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The cytoplasmic 'linker region' in Toll-like receptor 3 controls receptor localization and signaling

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Abstract

Toll-like receptor 3 (TLR3) recognizes double-stranded RNA and transmits signals to activate NF-κB and the interferon (IFN)-β promoter via the newly identified adaptor, TICAM-1. The extracellular LRR domain of TLR3 is engaged in the ligand recognition, while the intracellular TIR domain is crucial for the adaptor binding and signal transduction upon ligand stimulation. Here, we analyzed TLR3 localization in human monocyte-derived immature dendritic cells (iDCs) and stable transfectants expressing human TLR3 by immunofluorescence staining and confocal microscopy. TLR3 was predominantly localized in specific but as yet unidentified intracellular vesicles where TLR3 signaling was initiated. Expression analysis of TLR3-tail-truncated mutants revealed that the cytoplasmic 'linker' region (residues 730–755) determines the intracellular localization of TLR3. Site-directed mutagenesis of the linker region allowed us to identify the relevant determinants as Arg^{740} and Val^{741} residues for intracellular expression of TLR3. Furthermore, alanine scanning of the linker region demonstrated that the Phe^{732}, Leu^{742} and Gly^{743} in the TLR3 cytoplasmic linker region are essential for ligand-induced NF-κB and IFN-β promoter activation. Thus, the cytoplasmic linker region of TLR3 regulates receptor retention inside the organelle and signaling, which may be closely linked to TLR3 function in DCs.

Introduction

The innate immune system recognizes a wide variety of pathogens and induces a number of anti-microbial and inflammatory responses. Toll-like receptors (TLRs) recognize pathogen-associated molecular patterns and initiate anti-microbial immune responses (1–3). Ten members of the TLR family (TLR1–10) have been identified in humans (4–6). The TLR family proteins consist of an extracellular leucine-rich repeat (LRR), a transmembrane region (TM) and a cytoplasmic tail containing a Toll/IL-1 receptor homology (TIR) domain (7). After stimulation by distinct microbial components, each TLR elicits different, but sometimes overlapping, immune responses by recruiting specific adaptor molecules to the intracellular TIR domain (8).

TLR3 recognizes double-stranded (ds) RNA by its ectodomain, and triggers downstream signals leading to interferon (IFN)-β production (9,10). Recently, we and another group independently identified a novel adaptor protein, TIR-containing adaptor molecule (TICAM)-1 (also called as TRIF), which binds the TIR domain of TLR3 and activates NF-κB and the IFN-β promoter in response to poly(I)•poly(C), a synthetic analog of dsRNA (11–13). TICAM-1, when overexpressed, activates NF-κB, AP-1 and interferon regulatory factor (IRF)-3, a critically important transcription factor for IFN-β gene expression (11,14,15).

Upon viral infection, many anti-viral responses, including induction of IFN-inducing genes, are rapidly elicited in host cells (16). dsRNA generated in the cytoplasm during viral replication activates a cytoplasmic receptor, dsRNA-activated protein kinase (PKR), resulting in the induction of anti-viral responses (17,18). Although TLR3 is another receptor for dsRNA and is thought to play an important role in anti-viral immune responses through IFN-α/β production

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and dendritic cell (DC) maturation (19), how TLR3 recognizes viral dsRNA is currently unknown. TLR3 is a typical type I transmembrane protein, whose expression and localization are regulated in a cell-type specific manner (10,20–22). In human fibroblasts, TLR3 is localized both on the cell surface and inside the cells. A mAb against human TLR3 (TLR3.7) inhibits TLR3-mediated dsRNA-induced IFN-β secretion by fibroblasts, suggesting that TLR3 acts on the cell surface to sense viral infection (10). In contrast, monocyte-derived immature DCs (iDCs) and $CD11c^+$ blood DCs express TLR3 inside the cells, and anti-TLR3 mAb does not confer inhibition on dsRNA-induced IFN-β production by DCs (22). The mechanisms by which intracellular TLR3 recognizes dsRNA in DCs are still unknown.

In this study, we found that TLR3 localized to specific intracellular organelles in human monocyte-derived iDCs and HEK293 cells stably expressing human TLR3 by using anti-TLR3 mAb. Expression analysis of TLR3-tail-truncated mutants and site-directed mutants revealed that the determinants for the intracellular localization of TLR3 resided in the cytoplasmic 'linker region' (residues 730–755). Furthermore, we clearly showed that the linker region, in addition to the TIR domain, plays a critical role in dsRNA-induced TLR3-mediated signaling.

Methods

Reagents

Anti-human TLR2 mAbs (TLR2.45, TLR2.524) and anti-human TLR3 mAb (TLR3.7) were raised in our laboratory (10,23). Anti-human TLR3 mAb (IMG-315) was purchased from BioCarta (San Diego, CA). Anti-Rab5a mAb (S-19) and anti-Rab7 mAb (H-50a) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-Lamp-2 pAb was from Affinity Bioreagents, Inc. (Golden, CO), anti-Syntaxin 7 pAb was from Synaptic Systems (Gottingen, Germany), and anti-Calnexin pAb was from Stressgen (Victoria, Canada). Anti-FLAG M2 mAb, anti-HA pAb, lipopolysaccharide (LPS) and chloroquine were purchased from Sigma (St. Louis, MO). Anti-CD46 pAb was produced in our laboratory (24). MitoTracker (R) Red CMXRos and wheat germ agglutinin, Texas Red(R)-X conjugate (WGA-TR) and anti-dinitrophenyl-KLH, fluorescenine conjugate were purchased from Molecular Probes (Eugene, OR). DAMP [3-(2,4-denitroanilino)-3′-amino-*N*-methyldipropylamine] was purchased from Oxford Biomedical Research, Inc. (Oxford, MI). Bafilomycin A1 was from Wako Pure Chemical industries, Ltd (Osaka, Japan) and poly(I)•poly(C) was from Amersham Bioscience (Buckinghamshire, UK).

Cell cultures

HEK293 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated FCS (JRH Biosciences, Lenexa, KS). The IL-3-dependent murine Ba/F3 cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS, 5 ng/ml mouse IL-3, 100 μM 2-ME and antibiotics. $CD14^+$ human monocytes were isolated from human PBMCs using the MACS system (Mlitenyi Biotec, Germany). The monocytes were cultured for 6 days in RPMI 1640 supplemented with 10% heat-inactivated FCS and antibiotics in the presence of 500 U/ml GM-CSF (Peprotech EC Ltd, London, UK) and 100 U/ml IL-4 (Peprotech) to obtain monocyte-derived iDCs (22).

Plasmids

cDNAs for human TLR2, TLR3 and TLR4 were cloned in our labolatory by RT–PCR from mRNA of monocyte-derived iDCs and were ligated at the cloning site of the expression vector pEF-BOS (25). Various truncated TLR3 constructs (Fig. 3a) were generated by PCR with human TLR3 cDNA as a template. HA-tag was inserted to C-terminus of signal sequence of human TLR3delTIR (see Fig. 3a) by PCR with appropriate primers. Alanine-substituted constructs were generated by site-directed mutagenesis (Figs 4a and 5a). The mutated cDNAs were cloned and inserted into pEF-BOS. The N-terminal Flag-tagged TLR3 expression vector was constructed by inserting the cDNA fragment into the mammalian expression vector pFLAG-CMV-1 (Sigma). The expression vectors for MD-2 and CD14 were provided from K. Miyake and H. Nishimura, respectively. The TLR3-fluorescent protein chimera consists of the N-terminal signal sequence from TLR3 (1–26 aa) followed by the EGFP moiety fused to the N-terminus of TLR3 lacking its signal sequence (27–904 aa).

Stable transfectants

Stable transfectants expressing the various mutated constructs of human TLR3 were prepared as described previously (10). Briefly, HEK293 cells were transfected in 24-well plates using Lipofectamine 2000 reagent (Invitrogen) with the indicated expression vector together with pSV2neo plasmid (Riken GenBank, Tsukuba). The transfectants were selected with G418 (Sigma, final 600 μg/ml) for 2–3 weeks. Ba/F3 cells were transfected with the indicated expression vector together with pSV2neo plasmid by electroporation, and selected with G418 (final 750 μg/ml) for 10 days.

Flow cytometry

Cells were incubated with the indicated mAbs (0.5–1 μg) for 30 min at 4°C in FACS buffer (DPBS containing 0.1% BSA and 0.1% sodium azide). After the cells were washed twice with the above buffer, FITC-labeled secondary antibody (American

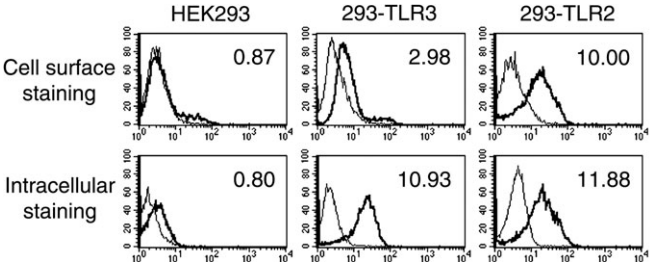

Fig. 1. Expression profiles of human TLR3 and TLR2 in HEK293 cells. Human TLR3 and TLR2 were stably expressed in HEK293 cells. TLR3.7 and TLR2.45 mAbs (10 μg/ml) were used for TLR3 and TLR2 detection, respectively (thick line). Mouse IgG1 was used as a control antibody (thin line). For intracellular staining, cells were treated in permeabilizing solution for 10 min before antibody treatment. Non-transfected HEK293 cells were stained with TLR3.7 as a control labeling. Inset values indicate the mean fluorescent intensities specific for the anti-TLR3 mAb or anti-TLR2 mAb. Upper panels, cell surface staining; lower panels, intracellular staining.

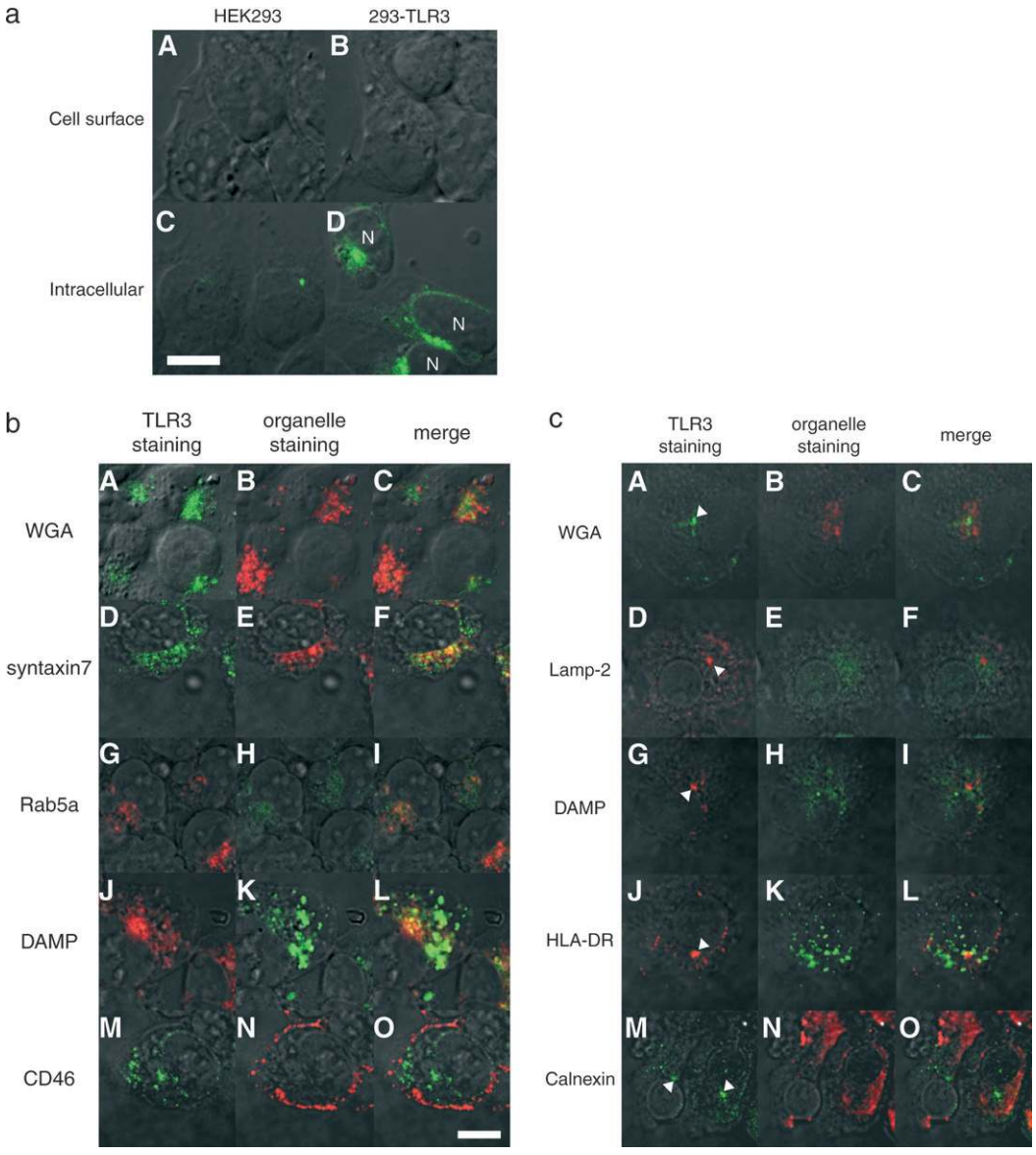

Fig. 2. Subcellular localization of TLR3 in human monocyte-derived iDCs and HEK293 transfectants. (a) TLR3 localizes in intracellular compartments in stable HEK293 transfectants. For intracellular staining, cells were fixed with 3% formaldehyde in PBS, permeabilized with 0.5% saponin in BSA–PBS, stained with TLR3.7 (20 μg/ml) followed by FITC-conjugated secondary antibody, and analyzed by confocal microscopy (C and D). For surface staining, cells were fixed with 0.5% paraformaldehyde after antibody treatment (A and B). Mouse IgG1 was used as an isotype control (data not shown). The nuclei (N) are marked in (D). Bar, 10 μm. (b) Confocal microscopic analysis of the subcellular localization of TLR3 in HEK293 cells. Fixed and permeabilized cells were stained with TLR3.7 mAb (green, A, D and M; red, G and J) and WGA-TR (red, B), anti-syntaxin 7 (red, E), or anti-Rab5a (green, H), followed by FITC-, rhodamine B- or Alexa-conjugated secondary antibody. For acidic organelle staining, cells were treated with DAMP (30 μM) for 60 min before fixation (green, K). For plasma membrane staining, cells were stained with anti-human CD46 pAb and Alexa 594-conjugated secondary antibody before fixation (red, N). The merged color (yellow) is indicative of co-localization. Bar, 10 μm. (c) TLR3 does not co-localize with well-characterized organelles in monocyte-derived iDCs. Fixed and permeabilized cells were stained with TLR3.7 mAb (green, A and M; red, D, G and J) and WGA-TR (red, B), anti-lamp-2 (green, E), anti-HLA-DR (green, K), or anti-calnexin (red, N) followed by FITC-, rhodamine B-, or Alexa 594-conjugated secondary antibodies. For acidic organelle staining, cells were treated with DAMP (30 μM) for 60 min before fixation (green, H). Anti-syntaxin 7 and anti-lamp-2 antibodies are used for late endosome and lysosome staining. Anti-Rab5a antibody was used for early endosome staining, and anti-calnexin antibody was for ER staining. The strong spot-like fluorescent signal observed in TLR3-staining of iDCs (part c, arrowheads in A, D, G, J and M) indicates that some unknown centrosomal molecule shares the relevant epitope with TLR3 (22). Bar, 10 μm.

Qualex, San Clemente, CA) was added and the cells were further incubated for 30 min at 4°C. For intracellular staining, cells were pretreated with permeabilizing solution (BD Bioscience, San Jose, CA) for 10 min at room temperature, washed once with FACS buffer and then incubated with the indicated mAbs (0.5–1 μg) for 30 min at room temperature. After washing, FITC-labeled secondary antibody (American Qualex) was added and the cells were further incubated for 30 min at room temperature. Ten percent goat serum was added to each reaction mixture to prevent non-specific

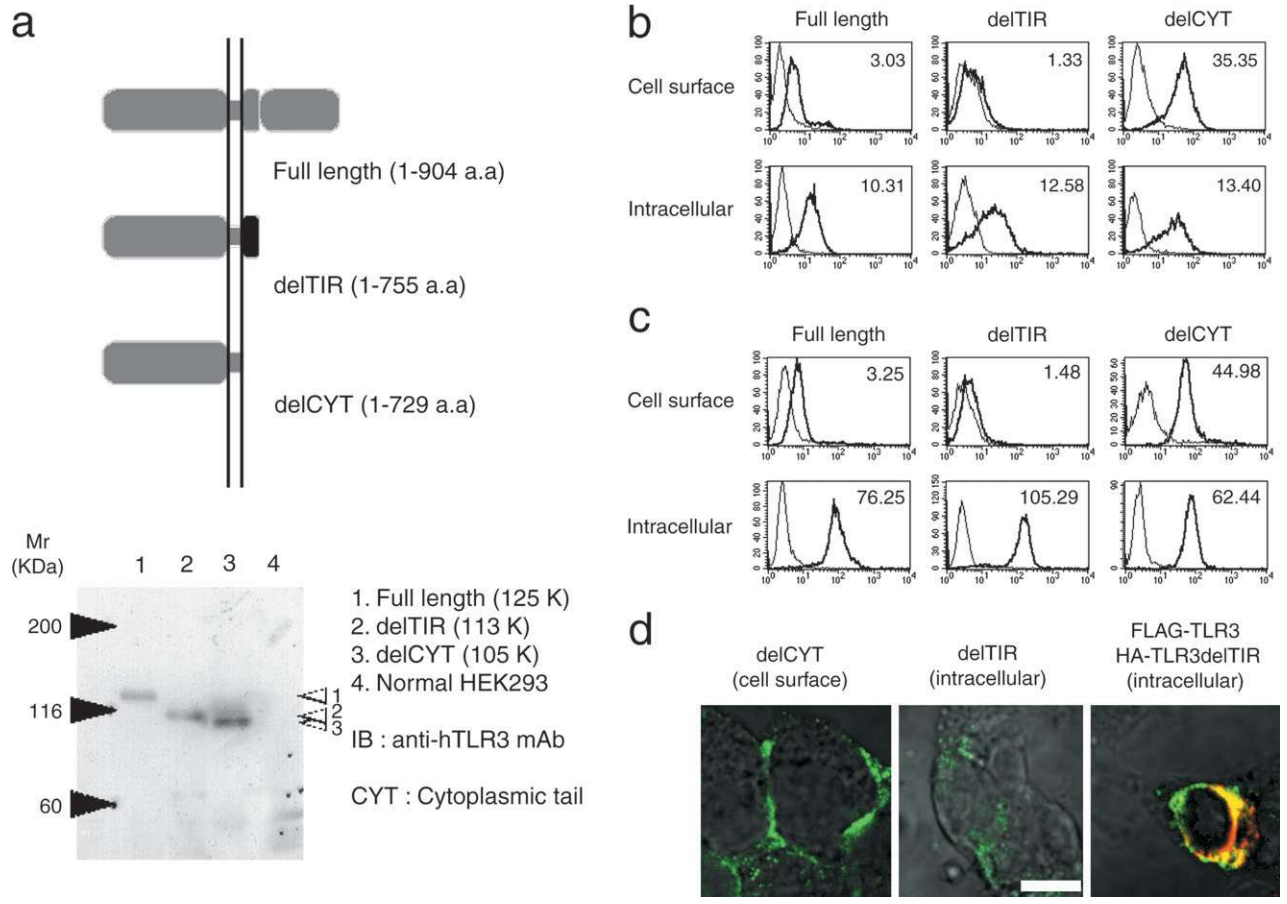

Fig. 3. The cytoplasmic linker region of TLR3 determines intracellular localization of TLR3. (a) Constructs of truncated form of TLR3. Each truncated construct was stably expressed in HEK293 cells. TLR3 proteins expressed in HEK293 cells were detected by immunoblotting with anti-TLR3 peptide mAb (2 μg/ml). Lane 1, full-length TLR3; lane 2, delTIR; lane 3, delCYT, lane 4, un-transfected HEK293 cells. Open arrowheads indicate intact and truncated forms of TLR3 proteins. (b) Expression profiles of TLR3delTIR and TLR3delCYT in HEK293 cells. Localization patterns of truncated forms of TLR3 were analyzed by flow cytometry using anti-TLR3 mAb (TLR3.7) as described in Fig. 1, and compared with intact TLR3. (c) Expression profiles of TLR3delTIR and TLR3delCYT in Ba/F3 cells. Full-length TLR3 and each truncated construct were stably expressed in Ba/F3 cells. Localization analysis was performed as described above. Inset values indicate the mean fluorescent intensities specific for the anti-TLR3 mAb. (d) TLR3delTIR co-localizes with full-length TLR3. Immunofluorescence staining of HEK293 cells stably expressing TLR3delCYT or TLR3delTIR was performed as described in Fig. 2(a). Left panel, cell-surface staining of TLR3delCYT; center panel, intracellular staining of TLR3delTIR. Right panel, HEK293 cells were transiently co-transfected with pCMV-FLAG-TLR3 and pEFBOS-HA-TLR3delTIR. After 24 h, transfected cells were harvested and plated on glass coverslips coated with poly-L-lysine. FLAG-TLR3 was stained with anti-FLAG mAb followed by Alexa 488-conjugated secondary antibody (green) and HA-TLR3delTIR was stained with rabbit anti-HA pAb followed by Alexa 594-conjugated secondary antibody (red). The stained cells were analyzed by confocal microscopy. TLR3delTIR co-localized with full-length TLR3 (indicated as yellow). Bar, 10 μm.

binding. Cells were then analyzed using a FACS Calibur flow cytometer (BD Bioscience).

Confocal microscopy

Monocyte-derived iDCs attached to glass slides were prepared using Cytospin (Thermo Shandon, Cheshire, UK). HEK293 cells were cultured on glass coverslips coated with poly-L-lysine (BD Bioscience). The adherent cells were fixed for 30 min with 3% formaldehyde in PBS and permeabilized with 0.5% saponin in 1% BSA/PBS for 30 min, and then washed four times with PBS. After the cells were soaked in 1% BSA/PBS, they were treated for 1 h at room temperature with anti-TLR3 mAb (TLR3.7) or normal mouse IgG (Sigma) (final 20 μg/ml) in 1% BSA/PBS. The cells were then washed with 1% BSA/PBS and treated for 30 min at room temperature with FITC-labeled goat anti-mouse IgG (American Qualex) (1:100), rhodamine B-conjugated goat anti-mouse IgG (Biosource) (1:100) or Alexa 488/594-conjugated goat anti-mouse IgG (Molecular Probes) (5 μg/ml) in PBS containing 10% (w/v) Block Ace (Yukijirushi, Sapporo, Japan). For dual staining with various organelle markers, after TLR3.7 staining, cells were treated with antibodies against organelle marker proteins and then FITC-, rhodamine B- or Alexa 488/594-conjugated secondary antibody. For mitochondoria and acidic organelle staining, cells were pretreated with Mytotracker (final 500 nM) for 45 min and DAMP (final 30 μM) for 60 min, respectively, before fixation. For plasma membrane staining, the adherent cells were stained with anti-CD46 pAb and Alexa 594-conjugated secondary antibody at 4°C before fixation and then stained with TLR3.7. For dual staining of epitope-tagged TLR3, transfected cells were harvested and plated on glass coverslips coated with poly-L-lysine. After fixation and

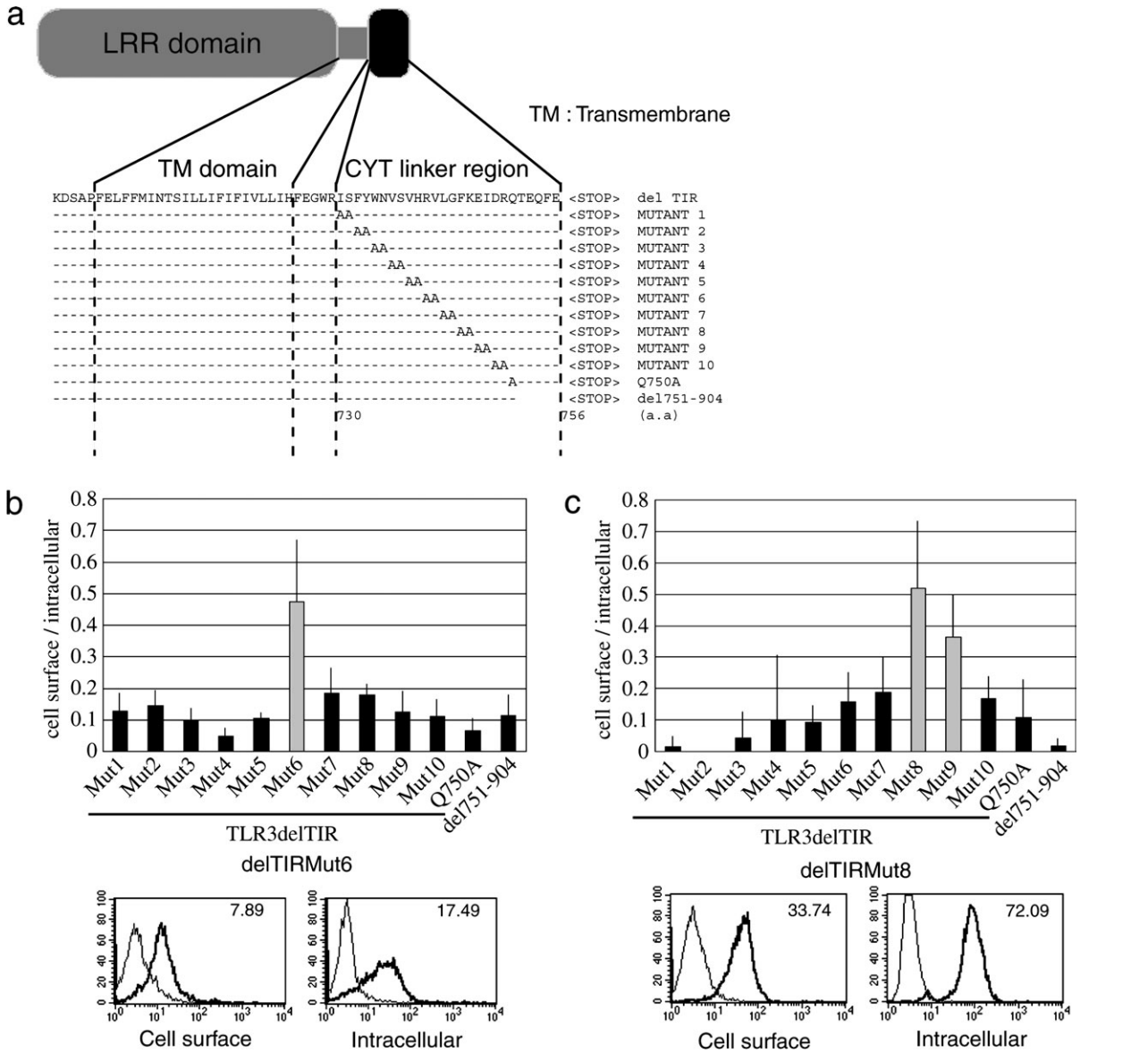

Fig. 4. The Arg^{740} and Val^{741} in the linker region are critical for intracellular localization of TLR3delTIR in HEK293 transfectants. (a) Constructs of various alanine-substituted mutants and one deletion mutant of TLR3delTIR. (b) Mutant 6 of TLR3delTIR (delTIRMut6) is transported to the cell surface in HEK293 transfectants. Upper panel, relative cell surface localization of TLR3delTIR mutants. Each mutant was stably expressed in HEK293 cells and the localization profiles were analyzed by flow cytometry. Cell surface expression of each mutant was quantified as a ratio of mean fluorescence intensity of cell surface vs that of cytoplasm. Flow cytometric analysis was performed on 3–10 separate clones in each mutant. Lower panel, expression profile of delTIRMut6 in HEK293 transfectants. Thick line, TLR3 staining; thin line, control mouse IgG1. Inset values indicate the mean fluorescence intensities specific for the anti-TLR3 mAb. Representative data from a minimum of three separate clones are shown. (c) Mutant 8 and Mutant 9 of TLR3delTIR (delTIRMut8 and delTIRMut9) are largely expressed on the cell surface in Ba/F3 transfectants. Upper panel, relative cell surface localization of TLR3delTIR mutants. Each mutant was stably expressed in Ba/F3 cells and the localization profiles were analyzed by flow cytometry. Lower panel, expression profiles of delTIRMut8 in Ba/F3 transfectants. Representative data from a minimum of three separate clones are shown.

permeabilization, cells were stained with anti-FLAG mAb (final 10 μg/ml) and anti-HA pAb (2.5 μg/ml). The stained cells were visualized at ×60 magnification under a FLUOVIEW (Olympus, Tokyo, Japan). Images were captured using the attached computer software, FLUOVIEW.

Reporter gene assay

HEK293 cells (1×10^6 cells/well) in 6-well plates were transiently transfected with p-125 luc (IFN-β promoter) (0.5 μg) or NF-κB reporter plasmid (0.5 μg; Stratagene, La Jolla, CA), pCMVβ or phRL-TK (internal control vector, 0.025 μg) and the indicated expression vectors (11). The total amount of DNA (4 μg) was kept constant by adding empty vector. In some experiments, HEK293 cells (2×10^5 cells/well) in 24-well plates were transfected with reporter plasmid (0.1 μg), internal control vector (2.5 ng) and indicated expression vectors. The total amount of DNA (0.8 μg) was kept constant by adding empty vector. After 24 h, cells were

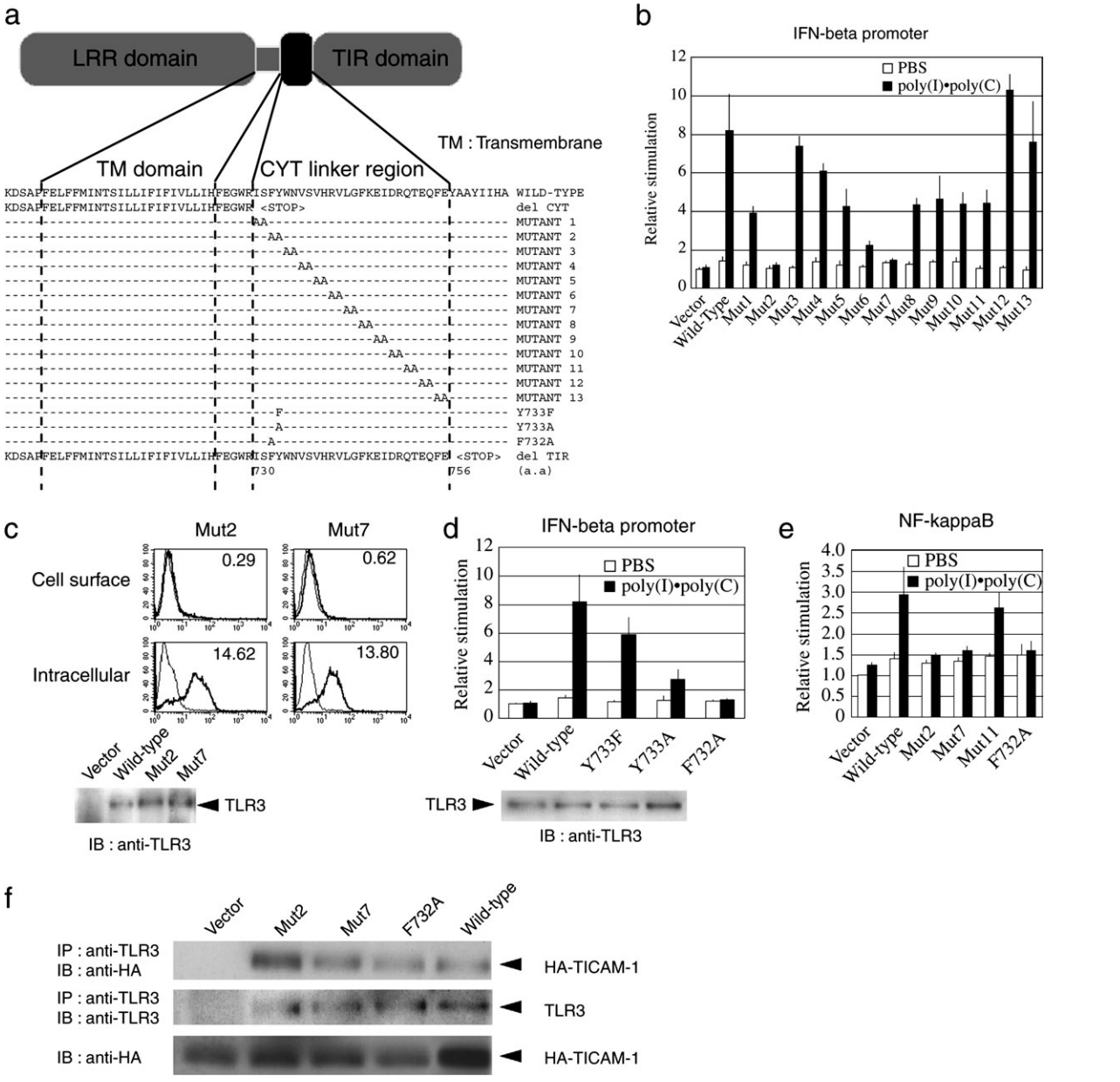

Fig. 5. Cytoplasmic linker region of TLR3 is involved in TLR3-mediated signaling. (a) Constructs of various alanine-substituted TLR3 mutants. (b) Mutant 2 (Mut2) and Mutant 7 (Mut7) completely lost ability to mediate poly(I)•poly(C)-induced IFN-β promoter activation. HEK293 cells were transiently transfected with plasmids encoding empty vector, TLR3, or various alanine-substituted mutants, together with p-125 luc (IFN-β promoter) reporter plasmid. After 24 h, the cells were treated with PBS or poly(I)•poly(C) (2 μg/ml). Luciferase activities are shown as mean relative stimulation plus S.D. Representative data from a minimum of three separate experiments are shown. (c) Localization profiles of dysfunctional alanine-substituted TLR3 mutants in HEK293 cells. HEK293 cells stably expressing dysfunctional TLR3 mutants (Mut2 and Mut7) were prepared. Localization of these TLR3 mutants was analyzed by flow cytometry using anti-TLR3 mAb (TLR3.7) as described in Fig. 1. Thick line, TLR3 staining; thin line, control mouse IgG1. Inset values indicate the mean fluorescent intensities specific for the anti-TLR3 mAb. Lower panel, immunoblotting of intact and mutated TLR3 expressed in HEK293 cells using anti-TLR3 peptide mAb. (d) Phe^{732} is essential for TLR3-mediated IFN-beta promoter activation. The luciferase assay was performed as in (b). Lower panel, immunoblotting of intact and mutated TLR3 expressed in HEK293 cells using anti-TLR3 peptide mAb. (e) The cytoplasmic linker region plays an important role in TLR3-mediated NF-κB activation. HEK293 cells were transiently transfected with plasmid encoding empty vector, TLR3, Mut2, Mut7, F732A, together with NF-κB reporter plasmid. The luciferase assay was performed as in (b). (f) Binding activity of dysfunctional TLR3 mutants to TICAM-1. HEK293 cells were transiently transfected with plasmids encoding empty vector, TLR3 or various alanine-substituted TLR3 mutants, together with HA-TICAM-1 expression plasmid. Intact and mutated TLR3 were immunoprecipitated with TLR3.7, and subjected to SDS–PAGE followed by immunoblotting. Co-precipitated TICAM-1 was detected by anti-HA pAb (upper panel). The membrane was reprobed with anti-TLR3 peptide antibody (middle panel). TICAM-1 expression was confirmed by immunoblotting of whole-cell lysates (lower panel).

harvested, added to 24-well plates (2×10^5 cells/well) and stimulated with PBS, poly(I)•poly(C) (2 μg/ml), or LPS (100 ng/ml) for 6 h. Cells were then lysed in lysis buffer (Promega) and assayed for dual luciferase activities (Firefly and Renilla luciferase activities) and β-galactosidase activity (internal control). Luciferase activity was normalized by β-galactosidase or Renilla luciferase activity and expressed as the fold stimulation relative to the activity in vector-transfected cells or PBS-stimulated cells.

Immunoprecipitation

HEK293 cells in 6-well plates were transfected with expression vectors containing various mutated TLR3 constructs (3 μg) together with TICAM-1-HA expression vector (0.5 μg) (11). The total amount of DNA (4 μg) was kept constant by adding empty vector. After 24 h, the cells were lysed in lysis buffer (20 mM Tris–HCl, pH 7.5, containing 150 mM NaCl, 1% NP-40, 10 mM EDTA, 25 mM iodoacetamide and 2 mM PMSF). TLR3 was immunoprecipitated with anti-TLR3 mAb (TLR3.7) and subjected to SDS–PAGE (7.5%) under reducing conditions, followed by immunoblotting with anti-HA pAb (Sigma) or anti-TLR3 mAb (BioCarta).

Results

TLR3 localizes to specific intracellular organelles

The localization of TLR3 was first analyzed by flow cytometry of HEK293 cells stably expressing human TLR3 (293-TLR3). TLR3 was predominantly present inside the cells and only slightly expressed on the cell surface (Fig. 1). In contrast, TLR2 was surface-expressed in HEK293 cells stably expressing human TLR2 (293-TLR2), as previously reported (Fig. 1) (26,27).

To identify the intracellular compartment where TLR3 is enriched, we performed immunofluorescence analysis using anti-TLR3 mAb, TLR3.7, in 293-TLR3. TLR3 was found to localize to the perinuclear compartment and scattered endosome-like vesicles (Fig. 2a). Cell-surface TLR3 could not be detected by this method, reflecting its low level. Dual staining and confocal microscopic analysis revealed that organelle markers, MytoTracker for mitochondria (data not shown) and Rab5a for early endosomes did not co-localize with TLR3-enriched compartments (Fig. 2b) (28). WGA, a marker for TGN and trans-Golgi, syntaxin 7 for late endosomes, and DAMP for an acidic organelle were only partly merged with TLR3-enriched compartments (29,30). To directly visualize TLR3 localization, we engineered a green fluorescent protein (GFP)–TLR3 chimeric construct and established stable HEK293 transfectants expressing GFP-fused TLR3. The localization pattern of the GFP–TLR3 fusion receptor in HEK293 cells matched that of wild-type TLR3 (data not shown).

Next, we analyzed the subcellular localization of endogenous TLR3 in monocyte-derived iDCs by immunofluorescence staining and confocal microscopy. Endogenous TLR3 was confined in scattered vesicles and did not co-localize with WGA-stained Golgi apparatus in iDCs (Fig. 2c). Interestingly, EEA-1 [early endosome marker, (31)] (data not shown), Lamp-2 [late endosome/lysosome marker, (32)] and DAMP did not co-localize with TLR3-positive vesicles. HLA-DR, which is highly expressed in the endosomal compartment in iDCs, did not co-localize with TLR3-positive vesicles. Recently, Latz *et al.* reported that endogenous and fluorescently tagged TLR9 are expressed in the endoplasmic reticulum (ER) of resting cells (33). In the case of TLR3, co-localization of TLR3 with calnexin, an ER-resident protein, was not observed in iDCs (Fig. 2c). These results suggest that TLR3 resides in as yet uncharacterized organelles in iDCs and 293-TLR3. The partial co-localization of transfected-TLR3 with WGA, syntaxin 7 and DAMP in HEK293 cells probably reflects overexpressed status in which TLR3 may overflow the transported pathway besides the targeted vesicles (Fig. 2b).

The cytoplasmic linker region determines intracellular localization of TLR3

The cytoplasmic tail of TLR3 contains 179 residues (725–904 aa) including the TIR domain (756–904 aa). To analyze the role of the cytoplasmic tail in the subcellular localization of TLR3, various truncated forms of TLR3 were stably expressed in HEK293 cells (Fig. 3a). Both TLR3delCYT lacking the cytoplasmic tail and TLR3delTIR lacking the TIR domain but retaining the proximal 31 amino acids were expressed with the expected molecular sizes to similar extents as intact TLR3 (Fig. 3a). Flow cytometric analysis showed that TLR3delCYT was predominantly expressed on the cell surface, while TLR3delTIR was expressed intracellularly, like intact TLR3 (Fig. 3b). Other truncated forms with a partially deleted TIR domain resided inside the cells (data not shown). Similar localization patterns were also observed in mouse B cell line Ba/F3 stably expressing the same constructs (Fig. 3c). The different distribution of TLR3delCYT and TLR3delTIR in HEK293 transfectants was confirmed by immunofluorescene staining (Fig. 3d, left and center panels). The localization pattern of TLR3delTIR matched that of full-length TLR3. Dual staining and confocal microscopic analysis of HEK293 cells transiently expressing TLR3delTIR and full-length TLR3 clearly showed the co-localization of TLR3delTIR with full-length TLR3 (Fig. 3d, right panel). Thus, the 26 amino acid residues (730–755) of the cytoplasmic tail outside the TIR domain (named the cytoplasmic 'linker' region) are critical for specifying the intracellular localization of TLR3.

To identify the residues determining intracellular localization of TLR3, we performed alanine scanning of the linker region. As shown in Fig. 4(a), alanine-substituted mutants of TLR3delTIR (Mutant 1~Mutant 10, Q750A) were stably expressed in HEK293 cells and their localization profiles were analyzed. Strikingly, Mutant 6 (RV740,741AA) appeared on the cell surface (Fig. 4b). Other TLR3 mutants behaved like the wild-type delTIR, being expressed inside the cells (Fig. 4b, upper panel). The C-terminal acidic residues (TExxE) in the linker region, a possible targeting signal for the endosomal membrane, is independent of TLR3 targeting, since the truncated mutant lacking these residues (del 751–904) was expressed intracellularly (Fig. 4b). These data demonstrated that the residues Arg^{740} and Val^{741} in the linker region are crucial for retaining TLR3 in the intracellular organelle. An interesting finding was that the critical residues for intracellular localization of TLR3 in Ba/F3 transfectants were different from those in 293 transfectants. When alanine-substituted mutants were stably expressed in Ba/F3 cells, Mutant 8 (FK744,745AA) and Mutant 9 (EI746,747AA) were surface-expressed (Fig. 4c). In Ba/F3

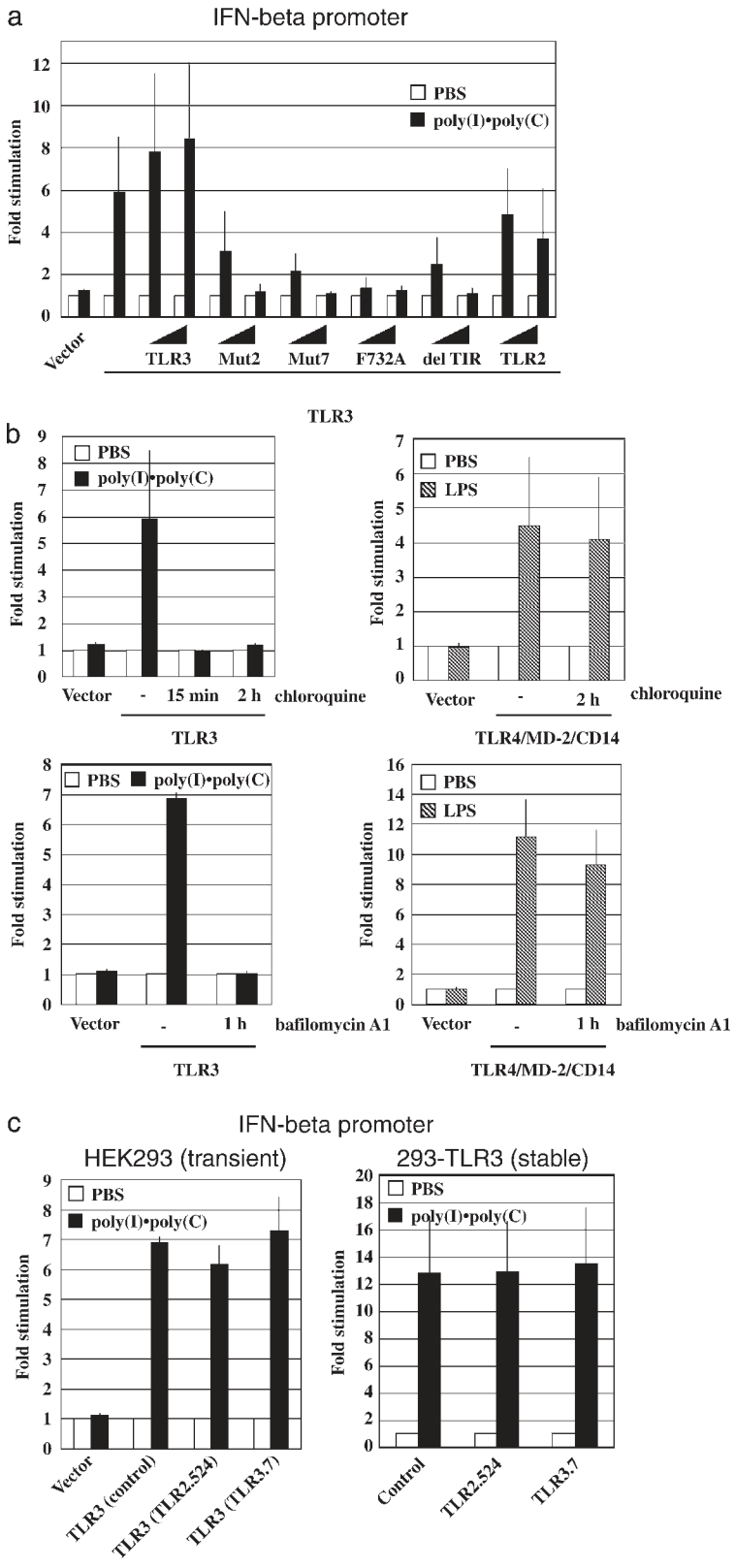

Fig. 6. TLR3-mediated signaling is initiated in the intracellular compartment that is enriched in TLR3. (a) Alanine-substituted mutants inhibit TLR3-mediated IFN-β promoter activation. HEK293 cells were transiently transfected with plasmids encoding TLR3 (0.5 μg) together with increasing amounts of expression vector for TLR3, Mut2, Mut7, F732A, delTIR, TLR2 (0.5 or 3.0 μg) and p-125 luc reporter plasmid. Luciferase activities are shown as mean relative stimulation plus S.D. Representative data from a minimum of three separate experiments are shown. (b) Chloroquine and bafilomycin A1 inhibit TLR3-mediated IFN-β promoter activation. HEK293 cells transiently transfected with plasmids encoding empty vector, TLR3

transfectants, TLR3 largely accumulated in the multivesicular bodies (MVBs), a subcellular compartment situated in endocytic trafficking pathways (22), which differed from TLR3 localization in iDCs and HEK293 transfectants. The residues FKEI at 744–747 in the linker region may specifically function as a sorting signal to MVBs in Ba/F3 transfectants, and the RV-containing region participates in sorting TLR3 to other vesicles.

TLR3 cytoplasmic linker region is essential for TLR3 signaling

To analyze the possible role of the linker region in TLR3 signaling, we prepared a series of alanine-substituted mutants of full-length TLR3 (Fig. 5a). All these mutants were normally expressed with the expected molecular sizes at similar levels as intact TLR3 (data not shown). First, we performed reporter gene assays in HEK293 cells transiently transfected with each mutated construct. Two mutants, Mutant 2 (Mut2; FY732,733AA) and Mutant 7 (Mut7; LG742,743AA), completely lost the ability to activate NF-κB and the IFN-β promoter upon poly(I)•poly(C) stimulation (Fig. 5b and e). These dysfunctional mutants localized intracellularly in stable HEK293 transfectants (Fig. 5c). The other mutants showed similar activity compared to wild-type TLR3 except Mutant 6, which had lower activity, presumably due to lower expression in the intracellular compartment.

Sarkar *et al.* reported that cytoplasmic tyrosine phosphorylation, especially Tyr^{759} in the TIR domain, affected dsRNA-TLR3 mediated signal transduction (34). As Tyr^{733} was substituted by alanine in Mut2 (FY732,733AA), we made additional single amino acid-substituted mutants, Y733F, Y733A and F732A, to identify the role of Tyr^{733} in TLR3 signaling (Fig. 5a). Western blot analysis showed that these mutated proteins were expressed to similar extents as wild-type TLR3 in HEK293 cells (Fig. 5d). Consistently with a previous report (34), the Y733F mutant had the ability to activate the IFN-β promoter, while the Y733A mutant had less such ability (Fig. 5d). The Tyr^{733} must not be a phospho-acceptor but partly contribute to steric organization of the linker region. Surprisingly, the F732A mutant did not activate NF-κB or the IFN-β promoter when expressed in HEK293 cells, indicating that the Phe^{732} is essential for TLR3-mediated NF-κB and IFN-β promoter activation by poly(I)•poly(C) stimulation (Fig. 5d and e).

The TIR domain of TLR3 is crucial for adaptor binding and signaling. The A795H mutant of TLR3, in which Ala^{795} in the TIR domain is substituted by histidine, has lost TICAM-1-binding ability (11). In reporter gene assays, the A795H mutant could not activate NF-κB or the IFN-β promoter upon poly(I)•poly(C) stimulation (11). We therefore investigated whether dysfunctional TLR3 mutants have the ability to bind to TICAM-1. Immunoprecipitation study using HEK293 cells revealed that all mutants could associate with TICAM-1 (Fig. 5f). Thus, mechanisms of the defectiveness of Mut2, Mut7 and F732A are completely different from that of A795H mutant.

TLR3-mediated signaling is initiated in the intracellular compartment in HEK293 transfectants

To clarify the role of the linker region in TLR3 function, experiments were performed using dominant–negative dysfunctional mutants, TLR3delTIR, Mut2, Mut7 and F732A. Poly(I)•poly(C)-induced IFN-β promoter activation mediated by wild-type TLR3 was inhibited by simultaneous expression of these mutants (Fig. 6a), suggesting that these mutated proteins were located in the same place as wild-type TLR3 and exhibited inhibitory function. Indeed, confocal microscopic analysis revealed that these TLR3 mutants co-localized with wild-type TLR3 in HEK293 cells (data not shown). The Phe^{732}, Leu^{742} and Gly^{743} in the TLR3 cytoplasmic linker region may play an important role in poly(I)•poly(C)-induced receptor dimerization followed by the recruitment of adaptor molecules.

In monocyte-derived iDCs, endosomal maturation is required for poly(I)•poly(C)-induced cytokine production (22). We tested the effects of chloroquine and bafilomycin A1, which inhibit endosomal maturation by different mechanisms, on TLR3-mediated IFN-β promoter activation in HEK293 cells expressing human TLR3. As shown in Fig. 6(b), poly(I)•poly(C)-induced IFN-β promoter activation was completely inhibited in the presence of chloroquine or bafilomycin A1. In contrast, LPS-induced IFN-β promoter activation was not affected, since TLR4/MD-2 complex was surface-expressed in HEK293 cells and mediated LPS signaling at the cell-surface (35). Furthermore, function-blocking mAb against TLR3, TLR3.7, did not block poly(I)•poly(C)-induced IFN-β promoter activation in HEK293 cells transiently or stably expressing human TLR3 (Fig. 6c). These results indicate that TLR3-mediated signaling occurs in the intracellular compartment, but not on the cell surface, in HEK293 transfectants, although a small number of TLR3 molecules were expressed on the cell surface (Fig. 1).

Discussion

The novel points we showed in the present study are that: (i) in monocyte-derived iDCs and HEK293 transfectants, TLR3 localizes to specific intracellular organelles where TLR3 signaling is initiated; (ii) the cytoplasmic linker region (residues 730–755) determines intracellular localization of TLR3; (iii) the determinants are within several amino acid residues in the linker region; (iv) Phe^{732}, Leu^{742} and Gly^{743} in the TLR3 cytoplasmic linker region are essential for ligand-induced NF-κB and the IFN-β promoter activation. Thus, the linker

(left panels) or TLR4 + CD14 + MD-2 (right panels) were pretreated with chloroquine (5 μg/ml) for 15 min or 2 h (upper panels) or bafilomycin A1 (1 μM) for 1 h (lower panels) and stimulated with PBS, 2 μg/ml of poly(I)•poly(C), or LPS (100 ng/ml) for 6 h. Luciferase activities are shown as mean relative stimulation plus S.D. Representative data from a minimum of three separate experiments are shown. (c) Anti-TLR3 mAb does not inhibit TLR3-mediated IFN-β promoter activation in HEK293 cells. HEK293 cells transiently transfected with plasmid encoding TLR3 together with p-125 luc reporter plasmid (left panel) or 293-TLR3 (see Fig. 1) transfected with only p-125 luc reporter plasmid (right panel) were pretreated with 20 μg/ml of TLR3.7 or TLR2.524 for 1 h, and stimulated with PBS or 2 μg/ml of poly(I)•poly(C) for 6 h. Luciferase activities are shown as mean relative stimulation plus S.D.

region of TLR3 plays an important role in receptor localization and signaling, suggestive of the presence of new modes for regulation of signal and distribution in TLRs.

Receptor localization and the nidus for signaling have been extensively studied with TLR4 and TLR9. TLR4, in association with MD-2, localizes to the plasma membrane and the Golgi apparatus in monocytes, macrophages and HEK293 cells stably expressing fluorescent TLR4 (35), and to the Golgi apparatus in intestinal epithelial cells (36). The TLR4-CD14-MD-2 complex signals the presence of LPS at the cell surface in stable HEK293-transfected cells, but this occurs instead at the Golgi apparatus in intestinal epithelial cells (35,36). On the other hand, TLR9, known to be a receptor for bacterial CpG-DNA (37), localizes to the lysosomal compartment where CpG-induced signaling starts (38). Endosomal acidification inhibitors such as bafilomycin A1 and chloroquine completely block CpG DNA-induced cytokine production in macrophages (39,40), indicating that TLR9-mediated signaling requires endosomal maturation. According to a recent report, TLR9 is expressed in the ER of resting cells and redistributes to the endosomal compartment in response to CpG DNA stimulation (33). The molecular mechanism of intracellular localization of TLR9, however, remains elusive.

In the case of TLR3, expression and localization are regulated in a cell-type specific manner. TLR3 localizes to the plasma membrane and intracellular compartment in human fibroblasts, while monocyte-derived iDCs and $CD11c^+$ myeloid blood DCs express TLR3 intracellularly, not on the cell surface (10,22). Immunoelectron microscopic analysis revealed that human TLR3, when stably expressed in mouse B-cell line Ba/F3, largely accumulated in MVBs (22). Here we showed by immunofluorescence analysis that TLR3 selectively accumulates in specific but as yet unidentified vesicles in DCs and 293-TLR3.

As expected, the function-blocking mAb against TLR3 (TLR3.7) could not inhibit TLR3-mediated signaling in these cells. In addition, dsRNA-induced cytokine production in iDCs and IFN-β promoter activation in HEK293 transfectants were completely blocked by pretreatment of the cells with chloroquine or bafilomycin A1 (22, Fig. 6b). These reagents are known to inhibit endosomal maturation by neutralizing the pH of endocytic organelles or specifically inhibiting vacuolar type H^+-ATPase, respectively (41). Thus, TLR3 signaling in iDCs and HEK293 transfectants occurs intracellularly in conjunction with endosomal maturation, as observed for TLR9 signaling. However, based on the fact that TLR3 does not localize in the late endosomes/lysosomes or ER in iDCs and 293-TLR3 before or after poly(I)•poly(C) stimulation (Fig. 2c, data not shown), signaling mechanisms between TLR3 and TLR9 must be different. Although how intracellular TLR3 recognizes dsRNA remains unknown, our preliminary data suggest the presence of another receptor that recognizes dsRNA on the cell surface in iDCs (M. Matsumoto, S. Nishikawa, K. Funami and T. Seya, unpublished data). Chloroquine treatment may affect the accessibility of internalized dsRNA to intracellular TLR3 by disturbing endosomal maturation. Further characterization of TLR3-enriched compartment will be needed to clarify how TLR3 recognizes dsRNA.

We first demonstrated in this study that the cytoplasmic linker region of TLR3 directed a receptor to intracellular compartment. Targeting signals placed in the cytoplasmic tail determine the localization of many transmembrane receptors. A tyrosine-based motif and dileucine-based motif are known to be endocytic/lysosomal targeting signals (42). TLR3 has no reported targeting sequences in its cytoplasmic tail except for the tyrosine-based motif (733–736, YWNV) in the linker region. However, these residues were not critical for the intracellular localization of TLR3, as their mutations in TLR3delTIR did not affect receptor localization (Mut2 and Mut3 in Fig. 4b). In contrast, mutations of Arg^{740} and Val^{741} altered localization of TLR3delTIR in HEK293 transfectants (Mut6 in Fig. 4b), indicating that these residues are critical for retaining TLR3 inside the cells. Notably, these amino acids are not included in any kind of motif found in proteins with subcellular localization, which suggests the presence of novel mechanism for distribution of TLR3.

In Ba/F3 transfectants, human TLR3 predominantly resides on the internal membrane of MVBs and a small number of TLR3 molecules were detected on the limiting membrane of MVBs (22). Recent studies have defined the MVB-sorting pathway, in which ubiquitin was identified as a signal for the efficient sorting of membrane proteins into inner vesicles of MVBs (43). One possible mechanism of intracellular localization of TLR3 in Ba/F3 cells is that Lys^{745} in the linker region may be ubiquitinated, resulting in sorting to the MVB pathway. Indeed, substitution of the residues FKEI at 744–747 in the linker region by alanines affected the intracellular localization of TLR3delTIR in Ba/F3 cells (Fig. 5c). Although the precise mechanisms of the intracellular localization of TLR3 in DCs and HEK293 cells are still unclear, unidentified molecules must be specifically associated with the linker region and control the intracellular localization of TLR3.

The functional roles of the TIR domain in TLR-mediated signaling have been well characterized. In contrast, the roles of the cytoplasmic region outside the TIR domain in TLR signaling have not been clarified. We found that Phe^{732}, Leu^{742} and Gly^{743} in the linker region were essential for TLR3-mediated signaling, since mutants of these residues could not transmit signals upon poly(I)•poly(C) stimulation. They possessed TICAM-1 binding ability but exhibited a dominant-negative effect. One possible interpretation of this finding is that the mutations in these residues result in abrogation of ligand-induced receptor dimerization secondary to TICAM-1 recruitment. Co-immunoprecipitation of these mutants and TICAM-1 observed when they were expressed in HEK293 cells may reflect the interaction between mutants-TIR and TICAM-1-TIR. Interestingly, essential residues for signaling (FY732,733; LG742,743) are conserved across human, mouse and *Fugu rubripes* TLR3 (44).

Among the TLR family proteins, there is less sequence similarity in the cytoplasmic linker region than in the TIR domain. TLR7, TLR8 and TLR9, however, have highly-conserved linker regions, which differ from that of TLR3. The different TLR linker regions may have different functions, as observed for TLR2, in which tyrosine phosphorylation was important for TLR2 signaling (45). Identification of molecules associated with the TLR3 cytoplasmic linker region is currently in progress in our laboratory and should yield insight into the molecular mechanism as to how TLR3 is targeted to the intracellular compartment and where TLR3 recognizes virus-derived dsRNA in DCs.

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Abbreviations

DAMP	3-(2,4-denitroanilino)-3′-amino-*N*-methyldipropylamine
DCs	dendritic cells
GFP	green fluorescent protein
iDC	immature DC
HA	hemagglutinin
IRF	interferon regulatory factor
LRR	leucine-rich repeat
MVB	multivesicular body
PKR	dsRNA-activated protein kinase
TICAM-1	TIR-containing adaptor molecule-1
TIR	Toll-IL-1 receptor homology
TLR	Toll-like receptor
TM	transmembrane
TRIF	TIR domain-containing adaptor-inducing IFN
WGA	wheat germ agglutinin

References

1 Medzhitov, R. and Janeway, C. A. Jr 1997. Innate immunity: the virtues of a nonclonal system of recognition. *Cell* 91:295.
2 Aderem, A. and Ulevitch, R. J. 2000. Toll-like receptors in the induction of the innate immune response. *Nature* 406:782.
3 Underhill, D. M. and Ozinsky, A. 2002. Toll-like receptors: key mediators of microbe detection. *Curr. Opin. Immunol.* 14:103.
4 Medzhitov, R., Preston-Hurlburt, P. and Janeway, C. A. Jr. 1997. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature* 388:394.
5 Rock, F. L., Hardiman, G., Timans, J. C., Kastelein, R. A. and Bazan, J. F. 1998. A family of human receptors structurally related to *Drosophila* Toll. *Proc. Natl Acad. Sci. USA* 95:588.
6 Takeda, K., Kaisho, T. and Akira, S. 2003. Toll-like receptors. *Annu. Rev. Immunol.* 21:335.
7 Dunne, A. and O'Neill, L. A. 2003. The interleukin-1 receptor/Toll-like receptor superfamily: signal transduction during inflammation and host defense. *Sci. STKE.* 171:3.
8 Imler, J. L. and Hoffmann, J. A. 2003. Toll signaling: the TIReless quest for specificity. *Nat. Immunol.* 4:105.
9 Alexopoulou, L., Holt, A. C., Medzhitov, R. and Flavell, R. A. 2001. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* 413:732.
10 Matsumoto, M., Kikkawa, S., Kohase, M., Miyake, K. and Seya, T. 2002. Establishment of a monoclonal antibody against human Toll-like receptor 3 that blocks double-stranded RNA-mediated signaling. *Biochem. Biophys. Res. Commun.* 293:1364.
11 Oshiumi, H., Matsumoto, M., Funami, K., Akazawa, T. and Seya, T. 2003. TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. *Nat. Immunol.* 4:161.
12 Yamamoto, M., Sato, S., Mori, K., Hoshino, K., Takeuchi, O., Takeda, K. and Akira, S. 2002. Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling. *J. Immunol.* 169:6668.
13 Yamamoto, M., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Sanjo, H., Takeuchi, O., Sugiyama, M., Okabe, M., Takeda, K. and Akira, S. 2003. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science* 301:640.
14 Taniguchi, T., Ogasawara, K., Takaoka, A. and Tanaka, N. 2001. IRF family of transcription factors as regulators of host defense. *Annu. Rev. Immunol.* 19:623.
15 Peters, K. L., Smith, H. L., Stark, G. R. and Sen, G. C. 2002. IRF-3-dependent, NFkappa B- and JNK-independent activation of the 561 and IFN-beta genes in response to double-stranded RNA. *Proc. Natl Acad. Sci. USA* 99:6322.
16 Muller, U., Steinhoff, U., Reis, L. F., Hemmi, S., Pavlovic, J., Zinkernagel, R. M. and Aguet, M. 1994. Functional role of type I and type II interferons in antiviral defense. *Science* 264: 1918.
17 Der, S. D. and Lau, A. S. 1995. Involvement of the double-stranded-RNA-dependent kinase PKR in interferon expression and interferon-mediated antiviral activity. *Proc. Natl Acad. Sci. USA* 92:8841.
18 Yang, Y. L., Reis, L. F., Pavlovic, J., Aguzzi, A., Schafer, R., Kumar, A., Williams, B. R., Aguet, M. and Weissmann, C. 1995. Deficient signaling in mice devoid of double-stranded RNA-dependent protein kinase. *EMBO J.* 14:6095.
19 Cella, M., Salio, M., Sakakibara, Y., Langen, H., Julkunen, I. and Lanzavecchia, A. 1999. Maturation, activation and protection of dendritic cells induced by double-stranded RNA. *J. Exp. Med.* 189:821.
20 Muzio, M., Bosisio, D., Polentarutti, N., D'Amico, G., Stoppacciaro, A., Mancinelli, R., van't Veer, C., Penton-Rol, G., Ruco, L. P., Allavena, P. and Mantovani, A. 2000. Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. *J. Immunol.* 164:5998.
21 Cario, E. and Podolsky, D. K. 2000. Differential alteration in intestinal epithelial cell expression of toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. *Infect. Immun.* 68:7010.
22 Matsumoto, M., Funami, K., Tanabe, M., Oshiumi, H., Shingai, M., Seto, Y., Yamamoto, A. and Seya, T. 2003. Subcellular localization of toll-like receptor 3 in human dendritic cells. *J. Immunol.* 171:3154.
23 Uehori, J., Matsumoto, M., Tsuji, S., Akazawa, T., Takeuchi, O., Akira, S., Kawata, T., Azuma, I., Toyoshima, K. and Seya, T. 2003. Simultaneous blocking of human Toll-like receptors 2 and 4 suppresses myeloid dendritic cell activation induced by *Mycobacterium bovis* bacillus Calmette–Guerin peptidoglycan. *Infect. Immun.* 71:4238.
24 Seya, T., Turner, J. R. and Atkinson, J. P. 1986. Purification and characterization of a membrane protein (gp45-70) that is a cofactor for cleavage of C3b and C4b. *J. Exp. Med.* 163:837.
25 Mizushima, S. and Nagata, S. 1990. pEF-BOS, a powerful mammalian expression vector. *Nucleic Acids Res.* 18:5322.
26 Underhill, D. M., Ozinsky, A., Hajjar, A. M., Stevens, A., Wilson, C. B., Bassetti, M. and Aderem, A. 1999. The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. *Nature* 401:811.
27 Ozinsky, A., Underhill, D. M., Fontenot, J. D., Hajjar, A. M., Smith, K. D., Wilson, C. B., Schroeder, L. and Aderem, A. 2000. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. *Proc. Natl Acad. Sci. USA* 97:13766.
28 Chavrier, P., Parton, R. G., Hauri, H. P., Simons, K. and Zerial, M. 1990. Localization of low molecular weight GTP binding proteins to exocytic and endocytic compartments. *Cell* 62:317.
29 Anderson, R. G., Falck, J. R., Goldstein, J. L. and Brown, M. S. 1984. Visualization of acidic organelles in intact cells by electron microscopy. *Proc. Natl Acad. Sci. USA* 81:4838.
30 Nakamura, N., Yamamoto, A., Wada, Y. and Futai, M. 2000. Syntaxin 7 mediates endocytic trafficking to late endosomes. *J. Biol. Chem.* 275:6523.
31 Mu, F. T., Callaghan, J. M., Steele-Mortimer, O., Stenmark, H., Parton, R. G., Campbell, P. L., McCluskey, J., Yeo, J. P., Tock, E. P. and Toh, B. H. 1995. EEA1, an early endosome-associated protein. EEA1 is a conserved alpha-helical peripheral membrane protein flanked by cysteine "fingers" and contains a calmodulin-binding IQ motif. *J. Biol. Chem.* 270:13503.
32 Karlsson, K. and Carlsson, S. R. 1998. Sorting of lysosomal membrane glycoproteins lamp-1 and lamp-2 into vesicles distinct from mannose 6-phosphate receptor/gamma-adaptin vesicles at the trans-Golgi network. *J. Biol. Chem.* 273:18966.
33 Latz, E., Schoenemeyer, A., Visintin, A., Fitzgerald, K. A., Monks, B. G., Knetter, C. F., Lien, E., Nilsen, N., Espevik, T. and Golenbock, D.

T. 2004. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. *Nature Immunol.* 2:190.
34 Sarkar, S. N., Smith, H. L., Rowe, T. M. and Sen, G. C. 2003. Double-stranded RNA signaling by Toll-like receptor 3 requires specific tyrosine residues in its cytoplasmic domain. *J. Biol. Chem.* 278:4393.
35 Latz, E., Visintin, A., Lien, E., Fitzgerald, K. A., Monks, B. G., Kurt-Jones, E. A., Golenbock, D. T. and Espevik, T. 2002. Lipopolysaccharide rapidly traffics to and from the Golgi apparatus with the toll-like receptor 4-MD-2-CD14 complex in a process that is distinct from the initiation of signal transduction. *J. Biol. Chem.* 277:47834.
36 Hornef, M. W., Frisan, T., Vandewalle, A., Normark, S. and Richter-Dahlfors, A. 2002. Toll-like receptor 4 resides in the Golgi apparatus and colocalizes with internalized lipopolysaccharide in intestinal epithelial cells. *J. Exp. Med.* 195:559.
37 Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K. and Akira, S. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature* 408:740.
38 Ahmad-Nejad, P., Hacker, H., Rutz, M., Bauer, S., Vabulas, R. M. and Wagner, H. 2002. Bacterial CpG-DNA and lipopolysaccharides activate Toll-like receptors at distinct cellular compartments. *Eur. J. Immunol.* 32:1958.
39 Hacker, H., Mischak, H., Miethke, T., Liptay, S., Schmid, R., Sparwasser, T., Heeg, K., Lipford, G. B. and Wagner, H. 1998. CpG-DNA-specific activation of antigen-presenting cells requires stress kinase activity and is preceded by non-specific endocytosis and endosomal maturation. *EMBO J.* 17:6230.
40 Yi, A. K., Tuetken, R., Redford, T., Waldschmidt, M., Kirsch, J. and Krieg, A. M. 1998. CpG motifs in bacterial DNA activate leukocytes through the pH-dependent generation of reactive oxygen species. *J. Immunol.* 160:4755.
41 Yoshimori, T., Yamamoto, A., Moriyama, Y., Futai, M. and Tashiro, Y. 1991. Bafilomycin A1, a specific inhibitor of vacuolar-type H(+)-ATPase, inhibits acidification and protein degradation in lysosomes of cultured cells. *J. Biol. Chem.* 266:17707.
42 Bonifacino, J. S. and Lippincott-Schwartz, J. 2003. Opinion: Coat proteins: shaping membrane transport. *Nat. Rev. Mol. Cell. Biol.* 4:409.
43 Katzmann, D. J., Odorizzi, G. and Emr, S. D. 2002. Receptor downregulation and multivesicular-body sorting. *Nat. Rev. Mol. Cell. Biol.* 3:893.
44 Oshiumi, H., Tsujita, T., Shida, K., Matsumoto, M., Ikeo, K. and Seya, T. 2003. Prediction of the prototype of the human Toll-like receptor gene family from the pufferfish, *Fugu rubripes*, genome. *Immunogenetics* 54:791.
45 Arbibe, L., Mira, J. P., Teusch, N., Kline, L., Guha, M., Mackman, N., Godowski, P. J., Ulevitch, R. J. and Knaus, U. G. 2000. Toll-like receptor 2-mediated NF-kappa B activation requires a Rac1-dependent pathway. *Nat. Immunol.* 1:533.