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Early gene *m18*, a novel player in the immune response to murine cytomegalovirus

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The identification of all antigenic peptides encoded by a pathogen, its T cell 'immunome', is a research aim for rational vaccine design. Screening of proteome-spanning peptide libraries or computational prediction is used to identify antigenic peptides recognized by CD8 T cells. Based on their high coding capacity, cytomegaloviruses (CMVs) could specify numerous antigenic peptides. Yet, current evidence indicates that the memory CD8 T cell response in a given haplotype is actually focused on a few viral proteins. CMVs actively interfere with antigen processing and presentation by the expression of immune evasion proteins. In the case of murine CMV (mCMV), these proteins are effectual in the early (E) phase of the virus replication cycle and should thus preclude the presentation of peptides derived from E proteins. Notably, the *m18* gene is here added to a growing list of mCMV E genes that encode antigenic peptides in spite of the E phase immune evasion strategies of the virus.

For more than a decade since its identification in 1989, the IE1 protein (pp89)-derived peptide ^{168}YPHFMPTNL^{176}, which is encoded by the immediate-early (IE) gene *m123* exon 4 (*ie1*) and presented by the major histocompatibility complex (MHC) class I molecule L^{d}, had remained the only known antigenic peptide of murine CMV (mCMV) (Reddehase *et al.*, 1989). Its immunodominance appeared to be explained conveniently by the later finding that mCMV encodes early (E) phase immune evasion proteins that interfere at various steps with the MHC class I pathway of antigen processing and presentation (reviewed by Hengel *et al.*, 1998, 1999; see also Reddehase, 2000). Specifically, the *m152* gene product gp37/40 mediates the retention of peptide-loaded MHC class I β2-microglobulin complexes in a cis-Golgi compartment (Del Val *et al.*, 1992; Ziegler *et al.*, 1997, 2000), the *m04* gene product gp34 serves as a chaperone for MHC class I molecules (Kleijnen *et al.*, 1997) and the *m06* gene product gp48 re-routes MHC class I molecules for lysosomal degradation (Reusch *et al.*, 1999). Collectively, these redundant mechanisms should preclude effectively the presentation of peptides derived from E proteins, whereas presentation of the IE1 peptide can occur during the IE phase and is prevented only after expression of the immune evasion proteins in the E phase (Reddehase *et al.*, 1986; Del Val *et al.*, 1989).

This smooth view of mCMV antigen presentation was called into question by the finding that CD8-positive *ex vivo* cytolytic T lymphocytes (CTL) isolated from pulmonary infiltrates during mCMV pneumonia lysed infected foetal fibroblasts preferentially in the E phase (Holtappels *et al.*, 1998). MHC restriction of that recognition predicted the existence of antigenic E phase peptides presented by MHC class I molecules. In addition, control of mCMV in the *L*^{d} gene-deletion mutant BALB/c-*H-2*^{dm2} (Alterio de Goss *et al.*, 1998) indicated the existence of antigenic peptide(s) presented by K^{d} and/or D^{d}. As a fancy of nature, the immune evasion protein pORF*m04* (gp34) was the first E protein of mCMV for which an antigenic peptide was identified, namely peptide ^{243}YGPSLYRRF^{251} presented by D^{d} (Holtappels *et al.*, 2000a). Further E proteins instantly followed. Specifically, mCMV homologues of human CMV (hCMV) p*UL83* (pp65), namely p*M83* (pp105) and p*M84* (p65) (Cranmer *et al.*, 1996; Morello *et al.*, 1999, 2000), were found to account for antigenic peptides ^{761}YPSKEPFNF^{769} (Holtappels *et al.*, 2001) and ^{297}AYAGLFTPL^{305} (Holtappels *et al.*, 2000b, 2001) presented by L^{d} and K^{d}, respectively. However, these three E peptides represented subdominant antigenic peptides. While CTL lines (CTLL) with the respective peptide specificities were all protective in preemptive cytoimmunotherapy of mCMV disease (Holtappels *et al.*, 2000a, 2001), the quantitative contribution of these peptides to the priming of an immune response during mCMV infection was minimal to undetectable (Holtappels *et al.*, 2000c, 2001). The idea that subdominance might be a characteristic feature of E phase peptides soon proved to be unfounded: very recent work identified a dominant peptide in E protein pORF*m164* (Holtappels *et al.*, 2002). Peptide ^{257}AGPPRYSRI^{265} is presented by D^{d} and elicits acute and memory CD8 T cells at a frequency that compares to IE1.

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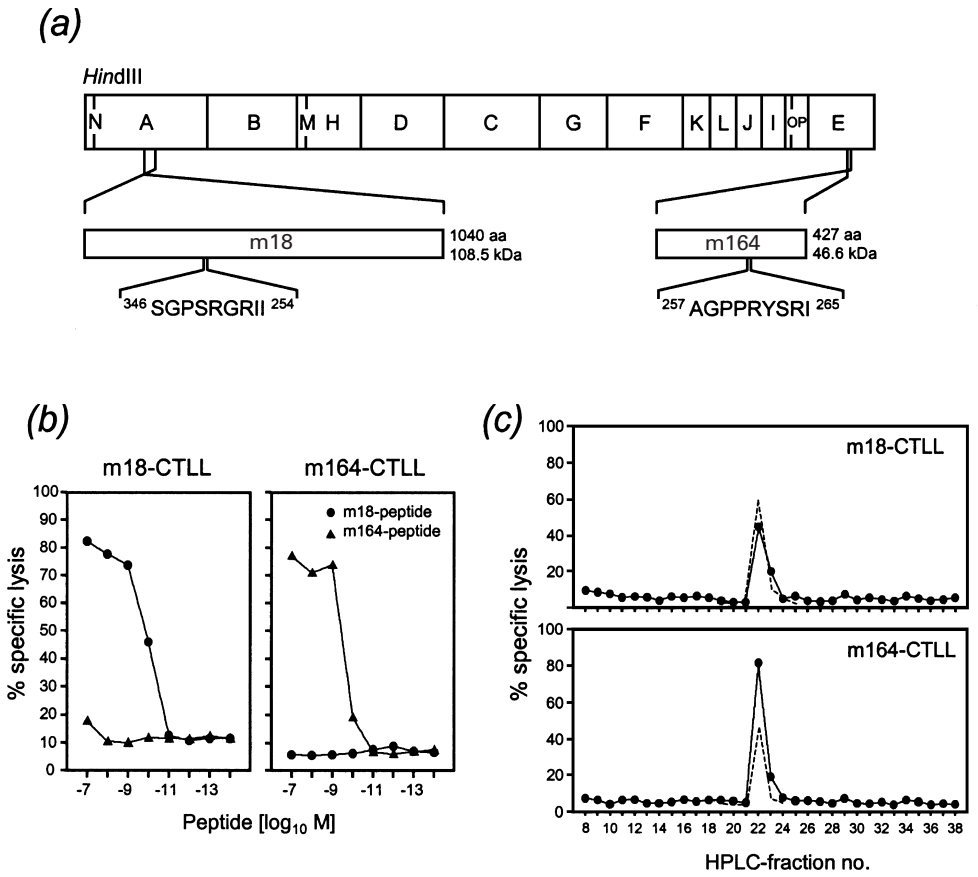

Fig. 1. Identification of an antigenic peptide in the ORF*m18* protein. (a) *Hin*dIII map of the mCMV Smith strain (ATCC VR-194/1981) genome showing the locations of the ORF*m18* (m18) and ORF*m164* (m164) proteins and the sequences of the antigenic peptides therein. Molecular masses were deduced from the amino acid sequence (Rawlinson *et al.*, 1996). So far, neither of the two proteins has been characterized biochemically. (b) Mutually exclusive recognition of the m18 and m164 peptides. CTLL were generated as described previously (Holtappels *et al.*, 2002). The cytolysis assay was performed with P815 (*H-2*^{d}) target cells pulsed with synthetic peptide at the indicated molar concentrations. (c) Co-elution of naturally processed peptides m18 and m164. m18-CTLL and m164-CTLL were used as responder cells in a cytolysis assay performed with P815 target cells that were pulsed with aliquots of HPLC fractions. Solid line, HPLC fractions containing naturally processed peptides derived from the acidic extract of foetal fibroblasts in the E phase of the virus replication cycle. Dashed line, HPLC fractionation of acidic extract of uninfected foetal fibroblasts supplemented alternatively with synthetic peptides m18 (top) or m164 (bottom). Throughout, the effector:target cell ratio was 15:1.

Naturally processed m164 peptide was found to elute in fractions 22 and 23 of a high performance liquid chromatography (HPLC) separation of peptides present in lysates of foetal fibroblasts in the late phase of infection (Holtappels *et al.*, 2002). Notably, in that study, an enzyme-linked immunospot (ELISPOT) assay performed with the naturally processed peptides present in fractions 22 and 23 revealed a frequency of responding memory CD8 T cells that was higher than the frequency obtained after stimulation with a saturating dose of synthetic m164 peptide. This finding had thus indicated the existence of at least one further antigenic peptide co-eluting with the m164 peptide.

How can this hidden peptide be disclosed? We knew from previous experience that mass spectrometry is not a promising approach, because an HPLC fraction represents a mixture of different peptides, including many self peptides, all present in a low absolute amount. Support came from the analysis of MHC restriction of antigenic activity. In a previous report, we had shown already that short-term microculture CTLL raised with the fraction 22 and 23 eluates lysed only L-D^{d} transfectants pulsed with the same eluates, but not L-L^{d} or L-K^{d} transfectants (Holtappels *et al.*, 2000a). As a consequence, like the m164 peptide, the unknown peptide co-eluting must be presented by D^{d}. In two previous reports in which the D^{d}-restricted m04 (Holtappels *et al.*, 2000a) and m164 (Holtappels *et al.*, 2002) peptides were identified, we had already performed genome-wide screenings of D^{d}-binding motifs based on the forecast developed by Rammensee and co-workers (reviewed by Rammensee *et al.*, 1997). Both screenings had indicated the existence of minor activities, which we had, at that time, not followed any further. One such minor activity was visible in both previous screenings and mapped to genomic position

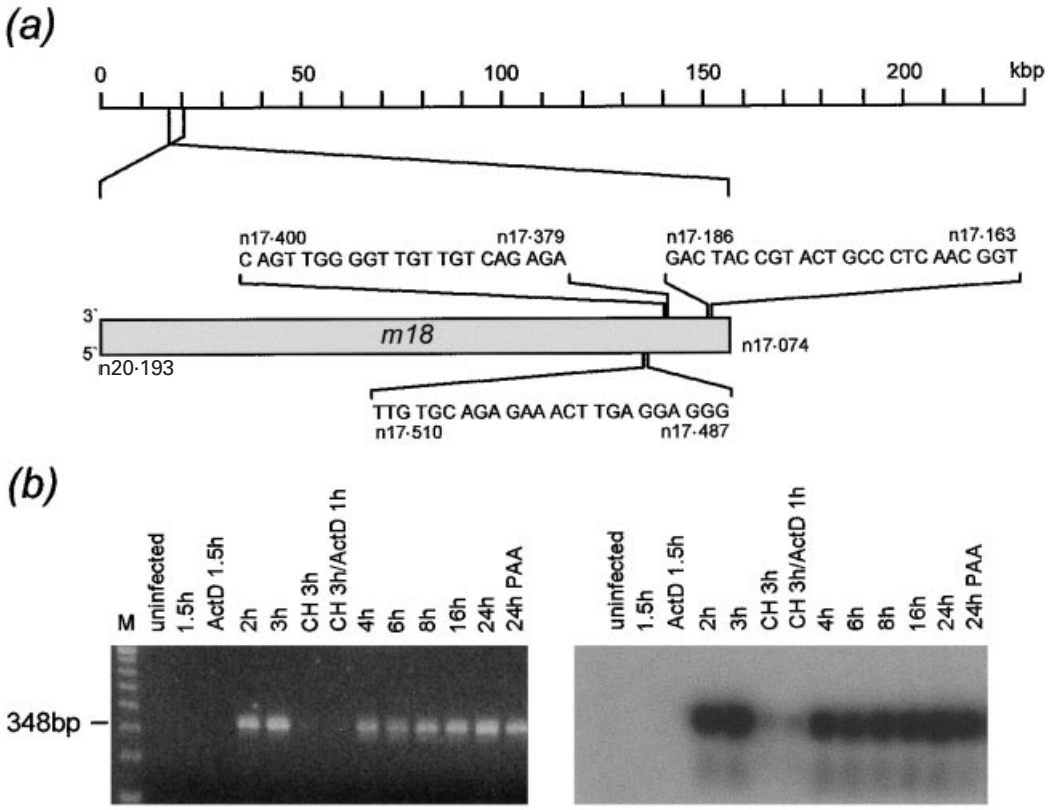

Fig. 2. Characterization of gene *m18* as an E phase gene. (a) Location map of gene *m18* and of the primers and probe for the PCR amplification and Southern blot detection of an *m18* cDNA fragment. Map positions refer to the sequence of mCMV strain Smith ATCC VR-194 (Rawlinson *et al.*, 1996; GenBank accession no. MCU68299). (b) Kinetics and drug sensitivity of gene *m18* transcription. RT–PCR was performed with purified poly(A)$^+$ RNA isolated from mouse foetal fibroblasts at the indicated times after infection (multiplicity of 4 IU per cell). Oligo(dT) priming was used for the RT reaction. Conditions for RT–PCR were as described previously (Holtappels *et al.*, 2001), except that the annealing temperature in the PCR amplification of the cDNA fragment was 64 °C. An ethidium bromide-stained gel is shown on the left; M, 100 bp size markers. A corresponding Southern blot autoradiograph obtained after hybridization with the γ-^{32}P-end-labelled oligonucleotide probe is shown on the right. ActD, actinomycin D (5 μg/ml); CH, cycloheximide (100 μg/ml); PAA, phosphonoacetic acid (250 μg/ml).

C^{19158} within *Hin*dIII fragment A of the mCMV Smith strain genome (Ebeling *et al.*, 1983; Rawlinson *et al.*, 1996; GenBank accession no. MCU68299). The corresponding peptide ^{346}SGPSRGRII^{254} is part of the ORF*m18* protein (Fig. 1a).

Then, we used successfully the synthetic m18 peptide in a restimulation concentration of 10^{-10} M for the generation of a long-term CTLL, here referred to as m18-CTLL, from memory spleen cells, according to a protocol described recently for the generation of m164-CTLL (Holtappels *et al.*, 2002). As it is documented in Fig. 1(b), recognition of the D^d-presented m18 and m164 peptides by m18-CTLL and m164-CTLL was mutually exclusive, which proved the specificity of these CTLL and excluded cross-reactive recognition of these two peptides. We emphasize this as being an important control, because cross-reactive recognition of the m164 peptide by m18-CTLL could have mimicked a false co-elution of the two peptides. D^d restriction of both CTLL was confirmed by the lysis of peptide-pulsed L-D^d transfectants and the absence of lysis with peptide-pulsed transfectants L-K^d and L-L^d as target cells (data not shown). On these firm grounds we could address finally the question of whether the m18 peptide was indeed the hidden fraction 22 peptide that we had been seeking.

Target cells were pulsed with the HPLC fractions obtained from lysates of foetal fibroblasts in the E phase of infection and the two CTLL were used as effector cells for the localization of the respective peptides (Fig. 1c). Both CTLL detected their cognate naturally processed peptide in fraction 22 and, to a lesser extent, fraction 23. In addition, HPLC retention was determined for the corresponding synthetic peptides and both were found to elute with a peak in fraction 22 (Fig. 1c, dashed line).

In conclusion, the m18 and m164 peptides do indeed co-elute and the hidden fraction 22 peptide is now identified as the m18 peptide ^{346}SGPSRGRII^{254}.

The *UL18* gene of hCMV, the positional homologue of the mCMV *m18* gene, has evoked great interest, as it is a sequence and structural homologue of MHC class I molecules proposed to be involved in the silencing of natural killer cells by binding

(a) List of currently known antigenic peptides of mCMV

ORF	Replication phase	Peptide sequence	MHC class I restriction	Reference
m04	E	^{243}YGPSLYRRF^{251}	D^d	Holtappels *et al.* (2000a)
m18	E	^{346}SGPSRGRII^{254}	D^d	This report
M83	E/L	^{761}YPSKEPFNF^{769}	L^d	Holtappels *et al.* (2001)
M84	E	^{297}AYAGLFTPL^{305}	K^d	Holtappels *et al.* (2000b)
m123ex4 (ie1)	IE	^{168}YPHFMPTNL^{176}	L^d	Reddehase *et al.* (1989)
m164	E	^{257}AGPPRYSRI^{265}	D^d	Holtappels *et al.* (2002)

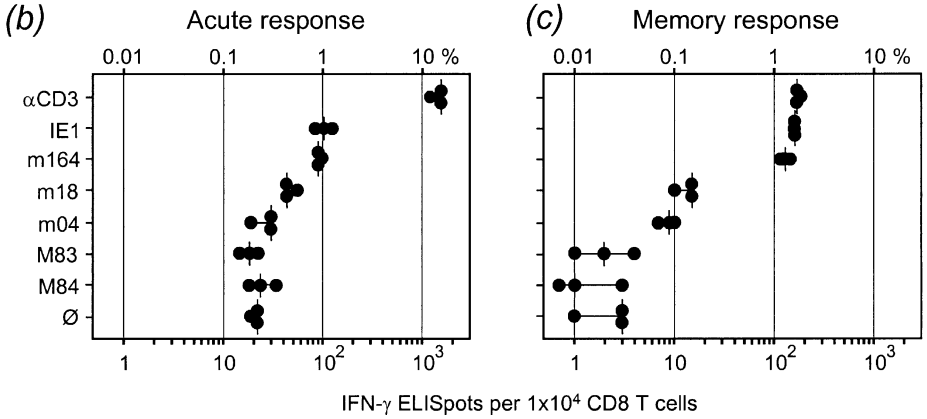

Fig. 3. Contribution of the m18 peptide to the CD8 T cell response. (a) Compilation of antigenic peptides of mCMV known currently. IE, immediate-early; E, early; L, late. Gene *M83* is transcribed in the E phase, but the protein is detected in significant amounts only in the L phase (Cranmer *et al.*, 1996). (b) Frequencies of acutely sensitized CD8 T cells present in the draining popliteal lymph node on day 8 after intraplantar mCMV infection (1×10^6 p.f.u.). IFN-γ-based ELISPOT assays were performed with immunomagnetically purified CD8 T cells. P815-B7 stimulator cells were pulsed with a saturating dose (10^{-8} M) of the indicated mCMV peptides. αCD3, polyclonal stimulation with 145-2C11 hybridoma cells that produce monoclonal antibody directed against mouse CD3ε; ∅, P815-B7 cells with no peptide added; ●, data from triplicate assay cultures. Median values are marked by vertical bars. (c) Frequencies of memory CD8 T cells present in the spleen at 8 months after intraplantar mCMV infection. Details are as in (b).

to inhibitory receptors (Reyburn *et al.*, 1997). Recently, it has also been identified as a ligand of the leukocyte immunoglobulin-like receptor LIR-1 (Chapman *et al.*, 1999; reviewed by Cosman *et al.*, 1999). However, the mCMV *m18* gene is not a sequence homologue of cellular MHC class I or hCMV *UL18* [indicated by the lowercase letter 'm', according to the nomenclature used by Rawlinson *et al.* (1996)].

So far, nothing is known about the biological role of the ORF*m18* protein in the virus life cycle. According to structure prediction algorithms, the protein is largely coiled and does not possess membrane-spanning α-helices (PredictProtein PHDhtm; Rost *et al.*, 1996).

The expression kinetics of the *m18* gene in productively infected foetal fibroblasts was studied by RT–PCR performed with purified poly(A)$^+$ RNA using oligo(dT) priming for reverse transcription. Map positions of the *m18* gene as well as PCR primers and probe for the detection of *m18* cDNA are illustrated in Fig. 2(a). The absence of transcripts after infection in the presence of actinomycin D confirmed that *m18* RNA is not virion RNA (Bresnahan & Shenk, 2000). Newly synthesized mRNA was detected first at 2 h post-infection. As indicated by sensitivity to cycloheximide, this transcription required preceding IE protein synthesis, whereas insensitivity to phosphonoacetic acid showed its independence of viral DNA replication. All in all, these data clearly identified gene *m18* as an E gene (Fig. 2b).

Finally, we asked for the quantitative contribution of the newly identified m18 peptide to the priming of an mCMV-specific immune response and to the establishment of CD8 T cell memory (Fig. 3). As a measure of this contribution, we determined the frequencies of immunomagnetically purified CD8 T cells responding in interferon (IFN)-γ-based ELISPOT assays. The total number of responsive cells was determined by polyclonal stimulation via the signal-transducing CD3ε molecule of the T cell receptor–CD3 complex (CD3ε-redirected ELISPOT assay; Holtappels *et al.*, 2001). Frequencies of mCMV-specific CD8 T cells were measured by stimulation with the antigenic peptides of mCMV known currently (listed in Fig. 3a). The primary immune response to mCMV was analysed in the draining popliteal lymph node on day 8 after

intraplantar infection (Fig. 3b). Memory was assessed for the CD8 T cell population in the spleen during latent infection (Reddehase *et al.*, 1994) at 8 months after priming (Fig. 3c). In accordance with previous results (Holtappels *et al.*, 2002), peptides IE1 and m164 co-dominated the immune response to mCMV during acute infection as well as during the memory state after clearance of productive infection. As discussed recently in greater detail (Holtappels *et al.*, 2002), the memory 'immunome' of mCMV, which is the repertoire of antigenic peptides involved in immunological memory, is more focused than the acute response immunome. 'Bystander activation' of CD8 T cells (Tough *et al.*, 1996; McNally *et al.*, 2001) with specificities for peptides unrelated to mCMV is likely to contribute to the gap between CD3ε-reactive cells and mCMV peptide-specific cells during the acute response. The new information is that the m18 peptide takes the third rank in immunogenicity among the antigenic peptides of mCMV known currently. Notably, while CD8 T cells specific for peptides IE1 and m164 are enriched in the memory cell pool during latency (Holtappels *et al.*, 2000c, 2002), this is apparently not the case for m18-specific CD8 T cells (Fig. 3c).

It was surmised previously that MHC allele preferences contribute to the immunodominance of peptides. Specifically, the finding that immunodominant peptides are frequently presented in the *H-2*^{d} haplotype by the L^d molecule was explained by a constitutively low loading of L^d with self peptides providing a higher number of accessible binding sites for foreign peptides (Lie *et al.*, 1991). The list of antigenic peptides of mCMV now available allows us to revisit that view. At least, there is no strict correlation between the presenting MHC molecule and peptide immunogenicity: L^d and D^d both present dominant as well as subdominant peptides of mCMV.

In conclusion, we have identified pORF*m18* as a further E protein of mCMV that contributes significantly to the CD8 T cell response to infection in spite of E phase immune evasion mechanisms. Thus, it appears that the prevention of antigenic peptide presentation by the 'immune evasion' proteins of mCMV, so far documented only for the presentation of the IE1 peptide in fibroblasts, represents an exception rather than the rule.

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