An Atypical Tumor Necrosis Factor (TNF) Receptor-associated Factor-binding Motif of B Cell-activating Factor Belonging to the TNF Family (BAFF) Receptor Mediates Induction of the Noncanonical NF-κB Signaling Pathway

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BAFF receptor (BAFFR) is a member of the TNF receptor (TNFR) superfamily that regulates the survival and maturation of B cells. BAFFR exerts its signaling function by inducing activation of NF-κB, although the underlying mechanism has not been well defined. By using a chimeric BAFFR, we show that BAFFR preferentially induces the noncanonical NF-κB signaling pathway. This specific function of BAFFR is mediated by a sequence motif, PVPAT, which is homologous to the way. This specific function of BAFFR is mediated by a sequence motif, PVPAT, which is homologous to the typical TRAF-binding sequence, PVQET, is sufficient to render the BAFFR capable of inducing strong and more rapid association with TRAF3. Interestingly, modification of the PVPAT sequence to the typical TRAF-binding sequence, PVQET, is sufficient to render the BAFFR capable of inducing strong NF-κB signaling. Further, this functional acquisition of the modified BAFFR is associated with its stronger and more rapid association with TRAF3. These findings suggest that the PVPAT sequence of BAFFR not only functions as a key signaling motif of BAFFR but also determines its signaling specificity in the induction of the noncanonical NF-κB pathway.

B cell-activating factor belonging to the TNF family (BAFF) is a critical factor that regulates the survival and maturation of peripheral B cells. BAFF interacts with three receptors of the TNF receptor (TNFR) superfamily: transmembrane activator, calcium modulator, and cyclophilin ligand interactor (TACI), B cell maturation antigen (BCMA), and BAFF receptor (BAFFR). Genetic evidence suggests that BAFFR is primarily responsible for mediating the role of BAFF in B-cell survival and maturation (2–5). It is generally believed that the BAFF/BAFFR system mediates B-cell survival by promoting the expression of anti-apoptotic genes belonging to the Bcl-2 family, such as Bfl-1 (also named Bfl-1) and Bcl-XL (6). The BAFFR signal appears to function in cooperation with other signals, especially those elicited by the B-cell receptor (6), because recombinant BAFF is insufficient to trigger significant expression of the anti-apoptotic genes in cell culture (7, 8).

A major signaling event induced by BAFF is activation of NF-κB, a family of inducible transcription factors that play pivotal roles in regulating diverse aspects of immune function (9–11). The NF-κB family is composed of five members: RelA, RelB, c-Rel, NF-κB1, and NF-κB2, which function as various dimeric complexes that transactivate specific target genes via binding to the κB enhancer (12). The function of NF-κB is normally suppressed by their physical association with inhibitory proteins, including IκBα and homologs as well as the NF-κB1 and NF-κB2 precursor proteins, p105 and p100 (13). Activation of NF-κB by most cellular inducers involves phosphorylation and subsequent degradation of IκBα and the concomitant nuclear translocation of p50/RelA and p50c/Rel NF-κB complexes (14). This so-called canonical pathway of NF-κB activation is mediated by a multisinubunit IκB kinase (IKK) composed of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ (14).

Recently, another mechanism of NF-κB activation has been identified, which is based on the inducible processing of the NF-κB2 precursor protein p100 (15), an IκB-like molecule specifically inhibiting the nuclear translocation of RelB (16). This mechanism, known as the noncanonical NF-κB pathway, is required for generation of p52 as well as the nuclear translocation of RelB-containing NF-κB complexes (17). Interestingly, activation of the noncanonical NF-κB pathway is specifically mediated by a subset of TNFR family members, including BAFFR, CD40, lymphotoxin β receptor, and receptor activator of NF-κB (18–22). Like BAFFR, CD40 plays an important role in regulating the function of B cells, although these two receptors regulate different aspects of B-cell function. Whereas BAFFR regulates the maturation of B cells, CD40 is particularly important for the survival and differentiation of antigen-activated B cells in the germinal centers (23).

Although the signaling mechanism of BAFFR remains poorly understood, the signaling function of CD40 has been extensively studied. The cytoplasmic domain of CD40 contains specific motifs for binding to members of the TNFR-associated factors (TRAF) (23). One such motif, PVQET, predominantly
with the pCLXSN retroviruses encoding wild type and mutant forms of the hCD40-BAFFR chimeras as previously described (32). After drug selection (G418), bulk infected cells were used in the experiments to avoid intercellular variations. All of these B-cell lines were cultured in RPMI medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine, antibiotics, and 10 μg 2-mercaptoethanol. The kidney carcinoma cell line 293T was cultured in Dulbecco’s medium with the same supplements except the lack of 2-mercaptoethanol. Transient transfection of 293 cells was carried out using Lipofectamine 2000 (Invitrogen). For retrovirus production (32), the cells were transfected with FuGENE 6 (Roche Applied Science).

Fluorescence-activated Cell Sorting—About 1 × 10^6 cells were resuspended in 100 μl of FACS buffer (2% fetal bovine serum in phosphate-buffered saline). The cells were stained with fluorescein isothiocyanate-conjugated anti-hCD40 antibody and subjected to FACS analysis using a BD Biosciences FACScan at the Pennsylvania State College of Medicine Cell Science/Flow Cytometry Core Facility.

RESULTS

BAFFR Preferentially Induces the Noncanonical NF-κB Signaling Pathway—To study the signaling function of BAFFR, we generated a chimeric receptor containing the cytoplasmic domain of murine BAFFR and the extracellular ligand-binding domain of human CD40 (hCD40) (Fig. 1A). Within this chimera, the signaling activity of BAFFR could be stimulated with an agonistic anti-hCD40 antibody without the interference of TACI or BCMA or the endogenous BAFFR. Further, this chimeric receptor allows direct comparison of the signaling functions of BAFFR and CD40 using the same stimulus. By retroviral infection, we stably expressed the hCD40-BAFFR chimera in a murine B cell line, M12 (30), which has been used as a model for studying the signaling function of both CD40 (36–38) and BAFFR (29). Because close to 100% of the cells expressed hCD40-BAFFR (Fig. 1B), we used the bulk-infected cells for the signaling studies. To compare the signaling activity of BAFFR with that of CD40, we also included M12 cells stably expressing the wild type (intact) human CD40 (M12-hCD40).

We examined the BAFFR-mediated activation of NF-κB signaling pathways by detecting the processing of p100 and degradation of IκBα, indicators of noncanonical and canonical NF-κB pathways, respectively (17). Consistent with the lack of cross-reactivity of the anti-hCD40 (G28–5) with murine CD40 (31), the parental murine M12 B cells did not respond to stimulation by the agonistic anti-hCD40 antibody (Fig. 1C, top...
A tubulin IB is included as a loading control (or stimulated with anti-hCD40 for 24 h followed by analyzing the processing of p100 does not recognize the endogenous murine CD40. Parental M12 murine B cells or M12 cells stably expressing M12-hCD40-BAFFR or M12-hCD40 were stained with a fluorescein isothiocyanate-conjugated anti-hCD40 antibody and subjected to FACS analysis. The background peak (thin line) was set using unstained cells. The parental M12 cells were stained negative, because the anti-hCD40 does not recognize the endogenous murine CD40. C, processing of p100 and degradation of TRAF3. The indicated cells were either untreated (−) or stimulated with anti-hCD40 for 24 h followed by analyzing the processing of p100 (top panel) and degradation of TRAF3 (middle panel) by IB. A tubulin IB is included as a loading control (bottom panel). D, degradation of IκBα. The indicated cells were stimulated with anti-hCD40 for the indicated times followed by IB analyses to detect IκBα and tubulin.

Because a primary consequence of p100 processing is nuclear translocation of RelB and p52 NF-κB heterodimer, we analyzed the expression of these two NF-κB members in the nucleus of panel, lane 2). On the other hand, the hCD40-BAFFR cells efficiently responded to the anti-hCD40, as demonstrated by the inducible processing of p100 to p52 (lane 4). A similar result was obtained with the M12-CD40 cells (lane 6). Because the induction of p100 processing by recombinant BAFF is associated with degradation of TRAF3 (29), we examined whether the BAFFR chimera also induces TRAF3 degradation. Indeed, TRAF3 was lost concomitantly with the induction of p100 processing by both CD40-BAFFR and the intact CD40 (Fig. 1C, middle panel). The loss of TRAF3 was not due to its translocation to the nucleus (data not shown) and could be detected at early time points of cell stimulation (see Fig. 2F).

Using the CD40-BAFFR chimera system, we next examined whether BAFFR targets the canonical NF-κB signaling pathway by analyzing degradation of IκBα. Consistent with the ability of CD40 to activate canonical NF-κB, stimulation of M12-hCD40 cells resulted in efficient degradation of IκBα (Fig. 1D, upper panel, lanes 5–8). In contrast, however, stimulation of the M12-hCD40-BAFFR cells only led to a weak loss of IκBα (lanes 1–4). This result was not due to the lower expression of hCD40-BAFFR than hCD40 (Fig. 1B). Further, because the p100 processing was even more strongly induced by the hCD40-BAFFR than hCD40 (Fig. 1C), it is unlikely that the ineffectiveness of hCD40-BAFFR in inducing IκBα degradation was due to its intrinsically weaker signaling activity. Thus, unlike CD40, BAFFR is a TNFR member that preferentially stimulates the noncanonical NF-κB signaling pathway.

An Atypical TRAF-binding Sequence Motif Is Critical for BAFFR Signaling—The signaling function of TNFRs requires TRAF molecules. A specific TRAF-binding motif has been identified in a number of TNFR members (39), with those in CD40 being the most extensively characterized (40). To understand the biochemical mechanism mediating the unique signaling function of BAFFR, we compared the amino acid sequences of the cytoplasmic domains of BAFFR and CD40. Interestingly, although BAFFR lacks a typical TRAF-binding motif, it possesses a sequence element (PVPAT) that shares significant homology, in both location and sequence, with the TRAF-binding motif of CD40 (Fig. 2A, bolded and underlined). To examine the role of this sequence element in BAFFR signaling, we performed site-directed mutagenesis to substitute the conserved amino acids within the hCD40-BAFFR chimera with alanines (Fig. 2B, M1). In parallel, we also mutated the BAFFR motif to the typical TRAF-binding sequence, PVQET, of CD40 (Fig. 2B, M2). FACS analyses revealed a comparable expression level between the wild type and mutant forms of hCD40-BAFFR (Fig. 2C). Importantly, the M1 mutant (harboring PVQAT to AVAAA mutations) completely lost the ability to induce p100 processing (Fig. 2D, top panel, lane 4). On the other hand, the M2 mutant remained competent in inducing p100 processing (lane 6). The mutations introduced into M1, but not M2, also abolished the induction of TRAF3 degradation (Fig. 2D, middle panel). A separate experiment using shorter stimulation time points revealed that the TRAF3 degradation by wild type BAFFR and M2 mutant occurred around 2 h following receptor cross-linking, but this response was not detected in cells expressing the M1 mutant (Fig. 2F).

Because a primary consequence of p100 processing is nuclear translocation of RelB and p52 NF-κB heterodimer, we analyzed the expression of these two NF-κB members in the nucleus of...
the different M12 derivative cells. Upon stimulation of the cells expressing wild type hCD40-BAFFR by anti-hCD40, both RelB and p52 efficiently expressed in the nucleus (Fig. 2E, top two panels, lane 2). In agreement with the result of p100 processing, the M1 mutant of the hCD40-BAFFR failed to mediate the inducible nuclear expression of RelB and p52 (lane 4), whereas the M2 mutant was effective in this signaling function (lane 6). Thus, these data establish the PVPAT sequence as a key signaling motif of BAFFR that mediates the induction of p100 processing and nuclear expression of the noncanonical NF-κB members.

The PVPAT Motif Also Determines the Signaling Specificity of BAFFR—An important question regarding BAFFR signaling is how its signaling specificity is regulated. As shown in Fig. 1 (C and D), BAFFR preferentially stimulates the noncanonical NF-κB signaling pathway, whereas CD40 stimulates both the canonical and noncanonical NF-κB pathways. One hypothesis we were considering was that the unique signaling function of BAFFR was likely due to its possession of an atypical TRAF-binding motif. To test this hypothesis, we examined the effect of PVPAT-to-PVQET conversion within the cytoplasmic domain of BAFFR on its ability to induce IκBα degradation. As expected, cross-linking of the wild type hCD40-BAFFR only led to a low level of IκBα degradation (Fig. 3A, upper panel, lanes 1–4), which was not detected in the M1 mutant of the receptor (lanes 5–8). Remarkably, however, conversion of the PVPAT sequence to PVQET rendered the BAFFR capable of stimulating efficient degradation of IκBα (lanes 9–12). Thus, a two-amino acid substitution in the putative TRAF-binding site of BAFFR is sufficient to convert it into a receptor capable of stimulating the canonical pathway of NF-κB.

One major difference between the canonical and noncanonical NF-κB pathways resides in their signaling kinetics. Whereas the canonical pathway is rapid and transient, the noncanonical pathway is slow and persistent. We performed EMSA to examine the acute and delayed phases of NF-κB activation by the wild type and mutant forms of BAFFR. During the early phase of cell stimulation, the wild type BAFFR only mediated a weak activation of NF-κB (Fig. 3B, lanes 2 and 3), but the M2 mutant of BAFFR caused a much stronger activation of NF-κB under the same conditions (lanes 6 and 7). This result was consistent with the elevated induction of IκBα degradation by the M2 mutant (Fig. 3A). In contrast to the acute NF-κB activation, no appreciable difference was detected in the late-phase NF-κB activation mediated by the wild type and M2 mutant of BAFFR (compare lanes 4 and 8). Further, the M1 mutant of BAFFR was defective in activation of both the acute and delayed phases of NF-κB (lanes 10–12). Thus, the PVPAT motif of BAFFR specifically mediates the delayed activation of NF-κB, which in turn is associated with the processing of p100 and nuclear translocation of RelB/p52 (Fig. 2, D and E).
To further assess the signaling specificity of BAFFR, we examined the expression of downstream genes. Previous studies suggest that the BAFFR signal alone is insufficient for triggering significant induction of anti-apoptotic genes, such as \( Bfl-1/A1 \) (7). In contrast, the \( Bfl-1/A1 \) gene can be potently stimulated by the CD40 signal (25, 41). We thus examined the mRNA levels of this downstream gene in cells expressing the chimeric BAFFR receptors. Consistent with the prior studies, stimulation of the wild type BAFFR did not appreciably induce the expression of \( Bfl-1/A1 \) mRNA (Fig. 3C, lanes 1–5). Interestingly, however, stimulation of the BAFFR harboring PVPAT-to-PVQET modifications led to potent induction of \( Bfl-1/A1 \) (lanes 11–15). Together, these data suggest that the atypical TRAF-binding motif of BAFFR not only serves as a key signaling motif but also regulates its signaling specificity.

The PVPAT Motif Regulates Recruitment of TRAF3 to BAFFR—A prior study demonstrated that BAFFR specifically interacts with TRAF3 (42). To directly determine the role of the PVPAT motif in TRAF binding, we examined the recruitment of TRAF3 to the hCD40-BAFFR receptor in cells stimulated with anti-hCD40. In agreement with the previous report, we found that TRAF3 was recruited to the wild type BAFFR upon ligation by anti-hCD40, although significant BAFFR/TRAF3 association was not detected until 1 h following anti-hCD40 stimulation (Fig. 4A, lanes 1–5).
treatment (Fig. 4A, upper panel, lane 3). More importantly, this physical interaction critically requires the PVPAT motif, because the M1 mutant (carrying the AVAAA mutation) failed to recruit TRAF3 (lanes 4–6). Interestingly, the M2 mutant (carrying the PVQET mutation) interacted with TRAF3 more strongly and more rapidly than the wild type BAFFR (lanes 7–9). This property of the BAFFR M2 mutant was reminiscent of the CD40 molecule, which potently and rapidly interacted with TRAF3 in response to the anti-hCD40 treatment (lanes 10–12). These biochemical results support the functional studies described above.

We next examined whether the PVPAT to PVQET conversion was sufficient to render the modified BAFFR capable of binding to TRAF2. These studies were performed using transfected 293 cells. Consistent with a previous study, the wild type BAFFR did not interact with TRAF2 even under the overexpression conditions (Fig. 4B, upper panel, lane 2). Similarly, the M1 mutant was inactive in this physical interaction (lane 3). Interestingly, however, the M2 mutant, which carries the PVQET motif, exhibited a significant TRAF2-binding activity (lane 4). Together, these results suggest that the atypical TRAF-binding motif of BAFFR specifically interacts with TRAF3 but not with TRAF2, which likely contributes to the signaling specificity of BAFFR. Further, conversion of the atypical TRAF-binding motif to a conserved one is sufficient to alter the specificity of BAFFR in TRAF binding and NF-κB signaling.

**DISCUSSION**

The results of this study provide an insight into the signaling mechanism of BAFFR. We have identified a signaling motif, PVPAT, within the cytoplasmic domain of BAFFR that shares significant sequence homology with the TRAF-binding motif, PVQET, of CD40. However, the BAFFR motif does not fall into the consensus sequence of the TRAF-binding sequence (PXVQXT) present in several TNFR superfamilly members. We propose that this atypical TRAF-binding motif is responsible for the unique signaling property of BAFFR that preferentially targets the noncanonical NF-κB pathway. We have obtained several lines of evidence that support this hypothesis. First, alanine substitutions of the conserved amino acids within the PVPAT motif abolishes the ability of BAFFR to induce p100 processing and nuclear translocation of RelB and p52 (Fig. 2), key steps in noncanonical NF-κB signaling (17). Second, a two-amino acid substitution that converts the PVPAT sequence present in the cytoplasmic tails of TNFR family members into the consensus sequence of the TRAF-binding motif (QTE or H9260) B (Fig. 2), but this manipulation markedly enhances the specificity of BAFFR in TRAF binding and NF-κB signaling. The specificity of BAFFR in TRAF binding and NF-κB signaling is critical for BAFFR-mediated induction of p100 processing. One possibility is that the receptor recruitment serves as a trigger for targeting the degradation of TRAF3, which appears to be an important step in the noncanonical NF-κB signaling pathway (29). In support of this idea, the functionally inactive BAFFR mutant (M1) fails to induce TRAF3 degradation (Fig. 2D). However, it also remains possible that the receptor recruitment of TRAF3 plays an active role in the initiation of the noncanonical NF-κB signaling. Examination of this latter hypothesis will need genetic manipulation B cells lacking TRAF3.

**TRAF2 and TRAF3** are generally thought to bind to the same sequence present in the cytoplasmic tails of TNFR family members (23). However, detailed mutagenesis analysis using the cytoplasmic domain of CD40 reveals that TRAF2 and TRAF3 exhibit subtle differences in target sequence requirement (40). Our data suggest that the atypical TRAF-binding sequence within BAFFR favors binding to TRAF3 but does not interact with TRAF2. This biochemical property may in turn contribute to the signaling specificity of BAFFR in the induction of noncanonical NF-κB activation. In support of this hypothesis, conversion of the atypical TRAF-binding motif to a conserved TRAF-binding motif renders the modified BAFFR competent to interact with both TRAF3 and TRAF2 and to target both the noncanonical and canonical NF-κB signaling pathways.

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