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E5 protein of Human Papillomavirus 16 down-regulates HLA class I and interacts with the heavy chain via its first hydrophobic domain

Ashrafi GH, Haghshenas M, Marchetti B and Campo MS

Division of Pathological Sciences

Institute of Comparative Medicine

University of Glasgow

Glasgow, Scotland, UK

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Corresponding author: MS Campo, Division of Pathological Sciences, Institute of Comparative Medicine, University of Glasgow, Garscube Estate, Glasgow G12 0PH

s.campo@vet.gla.ac.uk; tel: +44 141 330 2876; fax: +44 141 330 5602

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The manuscript shows that HPV-16 E5 down-regulates and physically interacts with HLA class I via the first 30 N-terminus amino acid residues. This N-terminus domain is capable by itself of retaining HLA class I in the Golgi apparatus and preventing the anterograde transport of the complex to the cell surface.

Abstract

Human papillomavirus type 16 E5 protein (HPV-16 E5) is expressed early in papillomavirus infection and is localised primarily in the cell Golgi apparatus and endoplasmic reticulum. E5 prevents transport of the major histocompatibility class I (MHC I; HLA class I in humans) to the cell surface and retains the complex in the Golgi apparatus. We report that these effects are due, at least in part, to the interaction between E5 and HLA I heavy chain. We also demonstrate that the down-regulation of surface HLA I, and interaction with HC are mediated by the first hydrophobic domain of E5. Although E5 down-regulates classical HLA selectively as it does not down-regulate non-classical HLA, the interaction with the HC of classical HLA I is not specific for a particular haplotype of HLA I. This suggests that E5 can interfere with antigen presentation by most, if not all, classical HLA I haplotypes, with potentially serious consequences as the ability of infected cells to present antigenic peptides to effector T cells would be compromised.

Introduction

Human papillomaviruses (HPVs) infect mucosal and cutaneous epithelia, and induce lesions that can persist and progress to cancer. The mildest forms of HPV disease are benign hyperproliferative lesions, known as warts or papillomas. In most cases these lesions are cleared after several months following activation of the host immune system against viral antigen [1]. However, due to the ability of certain types of HPVs to avoid immune clearance, occasionally the lesions do not regress and can progress to cancer. This is especially true for HPV type 16 which is involved in the majority of cases of HPV-induced cervical cancers [2]. The ability of the virus to avoid immune clearance is due to several factors dependent on the virus life cycle, but also on active mechanisms operated by the viral proteins to counteract the host immune attack. Elimination of virally infected cells requires cytotoxic T-lymphocytes (CTL) that can recognise and kill virally infected cells via ligation of their receptor to major histocompatibility class I complex (MHC I; HLA I in humans) bound to viral peptides on the surface of infected cells [3].

One of the potential effectors of HPV-16 escape from host immunosurveillance is the viral oncoprotein E5 [4]. E5 is a hydrophobic membrane protein 83 amino acids long, possessing three well-defined hydrophobic regions. E5 is expressed early in papillomavirus infection in the deep layers of the infected epithelium [5, 6] (Araibi et al., unpublished results) and is localised mainly in the endoplasmic reticulum (ER) and Golgi apparatus (GA) membranes [7]. We have previously shown that HPV-16 E5 down-regulates the expression of surface HLA I by retaining the complex in the GA, and this down-regulation is selective, as E5 does not interfere with non-classical MHC. This may allow the virus to establish itself by avoiding clearance of virus-infected cells by both CTL and natural killer cells (NK) [4].

Here we show that E5 physically interacts with the heavy chain (HC) of HLA I and that interaction and down-regulation of surface HLA I are mediated by the first hydrophobic domain of E5.

Materials and methods

Construction of HPV-16 E5 mutants

The plasmid pcDNA-Neo (Invitrogen, UK), encoding G418 resistance and containing the universal IE promoter of CMV, was used to express HPV-16 E5 wild type (wt) or mutant forms, all tagged with the hemagglutinin (HA) epitope YPYDVPDYA at their N-terminus. HPV-16 E5 deletion mutants R79, A54, V36 and R30 (gift of Dr Alonso, Deutsches Krebsforschungszentrum, Heidelberg, Germany) were generated by introducing double stop codons at the respective nucleotide positions of the HPV 16 E5 sequence [8]. HPV-16 E5 Del1 mutant protein was made by deletion of the first hydrophobic domain (amino acids 1-30) by PCR amplification using forward primer from nt 91 to 105 of the HPV-16 E5 ORF (5'cattgctagcatgtacccatacgatgttccagattacgctccgctgcttttgtct3') and reverse primer from nt 252 to 231 (5'tcgcgaattcttatgtaattaaaaagcgtg3') including sites for Eco RI and Nhe I respectively. The resulting PCR products were inserted between the Eco RI and Nhe I sites of pcDNA-Neo. All clones were verified by sequencing.

Construction of GFP-E5 fusion proteins

pEGFP-C1 (Clontech, UK) is a eukaryotic expression plasmid which encodes the green fluorescent protein (GFP). It was used to clone full length HPV-16 E5 and its mutants to generate GFP-E5 wt and GFP-E5 mutant fusion proteins by PCR. The forward primer was from nt 1 to 22 of the E5 ORF for E5 wt and mutants R79, A54, V36 and R30, and from nt

91 to 112 for the Del1 mutant. The reverse primers were from nt 252 to 231 for Del1, from 237 to 216 for R79, from 162 to 141 for A54, from 111 to 90 for V36, and from 93 to 72 for R30. The forward and reverse primers included an Eco RI and a Bgl II site respectively. The resulting PCR products were inserted between the Eco RI and Bgl II sites of pEGFP-C1. All clones were verified by sequencing.

Cell culture and transfection

The immortalised human keratinocyte (HaCaT) cell line was grown in Dulbecco's modified Eagle medium high glucose without calcium chloride (Life Technologies, UK), supplemented with 10% foetal calf serum, as previously described [9]. One x 10^6 cells were stably transfected with 4μg pcDNA, pc-16E5 or pc-16E5 mutants by using Lipofectamine Plus (Invitrogen). Following transfection, HaCaT cells were selected in medium containing 500μg/ml G418 for 21 days. G418-resistant colonies were marked, picked and expanded into cell lines for analysis.

Real time/quantitative RT-PCR

RNA was isolated from HaCaT cells using the RNAeasy Mini kit (Qiagen, Sussex, UK), and residual DNA was removed by DNAase I treatment according to the manufacturer's guidelines (Invitrogen, UK). Real-time RT-PCR for E5, E5 mutants and β-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. 100ng RNA was used per each reaction, done in triplicate. Primers were as follows: forward (F) primer for E5 wt, R79, A54, V36 and R30 was 5'tgacaaatcttgatactgcatcca3'; reverse (R) primer for E5, R79 and A54 was 5'tgacaaatcttgatactgcatcca3'; R primer for V36 and R30 was 5'taataggcagacacacaaa3'.

The probe was 5'aacattactggcgtgctttttgctttgct3' in all cases. For ß-actin, primers and probe were commercially available (Applied Biosystems). Standard curves were generated using 10-fold serial dilutions of each template DNA, to quantify the relative levels of E5 and β-actin mRNA.

Semi-quantitative RT-PCR

Real time RT-PCR did not work for E5 Del1 as no appropriate forward primer could be identified outside the first hydrophobic domain that matched the probe. Therefore, to quantify the levels of E5 Del1, semi-quantitative RT-PCR was performed[9]. The Del1 F primer was 5'ctgcttttgtctgtgtctacata3' and the R primer was 5'tataatatatacaataaaacacctaa3'. RT-PCR was performed using an ABI prism 7700 sequence detector for 20, 25, 30 or 35 cycles. The same amplification protocol was used with E5 wt and R30 as controls. The RT-PCR products were run on gels, the bands scanned with MagicScanner 32-v4.3 and quantified with ImageQuant v5.2 software.

Western blotting

Fifty μg of protein lysates from HaCaT cell harbouring empty vector (Control), expressing Del1 or E5, were electrophoresed, and transferred to nitrocellulose membrane. The membranes were blocked and incubated with mAb HA11 (1/500 dilution; Sigma) for 1 hour, washed and incubated with anti-mouse IgM-HRP (1/5000; Oncogene Calbiochem-Novabiochem International). After 1 hour the membranes were washed and bound antibody was detected by enhanced chemoluminescence staining (ECL) (Amersham Pharmacia Biotech).

Flow cytometry

Detection of HLA I by flow cytometry was performed essentially as previously described [4]. Briefly HaCaT cells and cells expressing E5 and mutants were incubated with anti-human HLA class I monoclonal antibody (mAb) W6/32 (1/100; Serotec), at 4°C for 30 min, and then with anti-mouse IgG-FITC (1/100; Sigma) at 4°C for 30 min in the dark.. After washing the cells were analysed by flow cytometry. If the flow cytometry analysis was not performed immediately, the cells were re-suspended in 500µl of 3.4% paraformaldehyde in PBS and kept at 4°C. For the detection of intracellular HLA I, the cells were permeabilised with 0.5% Saponin (Sigma) in PBS-B and incubated with the primary antibody 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.

Immunofluorescence

Detection of HLA I by immunofluorescence was performed essentially as previously described [4]. Briefly, cells were washed and fixed in fixing solution (19ml PBS, 1ml 37% formaldehyde and 0.4g sucrose) for 10 minutes at room temperature (RT). After fixation, cells were incubated in permeabilising solution (19ml PBS, 1ml 10% NP40 and 0.4g sucrose) at RT for 10 minutes, incubated with mAb W6/32 (1:50 dilution) for 1 hour at room temperature and then with anti-mouse IgG-FITC (1/500;Sigma) at 4°C for 1 h in the dark. Following three final washes with PBS, the slides were mounted in Citifluor^{TM} (glycerol/PBS solution, Sigma) and analysed with a Leica TCS SP2 fluorescence confocal microscope (Leica-microsystems, Heidelberg Germany).

Co-localisation of Golgi apparatus and E5

HaCaT cells were transiently transfected with 0.1 μg of either pEGFP-E5 or pEGFP-E5 mutants, or with control empty plasmid, using Lipofectamine Plus™ Reagent according to the manufacturer's instructions. Seventy-two hours after transfection, cells were plated approximately 25-50% confluent, and grown overnight. 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 GA, in DMEM-H for 30 min at 4^{o}C. Cells were washed twice with PBS and fixed in fixing solution as above, and washed three times with PBS. Cells were incubated with 4′6′-diamino-2-phenylindole (DAPI) for 10 min, to stain the nucleus, washed in PBS–FCS and then distilled water, dried and mounted in Citifluor^{TM} (Sigma). Cells were analysed with a Leica TCS SP2 confocal scanner microscope. Images were acquired and merged using Leica confocal software.

Co-immunoprecipitation of E5 and HLA heavy chain in HaCaT cells

Control or E5-HaCaT cells were lysed in RIPA buffer (50mM Tris HCl pH 7.5, 150mM NaCl, 1% Nonidet P40, 0.5% NaDoc, 0.1% SDS) containing a cocktail of protease inhibitors (Roche, Lewes, UK). 100 μg of protein lysate were immunoprecitated with 50 μl of mAb HA11 (1:10 diluition; Sigma) against the HA-tag of E5 and then incubated at 4°C overnight. After that, protein G-sepharose bead suspension (Sigma) was added for 1 h at 4^{o}C. Following four washes in RIPA buffer the sepharose beads were resuspended in 20 μl of SDS loading buffer, heated at 75^{o}C for 10 min, and then electrophoresed in 12% NuPAGE gels (Invitrogen). Proteins were transferred onto nitrocellulose membranes and immunoblotted with 10 μl mAb HC10 against HLA-A, B, C (1:1000 dilution; Cancer Research, UK).

In vitro transcription/translation and co-immunoprecipitation

HLA heavy chain A2 cDNA sequence in pAL356 was excised and re-cloned in pBluescript II SK (+) (Stratagene); HLA heavy chain A1 or B8 cDNA sequences in pRSV5-neo were excised and re-cloned in pGEM-11Zf (Promega, UK). All HLA heavy chain plasmids were a gift from Dr S. Man, University of Cardiff, UK. HPV-16 E5 or E5 mutants, and A1, A2 or B8 were in vitro transcribed–translated 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, and as described previously [10]. Half of each transcription/translation reaction product was immunoprecipitated with 3 µl of mAb HA11 (1:10 diluition; Sigma) or, in the case of R30, 3 µl of a polyclonal antiserum raised against the N-terminus of E5 (gift of Prof D. DiMaio, Yale University, USA; Hwang et al., 1995), or 10 µl mAb HC10 against HLA-A, B, C (1:250 dilution; Cancer Research, UK) and then incubated at 4°C overnight. The other half of each reaction was left without antibody as a negative control. For co-immunoprecipitation the individual transcription/translation products (25 µl each of HLA heavy chain and E5 reactions) were mixed in equivalent amounts and immunoprecipitated with double the amount of either antibody. For competition experiments, unlabelled R30 or Del1 was added to labelled HLA-A2 overnight before the addition of labelled E5 and antibody as above. After incubation overnight at 4^{o}C, protein G-sepharose bead suspension (Sigma) 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% Nonidet P40, 0.05% NaDoc) and one wash in a low salt buffer (50mM Tris HCl pH 7.5, 1% NonidetP-400, 0.05% NaDoc) the beads were resuspended in 20 µl of SDS loading buffer, heated at 75^{o}C for 10 min, and 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°C overnight or exposed to a screen in a Storm 840 apparatus using a ImageQuant v5.2 software.

Results

HPV-16 E5 and HLA I heavy chain interact physically

HPV-16 E5 prevents the transport of HLA class I to the cell surface by retaining the complex in the GA [4]. We have recently shown that BPV-4 E5 physically interacts with the HC of the bovine MHC I, providing an additional mechanism to the alkalinisation of the GA for the retention of the complex in the GA [10, 11]. To investigate whether this was the case also for HPV-16 E5, we performed co-immunoprecipitation experiments between E5 and HLA I HC with monoclonal antibody (mAb) HA11 (against the HA tag of E5), in E5-expressing and control HaCaT keratinocytes, which are known to express HLA-A3**,** A31 B60 B51. A clear band was present in the precipitate from E5-expressing cells, while no band corresponding to HC was detected in the precipitate from control cells (Figure 1A, lanes 4 and 7). In addition to being detected by mAb HC10, specific for HLA class I, the band corresponded to the HC detected in the protein lysate by western blotting (Figure 1A, lanes 1 and 2), leading to its identification as HC, and to the conclusion that E5 and HLA-A3 HC (and possibly A31 B60 B51, as suggested by the double HC band) exist in a physical complex in E5-expressing HaCaT cells. (Del1 is a non-interacting mutant of E5 which is described later). The relative faintness of the HC band co-precipitated with E5 is due to the very low levels of E5 in these cells: E5 is expressed at approximately four orders of magnitude lower than actin (see Figure 2B and [4]. Additionally we performed an *in vitro* co-immunoprecipitation experiment between ^{35}S-labelled E5 and HLA-A2 HC, as a similar experiment had already validated the interaction between BPV-4 E5 and bovine MHC I heavy chain [10]. Physical interaction

between E5 and HC was confirmed by the co-immunoprecipitation of ^{35}S-labelled E5 and HLA-A2 with mAb HC-10 (Figure 1B, lane 7). Moreover, the formation of a complex between E5 and HLA-A2 HC *in vitro* indicates that the interaction is not specific for a particular HLA I haplotype.

E5 interacts with the HC of different HLA I haplotypes

To investigate whether E5 interacts with different HC, and as the *in vitro* interaction between E5 and HC reflects the interaction occurring *in vivo*, we performed *in vitro* co-precipitation experiments with E5 and A1 or B8 HC. In both cases, E5 and HC co-precipitated when either mAb HC10 or mAb HA11 was used (Figure 1D-G, lanes 7). Although a faint band corresponding to A1 or B8 HC was detected with mAb HA11 even in the absence of E5, the band was stronger when E5 was present, particularly in the case of B8, giving confidence that the co-precipitation reflects a genuine interaction. As E5 interacts with A3 in HaCaT cells, and with A1, A2 and B8 *in vitro*, we conclude that the interaction between E5 and HC is likely to take part with most, if not all, classic HLA I alleles.

Characterisation of E5 mutants

HPV-16 E5 is a transmembrane protein containing three well defined hydrophobic regions, of which the first one is the longest [12, 13]. To determine the E5 domain responsible for down-regulation of surface HLA I, deletion mutants of E5, lacking helical domain 1, 2 or 3, were assayed for their ability to retain HLA I in the GA. Mutant R79 lacks the last five C-terminal amino acids, A54 lacks the complete third hydrophobic domain, V36 and R30 lack the second and third hydrophobic domains, and Del1 lacks the first hydrophobic domain (Figure 2A). Before any analysis of the E5 mutants was carried out, we made sure that their expression and cellular localisation were not different from those of E5 wt.

Expression of E5 in stably transfected cells. We verified that the E5 proteins were being transcribed to a similar extent using quantitative RT-PCR (Q-RT-PCR). RNA was isolated from three clones each of HaCaT keratinocytes stably transfected with E5 wt or mutants. The level of E5 or E5 mutant RNA (with the exception of E5 Del1, see below) was determined and compared to the level of ß-actin RNA. Representative results from one clone each of E5 wt and mutants are shown in Figure 2B. The levels of E5 wt or mutant RNA were very low, approximately four orders of magnitude less than actin RNA as previously found [4]; however, they were comparable among clones, ranging approximately from 0.02 to 0.03 pg per 100ng RNA.

Del1 RNA could not be amplified by Q-RT-PCR because no forward primer compatible with the probe could be identified outside the first hydrophobic domain, so semi-quantitative RT-PCR was used instead. Also in this case, there was no appreciable difference in the amounts of RNA between E5 wt and mutants, including Del1 (Figure 2C).

Cellular localisation of E5 proteins. Given the very low expression levels of E5, it is not possible to determine the cellular location of the protein by cytoimmunofluorescence. Therefore we decided to analyse E5 location by the use of GFP-E5 fusion proteins. The correct cellular localisation of GFP-E5 fusion proteins has been reported [14]. HaCaT cells were transiently transfected with plasmids encoding GFP-fusions of E5 or E5 mutants or empty EGFP vector; the GA was visualised by using the Golgi marker BODIPY-TR-ceramide and the nucleus was stained with DAPI. The localisation of the GFP-fusion proteins was determined by three-colour confocal microscopy. There was no discernible difference in localisation between E5 and its mutants; in all cases, the E5 proteins were localised in the endomembranes, mainly the GA, as shown by the merged images (Figure 2D).

The first hydrophobic domain of E5 is responsible for surface HLA I down-regulation

As E5 wt and mutants were expressed to comparable levels and localised to similar cellular compartments, we next investigated which E5 domain(s) was responsible for prevention of transport of MHC class I to the cell surface and retention of the complex in the GA. Using flow cytometry we determined the levels of both surface and total (surface plus intracellular) HLA I in parental HaCaT, control cells harbouring empty vector, cells stably expressing E5 wt or mutants**.** Control cells were no different from parental cells, with approximately half as much surface HLA I than total HLA I (Figure 3A). In clones expressing E5 wt the levels of surface HLA I were reduced to approximately half that of control cells, as previously reported [4]; likewise, clones expressing the E5 mutants containing the first hydrophobic domain (R79, A54, V36, R30) had reduced levels of surface HLA I to the same extent as E5 wt (Figure 3A). In contrast, expression of E5 Del1, lacking the first hydrophobic domain, did not have any effect on the levels of surface HLA I (Figure 3A). The failure of E5 Del1 to down-regulate surface HLA I could be due to impaired stability of the protein. However, western blotting of protein lysates from E5 wt- or E5 Del1-expressing HaCaT cells showed that this is not the case as E5 wt and E5 Del1 were present at comparable levels (Figure 1C, lanes 1 and 3).

From these results, we conclude that the first N-terminus 30 amino acids of E5 are responsible for the down-regulation of HLA I.

This conclusion was confirmed by the localisation of HLA I in HaCaT cells expressing the E5 proteins. Cells carrying empty vector, expressing E5 wt or E5 mutants were stained with mAb W6/32 and analysed for HLA I by cytoimmunofluorescence. In cells expressing E5 wt or mutants R79, A54, V36 or R30 (all containing the first hydrophobic domain), HLA class I

was detected only intracellularly (Figure 3B); in contrast, in cells expressing E5 Del1 HLA I was detected both on the cell surface and intracellularly, as in control cells (Figure 3B). These results, together with those obtained by flow cytometry, confirm that E5 amino acids 1-30 are responsible for the retention of HLA class I complex in the GA.

HPV-16 E5 binds HLA I heavy chain via its first helical domain

E5 interacts with HLA I HC (Figure 1) and prevents the transport of HLA I to the cell surface via its first hydrophobic domain (Figure 3). To investigate whether the same E5 domain was responsible for the interaction with HC, we performed *in vivo* and *in vitro* co-immunoprecipitation experiments as above. In HaCaT cells expressing E5 Del1, immunoprecipitation with mAb HA11 failed to precipitate HC (Figure 1A, lanes 5 and 6), although in immunoblots HC was easily detected by mAb HC10 (Figure 1A, lane 3) and E5 Del1 and E5 wt were expressed at comparable levels at both mRNA and protein levels (Figure 1C, lanes 1 and 3, and 2C).

In *in vitro* co-immunoprecipitations, E5 wt and all the mutants containing the first helical domain (including R30, which contains only the first N-terminus 30 amino acids) co-precipitated with HLA-A2 HC (Figure 4A, lane 7, and B, lane 2, shown only for A54 and R30). Despite the presence of non-specific bands also in the lanes without antibody (some deriving from the translation of E5 and its mutants, possibly representing multimers of E5; see input lanes), mAb HC-10 co-precipitated in each case both A2 and the E5 proteins (marked with a black dot in Figure 4). In contrast, Del1 failed to co-precipitate with HLA-A2 HC (Figure 4B, lane 5). Co-immunoprecipitation between R30 and HLA-A2 HC was confirmed by using an antibody raised against the N-terminus of E5 [15]. Also in this case, HLA-A2 HC co-precipitated with R30 (Figure 4C, lane 2), validating the results obtained with mAb HA11 and HC10. These data indicate that the interaction between E5 and HC takes

place via the N-terminus first 30 amino acids. This was confirmed by competition experiments. The addition of unlabelled R30 (containing the first 30 amino acids) to the reaction prevented the interaction between E5 and A2 HC (Figure 4D, lane 5), whereas the addition of unlabelled Del1 (amino acids 31-83) failed to compete with E5 and did not affect the E5-HC interaction (Figure 4D, lane 9).

Taken together, these results conclusively show that E5 both physically interacts with HC and down-regulates surface HLA I via its first hydrophobic domain.

Discussion

As is the case for the E5 oncoprotein of BPV [9-11, 16], the E5 oncoprotein of HPV-16 prevents the transport of HLA I to the cell surface and retains the complex in the Golgi apparatus [4]. We have argued that the Golgi retention of MHC I by BPV-4 E5 is due to at least two events: the impaired acidification of the Golgi apparatus and the physical interaction of E5 with the heavy chain of MHC I.

HPV-16 E5 interacts with HC. The interaction between E5 and the MHC I HC is not confined to BPV E5. HPV-16 E5 forms a stable complex with the HC of the HLA-A3 (and/or A31 B60 B51) of HaCaT keratinocytes, and *in vitro* also with the HC of HLA-A1, -A2 and -B8. This interaction is therefore not specific for a particular HLA I haplotype/allele but it is likely to occur with most, if not all, HLA I. Complex formation between E5 and HC is responsible for down-regulation of surface HLA I, as a mutant of E5 which fails to bind HC, also fails to prevent the transport of HLA I to the cell surface, as discussed below.

However, while the haplotype/allele of classical HLA I is not critical for E5-HC complex formation, E5 fails to down-regulate non-classical HLA I [4]. These findings parallel those obtained with BPV-4 E5. BPV-4 E5 down-regulates classical MHC I independently of its type [16] and interacts with different HC (Marchetti et al., 2005), but fails to down-regulate non classical MHC I and to interact with the HC of a non-classical MHC I (our unpublished results). It remains to be seen if HPV-16 E5 is capable of binding the HC of non-classical HLA I.

The first N-terminus hydrophobic domain of E5 is essential for down-regulation of HLA I. HPV-16 E5 interacts also with 16k subunit c, a component of the vacuolar H^+-ATPase [17]. Previously published deletion mutants of E5 have been proficiently used to map the domain

of E5 mediating this interaction (see below; [8]). These mutants and the new N-terminus deletion mutant of E5 showed the importance of the first N-terminus 30 amino acids in the interaction with, and down-regulation of, HLA I. The absence of the middle and last hydrophobic domains does not prevent down-regulation of surface HLA I, and the 30 amino acid-long N-terminus hydrophobic peptide is sufficient to prevent the traffic of HLA I to the cell surface. In contrast, the absence of the first hydrophobic N-terminus domain completely abolishes surface HLA I down-regulation. The impaired function of this Del1 mutant is not due to diminished expression or inappropriate cellular localisation, as both expression and localisation are comparable to those of E5 wt or the other mutants. Furthermore, Del1 fails to interact with the HLA I HC both in HaCaT cells and *in vitro*, the latter observation confirming that the inability of Del1 to complex with HC is not due to improper expression or location. Of importance is the fact that R30, comprising only the first 30 amino acids, is capable of interacting with and down-regulating HLA I to the same extent as E5 wt, thus largely eliminating the possibility that the failure of Del1 to down-regulate HLA I is due to improper configuration, instability or any other feature which may be peculiar to this deletion mutant.

We have recently reported that BPV E5 interacts with the HC of bovine MHC I via its C-terminus domain [10]. This contrasts with the results presented here with HPV-16 E5 but this difference is not surprising. Despite their differences in length and presumed conformation, BPV E5 and HPV E5 share numerous functional similarities, one of them being the interference with the proper function of 16k ductin (and therefore of the vacuolar ATPase) [7]. BPV E5 and HPV-16 E5 interact with 16k subunit c via different E5 domains: BPV E5 interacts via its 17th amino acid residue (Q in BPV-1 E5, N in BPV-4 E5; [18, 19] whereas HPV-16 E5 interacts via its second and third hydrophobic domains [8, 20]. Thus, the same effects,

such as down-regulation of MHC I and disablement of the proton pump, can be achieved by distinct physical interactions with HC and subunit c, respectively.

The interaction between HPV-16 E5 and 16k subunit c has been deemed responsible for the impaired functioning of the pump and the consequent lack of acidification of the endomembrane compartments [21, 22]. Alkaline pH in the endolysosomes leads to delayed degradation and faster recycling of the epidermal growth factor receptor [22, 23], thus promoting increased cell proliferation. Studies with deletion mutants, including those employed by us here, have mapped the 16k-interacting domain of HPV-16 E5 to a relatively large region of E5 encompassing the second and third hydrophobic domains [8, 20]. The observation that HPV-16 E5 interacts with HC and 16k via different domains suggests that E5 can in principle bind the two cellular partners at the same time, thus simultaneously promoting immunomodulation and cell transformation, partly explaining the pleiotropic effects of E5 expression.

We have commented before on the functional similarity between HPV-16 E5 and HIV-1 Nef. Both proteins prevent transport of classical HLA I to the cell surface in their natural host cells type [24], bind to the HLA I HC via the same domains needed for HLA I down-regulation [25] and neither down-regulate non-classical HLA I [26]; both bind 16k subunit c [27] and induce cytoskeletal rearrangements [28, 29], and induce epidermal hyperplasia when expressed in the basal layer of the skin of transgenic mice [30, 31]. These similarities are intriguing and point to a process of "convergent evolution" of these two proteins encoded by such different viruses.

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Figure Legend

Figure 1. E5 and HLA I HC interact physically. **A**, co-immunoprecipitation of E5 and HLA I HC in HaCaT cells. Proteins from HaCaT cells harbouring empty vector (C), expressing Del1 or E5, were either immunoblotted with mAb HC10 to detect HC, or first immunoprecipitated with mAb HA11 and immunoblotted with mAb HC10. Del1 is a deletion mutant of E5 which does not interact with HLA HC (see later). The double HC band, indicated by two black dots, more visible when co-immunoprecipitated with E5 (lane 7), may represent two or more of the HC alleles expressed by the cells. The heavy bands in the immunoprecipitations are Ig. Despite equal amounts of protein in the lanes, equal amounts/dilutions of antibody, and equal exposure time, the Ig bands have been consistently more pronounced in the immunoprecipitates from E5-expressing HaCaT cells. The reason for this effect is not known. Please note that the two Del1 IP lanes 5 and 6 represent two independent experiments. WB and IP of E5 and the second Del1 (lanes 2-5) were run in a separate but essentially identical gel and aligned for ease of comparison. **B**, In vitro co-precipitation between E5 and HLA-A2 HC. E5 and HC of HLA-A2 were transcribed/translated *in vitro* in the presence of ^{35}S-methionine and canine microsomes, mixed together and co-precipitated with mAb HC10 (anti HLA-A, -B, -C) (+ lanes). No precipitate was observed in the absence of the antibody (- lane 6) or of A2 HC (lanes 4 and 5). A faint A2 band is visible in lane 2, despite the absence of antibody. **C**, E5 wt and E5 Del1 are expressed at comparable levels. Immunoblots of the same protein lysates in A, probed with mAb HA11. **D-G**, E5 interacts with HC from different HLA I haplotypes. E5 and HC of HLA-A1 and -B8 were transcribed/translated *in vitro* as in panel B and co-precipitated either with mAb HC10 (anti HLA-A, -B, -C; panels D and E) or mAb HA11 (anti the HA epitope of E5; panels F and G). A1, B8 and E5, were precipitated by their specific antibodies (+ lanes); A1 and E5, or B8 and E5 co-precipitated when incubated with either mAb HC10 (panels D and E; + lanes 7) or mAb HA11 (panels F and G; + lanes

7), and the co-immunoprecipitated bands were much stronger than in the absence of the antibody (- lanes). The E5 and HA bands are indicated by black dots; the other bands originate from the translated E5 protein (see input lanes) and may represent multimers of E5.

Figure 2. Deletion mutants of E5 express to the same levels and have similar cellular localisation to E5 wt. **A**, Diagrammatic representation of E5 wt, showing the three hydrophobic domains, and E5 mutants, showing the missing domains of E5; **B**, Quantitative RT-PCR for E5 wt, E5 mutant and actin RNA, showing that the E5 mutants express at comparable levels; note that the right-hand Y axis represents the amount of E5 RNA and is $1x10^{-4}$ that of the left-hand Y axis which represents the amount of actin RNA; **C**, Semi-quantitative RT-PCR for E5 wt, E5 mutant and actin RNA, showing that E5 Del1 mutant express at levels comparable with E5 wt. The Y axis represents the band intensity after 25 amplification cycles as measured by ImageQuant v5.2 software. **D**, GFP-fusion forms of E5 wt or E5 mutants, transiently transfected in HaCaT keratinocytes, were visualised at the confocal microscope, along with the Golgi apparatus stained with BODIPY-TR-ceramide and the nucleus stained with DAPI. Only the merged images are presented (in greyscale) which show that E5 wt and mutants localise in the GA and ER. As the HaCaT cells were transiently transfected with the GFP plasmids, GFP-E5 fusion proteins are expressed only in a minority of cells.

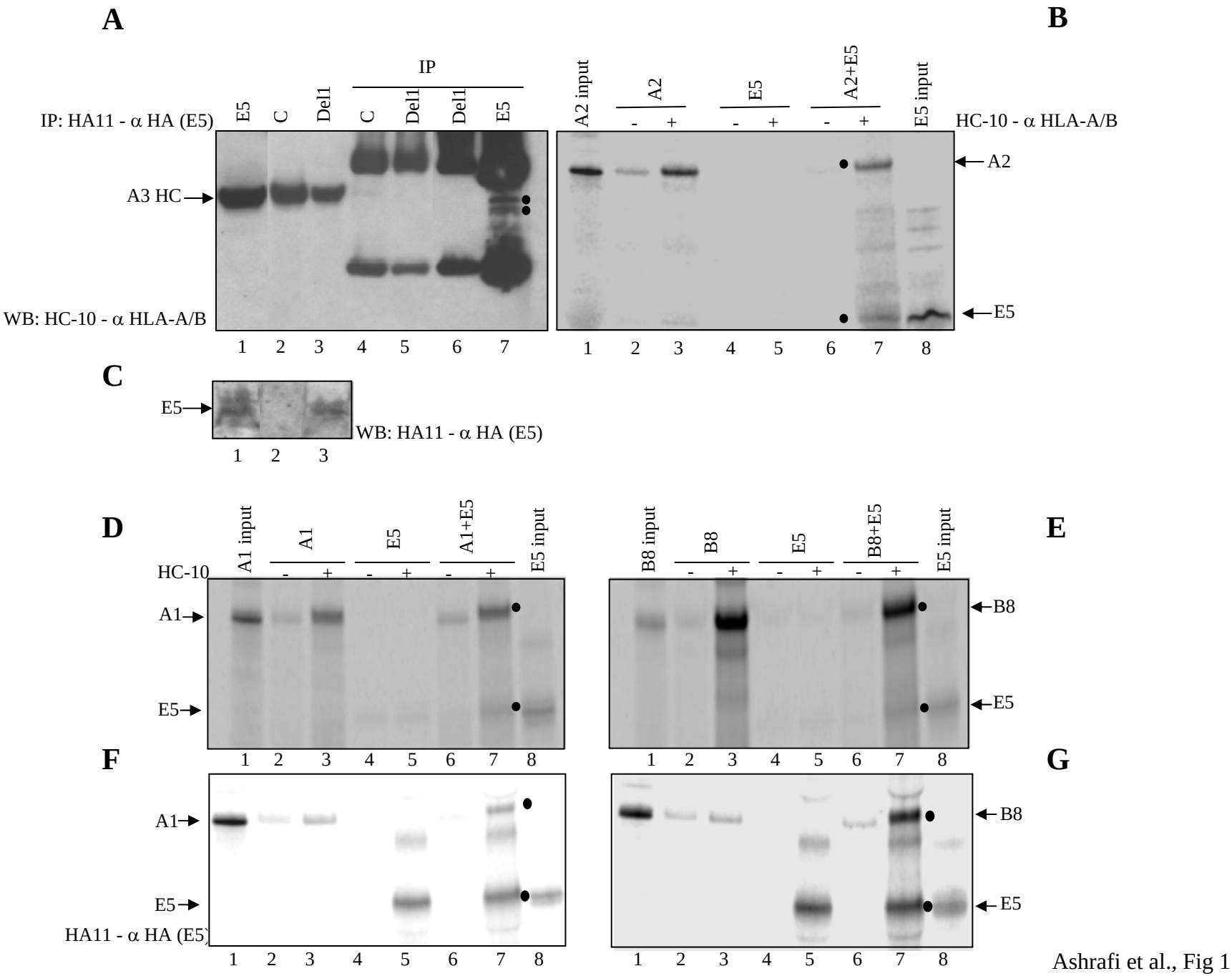
Figure 3. The first hydrophobic domain of E5 mediates the down-regulation of surface HLA I. **A,** HaCaT parental cells, HaCaT carrying empty vector, or expressing E5 wt or mutants were analysed by flow cytometry for surface (white bars) and total HLA I (grey bars). The average mean fluorescence was calculated from the flow cytometry analyses of two duplicate measurements from at least three clones of each cell line. The background (the reading of

cells stained with no primary antibody and only secondary antibody) was 0.4 in all cases. Standard deviation is shown. Del1 fails to down-regulate surface HLA I. The dotted line indicates the level of surface HLA I in parental and control cells. The HLA I values for Del1 are boxed.

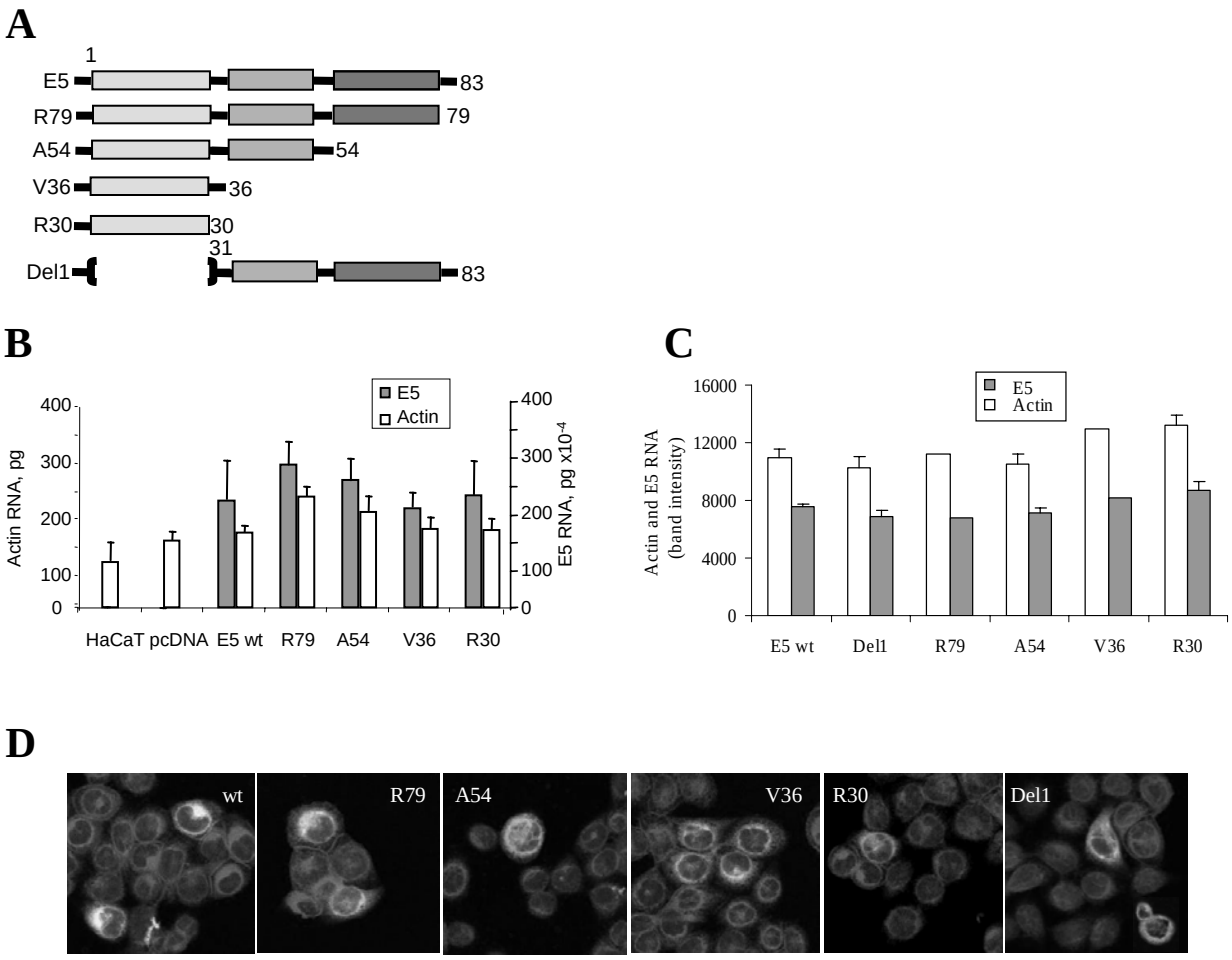
B, The same cells were stained with mAb W6/32 and analysed by cytoimmunofluorescence. Del1 mutant failed to retain HLA I in the GA (highlighted with *). Although only a few cells are shown, the localisation of HLA I was the same in all cells of each clone. N, nucleus.

Figure 4. Del1 does not bind HC. **A**, E5 wt, A54 and A2 HC were transcribed/translated *in vitro* in the presence of ^{35}S-methionine and canine microsomes, mixed together and co-precipitated with mAb HC10 (+ lanes). Co-precipitates were observed only in the presence of antibody and of A2 HC. **B,** R30, Del1 and A2 HC were processed as above. The only E5 mutant that did not interact with A2 was Del1 (lane 6). **C**, R30 and HLA-A2 HC were transcribed/translated *in vitro* as above and precipitated with an antibody against the N-terminus of E5 (10) (+ lanes). Co-precipitates were observed only in the presence of antibody. **D**, E5 wt and HLA-A2 HC were transcribed/translated and labelled *in vitro* as above; unlabelled R30 or Del1 were added to the reactions, and proteins precipitated with mAb HC10 (+ lanes). R30 competed with E5 wt and prevented its binding to HC (lane 4), while Del1 failed to do so (lane 9). Lanes 1 and 2 are the same as lanes 13 and 14, repeated for ease of comparison.

In all panels, the co-precipitated E5, E5 mutants and HC bands are indicated by black dots; the other bands originate from the translated E5 protein (see input lanes) and may represent multimers of E5.

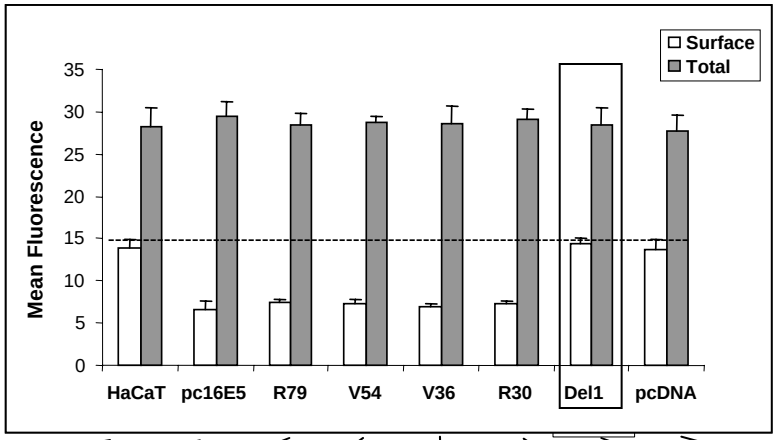


Ashrafi et al., Fig 1



Ashrafi et al., Fig 2

A



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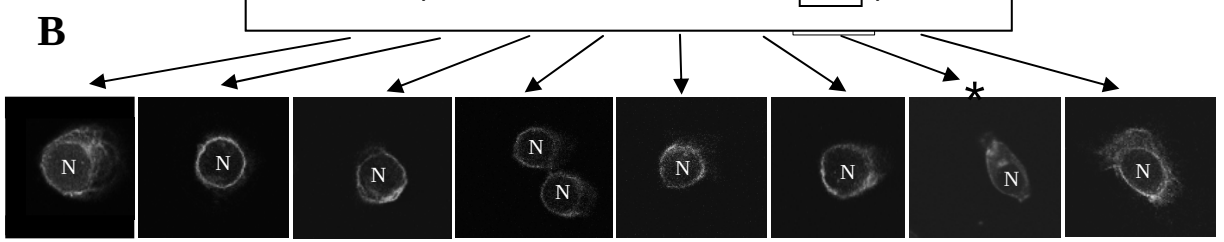
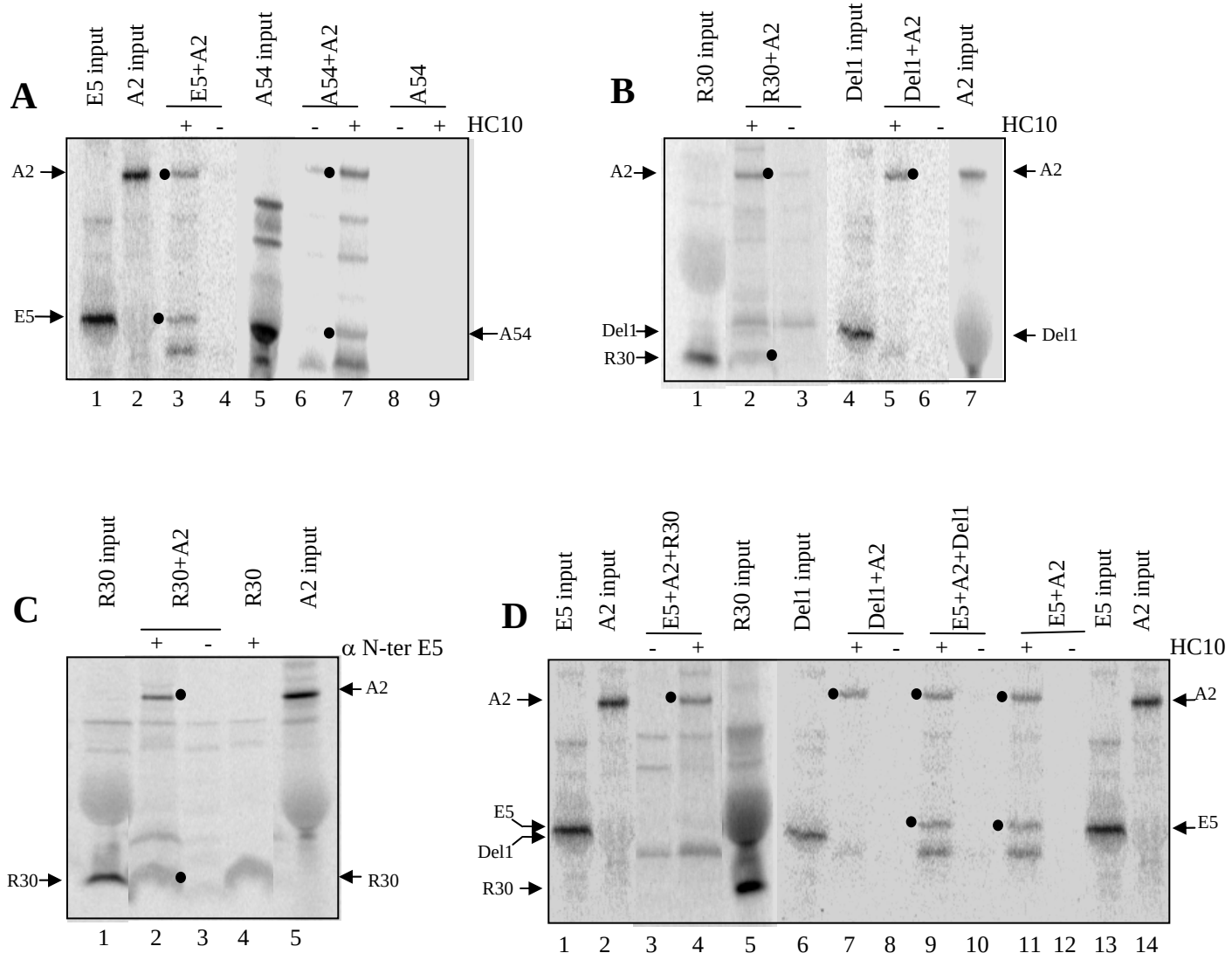


Ashrafi et al., Fig 3



Ashrafi et al., Fig 4