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Cutting Edge: The UNC93B1 Tyrosine-Based Motif Regulates Trafficking and TLR Responses via Separate Mechanisms

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Sensing of nucleic acids by TLRs is crucial in the host defense against viruses and bacteria. Unc-93 homolog B1 (UNC93B1) regulates the trafficking of nucleic acid–sensing TLRs from the endoplasmic reticulum to endolysosomes, where the TLRs encounter their respective ligands and become activated. In this article, we show that a carboxyl-terminal tyrosine-based sorting motif (YxxΦ) in UNC93B1 differentially regulates human nucleic acid–sensing TLRs in a receptor- and ligand-specific manner. Destruction of YxxΦ abolished TLR7, TLR8, and TLR9 activity toward nucleic acids in human B cells and monocytes, whereas TLR8 responses toward small molecules remained intact. YxxΦ in UNC93B1 influenced the subcellular localization of human UNC93B1 via both adapter protein complex (AP)1- and AP2-dependent trafficking pathways. However, loss of AP function was not caused for altered TLR responses, suggesting AP-independent functions of YxxΦ in UNC93B1. *The Journal of Immunology, 2014, 193: 3257–3261.*

One strategy to detect pathogens or tissue damage is nucleic acid recognition by TLR3, TLR7, TLR8, or TLR9 (1). Because nucleic acids are not unique to microbes, their sensing evokes the risk for autoimmunity (1). Unc-93 homolog B1 (UNC93B1) regulates endoplasmic reticulum (ER) to endolysosome trafficking of nucleic acid–sensing TLRs (2–4). A single point mutation in UNC93B1 (H412R) that prevents its ability to exit from the ER ablates endosomal TLR signaling (5), and patients lacking functional UNC93B1 are at risk for developing lethal HSV infections (6), a clinical phenotype resembling TLR3 deficiency (7).

Proteins can be directly delivered from the trans-Golgi network to endosomes or indirectly delivered via the plasma membrane (8). Recently, a tyrosine-based sorting motif (YxxΦ) in murine UNC93B1 was identified to interact with the major endocytic adapter protein complex (AP)2, which was suggested to be required for murine TLR9 function and delivery to endosomes (9). In contrast, the function of other murine endosomal TLRs was reported to be unimpaired by mutation of YxxΦ in UNC93B1 (9).

The cellular distribution and function of endosomal TLRs differ among species. Human TLR9 expression is restricted to plasmacytoid dendritic cells and B cells (10) and TLRs 11–13 are present in mice but not in humans (11). Furthermore, human TLR8 signaling serves important roles in monocytes, dendritic cells, and neutrophils, whereas murine TLR8 does not have the same functions (12).

In this study, we investigated the role of YxxΦ in human UNC93B1 on the activation of human TLR7, TLR8, and TLR9 by nucleic acids and small molecule agonists in different human cell types. We found that YxxΦ in human UNC93B1 bound to AP1 and AP2, both of which were involved in the correct localization of UNC93B1. Destruction of YxxΦ caused receptor- and ligand-specific defects in TLR responses. However,
knockdown of AP1 or AP2 did not mimic the observed TLR defects, suggesting that the tyrosine-based motif in UNC93B1 likely serves additional roles in regulating TLR signaling.

Materials and Methods

Cell lines and plasmids
TLR-expressing HEK cells were purchased from InvivoGen. EBV-immortalized B cells derived from an UNC93B1-deficient patient were described previously (6), and human UNC93B1-knockout THP-1 monocytes were generated using CRISPR/Cas9-based gene editing (13). Plasmids for mCitrine, human UNC93B1-mCitrine wild-type (WT), H412R or the YxxF-mutant UNC93B1 Y539A L542A (AxxA), and mCherry-KDEL were engineered by standard cloning techniques. Stable cells generated by transduction were sorted for similar expression levels of UNC93B1 versions.

Cell stimulation and analysis
RNA interference was performed by lipofection (RNAiMax; Life Technologies) of 5 nM Silencer Select small interfering RNAs (siRNAs; Life Technologies) for 72 h. Cells were stimulated for 14 h with CpG2006 (Metabion), R848 (InvivoGen), CL075 (InvivoGen), Pam2CSK4 (EMC microcollections), human TNF or IL-1β (R&D Systems), TLR7-specific RNA (5′-ACUG1-CG1AG1CUU-X-UUCG1AG1CG1UCA-5′, G1 is 7-deazaguanosine, X is 1,2,3-propanetriol) (14), or TLR8-specific RNA (5′-YYUGCUGCXGUU-XXGUUUCGCU-GUY-5′, Y is 1,3-propanediol, X is 1,2,3-propanetriol) (15) (Idera Pharmaceuticals). Supernatants were analyzed by ELISA for human IL-8 (BD Biosciences), human IL-6, or human TNF (both from R&D Systems). Efficiency of RNA interference was analyzed by SYBR Green quantitative PCR (qPCR) for MyD88, AP1M1, and AP2M1 expression normalized to HPRT.

Surface plasmon resonance spectroscopy
Soluble µ subunits of AP1 (mouse µ1A aa 158–423 in pET28b), AP2 (rat µ2 aa 158–435, pET28a), and AP3 (rat µ3a aa 166–418, pET28b) were expressed in E. coli BL21-DE3 and purified by Ni-NTA affinity, followed by size-exclusion chromatography (GE Healthcare Life Sciences). Surface plasmon resonance (SPR) was performed with a Biacore 3000 instrument (GE Healthcare Life Sciences). Biotinylated peptides containing the WT (YxxL) or mutant (AxxA) tyrosine-based motif of UNC93B1 or the tyrosine-based motif of TGN38 (21st Century Biochemicals) were immobilized on streptavidin sensor chips (GE Healthcare Life Sciences). Proteins were injected for 1 min at 40 µl/min flow rate at 25˚C in 10 mM HEPES (pH 7.4), 500 mM NaCl, 10 mM 2-ME, and 5 mM DTT. Data were analyzed by BIAevaluation 4.1.1 software.

Microscopy
Subcellular compartments were labeled using CellLight Reagents, LysoTracker Red (L7528), or Transferrin–Alexa Fluor 647 (T23366) (Life Technologies). Live cells were imaged using a Leica SP5 SMD confocal microscope with a 63×/1.2 water-immersion objective. Data quantification was performed with a custom-designed MATLAB-based analysis software.

Results and Discussion

YxxF in UNC93B1 regulates human TLR signaling in a cell type–, receptor-, and ligand-specific manner

YxxF in UNC93B1 is conserved between species and was suggested to regulate TLR9 function in murine cells (9). We assessed the effects of YxxF on TLR function in three human cell types. To study UNC93B1-mediated regulation of human
TLRs, we first performed RNA interference–mediated silencing of endogenous UNC93B1 or overexpression of WT or mutated UNC93B1 in HEK cells. As expected, knockdown of UNC93B1 abrogated TLR7, TLR8, and TLR9 signaling (Fig. 1A), whereas overexpression of WT UNC93B1, but not the nonfunctional H412R mutant of UNC93B1, resulted in increased TLR7, TLR8, and TLR9 responses (Fig. 1B). Cellular activation by TNF remained unchanged (Fig. 1A, 1B). We next tested UNC93B1 WT, H412R or YxxΦ-mutant UNC93B1 (UNC93B1 Y539A L542A, termed AxxA hereafter) in stable HEK cells also expressing either TLR7, TLR8, or UNC93B1 (9), human TLR9 signaling (Fig. 1C). In contrast with murine TLR7, which was reported to be unaffected by changes in YxxΦ of UNC93B1 (9), human TLR9 activity was also reduced (Fig. 1C). Conversely, signaling of TLR8 was increased in cells expressing UNC93B1 AxxA (Fig. 1C), suggesting that YxxΦ in UNC93B1 exerts differential effects on TLR7, TLR8, and TLR9 activation. Expression levels of all forms of UNC93B1 were comparable (Supplemental Fig. 1A), and TNFR signaling remained unaffected by the expression of either form of UNC93B1 (Fig. 1C). Previous studies on murine TLR9 showed cell-specific differences with regard to the subcellular distribution (16) and signaling requirements of nucleic acid–sensing TLRs (17). To study endogenous TLRs in human immune cells, we reconstituted EBV-transformed B cells from an UNC93B1-deficient patient (6) and UNC93B1-deficient human THP-1 monocytes (13) with fluorescent UNC93B1 WT, H412R, or AxxA and sorted cells for UNC93B1 expression (Supplemental Fig. 1B, 1C). Similar to what we observed in HEK cells, TLR7 and TLR9 signaling was largely reduced in B cells expressing UNC93B1 AxxA, whereas TLR2 signaling in the same cells remained intact (Fig. 1D). Human THP-1 monocytes naturally express TLR7 and TLR8 (12), both of which may contribute to responses toward dual-specific ligands. Because no strictly TLR8-specific small molecule activator is available, we chose to use R848 and CL075, which preferentially stimulate TLR7 and TLR8, respectively. THP-1 monocytes expressing UNC93B1 AxxA showed defective responses toward R848, whereas their response to CL075 was similar to cells expressing WT UNC93B1 (Fig. 1E), essentially reflecting the difference between TLR7 and TLR8 that we observed in HEK cells. However, unlike in HEK cells, no hyperresponsiveness of TLR8 toward the small molecule agonists was found. This difference could be explained by the mixed TLR7/8 response in monocytes or by cell type–specific differences in small molecule diffusion or accumulation in subcellular compartments. To further elucidate TLR7 and TLR8 signaling in monocytes, we stimulated UNC93B1-knockout THP-1 cells expressing different forms of UNC93B1 with TLR7- or TLR8-specific ssRNA (14, 15). Notably, both TLR7 and TLR8 responses toward RNA agonists were completely abolished in cells expressing UNC93B1 AxxA (Fig. 1E), revealing differential regulation of small molecule– versus RNA-activated TLR8. Cellular responses toward IL-1β remained intact (Fig. 1E). Unlike in THP-1 monocytes, TLR8 responses toward RNA were intact in HEK cells expressing UNC93B1 AxxA (Supplemental Fig. 1D). These results show that TLR7 and TLR8 responses are regulated in a cell type– and ligand-specific manner and that small molecule activators of TLRs should not be seen as simple surrogate ligands for nucleic acids.

The YxxΦ motif in human UNC93B1 mediates both AP1- and AP2-dependent trafficking of UNC93B1

Tyrosine-based sorting motifs bind to μ subunits of AP complexes, which are heterotetrameric protein complexes that connect cargo to the clathrin coat of transport vesicles (18). To determine whether YxxΦ in human UNC93B1 interacts with specific μ subunits of AP1, AP2, or AP3, we assessed the binding of purified proteins and peptides containing sorting motifs by SPR. This analysis showed that the peptide derived from UNC93B1 containing WT YxxΦ strongly interacted with the μ subunit of AP2 and, to a lesser extent, with the μ subunits of AP1 and AP3. These interactions were reduced when YxxΦ was destroyed (Fig. 2A). Further studies showed that the interaction with the μ subunit of AP2 was comparable to that of a YxxΦ-containing peptide derived from TGN38, one of the strongest AP2 interactors known (19). The μ subunit of AP2 is known to have the highest affinity and broadest specificity of all AP complexes, whereas the μ1

FIGURE 2. YxxL of UNC93B1 binds APs and functions as a trafficking motif. (A) SPR analysis of μ1, μ2, and μ3 (2 μM) binding to immobilized TGN38 YxxL (WT), UNC93B1 YxxL (WT), and UNC93B1 AxxA peptides. Specific binding to YxxΦ was determined by subtracting UNC93B1 AxxA from UNC93B1 WT SPR signals. Data are representative of three independent experiments. (B) HEK cells stably expressing UNC93B1-mCitrine WT, H412R, or AxxA were transiently transfected with the ER marker mCherry-KDEL and analyzed by confocal microscopy. The graph represents combined data of three independent experiments (mean + SEM). (C) HEK cells stably expressing UNC93B1-mCitrine WT or AxxA were transfected for 24 h with markers for early endosomes (Rab5-RFP), late endosomes (Rab7-RFP), or lysosomes (Lamp1-RFP) or incubated for 5 min with LysoTracker Red and analyzed by confocal microscopy. The graph shows combined data of four independent experiments (mean + SEM).
analyzed by confocal microscopy. The graph shows combined data of four control siRNA–treated samples. Transferrin–Alexa Fluor 647 (10 ng/ml) was incubated for 10 min with expressing UNC93B1-mCitrine WT or AxxA were incubated for 10 min with 4% paraformaldehyde, permeabilized with 0.5% saponin, stained for AP1 and AP2 are crucial for UNC93B1 trafficking. (Fig. 3A). We used fluorescent transferrin to elucidate whether human UNC93B1 entered the AP2-dependent trafficking pathway. Transferrin is endocytosed via the transferrin receptor in an AP2-dependent manner, thereby serving as a tracer molecule for AP2-dependent endosomes. Quantification of UNC93B1 and transferrin double-positive endosomes showed that UNC93B1 AxxA was largely absent from transferrin endosomes, whereas ~60% of WT UNC93B1 endosomes contained transferrin (Fig. 3B). Our data support the notion that both AP1 and AP2 are involved in the YxxΦ-mediated trafficking of UNC93B1.

AP1- and AP2-dependent trafficking pathways are dispensable for human endosomal TLR responses

To assess whether the inability of UNC93B1 AxxA to use AP1- and AP2-dependent sorting pathways was responsible for the observed TLR defects, we performed knockdown studies of AP1 and AP2 in HEK cells and analyzed TLR7, TLR8, or TLR9 responses. Knockdown of AP2 and, to a lesser extent, AP1 caused increased TLR8 activity (Fig. 4A), similar to the TLR8 phenotype observed in HEK cells expressing UNC93B1 AxxA. However, unlike mutating YxxΦ of UNC93B1, neither AP1 nor AP2 knockdown abolished TLR7 and TLR9 responses (Fig. 4A). In stark contrast, TLR9 responses were increased upon silencing of AP2 (Fig. 4A). These data clearly demonstrate that loss of AP1 or AP2 binding to UNC93B1 AxxA does not explain altered TLR responses upon destruction of YxxΦ in UNC93B1 and suggest that YxxΦ of UNC93B1 regulates UNC93B1 trafficking versus TLR responses via two separate mechanisms. TNFR activity was increased upon AP2 knockdown (Fig. 4A), which is in accordance with a shutdown of proinflammatory TNFR signaling via AP2-dependent TNFR endocytosis (20). As expected, silencing of MyD88 or AP1 did not change TNFR responses compared with control siRNA-treated cells (Fig. 4A). qPCRs for MyD88, AP1, and AP2 established that the knockdown was very efficient, with >90%

FIGURE 3. AP1 and AP2 are crucial for UNC93B1 trafficking. (A) HEK cells stably expressing UNC93B1-mCitrine WT or AxxA were fixed with 4% paraformaldehyde, permeabilized with 0.5% saponin, stained for AP1 and AP2, and analyzed by confocal microscopy. The graph represents combined data of two independent experiments (mean + SEM) analyzing copy. The graph shows combined data of four independent experiments (mean + SEM).

FIGURE 4. AP1 and AP2 are dispensable for TLR responses. (A) HEK TLR7, TLR8, or TLR9 cells were transfected with siRNA targeting MyD88, AP1 (AP1M1 and AP1G1), or AP2 (AP2M1) or control siRNA and stimulated with R848 (0.5 μM for TLR7, 10 μM for TLR8), 0.5 μM CpG2006, or 10 ng/ml TNF or were left unstimulated and analyzed for IL-8 secretion. All data are combined from three independent experiments (mean + SEM). (B) To validate knockdown efficiency, cells from (A) were analyzed for expression levels of MyD88, AP1M1, and AP2M1 by qPCR. Expression levels are shown relative to control siRNA–treated samples.
knockdown at the mRNA level (Fig. 4B). Furthermore, immunofluorescence staining of AP1 in control siRNA-treated cells versus AP1 siRNA-treated cells showed efficient silencing of AP1 at the protein level (Supplemental Fig. 2A). Loss of AP2 function was validated, because transferrin uptake was completely abolished upon AP2 silencing, leading to transferrin accumulation on the cell surface in AP2 siRNA-treated but not control siRNA-treated cells (Supplemental Fig. 2B). These results suggest that YxxΦ of UNC93B1 regulates TLR signaling independently of AP1 or AP2 and, therefore, must serve additional functions.

One possibility is that YxxΦ of UNC93B1 recruits other molecules that regulate TLR signaling. For example, YxxL of UNC93B1 could serve as a so-called “hemITAM motif.” Upon tyrosine phosphorylation, ITAM or hemITAM motifs recruit Src Homology 2 domain containing proteins, such as spleen tyrosine kinase, which was previously suggested to regulate endosomal TLR signaling (21, 22).

Our study identifies a complex and versatile regulatory role for YxxΦ in human UNC93B1, which is TLR-, ligand-, and cell type–specific. The notion that UNC93B1 could serve multiple functions in trafficking and, potentially, signaling should spark future investigations.

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Disclosures

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