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Chaperones of the class I peptide-loading complex facilitate the constitutive presentation of endogenous antigens on HLA-DP^{84GGPM87}

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A B S T R A C T

Recent work has delineated key differences in the antigen processing and presentation mechanisms underlying HLA-DP alleles encoding glycine at position 84 of the DPβ chain (DP^{84GGPM87}). These DPs are unable to associate with the class II-associated Ii peptide (CLIP) region of the invariant chain (Ii) chaperone early in the endocytic pathway, leading to continuous presentation of endogenous antigens. However, little is known about the chaperone support involved in the loading of these endogenous antigens onto DP molecules. Here, we demonstrate the proteasome and TAP dependency of this pathway and reveal the ability of HLA class I to compete with DP^{84GGPM87} for the presentation of endogenous antigens, suggesting that shared subcellular machinery may exist between the two classes of HLA. We identify physical interactions of prototypical class I-associated chaperones with numerous DP alleles, including TAP2, tapasin, ERp57, calnexin, and calreticulin, using a conventional immunoprecipitation and immunoblot approach and confirm the existence of these interactions *in vivo* through the use of the BioID2 proximal biotinylation system in human cells. Based on immunological assays, we then demonstrate the ability of each of these chaperones to facilitate the presentation of endogenously derived, but not exogenously derived, antigens on DP molecules. Considering previous genetic and clinical studies linking DP^{84GGPM87} to disease frequency and severity in autoimmune disease, viral infections, and cancer, we suggest that the above chaperones may form the molecular basis of these observable clinical differences through facilitating the presentation of endogenously derived antigens to CD4$^+$ T cells.

1. Introduction

The human leukocyte antigen (HLA) class I and II genes are well established in connecting the innate and adaptive arms of immunity. Textbook knowledge of HLA suggests that class I is critical in priming CD8$^+$ T cells with endogenously derived antigenic peptides, whereas class II mainly binds and presents exogenously derived epitopes to CD4$^+$ T cells. Classical antigen presentation in class II describes the synthesis of these molecules in the endoplasmic reticulum (ER), where they rapidly associate with the glycoprotein chaperone invariant chain (Ii), through both its class II-associated Ii peptide (CLIP) region and other, non-CLIP regions [1]. In addition to aiding in the folding of class II, Ii blocks the association of endogenous antigens in the ER via binding of its CLIP region and guides class II to the MIIC compartment, where Ii is degraded so that class II can be loaded with exogenous antigens [2–6]. In this context, the non-classical and non-polymorphic class II molecule HLA-DM acts to efficiently remove CLIP from the class II cleft and plays a peptide-editing role, to ensure that predominantly high affinity-antigens are bound to class II [7,8]. In addition to this canonical pathway, cross-presentation on class II molecules has been described, leading to the presentation of endogenously derived antigens on these HLAs. Importantly, this cross-presentation has demonstrated biological relevance in human diseases such as cancer [9] and can result from numerous distinct pathways, including various autophagy mechanisms [10,11], exosome-mediated antigen sharing [12], chaperone-specific guiding of endogenous antigens to endosomal compartments [13], and polymorphism in class II leading to the inability of CLIP to block the association of endogenous antigens in the ER [14,15], all of which have documented roles in contributing to the presentation of endogenously derived antigenic peptides on HLA class II molecules.

Our group has previously demonstrated the ability of certain HLA-DP alleles to constitutively present both endogenous and exogenous antigens on DPs [14,15]. DP alleles encoding aspartic acid at position 84 of their DPβ chain (DP^{84DEAV87}, such as DP5 and DP8) are able to associate with Ii as described in textbooks and present predominantly exogenous antigens. However, these alleles encoding glycine at position 84 (DP^{84GGPM87}, such as DP2 and DP4) cannot associate with Ii via the CLIP region, although non-CLIP interactions with these DP molecules

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are preserved. Consequently, early in the endocytic pathway, endogenous antigens can associate with these DP molecules, after which they traffic to the MIIC compartment by virtue of their preserved non-CLIP-Ii interactions, resulting in the presentation of both endogenous and exogenous antigens on these class II molecules. Though this presentation has been demonstrated to be robust even in the presence of the class II chaperones Ii and HLA-DM, little is known about how endogenous antigens are able to load onto these DP molecules early in the endocytic pathway. In particular, with the exception of Hsp-90-mediated trafficking of antigens to endosomal compartments in mice, very little is known about the chaperone support required to direct and load endogenous antigens onto these molecules in humans [13].

In this study, we investigate the chaperone support required to load endogenous antigens onto HLA-DP, particularly $DP^{84GGPM87}$. We identify the proteasome and TAP dependency of this pathway and note the ability of class I molecules to compete with these DP molecules for the use of subcellular machinery to facilitate this presentation. Turning our attention to specific members of the class I peptide-loading complex (PLC), we identify interactions between several of these chaperones, including TAP, tapasin (TPN), ERp57 (E57), calnexin (CNX), and calreticulin (CRT), and various DP alleles both *in vitro*, via immunoprecipitation (IP) and immunoblotting techniques, and *in vivo*, making use of BioID2 technology to detect proximate complexes in living cells. Finally, the functional role of these chaperones is investigated through T cell activation assays, revealing that these chaperones facilitate the presentation of endogenous, but not exogenous, antigens on $DP^{84GGPM87}$. Thus, we suggest that these traditionally class I-associated chaperones play a dual role in supporting the presentation of endogenous antigens on both class I and class II molecules early in the endocytic pathway, forming the molecular basis of cross-presentation on these DP alleles which are significantly associated with clinical outcomes in several human diseases, including autoimmune disease, viral infections, and cancer.

2. Materials and methods

2.1. Cell lines, culture conditions and cDNA

All cells lines were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). Human K562 cells are suspension cells of erythroleukemic origin and do not express HLA class I, class II, Ii, or HLA-DM. Human HEK293 cells are adherent cells of embryonic kidney origin and endogenously express HLA class I, but do not express class II, Ii, or HLA-DM. Human DU145 cells are adherent cells of prostate cancer origin and endogenously express HLA class I, but do not express class II, Ii, or HLA-DM. Suspension and adherent cells were cultured in RPMI 1640 or DMEM-based culture medium, respectively, supplemented with 10% FCS and gentamycin (Life Technologies, Carlsbad, CA). The cultures were frequently examined for mycoplasma contamination using the ATCC's Mycoplasma Detection Kit. The peripheral blood mononuclear cells (PBMCs) used in cytokine release experiments were collected from healthy volunteer donors following institutional board approval.

Human cell lines stably expressing genes of interest were generated through retroviral transduction of cDNAs for the genes in all cases. A PG13-derived virus was utilized for human T cell transduction, while a 293GPG-derived virus was utilized for all other cell lines. cDNAs encoding the indicated genes were fused to EGFP or a truncated human nerve growth factor receptor (ΔNGFR) as necessary using an enhanced internal ribosome entry site sequence (IRES), to monitor transduction efficiency or for sorting purposes, respectively. Magnetic bead sorting to purify transduced cells was carried out as described previously [16,17]. The DP heterodimers employed in this study were DP4 or DP5 heterodimers comprised of either the appropriate wild type DPβ chain, or a mutational derivative of either position 84 of the DPβ chain alone, or of all four amino acids of the 84–87 region, as indicated [14,15,18]. BioID2 enzymes were linked to either the DPα or DPβ chain (or both) as indicated using a flexible linker comprised of 13 repeats of the G_4S amino acid sequence, the maximum length previously reported for use with this system [19]. T cell receptor cDNAs transduced for the purpose of cytokine release experiments were comprised of TCR α and β chains specific for DP4/MAGE-$A3_{243\text{-}258}$ complexes, which were obtained from published gene sequences [20]. All cDNAs were cloned into the pMX backbone vector and sequenced to verify successful cloning.

2.2. Reagents

The peptides used as controls in T cell activation assays were the MAGE-$A3_{243\text{-}258}$ ($_{243}$ KKLLTQHFVQENYLEY$_{258}$) and tetanus toxin $TT_{947\text{-}967}$ ($_{947}$FNNFTVSFWLRVPKVSASHLE$_{967}$) peptides, which were purchased from GenScript (Piscataway, NJ). The Dynabeads used in the isolation of biotinylated target proteins were M − 280 streptavidin Dynabeads, purchased from Thermo Fisher Scientific (Canada).

2.3. T cell activation/cytokine release assays

T cells isolated from healthy donors were purified for $CD4^+$ T cells through magnetic bead sorting and transduced with the TCR genes discussed in Section 2.1 (Miltenyi Biotec). Cytokine ELISPOT analyses were performed in accordance with previous studies [21–23]. Briefly, PVDF plates (Millipore, Bedford, MA) were coated with the appropriate capture antibody (IFN-γ: 1:200 dilution of the 1D1K mAb purchased from MABTECH (Mariemont, OH); IL-2: 1:75 dilution of SEL202 (part #840606) purchased from R&D Systems (Minneapolis, MN). Plates were washed with 2% FCS/PBS, and 2×10^4 T cells were co-cultured with 5×10^4 of the indicated stimulator cells for 20–24 h at 37 °C. After washing with PBS, a biotin-conjugated detection Ab (IFN-γ: 1:2000 dilution of the 7-B6-1 mAb purchased from MABTECH; IL-2: 1:75 dilution of SEL202 (part # 840607) purchased from R&D Systems) was added, and the plates were incubated overnight at 4 °C. For IL-2 ELISPOT assays, after washing with PBS, plates were incubated with alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA) overnight at 4 °C, and after a final wash with PBS, they were treated with NBT and BCIP (nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate; Promega, Madison, WI) to develop IL-2 spots. For IFN-γ ELISPOT assays, after washing with PBS, plates were treated with streptavidin-HRP (DAKO, Carpenteria, CA) at a 1:5000 dilution overnight at 4 °C, and the resulting spots were subsequently developed.

2.4. Transient transfection

K562 cells were transiently transfected using 1.5μL/well of Lipofectamine 2000 in 24 well plates (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions, with 1ug of the following plasmids pMX (control), pMX/Native MAGE-A3, pMX/Endosomal MAGE-A3, CRISPR-Cas9/sgRNA plasmid (Santa Cruz, CA), and/or HDR Plasmid (0.1 μg) (Santa Cruz, CA) to identify knockout cells, as indicated in each experiment. HEK293 and DU145 cells were transiently transfected using 7.5μL/well of Transit293 in 6 well plates (Mitus, Madison, WI) according to the manufacturer's instructions, with 1ug of pMX (control), pMX/Native MAGE-A3, pMX/Endosomal MAGE-A3, CRISPR-Cas9/sgRNA plasmid (Santa Cruz, CA), and/or HDR Plasmid (0.1 μg) (Santa Cruz, CA) to identify knockout cells, as indicated in each experiment.

2.5. Flow cytometry

The staining protocols for flow cytometric analyses were performed as described previously [22,23]. The following mAbs were used in flow cytometry analyses: HLA-A/B/C (1:200 dilution, 311406, BioLegend), pan-HLA class II (1:500 dilution, 6604366, Beckman Coulter), and

NGFR (1:200 dilution, 557196, BD Biosciences). Appropriate isotype controls were purchased from BD Biosciences and used at a 1:500 or 1:200 dilution, as appropriate.

2.6. Immunoprecipitation and immunoblotting

For both immunoprecipitation and immunoblotting protocols, cells were lysed in cold 1% NP-40 lysis buffer (150 mM NaCl, 20 mM Tris-HCl, 2.5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM β-glycerophosphate, 1% NP-40, 1 μg/ml aprotinin and 1 mM PMSF at pH 7.5), except in the case of IP experiments involving calnexin, where a 1% digitonin lysis buffer was substituted (50 mM NaCl, 20 mM Tris-HCl, 2.5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM β-glycerophosphate, 1% digitonin, 1 μg/ml aprotinin and 1 mM PMSF at pH 7.5). Cells were incubated on ice for either 15 (NP-40) or 30 (digitonin) minutes, followed by centrifugation at 12,000 rpm for 10 min at 4 °C. In the case of IP samples, the lysates were then incubated with 20 μL of protein A or protein G Sepharose beads (Santa Cruz Biotechnology, Santa Cruz, CA) and 1 μg of the appropriate IP Ab overnight at 4 °C on a tube turner. The beads were then separated via pulse centrifugation and washed 3 times in cold lysis buffer. IP samples or lysates, as appropriate, were then subjected to SDS-PAGE and transferred to Immobilion P membranes (Millipore), which were subsequently blocked for 1 h at room temperature on a shaker with a 5% milk protein solution (5% milk protein w/v and 0.1% Tween 20 in TBS) and incubated with the appropriate primary Ab or streptavidin-HRP (1:10,000 dilution, 016-030-084, Jackson ImmunoResearch, West Grove, PA) overnight at 4 °C. The membranes were next washed three times, incubated with the appropriate HRP-conjugated secondary Ab, if any, for 1 h at room temperature on a shaker, and treated with ECL reagents (GE Healthcare) before image collection using photosensitive membranes (GE Healthcare). The following Abs were used for IP and as primary Abs in immunoblotting analyses: DPβ/DRβ (1:3000 dilution, MA1-19147, Thermo Fisher Scientific), β-actin (1:1500 dilution, sc-47778, Santa Cruz Biotechnology), TAP2 (1:3000 dilution, NBP1-54436, Novus Biologicals), TPN (1:600 dilution, sc-393552, Santa Cruz Biotechnology), E57 (1:3000 dilution, MA1-90869, Thermo Fischer Scientific), CRT (1:3000 dilution, sc-166837, Santa Cruz Biotechnology) and CNX (1:5000 dilution, ab92573, Abcam, Cambridge, MA; and 1:3000 dilution, ab22595, Abcam). The following Abs were used as secondary Abs in immunoblotting analyses: HRP-conjugated goat anti-mouse IgG (H + L) (1:75,000 dilution, W4021, Promega), HRP-conjugated goat anti-rabbit IgG (H + L) (1:50,000 dilution, W4011, Promega) and an HRP-conjugated rat anti-mouse IgG VeriBlot secondary antibody, capable of recognizing only non-denatured primary Abs, to eliminate contaminating signals from IP Abs (1:8000 dilution, ab131368, Abcam).

2.7. Proximal biotinylation using the BioID2 system

K562 cells were engineered to stably express BioID2 on both the DPα and DPβ chains. In our system, additional biotin supplementation beyond biotin-containing RPMI 1640 media was not required and did not enhance observable biotinylation. BioID2-expressing K562 cells were cultured in standard RPMI 1640 media until collection for lysate preparation, when 2×10^6 cells were lysed in 600 μL of 1% NP-40 lysis buffer. The lysates were then subjected to pull-down with 30 μL of M − 280 streptavidin Dynabeads (Thermo Fisher Scientific, Canada) to isolate biotinylated protein complexes. The beads were subsequently washed as described previously [19]. Briefly, the beads were washed twice with wash buffer 1 (2% SDS in dH_2O), once with wash buffer 2 (500 mM NaCl, 50 mM HEPES, 1 mM EDTA, 1% w/v Triton X-100, and 0.1% w/v deoxycholate at pH 7.5 in dH_2O), once with wash buffer 3 (250 mM LiCl, 10 mM Tris, 1 mM EDTA, 0.5% w/v NP-40 and 0.5% deoxycholate at pH 8.1 in dH_2O), and once with wash buffer 4 (50 mM Tris at pH 7.4 in dH_2O) to dissociate possible non-biotinylated proteins. The samples were then boiled, reduced, and subjected to SDS-PAGE and western blotting analysis as in section 2.6, using either chaperone-specific Abs as described or streptavidin-HRP (1:10,000 dilution, 016-030-084, Jackson ImmunoResearch) to detect biotinylated proteins.

3. Results

3.1. HLA-DP^{84GGPM87} constitutively presents endogenous antigens through a proteasome and TAP-dependent pathway, even in the presence of Ii and HLA-DM

Unlike HLA-DP^{84DEAV87} or canonical HLA-DR molecules, HLA-DP^{84GGPM87} molecules are capable of presenting endogenous antigens even in the presence of Ii [14,15]. To confirm these findings and assess the presentation of antigens derived from both endogenous and exogenous sources on these DP molecules in the presence of both Ii and HLA-DM, we generated stable K562 transfectants expressing DP4 or DP4^{84DEAV87}, whose four amino acid residues at positions 84–87 of the DPβ chain were exchanged as indicated, either in the presence or absence of co-expressed Ii and HLA-DM. K562 cells are deficient in endogenous class II, Ii, and HLA-DM expression, yet retain the remaining processing and presentation machinery; consequently, the introduction of class II results in functional antigen presentation [16,17,24]. To study endogenous vs. exogenous antigen processing and presentation pathways independently, we utilized two separate model antigens based on the cancer testis antigen MAGE-A3, which encodes a known DP4-restricted epitope that is naturally processed and presented, MAGE-A3$_{243\text{-}258}$ [14,25]. Full-length native MAGE-A3 is cytosolically expressed and is an endogenous model antigen, whereas a previously reported endosomally targeted form of MAGE-A3 was employed as a model exogenous antigen [14]. The above K562-based cell lines were transiently transfected with either form of MAGE-A3 or the control vector, and used as stimulator cells in ELISPOT analyses to stimulate DP4/MAGE-A3$_{243\text{-}258}$-specific T cells (Fig. 1). When native MAGE-A3 was the source of the MAGE-A3 antigen, only wild-type DP4 was able to process and present MAGE-A3$_{243\text{-}258}$ to T cells when Ii and HLA-DM were present; DP4^{84DEAV87}-expressing K562 cells were not able to stimulate T cells (Fig. 1a), due to the ability of the CLIP region of Ii to block the cleft of DP4^{84DEAV87} early in endocytic pathway, preventing endogenously derived MAGE-A3 from binding these class II molecules. On the other hand, exogenously targeted MAGE-A3 could be processed and presented on both DP alleles tested, even in the presence of Ii and HLA-DM (Fig. 1b), as expected. The increase in exogenously derived antigen presentation upon the addition of Ii and DM likely results from increased trafficking of class II molecules to endosomal compartments, as previously reported [14].

To confirm the origin and pathway of the presented antigens, the proteasome and TAP dependency of both forms of MAGE-A3 antigen presentation were investigated. To examine proteasome involvement, DP4-expressing K562 cells were transiently transfected with either form of MAGE-A3 or the control vector, and treated with various concentrations of the proteasome inhibitor bortezomib, before utilization as stimulator cells in ELISPOT analyses as indicated above (Fig. 1c and d). Only in the case of native MAGE-A3-transfected K562 cells (Fig. 1c) did bortezomib treatment decrease the ability of the stimulator cells to stimulate T cells; the presentation of antigens derived from endosome-targeted MAGE-A3 (Fig. 1d) was not affected by bortezomib treatment. Similar results were obtained using a different proteasome inhibitor, carfilzomib (Supplementary Fig. 1). To study TAP involvement in the presentation of both forms of MAGE-A3, K562/DP4 cells stably expressing one of the herpes virus-derived TAP inhibitor genes ICP47 and UL49.5, or the control vector, were transfected with either form of MAGE-A3 and used as stimulator cells in ELISPOT analysis as indicated above (Fig. 1e–g). Similar to proteasome involvement, the presentation of only MAGE-A3$_{243\text{-}258}$ derived from native MAGE-A3 (Fig. 1e), but not endosomal MAGE-A3 (Fig. 1f), was reduced by TAP inhibitor

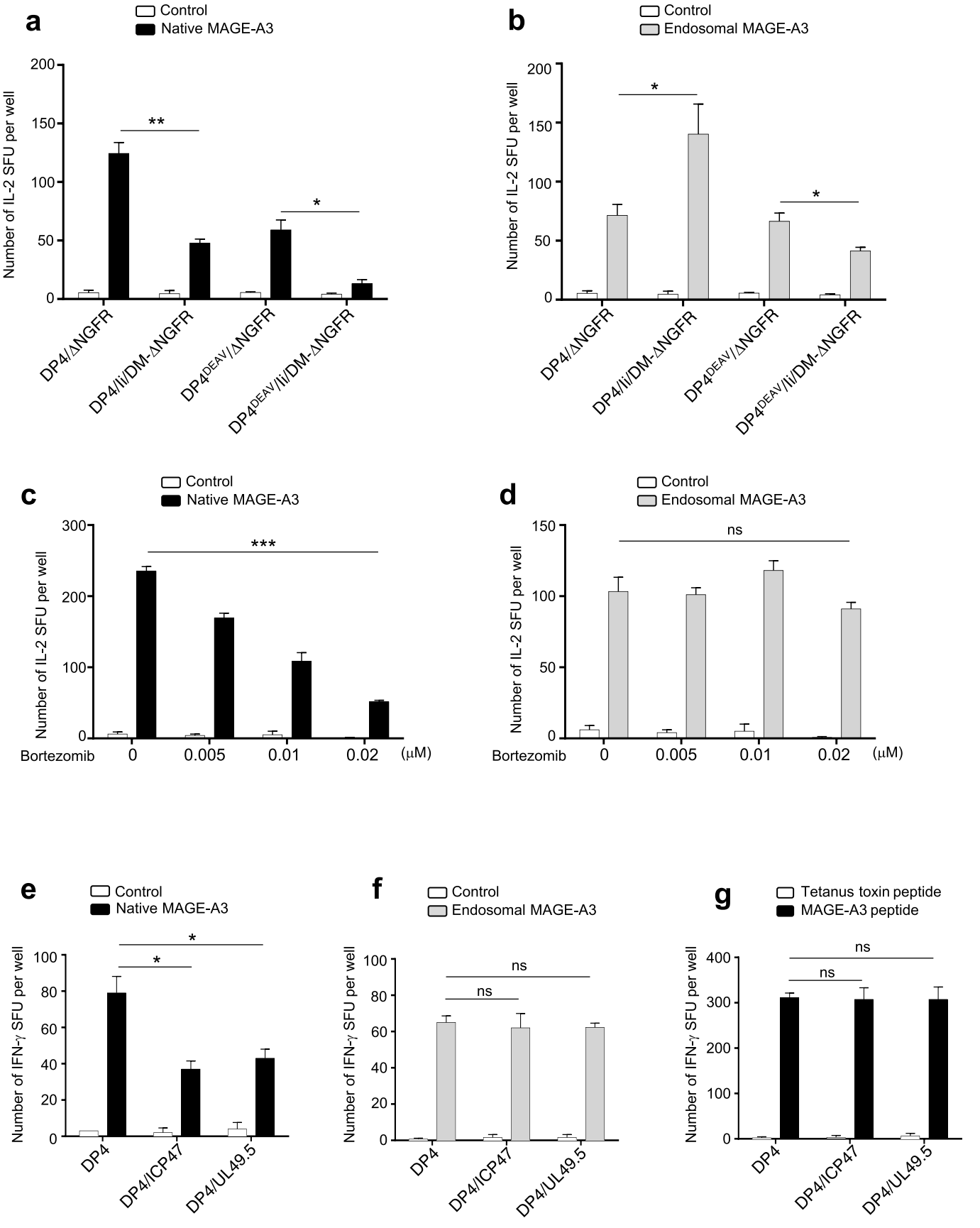

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Fig. 1. HLA-DP^{84GGPM87} constitutively presents endogenous antigens, regardless of the presence of Ii and HLA-DM, with the support of the proteasome and TAP. (**a-g**) DP4-MAGE-A3$_{243-258}$-specific CD4$^+$ T cells were stimulated using the indicated K562-based aAPCs, and IL-2 or IFN-γ responses, as indicated, were measured via ELISPOT analysis. (**a,b**) K562 cells expressing the indicated combination of genes were transiently transfected with native (**a**) or endosomally (**b**) targeted MAGE-A3, or the control vector, linked with IRES-EGFP and used to stimulate T cells. (**c,d**) DP4-expressing K562 cells were transiently transfected with native (**c**) or endosomally (**d**) targeted MAGE-A3, or the control vector, linked with IRES-EGFP, then cultured with bortezomib at the indicated concentration for 48 h and used to stimulate T cells. (**e,f**) K562 cells expressing DP4 and the indicated TAP inhibitor were transiently transfected with native (**e**) or endosomally (**f**) targeted MAGE-A3, or the control vector, linked with IRES-EGFP and used to stimulate T cells. (**g**) Stimulator cell immunogenicities were evaluated by stimulating T cells with the indicated stimulator cells pulsed with 10 μM of either the tetanus toxin (TT$_{947-967}$) or MAGE-A3$_{243-258}$ peptide. Transient transfection efficiencies were normalized based on EGFP, as measured by flow cytometry (Supplementary Fig. 5). The presented data represent the means ± SD of triplicates. The results are representative of a minimum of three experiments. ns: not significant; *p < 0.05; **p < 0.01; ***p < 0.001, by the unpaired two-tailed Welch's *t*-test.

activity. The equivalent immunogenicity of these stimulator cells was confirmed through equivalent T cell responses evoked by stimulator cells treated with non-saturating concentrations of the MAGE-A3$_{243-258}$ peptide (Fig. 1g). Together, these results confirm previous findings that DP^{84GGPM87}, unlike DP^{84DEAV87}, constitutively presents both endogenous and exogenous antigens, even in the presence of Ii and HLA-DM, and indicate that these intracellular antigens are generated via a proteasome and TAP-dependent pathway.

3.2. DP^{84GGPM87} competes with class I molecules for the use of subcellular machinery to present endogenous antigens

Considering the proteasome and TAP dependency of native MAGE-A3 presentation as discussed in section 3.1, we hypothesized that DP^{84GGPM87} may also make use of other components of the class I antigen processing and presentation pathway to present endogenous antigens. To study whether the presence of class I molecules could influence the efficiency of endogenous antigen presentation on DP^{84GGPM87} by competing for the same subcellular machinery, CRISPR-Cas9-mediated knockout of β2m in HEK293 cells, which endogenously express class I but are deficient in the expression of class II, Ii, and HLA-DM was performed (Fig. 2a). These cells were then transfected with native or endosomally targeted MAGE-A3 and utilized in ELISPOT analysis, as indicated in section 3.1 (Fig. 2b and c). Interestingly, when class I was not present in stimulator cells, a significantly greater T cell response was observed (Fig. 2b), suggesting that antigen presentation in these cells was somehow enhanced in the absence of class I. Importantly, the presence or absence of class I did not impact the efficiency of exogenous antigen presentation on these class II (Fig. 2c). Equivalent immunogenicity of the stimulator cells utilized was demonstrated using a non-saturating peptide pulse, as indicated in section 3.1 (Fig. 2d). Similar results were obtained using DU145 cells, a prostate cancer cell line that also endogenously expresses class I but is deficient in class II, Ii, and HLA-DM expression, indicating that this is not a cell line-specific phenomenon (Supplementary Fig. 2). This increase in endogenous antigen presentation also occurred without any detectable change in the expression levels of class I-associated chaperones (Fig. 2e). These results suggest that DP^{84GGPM87} may compete with class I molecules for the use of subcellular machinery early in the endocytic pathway, prompting us to focus our attention on the chaperones of the class I peptide-loading complex (PLC).

3.3. TAP, tapasin, ERp57, calnexin, and calreticulin of the class I PLC associate with HLA-DP in vitro and in vivo

To explore which components of the class I processing and presentation pathway may be involved in endogenous antigen presentation on DP^{84GGPM87}, we first employed IP/western experiments to detect physical associations between chaperone molecules and DP (Fig. 3). We transduced K562 cells with various DP alleles, including DP4, DP5 (a DP^{84DEAV87} allele), and both one and four amino acid substitution mutants of these alleles either at position 84 alone, or of the entire 84–87 region, to study whether polymorphism in this region could influence the ability of chaperone molecules to associate with DP molecules. We considered the class I peptide-loading complex (PLC) chaperones of TAP, tapasin (TPN), ERp57 (E57), calnexin (CNX), and calreticulin (CRT) to be of particular interest, considering their documented roles in the loading of endogenous antigens onto class I molecules in the same subcellular space. Lysates from these DP-expressing K562 cells, or parental K562, were obtained and subjected to IP and immunoblotting experiments to detect complex formation between DPβ and components of the class I PLC, including TAP2 (Fig. 3a), TPN (Fig. 3b), E57 (Fig. 3c), CNX (Fig. 3d), and CRT (Fig. 3e). The ability of the DPβ-chain specific antibody to equally IP all DP alleles tested was demonstrated through an IP and immunoblotting experiment in which the same antibody was used for both IP and blotting, to confirm that the same quantity of the DPβ chain was pulled down in all IP samples. (Fig. 3f). Total cell lysates were blotted with the above chaperone-specific antibodies to demonstrate equivalent lysate concentrations and chaperone expression across all cell lines (Fig. 3g). In all cases, across all alleles tested, all of the above chaperones exhibited the ability to form complexes with HLA-DP, although for some chaperones, such as E57 and CRT, differing quantities of chaperone were detectable during immunoblotting between the three DP^{84Gly} and three DP^{84Asp} alleles, suggesting that the nature of complex formation with these chaperones may differ depending on polymorphisms at position 84. Nevertheless, all of the above chaperones were observed to complex with both DP^{84GGPM87} and DP^{84DEAV87} *in vitro*.

To confirm that these associations are also formed in an *in vivo* context, we made use of BioID2 technology to facilitate proximal biotinylation within living cells. BioID2 is a version of enhanced sensitivity, specificity, and localization compared to the original BioID system, and is widely employed in the examination of proximate protein-protein interactions in living cells [19]. Using this technology, target proteins that physically associate, or complex, with a bait protein of interest will be labeled with biotin and can then be identified through downstream methods. Using a flexible G$_4$S linker of maximized length, as was employed both in this study and in previously reported work, the effective range of biotinylation can reach up to 25 nm from the site of fusion [19]. To confirm that the identified chaperone associations above are extant and relevant *in vivo*, we first generated BioID2-fused *DPA*0103*, *DPB*0401*, and *DPB*0401^{84DEAV87}* genes (Fig. 4a). To examine the efficiency of BioID2 linked to the DPα vs. DPβ chain, several combinations of DP heterodimers were engineered in K562 cells, encoding the BioID2 enzyme linked to either DPα alone, the DPβ chain alone, or both chains simultaneously (Fig. 4b). These three combinations of DPα and DPβ chains were generated for both DP4 and DP4^{84DEAV87} to study possible differences in chaperone proximity for both DP^{84GGPM87}- and DP^{84DEAV87}-type alleles. To determine which combination of BioID2 enzymes was optimal for the maximal detection of chaperone proteins *in vivo*, K562 cells expressing each of the six combinations of heterodimers, as indicated above, were lysed, and their lysates were subjected to blotting with streptavidin-linked HRP. Whereas DPα-linked BioID2 yielded numerous observable biotinylated targets, DPβ-linked BioID2 proved substantially less functional, possibly due to conformational constrictions of the fusion protein or steric hindrance of the BioID2 enzyme, which significantly limited the effective range of biotinylation (Fig. 4c). In our system using the improved BioID2 enzyme, additional biotin supplementation of culture media was not necessary to facilitate biotinylation and did not enhance

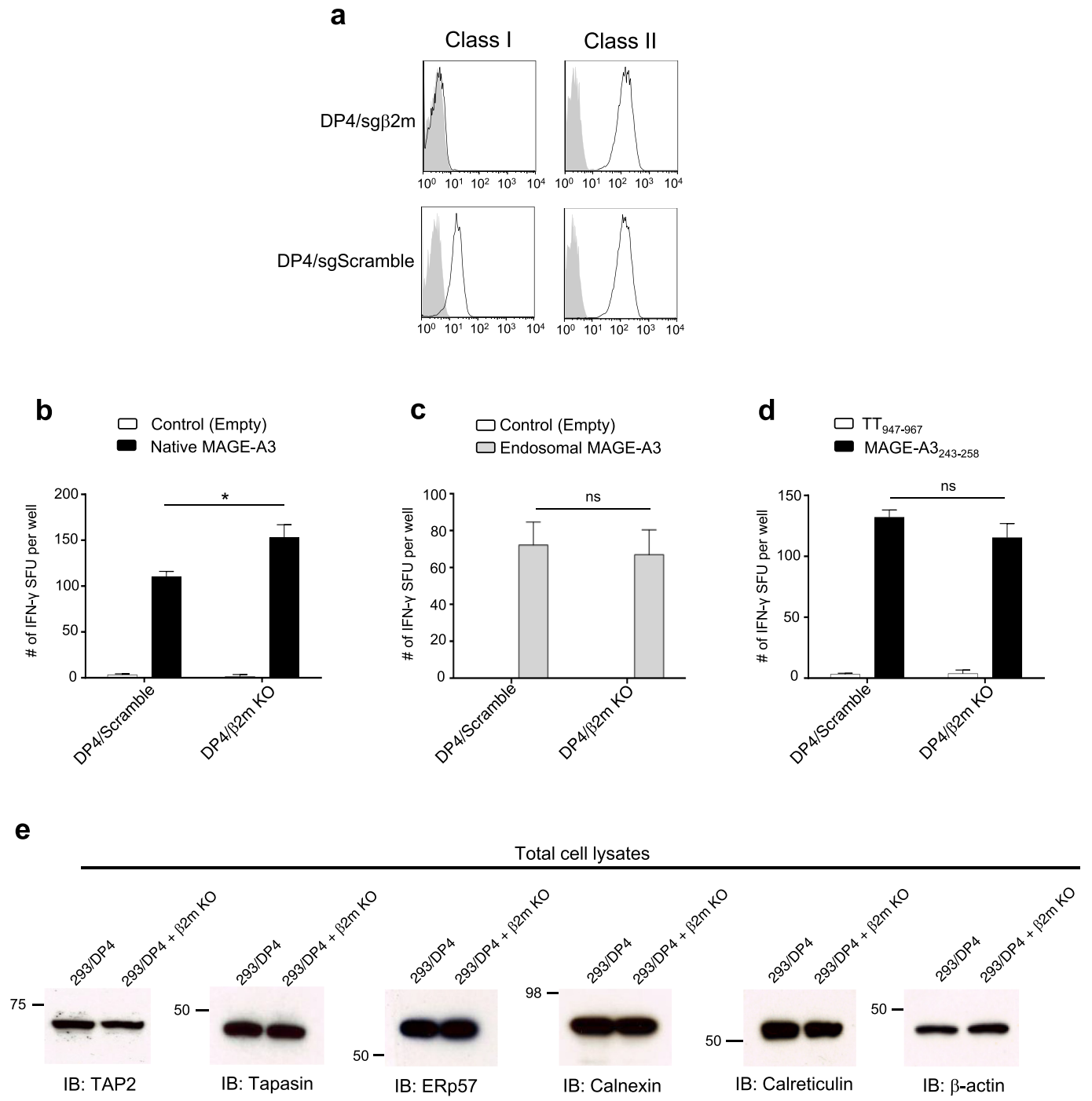

Fig. 2. DP^{84GGPM87} competes with class I molecules for the use of class I-associated molecular chaperones. (**a**) Surface expression of HLA class I and class II was measured in the indicated HEK293 transfectants by flow cytometry following staining with specific mAbs. (**b-d**) DP4-MAGE-A3$_{243-258}$-specific CD4$^+$ T cells were stimulated using the indicated HEK293-based aAPCs, and IFN-γ responses were measured through ELISPOT analysis. (**b,c**) HEK293/DP4 cells expressing β2m-specific sgRNA or the control sgRNA were negatively sorted for HLA class I, then transiently transfected with native MAGE-A3 (**b**), endosomal MAGE-A3 (**c**), or the control vector, linked with IRES-EGFP, and used as stimulator cells in ELISPOT analysis. (**d**) Stimulator cell immunogenicities were evaluated by stimulating T cells with the indicated stimulator cells pulsed with 10 μM of either the tetanus toxin (TT$_{947-967}$) or MAGE-A3$_{243-258}$ peptide. Transient transfection efficiencies were normalized based on EGFP, as measured by flow cytometry (Supplementary Fig. 5). The presented data represent the means ± SD of triplicates. The results are representative of a minimum of three experiments. ns: not significant; *$p < 0.05$, by the unpaired two-tailed Welch's *t*-test. (**e**) The indicated HEK293 transfectants were lysed, and their lysates were subjected to immunoblotting under reducing conditions with Abs specific for the indicated targets.

observable biotinylation in all cell lines tested (Supplementary Fig. 3). Considering that the constructs encoding the BioID2 enzyme linked to both DPα and DPβ chains yielded the greatest number of observable biotinylated proteins and that the nature of the complex formed between the identified chaperones and DP is not known, we selected these constructs with BioID2 fused to both chains for both DP4 and DP4^{84DEAV87} for further analysis (henceforth referred to as BioID2-DP4 and BioID2-DP4^{84DEAV87}). To assess whether the above identified chaperones can form proximal complexes with DP *in vivo*, K562 cells expressing either BioID2-DP4 or BioID2-DP4^{84DEAV87} were lysed, and their lysates were subjected to streptavidin-based pulldown using streptavidin-coated Dynabeads, followed by immunoblotting for each

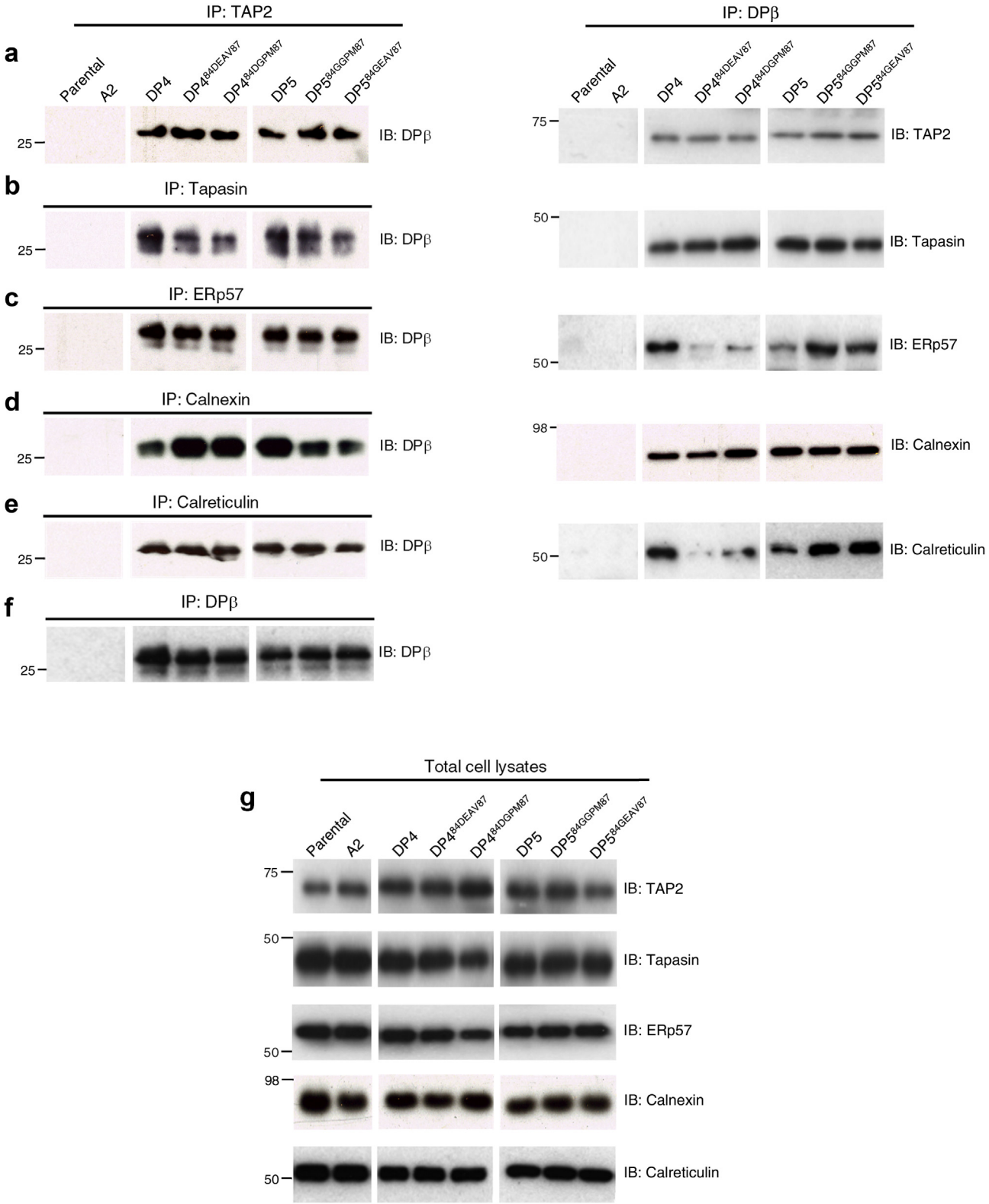

Fig. 3. TAP, tapasin, ERp57, calnexin, and calreticulin associate with HLA-DP *in vitro*. (**a-f**) K562 cells expressing the indicated HLA allele, or parental K562 cells, were lysed, and their total cell lysates were subjected to immunoprecipitation and immunoblotting under reducing conditions with the indicated Abs. (**g**) Total cell lysates from the indicated K562 transfectants were immunoblotted with the indicated Abs.

of the above identified chaperones, to assess proximal complex formation. In accordance with the results of the *in vitro* IP/immunoblotting analysis, TAP2, TPN, E57, CNX, and CRT were all recovered by streptavidin pulldown in both DP4- and DP4^{84DEAV87}-expressing K562 cells, indicating that each of these chaperones forms proximal complexes with both DP alleles tested *in vivo* (Fig. 4d). Together, these data demonstrate the ability of several chaperones of the class I PLC to form complexes with various HLA-DP alleles both *in vitro* and *in vivo* in

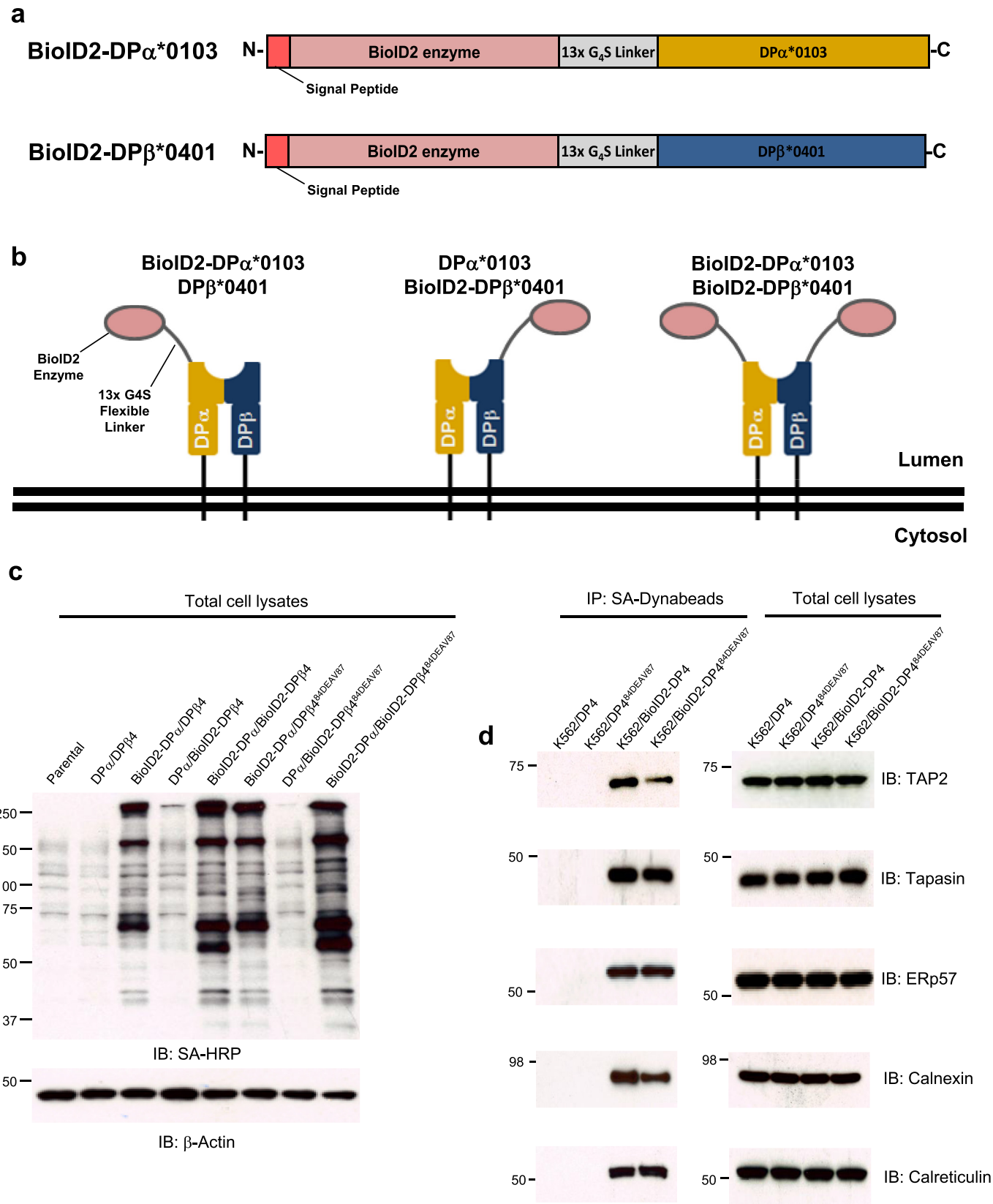

Fig. 4. TAP, tapasin, ERp57, calnexin, and calreticulin form proximal complexes with HLA-DP *in vivo*. (**a**) Schema depicting BioID2 fusion constructs for DPα and DPβ chains. Note that the BioID2 enzyme is preceded by an endoplasmic reticulum (ER)-targeting sequence and is fused upstream of class II by a flexible G_4S linker of maximized length. (**b**) Scheme depicting the three BioID2 fusion proteins generated for each DP4 and $DP4^{84DEAV87}$ embedded in the ER membrane. (**c**) K562 cells stably transduced with the indicated combinations of DPα and DPβ chains, with or without BioID2 fusion, were lysed, and their lysates were subjected to western blotting with streptavidin-HRP or β-actin mAb, as indicated, to detect the biotinylated protein fraction. (**d**) K562 cells stably transduced with either DP4 or $DP4^{84DEAV87}$ encoding BioID2 fused to both the DPα and DPβ chains were lysed, and their lysates were subjected to streptavidin-based pulldown followed by immunoblotting with Abs specific for TAP2, tapasin, ERp57, calnexin, or calreticulin. Cells were cultured in 100 ng/mL IFN-γ for 24 h prior to lysate collection to induce TAP2 expression.

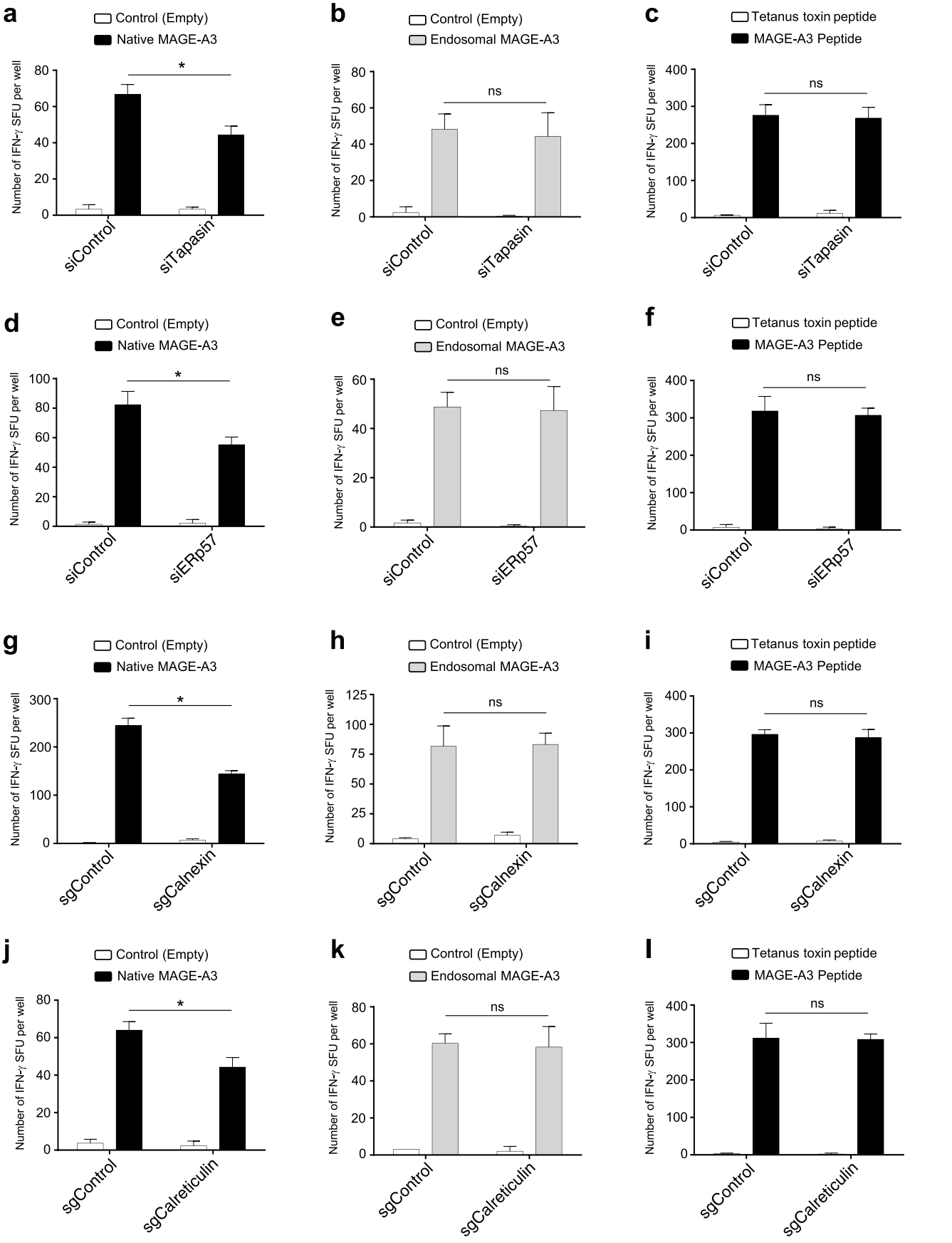

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Fig. 5. Endogenous antigen presentation on HLA-DP^{84GGPM87} is dependent on class I-associated PLC chaperones. (**a-l**) DP4-MAGE-A3$_{243-258}$-specific CD4$^+$ T cells were stimulated using the indicated K562-based aAPCs, and IFN-γ responses were measured through ELISPOT analysis. (**a-c**) K562/DP4 cells transduced with siRNA for tapasin or the control siRNA were transiently transfected with either native (**a**) or endosomally targeted MAGE-A3 (**b**), or the control vector, linked with IRES-EGFP, and used to stimulate T cells. The immunogenicity of stimulator cells was investigated by pulsing stimulator cells with 10 μM of either the MAGE-A3$_{243-258}$ or TT$_{947-967}$ peptide and then using these cells to stimulate T cells (**c**). (**d–f**) K562/DP4 cells transduced with siRNA for ERp57 or the control siRNA were transiently transfected with either native (**d**) or endosomally targeted MAGE-A3 (**e**), or the control vector, linked with IRES-EGFP, and used to stimulate T cells. The immunogenicity of stimulator cells was investigated by pulsing stimulator cells with 10 μM of either the MAGE-A3$_{243-258}$ or TT$_{947-967}$ peptide and using these cells to stimulate T cells (**f**). (**g–i**) K562/DP4 cells transfected with the CRISPR-Cas9 machinery and sgRNA specific for calnexin or the control sgRNA were transiently transfected with either native (**g**) or endosomally targeted MAGE-A3 (**h**), or the control vector, linked with IRES-EGFP, and used to stimulate T cells. The immunogenicity of the stimulator cells was investigated by pulsing stimulator cells with 10 μM of either the MAGE-A3$_{243-258}$ or TT$_{947-967}$ peptide and using these cells to stimulate T cells (**i**). (**j–l**) K562/DP4 cells transfected with the CRISPR-Cas9 machinery and sgRNA specific for calreticulin or the control sgRNA were transiently transfected with either native (**j**) or endosomally targeted MAGE-A3 (**k**), or the control vector, linked with IRES-EGFP, and used to stimulate T cells. The immunogenicity of the stimulator cells was investigated by pulsing stimulator cells with 10 μM of either the MAGE-A3$_{243-258}$ or TT$_{947-967}$ peptide and using these cells to stimulate T cells (**l**). Transient transfection efficiencies were normalized based on EGFP, as measured by flow cytometry (Supplementary Fig. 5). The presented data represent means ± SD of triplicates. The results are representative of a minimum of three experiments. ns: not significant; *$p < 0.05$, by the unpaired two-tailed Welch's *t*-test.

human cells.

3.4. Chaperones of the class I PLC participate in the presentation of endogenous, but not exogenous, antigens on DP^{84GGPM87}

To demonstrate that the observed chaperone associations described in section 3.3 are biologically relevant to antigenic peptide presentation, T cell activation assays were employed. siRNA or CRISPR-Cas9 mediated knockdown of each of the chaperones observed to complex with DP in section 3.3 were carried out in DP4-expressing K562 cells, and knockdown efficiency was confirmed (Supplementary Fig. 4). These chaperone-deficient cell lines were then transiently transfected with either native or endosomally targeted MAGE-A3 and used as stimulator cells in IFN-γ ELISPOT analyses, to assess whether the loss of chaperone molecules identified as associating with DP participate in the presentation of antigen from either source (Fig. 5). When K562/DP4 cells deficient in TPN expression were transfected with native MAGE-A3, a significant reduction in T cell responses was observed compared to TPN-sufficient cells (Fig. 5a), whereas no deficit of antigen presentation was noted when the source of the MAGE-A3 antigen was endosomally targeted (Fig. 5b). Similar reductions in native-MAGE-A3-derived antigen presentation were observed in the case of E57 (Fig. 5d)-, CNX (Fig. 5g)-, and CRT (Fig. 5j)-knockdown cells, while endosomal-MAGE-A3-transfected E57 (Fig. 5e)-, CNX (Fig. 5h)-, and CRT (Fig. 5k)-transfected stimulator cells did not exhibit a reduced ability to present antigen. Equivalent immunogenicity of these stimulator cells to stimulate T cells was demonstrated using peptide pulse experiments as indicated in section 3.1 (Fig. 5c,f,i,l). These data demonstrate the biological relevance of the associations demonstrated in section 3.3, suggesting the ability of TAP, TPN, E57, CNX, and CRT to participate in the presentation of endogenous antigens on DP^{84GGPM87}. Together, these results suggest a dual role of several canonically class I-associated chaperones early in the endocytic pathway, and indicate the possibility of competition between class I and class II molecules for the use of this subcellular machinery to facilitate endogenous antigen presentation on these DP molecules.

4. Discussion

In conjunction with our previous work detailing the molecular interactions between DP^{84GGPM87} and Ii, the data presented here provide a plausible mechanism for a novel mode of constitutive cross-presentation of endogenous antigens on these DP molecules. The proteasome and TAP dependency of this pathway, along with the apparent competition between class I and class II molecules for the use of subcellular machinery to facilitate the presentation of these antigens, suggests that the site of loading occurs early in the endocytic pathway and is likely in the ER, considering that this is the known site of the loading of endogenous antigens onto class I molecules. The further identification of several class I PLC chaperones, as implicated in this pathway, suggests novel roles, or at least novel targets, for these chaperones in the class II pathway of antigen presentation, at least for DP. To our knowledge, reports demonstrating functional roles of class I-associated chaperones in class II processing and presentation are limited, with the exception of TAP, which has a documented role in the cross-presentation of cancer testis antigen-derived peptides from NY-ESO-I and MAGE-A3, likely by importing proteasomally digested antigens from the cytosol into the ER, where they can load onto these class II molecules [9,14]. Although the lectins CNX [26] and CRT [27] have been shown to interact with HLA-DR and class II-associated antigens, respectively, CNX is thought to function only as a retention chaperone in the ER, prior to the completion and export of the classical nonameric class II-Ii complex [28], and no biologically relevant role for the binding of these antigens by CRT has yet been shown. This situation gives rise to the question of whether these chaperones perform similar roles for these DP molecules compared to those they perform for class I molecules, or if novel functions of these chaperones have yet to be revealed. Thus, this report demonstrates novel and biologically relevant interactions regarding several chaperones of the class I-associated processing and presentation pathway, which facilitate endogenous antigenic peptide presentation on HLA-DP.

The clinical relevance of the cross-presentation facilitated by these chaperones is supported by several clinical and genetic studies in various diseases which detail clinical distinctions observed between DP^{84GGPM87}- and DP^{84DEAV87}-encoding patients. For example, anti-neutrophil cytoplasmic autoantibody-associated (ANCA) vasculitis [29], rheumatoid arthritis (RA) [30], childhood acute lymphoblastic leukemia (ALL) [31], and hepatitis B infections [32] all exhibit differences in the prevalence or severity of disease specifically between DP^{84GGPM87}- and DP^{84DEAV87}-encoding patients. Considering that this cross-presentation results in increased presentation of all intracellular antigens on DP, including cancer, viral, and even self-derived peptides, which are then presented to CD4$^+$ T cells, one might expect a higher burden of autoimmune disease among DP^{84GGPM87}-expressing individuals compared to those encoding DP^{84DEAV87}, but lower disease burdens in regard to cancer and viral diseases, due to the enhanced targeting of these antigens; in fact, this is precisely what is observed across all of the aforementioned diseases. Critically, even in diseases in which specific DP^{84GGPM87}-encoding alleles act as risk alleles for particular diseases, such as DP2 in ALL, likely due to cleft conformation-specific differences in these DP molecules, when all DP^{84GGPM87}-encoding class II alleles are compared as a group to DP^{84DEAV87} alleles, the above-mentioned trends prevail [31,33]. Considering all of these findings together, we suggest that differences in disease phenotypes resulting from polymorphism in DP molecules result from two simultaneously acting, but discrete mechanisms: first, alteration of the DP cleft conformation to accommodate distinct repertoires of peptides, due to differences in antigen affinity between alleles; and second, modulation

of CLIP-dependent Ii interactions with DP early in the endocytic pathway, affecting the availability of the DP cleft to be bound by endogenous antigens with the help of class I-associated chaperone proteins, before transport to the MIIC compartment. It is also worth noting that minor polymorphisms in HLA-DM do exist, and seem to be able to mediate clinically relevant impacts on the peptide repertoire as well. Genetic studies have identified certain DM polymorphisms as risk alleles in psoriasis [34], and animal work has demonstrated the ability of H2-M polymorphisms to modulate the susceptibility of mice to both collagen-induced arthritis [35] and type 1 diabetes (T1D) [36], supporting the notion that the final repertoire of peptides presented to T cells can be influenced by many overlapping mechanisms to influence disease phenotypes.

Clearly, further work remains to elucidate the exact role of each of the chaperones identified here in the presentation of endogenous antigens on DP molecules. Furthermore, we argue that the characterization of this cross-presentation pathway in our recent studies [14,15], in conjunction with the work shown in this study, justifies future research aimed at identifying specific antigenic peptides presented by this cross-presentation pathway, which may give rise to the above-noted clinical differences. In this way, direct relationships between cross-presentation mechanisms on DP and human disease can be established, leading to a more complete understanding of the etiology of these diseases and facilitating the development of targeted therapies. Nevertheless, based on the present study, we suggest that the associations and functional relevance of these chaperones with HLA-DP form the basis of a novel form of cross-presentation, which may facilitate the presentation of antigenic peptides that are key in explaining the clinical distinctions noted in autoimmune diseases, cancer, and viral diseases for which no satisfying mechanistic explanation has yet been revealed.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2019.04.023.

Conflicts of interest

Declarations of interest

None.

Author contributions

M.A. and N.H. designed the project. M.A., K.S., Y.M., Y.Y., C–H.W., T.G., K.M., H.S., Y.K., and K.S. performed the experiments. M.O.B. provided critical samples. M.A. and N.H. analyzed the results and wrote the manuscript.

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