The human NKG2D ligand ULBP2 can be expressed at the cell surface either with or without a GPI anchor and both forms can activate NK cells

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Summary

The activating immune receptor NKG2D binds to multiple stress-induced ligands that are structurally different: MHC-class I-related Chain (MIC) A/B molecules have a transmembrane domain whereas most UL16 binding proteins (ULBPs) are glycosylphosphatidyl-inositol (GPI)-linked molecules. The significance of this variability in membrane anchor is unclear. Here, we demonstrate that ULBP2, but not ULBP1 or 3, can reach the cell surface without the GPI modification. Several proteins are expressed at the cell surface as both transmembrane and GPI-linked molecules, either via alternative splicing or by the expression of linked genes. However, to our knowledge, ULBP2 is the first single mammalian cDNA that can be expressed as either a transmembrane or a GPI-anchored protein. The rate of maturation and the levels of cell surface expression of the non-GPI linked form were lower than those of the GPI-linked ULBP2. Nonetheless, non-GPI ULBP2 was recognised by NKG2D and triggered NK cell cytotoxicity. These data show that differences in membrane attachment by NKG2D-ligands are more important for regulation of their surface expression than for cytotoxic recognition by NKG2D and emphasise that detailed characterization of the cell biology of individual NKG2D-ligands will be necessary to allow targeted modulation of this system.

Introduction

NKG2D is a C-type lectin-like receptor that can stimulate both cytotoxicity and cytokine secretion by NK cells, TCRγδ+ and CD8+ TCRαβ+ T cells [For review, (Lopez-Larrea et al., 2008; Nausch and Cerwenka, 2008)]. Receptor ligation can costimulate the activation of naïve T cells (Maasho et al., 2005) and can even trigger cytotoxicity by IL-2 activated T cells in the absence of TCR ligation (Verneris et al., 2004). The ligands for NKG2D are related to MHC class I molecules, but unlike these proteins, the expression of NKG2D-ligands (NKG2D-L) is restricted or absent on normal tissues, instead they are upregulated in situations of stress and disease such as pathogen infection or tumour transformation to render these cells susceptible to NK cell lysis (Gonzalez et al., 2006). In humans, eight ligands of NKG2D have been identified, which are members of either the MIC (MICA and MICB) (Bahram et al., 1994; Bahram et al., 1996) or the ULBP (ULBP1, ULBP2, ULBP3, ULBP4, RAET1G, and RAET1L) family (Chalupny et al., 2003; Cosman et al., 2001; Eagle et al., 2009). The biological significance of the existence of multiple ligands for one receptor is poorly understood, but may reflect differences in the biochemistry and cell biology of the different ligands leading to differential roles for these molecules in immune surveillance as an evolutionary response to selective pressures exerted by pathogens or cancer. Consistent with this hypothesis NKG2D-L are variable in both their amino-acid sequence and domain structure. All ligands share an MHC class-I-like α1-α2 domain that binds to NKG2D, but while the MICA/B proteins have an MHC class-I-like α3 domain, ULBP1-3 and ULBP6 (RAET1L) are anchored to the membrane through a glycosylphosphatidylinositol (GPI) moiety. ULBP4 (also known as RAET1E) and ULBP5 (RAET1G) proteins also have transmembrane domains and cytoplasmic tails of variable length and sequence. However, the functional significance of these extensive structural differences between NKG2D-L for immune activation is not clear. In particular, the significance of the presence of a GPI-anchor in some NKG2D-L while others contain transmembrane and cytoplasmic domains is unknown. In the mouse, GPI-anchored proteins have modest to low affinities for NKG2D, whereas the ligands possessing transmembrane/cytoplasmic domains have high affinity (O'Callaghan et al., 2001), but it is not known if the human NKG2D-L conform to this pattern. In any case, differences in cellular distribution between GPI-anchored and TM proteins may also affect the strength of the receptor-ligand

interaction since GPI-anchored proteins tend to be found in localized regions of the membrane, rich in sphingolipids and cholesterol, known as lipid rafts (Sharom and Lehto, 2002). Clustering of the GPI-anchored ULBPs within lipid rafts, that are known to polarise to the site of the interaction of an NK cell with a susceptible target cell (Lou et al., 2000), might increase the avidity of their interaction with the NKG2D receptor.

Another possible relevant difference between GPI-anchored and TM proteins is that within polarized epithelial layers GPI-anchored proteins have a greater tendency to be found at the apical surface (Paulick and Bertozzi, 2008), whereas TM proteins, are often targeted to the basal or lateral sides of the cell. Normal wild-type (wt) MICA is targeted to the basolateral membrane of gut epithelium, in proximity to NK or T cells, where it can signal cell destruction. However, a naturally occurring allele that has a TM motif, but no cytoplasmic tail, is found instead at the apical surface, where there is a greater concentration of lipid rafts and less contact with NKG2D-bearing cells (Suemizu et al., 2002). Thus the differences in anchorage of NKG2D-L could be associated with differential distribution of these molecules in the same cell and differential availability to lymphocytes.

We have shown differences in the shedding mechanisms of different ULBP molecules (Fernandez-Messina et al.) and now provide evidence to demonstrate that the NKG2D-L ULBP2, and not the other GPI-anchored ULBPs, while it is principally expressed at the cell surface as a GPI-linked molecule can reach the cell surface without this modification. The transmembrane form of ULBP2 is expressed at lower levels than the GPI form, however, its ability to stimulate NKG2D-dependent NK cell-mediated cytotoxicity is maintained.

Results

ULBP2 is expressed on the cell surface in the absence of GPI moiety

The sequence of the ULBP family of NKG2D-L predicted that ULBP1-3 would be expressed on the cell surface as GPI-anchored proteins. However, the first description of the ULBPs showed that while expression levels of ULBP1 and ULBP3 were reduced about 50% after treatment with phosphatidylinositol-specific phospholipase C (PI-PLC), PI-PLC treatment only reduced cell surface expression of ULBP2 by around 30% (Cosman et al., 2001). These data prompted us to re-evaluate the GPI-linkage of the ULBP proteins and for this purpose we used a genetic system: transfection of CHO cells (parental CHO G9PLAP, referred to as CHO) and mutagenised CHO cells (CHO G9PLAP.85, referred to as CHO ΔGPI) unable to synthesise GPI anchors because of a deficiency in PIG-L, a key enzyme in the second step of biosynthesis of GPI anchors. Initially, to avoid artifacts associated with the selection of particular clones during the generation of stable transfectants, these experiments were done by transiently transfecting parental CHO and CHO ΔGPI cells with expression constructs encoding ULBP1, ULBP2, ULBP3 and the transmembrane molecule MICB. As expected, the expression of MICB, a protein attached to the plasma membrane through a transmembrane region, was comparable between the parental and mutant cell lines. Strikingly, however, while ULBP1 and 3 were not expressed on the cell surface of the GPI-deficient CHO cells, significant levels of ULBP2 were detected by FACS analysis (Figure 1A). In control experiments, western blot analysis of cell lysates prepared from the transiently transfected cells demonstrates expression of the ULBP1, 2 and 3 proteins in both the wt and ΔGPI CHO transfectants (Figure 1B). The different bands observed on comparison of the CHO and CHO ΔGPI lysates presumably reflect the presence of unmodified and GPI-modified ULBP proteins in variable proportions. The presence of intracellular ULBP1 and 3, absent from the cell surface, was further confirmed by flow cytometry after fixation, permeabilisation and intracellular staining (data not shown). These data strongly suggest that ULBP1 and ULBP3 must be modified by the addition of a GPI moiety for surface expression, confirming that these molecules are preferentially expressed as GPI-linked proteins. In contrast, although the levels of surface expression of ULBP2 were higher in the CHO cells than in the ΔGPI cell line, some ULBP2 was expressed at the surface of these cells suggesting that modification by

GPI addition is not an absolute requirement for surface expression of ULBP2 and that the transmembrane sequence present in the C-terminal portion of full-length ULBP2 may be competent for expression at the plasma membrane. To explore further this hypothesis, chimaeras comprising the extracellular domain of CD8α and the C-terminus of either ULBP1 or ULBP2, including the predicted site of GPI modification were constructed (Supplementary Figure 1). Transient transfection of the wt CHO and CHO ΔGPI-deficient cells with the chimaeric proteins confirmed that the CD8/ULBP2 chimaera could be expressed at the cell surface while the CD8/ULBP1 chimaera was not (Figure 1C). These data demonstrate that the ability of ULBP2 to be present at the plasma membrane in the absence of a GPI anchor is determined by the C-terminal region, where the signal for the attachment of the anchor is located.

The observation that ULBP2 could be expressed on the cell surface of a cell line unable to synthesise GPI anchors was surprising, since it is thought that there is a tight correlation between the attachment of the GPI anchor and the ability of the GPI-anchored protein to progress from the ER to the Golgi (Moran et al., 1991). On translation, GPI-linked proteins are inserted in the ER membrane through a C-terminal hydrophobic region located after the GPI anchor signal site. These transmembrane proteins are then cleaved and a pre-assembled GPI moiety is attached to the so-called ω residue via the action of a multi-enzyme complex. The lack of an essential enzyme required for GPI anchor synthesis results in a non-cleaved protein precursor that still contains a hydrophobic region, but shorter than a typical transmembrane domain, that is believed to lead to the precursor being recognized and retained by the quality-control system of the ER (Mayor and Riezman, 2004). In this light it was important to rigorously confirm that the ULBP2 protein expressed in the CHO ΔGPI cells was not GPI-anchored and that it could still reach the cell surface. For this purpose, stable transfectants in CHO and CHO ΔGPI cells were produced (Figure 2A). Consistent with the data from the transient transfection experiments, ΔGPI-deficient cells expressed ULBP2 at a lower level than wt CHO cells. As shown in Supplementary Figure 2a, the proportion of mature and immature ULBP2 varies between the two cell lines: in wt cells, the majority of the expressed ULBP2 is in the upper band corresponding to mature protein whereas in GPI-deficient cells, ULBP2 is mainly immature and migrates faster in SDS-PAGE.

To analyse the presence of ULBP2 at the plasma membrane, surface proteins were labelled using sulfo-NHS-biotin that is unable to cross the plasma membrane. Total ULBP2 was immunoprecipitated and surface protein was analysed by western blot using streptavidin-HRP (Figure 2B). Control immunoprecipitations of actin demonstrated that the biotin labelling was cell surface specific since no actin was visible when the western was probed with streptavidin (Figure 2B, left side), but actin protein was detected when probing with anti-actin antibody (Figure 2B, right side). In these experiments, Endoglycosidase H (EndoH) was used to discriminate between proteins that are still in the ER (sensitive to Endo H digestion) and those that have progressed further through the secretory pathway. As expected, cell surface mature protein is resistant to Endo H cleavage. In total lysates, only the intracellular lower band was susceptible to cleavage by Endo H (Supplementary Figure 2b). These data confirmed the presence of ULBP2 at the cell surface of cells deficient for GPI synthesis.

To test for the presence of a GPI anchor, ULBP2 transfected CHO and CHO ΔGPI cells were treated with bacterial PI-PLC. Loss of ULBP2 from the membrane and its appearance in the supernatant was assayed, after incubation with PI-PLC, by using temperature-induced phase separation of Triton X-114 (Bordier, 1981). After incubation at 37°C, Triton X-114 separates in two phases: the lower, detergent-rich phase, where membrane-bound proteins are found; and the upper, aqueous phase, where soluble hydrophilic proteins are recovered. In these experiments, membrane bound GPI-anchored proteins are expected in the detergent phase and, after digestion with PI-PLC, cleaved hydrophilic proteins move to the aqueous phase. These data (Figure 2C) confirmed that while ULBP2 expressed in CHO cells was highly susceptible to cleavage with PI-PLC, the ULBP2 found in CHO ΔGPI cells was essentially unaffected by treatment with this enzyme.

The presence of ULBP2 as a non-GPI-linked protein in transfectants is not an artifact of the transfection system, since these data could be reproduced in a mutagenised K562 cell line defective in GPI anchor (Mohney et al., 1994), expressing ULBP1 and 2 endogenously. Indeed, while ULBP1 was unable to reach the cell surface in the mutant cell line, ULBP2 was still present at the plasma membrane (Figure 3). This result was not due to the absence of ULBP1 in the GPI-deficient cell line because it was possible to detect intracellular protein after staining of

permeabilised cells.

Transmembrane ULBP2 is poorly recruited to DRMs

Proteins with a GPI are usually recruited to domains of the membrane rich in cholesterol and sphingolipids, unable to be solubilised in the presence of detergents such as Brij58 and Triton X-100. ULBP3 has been shown to be included in lipid rafts (Eleme et al., 2004), however no data are available on ULBP2. As predicted, ULBP2 was recruited to lipid rafts in parental CHO cells, but excluded from the microdomains when the protein was expressed in GPI-deficient cells (Figure 4). It is interesting to note that the size of the majority of the protein in ΔGPI cells is apparently smaller than that seen in CHO cells, again reflecting that the more abundant biochemical species are different in the two cell lines.

Maturation of ULBP2

The modification of proteins by attachment of a GPI moiety is a process tightly regulated by the ER quality control machinery. Thus, it was of interest to analyse the ability of ULBP2 to progress in the secretory pathway in the presence and absence of the GPI anchor. Interestingly, pulse-chase experiments showed that wt ULBP2 progressed through the secretory pathway more slowly than ULBP3: while ULBP3 rapidly acquired resistance to Endo H digestion, almost 30% of ULBP2 was still immature even after 180 min of chase (Figure 5A). Consistent with these data, confocal microscopy showed the presence of significant amounts of intracellular protein (Figure 5B). These observations suggest that either the folding or the GPI modification occurs significantly more slowly for ULBP2 than for ULBP3. The absence of the GPI synthesis machinery slowed even more dramatically the rate of maturation of ULBP2 (Figure 5C). Most of the protein was immature (Figure 5C) and intracellular (Figure 5B), suggesting that the reduced levels of ULBP2 cell surface expression observed in the GPI-deficient cell line reflect the low efficiency of exit of non-GPI linked ULBP2 from the ER. The percentages of mature and immature protein in the two cell lines are summarised in Figure 5D.

Non-GPI linked ULBP2 is recognised by NKG2D

The contribution of the GPI anchor to the functional activity of ULBP2 was

explored next. First, the ability of the protein to be recognised by the NKG2D receptor was evaluated in flow cytometry experiments. NKG2D-Ig recombinant protein was used to stain the CHO and ΔGPI cell lines stably transfected with ULBP2 (Figure 6A). Although, in these experiments the total amount of ULBP2 protein expressed at the surface of these transfectants was quite different, the value for half-maximal binding of the NKG2D-Ig was comparable in both cell lines thus it seems reasonable to conclude that the affinity of the receptor-ligand interaction was similar in both instances.

The recognition of GPI-deficient ULBP2 was then analysed in cytotoxicity assays (Figure 6B). The absence of GPI anchor did not affect the recognition by NK cells significantly, suggesting that while differences in the form of membrane anchor of the ULBP2 molecule might be associated with differences in their responses to stress pathways, once at the cell surface both forms of ULBP2 are equally competent to trigger cytolysis by NK cells. In support of these data, wt CHO and CHO ΔGPI cells expressing ULBP2 were equally able to stimulate NK cell degranulation in experiments measuring the appearance of LAMP1 at the plasma membrane after two hours exposure to target cells (data not shown).

Discussion

In summary, the data presented here reveal that although the NKG2D-ligand ULBP2 is normally expressed as a GPI linked molecule, it can reach the cell surface without undergoing this modification, however, in these circumstances the level of cell surface expression is lower. While other proteins can also be expressed at the cell surface as both transmembrane and GPI-anchored molecules, they achieve this duality either as a result of alternative splicing *e.g.* CD58 (Dustin and Springer, 1991) or by expression from closely linked genes that encode either a transmembrane form or a GPI-linked form *e.g.* CD16 (Ravetch and Perussia, 1989). In contrast, our data show that a single cDNA clone of ULBP2 can be expressed at the cell surface in either a transmembrane or GPI-linked form. The data with CD8/ULBP2 chimaeras clearly show that the ability of ULBP2 to be present at the plasma membrane in the absence of a GPI anchor is determined by the C-terminal aminoacids of ULBP2, but mutagenesis studies will be required to further characterise this phenomenon. Functionally, the data show that both GPI-linked and transmembrane ULBP2, despite marked differences in levels of cell surface expression are equally able to stimulate NK cell cytotoxicity. The ligation of the NKG2D receptor by ligand is known to be a particularly potent stimulus to activate NK cell killing (Cosman et al., 2001) and this probably explains why the approximately 5-fold difference in cell surface expression does not lead to a significant difference in susceptibility of the CHO/ULBP2 and ΔGPI/ULBP2 cells to NK lysis. These data suggest either that GPI-anchored and transmembrane versions of the ULBP2 protein have equal lateral mobility in the membrane, or else that increased lateral mobility is not advantageous for this molecule in stimulating NK cytotoxic function.

The biological significance of the existence of multiple ligands for NKG2D is a recurrent question in the field and it has been proposed that it may reflect differences in the biochemistry and cell biology of the different ligands that have evolved in response to selective pressures exerted by pathogens or cancer. We have demonstrated previously that the different NKG2D-L are released from cells by different routes and these new data demonstrate that individual ULBPs have different trafficking rates through the secretory pathway and that the regulation of their surface expression depends on the C-terminal region of the protein. The fact that ULBP2 can reach the cell surface even in the absence of the complete machinery for synthesis of

GPI anchors supports the idea that different stress stimuli might affect the various ULBPs in different manners. In particular, our data suggest that while ULBP2 is normally expressed as a GPI-linked molecule, it can reach the cell surface as a transmembrane protein where it can activate NKG2D-dependent cytotoxicity by NK cells. Thus, while normally this alternative pathway for expression of ULBP2 is minimally utilised, ULBP2 expression could still be induced for immune surveillance, even after stresses or mutations that reduce or block GPI anchor synthesis (see model in Supplementary Figure 3).

It is interesting to note that NKG2D-L upregulation in cancer has been associated with very different outcomes for different types of cancer. For example, high tumour expression of MIC and/or ULBPs was associated with improved patient survival in colorectal cancer (McGilvray et al., 2009) whereas in ovarian cancer high expression of several NKG2D-ligands was inversely correlated with patient survival. Analysis of the NKG2D system in disease is complex because of the large number of distinct ligands for a single receptor and the possibility of regulation by released soluble ligands. Our work shows that there are significant differences in the cell biology of these various ligand molecules and that further study is needed to understand how the different stress situations affect the upregulation, release and function of the different NKG2D-L and how these factors impact on immune responses towards different types of cancer.

Materials and Methods

Cells and reagents

ULBP1, 2 and 3 constructs were obtained from Dr. Richard Apps (Apps et al., 2008). Chinese Hamster Ovary (CHO) G9PLAP cells and the GPI-deficient mutant CHO G9PLAP.85 (referred as CHO ΔGPI) (Stevens et al., 1996) (kind gifts of Dr. V.L. Stevens, Emory University and Dr. S. Vainauskas, Cornell University) were maintained in Hams F12 medium with supplements. CHO cells were transfected, using Lipofectamine 2000 (Invitrogen), with a mixture (9:1 ratio) of each ULBP expression plasmid with a vector conferring resistance to puromycin for stable transfectants (de la Luna and Ortin, 1992). Stable transfectants were sorted where necessary and grown in selective medium (8μg/ml puromycin, Calbiochem). The isolation and culture of human primary polyclonal NK cells was as previously described (Ashiru et al., 2010). K562 class K mutants (a kind gift of Dr. ME Medoff, Case Western Reserve University, Cleveland, Ohio) were maintained in RPMI 10% FCS (Mohney et al., 1994; Yu et al., 1997). Antibodies directed against ULBPs were purchased from R&D systems (Abingdon, UK) and the anti-KDEL antibody was from StressGen. B9.4 antibody directed against CD8 was a gift from Prof. J.L. Strominger. Leupeptin, Pepstatin A, 1-10 phenanthroline, PI-PLC were purchased from Sigma. NKG2D-Ig fusion protein was prepared as previously described (Vales-Gomez et al., 2003).

Flow Cytometry

10^5 cells were preincubated in PBS containing 1% bovine serum albumin, 0.1% sodium azide (PBA). Cells were then incubated with mouse mAbs and bound antibody was visualised using either phycoerythrin- or FITC-labelled $F(ab')_2$ fragments of goat anti-mouse Ig (Dako). For staining with the NKG2D-Ig fusion protein, bound protein was visualised using PE-labelled anti-human-Ig (Immunotech). Samples were analysed using a FACScan II flow cytometer (Becton Dickinson). Dead cells were excluded from all analyses by staining with propidium iodide.

Intracellular staining was performed by permeabilization using saponin: cells were fixed using 1% p-formaldehyde for 20 min at 4^oC, washed twice with PBS containing 0.1% saponin and 1% BSA and incubated with for 10 min at room

temperature. Cells were then washed and stained as usual using PBA buffer containing 0.1% saponin.

Western blot

Cell lysates were prepared by incubation in TNE buffer containing 1% NP-40 and the protease inhibitors leupeptin, pepstatin and 1,10 phenanthroline, for 30 min at 4ºC. Nuclei were eliminated by centrifugation at 13,000xg. Lysates and membrane/soluble fractionations were run on 12% SDS-PAGE gels and transferred to Immobilon-P (Millipore) membrane. The membrane was blocked using PBS containing 0.1% Tween-20 (PBS-T) and 5% non-fat dry milk. Detection of ULBP was performed by incubation with biotinylated goat polyclonal anti-ULBP antibody, followed by horseradish peroxidase-conjugated streptavidin. Proteins were visualized using the ECL system (Amersham Pharmacia). In some experiments, samples were treated with Endo-glycosidase H (Endo H) (New England Biolabs, Ipswich, MA), according to the manufacturer's instructions.

Preparation of CD8/ULBP chimaeras

The C-terminal portions of ULBP1 and ULBP2 were amplified by PCR using the oligonucleotides ULBP1for-GCATACAGCTGGATCCAACAAAACCACC; ULBP1rev-CGGAATTCATCTGCCAGCTAGAATGAAGC; ULBP2for-GCATACAGCTGGAGCCAAGTGCAGGAG and ULBP2rev-CGGAATTCAGATGCCAGGGAGGATGAAGC. The PCR products were then digested with *Pvu II* and *Eco RI*. The CD8/ULBP chimaeras were prepared in a three way ligation with pCDNA3 digested with *Eco RI* and *Hind III*, the extracellular domain of CD8 as a *Hind III – Eco RV* fragment and the C-terminal portion of either ULBP1 or ULBP2 as *Pvu II - Eco RI* fragments. All plasmids were sequenced to verify the integrity of the constructs (Supplementary Figure 1).

Triton X-114 fractionation

A 12% solution (w/v) of Triton X-114 was preconcentrated separating three times the aqueous and detergent phases by incubation at 30°C-37°C followed by centrifugation at 1,000 g for 5 min. The resulting detergent was used as the stock of Triton X-114 for experiments. Cells were lysed in 1% Triton X-114 in TBS with protease inhibitors. Extraction was performed for 1 hour at 4°C, rotating. After

removing insoluble debris by centrifugation at 13000 g at 4°C, phases were separated by incubating at 37°C for 5 min and centrifugation at 300 g at 25°C. The upper aqueous phase and the lower detergent phase were analysed separately by western blot after trichloroacetic acid (TCA) precipitation.

DRM fractionation

Detergent resistant and detergent soluble membrane fractions were prepared as previously described (Boutet et al., 2009). Western blot was performed using antibodies specific for ULBP2 and caveolin, as control for the fractionation.

^{35}S Pulse-Chase experiments.

$40 \cdot 10^6$ CHO cells transfected with ULBP2 were harvested and starved for 30 min in 2 ml of Met/Cys-free medium. Cells were then incubated in 2 ml of Met/Cys-free medium containing 1 mCi of [^{35}S]-methionine for 10 minutes and chased for intervals of 0, 30, 90, 180 minutes by addition of medium supplemented with 10% FCS as source of non-radioactive methionine. Cell lysates were prepared in 1% NP-40 lysis buffer. After preclearing the lysate with Pansorbin (Calbiochem), ULBP proteins were recovered by immunoprecipitation using goat polyclonal antibodies (R&D). Immunoprecipitated proteins were recovered using protein G beads (GE heathcare), washed three times in lysis buffer and digested with Endoglycosidase H (NEB), following manufacturer's instructions. Proteins were resolved in 12% SDS-PAGE. Two independent experiments were performed.

Confocal microscopy

Cells were either only fixed with 4% p-formaldehyde at 4ºC for 15 min or fixed and permeabilised by incubation with 0.1% saponin at room temperature for 10 min after fixation. The different preparations were stained with polyclonal anti-ULBP antibodies (R&D) and analysed by confocal microscopy as previously described (Aguera-Gonzalez et al., 2009). Fluorescence images were obtained using confocal microscopes (either Leica TCS-NT-UV confocal laser scanning microscope; Olympus IX81or Zeiss LSM510-Confocor 2). Images of fixed cells were taken using a 63x 1.32 NA objective with the confocal pinhole set to one Airy unit. Images were obtained by scanning series of single focal planes across the cell using either Leica TCS software,

FV10-ASW1.7 or LSM5 Image Examiner. To explore the whole intracellular area, series of sections (total interval z=2-4 μm) were acquired.

Cytotoxicity assays

Cytotoxicity assays were carried out using a one-step fluorimetric assay based on the use of AlamarBlue (Invitrogen) (Nociari et al., 1998). Effector cells alone, target cells alone and mixes of effectors and target cells at the indicated E:T ratios were incubated with AlamarBlue in 96 well flat-bottomed plates at 37^{o}C in a humidified 5% CO_2 incubator overnight. Following the incubation, the fluorescence of the AlamarBlue was read on a Synergy HT plate reader (Biotek) with excitation at 530nm and emission at 590nm at 37^{o}C. The percentage specific lysis was calculated using the following formula:

$$\% \text{ specific lysis} = 100 \times \{(AF \text{ of targets alone}) - [(AF \text{ of mix}) - (AF \text{ of effectors alone})]\} / AF \text{ of targets alone}$$

where AF = absolute fluorescence units.

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References

Aguera-Gonzalez, S., Boutet, P., Reyburn, H. T. and Vales-Gomez, M. (2009). Brief residence at the plasma membrane of the MHC class I-related chain B is due to clathrin-mediated cholesterol-dependent endocytosis and shedding. *J Immunol* **182**, 4800-8.

Apps, R., Gardner, L., Traherne, J., Male, V. and Moffett, A. (2008). Natural-killer cell ligands at the maternal-fetal interface: UL-16 binding proteins, MHC class-I chain related molecules, HLA-F and CD48. *Hum Reprod* **23**, 2535-48.

Ashiru, O., Boutet, P., Fernandez-Messina, L., Aguera-Gonzalez, S., Skepper, J. N., Vales-Gomez, M. and Reyburn, H. T. (2010) Natural killer cell cytotoxicity is suppressed by exposure to the human NKG2D ligand MICA*008 that is shed by tumor cells in exosomes. *Cancer Res* **70**, 481-9.

Bahram, S., Bresnahan, M., Geraghty, D. E. and Spies, T. (1994). A second lineage of mammalian major histocompatibility complex class I genes. *Proc Natl Acad Sci U S A* **91**, 6259-63.

Bahram, S., Mizuki, N., Inoko, H. and Spies, T. (1996). Nucleotide sequence of the human MHC class I MICA gene. *Immunogenetics* **44**, 80-1.

Bordier, C. (1981). Phase separation of integral membrane proteins in Triton X-114 solution. *J Biol Chem* **256**, 1604-7.

Boutet, P., Aguera-Gonzalez, S., Atkinson, S., Pennington, C. J., Edwards, D. R., Murphy, G., Reyburn, H. T. and Vales-Gomez, M. (2009). Cutting edge: the metalloproteinase ADAM17/TNF-alpha-converting enzyme regulates proteolytic shedding of the MHC class I-related chain B protein. *J Immunol* **182**, 49-53.

Chalupny, N. J., Sutherland, C. L., Lawrence, W. A., Rein-Weston, A. and Cosman, D. (2003). ULBP4 is a novel ligand for human NKG2D. *Biochem Biophys Res Commun* **305**, 129-35.

Cosman, D., Mullberg, J., Sutherland, C. L., Chin, W., Armitage, R., Fanslow, W., Kubin, M. and Chalupny, N. J. (2001). ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. *Immunity* **14**, 123-33.

de la Luna, S. and Ortin, J. (1992). pac gene as efficient dominant marker and reporter gene in mammalian cells. *Methods Enzymol* **216**, 376-85.

Dustin, M. L. and Springer, T. A. (1991). Role of lymphocyte adhesion receptors in transient interactions and cell locomotion. *Annu Rev Immunol* **9**, 27-66.

Eagle, R. A., Traherne, J. A., Hair, J. R., Jafferji, I. and Trowsdale, J. (2009). ULBP6/RAET1L is an additional human NKG2D ligand. *Eur J Immunol* **39**, 3207-16.

Eleme, K., Taner, S. B., Onfelt, B., Collinson, L. M., McCann, F. E., Chalupny, N. J., Cosman, D., Hopkins, C., Magee, A. I. and Davis, D. M. (2004). Cell surface organization of stress-inducible proteins ULBP and MICA that stimulate human NK cells and T cells via NKG2D. *J Exp Med* **199**, 1005-10.

Fernandez-Messina, L., Ashiru, O., Boutet, P., Aguera-Gonzalez, S., Skepper, J. N., Reyburn, H. T. and Vales-Gomez, M. Differential mechanisms of shedding of the glycosyl-phosphatidylinositol (GPI)-anchored NKG2D ligands. *J Biol Chem*.

Gonzalez, S., Groh, V. and Spies, T. (2006). Immunobiology of human NKG2D and its ligands. *Curr Top Microbiol Immunol* **298**, 121-38.

Lopez-Larrea, C., Suarez-Alvarez, B., Lopez-Soto, A., Lopez-Vazquez, A. and Gonzalez, S. (2008). The NKG2D receptor: sensing stressed cells. *Trends Mol Med* **14**, 179-89.

Lou, Z., Jevremovic, D., Billadeau, D. D. and Leibson, P. J. (2000). A balance between positive and negative signals in cytotoxic lymphocytes regulates the polarization of lipid rafts during the development of cell-mediated killing. *J Exp Med* **191**, 347-54.

Maasho, K., Opoku-Anane, J., Marusina, A. I., Coligan, J. E. and Borrego, F. (2005). NKG2D is a costimulatory receptor for human naive CD8+ T cells. *J Immunol* **174**, 4480-4.

Mayor, S. and Riezman, H. (2004). Sorting GPI-anchored proteins. *Nat Rev Mol Cell Biol* **5**, 110-20.

McGilvray, R. W., Eagle, R. A., Rolland, P., Jafferji, I., Trowsdale, J. and Durrant, L. G. ULBP2 and RAET1E NKG2D ligands are independent predictors of poor prognosis in ovarian cancer patients. *Int J Cancer*.

McGilvray, R. W., Eagle, R. A., Watson, N. F., Al-Attar, A., Ball, G., Jafferji, I., Trowsdale, J. and Durrant, L. G. (2009). NKG2D ligand expression in human colorectal cancer reveals associations with prognosis and evidence for immunoediting. *Clin Cancer Res* **15**, 6993-7002.

Mohney, R. P., Knez, J. J., Ravi, L., Sevlever, D., Rosenberry, T. L., Hirose, S. and Medof, M. E. (1994). Glycoinositol phospholipid anchor-defective K562 mutants with biochemical lesions distinct from those in Thy-1- murine lymphoma mutants. *J Biol Chem* **269**, 6536-42.

Moran, P., Raab, H., Kohr, W. J. and Caras, I. W. (1991). Glycophospholipid membrane anchor attachment. Molecular analysis of the cleavage/attachment site. *J Biol Chem* **266**, 1250-7.

Nausch, N. and Cerwenka, A. (2008). NKG2D ligands in tumor immunity. *Oncogene* **27**, 5944-58.

Nociari, M. M., Shalev, A., Benias, P. and Russo, C. (1998). A novel one-step, highly sensitive fluorometric assay to evaluate cell-mediated cytotoxicity. *J Immunol Methods* **213**, 157-67.

O'Callaghan, C. A., Cerwenka, A., Willcox, B. E., Lanier, L. L. and Bjorkman, P. J. (2001). Molecular competition for NKG2D: H60 and RAE1 compete unequally for NKG2D with dominance of H60. *Immunity* **15**, 201-11.

Paulick, M. G. and Bertozzi, C. R. (2008). The glycosylphosphatidylinositol anchor: a complex membrane-anchoring structure for proteins. *Biochemistry* **47**, 6991-7000.

Ravetch, J. V. and Perussia, B. (1989). Alternative membrane forms of Fc gamma RIII(CD16) on human natural killer cells and neutrophils. Cell type-specific expression of two genes that differ in single nucleotide substitutions. *J Exp Med* **170**, 481-97.

Sharom, F. J. and Lehto, M. T. (2002). Glycosylphosphatidylinositol-anchored proteins: structure, function, and cleavage by phosphatidylinositol-specific phospholipase C. *Biochem Cell Biol* **80**, 535-49.

Stevens, V. L., Zhang, H. and Harreman, M. (1996). Isolation and characterization of a Chinese hamster ovary (CHO) mutant defective in the second step of glycosylphosphatidylinositol biosynthesis. *Biochem J* **313 (Pt 1)**, 253-8.

Suemizu, H., Radosavljevic, M., Kimura, M., Sadahiro, S., Yoshimura, S., Bahram, S. and Inoko, H. (2002). A basolateral sorting motif in the MICA cytoplasmic tail. *Proc Natl Acad Sci U S A* **99**, 2971-6.

Vales-Gomez, M., Browne, H. and Reyburn, H. T. (2003). Expression of the UL16 glycoprotein of Human Cytomegalovirus protects the virus-infected cell from attack by natural killer cells. *BMC Immunol* **4**, 4.

Verneris, M. R., Karami, M., Baker, J., Jayaswal, A. and Negrin, R. S. (2004). Role of NKG2D signaling in the cytotoxicity of activated and expanded CD8+ T cells. *Blood* **103**, 3065-72.

Yu, J., Nagarajan, S., Knez, J. J., Udenfriend, S., Chen, R. and Medof, M. E. (1997). The affected gene underlying the class K glycosylphosphatidylinositol (GPI) surface protein defect codes for the GPI transamidase. *Proc Natl Acad Sci U S A* **94**, 12580-5.

Figure legends

Figure 1. ULBP2 can be expressed at the cell surface in the absence of GPI machinery in transient transfectants. Parental CHO and the GPI-deficient mutant CHO cell line (ΔGPI) were transiently transfected with ULBP1, 2, 3 and MICB and analysed by (A) flow cytometry and (B) western blot. Grey: isotype control; black line: ULBP/MIC staining. The same cell lines were also transiently transfected with CD8 and chimeric molecules containing the extracellular region of CD8 and the C-terminal region of the indicated ULBPs (as in Supplementary Figure 1). The percentage of positive cells assessed by flow cytometry (C) is shown.

Figure 2. ULBP2 can be expressed at the cell surface in the absence of GPI machinery in stable transfectants. A. Flow cytometry showing cell surface expression of ULBP2 stably transfected in parental CHO and in the GPI-deficient CHO cell line. B. Cell surface proteins were labelled by biotinylation. ULBP2 was immunoprecipitated and, after SDS-PAGE and western transfer, visualised with streptavidin-peroxidase and ECL. As a control for the cell-surface specificity of biotinylation, beta-actin was also immunoprecipitated. No beta-actin could be detected after probing with streptavidin-HRP (left panel), however actin protein was detected when the blot was probed with actin-specific mAb (right panel), demonstrating that the actin IP had worked correctly, but that only cell surface proteins had been biotinylated. C. CHO and GPI-deficient cells transfected with ULBP2 were treated with the enzyme PI-PLC to remove GPI-anchored proteins from the cell surface. After digestion, cells were lysed in Triton X-114 and membrane and soluble proteins were fractionated as described in materials and methods. The resulting detergent (membrane) and aqueous (soluble) fractions were analysed by western blot with ULBP2 specific polyclonal antibodies.

Figure 3. Endogenous ULBP2, but not ULBP1, is expressed at the surface of GPI-deficient K562 cells. K562 cells and K562 class K mutants were stained with mAbs specific for ULBP1, ULBP2 and an isotype control and analysed by flow cytometry. ULBP1 and ULBP2 are expressed in the cell, as shown by intracellular staining, but only ULBP2 appears at the cell surface of GPI-deficient cells.

Figure 4. The majority of ULBP2 is present in detergent resistant membranes (DRMs) of wild-type, but not GPI-deficient CHO cell lines. CHO cells and ΔGPI cells transfected with ULBP2 were lysed and fractionated by centrifugation on discontinuous sucrose gradients. Equal volumes of these fractions were analysed, by western blot, for the presence of ULBP2. As a positive control for fractionation, the presence of caveolin-1 in the different fractions was also analysed.

Figure 5. ULBP2 matures more slowly than ULBP3 in parental CHO cells and, in GPI-deficient cells, the rate of egress of ULBP2 from the ER is still slower. A. Stable transfectants of ULBP2 and ULBP3 were metabolically labelled with ^{35}S Met/Cys for 10 min and chased for the indicated times. After that, lysates were prepared and the ULBP molecules immunoprecipitated, one half of the immunoprecipitated material was digested with EndoH before being analysed by SDS-PAGE. B. CHO and ΔGPI cells transfected with ULBP2 were cultured on cover-slips, fixed, or fixed and permeabilised (as indicated) and stained with polyclonal antibody specific for ULBP2. Bar: 10 μm C. ΔGPI cells stably transfected with ULBP2 were pulse-labelled with ^{35}S Met/Cys and chased for the indicated times before immunoprecipitation, digestion with EndoH and analysis by SDS-PAGE. D. Quantitation of mature vs immature ULBPs. The percentage of mature protein present in each one of the bands of the experiments shown in A and C was analysed using Image J.

Figure 6. ULBP2 molecules expressed in either the CHO wild-type or GPI-deficient cells interact similarly with NKG2D. A. Increasing amounts of recombinant human NKG2D-Ig fusion protein were used to stain untransfected and ULBP2-transfected CHO and ΔGPI cells. Specific staining was calculated by subtracting the MFI of NKG2D staining of the untransfected cells from the MFI of NKG2D staining of the ULBP2 transfectant. One representative experiment, of three, is shown. B. Primary NK cells were used as effectors in a cytotoxicity assay to assess the susceptibility of the untransfected and ULBP2 transfected CHO and ΔGPI cells to lysis. The data shown are the mean ± SD values for % specific cytotoxicity from three experiments.

Figure 1

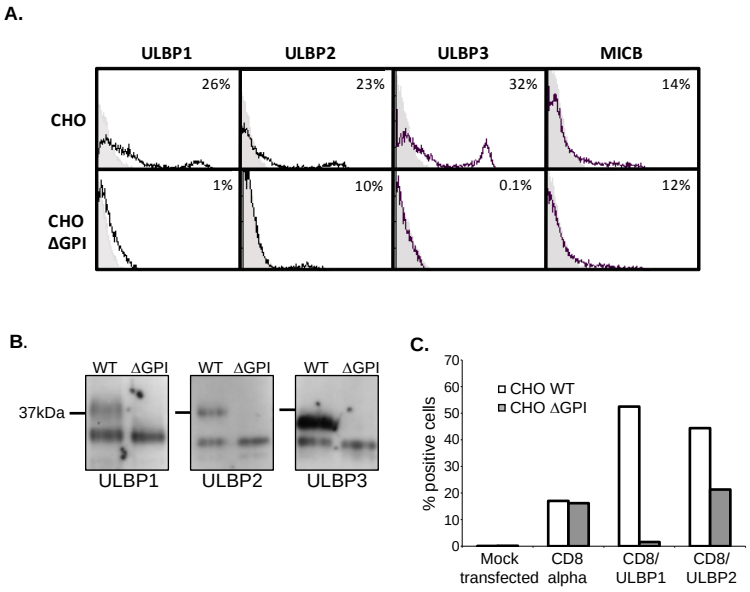
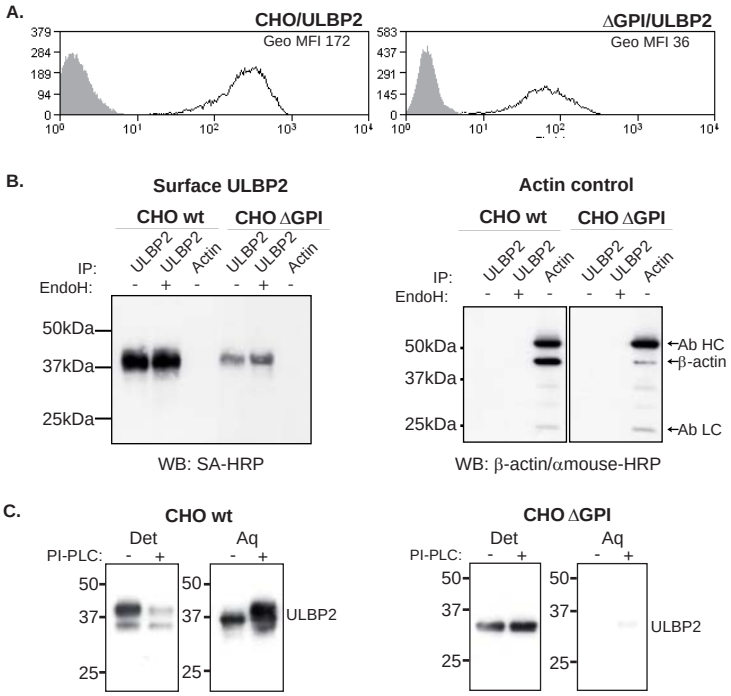

A.
ULBP1
ULBP2
ULBP3
MICB
CHO
CHO ΔGPI
26%
23%
32%
14%
1%
10%
0.1%
12%
B.
WT ΔGPI
WT ΔGPI
WT ΔGPI
37kDa
ULBP1
ULBP2
ULBP3
C.
CHO WT
CHO ΔGPI
% positive cells
70
60
50
40
30
20
10
0
Mock transfected
CD8 alpha
CD8/ ULBP1
CD8/ ULBP2

Figure 2


A.
CHO/ULBP2
Geo MFI 172
ΔGPI/ULBP2
Geo MFI 36
B.
Surface ULBP2
Actin control
CHO wt
CHO ΔGPI
IP:
EndoH:
ULBP2
Actin
50kDa
37kDa
25kDa
Ab HC
β-actin
Ab LC
WB: SA-HRP
WB: β-actin/αmouse-HRP
C.
CHO wt
CHO ΔGPI
Det
Aq
PI-PLC:
ULBP2

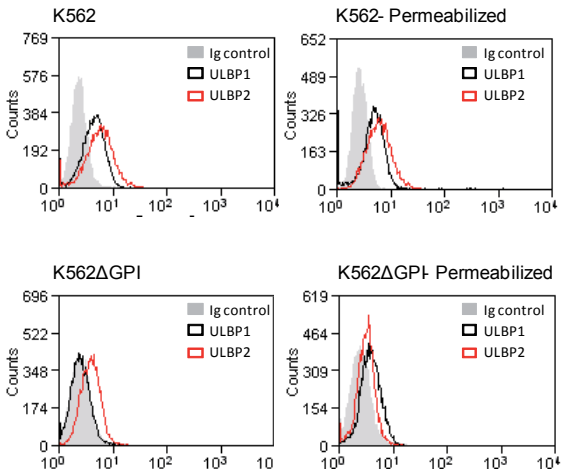
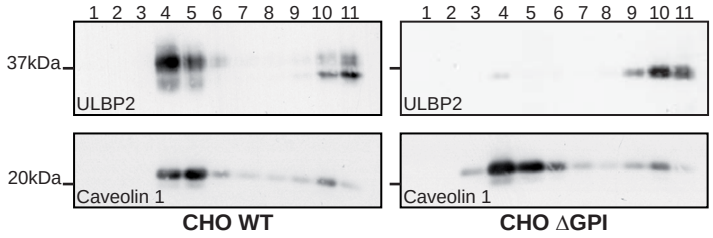
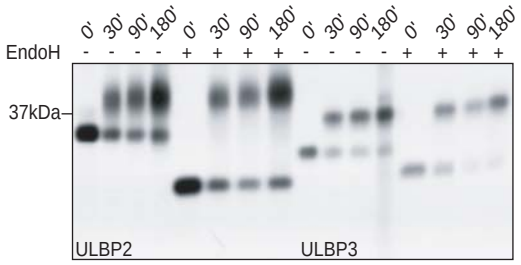

K562
K562- Permeabilized
K562ΔGPI
K562ΔGPI- Permeabilized
Counts
Ig control
ULBP1
ULBP2
769
576
384
192
0
652
489
326
163
696
522
348
174
619
464
309
154
10^0
10^1
10^2
10^3
10^4

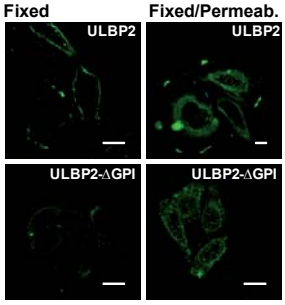
Figure 4


1 2 3 4 5 6 7 8 9 10 11
1 2 3 4 5 6 7 8 9 10 11
37kDa
ULBP2
ULBP2
20kDa
Caveolin 1
Caveolin 1
CHO WT
CHO ΔGPI

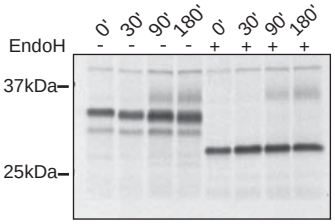
A. Pulse chase experiments CHO-ULBP2 and -ULBP3



B. Confocal microscopy



C. Pulse chase CHO ULBP2-ΔGPI



D. Quantification of pulse-chase experiments

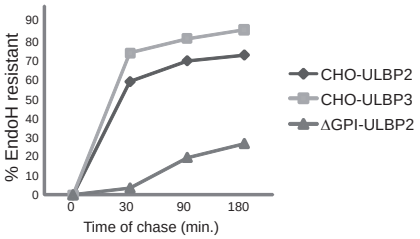
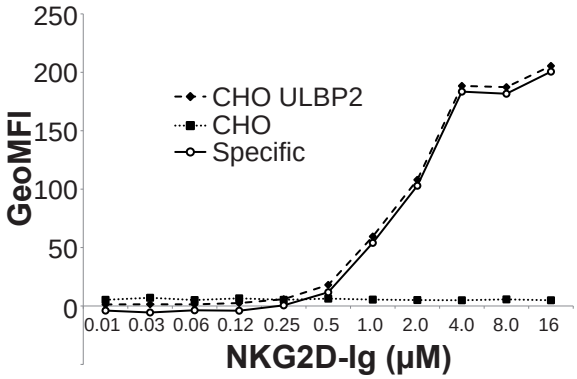
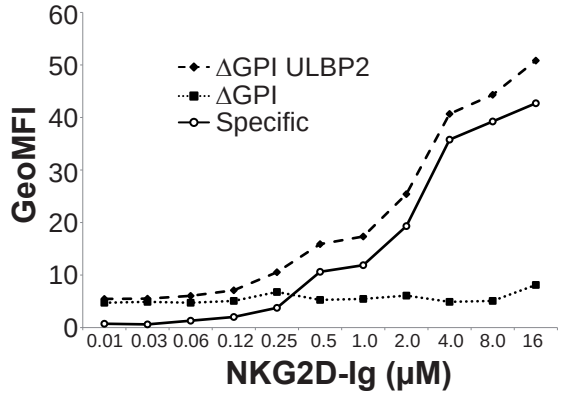


Figure 6

A. NKG2D-Ig binding





B. Cytotoxicity assay