

Presentation of CMV Immediate-Early Antigen to Cytolytic T Lymphocytes Is Selectively Prevented by Viral Genes Expressed in the Early Phase

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Summary

The regulation of antigen processing and presentation to MHC class I-restricted cytolytic T lymphocytes was studied in cells infected with murine cytomegalovirus. Recognition by cytolytic T lymphocytes of the phosphoprotein pp89, the immunodominant viral antigen expressed in the immediate-early phase of infection, was selectively prevented during the subsequent expression of viral early genes. The surface expression of MHC class I glycoproteins and their capacity to present externally added pp89-derived antigenic peptides were not affected. Because recognition of several other antigens occurred during the early phase, a general failure in processing and presentation was excluded. Since neither rate of synthesis, amount, stability, nor nuclear transport of pp89 was modified, the failure in recognition indicates a selective interference with pp89 antigen processing and presentation.

Introduction

Recognition of antigens by T lymphocytes requires their presentation by glycoproteins encoded in the major histocompatibility complex (MHC), a phenomenon referred to as MHC restriction (Schwartz, 1985). It is assumed that, after synthesis in infected cells, viral protein antigens have to be processed to yield peptides that, after binding to the MHC-encoded glycoproteins expressed in those cells, are presented at the cell surface to the T cell receptor. Processing is a poorly understood event that appears to involve limited proteolytic degradation (Shimonkevitz et al., 1983; Townsend et al., 1985). Antigens presented by MHC class II molecules are processed in low-pH endosomes, whereas antigens synthesized within the cell and presented by MHC class I molecules seem to be processed at a so far unknown, yet different, cytoplasmic site (Braciale et al., 1987; Germain, 1986, 1988). The location at which the physiological association of antigens with MHC class I molecules takes place is not known either, but it has been shown that externally added antigenic peptides can be presented at the cell surface (Townsend et al., 1986).

Viruses can interfere with antigen presentation by affecting the surface expression of cell membrane proteins required for the recognition of infected cells by cytolytic T lymphocytes (CTLs). For several viruses (Gooding, 1982; Bernardis et al., 1983), including herpesviruses (Jennings

et al., 1985; Masucci et al., 1987), defective antigen recognition has been associated with decreased surface levels of MHC class I glycoproteins. For adenoviruses, there is evidence that the underlying mechanism is an active interference with class I mRNA processing (Vaessen et al., 1987) or with glycosylation (Burgert and Kvist, 1985). A correlation of these findings with tumorigenicity and evasion of adenoviruses and SV40 from the immune response (Gooding, 1982; Bernardis et al., 1983; Tanaka et al., 1985) is a matter of controversy (Haddada et al., 1988). For Epstein-Barr virus, evasion from host-cellular immunity has been suggested to result from the establishment of a latent infection in cells that, owing to very low surface levels of accessory adhesion molecules, constitute poor targets for antiviral CTLs (Gregory et al., 1988). As expected, and as actually shown for class I molecules (Yewdell et al., 1988), the reduction in the surface levels of cell membrane proteins required for antigen presentation and target cell recognition by CTLs indiscriminately prevents efficient recognition of any antigen.

Previous observations made with murine cytomegalovirus (MCMV) infection suggest the existence of an interference of viral gene products with antigen processing and presentation that is not mediated through a modulation in the expression of MHC class I molecules. In permissive cells, cytomegalovirus (CMV) gene expression is regulated in a cascade fashion characteristic of herpesviruses (Stinski, 1983). Proteins expressed in the immediate-early (IE) phase of infection, such as the nonstructural regulatory phosphoprotein pp89, which is encoded by MCMV gene *ie1* (Keil et al., 1985, 1987; Koszinowski et al., 1986), are required for the subsequent expression of early-phase genes, which, after onset of DNA replication, are followed by late-phase genes. IE proteins also represent antigens recognized by the majority of antiviral CTLs (Reddehase and Koszinowski, 1984; Reddehase et al., 1984; Koszinowski et al., 1987; Borysiewicz et al., 1988). Vaccination of mice with the recombinant vaccinia virus MCMV-*ie1*-VAC, which expresses pp89 as the only MCMV gene product (Volkmer et al., 1987), sensitizes CD8⁺ T lymphocytes that protect mice against lethal challenge with MCMV (Jonjic et al., 1988). The L^d-restricted CTL clone IE1 lyses cells that selectively synthesize IE proteins (Reddehase et al., 1986a) and is specific for a peptide of pp89 (Del Val et al., 1988; Reddehase et al., 1989). Clone IE1 also recognizes pp89 when it is synthesized again during the late phase of infection, whereas, even though pp89 is present throughout the virus replication cycle, no recognition takes place during the early phase (Reddehase et al., 1986b). Because these experiments did not reveal any alteration in MHC class I expression, an as yet unknown mechanism that prevents pp89 antigen presentation to CTLs must be postulated to operate during the early phase.

In this article we describe that MCMV gene products interfere in a very selective way with the posttranslational steps of processing and presentation of pp89 for recogni-

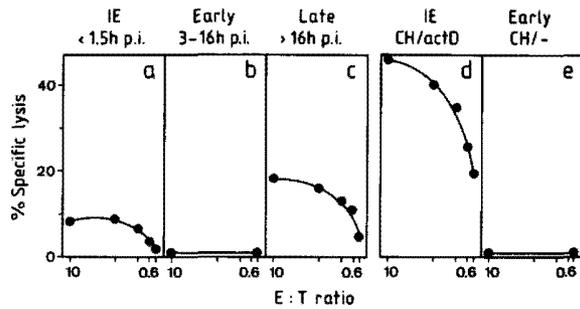


Figure 1. Stage-Specific Recognition of MCMV-Infected Cells by the pp89-Specific CTL Clone IE1

Recognition by clone IE1 of ^{51}Cr -labeled MCMV-infected MEF targets was tested in a cytolytic assay using graded numbers of effector CTLs. Infection conditions were as follows: (a) after 1.5 hr of infection, actinomycin D (act D) was added to prevent early gene expression and thus to achieve nonenhanced, selective IE protein synthesis; (b) cells were assayed at 3, 5, 8, 12, or 16 hr p.i. during the early phase of infection (summary of data); (c) cells were assayed at 24 hr p.i. during the late phase of infection; (d) infection was performed in the presence of cycloheximide (CH), which was replaced after 3 hr by act D to achieve enhanced and selective IE protein synthesis; (e) infection was performed in the presence of CH, which was removed after 3 hr, resulting in enhanced IE gene expression concomitant with early gene expression. In (a), (d), and (e), the cytolytic assay started at 7 hr p.i. E:T ratio, ratio of IE1 effector cells to target cells.

tion by MHC class I-restricted CTLs. Unlike the inhibitory mechanisms mentioned above, the surface levels of MHC class I molecules, as well as their capacity to present both externally added and other endogenously synthesized viral antigens, remain unaffected. The data demonstrate that the three conditions regarded as essential and sufficient for target cell formation, namely, the presence of a sufficient amount of an antigenic protein, the functional integrity of general processing mechanisms, and the presence of MHC molecules capable of presenting antigens, do not guarantee the recognition of that antigen by CTLs. These findings thus reveal the existence of hitherto unknown mechanisms regulating antigen processing and presentation.

Results

Transition to the Early Phase of MCMV Gene Expression Prevents Presentation of the IE Protein pp89 to CTL Clone IE1

The kinetics of the expression of the MCMV IE protein pp89 in permissively infected mouse embryo fibroblasts (MEFs) have been determined previously (Keil et al., 1984, 1985; Reddehase et al., 1986b). Gene *ie1* encoding pp89 (Keil et al., 1987) is transcribed during the IE phase of viral gene expression, becomes silent during the early phase, and is transcribed again during the late phase. The synthesis of pp89 and the recognition of infected MEFs by the L^d-restricted pp89-specific CTL clone IE1 also follow essentially these kinetics (Figures 1a-1c; Reddehase et al., 1986b). Specifically, infected cells are not recognized when virus gene expression proceeds into the early phase. This result was obtained when cells were arrested

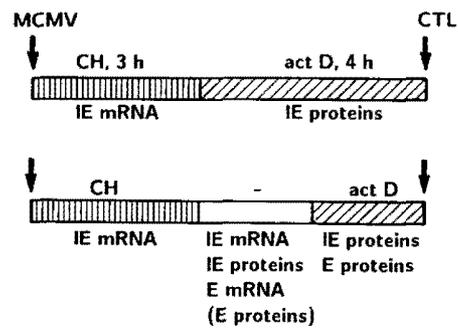


Figure 2. Experimental Design

(Top) Infection of cells in the presence of the protein synthesis inhibitor CH results in enhanced transcription of IE genes, including gene *ie1* encoding pp89. After replacement of CH by the inhibitor of transcription act D, IE mRNA is translated, leading to enhanced and selective IE protein synthesis and to enhanced recognition of IE antigens by CTLs 4 hr later (Reddehase et al., 1984).

(Bottom) If act D is not added immediately after removing CH, but at later time points, the synthesized IE proteins activate the transcription of early (E) genes. The duration of E gene transcription is defined by the time at which act D is added. Translation of early mRNAs leads to the synthesis of E proteins. With this protocol, conditions of limited E gene expression after enhanced IE gene expression are achieved.

in the early phase by performing the infection in the presence of phosphonoacetic acid (PAA) and tested 24 hr after infection (Reddehase et al., 1986b), and also when they were tested at different times during the early phase (data for 3, 5, 8, 12, and 16 hr postinfection [p.i.] are jointly shown in Figure 1b). The fact that infected cells were no longer recognized by clone IE1 after only 3 hr of permissive infection suggested that viral gene products expressed during the early phase negatively affected the presentation of pp89.

Because the rate of pp89 synthesis is low in the physiological IE phase, infection was performed in the presence of cycloheximide (CH) to prevent translation, which results in an accumulation of IE transcripts. Enhanced and selective synthesis of IE proteins, the most abundant of which is pp89, is then accomplished by replacing CH by actinomycin D (act D), which causes release from the translation block while preventing the transcription of early genes (see experimental design in Figure 2, top; Reddehase et al., 1986b). Under these conditions the synthesis of pp89 continues for at least 8 hr without detectable decline (Keil et al., 1985), and the recognition of the cells by clone IE1 is markedly increased (Figure 1d; Reddehase et al., 1984). If, however, the transition to early gene expression is not blocked by act D after the removal of CH, the synthesis of pp89 from the accumulated IE transcripts declines with time (Keil et al., 1985) and the cells no longer constitute targets for clone IE1 (Figure 1e). These results demonstrate the following: first, transition to the early phase, which shuts off IE translation, also prevents the expression of the antigenic determinant of pp89 defined by clone IE1; second, this prevention is posttranscriptional; and third, it cannot be overcome by enhancing the transcription of IE genes.

Since pp89 can be detected with specific antibodies

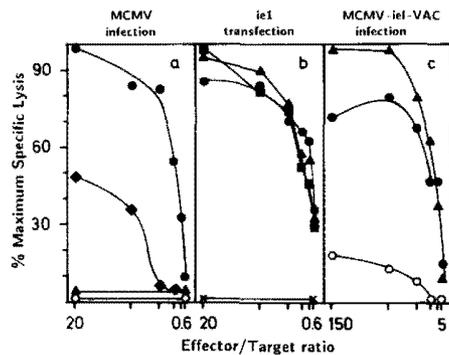


Figure 3. Transition to the MCMV Early Phase Prevents Presentation of pp89 to CTL Clone IE1

Recognition of pp89 was tested in a cytolytic assay using the pp89-specific CTL clone IE1 (a and b) or MCMV-specific polyclonal CTLs (c) as effector cells. The target cells were prepared as follows: (a) MEFs were infected with MCMV in the presence of CH for 3 hr. After removal of CH, act D was added either immediately (●), 20 min (◆), or 45 min (▲) later, as explained in Figure 2. Uninfected inhibitor-treated cells were used as a control (○). (b) L/ie1-L^d transfectants, expressing pp89 constitutively, were treated for 3 hr with CH. After removal of CH, act D was added immediately (●) or after 45 min (▲). Controls included L/ie1-L^d cells not incubated with metabolic inhibitors (■) and L/L^d cells that do not contain viral genes (x). (c) MEFs were infected with MCMV-*ie1*-VAC in the presence of CH. Following removal of CH 3 hr later, act D was added immediately (●) or after 45 min (▲). As a control, cells infected with wild-type vaccinia virus and treated with the metabolic inhibitors were used (○). In all cases, the cytolytic assay started 7 hr after addition of CH to target cells. Mean values of specific lysis are normalized for the maximum lysis obtained within each group (40% for [a], 30% for [b], and 24% for [c]).

throughout the early phase, the data were thus far consistent with the interpretation that recognition of the antigen by clone IE1 correlated with the synthesis of pp89 rather than with its presence (Reddehase et al., 1986b). The early phase lasts about 16 hr, during which transcription from the entire 235 kb genome, involving a high number of genes, is detected (Keil et al., 1984). None of these genes is defined as yet. Viral functions that cause the shutoff of pp89 synthesis and those that prevent the presentation of the IE1 determinant may be specified by the same gene or by different genes. In addition to early gene functions, an autoregulatory role of pp89 in antigen presentation has to be considered.

To dissect these events, the experimental regimen explained in Figure 2 (bottom) was employed: by adding act D not immediately after removal of CH but at defined time points later, a variable period permissive for early gene transcription was set. With this approach it was determined that an interval of 20 min of early gene transcription was sufficient to diminish the presentation of the pp89 antigen, and that an interval of 45 min completely interfered with it (Figure 3a). It has been demonstrated that pp89 can activate the transcription of cellular genes such as *c-fos* (Schickedanz et al., 1988). During the interval permissive for early gene transcription, the pp89 synthesized in the absence of act D could activate the transcription not only of viral early genes but also of cellular genes. Therefore, the possibility had to be considered that the inhibitory effect on pp89 antigen presentation was caused by cellular

proteins induced by pp89. The same schedule of treatment with the metabolic inhibitors of the transfectant cell line L/ie1-L^d, which expresses pp89 constitutively, as well as of MEFs infected with the recombinant virus MCMV-*ie1*-VAC, which expresses pp89 only upon infection, and both of which lack MCMV early genes, did not prevent or reduce lysis by CTLs (Figures 3b and 3c). These controls thus excluded the possibility that cellular proteins induced by pp89 itself or by related proteins translated from splicing derivatives in transcription unit *ie1* (Koszinowski et al., 1987) were responsible for the failure in pp89 antigen expression. In conclusion, the function that interferes with processing and presentation of the pp89 antigen is based on viral genes expressed in the first 45 min after transition to the early phase.

Inhibition of pp89 Antigen Presentation and of pp89 Synthesis Are Separate Events

pp89 is not a structural component of the virus particle; therefore, its synthesis in infected cells is required for recognition by pp89-specific CTLs. It was an open question whether the genes expressed during the first 45 min of the early phase, which abrogate pp89 antigen presentation, also caused the shutoff of pp89 synthesis or whether this effect was specified by genes expressed at later times during the prolonged early phase. To this end, protein synthesis after periods of 20 and 45 min of early gene transcription was analyzed by pulse-labeling and immunoprecipitation at 5, 7, and 9 hr p.i., i.e., after 2, 4, and 6 hr of translation. No difference was found at any time tested in the rate of synthesis of pp89 between cells expressing only IE genes (Figure 4A, lane b) and those that also express early genes (lanes c and d). Shown are only the data for 9 hr p.i., the time point that coincides with the end of the cytolytic assay. Posttranslational conversion of pp89 to pp76, which is mediated by cellular proteases (Keil et al., 1985), was not altered either. The only difference detected after immunoprecipitation between cells lysed by CTL clone IE1 (lane b) and those not recognized was the additional presence of early proteins (marked with E in Figure 4A, lanes c and d). These results indicated that the genes that negatively affect pp89 synthesis are not transcribed during the first 45 min of the early phase. It is worth noting that in spite of a significantly lower rate of pp89 synthesis in L/ie1-L^d transfectants (lane e), these cells are efficiently recognized by CTLs. The experimental dissection of the interference with pp89 antigen presentation and the down-regulation of pp89 synthesis proved that these are separate events operating at different times during the early phase. In addition, it was concluded from these data that the inhibitory effect on pp89 antigen presentation operates posttranslationally.

Expression of the First MCMV Early Genes Does Not Affect Amount, Stability, or Nuclear Transport of pp89

Deficient presentation to CTLs of stable influenza virus proteins expressed in recombinant vaccinia virus-infected cells has been shown recently to be overcome by constructing modified proteins with a reduced half-life

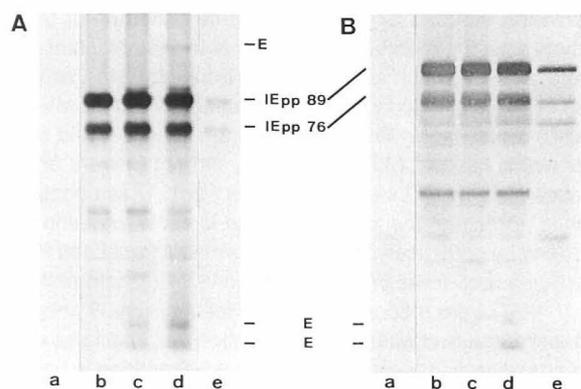


Figure 4. Expression of the First MCMV Early Genes Does Not Affect the Rate of pp89 Synthesis, the Posttranslational Conversion to pp76, or the Amount of pp89

(A) The rate of synthesis of pp89 was measured by metabolic labeling of infected cells with [³⁵S]methionine for 20 min starting at 9 hr p.i., followed by immunoprecipitation of cell lysates with anti-MCMV serum and polyacrylamide gel electrophoresis. Positions of the IE proteins pp89 and pp76 and of early proteins (E) are indicated. Lane a, uninfected MEFs. Lane b, MEFs infected with MCMV under enhanced and selective IE conditions (CH and act D treatment). Lane c, MEFs that were infected in the presence of CH were incubated for 20 min in the absence of metabolic inhibitors before act D was added. Lane d, same as lane c but with a 45 min incubation without inhibitors. Lane e, L/ie1-L^d transfectants.

(B) Amount of pp89 detected by Western blot of the cell lysates described in (A), lanes a-e, using the same antiserum as in (A).

(Townsend et al., 1988). This further strengthened the idea that processing of an antigen involves proteolytic degradation (Townsend et al., 1985, 1986). Following this reasoning, an increased stability of pp89 during the early phase could offer an explanation for the observed lack of recognition by CTLs. On the other hand, a different cleavage pattern of pp89 during the early phase might lead to the destruction of the epitope recognized by CTLs, and this would also result in a failure of antigen presentation. Therefore, the stability of pp89 during the IE and early phases was compared.

As a first step, the amount of pp89 synthesized after release from the CH block and present in infected cells at 5, 7, and 9 hr p.i. was measured by Western blot analysis in cells expressing only IE proteins and in cells also expressing early proteins. As shown in Figure 4B for 9 hr p.i., the amount of IE proteins was the same regardless of whether early genes were expressed (compare lane b with lanes c and d) and was clearly higher in infected (lanes b-d) than in transfectants (lane e). As a second step, the stability of pp89 under IE and early conditions was measured by pulse-labeling with [³⁵S]methionine at 3 hr p.i. followed by a period of chase in the absence of radioactive precursor (Figure 5). With the aim of identifying the relevant protein fragments containing the IE1 epitope, an antiserum raised against the 19 amino acid peptide of pp89 that contains this epitope (Del Val et al., 1988) was used for immunoprecipitation. Even though efficiently presented to CTLs, pp89 synthesized in the IE phase represents a very stable protein, since most of the labeled

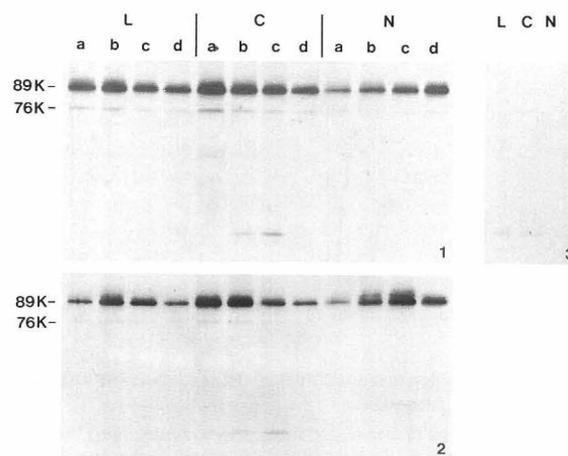


Figure 5. Stability and Nuclear Transport of pp89 Are Not Affected by MCMV Early Gene Expression

MEFs were infected in the presence of CH. After removal of CH at 3 hr p.i., act D was added either immediately (1) or after 45 min (2). Uninfected cells treated as in (1) were used as a control (3). Cells were labeled for 30 min with [³⁵S]methionine immediately after CH removal and then washed with an excess of unlabeled methionine. Whole-cell lysates (L) or cytoplasmic (C) and nuclear (N) fractions were prepared immediately (lanes a) and 3, 6, and 12 hr later (lanes b, c, and d, respectively). Immunoprecipitation was performed with a rabbit antiserum against peptide P(161-179), which contains the epitope recognized by CTL clone IE1 in pp89. Samples were analyzed by polyacrylamide gel electrophoresis. Positions of pp89 and pp76 are indicated.

pp89 was still detectable in the cell lysates even after 12 hr of chase (Figure 5, 1L, lanes a-d). The same stability was observed for pp89 when transcription was allowed for 45 min (Figure 5, 2L, lanes a-d). Low amounts of proteins smaller than pp89 that contained the IE1 epitope were detected. These might represent either degradation products or proteins related to pp89 and arising by differential splicing from transcription unit ie1, which encodes pp89 (Keil et al., 1984, 1985). The protein pattern was the same under IE or early conditions, and was also identical with the one detected when, instead of the epitope-specific antiserum, antiserum directed against the whole protein was used (data not shown), thus giving no indication of a selective destruction of the IE1 epitope. However, relevant fragments of small size or very low abundance may have passed undetected. The same results were obtained when radioactive labeling was performed at 6 hr p.i. (data not shown). Altogether, these findings demonstrated that MCMV early gene expression was not associated with an altered stability of pp89.

After synthesis, pp89 migrates into the nucleus, where it activates transcription (Koszinowski et al., 1986). From a deletion mutant of pp89 that lacks the nuclear transport signal and is therefore confined to the cytoplasm, it is known that migration of pp89 into the nucleus is not required for recognition by CTLs (H. Volkmer and M. D. V., unpublished data). This finding is in accordance with the widely accepted idea (Germain, 1988) that processing of endogenously synthesized antigens takes place in a cytoplasmic compartment. On the other hand, enhanced

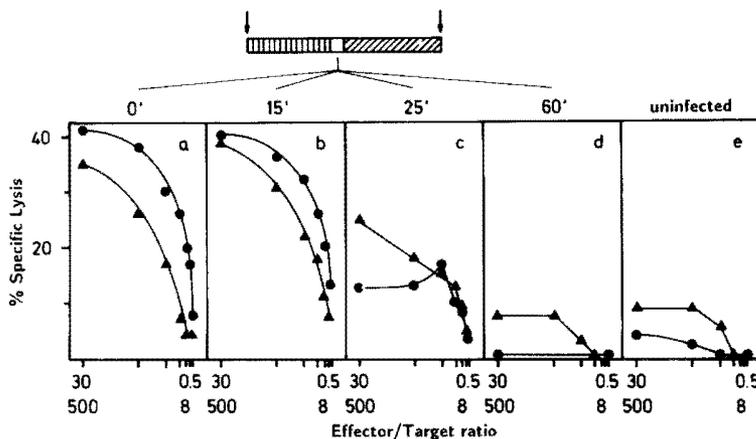


Figure 6. Transition to the Early Phase Abrogates Recognition of pp89 by Specific BALB/c Polyclonal CTLs

Recognition of pp89 was tested in a cytolytic assay using as effector cells the CTL clone IE1 (effector to target cell ratios from 30:1 to 0.5:1) (●) or pp89-specific polyclonal CTLs (effector to target cell ratios from 500:1 to 8:1) (▲). For the preparation of target cells, MEFs were infected with MCMV in the presence of CH, which was removed after 3 hr. Act D was then added either immediately (a) or after 15 (b), 25 (c), or 60 (d) min, as explained in Figure 2. The assay was performed at 7 hr p.i. Uninfected cells treated as in (a) were used for control (e).

transport of pp89 into the nucleus during the early phase might result in a withdrawal of pp89 from cytoplasmic antigen-processing pathways. The rate of nuclear transport of pp89 was therefore measured by tracing the labeled protein in the nuclear and cytoplasmic fractions of infected cells. With increasing periods of chase, the amount of labeled pp89 gradually decreased in the cytoplasm and was localized to the nuclear fraction (Figure 5, C and N). No influence of early gene expression on the rate of nuclear transport could be detected (Figure 5, compare 1C and 1N with 2C and 2N), thus excluding an enhanced nuclear trapping of pp89 as an explanation for the failure in pp89 antigen presentation during the early phase.

In summary, neither a general enhancement of pp89 stability, nor a detectable selective loss of the epitope, nor a different nuclear/cytoplasmic compartmentalization of the protein can account for the lack of pp89 antigen presentation to CTLs in the early phase.

The Fate of the IE1 Epitope Reflects the Recognition of pp89 by BALB/c Polyclonal CTLs

The data presented so far showed that the particular epitope of pp89 defined by clone IE1 was no longer detectable when viral gene expression proceeded into the early phase. To study whether the fate of this epitope was representative of the recognition of all pp89 epitopes, pp89 presentation to BALB/c pp89-specific polyclonal CTLs was analyzed. By using MCMV-*iel*-VAC for in vivo sensitization, pp89-specific polyclonal CTLs were generated that recognized cells arrested in the IE phase of MCMV infection (Figure 6a). Transition into the early phase prevented recognition of pp89 by these polyclonal CTLs with kinetics indistinguishable from those observed for clone IE1 (Figures 6b–6d). In addition, it was found that pp89-specific polyclonal CTLs did not lyse infected fibroblasts of the mutant strain BALB/c-H-2^{dm2}, in which the L^d gene is deleted (Table 1B). These results demonstrated that recognition of pp89 by BALB/c polyclonal CTLs was entirely restricted through L^d, the same class I molecule that serves as the antigen-presenting molecule for clone IE1.

In conclusion, the epitope recognized by clone IE1 truly reflected the recognition pattern of pp89 by polyclonal BALB/c CTLs.

Surface Expression and Functional Integrity of the MHC Class I Molecule L^d during the Early Phase

For recognition by CTLs of viral antigens synthesized in an infected cell, antigenic peptides have to be presented by MHC-encoded class I glycoproteins. Interference with the cell surface expression of class I molecules can diminish or even abolish recognition by CTLs. Cytofluorometric determination of the surface expression of the MHC class I molecules K^d, D^d, and L^d revealed no differences between cells that selectively synthesized IE proteins (Figure 7A, top panel), cells with restricted expression of early genes (center panel), and uninfected cells (bottom panel). When L^d served as a target antigen in a cytolytic assay using the L^d-specific CTL clone B6αL^d, no differences in target cell susceptibility to lysis were seen either (Figure 7B). It was thus concluded that inhibition of pp89 presentation was not associated with alterations in the surface expression of class I molecules.

Yet, these results did not exclude an alteration in the function of the class I molecules to serve as antigen-presenting molecules. It has been proposed that incubation of target cells with peptides circumvents the intracellular steps of processing, since it leads to target cell recognition by CTLs (Townsend et al., 1986). The synthetic nonapeptide YPHFMPTNL (one-letter code), which is deduced from the pp89 sequence, represents the optimal antigenic peptide for CTL clone IE1 (Reddehase et al., 1989). Incubation with this nonapeptide of cells that expressed MCMV early genes and did not present endogenously synthesized pp89 revealed that these cells were still able to present the externally added nonapeptide (Figure 7C, center panel). Identical results with the suboptimal heptapeptide PHFMPTN confirmed this conclusion (data not shown). Therefore, the block in pp89 presentation was caused neither by an alteration in the cell surface expression of L^d nor by a defect in its function to present antigenic peptides.

Table 1. Differential Presentation of MCMV Antigens to CTLs during the Early Phase of the Viral Replication Cycle

	Genes Expressed in Target Cells		CTLs [‡]				
	MHC*	MCMV [†]	Clone IE1	Polyclonal Anti-pp89	Clone E1	Clone E2	Polyclonal Anti-MCMV
A.	K ^d D ^d L ^d	IE phase	40 [§]	59	0	0	48
	K ^d D ^d L ^d	IE + E phase	1	7	<u>60</u>	<u>40</u>	74
	K ^d D ^d L ^d	—	0	0	0	0	3
B.	K ^d D ^d	IE phase	0	4	1	0	20
	K ^d D ^d	IE + E phase	0	0	4	0	<u>38</u>
	K ^d D ^d	—	0	0	0	0	5
C.	L ^d	EcoRI F	0	ND	29	0	ND
	L ^d	—	0	ND	7	0	ND
D.	K ^b D ^b	IE phase	0	21	0	ND	25
	K ^b D ^b	IE + E phase	0	9	0	ND	<u>32</u>
	K ^b D ^b	—	0	8	0	ND	8

* Target cells: MEFs from BALB/c mice (A), BALB/c-H-2^{dm2} mice (B), or C57BL/6 mice (D). L/L^d cells were transfected with fragment EcoRI F from MCMV DNA (C).

[†] Infection conditions: IE phase, enhanced and selective IE expression, as described in Figure 2, top. IE + E phase, infection performed in the presence of PAA to prevent late-phase gene expression.

[‡] CTL clones were derived from BALB/c (H-2^d) mice. Polyclonal CTLs were obtained either from BALB/c mice (for A and B) or from C57BL/6 (H-2^b) mice (for D).

[§] Percentages of specific lysis obtained at effector to target cell ratios of 20:1 (for CTL clones) or 100:1 (for polyclonal CTLs).

^{||} Underlined are results that revealed the presentation of viral antigens other than pp89 during the early phase of the MCMV replication cycle. ND, not done.

Selectivity of the Negative Regulation of pp89 Antigen Processing and Presentation

From all these findings it can be concluded that the interference with pp89 antigen expression involved the poorly defined intracellular steps of the antigen-processing and -presentation pathways. To study whether this negative regulation is selective for pp89 or concerns antigen presentation in general, the effect on the presentation of other MCMV antigens expressed concomitantly with pp89 was analyzed (Table 1). To this end, CTLs derived from infected mice were cloned and screened for recognition of cells infected with MCMV in the presence of PAA. Viral gene expression in such cells is restricted to IE and early genes, and, although they are not recognized by CTL clone IE1, they constitute targets for polyclonal MCMV-specific CTLs (Reddehase et al., 1984). With this approach, two new CTL clones were established, E1 and E2, both of which recognized MCMV early antigens since they were able to lyse cells infected in the presence of PAA but did not recognize cells infected under IE conditions (Table 1A). Antigen recognition by both clones was restricted through L^d, since no lysis of infected BALB/c-H-2^{dm2} cells, which lack L^d, was detected (Table 1B). CTL clone E1, but not clone E2, recognized L/L^d cells transfected with the EcoRI F fragment of MCMV DNA (Ebeling et al., 1983; B. Bühler et al., unpublished data), which indicated that these two CTL clones have different antigen specificities (Table 1C). Lysis of infected cells by clones E1 and E2 was not higher during the late phase than during the early phase (not shown). The objection that presentation of E1 and E2 antigens might also have been inhibited during the early phase, although to a lower extent than pp89, can thus be refuted. Presentation of these two antigens could

account for the recognition of early-phase infected cells by polyclonal MCMV-specific CTLs (Table 1A); however, additional viral antigens must be presented during the early phase by the K^d or D^d glycoproteins, because BALB/c-H-2^{dm2} cells infected in the presence of PAA were also recognized by these polyclonal CTLs (Table 1B). It was therefore concluded that, even though presentation of pp89 to BALB/c CTLs was prevented during the early phase, presentation of at least three other viral antigens, two of them L^d restricted, as is pp89 itself, was nevertheless very efficient.

When recognition of MCMV-infected cells by C57BL/6 (H-2^b) pp89-specific polyclonal CTLs was analyzed, it was found that, as was the case in the H-2^d haplotype, presentation of pp89 by H-2^b class I molecules did occur during the IE phase but was prevented during the early phase (Table 1D). Again, the testing of MCMV-specific polyclonal CTLs revealed further viral antigens presented during the early phase by H-2^b class I molecules (Table 1D).

Collectively, the results summarized in Table 1 demonstrate that the inhibitory effect on pp89 antigen presentation is highly selective, because at least two viral early antigens presented by L^d, at least one presented by K^d or D^d, and at least one presented by K^b or D^b were not subject to this inhibition. A general block affecting processing and presentation of all nascent viral antigens in the early phase of MCMV replication was thus excluded.

Inhibition of pp89 Presentation Cannot Be Explained by Antigen Competition for the Presenting Molecule L^d

Competition between different antigens presented by the

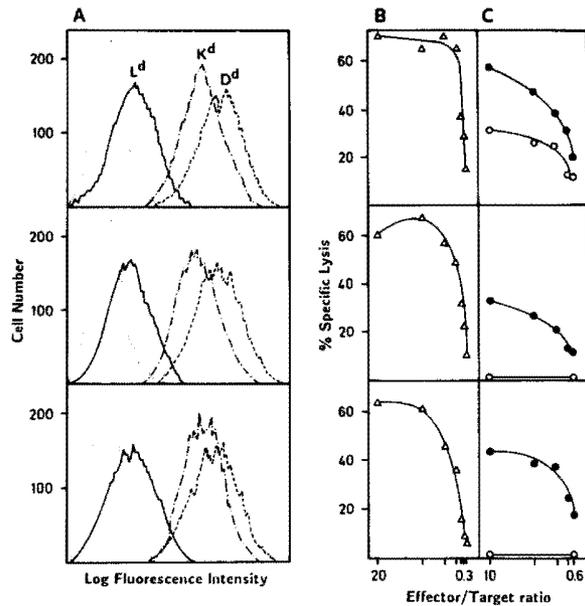


Figure 7. Expression of MCMV Early Genes Does Not Modify the Surface Levels or the Peptide Presentation Function of MHC Class I Molecules

MEFs were infected under enhanced and selective IE conditions (top panels) or under conditions in which early transcription was limited to 2 hr (center panels) as explained in Figure 2. Uninfected MEFs treated with the metabolic inhibitors are shown as controls in the bottom panels.

(A) Surface levels of H-2^d class I molecules were measured by flow cytometry at 7 hr after infection or mock infection on cells labeled with mouse monoclonal antibodies specific for L^d, K^d, and D^d, as indicated, followed by labeling with fluoresceinated anti-mouse antibody. The dotted lines represent background labeling obtained with the fluoresceinated anti-mouse antibody alone.

(B) Recognition of surface L^d molecules on ⁵¹Cr-labeled target cells by the L^d-specific CTL clone B6αL^d.

(C) Presentation of the pp89-derived synthetic peptide YPHFMPNTL after a 1 hr incubation of target cells with 100 μM peptide (●), measured in a cytolytic assay using CTL clone IE1 as effector cells. Control cells not incubated with peptide (○) were also tested.

same MHC class I molecule and recognized by CTLs can be demonstrated with synthetic peptides (Maryanski et al., 1987). In particular, it has been recently shown that peptides derived from the tum⁻ antigen P91A (De Plaen et al., 1988) and pp89-derived peptides, the only two antigenic sequences presented by the L^d glycoprotein that have been characterized so far, can mutually compete for L^d (Lurquin et al., 1989). During the early phase of infection, when the IE protein pp89 is not presented by L^d, other antigens are recognized by the L^d-restricted CTL clones E1 and E2. Competition for the L^d molecule effected by these early antigens could possibly prevent presentation of pp89 during the early phase.

To test this hypothesis, the kinetics of pp89 and E1 antigen expression were analyzed during the transition from the IE to the early phase. The duration of early gene transcription was limited by the addition of act D at different times after removal of CH, in order to detect the time at which the early gene encoding the E1 antigen is ex-

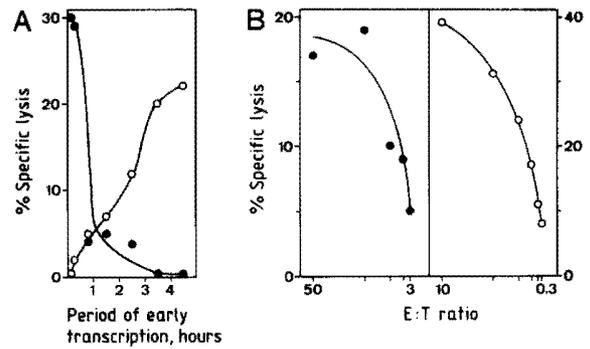


Figure 8. Presentation by L^d of the E1 Epitope and of pp89 Can Occur Simultaneously

(A) Kinetics of antigen expression in the transition from the IE to the early phase. MEFs were infected with MCMV in the presence of CH, which was washed out 3 hr later. The time points at which act D was added define the duration of early gene transcription. In all cases, 8 hr for protein synthesis, antigen processing, and antigen presentation was allowed before the target cells were assayed for recognition by pp89-specific L^d-restricted polyclonal CTLs at an effector to target cell ratio (E:T ratio) of 25:1 (●), and by the early-specific L^d-restricted CTL clone E1 at an E:T ratio of 5:1 (○).

(B) Antigen expression in the late phase. MEFs were infected with MCMV at a multiplicity of 2 in the absence of metabolic inhibitors, and tested at 20 hr p.i. with pp89-specific polyclonal CTLs (●) and with CTL clone E1 (○).

pressed. The results shown in Figure 8A indicate that 2–4 hr of early gene transcription was required eventually to obtain an amount of E1 antigen sufficient for detection by CTL clone E1. This period of early gene transcription was longer than the period required to prevent pp89 antigen presentation, thus suggesting that E1 peptide competition for L^d was unlikely. It could be argued, however, that the amount of E1 peptide required for competition with pp89 is lower than the amount required for target recognition by CTL clone E1. This possibility was excluded by the finding that simultaneous presentation of pp89 and E1 took place in the late phase of the virus replication cycle (Figure 8B). Recognition of infected cells by CTL clone E2 also occurred during the late phase (data not shown). Thus, sufficient amounts of E1 and E2 peptides were present in the late phase to produce a target; nevertheless, they did not exert an inhibitory effect on the presentation of pp89-derived peptides.

In summary, the selective prevention of pp89 antigen presentation that occurs during the early phase of the MCMV replication cycle cannot be explained by competition for binding to the L^d molecule exerted by the endogenously synthesized viral antigens E1 and E2.

Discussion

In response to infection with MCMV, BALB/c mice generate protective T lymphocytes of the CD8⁺ subset. These T lymphocytes predominantly recognize antigens synthesized during the IE phase of the viral replication cycle. Vaccination with the recombinant vaccinia virus MCMV-*ie1*-VAC, encoding the major IE protein pp89, renders mice

resistant to lethal challenge with MCMV. By using both polyclonal and cloned pp89-specific MHC class I (L^d)-restricted CTL lines, regulation of the presentation of pp89 was examined throughout the virus replication cycle. We present evidence for the following: first, similar to MCMV gene expression, which is temporally regulated in three phases—namely, IE, early, and late—processing and presentation of pp89 for recognition by CTLs is under strict control, displaying the same kinetics; second, arrest of the viral replication cascade in the IE phase, as well as selective expression of the isolated gene *iel* encoding pp89, provide efficient antigen presentation; third, release from the arrest followed by expression of the first viral early genes completely prevents pp89 antigen presentation; fourth, the late phase of gene expression is associated with reinitiation of pp89 synthesis and release from the interference with antigen presentation; fifth, the interference with pp89 antigen presentation occurs posttranslationally but does not involve alterations in protein amount, stability, or nuclear compartmentalization; sixth, the surface expression and antigen-presenting function of the MHC class I molecule L^d are not affected; and seventh, the intervention with pp89 antigen processing and presentation is highly selective in that it does not apply to other MCMV antigens synthesized concomitantly with pp89 in the infected cells and presented by several different class I molecules, including L^d.

This report gives a clear example for a highly selective, temporally regulated, and reversible prevention of viral antigen presentation to class I-restricted T lymphocytes. The results indicate that the interference takes place at an intracellular stage of pp89 antigen processing after translation and before the presentation of antigenic peptides at the cell surface.

Because of its selectivity, the inhibition of pp89 presentation is unlike other reported cases of interference with antigen recognition by CTLs, all of which could be explained by a general decrease in the surface levels of cellular presenting or adhesion molecules (Gooding, 1982; Bernardis et al., 1983; Jennings et al., 1985; Masucci et al., 1987; Gregory et al., 1988) that indiscriminately affects the presentation of any antigen.

An observation more closely related to the data presented in this article has been made in recombinant vaccinia virus-infected cells, in which presentation of influenza virus antigens to CTLs was influenced by the phase of vaccinia virus gene expression (Coupar et al., 1986). During the late phase of the virus replication cycle, and to some extent also during the early phase, partial or total inhibition of presentation of several influenza virus epitopes was observed (Townsend et al., 1988). Vaccinia virus-induced shutoff of cellular functions was discussed as a possible mechanism of the inhibition of antigen presentation. Because restoration of antigen recognition could be achieved by reducing the stability of influenza proteins, it was proposed, as an alternative explanation, that putative protease inhibitors encoded by vaccinia virus might be responsible for the observed inhibition of antigen processing.

Besides the replication-phase association, the inhibi-

tory effect observed here for MCMV has little in common with the inhibitory effect described for vaccinia virus, and the mechanisms postulated there may not apply. First, MCMV does not induce a strong host shutoff, in particular not at the beginning of the early phase. Second, in comparison with the IE phase, when pp89 presentation was optimal, no alterations in pp89 stability were observed during the early phase, when presentation was blocked. For technical reasons, an MCMV recombinant expressing an unstable pp89 has not been constructed to date. If, as the data presented by Townsend et al. (1988) convincingly suggest, the rate of protein degradation governs antigen processing, then an accelerated catabolism of pp89 resulting in its processing before the inhibitory function has had time to operate would restore antigen presentation in cells infected with such an MCMV recombinant. This, however, would not explain the mechanism of interference with pp89 presentation. Third, the highly selective nature of the inhibition of pp89 antigen presentation is not compatible with the more general effect of protease inhibitors. Recently the human CMV, a virus closely related to MCMV, has been completely sequenced. Comparison of open reading frames coding for polypeptides of 300 amino acids and larger with available data bases has, unlike the example of vaccinia virus (Kotwal and Moss, 1989), revealed no detectable homology to known serine protease inhibitors (B. G. Barrell, personal communication). In summary, as opposed to the vaccinia virus system, a mechanism of inhibition that selectively affects pp89 must be proposed for MCMV.

An alternative mechanism that we considered is competition at the peptide level for binding to the L^d molecule. Related pp89-derived synthetic peptides, differing only in length and all containing the antigenic core sequence HFMPT, differ considerably in their antigenic potency, with the nonapeptide YPHFMPTNL having a very high affinity for L^d (Reddehase et al., 1989). Since it is not known which of these peptides are actually generated in infected cells, it is highly possible that peptides derived from the early antigens E1 and E2 have a higher affinity for L^d. However, because simultaneous recognition of pp89, E1, and E2 was feasible in the late phase, peptide competition exerted by E1 or E2 is not a likely mechanism. It can be expected that, if pp89 were competed out by an unknown competitor stronger than E1 and E2, this competitor should also compete with E1 and E2. From all this, it appears that the number of L^d molecules available for binding of peptides is not the limiting factor for pp89 presentation. Finally, the fact that the inhibitory effect operated also in the H-2^b haplotype, in which the antigenic determinants of pp89 and of early antigens are most probably different from those presented by L^d, also disfavors competition.

In view of the evidence available, we hypothesize that inhibition of pp89 presentation is caused by a selective effect on pp89 exerted during the early phase by a viral protein or by a cellular factor induced by it. The selectivity of the effect may relate to the regulatory function of pp89. As a mechanism, one could imagine complex formation with pp89 or an enzymatic activity exerted on pp89, either of

which could prevent correct processing or transport to the cell surface. Studies with mutated pp89 may help to identify possible target sequences for the proposed interactions, and analysis of the genes transcribed during the first period of the early phase should contribute to the understanding of the underlying mechanism.

An interesting implication of our results is the fact that abundant expression of a protein of proven antigenicity in cells that express all molecules essential for antigen processing and presentation does not inevitably result in presentation of this antigen. It is worth considering that the presentation of certain cellular protein antigens may be subject to similar control mechanisms. Such regulatory mechanisms could add considerably to the complexity of self/nonself discrimination.

Under the control of the immune system, CMV persists for life in the infected host in a state of latency that is associated with a block in viral gene expression, a characteristic of herpesviruses. For human CMV there is evidence that IE genes, but not late genes, are expressed in latently infected cells (Rice et al., 1984; Schrier et al., 1985). Since in human CMV the gene IE1 corresponding to gene *ie1* of MCMV also codes for an immunodominant antigen for CTLs (Borysiewicz et al., 1988), it remains an unsolved question how latently infected cells expressing a protein of proven antigenicity can survive in the face of a specific CTL response. The kind of negative regulation of antigen presentation documented here for MCMV could provide the solution for this paradox.

Experimental Procedures

Mice

BALB/c mice (H-2^d haplotype), BALB/c-H-2^{dm2} mutant mice lacking L^d (Hansen et al., 1977), and C57BL/6 mice (H-2^b haplotype) were bred in our own colony under specific-pathogen-free conditions.

Cells and Viruses

Third-passage MEFs were used for virus infection. The L/L^d line of L fibroblasts (H-2^k haplotype), which is transfected with the gene for the MHC class I molecule L^d, and the L/*ie1*-L^d line, which is transfected in addition with MCMV transcription unit *ie1*, have been described previously (Koszinowski et al., 1987). MCMV of the strain Smith (VR-194; American Type Culture Collection, Rockville, MD) was employed as tissue-culture-grown virus. The production of the recombinant vaccinia virus MCMV-*ie1*-VAC, encoding pp89, has been reported before (Volkmmer et al., 1987).

Cytolytic Effector Cells

Generation of MCMV-specific and pp89-specific polyclonal CTLs was achieved by in vitro restimulation with MCMV of spleen cells from mice that were latently infected with MCMV and infected with 1×10^8 PFU of MCMV-*ie1*-VAC 3 weeks before, respectively (Del Val et al., 1988). The L^d-restricted pp89-specific CTL clone IE1 (Reddehase et al., 1986a, 1989) and the L^d-specific CTL clone B6 α L^d (Reddehase et al., 1986b) have been characterized previously. The L^d-restricted CTL clones E1 and E2, which are specific for MCMV early antigens, were cloned by limiting dilution from MCMV-specific polyclonal CTL cultures. Clones E1 and E2 were grown in medium containing 100 U/ml recombinant human interleukin 2 (Sandoz Forschungsinstitut, Vienna, Austria), and restimulated weekly with MCMV and stimulator cells as described for clone IE1 (Reddehase et al., 1986a).

Target Cells and Cytolytic Assays

For selective and enhanced expression of IE genes, MEF were infected with 0.5 PFU of MCMV per cell by centrifugation (800 \times g, 30 min), which results in a multiplicity of infection of 10–20 (Reddehase et al.,

1984). Infection was performed in the presence of CH (50 μ g/ml), which was removed 3 hr later by washing with medium containing act D (5 μ g/ml). Limited early gene transcription after the CH treatment was achieved by removal of CH using inhibitor-free medium and by adding act D to the final concentration of 5 μ g/ml after the intervals indicated. The same metabolic inhibitor treatment was performed on L/*ie1*-L^d cells. Infection of MEF with MCMV-*ie1*-VAC was done as with MCMV except that a multiplicity of 3 was used.

Restriction of gene expression to the IE and early phases of the MCMV replication cycle was achieved by infecting MEF with MCMV in the presence of PAA (250 μ g/ml) to prevent viral DNA replication and late-phase gene expression.

Target cells were trypsinized and labeled for 90 min with Na₂⁵¹CrO₄, and a standard 3 hr cytolytic assay was performed with 1000 target cells and graded numbers of effector cells in 2-fold dilution steps. Data represent the mean percentage of specific lysis from three replicate cultures. For preparing target cells with exogenously added peptides, cells were incubated with peptides at a concentration of 100 μ M for 1 hr at 37°C after the ⁵¹Cr labeling, followed by removal of excess peptide by washing the target cells before the addition of effector cells.

Immunoprecipitation and Western Blot Analysis

Experiments were performed essentially as described (Keil et al., 1985). In brief, the same number of L/*ie1*-L^d cells, uninfected MEF, and MEF infected with MCMV under the same conditions as used for target cell preparation were labeled with [³⁵S]methionine in methionine-free medium for 20 min, starting at 5, 7, and 9 hr p.i. Cell lysates were immunoprecipitated with a murine antiserum to MCMV and analyzed by 10% polyacrylamide gel electrophoresis and autoradiography. Western blot analysis was performed with the same lysates, which, after separation by electrophoresis, were blotted onto a nitrocellulose membrane and incubated with the same antiserum.

Pulse-Labeling, Chase, and Nuclear/Cytoplasmic Fractionation

Uninfected MEFs and MEFs infected with MCMV in the presence of CH were trypsinized at 3 hr p.i., that is, immediately after CH removal, and labeled with [³⁵S]methionine for 30 min in medium devoid of unlabeled methionine. Act D was added at the indicated times. After the labeling period, cells were washed twice with medium containing a 2000-fold excess of unlabeled methionine, followed by further incubation in medium containing the standard concentration of methionine. At given time points, aliquots were taken for the preparation of whole-cell lysates and for the separation of nuclear and cytoplasmic fractions (Keil et al., 1985). This separation was achieved by resuspending the sedimented cells for 5 min in ice-cold buffer I (10 mM Tris-HCl [pH 8.5], 0.14 M NaCl, 1.5 mM MgCl₂, 0.5% Nonidet P-40), followed by centrifugation (800 \times g, 4°C, 5 min) through a cushion of 24% sucrose in buffer I. Lysates were then prepared from the cytoplasmic fraction on top of the cushion and from the sedimented nuclei. Lysates were analyzed by immunoprecipitation and gel electrophoresis. A rabbit antiserum raised against the synthetic peptide P(161–179) of pp89 coupled to bovine serum albumin as described (Del Val et al., 1988) was used for immunoprecipitation.

Flow Cytometry

Uninfected MEFs and MCMV-infected MEFs were treated with CH and act D following the protocol for target cell preparation. They were then incubated with the monoclonal antibodies B22-249 (Lemke et al., 1979), 34.5.8S, and 31.3.4S (Cochet et al., 1986), specific for L^d, D^d, and K^d, respectively, followed by fluorescein-conjugated (F(ab)₂)₂ fragments of goat anti-mouse IgG antibodies (Jackson Immunoresearch Laboratories Inc., West Grove, PA) and analyzed on a FACS IV as described (Reddehase et al., 1986a).

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