NOTES

Mutational Analysis of the Cellular Receptor for Poliovirus

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To identify sequences of the cellular poliovirus receptor (PVR) involved in viral infection, mutant PVR cDNAs were constructed and assayed for biological activity in mouse L cells. To confirm that mutant PVRs reached the cell surface, an immunological tag, consisting of part of C_H3 from human immunoglobulin G1, was engineered into the PVR. Deletion of PVR amino acids 256 to 320 or 385 to the carboxy terminus yielded receptors that were able to support poliovirus infection. PVRs lacking amino acids 40 to 136 or 137 to 256 were expressed at the cell surface but were not active as receptors for poliovirus. The results show that immunoglobulin-type domain 3 and the extreme carboxy terminus of the PVR are not required for viral receptor function, but sequences within the two amino-terminal domains contribute to the initiation of poliovirus infection.

Poliovirus initiates infection of susceptible cells by specifically interacting with a cellular poliovirus receptor (PVR), a member of the immunoglobulin superfamily whose natural cellular function is unknown (13). PVR consists of three immunoglobulin-type domains (numbered from the amino-terminal, membrane-distal end), a transmembrane segment, and a short cytoplasmic tail. Mutational analyses of CD4, the receptor for human immunodeficiency virus (12); ICAM-1, the major receptor for human rhinovirus (8); and CR2, the receptor for Epstein-Barr virus (4, 15) have provided structural information about receptor sequences involved in interaction with virus. As a first step toward identification of PVR sequences required for poliovirus infection, a series of mutants were constructed and assayed for the ability to confer poliovirus susceptibility to resistant mouse L cells.

The domain structure of the PVR was used as a guide to construct deletion mutants (Fig. 1). Since the atomic structure of the PVR has not been determined, predicted boundaries of the immunoglobulin-type domains (domain 1, a V type, amino acids 35 to 140; domain 2, a C type, amino acids 153 to 236; domain 3, a C type, amino acids 250 to 330) were modeled after known immunoglobulin-type structures (19, 22, 23). PVR cDNAs were derived from a parental plasmid which expresses PVR from a simian virus 40 promoter (13). Two related PVR cDNAs, H20A (encoding 417 amino acids) and H20B (encoding 392 amino acids), were previously cloned from HeLa cell mRNA (13). These two cDNAs encode functional PVRs whose sequences diverge at their extreme C termini, 3' of the common *Xho*I site. A deletion mutant was constructed in which sequences which differ between the two PVRs were removed (PVR Δ LXho). The cytoplasmic tail from H20A cDNA was retained for the other mutants. Most of domain 3 (amino acids 257 to 320) was deleted (PVR Δ L3) by fusing a repaired *Asp*718I site to a trimmed *Sac*I site, leaving the transmembrane and cytoplas-

mic regions intact. To form the PVR/C_H3 chimera, the same two PVR restriction sites were used to insert an immunoglobulin G (IgG) C_H3 domain. The C_H3 domain was generated by polymerase chain reaction (20) of the DNS-VH-HuG1/pSV2DH-gpt plasmid (kindly supplied by S. Morrison) (3). The result was substitution of IgG amino acids 358 to 430 for PVR amino acids 257 to 319. The remaining two deletion mutants were generated in the PVR/C_H3 chimera. Deletion of domain 1 (PVR/C_H3 Δ L1) was accomplished by fusing an introduced *Pst*I site to an *Eco*RV site, removing codons for amino acids 40 to 136. Codons for amino acids 137 to 256 were deleted (PVR/C_H3 Δ L2) by ligating the *Eco*RV site near sequences encoding the end of domain 1 to the trimmed ends of the *Kpn*I site. To confirm the structure of the mutated plasmids, the nucleotide sequences of the junction regions were determined.

These plasmids were cotransformed into mouse thymidine kinase-negative L cells with a thymidine kinase selectable marker, and stable cell lines were generated under hypoxanthine-aminopterin-thymidine selection (13). The presence of transformed DNA was confirmed by polymerase chain reaction analysis of DNA from the stable cell lines (6). The susceptibility of the stable cell lines expressing mutant PVRs to poliovirus infection was tested (Fig. 1). The yield of poliovirus from cells transformed with unmodified PVR cDNA was almost 5 log₁₀ PFU per ml greater than the background observed with resistant L cells. Cells expressing mutant receptor PVR Δ LXho or PVR Δ L3 produced nearly as much poliovirus as cells expressing wild-type PVR, about a 4 log₁₀ PFU per ml increase over background. These results suggest that PVR amino acids 257 to 320 (domain 3), 385 to 392 in H20B, and 385 to 417 in H20A are not required for PVR activity.

In preliminary experiments, we found that cells expressing PVRs lacking domain 1 or 2 were unable to support poliovirus infection (6). However, it was not possible to demonstrate that the lack of susceptibility was due to a defect in receptor function rather than a defect in protein stability or

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Receptor		Virus Yield (log ₁₀ pfu per ml)
none		3.56
PVR		8.04
PVRdl Xho		7.71
PVRdl 3		7.57
PVR/CH3		7.43
PVR/CH3dl 1		3.46
PVR/CH3dl 2		3.35

FIG. 1. Infectivity assay of cell lines expressing mutant PVRs. A total of 10^6 L cells expressing the indicated PVRs were plated in 6-cm dishes or six-well plates. The following day, the cells were infected with type 1 (Mahoney) poliovirus at a multiplicity of infection of 10, in a volume of 0.1 ml. After a 45-min incubation in a 37°C CO₂ incubator, the unabsorbed virus was removed by rinsing, and 5 ml of medium was added. After 48 h of incubation, the virus yield was determined in a standard plaque assay (18) and is expressed as the log PFU produced per milliliter of culture supernatant after subtracting the virus titer at 0 h of incubation (time of addition of medium). Each titer is the average of at least three separate experiments. Results for PVR are from cells expressing H20A; H20B yielded similar results (6). The diagrams depict the mutant PVRs (with the N terminus, or membrane-distal end, to the left) as described in the text; specific domains (Dom) are labeled. Vertical stripes, signal peptide; horizontal stripes, transmembrane domain; rightmost open box, cytoplasmic tail. Putative disulfide bonds in each domain of the wild-type PVR are shown. Restriction sites used in the construction are *Pst*I (P), *Eco*RV (E), *Asp*718I plus *Kpn*I (isoschizomers; A/K), *Sac*I (S), and *Xho*I (X).

surface localization. Currently available anti-PVR polyclonal antisera are not suitable for cell surface detection, since the antisera react with PVR-negative L cells (5), possibly owing to the expression of a highly conserved mouse homolog (16). Anti-PVR monoclonal antibodies that have been isolated block viral infection and would be unlikely to react with functionally negative receptors (10, 14, 17). The finding that domain 3 was dispensable for virus receptor function (see above) suggested that an immunological tag could be inserted in this region of the PVR, enabling surface detection with appropriate antisera. A chimeric receptor, PVR/CH₃, was constructed in which PVR domain 3 was substituted with an IgG C_H3 domain. L cells transformed with PVR/CH₃ supported poliovirus infection nearly as efficiently as cells expressing wild-type PVR (Fig. 1). Domain 1 or 2 was then deleted from PVR/CH₃. Deletion mutants PVR/CH₃dl1 and PVR/CH₃dl2 yielded inactive receptors, since cells transformed with these cDNAs failed to produce poliovirus over the background observed with PVR-negative L cells (Fig. 1).

To show that the mutant receptors were expressed on the cell surface, stable cell lines expressing the mutant receptors were subjected to a rosetting assay (Fig. 2). Affinity-purified anti-human immunoglobulin antibodies (Boehringer Mannheim Biochemicals) were conjugated to sheep erythrocytes (7) (Cappel/Organon Technica). Sparse monolayers of each cell line were incubated with these erythrocytes, rinsed, and scored for rosette formation. Surface-expressed antigen binds antibody on the erythrocytes, causing them to cluster (rosette formation) around target cells. Cell lines expressing the chimeric receptors PVR/CH₃, PVR/CH₃dl1, and PVR/CH₃dl2 induced rosette formation, while L cells did not (Fig. 2). These results demonstrated that both nonfunctional PVRs are expressed on the cell surface.

The results presented here show that domain 3 is not necessary for virus receptor function. The finding that cells expressing PVRdlXho, which lacks the region 3' of the *Xho*I site, are susceptible to poliovirus implies that the differences between H20A and H20B are irrelevant for virus receptor function in cell culture. In addition, preliminary results indicate that cells expressing PVR lacking the entire cytoplasmic tail are susceptible to poliovirus (9). The results further suggest that amino acids 40 to 136 and 137 to 256 contribute to the ability of PVR to serve as a functional poliovirus receptor. A concern with interpreting the results of large deletion mutations is that function may be abrogated by conformational alterations and not by removal of specific functional regions. The reactivity of the mutant PVRs with anti-human immunoglobulin antibodies suggests that global conformational changes are not occurring. However, finer mutational analysis will be required to fully address this concern. A preliminary report implicating the first 165 amino acids of PVR in PVR activity is consistent with these results (11).

The organization of sequences required for the virus receptor function of PVR may be like that of other virus receptors with similar domain structures. Mutational studies of CD4, which has four immunoglobulin-type domains, show that areas in domain 1 (residues 38 to 52, which includes the region analogous to complementarity-determining region 2 [CDR2], 49, 55, 62, 67, 77, 81, and 85) are implicated in gp120 binding, and residues 87 to 89 (analogous to CDR3) are important for cell-cell fusion. Mutations in residues 122 and 164 in domain 2 have been shown to affect gp120 binding without affecting overall structure, although the mechanism is not clear (references 19 and 22 and references therein). Thus, in CD4, domain 1 mediates the primary interactions with gp120, while domain 2 makes further contributions. The outermost two of the five immunoglobulin-type domains of ICAM-1 mediate human rhinovirus binding, although at only 10% of the efficiency of full-length ICAM-1 (21). Mutation of putative β -turn residues revealed that amino acids within domains 1 and 2 interact with the virus. CR2, a member of the regulators of complement activation gene family with 12 to 15 domains, can mediate susceptibility to Epstein-Barr virus infection when the two most N-terminal domains are present on the cell surface (2). The primary interactions of virus with these similarly structured molecules may involve residues (analogous to the CDR regions in immunoglobulins) which are most exposed on the cell surface, but complete activity may require adjacent sequences. It will be of interest to determine whether distance from the membrane is a critical parameter by altering the order of domains in the PVR.

The finding that a chimeric PVR/CH₃ is active as a PVR can be applied to the construction of novel anti-PVR mole-

FIG. 2. Rosette assay of cell lines expressing PVR/CH₃ chimeric receptors. Approximately 10^4 cells from stable cell lines expressing the indicated receptors were plated in 24-well dishes. The following day, the medium was aspirated, and the cells were rinsed and incubated with 1 ml of 0.2% (by volume) sheep erythrocytes conjugated to affinity-purified goat anti-human immunoglobulin antibodies and diluted into Dulbecco's modified Eagle's medium with 2% fetal calf serum. After 1 h of incubation at room temperature with occasional gentle swirling, the cells were rinsed three times with warm medium and photographed.

cules, such as immunoadhesins (1). This work also establishes an assay system for detecting nonfunctional PVRs at the cell surface, since existing monoclonal antibodies that block virus infection likely only recognize functional PVRs. This assay should facilitate the characterization of PVR point mutants. Candidate residues for finer mutational analysis are those in predicted β -turns, particularly those in positions analogous to CDRs.

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