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**IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF HUMAN CYTOMEGALOVIRUS
SURFACE EXPOSED GLYCOPROTEINS**

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*“Quando ci si trova davanti un ostacolo,
la linea più breve tra i due punti,
può essere una linea curva”*

Berthold Brecht

A Mirko, Marc e Giuli

Summary

The reverse vaccinology approach to the human cytomegalovirus (HCMV) genome, one of the largest known mammalian viral genome with a coding potential of more than 166 proteins, led to the identification of 94 potential membrane-associated or secreted proteins. Many of the selected proteins have no assigned functions but share a high degree of conservation among different strains. These designed ORFs have been synthesized as eukaryotic codon-optimized sequences in frame with both hexa-histidine tail and c-myc tag coding elements. A more detailed analysis was performed on a certain number of these tagged proteins in both epithelial and fibroblast human derived cell lines, natural targets of HCMV. As starting point 12 hCMV proteins with antigenic properties were chosen for further characterization in terms of structural and functional features.

This work was aimed to the identification and functional characterization of two new HCMV surface exposed glycoproteins.

The study is divided in two parts: first the identification and characterization of ORFX*; second the identification and characterization of a novel HCMV Fc γ binding protein. Regarding the first part, we focused our attention on ORFX gene product, a putative ORF identified by in silico analysis. Biochemical analysis on both ORFX viral protein product or transient expressed single gene allowed us to conclude that ORFX is: a) heavily glycosylated protein; b) expressed as late product during infection; c) present in the assembly complex, the cellular site of virus assembly; d) present on the HCMV virion envelope and e) secreted in transient expression as by in silico prediction. These observations led us to search for ORFX partners among other HCMV envelope exposed glycoproteins. We found that gH was efficiently transported to plasma membrane only when co-expressed with ORFX. Moreover, co-expression of gH (and gH/gL) with ORFX and co-immunoprecipitation experiments, carried on both transfected and infected cells, showed that ORFX is part of a gH-based complex. These results suggest that ORFX could function as both gH “escort” protein but also be part of a cellular recognizing complex and invite to a deeper analysis of both its functions and immunological properties.

Regarding the second part of the study, we were able to identify two novels Fc γ binding protein coded by CMV: RL12 and RL13. The latter was also further characterized as recombinant protein in terms of cellular localization, Fc binding site and IgG internalization ability.

*Names and protein sequences are, actually, under revision of NVD Intellectual property office

Riassunto

L'approccio di Reverse vaccinology applicato all'intero genoma del cytomegalovirus umano (HCMV), uno dei genomi più estesi all'interno della famiglia degli herpesviruses umani, ha portato all'identificazione di 94 ORF predette come codificanti per proteine di membrana o proteine secrete. La gran parte delle ORF selezionate non ha alcuna funzione assegnata pur rimanendo altamente conservata in tutti i ceppi conosciuti di HCMV. Tali ORF sono state inserite in vettori d'espressione eucariotici aggiungendovi una porzione C-terminale codificante per i tags His e myc. L'analisi dettagliata riguardante le proprietà antigeniche dei prodotti proteici derivanti dalla trasfezione in cellule epiteliali e fibroblasti umane dei plasmidi in questione ha prodotto la selezione di 12 proteine virali.

Questo lavoro di tesi è stato incentrato sull'identificazione e caratterizzazione funzionale di due nuove glicoproteine presenti sulla membrana esterna di HCMV.

Lo studio è diviso in due parti: in prima analisi, l'identificazione e la caratterizzazione di ORFX*; in seconda analisi l'identificazione e caratterizzazione di RL13 come Fc binding protein.

Riguardo la prima parte del lavoro, l'attenzione è stata focalizzata sul prodotto genico di ORFX inizialmente identificata solo attraverso analisi *in silico*. Analisi biochimiche su ORFX prodotta in infezione e talvolta in trasfezione di cellule umane hanno portato i seguenti risultati: 1) ORFX è una proteina estesamente glicosilata 2) Viene prodotta nelle fasi tardive di espressione lungo il ciclo di replicazione virale, 3) è presente nel sito di assemblaggio virale (Assembly complex) in cellule infettate da HCMV, 3) è esposta sulla sua membrana esterna del virus, 4) Viene secreta dalle cellule che la esprimono in maniera transiente come da predizioni. Queste osservazioni ci hanno guidato verso l'idea di cercare dei possibili partner proteici tra quelli conosciuti come presenti sull'envelope esterno virale. A tal proposito abbiamo riscontrato che gH veniva trasportato nella membrana plasmatica cellulare solo quando co-espressa con ORFX e viceversa. La Co-immunoprecipitazione di ORFX e gH da lisati di cellule infettate e trasfettate con i singoli geni ha rivelato la diretta interazione tra le due proteine identificando ORFX come parte di un complesso "gH based". Questi risultati delineano un'ipotetica funzione di ORFX come proteina potenzialmente implicata nell'"escort" di gH e talvolta parte di un complesso virale possibilmente riconosciuto dall'host.

Riguardo la seconda parte del lavoro di tesi, i risultati sono riguardanti l'identificazione due nuove "Fc binding proteins" codificate da HCMV :RL 12 e RL13. Quest'ultima è stata sottoposta a caratterizzazione biochimica nella sua forma ricombinante in termini di localizzazione intracellulare, studio del sito di legame per le Fc e abilità della proteina a promuovere l'internalizzazione cellulare delle IgG nel contesto dei meccanismi virali di immuno-evasione.

*Nomi e sequenze proteiche sono attualmente sotto la revisione dell'ufficio brevetti NVD

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INTRODUCTION

1. Human Cytomegalovirus pathogenesis

Human Cytomegalovirus (HCMV) is a member of the β -herpesviridae family that includes the herpesviruses of mammals, birds and reptiles [1]. Human Cytomegalovirus (HCMV) is ubiquitous, infecting as many as 85% of human adults. HCMV establishes latency and persists, replicating even in the face of robust host immunity through effects of numerous immune evasion strategies (reviewed in [1]). HCMV infections are normally relatively benign, but immunocompromised individuals can experience severe disease. Infants infected in utero can be born with disseminated HCMV disease or be damaged in terms of neurologic development (reviewed in [2]). Among the cell types infected are placental trophoblasts, which spread virus from mother to baby, and neurons and glial cells in the developing nervous system. Transplant patients do not control HCMV well and experience infection in gut epithelial cells, liver hepatocytes, monocyte-macrophages that disseminate virus to endothelial cells and smooth muscle cells which can accelerate transplant rejection [1]. HCMV is also a major opportunistic pathogen in AIDS patients, frequently infecting retinal epithelial cells and causing blindness. Nevertheless complete clearance of the virus by the organism is never achieved and lifelong lasting latency with recurrent and spontaneous viral reactivation is always observed. Thus in the presence of an impaired or absent immune system, antiviral therapies are necessary to counteract the severe disease associated to the HCMV primary infection or reactivation. However almost all the antiviral agents actually on the market showed a significant number of potential side effects including marrow and organ toxicity and increasing drug resistant viral strains [1]. Passive immunization using hyperimmune globulin preparations with high levels of antibodies against HCMV (CMVIG) are usually implemented as prophylactic tools to reduce the morbidity and mortality associated to HCMV infection in immunocompromised patients. For example combination of antiviral drug gancicovir and CMVIG proved to be effective in preventing HCMV infection in patients undergoing solid organ transplant [29]. Moreover a study suggests their effectiveness in the treatment and prevention of severe diseases associated to congenital HCMV infection [30]. Nevertheless, rather than a general prophylactic tool, the efficacy and effectiveness of CMVIG in reducing HCMV infection seems to be restricted to specific clinical cases while only modest beneficial effects were shown upon CMVIG treatment in other settings such as hematopoietic cell transplantation (HCT) and blood transfusion in premature newborns [31]. Additionally CMVIGs exhibit low potency in vitro [32] and must be administrated at high

doses to reach the amount of neutralizing antibodies required to exert a beneficial effect. To increase the efficacy of a passive immunization therapy, a series of neutralizing monoclonal antibodies against immunodominant HCMV envelope glycoproteins were developed. For example, a highly in vitro neutralizing antibody against gH was isolated from the spleen of a CMV seropositive patient. The monoclonal antibody, named MSL-109 or sevirumab, was tested in phase II clinical trials as treatment for CMV-induced retinitis in AIDS patients [33]. Unfortunately the tests were stopped due to lack of efficacy [34].

2. HCMV structure and genome organization

Human Cytomegalovirus is a β -herpesvirus. Although more recently validated by genome sequences, historically the members of the family were classified on the basis of the virion structure, composed of an envelope, tegument, capsid and an inner core containing the viral genome. The virus envelope is composed of a double host cell-derived lipid layer and contains the viral glycoproteins necessary for the cellular tropism. Compared to the other members of the family, the envelope is very irregular and generates a viral shape whose diameter reaches up to 300 nm with an average size of 230 nm diameter. Under the envelope is present an amorphous matrix referred as tegument. This compartment carries the majority of the HCMV expressed proteins plus a huge number of host species including proteins and nucleic acids. Several crucial viral functions are incorporated in the tegument including kinases, trans-activating factors and the most abundant and immunogenic protein pp65. The HCMV capsid is embedded in the tegument. The core of the capsid contains a tightly packed linear double stranded DNA genome. In line with the huge virus dimension, HCMV has the largest genome among its family with approximately 230 kb, 50% larger than HSV, despite the capsids of both viruses being roughly of the same size (110-125 nm) [1]. As for the other herpesviruses, genome organization reflects the common structure composed of unique long (UL) and unique short (US) genetic regions flanked by two sets of inverted repeats (RL, repeated long and RS, repeated short). Recombination phenomenon can occur among identical terminal and internal repeats, leading to genome isomerization.

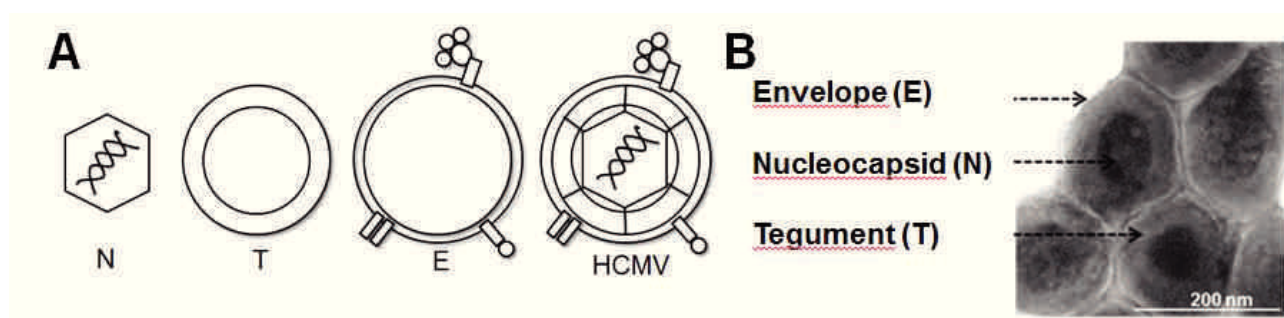


Fig. 1: **A.** Schematic representation of HCMV dissected virion **B.** Negative stained purified virion preparation analyzed through electron microscopy.

Thus, genetic material isolated from a viral population consists of equal amounts of four different genomic isomers pooled together [2]. While the general genomic arrangement consistent of repeated and unique sequences is conserved among HCMV strains, a major difference in the open

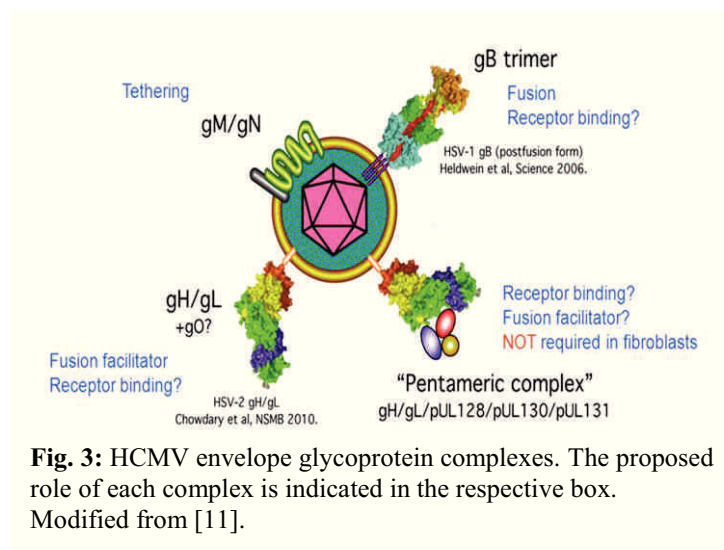
reading frame (ORF) composition and organization can be observed among “laboratory strains” and “clinical isolates”. The latter are generally defined as viruses that underwent through none or limited passages in fibroblast cells before being clones as bacterial artificial chromosomes (BACs) and/or sequenced. On the contrary, laboratory strains indicate all the strains extensively passaged and adapted to growth in human fibroblasts. These latter have been selected for rapid replication, fast growing and high yields of produced virus. Consequently to the fibroblast adaptation severe genomic rearrangements occurred in these viruses. Both AD169 and Towne laboratory strains acquired a large deletion in a multilocus of the UL segment, concomitantly being replaced by duplicated RL region. An impaired tropism, tightly restricted to fibroblast cells only, resulted from the abrogation of several envelope glycoproteins encoded in the deleted segment. Due to these rearrangements, a difference in the coding potential can be observed between laboratory strains and clinical isolates. In particular, while the AD169, a laboratory strain prototype, is predicted to encode for 208 ORFs (including the repeated segments), the coding potential of a clinical isolate is estimated around 252 ORFs [3]. Recently, through an integrated approach that combines ribosome footprinting assays, high-resolution mass spectrometry and analysis of mutated viruses, Stern-Ginossar and co-workers were able to identify a total of 751 translated ORFs including short peptides [4]. In spite of this huge coding potential, only a small subset of these proteins constitutes the mature virion: it has been estimated that around 50 [1,5] proteins are inserted in the virion, divided through the different compartments.



Fig. 2: HCMV genome organization. ORFs map of conventional laboratory strain AD169 and clinical isolate. The AD169 genome (upper) carries TRL1-14 (green arrow), UL1-132 (dark blue arrow), IRL14-1 (green arrow), IRS1 (red arrow), US1-36 (light blue arrow), and TRS1 (red arrow). In the clinical isolate RL1-14, (green arrow), UL1-151 (dark blue arrow), IRS1 (red arrow), US1-36 (light blue arrow), and TRS1 (red arrow) are present. Modified from [3].

3. HCMV Envelope glycoproteins

The HCMV genome includes over 230 genes that encode 54 membrane proteins at least 25 membrane glycoproteins found in the virion envelope [3–7]. Being the modulator of viral entry, envelope glycoproteins play a key role in HCMV pathogenesis. Of the total number of proteins composing the virion, around 20 [1,5–7] were identified as envelope associated proteins. Notably, only 8 were demonstrated as essential for virus replication *in vitro* in different cell types: glycoproteins B (gB), M (gM), N (gN), L (gL), H (gH), UL128, UL130, UL131 [8]. All these proteins act as complexes on the viral surface. gB is an homotrimeric complex mediating virus attachment and fusion, involved in the first step of heparin sulfate proteoglycans (HSPG) tethering. The most abundant envelope glycoprotein, gM, forms an heterodimeric complex associating with gN that is present on the virion only as 1:10 ratio to gM. Like gB, gM:gN complex is involved in HSPG tethering and stabilization of virus-cell contact. The complex that has been involved in the fusion event is composed by the glycoprotein gH and gL. This complex is sufficient to mediate fibroblast infection while formation of a pentameric complex through additional binding of UL128, UL130 and UL131 gene products is necessary for the infection of epithelial, endothelial, polymorphonuclear leukocyte and dendritic cells (DC). Indeed, the attenuated tropism observed in laboratory strains can be fully explained by the mutations of the UL128-UL131 locus [3,9,10].



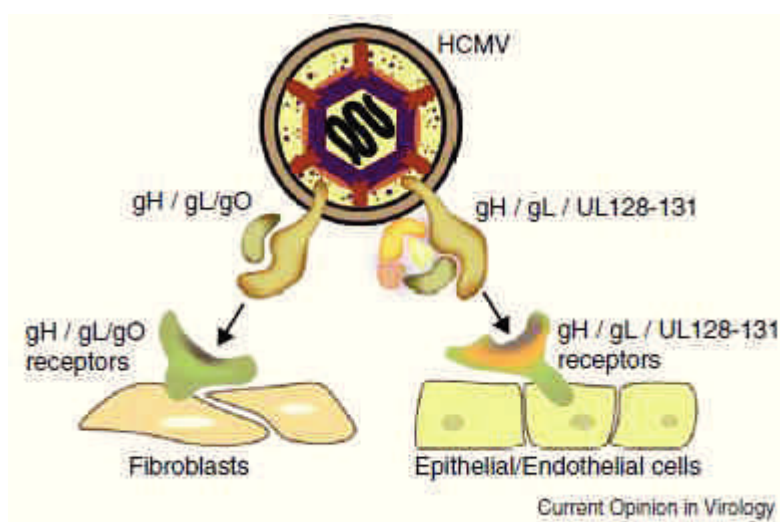
Various works describing the HCMV entry step suggest that the virus uses distinct cellular receptors, and consequently different entry pathways, depending on the target cell. HCMV entry consists of a first low specificity - high avidity tethering step of the cell surface HSPGs mediated by both gB and gM/gN [11,12]. The interaction with high avidity receptors has been shown to follow the first attachment

step. A series of “post-attachment” receptors were proposed over the time, even if none of them completely fulfilled this role. The most accredited cellular interactors are $\beta 1$ and, to a lesser extent, $\beta 3$ integrins. Engagement of gB by these molecules induce receptors clustering and intracellular signaling. The entry step culminates with gH/gL mediating the fusion of viral and cellular membranes. While direct fusion at neutral pH has been observed *in vitro* in fibroblast cells [13], low pH dependent receptor-mediated endocytosis is required for viral entry in epithelial and endothelial

cells [14–16]. Moreover, a recent report suggests that HCMV entry into DCs relies on macropinocytosis-like pathway in a cholesterol-dependent and pH-independent manner [17]. Interaction between gB and dendritic cell specific C-type lectin DC-SIGN (DC specific ICAM-grabbing non-integrin) has been indicated to have a prominent role in DCs infection [18].

3.1 gH/gL/gO and gH/gL/UL128-131

HCMV gH/gL assembles with a third glycoprotein, gO, forming a disulfide linked trimer [26]. Studies of HCMV laboratory strain AD169 found gH/gL/gO in the virion envelope [26,27]. gO-null mutants produced small plaques in fibroblasts and titers <1000 lower than wild type HCMV [8,9,28,29]. A distinct HCMV gH/gL complex denoted gH/gL/UL128-131 is formed by the binding of three proteins UL128, UL130 and UL131. Recognition of the existence of gH/gL/UL128-131 began with observations that HCMV laboratory strains, for example AD169 and Towne, were passaged many times in fibroblasts and no longer infected epithelial and endothelial cells and monocyte-macrophages. Sequencing of these lab HCMV showed that there were mutations in a block of 22 genes extending from UL128 to UL151 [5]. Repair of just the UL128, UL130 and UL131 genes in laboratory strains was sufficient to restore infection of epithelial and endothelial cells [30,31]. The UL128, UL130 and UL131 proteins were found to assemble onto gH/gL producing gH/gL/UL128-131 that was found in the virion envelope and mediated infection of epithelial and endothelial cells and monocyte-macrophages [32–34]. Assembly of all of gH, gL, UL128, UL130 and UL131 was necessary for a function complex; omitting any one protein produced a misfolded complex that was stalled in ER [35]. That gH/gL/UL128-131 was required for HCMV entry into cells, rather than some other step in virus replication, was demonstrated using

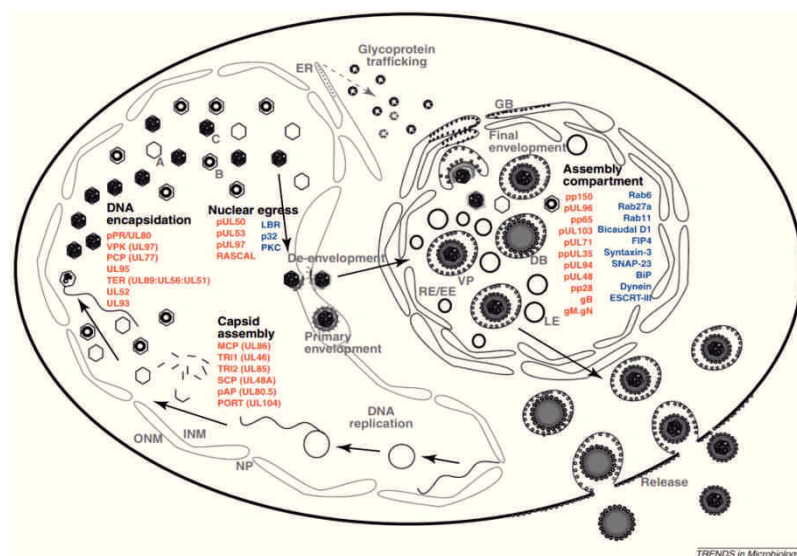


PEG to overcome entry defects with HCMV UL128-131 mutants [36]. Combined with evidence that HCMV gO-mutants produced small plaques on fibroblasts these data suggested a model in which gH/gL/UL128-131 mediates entry into epithelial/endothelial cells and gH/gL/gO mediates entry into fibroblasts (Figure 4)

Fig. 4: HCMV envelope glycoprotein complexes. The proposed model of tropism of each complex is indicated.

4. HCMV viral replication

HCMV entry is followed by the delivery of both the tegument and DNA containing capsid into the cytoplasm. Tegument proteins with regulatory function dissociate from the capsid and remain in the cytoplasm or migrate independently in the nucleus, where they modulate cellular and viral genes expression. A thick layer of tegument proteins remains tightly associated with the capsid and contributes to the delivery of the DNA to the nucleus [19]. For the efficient delivery of HCMV DNA, an intact microtubular network (MT) is essential. The MT spans from cellular periphery up to the perinuclear MT organizing center (MTOC). HCMV moves along MT branches to reach the nuclear pore complex and to inject the DNA into the nucleus [19]. Viral replicative cycle starts shortly after with the expression of immediate early (IE) genes, that can be detected as short as 4 hour post infection [20–22]. During the replicative cycle, genes expression can be divided in distinct temporal phases, defined as immediate early, early and late, that are linked together by a tightly regulated temporal cascade. A set of genes expressed with immediate early kinetic, whose transcription do not require de novo protein synthesis, act as global regulators of viral genes expression. Early genes protein products are responsible for DNA replication, while late genes



encode for structural proteins and are transcribed following viral DNA replication [19].

Fig. 5: HCMV maturation. The model illustrates the HCMV virion particles formation, maturation and budding processes. Major cellular and viral proteins involved in these processes are reported (red and blue, respectively). List of abbreviations: DB, dense body; VP, virus particle; EE/RE, early endosome/recycling endosome; LE, late

endosome; GB, Golgi body; ER, endoplasmic reticulum; NP, nuclear pore; INM, inner nuclear membrane; ONM, outer nuclear membrane; A, B and C, types of nuclear capsids. From [23]

5. HCMV Immune response manipulation and evasion mechanisms

Primary infection in immunocompetent individuals is accompanied by a period of viral dissemination (viremia). In this phase, virus excretion in bodily fluids, such as breast milk, blood, urine, saliva and seminal secretions lasts from months to years depending on the host age, and is the primary cause of viral transmission. Drop and clearance of the acute infection phase correlates with the mounting of strong humoral and, more importantly, cellular adaptive immune responses. Despite the setting up of a strong response, a complete clearance of infected cells is never observed. HCMV is able to establishing lifelong latency remaining silent in myeloid lineage cells [35]. To escape from the immune system surveillance, HCMV has developed a huge arsenal of genetic functions committed to alter and modulate both innate and adaptive arms of the immune response. In particular, HCMV coevolution within its host has led to the incorporation of a repertoire of functions with strong homologies to host genes. Thus the virus is able to subvert the immune system mimicking the same strategies and mechanisms used by hosts to clear the infection. Inhibition of complement cascade and natural killer (NK) cells activation, attenuation of interferon (INF) response and disruption of antigen presentation are only few examples of the functions hijacked by virus encoded chemokines, cytokine and cellular receptors homologues [35].

Table 1: List of selected protein functions encoded by cytomegalovirus that interfere with adaptive and innate immune response. Modified from [35].

| Adaptive immune response | Innate immune response |
|--|--|
| Inhibits MHC I and II expression (<i>US3</i>) | Fcγ Receptor Homologs (<i>UL119, RL11</i>) |
| MHC I and II degradation (<i>US2</i>) | Interferon-Mediated Immunity (<i>IE2, UL83</i>) |
| MHC I degradation (<i>US11</i>) | Natural Killer Cells (<i>UL18, UL142, UL141, UL40</i>) |
| Binds MHC I molecules (<i>US8, US10</i>) | Cytokine Homologs (<i>UL111</i>) |
| Inhibits TAP peptide transport (<i>US6</i>) | Viral Chemokine Homologs (<i>UL146, UL147, UL128-131</i>) |
| Inhibit MHC II expression (<i>UL83</i>) | Apoptosis (<i>UL36, UL37x1</i>) |

5.1 Fc receptors

Fc receptors (FcR) are cellular surface exposed proteins present on almost every kind of immune cell and on some epithelial, endothelial and hepatocytic cells. Fc receptors are involved in the process of antigen recognition but, unlike the B and T cell receptors (BCR and TCR, respectively) they do not directly bind the antigen: instead the Fc receptor ligands are the constant region of immunoglobulins. Each Ig class has one or more dedicated Fc receptors and they are named according to the type of bound Ig: Fc α R, Fc γ R, Fc δ R, Fc μ R and Fc ϵ R bind respectively Ig of the A, G, D, M and E class.

The Fc receptors belong to the immunoglobulin superfamily (IgSF) and possess at least one immunoglobulin domain. The only exception is the Fc ϵ RII receptor that belongs to the lectin family and possesses a C-type Lectin Domain (CLTD). Two layers of β -sheets composed from seven to nine antiparallel β -strands connected by a loop compose the immunoglobulin fold common to all the members of the Ig superfamily [52].

Fc receptor functions can be divided in three main categories: regulation of immune-cell responses through their activation or inhibition; antigen-antibodies immunocomplexes (IC) uptake; immunoglobulin transport and stabilization.

Regulation of immune cells functions can be initiated through the recruitment of surface FcRs by antigen bound Igs. Depending on the kind of FcRs recruited and on the type of cell, activatory or inhibitory stimuli can be triggered. Many different downstream effects can result from the signaling cascade including up- or down- modulation of cellular proliferation, phagocytosis, degranulation, cytokine production. Most FcRs do not possess intrinsic signaling motifs thus they must associated with other proteins able to mediate the signals transduction. The Immunoreceptor Tyrosine-based Activatory or Inhibitory Motifs (ITAM and ITIM, respectively) are regulatory motifs that are present in the cytoplasmic tails of the receptor or in the associated units and regulate the signaling cascade. Receptor engagement induces a phosphorylation event on the tyrosine present in both motifs that culminates with the activation, in the case of ITAM triggered signaling, or in the inhibition, in the case of ITIM signaling, of the cell functions [52].

Another important function mediated by the FcRs is the immune complexes internalization. FcRs mediate ICs endocytosis and delivery into lysosomes for degradation. Proteolytically cleaved antigen-derived peptides can then be loaded and presented on MHC class I and II molecules [52].

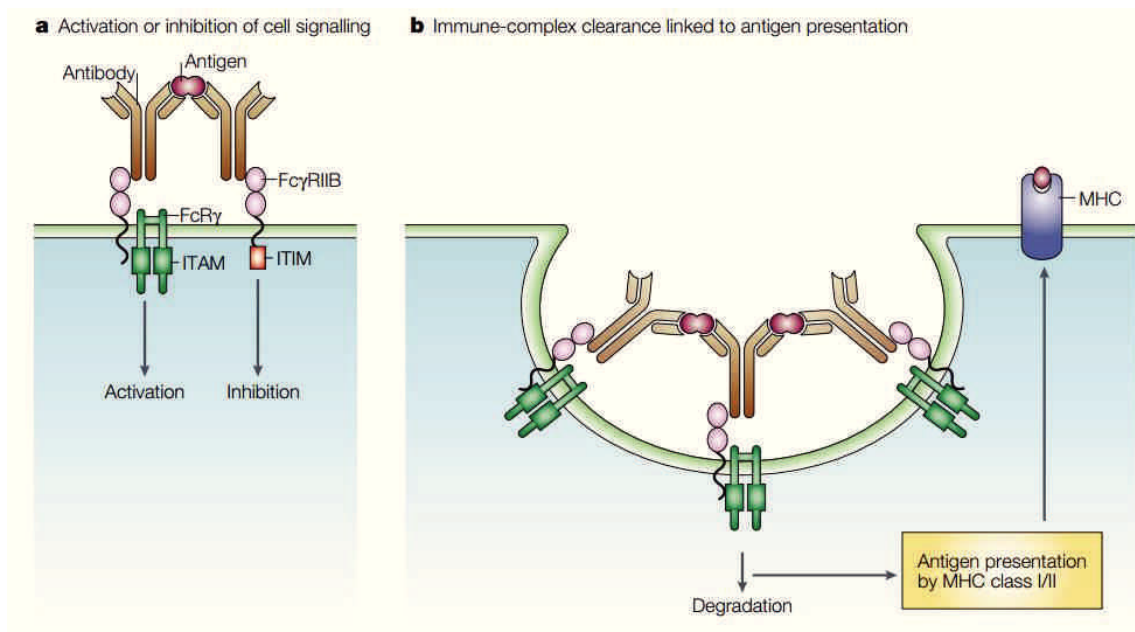


Fig. 6: Principal functions of Fc receptors. **a:** Fc receptors modulate positive or negative cellular signaling upon IC binding. Activation can lead to cellular proliferation, phagocytosis, degranulation and is mediated by the ITAM motif present in the FcR associated γ -chain. By contrast, an inhibitory signaling cascade can be initiated through engagement of the ITIM motif possessing Fc γ RIIB. **b:** FcR can mediate both IC and Ig-coated pathogens phagocytosis. The internalization process can lead to the clearance of the pathogen/IC and to MHC class I and class II antigen-presentation of the resulting antigenic peptides. From [52].

5.2 Bacterial and Viral Fc receptor homologues

Among the mechanisms used by pathogens to escape the immune system response, the acquisition of FcR homologues function seems to be highly diffuse. While the host cells use the FcR as a mean to sense and dispose of antibodies marked intruders, pathogens have developed similar functions in order to counteract immune system recognition. *Staphylococcus aureus*, a Gram positive bacterium, covers its membrane with the Fc binding protein A that captures and fixes immunoglobulins in the wrong orientation on the bacterium surface, disguising it and impairing its identification [55]. Fc binding proteins are highly conserved among the human herpesviruses: genes coding for FcR homologue functions were found almost in all genomes of the herpesviridae family suggesting a prominent role for these functions in pathogen survival [56].

Most of the viral FcRs (vFcR) recognize and bind the constant region of IgG probably impairing and inhibiting host effector function. Different mechanisms of action were proposed depending on the localization of the vFcR. Disguising mechanisms, similar to those observed for *S. aureus* protein A, can be put in place by vFcR present on the virions while circulating antibodies clearing can start from vFcR exposed on infected cells membrane. While the latter can inhibit the ADCC of the infected cells, the former can contribute to counteract the neutralization activity of antibodies bound to their target on the viral surface. The blockage of the free Fc portion can avoid the recognition of the viral particles and the activation of mechanisms such as the complement cascade or phagocytosis processes. This mechanism, named antibody bipolar bridging, has been described for Herpes Simplex Virus (HSV) heterodimeric FcR gE:gI.

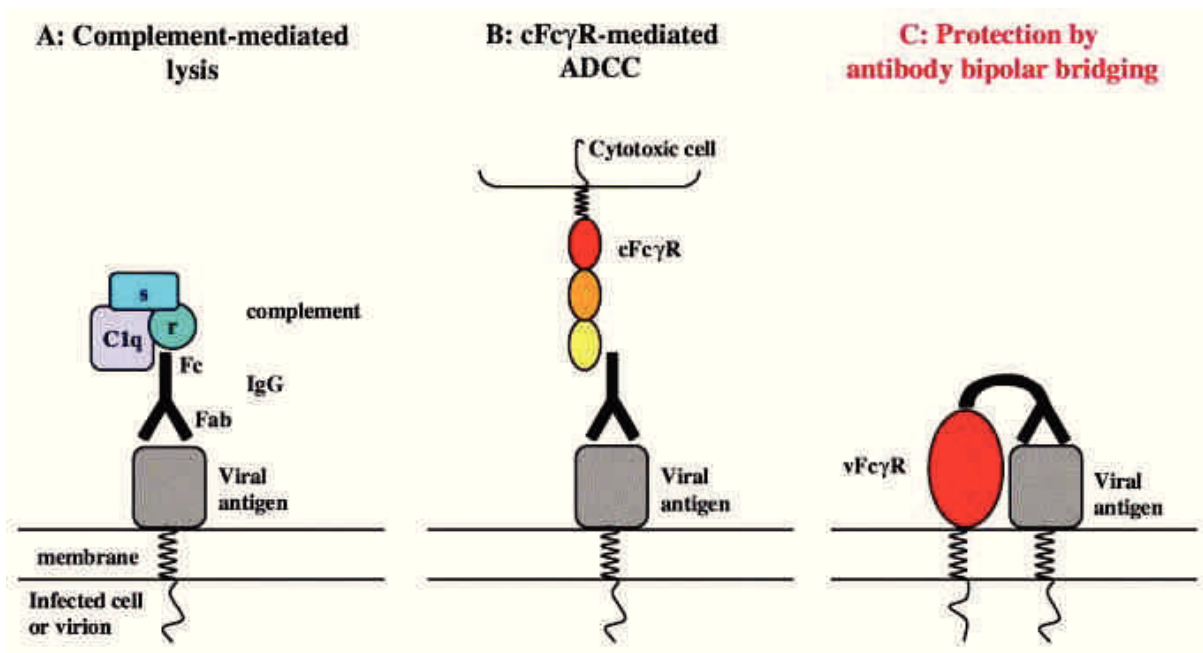


Fig. 7: Herpesviral FcγRs mediated mechanism of protection against antiviral IgG. The Fc portion of human IgG bound to viral antigen on infected cell surface or on virus particle can recruit complement proteins (A) or host FcγRs (B). The former mechanism culminates with the complement-mediated lysis (ADCML) of the infected cells/viral particle. Recruitment of host FcγRs present on the surface of cytotoxic effector cells results in the antibody-dependent cellular cytotoxicity (ADCC). (C) Viral FcγR impairs both mechanisms through the blockage of the Fc portion of the IgG. This mechanism of antibody bipolar bridging protects the virus particle or infected cell from recognition and destruction. From [56].

Moreover, several reports underline the importance of the vFcγR endocytic activity of HSV, Pseudorabies Virus (PrV) and Varicella Zoster Virus (VZV) in the contest of the infected cells [57–60]. For example gE:gI vFcγR complex in PrV infected monocytes internalizes antibodies-viral antigens complexes from the cell surface preventing the ADCML of the infected cell [61].

HCMV is known to encode for two different FcγRs: glycoprotein gp68, encoded from the UL118-119 locus, and gp34, encoded from the RL11 locus [62,63]. Both gp68 and gp34 are type I membrane proteins with a predicted IgSF domain and extensive glycosylation and localize on the membrane of transfected cells. gp68 is a 347 amino acid long protein generated through a splicing event between the UL119 and UL118 open reading frames (ORFs). It is predicted to possess up to 12 potential N-linked and several O-linked glycosylations sites. *In silico* alignment showed that the IgSF domain of gp68 displays a high degree of similarity to the third IgSF domain of cellular FcγRI. Moreover an almost consensus ITIM motif (WSYKRL₃₂₈) is present in gp68 cytoplasmic tail. Studies conducted on the purified ectodomain showed that gp68 binds Fcγ with a 2:1 stoichiometry. Thus a single IgG can crosslink two molecules of gp68 allowing receptor clusterization in the absence of antigen binding. Surface plasmon resonance studies demonstrated a high affinity between gp68 and Fcγ with a K_D in the nanomolar range. Furthermore the binding

remained stable at acidic pH, a major difference from the previously characterized gE:gI- Fc γ complex that dissociate at pH lower than neutrality [64].

Less experimental information is available for RL11 gene product gp34. It consists of 234 amino acids and possesses only N-linked glycosylations on three predicted sites. Multiple alignments showed the presence of determinants that are conserved in the Fc binding region of host Fc γ IIIRs. The cytoplasmic tail contains a predicted dileucine internalization motif (DXXXLL₂₂₆) that could indicate a propensity of the protein to enter the endocytic route upon Fc γ binding. Differential binding propensity to the IgG subclasses for gp68 and gp34 are still unknown, but the different reported affinities for IgG from various species suggest a diverse mechanism of binding for the two proteins. In particular, both gp68 and gp34 were able to bind to the same levels IgG1, IgG2, IgG3 and IgG4 while only gp34 showed affinity for non-human IgG binding both rabbit and rat IgGs [62]. Moreover the HCMV FcRs seem to be diverse from HSV gE:gI not only for what concern pH requirement but also in terms of binding sites. In fact, while gE:gI binding site to the Fc fragment competes with protein A, HCMV vFc γ Rs bound to the Fc can be immunoprecipitated with the *S. aureus* Fc binding protein. Overall the structural and topological similarities between HCMV Fc γ R and the Fc binding proteins coded by other herpesviruses may indicate a mechanism of action similar for these classes of proteins [56]. Nevertheless some distinctive features can evidence a mode of action that do not reside in the mechanisms previously described, suggesting a diverse and peculiar role for the HCMV Fc γ R in the contest of viral infection.

1. Reverse vaccinology approach

At the end of the 20th century, most of the vaccines that could be developed by traditional technologies had been developed, and new technologies were required to conquer the remaining pathogens. Remarkable progress was made during this period by the introduction of new technologies such as recombinant DNA and chemical conjugation of proteins to polysaccharides, as well as advances in the use of novel adjuvants. Additionally, a powerful tool came from the ability to access the genomes of microorganisms, a new technology that became available in 1995 when Craig Venter published the genome of the first free living organism (Fleischmann et al., 1995). This technological revolution allowed for the first time the capacity to move beyond the rules of Pasteur, using the computer to rationally design vaccines starting with information present in the genome, without the need to grow the specific microorganisms. This new approach was denominated “reverse vaccinology” (Rappuoli, 2000). More recently, the power of the genome has been complemented by the ability to interrogate the entire antigenic repertoire. This has been done by using libraries of expressed antigens and screening for the immunogenicity of the proteins (referred to as the “antigenome”) during infection (Giefing et al., 2008), or by testing directly for the presence and amount of antigens on the surface of bacteria. This latter technology takes advantage of the power of mass spectrometry to identify and quantify those antigens that are present on the bacterial surface (Rodríguez-Ortega et al., 2006). Surface proteins of gram-positive bacteria are first partially digested by treatment with proteases, and then the resulting peptides are loaded on a mass spectrometer that provides information on the identity and quantity of surface exposed proteins. In the case of the gram-negative bacteria, antigens on the surface are identified by mass spectrometry of membrane fragments free of cytoplasmic material that are released by gram-negative bacteria, which have been genetically modified to weaken the outer membrane stability (Berlanda Scorza et al., 2008). In addition to the discovery of many previously unknown antigens which have led to successful vaccine development in several instances, reverse vaccinology has made possible studies on antigen function, leading to an understanding of the biology of the pathogen. In conclusion, reverse vaccinology uses the entire protein repertoire of each pathogen to select the best candidate vaccine antigens. This allows the development of vaccines that were previously difficult or impossible to make and can lead to the discovery of unique antigens that may improve existing vaccines. Processes affecting vaccine development during reverse vaccinology are reported in Figure 8.



Figure 8. Schematic Diagram Summarizing the Pathway of Vaccine Development Starting from Reverse Vaccinology

(1) First, computer analysis of the whole genome identifies the genes coding for predicted antigens and eliminates antigens with homologies to human proteins. (2) Then the identified antigens are screened for expression by the pathogen and for immunogenicity during infection. (3) The selected antigens are then used to immunize animals and test whether immunization induces a protective response. (4) Protective antigens are then tested for their presence and conservation in a collection of strains representative of the species (molecular epidemiology). (5) Finally, selected antigens are manufactured in large scale for clinical trials, and candidate vaccines are tested for safety and protective immunity in humans using established correlates of protection or efficacy studies. (6) Scientific, clinical, and technical information is then analyzed and approved by regulatory agencies, such as the Food and Drug Administration (FDA) or the European Medicinal Agency (EMA). (7) Policy-making bodies, such as the ACIP and equivalent bodies from other nations, make the recommendation on how the vaccine should be used. (8) The approved vaccine is then commercialized and used in large scale. At this point, phase IV clinical studies confirm safety. (from *Reverse Vaccinology: Developing Vaccines in the Era of Genomics*, 2010 R.Rappuoli)

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OUTLINE OF THESIS

The reverse vaccinology approach to the human cytomegalovirus (HCMV) genome, one of the largest known mammalian viral genome with a coding potential of more than 166 proteins, led to the identification of 94 potential membrane-associated or secreted proteins. Many of the selected proteins have no assigned functions but share a high degree of conservation among different strains. These designed ORFs have been synthesized as eukaryotic codon-optimized sequences in frame with both hexa-histidine tail and c-myc tag coding elements. A more detailed analysis was performed on a certain number of these tagged proteins in both epithelial and fibroblast human derived cell lines, natural targets of HCMV. As starting point 12 hCMV proteins with antigenic properties were chosen for further characterization in terms of structural and functional features.

This work was aimed to the identification and functional characterization of two new HCMV surface exposed glycoproteins.

The study is divided in two parts: first the identification and characterization of ORFX; second the identification and characterization of a novel HCMV Fc γ binding protein. Regarding the first part, we focused our attention on ORFX gene product, a putative ORF identified by *in silico* analysis. Biochemical analysis on both ORFX viral protein product or transient expressed single gene allowed us to conclude that ORFX is: a) heavily glycosylated protein; b) expressed as late product during infection; c) present in the assembly complex, the cellular site of virus assembly; d) present on the HCMV virion envelope and e) secreted in transient expression as by *in silico* prediction. These observations led us to search for ORFX partners among other HCMV envelope exposed glycoproteins. We found that gH was efficiently transported to plasma membrane only when co-expressed with ORFX. Moreover, co-expression of gH (and gH/gL) with ORFX and co-immunoprecipitation experiments, carried on both transfected and infected cells, showed that ORFX is part of a gH-based complex. These results suggest that ORFX could function as both gH “escort” protein but also be part of a cellular recognizing complex and invite to a deeper analysis of both its functions and immunological properties.

Regarding the second part of the study, we were able to identify two novel Fc γ binding protein coded by CMV: RL12 and RL13. The latter was also further characterized as recombinant protein in terms of cellular localization, Fc binding site and IgG internalization ability. Finally binding specificity of both RL12 and RL13 seems to be confined to human IgG1 and IgG2.

Characterization of the newly identified HCMV-TR protein ORFX, an envelope protein forming complex with glycoprotein H (gH)

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Running Head: The envelope HCMV TR-ORFX protein interact with gH in infected human fibroblasts

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ABSTRACT

Human cytomegalovirus (HCMV) attachment and invasion of cells rely on the recognition of a still unknown cellular receptor with a viral envelope complex including gH. Recently it has been shown that, while gH/gL/gO complex appears to be important for HCMV entry into or spread between fibroblasts, formation of a pentameric complex including gH/gL, UL128, UL130 and UL131 is required for epithelial/endothelial tropism. Many HCMV-specific and conserved surface-exposed glycoproteins are required to be expressed in complexes for their correct final folding and function.

In this study we focused our attention on ORFX gene product, a putative ORF identified by *in silico* analysis. Biochemical analysis on both ORFX viral protein product or transiently expressed single gene allowed us to conclude that ORFX is: a) heavily glycosylated protein; b) expressed as late product during infection; c) present in the assembly complex, the cellular site of virus assembly; d) present on the HCMV virion envelope. These observations led us to search for ORFX partners among other HCMV envelope exposed glycoproteins. We found that gH was efficiently transported to plasma membrane only when co-expressed with ORFX. Moreover, co-expression of gH (and gH/gL) with ORFX and co-immunoprecipitation experiments, carried on both transfected and infected cells, showed that ORFX is part of a gH-based complex.

In summary, the protein product of the ORFX open reading frame represents a novel HCMV envelope glycoprotein and is found to be a direct gH interaction's partner during the virus infection cycle.

INTRODUCTION

HCMV is a ubiquitous betaherpesvirus infecting the majority of the world's population. HCMV infection is usually asymptomatic in healthy individuals, but the viral infection causes severe disease in immunocompromised adults and birth defects in newborns. Infants infected in utero can be born with disseminated HCMV disease or be damaged in terms of neurologic development and transplant patients do not control HCMV well and experience infection in gut epithelial cells, liver hepatocytes, monocyte-macrophages that disseminate virus to endothelial cells and smooth muscle cells which can accelerate transplant rejection (1).

The HCMV genome from clinical isolates includes over 230 genes that encode for \approx 54 membrane proteins at least 25 membrane glycoproteins found in the virion envelope(2). Recently, through an integrated approach combining ribosome footprinting assays, high-resolution mass spectrometry and analysis of mutated viruses, Stern-Ginossar and colleagues were able to identify a total of 751 translated ORFs including short peptides(3).

HCMV structural glycoproteins can be divided into two broad classes, those conserved between members of the family Herpesviridae (including gB, gH, gL, gM, and gN) and subgenus-specific glycoproteins without homology to other herpesviruses. These latter include, among others, gpRL13 (4, 5), gpTRL10(6), gpUL132(7), UL74 encoded gO(8), UL4-encoded gp48 (9), US27 (10), and UL33(11).

Out of the total number of proteins composing the virion, around 20 were identified as envelope associated proteins(5, 12, 13). Notably, only 8 were demonstrated as essential

for virus replication *in vitro* in different cell types: glycoproteins B (gB), M (gM), N (gN), L (gL), H (gH), UL128, UL130, UL131 (14). Apart from the gB homotrimers, the HCMV most studied glycoproteins complexes include gH. The gH/gL/gO complex appears to be important for HCMV entry into fibroblasts as well as for cell-to-cell spreading among the culture (8, 15). The more recently discovered gH complex is the so called pentameric complex that include gH/gL, UL128, UL130 and UL131(16). The particular association of these five species on the viral envelope is required for epithelial/endothelial tropism (17). HCMV attachment begins with a low affinity tethering to the cell surface HSPGs, an event mediated by both gB and gM/gN(14, 18). Although there is a broad list of “post-attachment” receptors and none of them completely fulfilled the role, gH/gL complex has nevertheless essential to mediate the fusion of viral and cellular membranes. The presence of other associated species, and likely the pattern of receptor-like host molecules, determine the cell tropism and the mechanism of entry(19-22). Historically, gB has been the first target as antigen for a protective HCMV vaccine (23, 24). More recently, antibodies directed against pentameric complex appear to be a more robust tool in neutralizing HCMV infection(25). Although almost all subunit of the pentamer are target of specific antibodies, anti-gH antibodies represent the prevalent species accounting for neutralizing activity (26-28).

Many of the HCMV-specific glycoproteins, in contrast to the conserved glycoproteins, are not essential for *in vitro* replication in fibroblasts and presumably participate in cell and tissue tropism or pathogenicity (29). Structural glycoproteins that are not conserved between herpesviruses potentially add specific functions to individual genera or viruses and viral ligands that bind to cell surface receptors differ between the viruses and may

thus in part contribute to the differences in cell and tissue tropism. For example, Epstein-Barr virus uses the two nonconserved glycoproteins gp350 and gp220 for initial interaction with the target cell(30).

In the present study, we have approached the molecular characterization of HCMV-specific ORFX. To this end, an HCMV (TR-derived BAC) recombinant with Flag epitope-tagged ORFX strain was generated.

Our data reveal that the ORFX gene protein product is expressed with late kinetics. pORFX is highly glycosylated and localizes at the site of virus assembly and secondary envelopment in infected cells, forming part of the envelope of HCMV virion. These observations led us to search for ORFX partners among other HCMV envelope exposed glycoproteins. We found that gH was efficiently transported to plasma membrane only when co-expressed with ORFX. Moreover, co-expression of gH (and gH/gL) with ORFX and co-immunoprecipitation experiments, carried on both transfected and infected cells, showed that ORFX interacts with gH during the viral replication cycle.

MATERIALS & METHODS

Cell lines, plasmids and viruses.

TR is a clinical HCMV strain that was derived from a ocular vitreous fluid sample from a patient with HIV disease (31) and was cloned into a BAC after limited passage in fibroblasts (32).

HCMV strain TR and recombinant ORFX Flag-TR virus were propagated in human foreskin fibroblasts HFF-1 (ATCC: SCRC-1041) grown in minimal essential medium (Invitrogen) supplemented with 10% fetal calf serum, glutamine (100 mg/liter), and gentamicin (350 mg/liter). Virions were isolated by glycerol-tartrate gradient centrifugation as described (33). HEK293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, glutamine and gentamicin. Lipofectamine 2000 (Invitrogen) was used to transfect HEK293T cells. Human codon-optimized ORFX, gH(UL75), gL(UL115) and gB (UL55) HCMV genes based on the TR strain sequence were synthesized by Geneart and cloned in plasmid pcDNA3.1(-)/myc-His C (Invitrogen) in frame with C-terminal myc and six histidine tag sequences. Single point-mutations were performed with the Quick Change Mutagenesis Kit (Stratagene) resulting in the mono-tag versions and the tag-less versions of these genes.

Construction and generation of ORFX-Flag TR virus. Insertion of the Flag tag at the C-terminus of ORFX ORF on the TR BAC was achieved using the method of Two-step Red-mediated recombination as described in the short technical report (34). The primer pair used to amplify the kanamycin insertion cassette are the forward 5'-TTC GGC GCC AAC TGG CTC CTT ACC GTC ACA CTC TCA TCG TGC CGC AGA CTG ATT ACA AGG ATG ACG ACG ATA AG-3' and the reverse 5'-TAT CAC CGG TCC AGG

TGA GAA AGA GAA GCC GCA ATC CGG GCG GCG GCA CA
TCA CTT ATC GTC GTC ATC CTT GTA ATC AGT CTG CGG CAC GAT GAG
CAA CCA AAT TAA ACCA ATT CTG ATT TAG-3' where the underlined base pairs
encode for the Flag peptide.

Reconstitution of infectious virus. To reconstitute the virus, 2 ug of the BAC-HCMV DNA and 1 ug of the pp71 expression plasmid were transfected into MRC-5 cells by electroporation as described in the current protocols. Culture medium was changed 24 h later and the cells were split and cultured until the appearance of virus plaques.

The virus stock was prepared by harvesting cell-free culture supernatant when the entire monolayer of cells was lysed or the cells were split and cultured until the appearance of virus plaques.

Purification of HCMV-TR virions. HCMV particles in cell supernatants were separated into virion, dense body, and noninfectious enveloped particle (NIEP) fractions by positive density/negative viscosity gradient centrifugation as described previously (35). Particle concentrations in the preparations were estimated by counting negatively stained samples by EM in relation to a standard concentration of latex beads. To separate virion envelope proteins from capsid and tegument proteins, 10⁸ particles were mixed 1:1 with envelope stripping buffer (2% Nonidet-P40 in PBS) and incubated for 15 minutes at 4°C. Particles were pelleted (12,000 g for 5 minutes at 4°C), and the soluble envelope fraction was harvested. The insoluble capsid/tegument material was washed twice with envelope-stripping buffer and once in PBS before being solubilized in SDS-PAGE sample buffer.

Flow cytometry. For detection of membrane exposed ORFX, HEK293T transiently transfected with vectors coding for ORFX, gH, gB and empty vector were trypsin

detached 48 h post-transfection, incubated 30 min at RT with Live&Dead Aqua (Invitrogen), diluted 1:400 in PBS then incubated with different dilutions of anti-ORFX polyclonal mouse sera for 60 min on ice. As secondary antibody, Alexa Fluor 647-conjugated goat anti-mouse was used for 30 min on ice at 1:200 dilution. A total of 10^4 cells were analyzed for each curve using FACSCanto II (Becton Dickinson, Heidelberg, Germany). Experiments were performed in triplicate for statistical consistency, means and standard deviations were analyzed and plotted using Graphpad Prism software.

Glycosidase treatment. Samples were treated with PNGase F (P0705S; New England BioLabs) or ENDO H (P0703S; New England BioLabs) according to the manufacturer's instructions. Briefly, the samples were denatured in glycoprotein-denaturing buffer at 100°C for 10 minutes and cooled to 0°C for 5 minutes. The samples were then digested overnight at 37°C with PNGase F or ENDO H before being analyzed by Western immunoblotting.

Immunogold electron microscopy. Purified virions were air-dried to the surface of Formvar-coated EM grids. The grids were treated with mouse Anti-Flag antibody (Sigma-F3165) for 4 hours at room temperature, washed 3 times with PBS, and incubated with goat anti-mouse antibody conjugated to 5-nm gold particles for 1 hour at room temperature. After further washing in PBS, the grids were negatively stained with phosphotungstic acid and subjected to EM.

SDS-PAGE and immunoblotting. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10 to 15% polyacrylamide gels under standard conditions. Proteins were transferred to nitrocellulose membranes, and membranes were blocked with PBS containing 0.1% Tween 20 and 5% powdered milk.

Antibodies and sera were diluted in PBS containing 0.1% Tween 20. For detection of primary antibody binding, horseradish peroxidase- conjugated anti-rabbit or anti-mouse immunoglobulin G antibody (Perkin Elmer) and the enhanced chemiluminescence detection system (Pierce) were used according to the manufacturer's instructions.

Immunofluorescence. Cells were plated on glass coverslips and infected with HCMV. At 7 days PI, the cells were fixed in 4% paraformaldehyde, permeabilized with 0.5% NP-40, pre-blocked with HCMV seronegative human IgG and incubated with primary antibody for 1 hour at RT°C. Following washing, secondary antibodies were incubated for 1 hour at 37°C, washed again, and mounted with DAPI Pro-long Safe Stain mounting media (Invitrogen). Primary antibodies were rabbit anti-Flag (F7425; Sigma), sheep anti-human TGN46 (AHP500; Serotec), mouse MAb anti-pp28 (6502; Abcam), and mouse anti-gH (2470-5437; Adb Serotech). Secondary antibodies were Alexa Fluor 488-, 568-, and 647-conjugated goat anti-mouse and anti-rabbit (Invitrogen). The intracellular locations of antibody-tagged proteins were examined under laser illumination in a Zeiss LMS 700 confocal microscope, and images were captured using ZEN 2009 software.

Immunoprecipitations. HFF-1 cells were infected with wtTR and UL-116Flag-TR stocks. Protein expression was allowed to proceed for 72hrs before the cells were washed in 1xPBS (0.137M NaCl, 0.0027M KCl, 0.1M, Na₂HPO₄, 0.002M KH₂PO₄, pH7.4) and lysed (0.025M Tris, 0.15M NaCl, 0.001M EDTA, 1%NP-40, 5% glycerol, pH 7.4) in the presence of protease inhibitors. 500ug of protein was incubated with 5ug of MSL-109 (courtesy of A.Feire) antibody for 16hrs at 4°C. Complexes were immunoprecipitated using the Protein G Dynalbeads (Invitrogen) according to the manufacturer's protocol. The complexes were washed four times in lysis buffer. Samples were boiled for 5 mins at

100°C before immunoblotting. The same procedure described above was adopted for the immunoprecipitation experiments carried out on transiently expressing HEK293T cells. Complexes were captured in parallel experiments with covalently linked anti-His antibody magnetic beads (Genescript) and anti-c-myc magnetic beads (Pierce) to avoid background signals in resulting eluted materials.

RESULTS

Primary structure of ORFX gene product

In all sequenced HCMV genomes, the ORFX ORF is located in the unique-long (UL) region between the ORFY and ORFW genes on the antisense coding strand (figure 1A not shown under NVD IP department revision). The ORFX mRNA was previously shown to arise in the true-late stage of AD169 infection as part of the ORFY-ORFW transcription unit but no analysis were on the gene translation that remained a putative glycoprotein (36). A multi-alignment of ORFX translation primary sequences derived from gene sequences of HCMV clinical and lab adapted strains is shown in figure 1B. The conservation degree among most representative HCMV strains is 98% resulting in a very strong conservation of this protein among the cytomegalovirus population. The ORFX ORF is predicted to encode a 313 amino-acid glycoprotein comprising a signal-peptide (1 to 24 amino acids, SP in figure 1B), a threonine-rich domain (27 to 157 amino acids) with 14 predicted N-linked glycosylation. The resulting polypeptide would have a molecular mass of 34.2 kDa without the contribution of the carbohydrate moiety and the absence of membrane anchor designates a secreted protein.

Kinetics of ORFX expression during HCMV replication.

To investigate the expression kinetics of ORFX during productive HCMV infection we had to generate a recombinant HCMV (TR-derived BAC) with a Flag-tag fused to the ORFX C-terminal end (see materials and methods). The reconstituted TR ORFX-Flag was used to infect HFF-1 cells where the kinetic of pORFX expression was monitored by immunoblot using an anti-Flag antibody in cell extracts harvested at different time points ranging from 4 to 120 h p.i.. As control, expression of the major immediate protein IE1 pp72 (UL123) and of the late pp28 (UL99) phosphoprotein were detected. Results are shown in figure 2A. In extracts of infected human fibroblasts, ORFX migrated as two species, a faster-migrating protein of 76 kDa and a slower migrating protein of about 125 kDa. The latter appeared to be the mature product of the 76 kDa species as indicated by the accumulation of the intracellular products starting from 72 h p.i. through 120 h p.i. coincident with the expression kinetic of pp28 (37). At time points after infection when signals for pORFX were not observed, IE1 pp72 was present, as expected, already being expressed after 4 h p.i. and remaining for the duration of the HCMV replication cycle. Consistently with the observed kinetic pattern, a metabolic blockade with PAA resulted in the disappearance of the 125\76-kDa bands (Fig. 2B), indicating that the ORFX protein is expressed late during HCMV replication. Trafficking of herpesvirus glycoproteins to the Golgi apparatus is associated with the processing of N-linked oligosaccharides to complex oligosaccharides that are resistant to endo H (38). The high number of putative N-linked glycan sites (n = 14) present in pORFX, the difference between the apparent molecular mass (125 kDa) observed for this protein by immunoblotting and the

calculated molecular mass (34.2 kDa) of the 313- amino-acid polypeptide backbone suggested that pORFX undergoes an extensive post-translational glycosylation process. Indeed, digestion with endoH which removes only high-mannose carbohydrates and PNGase F, which removes all the complex and high-mannose N-linked sugars showed the faster migrating 76kDa band sensitive to endoH treatment while the 125kDa band as PNGase F-sensible producing a band of approximately 35kDa in line with the expected molecular weight (Fig 2C). It appears that a fraction of pORFX (125kDa) produced in TR-HCMV-ORFXFlag-infected cells acquires substantial endo H resistance, consistent with all or most of the glycoproteins reaching the Golgi apparatus.

Subcellular localization and colocalization of pORFX with tegument and envelope HCMV proteins. In order to investigate the localization of pORFX in HCMV-infected cells, the Flag tag of the TR ORFX-Flag virus was employed to trace its subcellular distribution by confocal microscopy showing at 96 h p.i. a specific colocalization for the Trans-Golgi marker TGN 46 among a viral HFF-1 syncytia present at this time point (Fig 4A). Based on the cytoplasmic compartmentalization of pORFX, we explored whether other structural HCMV proteins gathered at the pORFX-containing site. To this purpose, in HFF-1 TR-ORFX Flag infected cells following fixation at 72 h p.i. , antibodies against the tegument phosphoprotein pp28 and the envelope glycoprotein gL were employed. HCMV pp28 was previously reported to localize with other tegument and viral envelope proteins at the virus assembly complex (AC) site during the late phase of the infectious cycle (39, 40), and to be acquired by the virion in the cytoplasm. Interestingly, pORFX

colocalized with pp28 (Fig. 4B) and gL (Fig. 4C), consistent with its presence at the site of viral AC, thus suggesting its possible integration into the HCMV virion.

pORFX is a component of the HCMV virion envelope. The finding that pORFX is a glycoprotein that localizes to the intracellular virion assembly complex raised the hypothesis that it might be a virion surface envelope protein. With this rationale, we purified

TR ORFX-Flag particles by negative-viscosity–positive-glycerol/tartrate gradient centrifugation then performed a detergent extraction to separate envelope and tegument fractions and analyzed them by immunoblotting with the anti-Flag antibody. In this virion fractionation studies, ORFX protein copurified with glycoprotein B (UL55) and glycoprotein L (UL115) in the soluble envelope fraction, rather than with pp65 (UL83) in the tegument. IE1 (pp72), a nonstructural protein present only in preparations of infected cells antibody was used as control of virion purity (Fig. 5A). The presence of pORFX on the virus envelope was supported by immunoelectron microscopy, in which 5nm gold-labeled secondary antibody against the anti-Flag exhibited the labeling around the inner surface of the envelope (Fig 5B).

pORFX co-immunoprecipitates with glycoprotein H in transient expressing HEK-293T cells The pORFX viral envelope localization together with the intracellular staining showing the ORFX completely ER-retained in MRC-5 transient expression experiments (data not shown) suggested us the idea that it might be associated with one or more of the major reported HCMV envelope glycoproteins as showed in literature for the pentameric complex members(17).

To investigate this possibility we performed a cytofluorimetric assay in which HEK-293T cells were co-transfected with a TR-ORFX encoding plasmid in binary combinations with different known trans-membrane HCMV glycoproteins. In particular we show the representative experiment runned with the couple ORFX/gH using gB as negative control of interaction. After a plasma-membrane staining of these cells with a polyclonal anti-ORFX mice sera at different dilutions, we used FACS analysis to measure the mean fluorescence intensity coming from the FITC conjugated secondary Ab. To clearly exclude possible aspecific binding coming from the single protein contributions we included the single transfectant populations as negative controls. Experiments were performed in triplicate to have a statistically consistent data set. Mean fluorescence intensities plotted in Fig. 6A with respective standard deviations show a strong ORFX plasma membrane signal only for the ORFX-gH co-expressing cells. This ORFX plasma membrane export event led us to consider the possibility of a physical interaction between the two proteins since, in this configuration, at least a fraction of ORFX became exported from the ER.

To test the formation of a gH-ORFX complex, both proteins were immunoprecipitated from ORFX-his/gH-Myc transfected cell lysates in parallel and complementary experiments with anti-His and anti-Myc antibodies. Complexes were captured with magnetic beads covalently linked to the respective Abs; eluted material was separated by SDS-PAGE and analyzed by immunoblot using anti-tag antibodies (Fig. 6B). Both ORFX and gH proteins were successfully co-immunoprecipitated from cell lysates meanwhile gB used as negative control was not captured by any of the two proteins as expected and shown in Fig 6B .

pORFX interacts with glycoprotein H in infected HFF-1 and colocalize with it in the intracellular AC. To further confirm the ORFX/gH interaction in our infection system we used the anti-gH human monoclonal MSL-109 Ab to immunoprecipitate gH from TR-ORFX-Flag infected HFF-1 cell lysates at 96 h p.i. As negative control we used a wild-type TR infected HFF-1 cells lysate. Meanwhile as positive control we tested the eluted materials with an anti-glycoprotein L rabbit-polyclonal antibody since gL is known to be the gH direct interactor (17). The interaction between gH and ORFX was confirmed by the anti-Flag signal present in TR-ORFX Flag elution lane (Fig.7A).

The direct association between pORFX and gH was then supported by a colocalization experiment carried out on TR-ORFX-Flag infected HFF-1 cells using confocal laser scanning microscopy. Confocal microscopy analysis of permeabilized TR-ORFX-Flag infected HFF-1 cells stained with both anti-gH mAb and anti-Flag mAb revealed a strong accumulation of ORFX on the viral Assembly Complex site that extensively overlapped with gH signals as shown also by cell orthogonal projections in Fig. 7B (yellow color in right merge panel).

These datas reflect the ORFX/gH association during the TR-HCMV replication cycle.

DISCUSSION

Although a great deal is known about the envelope of betaherpesviruses, our understanding of the composition of the HCMV envelope is still incomplete. Growing numbers of viral glycoproteins have been reported to be components of the HCMV envelope, including molecules conserved between members of the Herpesviridae family (gB, gH, gL, gM, and gN) and others that are HCMV specific (57) (e.g., gO (8), gpTRL10 (6), gpRL13 (4, 5), UL4/gp48(9), gpUL128 (17), gpUL130 (41), gpUL132 (7), US27 (42), and UL33 (11)).

It is reported that genus-specific envelope glycoproteins contribute to the broad cell/tissue tropism of HCMV and its pathogenicity.

In this study, we have characterized the HCMV specific ORFX-encoded protein product in infected human fibroblasts, constructing a ORFX-Flag tagged version of the BAC-reconstituted strain TR. Early studies have identified the late 2.1-kb and 1.2-kb transcripts, rising from the UL119-UL115 transcriptional unit, to encode the viral mRNAs of ORFX and UL115 (i.e. gL) (36). Further experiments were carried out on a AD169 deletion mutant virus for ORFX gene that was reported to be produced at wild-type levels by human fibroblasts indicating this gene as dispensable for HCMV growth in HF (43).

During our study we found that pORFX is a PNGase F-sensitive glycoprotein, synthesized late during productive HCMV infection. In infected human fibroblasts, pORFX is targeted to the intracellular virion assembly complex site together with several HCMV tegument and envelope proteins. Consequently, we found that pORFX can be incorporated into purified TR virions being exposed on the viral envelope and showing

that it interacts with gH in human fibroblasts during the HCMV-TR viral replication cycle. Based on these results, we propose that pORFX constitutes a novel HCMV structural envelope glycoprotein. Several studies have investigated antibody specificities against HCMV structural proteins in detail and recently the pentameric gH based complex (gH, gL, UL128, UL130, and UL131) has been reported to induce at least 45 monoclonal antibodies (mAbs) in rabbits immunized with an experimental vaccine virus in which the expression of the pentameric gH complex was restored. Over one-half of the mAbs have shown neutralizing activity (25). Because of its location in the virion envelope and molecular interaction with gH, pORFX is potentially capable of inducing neutralizing antibodies and thus could represent a target of the humoral immune response during natural infection. In addition, since induction of strain-specific antibodies and antigenic variation is a well-known phenomenon in HCMV biology proteins like ORFX, showing high degree of primary aminoacidic sequence conservation among different lab and clinical strains (Fig 1B), could potentially represent an antigenic solution in this scenario.

Moreover, the advances made in the attempt to understand HCMV entry led to the statement that gH/gL/UL128–131 is required for entry into epithelial and endothelial cells, but not for fibroblast entry where gH/gL/gO seems to be the main protagonist complex (16, 21, 44). However, the accepted hypothesis is that HCMV entry into fibroblasts could involve viral proteins other than gH/gL/UL128-UL131. In addition, is well assessed that gH and gL are essential for viral production in fibroblasts (29). By contrast, we wouldn't exclude, among all the different HCMV strains, the presence of alternative complexes formation that could help to the correct protein folding along the

secretory pathway or give the virus different tropisms for diverse cellular targets. With this aim, given that ORFX is present at the viral envelope site, we investigated its possible HCMV envelope's partners finding that the co-expression of ORFX and gH in transfected HEK 293T cells allowed the ORFX delivery on the cellular membrane (Fig), by contrast ORFX expressed alone remain ER retained (data not shown) as for other HCMV glycoproteins such as gH, gL, gO, gM, and gN, which also require complex formation for proper folding and transport to the cell surface (45-47); later we found the ORFX physically interacts with gH during the TR replication cycle in human fibroblasts. Unfortunately, we were not able to report data showing the presence of the gH/ORFX complex on the viral envelope possibly due to the low abundance of this complex in relation to the well characterized gH/gL based complexes. However, our data on the gH/ORFX complex, obtained from single particle analysis by electron microscopy, revealed ORFX sharing the same gH binding site usually adopted by gL. This data was confirmed by co-immunoprecipitation experiments done using lysates of HEK 293T cells co-expressing gH-myc, gL and ORFX-His in which the ORFX/gH complex was captured by anti-His tag antibodies; as result, the anti-gL immunoblotted eluted material showed complete absence of signal for the well known gH partner (data not shown). Speculations on this results could suggest that ORFX can function as both gH "escort" protein and eventually be part of a cellular recognizing complex in alternative to gL. Whether the gH/ORFX complex or the ORFX protein alone plays an essential role in the biology of HCMV remains to be determined; the maintenance of the ORFX ORF in the genome of clinical isolates strongly argues for an essential role in the replication of this virus in the natural host. A more complete study of the function of envelope

glycoproteins as ORFX whose expression is restricted to individual members of a virus group could provide insight into the role of these virion envelope components in specific aspects of infection, such as cell tropism and disease induction, which in turn define the unique phenotypes of individual herpesviruses.

FIGURES

Figure 1 A-B (under NVD Intellectual Property department revision)

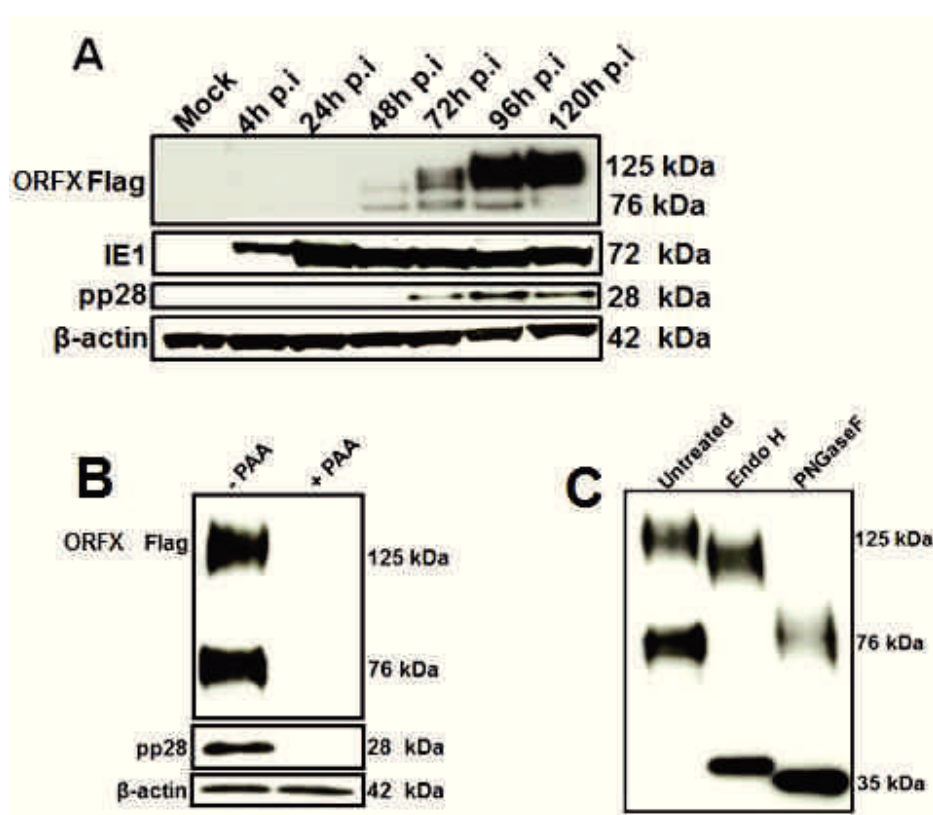


Fig. 2. Expression of the ORF X glycoprotein in HCMV-infected fibroblasts. (A) Uninfected (mock) and TR-ORF X-Flag-infected HFF-1 cells at an MOI of 5 were harvested at the indicated times p.i. Equivalent amounts of cell lysates were subjected to SDS-PAGE and analyzed by immunoblotting with antibodies reactive with Flag or the HCMV IE1 and pp28 proteins. Actin detection was used as a protein loading control. (B) Experiments were performed as described above for panel A to assess the effect of PAA treatment (PAA) on late-phase protein expression. (C) Whole-cell lysates of TR ORF X-Flag-infected HFF-1 cells (72 h p.i.) were left untreated or digested with EndoH and PNGase F. The preparations were subjected to immunoblot analysis using an anti-Flag antibody.

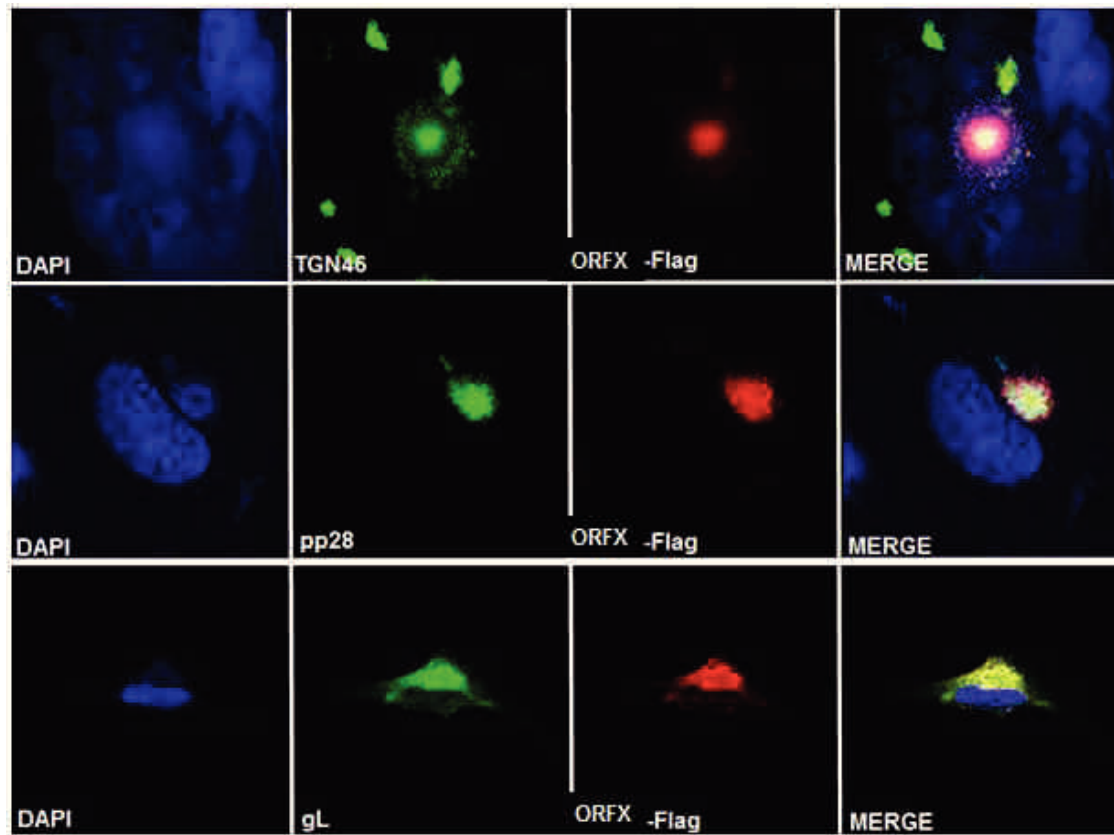


Fig. 3. Intracellular localization of ORF X in infected HFF-1 cells. Fibroblasts were infected with the recombinant virus TR-ORF X-Flag for 72 h. The intracellular localization of the individual proteins was determined by comparing the signal from anti-Flag-specific antibody (ORF X) with those of antibodies specific for HCMV envelope glycoprotein L, tegument protein pp28 and a component of the secretory pathway trans-Golgi network [TGN46]. The merge panel shows colocalization of the signals, cell nuclei are also stained blue.

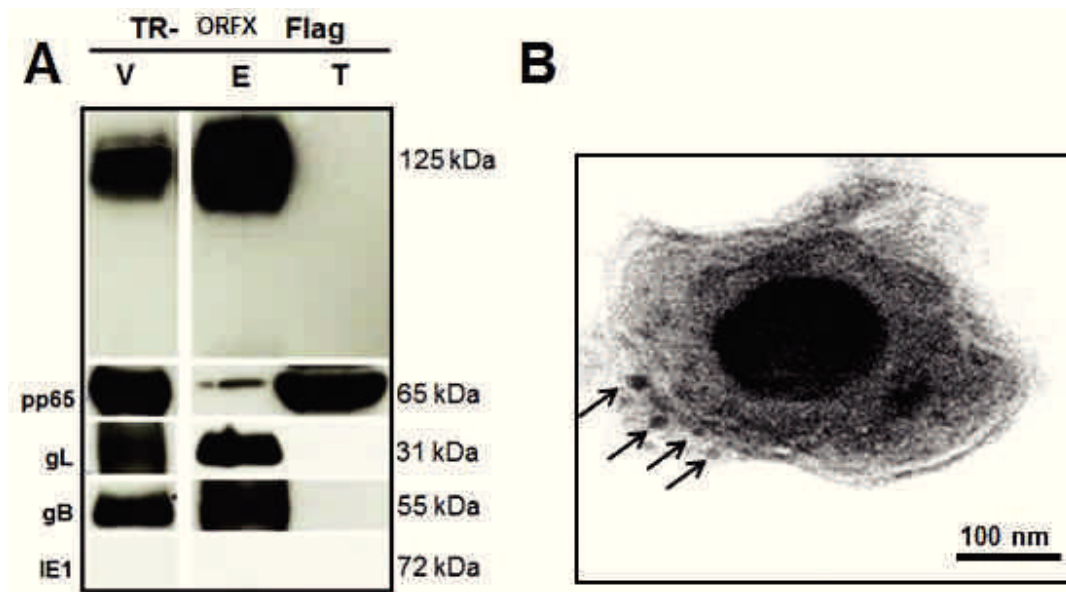


Fig. 4. Localization of ORF X on the virion envelope. (A) Western blot performed on purified virions from virus expressing a full-length ORF X with a C-terminal epitope Flag tag. Complete virions (V), envelope fraction (E), and tegument/capsid fraction (T) were probed for the antigens indicated. (B) Immunogold EM staining for anti-Flag mAb in purified TR-ORF XFlag virions. Original magnification, $\times 40,000$

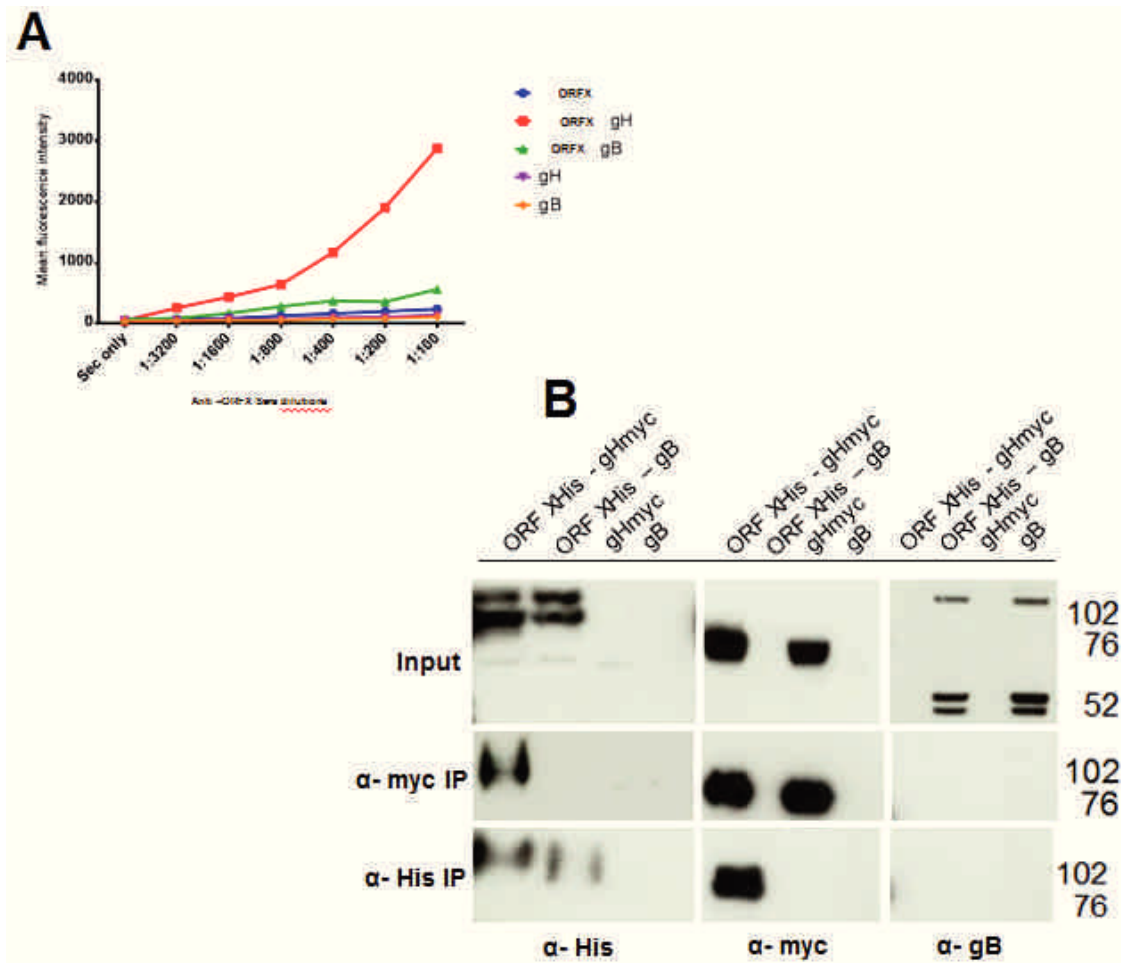


Fig. 5. ORF X interaction with gH in HEK 293T transfected cells. (A) Binding of Anti-ORF X mouse sera to surface-exposed ORF X in presence of gH. HEK293T cells transfected with expression vectors for ORF X, gH and gB in different combinations were first stained at 4°C with anti-ORF X polyclonal mouse sera at different dilutions. Excess of probe was removed by washing in PBS and then cells were fixed and stained with AlexaFluor-488 conjugated anti-mouse antibody. 488-fluorescent positive cells were found only in the ORF X/gH co-transfection setup and compared to the single transfectants for ORF X, gH, gB and the couple gB/ORF X used as negative controls. (B) ORF X co-immunoprecipitation with gH. HEK 293T transiently expressing ORF X-his/gH-myc, ORF X-his, gB and ORF X-his/gB. Cell lysates were subjected to parallel immunoprecipitation experiments with both covalently linked magnetic anti-His and anti-myc beads. The respective eluted materials were separated by SDS-PAGE and together with the inputs analyzed by immunoblot for both the His and myc tag.

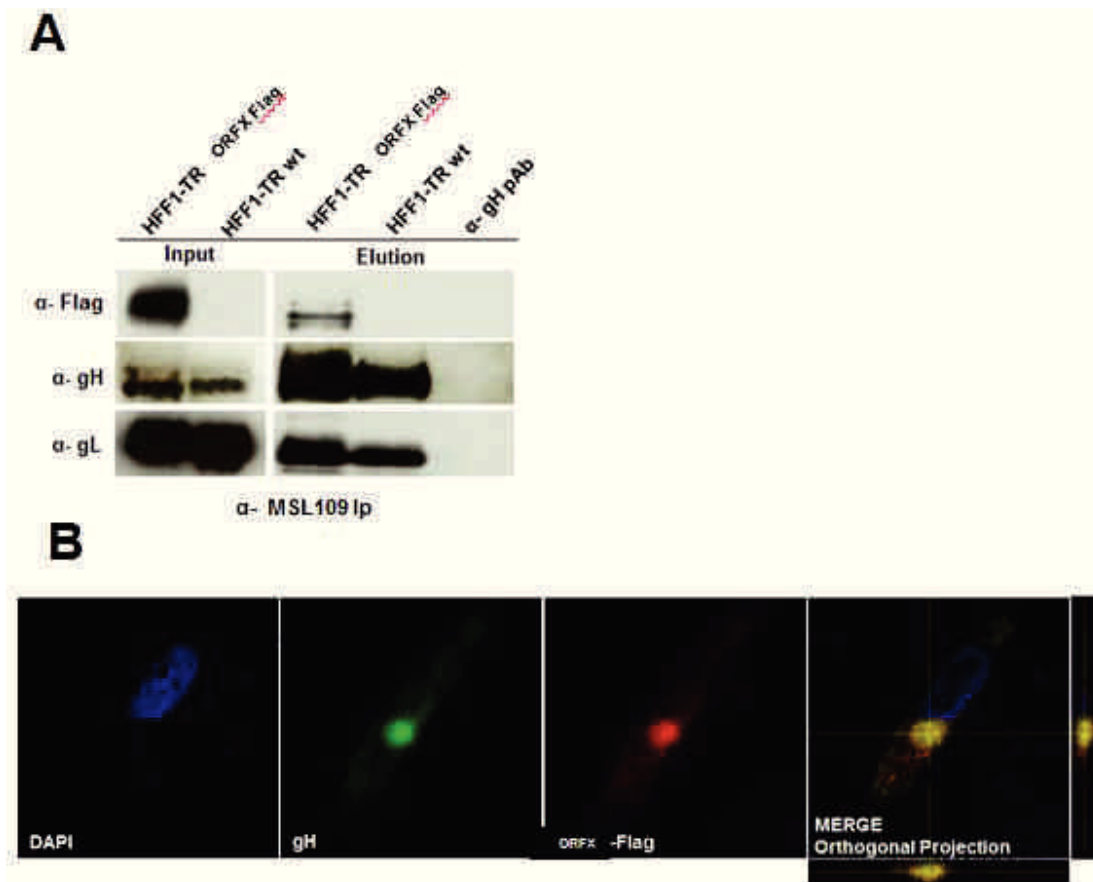


Fig. 6. ORF X interaction with gH in infected HFF-1 cells.(A) Co-immunoprecipitation (Co-IP) of the ORF X - gH complex. Co-immunoprecipitation studies were carried out with lysates prepared from HFF-1 infected with HCMV-TR ORF X Flag and wt TR separately. Complexes were captured using the human monoclonal antibody MSL-109. Elutions and crude extracts were subjected to immunoblot using a rabbit anti-gH polyclonal sera, an anti-Flag M2 clone monoclonal mAb and for control purposes a polyclonal antibody specific for gL. (B) Intracellular co-localization between signals derived from anti-Flag mAb (red) and anti gH pAb (green) among all the z planes showed on the left merge panel with the respective orthogonal projections.

SUPPLEMENTARY FIGURES

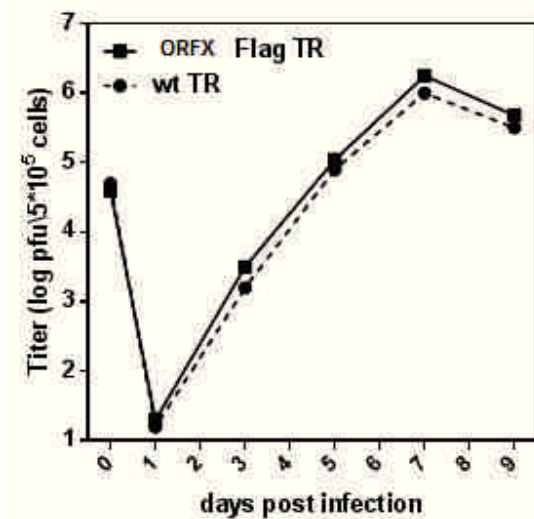


Fig S1. Multiple-step growth curve analysis of the reconstituted virus ORF X-Flag TR and of the parental HCMV strain TR. HFF cells seeded in six-well dishes (5×10^5 cells/well) were infected with an MOI of 0.1. At the indicated time points (days post infection) supernatants from the infected cultures were harvested, and total PFU of infectious virus in the culture supernatants were determined by plaque assay on HFF cells. Time point 0 titers represent input inocula and each data point represents the average of three independent wells.

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Recombinant Human Cytomegalovirus (HCMV) RL13 binds human immunoglobulin G Fc

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ABSTRACT

The human cytomegalovirus (HCMV) protein RL13 has recently been described to be present in all primary isolates but rapidly mutated in culture adapted viruses. Although these data suggest a crucial role for this gene product in HCMV primary infection, no function has so far been assigned to this protein. Working with RL13 expressed in isolation in transfected human epithelial cells, we demonstrated that recombinant RL13 from the clinical HCMV isolates TR and Merlin have selective human immunoglobulin (Ig)-binding properties towards IgG1 and IgG2 subtypes. An additional Fc binding protein, RL12, was also identified as an IgG1 and IgG2 binding protein but not further characterized. The glycoprotein RL13 trafficked to the plasma membrane where it bound and internalized exogenous IgG or its constant fragment (Fc γ). Analysis of RL13 ectodomain mutants suggested that the RL13 Ig-like domain is responsible for the Fc binding activity. Ligand-dependent internalization relied on a YxxL endocytic motif located in the C-terminal tail of RL13. Additionally, we showed that the tyrosine residue could be replaced by phenylalanine but not by alanine, indicating that the internalization signal was independent from phosphorylation events. In sum, RL13 binds human IgG and may contribute to HCMV immune evasion in the infected host, but this function does not readily explain the instability of the RL13 gene during viral propagation in cultured cells.

INTRODUCTION

Human cytomegalovirus (HCMV) infection is common and, although typically subclinical in the healthy population, it can cause severe disease in congenitally infected infants and in individuals with suppressed immunity [1]. In immunocompetent individuals, infection is controlled by both cellular and humoral immune responses, defenses that are weakened in immunocompromised patients leading to infection of several tissues and a vast range of cell types [2].

Characterization and use of clinical and low passage isolates of cultured HCMV strains have led to a reconsideration of the viral coding potential and suggested the presence of new unidentified functions (reviewed in [3]). Comparative sequencing analysis of unpassaged clinical isolates versus cell culture adapted viruses allowed a more refined identification of the genetic changes corresponding to functions that are lost in *in vitro* cultures. This is the case of RL13 and UL128 gene products, both possessing a suppressive phenotype on tissue culture adapted viruses [4,5]. For RL13 in particular, Stanton *et al.* recently reported rapidly emerging genetic mutations following a few passages of BAC-derived Merlin strain virus in cultures of human cells of different origins [6]. These authors, using an elegant BAC system of conditional gene repression during virus propagation, were able to show that virus with reconstructed wild type RL13 repressed cell culture growth while the emerging deletion mutants allowed the virus to adapt to cell culture growth [6]. Providing that a functional RL13 gene appears to be carried by all sequenced clinical isolates, the authors hypothesized that this protein is critical for productive HCMV replication *in vivo*, perhaps increasing the repertoire of HCMV cell tropism [6].

HCMV has evolved a number of different ways to evade the immune system, often employing seemingly redundant factors and mechanisms to maintain the lifelong infection of the host [7]. Limitation of the host antibodies and complement activities through the expression of viral proteins able to bind the constant region of immunoglobulin G (Fc γ) is a mechanism common to several

herpesviruses [8,9]. These viral proteins interfere with the host receptors for the Fc portion (FcγRs) of immunoglobulin G (IgG), expressed on the surface of all cell types in the innate and adaptive parts of the immune system [10].

FcγRs are a complex family of proteins with several distinct classes and subclasses that function at the interface of the adaptive and innate immune systems [10]. They sense immune complexes in the extracellular environment and regulate signaling cascades in effector cells, which may contribute significantly to balancing pro- and anti-inflammatory responses to infection. HCMV expresses two proteins of 34 and 68 kDa that bind the Fc region of IgGs [11–13]. gp34 is encoded in clinical isolates by RL11 and in laboratory isolates by the duplicate genes TRL11 and IRL11 [12,14]. gp68 is translated from the spliced mRNA encoded by UL119 and UL118 [11] and has been detected in preparations of purified virions [15]. Both gp34 and gp68 are glycosylated type I membrane proteins predicted to form immunoglobulin supergene family (IgSF)-like domains [11]. They bind all classes of human IgG with approximately equal affinity, whereas gp34 also binds rabbit IgG and, to a lesser extent, rat IgG1.

In this report we identified two additional Fcγ binding activities encoded by the HCMV genome, RL12 and RL13, and characterized this property of recombinant RL13 from the TR and Merlin strains of HCMV. We demonstrated that RL13 transfected into cultured human epithelial and fibroblast cells binds and internalizes the Fc portion of human IgGs.

RESULTS

Fc γ binding ability of selected members of the RL11 family

RL11 was the first identified Fc γ binding protein of HCMV [12] and is hypothesized to function through its Ig-like domain [11]. Using RL11 as positive control, we sought to test if RL10, RL12 and RL13 were also able to bind human IgGs. We included gB (UL55) as a negative control. HEK293T cells were transfected with expression plasmids for myc-His tagged gB, RL10, RL11, RL12, RL13. 48 h after transfection, flow cytometry analysis was performed with DyLight 649-conjugated human Fc γ and FITC-conjugated anti-myc. Permeabilized (Fig. 1A) and non-permeabilized (Fig. 1B) FITC-positive cells were tested for ability to bind human Fc γ . RL11, RL12 and RL13 bind the Fc portion of human IgG while RL10 and gB are comparable to negative control (Fig. 1A and 1B). It appears that a large fraction of the Fc γ -binding activity of RL13 (brown trace) is surface exposed, whereas a large fraction of RL11 and RL12 (blue and green trace, respectively) is not (Fig. 1A and 1B). Expression of the different proteins was equal as demonstrated by comparable percentage of FITC positive populations and their mean fluorescence intensity (data not shown). Taken together, these data suggested that RL12 and RL13 are two previously unidentified human Fc γ binding proteins encoded by HCMV.

RL11 has been shown to bind all different subclasses of human IgGs [11]. To assess if RL12 and RL13 differentially recognized human IgG subclasses, flow cytometry on permeabilized HEK293T cells expressing RL11, RL12, or RL13 was performed using individual human IgG subclasses as probes. Cells expressing RL11 bind all IgG subclasses whereas cells expressing RL13 appear to specifically bind IgG1 and IgG2 (Fig. 1C).

These data and similar analysis of RL12 binding to human Ig isotypes and binding of RL12 and RL13 to Igs from different species are summarized in Table 1. Although the finding that RL12 binds Fc γ was novel, we focused the rest of this report on RL13.

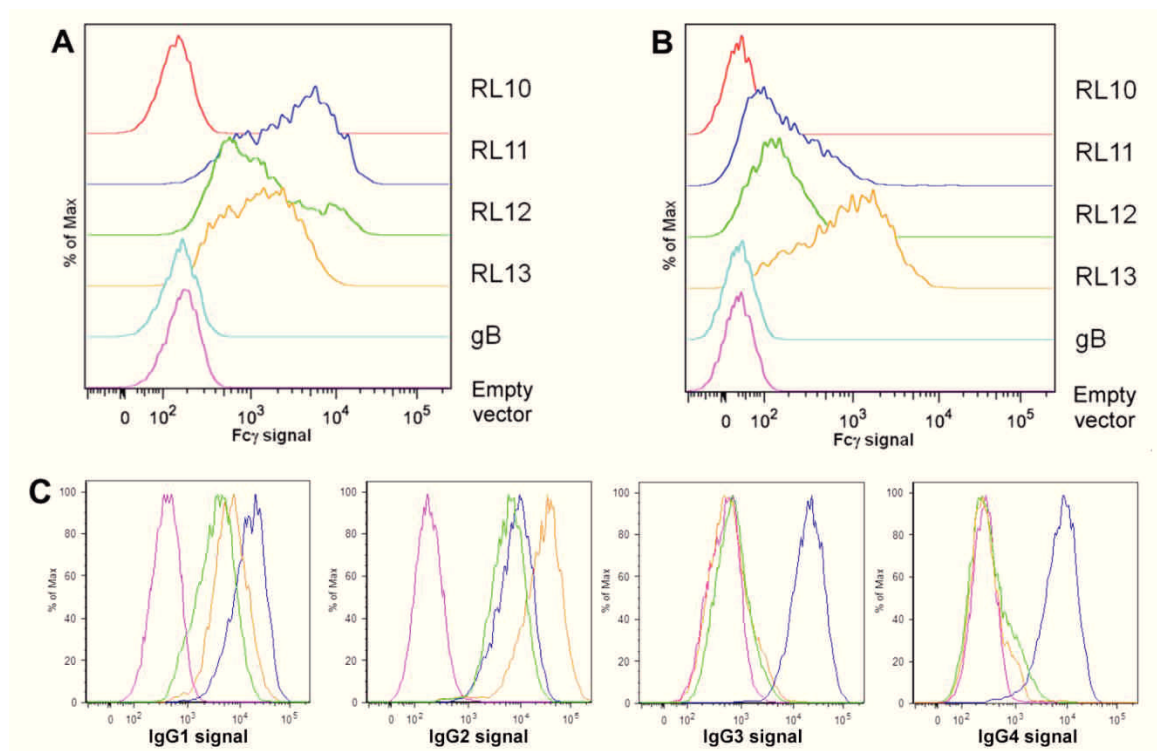


Figure 1: FACS analysis of Fc γ and IgG binding.

A: Binding of Fc γ to RL13 in permeabilized cells. HEK293T cells transfected with empty vector or expression vectors for myc-tagged gB, RL10, RL11, RL12 or RL13 were fixed, permeabilized and stained using FITC-conjugated anti-myc and 25 μ g/ml DyLight 649-conjugated human IgG Fc fragment (Fc γ). FITC positive cells were compared to mock transfected cells for their ability to bind Fc γ .

B: Binding of Fc γ to surface-exposed RL13. HEK293T cells transfected with empty vector or expression vectors for myc-tagged gB, RL10, RL11, RL12 or RL13 were first stained at 4°C with DyLight 649-conjugated human IgG Fc fragment (25 μ g/ml). Excess of probe was removed by washing in PBS and then cells were fixed and stained with FITC-conjugated anti-myc. FITC positive cells were compared to mock transfected cells for their ability to bind Fc γ .

C: Binding specificity of RL10-RL13 towards different human immunoglobulin subclasses. HEK293T cells were transiently transfected with myc-tagged RL11 (blue), RL12 (green), RL13 (brown) or with empty vector (magenta). Cells were fixed, permeabilized and stained with FITC-

conjugated anti-myc together with 10 $\mu\text{g/ml}$ of human Ig of the different subclasses. Alexa fluor 647-conjugated goat anti-human was used as secondary antibody. FITC positive cells were compared to mock transfected cells for their ability to bind $\text{Fc}\gamma$.

| Species | Ig subtype | Ig binding by | | |
|---------|------------|---------------|------|------|
| | | RL11 | RL12 | RL13 |
| Human | IgG1 | ++ | + | + |
| Human | IgG2 | ++ | ++ | +++ |
| Human | IgG3 | ++ | - | - |
| Human | IgG4 | ++ | - | - |
| Human | IgM | - | - | - |
| Human | IgA | - | - | - |
| Human | IgE | - | - | - |
| Rabbit | IgG | + | ++ | ++ |
| Mouse | IgG | - | - | - |
| Rat | IgG | - | - | - |
| Goat | IgG | - | - | - |

Table 1: Ig-binding specificity of Fc-binding proteins RL11, RL12 and RL13.

HEK293T cells were transfected with expression plasmids encoding a tagged version of RL11, RL12, and RL13 genes, stained with the indicated immunoglobulins, and analyzed by flow cytometry. +/++/+++ indicates efficiency of Fc-binding observed as mean fluorescent intensity; - indicates no binding.

Amino acid residues 109 to 218 of TR strain RL13 are necessary for Fcγ binding

The RL13 protein possesses strong topology similarities with the previously characterized HCMV Fcγ binding proteins coded by the RL11 and UL119 genes (gp34 and gp68 respectively) [11]. HCMV RL13 is a member of the RL11 multigene family [16] encoding a characteristic domain, known as RL11D or CR1 [17]. RL13 from HCMV strain TR has the typical features of a type I membrane glycoprotein (Fig. 2A): a 20 amino acids (aa) long signal peptide, a transmembrane domain in the 248-268 aa region, and a predicted cytoplasmic tail of 26 aa in accordance with previous studies [6]. Further analysis suggests the likely presence of 11 N-linked glycosylation sites (pos. 21, 32, 37, 41, 90, 108, 120, 167, 201, 208, 215 aa) and 22 O-linked glycosylation sites along the sequence, all restricted to the N-terminal region between residues 40 and 89. gp34 and gp68 contain a DxxxLL dileucine consensus motif for internalization and a potential I/V/L/SxYxxL intracytoplasmic immunoreceptor tyrosine based-like inhibition motif, respectively [11,12]. Similarly, we noted the presence of a tyrosine-based motif (YxxL) for intracellular targeting of transmembrane proteins [18] in the C-terminal cytoplasmic domain of the RL13 sequence (Fig. 2A). A search for conserved patterns in the TR RL13 ectodomain revealed an immunoglobulin (Ig)-like domain (Fig. 2A) identified as SM00409 (IG) in the SMART family classification (106-205 aa, with an HMMSmart E-value of 6.16e-04) [19]. A multiple sequence alignment generated from 15 different HCMV strains reported a very high conservation along the entire domain except 3 short regions predicted to form loops (Fig. 2B). All 15 sequence variants contain an immunoglobulin domain identified as SMART SM00409 or Interpro IPR013783 (E-values ranging from 9.9e-07 to 3.4e-04 and from 4.70E-07 to 6.10E-04, respectively). The secondary structure predictions suggested a propensity of the identified TR RL13 Ig-like domain to form a series of 8 beta-strands (Fig. 2B).

To investigate the involvement of the Ig-like domain in the Fc γ binding ability, we produced 4 different mutants of the RL13 ectodomain (Fig. 2C): RL13 Δ NT, lacking 76 residues (aa 24-99) from the N-terminal region predicted to contain the O-linked glycosylation sites; RL13 Δ E1, lacking 29 (aa 218-246) residues between the Ig-like domain and the transmembrane region; RL13 Δ E2 (aa 159-246), with the same deletion as above plus 57 residues of the C-terminus of the Ig-like domain; and RL13 Δ Ig, lacking the entire Ig-like domain (aa 109-218). The mutant proteins were transiently expressed fused to myc and His tags in HEK293T cells with comparable expression levels as judged by flow cytometry analysis using anti-myc antibody (data not shown).

Fc γ binding ability was assayed by flow cytometry of myc-expressing permeabilized cells after staining with different concentrations of DyLight 649-conjugated Fc γ (Fig. 3A, only 12.5 μ g/ml concentration is shown). Quantification of the results is reported as percentage of Fc γ stained cells in each sample compared to wild type RL13 (Fig. 3B). Values from RL13 Δ NT were comparable to the wild type RL13, while a substantial reduction was observed in the RL13 Δ E1 and RL13 Δ E2 samples. Deletion of the Ig-like domain carried by the RL13 Δ Ig (RL13 Δ E1 and RL13 Δ E2) mutants completely abolished specific Fc γ binding since the number of positive cells was comparable to negative controls, cells expressing RL10 or transfected with empty vector (Fig. 3A-B).

These data indicated that the Fc γ binding activity of RL13 maps to amino acids 109-218. Removal of the region located between the Ig-like domain and the transmembrane domain severely impaired Fc γ binding but did not completely abrogate it. This observation suggests that this region could be important for the stability of the binding and/or the correct protein fold.

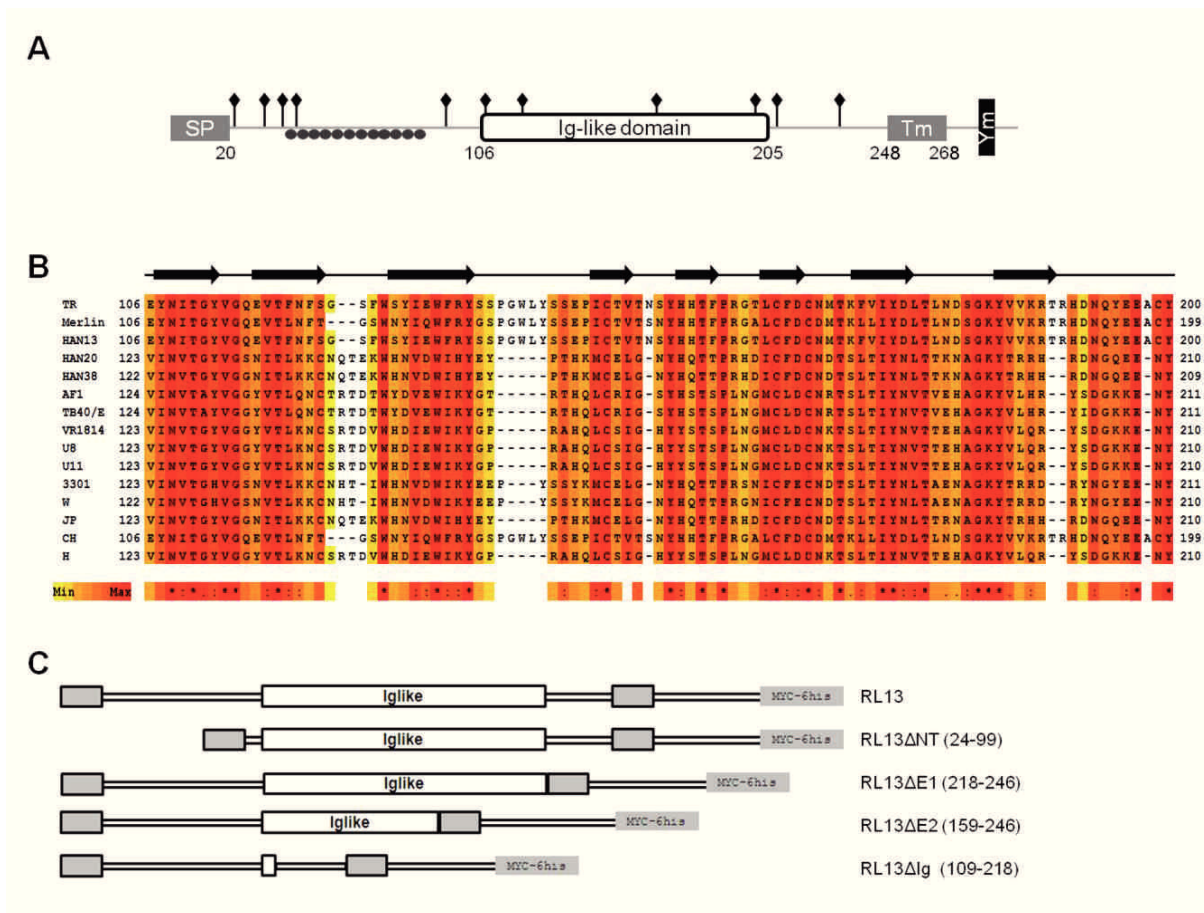


Figure 2: Predicted structure of the RL13 protein and schematic representation of TR RL13 ectodomain mutants.

A: Schematic representation of the HCMV TR RL13 full length protein. The signal peptide at the N-terminus (SP), the Ig-like domain, the transmembrane domain (Tm), the tyrosine-based motif YxxL (Ym) sorting signal, 11 potential N-linked glycosylation sites (diamonds), and the O-linked glycosylation region (closed circles) are indicated.

B: Multiple sequence alignment of the predicted RL13 Ig-like domain from the indicated strains of HCMV. The residues are colored according to the conservation level (red for higher conservation). Asterisks (*) below the alignments represent conserved amino acid in all sequences; colons (:) represent residues with similar physicochemical properties; dots (.) semi-conserved residues. The black arrows represent the positions of predicted β strands along the sequence.

C: Graphic representation of TR RL13 and the ectodomain mutants used in this study. The amino-terminal signal peptide, the carboxy-terminal transmembrane (gray boxes) and the Ig-like domain (white box) are indicated.

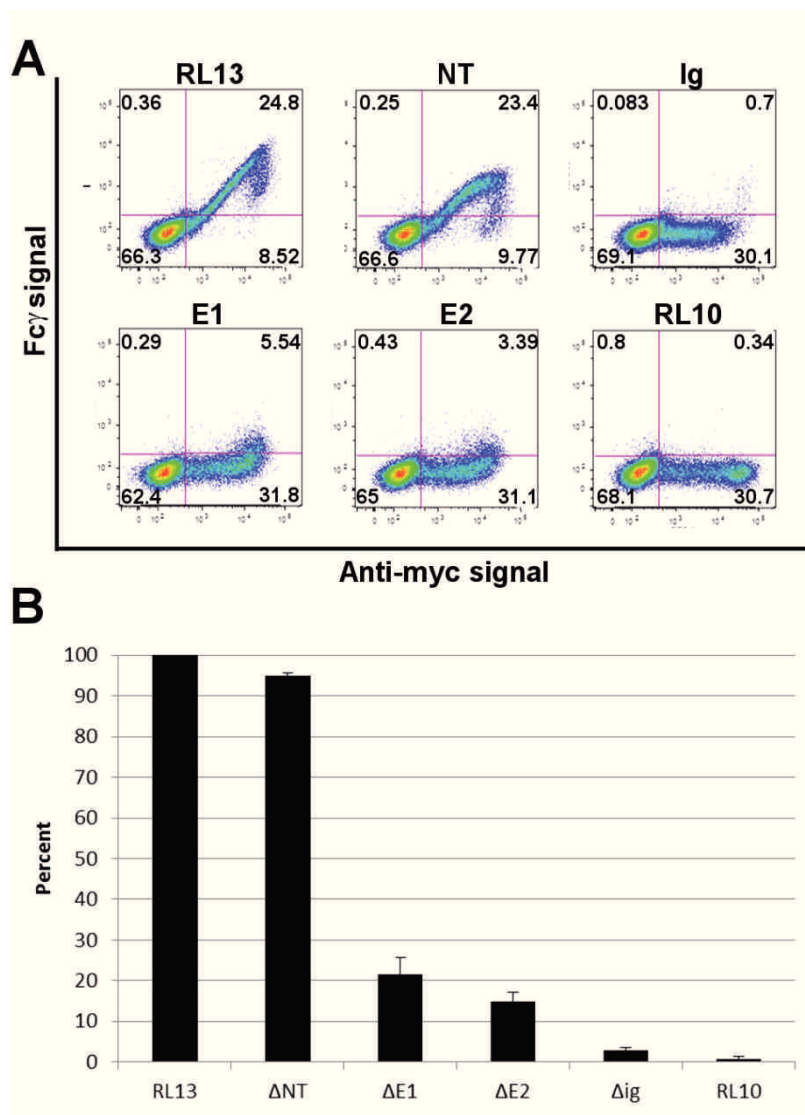


Figure 3: Fc γ -binding by RL13 ectodomain mutants.

A: Cytofluorometric analysis of Fc γ binding by RL13 ectodomain mutants. HEK293T cells transfected with the indicated constructs were permeabilized and stained with DyLight 649-conjugated human IgG Fc fragment (Fc γ , 12.5 μ g/ml) and Alexa Fluor 488-conjugated mouse anti-myc. Signals from 10,000 myc-positive cells are shown in each graph. The percentage of cells in each quadrant is indicated.

B: Quantitative analysis of the FACS data. Each histogram shows the percentage of Fc γ positive cells relative to the RL13 wild type positive population (RL13). Values and error bars represent the mean and range of three independent experiments.

Subcellular localization of RL13 in transfected cells

Stanton *et al.* recently reported that adenovirus-expressed RL13 transits through the Golgi and traffics mainly to Rab5-positive cytoplasmic vesicles [6]. We sought to verify the intracellular localization of RL13 expressed transiently in human cells. Confocal microscopy images were collected from epithelial (ARPE-19) and fibroblast (MRC-5) cells transfected with two different constructs, the previously described myc-His tagged construct and a second construct coding for RL13 with a C-terminal YFP-tag. Since sub-cellular localization of RL13 with different tags was identical in both cell types (data not shown), only representative data obtained in ARPE-19 human epithelial cells with RL13-YFP are shown.

48 h after transfection, cells were fixed, permeabilized and stained with different markers of cellular compartments and with fluorophore-conjugated Fc γ . The confocal microscopy analysis of these samples is shown in Fig. 4A. RL13 co-localized to a limited extent with Golgi markers and EEA1-positive endosomes and more extensively with markers of the trans-Golgi network (TGN) (Fig. 4A, left panels). Fc γ co-localized extensively with RL13 (Fig. 4A, far right panels), although a population of RL13 that does not bind to the Fc of human IgGs was consistently observed (Fig. 4A, green color in far right panels). Although it is tempting to speculate that this pool represents an immature ER population, also suggested by the shape of the RL13 distribution, we have been unable to find any co-localization of RL13 with the ER marker PDI (not shown).

The presence of RL13 on the surface of both HEK293T and ARPE-19 cells was further verified by the use of a mouse monoclonal antibody (mAb 5H3/B10) directed against the ectodomain portion of RL13. Analysis by flow cytometry was performed on transiently transfected non-permeabilized HEK293T cells (Fig. 4B). A clear increase in fluorescence was obtained on RL13 expressing cells stained with mAb 5H3/B10 compared to mock transfected cells or isotype control. Confocal microscopy analysis of non-permeabilized RL13 expressing ARPE-19 cells stained with

both Fc γ and 5H3/B10 revealed a strong accumulation of RL13 on plasma membrane clusters (Fig. 4C) that extensively overlapped with Fc γ signals (Fig. 4C, white color in right merge panel).

These observations are consistent with RL13 trafficking through the secretory pathway and recycling from the plasma membrane, although other possible explanations for this pattern have not been excluded.

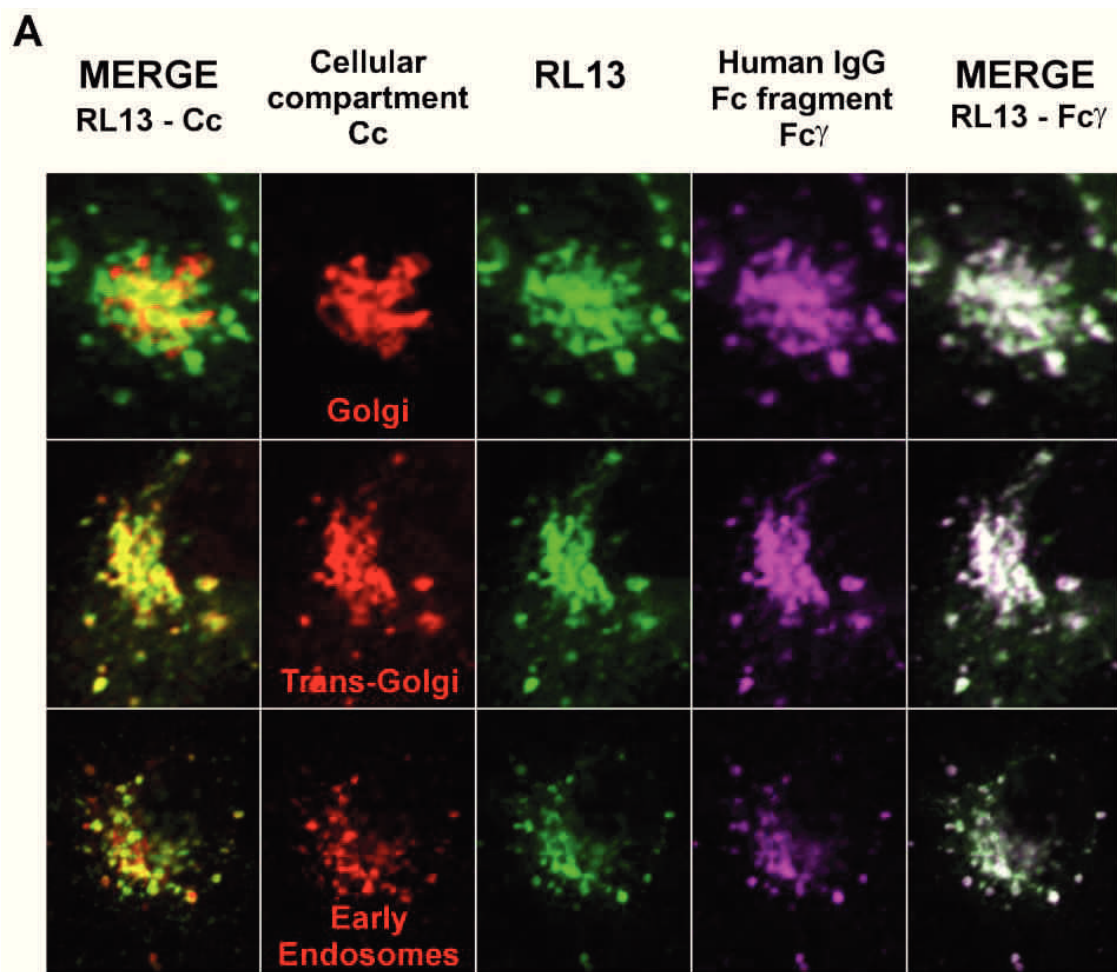
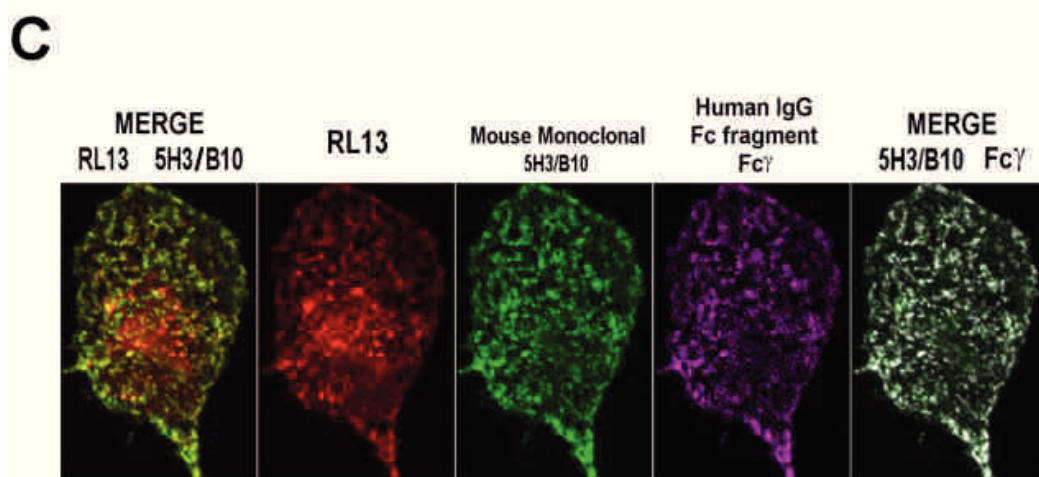
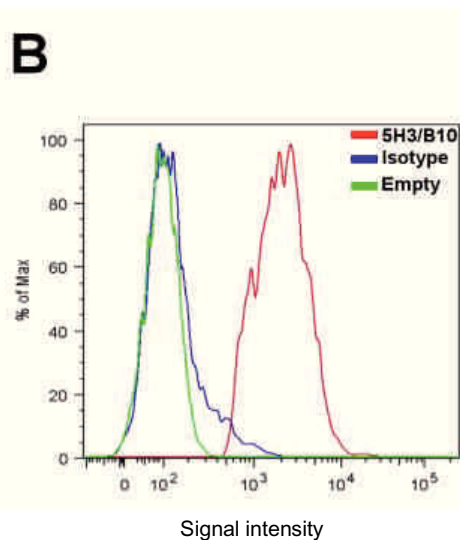


Figure 4: Localization of RL13 in transfected cells.

A: Co-localization of RL13 with organelle markers and Fc γ in ARPE-19 cells. ARPE-19 epithelial cells were transfected with RL13-YFP fusion protein (green color, central column) and treated for confocal analysis 48 h later. Cells were fixed, permeabilized and stained with antibodies against either GM130, TGN46 or EEA1 intracellular markers (red color, second column) and with 20 $\mu\text{g/ml}$ of DyLight 649-conjugated human IgG Fc fragment (magenta color, fourth column). The merged panels on the far left show co-localization between RL13 and, from top to bottom, markers of Golgi (GM130), trans-Golgi (TGN46) and early endosomes (EEA1) respectively (co-localization in yellow). The merged panels on the far right show co-localization of Fc γ with RL13 (co-localization in white).



B: Flow cytometry analysis of RL13 surface-expression. HEK293T cells were transiently transfected with vector coding for YFP (green line) or RL13-YFP fusion protein (red and blue lines). Cells were allowed to recover for 48 h and then surface-exposed RL13 was stained either with 5 $\mu\text{g}/\text{ml}$ of mouse monoclonal antibody directed against RL13 ectodomain (clone 5H3/B10, red and green lines) or isotype control at the same concentration (isotype, blue line) on ice. Alexa fluor 647 conjugated goat anti-mouse was used as secondary antibody. YFP positive cells were compared to empty vector transfected cells for their ability to bind 5H3/B10 antibody.

C: Immunofluorescence analysis of RL13 surface expression on ARPE-19 cells. ARPE-19 cells expressing RL13-YFP fusion protein (red color) were stained on ice with 12.5 $\mu\text{g}/\text{ml}$ of 5H3/B10 monoclonal antibody (green color) and 20 $\mu\text{g}/\text{ml}$ of DyLight 649-conjugated human IgG Fc fragment (magenta color) without permeabilization. Merge panels show co-localization between 5H3/B10 and either RL13-YFP or Fc γ signals (yellow color in the far left panel and white color in the far right panel, respectively).

Internalization of RL13-Fc γ complex

The presence of an internalization motif in the C-terminal tail, together with the intracellular localization data in Fig. 4, suggested that RL13 could traffic to the cell surface where it may bind and internalize extracellular IgGs. To test this hypothesis, we performed a short time-course characterization of the internalization of YFP-tagged RL13 in transfected ARPE-19 cells. Cells were initially placed on ice to reduce lateral diffusion of membrane proteins and block potential internalization of the ligand. Fluorescently labelled Fc γ was added and allowed to bind for 30 min on ice. Following extensive washing of the Fc γ excess and fixation of the cells, fluorescence analysis at time 0 showed that RL13 partially co-localized with Fc γ on the surface of transfected cells (Fig. 5A). Restoring the internalization processes at 37°C induced the uptake of the RL13- Fc γ complex, the majority of which accumulated mostly in large ring-shaped structures after 30 min (Fig. 5B). These RL13-Fc γ structures persisted 90 min after the shift to 37°C and stained positive for the early endosome marker Rab5 (Fig. 5C).

These data demonstrate that RL13 binds to the Fc of human IgGs on the cell surface and that the internalized complex remains associated with large membranous vesicles containing markers of early endosomes.

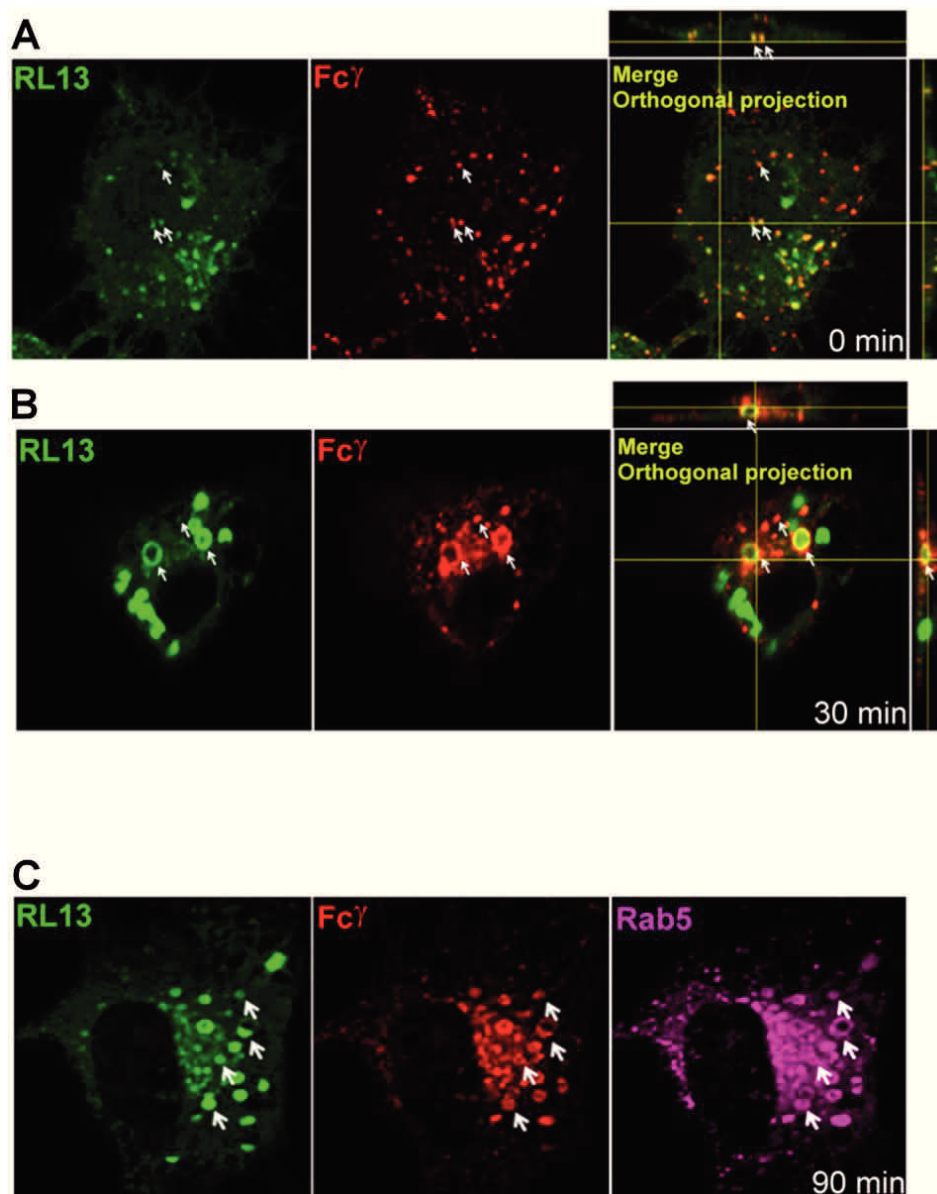


Figure 5: RL13 binds and internalizes Fc γ .

ARPE-19 epithelial cells were transfected with RL13-YFP (green) and allowed to recover for 48 h. Cells were placed on ice 5 min before adding DyLight 649-conjugated human IgG Fc fragment (red) and incubated on ice for 30 min. Then, cells were washed and **A**: immediately fixed, **B**: shifted to 37°C for 30 min, or **C**: shifted to 37°C for 90 min before proceeding to fixation and confocal analysis. Rab5 (purple) was revealed by specific antibodies after permeabilization of the samples. Examples of co-localization between signals are indicated by white arrows. Orthogonal projections of the optical sections acquired from Z-stack are shown. White arrows indicate examples of co-localized spots. Fluorescent Fc γ was absent on the membrane of ARPE-19 cells transfected with empty expression vector used as control and no staining was detected following 30 min switch at 37°C (data not shown).

Mutation of RL13 sorting motif affects the internalization of Fc γ

As shown by the *in silico* analysis, the carboxy-terminal domain of RL13 contains a tyrosine-based sorting signal (YxxL₂₈₂) that could be responsible for the internalization of the RL13-Fc γ complex. A local multiple sequence alignment of the C-terminal region of RL13 from 15 HCMV strains showed that the YxxL motif was highly conserved (Fig. 6A, residues 279-282). To test the contribution of the cytoplasmic tail, and in particular of the tyrosine motif, to the internalization of the RL13-Fc γ complex, a panel of mutants were built (Fig. 6B). Plasmids encoding RL13 with three single amino acid substitution mutants, Y279F, Y279A, T280A, one double substitution mutant Y279A/L282A (indicated as AA), one triple substitution mutant Y279A/R281A/L282A (AAA), and one quadruple substitution mutant with all amino acids of the motif replaced by alanines (AAAA) were produced. Additionally, plasmids encoding a YxxL deletion mutant (Δ 279-282), a deletion of the motif and the entire C-terminal (Δ 279-294), and a deletion of 12 amino acids immediately C-terminal to the sorting motif (Δ 283-294) were generated. 48 h after transfection, internalization of the mutated/deleted proteins was assessed by flow cytometry on HEK293T cells incubated with Fc γ at 4°C for 30 min and, after removal of the ligand by extensive washing, shifted to 37°C. The percent of internalized Fc γ was calculated from the mean fluorescence intensity of the samples incubated at 37°C for 30 minutes and the samples submitted to the same treatment but kept on ice. As shown in Fig. 6C, the YxxL motif is critical for protein internalization since removing the region downstream did not alter the RL13 Fc γ internalization ability while deletion of the motif, both individually or with the C-terminus sequence, strongly reduced the internalization ability of RL13 (Fig. 6C). Substitution of the tyrosine 279 with phenylalanine, an alternative aromatic side chain, did not reduce the ability of RL13 to internalize. In contrast, uptake was reduced by approximately 1/3 when an alanine was placed in this position. Substitution of the adjacent threonine 280 with alanine did not show a difference in internalization compared to the wild type. However, leucine 282 appeared to play a role as shown by the further internalization reduction of

the Y279/L282 double mutant (Fig. 6C). Substitution of multiple residues with alanine reduced internalization to the same extent as complete deletion of the YxxL motif (Fig. 6C).

Taken together these results are consistent with the YxxL₂₈₂ motif being necessary for Fcγ internalization by RL13 and suggest that an aromatic residue at position 279 is required.

Fcγ binding ability is conserved in the HCMV Merlin strain

Due to the high variability of the RL13 genes among different HCMV strains [20] we also tested the Merlin RL13 protein (87% aa identity with its TR counterpart) for its Fc γ binding ability. *In vitro* synthesized Merlin RL13 gene was cloned in pcDNA3.1(-)/myc-His expression vector and used to transfect HEK293T cells. Intracellular staining was performed as previously described and cells were analyzed through flow cytometry. Both the expression levels (data not shown) and the Fc γ binding ability of cells expressing RL13 from Merlin and TR strains were comparable (Fig. 7A). To further confirm the specificity of the binding, both proteins were immunoprecipitated from transfected cell lysates with biotinylated Fc γ . Complexes were captured with magnetic streptavidin beads and eluted material was separated by SDS-PAGE and analyzed by immunoblot using anti-tag and anti-human IgG antibodies (Fig. 7B). Both Merlin and TR RL13 proteins were successfully immunoprecipitated from cell lysates. The observed molecular weights of the two proteins in denaturing and reducing conditions appeared to be slightly different: TR RL13 was composed of three major isoforms of around 123 kDa, 111 kDa and 76 to 66 kDa, while Merlin RL13 gave three bands of 116 kDa, 108 kDa and 66 to 52 kDa. These differences in the molecular weight could reflect the different glycosylation profiles, with 7 and 11 predicted N-linked glycosylations for RL13 from Merlin and TR strains, respectively.

These experiments support the conclusion that the Fc γ -binding ability of RL13 is conserved in at least two different clinical HCMV isolates.

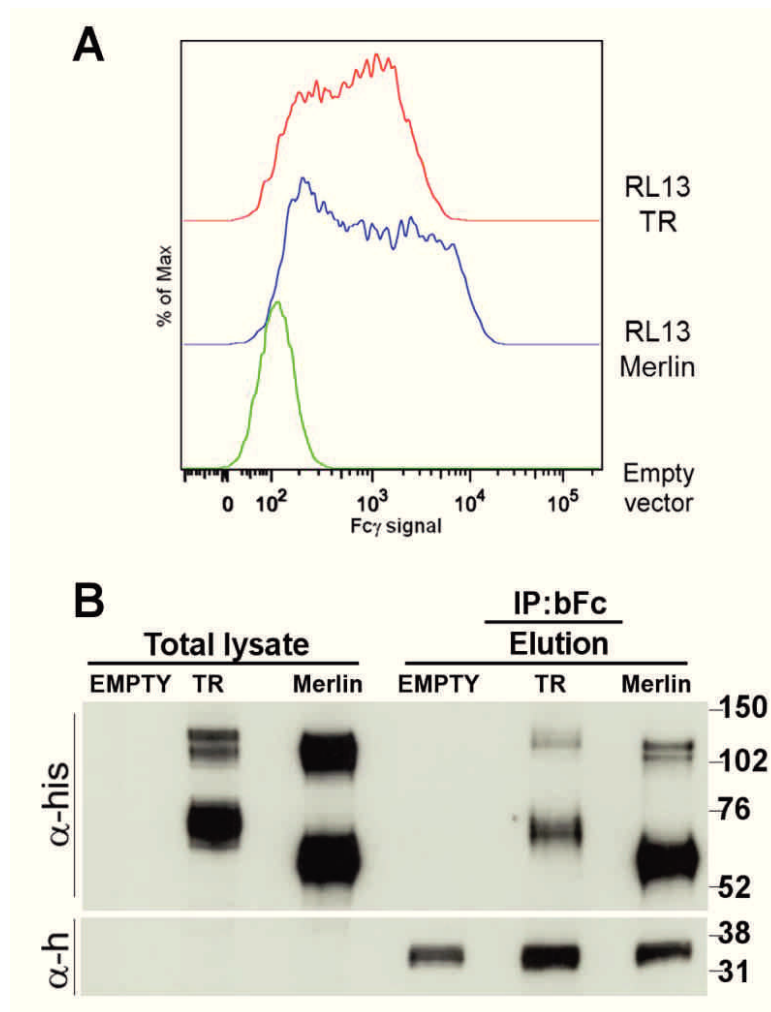


Figure 7: Fc γ binding is a conserved function for recombinant RL13 from Merlin and TR clinical strains.

A: HEK293T cells were transfected with plasmids coding for myc-His tagged RL13 from TR (red), Merlin (blue) strains and empty vector (green). 48 h post-transfection cells were collected, permeabilized and stained with FITC-conjugated mouse anti-myc and 25 μ g/ml of DyLight 649-conjugated human IgG Fc fragment. 10,000 FITC-positive cells were compared to empty vector transfected cells.

B: HEK293T cells were transfected with empty vector or plasmids coding for myc-His tagged RL13 from TR and Merlin strains. 48 h post-transfection cells were collected, lysed and subjected to immunoprecipitation with biotinylated Fc γ fragment. Total lysates and elution fractions were subjected to western blot analysis using HRP-conjugated anti-his tag (α -his) and anti-human IgG (α -h) antibodies. TR: sample expressing myc-His tagged RL13 from TR strain; Merlin: sample expressing myc-His tagged RL13 from Merlin strain; Empty: empty vector transfected cells.

DISCUSSION

The low fitness of wild-type RL13-carrying strains cultured *in vitro*, leading to the destruction of the ORF present in the clinical isolates within a few passages [5,6], make functional studies of this protein difficult. To better understand its function, we used synthetic RL13 genes reproducing the sequences of the clinical isolates TR and Merlin. In the absence of an unpassaged reference sample of TR, we cannot say with certainty that the sequence and function of the TR RL13 we used is identical to the originally isolated, naturally occurring virus. However, we do not detect functional differences between RL13 from the TR and Merlin strains. Expression of this recombinant protein in human-derived epithelial cells allowed us to demonstrate that RL13: a) binds Fc from IgG of human (and rabbit) origin; b) preferentially binds to Fc from IgG1 and IgG2 subclasses; c) is at least transiently exposed on the cell surface and is subsequently internalized with (and likely without) bound Fc γ ; d) internalization relies on a YxxL motif and e) its Ig-like extracellular domain is necessary for Fc γ binding. Similar analysis of RL13 in human fibroblasts produced identical results (data not shown). Furthermore, we revealed that RL12 also binds IgG1 and IgG2, although we did not study this interaction in detail. Our data does not offer an explanation for why RL13 is so rapidly mutated when clinical isolates of HCMV are grown in cultured cells [5].

Apart from HCMV, viral Fc γ binding proteins (vFcBPs) are present on herpes simplex virus type 1 (HSV-1), HSV-2, and varicella zoster virus and on the non-human herpesviruses pseudorabies virus and murine cytomegalovirus [21–25]. The most accepted model to explain how those activities promote immune evasion is the so called “antibody bipolar bridging” in which the neutralizing potential of the IgG Fab domain is counteracted by simultaneous binding of vFcBPs to the Fc domain of the same immunoglobulin molecule [26,27]. The possibility of this mechanism has been supported at a molecular level by Sprague and coworkers [27], who crystallized a portion

of HSV gE ectodomain and gE-gI complex bound to an human IgG Fc fragment. Alternatively, it has been hypothesized that HSV, as well as HCMV vFcBPs, function once expressed on infected cell surface by endocytosis and subsequent degradation of complexes including human IgG and viral antigens [11,27]. Consistent with this hypothesis, we observed that RL13 is able to internalize human IgG Fc fragment from the surface of transfected cells trafficking at early times in Rab5 and EEA1 positive endosomes. The well-recognized YxxL motif is essential for RL13 endocytosis through the aromatic nature of the tyrosine and not its property of phosphate acceptor. As already described for the RID receptor of adenovirus [28], this type of sorting signal does not affect signal transduction pathways. On the other hand, the fact that RL13 is located on the envelope of the virus [6] suggests that antibody bipolar bridging could be an important function of RL13 that would help protect HCMV virions from complement- and antibody-dependent neutralization.

RL13 and RL12 discriminate among human IgG subtypes showing no association with IgG3 and IgG4. To our knowledge, this observation remains the only example of vFcBPs selecting not only among immunoglobulin isotypes but also IgG subtypes. IgG2 represents roughly 25% of human total plasma IgGs in adults and has been traditionally associated with protection from infection with encapsulated bacteria since their immune function is primarily directed against polysaccharide antigens [29]. Recent reports, however, have pointed out a significant association between IgG2 deficiency and severe or even fatal clinical outcome of the pandemic influenza A (H1N1), although IgG2 unbalance has been suggested to be a consequence of cytokine deregulation rather than a predisposing risk factor [30,31]. Due to the low number of studies performed on human IgG2 function related to viral infection, the meaning of such observation remains elusive and needs further analysis. Compared to IgG1, subtype 2 immunoglobulins cannot mediate complement-dependent cytotoxicity or NK-mediated antibody-dependent cellular cytotoxicity (ADCC) but do engage FcγRIIIa receptors and trigger ADCC mediated by cells of the myeloid lineage [32] (9) (Schneider-Merck, Lammerts van Bueren et al. 2010). Again, the relative

importance of IgG2 subtype in protecting from viral infection just begun to be addressed and it is difficult to evaluate the importance of a viral factor able to counteract such mechanism, if any, but it is noteworthy that HCMV has a latent reservoir in myeloid cells [33]. It is tempting to speculate that a viral IgG2-binding receptor could compete with cellular receptors for binding to cellular antibodies to viral proteins and prevent ADCC. It would be important to determine the affinity of RL13, as well as of the other HCMV Fcγ binding proteins, toward IgG2 to evaluate if this protein represents a function more dedicated to a particular IgG subtype.

Atalay and coworkers described the presence of immunoglobulin supergene family (IgSF)-like domains in the products of both RL11 and UL119 genes [11]. We have mapped the RL13 Fcγ binding activity to the Ig-like domain in the extracellular region, while the N-terminal proximal region composed of approximately 70 amino acids is not crucial for the binding. Indeed, Sprague *et al.* showed that removing the N-terminal part of the gp68 ectodomain did not impair the Fcγ binding [13] (10) (Sprague, Reinhard *et al.* 2008). These data are consistent with the presence of an immunoglobulin supergene family (IgSF)-like domains in all the described HCMV Fc binding proteins. Our findings do not rule out the possibility that RL13 could carry out other functions. The best characterized viral Fc binding protein, the gE product of HSV-1, is also involved in cell-to-cell spread or trafficking of viral proteins from neuron body cell into axon independently of its Fc binding capability [34,35]. We are currently investigating other possible roles of RL13 during HCMV pathogenesis.

Our preliminary characterization of transiently transfected RL13 agrees only partially with what was reported by Stanton *et al.* [6]. They found significant differences in cellular localization and apparent SDS-PAGE MW between adenovirus-expressed RL13 and the protein expressed in the context of viral infection. In their report, adenovirus-expressed RL13 showed a lower MW (80 and 55 kDa) compared to the species found in infected cells (100 and 55 kDa) and the protein only co-localized with TGN in the context of infection [6]. The behavior of RL13 observed in this report is

more similar to Stanton *et al.*'s data for infected cells, showing a MW above 100 kDa and a sub-cellular co-localization with early endosomes and the TGN. This last observation would be consistent with a default mechanism of internalization similar to what was described for other HCMV envelope proteins [36]. We currently have no explanation for the slight discrepancies between Stanton *et al.* and our report that may be due to the different expression system used or, at least for the confocal analysis, to the protein derived from a different strain. However, characterization of the expressed RL13 remains preliminary and further studies will elucidate this point.

To conclude, we have shown that RL13 encoded by HCMV TR binds the Fc portion of human IgG1 and IgG2 and propose that this protein is used by the virus to circumvent the humoral immune response in the host.

MATERIALS & METHODS

Sequence data, in silico analysis and predictions

Start to stop open reading frames (ORFs) in the complete genome sequence of the TR strain (Genbank: AC146906.1) were identified using the Getorf program from the EMBOSS suite 5.0.0 [37]. The sequence similarity searching algorithm FASTA 35.4.3 [38]. was exploited to compare the protein sequences of RL13 gene product from the Merlin strain (Genbank: YP_081461.1) to all the TR ORFs and select homologous ones with BLOSUM50 as substitution matrix and imposing an expectation value (E) upper limit of 1e-05. The putative TR RL13 sequence was searched for specific conserved signatures using InterProScan 4.8 [39] with databases v.34.0. Multiple sequence alignments were performed using the PSI-Coffee mode of T-Coffee v.9.01 [40], comparing the TR RL13 with an additional 14 different RL13 protein sequences (Genbank: YP_081461.1, AEI84615.1, ACS91939.1, AAR31271.1, AAR31220.1, ACT81850.1, ACT81685.1, ACS92104.1, ACZ79760.1, ACZ79925.1, ADI46773.1, ACZ80255.1, ABV71530.1, ACZ80090.1). Signal peptides and transmembrane regions were predicted by Phobius [41], while secondary structures were predicted using PSIPRED [42]. N-linked and O-linked glycosylation sites were predicted using NetNGlyc 1.0 (<http://www.cbs.dtu.dk/services/NetNGlyc/>) and NetOGlyc 3.1 [43] (<http://www.cbs.dtu.dk/services/NetOGlyc/>) respectively.

Cells, plasmids and antibodies

ARPE-19, MRC-5 and HEK293T cell lines were brought from ATCC and cultured according to the supplier's instructions. DMEM high glucose and DMEM:F12 media (Gibco, Invitrogen) were supplemented with 10% fetal calf serum (FCS) and penicillin streptomycin glutamine (Gibco, Invitrogen). Lipofectamine 2000 (Invitrogen) was used to transfect HEK293T cells while Fugene 6 (Roche) and Nucleofector kit V (Amaxa) were used to transfect ARPE-19 cells as suggested by

manufacturer. Human codon-optimized RL10, RL11, RL12, RL13 and gB (UL55) HCMV genes based on the TR strain sequence and RL13 from Merlin strain (NCBI Reference Sequence: YP_081461.1) were synthesized by Geneart and cloned in plasmid pcDNA3.1(-)/myc-His C (Invitrogen) in frame with C-terminal myc and six histidine tag sequences. Fluorescent fusion proteins were obtained by subcloning a gene of interest upstream of the EYFP sequence in pEYFP-N1 vector (Clontech). Ectodomain and C-terminal tail RL13 mutants were cloned using QuickChange™ Site-directed Mutagenesis kit and instructions therein (Stratagene). Primary antibodies used in this work were mouse anti-His (C-term) and mouse anti-PDI (Invitrogen), mouse anti-Rab5 mouse, anti-GM130, mouse anti-TGN46 and mouse anti-EEA1 (Abcam). Mouse monoclonal antibody against RL13, clone 5H3/B10, was developed by Areta International (Gerenzano, Italy). Mice immunization was performed with peptides encompassing the amino acid region 111-246 of TR strain RL13. Secondary antibodies were Alexa Fluor 488-, 568-, and 647-conjugated goat anti-mouse (Invitrogen) and HRP-conjugated secondary antibodies from Perkin Elmer. Chrompure DyLight 649-conjugated rat, rabbit, mouse, goat IgG and human Fc fragment were from Jackson Immunoresearch, human IgG subclasses from SIGMA, and human IgA, IgE, IgM from Calbiochem.

Flow cytometry

For intracellular staining, HEK293T cells transfected with the plasmids of interest were trypsin detached 48 h post-transfection, incubated 30 min at RT with Live&Dead Aqua (Invitrogen), diluted 1:400 in PBS, fixed and permeabilized with Cytotfix/Cytoperm kit (BD) and stained as instructed by the manufacturer. For cells expressing the myc-tagged proteins, mouse anti-myc-FITC antibody was used at 1:500 dilution. Binding of the Fc portion of the human IgGs (Fc γ) was assessed using DyLight 649-conjugated human IgG Fc fragment or different human immunoglobulin isotypes and human IgG subclasses at different concentrations (5-50 μ g/ml). Alexa Fluor 647 fluorophore-conjugated secondary antibodies against the above mentioned species were

used at 1:200 dilution. For membrane staining, transfected cells were detached with trypsin 48 h post-transfection and incubated for 30 min on ice with DyLight 649-conjugated human Fc γ . When needed, cells were subsequently subjected to intracellular staining. A total of 10^4 cells were analyzed for each histogram using FACSCanto II (Becton Dickinson, Heidelberg, Germany). For detection of membrane exposed RL13, HEK293T were transiently transfected with vectors coding for YFP or RL13-YFP fusion protein, were incubated with different dilutions of mouse monoclonal 5H3/B10 antibody or mouse IgG1 isotype control (Sigma) for 60 min on ice. As secondary antibody, Alexa Fluor 647-conjugated goat anti-mouse was used for 30 min on ice at 1:200 dilution. Internalization of Fc γ in HEK293T cells was quantified through flow cytometry. 48 h post-transfection cells were detached with trypsin and transferred to round bottom 96 well plates for staining. Cells were washed in cold PBS and incubated on ice with human IgG Fc fragment at different concentrations (50, 25, 12.5 μ g/ml). After 30 min incubation, cells were extensively washed and incubated with warm medium to allow for endocytosis (Fc $\gamma_{37^\circ\text{C}}$). Half of the samples were kept on ice for the duration of the experiment (Fc $\gamma_{4^\circ\text{C}}$). At the end of the endocytosis, cells were washed with cold medium and Fc γ remaining on the surface was stained with Alexa fluor 488 conjugated goat anti-human antibody. The percentage of internalized Fc γ was calculated as: $(\text{Fc}\gamma_{4^\circ\text{C}} - \text{Fc}\gamma_{37^\circ\text{C}})/\text{Fc}\gamma_{4^\circ\text{C}} \times 100$. Values shown are relative based on internalization by RL13 wild type.

Confocal microscopy analysis

Cells transfected with plasmids of interest were trypsin detached and plated on glass coverslips for 24 h post transfection. For intracellular staining, cells were fixed 48 h post transfection with 3.7% paraformaldehyde, permeabilized with 0.1% Triton X-100 (Sigma), blocked with PBS with 1% BSA and stained at RT by incubating for 1 h with primary antibodies in PBS/1% BSA, washed in PBS, and further incubated with secondary antibodies in PBS/1% BSA for 1 h. The ProLong

Gold with DAPI (Invitrogen) was used as mounting solution. For membrane staining, the permeabilization step was omitted and fixation was performed after staining with primary and secondary antibodies for 30 min on ice. For human Fc γ internalization, cells expressing RL13-YFP, YFP, or empty vector (controls) were plated on glass coverslips 24h post transfection. 24 h later, cells were washed in cold PBS and incubated at 4°C with 20 μ g/ml of DyLight 649-conjugated human Fc γ for 30 minutes. After extensive washes, temperature was switched to 37°C to allow for internalization. When required, cells were fixed, permeabilized and stained before mounting. The intracellular locations of antibody-tagged or fluorescent fusion proteins were examined under laser illumination in a Zeiss LMS 710 confocal microscope and images were captured using ZEN software (Carl Zeiss).

Immunoprecipitation

HEK293T cells were transfected with empty vector or myc-His tagged RL13 from TR or Merlin strains. 48 h post-transfection, cells were harvested, washed in cold PBS several times and lysed in lysis buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% glycerol, 1 mM EDTA) containing 1% NP-40 (Roche) and supplemented with complete EDTA-free protease inhibitors (Roche). Supernatants were collected after centrifugation at max speed for 30 min at 4°C in a tabletop centrifuge. 100 μ g of lysate was incubated with 2 μ g of biotinylated human IgG Fc fragment (Jackson immunoresearch) for 1 h at 4°C, then with 30 μ l of Streptavidin Dynabeads (Invitrogen) prewashed in lysis buffer. Precipitation was carried out at 4°C with overnight rotation. Immunocomplexes were collected using magnetic beads, washed 4 times with lysis buffer and eluted by adding 30 μ l of LDS-buffer and heating at 96°C for 5 minutes. Lysates were mixed with 100 mM DTT (Sigma) and heated at 96°C for 3 min. Proteins were then separated by SDS-PAGE and blotted on a nitrocellulose membrane. Membranes were blocked in blocking buffer (5% w/v

nonfat dry milk in PBS with 0.1% Tween 20). All incubations with antibodies were done at room temperature for 1 h in blocking buffer. Mouse anti-His (Invitrogen) was used at 1:1,000 dilution. Secondary antibodies were diluted 1:10,000 in blocking buffer.

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1) Characterization of a newly identified HCMV envelope protein interacting with glycoprotein H (gH)

Calò S., M.Cortese, L.Bruno, Y.Uematsu, A.Lilja, C.Ciferri, A.Carfi, M.Merola
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2) Recombinant human cytomegalovirus (HCMV) RL13 binds human immunoglobulin G Fc

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