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The E5 protein of BPV-4 interacts with the heavy chain of MHC class I and irreversibly retains the MHC complex in the Golgi apparatus

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Abstract

BPV-4 E5 inhibits transcription of the bovine MHC class I heavy chain gene, increases degradation of heavy chain and down-regulates surface expression of MHC class I by retaining the complex in the Golgi apparatus. Here we report that transcription inhibition can be alleviated by interferon treatment and the degradation of heavy chain can be reversed by treatment with inhibitors of proteasomes and lysosomes. However, the inhibition of transport of MHC class I to the cell surface is irreversible. We show that E5 is capable of physically interacting with heavy chain. Together with the inhibition of the vacuolar ATPase (due to the interaction between E5 and 16k subunit c), the interaction between E5 and heavy chain is likely to be responsible for retention of MHC class I in the Golgi apparatus. C-terminus deletion mutants of E5 are incapable of either down-regulating surface MHC class I or interacting with heavy chain, establishing that the C-terminus domain of E5 is important in the inhibition of MHC class I.

Introduction

MHC class I presents antigenic peptides, including viral peptides, to cytotoxic T lymphocytes (CTL), effector cells capable of recognising and destroying transformed or infected cells. MHC class I is therefore a central player of the adaptive immune response. Numerous viruses have developed the ability of down-regulating MHC class I (Piguet, 2005) either through direct interaction between viral proteins and MHC class I, as in the case of HIV and HCMV (Chevalier et al., 2002; Furman et al., 2002; Kasper & Collins, 2003; Tirabassi & Ploegh, 2002; Williams et al., 2002), or indirectly, as in the case of Adenovirus (Ad) 12 (Piguet, 2005). Similarly, the E5 oncoprotein of both BPV-1 and BPV-4 inhibits the transport of MHC class I to the cell surface and retains the complex in the Golgi apparatus (GA) (Ashrafi et al., 2002; Marchetti et al., 2002). BPV-4 E5-induced down-regulation of MHC class I takes place not only in cultured cells but crucially also in naturally occurring papillomas (Araibi et al., 2004). We have argued that the down-regulation of MHC class I is an important step in the establishment and persistence of papillomavirus infections and neoplastic progression of pre-malignant lesions to squamous carcinoma (Ashrafi et al., 2005). While both BPV and HPV E5 proteins retain MHC class I in the GA, only BPV E5 induces a general down-regulation of MHC class I expression (Ashrafi et al., 2002; Marchetti et al., 2002). Here we show that BPV-4 E5-induced transcriptional

inhibition of the MHC class I heavy chain (HC) gene and degradation of the HC peptide can be alleviated by treatment with interferon (IFN) or proteasome and lysosome inhibitors respectively, but that MHC class I transport to the cell surface is irreversibly compromised, probably due to a physical interaction between E5 and HC.

Results

The down-regulation of MHC class I by BPV E5 takes place at several levels: transcription of the heavy chain (HC), degradation of the HC and transport of the MHC complex to the cell surface (Ashrafi et al., 2002; Marchetti et al., 2002).

Transcription of the heavy chain of the MHC class I complex in E5-expressing cells increases with interferon treatment.

In PalF-4E5 cells, including cells expressing N17A, a hypertransforming mutant of BPV-4 E5 (4E5), the levels of the HC RNA are drastically reduced compared to control cells (Figure 1A, white bars). To investigate whether the reduction in HC RNA was due to transcriptional repression or to increased degradation of the RNA, pBoLa-Luc, containing the reporter gene for luciferase under the transcriptional control of the promoter/enhancer of a bovine MHC class I HC gene, was transiently introduced into PalF cells along with pZip-4E5, a plasmid expressing BPV-4 E5. Expression of luciferase was less than half the level than when pBoLa-Luc was co-transfected with an empty pZip vector (Figure 1B, white bars). This result indicates that the reduction in HC RNA in PalF-4E5 cells is due to the E5-induced inhibition of the transcriptional promoter of the HC gene, similar to the repression of class I transcription by Ad12 E1A (Piguet, 2005).

The transcription of genes encoding MHC HCs is stimulated by both class 1 and class 2 interferons (IFN) (Agrawal & Kishore, 2000). The bovine HC promoter used in these experiments contains responsive elements for both βIFN and γIFN (data not shown). Treatment of the PalF control and PalF-4E5 cell lines with either βIFN or γIFN lead to a noticeable increase of HC RNA: between 2 and 3 fold in control cells, and between 5 and 10 fold in the PalF-4E5 cells (Figure 1A, grey and black bars). Similar increases in transcription of the HC gene upon IFN treatment were observed in parental PalF cells (data not shown). However, despite the IFN-induced increase in

HC gene transcription, the levels of HC RNA in the PalF-4E5 cells were 2-3-fold lower than in the control cells. Additionally, γIFN-treatment stimulated the expression of luciferase from pBoLa-Luc by approximately 2-fold both in the presence or absence of 4E5 (Figure 1B, grey bars). Even with γIFN-treatment, in cells expressing 4E5 luciferase expression did not achieve the levels observed in cells that did not express E5 (Figure 1B). Treatment with IFN did not increase transcription of the 4E5 gene (data not shown).

We conclude from these results that 4E5 inhibits transcription of cattle class I HC genes, and that IFN treatment rescues transcription, but without completely overcoming 4E5-induced repression.

Lysosome and proteasome inhibitors increase the stability of the heavy chain in E5-expressing cells.

MHC class I stability is regulated by proteasomes and lysosomes, which degrade mis-located or mis-folded MHC class I molecules (Bartee et al., 2004; Hewitt et al., 2002; Hughes et al., 1997). The levels of HC protein are extremely low in PalF-4E5 cells, but increase when transcription of the HC gene is stimulated by IFN treatment, without however reaching the levels of HC in IFN-treated control cells (Figure 2). To investigate further the cause of this reduction in HC protein, the control and 4E5 cell lines were treated with inhibitors of lysosomes (bafilomycin or ammonium chloride) or of proteasomes (MG132 or ALLN). Levels of MHC class I were assessed by immunoblotting. Treatment increased the amounts of HC in control PalF cells but only marginally in PalF-4E5 cells (Figure 2, lanes with no IFN), probably because of the preceding block of HC gene transcription. Given that the transcriptional block could be relieved by IFN, we treated cells with both IFN and inhibitors. After the combined treatment, the level of HC protein in PalF-4E5 cells was comparable with that of control PalF cells (Figure 2). We conclude therefore that in 4E5 cells, as in control cells, the degradation of the HC protein via the lysosomes and proteasomes can be abrogated by treatment with inhibitors.

Transport of MHC class I complex to the cell surface is irreversibly inhibited by E5.

To see whether the increase in MHC class I HC induced by treatment with IFN and lysosome or proteasome inhibitors resulted in increased levels of MHC class I complex on the cell surface, we performed flow cytometry analyses on the treated cells. Treatment with IFN+bafilomycin or IFN+MG132 increased the amount of surface MHC class I in control cells by 5-6 fold, but not in PalF-4E5 cells, where the increase was so negligible that the shift in forward fluorescence could not even be detected (Figure 3). We conclude that the 4E5 protein prevents the traffic of MHC class I complex to the cell surface in an irreversible manner.

E5 and heavy chain protein interact physically.

We have shown that the retention of MHC class I in the Golgi apparatus is due at least in part to the alkalinisation of the organelle (Marchetti et al., 2002), attributed to the binding of E5 to the 16k subunit c of the vacuolar H^+ ATPase (Goldstein et al., 1991). However, the almost complete co-localisation of 4E5 and the residual MHC class I in the Golgi apparatus (Marchetti et al., 2002) would suggest an interaction between 4E5 and the complex. To investigate if any such interaction exists, we performed co-immunoprecipitation experiments both in vitro and in vivo. 4E5 and the bovine HD6 HC (Ellis et al., 1996; Gaddum et al., 1996) were separately transcribed/translated in vitro in the presence of ^{35}S-methionine (Faccini et al., 1996) and then kept separately or mixed together. The proteins were precipitated either with Ab 274 against the C-terminus of BPV-4 E5 (Anderson et al., 1997; Araibi et al., 2004) or mAb IL-A88 against bovine HC. There was no precipitate in the absence of antibody (Figure 4C, lanes 3,5,7); the anti-E5 antibody precipitated E5 but not HD6 HC (Figure 4A, lanes 1,5), and the mAb IL-A88 precipitated HC but not E5 (Figure 4C lanes 4,6). However, when the two proteins were mixed together, they co-precipitated with either antibody (Figure 4A, lane 2 and 4D, lane 3), indicating that E5 and HC interact.

To see whether the interaction between E5 and HC was specific for a particular MHC haplotype, we repeated the experiment with a different HC, JSP.1 (Pichowski et al., 1996). Also in this case, E5 and JSP.1 HC co-precipitated when incubated with mAb IL-A88 (Figure 4C, lane 8), showing that interaction is not restricted to certain MHC alleles.

To confirm that E5 and HC interact also in cells, co-immunoprecipitation experiments were performed in PalF control cells and in PalF-4E5 cells. Both cell lines were

treated with IFN and MG132 as described above to bring the amount of heavy chain in PalF-4E5 cells to detectable levels. Protein lysates from both cell lines were incubated with Ab 274 and the immunoprecipitates were run on gel and immunoblotted with mAb IL-A88. A band of the appropriate MW for HC (approximately 45 kDa) was visible in a Comassie blue stained gel in the immunoprecipitate from PalF-4E5 cells, which was not visible in the immunoprecipitated from control cells (Figure 4D). This band reacted with mAb IL-A88 and corresponded to bona fide HC detected by direct immunoblotting of protein lysates (Figure 4D). Despite the combined IFN+bafilomycin treatment, the HC band co-precipitated with E5 is faint because the treatment does not increase expression of E5 (data not shown) and E5 is expressed at almost undetectable levels (O'Brien at al., 1999; O'Brien et al., 2001); therefore the amount of E5 is limiting for the amount of bound HC. However, the reactivity with mAB IL-A88 specific for bovine HC only in E5-expressing cells, the correspondence of the band with bona fide HC and the results obtained in vitro, establish the identity of this band as MHC class I HC and confirm that the interaction between E5 and HC takes place also in cells.

E5 inhibits MHC class I transport and binds to MHC heavy chain via its C-terminus.
In a study of the cell transforming properties of BPV-4 E5 (O'Brien V et al., 1999), we established that mutation of the asparagine residue 17 to tyrosine (N17Y) lead to loss of cell transformation and so did deletion of the C-terminus (E5T) of the protein. On the contrary, mutation of N17 of BPV-4 E5 to alanine (N17A) resulted in a hyper-transforming mutant. The ability to down-regulate MHC class I expression co-segregated with the ability to transform cells (Ashrafi et al., 2002; O'Brien & Campo, 2003). BPV E5 mutants that did not transform did not down-regulated MHC class I as assessed by flow cytometry (Figure 5A) and by immunofluorescence (Figure 5B) and both the total and cell surface levels of MHC class I were similar to those of control cells. In contrast the hyper-transforming mutant of BPV-4 E5 down-regulated surface MHC class I to the same extent as wild type E5 (Figure 5A). To investigate whether the BPV-4 E5 mutants were capable of interacting with HC we performed co-immunoprecipitation experiments in vitro as above. Both N17Y and N17A were co-precipitated with HC by Ab 274 (Figure 4A, lanes 6,8) and mAb IL-A88 (Figure 4E, lanes 5,7), while E5T was not co-precipitated with HD6 HC by mAb IL-A88 (Figure 4E, lane 9). E5T lacks the C-terminal domain and therefore could not be precipitated

by Ab 274. As E5 is tagged at its N-terminus with the HA epitope, we performed co-immunoprecipitation experiments with mAb HA.11 raised against the HA epitope. mAb HA.11 co-precipitated E5 and HC, although not as effectively as Ab 274, (Figure 4B, lanes 1,2) but did not co-precipitate HC with E5T (Figure 4B, lanes 3,4). These data indicate that the interaction between E5 and HC takes place via the C-terminus of E5. To confirm this, we performed competition experiments with a well characterised peptide corresponding to the C-terminus of E5, the ability of which to compete with E5 had been validated in immunocyto- and immunohisto-staining experiment (Anderson et al., 1997; Araibi et al., 2004; Pennie et al., 1993). The peptide did successfully compete with E5 in co-immunoprecipitation experiments (Figure 4E, lane 11); its addition to the reaction prevented the interaction between E5 and HD6, proving conclusively that the C-terminus of E5 interacts with MHC class I HC.

Three conclusions can be reached from these experiments: one, binding of E5 to HC is *necessary* for down-regulation of MHC class I as shown by E5T, but not *sufficient* as shown by N17Y; two, the nature of residue 17 is not important for the interaction with HC, and three, the C-terminus of E5 is responsible for the interaction between E5 and HC.

Discussion

The E5 oncoprotein of BPV-4 has a profound effect on MHC class I expression and transport. E5 disruption of the normal metabolism of MHC class I takes place at several levels: transcription, protein degradation and complex transport.

Inhibition of transcription of the MHC class I heavy chain gene by BPV-4 E5 can be alleviated by IFN treatment. In PalF-4E5 cells, the levels of HC RNA are barely detectable (Ashrafi et al., 2002), and by quantitative RT-PCR we estimate that the amount of HC RNA in these cells is between 10 and 30-fold less than in control cells (Figure 1A, white bars). The reduction of HC RNA is due to a transcriptional inhibition of the HC gene. This conclusion is supported by two observations: E5 down-regulates a BoLa HC gene promoter/enhancer (Figure 1B, white bars) and

treatment with either β or γIFN, both of which increase HC gene transcription (Agrawal & Kishore, 2000), leads to accumulation of HC RNA also in PalF-4E5 cells, and to increased activity of the BoLa HC promoter/enhancer (Figure 1, grey and black bars). However, even in the presence of IFN, the levels of HC RNA in PalF-4E5 cells are still approximately 2-3-fold lower than in control cells. This suggests that E5 expression causes a transcription inhibitor to bind to the BoLa promoter/enhancer, and that this putative inhibitor exerts its action even when IFN-induced transcription activators promote transcription. This hypothesis however requires confirmation and other explanations are possible. BPV E5 is not present in the nucleus (Burkhardt et al., 1989; Pennie et al., 1993; Zago et al., 2004) and its effect on transcription is therefore not direct. Rather, BPV E5 interferes with several signal transduction pathways (DiMaio & Mattoon, 2001; Grindlay et al., 2005; O'Brien V et al., 2001; Zago et al., 2004) and it is therefore likely that inhibition of the BoLa promoter/enhancer is achieved by interference with one or more of these pathways. We have already shown that BPV-4 E5 affects the expression of cyclin A by acting indirectly on the transcriptional promoter of the cyclin A gene (Grindlay et al., 2005; O'Brien V et al., 1999) and BPV-4 E5 inhibition of the BoLa promoter/enhancer is another example of E5 interference with transcription regulation.

Degradation of MHC class I heavy chain can be inhibited in PalF cells.

MHC class I half-life is regulated by degradation in the proteasomes and lysosomes (Bartee et al., 2004) and several viral proteins contribute to the destruction of MHC class I by favouring HC ubiquitination, or by inducing peptide mis-folding or complex mis-location (Bartee et al., 2004; Hewitt et al., 2002; Hughes et al., 1997). In PalF-4E5 cells treatment with either proteasome or lysosome inhibitors rescues HC to levels comparable to those of control cells (Figure 2). In 4E5-expressing cells the half-life of HC is drastically reduced (data not shown) and the virtual absence of HC in PalF-4E5 cells is in agreement with the retention of MHC class I in the GA by E5 and with the degradation of mis-folded, mis-located or improperly processed MHC class I by both proteasomes and lysosomes.

BPV E5 interacts with 16k subunit c of the V0 sector of the V-ATPase (Faccini et al., 1996; Goldstein et al., 1991) and this interaction results in alkalinisation of the GA

(Schapiro et al., 2000), which in turn we have argued is in part responsible for retention of MHC class I in the GA (Marchetti et al., 2002). The V-ATPase pump is ubiquitous in the endomembrane compartments, including the lysosomes, so it would have been expected that its inhibition by E5 would not have lead to HC degradation, or that lysosome inhibitors would have no effect on HC degradation. This apparent discrepancy may be due to different reasons. First, E5 resides mainly in the GA and may inhibit the pump only in this organelle, similar to HPV-16 E5 which alters endosomal pH but not GA pH (Disbrow et al., 2005); second E5 may disable the pump only transiently (Ashby et al., 2001), and third, E5 interference with the pump is not complete and stronger inhibition is needed for blocking protein degradation in the lysosomes.

BPV-4 E5 irreversibly blocks transport of MHC class I to the cell surface.

The arguments discussed above point to the fact that both E5-induced transcriptional inhibition of the HC gene and degradation of HC peptide can be overcome. However, the transport of MHC class I from the endomembranes to the cell surface is irreversibly compromised by E5. Although after treatment with IFN and proteasome/lysosome inhibitors the amount of HC in PalF-4E5 cells is similar to that of control cells, the increase in surface MHC class I is negligible (Figure 3). The alkalinisation of the GA by E5 is in part responsible for the intracellular retention of MHC class I, as it is also observed in cells treated with the ionophore monensin which disperses the proton gradient of the GA (Marchetti et al., 2002). However, we found it surprising that treatment with lysosome inhibitors resulted in a large increase in surface MHC class I in control cells but not in PalF-4E5 cells (Figure 3), and hypothesised that an additional reason(s) would be responsible for the inhibition of MHC class I traffic in PalF-4E5 cells.

BPV-4 E5 interacts physically with HC.

BPV-1 E5 interacts physically with the receptor for PDGF (Petti & DiMaio, 1994) and both BPV-1 and BPV-4 E5 interact with 16k subunit c (Faccini et al., 1996; Goldstein et al., 1991). Given the established interaction between E5 and membrane proteins, we hypothesised that BPV-4 E5 interacts also with MHC class I HC. Indeed this is the case and the two proteins co-precipitate in vitro and in vivo (Figure 4). This interaction is not an artefact, as E5 is precipitated by an antibody against HC even

when HC is not labelled (not shown), and E5T, a C-terminal truncated version of E5, does not interact with HC (Figure 4). E5 and HC interact also in PalF-4E5 cells as shown by the co-immunoprecipitation of HC with an antibody against E5 only in PalF-4E5 cells and not in control cells. The reason for the low levels of the HC co-precipitated with 4E5 resides in the fact that, although the levels of HC can be boosted many folds by the combined treatment of IFN and proteasome/lysosomes inhibitors, expression of E5 is not affected; E5 is present at barely detectable levels (O'Brien V et al., 1999) and is therefore rate-limiting for complex formation with HC. The interaction between E5 and HC reflects a genuine relationship between the two proteins as there is agreement between interaction in vivo and in vitro and a good correlation between interaction in vitro and down-regulation of MHC class I in vivo. Furthermore, interaction takes place also between HPV-16 E5 and HLA-A in human keratinocytes (Ashrafi et al., in preparation), suggesting that the physical relationship between E5 proteins and HCs is a common mechanism to down-regulate surface MHC/HLA class I. We do not know the MHC class I phenotype of the PalF cells but, as the in vitro co-precipitation experiments have been done with two different class I alleles, encoded at different loci, and E5 down-regulates MHC class I in papillomas from calves of different breeds (Araibi et al., 2004), it is reasonable to conclude that BPV-4 E5 can interact with and down-regulate most, if not all classical class I HCs. This is indeed the case for HPV-16 E5 (Ashrafi et al., in preparation).

The interaction between BPV-4 E5 and HC is mediated by the C-terminus domain of E5. The conclusion is based on the three observations that E5T does not inhibit the expression of MHC class I or its transport to the cell surface (Ashrafi et al., 2002), that E5T does not interact with HC and that the interaction between E5 and HC is prevented by a peptide corresponding to the C-terminus of E5 (Figure 4). The C-terminus tail of E5 is clearly critical to the function of the protein: E5T is not transforming (O'Brien V et al., 1999), does not induce the typical morphological changes brought about by E5, and does not distort the GA (Ashrafi et al., 2000). The ability to bind MHC class I HC has to be added to the functions of E5 C-terminus.

However, the physical interaction between 4E5 and HC appears to be *necessary* but not *sufficient* for down-regulation of MHC class I as the E5 mutant N17Y binds HC but does not inhibit MHC class I expression (Figures 4, 5). This situation is reminiscent of the fact that interaction between E5 and 16k subunit c is not sufficient for cell transformation (Ashby et al., 2001; Ashrafi et al., 2000) and points to a

complex interaction of E5 with cellular mechanisms/pathways. Nevertheless, we confirm here our previous observation that only transformation-competent E5 proteins are capable of inhibition of MHC class I.

It is interesting that BPV E5 can potentially interact with more than one membrane protein simultaneously. E5 interacts with 16k subunit c trough the transmembrane residue 17 (glutamine in BPV-1 E5, asparagine in BPV-4) (Goldstein et al., 1992), and could therefore interact with HC through its C-terminus at the same time. BPV-1 E5 interacts with the PDGF receptor through glutamine 17 and the juxtamembrane aspartate 33 (Klein et al., 1999; Klein et al., 1998; Petti et al., 1997) in a multi-protein complex that can include 16k ductin (Goldstein, 1992; Lai et al., 2000). Residue 33 is not present in either 1-E5T or 4-E5T (O'Brien V et al., 1999), and we cannot rule out that it plays a role in the interaction with HC. However, although we have not investigated any interaction between BPV-4 E5 and PDGF receptor, cell transformation by BPV-4 E5 does not appear to depend on signalling from growth factor receptors (Grindlay et al., 2005; Zago et al., 2004), and it is therefore unlikely that receptor binding would compete with HC binding.

Other viral proteins, such as HIV Nef (Williams et al., 2002) and HCMV US2 and US8 (Chevalier et al., 2002; Tirabassi & Ploegh, 2002), to name but a few, bind HC and down-regulate surface MHC class I, and E5 now joins this large number. It is interesting to point out the parallels between E5 and Nef. Nef binds both Vma13, a component of the V1 sector of the vacuolar ATPase, facilitating the internalisation of surface receptors (including MHC class I), and MHC class I HC, disrupting MHC class I traffic (Lu et al., 1998; Williams et al., 2002). Furthermore, as for E5, the same domain of Nef necessary for disruption of MHC class I traffic, mediates the binding to the HC (Williams et al., 2005). Thus, although papillomavirus and HIV are very different viruses, each encodes a protein with similar functions.

Conclusions.

We have demonstrated that BPV-4 E5-induced retention of MHC class I in the GA is linked to cell transformation (Ashrafi et al., 2002; O'Brien P & Campo, 2003) and is due to the alkalinisation of the GA (Marchetti et al., 2002). Here we suggest that direct binding between E5 and HC contributes to (but is not sufficient for) the inhibition of MHC class I transport to the cell surface. The expression of E5 is much lower than that of HC, but this stoichiometric imbalance is offset by E5-induced

transcriptional inhibition of the HC gene and degradation of the HC peptide. However, E5 prevents traffic of the MHC class I complex even when the amount of HC is greatly increased by drug treatment, therefore, for E5 to exert any effect on HC, the binding must be dynamic. We propose the following hypothesis, consistent with our results: E5 retains newly assembled MHC class I complex in the GA by preventing GA acidification and by physically interacting with HC; the mis-located complex is shunted to lysosomes for degradation, and E5 is free to interact with new MHC class I and re-start the process. While aspects of this hypothesis need confirming, the absence of MHC class I in the E5-expressing cells of naturally occurring BPV-4 induced papillomas supports our suggestion that E5 plays an important role in the establishment and persistence of viral infection by allowing the infect cells to escape host immunosurveillance (Araibi et al., 2004).

Materials and Methods

Cells.

The bovine foetal PalF cells and their transformed derivatives have been described before (Pennie et al., 1993). Briefly, they are as follows: "parental" PalF cells are normal foetal primary cells; "control" cells are cells transformed by BPV-4 E7, HPV-16 E6 and activated ras; PalF-1E5 or PalF-4E5 are "control" cells additionally expressing either BPV-1 E5 or BPV-4 E5 respectively. The latter cells are referred to as PalF E5 cells when a particular description applies to both PalF-1E5 and PalF-4E5. PalF-N17Y and -N17A express a non-transforming and a hypertransforming mutant of BPV-4 E5 respectively (O'Brien V et al., 1999). All the clones analysed within each type exhibit the same characteristics (Pennie et al., 1993; O'Brien V et al., 1999; Ashrafi et al., 2002; Marchetti et al., 2002). Cells were grown in DMEM, 10% foetal calf serum (FCS) at 37^{o}C in 5% CO_2 as previously described (Ashrafi et al., 2002).

Plasmids.

pcDNA3.1(-)/HA4E5 contains the BPV-4 E5 ORF (nt 332-460) tagged at the 5'end with the sequence for the HA epitope inserted into the BamHI/HindIII sites of pcDNA3.1(-) (Invitrogen, UK). pcDNA 3.1(-)/4E5T contains the HA-tagged BPV-4 E5 ORF in which a premature stop codon has been introduced to terminate the protein

at residue 32 (O'Brien V et al., 1999). pcDNA3-HD6 and pcDNA-JSP.1 contain cDNA for 2 *Bos taurus* MHC class I heavy chain alleles, HD6 and JSP.1 (GenBank accession numbers X80934 and X92870 respectively) (Ellis et al., 1996; Pichowski et al., 1996) inserted in the HindIII/EcoRI sites of pcDNA3 (Invitrogen, UK). Both pcDNA 3.1(-) and pcDNA3 contain the cloned gene of interest under the control of the T7 promoter for in vitro transcription/translation experiments (see below).

pBoLa-Luc was derived from pBoLa-19, a plasmid containing the promoter/enhancer, exon 1 and the 3'UTR of another cattle MHC class I allele (Sawhney et al., 1995; Sawhney et al., 1996). The promoter sequences were amplified by PCR using the forward primer 5'GTTGAAGGCTCTCGAGGGCATCGGTCGAC3' and the reverse primer 5'TGCAAAGCTTCCTCTGGGTCTGGGAAGAAGC3'. The resulting amplicon of 1100 base pairs was digested with Xho I and Hind III and inserted between the Xho I and Hind III sites of pGL3 ahead of the luciferase gene sequences. pBoLa-19 was a kind gift of Dr G Russell.

Treatment of cells with interferon.

10^6 parental, control and PalF-4E5 were seeded in tissue culture flasks. The following day, the medium was replaced with fresh medium with or without 500U/ml βIFN (Sigma, UK) or 250U/ml recombinant ovine γIFN (Graham et al., 1995) for 48 h. Ovine recombinant γIFN was a kind gift of Dr G Entrican (Moredun Research Institute, Penicuik, UK).

Treatment of cells with lysosome and proteosome inhibitors.

Cells were treated either with the lysosome inhibitors Bafilomycin A1 (1μM) (Calbiochem, UK) or NH_4Cl (40mM) (Calbiochem, UK) for 24 h, or with the proteosome inhibitors MG-132 (5μM) (Calbiochem, UK) or ALLN (100μM) (Merck Biosciences, UK) for 8 h. For combined γIFN and inhibitors treatment, lysosome inhibitors were added for the last 24hr of γIFN treatment, and proteosome inhibitors for the last 8 h of γIFN treatment. The cells were then harvested for detection of MHC class I by immunoblotting or flow cytometry analysis.

Transient transfection and luciferase transcription assays.

10^5 PalF cells were plated in each well of 6 well plates, in duplicate, with 5 ml of DMEM, 10% FCS at 37°C in 5% CO_2. Twenty four hours later, the cells were transiently co-transfected with 1μg of pBoLa-Luc and 1μg of pZipneo or 1μg of pZip-4E5 (O'Brien V et al., 1999), using the standard Lipofectamine Plus (Invitrogen, UK) method. After 3 hr, cells were washed twice with 2 ml PBS and incubated in medium for further 48 hr, with or without 250U/ml γIFN, before being harvested and lysed in reporter lysis buffer. The lysates were either assayed for reporter enzyme activity immediately or stored at -20°C. Luciferase activity was determined as described previously (O'Brien V et al., 1999) and activity was normalised for protein content determined using the BCA assay (Pierce Chemical Co).

Detection of MHC class I HC RNA by quantitative RT-PCR.

Total RNA was extracted from parental, control and PalF-4E5 cells using the RNeasy Mini kit (Qiagen, Sussex, UK), and residual DNA was removed by DNase I treatment according to the manufacturer's guidelines (Invitrogen, UK).

Real-time RT-PCR for MHC I heavy chain and bovine actin mRNA was carried out using the Taqman EZ RT-PCR kit (Applied Biosystems, Foster City, CA, USA) with gene-specific primers and FAM/TAMRA probe designed by primer express v1.7 software. 100 ng RNA was used per each reaction, done in triplicate. The primers, spanning exons 2 and 3, were BovMHC_Taq_Forward 5'-TCCGGGCGAACCTGAAC-3' and BovMHC_Taq_Reverse 5'-ACATCTCCTGGAAGGTGTGAGAC-3' and the probe was 5'-CCGCACTCGGCTACTACAACCAGAGC-3'. Bovine actin primers were BovACT_Taq_Forward 5'-CCTCACGGAACGTGGTTACAG-3' and BovACT_Taq_Reverse 5'-TCTCCTTGATGTCACGCACAA-3' and the probe was 5'-TTACCACCACAGCCGAGCGGG-3'. RT-PCR was performed using an ABI prism 7700 sequence detector. In each experiment, additional reactions with 10 ten-fold serial dilutions of template DNA were performed with each set of primers and probes on the same 96-well plates to generate standard curves. All samples were amplified in triplicate. The relative amounts of MHC I heavy chain and bovine actin mRNA were determined by using the standard curves.

Detection of MHC class I HC by immunoblotting.

Cells were removed from the flasks by trypsinisation, washed with PBS, then lysed in lysis buffer (100mM Tris HCl, pH 7.5, 2% SDS, 2% glycerol) and insoluble material was removed by centrifugation at 20,000g. Ten µg of lysate were electrophoresed in 4-12% NuPAGE gels (InVitrogen), and proteins transferred to nitrocellulose membrane (InVitrogen) using a semidry blotting apparatus at 20V/150A for 1 h. The membranes were blocked in 5% milk/TBS/Tween 20 (0.5%) at room temperature for 1 h before incubation with mAb IL-A88 specific for bovine HC (Toye et al., 1990) or mAb AB-1 (Calbiochem) specific for actin. After repeated washing with TBS/Tween 20 (0.5%) the membranes were incubated with anti-mouse Ig-HRP (Amersham Pharmacia Biotech, UK) for mAb IL-A88, and anti-mouse IgM-HRP (Oncogene Calbiochem-Novabiochem International) for mAb AB-1, in 5% milk/TBS/Tween 20 (0.5%) for 1 h at room temperature. The membranes were washed three times with TBS/Tween 20 (0.5%) and bound antibody was detected by enhanced chemoluminescence staining (ECL) (Amersham Pharmacia Biotech).

Detection of MHC class I by flow cytometry.

After removal of the medium, the cells were washed once with PBS, then detached from the flask with trypsin/EDTA and pelleted at 200g for 5 min at room temperature. The cell pellet was resuspended in DMEM, 10% FCS, for 1h at 37^{o}C to allow surface antigens to be re-expressed. The cells were washed and re-suspended in PBS, 1%BSA (PBS-B) at 10^{7}cells/ml. For the detection of surface MHC class I, 100µl of cells were aliquoted and incubated with an equal volume of mAb IL-A19 (Bensaid et al., 1989) for 30 min at 4^{o}C. The cells were washed three times in PBS-B and incubated with anti-mouse IgG-FITC (Sigma) at 4^{o}C for 30 min in the dark. The cells were washed and resuspended in 500µl PBS-B and analysed by flow cytometry. If the flow cytometry analysis was not carried out immediately, the cells were resuspended in 500µl of 3.4% paraformaldehyde (PFA) in PBS and kept at 4^{o}C.

For the detection of intracellular MHC class I, the cells were washed in PBS-B and permeabilised with 0.1% saponin in PBS-B for 30 minutes at room temperature. Following a further wash in PBS-B, the permeabilised cells were stained with mAb IL-A19 as described above. All samples were examined in a Beckman Coulter EPICS

Elite analyser equipped with an ion argon laser with 15 mV of excitation at 488 nm. The data were analysed using Expo 2 software.

Detection of MHC class I and Golgi apparatus by two-colour immunofluorescence.
Cells were grown until 80% confluent in single well chamber slides. After removal of medium cells were washed twice with serum free DMEM, 25mM Hepes (DMEM-H) and incubated in 200µl of 5µM BODIPY-TR-ceramide, which localises to the Golgi apparatus, in DMEM-H for 30 min at 4^{o}C. Cells were then washed with DMEM-H for 30 min at 37^{o}C. After removal of the medium, cells were washed twice with PBS and fixed in fixing solution (19ml PBS, 1ml 37% formaldehyde and 0.4g sucrose) for 10 minutes at room temperature (RT). For the detection of endogenous MHC class I, after fixation, cells were washed twice and incubated in permeabilising solution (19ml PBS, 1ml 10% NP40 and 0.4g sucrose) at RT for 10 minutes and washed as above, then incubated with mAb IL-A88 for 1 h at room temperature and washed three times as above. The cells were then incubated with anti-mouse IgG-FITC (Sigma) at 4^{o}C for 1 hr in the dark. Following three final washes with PBS, the slides were mounted in Citifluor^{TM} and analysed with a Leica TCS SP2 fluorescence confocal microscope. The merge between FITC and BODIPY-TR-ceramide fluorescent signals was achieved with the Leica TCS SP2 accompanying software.

In vitro transcription/translation and co-immunoprecipitation.
In vitro transcription/translation reactions were performed using the TNT® T7 Quick Coupled Transcription/Translation System (Promega, UK) in presence of Redivue L-[^{35}S] Methionine (Amersham Pharmacia Biotech, UK) following the manufacturer instructions. Briefly, 1 µg of pcDNA3.1(-)/HA4E5, pcDNA 3.1(-)/4E5 mutants, pcDNA3-HD6 or pcDNA3-JSP.1 was mixed in a 50µl reaction containing TNT® mix (TNT® lysate with energy generating system, T7 RNA polymerase, nucleotides, salts, recombinant RNasin® ribonuclease inhibitors) in presence of canine microsomal membranes (CMM) (Promega, UK) at 30^{o}C for 1.5 h. Half of each transcription/translation reaction product was immunoprecipitated with either 10 µl rabbit antiserum Ab 274, raised against the C-terminus of the BPV-4 E5 protein (Anderson et al., 1997; Pennie et al., 1993) or with 5 µl of mAb HA.11 (Cambridge Bioscience, UK) against the HA epitope tagging E5, or with 3 µl mAb IL-A88. The

other half of each reaction was left without antibody as a negative control. For co-immunoprecipitation experiments the individual transcription/translation products were mixed in equivalent amounts and immunoprecipitated with double the amount of either antibody. For competition experiments, 10μg of a synthetic peptide corresponding to the C-terminal 12 amino acid residues of E5 (Anderson et al., 1997; Araibi et al., 2004) were added to labelled HD6 for an hour before the addition of labelled E5 as above. After incubation overnight at 4^{o}C, protein G-sepharose bead suspension (Sigma, UK) was added for 1 h at 4^{o}C. Following two washes in a high salt buffer (50mM Tris HCl pH 7.5, 500mM NaCl, 1% NonidetP-40, 0.05% NaDoc) and one wash in a low salt buffer (50mM Tris HCl pH 7.5, 1% NonidetP-400, 0.05% NaDoc) the sepharose beads were resuspended in 20 μl of SDS loading buffer, heated at 75^{o}C for 10 min, and then were electrophoresed in 4-12% NuPAGE gels (Invitrogen). Gels were fixed with glacial acetic acid and methanol, incubated for 15 min in Amplify^{TM} Fluorographic reagent (Amersham, UK), dried and exposed for autoradiography at –70^{o}C overnight or exposed on a screen for quantification on a Storm 840 apparatus using a ImageQuant v5.2 software.

In vivo co-immunoprecipitation. Control PalF cells and PalF-4E5 cells were treated with IFNγ and MG132 as described above. Cells were lysed in RIPA buffer containing a cocktail of protease inhibitors (Roche, Lewes, UK). 100μg of protein lysate were immunoprecitated with Ab 274 as described above. 10μg of the immunoprecipitate were run in NuPAGE gels, transferred to nitrocellulose membranes and immunoblotted with mAb IL-A88 as described above. A parallel gel was stained with Comassie blue.

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The authors declare they have no conflict of interest.

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Figure Legends

Figure 1. BPV-4 E5 inhibits transcription from the BoLa promoter. Quantitative RT-PCR for MHC class I HC RNA was performed on RNA from PalF control cells and PalF-4E5 cells **(A)**, before (white bars) or after treatment with 500U/ml βIFN (grey bars) or 250U/ml γIFN (black bars). The extent of amplification was normalised to that of the actin RNA and is plotted as arbitrary units. The panel shows the averages with standard deviations of at least three experiments. Both βIFN and γIFN increased transcription of HC RNA in control and E5 cells. **B**, BoLa promoter transcriptional activity in PalF parental cells transiently co-transfected with pBoLa-Luc and pZipneo empty vector, or with pBoLa-Luc and pZip-4E5, either without (white bars) or with 250U/ml γIFN treatment (grey bars). Luciferase activity is plotted as arbitrary transcription units, with the activity of pBoLa in PalF parental cells taken as 1. The values are the average of three almost identical measurements. γIFN increases the transcriptional activity of pBoLa also in the presence of E5.

Figure 2. Proteasome and lysosome inhibitors prevent degradation of MHC class I HC in PalF control and PalF-4E5 cells. Cells were left untreated or treated with either 250U/ml γIFN for 48 h, the lysosome inhibitors Bafilomycin A1 (1μM) or NH_4Cl (40mM) for 24 h, or the proteosome inhibitors MG-132 (5μM) or ALLN (100μM) for 8 h. For combined treatment with γIFN and inhibitors, lysosome inhibitors were added for the last 24 h of γIFN treatment, and proteosome inhibitors for the last 8 h. Protein lysates were probed with mAb IL-A88 against HC, or with mAb AB-1 against actin. Combined γIFN and inhibitors treatment increases levels of MHC class I HC in both control and E5 cells. The experiment shown is representative of at least three experiments giving essentially the same results.

Figure 3. MHC class I transport to the cell surface is irreversibly inhibited by BPV-4 E5. Cells were treated with γIFN + MG-132 or γIFN + Bafilomycin A1, as in Figure 2, and expression of surface MHC class I was analysed by flow cytometry with mAb IL-A19. **A**, FACS profiles of control and 4E5 cells with no treatment (white), γIFN + MG-132 (dark grey) and γIFN + Bafilomycin A1 (light grey). **B**, The extent of surface MHC class I expression is plotted as Mean Forward Fluorescence. In control cells,

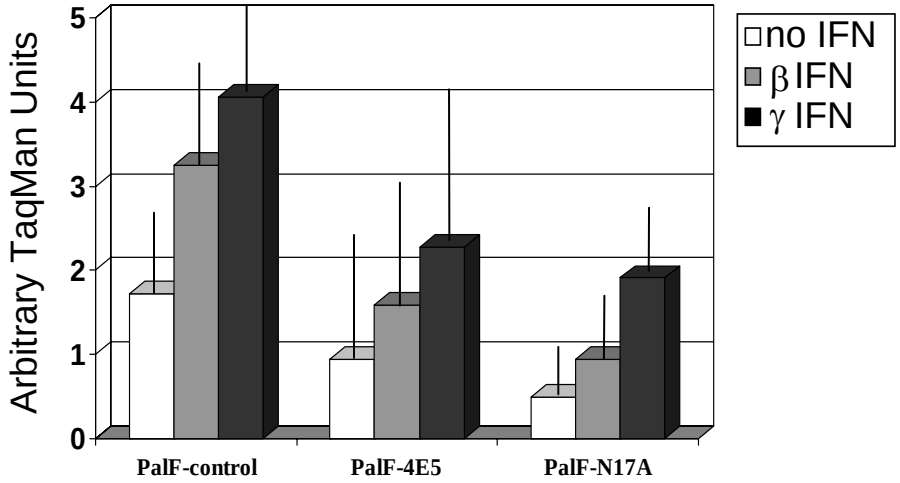
combined γIFN and inhibitors treatment increases surface MHC class I, whereas in E5 cells, surface MHC class I does not increase. The experiment shown is representative of three experiments giving essentially identical results.

Figure 4. BPV-4 E5 and MHC class I HC interact via the C-terminus domain of E5. **A**, ^{35}S-labelled in vitro transcribed/translated E5, N17Y or N17A, and HD6 HC were immunoprecipitated with Ab 274, against the C-terminus of E5; the precipitate was run in a NuPAGE gel and the dried gel exposed on a screen on a Storm 840 apparatus. Note that lanes 1-3 and lanes 4-9 were run in separate gels and aligned together for ease of comparison. **B**, ^{35}S-labelled in vitro transcribed/translated E5 or E5T and HC were immunoprecipitated with mAb HA.11 against the HA epitope tag of E5 and E5T and processed as in A. E5T does not interact with HD6 HC. **C**, ^{35}S-labelled in vitro transcribed/translated E5 and JSP.1 HC were immunoprecipitated with mAb IL-A88 against bovine MHC class I HC and processed as in A. E5 and JSP.1 HC interact physically. **D**, Co-immunoprecipitation of E5 and HC in PalF-4E5 cells. Left panel: Comassie blue stained gel of protein immunoprecipitates with Ab 274 from PalF-4E5 and PalF control cells. Right panel: immunoblot with mAb IL-A88 of Ab 274-immunoprecipitates from PalF-4E5 and control cells. A band corresponding to HC and reacting with IL-A88 is visible only in the immunoprecipitated from PalF-4E5 cells. **E**, ^{35}S-labelled in vitro transcribed/translated E5, N17Y, N17A or E5T and HD6 HC were immunoprecipitated with mAb IL-A88 against bovine MHC class I HC and processed as in A. E5, N17Y and N17A interact with HC, whereas E5T, deleted in the C-terminus domain does not. The C-terminus peptide prevents E5 binding to HD6 HC. Note that lanes 5-10 were run in a separate gel and aligned with the other lanes for ease of comparison.

Figure 5. C-terminus deletion mutants of BPV E5 do not down-regulate surface MHC class I. **A**, the expression of MHC class I in control cells, and in cells expressing wild type or mutant E5 was assessed by flow cytometry analysis with mAb IL-A19. The extent of MHC class I expression is plotted as Mean Forward Fluorescence. **B**, Surface localisation of MHC class I in parental PalF cells and in cells expressing BPV-4 E5T. MHC class I was detected with mAb IL-A88 and FITC-conjugated secondary antibody (green) and the Golgi apparatus with BODIPY-TR-ceramide (red)

in a Leica TCS SP2 confocal scanner microscope. The merge between the FITC and Texas Red fluorescent signals was achieved using the Leica TCS SP2 accompanying software. The non-transforming E5 mutants N17Y and E5T fail to down-regulate surface MHC class I.

a



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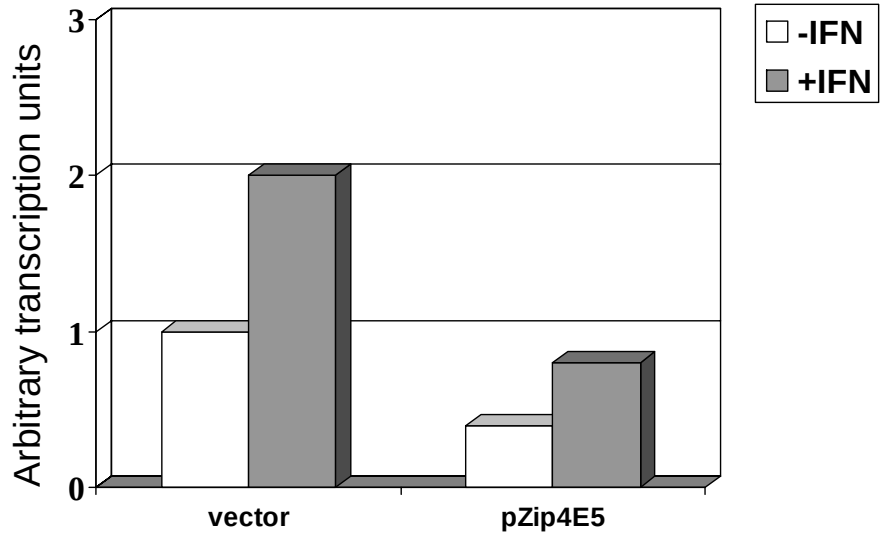
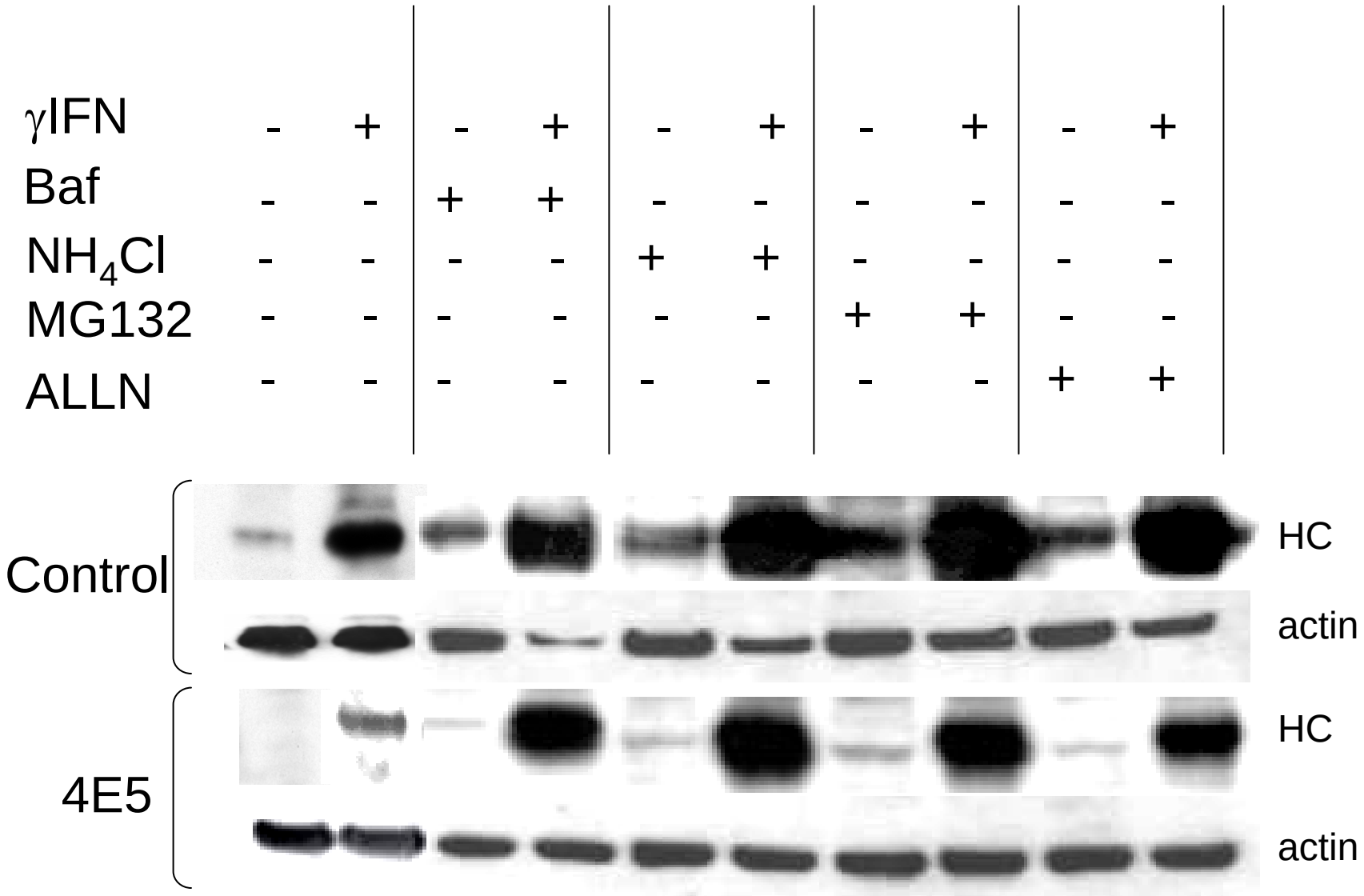
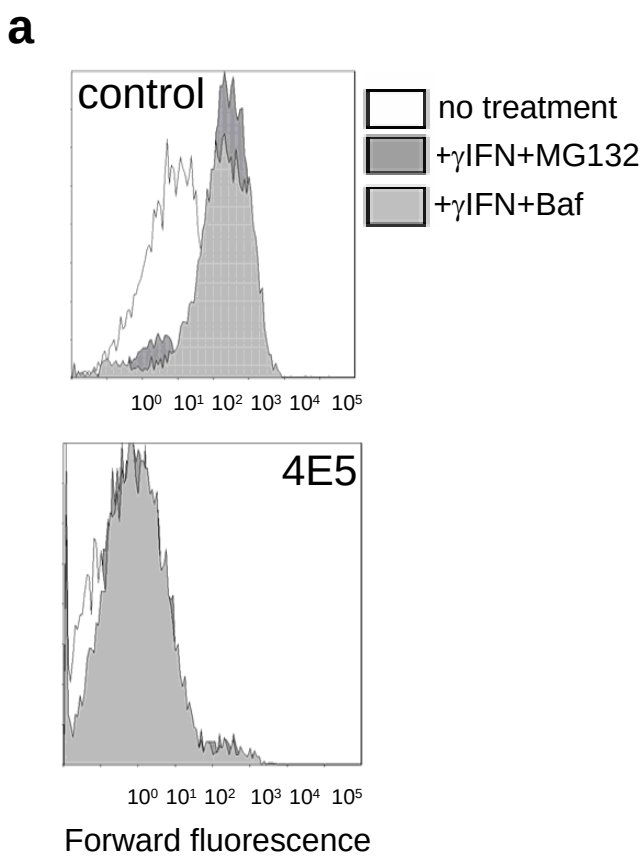
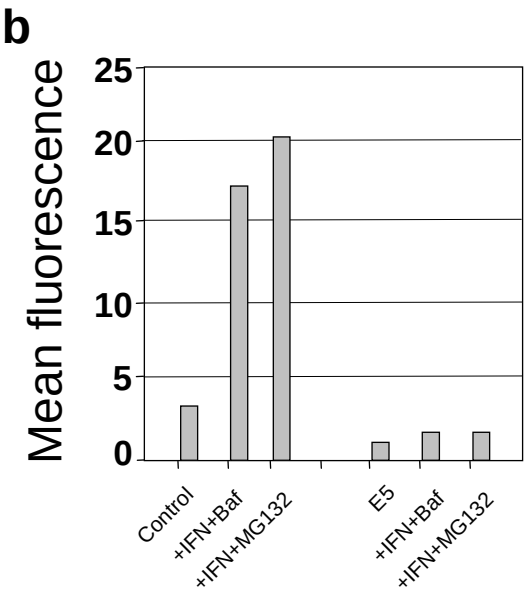


Marchetti et al., Fig 1

Marchetti et al., Fig 2

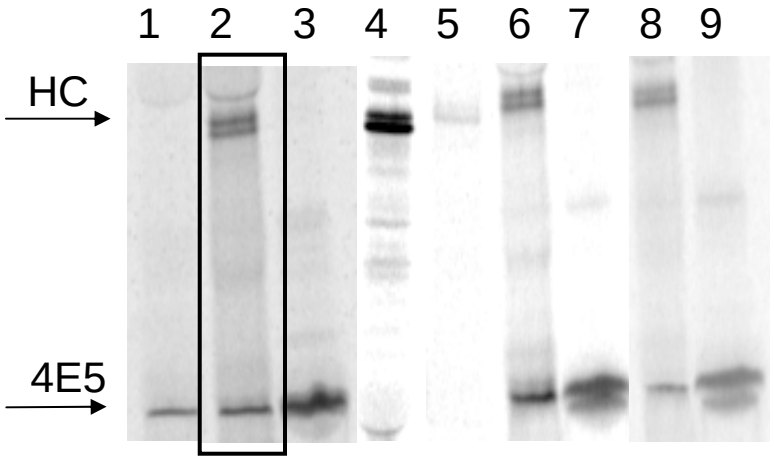






Marchetti et al., Fig 3

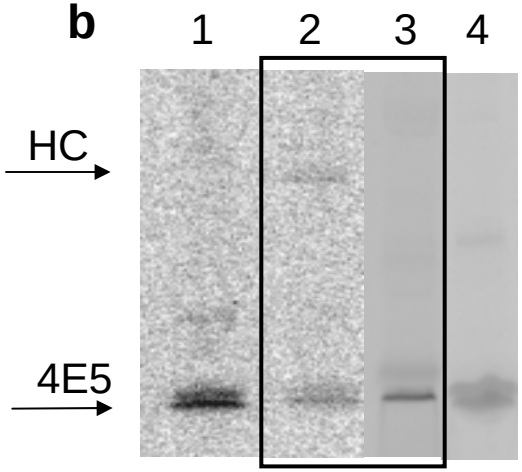
a



Lanes: 1, E5+Ab
2, E5+HD6+Ab
3, input E5
4, input HD6
5, HD6+Ab
6, N17Y+HD6+Ab
7, N17Y input
8, N17A+HD6+Ab
9, N17A input

IP: Ab 274: anti-E5 C-terminus

b

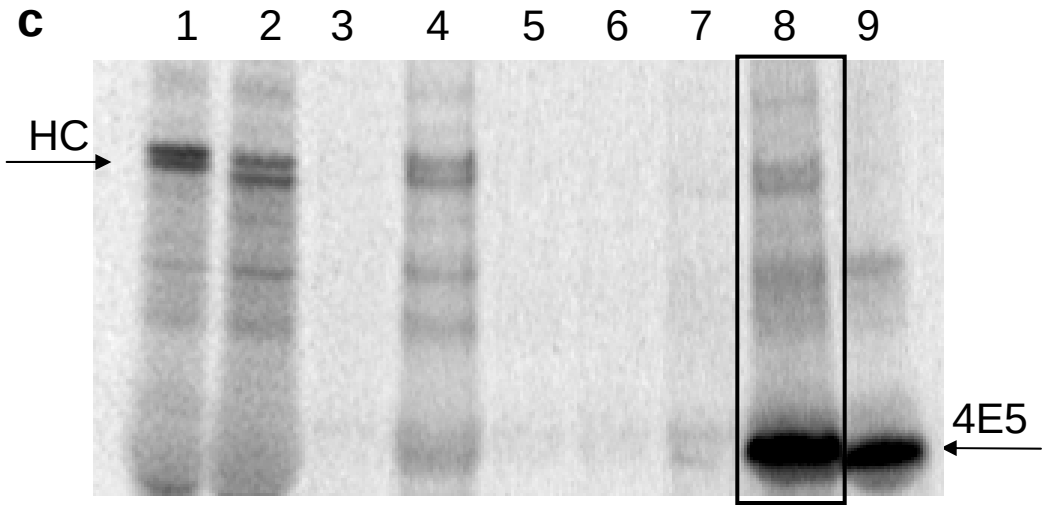


Lanes: 1, E5+Ab
2, E5+HD6+Ab
3, E5T+HD6+Ab
4, E5T+Ab

IP: Ab HA.11: anti-HA epitope

Marchetti et al., Fig 4

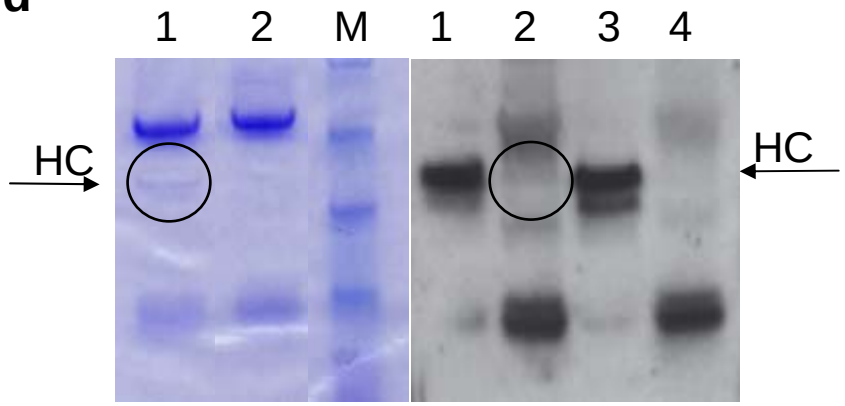
c



IP: mAb IL-A88: anti-bovine MHC I HC

Lanes: 1, input HD6
2, input JSP.1
3, JSP.1-Ab
4, JSP.1+Ab
5, E5-Ab
6, E5+Ab
7, JSP.1+E5-Ab
8, JSP.1+E5+Ab
9, E5 input

d

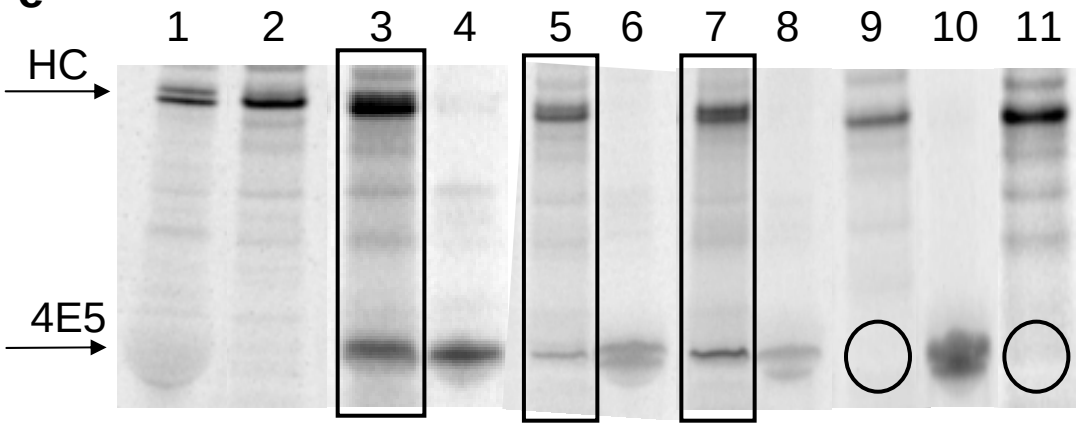


IP: Ab 274; IB: mAB IL-A88

Left panel: Comassie blue stained gel
Lanes: 1, co-IP from PalF-4E5
2, co-IP from control cells
M, markers
Right panel: immunoblot
Lanes: 1, protein lysate from PalF-4E5
2, co-IP from PalF-4E5
3, protein lysate from control cells
4, co-IP from control cells

Marchetti et al., Fig 4

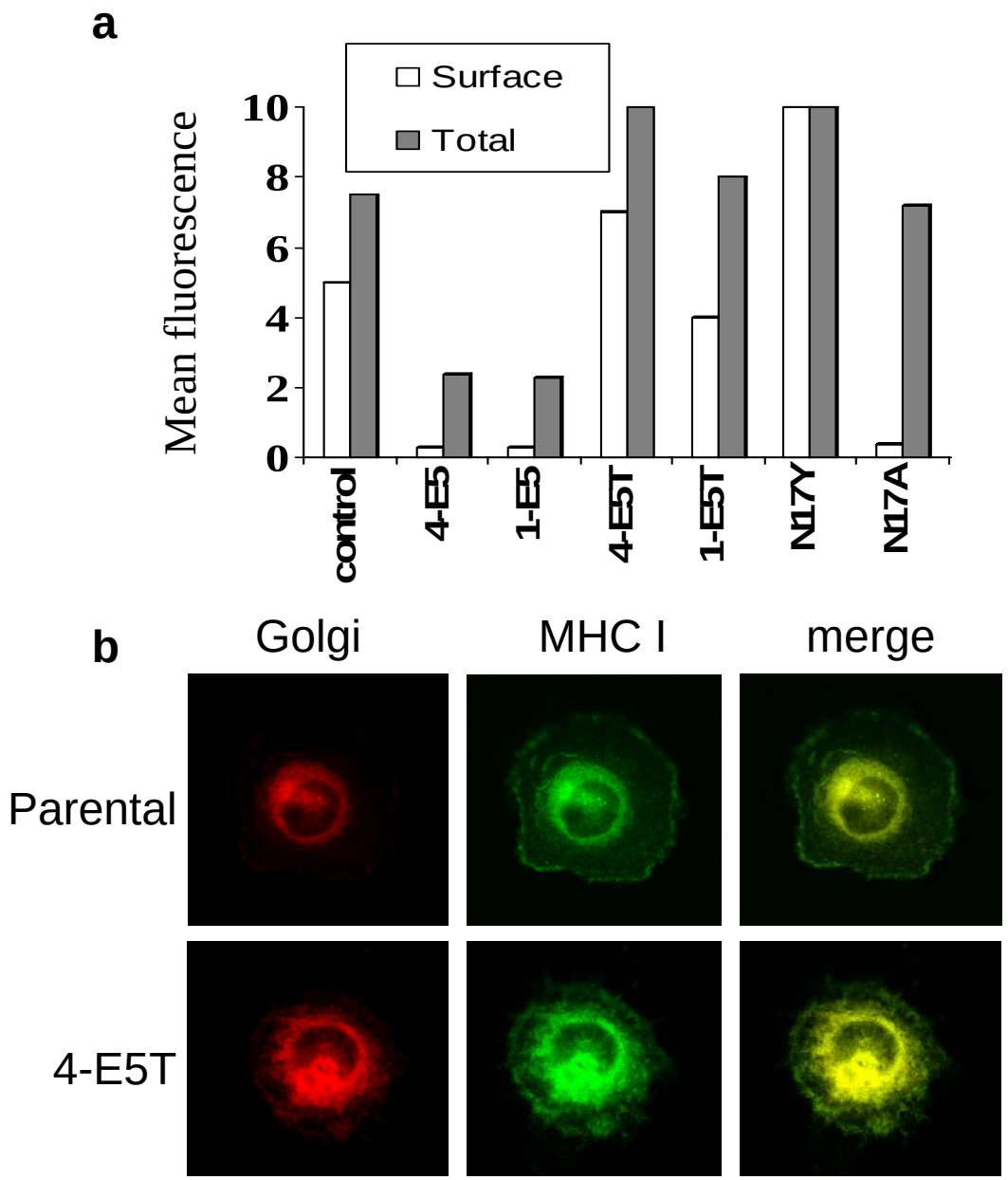
e



IP: mAb IL-A88: anti-bovine MHC I HC

Lanes: 1, input HD6
2, HD6+Ab
3, HD6+ E5+Ab
4, input E5
5, HD6+N17Y+Ab
6, N17Yinput
7, HD6+N17A+Ab
8, N17A input
9, HD6+E5T+Ab
10, E5T input
11, HD6+E5+peptide

Marchetti et al., Fig 4



Marchetti et al., Fig 5