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Identification of a Cytoplasmic Tyr-X-X-Leu Motif Essential for Down Regulation of the Human Cell Receptor CD46 in Persistent Measles Virus Infection

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To investigate the sequence requirements for measles virus (MV)-induced receptor down regulation, we transfected the human CD46 gene into simian cells persistently infected by the Biken strain of MV. Surface expression of CD46 is drastically reduced in these cells. Deletion analysis has shown that the juxtamembrane region of the CD46 cytoplasmic domain is essential for down regulation. Deleting a Tyr-Arg-Tyr-Leu sequence in this region or changing these residues to Ala prevents CD46 down regulation from the infected cell surface. Alanine-scanning mutagenesis has identified two amino acid residues, Tyr and Leu, forming a Tyr-X-X-Leu motif critical for CD46 down regulation. Mutations that prevent CD46 down regulation enhance syncytium formation. These results indicate that CD46 down regulation limits the cytopathic effects in a persistent MV infection and that CD46 down regulation requires a cytoplasmic Tyr-X-X-Leu sequence which resembles known motifs for membrane protein trafficking and receptor signalling.

Measles virus (MV) is responsible for an acute infection that causes about a million deaths annually, especially among infants and children in developing countries (6). A large number of these deaths are due to secondary opportunistic infections that occur during a period of MV-induced immunosuppression (25). In rare instances, persistent infection can lead to lethal disorders of the central nervous system (39).

MV is an enveloped negative-strand RNA virus that attaches to human cells through interactions between the virus-encoded hemagglutinin (H) protein and a specific cellular receptor. After binding to host cells, the viral fusion (F) protein, together with the H protein, induces fusion between the viral envelope and the host cell plasma membrane to allow virus entry (42). These protein interactions also permit fusion between MV-infected and uninfected neighboring cells to facilitate cell-to-cell transmission of the virus.

CD46, or membrane cofactor protein (MCP), has been identified as the major human cell receptor for laboratory-adapted MV strains (3, 24). CD46 is a complement regulatory protein whose normal function is to protect the host from autologous complement attack by serving as a cofactor for factor I-mediated degradation of C3b/C4b complement proteins (17). Human CD46 (Fig. 1) consists of four short consensus repeats (SCR1, -2, -3, and -4); three regions rich in serine, threonine, and proline (STP-A, -B, and -C); a short region with no known function; a transmembrane anchor (TM); and a common juxtamembrane cytoplasmic sequence (JxM), followed by two alternative distal cytoplasmic sequences (Cyt 1 or Cyt 2) derived from differential mRNA splicing (17, 28, 29). All of the common CD46 isoforms contain both N- and O-linked glycosylation sites and can serve as receptors for MV (7, 19). The SCR1 and SCR2 domains constitute the MV-binding determinant, whereas SCR3, SCR4, and, to a lesser extent, SCR2 are responsible for the complement-regulating function (13, 20).

Infection by laboratory-adapted MV causes a reduction of CD46 from the cell surface (23), which enhances the susceptibility of the cell to complement-mediated lysis (35). However, many non-laboratory-adapted wild-type MV strains do not down regulate CD46 (16, 32, 33), suggesting an inverse correlation between CD46 down regulation and MV pathogenicity. Recently, cross-linking of CD46 has been shown to cause the suppression of interleukin-12 production from monocytes (14). This raises the possibility that CD46 down regulation by laboratory-adapted and vaccine MV strains may serve to limit the CD46-mediated immunosuppressive effects. Therefore, understanding how MV induces CD46 down regulation may provide insight into the viral and host determinants of MV pathogenesis. Previous studies have shown that the MV H protein can trigger CD46 down regulation (15, 23, 34). However, the molecular mechanism of this down regulation remains to be determined.

One approach to gain insight into this down regulatory mechanism is to study the expression of a human CD46 gene transfected into simian cells persistently infected by MV. Biken-CV-1 cells are African green monkey kidney (CV-1) cells that are 100% infected with the Biken strain of MV and constitutively express the Biken viral H and F proteins (9). Previously, we examined the expression of CD46 cytoplasmic deletion mutants in these cells and found that sequences contained within a membrane-proximal region of CD46 are required for MV-induced CD46 down regulation from the cell surface (10). In the present study, we used site-directed mutagenesis to define the precise cytoplasmic sequences essential for CD46 down regulation.

Site-directed mutagenesis of CD46 juxtamembrane region. The six amino acid residues proximal to the membrane contain sequences required for CD46 down regulation in Biken-CV-1 cells (10). Interestingly, this region and the adjacent predicted transmembrane domain contain a Tyr-X-X-Leu sequence resembling a signal for the internalization and lysosomal targeting of other membrane proteins from the cell surface or *trans*-Golgi network (TGN) (1, 12, 21, 27, 31, 41, 43, 44). Computer analyses predict that the first tyrosine in this sequence, Tyr 336, is part of the transmembrane domain of CD46. However, it is

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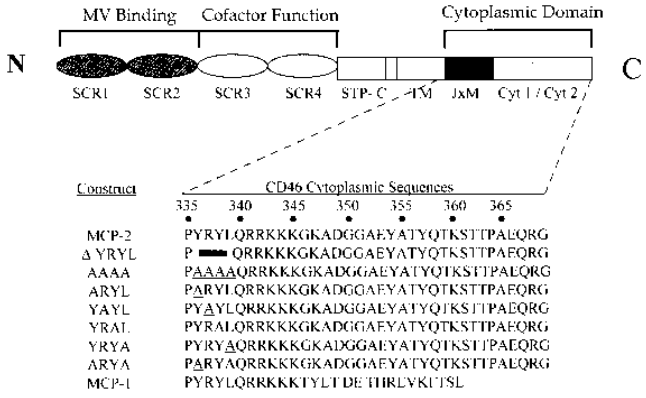

FIG. 1. Cytoplasmic sequences of CD46 and mutants. Amino acid sequences (in single-letter amino acid code) are shown for the Cyt 1 (MCP-1) and Cyt 2 (MCP-2) CD46 cytoplasmic domains. Numbers indicate positions of amino acid residues in the CD46 isoform encoded by the MCP-STP^c-2 clone. The boldface bar represents a deleted region, and alanine substitutions are underlined. TM, transmembrane domain; JxM, juxtamembrane region, which is conserved between the Cyt 1 and Cyt 2 cytoplasmic tails.

possible that Tyr 336 is actually exposed on the cytosolic surface, since the 22 amino acid residues proximal to Tyr-336 are sufficient to span the plasma membrane (30) and the proline residue immediately preceding this tyrosine is predicted to destabilize a membrane-spanning α-helical region (40). To determine whether this Tyr-Arg-Tyr-Leu sequence plays any role in CD46 down regulation, we generated a deletion (ΔYRYL) and alanine substitution (AAAA) mutant by overlap extension PCR by a previously described procedure (11) (Fig. 1). These mutations were introduced into the MCP-2 cDNA clone (MCP-STP^c-2) that encodes a human CD46 isoform with STP^c and Cyt 2 domains (18) and were expressed under the control of a modified simian virus 40 early promoter, SRα (38). We transfected the wild-type (MCP-2) and mutant constructs (12 μg) into 7×10^5 uninfected (CV-1) or persistently infected (Biken-CV-1) cells by the calcium phosphate method (8) and isolated surface and intracellular populations of CD46 protein from both cell types approximately 45 h posttransfection by a sequential immunoprecipitation procedure described previously (10). Briefly, CD46 proteins expressed on the surface of transfected cells were labeled with anti-CD46 monoclonal antibody (MAb) M177, excess antibody was removed by washing, and the cells were lysed in radioimmunoprecipitation assay buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, and 0.1% sodium dodecyl sulfate [SDS]). Antibody-bound proteins were precipitated with protein G-Sepharose (Pharmacia), and intracellular CD46 protein was recovered from the supernatant by the sequential addition of more antibody and protein G-Sepharose. Protein samples were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and detected by enhanced chemiluminescence (Amersham) Western blotting analysis with the M177 MAb (36). This assay detects both surface and intracellular CD46 protein and thus can provide a control for possible differences in expression between the transfectants.

Uninfected CV-1 cells expressed the wild-type and mutant CD46 proteins from the cloned DNA equally well on both the cell surface and intracellularly (Fig. 2, CV-1, lanes 2 to 4 and 6 to 8). However, in the transfected Biken-CV-1 cells, CD46 protein expression was drastically reduced at the cell surface compared with intracellular expression (Fig. 2, Biken-CV-1, lanes 2 and 6). Northern blot analyses showed that the transfected CV-1 and Biken-CV-1 cells expressed comparable levels of CD46 RNA (data not shown). Pulse-chase labeling studies demonstrated that any single CD46 gene construct produced equivalent levels of CD46 protein in 1 h in both cell types (10). Therefore, the down regulation of surface CD46 in Biken-CV-1 cells is not due to differences in transfection efficiency or protein synthesis. The 40-kDa band and the additional lower band represent incompletely glycosylated CD46 species, probably generated by interaction with the Biken H protein during exocytic transport of CD46 (reference 10 and unpublished data). Interestingly, CD46 mutants lacking the membrane-proximal Tyr-Arg-Tyr-Leu sequence were not reduced at the Biken-CV-1 cell surface compared with intracellular expression (Fig. 2, Biken-CV-1, lanes 3, 4, 7, and 8). These results confirm our previous conclusions that the cytoplasmic domain of CD46 contains sequences important for the down regulation of this receptor from the cell surface. These data further show that a sequence between amino acids 336 and 339 is required for CD46 down regulation.

Alanine scanning reveals a cytoplasmic sequence motif required for efficient CD46 down regulation. To further define the residues within the region encompassing amino acids 336 to 339 of CD46 that are critical for receptor down regulation, we individually replaced each of these amino acid residues with alanine by using mutagenization primers. We also created a double mutant with alanine substitutions at Tyr-336 and Leu-339 (Fig. 1). These mutants were expressed in CV-1 and Biken-CV-1 cells by transient transfection.

In CV-1 cells, each of the alanine substitution mutants was expressed equally well on both the cell surface and intracellularly (Fig. 3, CV-1), with the exception of the Arg-337 mutant (YAYL), which was not expressed efficiently in either CV-1 or Biken-CV-1 cells (Fig. 3, lanes 3 and 9). In Biken-CV-1 cells, mutation of Tyr-336 (ARYL) was sufficient to partially restore CD46 surface expression (Fig. 3, Biken-CV-1, lanes 2 and 8),

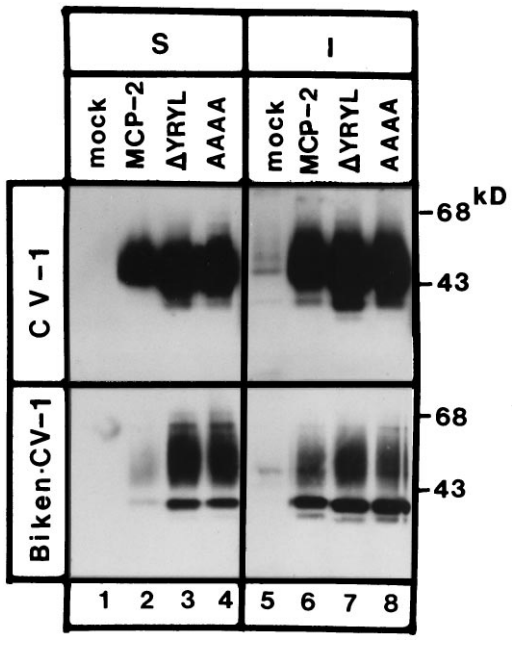

FIG. 2. Surface and intracellular expression of CD46 and Tyr-Arg-Tyr-Leu mutants. CV-1 or Biken-CV-1 cells were transfected with no DNA (lanes 1 and 5), MCP-2 (lanes 2 and 6), ΔYRYL (lanes 3 and 7), or AAAA (lanes 4 and 8). Surface (S) and intracellular (I) proteins were immunoprecipitated with MAb M177, resolved by SDS-PAGE, and detected by immunoblotting with the same MAb.

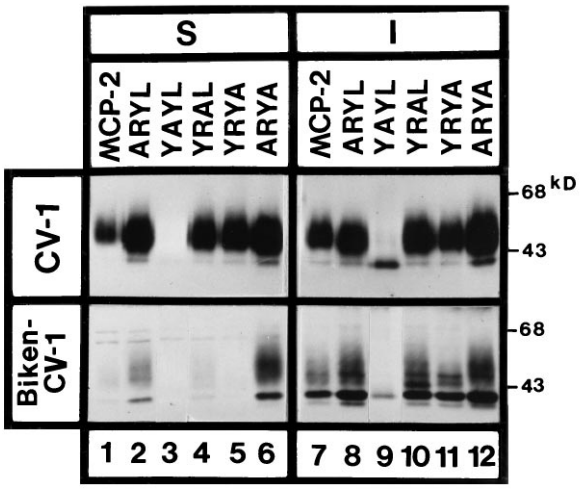

FIG. 3. Surface and intracellular expression of CD46 and alanine-substitution mutants. CV-1 or Biken-CV-1 cells were transfected with MCP-2 (lanes 1 and 7), ARYL (lanes 2 and 8), YAYL (lanes 3 and 9), YRAL (lanes 4 and 10), YRYA (lanes 5 and 11), or ARYA (lanes 6 and 12) mutants. Surface (S) and intracellular (I) proteins were immunoprecipitated with MAb M177, resolved by SDS-PAGE, and detected by immunoblotting with the same MAb.

whereas mutation of Tyr-338 had little effect on CD46 down regulation (Fig. 3, Biken-CV-1, lanes 4 and 10). Interestingly, while mutation of Leu-339 alone (YRYA) did not inhibit CD46 down regulation (Fig. 3, Biken-CV-1, lanes 5 and 11), mutation of Leu-339 in combination with Tyr-336 (ARYA) markedly inhibited CD46 down regulation from the cell surface (Fig. 3, Biken-CV-1, lanes 6 and 12). These data demonstrate that two amino acids, Tyr-336 and Leu-339, near the transmembrane-cytosolic junction of CD46 are required for efficient down regulation of surface CD46 in cells persistently infected with MV.

Mutations that reduce CD46 down regulation increase syncytium formation in persistently infected cells. To test the biological consequence of CD46 down regulation, we examined the morphology of the persistently infected cells expressing the various CD46 mutants. Biken-CV-1 cells expressed very little CD46 at the cell surface (10) (Fig. 2, Biken-CV-1, lane 1) and showed no sign of cytopathic effects (9, 10) (Fig. 4A). However, when these cells were transfected with a wild-type CD46 cDNA clone, there was a transient increase in CD46 surface expression, which facilitated the formation of multinucleated syncytia via interactions with the H and F proteins on neighboring infected cells (Fig. 4B) (10). Interestingly, when these cells were transfected with CD46 mutants in which the Tyr-Arg-Tyr-Leu sequence was deleted (ΔYRYL) or changed to alanines (AAAA), the transfected cells developed much more extensive syncytia (over 90%) in the same time period when wild-type CD46-transfected cells showed less than 50% syncytia (Fig. 4C, D, and B, respectively). Furthermore, the extent of syncytium formation correlated inversely with the down regulation of the individual alanine mutants. Biken-CV-1 cells expressing the Tyr-336 mutant (Fig. 4E) showed intermediate degrees of cell fusion between the wild-type CD46 and the ΔYRYL or AAAA transfectants, confirming an important role for Tyr-336 in CD46 surface expression. Since the Arg-337 mutant was found to be poorly expressed (Fig. 3, Biken CV-1, lanes 3 and 9), it was not surprising that cells transfected with this clone showed very few syncytia (Fig. 4F). Cells expressing the Tyr-338 mutant (Fig. 4G) or the Leu-339 mutant (Fig. 4H) showed lower syncytium-forming activities than those of cells expressing the Tyr-336 mutant (Fig. 4E). However, Biken-CV-1 cells transfected with the Tyr-336–Leu-339 double mutant (ARYA) showed cytopathic effects approaching those seen in the ΔYRYL and AAAA transfectants (Fig. 4I, C, and D, respectively). We have monitored the fusion morphology of these cells at different time points and reproducibly found a greater number of syncytia in cells expressing the ARYA double mutant compared with the ARYL mutant,

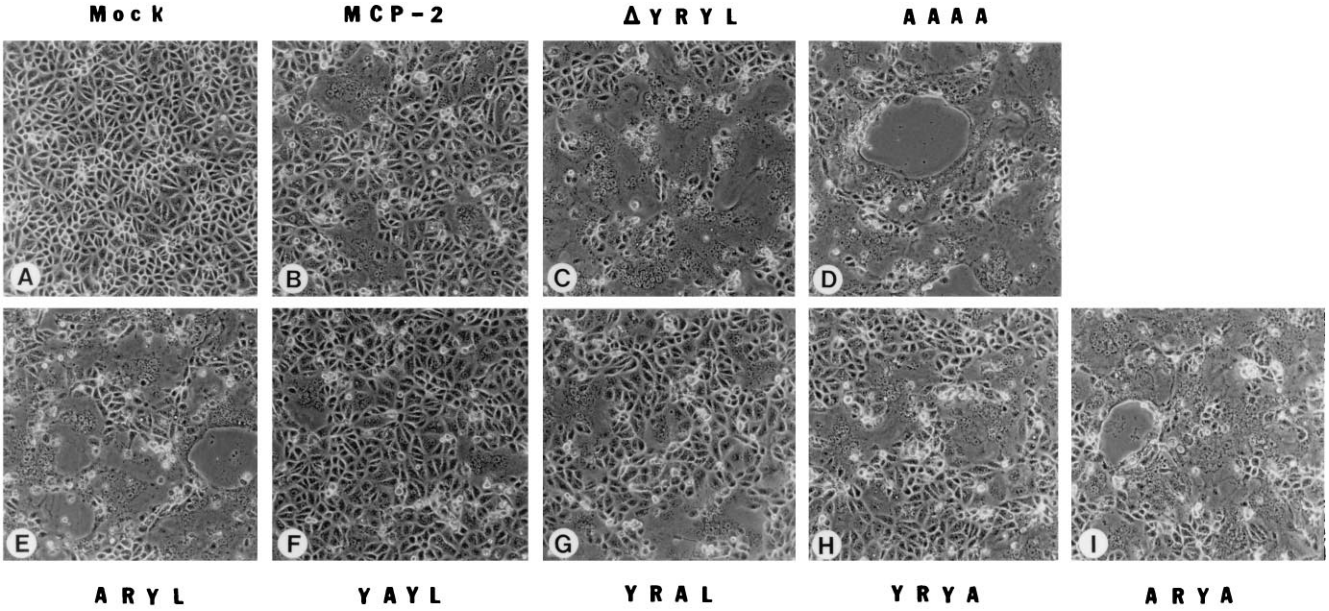

FIG. 4. Syncytium formation induced by human CD46 in Biken-CV-1 cells. Cells transfected with cDNAs encoding wild-type CD46 (MCP-2) (B), ΔYRYL (C), AAAA (D), ARYL (E), YAYL (F), YRAL (G), YRYA (H), or the ARYA mutant (I) were studied by light microscopy approximately 45 h posttransfection, along with mock-transfected Biken-CV-1 cells (A).

even though this difference is not apparent in the photographs shown.

A role for receptor down regulation in persistent infection by paramyxovirus has been suggested in the human parainfluenza virus type 3, which maintains a noncytopathic infection by removing neuraminic acid from the cell surface (22). The present study confirms our finding that CD46 down regulation plays a significant role in the maintenance of a noncytopathic persistent MV infection (10). Our data further show that Tyr-336 and Leu-339 in the CD46 cytoplasmic domain are critical determinants for CD46 down regulation. These critical residues form a Tyr-X-X-Leu motif which resembles a sequence motif required for the internalization of many membrane proteins, including the mammalian endoprotease furin (31, 41), the human nerve growth factor receptor (21), TGN38 (43), and the epidermal growth factor receptor (1). Overexpression of the H protein accelerates CD46 internalization from the cell surface (23). We have found that nearly 10-fold more CD46 is internalized in persistently infected cells constitutively expressing the H protein than in uninfected cells (unpublished data). It is therefore conceivable that binding of the Biken viral H protein to CD46 results in CD46 internalization. Interestingly, by use of a yeast (*Saccharomyces cerevisiae*) two-hybrid system for studying protein interactions, a Tyr-X-X-Leu sequence has been shown to interact with different adaptin subunits involved in membrane protein internalization or protein sorting in the TGN (26). Whether the juxtamembrane Tyr-X-X-Leu motif in CD46 interacts with cellular factors to mediate CD46 down regulation is an interesting question.

Besides cell surface interactions, MV may trigger CD46 down regulation by intracellular mechanisms. Notably, Tyr-X-X-Leu motifs have also been implicated in the lysosomal targeting of various membrane proteins, including rat and human lamp-1 (12, 43) and lysosomal acid phosphatase (27). Chimeric studies have shown that these cytoplasmic sequences are sufficient to target a recombinant protein to the lysosome from either the TGN or cell surface after endocytosis (27). The transferrin receptor, which contains a similar tyrosine-based tetrapeptide, can be detected in endosomal compartments prior to its appearance at the cell surface (5). This raises the possibility that newly synthesized CD46 containing the Tyr-X-X-Leu motif may also be delivered directly to the lysosome in persistently infected cells. In support of this hypothesis, we have found that although very little CD46 reaches the surface of MV-infected cells, CD46 does not accumulate intracellularly (10). This suggests that CD46 may be degraded intracellularly prior to its presentation on the cell surface. Alternatively, CD46 degradation may also occur in intracellular compartments distinct from the lysosome. For instance, intracellular binding of MV H and CD46 in the endoplasmic reticulum could interfere with CD46 maturation and transport to the cell surface. Such an interaction is analogous to the way gp120 of human immunodeficiency virus type 1 interferes with the transport of its cognate receptor CD4 from the endoplasmic reticulum (2). Whether MV can induce such changes in the intracellular trafficking of CD46 is currently under investigation.

In addition to a role in protein trafficking, Tyr-X-X-Leu motifs have been implicated in receptor signalling. The cytoplasmic domains of many receptors on immune cells contain Tyr-X-X-Ile/Leu motifs that are necessary for mediating signal transduction events important for immune responses (4, 37). Recently, Karp and coworkers showed that cross-linking CD46 leads to suppression of interleukin-12 production by activated monocytes (14), suggesting a cross-talk between CD46 and macrophage activation pathways. We have found that the cytoplasmic domain of CD46 is also important for CD46 down regulation in acute MV infection (unpublished data). Studying the cellular factors that interact with the CD46 cytoplasmic sequences will provide insight into the mechanisms of CD46 down regulation and may shed light on the possible role of these sequences in MV-induced immunosuppression.

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