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Spatiotemporal Mobilization of Toll/IL-1 Receptor Domain-Containing Adaptor Molecule-1 in Response to dsRNA

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TLR3 recognizes viral dsRNA and induces antiviral immune responses. TLR3-mediated cell activation relies on Toll/IL-1R (TIR) domain-containing adaptor molecule-1 (TICAM-1), also named TIR domain-containing adaptor inducing IFN-β or TRIF), which recruits downstream signaling molecules to activate the transcription factors IFN regulatory factor 3 (IRF-3) and NF-κB. The mechanisms by which TICAM-1 is activated and transmits signals remain largely unknown. In this study we show that TICAM-1 alters its distribution profile from a diffuse cytoplasmic form to a speckle-like structure in response to dsRNA. The receptor-interacting protein 1 (RIP1), a crucial signaling molecule for TICAM-1-mediated NF-κB activation, accumulated in the TICAM-1 speckles. In addition, NF-κB-activating kinase-associated protein 1 (NAP1), a downstream molecule linking TICAM-1 and the IRF-3-activating kinase TBK1 (TANK-binding kinase 1), was also recruited to the TICAM-1 speckles. Notably, a transient colocalization of TICAM-1 and TLR3 was observed before the extensive formation of the TICAM-1 speckles. Thus, the spatiotemporal mobilization of TICAM-1 in response to dsRNA and the formation of the TICAM-1 speckles containing RIP1 and NAP1 are important for the activation of the TLR3-TICAM-1 pathway.

signaling molecules in human epithelial cell lines by live cell imaging and confocal immunofluorescence analyses. TICAM-1 appears to be mobile depending on the activation state of its coupling receptor, TLR3. Here we demonstrated that TICAM-1 alters the distribution profile concomitant with its activation from a diffuse cytoplasmic form to a speckle-like structure containing RIP1 and NAP1 where TICAM-1-mediated signaling is initiated.

Materials and Methods
Reagents and cell culture
Anti-human TLR3 mAb (clone TLR3.7) was generated in our laboratory (3). Anti-RIP1 and anti-IRF-3 mAbs were purchased from BD Biosciences. Anti-early endosome Ag 1 (EEA1) polyclonal Ab (pAb) was from Abcam. Bio-reagents, anti-calnexin pAb was from StressGen Biotechnologies, anti-FLAG M2 mAb, anti-HE pAb, 4’6’-diamidino-2-phenylindole (DAPI) dihydrochloride and benzoyloxyacetyl-val-Ala-Asp-fluoromethyl ketone (z-VAD-fmk) were from Sigma-Aldrich, anti-mannose-6-phosphate receptor (MPR) pAb was from Abcam, anti-Myc mAb was from NeoMarkers (Lab Vision), and anti-p115 pAb was from Calbiochem. LysoTracker Red DND-99 and Alexa Fluor 568- and Alexa Fluor 488-conjugated secondary Abs were from Invitrogen Life Technologies. Polyribosinosinic/polyribocytidylic acid (poly(I:C)) was from Ambion Biosciences, and peptidoglycan (PGN) from Staphylococcus aureus was from Fluka Chemie. Rabbit anti-human TICAM-1 pAb was generated in our laboratory using recombinant TICAM-1 (aa 1–361) as the immunogen. HeLa cells were maintained in MEM (Nissui Pharmaceutical) supplemented with 10% heat-inactivated FCS (Invitrogen Life Technologies).

Plasmids
Complementary DNAs for human TLR3, TICAM-1, and RIP1 were cloned in our laboratory by RT-PCR and ligated at the cloning site of the expression vector, pFLAG-CMV, pEF-BOS, p3FLAG14 (C-terminal 3xFLAG tag), pECFP-N1 (C-terminal cyan fluorescent protein (CFP) tag), or pEYFP-N1 (C-terminal yellow fluorescent protein (YFP) tag). pECFP-C1 (N-terminal CFP tag) was used to make TICAM-1 tagged with CFP. cDNAs of NAP1 were kindly provided by Dr. M. Nakanishi (Nagoya City University, Nagoya, Japan).

Confocal microscopy
HeLa cells (1.0 × 10^5 cells/well) were plated onto a micro cover glass (Matsumani Glass) in a 24-well plate. The following day, cells were transfected with the indicated plasmids using Lipofectectamine Plus (Invitrogen Life Technologies) or FuGENE HD (Roche). The total amounts of DNA were kept constant by adding empty vector. In some cases transfection was performed in the presence of z-VAD-fmk (final concentration, 20 μM) to inhibit apoptosis. After 24 h (under the presence of z-VAD-fmk) or 12 h (under the absence of z-VAD-fmk), cells were fixed in acetone or 1% paraformaldehyde in PBS. In the case of formaldehyde fixation, cells were then permeabilized with PBS containing 0.2% Triton X-100 for 15 min. Fixed cells were blocked in PBS containing 1% BSA and labeled with the indicated primary Abs for 1 h at room temperature. Alexa Fluor 488- or Alexa Fluor 568-conjugated secondary Abs (×400 dilution) were used for the visualizing staining of the primary Abs. For acidic organelle staining, cells were treated with LysoTracker (500 μM) for 30 min before fixation. For nucleus staining, cells were treated with DAPI (final concentration, 2 μg/ml) in PBS for 10 min before mounting. After all staining procedures were finished, glass cover glasses were mounted onto a slide glass using PBS containing 2.3% 1,4-diazabicyclo[2.2.2]octane (DABCO) and 50% glycerol. Cells were visualized at ×63 magnification under an LSM510 META microscope (Zeiss).

Time lapse analysis
Time-lapse analyses were performed using AquaCosmos 2.0 software (Hamamatsu Photonics) to control a digital imaging system coupled to an inverted microscope (IX-70; Olympus). Briefly, HeLa cells (5 × 10^5/dish) were plated onto a 36-mm glass dish (IWAKI; Gene Company). The following day, cells were transfected with indicated plasmid using FuGENE HD (Roche) in the presence of z-VAD-fmk. The total amount of DNA (2 μg) was kept constant by adding empty vector. After 18–24 h from transfection, cells were stimulated with 20 μg/ml poly(I:C) or PGN for the indicated times and images were acquired through a cooled charge-coupled device camera (ORCA ER; Hamamatsu Photonics).

Fluorescence resonance energy transfer (FRET) analysis
The sensitized FRET measurement was performed as described previously (17). Fluorescent images were acquired sequentially through YFP, CFP, and FRET filter channels. Filter sets used were YFP (excitation, 500/25 nm; emission, 535/26 nm), CFP (excitation, 440/21 nm; emission, 480/30 nm), and FRET (excitation, 440/21 nm; emission, 535/26 nm). An XF2034 (455DRLP) dichroic mirror was used throughout the experiments. Integration times were 100–200 ms with a 2 × 2 binning mode. The background was subtracted from raw images before FRET calculations. Corrected FRET (FRETc) was calculated on a pixel-by-pixel basis for the entire image using the following equation: FRETc = FRET – (α × CFP) – (β × YFP), where FRET, YFP, and CFP correspond to background-subtracted images of cells coexpressing CFP and YFP acquired through the FRET, YFP, and CFP channels, respectively. α and β are the fractions of bleed-through of CFP and YFP fluorescence, respectively, through the FRET channel that were ordinarily 0.52 and 0.48, respectively, under our experimental conditions.

Reporter gene assay
HeLa cells in a 96-well plate were transiently transfected with the expression vector for TICAM-1 (p125 Luc reporter plasmid (15 ng) and an internal control vector, phRL-TK (0.5 ng) using FuGENE HD. The p125 Luc reporter that contains the human IFNβ promoter region (~125 to +19) was provided by Dr. T. Taniguchi (University of Tokyo, Tokyo, Japan). The total amount of DNA (100 ng) was kept constant by adding empty vector. After 24 h, cells were lysed in lysis buffer (Promega) and firefly and Renilla luciferase activities were determined according to the manufacturer’s instructions. Luciferase activity was normalized by Renilla luciferase activity and expressed as the fold stimulation relative to the activity in vector-transfected cells.
Immunoblotting

HeLa cells (1 × 10^5 cells/well) in a 24-well plate were cotransfected with TICAM-1^YFP plasmid (0.5, 50 ng) or empty vector using FuGENE HD in the presence of z-VAD-fmk. The total amount of DNA (500 ng) was kept constant by adding empty vector. After 24 h the cells were fixed and TLR3 was stained with anti-FLAG mAb and Alexa Fluor 568-conjugated secondary Ab. Upper panels, HeLa cells were transfected with a high dose of the TICAM-1 plasmid. TICAM-1^YFP forms speckle-like structures in the cytosol. Lower panels, HeLa cells were transfected with a low dose of the TICAM-1 plasmid. TICAM-1^YFP localizes diffusely in the cytosol. Green, TICAM-1^YFP; red, FLAG-TLR3; blue, nuclei with DAPI; bar, 10 μm. B, Upper panel, Luciferase reporter activity of TICAM-1^YFP. HeLa cells in a 24-well plate were transiently transfected with TICAM-1^YFP plasmid (0.5 ng or 50 ng/well) or empty vector together with the p-125-Luc reporter plasmid and the phRL-TK reporter plasmid using FuGENE HD. After 24 h, luciferase activities were measured and expressed as the fold stimulation relative to the activity in vector-transfected cells. Representative data from a minimum of three separate experiments are shown. Lower panel, Protein expression of TICAM-1^YFP was assessed by immunoblotting with anti-TICAM-1 pAb. C, TICAM-1 forms speckle-like structures in response to poly(I:C). HeLa cells expressing a low level of TICAM-1^YFP were placed on a time-lapse microscope and stimulated with poly(I:C) (20 μg/ml) (center and lower panels) or PGN (20 μg/ml) (upper right panels) for up to 60 min or left unstimulated (upper left panels). Cell images were taken every 10 min. YFP images at the indicated periods are shown.

Results

TLR3 localizes to the early endosome in the human epithelial cell line HeLa

We previously showed that TLR3 localizes to intracellular compartments in human monocyte-derived DCs (7). To identify the organelle in which TLR3 resides, we extended the analysis of the subcellular localization of endogenous TLR3 in HeLa cells with a low level of expression of TLR3 on the plasma membrane and a higher level of expression in intracellular organelles. TLR3-positive compartments were partially merged with EEA1 (Fig. 1). Late endosome (MPR), lysosome (LysoTracker), Golgi (p115), and endoplasmic reticulum (calnexin) markers were not colocalized with TLR3. Thus, in steady-state conditions some TLR3 molecules localize to the early endosome in epithelial cells, consistent with a previous study (18).

TICAM-1 alters the distribution profile in the cytosol in response to dsRNA

To clarify how TLR3 transmits signals to the adaptor protein TICAM-1, we analyzed the subcellular localization of TICAM-1 in HeLa cells. To visualize the expression of TICAM-1, we prepared a TICAM-1 construct tagged with YFP, TICAM-1^YFP, which
proteins level was sufficiently high to induce IFN-
with a high dose of the TICAM-1-YFP plasmid, the TICAM-1-YFP pro-
(Fig. 2B). HeLa cells were cotransfected with TICAM-1-YFP and FLAG-TLR3 and stimulated with poly(I:C) (20 μg/ml) for up to 60 min. At the indicated periods, cells were fixed and stained with anti-
FLAG mAb (5 μg/ml) and Alexa Fluor 568-conjugated secondary Ab. Upper panels show ×1.5 magnified images of the insets in the lower panels. Green, TICAM-1-YFP; red, FLAG-TLR3; blue, nuclei with DAPI; bar, 10 μm.

possesses IRF-3- and NF-κB- activating abilities. We first coex-
pressed FLAG-tagged TLR3 and TICAM-1-YFP in HeLa cells and
subjected these cells to confocal microscopic analysis. As shown in Fig. 2A, overexpressed TICAM-1 was localized to the speckle-
like structures in the cytosol and did not colocalize with TLR3 (upper panels). Because overexpressed TICAM-1 induces apopto-
sis in a caspase-dependent manner, transfected cells were cultured in the presence of a caspase inhibitor (z-VAD-fmk) for 24 h or
cultured for only <12 h in the absence of z-VAD-fmk. Under both cell culture conditions TICAM-1 formed a speckle-like localization pattern (data not shown). In contrast, by transfecting a limited dose of the TICAM-1 plasmid into HeLa cells TICAM-1 emerged diffusely in the cytosol and did not colocalize with TLR3 (Fig. 2A, lower panels). In this experimental condition, TICAM-1-YFP protein level was so low that it only weekly activated the IFN-β promoter (Fig. 2B, middle column). In contrast, when cells were transfected with a high dose of the TICAM-1-YFP plasmid, the TICAM-1-YFP protein level was sufficiently high to induce IFN-β promoter activation (Fig. 2B, right column). It has been found that overexpressed TICAM-1 strongly induces IFN-β promoter activation in HEK293 cells irrespective of TLR3 stimulation (5, 19). We therefore hypothesized that the distribution profile of TICAM-1 depends on its activation stage. To test this issue, HeLa cells expressing a low level of TICAM-1-YFP were stimulated with poly(I:C), a synthetic dsRNA, and subjected to a time-lapse analysis using an inverted fluorescence microscope. In response to poly(I:C), TICAM-1-YFP formed speckle-like structures in a time-dependent manner (Fig. 2C, middle and lower panels). The speckle-like structures appeared 30 min after poly(I:C) stimulation and were gradually increased and concentrated. The tendency of TICAM-1 to form speckles increased to a level comparable to that of overexpression.

In the absence of poly(I:C), TICAM-1 remained diffuse in the cytosol (Fig. 2C, upper left panels). Also, TICAM-1 did not alter the distribution profile upon PGN (TLR2 ligand) stimulation, suggesting that TICAM-1 speckle formation specifically depends on TLR3 stimulation (Fig. 2C, upper right panels).

Transient colocalization of TICAM-1 and TLR3 in response to dsRNA

When poly(I:C) is exogenously added to the cells it is delivered to the intracellular compartments in which TLR3 resides and activates the latter (20, 21). To visualize the association of TICAM-1 with TLR3 upon poly(I:C) stimulation, HeLa cells were cotransfected with TICAM-1-YFP and FLAG-TLR3 and stimu-

ated with poly(I:C) for 60 min. Cells were subjected to confocal immunofluorescence analyses. TICAM-1 and TLR3 were localized distinctly in unstimulated cells (Fig. 3). Partial colocal-
ization of TICAM-1 and TLR3 was observed within 30 min after poly(I:C) stimulation. However, 60 min after poly(I:C) stim-
ulation TICAM-1 dissociated from TLR3, forming speckles. Hence, TICAM-1 in the cytosol is transiently recruited to the endo-
somal TLR3 in response to dsRNA and thereafter moves away from TLR3 to form speckle-like structures.

FIGURE 3. Transient association of TICAM-1 with TLR3 in response to poly(I:C). Confocal images of HeLa cells coexpressing TICAM-1-YFP with FLAG-TLR3 are shown. HeLa cells were cotransfected with TICAM-1-YFP and FLAG-TLR3 and stimulated with poly(I:C) (20 μg/ml) for up to 60 min. At the indicated periods, cells were fixed and stained with anti-
FLAG mAb (5 μg/ml) and Alexa Fluor 568-conjugated secondary Ab. Upper panels show ×1.5 magnified images of the insets in the lower panels. Green, TICAM-1-YFP; red, FLAG-TLR3; blue, nuclei with DAPI; bar, 10 μm.

FIGURE 4. Subcellular localization of RIP1, NAP1, and IRF-3. A and B, RIP1 and NAP1 accumulate in the speckle-like structures in response to poly(I:C). HeLa cells coexpressing RIP1-YFP (A) or NAP1-YFP (B) with TI-
CAM-1-CFP were placed on a time-lapse microscope and imaged every 10 min. Cells were stimulated with poly(I:C) (20 μg/ml) for up to 60 min (lower panels) or left unstimulated (upper panels). Fluorescent images of YFP at the indicated time periods are shown. CFP images indicate the expression level of TICAM-1. C, Nuclear translocation of IRF-3 induced by poly(I:C) stimulation. HeLa cells were seeded onto cover glass and stimulated with poly(I:C) (40 μg/ml) for up to 120 min. At the indicated periods, cells were fixed, stained with anti-IRF-3 mAb and Alexa Fluor 568-conjugated secondary Ab, and subjected to confocal analyses. Green, IRF-3; blue, nuclei with DAPI; bar, 10 μm.
FIGURE 5. RIP1 and NAP1 are recruited to TICAM-1-positive speckles. A, Interaction of TICAM-1 with RIP1 or NAP1. HeLa cells coexpressing RIP1-YFP or NAP1-YFP and TICAM-1-YFP were stimulated with poly(I:C) (20 μg/ml) for 60 min. FRET, YFP, and CFP images were collected by using an inverted fluorescence microscope equipped with a cooled charge-coupled device camera and FRET was determined as described in the text. Upper panels show FRET images of RIP1-YFP and TICAM-1-YFP. The lower graph shows the normalized FRET values of 60 individual regions of cells calculated by dividing FRET* for each region by the mean intensity of RIP1-YFP or NAP1-YFP. B and C, Confocal images of HeLa cells expressing TICAM-1-YFP only (B) or TICAM-1-YFP and NAP1-Myc (C). Cells were fixed and stained with anti-RIP1 mAb for detecting endogenous RIP1 (B) or with anti-Myc mAb (C) followed by an Alexa Fluor 568-conjugated secondary Ab. Arrowheads indicate merged speckles. Green, TICAM-1; red, RIP1 or NAP1; blue, nuclei with DAPI; bar, 10 μm.

RIP1 and NAP1 are recruited to TICAM-1-positive speckles

To study the relationship between the translocation of TICAM-1 and its functionality, we next analyzed the subcellular localization of the signaling molecules downstream of TICAM-1. RIP1 is crucial for TICAM-1-mediated NF-κB activation. When RIP1 tagged with YFP (RIP1-YFP) was coexpressed with a low dose of TICAM-1 tagged with cyan fluorescent protein (CFP; TICAM-1-CFP) in HeLa cells, RIP1-YFP was uniformly distributed in the cytosol except for constitutive basal RIP1 accumulation (Fig. 4A, upper panels). Upon poly(I:C) stimulation, RIP1-YFP accumulated in the speckle-like structures in a time-dependent manner (Fig. 4A, lower panels). The speckle formation was detected 30 min after poly(I:C) stimulation and then gradually increased up to 60 min, concomitant with TICAM-1 translocation. In the absence of poly(I:C) stimulation, RIP1-YFP remained diffusely in the cytosol (Fig. 4A, upper panels).

Similar results were obtained with NAP1 tagged with YFP (NAP1-YFP). NAP1 is located downstream of TICAM-1 and engages in TICAM-1-mediated IRF-3 activation (13). The N-terminal region of TICAM-1 is responsible for the NAP1–TICAM-1 association. When HeLa cells were transfected with NAP1-YFP together with TICAM-1-YFP and stimulated with poly(I:C), NAP1 formed the speckle-like structures 30 min after poly(I:C) stimulation, as observed in RIP1-YFP localization analyses (Fig. 4B). The nuclear translocation of IRF-3 was detected ~60 min after poly(I:C) stimulation, which followed the formation of TICAM-1-positive speckles (Fig. 4C).

These results suggest that TICAM-1, once activated by dsRNA-TLR3, interacts with downstream signaling molecules and forms speckle-like structures in the cytosol apart from TLR3. To carry out a precise examination of the molecular interaction between TICAM-1 and RIP1 or NAP1, these molecules tagged with YFP or CFP were coexpressed in HeLa cells and subjected to FRET analysis after poly(I:C) stimulation. As shown in Fig. 5A, FRET images revealed a strong energy transfer from RIP1-YFP to TICAM-1-YFP, whereas energy transfer from NAP1-YFP to TICAM-1-YFP was relatively weak. These results indicate that RIP1 makes direct contact with TICAM-1 in response to poly(I:C). NAP1 also associates with TICAM-1 in response to poly(I:C), although direct binding was not detected by the yeast two-hybrid technique (data not shown). Thus, TICAM-1 seems to recruit both RIP1 and NAP1 to the putative speckle signalosome. Notably, when TICAM-1 was overexpressed, endogenous RIP1 was highly concentrated in the TICAM-1-positive speckles (Fig. 5B). Also, colocalization of TICAM-1 and NAP1 was partly detected in an overexpression study (Fig. 5C). At a high TICAM-1 expression level, TICAM-1 might be oligomerized in a TLR3-independent manner and activate downstream signaling molecules to form speckle-like structures. These TICAM-1-positive speckles containing RIP1 and NAP1 barely merged with TLR3 or any organelle markers (data not shown). Because recent reports demonstrated that the internalized dsRNA was trafficked with TLR3 from the endosome to the lysosome in both DCs and epithelial cells (20, 21), the TICAM-1 signaling complex appears to reside in the cytosol distinctly from the dsRNA-TLR3-positive compartments.

Discussion

TLR signaling is mediated by the recruitment of distinct combinations of adaptor molecules to the cytoplasmic TIR domain of each TLR. Distribution and localization of the adaptor molecules are important factors controlling TLR-mediated signaling. TIRAP/Mal was found to be associated with the plasma membrane via a phosphatidylinositol 4,5-bisphosphate-binding domain and facilitates MyD88 delivery to activated TLR4 (22). Also, myristoylation of TICAM-2/TRAM targets it to the plasma membrane and the Golgi apparatus, where it colocalizes with TLR4 (23). Thus, the role of these adaptor proteins other than TICAM-1 has been clearly demonstrated; TIRAP/Mal and TICAM-2/TRAM mainly function as bridges between TLR4 and the signaling adaptors, whereas MyD88 restricts its localization by TIRAP to assemble signal molecules around the TLR4 complex (9, 10, 24, 25). In this study we demonstrated that TICAM-1/TRIF delivers a signal in a unique fashion distinct from that of MyD88.

This is the first study to visualize the molecular dynamics of the TICAM-1 pathway that leads to activation of IRF-3 and NF-κB. When TICAM-1 expression is controlled to a low level, TICAM-1 emerges diffusely in the cytosol, whereas overexpressed TICAM-1, which strongly induces IFN-β promoter activation and apoptosis, forms speckles. Neither the diffuse nor speckle form of TICAM-1 co-localizes with endogenous and overexpressed TLR3. Once TLR3 is stimulated with dsRNA in cells with diffuse TICAM-1, TICAM-1 is recruited to the TLR3 inside the cells. Thus, TICAM-1 exists unrelated to TLR3 in resting cells. Whereas dsRNA comes over the cells, TICAM-1 is activated along endosomal TLR3 and then the activated TICAM-1 dissociates from the TLR3 to form a speckle-like structure. The speckle-like structure gathers multiple TICAM-1 molecules according to the confocal feature, suggesting the event of the receptor-induced TICAM-1 oligomerization. This TICAM-1 trip facilitates the focusing of the signaling molecules on the speckle containing TICAM-1 and finally activates NF-κB and IRF-3. In fact, the speckles are comprised of TICAM-1 and the signaling molecules, NAP1 and RIP1. Thus far, however, the intriguing issue as to whether RIP1 and/or NAP1 are recruited to TICAM-1 before or after the dissociation of TICAM-1 from TLR3 remains undetermined. Furthermore, the...
mechanism by which TICAM-1 is activated by TLR3 also remains undetermined.

TLR3-TICAM-1 signaling results in various cellular responses, including the production of type I IFN and inflammatory cytokines, DC maturation (28), cross-presentation of exogenous Ags for the proliferation of CD8+ T cells (29, 30), NK cell activation (31, 32), and apoptosis (33). TICAM-1 may act as a platform recruiting signaling molecules for DC output. The signaling components participating in the TICAM-1-mediated type I IFN production have been well characterized. Our current focus is to clarify how these components are orchestrated in undefined molecular cascades. It is becoming clear that the TICAM-1 pathway in myeloid DCs is involved in CTL induction and NK cell activation (29, 32). Through these investigations, the cascades by which the TLR3-TICAM-1 pathway induces cross-presentation and NK activation in myeloid DCs will be defined.

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Disclosures

The authors have no financial conflict of interest.

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