Cytomegalovirus selectively blocks antigen processing and presentation of its immediate–early gene product

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Recognition of virus-infected cells by CD8⁺ cytotoxic T lymphocytes requires that the viral proteins be processed into peptides, the derived peptides transported into the endoplasmic reticulum and inserted into the binding groove of a major histocompatability complex class I molecule, and the antigenic complex exported to the cell surface[1]. However, viral pathogens can disrupt this process and interfere with immune recognition[1–4]. These mechanisms may be vital to large viruses such as human cytomegalovirus (CMV), which causes persistent infection despite producing over 200 potentially antigenic proteins during the sequential immediate–early, early and late phases of viral gene expression[5,6]. Products of CMV early-phase gene expression can globally block class I presentation[7–10] and prevent recognition of infected cells by cytotoxic T lymphocytes, but an essential viral transcription factor, the 72K principal immediate–early protein, is abundantly expressed before this blockade. However, only a few host CD8⁺ cytotoxic T lymphocytes specific for immediate–early protein are present in seropositive individuals, and these lyse CMV-infected cells poorly[11]. Here we demonstrate selective abrogation of immediate–early peptide presentation by a CMV matrix protein with associated kinase activity and suggest that modification of a viral protein can result in limiting access to the processing machinery and evasion of cytotoxic-T-cell recognition.

Immediate–early-peptide(IE)cytotoxic lymphocytes (CTLs) efficiently lyse autologous cells infected with recombinant vaccinia virus encoding this protein (vac/IE) but not cells infected with CMV, although both targets express the protein[11]; this could be the result of an inhibitory effect by other CMV proteins or of help from vaccinia proteins in the recombinant virus. We therefore infected autologous fibroblast targets with either a single virus or with combinations of CMV, vac/IE, or a control virus vac/pp150 (encoding the CMV pp150 matrix protein)[12]. IE-specific CTLs lysed targets infected with vac/IE alone but failed to lyse CMV-infected targets or targets co-infected with CMV and vac/IE, despite comparable levels of IE expression (Figs 1*a*, 2*a*). By contrast, CTLs specific for pp150 lysed with equal efficiency targets infected with vac/pp150 or CMV alone, or targets co-infected with vac/pp150 and CMV or vac/IE and CMV (Fig. 1*a*). Thus, a CMV protein appears selectively to interfere with IE presentation, but not with presentation of other viral proteins.

Several CMV virion matrix proteins are introduced into the cell during viral entry and are detectable before IE gene expression starts[12–14]. CMV-infected cells treated sequentially with metabolic inhibitors of translation and transcription to permit expression of only IE genes are efficiently lysed by CTLs specific for introduced matrix proteins but not those specific for IE, suggesting that virion proteins are candidates for inhibiting presentation of IE (data not shown)[15,16]. As the most abundant CMV matrix proteins are pp150 and pp65 (ref. 17), we co-infected fibroblasts with combinations of vac/IE, vac/pp150, vac/pp65 and wild-type vaccinia to assess blockade of IE presentation[11,12]. We found that IE-specific CTLs lysed cells co-infected with vac/IE and either vaccinia or vac/pp150, but not cells co-infected with vac/IE and vac/pp65 (Fig. 1*b*). By contrast, CTLs specific for pp65 lysed targets co-infected with vac/IE and vac/pp65, and pp65 expression did not interfere with lysis of pp150-expressing targets by pp150-specific CTLs (Fig. 1*b*). This selective inhibition by pp65 was not limited to a single IE epitope as other IE-specific CTL clones, which were restricted to A24, B18, A2, B8, B35 and B57 and recognized epitopes dispersed throughout the 491-amino-acid IE protein[16,18–20], also failed to recognize IE if pp65 was co-expressed in target cells (data not shown). Moreover, pp65 did not interfere in co-infection experiments with presentation to CTL of a CMV envelope protein, gB, or a human immunodeficiency virus-1 structural protein, p55 Gag (data not shown). The effect of pp65 did not result from modification of IE protein expression or stability, because IE synthesis and degradation rates were equivalent in fibroblasts infected with either vac/IE or CMV alone, or co-infected with either vac/IE and vac/pp65 or vac/IE and vac/pp150 (Fig. 2*a*, *b*).

To determine whether the small amount of pp65 introduced with the CMV virion is sufficient to interfere with IE presentation, a pp65-deletion mutant of CMV strain AD169 (RVAd65) was constructed. This mutant contains a matrix devoid of pp65 but expresses IE in amounts comparable to wild-type CMV[21]. Fibroblasts were infected with wild-type and RVAd65 CMV virus in the

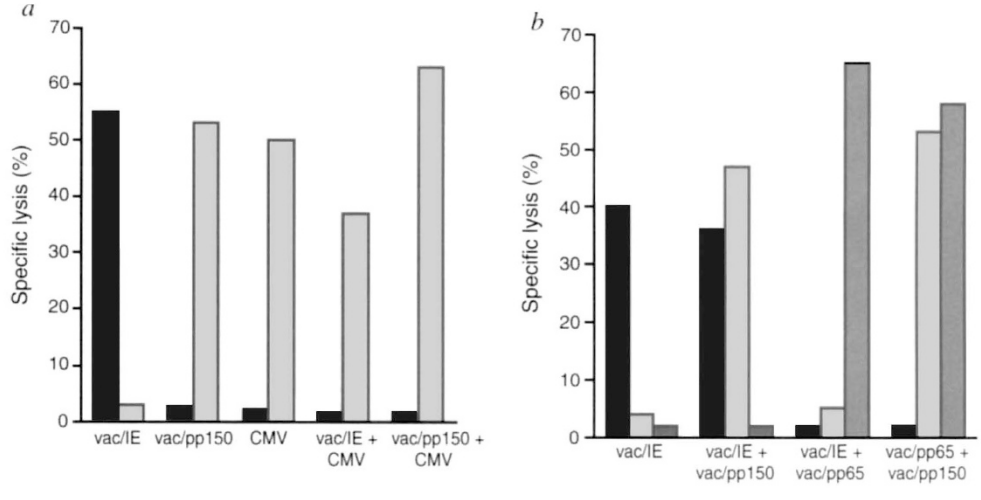

FIG. 1 *a*, Class-I-restricted presentation of IE protein in cells expressing IE alone or in the presence of other CMV proteins. Autologous fibroblasts were infected with either vac/IE, vac/pp150 or CMV alone or co-infected with vac/IE and CMV or vac/pp150 and CMV, and analysed for cell lysis by CTL clones specific for IE (TM2A3; black bars) or pp150 (TM3C11; grey bars) in a 5-h chromium-release assay[11]. *b*, Class-I-restricted presentation of IE in the presence or absence of matrix proteins pp65 and pp150. Fibroblasts were co-infected with combinations of vaccinia viruses and analysed for cell lysis by CTL clones specific for IE (TM2A3; black bars), pp150 (TM3C11; light grey bars) or pp65 (CM7C3; dark grey bars)[22]. Data are presented at an effector:target (E:T) ratio of 10:1; similar results were obtained at an E:T ranging from 5:1 to 20:1.

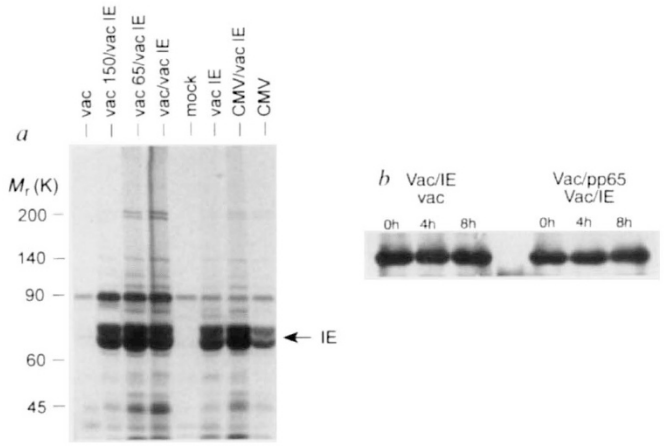

FIG. 2 *a*, IE protein expression in targets expressing IE alone or in the presence of other CMV proteins. Fibroblasts were infected with CMV and vaccinia viruses, radiolabelled with ^{35}S-methionine, immunoprecipitated with IE-specific antibody, and analysed for IE protein expression by SDS–PAGE. *b*, Comparison of IE degradation rate in the presence and absence of pp65. Cells were co-infected with either vac/IE and vac or vac/IE and vac/pp65, pulsed with ^{35}S-methionine, then incubated for the indicated times after addition of unlabelled methionine; cell lysates were then immunoprecipitated with antibody specific for IE and analysed by SDS–PAGE.

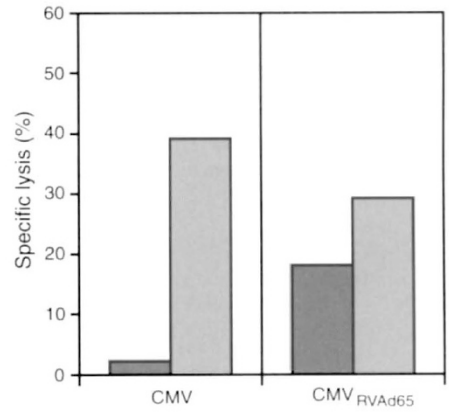

FIG. 3 IE-protein presentation in cells infected with a mutant CMV bearing a deletion in the pp65 gene. Fibroblasts were infected with either wild-type CMV or CMV RVAd65 lacking the *UL83* gene encoding pp65, under conditions in which metabolic inhibitors permit new transcription and translation of only viral IE genes, and then evaluated for lysis by CTLs specific for IE (dark grey bars) or pp150 (light grey bars) at E:T of 10:1.

presence of metabolic inhibitors of translation and transcription added sequentially to permit introduction of viral matrix proteins but new expression of only IE genes products[11,12]. IE-specific CTLs recognized targets infected with RVAd65 but did not lyse CMV-infected targets (Fig. 3). By contrast, CTLs specific for the introduced pp150 matrix protein lysed targets infected with either virus.

pp65 expression results in phosphorylation of several CMV proteins[21], and pp65 isolated from infected cells phosphorylates *in vitro* the casein kinase II substrates phosvitin and casein[20,22,23], so pp65 or a cellular kinase that interacts with pp65 might modify IE by phosphorylation. The carboxy terminus of pp65 contains conserved sequences of serine/threonine kinases[22], and a recombinant vaccinia vector was constructed to contain a mutant pp65 (vac/Δ65) with 174 base pairs (bp) deleted, including the putative ATP-binding site and the invariant lysine required for phosphotransfer reaction[22]. After infection of cells with vac/Δ65, the immunoprecipitated mutant protein did not show any kinase activity *in vitro* (data not shown). Fibroblasts were co-infected with vac/IE and vac, vac/IE and vac/pp65, or vac/IE and vac/Δ65, and the expressed IE proteins immunoprecipitated. IE protein expression and tyrosine phosphorylation were similar in cells co-infected with vac/IE and either vac/pp65 or vac/Δ65 (Fig. 4*a*). No serine-phosphorylation of IE was detected in any of these cells (data not shown). However, expression of full-length pp65, but not Δ65, resulted in increased threonine-phosphorylation of IE (Fig. 4*a*). These targets were also analysed for lysis by IE-specific CTL clones and by a CTL clone specific for an epitope on pp65 present in both the wild type and Δ65 mutant. Co-infected targets expressing either pp65 or Δ65 were lysed by pp65-specific CTLs, but the Δ65 deletion mutant (in contrast to wild-type pp65) did not prevent IE presentation (Fig. 4*b*).

These results show that structural viral proteins released into the cytosol after virion entry can modify presentation of endogenously synthesized viral antigens, and that presentation of a viral protein can be selectively blocked at a processing step before peptide loading, without general blockade of class I antigen presentation in the infected cell. In the case of CMV, pp65 expression results in increased phosphorylation of IE in threonine residues and interferes with IE presentation without detectable changes in either the stability of the IE protein or its ability to

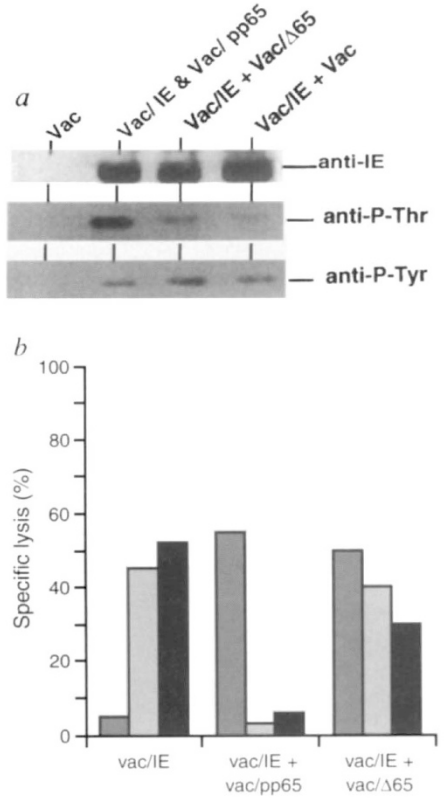

FIG. 4 *a*, Phosphorylation of IE protein in the presence and absence of pp65 and mutant pp65. Fibroblasts were infected with recombinant vaccinia viruses, immunoprecipitated with IE-specific antibody, transferred to nitrocellulose, and probed with antibodies specific for IE, phosphothreonine or phosphotyrosine. *b*, Presentation of IE in association with two class-I-restricting elements in cells co-expressing IE and either pp65 or Δ65. Fibroblasts expressing HLA-A24 or HLA-B18 were co-infected with recombinant vaccinia viruses and analysed for lysis by CTLs specific for pp65 (CM7C3; dark grey bars) or CTLs specific for IE (TM2A3, light grey bars; MR3H9, black bars) in a standard chromium-release assay. Data are presented at an E:T of 10:1.

activate transcription[21,24]. This suggests that phosphorylation of IE residues may either severely restrict access of the protein to the antigen-processing machinery or divert the protein to a different degradative pathway, providing an evasion strategy that could be crucial for CMV persistence. Similar modification of other viral or cellular proteins may regulate the access of protein antigens to processing/presentation for recognition by CTL. □

Methods

Viruses. CMV strain AD169 was obtained from the American Tissue Culture Collection[11]; the mutant AD169 strain, RVAd65, with a deleted *UL83* gene, was constructed by B. Plachter[21]. Vac/IE, a recombinant vaccinia virus encoding the major immediate-early protein of CMV strain AD169, was obtained from E. Paoletti[11,12]. Vac/pp150, a recombinant vaccinia virus encoding the gene for CMV pp150 was constructed by homologous recombination between vaccinia strain WR and DNA from a plasmid, pSC11, encoding the full-length pp150 gene. Vac/pp65, a recombinant vaccinia virus encoding CMV pp65 of CMV strain AD169, was obtained from W. Britt. Vac/Δ65 contains a mutant pp65 deleted of a 174-base segment between base pairs 1,194 and 1,368, which was constructed by digestion first with *Afl*II, then mung-bean nuclease, then with *Tth*III, after which protruding ends were filled in using Klenow polymerase and blunt-end ligation of the 3′-coding sequence in-frame[25]. The Δ65 mutant gene was inserted into vaccinia by homologous recombination[11–14].

T cells. T-cell clones specific for IE (TM2A3 restricted to HLA-A24 and MR3H9 restricted to HLA-B18), pp65 (CM7C3 restricted to HLA-B35) and pp150 (TM3C11 restricted to HLA-A24) were isolated from CMV-seropositive individuals as described[11,12,15]. Cytolytic function was assessed by 5-hour chromium release assay using low-passage fibroblasts from donor M.R. (HLA: A24, 25; B18, 35), infected for 12 h with combinations of recombinant vaccinia viruses at a multiplicity of infection (MOI) of 10, CMV at MOI 5, or ΔCMV at MOI 25 (ref. 11).

Radioimmunoprecipitation. 10^6 fibroblasts were either mock-infected, infected with CMV, or co-infected with CMV and vac/IE, vac/IE and vac, vac/IE and vac/pp65, or vac/IE and vac/pp150. After 12 h, cells were incubated with 200 μCi ^{35}S-methionine per 10^6 cells for 30 min, detergent-lysed by Nonidet P-40, immunoprecipitated with an IE-specific monoclonal antibody 6E3 (ref. 26) and staphylococcal protein A cells, fractionated by SDS–PAGE, and analysed by autoradiography[11].

Pulse–chase experiments. Human fibroblasts (10^6) were infected for 12 h with vac/IE and vac, or vac/IE and vac/pp65, and radiolabelled with ^{35}S-methionine. Following a 30-min pulse, infected fibroblasts were washed with Dulbecco's MEM medium with 10% fetal calf serum and unlabelled methionine and either lysed and immunoprecipitated with anti-IE monoclonal antibody (6E3)[26] (time point '0')[11] or incubated for a further 4 or 8 h before lysis and immunoprecipitation.

Sequential metabolic blockade of translation and transcription of CMV-infected cells. Fibroblasts were pretreated for 30 min with 50 μg ml^{-1} cyclohexamide to block translation, infected with wild-type CMV (AD169) or mutant CMV lacking the *UL83* gene encoding pp65 (RVAd65)[21] at MOI 25. Four hours after cyclohexamide exposure, cells were washed with PBS containing 100 μg ml^{-1} actinomycin D to block transcription and then incubated for 3 h in medium containing actinomycin D to block transcription but permit translation of existing IE mRNA. Cells were washed twice with PBS containing actinomycin D before chromium-release assay[12].

Immunoblots for IE, phosphothreonine, phosphotyrosine and phosphoserine. 10^6 late-passage fibroblasts were infected at MOI 10 with vac/IE and vac, vac/IE and vac/Δ65, vac/IE and vac/pp65, or with vac alone as control, incubated for 12 h, and then lysed and immmunoprecipitated with the IE-specific monoclonal antibody (mAb) 810 (Chemicon) as already described. Samples were fractionated on 10% SDS–PAGE, transferred to nitrocellulose, and analysed by immunoblotting for IE protein and for phosphorylation on serine, threonine and tyrosine residues, with monoclonal antibodies 810, P-3555 (specific for phosphothreonine residues; Sigma), P-3300 (specific for phosphothreonine residues; Sigma), and P-3430 (specific for phosphoserine; Sigma). Blots were enhanced with goat anti-mouse secondary antibody (Biosource), and chemiluminescence reagent (NEN), and analysed by autoradiography.

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Bidirectional signalling through the EPH-family receptor Nuk and its transmembrane ligands

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RECEPTOR tyrosine kinases of the EPH class have been implicated in the control of axon guidance and fasciculation[1–7], in regulating cell migration[8], and in defining compartments in the developing embryo[9–11]. Efficient activation of EPH receptors generally requires that their ligands be anchored to the cell surface, either through a transmembrane (TM) region or a glycosyl phosphatidylinositol (GPI) group[12]. These observations have suggested that EPH receptors can transduce signals initiated by direct cell–cell interaction. Genetic analysis of Nuk, a murine EPH receptor that binds TM ligands, has raised the possibility that these ligands might themselves have a signalling function[6]. Consistent with this, the three known TM ligands have a highly conserved cytoplasmic region, with multiple potential sites for tyrosine phosphorylation[12–17]. Here we show that challenging cells that express the TM ligands Elk-L or Htk-L with the clustered ectodomain of Nuk induces phosphorylation of the ligands on tyrosine, a process that can be mimicked both *in vitro* and *in vivo* by an activated Src tyrosine kinase. Co-culture of cells expressing a TM ligand with cells expressing Nuk leads to tyrosine phosphorylation of both the ligand and Nuk. These results suggest that the TM ligands are associated with a tyrosine kinase, and are inducibly phosphorylated upon binding Nuk, in a fashion reminiscent of cytokine receptors[18]. Furthermore, we show that TM ligands, as well as Nuk, are phosphorylated on tyrosine in mouse embryos, indicating that this is a physiological process. EPH receptors and their TM ligands therefore mediate bidirectional cell signalling.

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