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A Novel Interaction between Protein Kinase D and TNF Receptor-Associated Factor Molecules Regulates B Cell Receptor-CD40 Synergy

Sokol A. Haxhinasto* and Gail A. Bishop*‡

Signaling by Ag to the B cell Ag receptor (BCR) plays a critical role during B lymphocyte development and activation. BCR signaling is mediated directly by protein tyrosine kinases of the Src, Syk/Zap-70, and Tec families, as well as protein tyrosine phosphatases, such as CD45. Activation of protein tyrosine kinases triggers a cascade of secondary signals, resulting in activation of several intracellular effector signaling molecules, including members of the protein kinase C (PKC) family of serine-threonine kinases. A downstream target of second messengers, primarily DAG, is PKD, and represents a novel family of serine/threonine kinases. Structurally, PKD consists of an N-terminal region rich in alanines and prolines, two cysteine-rich zinc-finger regions, followed by a pleckstrin homology (PH) domain and a protein Ser/Thr kinase catalytic domain.

The role of PKD was dependent upon the association of CD40 with TRAF2, and was inhibited by the binding of TRAF3, revealing a novel functional link between these two classes of signaling molecules. The Journal of Immunology, 2003, 171: 4655–4662.

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ignals delivered by the B cell Ag receptor (BCR) play a critical role during B lymphocyte development and activation. BCR signaling is mediated directly by protein tyrosine kinases of the Src, Syk/Zap-70, and Tec families, as well as protein tyrosine phosphatases, such as CD45. Activation of protein tyrosine kinases triggers a cascade of secondary signals, resulting in activation of several intracellular effector signaling molecules, including members of the protein kinase C (PKC) family of serine-threonine kinases (4). BCR-mediated PKC activation influences diverse aspects of BCR signaling, including activation of transcription factors, such as NF-κB (5) and CREB (6).

The PKC family can be divided into three subfamilies, depending on the second messengers required. The conventional PKCs (PKC-α, β, βγ, γ) use both diacylglycerol (DAG) and Ca2+ for their activation, relying primarily on the phospholipase C-γ (PLC-γ) pathway. The activation of novel PKCs (PKC-δ, ε, η, θ, ι) is DAG dependent and Ca2+ independent. The atypical PKCs (PKC-ζ, λ, κ) are activated by neither DAG nor Ca2+ and have been shown to be downstream of phosphatidylinositol 3 kinase (7).

More recently, the protein kinase D (PKD) family has been described, with PKC-μ/PKD as its main member (8). B lymphocytes express PKC-α, β, γ, δ, ε, ζ, η, and μ isoforms, and BCR engagement has been shown to induce their activation (9–11).

PKD is a downstream target of second messengers, primarily DAG, and represents a novel family of serine/threonine kinases. Structurally, PKD consists of an N-terminal region rich in alanines and prolines, two cysteine-rich zinc-finger regions, followed by a pleckstrin homology (PH) domain and a protein Ser/Thr kinase catalytic domain (reviewed in Ref. 8). Different from the other PKCs, PKD does not possess an autoinhibitory pseudo-substrate sequence, and is not inhibited by PKC inhibitors, including Gö6983 (12). PKD is activated upon phosphorylation of two serines in the activation loop (Ser744/748) upon various stimuli, including BCR engagement (13–15).

Extensive work has been done to determine the signaling pathways that activate PKD. These studies showed that PKD is activated by multiple signals (review in Ref. 8), including BCR engagement in B lymphocytes (10, 16, 17). To date, the contribution of PKD in BCR signaling is not clear. It has been suggested that PKD negatively regulates BCR signaling by phosphorylating Syk and reducing its ability to phosphorylate PLC-γ (10).

Although BCR engagement by Ag is necessary for B lymphocyte activation, it is not sufficient for full activation; this requires contact-mediated interaction with Ag-specific Th cells and engagement of additional signaling receptors, including CD40 (18). CD40 is a member of the TNFR family that plays a crucial role in B cell proliferation, differentiation, isotype switching, up-regulation of surface molecules, development of germinal centers, and immunological memory. The critical role of CD40 and its interaction with its ligand, CD154, are evident in humans with X-linked hyperIgM syndrome (19–21), as well as in mice lacking either CD40 (22, 23) or its ligand CD154 (24). The CD40 cytoplasmic tail does not contain any tyrosine residues to serve as docking sites for tyrosine-dependent downstream signaling molecules. However, CD40 relies on adapter molecules, such as TNFR-associated factors (TRAFs), in transmission of its signals (25). CD40 engagement in B lymphocytes leads to CD40 association with several TRAFs (26), and activation of various kinases and transcription factors (27, 28).

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3 Abbreviations used in this paper: BCR, B cell Ag receptor; DAG, diacylglycerol; EE, constitutively active; hCD40, human CD40; IPTG, isopropyl-β-D-thiogalactopyranoside; KD, kinase dead; mCD40, mouse CD40; PH, pleckstrin homology; PHD, PH domain deleted; PKC, protein kinase C; PKD, protein kinase D; PLC, phospholipase C; TRAF, TNFR-associated factor; WT, wild type.
It is evident from several studies that coengagement of the BCR and CD40 has synergistic effects on B lymphocyte function, including B cell proliferation and production of IgM, TNF-α, and IL-6 (29–33). However, the molecular mechanisms responsible for this synergy are not completely understood. Despite the ability to activate some of the same kinases and transcription factors, the BCR and CD40 are also able to activate distinct signaling molecules, which may contribute to the synergy between these receptors. Recently, we showed that the BCR is able to enhance the response of B lymphocytes to CD40 in a TRAF2-dependent manner. Mutant CD40 molecules that bind TRAF3 in the absence of TRAF2 are not able to synergize, implying a positive role for TRAF2 and a negative or neutral role for TRAF3 in the BCR/CD40 cross talk (30). However, it is still unclear what signaling molecule(s) downstream of BCR engagement is important for the enhancement of the CD40 response. We hypothesized that the BCR is able to augment the CD40 response by activating unique signaling molecule(s) that CD40 is unable to activate. Previous studies have clearly shown that the BCR activates several isoforms of PKC, while CD40 signaling is PKC independent (34–36). We thus investigated the role of PKC in the molecular interactions between BCR and CD40. We found that activation of the PKC isoform PKD was necessary for the synergy between BCR and CD40. Unexpectedly, the action of PKD was TRAF2 dependent, and was inhibited by TRAF3, demonstrating a critical novel functional link between PKD and TRAF molecules in bridging the signaling pathways of the BCR and CD40 in B lymphocytes.

Materials and Methods

Cells

The mouse B cell line CH12.LX expresses surface IgM specific for phosphatidylcholine, an Ag found on the surface of SRBC (37), and has been well characterized (38). The CH12.LacR subclone used for the inducible expression of stably transfected molecules has been previously described (39). Subclones of CH12.LX lacking either TRAF2 or TRAF3 were produced by homologous recombination-based gene targeting, and are described in detail elsewhere (58). All B cells were cultured in RPMI 1640 supplemented with 10% FCS, 10 μM 2-ME, and antibiotics (B cell medium 60/10/B cell medium). Spodoptera frugiperda (Sf9) cells infected with wild-type (WT) or a recombinant baculovirus expressing mCD154 were from the indicated individuals: anti-hCD40 (G28-5, mIgG1) from ATCC, American Type Culture Collection (ATCC, Manassas, VA) or were gifts from T. Waldschmidt (University of Iowa, Iowa City, IA). Goat anti-mouse (IgG1 isotype control mAb (MOPC-21) was purchased from Sigma-Aldrich. All chemical inhibitors, staurosporine, H-7, Go6976, and Go6983 were purchased from Calbiochem (San Diego, CA).

Assays for B cell function

IgM-secreting cells per million recovered viable cells were measured by direct plaque-forming cell assay as previously described (42). Inducible TRAF expression was initiated by addition of IPTG to cultures 18 h before addition of stimuli. All stimulations proceeded for a total of 72 h, including the time of IPTG incubation. In all experiments, control stimulation included Ag and isotype controls for respective Abs. Cytokine ELISAs were performed, as described previously (30). For proliferation studies, 1 × 10^6 cells were stimulated with 1 μg/ml of anti-CD40 and/or anti-CD40 Abs for 72 h. Five to six hours before harvesting, 1 μCi of [3H]thymidine was added to each well, and incorporation was determined by liquid scintillation counter. When using chemical inhibitors, cells were pretreated for 30 min before the stimuli were added. Concentrations of inhibitors that showed no detectable toxicity to our cells, as measured by trypan blue exclusion, were chosen for longer assays.

Western blotting and immunoprecipitation

CH12.LX cells (5 × 10^6) were stimulated for 30 min with 10 μg/ml of anti-mouse μ-chain-specific Fab’2, 100 ng/ml of PMA, or mCD154-expressing cells (1 mCD154-expressing cell:4 B cells). Total cell lysates were subjected to SDS-PAGE and electroblotted onto nitrocellulose paper, and Western blotting was performed. To detect the PKD protein upon IPTG induction, cells were lysed in 1% Nonidet P-40 lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris (pH 7.5), 0.02% NaN3, and protease and phosphatase inhibitors) for 30 min. The supernatants were incubated with protein G beads preconjugated with anti-Flag Ab. Immunoprecipitated proteins were separated by SDS-PAGE, and Western blotting was performed.

Results

Pharmacologic inhibition of PKD activity abrogated BCR-CD40 synergy

Engagement of CD40 on normal B cells or on the B cell line CH12.LX results in production of IgM, TNF-α, and IL-6, and BCR signals synergize with these CD40 functions (32, 43–45). We hypothesized that the BCR enhances the CD40 response by activating distinct signaling molecules unique to the BCR pathway. To test the role of serine/threonine kinases in the synergy, two broad-spectrum serine/threonine kinase inhibitors, staurosporine and H-7, were examined. Interestingly, synergy between the BCR and CD40 was abrogated upon treatment of cells with these kinase inhibitors, while the CD40 response was not affected (Fig. 1). BCR signaling alone does not stimulate IgM secretion (40). A candidate serine/threonine kinase, activated by the BCR, but not CD40, is PKC (34–36). BCR engagement leads to activation of several isoforms of PKC (9–11). To test the role of PKCs in the synergy between BCR and CD40, cells were treated with two highly specific staurosporine-derived PKC inhibitors, Go6976 and Go6983 (12, 46, 47), and the ability of the BCR to enhance the CD40 response was examined. TNF-α is produced within 3 h of CD40 stimulation, so in the experiments shown in Fig. 2C, a higher concentration of inhibitors could be used. However, higher concentrations show toxicity during the longer time periods (48–72 h) required for IL-6 or IgM production, so doses above 200 nM were not used in these experiments. As in Fig. 1, the CD40 response was not affected by treatment of cells with PKC inhibitors (Fig. 2). Interestingly, Go6976 abrogated the synergy between BCR and CD40 in splenic B cell proliferation (Fig. 2A), IgM secretion (Fig. 2B), and TNF-α and IL-6 production (Fig. 2, C and D), while Go6983 did not affect synergy. Both inhibitors have been reported to inhibit conventional PKC isoforms such as PKCα and

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PKCβ, but only G6976 has been shown to additionally inhibit PKD (12, 47). We examined the effect of G6976 and G6983 on PKD activation in CH12.LX B cells and splenocytes. BCR engagement has been shown to activate PKD in freshly isolated B lymphocytes (10). Fig. 3 shows that BCR engagement also led to PKD activation in CH12.LX B cells (Fig. 3A) and splenocytes (Fig. 3B), but stimulation of CD40 (mCD154) did not. G6976, but not G6983, inhibited PKD activation in CH12.LX cells (Fig. 3A) and splenocytes (Fig. 3B), indicating that the effects of these drugs on B cell effector functions (Fig. 2) are most likely due to their specific effects on PKD activation. Previous studies have suggested that PKD is activated in a PKC-dependent manner (17, 48); however, these authors used different PKC inhibitors than the drugs we used, so the results cannot be compared directly. In addition, previous studies (47) have indicated a differential effect of G6976 and G6983 on PKC vs PKD. It is possible that the inhibition of classical PKC by G6983 is not complete, and therefore some PKD activation still occurs in the presence of G6983, or that other PKC-independent pathways exist to activate PKD in B lymphocytes. Regardless of what is upstream of PKD, our data strongly suggested that PKD is critical for the synergy between BCR and CD40.

**Requirement for PKD activity in BCR-CD40 synergy demonstrated by inactive or constitutively active PKD expression**

Chemical inhibitors, while providing important clues, have limitations due to potentially unknown, nonspecific effects. To more directly address the role of PKD in the cross talk between BCR and CD40, we used an IPTG-inducible system (described in Ref. 39) to express various PKD-expressing constructs in CH12.LX B cells. This allowed controlled expression of the PKD. Previous studies have shown that substitution of lysine 618 with asparagine (K618N) in PKD’s catalytic domain prevents ATP from binding and creates a catalytically inactive PKD that serves as a dominant-negative molecule (15, 48, 49). Replacement of serines at positions 744 and 748 with alanines (AA) also leads to inactivation of PKD, while replacement of these residues with glutamic acid leads to a constitutively active PKD (EE) (13). We combined K618N and the SS744/748AA mutations to generate the inactive (KD) PKD.

We inducibly expressed WT, the PKD-EE, and PKD-KD (Fig. 4A) in CH12.LacR cells. The constructs were Flag tagged at the N terminus to differentiate the exogenous, inducible PKD from the endogenous enzyme. Expression of the recombinant proteins was confirmed by intracellular staining and flow cytometry (data not shown) as well as Western blot analyses (Fig. 4B). The effects of expression of WT (Fig. 4C) and mutant (Fig. 4D) PKD molecules on CD40-mediated IgM production were tested. WT PKD did not affect the CD40 response alone and slightly increased the magnitude of synergy between BCR and CD40. Expression of PKD-KD

**FIGURE 1.** Effects of serine/threonine kinase inhibitors on BCR/CD40 synergy. CH12.LX cells were cultured with the indicated stimuli (2 μg/ml 1C10 anti-CD40 mAb and 0.1% SRBC as Ag), and IgM secretion was measured after 72 h of culture, as described in Materials and Methods. Serine/Threonine kinase inhibitors were included in cultures at final concentrations equal to their Ki for PKC of 6 μM for H7 and 700 pM for staurosporine.

**FIGURE 2.** BCR/CD40 synergy was abrogated by G6976, but not G6983. Cells were stimulated as indicated in the presence or absence of the pharmacologic inhibitors. A, Splenic B cell proliferation was measured after 72 h by the uptake of [3H]thymidine, as in Materials and Methods. Inhibitors were included in cultures at a final concentration of 100 nM. B, IgM secretion was measured as in Fig. 1. Inhibitors were included in cultures at a final concentration of 100 nM. C, TNF-α secretion was measured after 3 h of culture with the indicated stimuli, using ELISA, as in Materials and Methods. B cells were pretreated for 30 min with the indicated concentrations of inhibitors before the stimuli were added. Sf9 insect cells are used as a control for insect cells infected with mCD154-expressing recombinant baculovirus, as described in Materials and Methods. DMSO controls for the diluent used to make a concentrated stock of the inhibitors. D, IL-6 secretion was measured after 48 h of culture, by ELISA, as described in Materials and Methods. Inhibitors were included in cultures at the indicated final concentrations. Results presented in all panels are mean ± SD of replicate cultures and are representative of at least three similar experiments.
(Fig. 4D) abrogated the synergy between BCR and CD40, without affecting the CD40 response, demonstrating that BCR-induced PKD activation is necessary for the synergy between BCR and CD40. This demonstrates that functional PKD is specifically required for BCR-CD40 synergy.

Although PKD-EE expression substantially enhanced the cooperation between BCR and CD40, PKD-EE expression alone, without BCR engagement, was not sufficient to effect synergy (Fig. 4D). A possible explanation is that substitution of Ser744/748 with Glu is not sufficient for PKD activation in vivo, and that BCR engagement is still necessary to remove or modulate any inhibitory elements within PKD (50). BCR engagement might also be important for the subcellular localization of PKD, which could regulate its role in synergy. It has been reported that PH domain of PKD serves as a negative regulator of PKD activity (8, 50). To test the role of the PH domain of PKD in BCR/CD40 synergy, the PH domain was deleted (PHD) from PKD-WT and PKD-EE, and both were expressed inducibly in CH12.LacR cells. The expression of the PHD-PKD proteins was confirmed by intracellular staining (data not shown) and Western blotting (Fig. 5A). Interestingly, expression of the PHD-PKD (WT or EE) was itself sufficient for the synergy between the two receptors and substituted for BCR engagement (Fig. 5, B and C). PKD activation is not required in the absence of TRAF3 binding to CD40

Previously, we demonstrated that BCR signaling is not able to synergize with CD40 molecules that bind TRAF3 in the absence of TRAF2 (30), although BCR can synergize with mutant CD40 molecules that bind neither TRAF2 nor TRAF3 (30). We thus hypothesize that TRAF3 binding to CD40 plays a negative role in BCR/CD40 synergy, and the major role of TRAF2 is to prevent TRAF3 from exerting its negative effects, so TRAF2 is not required for synergy if CD40 is not binding TRAF3. PKD inactivation in CH12.LX cells, either by chemical inhibitors or genetic manipulation, has the same effect on the BCR/CD40 synergy as does CD40 binding of TRAF3 in the absence of TRAF2, suggesting the unexpected, but intriguing possibility that PKD interacts with TRAFs to regulate BCR/CD40 synergy. To address this possibility, we tested whether PKD activation is required for synergy in the absence of TRAF3 binding to CD40. We used an hCD40 mutant (hCD40Δ22) that binds neither TRAF2 nor TRAF3 (30) as well as TRAF3-deficient CH12.LX cells.4 If PKD inhibition plays...
no role in the synergy between BCR and hCD40, or between BCR and endogenous CD40 in the TRAF3/WT cells, it would indicate that in the absence of TRAF3, PKD is unnecessary for BCR/CD40 synergy. This finding would imply that PKD contributes to the synergy by inhibiting TRAF3 from exerting its negative effect. The second possibility is that PKD inhibition leads to abrogation of synergy, and this would suggest that PKD effects on synergy are independent from TRAFs 2 and 3, but act through other molecules downstream of PKD.

As before, treatment of CH12.LX B cells with the PKD inhibitor, Gö6976, abrogated the synergy between BCR and endogenous WT mouse CD40 (mCD40) (which binds TRAF3 and TRAF2), WT hCD40 signals to mouse B cells indistinguishably from endogenous mCD40 (Fig. 6, C and D). However, Gö6976 treatment had no effect on the synergy between BCR and hCD40Δ22, which does not bind TRAF3 or TRAF2 (Fig. 6A). Similar findings were observed using cells that express hCD40Δ22 and the PKD-KD (Fig. 6B). These data support the hypothesis that PKD activation exerts its effects on synergy via the binding of TRAFs to CD40. Whether PKD activation plays an important role in BCR-CD40 synergy in the absence of TRAF3 binding to CD40 was also tested in TRAF3−/− cells. TRAF3−/− CH12.LX cells were generated using gene targeting by homologous recombination. Supporting the findings in Fig. 6, A and B, PKD inactivation either by inhibitors (Fig. 7A) or by expression of inactive PKD (Fig. 7B) did not affect synergy in the TRAF3-deficient B cells. These findings indicate that PKD exerts a novel BCR-mediated effect on TRAF3 that prevents its negative effect on BCR-CD40 synergy.

**PKD enhancement of the CD40 response is TRAF2 dependent**

We have previously shown that the hCD40 mutant T234A, when expressed in B cells, binds TRAF3 normally, but shows reduced binding to TRAF2 (30). This mutant also fails to synergize with BCR signals (30, 40), suggesting that PKD activation must cooperate with TRAF2 to promote synergy; the apparent direct or indirect effects of PKD on TRAF3 are either insufficient or not realized in the absence of TRAF2 binding. To test this hypothesis,
IgM secretion was measured, as in Fig. 1. B mean cells were enumerated, as in Fig. 1. Results for both panels indicate calculated as indicated in the presence and absence of Go stimulated as indicated in the presence or absence of 100 CH12.LacR cells transfected with PKD-PHD-EE and hCD40T234A were PKD enhancement of CD40 is TRAF2 dependent.

FIGURE 8. PKD enhancement of CD40 is TRAF2 dependent. CH12.LacR cells transfected with PKD-PHD-EE and hCD40T234A were stimulated as indicated in the presence of absence of 100 μM IPTG, and IgM-secreting cells were enumerated, as in Fig. 1. Results indicate mean ± SE of replicate cultures, and are representative of two similar experiments.

we expressed hCD40T234A in CH12.LX cells inductively expressing PKD-PHD-EE, and asked whether induced expression of the constitutively active PKD could overcome the synergy defect of the hCD40 mutant. As predicted by Fig. 5, induced expression of PKD-PHD-EE substituted for the BCR signal in synergy with the WT endogenous mCD40. However, the kinase activation was insufficient to replace the BCR signal in cooperating with hCD40T234A (Fig. 8). Thus, PKD appears to inactivate a negative effect of TRAF3 on synergy, in a manner dependent upon the binding of TRAF2 to CD40.

Discussion

The integration of signaling pathways relies on particular molecules that bridge the different pathways, providing the necessary link for full activation. Identification of molecules involved in the interactions between BCR and CD40 is necessary for better understanding of the mechanisms involved in B lymphocyte activation. In this study, we focused on the contribution of signals specific to the BCR, and identified PKD as a necessary component in the synergy between BCR and CD40. We further identified the PH domain of PKD as a negative regulator of this function.

Previous studies have shown that BCR engagement leads to PKD activation in B lymphocytes, and suggested a negative role for PKD in BCR signaling by down-regulating Syk-mediated PLC-γ2 activation (10). Overexpression studies, using 293 epithelial cells, have linked PKD to several kinases, including extracellular signal-regulated kinase (52) and c-Jun N-terminal kinase (53); however, the physiological relevance of these results to normal PKD levels in lymphocyte signaling is unclear. In the present study, PKD-specific chemical inhibitors as well as genetic experiments showed that PKD activation plays a necessary positive role in the synergy between BCR and CD40. Although PKD inactivation did not block CD40 signaling, it abrogated the ability of the BCR to enhance the CD40 response. Further analysis demonstrated a negative role for the PH domain of PKD in BCR-induced activation. These data extend previous reports of the autoinhibitory role of the PH domain (50, 54) and suggest that BCR-induced PKD activation occurs via relief of this autoinhibition. This relief seems to be necessary and sufficient for PKD to enhance the CD40 response, thus revealing a novel role for PKD in B lymphocyte activation.

To integrate the BCR and CD40 signaling pathways, the possible link between PKD and TRAF molecules was explored. There are several ways that PKD activation could enhance the CD40 response. PKD activation could be responsible for activation of downstream signaling molecules that could in turn cooperate with signals initiated by CD40. An additional intriguing possibility is that PKD links BCR and CD40 pathways by directly affecting CD40-signaling components. Interestingly, we previously showed that the contribution of BCR-induced activation in synergy is TRAF2 dependent, because CD40 mutants that bind TRAF3 in the absence of TRAF2 do not synergize with the BCR, although these mutants stimulate CD40 responses (30). In addition, synergy-defective mutants that bind TRAF3 in the absence of TRAF2 are able to synergize when expressed in TRAF3-deficient cells (Haxhinasto and Bishop, data not shown), pointing to the negative effect of TRAF3 in BCR-CD40 synergy. The findings in Fig. 8 further support a direct link between PKD and TRAF2. The PH-PKD molecule did not synergize with an hCD40 molecule that does not bind
TRAF2, although it synergizes with endogenous WT mCD40, which binds TRAF2.

Of particular interest was the finding that in the absence of TRAF3 binding to CD40, PKD activation was not required for synergy (Figs. 6 and 7), indicating that PKD activation directly or indirectly prevents TRAF3 from inhibiting CD40-BCR synergy. Our data support a model (Fig. 9) in which BCR engagement leads to PKD activation, which in turn prevents TRAF3 from exerting negative effects on BCR-CD40 synergy, in a TRAF2-dependent manner. Our studies support a critical role for PKD-TRAF2-TRAF3 interaction in integration of the signals emanating from the BCR and CD40. PKD activation could lead to modifications of TRAF molecules, such as phosphorylation, that could affect their CD40-induced degradation (55, 56). Additionally, PKD could exert its effects via other molecules, such as Syk, PLC-γ (10), or Btk (57). These and additional possibilities are under current investigation.

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References


