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Key Molecular Contacts Promote Recognition of the BAFF Receptor by TNF Receptor-Associated Factor 3: Implications for Intracellular Signaling Regulation

Chao-Zhou Ni,* Gagik Oganesyan,† Kate Welsh,* Xiwen Zhu,* John C. Reed,* Arnold C. Satterthwait,* Genhong Cheng,‡ and Kathryn R. Ely‡‡

B cell-activating factor belonging to the TNF family receptor (BAFF-R), a member of the TNFR superfamily, plays a role in autoimmunity after ligation with BAFF ligand (also called TALL-1, BLyS, THANK, or zTNF4). BAFF/BAFF-R interactions are critical for B cell regulation, and signaling from this ligand-receptor complex results in NF-κB activation. Most TNFRs transmit signals intracellularly by recruitment of adaptor proteins called TNFR-associated factors (TRAFs). However, BAFF-R binds only one TRAF adaptor, TRAF3, and this interaction negatively regulates activation of NF-κB. In this study, we report the crystal structure of a 24-residue fragment of the cytoplasmic portion of BAFF-R bound in complex with TRAF3. The recognition motif 162PVPAT166 in BAFF-R is accommodated in the same binding crevice on TRAF3 that binds two related TNFRs, CD40 and LTβR, but is presented in a completely different structural framework. This region of BAFF-R assumes an open conformation with two extended strands opposed at right angles that each make contacts with TRAF3. The recognition motif is located in the N-terminal arm and intermolecular contacts mediate TRAF recognition. In the C-terminal arm, key stabilizing contacts are made, including critical hydrogen bonds with Gln379 in TRAF3 that define the molecular basis for selective binding of BAFF-R solely to this member of the TRAF family. A dynamic conformational adjustment of Tyr377 in TRAF3 occurs forming a new intermolecular contact with BAFF-R that stabilizes the complex. The structure of the complex provides a molecular explanation for binding affinities and selective protein interactions in TNFR-TRAF interactions. The Journal of Immunology, 2004, 173: 7394–7400.

In human autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, and Sjögren’s syndrome (1–3), levels of B cell-activating factor belonging to the TNF family (BAFF), a TNF family ligand (also known as BLyS, TALL-1, THANK, TNFSF13B, or zTNF4) are elevated. Studies with transgenic mouse models have shown that overexpression of BAFF leads to the development of severe autoimmune disorders as well as B cell hyperplasia (4–6). Because high BAFF levels appear to correlate with the severity of the disease, it has been proposed that this ligand may play a direct role in autoimmune pathogenesis by influencing B cell survival and maturation (7).

BAFF signals by binding to three receptors, which are each members of the TNFR superfamily: transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI), B cell maturation Ag (BCMA), and BAFF-R (also known as BR3) (5, 8–10). Although TACI and BCMA also bind to a related ligand (TANK, TRAF-associated NF-κB activator).

BAFF-R but not TACI or BCMA produced a BAFF-deficient phenotype (10–12). Moreover, a naturally occurring BAFF-R mutation (WySnl strain) phenocopies the defect in BAFF knockout mice, suggesting that BAFF/BAFF-R interactions are critical in B cell regulation (8, 10). In addition, BAFF-induced signaling results in strong activation of the type 2 NF-κB pathway and to a lesser extent that of type 1. This is unlike other TNFR family members where type 1 NF-κB activation predominates.

BAFF-R is a type III transmembrane protein expressed on B cells. The extracellular domain of this unusual member of the TNFR family is significantly shorter than the comparable region in other TNFR family members. Typically, the extracellular domains of TNFRs consist of three to four cysteine-rich domains (CRD) that each are stabilized by three disulfide bridges. In contrast, BAFF-R bears only one CRD, stabilized by two noncanonical disulfide bridges in the extracellular region, suggesting that ligand-receptor recognition is novel in this system. Structural studies of the BAFF/BAFF-R complex have revealed that the CRD of BAFF-R is atypical, while the monomeric BAFF ligand assumes a jelly-roll folding pattern, similar to other TNF family ligands. In the complex, residues from a hairpin loop in BAFF-R provide the key contacts with the large trimer BAFF ligand (13, 14).

BAFF signaling mirrors that of other TNF family ligands in many ways; i.e., trimeric ligand induces clustering of BAFF-R at the membrane and the signal is then transmitted intracellularly by recruitment of adaptor proteins called TNFR-associated factors (TRAFs). BAFF-R and other TNFRs have no intrinsic enzymatic activity but rather direct intermolecular contacts with TRAFs or other downstream signaling molecules. Interestingly, while six TRAFs have been identified in humans, BAFF-R binds only to TRAF3 (15), which may negatively regulate activation of NF-κB by BAFF-R. This is in contrast to other TNFRs that bind to
several TRAFs. TNFRs, including BAFF-R, participate in a complex set of related interactions involving binding of short four to six residue motifs that are specifically recognized by TRAFs (reviewed in Ref. 16).

The protein interactions of several TNFRs, e.g., CD40 and lymphotixin β receptor (LTβR), have been defined in crystal structures of their complexes with TRAF3 (17, 18). Likewise, peptides representing the binding motif from a number of TNFRs have been co-crystallized with TRAF2, defining a common recognition sequence PXQXT or (P/S/T/A)X(Q/E)E (19). The crystal structures revealed that binding recognition involves specific contacts of the recognition sequence in a hydrophobic crevice on the surface of the TRAF molecule. The binding motifs from CD40, LTβR, or the downstream regulator TRAF-associated NF-κB activator (TANK) (20) are each accommodated in the same binding crevice.

The cytoplasmic domain of BAFF-R is 63 residues in length and shows little homology with the intracellular portion of other TNFRs. However, deletion mutants were used to map the sequences in this domain that are required for interaction with TRAF3 (15). The study suggested that a six-residue membrane proximal region and the C-terminal 35 residues of the domain were involved in binding recognition. Within the latter segment, a sequence PVPAT167 bears recognizable homology with the TRAF3 recognition motif in CD40 (PVQET) and the downstream regulator TANK (PIQCT). For this reason, in this study, a 24-aa fragment of the cytoplasmic domain of BAFF-R (C24) was crystallized in complex with TRAF3 to examine binding contacts. The crystal structure revealed that the PVPAT motif binds in the same surface binding crevice on TRAF3 that accommodates the homologous sequences in CD40 (17) and TANK (20), as well as the more distantly related motif IPEEGD in LTβR (21). Binding contacts observed in the complex were confirmed by mutational analyses. Unique contacts were also identified that may provide the molecular basis for binding of BAFF-R to TRAF3, but not TRAF2.

**Materials and Methods**

**Crystallographic analysis**

Crystals of TRAF3 were grown as described (18) using a protein preparation where tryptic digestion of the long N-terminal 

162PVPAT167 bears recognizable homology with the TRAF3 recognition motif in CD40 (PVQET) and the downstream regulator TANK (PIQCT). For this reason, in this study, a 24-aa fragment of the cytoplasmic domain of BAFF-R (C24) was crystallized in complex with TRAF3 to examine binding contacts. The crystal structure revealed that the PVPAT motif binds in the same surface binding crevice on TRAF3 that accommodates the homologous sequences in CD40 (17) and TANK (20), as well as the more distantly related motif IPEEGD in LTβR (21). Binding contacts observed in the complex were confirmed by mutational analyses. Unique contacts were also identified that may provide the molecular basis for binding of BAFF-R to TRAF3, but not TRAF2.

**Peptide synthesis**

The peptide representing a segment of the C-terminal region of the cytoplasmic domain of BAFF-R acetyl-SVPVATELGSTELVTTKTAGPEQ-amide was synthesized using Fmoc chemistry with diisopropylcarbodiimide/hydroxybenzotriazole (DIC/HOBt) coupling on Rink’s amide MBHA resin (loading 0.66 mmol/g resin) with an Advanced ChemTech 350 multiple peptide synthesizer. The peptide was cleaved from the resin and deprotected by treatment with 95:2.5:2.5 TFA:H₂O:TIS for 2 h at room temperature. The cleaved peptide was precipitated and washed with cold diethyl ether. After drying, the peptide was dissolved in aqueous acetonitrile, and purified with a Gilson HPLC apparatus (Middleton, WI) on a Cosmosil column (5C18-AR, 20 x 250 mm; Phenomenex, Torrance, CA) with detection at 210 nm. The peptide was separated from impurities using a linear gradient of 20–50% B over 30 min (solvent A: 0.1% trifluoroacetic acid in water, solvent B: 0.1% trifluoroacetic acid in 90% acetonitrile) at a flow rate of 8 ml/min and eluted as a single peak at 22 min. The purified peptide was analyzed and confirmed by MALDI-TOF mass spectrometric analysis with an Applied Biosystems Voyager System 6264 (Foster City, CA).

**Constructs and expression**

To generate single amino acid mutations of residues 162, 166, 170, and 175 to alanine in the C-terminal fragment of BAFF-R (160SVPVATELGSTELVTTKTAGPEQ183), oligonucleotides bearing the corresponding changes were annealed and cloned into BamHI and EcoRI sites of the pGex2T expression vector (Pharmacia, Peapack, NJ). Similarly, the full BAFF-R cytoplasmic region was amplified by PCR from a B cell cDNA library and cloned into pGex2T. The constructs were expressed in Top10 Escherichia coli cells, and the GST-fused proteins were purified as described previously (28). Construction of the pEBB-flu-tagged TRAF3 vector was also previously described (29).

**Cell culture and transfections**

293T human embryonic kidney cells were grown in DMEM with 10% FBS and 1% penicillin-streptomycin (Invitrogen Life Technologies, Carlsbad, CA). 293T cells were transfected with 10 μg of flu-tagged TRAF3 DNA in 100-mm plates by the calcium phosphate method and harvested 36–48 h after transfection.

**Binding assays**

The binding of BAFF-R to TRAF3 was tested in physical binding assays, but the binding was weak and not measurable by isothermal titration calorimetry, similar to what was observed in previous studies of the binding of LTβR to TRAF3 (21). Consequently, in vitro binding assays were used which have the capability to measure significant differences in binding. TRAF3-transfected 293T cells were lysed in 10 ml of lysis buffer (150 mM NaCl, 20 mM HEPES, pH 7.2, 10 mM NaF, 0.4 mM EDTA, 1% Triton X-100, 0.5 mM Na$_2$VO$_4$, 1 mM PMSF, 1 mM aprotinin, 0.5 μg/ml leupeptin). Membranes were pelleted by centrifugation (14,000 rpm for 10 min) and the supernatant was used for binding assays. Equal amounts of
Results
Structure of the BAFF-R/TRAF3 complex

TRAF3 is an adaptor molecule that functions as a trimer in solution. The trimer is stabilized by coiled-coil interactions between the long N-terminal α-helices of the protein (Fig. 1, A and B). In the crystal, one monomer is the asymmetric unit, with the structurally identical subunits in the trimer related by 3-fold crystallographic symmetry. At the end of the long helices, the TRAF C-domain (residues 348–504) is independently folded into an eight-stranded β-sandwich that consists of two layers of anti-parallel β-sheets (Fig. 1). This folding pattern is conserved in TRAF3 (17), TRAF2 (30, 31), and TRAF6 (32).

The TRAF C-domains are involved in intermolecular contacts in the crystal lattice, and therefore, because the trimer is shaped like a mushroom, large solvent channels exist along the length of the extended helices in the N-terminal region of the molecules. To form the complex, a 24-residue synthetic peptide that corresponds to the C-terminal sequence of the intracellular domain of BAFF-R was dissolved and soaked into existing crystals of TRAF3. The sequence 160GSPVPATELGSTELVTYDAGPEQ183 contains a motif PVPAT which bears recognizable homology to the recognition motifs that promote binding of CD40, CD30, and TANK to TRAF3 (17, 20). This segment of the cytoplasmic domain of BAFF-R was shown to be required for binding by deletion mapping (15). In that study, full-length BAFF-R with a deletion of 31 residues at the C terminus was not able to associate with TRAF3 in 293T cells. Although an additional membrane-proximal sequence of six residues was also implicated in that study, our results indicate that core binding recognition is mediated by the C24 segment used in the present study (see Fig. 3A).

The dissolved peptide diffused through the solvent channels in the crystal lattice and bound to a surface crevice on the β-sandwich of the TRAF3 domain, as shown in Fig. 1. The binding stoichiometry was 1:1 and because of the crystallographic symmetry, the structure of the peptide is identical in each of the three sites in the complex. After refinement, there was clearly defined electron density for residues 160–176. Strong intermolecular interactions stabilize the complex within this segment, but apparently the C-terminal seven residues do not make contact with TRAF3, and thus are flexible and not clearly defined in the density. It should be noted that the long BAFF-R peptide is accommodated in a crevice on the surface of TRAF3 and this crevice is located in a restricted solvent “cave” that exists in the TRAF3 crystals. The overall dimensions of this cave are 15 × 19 × 22 Å and the space in this portion of the crystal lattice is sufficient to accommodate the peptide so that the conformation is not affected by crystal packing (17, 20, 21). The results of the refinement of the complex are presented in Table I.

Structure of BAFF-R

In the BAFF-R/TRAF3 complex, residues 160–176 were clearly visible in the electron density maps and the side chains for all residues could be placed with confidence (Fig. 1C). In particular, the pattern of density for the sequence 160GSPVPATELGSTEL177 was unambiguous for assignment. BAFF-R residues 163PVPAT166 are accommodated in the binding crevice across the edge of the β-sandwich domain of TRAF3. Because this is precisely the same crevice that binds CD40 and LTβR, the structure reveals that the sequence PVPAT serves as the recognition motif in BAFF-R for interaction with TRAF3. Unlike CD40 and LTβR, which assume hairpin configurations when binding to TRAF3, the cytoplasmic domain of BAFF-R binds to TRAF3 in an open extended conformation (see Fig. 1). The structure consists of two extended strands that directly contact the TRAF domain, linked by two residues (Leu160–Gly169) that do not make contact with TRAF3 (see Fig. 1D). The two extended strands are opposed at right angles through the connector residues. Residues 160–167 in the first β-strand encompass the TRAF recognition motif and make several important intermolecular contacts. In the carboxyl segment, residues 170–176 form the second extended β-strand of the molecule. This strand lies in a parallel arrangement with consecutive main chain-main chain hydrogen bonds. Residues Glu172 and Thr175 participate in key contacts with TRAF3.

BAFF-R/TRAF3 intermolecular contacts

In the N-terminal strand, the first residue in the recognition motif, Pro162, lies within van der Waal’s contact of Phe148 in TRAF3 in the upper end of the hydrophobic crevice. The last residue in the motif, Thr166, participates in a hydrogen bond network with TRAF residues Tyr395 and Asp399. Glu167 lies within distance to form a salt bridge with Arg193 in TRAF3, although in the final refined complex the guanidinium group of Arg193 is rotated away. For the second strand, in addition to the backbone hydrogen bonds aligning the parallel intermolecular β-sheet interactions, Glu172 forms a salt bridge with Arg164 in TRAF3 and a critical hydrogen bond is made between the side chains of Thr175 and Gln379 in TRAF3. Thus, several key intermolecular contacts are made (Fig. 1D) on both strands of this BAFF-R fragment that contribute to formation of the complex. Few contacts are likely to involve BAFF-R residues beyond residue 176, because these residues remain flexible in the complex and are not ordered in the electron density maps.

The intermolecular contacts observed in the crystal structure were tested by site-directed mutagenesis. Residues within the recognition motif, as well as residues in the connecting linker and the second strand, were mutated to alanine to evaluate these sequences for their individual contributions to binding (see Fig. 3). Binding was only marginally diminished by substitution of alanine for Pro162 or Thr166, in the recognition motif. For Pro162, the change to alanine maintains the hydrophobic character of the side chain. Because binding in that part of the crevice is primarily due to hydrophobic interactions, loss of binding would not be expected as a result of this substitution. For the T166A mutation, the primary intermolecular contact is a main chain-side chain hydrogen bond formed between the polypeptide amide of Thr166 and the side chain carboxylate of Asp399. Substitution of a different side chain would not affect this molecular contact. Changes of residues in the linker, e.g., Ser170Ala, did not affect binding. However, substitution of alanine for Thr175 completely abolished binding of BAFF-R to TRAF3 (Fig. 3). Interestingly, Thr175 has the potential to make two hydrogen bonds with Gln379 in TRAF3. In contrast to the situation described for Thr166, the side chain of this threonine is located within hydrogen bonding distance of the polypeptide amide as well as the side carboxylate of Gln379 on TRAF3. Loss of the side chain hydroxy1 group through substitution of alanine for threonine would therefore have serious consequences for recognition, confirming the loss of binding seen in the mutagenesis experiments (Fig. 3). These results suggest that the distal intermolecular contact is essential for binding of BAFF-R to TRAF3. Residues in the recognition motif may be critical for initial docking in formation of the protein interactions, but Thr175 may provide the key hydrogen bonds that stabilize the complex.
FIGURE 1. Structure of the BAFF-R/TRAF3 complex. A, The TRAF3 trimer is shown schematically with each subunit colored separately as a ribbon model. The subunits are stabilized by coiled-coil interactions between the elongated α-helices at the N terminus of each monomer. Each monomer binds one BAFF-R molecule, which is shown in a white ball-and-stick model. The subunits and bound BAFF-R are identical in structure and related by crystallographic 3-fold symmetry. BAFF-R binds to a crevice at the edge of the β-sandwich conserved TRAF domain, and wraps around the domain in an extended configuration. For orientation, in this image the cell membrane is located above the trimer. B, View from the top of the trimer. The 3-fold symmetry is clearly apparent from this view. BAFF-R binds to TRAF3 in an extended conformation. C, The model of the C-terminal 24 residues of BAFF-R is displayed in the electron density map. The 2Fo-Fc density map, contoured at 2.7-Å resolution, clearly defined the polypeptide backbone and orientation of side chains so that the peptide was fitted unambiguously to the density. For clarity, the residues in the TRAF recognition motif PVPAT are labeled. D, Molecular contacts for BAFF-R/TRAF3 interaction. In this view, the principal hydrogen bonds and ionic interactions that mediate and stabilize the formation of the BAFF-R/TRAF3 complex are drawn as pink lines. TRAF3 is shown in orange as ribbon model, with contact residues shown as gray stick models. BAFF-R (green stick model) binds TRAF3 in an open conformation and key intermolecular contacts are made along the length of this BAFF-R cytoplasmic fragment. Residues participating in intermolecular interactions are labeled, and the residue numbers for BAFF-R are in green. Glu167 (BAFF-R) and Arg393 (TRAF3) are also labeled because these two residues are clearly in position to participate in an intermolecular salt bridge, although this contact was not actually formed in the crystal structure presented here.
The recognition motif of BAFF-R is presented in a structural context that is completely different from the folding pattern seen in the TRAF3-bound state of CD40 or LTβR. The binding portion of these two TNFRs forms a hairpin or reverse turn configuration with the recognition motif located in the first strand of the hairpin (17, 21). In contrast, BAFF-R forms an extended and open secondary structure, shaped not unlike a boomerang. This open structure provides extensive surface area to contact TRAF3 and critical protein-protein contacts are made by both extended strands of the boomerang. It is extremely interesting to note that the boomerang structure is closely similar to that assumed by another molecule that binds to TRAF3, TANK (Ref. 29; inhibitory TRAF, Ref. 34), a modulator of TRAF signaling, binds to TRAF3 and the recognition motif in TANK that promotes this interaction is PIQCT. This motif is presented in a boomerang structure that is quite similar to that seen in BAFF-R and reported here.

**Comparison of BAFF-R and TANK molecular contacts with TRAF3**

Both BAFF-R and the downstream regulator protein TANK bind to TRAF3 in a boomerang-like configuration, with contacts covering a broad area around the girth of the TRAF3 β-sandwich. A comparison of these two complexes reveals a set of contacts that may represent a pattern for this type of extensive interaction with TRAF3, similar to the pattern of hot spots that have been identified in the main binding crevice. The protein-protein interactions occur in two areas with contacts contributed by residues in the two extended strands. The connecting linker in the boomerang is two residues in length in BAFF-R, but in contrast is four residues long in TANK (20). The first contact region is the binding crevice where the pentapeptide motifs are bound; PVPAT in BAFF-R and PIQCT in TANK. The motifs are accommodated in a similar manner in this binding crevice (see Fig. 4 in Ref. 20). The first residue in the motif, proline, is located in the same hydrophobic pocket adjacent to Phe in TRAF3. The third residue in the motif, glutamine (Gln) in TANK, forms hydrogen bonds with a serine cluster in the TRAF3 pocket, but these contacts are not made in BAFF-R because this residue is proline. The last residue in the
motif, threonine, is located within hydrogen-bonding distance of the side chain carboxyl group of Asp$^{399}$ in TRAF3. This hydrogen bond is actually formed in the TANK/TRAF3 complex but not in the BAFF-R/TRAF3 complex.

The second contact region is the β-strand located at the edge of one layer of β-sheet in the TRAF3 domain. For this interaction, the second strand of the boomerang aligns next to residues 375–377 in TRAF3 with intermolecular hydrogen bonds forming in a parallel β-sheet pattern. At the end of this strand, a critical hydrophobic interaction is made with Trp$^{156}$ in TRAF3. In TANK, the participating residue is phenylalanine (Phe$^{194}$), but its equivalent in BAFF-R is valine. In the BAFF-R complex, because the side chain of valine is smaller than phenylalanine, the two residues are within van der Waal’s contact. The next residue makes a hydrogen bond with Gln$^{379}$ in TRAF3. In TANK this residue, Lys$^{195}$, makes a water-mediated hydrogen bond with the TRAF3 glutamine, but in BAFF-R, the equivalent, Thr$^{175}$ is in position to form a direct hydrogen bond with Gln$^{379}$. Interestingly, Lys$^{195}$ (TANK) and Thr$^{175}$ (BAFF-R) correspond to the last or penultimate residue that is visible in the electron density map for each complex, suggesting that the structures have captured the pattern of intermolecular contacts required to form a stable complex.

It is also of interest to note that the core sequence of BAFF-R that has been shown here to bind to TRAF3 (24 residues at the C-terminal region) is 100% conserved between human and mouse BAFF receptors, compared with only ~50% or less homology for the rest of the molecule (8). Strict conservation of the TRAF3 binding region across species (mouse and human) suggests functional significance and an important regulatory role for this interaction.

**BAFF-R binds only to TRAF3**

Unlike many TNFRs, BAFF-R binds only to TRAF3 and not to other related TRAFs. The structure of the BAFF-R/TRAF3 complex provides a molecular understanding for this specific recognition. BAFF-R Thr$^{175}$ is implicated in two key contacts with TRAF3 by forming hydrogen bonds with the side chain oxygen as well as the backbone amide of Gln$^{379}$. In TRAF2, the equivalent of Gln$^{379}$ is proline. This difference is significant because with proline at that position in TRAF2, neither of the key intermolecular contacts could be made with Thr$^{175}$ in BAFF-R. A proline side chain is cyclized back to the polypeptide backbone, thus eliminating the potential for hydrogen bonds with the side chain or the backbone amide. Without this critical contact, it appears that a stable complex with TRAF2 could be formed. Docking simulations with the known structure of TRAF2 (19) suggest that other contacts seen in the BAFF-R/TRAF3 complex and illustrated in Fig. 1D could also be made with TRAF2, suggesting that a single amino acid difference in TRAF3 and TRAF2 has considerable consequence for specific recognition and signaling. For TRAF6, in contrast, docking experiments with BAFF-R revealed numerous collisions and steric overlaps. This was not unexpected because the recognition pocket on TRAF6 is very different from other known TRAF structures (32). For TRAF5, simple homology alignments with TRAF3 do not provide an explanation for the failure of BAFF-R to bind to TRAF5. Residues corresponding to TRAF3 contact residues for BAFF-R are identical or closely conserved. Interestingly, the residue that corresponds to Gln$^{379}$ in TRAF3 is also glutamine in TRAF5, so the potential to form key hydrogen bonds with BAFF-R Thr$^{175}$ exists. Therefore, in the absence of a crystal structure of TRAF5, it is difficult to understand why BAFF-R does not bind to this TNFR.

**Molecular adaptation and stabilization of an extended protein-protein interface**

A paucity of information is available to define a pattern of contacts that promote molecular recognition at protein-protein interfaces. The patterns are difficult to predict when the interacting surfaces are large or extended and thus structural data are still being collected to understand the mechanisms that foster and stabilize the binding. In the case of the BAFF-R/TRAF3 complex, two major contact regions involving extended polypeptide strands in BAFF-R are involved, each extending through six residues. In the first case, there is significant understanding of the recognition, after identification of hot spots for recognition (33). Now with a comparison of similar interactions in hand for BAFF-R/TRAF3 and TANK/TRAF3 complexes, we are able to look for contacts that promote the docking as well as stabilize the formed complex. In comparing the structure of the TRAF3 domain bound to CD40 and LTβR (hairpin/reverse turn loop configuration) with the structure bound to BAFF-R and TANK (open boomerang configuration), one notable difference is seen. Tyr$^{377}$ in TRAF3 undergoes a striking conformational shift between the two structures (Fig. 2). In unliganded TRAF3 and TRAF3 bound to complex to CD40 or LTβR, the hydroxyl of this tyrosine forms a hydrogen bond with the guanidinium group of Arg$^{393}$. When bound to BAFF-R or TANK, this hydroxide does not form and the phenyl ring is rotated away from the domain so that the side chain is positioned close to Leu$^{174}$ in BAFF-R. The same is true for TANK/TRAF3 where Tyr$^{377}$ is within van der Waal’s distance of Leu$^{193}$ in TANK (see Fig. 3 in Ref. 20). It is possible that binding of BAFF-R or TANK to TRAF3 weakens the hydrogen bond between Arg$^{393}$ and Tyr$^{377}$ (TRAF3), through other intermolecular contacts with Arg$^{393}$. In this case, Tyr$^{377}$ may flip down to produce a new hydrophobic intermolecular contact with leucine in the partner, thus contributing to stabilization of the complex. Interestingly, the electron density for the phenyl ring of Tyr$^{377}$ is found in both positions, suggesting that in this complex the two orientations of the side chain occur with equal occupancy. This density pattern persisted even after final extensive cycles of refinement. For the unliganded TRAF3, no density is found in the flipped-down position, but solely and clearly for the orientation when the side chain is hydrogen-bonded to Arg$^{393}$. This is consistent with the observation in the BAFF-R/TRAF3 complex that although the potential exists for formation of an intermolecular salt bridge between the side chains of Gln$^{167}$ in BAFF-R with Arg$^{393}$ in TRAF3, the arginine side chain is not positioned close enough for the bond to form (see Fig. 1D). For the BAFF-R/TRAF3 complex, the tyrosine ring is found in two orientations, serving as a graphic example of the dynamic molecular adaptation that occurs in these protein-protein complexes. Such conformational adjustments are likely to be essential for binding affinity in systems such as the TNFR signaling pathways, where binding affinities have important functional implications. The results also provide valuable new insights for design of small molecule drugs directed against the ligand-binding pockets of TRAFs.

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4 Interestingly, the comparable intermolecular hydrogen bond does form in the TANK/TRAF3 complex between Asp$^{185}$ in TANK and Arg$^{393}$ in TRAF3 (20).
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