A New Domain in the Toll/IL-1R Domain-Containing Adaptor Inducing Interferon- β Factor Protein Amino Terminus Is Important for Tumor Necrosis Factor- α Receptor-Associated Factor 3 Association, Protein Stabilization and Interferon Signaling

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Abstract

Toll/IL-1R domain-containing adaptor inducing interferon- β (IFN- β) factor (TRIF) is a key adaptor for Toll-like receptor (TLR) 3 and TLR4 signaling. Using a novel cDNA isolate encoding a TRIF protein with a 21-residue deletion (Δ 160–181) from its amino-terminal half, we investigated the impact of this deletion on TRIF functions. Transfection studies consistently showed higher expression levels of the (Δ 160–181) TRIF compared to wild-type (wt) TRIF, an effect unrelated to apoptosis, cell lines or plasmid amplification. Colocalization of wt and (Δ 160–181) TRIF proteins led to a dramatic reduction of their respective expressions, suggesting that wt/ (Δ 160–181) TRIF heterocomplexes are targeted for degradation. We demonstrated that wt TRIF associates with tumor necrosis factor- α receptor-associated factor 3 (TRAF3) better than (Δ 160–181) TRIF, culminating in its greater ubiquitination and proteolysis. This explains, in part, the differential

expression levels of the two TRIF proteins. Despite higher expression levels in transfected cells, (Δ 160–181) TRIF inefficiently transactivated the IFN pathway, whereas the nuclear factor- κ B (NF- κ B) pathway activation remained similar to that by wt TRIF. In coexpression studies, (Δ 160–181) TRIF marginally contributed to the IFN pathway activation, but still enhanced NF- κ B signaling with wt TRIF. Therefore, this 21 amino acid sequence is crucial for TRAF3 association, modulation of TRIF stability and activation of the IFN pathway.

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Introduction

Recognition of invading microorganisms via their pathogen-associated molecular patterns (PAMPs) is crucial to activate innate immunity. In mammals, pathogens are detected by members of three different families of proteins: Toll-like receptors (TLRs), RNA-like helicases (RLHs) and NOD-like receptors (NLRs). Activation of innate immunity leads to production of type I interferons (IFNs), chemokines and inflammatory cytokines to limit the initial spread of pathogens and is paramount in the development

of antigen-specific adaptive immunity. Consequently, this ensures long-lasting protection for the host [1, 2].

Toll-like receptors (TLRs) are an evolutionarily conserved family of receptors found in organisms from plants to humans. In their cytoplasmic tails, all TLRs possess a well-conserved motif, the Toll/IL-1 receptor (TIR) domain crucial for interactions between TLRs and their adaptors [3]. Upon engagement with their cognate ligands, conformational rearrangements in TLRs juxtapose their TIR domains to create a molecular scaffold that recruits additional factors to activate downstream signaling pathways. Three main signaling pathways have been linked to TLR activation: IFN regulatory factor-3 (IRF-3), nuclear factor- κ B (NF- κ B) and activator protein-1 (AP-1). These pathways lead to the production of type I IFNs, proinflammatory cytokines and chemokines, respectively.

TLRs rely on a family of five cytoplasmic adaptors for downstream signaling. A critical adaptor is the myeloid differentiation factor 88 (MyD88) which binds to many TLRs, including TLR4 [4]. However, macrophages from MyD88-deficient mice still show activation of mitogen-activated protein kinase (MAPK) and NF- κ B pathways, though with delayed kinetics, in response to its cognate ligand, lipopolysaccharide [5]. Moreover, dendritic cell maturation and IFN- β production from the same mice remained unaffected [6, 7]. These observations suggested the presence of a MyD88-independent signaling pathway, which culminated in the molecular cloning of TRIF, also known as TICAM-1 (TIR-containing adaptor molecule-1) [8, 9]. TRIF directly interacts with TLR3, and is now established as the sole, essential adaptor for TLR3 to mediate both IRF-3 and NF- κ B signaling pathways. TRIF is also involved in the TLR4 pathway but indirectly through another adaptor, TRAM (also known as TICAM-2) [10, 11]. Gene-deletion studies indicated that TRIF-deficient mice are more susceptible to viral and bacterial infections, emphasizing the role of TRIF as a key signaling component of the TLR3 pathway against microbes *in vivo* [12, 13].

TRIF is characterized by a long N-terminus, a central TIR domain and a C-terminus involved in mediating NF- κ B activation via its receptor homotypic interacting motif (RHIM) [14, 15]. In unstimulated cells, TRIF shows a diffuse cytoplasmic distribution. During TLR3 signaling, TRIF transiently colocalizes with TLR3 in endosomes before dissociating from the receptor to relocalize with other signaling factors in speckle-like structures [16]. Trypsin digestion studies suggest that TRIF is composed of two protease-resistant domains, the N-terminal domain and the combined TIR and C-terminal

domains [17]. Prior to activation, the TRIF N-terminal domain is postulated to fold back on the TIR domain, keeping TRIF in a 'closed' conformation. By a yet undefined mechanism, ligand binding by TLR3 induces TRIF to unfold and oligomerize in high-molecular complexes. Such conformational rearrangements expose binding sites for signaling mediators on individual TRIF molecules, and oligomerization further allows TRIF to act as a platform to initiate downstream signaling. Funami et al. [18] recently demonstrated that a critical proline at position 434 (P⁴³⁴) in the TIR domain and the C-terminus are required for TRIF oligomerization. The TRIF amino-terminus was initially defined as essential for activation of the IFN- β promoter [8]. Mutagenesis studies further defined critical residues within the TRIF amino-terminus important for IRF-3 activation [17], and for binding to members of the tumor necrosis factor- α receptor-associated factor (TRAF) family [19–21]. TRAF3 is the most recent TRAF family member implicated in the TLR signaling apparatus [22]. The importance of TRAF3 is underscored by its essential role in type I IFN production, and protection against viral infections [23–25]. With its RING finger domain, TRAF3 acts as an E3 ubiquitin ligase, mediating the polyubiquitination of interacting proteins to subsequently alter their functions or to target them for proteolysis in a proteasome-dependent manner. To date, different groups have reported conflicting data about the physical association between TRAF3 and TRIF [20, 25]. Therefore, its specific binding site has not been definitively mapped. Moreover, TRAF3-mediated TRIF ubiquitination has not been directly demonstrated.

We report here the cloning and molecular characterization of a TRIF variant that lacks 21 amino acids (aa) from its N-terminal domain (Δ 160–181). Noteworthy, this segment is located apart from all previously identified binding sites in the TRIF amino-terminal domain. Focusing on this novel TRIF variant as a molecular tool, we investigated the potential role of this segment in TRIF functions. Transfection studies consistently showed that the (Δ 160–181) TRIF was expressed at higher levels than wild-type (wt) TRIF when equal amounts of DNA were transfected in various cell lines. The (Δ 160–181) TRIF higher expression was due in part to a reduced association with TRAF3, making this protein less susceptible to ubiquitination thereby resulting in a higher accumulation over its wt counterpart. Despite a higher expression level, (Δ 160–181) TRIF inefficiently transactivated IRF-3, but readily activated the NF- κ B, signaling pathway. These results suggest that although lacking an apparent consensus

TRAF-binding site, aa 160–181 of the TRIF protein appear to be involved in TRAF3 association, and modulate TRIF ubiquitination, and degradation. Finally this segment also appears to be important for TRIF-mediated IFN signaling. Therefore, this segment may be a novel subdomain critical for modulating selective TRIF functions.

Materials and Methods

Cell Lines and Antibodies

The following cell lines were used: 293T (H. Young, Columbia University, College of Physicians and Surgeons, New York, N.Y., USA), HeLa, and COS-7 (ATCC, Manassas, Va., USA). They were maintained in DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS). Suspension T lymphocyte cell lines MT1, MT2, MT4 and HuT102 were maintained in RPMI 1640 (Gibco) supplemented with 10% FCS. The CaGT cell line was established by culturing the fresh peripheral blood mononuclear cells of a patient with adult T cell leukemia in a medium consisting of 45% RPMI 1640 (Gibco), 45% HD SFM (Gibco) and 10% FCS without any exogenous cytokines or mitogens. Following 4 months in culture, clones were isolated by limiting dilution. Phenotypic analyses revealed a T cell origin expressing CD2, CD4, CD3^{dim}, CD25, CD122 and CD132, and negative for CD8, CD7, CD19 and CD20. The following antibodies were used to detect epitope-tagged proteins: Flag (F-7425) (Sigma, St Louis, Mo., USA), HA (sc-805) (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) and V5 (R960-25) (Invitrogen, Carlsbad, Calif., USA). Antibodies against caspase-3 (9662) (Cell Signaling Technologies, Beverly, Mass., USA) and PARP (556494) (BD Pharmingen, San Diego, Calif., USA) were used to detect apoptosis.

Expression Constructs

To construct the CaGT cDNA library, total RNA was extracted using FastTrack 2.0 kit (Invitrogen) and converted to cDNA using the SuperScript Plasmid system kit (Invitrogen). Resulting cDNA inserts were cloned in the pMT2T expression vector. Total RNA from HeLa and NK-92 cells extracted with Trizol (Invitrogen) was used to clone TRAF2 and TRAF3 cDNAs, respectively, by RT-PCR using the SuperScript III with Platinum Taq high-fidelity kit (Invitrogen) and gene-specific primers. When necessary, specific epitope tags were added at the cDNA 3'-ends using PCR mutagenesis and specific primers. All the constructs were sequenced to ensure the absence of spurious mutations. Luciferase reporter constructs containing IFN-stimulated responsive elements (ISRE) and NF- κ B sequences were purchased from Stratagene (Stratagene, Santa Clara, Calif., USA).

Transfection

Cells seeded in 6-well plates or 6-cm dishes as indicated in figure legends, were transfected with Lipofectamine 2000 (Invitrogen) or TurboFect (Thermo Scientific, Pittsburgh, Pa., USA) following the manufacturer's recommendations. An empty expression vector was used to maintain constant amounts of DNA transfected between samples. Cells seeded in 10-cm dishes for coimmunoprecipitation were transfected by calcium phosphate precipitation as previously described [26].

Proteasome and Caspase Inhibitors

At 19 hours after transfection, transfected 293T cells were treated for 5 h with 20 μ M of MG132 (Calbiochem, Billerica, Mass., USA), or 50 μ M of Z-VAD-FMK (BD Pharmingen) or a combination of the two inhibitors. Sham (DMSO)-treated cells served as a control. Clarified lysates were analyzed by immunoblotting with appropriate antibodies.

Immunoblotting and Immunoprecipitation

For direct immunoblotting, cells were lysed in TBES/1%NP40/0.5%DOC lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA (pH 8.0), 1% Nonidet-P40, 0.5% sodium deoxycholate]. The protease inhibitor cocktail ProteoBlockTM (Fermentas Inc., Glen Burnie, Md., USA) and/or phosphatase inhibitor tablets (Roche, Indianapolis, Ind., USA) were added to all lysis buffers. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes and probed with appropriate primary and horseradish peroxidase-linked secondary antibodies. Bands were visualized by chemiluminescence (Pierce Super Signal West Pico substrate kit). For coimmunoprecipitation studies, a milder buffer TBES/0.5% NP40 buffer was used to isolate protein complexes.

Luciferase Reporter Gene Assay

Cells seeded in triplicate in 6-well plates were cotransfected with appropriate TRIF plasmids, the ISRE-controlled or NF- κ B-controlled firefly luciferase reporter and a Renilla luciferase construct as an internal control to normalize transfection efficiency between samples. At approximately 24 hours after transfection, cells were lysed using the dual-luciferase reporter assay kit (Promega, Madison, Wisc., USA) and luciferase activities were measured with a MicroBeta 2 plate counter (Perkin Elmer). Fold-induction was calculated by dividing normalized luciferase activity in TRIF-transfected cultures over the activity in empty vector-transfected cultures. Each experiment was repeated 3 times ($n = 9$) with similar results and was reported as a mean.

Statistical Analysis

Data from the luciferase reporter gene assays were analyzed by an unpaired Student t test employing GraphPad InStat software (GraphPad software, Calif., USA).

Confocal Microscopy

HeLa cells grown in glass chambers (Lab-Tek II) (Nalge Nunc Int.) were transfected with plasmids encoding Flag-wt TRIF, HA-(Δ 160–181) TRIF or HA-Stat4 proteins using TurboFect (Thermo Scientific) according to the manufacturer's instructions. Methanol-fixed cells were stained with fluorophore-conjugated anti-Flag (Alexa Fluor AF555) (3768) (Cell Signaling Technologies) and anti-HA (Alexa Fluor AF488) (2350) (Cell Signaling Technologies) antibodies. Nucleus staining was performed with DAPI. Confocal images of cells were acquired using a Zeiss LSM510 META laser-scanning microscope (Carl Zeiss MicroImaging, Thornwood, N.Y., USA) equipped with a $\times$ 40 C-apochromat objective lens (N.A.1.2). Image z-stacks were collected with consistent settings including an optical slice thickness of 1.0 μ m, 0.11 μ m x-y pixel size and 0.40 μ m z-axis step size. Maximum intensity projections of each image stack were calculated using the LSM Image Browser software (v 4.0). Alternatively, HeLa cells were cotransfected with expression constructs encoding mCherry-wt TRIF and eGFP-(Δ 160–181) TRIF fusion proteins. Cells were kept in a humidified,

CO₂-fed stage-top chamber, and live cell imaging was acquired using the Zeiss LSM510 META laser-scanning microscope with images collected using the same confocal optical slice parameters.

Results

Transfected (Δ 160–181) TRIF Is Expressed at Higher Levels than Wild-Type TRIF in Various Cell Lines

Using an expression cDNA library constructed from an adult T cell leukemia patient-derived cell line CaGT, we isolated a novel cDNA encoding a variant TRIF protein. Compared to wt TRIF cDNA [9, 12], this clone exhibited an in-frame 63-nucleotide deletion (nucleotides 480–543) (fig. 1a, top panel). This deletion appeared to result from alternative splicing, though the predicted donor/acceptor sites of the transcript did not abide by the GT/AG rule. Regardless, the truncated transcript, though very rare, was detected in other cell lines (data not shown), suggesting that it was not a cloning artifact. The variant cDNA encodes a TRIF protein with a 21-aa deletion, from aa 160–181, in its amino-terminal half (fig. 1a, middle panel). At present, the physiological significance of such a TRIF transcript and its encoded product remain undefined. More importantly, the missing segment was distinct from all the sites that TRIF-associated factors have been identified to bind [17, 20, 27] (fig. 1a, bottom panel). This prompted us to examine whether this sequence is a novel element and/or exerts some functions so far unidentified. Using this variant TRIF protein as a molecular tool, our study focused on uncovering the potential role(s) of the aa sequence 160–181 in TRIF functions.

To determine if the lack of aa 160–181 exerted any effect on the TRIF protein expression, we employed an overexpression system to transfect different amounts of HA-tagged wt and (Δ 160–181) TRIF plasmids in HEK293T cells. Immunoblotting analysis showed that expression levels of (Δ 160–181) TRIF were higher than those of wt TRIF, especially when identical low amounts of DNA were transfected onto cells (fig. 1b, upper panel, compare lanes 5–6 to lanes 2–3). At higher amounts of transfected DNA, the differential expression levels of two TRIF proteins remained noticeable, albeit not as pronounced, likely due to the signal saturation of immunoblotting assays (fig. 1b, e, f, compare lane 7 to lane 4). Reprobing for Tyk2 served as an internal control and showed that similar amounts of total protein were loaded (fig. 1b, bottom panel).

TRIF activation has been shown to induce apoptosis [28–30]. Cleavage of PARP and caspase-3, two apoptotic

markers, occurred similarly in cells transfected with wt or (Δ 160–181) TRIF, strongly suggesting that the higher expression level of (Δ 160–181) TRIF was unlikely to be due to a lower apoptosis induction in transfected cells (fig. 1c, d).

To determine whether the previously observed differential expression levels were not uniquely restricted to HEK293T cells, transfections were carried out in COS-7 and HeLa cells. For COS-7 cells, similar higher expression levels of (Δ 160–181) TRIF protein were observed, ruling out any cell line-specific influence (fig. 1e). A similar result was also observed in HeLa cells, which are devoid of SV40 large T antigen, therefore ruling out plasmid amplification-related bias (fig. 1f). These results indicated that the (Δ 160–181) TRIF was expressed at a higher level than wt TRIF, independently of the cell lines used, apoptosis induction or plasmid amplification. These results also provide strong evidence that residues 160–181 impacted expression of the TRIF protein, prompting us to investigate the underlying mechanisms leading to the differential expression levels between the two TRIF proteins. We also examined if deletion of this sequence affects some general TRIF functions.

Wild-Type and (Δ 160–181) TRIF Proteins Colocalized in the Same Complexes

To determine whether deletion of aa 160–181 affects the ability of the (Δ 160–181) TRIF to interact with the wt counterpart, coimmunoprecipitation studies were performed in cells cotransfected with both TRIF plasmids. To distinguish them from each other, wt and (Δ 160–181) TRIF proteins were tagged with Flag and HA epitopes at their C-termini, respectively. Complexes consisting of wt and (Δ 160–181) TRIFs were readily detected in cells co-expressing both proteins (fig. 2a, top panel, lane 4). Thus the deletion does not seem to interfere with TRIF oligomerization. Interestingly, expression levels of either wt or (Δ 160–181) TRIF protein were substantially lower in cotransfected cells than those transfected with a single TRIF, in particular the cotransfected (Δ 160–181) TRIF showed a much greater decrease of expression than wt TRIF (fig. 2a, middle and bottom panels, compare lane 4 to lanes 2–3).

Wild-Type and (Δ 160–181) TRIF Proteins Overlapped in Their Subcellular Distribution

To support our coimmunoprecipitation data, we assessed the subcellular distribution of wt and (Δ 160–181) TRIF proteins in cotransfected HeLa cells using confocal microscopy. Both wt and (Δ 160–181) TRIF proteins dis-

Fig. 1. Expression levels of transfected ($\Delta 160$ –181) TRIF are higher than those of transfected wt TRIF in different cell lines. **a** Schematic representation of wt TRIF and ($\Delta 160$ –181) TRIF cDNAs with nucleotides surrounding positions 480 and 543 (upper panels). Different binding sites or domains on wt TRIF are shown with their corresponding aa (grey boxes). The aa 160–181 sequence with flanking residues is illustrated by the hatched box (lower panel). **b** 293T cells in 6-well plates were transfected with different amounts of DNA of empty vector (EV), HA-wt, or HA-($\Delta 160$ –181) (var) TRIF plasmid as indicated. Cell extracts were processed for immunoblotting with anti-HA antibody (upper panel), and reprobed with anti-Tyk2 antibody (lower panel). Standard markers were indicated on the left side of the panels. **c** Cell extracts from **b** were immunoblotted with anti-PARP antibody. Positions of full-length (FL) and cleaved (p85 fragment) PARP are indicated on the right side of the panel. **d** Same as **c** but with caspase-3 antibody that recognizes full-length (FL) and cleaved fragments (p19 and p17) of caspase-3. **e, f** Same as **b** with COS-7 and HeLa cells used, respectively.

played cytoplasmic punctuate staining in transfected cells (fig. 2b, first row, two left panels). More importantly, wt and ($\Delta 160$ –181) TRIF proteins showed extensive overlapping distribution, strongly suggesting that they colocalized to the same complexes (fig. 2b, 1st row, rightmost ‘merge’ panel). As an internal positive control, coexpression of wt TRIF tagged with 2 different epitopes, Flag and HA respectively, showed similar staining and colocalization patterns (fig. 2b, compare the ‘merge’ panels of rows 1 and 2). The data were further validated by live-cell imaging using the mCherry-wt TRIF and eGFP-($\Delta 160$ –181) TRIF fusion proteins (fig. 2b, row 4). On the other hand, an unrelated protein, Stat4, showed very little or no colo-

calization with wt TRIF, strongly suggesting specificity in TRIF complex formation and subcellular localization (fig. 2b, row 3). Taken together, these results indicate that, in our overexpression system, the ($\Delta 160$ –181) TRIF colocalized with wt TRIF and that this colocalization was indistinguishable from that of wt TRIF when the latter was expressed alone. The extensive overlap in their respective cytoplasmic distributions strongly suggests that both wt and ($\Delta 160$ –181) TRIF proteins are very likely part of the same oligomeric complexes. Therefore, the 21-aa deletion in ($\Delta 160$ –181) TRIF does not appear to aberrantly affect its ability to oligomerize with wt TRIF and/or its subcellular localization.

Decrease of Expression Level of (Δ160–181) TRIF Protein Occurred when Coexpressed with Wild-Type TRIF

Based on their differential expression levels, complexes consisting of different ratios of wt and (Δ160–181) TRIFs are likely to form within transfected cells, and may differ in their ability to activate downstream signaling. To follow up on this observation, coexpression assays were performed where increasing amounts of (Δ160–181) TRIF DNA were transfected in the presence of a stable amount of wt TRIF plasmid. In cotransfected cells, expression levels of wt and (Δ160–181) TRIF proteins were dramatically lower than those of singly TRIF-transfected cells. Again the relative decrease in the expression level was more pronounced with the (Δ160–181) TRIF than with its wt counterpart (fig. 2c, compare lanes 6–9 to lanes 2–3 and lanes 4–5, respectively). This suggests that wt TRIF provides the primary or dominant signal to target the wt/(Δ160–181) TRIF heterocomplexes for degradation, thereby resulting in lower expression levels of the (Δ160–181) TRIF protein.

In similar coexpression assays, the Stat4 protein, in place of (Δ160–181) TRIF, was expressed at comparable levels in singly or cotransfected cells (fig. 2d, bottom panel, compare lanes 6 and 8 to lanes 3–4). On the other hand, wt TRIF exhibited a reduced expression in cells cotransfected with Stat4 (fig. 2d, top panel, lanes 6–9), similar to previous data (fig. 2c, d, top panels, lanes 6–9). Therefore, this lack of an effect on Stat4 emphasizes the specificity of TRIF oligomerization followed by degradation of both wt and (Δ160–181) TRIF proteins.

To further support the requirement of TRIF oligomerization prior to its degradation, a coimmunoprecipitation assay was performed on cells cotransfected with appropriate TRIF and Stat4 constructs. Interaction between wt and (Δ160–181) TRIF proteins, but not TRIF-Stat4, was readily detected in the appropriately cotransfected cells (fig. 2e, top panel, lanes 7–8). This was not due to a noticeable difference in the amount of TRIF immunoprecipitated (fig. 2e, 'reprobe' panel) or lack of Stat4 expression (fig. 2e, bottom panel).

Association with TRAF3 Leads to Ubiquitination of TRIF Protein

TRAF3 has recently been implicated in TLR signaling pathways [22, 31]. Furthermore, TRAF3 E3 ubiquitin ligase activity was implicated in the degradation of its interacting targets, notably NF-κB-inducing kinase (NIK) [32]. Our results strongly suggest that aa 160–181 of wt TRIF may serve as a signal to recruit an additional factor that, in turn, directs TRIF protein oligomers to degradation (fig. 2). Coimmunoprecipitation assays readily demonstrated complexes consisting of wt TRIF and TRAF3 (fig. 3a, top panel, lane 6). However, under the same conditions, in spite of its higher expression level, the (Δ160–181) TRIF still showed a much weaker association with TRAF3 (fig. 3a, top panel, compare lanes 7–8 to lane 6). In addition, TRAF3 levels decreased in cells cotransfected with wt, but not with (Δ160–181) TRIF (fig. 3a, bottom panel, compare lanes 6–8 to lane 2). This suggests that, following its

Fig. 2. wt TRIF associates with (Δ160–181) TRIF in vivo. **a** Empty vector (EV), Flag-wt, HA-(Δ160–181) TRIF plasmids were transfected alone or in combination in 293T cells seeded in a 10-cm dish, as indicated. wt TRIF in cell extracts was immunoprecipitated with anti-Flag antibody, and associated (Δ160–181) TRIF protein was revealed by immunoblotting with anti-HA antibody (top panel). A fraction of cell extracts was directly analyzed by immunoblotting with anti-Flag (middle), or anti-HA antibody (bottom panel) to confirm expression of wt and (Δ160–181) TRIF, respectively. **b** HeLa cells were cotransfected with expression constructs encoding Flag-tagged wt TRIF and HA-tagged (Δ160–181) TRIF proteins (1st row). Following fixation with methanol, cells were stained with appropriate fluorophore-conjugated antibodies as indicated in the Materials and Methods section. DAPI was used for nuclear counterstaining. Cells were examined using a Zeiss LSM510 META laser-scanning confocal microscope. Image stacks (10) were collected and maximum intensity projections were calculated using the LSM Image Browser software (v 4.0). Similar experiments were performed by cotransfecting Flag-wt TRIF and HA-wt TRIF expression constructs (2nd row) or with Flag-wt TRIF and HA-Stat4 constructs (3rd row). HeLa cells were cotrans-

fected with expression constructs encoding mCherry-wt TRIF and eGFP-(Δ160–181) TRIF fusion proteins, and confocal images were acquired from live cells (4th row). Scale bar: 10 μm. **c** EV, Flag-wt, HA-(Δ160–181) TRIF vectors were transfected in 293T alone or together using different amounts of DNA, as indicated. An EV served as a negative control (lane 1), and was added to keep the DNA amounts constant among samples (lanes 2–9). Expression levels of wt and (Δ160–181) TRIFs were detected by immunoblotting with anti-Flag (top panel) and anti-HA antibody (bottom panel). **d** Similar assay to that in **c** except that HA-Stat4-encoding vector was substituted for the (Δ160–181) TRIF construct. **e** Plasmids encoding Flag-wt TRIF, HA-(Δ160–181) TRIF or HA-Stat4 were transfected alone or in combination, as indicated. Protein complexes were immunoprecipitated with anti-Flag antibody and coimmunoprecipitated proteins were revealed by immunoblotting with anti-HA antibody (top panel) and reprobated with anti-HA antibody to confirm immunoprecipitated wt TRIF (2nd panel). NS = unrelated proteins immunoprecipitated by Flag antibody and nonspecifically recognized by HA antibody. Aliquots of the same extracts were directly analyzed by immunoblotting with anti-Flag or anti-HA antibody (3rd and 4th panels, respectively).

(For figure see next page.)

Fig. 3. TRAF3 associates with wt TRIF and (Δ160–181) TRIF proteins. **a** Different amounts of DNA of vectors encoding V5-TRAF3, HA-wt TRIF and HA-(Δ160–181) TRIF were transfected in 293T as indicated. Empty vector (EV) was added to keep total amounts of DNA constant among samples. Extracts were subjected to immunoprecipitation with anti-V5 antibody, and associated TRIF proteins were revealed by immunoblotting with anti-HA antibody (top panel). Fractions of lysates were also analyzed by immunoblotting with anti-HA (middle) or anti-V5 antibody (bottom panel) to confirm expression of TRIF and TRAF3 proteins, respectively. **b** Same as **a** with the addition of a Flag-ubiquitin (Flag-Ub) vector. Following immunoprecipitation with anti-HA antibody, ubiquitinated wt or (Δ160–181) TRIFs were revealed by immunoblotting with anti-Flag antibody (top panel), and reprobe with

anti-HA antibody to confirm immunoprecipitation (2nd lower panel). Expression of TRIF and TRAF3 proteins in cell extracts was confirmed by immunoblotting aliquots of the same extracts with anti-HA (3rd lower panel) and anti-V5 antibodies (bottom panel), respectively. **c** 293T cells were transfected with either TRIF-encoding or Stat4-encoding plasmids. TRAF3 and Flag-tagged ubiquitin plasmids were included in various combinations as indicated. Immunoprecipitation with anti-HA antibody was performed, followed by immunoblotting with anti-Flag antibody to detect ubiquitin-linked TRIF or Stat4. **b, c** Lysates were probed in parallel by direct immunoblotting with anti-HA and anti-V5 antibodies to confirm expression of TRIF and Stat4 in transfected cells, respectively.

Fig. 4. TRIF ubiquitination is mediated by lysine (K)48 linkage. **a** Subconfluent 293T cells were transfected with an empty expression vector (EV) or cotransfected with different expression constructs as indicated. Lysine 48, 63 or both residues were substituted with arginine (K->R) in the wt ubiquitin-encoding construct. Lysates were subjected to immunoprecipitation with anti-HA antibody and Flag ubiquitin-linked TRIF were revealed

by immunoblotting with anti-Flag antibody (top panel). Lysates were directly analyzed by immunoblotting with anti-HA and anti-V5 antibodies to confirm TRIF, Stat4 and TRAF3 expression, respectively, in transfected cells (2nd and bottom panels). **b** Similar ubiquitination assay to that in **a** except that lysine residue was changed to alanine (K->A) in the wt ubiquitin construct.

interaction with wt TRIF, TRAF3 was subjected to a fate similar to the (Δ 160–181) TRIF protein because the latter complexes with wt TRIF to form TRIF heterocomplexes.

We next examined whether association with TRAF3 leads to TRIF ubiquitination. In cells coexpressing wt TRIF, TRAF3 and Flag-tagged ubiquitin, high-molecular weight species were detected from immunoprecipitates of wt TRIF, suggesting these were Flag-tagged, ubiquitinated forms of wt TRIF. Similar smears were detected, but to a lesser extent, from the immunoprecipitates of (Δ 160–181) TRIF, despite higher expression levels of (Δ 160–181) TRIF over the wt counterpart (fig. 3b, top panel, lanes 5–6). Thus, due to its weaker association with TRAF3 that became apparent, (Δ 160–181) TRIF was less ubiquitinated, therefore less targeted for proteasome-mediated proteolysis. Consequently, the (Δ 160–181) TRIF protein accumulated to a higher level than wt TRIF.

A lower level of TRIF ubiquitination was detected in the absence of exogenously expressed TRAF3, likely me-

diated by endogenous TRAF3 or other TRIF-specific E3 ubiquitin ligases (fig. 3c, top panel, compare lane 3 to lane 4 and fig. 4a, b, top panel, compare lane 4 to lane 6). To ascertain the selectivity of TRAF3 as a TRIF-specific E3 ubiquitin ligase, we also demonstrated that Stat4 was not ubiquitinated in the presence of coexpressed TRAF3 (fig. 3c, top panel, lane 8). The lack of ubiquitination occurred in spite of an expression level of Stat4 many-fold higher than that of wt TRIF under the same conditions (fig. 3c, middle panel, compare lanes 5–8 to lanes 2–4), and similar TRAF3 expression (fig. 3c, bottom panel). This result is in agreement with our coimmunoprecipitation (fig. 2e) and confocal microscopy data (fig. 2b). Specific lysines on the ubiquitin are utilized to link ubiquitin to its target, and confer different outcomes for the ubiquitinated protein. The two best characterized models of lysine linkage are with lysine (K)48 and (K)63. Lysine 48 linkage regulates the protein stability as it directs an ubiquitinated protein to the proteasome-dependent proteoly-

sis. On the other hand, lysine 63 linkage has nonproteolytic functions such as regulating DNA damage repair, membrane trafficking, chromatin remodeling and kinase activation [33]. Coexpression of a lysine 48-to-arginine (K48R) ubiquitin mutant (but not a K63R mutant) with TRIF greatly reduced TRIF ubiquitination (fig. 4a, top panel, compare lanes 7–8 to lane 6). Similar results were also obtained by using a lysine 48-to-alanine (K48A) ubiquitin mutant (fig. 4b, top panel, compare lanes 7–8 to lane 6). This indicates that TRIF ubiquitination was predominantly K48-linked. Thus, our data demonstrated that TRIF complexes interact with TRAF3 and undergo lysine 48 linkage ubiquitination. Such ubiquitinated complexes are subsequently shuttled to the proteasome degradation pathway.

Involvement of the Proteasome and Caspases in Degrading TRIF Oligomers

To evaluate a role for proteasome-mediated degradation of TRIF oligomers, we treated 293T cells, previously transfected with wt and (Δ 160–181) TRIF constructs, with different concentrations of the proteasome inhibitor MG132. In the absence of MG132, coexpression of wt and (Δ 160–181) TRIF proteins resulted in a decrease of their respective levels (fig. 5a, top panel, compare lane 2 to lane 4 and fig. 5b, top panel, compare lane 3 to lane 4). Treatment with MG132 did not lead to a significant accumulation of full-length, wt or (Δ 160–181) TRIF proteins (fig. 5a, b, top panels, lanes 5–8). At the highest MG132 concentration tested (50 μ M), the treatment even induced a decrease in TRIF protein levels, likely from the drug toxicity-mediated cell death (fig. 5a, b, top panels, lane 8). However, this led to the detection of small, approximately 30-kDa, C-terminal fragments, with 10–20 μ M of MG132 seeming to be the optimal concentration range (fig. 5a, b, bottom panels, lanes 6–7). We did not detect the corresponding N-terminal fragments since both wt and (Δ 160–181) TRIF proteins were constructed with only C-terminal Flag and HA epitopes, respectively. These approximately 30-kDa fragments were likely generated from the cleavage of full-length TRIF proteins, and were protected from the proteasome-mediated degradation by MG132, so they could be detected by immunoblotting. These cleaved TRIF fragments agree with data from Rebsamen et al. [34] who postulated a role for some cellular enzymes involved in the cleavage of TRIF in virus-infected cells.

Thus, our results suggest that at least two distinct, separate mechanisms operate to terminate TRIF-mediated

functions: cleavage and degradation. In addition to their roles in apoptosis induction, caspases are also well known for their cleavage of various effectors to terminate signaling cascades. To examine whether caspases contribute to the cleaving of TRIF, we treated TRIF-transfected cells with a pan-caspase inhibitor Z-VAD-FMK, alone or together with MG132. Individual use of MG132 or Z-VAD resulted in a partial increase of the full-length TRIF proteins (fig. 5c, 1st and 3rd panels, lanes 5 and 6). A slight increase of the full-length TRIF proteins was detected with the combined treatment of MG132 and Z-VAD (fig. 5c, 1st and 3rd panels, lane 7). This also correlated with a reduction of the smaller 30-kDa fragments, suggesting that cleavage of the full-length TRIF proteins was strongly inhibited (fig. 5c, 2nd and 4th panels, lane 7). Our results indicated that caspases and the proteasome pathway are involved in TRIF cleavage and proteolysis, respectively. However, other enzymes and/or regulatory mechanisms targeting the TRIF oligomers should also be considered since the combined use of MG132 and Z-VAD did not restore the expression levels of TRIF proteins in cotransfected cells compared to the levels when either wt or the (Δ 160–181) TRIF protein was expressed alone (fig. 5c, 1st and 3rd panels, compare lane 7 to lanes 2–3).

(Δ 160–181) TRIF Is Relatively Defective in Mediating IFN Signaling but Not NF- κ B Signaling

To induce type I IFNs and proinflammatory cytokines, TRIF activates IRF-3-mediated, NF- κ B-mediated and AP-1-mediated signaling pathways [35, 36]. To examine the effects of point mutation(s) or deletion(s) of TRIF sequence on TRIF functions, previous reports demonstrated that TRIF overexpression alone was sufficient to activate these signaling pathways, bypassing the requirement for ligand-induced signaling [8, 9, 37, 38]. To determine whether the (Δ 160–181) TRIF activates these signaling pathways as efficiently as the wt counterpart, transfected cell lysates were analyzed for the phosphorylation of key signaling mediators such as p65/RelA, I κ B, TANK-binding kinase 1 (TBK1) and IRF-3. Immunoblotting analysis revealed that in correlation with a higher expression level of (Δ 160–181) TRIF in transfected cells (fig. 6a, top left panel), phosphorylation of p65/RelA and its downstream target, I κ B, were moderately higher than those in wt TRIF-transfected cell lysates (fig. 6b, c, left panels, respectively). Similar amounts of total protein were loaded on gels upon reprobing of the same filters (fig. 6b, c, right panels). This suggests that (Δ 160–181) TRIF mediated activation of the NF- κ B pathway as well, if not better, than

Fig. 5. Inhibition of proteasome degradation and caspase cleavage activities partially protects the full-length TRIF protein. **a** After 19 h of posttransfection, transfected 293T were treated with different concentrations of MG132 for 5 h as indicated. Lysates were analyzed by immunoblotting with anti-Flag antibody (**a**) and anti-HA antibody (**b**) to detect wt TRIF and (Δ 160–181) TRIF proteins. **c** Similar assay to that in **a** except that MG132 (20 μ M) or Z-VAD-

FMK (50 μ M) were used individually or in combination as indicated. Sham (DMSO)-treated 293T cells served as a negative control. Enlargement of the 1st and 3rd panels was shown to better illustrate the full-length (FL) and cleaved C-terminal fragment of wt TRIF as indicated by the arrow. Some cellular factors were non-specifically detected in all samples by the anti-Flag antibody (indicated by an asterisk).

Fig. 6. Wt TRIF and (Δ160–181) TRIF-mediated activation of IRF-3 and NF-κB signaling pathways. **a** Different amounts of DNA of vectors encoding HA-wt or HA-(Δ160–181) (var) TRIF were transfected in 293T as indicated. Empty vector (EV) was also included as a negative control and to keep total amounts of DNA constant. Expression of wt TRIF and (Δ160–181) TRIF was detected by immunoblotting with HA antibody (left panel), and reprobed with Tyk2 antibody (right panel). **b** Extracts in **a** were analyzed with phospho-p65 antibody (left panel). **c** Same as **b** but with phospho-IκB antibody (left panel). **d** Same as **b** but with phospho-TBK1 antibody (left panel). **e** Same as **b** but with phospho-IRF3 antibody (left panel). **b–e** All filters were reprobed with appropriate antibodies to confirm loading of similar amounts of proteins (right panels). **f** 293T cells were cotransfected with an EV or constructs encoding wt TRIF or (Δ160–181) TRIF, an ISRE firefly luciferase, a vector constitutively expressing

Renilla luciferase and a wt TRIF or (Δ160–181) TRIF. Different amounts of DNA of wt TRIF and (Δ160–181) TRIF were used as indicated. At 18 h after transfection, cell extracts were prepared and analyzed for luciferase activity as described in Materials and Methods. Fold-induction indicates normalized luciferase activities of TRIF-transfected cells over that of EV transfectants. Error bars represent the standard error of the mean. Results were reported as the means of 3 experiments where each sample was performed in triplicate, and experiments were repeated 3 times with similar results ($n = 9$). **g** Similar to **f** except that an NF-κB firefly luciferase was used in place of the ISRE firefly luciferase. Asterisks indicate that differences in the luciferase reporter gene activities induced by wt TRIF and (Δ160–181) TRIF proteins were statistically significant ($p \leq 0.0001$), as analyzed by the Student unpaired t test.

wt TRIF. Based on these results combined with the higher expression level of (Δ 160–181) TRIF (fig. 6a), we reasoned that activation of the IRF-3 signaling pathway should also reach higher levels in (Δ 160–181) TRIF transfectants. Surprisingly, TBK1 and its downstream mediator, IRF-3, were slightly less phosphorylated than their counterparts in wt TRIF-transfected cells (fig. 6d, e, left panels, respectively).

For a better quantification, cells were cotransfected with appropriate TRIF plasmids and an ISRE-controlled or NF- κ B-controlled luciferase reporter construct. Wild-type TRIF but not (Δ 160–181) TRIF, activated ISRE-mediated luciferase activity in a DNA dose-dependent manner (fig. 6f). Interestingly, overexpression of (Δ 160–181) TRIF triggered a basal level of ISRE induction, but this level did not increase with increasing amounts of (Δ 160–181) TRIF plasmid DNA. The differences in ISRE reporter gene activities induced by the overexpressed wt and (Δ 160–181) TRIF proteins were proven to be statistically significant ($p < 0.0001$) (fig. 6f). On the other hand, transfection of increasing amounts of (Δ 160–181) TRIF DNA induced an increase of NF- κ B-mediated luciferase activity equal to or better than wt TRIF (fig. 6g), which parallels the phosphorylation results. Thus, in spite of higher expression levels in transfected cells, the (Δ 160–181) TRIF showed a statistically significant partial defect in inducing IRF-3 signaling, but retained its ability to transactivate the NF- κ B pathway as well as wt TRIF.

The (Δ 160–181) TRIF Decreases Wild-Type TRIF-Mediated IFN Signaling

Differences in their respective expression levels in cotransfected cells suggested that TRIF heterocomplexes are likely composed of various ratios of wt TRIF to (Δ 160–181) TRIF (fig. 2). Furthermore, the (Δ 160–181) TRIF was shown to be a weaker transactivator of the IRF-3 signaling pathway than wt TRIF (fig. 6). Therefore, depending on the ratio of wt/(Δ 160–181) TRIF monomers in these heterocomplexes, such TRIF oligomers may have different effects on the transactivation of downstream gene expressions. Consistent with our previous data (fig. 6f), transfection of increasing amounts of (Δ 160–181) TRIF DNA alone increased the IFN-mediated luciferase reporter activity only minimally (fig. 7a, black bars). When cells were cotransfected with increasing amounts of (Δ 160–181) TRIF DNA with a fixed amount of wt TRIF plasmid, they displayed a decrease of ISRE-mediated reporter gene activity compared to that obtained by transfection of wt TRIF plasmid alone [fig. 7a, compare the 2 top white bars with the grey bar of wt TRIF DNA alone (1.5 μ g)].

Under similar conditions, the NF- κ B luciferase activity increased in cells cotransfected with both TRIF plasmids (fig. 7b, white bars). This suggests that while (Δ 160–181) TRIF could enhance wt TRIF-mediated NF- κ B gene activation, it rather ‘diluted’ the effectiveness of wt TRIF-mediated IFN signaling, decreasing luciferase activity in cells expressing both TRIF proteins. On the other hand, cotransfection of increasing amounts of wt TRIF DNA with a fixed amount of (Δ 160–181) TRIF construct showed an increase of the ISRE-mediated luciferase activity (fig. 7c). Under the same conditions, a modest increase of the NF- κ B-mediated luciferase activity was also detected in cells cotransfected with both TRIF plasmids (fig. 7d). In summary, these data indicate that due to a partial defect of (Δ 160–181) TRIF in inducing IFN signaling, the ratio of this variant protein and its wt counterpart found in TRIF oligomers gave rise to different outcomes of downstream gene expression, in particular with the IFN signaling pathway.

Discussion

TRIF is now accepted as the sole adaptor for TLR3 and gene deletion studies have established it to be a pivotal mediator of TLR3 signaling pathways. We report the isolation and characterization of a novel TRIF cDNA from an HTLV-1-infected cell line, CaGT. This cDNA has an in-frame 63 nucleotide deletion, nucleotides 480–543, which corresponds to the loss of encoded aa 160–181. The nucleotides flanking the deletion in the (Δ 160–181) TRIF cDNA do not show typical donor/acceptor splice junctions (fig. 1); nevertheless, we detected its low abundance transcripts in several cell lines, making it unlikely that its presence was a cloning artifact (data not shown). The retrovirus HTLV-1 is well known for dysregulating many host cell functions, ultimately leading to uncontrolled cell growth. Therefore, it is of future interest to examine whether HTLV-1 also affects the host cell splicing machinery as another subversion mechanism contributing to neoplasia. In separate studies, it would perhaps be informative to determine if this TRIF transcript isolate exists more prevalently in leukemic cells of other adult T cell leukemia patients, and whether it correlates with the disease progression.

We chose to focus on the potential functional role of aa 160–181 in TRIF functions. An extensive review of past reports revealed that these aa are linearly distal to other binding sites previously identified in the TRIF amino-terminal domain. This suggests that residues 160–181 may be part of a novel element. Transfection

Fig. 7. Coexpression of wt TRIF and (Δ160–181) TRIF induces different effects on IRF-3 and NF-κB signaling pathways. **a** 293T cells were cotransfected with an empty expression vector (EV) or different amounts of DNA of (Δ160–181) TRIF plasmid with a constant amount of wt TRIF plasmid DNA as indicated, an ISRE firefly luciferase and a vector constitutively expressing Renilla luciferase. At 18 h after transfection, cell extracts were prepared and analyzed for luciferase activity as described in Materials and Methods. Fold-induction indicates the normalized luciferase activities of TRIF-transfected cells over those of EV transfectants. Error bars

represent the standard error of the mean. Results were reported as the means of 3 experiments where each sample was performed in triplicate and experiments were repeated 3 times with similar results ($n = 9$). **b** Similar to **a** except that an NF-κB firefly luciferase was used in place of the ISRE firefly luciferase. **c** Similar to **a** except that different amounts of DNA of wt TRIF plasmid were cotransfected with a constant amount of (Δ160–181) TRIF plasmid as indicated. **d** Similar to **c** except that an NF-κB firefly luciferase was used in place of the ISRE firefly luciferase.

studies consistently showed that the lack of this sequence conferred an unexpected phenotype to the corresponding (Δ160–181) TRIF protein. Expression levels of (Δ160–181) TRIF were many-fold higher than those of wt TRIF, independent of the cell lines used, apoptosis or plasmid amplification (fig. 1). It remains plausible that the higher expression levels of (Δ160–181) TRIF could be attributed at the transcriptional level. However, this

seemed unlikely since both wt and (Δ160–181) TRIF proteins were overexpressed from the same vector containing identical regulatory elements. Furthermore, our studies using a proteasome and caspase inhibitors showed that TRIF was regulated by degradation and/or cleavage at the posttranslational level (fig. 5).

The (Δ160–181) TRIF phenotype has not been reported in any previous studies examining different sites in the

TRIF amino-terminal domain. Our data are reminiscent of that of the SARM protein, as a SARM mutant lacking part of its amino terminus exhibited a higher expression level as well as increased inhibitory functions [38]. Therefore our data suggested that this 21-aa sequence could also modulate TRIF signaling functions.

Deletion or substitution of as little as 1 aa has been shown to disrupt protein-protein interactions, and to thereby alter functions of the mutated protein [26, 39]. However, several lines of evidence suggested that deletion of 21 residues in (Δ 160–181) TRIF induced only specific local conformational changes and affected selective TRIF functions. First, we readily detected complexes of (Δ 160–181) and wt TRIFs in transfected cells (fig. 2). Second, both (Δ 160–181) and wt TRIFs interacted equally well with TLR3 (data not shown). These data suggested that aa 160–181 are unlikely to be involved in TRIF oligomerization or receptor interaction. This was not surprising since TRIF oligomerization requires the proline at position 434 in the TIR domain and other undetermined residues of the carboxy-terminal domain of TRIF [18]. Third, apoptosis occurred to a similar extent in cells overexpressing either wt TRIF or (Δ 160–181) TRIF (fig. 1). Finally, (Δ 160–181) TRIF transactivated NF- κ B signaling as well as wt TRIF (fig. 6). Both apoptosis induction and NF- κ B activation have been assigned to the TRIF carboxy-terminal domain [15, 27, 28]. Taken together, our data strongly implied that folding of the TIR and C-terminal domains of (Δ 160–181) TRIF was likely similar to that of the wt counterpart. Noteworthy, the TRAF6-interacting domain delineated by residues 250–255 remains intact in (Δ 160–181) TRIF. Therefore, TRAF6 binding to (Δ 160–181) TRIF was unlikely to be affected [27, 40]. Furthermore, though TRAF6 has initially been shown to be involved in TRIF-mediated NF- κ B activation [40, 41], gene knockout studies suggested that its precise contribution to this signaling pathway may be cell type-dependent and less critical than RIP1 kinase, the binding site of which is located at the TRIF C-terminal domain [14, 42].

Conflicting data from different groups have been reported about an interaction between TRIF and TRAF3 [20, 23, 43]. At present, the exact causes remain unclear, but differences in the experimental protocols and/or reagents used may explain such discrepancies. Moreover, the precise boundaries and critical residues within such an interacting domain have not been defined so far. Our data strongly suggested that deletion of aa 160–181 impacted TRIF association with TRAF3, for the (Δ 160–181) TRIF inefficiently associated with TRAF3 (fig. 3). In addition, we demonstrated that in the presence of TRAF3,

TRIF underwent lysine 48-linked ubiquitination (fig. 4). The selectivity of TRAF3 as a TRIF-specific E3 ubiquitin ligase was underscored by the lack of similar ubiquitination on the Stat4 protein expressed under the same experimental conditions (fig. 3). Thus, the (Δ 160–181) TRIF protein was less ubiquitinated than its wt counterpart when overexpressed alone, and stably accumulated to higher levels in transfected cells. However, when coexpressed with wt TRIF, the levels of (Δ 160–181) TRIF decreased because following its oligomerization with wt TRIF, the (Δ 160–81) TRIF was targeted for degradation by caspase cleavage and the proteasome degradation pathway. Therefore, our data not only provided a molecular mechanism to explain in part the higher expression phenotype of (Δ 160–181) TRIF observed in transfection studies, but also narrowed the TRAF3-interacting domain to the vicinity of residues 160–181 of TRIF amino-terminal domain. Interestingly, closer examination of this 21-residue sequence shows a lack of an apparent consensus TRAF-binding motif [44]. This deletion (aa 160–181) may directly destroy the TRAF3-binding domain on TRIF. Alternatively, it could disrupt the binding of an intermediate factor which bridges TRAF3 to TRIF. We speculated that perhaps in contrast to the binding sites of other TRAFs, the TRAF3-interacting domain might be created through secondary or ternary TRIF conformation, therefore not being apparent in the linear aa sequence.

Our data also highlighted the fact that TRIF is cleaved as shown by the appearance of smaller fragments, and cleavage occurs independently of proteasome-mediated degradation (fig. 5). Rebsamen et al. [34] previously suggested the involvement of caspases to cleave TRIF is a mechanism to terminate signaling. The TRAF3 expression level was also decreased when TRAF3 was coexpressed with TRIF, implying that it was subjected to the same fate being part of TRIF complexes (fig. 3). This was unexpected because until now TRAF3 itself has only been shown to undergo K63-linked ubiquitination, which primarily regulates protein functions and/or localization, and not stability. Nevertheless, cIAP and TRIAD3A have been reported to add K48-linked ubiquitin moieties to TRAF3, thereby promoting TRAF3 degradation. However, these observations were made in the context of CD40 signaling and virus infection, respectively [45–47], thus they needed to be confirmed specifically for TLR signaling.

The importance of TRAF3 in TLR signaling has recently been appreciated, as TRAF3-deficient macrophages produced lower levels of type I IFNs and IL-10 in response to various TLR agonists and were less resistant to virus infec-

tion [23, 25]. Specifically related to TLR3/TRIF signaling, TRAF3 has been shown to be required for marshaling the TBK1 protein into the TRIF signalosome [23]. Consistent with this paradigm, our results showed that in (Δ 160–181) TRIF-transfected cells, TBK1 and its downstream mediator IRF-3 were less phosphorylated than in wt TRIF-transfected cells. This was also confirmed by the analyses of IRF-3-mediated luciferase activity (fig. 6). In contrast, Tatamatsu et al. [17] demonstrated that leucine at position 194 of TRIF was crucial in the recruitment of TBK1 to TRIF in a TRAF3-independent manner. Furthermore, a TRIF mutant lacking the amino-terminal 180 residues exhibited greater transactivation of the IFN- β promoter than wt TRIF, implying an inhibitory role for the TRIF amino-terminal domain [17]. Since IRF-3 is required for type I IFN production and all IFN-responsive genes, which are crucial in the innate antiviral response, different mechanisms may operate in parallel to ensure optimal TRIF-mediated IRF-3 activation. Finally, involvement of other TRAF members, notably TRAF2, should not be discounted since we still observed a basal level of IRF-3 signaling as well as TRIF ubiquitination with the (Δ 160–181) TRIF (fig. 4, 6).

In addition to its role in antiviral responses, TRIF is also involved in tumor eradication by participating in dendritic cell-mediated antitumor natural killer (NK) ac-

tivation or as a genetic adjuvant to enhance immune responses against some tumors [48, 49]. The importance of TRIF is further underscored because some viruses target it for inactivation to avoid immune detection [34, 50]. TRAF3 proved to be a critical regulator of B lymphocyte homeostasis [51]. Recently, the mutation of a single aa in TRAF3 has been shown to be responsible for impaired TLR3 responses and increased susceptibility to HSV-1 encephalitis in humans [24]. In addition, TRAF3 signaling was also subverted to benefit virus-induced cell transformation, notably by Epstein-Barr virus latent membrane protein-1 (LMP-1) [52–54].

Our study highlights the presence of a novel element in the TRIF amino-terminal domain which appears to be important for TRAF3 association, TRIF stability and TRIF-mediated IFN signaling. Our results contribute to further the understanding of the TRIF-TRAF3 interactions. This is a crucial aspect of innate immunity and it may lead to the development of new therapeutic agents to combat virus infections or even cancer.

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