 infection by facilitating AAV2 internalization into the cytoplasm.

Gene transfer vectors based on adeno-associated virus (AAV) show great promise for use in human gene therapy. Vectors based on AAV type 2 (AAV2) have been widely used in preclinical studies and clinical trials. Recently, with a clinical trial of intramuscular injection of an AAV2 vector expressing blood coagulation factor IX in adult patients with severe hemophilia B, the safety and the potential of AAV2-mediated gene therapy have been documented (19). Further, liver-targeted delivery of recombinant AAV2 (rAAV2) resulted in significantly higher levels of factor IX than those observed with intramuscular injections of an equivalent dose of vector in dogs with hemophilia B (24).

To date, eight primate isolates of wild-type AAV have been reported, and their tropism has been studied with laboratory cell lines in vitro and with murine tissues in vivo (10, 32). AAV2 has a broad tropism in vitro and can transduce both dividing and nondividing cells in vivo, including muscle, central nervous system, and liver (5, 21, 23). The main requirement for efficient entry into target cells is the presence of coreceptors on the cell surface, although the natural tropism is a fundamental limitation to efficient gene transfer. For instance, adenovirus attaches to host cells through the coxsackievirus adenovirus receptor and subsequently interacts with αVβ5 integrin as a coreceptor that facilitates virus internalization (3, 11, 34). Human immunodeficiency virus type 1 also attaches to host cells through the CD4 receptor, and it uses a chemokine receptor as a coreceptor that mediates its internalization (13).

Recently, limitations to efficient rAAV2 transduction have become apparent. Cell surface heparan sulfate proteoglycan (HSPG) serves as the primary attachment receptor for AAV2 (30). αVβ5 (29) and FGFR1 (27) were proposed as coreceptors. A novel 150-kDa glycoprotein was also proposed to be involved in AAV2 infection, although the identity of that glycoprotein is still uncertain (22).

The receptor of hepatocyte growth factor, c-Met, is composed of a 50-kDa α chain that is disulfide-linked to a 145-kDa β subunit in a complex of 190 kDa (4, 20, 25). In normal tissue, c-Met is predominantly expressed in epithelial cells (8, 26) and in several nonepithelial cells, such as liver, neural, and skeletal muscle cells. Those cells are prone to successful transduction by rAAV2 (1, 14, 31). Hepatocyte growth factor (HGF) is bound to heparin-like molecules, such as HSPG, on the cell surface of hepatocytes and soluble heparin in serum. HSPG on the cell surface plays important roles in the regulation of HGF/Met interaction. The dynamic state of HGF on the cell surface seems to be similar to that of AAV2 (27, 29, 30), especially with respect to using HSPG as a reservoir and then utilizing chemokine receptors for cell entry (18). Thus, we hypothesized that c-Met plays a role as a coreceptor for AAV2 infection.

AAV2-mediated transgene expression in 3T3/met and 3T3/neo cells. To evaluate the role of c-Met in AAV2 infection, we made individual clone cells (3T3/met) that express human c-Met constitutively and control neomycin-resistant cells (3T3/neo) by using human c-Met-encoding plasmid (28) and neomycin phosphotransferase-encoding plasmid (pMC1neo; Stratagene). The expression of c-Met was analyzed by a fluorescence-activated cell sorter (FACS) system using anti-human c-Met antibody (R&D Systems) (Fig. 1A). Originally, 3T3 cells hardly expressed endogenous c-Met on the cell surface (6, 7) and were only slightly transduced by AAV2. In an adenovirus-free system as described previously (33), rAAV2-GFP and rAAV2-ALP virus vector were propagated. We first analyzed rAAV2-mediated gene transfer into 3T3/met and 3T3/neo

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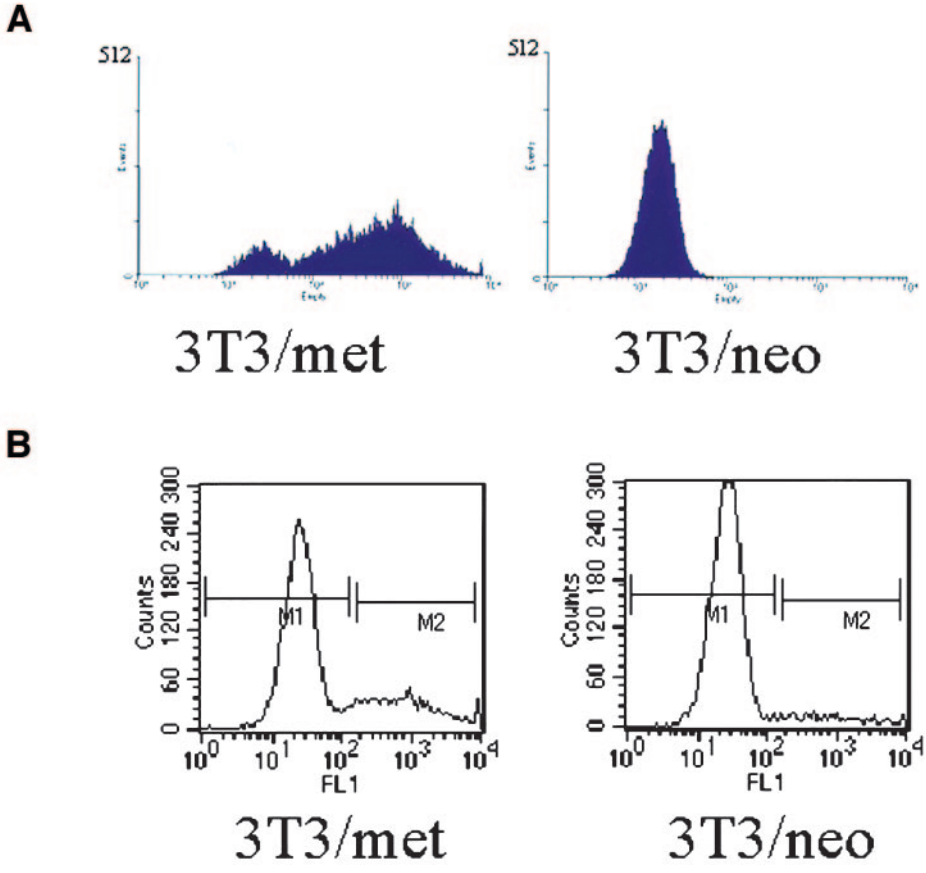

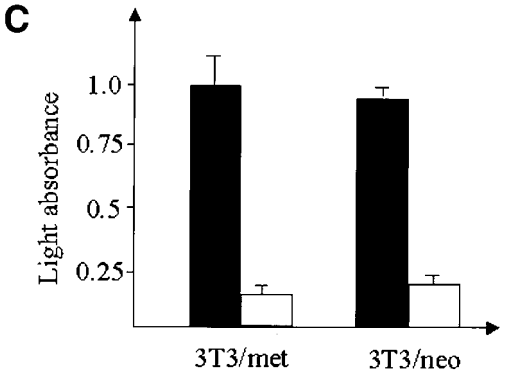

FIG. 1. c-Met expression in 3T3/met and 3T3/neo cells. (A) FACS analyses of c-Met expression in 3T3/met and 3T3/neo cells. Control data are not shown. (B) AAV2-mediated transgene expression in 3T3/met and 3T3/neo cells. Equivalent numbers of 3T3/met and 3T3/neo cells were transduced by 7×10^4 particles/cell of the rAAV2-GFP vector in identical conditions and analyzed after 48 h by FACS. FL1, fluorescence intensity. (C) Binding of AAV2 to 3T3/met and 3T3/neo cells. Binding AAV was reacted with horseradish peroxidase-conjugated antibody and then with luminol-based chemiluminescent substrate which specifically binds peroxidase labels. Finally, AAV binding was quantified by a luminometer and shown as relative light absorbance. Black bars, total binding. White bars, nonspecific binding. Data are means and standard deviations for two experiments performed in duplicate.

cells. These cells were transduced by the rAAV2-GFP or rAAV2-ALP vector and analyzed 48 h after transduction. FACS analyses demonstrated that green fluorescent protein (GFP)-positive cells were detected among rAAV2-GFP-transduced 3T3/met cells (15.6%) about four times more frequently than among rAAV2-GFP-transduced 3T3/neo cells (3.6%) (Fig. 1B). Furthermore, alkaline phosphatase (ALP) activity mediated by the rAAV2-ALP vector was 30-fold higher in 3T3/met cells than in 3T3/neo cells (data not shown). These data demonstrate that the presence of c-Met renders cells more susceptible to rAAV2 transduction.

AAV2 binding to 3T3/met and 3T3/neo cells. We next examined whether c-Met expression would affect cell attachment of AAV2. rAAV2 particles on the cell surface were detected by enzyme-linked immunosorbent assay systems as previously described (12) with minor modifications. Briefly, cells (10^4/well) in a 96-well plate were washed twice with phosphate-buffered saline (PBS) with 1% bovine serum albumin, and nonspecific binding sites were blocked by Blockace (Yukijirushi) for 90 min at 37°C. After rAAV2 (10^9 particles) was preincubated with an antibody against AAV2 capsid protein (A20, 200 ng/well; PROGEN, Wieblingen, Germany) for 30 min on ice in HEPES-buffered saline containing 1% bovine serum albumin (HBSB), cells were incubated with the rAAV-antibody solution at 4°C. Thereafter, cells were fixed in methanol for 15 min at −20°C and then incubated with sheep anti-mouse immunoglobulin G (IgG)-horseradish peroxidase (Amersham, Piscataway, N.J.) for 1 h. The horseradish peroxidase activity was measured by a luminometer using the SuperSignal enzyme-

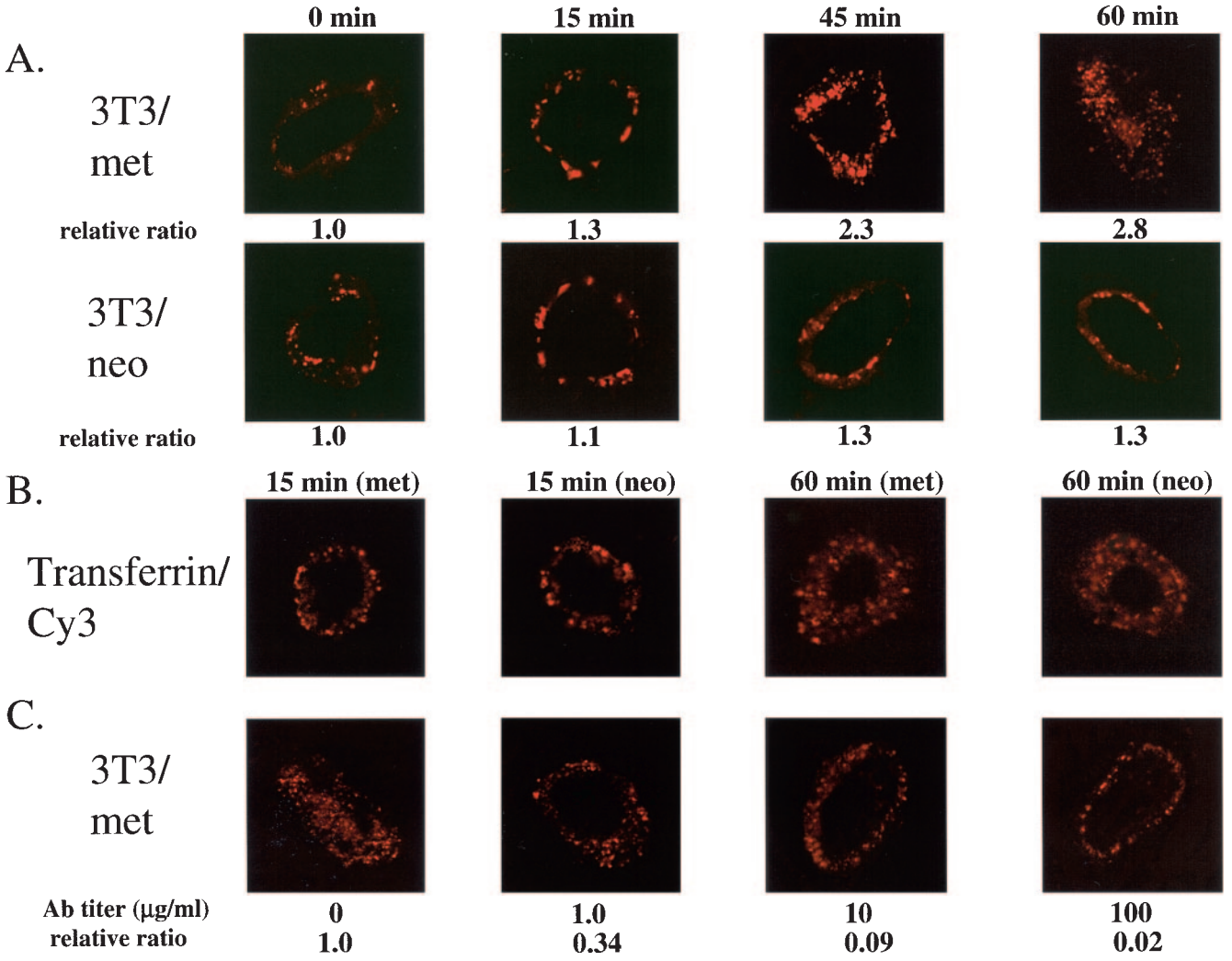

FIG. 2. (A) Internalization of Cy3-rAAV2 by 3T3/met cells and 3T3/neo cells. Images were obtained by confocal microscopy from cross sections representative of the centers of the cells. The relative Cy3 fluorescence intensities within the cells are shown under the panels as relative ratios (fluorescence intensity at starting time = 1.0). The 3T3/met cell shows an increase in the rate of internalization of virus compared with that of the 3T3/neo cell. (B) Internalization of Cy3-transferrin by 3T3/met cells and 3T3/neo cells. Images of transferrin internalization at 15 and 60 min are shown. (C) Internalization of Cy3-rAAV2 by 3T3/met cells with anti-c-Met antibody. Images of rAAV2 internalization at 60 min with different concentrations of anti c-Met antibody are shown. Relative Cy3 fluorescence intensities within cells are shown under the panels as relative ratios (fluorescence intensity for antibody-free condition = 1.0), together with antibody (Ab) concentrations.

linked immunosorbent assay Femto Maximum Sensitivity Substrate system (Pierce). The results revealed that the number of rAAV2 particles attached to 3T3/met cells was almost the same as that for 3T3/neo cells (Fig. 1C), suggesting that c-Met had no influence on cell attachment of AAV2.

AAV2 internalization into 3T3/met and 3T3/neo cells. To directly demonstrate that c-Met promotes AAV2 cell entry, we investigated the virus internalization with 3T3/met cells and control 3T3/neo cells. Internalization was monitored by using rAAV2 vector tagged with fluorescent (Cy3) dye as previously described (29), and Cy3 fluorescence intensity was measured. Briefly, Cy3-labeled rAAV2 was incubated with cells for 1 h at 4°C in HBSB at a concentration of 10^5 rAAV2 particles/cell. At various times after warming to 37°C, cells were placed on ice, washed once with ice-cold HBSB, and resuspended in PBS containing 4% paraformaldehyde. Images were obtained by confocal microscopy, and Cy3 fluorescence intensity was measured by using Meta imaging software version 6.1 (Universal Image Corporation). The Cy3 fluorescence intensity of individual cells was calculated by subtracting the fluorescence intensity of the cell surface from that of the whole cell, and the integrated intensity of each cell was determined after the setting of a consistent intensity threshold. The relative ratio based on the control cells was calculated and expressed as the average for 10 cells at the indicated time. As shown in Fig. 2A, rAAV2 was internalized into 3T3/met cells at a substantially faster rate than into 3T3/neo cells, although rAAV2 was able to bind both 3T3/met and 3T3/neo cells. Further, Cy3 fluorescence intensity of 3T3/met cells increasingly became higher over time while that of 3T3/neo cells hardly changed (Fig. 2A). These results demonstrated that c-Met expression facilitates AAV2 cell entry and has no effect on AAV2 cell binding. The

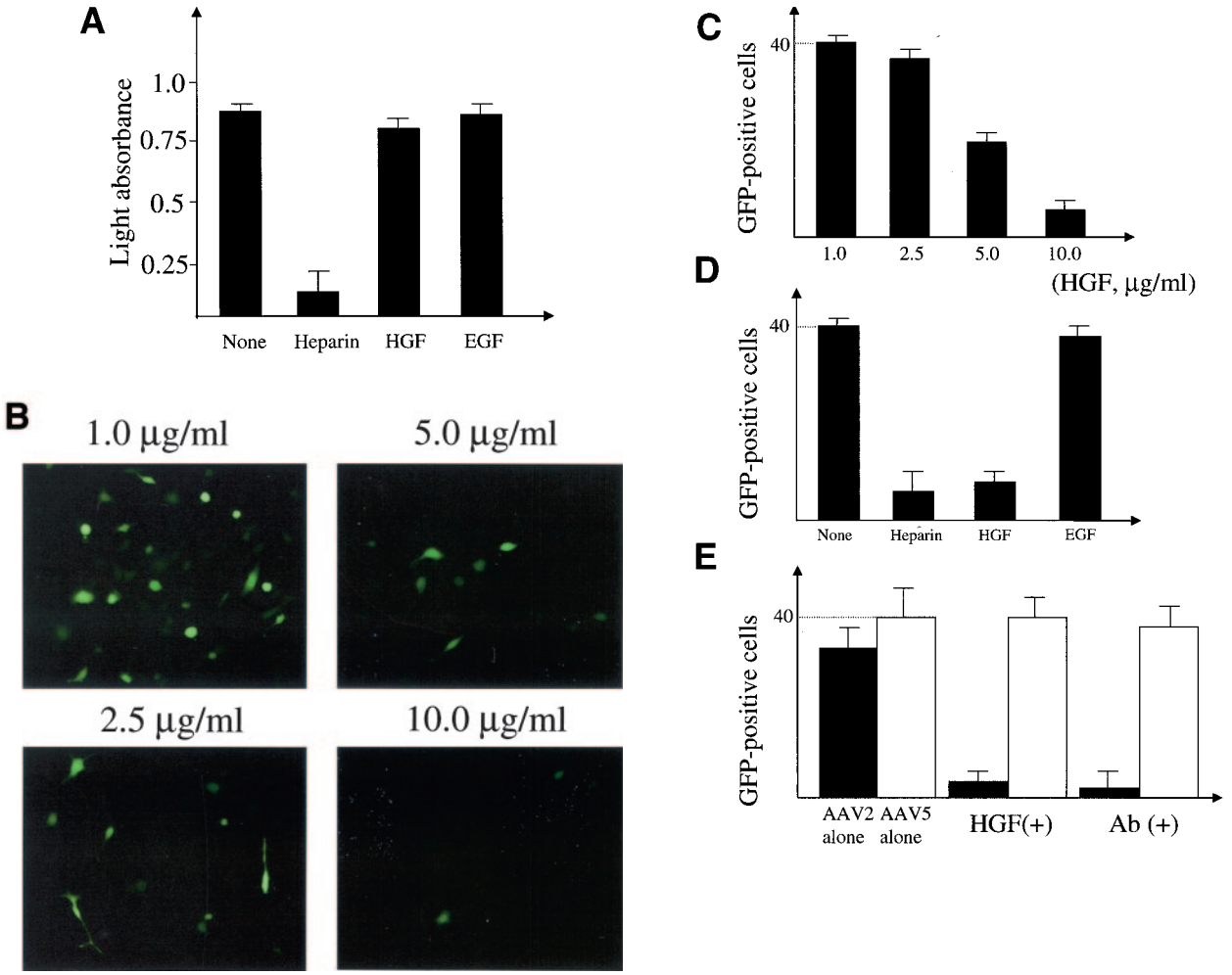

FIG. 3. (A) Binding of AAV2 to HeLa cells under competition with several competitors. With 10 μg of heparin, HGF, and EGF, AAV binding assays were performed with equivalent numbers of HeLa cells. Data are means and standard deviations for two experiments performed in duplicate. (B) AAV2-mediated transgene expression in HeLa cells incubated with different concentrations of HGF. Equivalent numbers of HeLa cells were transduced by rAAV2-GFP vector coincubated with HGF at 1, 2.5, 5.0, or 10.0 μg/ml. GFP-positive cells were detected 48 h after transduction by fluorescence microscopy. Magnification, ×31. (C) AAV2-mediated transgene expression in HeLa cells incubated with different concentrations of HGF. GFP-positive cells were counted 48 h after transduction in one microscopic field (×40). Data are means and standard deviations for an experiment performed in triplicate. (D) AAV2-mediated transgene expression in HeLa cells coincubated with 10 μg of heparin, HGF and EGF. GFP-positive cells were counted 48 h after rAAV2-GFP vector transduction in one microscopic field (×40). Data are means and standard deviations for experiments performed in triplicate. (E) AAV5-mediated transgene expression in CHO cells. CHO cells were transduced by rAAV2-GFP or rAAV5-GFP vector coincubated with HGF (100 μg/ml) and the c-Met antibody (100 μg/ml). GFP-positive cells were counted 48 h after transduction in one microscopic field (×40). Black bar, rAAV2. White bar, rAAV5. Data are means and standard deviations for an experiment performed in triplicate.

enhanced rate of virus entry into the 3T3/met cells was specific for rAAV2, as internalization of an unrelated molecule (Cy3-transferrin) was equivalent for each cell type (Fig. 2B). Because of the crucial role of c-Met in AAV2 cell entry, we further examined whether anti c-Met antibody (goat anti-human, polyclonal; R&D Systems) could inhibit AAV2 internalization in 3T3/met cells. We focused on the internalization at 60 min after warming to 37°C under competition with the antibody at various concentrations and compared respective fluorescence intensities with that for the antibody-free condition. These results revealed that AAV2 cell entry was inhibited by the c-Met antibody in a dose-dependent manner (Fig. 2C).

AAV2 cell binding under competition with HGF. Further, to examine whether HGF (10 μg/ml) prevents binding of AAV2 to HeLa cells, competition assays were performed with heparin (10 μg/ml; positive control) and epithelial growth factor (EGF) (10 μg/ml; negative control). rAAV2-GFP was incubated with respective competitors in the transduction medium for 30 min at 4°C. AAV2 binding was determined by a luminometer as described above. These results demonstrated that AAV2 cell binding was inhibited by heparin but not by EGF, as expected. Although HGF slightly decreased AAV2 cell attachment compared to vehicle control, it was not significantly different between the two groups (Fig. 3A). These results suggest that AAV2 cell binding may be regulated mainly by a primary attachment receptor, not by a coreceptor.

AAV2-mediated transgene expression under competition with HGF. To investigate whether HGF discourages rAAV2-mediated transgene expression, HeLa cells were transduced by rAAV2-GFP coincubated with HGF at different concentra-

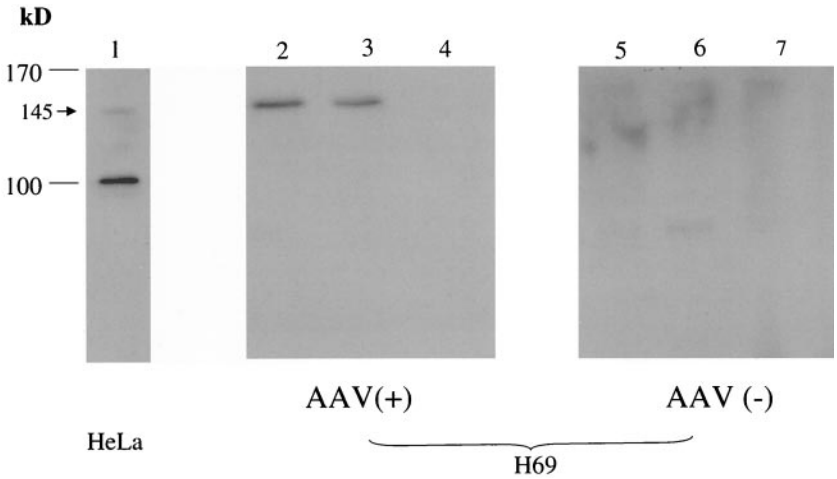

FIG. 4. Viral overlay assay. Proteins were transferred to nitrocellulose and probed with (lanes 1 to 4) or without (lanes 5 to 7) rAAV particles. H69 cells (lanes 2 to 7) are known to lack the β subunit of integrin. Proteins with a molecular mass of 100 kDa (presumed to be a β5 integrin) and 145 kDa (β chain of c-Met) were recognized (lanes 1 to 3). Without rAAV2 particles (lanes 5 to 7), no bands were detected. Plasma membrane proteins (lanes 1, 2, and 5); immunoprecipitated β chain protein of c-Met (lanes 3, 4, 6, and 7); control isotype-matched IgG antibody (lanes 4 and 7).

tions at 37°C for 3 h and then rinsed with PBS three times. Forty-eight hours after transduction, transgene expression was analyzed by counting of GFP-positive cells in one microscopic field. These results showed that HGF had an antagonistic effect on AAV2-mediated transgene expression and reduced transgene expression in a dose-dependent manner (Fig. 3B and C). Next, we evaluated AAV2-mediated transgene expression with several competitors (concentration of all competitors was 10.0 μg/ml). These data showed that both a primary receptor and a coreceptor were necessary for AAV2 to enter host cells (Fig. 3D).

AAV5-mediated transgene expression in CHO cells. To examine whether other AAV serotypes use c-Met in cell entry, we investigated rAAV5 transgene expression in CHO cells (known to be susceptible to AAV5 and to express endogenous c-Met). Cells were transduced by rAAV2-GFP or rAAV5-GFP coincubated with HGF (100 μg/ml) or anti-c-Met antibody (100 μg/ml, goat anti-mouse, polyclonal; R&D Systems). Forty-eight hours after transduction, transgene expression was analyzed by counting of GFP-positive cells in one microscopic field. These results showed that neither the c-Met antibody nor an excess of HGF inhibited rAAV5-mediated transgene expression, whereas rAAV2 transgene expression was down-regulated by the competitors (Fig. 3E), suggesting that AAV5 infects cells via its unique pathway independent of c-Met expression. We also analyzed rAAV5-mediated transgene expression in c-Met transient-transfected COS cells (known to be permissive cells for AAV5 infection) and confirmed that c-Met had no effect on AAV5 infection (data not shown).

Physical interaction between AAV2 and c-Met. To confirm the direct physical interaction between AAV2 particles and c-Met, we performed a viral overlay assay. Briefly, we prepared the immunoprecipitated β chain proteins of c-Met from H69 cells (known to lack the β5 subunit of αVβ5 integrin) (9, 21) and plasma membrane proteins from HeLa and H69 cells by a sucrose gradient flotation method as described previously (15, 29). Under reducing conditions, the samples were subjected to 5 to 20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. After incubation with rAAV2 (5×10^{11} particles/ml), the membrane was reacted with the first antibody (B1; PROGEN) against AAV2 capsid for 1 h, followed by incubation with sheep/anti-mouse IgG-horseradish peroxidase (Amersham) for 30 min. Finally, chemiluminescence analyses were performed by using an enhanced chemiluminescence system (Amersham). Using this assay, we determined that rAAV2 bound a αVβ5 integrin protein (about 100 kDa) and an approximately 150-kDa-molecular-mass protein of the purified plasma membrane proteins from HeLa (Fig. 4, lane 1). On the other hand, rAAV2 bound only an approximately 150-kDa protein of whole plasma membrane from H69 cells, which are known to express c-Met abundantly but hardly express the β5 subunit of integrin (lane 2). rAAV2 bound the immunoprecipitated β subunit protein of c-Met (lane 3). This protein had the same molecular weight as that of the whole plasma membrane which rAAV2 bound (lanes 2 and 3). Because previous reports demonstrated that AAV2 bound the β subunit protein of αVβ5 integrin (100 kDa) or an approximately 150-kDa protein in viral overlay assays (22, 29), it is highly likely that the approximately 150-kDa protein previously reported is the β subunit of c-Met (145 kDa) in our viral overlay assay. In the absence of rAAV2, this common band at 145 kDa was not detected in plasma membrane proteins or in the immunoprecipitated β subunit protein of c-Met (lanes 5 and 6).

Here, we demonstrate that c-Met is a coreceptor for AAV2 infection. HSPG on cell surface binds HGF by its heparan sulfate side chains. HSPG presumably acts by increasing the effective concentration of HGF on the plasma membrane and protecting HGF from degradation, whereas the binding of several HGF molecules to HSPG may promote dimerization and oligomerization of c-Met, leading to enhanced receptor activation. Upon HGF stimulation, c-Met is internalized in clathrin-coated pits which traffic, accumulate near the nucleus, and are associated with the Golgi apparatus (16, 17). As reported earlier, AAV2 clusters on cell surface and internalizes by receptor-mediated endocytosis from clathrin-coated pits

(2). The infectious entry pathway of AAV2 resembles the intracellular trafficking of c-Met as mentioned above. Our study provides justification for using AAV2 vectors with target cells that express high levels of c-Met. In addition, conditions that up-regulate c-Met expression should also enhance AAV2 vector transduction.

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