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ULBP4 is a novel ligand for human NKG2D

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Abstract

The ULBPs are a family of MHC class I-related molecules. We have previously shown that ULBPs 1, 2, and 3 are functional ligands of the NKG2D/DAP10 receptor complex on human natural killer (NK) cells. Here, we describe a new member of the ULBP family, ULBP4, which contains predicted transmembrane and cytoplasmic domains, unlike the other ULBPs, which are GPI-linked proteins. Transduction of ULBP4 into EL4 cells confers the ability to bind recombinant NKG2D and mediates increased cytotoxic activity by human NK cells, consistent with the role of ULBPs as ligands for the NKG2D/DAP10 activating receptors. Tissue expression of ULBP4 differs from other members of the family, in that it is expressed predominantly in the skin.
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The ULBPs, or UL16-binding proteins, are so named because the initial member, ULBP1, was identified by virtue of its binding to the HCMV glycoprotein UL16 [1]. ULBP2 and ULBP3 were later identified as expressed sequence tags with homology to ULBP1. Like ULBP1, ULBP2 also binds to UL16, whereas ULBP3 does not. Together they comprise an unusual family of human, MHC class I-related cell surface proteins. ULBPs differ from classical MHC class I molecules in several ways. ULBPs contain α1 and α2 domains, but lack an α3 domain, do not associate with β2-microglobulin, and are GPI-linked [1]. UL16 also binds to MICB [1,2], a member of a distantly related family of non-classical MHC class I proteins (MICs).

The ULBPs and MICs have been shown to be ligands for the activating NKG2D/DAP10 receptor complex, consisting of the C-type lectin-type molecule NKG2D and the signal transducing adapter molecule DAP10 [1,3–5]. More recently mouse NKG2D has been shown to additionally associate with DAP12/KARAP in some cell types [6,7]. In humans NKG2D is expressed on NK cells, γδ T cells, and CD8+ αβ T cells [3]. In the mouse NKG2D is expressed on NK cells and on activated CD8+ αβ T cells and macrophages [8]. A number of ligands for mouse NKG2D have been identified, including the retinoic acid-inducible early gene (RAE)-1 protein family [8–11], the H60 minor histocompatibility antigen [12], and MULT-1 [13]. The RAE-1 family consists of five isoforms, which resemble the ULBP1, 2, and 3 in structure. They contain α1 and α2 domains and are GPI-linked. H60 and MULT-1 also contain α1 and α2 domains, but additionally possess transmembrane and cytoplasmic domains. Binding of both human and mouse NKG2D ligands to NK cells has been shown to stimulate NK cytotoxicity [1,3,8,9].

NK cells are important components of the innate immune system [14]. NK cells mount cytotoxic responses against certain tumors and virally infected cells. NK cytolysis against target cells is determined by a balance between inhibitory and stimulatory receptors on the NK cells that are engaged by ligands on the target cells [15,16]. Cells which have down-regulated MHC class I expression are generally sensitive to NK cytolysis due to decreased engagement of NK cell inhibitory receptors. NKG2D ligands have been shown to be induced in response to infection, stress, and transformation [17–19]. Engagement of NKG2D on NK cells by ULBPs and MICs has been shown to mediate an activating signal, which can override the signal sent by binding of MHC

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class I to NK cell inhibitory receptors, resulting in the lysis of cells expressing normal MHC class I levels [1,3]. Therefore, the ULBPs and MICs may play an important role in immune surveillance by NK cells or NKG2D-expressing γδ T cells or CD8+ T cells, allowing cytolysis of abnormal cells which have upregulated ULBP or MIC expression, but remain MHC class I positive. Cytokines and chemokines produced by NK cells can regulate the immune responses of cells involved in both innate and adaptive immunity [20]. The binding of ULBPs to NKG2D/DAP10 on human NK cells has also been shown to induce production of multiple cytokines and chemokines, either alone or in synergy with IL-12 [1,21]. These factors may contribute to recruitment and activation of other immune effector cells.

Here, we describe the initial characterization of a novel member of the ULBP family, ULBP4. Like ULBP1, 2, and 3, ULBP4 is a functional ligand for NKG2D. ULBP4 is predominantly expressed in the skin.

Materials and methods

Identification of ULBP4 sequence. The ULBP4 sequence was identified by searching the NCBI human genomic sequence database, using the amino acid sequences of ULBP1, 2, and 3 and the tBLASTn algorithm. A bacterial artificial chromosome (BAC), AL355312, was identified that contained sequences that potentially encoded a ULBP-related protein. This BAC mapped to chromosome 6q24-25, adjacent to BACs encoding the other three ULBPs. To verify the prediction, primers were designed from the 5′ and 3′ ends of the predicted coding region, which we designated as ULBP4, and used to amplify ULBP4 cDNA by RT-PCR from esophageal mRNA (forward primer 5′-TAT GTCGACCTCCACAGTATGCGAAGAATATCCCTG-3′; reverse primer 5′-ATAGGCGGCCGCAGACTAAGACGTCCTCAA-3′). The predicted and observed sequences were identical (GenBank Accession No. AY252119).

TaqMan. Commercially available RNAs (Ambion, Clontech Laboratories, Stratagene) were DNase treated (Ambion) and reverse transcribed, using TaqMan Reverse Transcription Reagents (Applied Biosystems) according to the manufacturer's specifications, using random hexamers. Samples were distributed on plates at 20 ng per well and run in triplicate. TaqMan primer/probe sets were designed using Primer Express software (Applied Biosystems). Forward and reverse primer concentrations for ULBP4 were optimized and determined to be 900 nM each. 6-FAM/TAMRA labeled probe (Applied Biosystems) was used at 200 nM. GAPDH housekeeping gene (Applied Biosystems) was used on an Applied Biosystems Prism 7700 Sequence Detection System. Threshold cycle values (C_T) were determined, using Sequence Detector software version 1.7a (Applied Biosystems) and transformed to $2^{-\Delta CT}$ for relative expression comparison of ULBP4 to GAPDH.

Cell lines and purification of cells. EL4, a murine thymoma cell line (ATCC TIB-39), was grown in RPMI-1640 supplemented with 5% fetal calf serum (FCS), 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, and 0.055 mM 2-mercaptoethanol. The human NK cell line NKL was a generous gift from Dr. Michael J. Robertson and was cultured as described [22].

Mouse NK cells were obtained from the spleens of C57BL/6 SCID mice (Jackson Labs) as previously described [23]. On day 3 cells were stained with anti-NK1.1 to determine the percentage of NK cells in the culture. Cultures containing 90% NK cells or greater were used in cytotoxicity assays. CV-1 cells were grown and transfected as previously described [24].

Construction and expression of Fc fusion proteins. The NKG2D-Fc and muNKG2D-Fc fusion proteins were constructed as previously described [23]. The UL16-Fc fusion protein was constructed as previously described [1]. The control Fc protein, hCD40-Fc, has been previously described [25].

Plasmids encoding the Fc fusion proteins were transfected into CV-1/EBNA (ATCC CRL-10478) cells, and the fusion proteins were purified from culture supernatants by chromatography on protein A-Poros columns (PerSeptive Biosystems) as described [25].

Flow cytometric analysis. The following monoclonal antibodies and proteins were used for flow cytometric analysis: M90, anti-hCD40L used as a mouse IgG1 isotype control; M360, mouse IgG1 anti-MICB; M550, mouse IgG1 anti-ULBP3, UL16-Fc, NKG2D-Fc, and muNKG2D-Fc used to detect ULBP4, hCD40-Fc as a negative control Fc protein.

Cells (5×10^5) were incubated in 100 μl staining buffer with one ug antibody or fusion protein for 30 min on ice. Specific binding was detected with either a PE-conjugated F(ab′)2 fragment goat anti-mouse IgG (Jackson Immunoresearch) or a PE-conjugated F(ab′)2 fragment goat anti-human IgG1 (Fc specific) (Jackson Immunoresearch). After staining, cells were analyzed on a Becton–Dickinson FACScan.

Phosphatidyl-inositol-specific phospholipase C treatment. Cells transfected with cDNAs encoding MICB, ULBP4, or ULBP3 were treated with 2 U/ml phosphatidyl-inositol-specific phospholipase C (PI-PLC) (Sigma) at 37 °C for 1 h. Subsequently, cells were washed with 1× PBS and stained on ice by indirect immunofluorescence, using specific antibodies as described above.

Retroviral constructs and transduction. EL4 cells were transduced with amphotropic retroviruses generated by insertion of cDNAs encoding ULBP1, ULBP2, ULBP3, or ULBP4 into the LZRSpB MN-Z vector [26] followed by transfection into the Phoenix packaging line (provided by the Nolan lab, Stanford University). Retrovirally transduced cells expressing ULBP1, ULBP2, or ULBP3, were stained with monoclonal antibodies specific for the antigens of interest followed by PE-conjugated F(ab′)2 fragment of goat anti-mouse IgG, and sorted by flow cytometry. Cells expressing ULBP4 were stained with NKG2D-Fc followed by PE-conjugated F(ab′)2 fragment of goat anti-human IgG (Fc specific) and sorted by flow cytometry.

When stained with monoclonal antibodies specific for the ULBPs 1, 2, and 3 or with soluble NKG2D for ULBP4, the following MFIs were measured on the EL4 transductants: ULBP1 + EL4 = 1080; ULBP2 + EL4 = 1801; ULBP3 + EL4 = 1600; and ULBP4 + EL4 = 710. The MFIs for non-transduced EL4s were: ULBP1 = 4; ULBP2 = 7; ULBP3 = 10; and ULBP4 = 20.

Cytotoxicity assay. The ^{51}Cr release cytotoxicity assay was performed as previously described [1]. Proper shielding procedures for ^{51}Cr were used (according to the manufacturer's specifications).

Results and discussion

Identification of ULBP4

The MHC class I-related ULBP1, 2, and 3 genes map to human chromosome 6, but outside of the MHC region at 6q25 [27,28]. To identify other potential ULBP family members, we searched publicly available human genomic sequences for homologies to ULBP1, 2, and 3. This search identified a BAC that mapped adjacent to BACs encoding ULBP1, 2, and 3 genes. Analysis of sequences within this BAC identified a potential gene

encoding a predicted ULBP-related protein that we call ULBP4. We verified this prediction by amplification and sequencing of ULBP4 cDNA from esophageal mRNA.

The predicted α1 and α2 domain structure of this protein is similar to ULBP1, 2, and 3 (Fig. 1). However, unlike ULBP1, 2, and 3 which are GPI-linked proteins, ULBP4 appears to have both a transmembrane and a cytoplasmic domain (Fig. 1). ULBP4 retains most of the highly conserved residues throughout the α1 and α2 domains (Fig. 1), which identify the ULBPs as members of the extended MHC class I family. While the amino acid sequences of ULBP1, 2, and 3 are 55–60% identical, ULBP4 is more divergent, with greater similarity to ULBP3 than to ULBPs 1 or 2. When compared to the known ligands for mouse NKG2D, ULBP1, 2, and 3 resemble the RAE1 family due to their GPI-linkage, whereas ULBP4 appears to be more structurally homologous to H60 and MULT-1, both of which contain transmembrane and cytoplasmic domains. The functional significance of transmembrane and cytoplasmic domains for NKG2D ligands is unclear, but does suggest the possibility of "reverse signaling" through the ligands.

The possibility of an extensive family of ULBP-related genes has been supported by a recent report that the region from chromosome 6q24.1–6q25.3 contains as many as 10 ULBP-related sequences [28]. Many of these are clearly pseudogenes, while others may encode functional glycoproteins. The genomic sequence identified as RAET1E [28] is identical to that of ULBP4.

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ULBP1       MAAAA SPAFLLCLPL L.HLLSGWSR AGWVDTHCLC YDFIITPKSR
ULBP2       MAAAA ATKILLCLPL L.LLLSGWSR AGRADPHSLC YDITVIPKFR
ULBP3       MAAAA SPAILPRLAI LPYLLFDWSG TGRADAHSLW YNFTIIHLPR
ULBP4       MRRIS LTSSPVRLL. LFLLLLLIAL EIMVGGHSLC FNFTIKSLSR

                                  α1
ULBP1  PEPQWCEVQG LVDERPFLHY DCVNHKAKAF ASLGKKVNVT KTWEEQTETL
ULBP2  PGPRWCAVQG QVDEKTFLHY DCGNKTVTPV SPLGKKLNVT TAWKAQNPVL
ULBP3  HGQQWCEVQS QVDQKNFLSY DCGSDKVLSM GHLEEQLYAT DAWGKQLEML
ULBP4  PGQPWCEAQV FLNKNLFLQY NSDNNMVKPL GLLGKKVYAT STWGELTQTL

ULBP1  RDVVDFLKGQ LLDIQVENLI PIEPLTLQAR MSCEHEAHGH GRGSWQFLFN
ULBP2  REVVDILTEQ LRDIQLENYT PKEPLTLQAR MSCEQKAEGH SSGSWQFSFD
ULBP3  REVGQRLRLE LADTELEDFT PSGPLTLQVR MSCECEADGY IRGSWQFSFD
ULBP4  GEVGRDLRML LCDIK.PQIK TSDPSTLQVE MFCQREAERC TGASWQFATN

                                  α2
ULBP1  GQKFLLFDSN NRKWTALHPG AKKMTEKWEK NRDVTMFFQK ISLGDCKMWL
ULBP2  GQIFLLFDSE KRMWTTVHPG ARKMKEKWEN DKVVAMSFHY FSMGDCIGWL
ULBP3  GRKFLLFDSN NRKWTVVHAG ARRMKEKWEK DSGLTTFFKM VSMRDCKSWL
ULBP4  GEKSLLFDAM NMTWTVINHE ASKIKETWKK DRGLEKYFRK LSKGDCDHWL

ULBP1  EEFLMYWEQM LDPT..KPPS LAPGTTQPKA MATTLSPWSL LIIFLCFILA
ULBP2  EDFLMGMDST LEPSAQAPLA MSSGTTQLRA TATTLILCCL LIILPCFILP
ULBP3  RDFLMHRKKR LEPT..APPT MAPGLAQPKA IATTLSPWSF LIIL.CFILP
ULBP4  REFLGHWEAM PEPT....VS PVNASDIHWS SSSLPDRWII LGAFILLVLM

ULBP1  GR*~~~~~~~ ~~~~~~~~~~ ~~~~~
ULBP2  GI*~~~~~~~ ~~~~~~~~~~ ~~~~~
ULBP3  GI*~~~~~~~ ~~~~~~~~~~ ~~~~~
ULBP4  GIVLICVWWQ NGEWQAGLWP LRTS*
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Fig. 1. Amino acid sequence alignment of the ULBPs. Residues shown in bold are identical in all ULBPs. The open box encloses the GPI anchor signal of ULBPs 1, 2, and 3. The underlined region of ULBP4 marks its predicted transmembrane domain. The bold line above the sequences marks the α1 domain. The dotted line above the sequences marks the α2 domain.

NKG2D binding of ULBP4

In order to determine whether ULBP4 was able to bind to human NKG2D, we stained the ULBP+ EL4 transductants with a soluble form of human NKG2D, NKG2D-Fc. Like the ULBP1+, 2+, and 3+ EL4s, the ULBP4+ EL4 showed significant binding to NGK2D-Fc, relative to binding of a control Fc protein (Fig. 2).

Further study of the interaction of the ULBPs with NKG2D in mouse models may be useful in understanding the biology of this system. Therefore, we assessed the ability of all of the ULBPs to bind to murine NKG2D. A soluble form of mouse NKG2D, muNKG2D-Fc, was used to measure binding to the ULBP+ EL4 cells. The ULBP1+ and ULBP2+ EL4 bound strongly to muNKG2D-Fc, whereas muNKG2D-Fc bound weakly to ULBP4+ EL4 and did not bind detectably to ULBP3+ EL4 (Fig. 2). Cross-species binding of human NKG2D ligands to mouse NKG2D is notable, considering the low sequence identity between the ULBPs and any of the mouse NKG2D ligands, and illustrates the remarkable capacity of NKG2D to recognize diverse ligands.

Activation of NK cytotoxicity by ULBP4

We have previously shown that expression of ULBP1, 2, and 3 on mouse EL4 cells can trigger cytotoxicity by human NK cells [23]. In order to determine whether the ULBP4 molecule would function similarly, ULBP4+ EL4 were used as targets in NK cytotoxicity

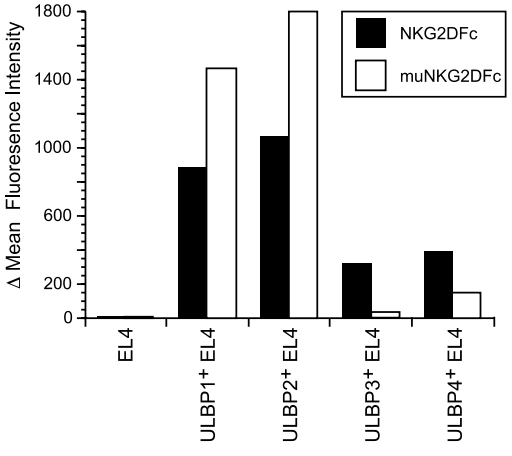

Fig. 2. ULBP4 binds to soluble forms of both human and murine NKG2D. Parental EL4 cells or EL4 cells transduced with amphotropic retroviruses encoding ULBP1, ULBP2, ULBP3, or ULBP4 were stained with NKG2D-Fc (human) or muNKG2D-Fc (murine) or a control Fc construct (hCD40-Fc) (10 μg/ml) followed by PE-conjugated F(ab′)2 goat anti-human IgG (Fc specific). The difference in mean fluorescence intensity values was calculated after subtraction of the background staining values obtained with the negative control Fc protein plus the secondary antibody. This experiment is representative of three independent experiments.

assays. The parental EL4 cells were modestly killed by the human NK cell line, NKL, while the ULBP4+ EL4 were killed much more efficiently (Fig. 3A). EL4 cells transduced with the mouse NKG2D ligand, Rae-1β, were not killed more efficiently than that of the non-transduced parental EL4 cells (data not shown).

ULBP1 and 2, but not 3 bind to mouse NKG2D, ULBP4 binds weakly, and ULBP3 does not bind detectably (Fig. 2). ULBP transduced EL4 cell lines were used in cytotoxicity studies with IL-15-activated mouse NK cells as effectors. Mouse NK cells were able to kill EL4 targets and expression of ULBP4 on the targets yielded a modest increase in cytotoxicity (Fig. 3B). A greater increase in cytotoxicity was observed in targets expressing ULBPs 1 and 2, while expression of ULBP3 had no effect (data not shown). Killing of ULBP4+ targets by mouse NK cells was efficiently blocked after the incubation of the effector cells with a rat antibody to mouse NKG2D, but not by incubation with an unrelated rat antibody (Fig. 3C). These results establish ULBP4 as a functional ligand for NKG2D.

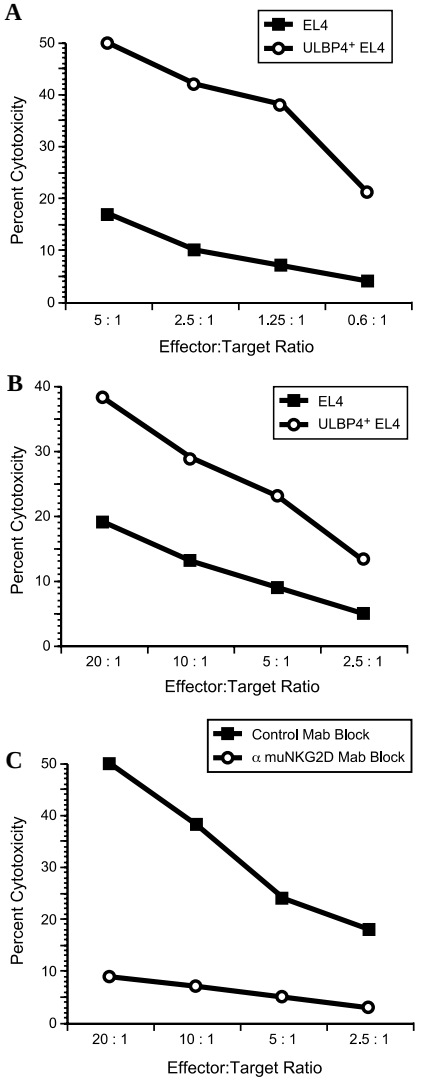

Fig. 3. Expression of ULBP4 enhances cytotoxicity of human and mouse NK cells against EL4 cells, and this effect can be blocked by anti-NKG2D. Parental EL4 cells or EL4 cells expressing ULBP4 were tested as targets in 3-h ^{51}Cr release cytotoxicity assays at the indicated E:T ratios. Individual data points are calculated from the averages of triplicate samples. (A) The human NK cell line, NKL, was applied as effectors. The results shown are representative of three separate experiments. (B) NK cells isolated from the spleens of C57BL/6 SCID mice cultured with 200 ng/ml rhuIL-15 for 4 days were applied as effectors. The results shown are representative of three separate experiments. (C) NK cells isolated from the spleens of C57BL/6 SCID mice cultured with 200 ng/ml rhuIL-15 for 4 days were pre-incubated with the M315 (rat anti-muNKG2D) or a control rat anti-mouse antibody (rat anti-mouse IL17R) (20 μg/ml) and applied as effectors. The results shown are representative of two separate experiments.

UL16 does not bind to ULBP4

UL16 shows detectable binding to ULBP1, ULBP2, and MICB, but not to ULBP3 or MICA [1]. Intracellular binding of UL16 to these three NKG2D ligands can down-regulate their surface expression, protecting against NK or T cell cytotoxicity [23,29,30]. The basis for selective targeting of these ligands is unclear, particularly given the high degree of sequence identity between MICA and MICB.

In order to determine whether ULBP4 was able to bind to UL16, EL4 cells were transduced with retroviruses containing cDNAs encoding ULBP1, 2, 3, or 4. Cell populations expressing the transduced cell-surface molecules were obtained as described in Materials and methods. The ability of these transduced EL4 cells to bind to a soluble form of UL16, UL16-Fc, was assessed. The ULBP4+ EL4 showed no detectable binding above the negative controls (non-transduced EL4 and ULBP3+ EL4), while ULBP1+ and ULBP2+ EL4 bound well to UL16-Fc (Fig. 4). The lack of UL16-Fc binding by ULBP3+ and ULBP4+ EL4 was not due to low level expression of these proteins on the transduced EL4 cells, because specific mAb or NKG2D-Fc bound to these cells and could be quantified by flow cytometry (see Materials and methods).

ULBP4 is not a GPI-linked protein

ULBPs 1–3 are GPI-linked proteins [1], however the sequence of the ULBP4 protein predicts a transmembrane glycoprotein. To determine whether the ULBP4 protein is transmembrane or GPI-linked, cells expressing the transiently transfected ULBP4 or MICB or ULBP3 were treated with phosphatidylinositol-specific phospholipase C (PI-PLC). NKG2D-Fc was used to measure cell surface expression of ULBP4. Cell surface expression of MICB or ULBP3 was assessed, using monoclonal antibodies specific for these proteins. Compared to cells undergoing a mock enzyme treatment, expression levels of ULBP3 were significantly reduced as has been previously shown [1]. Expression of

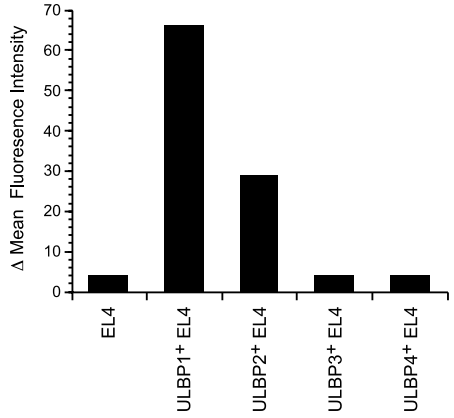

Fig. 4. ULBP4 does not bind to soluble UL16. Parental EL4 cells or EL4 cells transduced with amphotropic retroviruses encoding ULBP1, ULBP2, ULBP3, or ULBP4 were stained with UL16-Fc or a control Fc construct (hCD40-Fc) (100 μl at 10 μg/ml), followed by PE-conjugated secondary reagent. The difference in mean fluorescence intensity values was calculated after subtraction of the background staining values obtained with the negative control Fc protein plus the secondary antibody. This experiment is representative of two independent experiments.

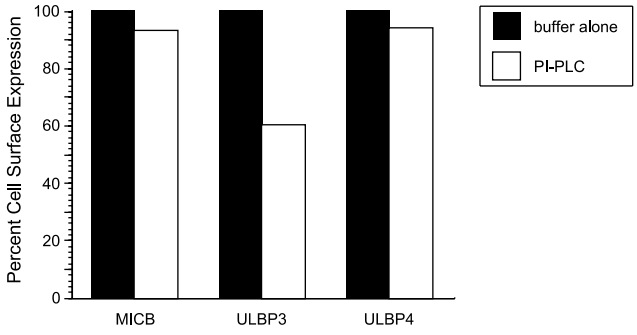

Fig. 5. ULBP4 is not GPI-linked. CV-1 cells transfected with cDNAs encoding the full-length MICB (negative control), ULBP3 (positive control), or ULBP4 were mock treated (black bars) or treated with PI-PLC (white bars). Subsequently, cells were stained with specific monoclonal antibodies or with NKG2D-Fc, followed by PE-conjugated goat anti-mouse IgG or PE-conjugated goat anti-human IgG, respectively. Percent cell surface expression was calculated, using the MFI of antibody or protein binding to the untreated samples, after subtraction of background staining values obtained with the secondary antibody alone, as 100%.

the type 1 transmembrane protein MICB was not altered by PI-PLC treatment, nor was the expression of ULBP4 (Fig. 5), demonstrating that ULBP4 is likely not a GPI-linked cell-surface molecule.

Expression of ULBP4 in tissues

MIC expression is up-regulated in certain epithelial tumors, in pathogen infected cells, and in response to stress [17–19]. The ligands for mouse NKG2D are expressed on skin epithelia in response to carcinogens, inducing killing by NKG2D+ γδ T cells [31]. The ligands for murine NKG2D are expressed on epithelial cells [31], thymocytes [6], and activated T cells [32]. ULBP messages are expressed in a wide range of tissues and ULBP proteins are expressed on a variety of tumor-derived cell lines [1]. Comparison of ULBP message levels in several matched normal and tumor tissue samples has revealed no consistent differences [1], but expression of ULBPs on a number of hematopoietic tumor lines has recently been described [33].

In order to rapidly survey the expression of ULBP4 in different tissue types, a set of primers was designed that would detect ULBP4, but not ULBP1, 2, or 3, in PCR experiments using a panel of cDNAs isolated from different tissues. An amplification product of the expected size was detected from an esophageal cDNA, but not when genomic DNA was used as a template (data not shown). Subsequently, real-time PCR was done on cDNAs from a larger number of tissue types, and transcripts were detected in skin, testis, and trachea (Table 1). No significant message was detected in a large number of tissue types (Table 1). The expression of ULBP4 transcripts seems to be more restricted than that of the other ULBPs [1] or the murine NKG2D ligands [8,9,13]. Further studies are required to fully understand the expression and regulation of all of the human and mouse NKG2D ligands.

In summary, we have identified ULBP4, a novel functional member of the ULBP family capable of binding NKG2D and transducing a stimulatory signal to human and mouse NK cells. ULBP4 is most similar in sequence to ULBP3, and like ULBP3, does not bind UL16 detectably. Unlike ULBP1, 2, and 3, ULBP4 is most likely a transmembrane protein, and is predominantly expressed in the skin. This work further extends

Table 1
Quantitative RT-PCR analysis of ULBP4 expression

Tissue type	$2^{-\Delta CT} \times 10^3$
Testis	0.39
Fetal stomach	0.10
Fetal colon	0.05
Skin	12.87
Fetal kidney	0.10
Fetal skeletal muscle	0.06
Kidney	0.15
Trachea	0.99
Small intestine, stomach, colon, prostate, adrenal, ovary, pancreas, skeletal muscle, spleen, thymus, fetal spleen, fetal lung, thyroid, uterus, brain, liver, heart, lung, cerebellum, fetal brain, spinal cord, fetal liver, placenta	0.00

Minimal expression of ULBP4 is detected in fetal stomach, fetal colon, kidney, fetal kidney, and fetal skeletal muscle. Above values are expression of ULBP4 relative to expression of GAPDH when multiplexed in the same sample. Relative expression is calculated as: $2^{-\Delta CT}$ (where $\Delta CT = CT_{ULBP4} - CT_{GAPDH}$).

the remarkable diversity of ligands recognized by mouse and human NKG2D.

Acknowledgments

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