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The HeLa Cell Receptor for Enterovirus 70 Is Decay-Accelerating Factor (CD55)

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Enterovirus 70 (EV70) is a recently emerged human pathogen belonging to the family *Picornaviridae*. The ability of EV70 to infect a wide variety of nonprimate cell lines in vitro is unique among human enteroviruses. The importance of virus receptors as determinants of viral host range and tropism led us to study the host cell receptor for this unusual picornavirus. We produced a monoclonal antibody (MAb), EVR1, which bound to the surface of HeLa cells and protected them against infection by EV70 but not by poliovirus or by coxsackievirus B3. This antibody also inhibited the binding of [^{35}S]EV70 to HeLa cells. MAb EVR1 did not bind to monkey kidney (LLC-MK$_2$) cells, nor did it protect these cells against virus infection. In Western immunoassays and in immunoprecipitations, MAb EVR1 identified a HeLa cell glycoprotein of approximately 75 kDa that is attached to the cell membrane by a glycosyl-phosphatidylinositol (GPI) anchor. Decay-accelerating factor (DAF, CD55) is a 70- to 75-kDa GPI-anchored membrane protein that is involved in the regulation of complement and has also been shown to function as a receptor for several enteroviruses. MAb EVR1 bound to Chinese hamster ovary (CHO) cells constitutively expressing human DAF. Anti-DAF MAbs inhibited EV70 binding to HeLa cells and protected them against EV70 infection. Transient expression of human DAF in murine NIH 3T3 cells resulted in binding of labelled EV70, and stably transformed NIH 3T3 cells expressing DAF were able to support virus replication. These data indicate that the HeLa cell receptor for EV70 is DAF.

Viruses belonging to the family *Picornaviridae* are responsible for a wide range of illnesses of humans and animals. Enterovirus 70 (EV70), a human enterovirus, is the etiologic agent of acute hemorrhagic conjunctivitis, a highly contagious form of conjunctivitis (55) that is distinct from ocular infections caused by other human enteroviruses (11). In rare instances, acute hemorrhagic conjunctivitis is followed by infection of the central nervous system, leading to poliomyelitis-like paralysis (17, 54). Since its emergence as a human pathogen in West Africa in 1969 (24), EV70 has been responsible for two pandemics and has demonstrated the potential to cause widespread outbreaks of acute hemorrhagic conjunctivitis throughout the world (54).

EV70 possesses biological and pathogenic properties that are unique among human enteroviruses (55). Among these properties is the ability to replicate in a wide variety of nonprimate-derived cell lines. Cell lines of porcine, murine, leporine, crecitine, and bovine origin can support the growth of EV70 in vitro (56).

Viruses initiate infection by binding to specific receptors on the surface of susceptible cells (2, 38, 41). Although factors affecting steps after this interaction may influence infectivity and replication, the nature and distribution of host cell receptors are recognized as major determinants of viral host range and of cell and tissue tropism (33).

A growing number of picornavirus receptors have been characterized, and it appears that most are molecules involved in cell-cell interactions or in the regulation of cell function (4, 53). Molecules belonging to the immunoglobulin (Ig) superfamily serve as receptors for the major-group human rhinoviruses (intercellular cell adhesion molecule 1 [15, 47]), for poliovirus (32), and for a variant of encephalomyocarditis virus (20). Echoviruses 1 and 8 attach to integrin VLA-2 (6, 7, 22), and $\alpha_v\beta_3$ integrin is reported to be the receptor for coxsackievirus A9, echovirus 22 (40), and foot-and-mouth disease virus (8). Minor-group human rhinoviruses bind to cells via members of the low-density lipoprotein receptor family (18). Decay-accelerating factor (DAF, CD55) is recognized as the cellular receptor for at least six echovirus serotypes (3, 51) and as a receptor for coxsackieviruses B1, B3, and B5 (5, 42). Interaction of group B coxsackieviruses with permissive cells may also involve a nucleolin-like membrane protein (39). Human DAF is a 70- to 75-kDa membrane glycoprotein involved in protecting cells against lysis by homologous complement (37). The molecule has five extracellular domains (27, 29): four contiguous short consensus repeat (SCR) domains of approximately 60 amino acids each, followed by a serine/threonine-rich, heavily O-glycosylated C-terminal domain. A glycosyl-phosphatidylinositol (GPI) anchor attaches the molecule to the outer leaflet of the cell membrane.

Here, we demonstrate that DAF is also the human (HeLa) cell receptor for EV70. A monoclonal antibody (MAb), EVR1, which blocks infection of HeLa cells by EV70, was produced. MAb EVR1 reacts with a 75-kDa cell surface glycoprotein that shares a number of properties with DAF, including sensitivity to phosphatidylinositol-specific phospholipase C (PI-PLC). Several anti-DAF MAbs also protect HeLa cells against EV70 infection and interfere with virus binding. The ability to bind virus is conferred to NIH 3T3 cells following transient surface expression of human DAF in these cells, and stably transformed NIH 3T3 cells expressing human DAF support EV70 replication. We therefore propose that DAF is the HeLa cell receptor for EV70.

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MATERIALS AND METHODS

Cells. HeLa cells were obtained from the National Institute of Allergy and Infectious Diseases AIDS Research and Reference Reagent Program, Bethesda, Md. Rhesus monkey (*Macaca mulatta*) kidney cells, LLC-MK_2, were purchased from Flow Laboratories, Rockville, Md. African green monkey (*Cercopithecus aethiops*) kidney cells, CV-1, were obtained from the American Type Culture Collection, Rockville, Md., and murine NIH 3T3 cells were provided by E. G. Brown, Department of Microbiology and Immunology, University of Ottawa. Growth medium consisted of Eagle's minimal essential medium containing Earle's salts (MEM) supplemented with 0.15% (wt/vol) sodium bicarbonate, 2 mM L-glutamine, 50 μg of gentamicin (Roussel Canada, Montreal, Quebec, Canada) per ml, and either 5% (vol/vol) (for LLC-MK_2 and NIH 3T3 cells) or 10% (vol/vol) (for HeLa and CV-1 cells) heat-inactivated fetal bovine serum (FBS). Cells were grown as monolayer cultures at 37°C in a 5% CO_2 atmosphere. Chinese hamster ovary (CHO) cells and CHO cells stably expressing human DAF (CHO-DAF cells) were grown in Ham's F12 medium (28). NIH 3T3 cells transfected with the gene for human DAF under the control of the simian virus 40 early promoter (3T3-DAF) and NIH 3T3 cells transfected with the DAF gene in reverse orientation with respect to the promoter (3T3-RDAF) were gifts of J. Atkinson, Washington University School of Medicine, St. Louis, Mo., and were maintained as described previously (52). All media and supplements were from Gibco/BRL Life Technologies Canada, Burlington, Ontario, Canada, unless otherwise stated.

Viruses. EV70 prototype strain J670/71 was obtained from M. Hatch and M. Pallansch, Centers for Disease Control and Prevention, Atlanta, Ga. Poliovirus Sabin 1 and coxsackievirus B3 were provided by S. A. Sattar, Department of Microbiology and Immunology, University of Ottawa. Vaccinia virus vTF7-3 was obtained from the American Type Culture Collection and was propagated and subjected to titer determination in CV-1 cells.

Growth and purification of EV70. Concentrated stocks of EV70 were prepared as follows. LLC-MK_2 cells were infected at a multiplicity of infection (MOI) of 0.1 PFU per cell for 1 h in serum and antibiotic-free growth medium and then incubated at 33°C for 30 h in complete medium. Virus was collected by harvesting the medium and cells, performing three cycles of freezing and thawing, and subjecting the mixture to clarification at 4,000 × *g* at 4°C. Pooled supernatants were concentrated with a Minitan ultrafiltration apparatus (Millipore Ltd., Mississauga, Ontario, Canada), and the virus was pelleted in a Beckman SW28 rotor at 110,000 × *g* for 4 h at 4°C. The virus pellet was resuspended in R buffer without glycerol and placed on a linear density gradient of 10 to 40% (wt/vol) sucrose in R buffer (1). After centrifugation at 154,000 × *g* at 4°C in a Beckman SW41 rotor for 3.5 h, gradient fractions were recovered and portions of each were tested by plaque assay. Peak fractions were pooled, virus titers were determined, and aliquots were stored at −80°C.

Purification of radiolabelled EV70. LLC-MK_2 cells were infected with EV70 at a MOI of 0.1 for 1 h at 33°C. The inoculum was removed, and serum-free, methionine-free growth medium (ICN Biomedicals Canada Ltd., Mississauga, Ontario, Canada) was added to the monolayers. After 3 h, 5 μCi of Tran^{35}S-label (ICN) was added per ml of medium. Incubation was continued for a further 30 h, and virus was harvested as described above. Sucrose gradient fractions were recovered and analyzed for the presence of ^{35}S by scintillation counting. Peak fractions were pooled, divided into aliquots, and stored at −80°C.

Membrane preparation. Cell monolayers were incubated for 15 min at 37°C with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 [pH 7.4]) containing 50 mM EDTA, removed, pooled, washed three times with cold PBS, resuspended in 10 mM sodium phosphate buffer, and swollen on ice for 15 min. The cells were disrupted in a Dounce homogenizer in the presence of protease inhibitors (1 mM benzamidine-HCl, 1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA), and centrifuged at 1,400 × *g* for 5 min. The supernatant was transferred to fresh tubes and centrifuged at 200,000 × *g* for 2 h in a TH641 rotor (DuPont Inc., Mississauga, Ontario, Canada). The pellet was resuspended in PBS, the protein concentration was adjusted to 10 mg/ml (Bradford assay; Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada), and the membranes were stored at −80°C.

Antibodies. (i) Production of anti-receptor antibodies. Inbred female BALB/c mice (6 to 8 weeks old) (Charles River Laboratories, St. Constant, Quebec, Canada) were immunized by intraperitoneal injection of HeLa cell membranes at 3-week intervals. The first injection consisted of 50 μg of membrane protein emulsified in complete Freund's adjuvant, the second consisted of 80 μg of antigen in incomplete Freund's adjuvant, and the third consisted of 120 μg of antigen in PBS. Antibody production against HeLa cell membranes was demonstrated by indirect enzyme-linked immunosorbent assay (ELISA), and 3 days prior to cell fusion, mice were primed by tail vein injection of 175 μg of membrane protein in PBS. Cells were prepared from the spleens of two mice and fused to SP2/0 myeloma cells (44) by a standard method (23). Supernatants of viable hybridoma cultures were screened for the presence of antibodies that inhibited EV70 infection of HeLa cells as determined in the cell protection assay described below. Hybridomas producing protective antibodies were cloned, and their supernatants were retested. Isotyping was performed by indirect ELISA, using standard methods (14). HeLa cell membrane protein was adsorbed to ELISA microplate wells, and undiluted tissue culture supernatants from cloned cells were used as the primary antibody. Horseradish peroxidase-conjugated goat anti-mouse isotype-specific antibodies (Caltag, South San Francisco, Calif.) at a dilution of 1:2,000 were used as secondary antibodies. Ascites fluid containing MAb was obtained from retired breeders. Antibodies in culture supernatants and ascites fluids were quantitated by antibody sandwich ELISA (19). Briefly, ELISA plates were coated with 1 μg of rat-anti mouse IgG (heavy plus light chains) (Jackson Immunoresearch Laboratories Inc., West Grove, Pa.) capture antibody per ml, incubated with dilutions of hybridoma culture supernatant or ascites fluid, and then reacted with a biotinylated mouse-specific IgG1 MAb. After incubation with peroxidase-conjugated streptavidin (Jackson) and *o*-phenylenediamine dihydrochloride substrate (OPD; Sigma Chemical Co., St. Louis, Mo.), the A_{490} was determined. Antibody concentrations were determined from a standard curve constructed with known amounts of mouse IgG1 (Coulter Electronics, Burlington, Ontario, Canada) as the primary antibody. EVR1 hybridoma supernatant contained 17.5 μg of Ab/ml, and EVR1 ascites contained 11 mg of Ab/ml.

(ii) Sources of other antibodies. DAF-specific MAbs 1H4 (35 μg/ml), 8D11 (36 μg/ml), 11D7 (40 μg/ml) (10), and 1F7 (1.2 mg/ml) (3) were used. 8D11 and 11D7 hybridoma culture supernatants were gifts from W. Rosse, Department of Medicine, Duke University Medical Center, Durham, N.C., and 1F7 ascites fluid was from R. W. Finberg, Dana-Farber Cancer Institute, Boston, Mass. Anti-lymphocytic choriomeningitis virus (LCMV) ascites fluid (IgG1; 4 mg/ml) was provided by K. Wright, Department of Microbiology and Immunology, University of Ottawa. Fluorescein isothiocyanate (FITC)-conjugated and nonconjugated mouse antibodies specific for human HLA ABC class 1 IgG1 (anti-MHC 1) were from Serotec Canada, Toronto, Ontario, Canada.

Cell protection assay. (i) Assessment of antibody EVR1. HeLa cells were grown in 96-well plates (Corning Science Products, Richmond Hill, Ontario, Canada). The growth medium was removed, and the monolayers were incubated with 100 μl of undiluted hybridoma cell culture supernatant for 1 h at 37°C. The cells were washed with PBS, and EV70 was added at a MOI of 1 in serum-free growth medium. After a 45-min incubation at 33°C, the inoculum was removed, fresh medium containing 10% FBS was added, and the cells were placed at 33°C. Monolayers were monitored at regular intervals by light microscopy for signs of virus-induced cytopathic effects (CPE). Results of antibody screening were confirmed on duplicate plates by a colorimetric assay based on the ability of viable cells to metabolize the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (16). Cell protection assays with poliovirus and coxsackievirus were performed in an identical manner, except that incubations were performed at 37°C.

(ii) Assessment of DAF-specific MAbs. DAF-specific MAbs 1H4, 8D11, 11D7, and 1F7 were tested for their protective ability as described above. Antibodies were diluted appropriately in RPMI 1640 medium containing 2 mM glutamine. For MAb 1F7, 2 mM $CaCl_2$ and 2 mM $MgCl_2$ were added (3).

Binding and binding inhibition assays. Cells were grown to confluency in 24-well dishes (Corning). The medium was aspirated, the monolayers were washed with PBS, and [^{35}S]EV70 was added in serum-free MEM. After a 45-min incubation at 33°C, the inoculum was removed and the monolayers were washed with PBS. The inoculum and PBS wash were pooled, and they represent the unbound fraction of input virus. The monolayers were then disrupted with PBS containing 1% (wt/vol) sodium dodecyl sulfate (SDS), and the wells were washed. These samples, representing the bound fraction of input virus, were also pooled. The samples were analyzed by scintillation counting. The percentage of input virus bound to cells was determined by dividing the counts per minute (cpm) of labelled EV70 in the bound fraction by the total number of cpm recovered. For binding inhibition studies, after removal of growth medium and the PBS wash, monolayers were treated with 100 μl of test sample (MAb EVR1, 1H4, 8D11, or 11D7; anti-major histocompatibility complex class [MHC] I; or growth medium) for 1 h at 37°C. The test sample was then removed, monolayers were washed with PBS, and virus-binding assays were performed as described above.

Indirect immunofluorescence. Cells were removed from 100-mm plates by scraping or by treatment with 0.05% trypsin–0.5 mM EDTA (Gibco/BRL) in PBS and washed in PBS–2% (wt/vol) bovine serum albumin (BSA). Approximately 5 × 10^5 cells were dispensed into separate tubes, pelleted, and resuspended in 25 μl of hybridoma cell culture supernatant or ascites fluid diluted 1:200 in PBS-BSA. After incubation on ice for 1 h, the cells were washed in PBS-BSA and the cell pellets were resuspended in 20 μl of PBS-BSA containing a 1:5 dilution of FITC-conjugated sheep anti-mouse Ig (Amersham Canada, Oakville, Ontario, Canada). After 1 h on ice, the cells were washed, resuspended in 90% glycerol–PBS (pH 9.6), deposited on glass slides under coverslips, and examined under a Zeiss Aristoplan epifluorescence microscope. To examine the susceptibility of the EVR1 ligand to PI-PLC, HeLa cells were trypsinized and washed three times prior to resuspension in PI-PLC buffer (RPMI 1640 medium, 0.2% BSA, 50 μM β-2-mercaptoethanol, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES], 0.1% [wt/vol] sodium azide). The cells were then divided into two fractions. One fraction received 0.5 U of *Bacillus thuringiensis* PI-PLC (Oxford Glycosystems, Rosedale, N.Y.) per 10^6 cells, and the other did not. Following incubation at 37°C for 1 h, both sets of cells were washed and processed for surface immunofluorescence as described above (EVR1 and 1H4 culture supernatant, neat; anti-MHC 1, 1:10). All photography was performed at 40× power with Kodak Ektachrome 400 ASA film.

Radioimmunoprecipitations. Cells grown on 100-mm tissue culture dishes were starved of sugars for 2 h and then incubated in 10 ml of medium containing 15 μCi of D-[6-^{3}H]glucosamine hydrochloride (Amersham) per ml. Labelling periods of 4 and 16 h gave identical results. The cells were then scraped and resuspended in 600 μl of RIPA buffer (50 mM Tris-HCl [pH 7.2], 150 mM NaCl, 1% sodium deoxycholate [Difco Laboratories, Detroit, Mich.], 1% Nonidet P-40) containing 1 mM phenylmethylsulfonyl fluoride and 1 mM benzamidine-HCl and given a short pulse in a benchtop centrifuge to pellet the cellular debris. Protein G-Sepharose 4 Fast Flow beads (Pharmacia Canada, Baie d'Urfé, Quebec, Canada) were washed in RIPA buffer containing 2% ovalbumin, and then 200 μl of cell lysate and 10 μl of undiluted ascites fluid were added. After an overnight incubation with mixing at 4°C, the samples were analyzed by electrophoresis in 8% polyacrylamide gels containing SDS (13, 26). Prestained high-molecular-weight markers were from Amersham.

Western blots (immunoblots). Lysates of unlabelled cells were prepared as described above for radiolabelled lysates. Proteins (25 μg per lane) were separated by electrophoresis at 4°C in 8% polyacrylamide gels by using standard protocols (26) with the following modifications. Gels and running buffers contained 0.05% SDS, and the samples were prepared, without being heated, in electrophoresis sample buffer containing 0.05% SDS and no β-mercaptoethanol. Proteins were transferred at 4°C to Schleicher & Schuell NC nitrocellulose membranes (Mandel Scientific Co. Ltd., Guelph, Ontario, Canada) with a Bio-Rad Mini Trans-Blot apparatus (100 V for 2 h) and the transfer buffer of Towbin et al. (49). The membranes were blocked in PBS–3% (wt/vol) BSA for 30 min at 37°C. After three 37°C washes in PBS–0.02% Tween 20 (PBS-Tween), the membranes were incubated for 1 h at 37°C with MAb as either hybridoma supernatant (1H4, 1:3,000) or ascites fluid (EVR1, 1:1,000; anti-LCMV IgG1, 1:300) diluted in PBS–3% BSA. After three washes in PBS-Tween, the membranes were incubated for 1 h at 37°C with peroxidase-conjugated goat anti-mouse IgG (heavy plus light chains) (Jackson) diluted 1:20,000 in PBS–3% BSA. The membranes were washed with PBS-Tween, and the color was developed in a solution consisting of 0.01 M $(NH_4)_2SO_4$, 0.01 M KH_2PO_4 (pH 6.0), 0.03% (vol/vol) H_2O_2, and 0.005% (wt/vol) *o*-dianisidine (Sigma). The reaction was stopped with repeated washes in distilled water.

Transient expression of DAF. A human DAF cDNA fragment (28) was subcloned into the *Eco*RI site of plasmid pcDNA3 (Invitrogen Corp., San Diego, Calif.). Twenty-four-well tissue culture plates (Corning) seeded with 8×10^4 NIH 3T3 cells were transfected 24 h later with 1 μg of plasmid DNA per well, using OptiMEM and Lipofectamine reagent (Gibco/BRL), by using protocols provided by the manufacturer. An equal volume of MEM containing 10% FBS was added to the transfection mix 9 h later, and the cells were cultured for a further 15 h. Transfected cells were then infected with vaccinia virus vTF7-3 at a MOI of 20 for 1 h at 37°C. The inoculum was removed, and the cells were incubated for a further 18 h in complete growth medium containing 5% FBS. Monolayers were then used in binding assays or in immunofluorescence studies, as described above.

RESULTS

Identification of a MAb that blocks EV70 infection of HeLa cells. To identify a HeLa cell membrane component that acts as the receptor for EV70, we chose to produce a MAb that would bind to the surface of HeLa cells and inhibit their infection by EV70. Mice were immunized with either whole HeLa cells or membrane preparations of HeLa cells. Hybridomas were generated by fusion of spleen cells with SP2/0 cells, and hybridoma culture supernatants were screened for their ability to protect HeLa cell monolayers from EV70 infection, as evidenced by the absence of CPE. Under the conditions of the cell protection assay, EV70 at a MOI of 1 resulted in complete destruction of HeLa monolayers in control cultures (no antibody) within 48 h postinfection. Of more than 900 hybridomas tested, one hybridoma (derived from mice immunized with HeLa cell membranes) produced Ab that prevented the appearance of CPE for the duration of the screening period (72 h). Nine clones were isolated from this culture, all of which were found to secrete IgG1. Ascites fluids generated from these clones were retested by the cell protection assay, and all demonstrated concentration-dependent protection of HeLa cell monolayers. MAb in ascites fluid or in the culture supernatant of cloned hybridoma cells was designated EVR1.

TABLE 1. Specificity of MAb EVR1 cell protection

Virus	Cell protection[a]	
	HeLa	LLC-MK_2
EV70	+	−
Poliovirus Sabin 1	−	−
Coxsackievirus B3	−	−

[a] +, no observable CPE for a minimum of 96 h postinfection; −, CPE observed within 45 h postinfection. EVR1 was used at 17.5 μg/ml.

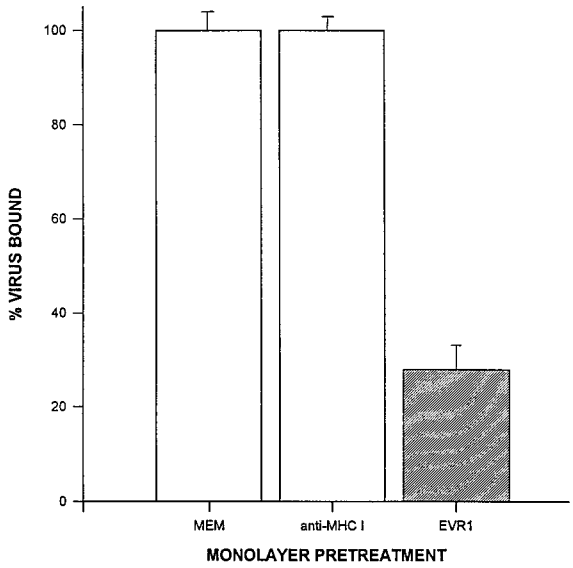

FIG. 1. MAb EVR1 inhibits binding of EV70 to HeLa cells. HeLa cell monolayers in 24-well plates were treated as indicated for 1 h at 37°C before incubation with 5×10^3 cpm of [^{35}S]EV70 for 45 min at 33°C. The amount of virus bound to cells was determined as described in Materials and Methods. MEM, growth medium alone; anti-MHC I, growth medium containing 10 μg of MHC 1-specific IgG1 per ml; EVR1, EVR1 hybridoma culture supernatant (17.5 μg/ml). Results are shown as the mean percentage of virus bound relative to the MEM control ± standard deviation for four samples.

Virus and cell specificity of MAb EVR1. In binding experiments with [^{35}S]EV70 and unlabelled competitor viruses, we previously determined that EV70 does not compete with poliovirus (strain Sabin 1) for binding sites on LLC-MK_2 cells and that coxsackievirus B3 can partially inhibit the binding of radiolabelled EV70 (data not shown). To rule out the possibility

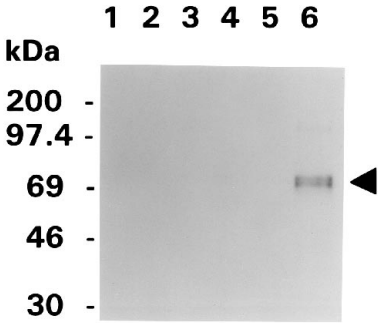

FIG. 2. MAb EVR1 immunoprecipitates a HeLa cell glycoprotein. Proteins immunoprecipitated from lysates of cells labelled for 16 h with [^{3}H]glucosamine were analyzed by SDS-PAGE as described in Materials and Methods. Lanes: 1, LLC-MK_2 lysate without Ab; 2, LLC-MK_2 lysate with LCMV-specific IgG1; 3, LLC-MK_2 lysate with EVR1; 4, HeLa lysate without antibody; 5, HeLa lysate with LCMV-specific IgG1; 6, HeLa lysate with EVR1. Positions of molecular mass standards are indicated on the left.

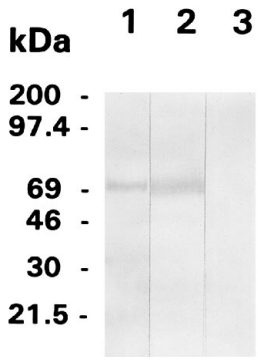

FIG. 3. Immunoblot analysis of HeLa cell lysates with MAbs EVR1 and 1H4. HeLa cell lysates were separated by electrophoresis in 8% polyacrylamide gels containing 0.05% SDS under nonreducing conditions and transferred to nitrocellulose. Membrane strips were incubated with MAbs as indicated and developed as described in Materials and Methods. Lanes: 1, EVR1, 2, 1H4, 3, LCMV-specific IgG1. Positions of molecular mass standards are indicated on the left.

that MAb EVR1 protects HeLa cells by nonspecific masking of the cell surface, we tested the ability of the antibody to protect HeLa and LLC-MK_2 monolayers against infection by these two other enteroviruses (Table 1). Undiluted EVR1 hybridoma supernatant completely protected HeLa cell monolayers against EV70 infection, with monolayers remaining free of CPE for 96 h, at which time monitoring was terminated. Neither HeLa nor LLC-MK_2 cells were protected against poliovirus or coxsackievirus B3. In all cases, the monolayers were completely destroyed within 18 h following infection. MAb EVR1 also failed to protect LLC-MK_2 monolayers from EV70 infection, as evidenced by the appearance of extensive CPE within 45 h after infection. To show that MAb EVR1 blocks the attachment of EV70, HeLa cell monolayers were incubated with various dilutions of hybridoma culture fluid and then with [^{35}S]EV70 in binding inhibition assays. At 10 μg/ml, inhibition by MAb EVR1 was maximal, reducing the binding of radiolabelled EV70 by 74 to 84% compared with controls (Fig. 1). Lower concentrations of antibody resulted in less inhibition. The cell line specificity of binding was confirmed by immunofluorescence with live cells. MAb EVR1 recognized an epitope that was evenly distributed over the surface of HeLa cells (see Fig. 4A). Dispersal of HeLa cells by trypsin treatment prior to incubation with antibody had no effect on MAb EVR1 binding, whereas LLC-MK_2 cells, dispersed either manually or by trypsin treatment, did not bind MAb EVR1 (data not shown). Binding inhibition assays and immunofluorescence confirmed the specificity observed in cell protection assays and indicated that MAb EVR1 binds to an epitope that is present on the surface of human (HeLa) but not monkey (LLC-MK_2) cells.

Immunoprecipitation of a HeLa cell glycoprotein by MAb EVR1. To identify the protein recognized by MAb EVR1, lysates of [^{3}H]glucosamine-labelled HeLa and LLC-MK_2 cells were incubated with Ab and the immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 2). MAb EVR1 reacted specifically with a HeLa cell protein of approximately 70 to 75 kDa that appeared to migrate as a doublet in polyacrylamide gels (Fig. 2, lane 6). Successful labelling after a 4-h (data not shown) or a 16-h

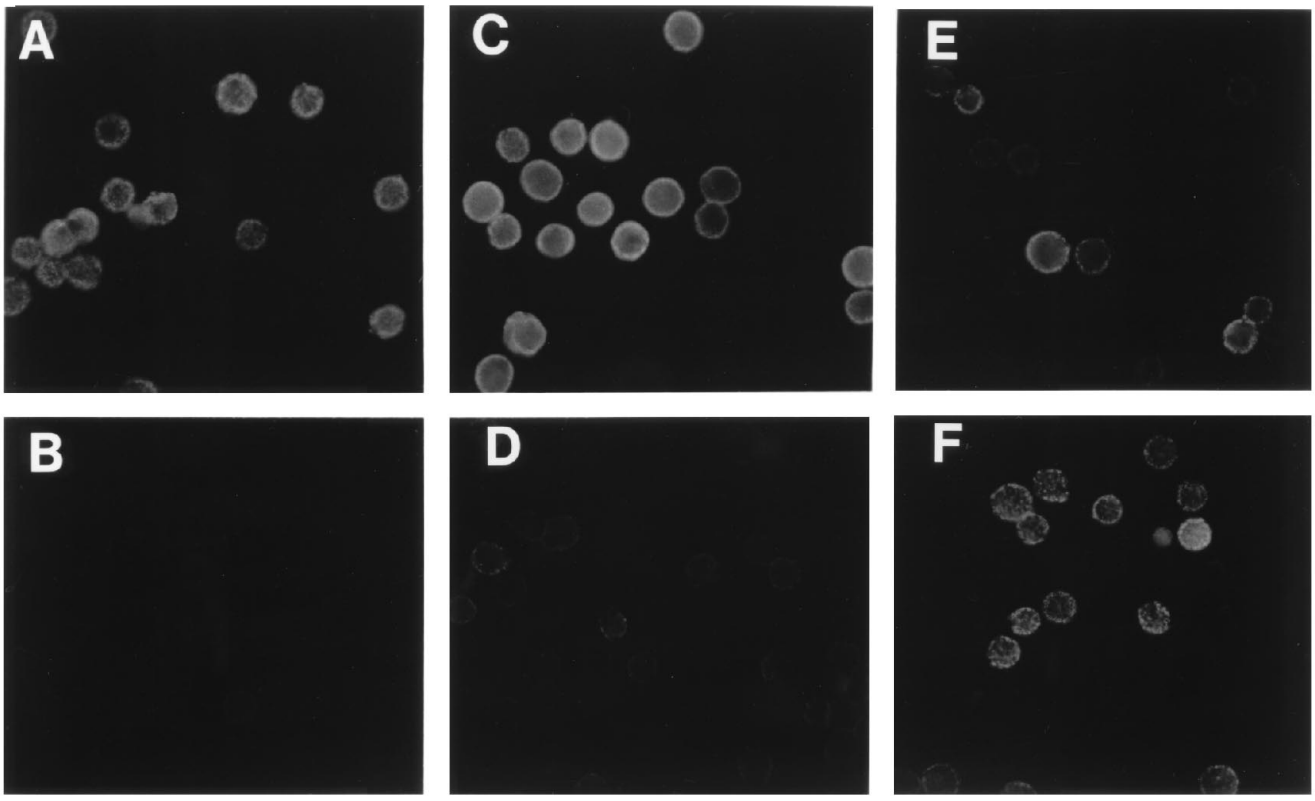

FIG. 4. The ligand of EVR1 is sensitive to PI-PLC. Cell monolayers were dispersed by trypsin treatment, washed, treated, and incubated with Abs as indicated. Ab binding was detected by indirect immunofluorescence with an FITC-conjugated sheep anti-mouse Ab, as described in Materials and Methods. (A) HeLa cells, MAb EVR1; (B) PI-PLC-treated HeLa cells, MAb EVR1; (C) HeLa cells, MAb 1H4; (D) PI-PLC-treated HeLa cells, MAb 1H4; (E) HeLa cells, anti-MHC I IgG1; (F) PI-PLC-treated HeLa cells, anti-MHC I IgG1.

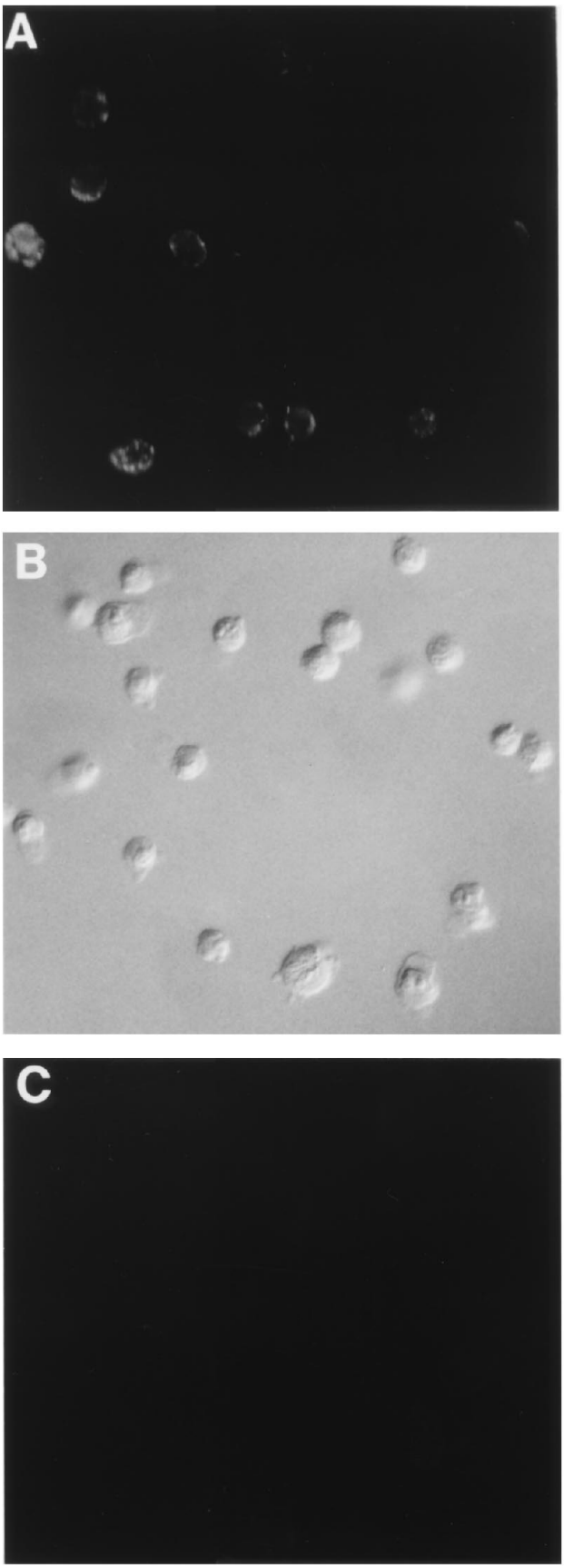

FIG. 5. MAb EVR1 recognizes DAF. Cell monolayers were dispersed by trypsin treatment, washed, and incubated with MAb EVR1. Ab binding was detected by indirect immunofluorescence with an FITC-conjugated sheep anti-mouse Ab, as described in Materials and Methods. (A) CHO-DAF cells; (B) CHO cells with Nomarski optics; (C) CHO cells, same field as in panel B.

incubation with [^{3}H]glucosamine indicated that the protein was glycosylated. No protein was immunoprecipitated from lysates of LLC-MK$_2$ cells (lane 3) or from HeLa or LLC-MK$_2$ lysates incubated with mouse LCMV-specific IgG1 or protein G beads alone (lanes 1, 2, 4, and 5). Preliminary immunoblotting studies demonstrated that the epitope recognized by MAb EVR1 was both heat labile and sensitive to reducing agents in electrophoresis sample buffer (β-mercaptoethanol) (data not shown). When membrane proteins were separated on 8% polyacrylamide gels containing 0.05% SDS under nonreducing conditions, MAb EVR1 recognized a HeLa cell protein of approximately 75 kDa (Fig. 3, lane 1), but did not react with proteins from LLC-MK$_2$ cells (data not shown).

The EVR1 ligand on HeLa cells is DAF. A survey of the literature suggested that the protein recognized by MAb EVR1 might be DAF (CD55). Because DAF is anchored to cells by a GPI moiety, we wished to determine if the ligand of EVR1 was also attached to HeLa cells by a GPI tail (12, 30). Therefore, prior to reaction with antibody, HeLa cells were treated with PI-PLC, an enzyme known to release GPI-linked proteins from the surface of cells. As shown in Fig. 4, the ability of MAb EVR1 to bind to HeLa cells treated with PI-PLC was greatly diminished (Fig. 4A and B), as was binding of the DAF-specific MAb, 1H4 (Fig. 4C and D). Binding of a MAb specific for human MHC 1 (a non-GPI-linked integral membrane glycoprotein) was not affected by PI-PLC (Fig. 4E and F). In Western blots, MAb 1H4, like MAb EVR1, reacted specifically with a HeLa cell protein of approximately 75 kDa (Fig. 3, lane 2).

Although we previously observed that CHO cells were able to bind EV70 and to support EV70 replication (data not shown), we exploited the availability of CHO cells transfected with a human DAF cDNA and constitutively expressing DAF (CHO-DAF [28]) to confirm the specificity of MAb EVR1 for human DAF. In immunofluorescence studies (Fig. 5), MAb EVR1 bound to CHO-DAF but not to CHO cells. MAb 1H4 behaved similarly (data not shown).

DAF-specific MAbs block EV70 binding to and infection of HeLa cells. To demonstrate that DAF acts as the HeLa cell receptor for EV70, monolayers of HeLa and LLC-MK$_2$ cells were treated with MAb 1H4 (10 to 35 μg/ml), prior to challenge with EV70, in a cell protection assay. As observed with MAb EVR1, MAb 1H4 protected HeLa cells (Table 2) but not LLC-MK$_2$ cells (data not shown) against EV70 infection, in a concentration-dependent manner (data not shown). We subsequently assessed the ability of other anti-DAF MAbs to protect HeLa cells (Table 2). Antibodies directed against SCRs 1 and 3 of DAF (MAbs 11D7 and 1H4, respectively [10]) protected cells, while MAbs specific for SCRs 2 and 4 (1F7 and 8D11, respectively [3, 10]), did not. MAbs EVR1 (17.5 μg/ml), 1H4 (35 μg/ml), and 11D7 (4 μg/ml) prevented the appearance of CPE for 96 h, whereas cells treated with MAb 8D11 (40 μg/ml) began showing CPE by 48 h postinfection. The ability of DAF-specific Abs to prevent EV70 infection of HeLa cells correlated with their ability to inhibit virus binding (Fig. 6).

The HeLa cell receptor for EV70 is DAF. NIH 3T3 cells, which do not bind EV70 or support virus replication, were used in transient-expression experiments to evaluate the ability of DAF to act as a receptor for EV70. Human DAF cDNA

TABLE 2. Anti-DAF MAb protection of HeLa cells against EV70 infection

MAb[a]	SCR	Protection[b]
11D7	1	+
1F7	2	−
1H4	3	+
8D11	4	−
EVR1	?	+

[a] 11D7, 4 μg/ml; 1F7, 240 μg/ml; 1H4, 35 μg/ml; 8D11, 36 μg/ml; EVR1, 17.5 μg/ml.
[b] +, no observable CPE for a minimum of 96 h postinfection; −, CPE observed within 48 h postinfection.

subcloned into vector pcDNA3 (pcDNA3-DAF) was introduced into NIH 3T3 cells, and transfected cells were infected with vaccinia virus vTF7-3, to drive DAF expression. As shown in Fig. 7, cells transfected with pcDNA3-DAF expressed DAF at their surfaces and reacted with MAbs EVR1 and 1H4 (Fig. 7B and F) whereas cells transfected with pcDNA3 alone did not (Fig. 7C and G). As shown in Fig. 8, NIH 3T3 monolayers transfected with pcDNA3-DAF were also able to bind [^{35}S]EV70. Although the amount of virus binding to pcDNA3-DAF-transfected cells varied among experiments, DAF transfectants consistently bound 50 to 100% of the amount of virus that bound to HeLa cells and bound two to four times as much virus as did cells transfected with pcDNA3.

Flow cytometric analysis (data not shown) indicated that transfected monolayers were a mixed population of cells displaying a range of DAF expression levels. For the experiments represented in Fig. 7 and 8, 10% of cells transfected with pcDNA3-DAF expressed approximately 10 times as much DAF on their surfaces as did HeLa cells and approximately 40% of cells expressed low to moderate levels of DAF. The remaining cells were negative for DAF expression.

NIH 3T3 cells constitutively expressing human DAF (3T3-DAF) have been described previously (51). In immunofluorescence assays, MAbs EVR1 and 1H4 reacted much more weakly with 3T3-DAF cells than with HeLa cells (data not shown). In binding assays, we were unable to detect EV70 binding to 3T3-DAF cells above the background levels observed in 3T3-RDAF cells (data not shown), which do not express DAF (51). Nevertheless, this 3T3-DAF cell line was able to support low-level EV70 replication. Monolayers were infected with EV70 at a MOI of 5 and assayed for the presence of infectious virus at regular intervals, for a period of 48 h (Fig. 9). The difference in the amount of virus associated with 3T3-RDAF and 3T3-DAF cells at time zero may indicate increased binding of EV70 to 3T3-DAF cells. Virus yield from DAF-expressing cells increased to a peak value of 1.6×10^3 PFU/ml by 24 h, representing a 2.5-fold increase from time zero, and began to decline thereafter. 3T3-RDAF cells could not be infected. EV70 titers from these cells remained low (approximately 300 PFU/ml) for 24 h and then, as with 3T3-DAF cells, began to fall. Experiments in which samples were assayed at 24-h intervals for 120 h (data not shown) indicated that this decline in virus titer continued, reflecting a decay of progeny virus following one cycle of replication (3T3-DAF cells) and decay of input virus (3T3-RDAF cells). No CPE was observed in EV70-infected 3T3-DAF monolayers. At this time, it is not known if these cells become persistently infected.

DISCUSSION

The initial interaction of a virus with specific host cell surface components is recognized to be an important determinant of viral host range and tissue tropism (33, 38). This suggested to us that the unique in vitro and in vivo replication characteristics of EV70 may be determined by its receptor and would reflect differences between the receptor for EV70 and those identified for other picornaviruses. In view of this information, we undertook studies to identify and characterize the HeLa cell receptor for EV70.

A MAb that exhibited specific receptor-blocking activity in both biological and physical assays was isolated. MAb EVR1 prevented infection of HeLa cells by EV70 but not by the closely related picornaviruses poliovirus and coxsackievirus B3 (46). Binding inhibition assays confirmed that this antibody acts at the level of virus-receptor interaction. Immunoprecipitations, Western blots, and immunofluorescence assays demonstrated that MAb EVR1 reacted with a GPI-anchored, 70- to 75-kDa protein on the surface of HeLa cells.

Our data were consistent with the possibility that MAb EVR1 identified DAF (CD55). DAF is an important modulator of the complement system and, together with membrane cofactor protein, complement receptor types 1 and 2, and C4-binding protein, is a member of the family of regulators of complement activation (RCA) (27, 34, 37). The human DAF molecule is typically expressed as a 70- to 75-kDa glycoprotein on the surface of many cell types, including HeLa cells (27, 30). The nucleotide sequence of DAF predicts a structure with five extracellular domains (27, 29). Proximal to the cell membrane is a serine/tyrosine-rich domain, which is extensively O glycosylated, and distally there are four SCR domains characteristic of RCA family members. Each SCR contains four cysteine residues that are predicted to form internal disulfide bonds (27). The sensitivity of the interaction between MAb EVR1 and its ligand to both heat and reducing agents suggests that the antibody recognizes a conformational epitope on one of

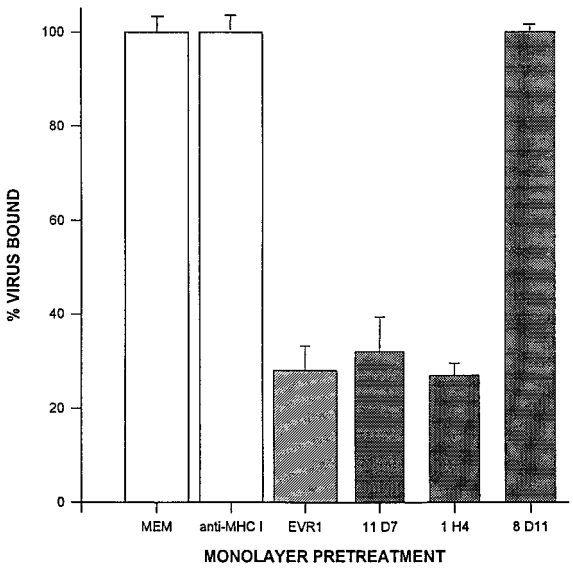

FIG. 6. DAF-specific MAbs inhibit the binding of EV70 to HeLa cells. HeLa cell monolayers in 24-well plates were treated with MAbs as indicated for 1 h at 37°C, before incubation with 5×10^3 cpm of [^{35}S]EV70 for 45 min at 33°C. The amount of virus bound to cells was determined as described in Materials and Methods. MEM, growth medium alone; anti-MHC I, 10 μg of MHC I-specific MAb per ml; EVR1, 10 μg of EVR1 per ml; 11D7, 10 μg of 11D7 per ml; 1H4, 10 μg of 1H4 per ml; 8D11, 20 μg of 8D11 per ml. Results are shown as the mean percentage of virus bound relative to the MEM control ± standard deviation for two samples.

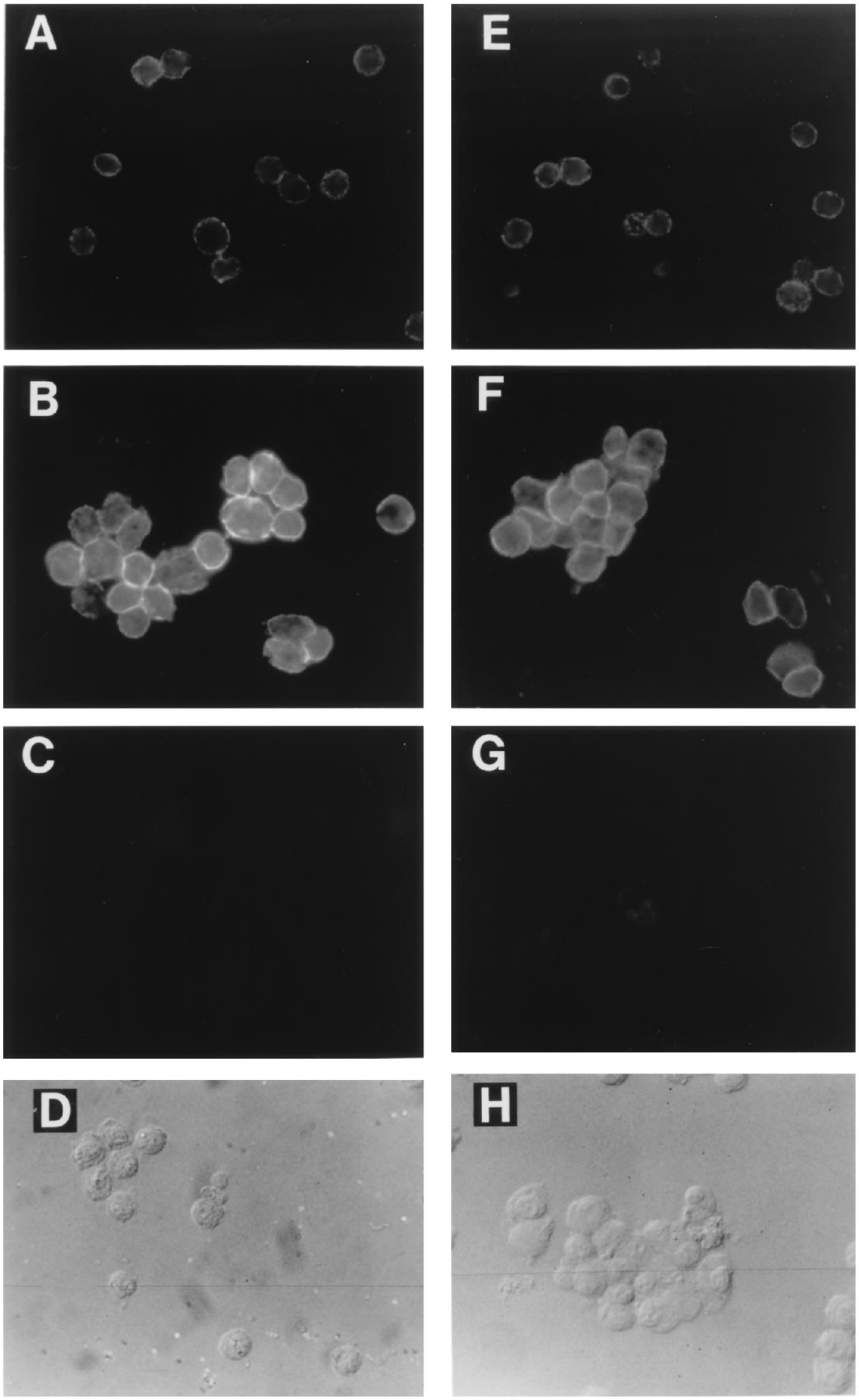

FIG. 7. Transfection of NIH 3T3 cells results in surface expression of DAF. Monolayers of transfected NIH 3T3 cells were dispersed by trypsin treatment, washed, and incubated with Abs as indicated. Ab binding was detected by indirect immunofluorescence with an FITC-conjugated sheep anti-mouse Ab, as described in Materials and Methods. (A through D) Incubation with EVR1 ascites fluid (1:200). (A) HeLa cells; (B) NIH 3T3 cells transfected with pcDNA3-DAF; (C) NIH 3T3 cells transfected with pcDNA3; (D) same field as in panel C with Nomarski optics. (E through H) Incubation with MAb 1H4 (1:5). (E) HeLa cells; (F) NIH 3T3 cells transfected with pcDNA3-DAF; (G) NIH 3T3 cells transfected with pcDNA3; (H) same field as in panel G with Nomarski optics.

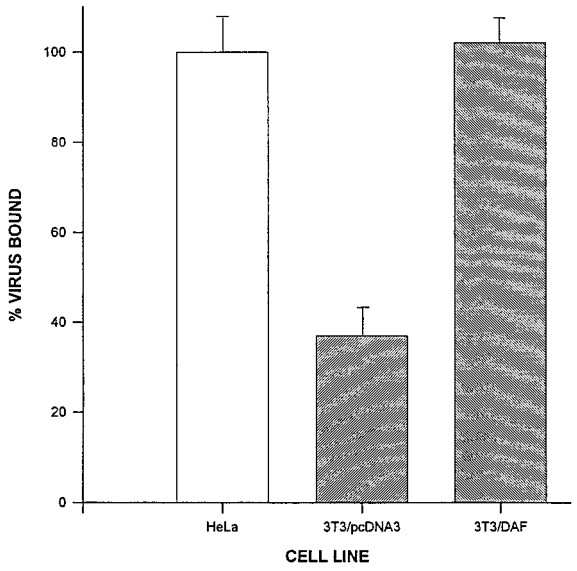

FIG. 8. Transient expression of DAF in NIH 3T3 cells results in binding of EV70. Monolayers of cells in 24-well plates were incubated with 3×10^3 cpm of [^{35}S]EV70 for 45 min at 33°C. The amount of virus bound to cells was determined as described in Materials and Methods. HeLa, HeLa cell control; 3T3/pcDNA3, NIH 3T3 cells transfected with pcDNA3; 3T3/DAF, NIH 3T3 cells transfected with pcDNA3-DAF. Results are shown as the mean percentage of virus bound relative to the HeLa cell control ± standard deviation for three samples.

the SCR domains. The appearance of a doublet in immunoprecipitations may reflect variable glycosylation of the protein.

The specificity of MAb EVR1 for DAF was substantiated by the observation that CHO-DAF (28) but not CHO cells reacted with antibody. Demonstration that DAF-specific MAbs 11D7 and 1H4 (directed against SCRs 1 and 3, respectively) blocked virus attachment to HeLa cells and were cytoprotective strongly suggested that DAF acts as the HeLa cell receptor for EV70.

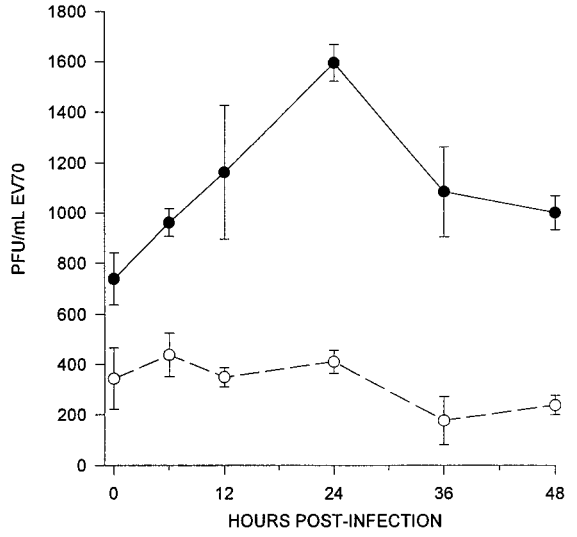

FIG. 9. DAF-expressing NIH 3T3 cells support EV70 replication. 3T3-DAF cells (●) and control 3T3-RDAF cells (○) were grown in 24-well plates and infected at an MOI of 5 for 1 h at 33°C. Following infection, monolayers were incubated at 33°C in growth medium containing 10% FBS. At the indicated intervals, cells and supernatants were recovered and frozen and thawed twice to liberate infectious virus. EV70 titers were measured by plaque assay on LLC-MK_2 monolayers. Results are expressed as means ± standard deviation for two duplicate independent determinations.

Conclusive evidence of this was provided by expression of human DAF in NIH 3T3 cells. Transient expression of DAF on the surface of NIH 3T3 cells resulted in virus binding. We were unable to detect significant EV70 binding to NIH 3T3 cells stably expressing human DAF (52). These 3T3-DAF cells also reacted weakly in immunofluorescence studies with MAbs EVR1 and 1H4. Since this cell line has been shown to express approximately seven times less DAF than reported for HeLa cells (3×10^4 versus 2×10^5 DAF molecules per cell [31, 52]), our results may indicate that the level of DAF expression was too low for us to detect virus binding above background levels. Nevertheless, we were able to detect virus replication in these 3T3-DAF cells, indicating that DAF expression is sufficient for EV70 to productively infect NIH 3T3 cells. Our findings suggest that DAF-expressing cells were able to support one round of virus replication, after which we observed the gradual decay of progeny virus. Control 3T3-RDAF cells were refractory to EV70 infection. An explanation for the low yield of virus recovered from 3T3-DAF cells may be that EV70 replication is poorly supported in NIH 3T3 cells. As yet unidentified cellular factors required for efficient growth of EV70 in these cells may be lacking, and their absence may contribute to a partial block in the viral replication cycle. Furthermore, our EV70 stocks were propagated on LLC-MK_2 monolayers, and the virus inocula used in NIH 3T3 infectivity assays may contain only a small proportion of variants able to replicate in this murine cell line. Adaptation of EV70 to this new host by repeated passage may result in more productive infection.

Human (36), guinea pig (35), rabbit (48), and mouse (45) DAF molecules have been identified. The presence of DAF-like molecules in different mammalian species is consistent with the ability of EV70 to infect a wide range of mammalian cell lines in vitro. MAb EVR1 did not bind to monkey kidney (LLC-MK_2) cells or protect them against EV70 infection. Although the predicted amino acid sequence of the DAF molecule of rhesus monkeys shares approximately 95% amino acid identity with that of human DAF (21a), the sequence differences might account for our observations. In addition, CHO cells were able to bind labelled EV70 and to support EV70 replication. This is also consistent with the broad in vitro host range of this virus, which is known to include cells of hamster origin (56). It will be of interest to determine if DAF homologs expressed on other mammalian cell lines function as receptors for EV70 or if an altogether different receptor molecule is used.

In addition to its role in the regulation of complement, DAF has recently been shown to act as a cellular receptor for at least six echovirus serotypes (3, 51) and for coxsackieviruses B1, B3, and B5 (5, 42). Regions of DAF involved in binding these different picornaviruses have been identified. Antibody blockade experiments showed that echovirus 7 and related viruses (3) and RD cell-adapted coxsackievirus B3 (5) interact with SCRs 2 and 3. Transfection experiments with DAF cDNAs containing deletions of specific SCRs subsequently determined that SCRs 2, 3, and 4 are required for echovirus 7 binding (9) and that SCR 2 is essential for coxsackievirus B3-RD attachment (5). Antibody blockade experiments also identified a site within or near SCR 3 as the possible binding region for coxsackievirus B5 (42). As reflected in these reports, mapping virus-binding sites by antibody blockade alone has certain limitations. The large size of antibodies relative to the DAF molecule and the absence of information regarding both the sequences of the epitopes recognized by the antibodies and the

precise location of these epitopes in the folded DAF molecule make definitive interpretation of data difficult. Our antibody-blocking data suggest that regions within or proximal to SCRs 1 and 3 are involved in the binding of EV70 and therefore that the EV70-binding site(s) on DAF may be different from those used by other enteroviruses.

EV70 and the echoviruses and coxsackieviruses that use DAF for attachment are all able to agglutinate human erythrocytes (3, 5, 25, 42, 50). An early study of the interaction between EV70 and human erythrocytes (50) demonstrated that the hemagglutinating ability of EV70 was sensitive to neuraminidase. However, treatment of erythrocytes with neuraminidase did not eliminate their capacity to adsorb echoviruses 7 and 11. This also would suggest that different regions of DAF are involved in binding these viruses. Experiments designed to more precisely define the DAF sequences involved in EV70 binding are under way.

DAF is expressed on conjunctival epithelial cells, and DAF antigen has been detected in cerebrospinal fluid (31). Thus, the receptor for EV70 is found at sites of virus replication, in tissues involved in the pathogenesis of EV70 and the clinical manifestations of acute hemorrhagic conjunctivitis (17, 21, 55). The presence of DAF on cells and tissues that are not recognized as sites of EV70 replication and the fact that DAF functions as a receptor for several other enteroviruses suggest that as with poliovirus (43), the tropism of EV70 is probably determined by the concomitant expression of receptor and additional host cell-specific proteins. Clearly, the disparate host range, tropism, and pathogenicity of human enteroviruses cannot simply be explained by differences in the nature and distribution of their receptors.

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