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INTERNALIZATION OF A MAJOR GROUP HUMAN RHINOVIRUS DOES NOT REQUIRE CYTOPLASMIC OR TRANSMEMBRANE DOMAINS OF ICAM-1[1]

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Intercellular adhesion molecule-1 (CD54), a cell adhesion molecule and the receptor for the major group of rhinoviruses, is a class 1 membrane protein with five Ig-like domains in its extracellular region, a transmembrane domain, and a short cytoplasmic domain. The amino-terminal domains (D1 and D2) are sufficient for virus binding and the first is most important (1). We have investigated whether other extracellular domains, transmembrane or cytoplasmic domains are required for virus entry as determined by postinfection virion protein biosynthesis. We demonstrate that cytoplasmic, transmembrane, and Ig-like domains 3, 4, and 5 are not essential for rhinovirus entry into transfected COS cells. The efficiency of rhinovirus infection directly correlates with the efficiency of rhinovirus binding and a form of intercellular adhesion molecule-1 that is glycophosphatidyl-inositol anchored, and thus does not extend into the inner leaflet of the membrane bilayer or the cytoplasm efficiently supports virus entry.

The steps that lead to virus internalization and entry into the cytoplasm of its host cell are not completely understood. Binding of rhinovirus, poliovirus, and HIV to their receptors demonstrate common features. These receptors all belong to the Ig supergene family and the most accessible amino terminal Ig-like domain forms the virus binding site that overlaps the site binding some of their counter-receptors in cell-cell interactions. Receptor binding may trigger or support the first step of virus uncoating. Fusion of enveloped viruses such as HIV with host cell membranes (2–4) is dependent (3, 5) or independent (4) of low pH. After receptor binding, nonenveloped viruses such as poliovirus, undergo capsid conformation changes and viral protein sequences that mediate liposome binding are extruded that may then increase membrane association (6).

Viral entry may also require linkage of receptor to host cell processes. Semliki Forest (2) and poliovirus (7, 8) internalize in coated vesicles and thus may be dependent on linkage of receptor to cytoplasmic proteins. However, direct association between poliovirus receptor and cytoplasmic proteins is not essential to poliovirus internalization (9). Other viruses such as SV40 (10), and a minor group rhinovirus, HRV2[3] (8), however, are found primarily in uncoated vesicles early after binding. Hence, internalization of nonenveloped viruses, poliovirus, HRV2, and SV40, may involve different processes the study of which will be greatly facilitated by the availability of the receptor genes. Two receptors for nonenveloped viruses have been identified; ICAM-1, the receptor for major group rhinoviruses and coxsackie A viruses (11–13), and PVR (14).

ICAM-1 (CD54) has a broad tissue distribution and binds several natural ligands and human pathogens. ICAM-1 is constitutively expressed on lymphoid cells and binds to the leukocyte integrins LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) to support cell-cell adhesion and induction and effector functions in the immune response (15–17). ICAM-1 expression is rapidly induced by cytokines in cells that possess low basal levels including epithelial, endothelial, and fibroblast cells (18). Adherence of leukocyte integrins to ICAM-1 on endothelial cells supports emigration of leukocytes from the circulatory system (17). ICAM-1 on endothelial cells also binds *Plasmodium falciparum*-infected E and may be involved in the pathogenicity of malaria (19). Furthermore, ICAM-1 has been subverted as a receptor for the major group (90%) of rhinoviruses.

Structural characteristics of ICAM-1 that are important to its binding to rhinovirus have been defined (1). ICAM-1 sequence analysis predicts five Ig-like C domains, a membrane spanning domain and a short cytoplasmic domain (20, 21). ICAM-1 is a long narrow rod with dimensions, 19 × 2.5 nm, that suggest that the Ig-like domains are unpaired and arranged end-to-end (1). These dimensions are consistent with ICAM-1 binding to the proposed binding site on HRV that is predicted to be located in a cleft 3 nm wide and 2.5 nm deep. Characterization of ICAM-1 amino acid substitution mutants demonstrates that contact occurs primarily between the amino terminal domain of ICAM-1(D1) and HRV14 (1). The binding site for HRV is distinct from but overlaps the binding site for LFA-1. ICAM-1 mutants with domain 3, domain 5, domains 4 and 5, or domains 3, 4, and 5 deleted bind HRV but with decreased efficiency. This may reflect a role in orienting or elevating the binding site of ICAM-1 from the cell surface (1).

In contrast to binding requirements, the structural features of ICAM-1 that are required for internalization and infectivity of rhinovirus have not been defined. We ex-

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[3] Abbreviations used in this paper: HRV, human rhinovirus; ICAM-1, intercellular adhesion molecule-1; LFA-1, leukocyte function antigen-1; GPI, glycosylphosphotidyl-inositol; PVR, poliovirus receptor.

amine involvement of ICAM-1 features including potential interaction between cytoplasmic domain and cytoskeleton and distance of binding site from the membrane. This characterization yields insight into the structural requirements of HRV internalization and what features may be common to other nonenveloped viruses.

MATERIALS AND METHODS

Construction of GPI-anchored ICAM-1. A GPI-anchored form of ICAM-1 was generated by replacing ICAM-1 cDNA encoding transmembrane and cytoplasmic domains with the GPI signal sequence from LFA-3. ICAM-1 and LFA-3 expression constructs were described previously (22, 23). A unique *Afl*II site was introduced by oligonucleotide-directed mutagenesis into ICAM-1 centered around sequence coding for residue L448 (silent mutation), amino-terminal to the transmembrane domain. The *Afl*II- *Bam*HI fragment (unique *Bam*HI site in the CDM8 vector) was then exchanged with a similar fragment from an LFA-3-CDM8 construct that contains an *Afl*II site introduced into sequence centered around C177 (24), N-terminal to the predicted GPI-anchored attachment site at S180 (25) and the construct was designated IC1/GPI1.4.

COS cell transfection and quantitation of HRV14 viral protein synthesis. COS cells at 50% confluency were transfected as described previously by DEAE-dextran method using approximately 4 μg of plasmid per 10-cm dish (22). Two days posttransfection COS cells were suspended using trypsin-EDTA and reseeded into 6-cm dishes. Three days posttransfection confluent COS cells in 6-cm dishes were washed with RPMI 1640/10 mM $MgCl_2$/25 mM HEPES pH 7.3 (binding buffer) and then incubated with HRV14 (multiplicity of infection = 20) in binding buffer for 60 min at 35°C with rotation. Binding buffer was aspirated and 2 ml of complete media added and incubation continued for 2 h. Media was then aspirated and 120 μCi of ^{35}S-methionine and ^{35}S-cysteine was added in 1.5 ml of methionine and cysteine-free RPMI 1640 with 10% dialyzed FCS and gentamicin. After 6 h at 35°C media was aspirated, cells were washed once, and lysed by three cycles of rapid freezing and thawing in 1 ml of DMEM/40 mM HEPES, pH 7.3. Cell debris was removed by centrifugation in a microfuge at 13,000 rpm for 10 min. Supernatants were layered on 30% sucrose with 1 M NaCl and 20 mM Tris-acetate, pH 7.5, and then centrifuged at 35,000 rpm for 2 h at 15°C (26). The radiolabeled virus pellet was resuspended in 20 μl of DMEM/40 mM HEPES, pH 7.3, and an aliquot was subjected to reducing SDS 12% PAGE. Autoradiograms were scanned on a densitometer (Bio-Rad, McLean, VA). The area under VP1, VP2, and VP3 peaks was added and divided by the area under VP peaks from wild-type ICAM-1-expressing cells.

RESULTS

A GPI-anchored form of ICAM-1 was generated by the exchange of ICAM-1 transmembrane and cytoplasmic domain sequence for LFA-3 GPI signal sequence (Fig. 1A). When expressed in COS cells, ICAM-1/GPI but not wild-type (WT) ICAM-1 was specifically released into culture supernatant by phosphatidyl inositol-specific phospholipase C digestion (Fig. 1B).

HRV14 internalization was demonstrated by infecting COS cell transfectants and radiolabeling de novo synthesized viral proteins with [^{35}S] cysteine and methionine. Virus was partially purified by sedimentation and the purified labeled viral capsid proteins (VP1, VP2, and VP3) visualized by SDS-PAGE (Fig. 2) migrated identically to viral proteins of highly purified HRV14 (data not presented). COS cells expressing human wild-type ICAM-1 clearly supported HRV14 VP synthesis. In contrast, no viral protein synthesis was detected in mock-transfected cells or cells expressing mouse ICAM-1, which does not bind HRV14 (1). In addition, a human-mouse chimera possessing the amino-terminal Ig-like domains of human ICAM-1 (1) but not the reciprocal chimera, supports VP synthesis in COS cells. Thus, HRV14 entry into COS cells is dependent on expression of ICAM-1 and the presence of residues 1–168 of human ICAM-1.

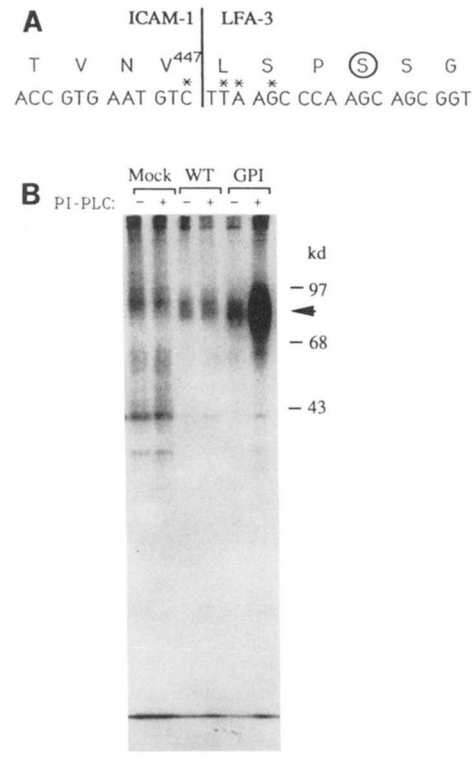

Figure 1. Construction and expression of a GPI-anchored form of ICAM-1. *A*, Sequence of ICAM-1/GPI at point of fusion (*Afl*II site) between ICAM-1 and LFA-3 mutant cDNA. The *asterisk* indicates nucleotides that differ from wild-type. The predicted carboxyl-terminal residue of the mature protein is circled. *B*, ICAM-1 was immunoprecipitated from the supernatant of [^{35}S] cysteine- and methionine-labeled mock- transfected COS cells or COS cells expressing wild-type (*WT*) or GPI- anchored forms that had been treated with (+) or without (-) PI-PLC as described (30). Immunoprecipitates were subjected to SDS 10% PAGE and fluorography.

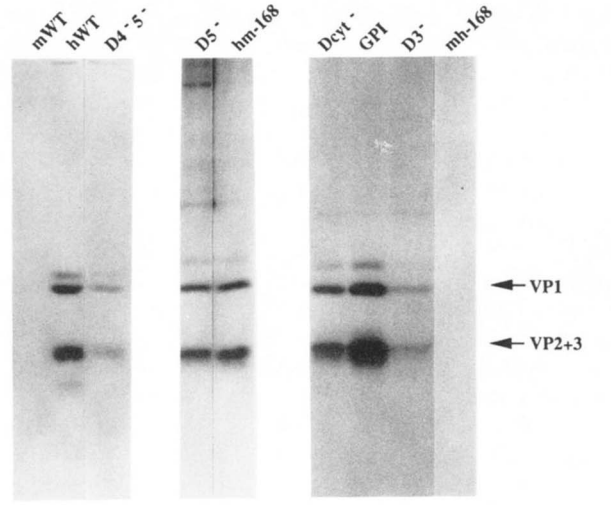

Figure 2. HRV14 viral protein synthesis in COS cells expressing ICAM-1 mutants. COS cells expressing wild-type or mutant ICAM-1 as indicated were infected with HRV14 (MOI = 20) and de novo synthesized proteins labeled with [^{35}S] methionine and cysteine. HRV was isolated from cells 9 h postinfection and subjected to SDS 12% PAGE and fluorography.

To determine if the cytoplasmic and transmembrane domains of ICAM-1 are required for HRV14 internalization a GPI anchored form of ICAM-1(ICAM-1/GPI) and a cytoplasmic domain deletion mutant (1) were tested for their ability to support virus entry when expressed in COS cells.

HRV14 internalization is supported by ICAM-1 with its

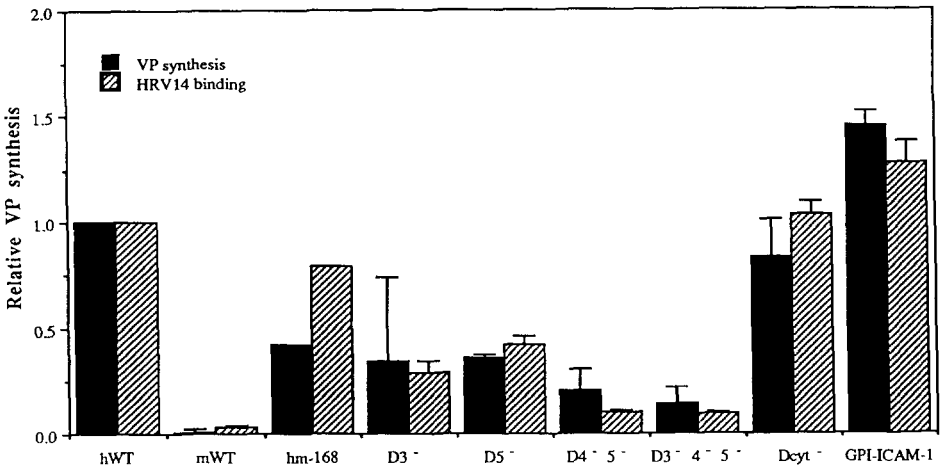

Figure 3. Comparison of HRV14 binding and viral protein synthesis supported by ICAM-1 mutants. ICAM-1 domain deletion mutants (D3⁻, D5⁻, D4⁻5⁻, D3⁻4⁻5⁻, and Dcyt⁻), GPI anchored ICAM-1 (*GPI-ICAM-1*) and human-mouse chimeric ICAM-1 (*hm-168*) were expressed in COS cells. These cells were infected with HRV-14 and viral protein synthesis was quantitated by scanning densitometry of autoradiograms similar to that presented in Figure 2. Viral protein synthesis and binding (1) was normalized for percent expressing cells and is relative to wild-type. Binding to ICAM-1 mutants was previously published (1), except for GPI-ICAM-1, and is shown for comparison purposes.

entire cytoplasmic domain deleted (Dcyt⁻) as well as by ICAM-1/GPI (Fig. 2). The cytoplasmic and transmembrane domains of ICAM-1 are therefore not essential to HRV14 infection.

Previously, we have demonstrated that binding of HRV14 to ICAM-1 with Ig-like domains D3, D4, and D5 deleted was less than that to wild-type (1). The possibility that deletion of these domains might restrict HRV internalization was tested in COS cell transfectants. All ICAM-1 domain deletion mutants conferred to COS cells the ability to internalize HRV14 (Fig. 2). Hence, these membrane proximal domains are not essential to the mechanism of HRV internalization.

To compare the efficiency of HRV14 internalization supported by different ICAM-1 mutants, viral protein synthesis was quantitated by densitometry and normalized for percent of transfected cells expressing ICAM-1. The level of VP synthesis was decreased for domain deletion mutants, correlating with the previously determined efficiency of HRV14 binding of these mutants (Fig. 3) (1). In contrast, Dcyt⁻ supported wild-type levels of VP synthesis and ICAM-1/GPI resulted in greater than wild-type levels of VP synthesis. This reflected the increased efficiency of HRV14 binding to these mutants (Fig. 3).

DISCUSSION

We have demonstrated that the first two amino terminal domains of ICAM-1 and a membrane anchor are sufficient to support HRV internalization and that ICAM-1 Ig-like domains 3, 4, 5, and transmembrane and cytoplasmic domains are not required. Truncation of the cytoplasmic domain and reanchoring on GPI did not decrease the efficiency of virus entry into the cytoplasm. GPI reanchoring results in replacement of the polypeptide membrane anchor with the GPI anchor, and this anchor is equivalent to a phospholipid and is present only in the outer leaflet of the membrane bilayer. The efficient internalization supported by the Dcyt⁻ mutant and ICAM-1/GPI demonstrates that interaction between the cytoplasmic domain of ICAM-1 and actin (27) or a cytoskeletal protein, e.g., α-actinin (O. Carpen, P. Pallai, D. E. Staunton, and T. A. Springer, manuscript submitted) is not required in this process.

Poliovirus is a picornavirus that binds a receptor (PVR) possessing three Ig-like domains. The cytoplasmic domain of PVR is also not necessary for poliovirus internalization (9). Furthermore, CD4 with cytoplasmic domain deleted (28) or GPI anchored (29), supports HIV infection. Hence, surprisingly there appears to be no role of the cytoplasmic domain in internalization of HRV, poliovirus or HIV. However, HIV possesses an envelope and cell entry occurs by, and may be entirely dependent on, direct fusion with the plasma membrane (4).

Why are particular surface molecules subverted as virus receptors? This could be related to receptor cellular distribution or accessibility. Alternatively, receptor ligation by the virus might trigger a signal through the membrane or cytoplasmic domain of the receptor that mimics signalling by a biologic ligand and that is advantageous for the virus interaction with the host. We have only studied one aspect of this host interaction, entry into the cytoplasm, and it is possible that other aspects are important in vivo.

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