

## The poxviral scrapin MV-LAP requires a myxoma viral infection context to efficiently downregulate MHC-I molecules

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### Abstract

Downregulation of MHC class I molecules is a strategy developed by some viruses to escape cellular immune responses. Myxoma virus (MV), a poxvirus causing rabbit myxomatosis, encodes MV-LAP that is known to increase MHC-I endocytosis and degradation through a C<sub>4</sub>HC<sub>3</sub> motif critical for an E3 ubiquitin ligase activity. Here, we performed a functional mapping of MV-LAP and showed that not only the C<sub>4</sub>HC<sub>3</sub> motif is necessary for a marked downregulation of MHC-I but also a conserved region in the C-terminal part of the protein. We also showed that the putative transmembrane domains are responsible for a specific subcellular localization of the protein: they retain MV-LAP in the ER in transfected cells and in the endolysosomal compartments in infected cells. We observed that a specific MV infection context is necessary for a fully efficient downregulation of MHC-I. Our data suggest that the functionality of viral LAP factors, inherited by herpes- and poxviruses from mammalian cells, is more complex than anticipated.

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### Introduction

DNA viruses take advantage of their large genomes to encode many factors that subvert the immune response. Downregulation of cellular membrane glycoproteins is one of the many strategies developed by viruses to disturb cellular trafficking and to escape from the host immune response. One of the most critical actions is the downregulation of MHC class I molecules from the cell surface which is associated with the diminution or abrogation of CTL activity and allows viruses to persist and replicate more easily in the host. Adenovirus E19 glycoprotein (Bennett

et al., 1999), US2, US6 and US11 proteins of human cytomegalovirus (Ahn et al., 1997; Hengel et al., 1997; Lehner et al., 1997; Wiertz et al., 1996) and m06 protein of mouse cytomegalovirus (Reusch et al., 1999) are examples of factors responsible for such an MHC-I downregulation. Recently, a new family of herpes- and poxviral factors involved in the downregulation of immune markers (such as MHC class I) was identified: MK3 of mouse herpesvirus 68 (Stevenson et al., 2000); Mir1 and Mir2 (or K3 and K5, respectively) of human herpesvirus 8 (Coscoy and Ganem, 2000; Ishido et al., 2000a); and MV-LAP (m153R protein) of myxoma virus (MV) (Guerin et al., 2002) share a conserved primary structure with a C<sub>4</sub>HC<sub>3</sub> motif in the N-terminus of the protein and two putative transmembrane domains. In our previous work, the name scrapins was proposed to designate these *surface cell receptor abductor proteins* (Guerin et al., 2002). Other authors refer to these

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proteins as the MIR (modulator of immune recognition) (Coscoy et al., 2001) or K3 (Fruh et al., 2002) family.

Some members of this family have recently been described to be able to promote the ubiquitination of the cytoplasmic tail of their targets. This phenotype is attributed to an E3 ligase activity, critically related to the presence of a functional C<sub>4</sub>HC<sub>3</sub> domain (Boname and Stevenson, 2001; Coscoy et al., 2001; Mansouri et al., 2003). Ubiquitination promoted by the ER-localized MK3 protein of MHV-8 leads to the degradation of MHC-I by the proteasome (Boname and Stevenson, 2001). Interestingly, although Mir1 and Mir2 of HHV8 are also ER residents (Coscoy and Ganem, 2000; Haque et al., 2001; Ishido et al., 2000a), ubiquitination of their targets (MHC-I, B7.2, ICAM-1 for Mir2, MHC-I for Mir1) is associated with degradation in the endolysosomal pathway after enhanced endocytosis (Coscoy and Ganem, 2000; Ishido et al., 2000b). This highlights the fact that at least two distinct mechanisms are used by herpesviral scrapins to interfere with the surface expression of cellular proteins.

Among poxviruses, only MV-LAP (m153R protein) of MV has been characterized so far: This scrapin, previously described to be localized in the ER, downregulates MHC-I, CD95 (Guerin et al., 2002) and CD4 (Mansouri et al., 2003) molecules. It was demonstrated that MV-LAP possesses an E3 ubiquitin ligase activity, like its herpesviral counterparts (Mansouri et al., 2003). However, many aspects of the intimate relationship between localization and function of the protein remain unclear. Moreover, it is not known whether poxviral scrapins and their herpesviral homologs share the same molecular mechanisms to downregulate surface molecules.

In order to investigate MV-LAP functionality and determine how closely related are herpes- and poxviral scrapins, we decided to perform a molecular mapping of the protein. In this study we demonstrate that MV-LAP shares all the structural characteristics of herpesviral immune modulators; however, it requires a particular context to be fully functional: This is the first member of the scrapins (or MIR family) described to require the help of a specific viral partner to optimize the downregulation of MHC-I molecules.

## Results

### Identification of a conserved region (CR) within MV-LAP

M153R codes for the 206 amino acids (aa) MV-LAP protein which contains a C<sub>4</sub>HC<sub>3</sub> domain between aa 23 and 68 and two putative transmembrane domains between aa 95 and 115 and aa 135–153 (Fig. 1A). A previous study performed on herpesviral scrapins Mir1 and Mir2 of HHV8 showed the existence of a conserved region (designated CR) in the C-terminal domain (Fig. 1B, boxed region) (Sanchez et al., 2002). An independent analysis revealed four critical clusters for Mir1 activity: three belong to the CR and one lies downstream (Fig. 1B, clusters represented by stars) (Means et al., 2002).

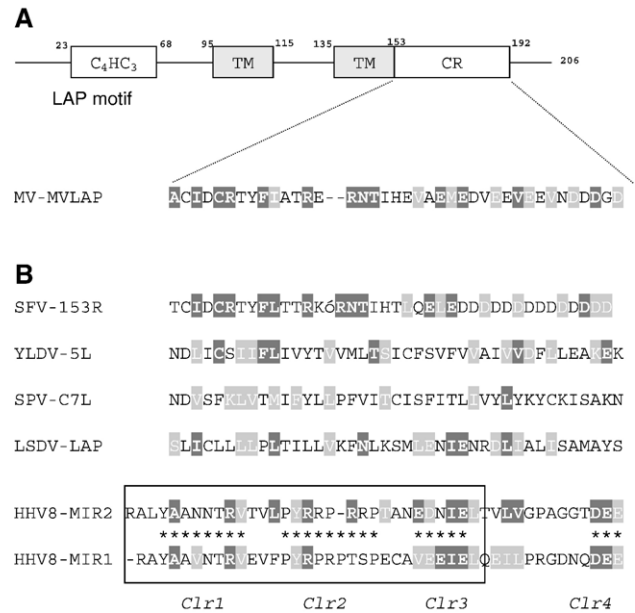


Fig. 1. Structure of MV-LAP and identification of a C-terminal conserved region. (A) General structure of MV-LAP. TM, transmembrane domain; CR, conserved region. Numbers above the diagrams, positions of amino acids flanking the motifs. (B) Sequence alignments of the newly defined CR of viral Scrapins. HHV8, human herpesvirus 8; SFV, Shope fibroma virus; YLDV, Yaba like disease virus; SPV, swinepox virus; LSDV, lumpy skin disease virus. Shaded letters, identical amino acids (factor of 0.45); light gray letters, related amino acids. Boxed letters are CR region previously described in herpesviral Mir-1 and Mir-2 proteins (Sanchez et al., 2002). Stars represent four critical clusters (Clr1-4) for Mir1 function (Means et al., 2002).

In order to assess if such a domain is present in the poxviral scrapin, we performed a bioinformatic analysis of MV-LAP. As shown in Fig. 1, we found a relatively conserved CR domain adjacent to the second transmembrane domain of MV-LAP, reminiscent of the CR of Mir1 and Mir2. We decided to enlarge the CR including the subsequent 13 aa in order to integrate the fourth cluster of Mir1 (which is an acidic cluster).

### A context of infection is critical for MHC-I downregulation by MV-LAP

Our next step was to determine the role of each domain in the function of MV-LAP. A previous work indicated a dramatic decrease of MHC-I molecules at the surface of BGMK or Malme 3 M cells infected with wild-type MV (Guerin et al., 2002). Surprisingly, our first attempts using A375 cells (and other cell types) transfected with pEGFP-MVLAP showed that MHC-I molecules were downregulated only weakly, far below the level obtained in infection experiments (Figs. 2b and c). Several hypotheses were formulated to explain this discrepancy, e.g., the potential need for a minimum strength of expression or the influence of GFP protein co-production. First, we decided to construct a pmyxGFP-MVLAP plasmid, in which the promoter region of MV-LAP replaced the CMV promoter

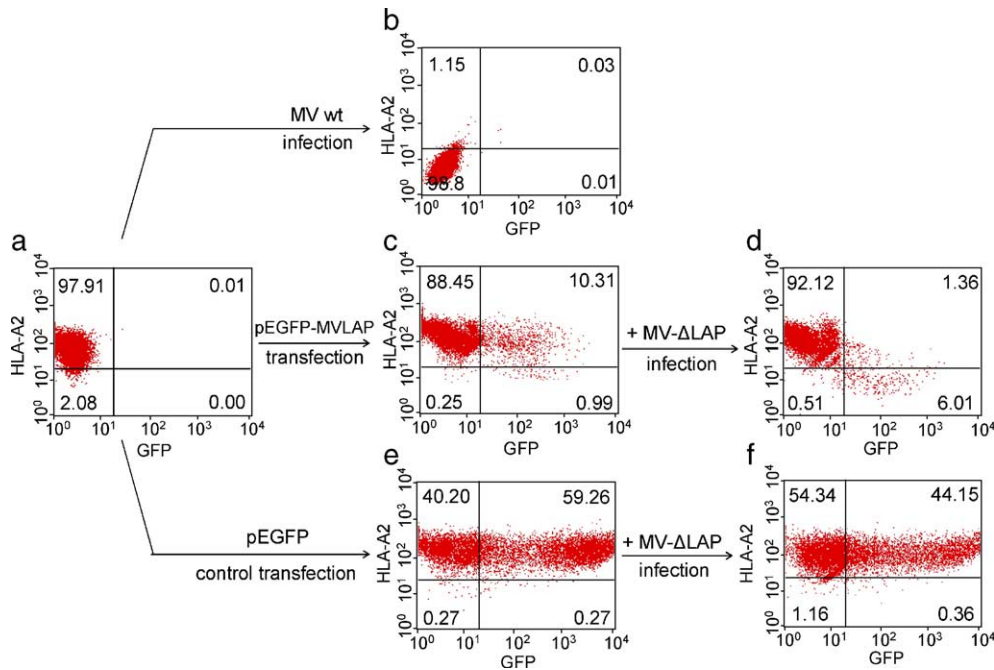


Fig. 2. A context of infection is critical for MHC class I downregulation by MV-LAP. A375 cells were either mock infected (a), infected with wild-type MV (b), transfected by pEGFP-MVLAP (c), transfected by pEGFP-MVLAP and infected with MV-ΔLAP (d). As a control, cells were also transfected with pEGFP in the absence (e) or presence (f) of MV-ΔLAP infection. MHC-I class I surface expression was then measured using HLA A2.1 antibodies. Displayed events: 10,000 cells/dot plot.

of pEGFP-MVLAP. Since poxviral genes require the poxviral transcription machinery, we realized a transfection/infection assay with pmyxGFP-MVLAP and MV-ΔLAP. MV-ΔLAP is derived from the wild-type MV by deletion of the *MV-LAP* open reading frame (Guerin et al., 2002). Only the cells that were both transfected and infected could express MV-LAP (and GFP): in those cells, the downregulation was restored to the level observed in

infection (data not shown). Interestingly, infection with MV-ΔLAP of A375 cells transfected with pEGFP-MVLAP gave similar results (Fig. 2d). These data indicate that neither the presence of GFP in the N-terminus of the protein nor the promoter driving MV-LAP expression influences the activity of the scrapin. Rather, a context of viral infection seems crucial for complete functional activity.

Table 1  
Functional mapping of MV-LAP

Construction	MHC-I Downregulation	Subcellular localization
MV-LAP 	YES	Punctuated
ΔC <sub>4</sub> HC <sub>3</sub> 	NO	Punctuated
Nterm 	NO	Diffuse
Cterm 	NO	Punctuated
ΔCR 	NO	Punctuated
ΔC <sub>4</sub> HC <sub>3</sub> ΔCR 	NO	Punctuated

Mutants of MV-LAP lacking PHD/LAP, or partially or fully CR region were cloned in pEGFP vector in order to generate GFP fusion proteins. After transient transfection of A375 cells infected with an MV-ΔLAP virus (MOI = 10), MHC class I surface expression was measured. Subcellular localization of each fusion protein was determined by confocal microscopy. The truncations were stably expressed, as judged by immunofluorescence analysis and flow cytometry (not shown).

### Functional mapping of MV-LAP

Since the more dramatic effects are obtained in transfection/infection experiments, we decided to perform the functional mapping of MV-LAP by complementing pEGFP-MVLAP-derived plasmids with MV- $\Delta$ LAP virus. In order to investigate the role of the LAP, TM and the newly identified CR domains in the downregulation of MHC-I molecules, we constructed a series of plasmids with the gene coding for GFP fused to various deletions of MV-LAP (Table 1). The full-length MV-LAP generates significant downregulation of MHC-I molecules, whereas each of the mutants we tested had no effect, suggesting that every domain of the scrapin is critically required for its function. In parallel, we observed the subcellular localization of the GFP-tagged proteins and determined that in the absence of the TM domains the proteins were diffusely distributed in the cell (similar to GFP alone, data not shown). The presence of the TM domains restored the targeting of MV-LAP constructs to punctuated substructures (Table 1). Hence, the TM domains are necessary for the specific localization of MV-LAP, where the C<sub>4</sub>HC<sub>3</sub> and CR are both essential for downregulating MHC-I molecules. These results are reminiscent of those observed with the herpesviral Mir2 protein (Sanchez et al., 2002), demonstrating that the poxviral and herpesviral scrapins share very similar molecular characteristics.

### A MV cofactor is required for MHC-I downregulation by MV-LAP

To understand why infection was favorable to a proper downregulation of MHC-I molecules in A375 cells, we compared the effects of MV with modified vaccinia virus Ankara (MVA), an Orthopoxvirus which does not code for an equivalent of MV-LAP (Antoine et al., 1998). A MVA-

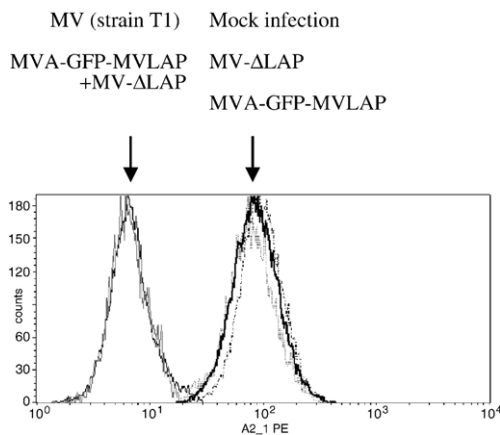


Fig. 3. A myxoma cofactor is necessary to achieve maximal downregulation of MHC class I molecules by MV-LAP. A375 cells were mock infected, infected with MV, MV- $\Delta$ LAP, MVA-GFP-MVLAP or both MVA-GFP-MVLAP and MV- $\Delta$ LAP. 15 h post-infection, cells were treated with HLA A2.1. antibodies and analyzed by FACS (gated for GFP).

GFP-MVLAP recombinant virus expressing a fluorescent MV-LAP protein through transcriptional control of the vaccinia virus early/late P7.5 promoter was used in infection assays of A375 cells. Fifteen hours post-infected, we quantified the MHC-I molecules at the cell surface and found that when MV-LAP was expressed by MVA the downregulation was very weak, in comparison with infection with the wild-type MV (Fig. 3). Alternatively, we complemented MVA-GFP-MVLAP-infected A375 cells with MV- $\Delta$ LAP. Now, cells that were co-infected displayed a dramatic decrease of MHC-I molecules at their surface, comparable to that observed in wild-type MV-infected cells (Fig. 3). This surprising result suggests that one or several factors specific of MV are required.

As a control, we also tried to complement transfected pmyxGFP-MVLAP plasmid with a wild-type MVA infection. Indeed, MVA is able to activate the early promoter of MV-LAP. However, MVA could not complement the effect of MV-LAP and MHC-I levels remained as poorly modified as in transfection experiments only (data not shown). Our results demonstrate that a factor expressed by MV, but not MVA, is necessary for the activity of MV-LAP.

### MV-LAP cofactor does not influence its subcellular localization

Since our previous data had shown that the intracellular localization can determine the function of MV-LAP, we explored the possibility that MV – but not MVA – could properly redirect the scrapin in the cell. To address this question, we determined the localization of GFP-tagged MV-LAP expressed in various experimental conditions. When pEGFP-MVLAP was transfected in A375 cells, the green fluorescence colocalized with calnexin, indicating that the viral protein was in the endoplasmic reticulum (Fig. 4). When the transfection experiment was complemented by transfection with MV- $\Delta$ LAP, the green fluorescence was localized in the late endosomal compartment, revealed by EEA-1 staining (Fig. 4). In another assay, we observed that MVA-GFP-MVLAP also directed the scrapin to endosomes. Coinfecting A375 cells with MVA-GFP-MVLAP and MV- $\Delta$ LAP did not change its distribution. These observations suggest that the endosomal localization of the protein is a consequence of the infected status of the cell, whether the infection results from MV or MVA.

### Discussion

A conserved family of viral proteins is used by poxviruses and herpesviruses to downregulate surface molecules of immune cells (for a review, see Fruh et al., 2002). While MIR or scrapin proteins of herpesviruses are being extensively studied, little is known about their counterparts in poxviruses. Indeed, like herpesviral proteins, MV-LAP is expressed early in the viral cycle,



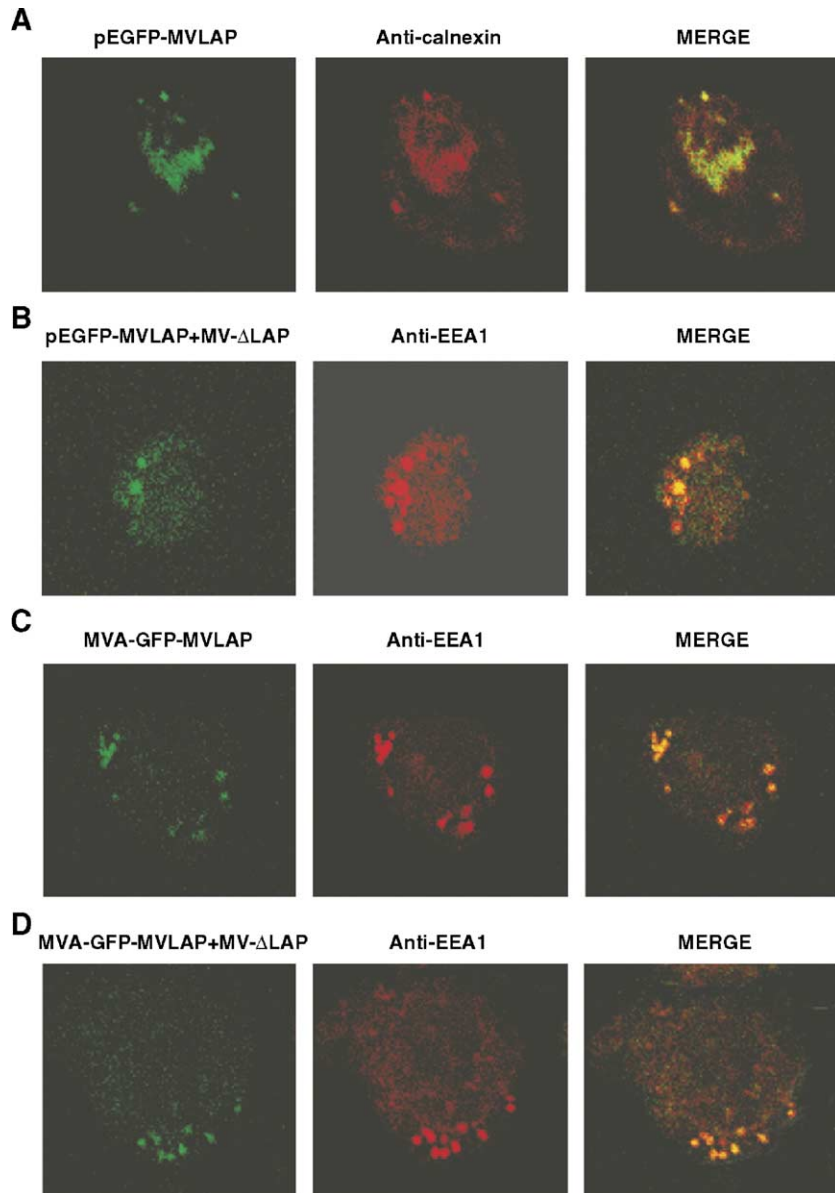


Fig. 4. Subcellular localization of GFP-MV-LAP. A375 cells were transfected with pEGFP-MVLAP (A, B) or infected with MVA-GFP-MVLAP (C, D) in the absence (A, C) or presence (B, D) of MV- $\Delta$ LAP. 15 h post-infection, cells were treated with anti-calnexin or anti-EEA1 antibodies and analyzed by confocal microscopy.

localizes in membrane compartments and downregulates MHC-I molecules by enhancing their endocytosis (Boshkov et al., 1992; Guerin et al., 2002; Mansouri et al., 2003). Among the structural features shared by the two families of viruses are the presence of a  $C_4HC_3$  domain which possesses a ubiquitin ligase activity (Coscoy and Ganem, 2003; Mansouri et al., 2003) and two transmembrane (TM) domains (Guerin et al., 2002). The TM domains of Mir2 are responsible for the oligomerized status of the protein as well as target selection (Sanchez et al., 2002). Mir1 and Mir2 also possess a critical region with conserved motifs downstream of the TM domains (Means et al., 2002; Sanchez et al., 2002). This conserved region (CR) has a tyrosine-based motif adjacent to conserved residues, fol-

lowed by a proline-rich motif and two diacidic clusters. We performed an alignment between MV-LAP, other scrapins of poxviruses and Mir1 and Mir2 of HHV-8, and showed that poxviruses also have conserved residues at the C-end of the protein. This newly defined CR of poxviruses based on sequence similarities has conserved some key residues, and acidic clusters are present. The CR of Mir1 and Mir2 are required for MHC-I downregulation (Means et al., 2002; Sanchez et al., 2002): the tyrosine-sorting motif and the conserved residues direct the MHC-I molecules to the TGN, where they are retargeted to the lysosomal compartment via the diacidic regions (Means et al., 2002). Detailed mechanism remains speculative. Our analysis of deletion mutants supports the notion that MV-LAP is composed of multiple

domains, including a CR, each of which is required to achieve efficient downregulation of MHC-I molecules. While the C<sub>4</sub>HC<sub>3</sub> domain is a ubiquitin ligase that transfers ubiquitin groups to the cytosolic tail of target molecules (Mansouri et al., 2003), the TM domains are necessary for a proper subcellular localization of the protein.

This raises a very intriguing aspect of scrapin functionality, which enhances the internalization of molecules exposed to the plasma membrane, while residing in the ER (Coscoy and Ganem, 2000; Haque et al., 2001; Ishido et al., 2000a). How this is achieved is debated, and some authors suggest that a small proportion of Mir2 escapes the ER compartment to the plasma membrane (Sanchez et al., 2002). While previous experiments with GFP-tagged proteins expressed by a plasmid had indicated that MV-LAP resides in the ER (Guerin et al., 2002), others observed that the protein is localized in the endosomal and TGN compartments when expressed by a vaccinia virus vector (Mansouri et al., 2003). We constructed an MVA-GFPLAP recombinant virus, which expresses MV-LAP under the control of the early/late P7.5 promoter. This helped confirm the observation that MV-LAP localizes to the ER when expressed in transfected cells but to endosomes when expressed by a poxviral vector. MV-LAP, when expressed by a plasmid, also localizes to endosomes in cells infected with a recombinant MV-ΔLAP virus. Hence, either some viral protein tethers MV-LAP and drives it from the ER to endosomes, or poxviral infection remodels cell architecture and trafficking so that MV-LAP localizes in endosomes. Without anticipating on the mechanisms, one can safely assume that the biological compartment of MV-LAP is the endosomes/TGN rather than the ER. Since localization experiments of herpesviral proteins were interpreted in a context of transfection, it would be informative to reproduce them after complementation with herpes infection.

While both MVA and MV target MV-LAP to endosomes, the biological effects of MV-LAP are not equivalent in both contexts: In A375 cells the downregulation of MHC-I was far more dramatic when cells were infected with MV than with MVA expressing MV-LAP. MVA does not replicate in A375 cells but can still express both early and late genes (data not shown). Hence, it is very unlikely that the difference between MVA and MV-ΔLAP can be attributed to different levels of expression of the same gene products made by both viruses. Obviously, one or more proteins specific of MV infection act as cofactors to ensure maximal activity of MV-LAP.

Our hypothesis of the existence of a cofactor is strengthened by previous observations: Although MV-LAP is expressed as an early gene product (Guerin et al., 2002) MHC-I downregulation is only moderate in the presence of Ara C, an inhibitor of DNA replication hence of late gene expression (Boshkov et al., 1992). More recently, it was shown that redistribution of MHC-I molecules from the ER is influenced by the addition of Ara C, although this drug does not affect their accumulation and degradation in late endosomes (Zuniga et al., 1999). These data suggest the require-

ment for one or more intermediate and/or late gene products to achieve maximal effect. This additional factor alone is unable to affect MHC-I surface expression, since a recombinant MV-ΔLAP virus has no effect on the level of MHC-I molecules (Guerin et al., 2002; Mansouri et al., 2003).

Structural and functional homologs of poxvirus and herpesvirus modulators of immune recognition have been discovered in mammalian genomes where they are designated as MARCH (membrane-associated RING-CH) proteins (Bartee et al., 2004). MARCH proteins regulate endocytosis of various cell surface receptors via ubiquitination. All the information that can be obtained on the function of Mir/Scrapin proteins will be valuable for a deeper knowledge of how MARCH function. Since MV can be easily grown on a wide variety of cell line as well as it infects the European rabbit with a dramatic clinical outcome, such information might be easier to obtain in a poxviral than a herpesviral model system.

## Materials and methods

### *Cells, viruses, transfections and infections*

The wild-type strain T1 and the MV-ΔLAP mutant virus of MV were grown in the rabbit kidney cell line RK13 maintained in OPTI minimum essential medium supplemented with 2% fetal calf serum. The MV-ΔLAP mutant is derived from T1 after deletion of the *MV-LAP* gene (*m153R*) by insertion of a *lacZ* cassette (Guerin et al., 2002). Human skin melanoma A375 human cells (ATCC CRL-1619) were maintained in RPMI medium with 10% calf serum and 25 mM HEPES. Subconfluent layers of A375 cells were transfected with plasmids encoding green fluorescent protein (GFP) fusion proteins by using liposomes (lipofectamine; Invitrogen). 5 h later, the transfection medium was replaced by fresh medium or a viral inoculum at a multiplicity of infection (moi) of 10. At 15 h post-transfection or post-infection, cells were rinsed twice with phosphate-buffered saline (PBS) for subsequent use in fluorescence-activated cell sorter (FACS) analysis or confocal microscopy.

### *Cloning, sequencing and computer analysis of DNA and protein sequences*

DNA and protein sequences were analyzed using DNA Strider 1.3 software (Marck, 1988), the BLAST program (GenBank) and the Web site <http://www.poxvirus.org>. Sequence alignments of scrapins were performed using Clustal software.

### *Construction of GFP fusions*

Fusion plasmids were obtained by subcloning specific MV-LAP fragments into the pEGFP plasmid vector (BD Biosciences Clontech), which contains a jellyfish GFP gene

optimized for maximum fluorescence downstream of a CMV promoter. MV-LAP DNA comprising nucleotides (nt) 1–621,  $\Delta$ CR (nt 1–459), N-MVLAP (nt 1–285),  $\Delta$ C<sub>4</sub>HC<sub>3</sub> (nt 1–69 fused to nt 204–621), C-MVLAP (nt 285–621) and  $\Delta$ C<sub>4</sub>HC<sub>3</sub> $\Delta$ CR (nt 1–69 fused to nt 204–459 then to nt 540–621) were generated by PCR using purified MV DNA as a template (Petit et al., 1996). PCR products were digested with the restriction enzymes *Bgl*II and *Pst*I and cloned into the corresponding sites of pEGFP, yielding fusions containing GFP at the N-terminus of the chimeric polypeptide (see Table 1). The integrity of MV fragments was assessed by sequencing.

pmyxGFP-MVLAP was derived from pEGFP-MVLAP after amplification of the promoter region of the M153R gene using primers 5'-*Ase*I-pmyx (5'-CAGATTAATACGGCC-TACGGATGTTCTAACG-3') and 3'-*Nhe*I-pmyx (5'-GACGCTAGCGTTTACACAACCTATTTACTTTAATTGAC-3'). The resulting fragment was inserted between the *Ase*I and *Nhe*I sites in pEGFP-MVLAP, thus replacing the CMV promoter of pEGFP-MVLAP by the natural promoter of *MV-LAP*.

#### Confocal microscopy observations

A375 cells were plated onto LabTek multichamber slide flasks (Falcon). At 15 h post-transfection and/or post-infection, cells were rinsed twice in PBS, fixed for 90 min at room temperature with 4% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100 for 30 min at room temperature. Cells were then incubated with mouse anti-human EEA1 antibodies (1/100; BD Biosciences Pharmingen), rabbit anti-calnexin antibodies (1/100; StressGen) or mouse anti-human Lamp1 antibodies (1/100; BD Biosciences Pharmingen) in 0.05% Tween in PBS for 1 h at 37 °C, rinsed three times in PBS-Tween and incubated in biotinylated goat anti-rabbit antibodies (1/500; Sigma) or biotinylated goat anti-mouse antibodies (1/200, Vector) for 1 h at 37 °C. Cy3-conjugated streptavidin (Jackson ImmunoResearch) was added at a dilution of 1/10,000 in PBS and incubated for 30 min at 4 °C. After three PBS washes, the samples were mounted with PBS–glycerol (1:1) and observed with a confocal LSM Olympus microscope fitted with a 60× Olympus objective.

#### Construction of *MVA-gfpMVLAP* recombinant

The pEGFP-MVLAP plasmid containing a jellyfish *gfp* gene fused at the N-terminus of the *MV-LAP* (Guerin et al., 2002) was used as a template to generate a PCR product comprising the chimeric *GFP/LAP* fusion gene. The primers were GFP-fusLAP-sense-*Bam*HI (5'-GGGC-GGATCCATGGTGAGCAAGGGCGAG-3') paired with *MV-LAP*-antisense-*Pme*I (5'-GGGCGTTTAAACCTAAGCGGGTACTCCACGAC-3'). The resulting PCR fragment was digested with the restriction enzymes *Bam*HI and *Pme*I and cloned between the corresponding unique sites

of pIII $\Delta$ HR-P7.5 to generate the MVA transfer vector plasmid pIII $\Delta$ HR-P7.5-GFP/LAP. The integrity of the amplified GFP/LAP fusion fragment was assessed by sequencing. Recombinant MVA expressing the *MV-LAP* gene fused to the *gfp* gene under control of the vaccinia virus-specific early/late P7.5 promoter, which was termed MVA-GFP-MVLAP, was constructed as described recently (Staib et al., 2000, 2004). Recombinant virus was propagated and titered following standard methodology. To generate stock preparations, virus was routinely purified by ultracentrifugation through sucrose and reconstituted in 10 mM Tris pH 7.4, 120 mM NaCl saline buffer (Staib et al., 2000, 2004).

#### Flow cytometry analysis of transfected and infected cells

Cells were washed in PBS/BSA (1%) on 96-well plates and pre-incubated for 30 min with purified rat anti-mouse CD16/CD32 (Fc $\gamma$ III/II receptor) monoclonal antibody (1/100; 2.4G2 (FcBlock); BD Biosciences Pharmingen). They were then washed, stained with mouse anti-human HLA A2.1 hybridoma supernatant IgG2b (BB7.2 (ATCC HB-82)), diluted 1:5 in PBS/BSA (1%) for 45 min, washed again and stained with F(ab')<sub>2</sub> goat anti-mouse IgG-PE antibody (1/500, Jackson ImmunoResearch) for 30 min. They were then washed again and analyzed using a Becton Dickinson FACScalibur. To determine viability of the cells, propidium iodide (BD Biosciences Pharmingen) was added to cells (50  $\mu$ g/ml). Graphs were plotted using CellQuestPro Software.

#### Note added in proof

Our co-author and friend, Frédérique Messud-Petit, suddenly passed away on August 29, 2005, much too soon for her family and for us, and before the publication of this paper. Our thoughts go with her and her family.

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