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Myxoma Virus Induces Extensive CD4 Downregulation and Dissociation of p56^{lck} in Infected Rabbit CD4$^+$ T Lymphocytes

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Myxoma virus is a pathogenic poxvirus that induces extensive dysregulation of cellular immunity in infected European rabbits. Infection of a rabbit CD4$^+$ T-cell line (RL-5) with myxoma virus results in dramatic reductions of cell surface levels of CD4 as monitored by flow cytometry. The virus-induced downregulation of CD4 requires early but not late viral gene expression and could not be inhibited by staurosporine, an inhibitor of protein kinase C, which effectively blocks phorbol 12-myristate-13-acetate-induced downregulation of CD4. The decrease in total cellular levels of CD4 during myxoma virus infection could be inhibited by the lysosomotrophic agent NH_4Cl, suggesting a lysosomal fate for CD4 during myxoma virus infection. Steady-state levels of the CD4-associated protein tyrosine kinase p56^{lck} remained unchanged during myxoma virus infection, suggesting that p56^{lck} dissociates from CD4 prior to CD4 degradation in virus infected cells. Total p56^{lck} kinase activity was unaffected during myxoma virus infection, although the amount of p56^{lck} physically associated with CD4 declined in parallel with the loss of CD4. Thus, myxoma virus infection of CD4$^+$ T lymphocytes triggers CD4 downregulation via a protein kinase C-independent pathway, causing the dissociation of p56^{lck} and the degradation of CD4 in lysosomal vesicles.

Poxviridae is the only family of DNA viruses that replicate autonomously in the cytoplasm of infected cells (18, 33). They are among the largest eucaryotic viruses and contain large linear double-stranded genomes of approximately 200 kb with covalently closed hairpin termini (32). In general, virus genes encoding proteins essential for viral propagation tend to cluster within the central portion of the genome, whereas those encoding nonessential virulence factors are preferentially located in the terminal regions of the genome (51). Myxoma virus is a leporipoxvirus and is the causative agent of myxomatosis, a rapidly lethal disease in the European rabbit (*Oryctolagus cuniculus*) (10, 28), which is characterized by the induction of a generalized systemic immunosuppression and dissemination via infected lymphocytes (48).

It is becoming increasingly evident that the genomes of many poxviruses encode a number of proteins that enable the virus to evade the host's immune system (29, 38, 47). In particular, myxoma virus has been found to encode multiple virulence factors that serve this specific function (29–31). One mechanism of viral immune evasion is the downregulation on infected cells of host surface proteins necessary for an efficient immune response. Previous experiments have indicated that major histocompatibility complex (MHC) class I molecules can be modulated in nonlymphocytic cells by infection with myxoma virus and malignant rabbit fibroma virus, a related leporipoxvirus (4). In view of the fact that T lymphocytes are known to play an important role in poxvirus infections (2, 3, 5, 15) we investigated whether infection of rabbit T lymphocytes with myxoma virus affected the levels of a variety of relevant T-cell-specific antigens and report here that CD4 was reproducibly downregulated from the cell membrane. Since the loss of CD4 molecules from the surface of CD4$^+$ T lymphocytes infected by other lymphotropic viruses, such as human immunodeficiency virus type 1 (HIV-1), is believed to be an important component of viral immunosuppression, we were therefore interested in characterizing the mechanism whereby myxoma virus mediates the modulation of CD4 from the surface of infected T cells.

CD4 is a transmembrane glycoprotein of 55 kDa that is expressed on a subset of mature T lymphocytes designated CD4$^+$ T helper cells and plays a pivotal role in T-cell ontogeny and T-cell activation (35). In addition, CD4 functions as the receptor for HIV (7, 21). During T-cell activation, the T-cell receptor (TCR) on CD4$^+$ cells recognizes exogenous antigen processed and presented in the form of a peptide complexed to the MHC class II molecule. During the recognition by the TCR, the extracellular domain of CD4 interacts with a nonpolymorphic region of the MHC class II molecule (9, 46). T-cell activation is triggered through several tyrosine kinases associated with the TCR, as well as the tyrosine kinase p56^{lck}, which is associated with the cytoplasmic domain of CD4 (11, 19).

Loss of surface CD4 can be triggered in vitro by exposing CD4$^+$ T lymphocytes to either monosialoganglioside G_{M1} or the phorbol ester phorbol 12-myristate-13-acetate (PMA) (16, 17, 41). PMA induces the activation of protein kinase C, which mediates the phosphorylation of serine residues in the cytoplasmic domain of CD4 (44), and triggers the dissociation of p56^{lck} from CD4, resulting in the endocytosis of CD4 via clathrin-coated pits (36). Endocytosis is followed by the subsequent degradation of CD4 in lysosomal vesicles (37, 40). G_{M1} has also been shown to cause the rapid internalization and degradation of CD4, which is preceded by the dissociation of p56^{lck} (41). The mechanism, however, is independent of protein kinase C activation.

The downregulation of CD4 can also be achieved by infection of CD4$^+$ T lymphocytes with HIV, simian immunodeficiency virus, or human herpesvirus 7 (12, 27, 42). In HIV-infected cells, the downregulation of CD4 has been attributed to two viral genes, *nef* and *vpu* (1, 54). The *nef* gene product is a 27-kDa protein that is expressed early in infection and is modified by both N-terminal myristoylation and phosphoryla-

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tion (13, 22). N-terminal myristoylation is necessary for membrane localization and also the subsequent downregulation of CD4 from the cell surface (1, 56). Nef functions by promoting CD4 endocytosis, which is subsequently followed by the degradation of CD4 in lysosomal vesicles (1, 39). In contrast, Vpu is a 16-kDa transmembrane phosphoprotein that is expressed late after infection and localizes to the endoplasmic reticulum of infected cells. Vpu facilitates the degradation of CD4 (54), and the phosphorylation state of Vpu is important for efficient degradation of CD4 (43).

In this study, cell surface levels of rabbit CD4 were investigated in RL-5, a rabbit $CD4^+$ T-cell lymphoma line, by flow cytometry using monoclonal antibody Ken 4, which is specific for rabbit CD4. We have observed that when myxoma virus infects RL-5 cells, the immune molecule CD4 is rapidly and reproducibly downregulated from the cell surface. We have investigated the mechanism of myxoma virus-induced downregulation of CD4 from infected $CD4^+$ T lymphocytes and show that CD4 is internalized and degraded in lysosomal vesicles during virus infection. We also find that the downregulation of CD4 in myxoma virus-infected cells, unlike that induced by PMA, is independent of protein kinase C activation. The fate of the CD4-associated tyrosine kinase $p56^{lck}$ was also investigated during myxoma virus infection, and experiments indicate that during infection, $p56^{lck}$ dissociates from CD4 prior to the degradation of CD4 in lysosomal vesicles. Thus, myxoma virus is the only other virus, aside from HIV, simian immunodeficiency virus, and human herpesvirus 7, and the first poxvirus currently known to induce the downregulation of CD4 in infected T lymphocytes.

MATERIALS AND METHODS

Cells and viruses. vMyxlac is a derivative of myxoma virus strain Lausanne containing the *Escherichia coli lacZ* gene inserted at an innocuous site between the myxoma virus growth factor and M9 genes, and its construction has been described elsewhere (34). vMyxlac was routinely propagated in baby green monkey kidney cells (a gift from S. Dales, University of Western Ontario, London, Ontario, Canada) grown in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum (Gibco BRL Life Technologies Inc.). Viruses were prepared as described elsewhere (50). RL-5 cells were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (20). RL-5 cells were routinely cultured in RPMI 1640 (Gibco BRL Life Technologies Inc.) supplemented with 200 U of penicillin (Ayerst Laboratories) per ml, 200 μg of streptomycin (Sigma Chemical Co.) per ml, and 10% fetal calf serum (Gibco BRL Life Technologies Inc.). Where indicated, cells were cultured in the presence of either 5 μg of brefeldin A (Sigma) per ml, 100 ng of PMA (LC Services Corp.) per ml, 20 μM staurosporine (Sigma), or 200 μM NH_4Cl (Sigma). For lysosomal degradation analysis, RL-5 cells were treated with 200 μM NH_4Cl for 1 h prior to the addition of PMA or added 1 h after the adsorption of virus, and NH_4Cl remained in the media for the duration of the experiment. To quantitate total cellular CD4 levels, the cells were washed twice in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and then fixed in 200 μl of PBS containing 2% paraformaldehyde for 30 min at room temperature with constant agitation. Cells were then washed once in PBS containing 1% BSA and permeabilized with 100 μl of 0.1% Triton X-100–0.1% sodium citrate for 2 min at 4°C.

Virus infection. RL-5 cells were either mock infected or adsorbed with virus for 1 h at 37°C at a multiplicity of infection of 10 PFU per cell. Following adsorption, cells were diluted to 5×10^5 cells per ml with fresh medium, and the infection was allowed to proceed at 37°C for the indicated time. Infected RL-5 cells were harvested with 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate). Cell integrity was routinely determined by trypan blue exclusion, and in all experiments, greater than 90% of the cells excluded the dye. The efficiency of virus infection was routinely quantitated by colorimetric analysis using the *lacZ* gene and in all experiments was found to be greater than 95%. When necessary, virus was UV inactivated for 60 min prior to infection.

Antibodies. Murine anti-rabbit antibodies CD4, CD4-biotin, and MHC class I were obtained from Spring Valley Laboratories (Sykesville, Md.) (23). The hybridoma L11/135 (murine anti-rabbit CD43) was obtained from the American Type Culture Collection, and antibody-containing supernatants were prepared (53). An anti-$p56^{lck}$ antiserum generated against a TrpE-Lck fusion protein containing amino acids 2 to 148 of the murine Lck sequence was generously provided by A. Veillette, McGill University. Unconjugated primary antibodies were visualized by using R-phycoerythrin-conjugated $F(ab')_2$ goat anti-mouse immunoglobulin G (Dako, Dimension Laboratories), and biotinylated antibodies were visualized by using fluorescein isothiocyanate-conjugated streptavidin (AMAC; BIO/CAN). For immunofluorescence, rhodamine-conjugated goat anti-mouse antibodies were obtained from Jackson ImmunoResearch Laboratories Incorporated.

Flow cytometry. Two-color staining was used. Cell concentrations were adjusted to 1.25×10^7/ml in Dulbecco's PBS (DPBS) containing 0.2% (wt/vol) BSA and 0.1% (wt/vol) sodium azide, pH 7.4 (binding buffer). All antibodies were used in excess of predetermined saturating concentrations. To 40-μl cell suspensions was added 5 μl of the appropriate unconjugated primary monoclonal antibody (20 to 50 μg/ml). Samples were incubated at 4°C for 20 min, washed with DPBS, and resuspended in 100 μl of binding buffer. Following this procedure, 5 μl of R-phycoerythrin-conjugated $F(ab')_2$ goat anti-mouse immunoglobulin G was added, and the mixture was incubated at 4°C for 20 min. After washing with DPBS, excess mouse immunoglobulin G (Zymed, Dimension Laboratories, Mississauga, Ontario, Canada) was added to block the remaining binding sites (4°C, 10 min), and the cells were incubated with 5 μl of biotinylated primary antibody (25 to 50 μg/ml) at 4°C for 20 min. The samples were then incubated with fluorescein isothiocyanate-conjugated streptavidin at 4°C for 20 min, washed, and fixed with 1% (wt/vol) paraformaldehyde in PBS before flow cytometric analysis. Flow cytometric analysis was performed on a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif.) equipped with an argon ion laser with 15 mW of excitation at 488 nm. The standard optical filter assembly for FACScan is 530/30 bandpass, 585/42 bandpass, and 650 long pass filter. Fluorescence compensation was set to eliminate any spectral overlap of the emitted signal. Data were acquired on 10,000 cells per sample with light scatter signals at linear gain and fluorescence signals at logarithmic gain.

Immunofluorescence. For immunofluorescence, 10^6 cells were washed twice in PBS containing 1% BSA. After washing, cells were fixed in 200 μl of PBS containing 2% paraformaldehyde for 30 min at room temperature with constant agitation. Cells were then washed once in PBS containing 1% BSA and permeabilized with 100 μl of 0.1% Triton X-100–0.1% sodium citrate for 2 min at 4°C. The cell pellet was then washed twice in PBS containing 1% BSA and resuspended in 100 μl of PBS containing 1% BSA with a 1:100 dilution of the anti-rabbit CD4 antibody. Following a 1-h incubation, the cells were once again washed, and the rhodamine-conjugated anti-mouse secondary antibody was added at a 1:200 dilution. Cells were washed several times in PBS containing 1% BSA and then resuspended in 50% glycerol before being transferred to slides and analyzed with a Zeiss Axioskop microscope.

Immunoprecipitation. One million cells were lysed in 500 μl of Nonidet 40 (NP-40) lysis buffer containing 150 mM NaCl, 50 mM Tris HCl (pH 8.0), and 1% NP-40. Lysates were cleared by centrifugation for 30 min at $12,000 \times g$. The supernatants were collected, and 5 μg of anti-rabbit CD4 (Spring Valley) was added. Following 1 h of incubation at 4°C, 25 μl of 10% (vol/vol) protein A-Sepharose (Sigma) was added, and the lysates were incubated for an additional 30 min at 4°C with constant agitation. The immune complexes were collected by centrifugation for 1 min at $12,000 \times g$, and the subsequent pellet was washed three times with NP-40 lysis buffer. The pellets were resuspended in 50 μl of sodium dodecyl sulfate (SDS-PAGE) sample buffer and boiled at 90°C for 5 min, and supernatants were subjected to SDS-PAGE.

Immunoblotting. Protein concentrations of total cell lysates and immunoprecipitation lysates were quantified by using the Lowry protein assay. Proteins were transferred to nitrocellulose (Micron Separations Inc.) by using a semidry transfer apparatus (Tyler Corp.) for 1 h at 50 mA. Membranes were blocked in PBS containing 0.1% Tween 20 (ICN Biomedicals Inc.) and 5% skim milk for at least 2 h. The anti-$p56^{lck}$ antiserum was added to a final concentration of 1 μg/ml. After 1 h, the blot was washed three times in PBS containing 0.1% Tween 20. The membranes were probed with horseradish peroxidase-conjugated mouse anti-rabbit secondary antibody at a 1:3,000 dilution and developed with Amersham's chemiluminescence detection system (Amersham Inc.).

In vitro $p56^{lck}$ kinase reactions. Following immunoprecipitation with the anti-rabbit CD4 monoclonal antibody or $p56^{lck}$ antiserum, the immune complexes were washed three times with NP-40 lysis buffer and then twice with double-distilled H_2O. The pellet was resuspended in 30 μl of 20 mM Tris-HCl (pH 7.5)–10 mM $MnCl_2$. To this suspension was added 10 μCi of [γ-^{32}P]ATP (ICN Biomedicals Inc.), and the samples were incubated at room temperature for 10 min to induce autophosphorylation. The immune complexes were then washed three times in double-distilled H_2O, the pellets were resuspended in 50 μl of SDS-PAGE sample buffer and boiled at 90°C for 5 min, and supernatants were subjected to SDS-PAGE.

RESULTS

Myxoma virus infection of a $CD4^+$ rabbit T-cell line (RL-5) induces the downregulation of CD4. Since T lymphocytes play a pivotal role in determining the outcome of poxviral infection and because myxoma virus is able to productively infect a variety of lymphoid cells (2, 3, 5, 15, 49), we investigated whether infection with a recombinant myxoma virus (vMyxlac)

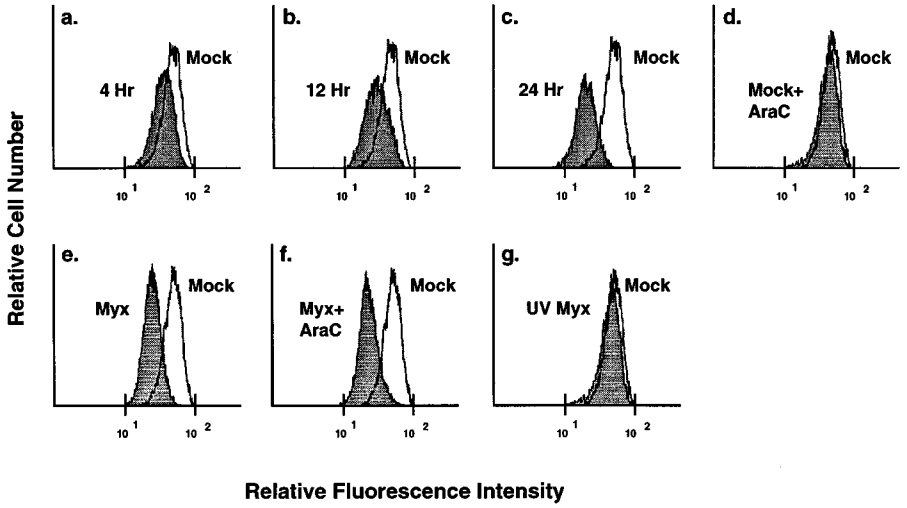

FIG. 1. Cell surface CD4 is downregulated in RL-5 cells infected with myxoma virus. RL-5 cells were infected with myxoma virus for 4 h (a), 12 h (b), or 24 h (c), and surface levels of CD4 were determined by flow cytometry with monoclonal antibody Ken 4, which is specific for rabbit CD4. RL-5 cells were mock infected in the presence of 40 μg of araC per ml (d), infected with myxoma virus (Myx) (e), infected with myxoma virus in the presence of araC for 24 h (f), and infected with UV-inactivated myxoma virus (g).

that contains the *E. coli lacZ* gene as a tag in a nonessential region of the viral genome (34) affected the cell surface expression of a number of rabbit T-cell surface proteins following infection of a rabbit T-cell line (RL-5). RL-5 is a $CD4^+$ rabbit T-lymphoma cell line derived from a herpesvirus ateles-induced rabbit tumor (20). The glycoproteins expressed on the surface of RL-5 cells include CD18, CD43, CD44, CD45, and CD4, but not CD8, suggesting that RL-5 cells probably represent a subset of rabbit $CD4^+$ helper T cells (52).

With monoclonal antibody Ken 4, which is specific for rabbit CD4, mock-infected RL-5 cells show strong positive surface fluorescence (Fig. 1). However, after infection with vMyxlac, there is a dramatic and reproducible decrease in the amount of CD4 antigen present on the cell surface, as represented by a decrease in the relative fluorescence intensity at 4, 12, and 24 h after myxoma virus infection (Fig. 1a to c). RL-5 cells were routinely assayed for the expression of *lacZ* as an indicator of viral infection, and we have found that 95% of RL-5 cells are routinely infected with vMyxlac in these experiments (unpublished data). Comparison of wild-type myxoma virus (strain Lausanne) and vMyxlac recombinant virus indicated similar levels of CD4 modulation from the surface of infected RL-5 cells (unpublished data).

A change in cell surface levels of CD4 could be detected as early as 4 h postinfection (Fig. 1a) with myxoma virus, suggesting that an early gene product may be involved in the downregulation of CD4. To assess this possibility, the reagent cytosine arabinoside (araC), an inhibitor of poxvirus DNA replication and late viral gene expression (32), was used to distinguish between early and late gene involvement in cell surface CD4 downregulation. If only late viral gene products were involved in the downregulation of CD4 by myxoma virus, then araC would be predicted to inhibit the loss of these molecules from the cell surface. In mock-infected RL-5 cells, the presence of araC showed no effect on the levels of CD4 (Fig. 1d). Furthermore, the presence of araC had no effect on the ability of myxoma virus to induce the downregulation of CD4 (Fig. 1e and f), indicating that early gene expression is sufficient for the complete downregulation of CD4 by myxoma virus. To ascertain whether viral gene expression was necessary for CD4 downregulation, virus-infected cells were treated with cycloheximide; however, the presence of cycloheximide induced apoptosis in RL-5 cells (unpublished data). Upon UV inactivation of the virus, the downregulation of CD4 was completely abolished (Fig. 1g), indicating that the virus-mediated modulation of CD4 was not simply due to virus fusion with the cell membrane and that early gene expression is required to mediate the downregulation of CD4.

We also monitored the expression levels of other rabbit cell surface molecules on RL-5 cells following myxoma virus infection. No significant change in cell surface fluorescence was detected with monoclonal antibodies specific for rabbit CD18 (Fig. 2a), CD43 (Fig. 2b), and CD45 (Fig. 2c). These results suggest that the loss of CD4 molecules from the cell surface is probably not a function of overall membrane glycoprotein disruption due to viral infection. Membrane integrity was also suggested by the exclusion of trypan blue in greater than 90% of RL-5 cells infected with myxoma virus after 24 h of infection (unpublished data).

Cell surface CD4 is downregulated following infection of RL-5 cells with myxoma virus in a protein kinase C-independent fashion. In vitro modulation of CD4 can be accomplished by exposing $CD4^+$ T cells to the phorbol ester PMA (16, 17), and the mechanism of PMA-induced CD4 downregulation is dependent on protein kinase C activation (44). To test if myxoma virus and PMA utilize a common mechanism of CD4 modulation, cell surface levels of CD4 were monitored by flow cytometry following the addition of PMA. The addition of PMA to RL-5 cells resulted in a very rapid loss of cell surface CD4, which could be detected as early as 1 h after the addition of PMA (unpublished data). A maximum decrease in CD4 levels could be detected after 12 h of PMA treatment (Fig. 3a). Thus, the kinetics of PMA-mediated CD4 modulation were found to vary considerably from the kinetics of modulation mediated by infection with myxoma virus. In addition, continuous treatment (24 to 72 h) of RL-5 cells with PMA resulted in the gradual recovery of CD4 at the cell surface such that by 72 h, cell surface levels of CD4 returned to almost normal (Fig. 3b). No such recovery, however, was ever detected in RL-5 cells infected with myxoma virus. This reemergence of cell surface CD4 in cells chronically treated with PMA is thought

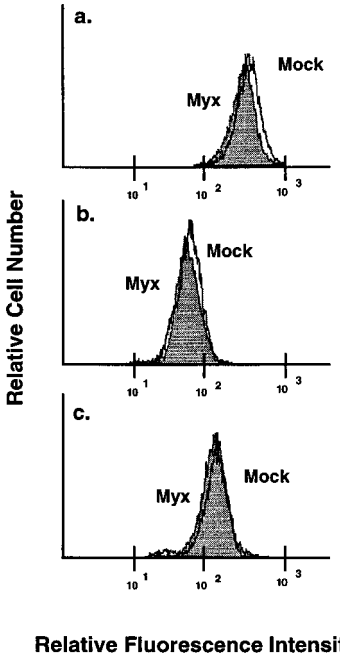

FIG. 2. Surface levels of CD18, CD43, and CD45 are unaffected during myxoma virus infection of RL-5 cells. Cell surface levels of CD18 (a), CD43 (b), and CD45 (c) were monitored by flow cytometry 24 h after infection with myxoma virus (Myx).

to involve the downregulation of protein kinase C steady-state levels via increased protein degradation (55).

Since cells which have been chronically treated with PMA and allowed to recover to almost normal levels of CD4 are no longer able to modulate CD4 via protein kinase C inhibition, we were interested in determining if these PMA-recovered RL-5 cells were susceptible to CD4 modulation by myxoma virus. RL-5 cells were cultured in the presence of PMA for 72 h, at which point they were infected with myxoma virus. Results indicate that RL-5 cells treated in this way are no longer susceptible to CD4 downregulation by myxoma virus (Fig. 3c). However, MHC class I molecules, which are also known to be modulated from the cell surface during myxoma virus infection (4), are not affected by PMA pretreatment and are still downregulated from the infected cell surface (Fig. 3d), indicating that PMA did not affect virus replication or the downregulation of at least one other cellular surface protein by myxoma virus. One possible explanation is that pretreatment of RL-5 cells with PMA results in the abrogation of CD4 turnover from the membrane. When untreated RL-5 cells were incubated in the presence of brefeldin A, a fungal metabolite that has been shown to block protein transport from the endoplasmic reticulum (8, 25), CD4 molecules were extensively downregulated from the cell surface (Fig. 3e). This result indicates that when nascent proteins cannot reach the plasma membrane, the levels of CD4 progressively turn over from the cell surface. In addition, when PMA-pretreated RL-5 cells were exposed to brefeldin A, a similar loss of cell surface CD4 was detected (Fig. 3f), suggesting that although PMA pretreatment inhibits myxoma virus-induced CD4 downregulation, it does not affect the recycling pathway that normally governs surface CD4 turnover.

The fact that myxoma virus was unable to induce CD4 downregulation in these PMA-pretreated RL-5 cells suggested that virus-induced modulation might be explained by the activation of protein kinase C during viral infection. To determine whether this was the case, RL-5 cells were infected in the presence of staurosporine, a potent inhibitor of protein kinase C activity. As expected, the modulation of CD4 by PMA was reduced in the presence of staurosporine in RL-5 cells (Fig. 3g). The presence of staurosporine, however, had no affect on the ability of myxoma virus to mediate the downregulation of CD4 (Fig. 3h), suggesting that the inappropriate activation of protein kinase C could not explain the loss of cell surface CD4 by myxoma virus. In view of the fact that the kinetics of CD4 modulation differ substantially for induction by PMA and by myxoma virus and the fact that inhibition of protein kinase C has no effect on CD4 modulation by myxoma virus, we conclude that the downregulation of CD4 by myxoma virus is distinct from PMA-induced downregulation of CD4 and is not due to the inappropriate activation of cellular protein kinase C.

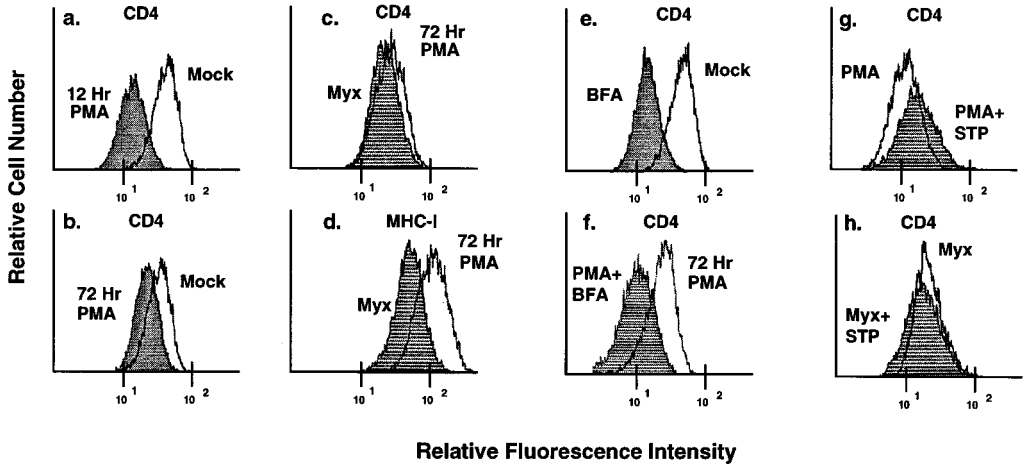

FIG. 3. The mechanism of myxoma virus-mediated CD4 downregulation in RL-5 cells is distinct from that of PMA-mediated CD4 downregulation. RL-5 cells treated with 100 ng of PMA per ml for 12 h (a) indicated a decline in cell surface CD4, which was found to recover after 72 h of PMA treatment (b). After PMA pretreatment (c), myxoma virus (Myx) does not induce the downregulation of CD4, but MHC class I molecules are downregulated (d). In the presence of brefeldin A (BFA), RL-5 cells show decreased levels of cell surface CD4 (e), and PMA-pretreated RL-5 cells also show decreased levels of cell surface CD4 (f). RL-5 cells were treated with PMA in the presence or absence of staurosporine (STP) (g) or infected with myxoma virus in the presence or absence of staurosporine (h).

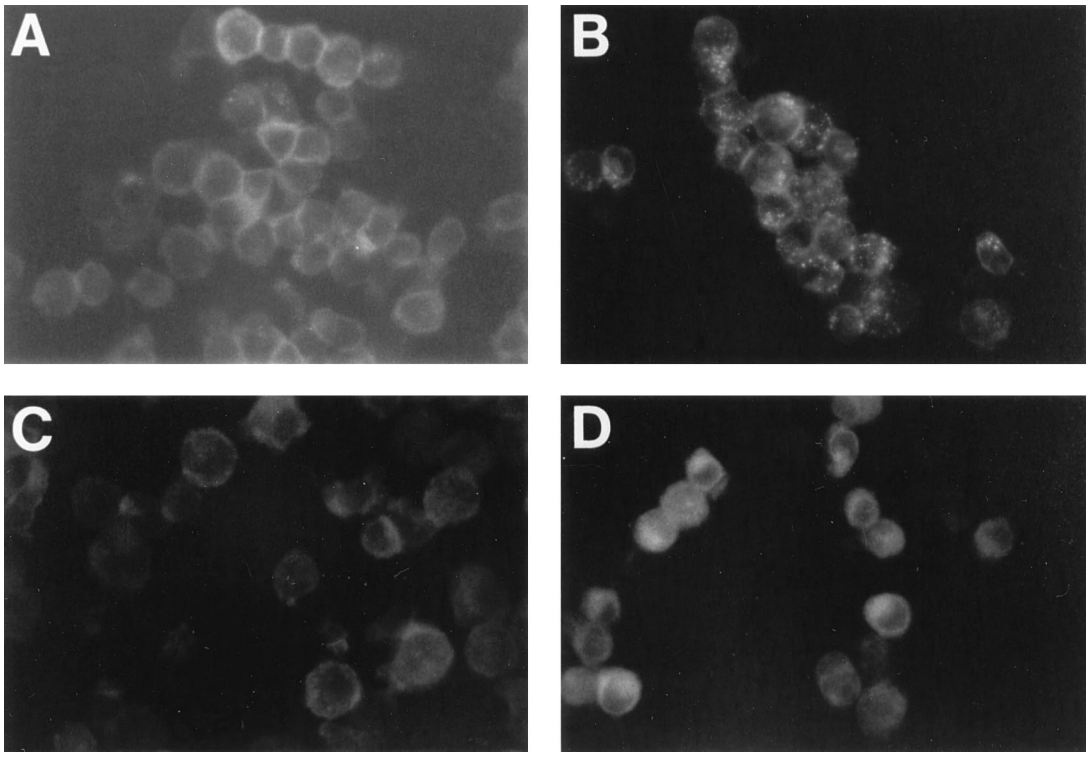

FIG. 4. Intracellular distribution of CD4 in response to PMA, brefeldin A, or myxoma virus. RL-5 cells were either untreated (A), treated with 100 ng of PMA per ml for 12 h (B), infected with myxoma virus for 24 h (C), or treated with 5 μg of brefeldin A for 12 h (D). Cells were then fixed with paraformaldehyde, permeabilized with Triton X-100, and stained with anti-mouse rhodamine-conjugated second antibody to visualize internalized CD4.

Distribution of intracellular CD4. To further investigate the downregulation of CD4 by myxoma virus, we examined the distribution of intracellular CD4 by immunofluorescence. Previous studies have shown that when $CD4^+$ cells are treated with PMA, internalized CD4 becomes sequestered within intracellular vesicles, which have been identified as late endosomes by colocalization with late endosomal markers (36). Thus, after infection with myxoma virus or treatment with PMA, RL-5 cells were fixed with paraformaldehyde and permeabilized with Triton X-100 prior to immunofluorescence. In mock-infected RL-5 cells, intracellular CD4 was observed at the cell membrane as well as in small vesicles throughout the cytoplasm (Fig. 4A). Upon treatment with PMA, the sequestration of CD4 into intracellular endosomal vesicles was evident by immunofluorescence (Fig. 4B). In contrast, comparable levels of CD4 positive endosomal vesicles were not detected in the cytoplasm of myxoma virus-infected RL-5 cells (Fig. 4C). Thus, when myxoma virus-infected cells were compared with PMA-treated cells, it was evident that the virus was not mimicking the same intracellular compartmentalization of CD4 by PMA.

We were also interested in investigating whether myxoma virus interferes with normal transport of CD4 from the endoplasmic reticulum to the surface. We have observed that treatment of RL-5 cells with brefeldin A mimics the kinetics of cell surface downregulation of CD4 by myxoma virus (Fig. 3e), suggesting that myxoma virus may take advantage of this same mechanism to induce the loss of cell surface CD4 in infected cells. RL-5 cells that were treated with brefeldin A, however, showed an intracellular distribution pattern for CD4 very different from that of cells infected with myxoma virus. Cells treated with brefeldin A for 12 h showed very intense immunofluorescence throughout the entire extranuclear region, indicating CD4 accumulation in the endoplasmic reticulum (Fig. 4D). This is in contrast to cells infected with myxoma virus (Fig. 4C), indicating that the downregulation of CD4 by myxoma virus is not due to the sequestration of CD4 in the endoplasmic reticulum.

CD4 is degraded in lysosomal vesicles during myxoma virus infection. To further examine the mechanism of myxoma virus-induced CD4 downregulation, total cellular levels of CD4 were quantitated by flow cytometry on fixed permeabilized cells following treatment with PMA or brefeldin A or infection with myxoma virus. With this approach, PMA-treated permeabilized RL-5 cells showed lower levels of total cellular CD4 than did untreated cells (Fig. 5a). This decrease in total cellular levels of CD4 is indicative of CD4 degradation via a previously defined lysosomal pathway (37, 40). In contrast, RL-5 cells treated with brefeldin A did not show a decrease in total cellular levels of CD4, but instead total cellular levels of CD4 were found to actually increase somewhat above the level in untreated cells (Fig. 5b), indicating that within brefeldin A-treated cells, CD4 is being intracellularly sequestered but not degraded. In myxoma virus-infected RL-5 cells, however, total cellular levels of CD4 were found to be significantly lower than in mock-infected cells (Fig. 5c), suggesting that the downregulation of CD4 in myxoma virus-infected cells is not due to sequestering of CD4 within infected cells, a result consistent with the immunofluorescence data in Fig. 4.

Since previous research has revealed a lysosomal fate for the degradation of CD4 following phorbol ester-induced and Nef-induced downregulation of CD4 (1, 37, 40), we wished to ascertain whether myxoma virus-induced CD4 downregulation resulted in the lysosomal degradation of CD4. Lysosomotro-

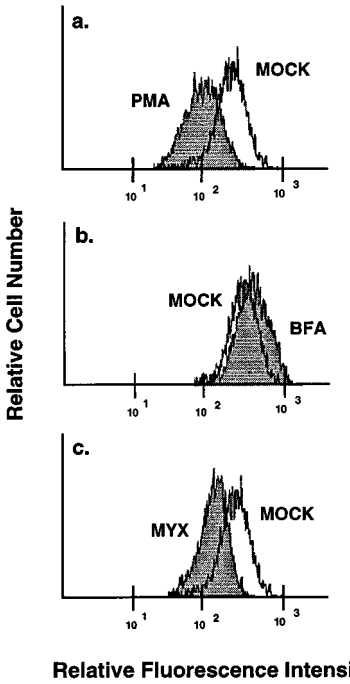

FIG. 5. Total cellular levels of CD4 in response to PMA, brefeldin A, or myxoma virus. RL-5 cells were either treated with 100 ng of PMA per ml for 12 h (a), treated with 5 μg of brefeldin A (BFA) for 12 h (b), or infected with myxoma virus (Myx) for 24 h (c), fixed, and permeabilized prior to quantitation by flow cytometry.

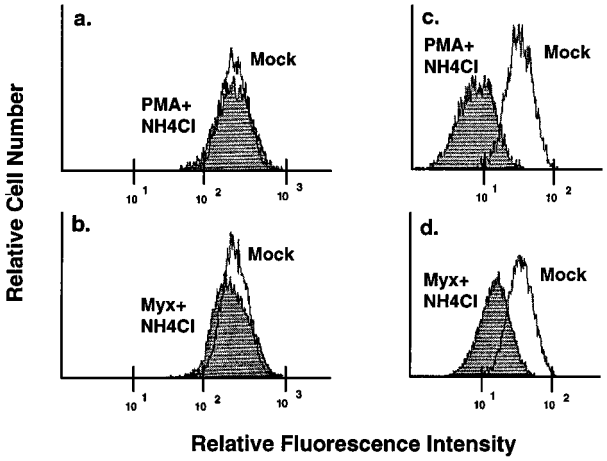

FIG. 6. NH_4Cl inhibits the degradation of CD4 by myxoma virus or PMA. Total cellular CD4 levels were monitored by flow cytometry following fixation and permeabilization of RL-5 cells after treatment with PMA for 12 h in the presence of NH_4Cl (a) or infection of RL-5 cells by myxoma virus (Myx) for 24 h in the presence of NH_4Cl (b). Cell surface modulation of CD4 was also monitored in the presence of NH_4Cl plus PMA treatment (c) and myxoma virus infection (d).

phic agents, such as NH_4Cl, inhibit the degradation of proteins in lysosomal vesicles by neutralizing the pH in lysosomal vesicles. In this study, RL-5 cells were pretreated with NH_4Cl 1 h prior to the addition of PMA or treated with NH_4Cl 1 h after the adsorption of myxoma virus. Following PMA treatment or myxoma virus infection, cells were fixed with paraformaldehyde and permeabilized with Triton X-100 prior to analysis by flow cytometry. PMA treated permeabilized cells in the presence of NH_4Cl showed no change in total cellular levels of CD4 compared with mock-treated cells, indicative of the predicted inhibition of CD4 degradation in RL-5 cells (Fig. 6a). Moreover, the degradation of CD4 in myxoma virus-infected cells was also blocked by the presence of NH_4Cl (Fig. 6b), suggesting a lysosomal fate for the degradation of CD4 in myxoma virus-infected cells. To ensure that the presence of NH_4Cl did not interfere with infection or the modulation of CD4 at the cell surface, NH_4Cl-treated RL-5 cells were monitored for CD4 surface expression by flow cytometry. In the presence of NH_4Cl, cell surface CD4 was downregulated as usual following PMA treatment (Fig. 6c) and myxoma virus infection (Fig. 6d), indicating that the presence of NH_4Cl did not alter the downregulation of cell surface CD4 by either PMA or myxoma virus. Even though inhibition of lysosomal degradation by NH_4Cl clearly resulted in an inhibition of CD4 degradation during myxoma virus infection, the loss of CD4 proceeded normally from the cell surface, suggesting that once CD4 is routed to the lysosomal pathway during myxoma virus infection, it is unable to recycle back to the cell surface.

Myxoma infection induces the dissociation of p56^{lck} from CD4 prior to degradation. Since the tyrosine kinase p56^{lck} associates with the cytoplasmic domain of CD4, and previous investigators have found that in PMA-treated or Nef-transfected cells, p56^{lck} dissociates from CD4 prior to the degradation of CD4 in lysosomal vesicles (1, 36), we wanted to ascertain if myxoma virus infection had a direct effect on p56^{lck}. Steady-state levels of p56^{lck} and the association of p56^{lck} with CD4 were monitored by direct immunoblotting of total cell lysates and immunoblotting after immunoprecipitation with anti-CD4, respectively. Using a cross-reactive antibody specific for the N-terminal region of murine Lck, we observed that rabbit RL-5 cells express equal amounts of the 56-kDa form and the 59-kDa phosphorylated form of p56^{lck}. No change in total steady-state levels of these two forms of p56^{lck} was detected by immunoblotting of total cell lysates harvested during myxoma virus infection compared with mock-infected lysates (Fig. 7). However, when levels of CD4-associated p56^{lck} were measured by immunoprecipitating myxoma virus-infected RL-5 cell lysates with an anti-CD4 antibody and then immunoblotting with an anti-p56^{lck} antiserum, the amount of p56^{lck} associated with CD4 declined in a fashion that paralleled the decrease in the steady-state levels of CD4 (Fig. 8). Since no change in steady-state levels of p56^{lck} was detected under these conditions, we conclude that p56^{lck} must dissociate from CD4 prior to the degradation of CD4 in lysosomal vesicles during myxoma virus infection. In addition, when CD4 was immunoprecipitated from cell lysates and in vitro kinase assays were

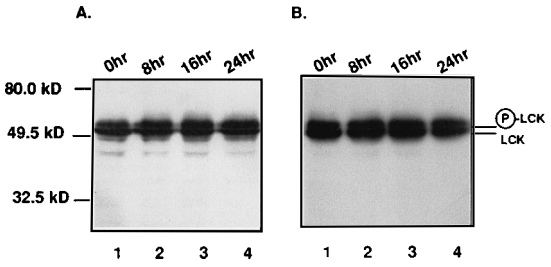

FIG. 7. Total cellular steady-state levels of the tyrosine kinase p56^{lck} are unaltered in myxoma virus infected cells. Steady-state levels of p56^{lck} were assessed by Western blotting of total cell lysates with an anti-p56^{lck} antiserum. RL-5 cells were either mock infected (A) or infected with myxoma virus (B) for 0, 8, 16, and 24 h. The unphosphorylated 56-kDa Lck and the 59-kDa phosphorylated Lck species are indicated.

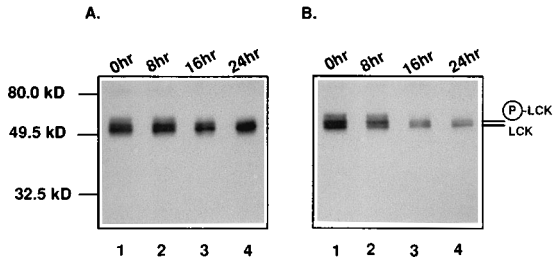

FIG. 8. The CD4-p56^{lck} complex is altered in myxoma virus-infected RL-5 cells. CD4 was immunoprecipitated from cell lysates of mock-infected (A) and myxoma virus-infected (B) RL-5 cells at 0, 8, 16, and 24 h, using monoclonal antibody Ken 4, and then subjected to Western blot analysis with an antiserum specific for the tyrosine kinase p56^{lck}. The unphosphorylated 56-kDa Lck and the 59-kDa phosphorylated Lck species are indicated.

performed, the kinase activity of p56^{lck} associated with CD4 was also found to markedly decline during myxoma virus infection (Fig. 9A and B). However, when total p56^{lck} activity was monitored by immunoprecipitation with an anti-p56^{lck} antiserum followed by an in vitro kinase assay, no detectable loss of total p56^{lck} kinase activity was observed (Fig. 9C). In fact, 24 h postinfection, the total cellular p56^{lck} kinase activity remained essentially unchanged, but the p56^{lck} detectable in CD4 immunoprecipitates possessed virtually no kinase activity (Fig. 9B, lane 4). We conclude that, similar to PMA treatment or transfection with HIV Nef, infection with myxoma virus induces the dissociation of p56^{lck} from CD4 prior to degradation of CD4 in lysosomes.

DISCUSSION

The specific downregulation of cell surface CD4 in response to viral infection of CD4$^+$ T lymphocytes has been described to date only in cells infected with HIV, simian immunodeficiency virus, and human herpesvirus 7. CD4 downregulation has been attributed to the expression of the *nef* gene in the genomes of HIV and simian immunodeficiency virus and the *vpu* gene of HIV (12, 42, 54), indicating that some lymphotropic viruses have been found to encode specific proteins that induce the downregulation of cell surface CD4. CD4 can also be modulated in vitro by treatment of cells with PMA or G_{M1} (16, 17, 41). In this communication, we report that CD4 is specifically downregulated from the surface of RL-5 (rabbit CD4$^+$ T-lymphoma) cells infected with myxoma virus, a poxvirus and the agent of myxomatosis in rabbits.

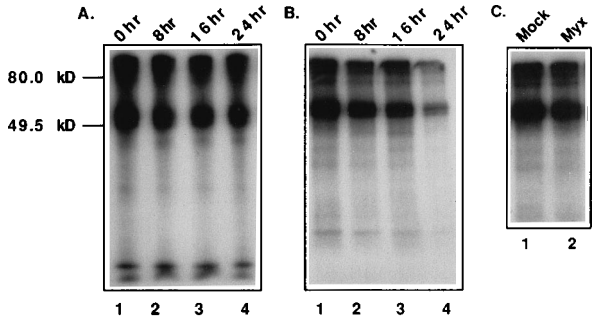

FIG. 9. p56^{lck} kinase activity is progressively lost in CD4 immunoprecipitates from myxoma virus-infected RL-5 cells. RL-5 cell lysates were immunoprecipitated with monoclonal antibody Ken 4, and in vitro protein kinase assays performed on mock-infected (A) or myxoma virus-infected (B) RL-5 cells at 0, 8, 16, and 24 h. RL-5 cell lysates were immunoprecipitated with an anti-p56^{lck} antiserum, and in vitro protein kinase assays were performed on mock-infected and myxoma virus-infected lysates 24 h postinfection (C).

Since the mechanism of PMA-induced CD4 modulation is known to occur via the activation of protein kinase C, we examined whether myxoma virus could exploit the same mechanism. Our results indicate that myxoma virus downregulation of CD4 is not dependent on protein kinase C because PMA-induced but not myxoma-specific modulation of CD4 could be inhibited by the presence of staurosporine, an inhibitor of protein kinase C. The kinetics of CD4 downregulation also varied significantly between PMA-treated RL-5 cells and myxoma virus-infected RL-5 cells. Most notable, however, was the observation that RL-5 cells continuously treated with PMA (24 to 72 h) showed a progressive recovery in surface levels of CD4 to near normal levels as a result of the downregulation of protein kinase C (55). In addition, PMA-pretreated RL-5 cells were no longer susceptible to CD4 downregulation by infection with myxoma virus, suggesting that chronic PMA treatment must have pleiotropic effects since the staurosporine experiments indicated that the activation of protein kinase C played no role in the downregulation of CD4 by myxoma virus. One possibility is that cell surface CD4 no longer undergoes normal turnover from the membrane in these PMA-pretreated cells. PMA pretreatment, however, had no effect on surface CD4 downregulation by brefeldin A, indicating that the normal membrane turnover machinery for CD4 is not affected by chronic exposure to PMA. Myxoma virus-induced CD4 downregulation, however, does not occur in these PMA-pretreated RL-5 cells, suggesting that the downregulation of CD4 by myxoma virus is not due to a virus block of nascent CD4 egress to the cell membrane but rather is due to a direct perturbation of surface CD4 turnover by the virus. The loss of CD4 modulation by myxoma virus in PMA-pretreated RL-5 cells also indicates that it is unlikely that the downregulation of CD4 mediated by myxoma virus is simply due to the masking of the CD4 epitope by a viral protein.

To further substantiate the argument that myxoma virus does not alter the egress of nascent CD4 from the endoplasmic reticulum, which would be predicted to cause accumulation of CD4 with in the endoplasmic reticulum, brefeldin A was used to interfere with transport of CD4 from the endoplasmic reticulum and cause the accumulation of trapped CD4 in this organelle (8, 25). Treatment of uninfected RL-5 cells with brefeldin A results in the downregulation of cell surface CD4 comparable to that observed with infection by myxoma virus. Immunofluorescence data for permeabilized cells, however, indicated a CD4 localization pattern for RL-5 cells treated with brefeldin A very different from that for cells infected with myxoma virus. Although RL-5 cells treated with brefeldin A were rapidly depleted of cell surface CD4, intracellular CD4 was found to accumulate in the endoplasmic reticulum throughout the extranuclear region in permeabilized cells. In contrast, comparable cytoplasmic CD4 fluorescence was not detected in cells infected with myxoma virus. Moreover, when cells were permeabilized and analyzed by flow cytometry for total cellular levels of CD4, it was obvious that RL-5 cells infected with myxoma virus contained substantially lower levels of total cellular CD4 than did cells treated with brefeldin A, which actually demonstrated an increase in total CD4. This reduction in steady-state levels of CD4 in myxoma virus-infected cells could be completely inhibited by NH_4Cl, an inhibitor of lysosomal degradation, indicating that the decrease in steady-state levels detected during myxoma virus infection is due to the physical degradation of CD4 in lysosomal vesicles.

It has been suggested that the short cytoplasmic tail of CD4 is essential for the endocytosis induced by PMA, G_{M1}, and Nef

(1, 41, 45). Nef-induced targeting of CD4 to lysosomal vesicles requires the presence of a dileucine motif within the cytoplasmic tail of CD4 (1), and this motif is also necessary for phorbol ester-induced CD4 downregulation (45). In addition, a dileucine motif has also been implicated in the endocytosis and lysosomal degradation of CD3, suggesting that this motif acts as a signal for endocytosis (24). This motif may be normally masked, but upon phosphorylation of the cytoplasmic tail of CD4 by protein kinase C or interaction with Nef, this motif becomes more accessible to the endocytic machinery. Our data are consistent with the model that upon myxoma virus infection, this dileucine motif of CD4 becomes unmasked, for example by the interaction with an early viral gene product, resulting in the accelerated endocytosis and degradation of CD4. As indicated, we have ruled out the phosphorylation of CD4 by protein kinase C as a possible mechanism in myxoma virus-infected cells, but experiments to assess the possibility that myxoma proteins directly interact with CD4 are in progress.

The cytoplasmic tail of CD4 is also important for association with the tyrosine kinase $p56^{lck}$. Although infection by myxoma virus reduces total cellular levels of CD4, no change in the steady-state level or the associated kinase activity of $p56^{lck}$ was detected during infection, suggesting that prior to the degradation of CD4 in lysosomal vesicles, $p56^{lck}$ must dissociate from CD4. A similar observation was made when cells were treated with PMA (36) or transfected with Nef (1). Recent studies have suggested that association of $p56^{lck}$ with CD4 serves a regulatory role for the activity of the kinase (14). Thus, when CD4 becomes degraded, the liberation of active $p56^{lck}$ kinase may result in enhanced T-cell responsiveness. One potential function of this stimulation would be to increase signal transduction and therefore raise the level of virus production in infected T cells (6, 26). The fact that myxoma virus downregulation of CD4 from the surface of infected cells also results in the liberation of $p56^{lck}$ suggests that this latter effect may play an important positive role in viral lymphotropisms, perhaps through a similar mechanism.

In conclusion, we have determined that during infection of a rabbit $CD4^+$ T-lymphoma cell line with myxoma virus, cell surface levels of CD4 are rapidly reduced. The significance of these observations and their relevance to viral virulence in vivo are currently being investigated. The ability of immunosuppressive viruses to interact directly with lymphocytes is an important determinant of virus-host interactions. In the case of myxoma virus, the natural evolutionary host is the South American rabbit (*Sylvilagus brasiliensis*), in which it forms a benign symbiotic relationship associated with little or no immune recognition but relatively minor pathogenicity. However, when myxoma virus infects the European rabbit (*O. cuniculus*), it causes a rapidly disseminating lethal infection associated with severe lymphocyte dysfunction and secondary supervening bacterial infections (10, 28, 48). Therefore, the study of the mechanisms by which viruses interact with lymphocytes involved in the mediation of cellular immunity is critical to our understanding of why viral infections can in some circumstances lead to silent chronic infections and in others result in overt viral pathogenesis and disease. In addition, the study of this phenomenon should also reveal important information concerning the function and biology of CD4 during the response to pathogenic challenge in general.

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