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BLNK Binds Active H-Ras to Promote B Cell Receptor-mediated Capping and ERK Activation*

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Cross-linked B cell receptor (BCR) aggregates on the cell surface, then assembles into the "cap" where Ras is co-localized, and transduces various intracellular signals including Ras-ERK activation. BCR signals induce proliferation, differentiation, or apoptosis of B cells depending on their maturational stage. The adaptor protein BLNK binds various signaling proteins and Igα, a signaling subunit of the BCR complex, and plays an important role in the BCR signal transduction. BLNK was shown to be required for activation of ERK, but not of Ras, after BCR cross-linking, raising a question how BLNK facilitates ERK activation. Here we demonstrate that BLNK binds the active form of H-Ras, and their binding is facilitated by BCR cross-linking. We have identified a 10-amino acid Ras-binding domain within BLNK that is necessary for restoration of BCR-mediated ERK activation in BLNK-deficient B cells and for anti-apoptotic signaling. The Ras-binding domain fused with a CD8α-Igα chimeric receptor could induce prolonged ERK phosphorylation, transcriptional activation of Elk1, as well as the capping of the receptor in BLNK-deficient B cells. These results indicate that BLNK recruits active H-Ras to the BCR complex, which is essential for sustained surface expression of BCR in the form of the cap and for the signal leading to functional ERK activation.

Signals from the B cell antigen receptor (BCR),[5] either bound with antigen or unbound, and the pre-B cell receptor (pre-BCR) play a critical role in B cell development, activation, and immune responses (1). Such signals are primarily transduced from Igα/Igβ subunits, transmembrane proteins each containing the immunoreceptor tyrosine-based activation motif (ITAM) in their cytoplasmic regions (2). Tyrosine residues in the ITAM are phosphorylated by Src family kinases, such as Lyn, and serve as a docking site for Syk, a pivotal tyrosine kinase for this signaling. The ITAM-bound Syk becomes activated and phosphorylates and activates key signaling proteins, such as another tyrosine kinase Btk, phospholipase C (PLC) γ2, and the adaptor protein BLNK (3). Upon phosphorylation by Btk, PLCγ2 produces inositol 1,4,5-triphosphate and diacylglycerol, which induce Ca^{2+} mobilization and activation of enzymes such as protein kinase C (PKC), respectively.

BLNK (also known as SLP-65 or BASH) is an important adaptor protein selectively expressed in B-lineage cells (4–7). BLNK is a cytoplasmic protein, but a part of it is constitutively bound to the plasma membrane through an N-terminal leucine zipper motif (8) and transiently to a cytoplasmic domain of Igα through its C-terminal SH2 domain upon BCR-stimulation (9, 10). Previous reports indicated that a non-ITAM phosphotyrosine in Igα is necessary for the binding with the BLNK SH2 domain and/or for normal BLNK function in signaling and B cell activation (9–12). Upon phosphorylation on tyrosines, BLNK binds Btk and PLCγ2 through their SH2 domains and mediates PLCγ2 activation by Btk (13). BLNK also binds other signaling molecules such as Vav, Grb2, Syk, and HPK1 (4–6, 14). BLNK has been shown to be necessary for BCR-mediated Ca^{2+} mobilization, for the activation of mitogen-activated protein kinases such as ERK, JNK, and p38 in a chicken B cell line DT40 (7), and for activation of transcription factors such as NF-AT and NF-κB in human or mouse B cells (4, 15). BLNK plays a crucial role in pre-BCR-dependent progression of B cell development, BCR-mediated B cell survival, activation, proliferation, and T-independent immune responses (16–20).

A small guanosine triphosphatase (GTPase) Ras is also implicated in BCR signal transduction. The earliest report demonstrated that, after BCR cross-linking, Ras is co-localized with the aggregates of BCR (patches), and then with the cap, an assembly of patches at one pole of the cell (21). Then followed reports indicating rapid activation of Ras upon BCR cross-linking (22, 23). Ras is constitutively bound to the inner surface of the plasma membrane through post-translational modifications initiated predominantly by farnesyltransferase. Upon receptor stimulation, Ras-bound GDP is rapidly replaced with

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[5] The abbreviations used are: BCR, B cell receptor; ERK, extracellular signal-regulated protein kinase; RBD, Ras-binding domain; ITAM, immunoreceptor tyrosine-based activation motif; PLC, phospholipase C; PKC, protein kinase C; SH2, Src homology 2; GTPase, guanosine triphosphatase; GST, glutathione S-transferase, ECFP, enhanced cyan fluorescent protein; EYFP, enhanced yellow fluorescent protein; MCS, multi-cloning site; FITC, fluorescein isothiocyanate; CLSM, Confocal laser scanning microscopy; TRITC, tetramethyl rhodamine isothiocyanate; WT, wild type; JNK, c-Jun N-terminal kinase; MEK, mitogen-activated protein kinase/ERK kinase; aa, amino acid(s).

GTP by Ras guanyl nucleotide exchange factors, and Ras becomes active. The GTP-bound Ras recruits several cytoplasmic enzymes to the plasma membrane, including Raf kinases such as Raf-1 and B-Raf. The membrane-recruited Raf is activated and stimulates downstream MEK-ERK signaling cascade leading to transcriptional activation of downstream genes. The active state of Ras is only transient because its own GTPase activity hydrolyzes GTP into GDP with the aid of GTPase-activating proteins. In BCR signal transduction, Ras is mainly activated by RasGRP3 with a minor contribution of RasGRP1, both being Ras guanyl nucleotide exchange factors activated upon binding to diacylglycerol (24, 25). Diacylglycerol-activated PKC also contributes to the activation of RasGRP3 by a site-specific phosphorylation (26–28). In B cells from RasGRP1/3 double null mutant mice, BCR ligation fails to induce activation of Ras and ERK as well as cell proliferation, indicating that BCR signaling through Ras is essential for B cell activation (25). Although BLNK has been shown to be necessary for PLCγ2 activation in BCR signal transduction, BLNK-deficient ($BLNK^-$) DT40 cells displayed normal Ras activation upon BCR cross-linking (7),[6] suggesting alternative pathways for RasGRP activation. Despite the normal Ras activation, BCR-induced ERK activation was markedly attenuated in the $BLNK^-$ DT40 cells (7). It was also shown that BLNK is required for efficient ERK activation in mouse splenic B cells when BCR stimulation is modest (29). These results suggest that BLNK is required for ERK activation in a Ras-independent pathway or, alternatively, that BLNK may link the active Ras to a downstream signaling pathway leading to full ERK activation. Here we demonstrate that BLNK directly binds active Ras and that this binding is critical for BCR-Ras co-capping and prolonged ERK activation leading to Elk1 activation upon BCR cross-linking.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—pGST-cBLNK(1–62), pECFP-cBLNK, pECFP-cBLNK(Δ62), and pAT7-mBLNK have been described previously (14, 30). Other vectors were constructed as follows. For pGST-cBLNK(1–158), the EcoRI-XhoI fragment from pHybLex/Zeo-cBLNK(1–158) (31) was inserted into the same sites of pGEX-5X-1 (Amersham Biosciences). For pApuroT7-cBLNK, the SnaBI-SalI fragment from pCAT7 was inserted into the same sites of pApuro2 vector to generate pApuroT7. The EcoRI-SalI cBLNK fragment from pCAT7-cBLNK was ligated into the EcoRI and SmaI sites of pApuroT7. For pApuroT7-cBLNK(Δ62) and pApuroT7-cBLNK(Δ158), cBLNK fragments were prepared by PCR using pCAT7-cBLNK as a template and primers cBLNK63 and cBLNK-A or primers cBLNK159 and cBLNK-A, respectively (Table 1) and cloned into the pApuroT7. For pApuroT7-cBLNK-S and pApuroT7-cBLNK(Δ133), a short form (cBLNK-S) (6) and an incomplete cDNA encoding a chicken BLNK lacking first 133 aa (Δ133)[6] were cloned into pApuroT7. The following internal deletion mutants of cBLNK were created by the two-step PCR. Two neighboring fragments were amplified independently using pCAT7-cBLNK as a template and each pair of primers (Table 1) in the first step, and the two fragments (1 ng each) were annealed through their 3′-end homologies, elongated to the 3′ ends, and amplified by PCR with the primers cBLNK1–1 and cBLNK-A in the second step. The resultant fragments were cloned into pApuroT7. The following primers were used in the first PCR: for pApuroT7-cBLNK(Δ134–158), cBLNK1–1 and cBLNK163–128, cBLNK129–164, and cBLNK-A; for pApuroT7-cBLNK(Δ135–144), cBLNK1–1 and cBLNK150–128, cBLNK129–151, and cBLNK-A; and for pApuroT7-cBLNK(Δ153–157), cBLNK1–1 and cBLNK162–146, cBLNK148–164, and cBLNK-A.

TABLE 1
Primers used in this study

Primer name	Sequence (5′ to 3′)
cBLNK63	GGGAATTCACCTCCAAGTCTACCACGAAGGG
cBLNK-A	AGTAGGGAGGGCTGATTTTGCGGG
cBLNK159	GGGAATTCACCCAGTTCAGCCTTGCCCAGAC
cBLNK1–1	GGGAATTCTATGGACAAGCTGAACAAAC
cBLNK163–128	CAAGGCTGAACTGGGAATAGGGAATGAGGAGGG
cBLNK129–164	TCCTCATTCCCTATTCCCAGTTCAGCCTTGCCC
cBLNK150–128	GGGAGGAAGCTGATGGTGAGAAATAGGGAATGAGGAGGG
cBLNK129–151	TCCTCATTCCCTATTTCTCACCATCAGCTTCCTCCCATC
cBLNK162–146	GGCTGAACTGGGTGTGTTGATGGGAGGAAGCTGATG
cBLNK148–164	CTTCCTCCCATCAACACACCCAGTTCAGCCTTGCCC

The following vectors were made by general insert vector ligation. pECFP-cBLNK(Δ158) and pECFP-cBLNK(Δ134–158). EcoRI-SalI fragments of pApuroT7-cBLNK(Δ158) or pApuroT7-cBLNK(Δ134–158) were inserted into the same sites of pECFP-C1 (Clontech). For pEYFP-H-Ras and pEYFP-H-Ras(61L), an EcoRI-SalI fragment of pCMV-H-Ras or a KpnI-SmaI fragment of pCMV-H-Ras(61L) were inserted into the same sites of pEYFP-C1 (Clontech), respectively. For pEYFP-H-Ras(17N), an EcoRI-BamHI fragment of pCMV-H-Ras(17N) was first cloned into pBluescript II SK(−), and the KpnI-BamHI fragment thereof was inserted into the same sites of pEYFP-C1.

CD8α:Igα^{F3}RBD and CD8α:Igα^{F3} were constructed as follows: Cla12L-mCD8α:chIgα^{F3} (11) was digested with NgoMI and HindIII, and the smaller fragment was replaced with a synthesized fragment (MCS: 5′-GCCGGCCTGGAGAAACCTCGAGCTAGCGGATCCAAGCTT-3′; the NgoMI, XhoI, BamHI, and HindIII sites are underlined). The resultant plasmid, Cla12L-mCD8α:chIgα^{F3}-MCS, was digested with XhoI and BamHI, and the smaller fragment was replaced with a BLNK-RBD fragment made by PCR with primers (5′-CCGCTCGAGCATTCCCTATTTCTAGAGGTG-3′; the XhoI site is underlined; and 5′-CGGGATCCGTCGACTACTGATGGTGACTGGTGCGA-3′; the BamHI and SalI sites are underlined) and chicken BLNK cDNA as a template. By these procedures, a chicken BLNK sequence including the RBD, FPISRGEYADNRTSHHQ(stop), was fused to the C-terminal end of the chicken Igα^{F3} with an insertion of two linker amino acids (Arg and Ala). A SacI-SalI fragment from the resultant plasmid (mCD8α:chIgα^{F3}-RBD), EcoRI/SalI-digested pApuro2, and annealed oligonucleotides (5′-AATTCAGATCTACTAGTGAGCT-3′ and 5′-CACTAGTAGATCTG-3′; the EcoRI and SacI protruding ends are underlined) were ligated together to generate pApuro2-CD8α:Igα^{F3}RBD. A ClaI-ClaI fragment from Cla12L-mCD8α:chIgα^{F3} was inserted into

[6] Y. Imamura, A. Oda, T. Katahira, K. Bundo, and D. Kitamura, unpublished result.

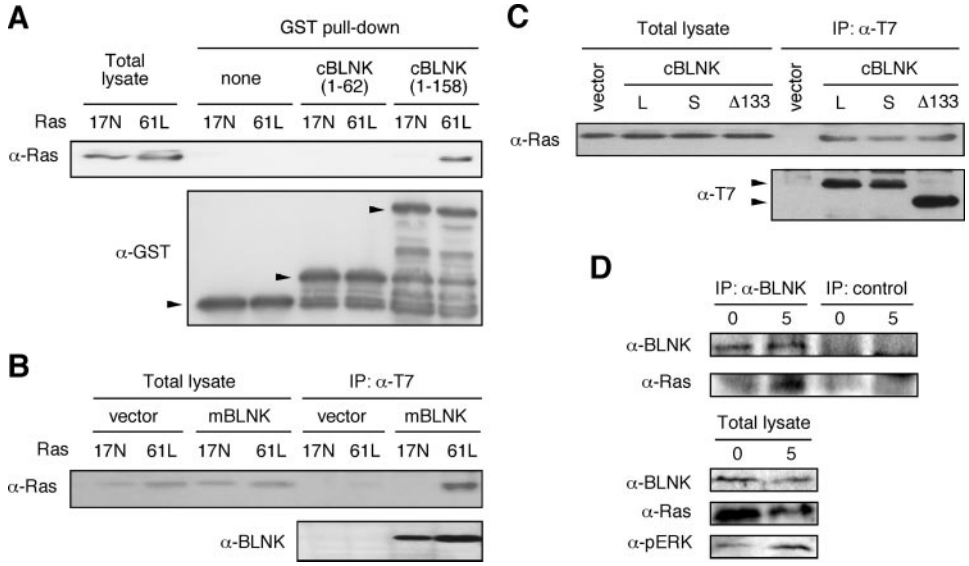

FIGURE 1. **Binding of BLNK and the active Ras.** *A*, GST pull-down assay. GST, GST-cBLNK(1–62), or GST-cBLNK(1–158) proteins immobilized on glutathione-Sepharose 4B were mixed with lysates from Cos-7 cells transfected with either pCMV-H-Ras(61L) or pCMV-H-Ras(17N), respectively. Bound proteins (GST pull-down) and cell lysates (total lysate) were analyzed by 12.5% SDS-PAGE followed by Western blotting with anti-Ras antibody (α-Ras). The filter was reprobed with anti-GST antibody (α-GST). The *arrowheads* indicate the position of each GST fusion protein. *B*, immunoprecipitation analysis between mouse BLNK and H-Ras proteins. The expression vectors pAT7-mBLNK and the empty vector, respectively, were co-transfected into Cos-7 cells with either pCMV-H-Ras(17N) or pCMV-H-Ras(61L). The cell lysates were subjected to immunoprecipitation with anti-T7 (α-T7) antibody and analyzed by Western blotting with α-Ras or with anti-BLNK antibody (α-BLNK), as indicated. *C*, binding of active H-Ras to various forms of chicken BLNK. The expression vectors, pApuroT7-cBLNK (*L*), pApuroT7-cBLNK-S (*S*), and pApuroT7-cBLNK(Δ133) (*Δ133*) and the empty vector (vector), respectively, were co-transfected with pCMV-H-Ras(61L) into Cos-7 cells. The cell lysates were subjected to immunoprecipitation with α-T7, followed by Western blotting with α-Ras and α-T7, as indicated. The *arrowheads* indicate the positions of the BLNK proteins. *D*, binding of endogenous BLNK and Ras proteins in B cells upon BCR cross-linking. Splenic B cells were incubated with (5) or without (0) anti-IgM antibody for 5 min, and the lysates from these cells were immunoprecipitated (*IP*) with α-BLNK or a normal rabbit serum (control). The precipitates and the total cell lysates were subjected to Western blotting using α-BLNK, α-Ras, or anti-phospho-ERK (α-pERK) antibodies, as indicated.

an EcoRI site of pApuro2 through end blunting to generate pApuro2-CD8α:Igα^{F3}. An Aor51HI-SalI fragment from the mCD8α:chIgα^{F3}-RBD or an Aor51HI-XhoI fragment from the mCD8α:chIgα^{F3}-MCS was cloned into the pCAT7-neo vector (32) between the Klenow-blunted EcoRI and SalI sites, to generate T7-Igα^{F3}RBD and T7-Igα^{F3}, respectively.

Cell Culture and Stimulation—DT40 cells and BLNK$^-$ DT40 cells were cultured and stimulated with anti-chicken IgM antibody (M4) as described previously (7). Cos-7 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. BLNK$^-$ DT40 cells were electroporated with pApuroT7- or pApuro2-based vectors and selected with puromycin as described previously (32). BLNK$^-$ DT40 cells expressing mCD8α fusion receptors were stimulated with preincubation for 20 min on ice with rat anti-mouse CD8α monoclonal antibody (53–6.72), followed by incubation at 40 °C with goat anti-rat IgG F(ab′)$_2$ (Jackson ImmunoResearch) for the indicated time periods.

Luciferase Assay—Twenty μg of a vector expressing either the wild type or one of the mutant cBLNKs, as well as a mixture of the reporter vectors, 1 μg of pFA2-Elk1, 10 μg of pFR-Luc, and 1 μg of pRSV-β-gal were transfected into 5 × 10^6 BLNK$^-$ DT40 cells by electroporation. DT40 cells, BLNK$^-$ DT40 cells, and the latter stably transfected with BLNK-derived constructs or CD8α-Igα^{F3} constructs were transfected with the reporter vectors similarly. After 24 h, surface BCR or CD8α was stimulated as described above for 6 h, and the luciferase and β-galactosidase activities were measured as described previously (30, 33).

Protein Analysis—Expression vectors carrying either the wild type or one of the various mutant cBLNKs or one of the Igα constructs, all tagged with the T7 epitope, were mixed with either pCMV-H-Ras(61L) or pCMV-H-Ras(17N), tagged with the FLAG epitope, and with TransIT-LT1 reagents (Mirus). These mixtures were co-transfected into 2 × 10^6 Cos-7 cells. Two days after transfection, the cells were harvested, and the cell extracts were immunoprecipitated with anti-T7 or anti-FLAG antibodies and analyzed by Western blotting as described previously (14, 30). GST fusion proteins of cBLNK were produced in *Escherichia coli* and used in GST pull-down assays and Western blotting as described previously (14, 30). To examine the association of endogenous BLNK and Ras proteins, the lysates of B cells purified from mouse spleens using MACS B cell isolation kit (Miltenyi Biotec) were subjected to immunoprecipitation using rabbit anti-BLNK antibody (18) or control rabbit serum, and the precipitates were analyzed by Western blotting.

Antibodies—The following antibodies were purchased: mouse monoclonal anti-T7 tag (Novagen), goat anti-GST (GE Healthcare), mouse monoclonal anti-FLAG (M5; Sigma-Aldrich), anti-pan Ras (Ab-3; Oncogene), anti-phospho-specific ERK (Cell Signaling), mouse monoclonal anti-Bcl-2 (Transduction Laboratories), anti-mouse CD8α (53–6.72), goat anti-rat IgG or anti-mouse IgM F(ab′)$_2$ fragments (Jackson ImmunoResearch).

Flow Cytometry—DT40-derived cells were stained with FITC-conjugated goat anti-chicken IgM antibody (Bethyl Laboratories) or FITC anti-mouse CD8α (53–6.72) and analyzed with FACSCalibur™ (Becton Dickinson) as described previously (34). For the analysis of BCR-induced apoptosis, DT40 and DT40-derived cells (2 × 10^5/well) were stimulated in culture medium with or without anti-IgM (M4; 1.25 μg/ml) for 48 h in 12-well plates and then washed with the incubation buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$). Then the cells were stained with annexin V-biotin labeling solution (Roche Applied Science) and propidium iodide (1 μg/ml) for 15 min on ice. Then the cells were washed, stained with FITC-conjugated streptavidin (eBioscience) for 20 min on ice, and analyzed by flow cytometry.

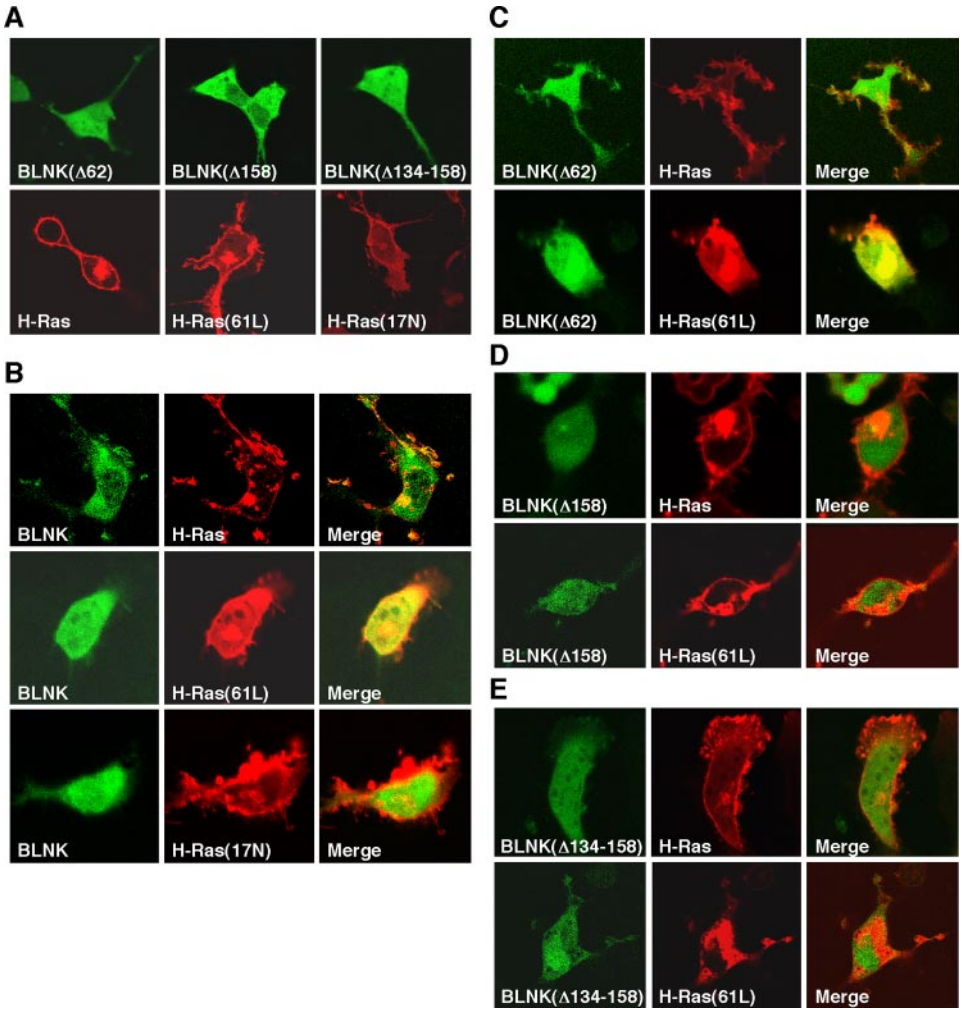

FIGURE 2. **Co-localization of BLNK and active Ras in living cells.** CFP-tagged BLNK-derivatives and YFP-tagged Ras-derivatives were transfected into Cos-7 cells alone or in combination as indicated below. Two days later, the cells were examined by CLSM. CFP fluorescence was digitally colored *green*, and YFP fluorescence is *red*. In the merged images (*Merge*), *yellow* signals indicate the co-localization of CFP and YFP fluorescence. Transfected vectors are as follows. *A*, pECFP-cBLNK(Δ62), pECFP-cBLNK(Δ158), pECFP-cBLNK(Δ134–158), pEYFP-H-Ras, pEYFP-H-Ras(61L), or pEYFP-H-Ras(17N). *B*, pECFP-cBLNK and either pEYFP-H-Ras (*top row*), pEYFP-H-Ras(61L) (*middle row*), or pEYFP-H-Ras(17N) (*bottom row*). *C*, pECFP-cBLNK(Δ62) and either pEYFP-H-Ras (*top row*) or pEYFP-H-Ras(61L) (*bottom row*). *D*, pECFP-cBLNK(Δ158) and either pEYFP-H-Ras (*top row*) or pEYFP-H-Ras(61L) (*bottom row*). *E*, pECFP-cBLNK(Δ134–158) and either pEYFP-H-Ras (*top row*) or pEYFP-H-Ras(61L) (*bottom row*).

Confocal Laser Scanning Microscopy (CLSM)—CLSM was performed for cells transfected with the CFP- and YFP-tagged constructs as described previously (30). DT40 cells and their transfectants were stimulated as described above, washed in cold phosphate-buffered saline, fixed with paraformaldehyde, and stained with anti-chicken IgM-FITC (Bethyl Laboratories) or with anti-rat IgG-FITC (Southern Biotechnology Associates) for staining of rat anti-mouse CD8-labeled cells. Then the cells were permeabilized with 0.1% Triton X-100 in phosphate-buffered saline, incubated with the blocking reagent (0.1% goat serum), and stained with anti-Ras (Ab-3), followed by and goat anti-mouse IgG-TRITC (Jackson ImmunoResearch). The stained cells were placed onto glass slides, covered with thin glass, and inspected with CLSM. Excitation wavelengths for FITC and TRITC were 488 and 568 nm, respectively. Emission signals were detected between 495 and 540 nm for FITC and between 585 and 610 nm for TRITC. Each result shown here is a representative of the data from the same experiments repeated at least and mostly more than twice.

RESULTS

Identification of H-Ras as a Binding Partner of BLNK—To elucidate the mechanism of BLNK action in the BCR signal transduction, we sought to identify proteins interacting with BLNK by the yeast two-hybrid system. We screened the cDNA library from the chicken B cell line DT40 with a "bait" consisting of a 158-aa region of chicken BLNK (BLNK(1–158) and identified several independent clones as reported previously (31). From these clones, we identified one that interacts with BLNK(1–158), but not with a 62-aa region of BLNK (BLNK(1–62)), in the two-hybrid system, and the insert of this clone turned out to be a cDNA encoding an H-Ras protein with a truncation of the first three amino acids. To verify the binding of BLNK and H-Ras *in vitro*, we performed a GST pull-down assay. Human H-Ras proteins, either constitutively active (61L) or inactive (17N) mutant forms, were transiently expressed in Cos-7 cells, and the cell lysates were subjected to binding with GST-fused BLNK(1–158) or BLNK(1–62) proteins. As shown in Fig. 1*A*, only the active form bound to the GST-BLNK(1–158) protein, and neither forms bound to GST-BLNK(1–62) or GST alone. We next expressed the H-Ras proteins and T7-tagged mouse BLNK protein transiently in Cos-7 cells, and the BLNK-bound complex was immunoprecipitated with anti-T7 tag antibody (α-T7). As shown in Fig. 1*B*, only the active form of H-Ras was co-precipitated with BLNK. With the same system, we showed that the active H-Ras also bound to a full-length chicken BLNK (long form) as well as the shorter form of chicken BLNK that lacks the 40–58 aa region (6) and the BLNK with an N-terminal 133-aa deletion (Fig. 1*C*). These results indicate that the 134–158 aa region of chicken BLNK is responsible for the binding with the active H-Ras. We further examined the binding of endogenous Ras and BLNK proteins in mouse splenic B cells. As shown in Fig. 1*D*, immunoprecipitation with anti-BLNK antibody (α-BLNK) co-precipitated Ras protein from the cells stimulated with anti-IgM antibody but not from unstimulated cells. A control rabbit serum precipitated neither BLNK nor Ras. Thus, endogenous

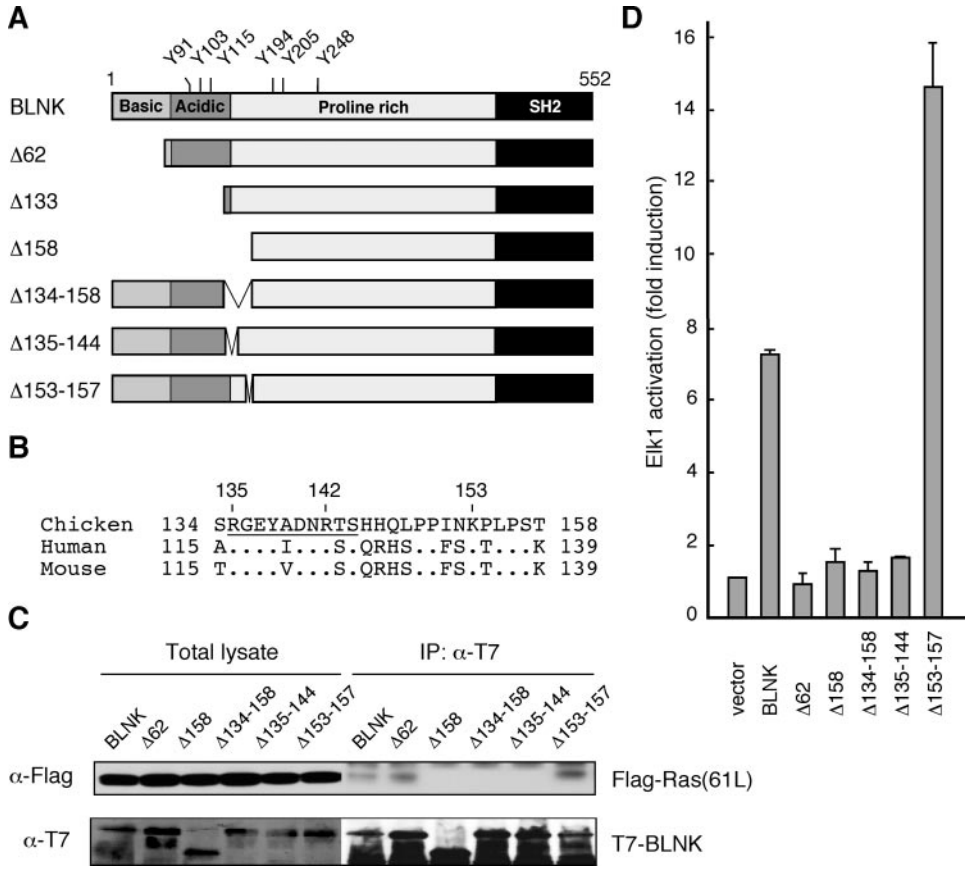

FIGURE 3. **Determination of a Ras-binding domain of BLNK that is responsible for BCR-mediated Elk1 activation.** *A*, schematic representation of wild type and mutated BLNK proteins encoded in the expression vectors used in the experiments demonstrated here. *B*, amino acid sequence alignment of chicken (134–158), human (115–139), and mouse (115–139) BLNK. The determined RBD is *underlined*. *C*, expression vectors encoding FLAG-H-Ras(61L) and one of the indicated BLNK proteins were transfected into Cos-7 cells, and the cell lysates were immunoprecipitated with α-T7. The precipitates and total cell lysates were analyzed by Western blotting with anti-FLAG antibody (α-FLAG, *top panel*). The filter was then reprobed with α-T7 (*bottom panel*). *D*, luciferase assay demonstrating the BLNK RBD-dependent Elk1 activation upon BCR cross-linking. The indicated BLNK-expression vectors were transfected with Elk1 reporter plasmids into BLNK$^-$ DT40 cells. The cells were stimulated with anti-IgM antibody and subjected to luciferase and β-galactosidase assays. *vector* indicates an empty vector. The values are indicated as fold induction of the standardized luciferase activity over the unstimulated control. The *bars* represent the means ± S.D. of duplicated samples. Shown is a representative of three-times repeated experiments with essentially identical results.

BLNK binds endogenous Ras in normal B cells after BCR stimulation.

Co-localization of BLNK and the Constitutively Active H-Ras in Living Cells—To examine the binding of BLNK and Ras in living cells, we transfected expression vectors encoding CFP-tagged BLNK and YFP-tagged H-Ras proteins into Cos-7 cells and examined their intracellular localization with CLSM (Fig. 2). The CFP-tagged full length as well as deletion mutants of chicken BLNK lacking 1–62 (Δ62), 1–158 (Δ158), or 134–158 (Δ134–158) aa regions, respectively, were diffusely distributed throughout the cells with occasional exclusion from nuclei (Fig. 2*A* and Ref. 30). In contrast, YFP-tagged wild type or the constitutively active (61L) or inactive (17N) forms of H-Ras were localized at plasma membrane, with accumulation in ruffles and lamellipodia, as well as at Golgi apparatus, as described previously (35, 36). When BLNK and wild type H-Ras were co-expressed, BLNK was distributed in the cytoplasm but with a noticeable accumulation at lamellipodia and Golgi area where H-Ras was co-localized, suggesting that the active H-Ras at these sites is associated with BLNK (Fig. 2*B*, *top row*). When BLNK was co-expressed with H-Ras(61L), H-Ras(61L) was mostly co-localized with BLNK in a diffuse cytoplasmic pattern (Fig. 2*B*, *middle row*), suggesting that the active Ras was trapped inside of the cell through binding with BLNK. By contrast, inactive H-Ras was mostly distributed at the periphery of the cells where BLNK was largely excluded (Fig. 2*B*, *bottom row*).

The cellular localization pattern of co-expressed BLNK(Δ62) and H-Ras proteins was almost the same as that of wild type BLNK and H-Ras; BLNK(Δ62) was co-localized with wild type H-Ras at the peripheral lamellipodia, with the H-Ras(61L) diffusely throughout the cells and not co-localized with H-Ras(17N) (Fig. 2*C*; data not shown). By contrast, BLNK(Δ158) and BLNK(Δ134–158) proteins were diffusely distributed in the cytoplasm and the nucleus and not apparently co-localized with either wild type or the active H-Ras. Localization of these H-Ras proteins was mainly at the plasma membrane and Golgi area and not affected by the expression of these BLNK variants (Fig. 2, *D* and *E*). The same holds true for the inactive H-Ras(17N) protein co-expressed with any of these BLNK variants (data not shown). These results indicate that BLNK specifically binds the active form of H-Ras in living cells and that the 134–158 aa region of BLNK is necessary for this binding.

Determination of a Ras-binding Domain of BLNK—To narrow down the region of BLNK that is necessary for the binding with H-Ras, we prepared additional deletion mutants of chicken BLNK cDNA and constructed their expression vectors (Fig. 3*A*). A sequence alignment of the 134–158 aa region of chicken BLNK with the corresponding regions of human and mouse orthologs showed two conserved sequences (135–144 and 153–157) (Fig. 3*B*). Each of these sequences was deleted to derive BLNK(Δ135–144) and BLNK(Δ153–157) constructs, respectively. These T7-tagged BLNK expression vectors were co-transfected into Cos-7 cells with the FLAG-tagged H-Ras(61L) expression vector, and their binding was examined by α-T7 immunoprecipitation. As shown in Fig. 3*C*, a deletion of a 1–158 or 134–158 region of BLNK resulted in a loss of the H-Ras binding, in accord with the result of *in vivo* co-localization (Fig. 2, *D* and *E*). Deletion of the 135–144 aa region also lost this binding, but a deletion of the 153–157 aa region did not

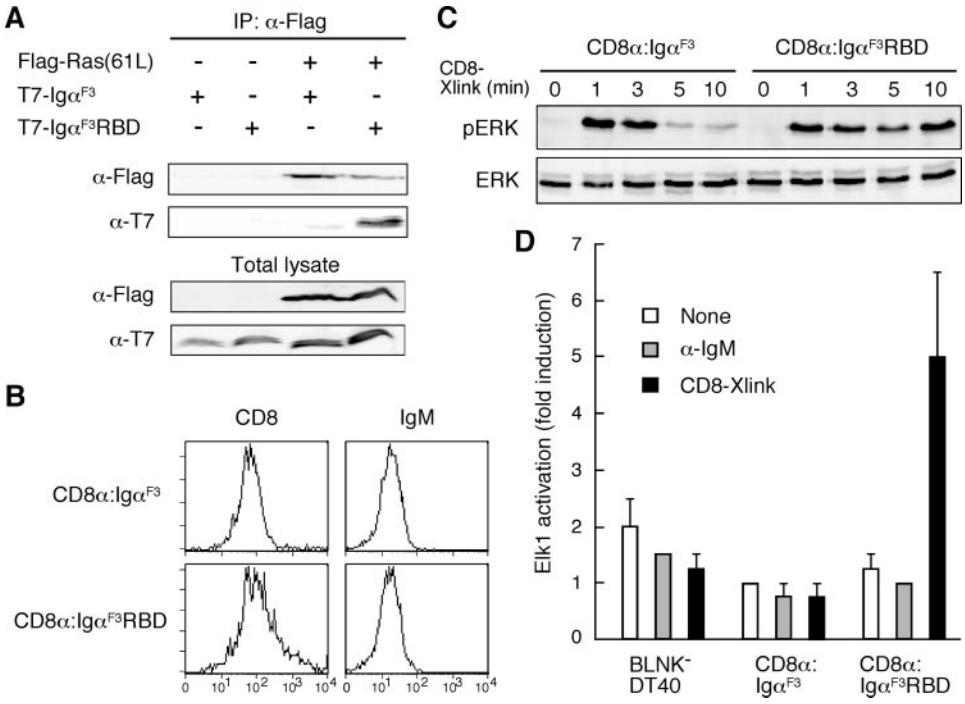

FIGURE 4. **The BLNK RBD restores BCR-signaled ERK activation in BLNK-deficient B cells.** *A*, a binding of the BLNK RBD and Ras. Expression vectors encoding FLAG-Ras(61L) and T7-Igα^{F3} or T7-Igα^{F3}RBD were transfected into Cos-7 cells as indicated by '+'. The cell lysates were immunoprecipitated (*IP*) with α-FLAG, and the precipitates as well as the lysates were analyzed by Western blotting with α-FLAG and α-T7, as indicated. *B*, surface expression of CD8α and IgM on BLNK$^-$ DT40 cell lines stably expressing CD8:Igα^{F3} or CD8:Igα^{F3}RBD analyzed by flow cytometry. *C*, cells shown in *B* were stimulated through CD8 cross-linking (*Xlink*) for the indicated time periods. The cells were lysed, and analyzed by Western blotting with α-pERK. The filter was reprobed with α-ERK. *D*, BLNK$^-$ DT40 cells, and the cells shown in *B* were transfected with the Elk1 reporter plasmids and stimulated through IgM (α-IgM) or CD8 cross-linking (CD8-Xlink) or not stimulated (None). Luciferase activity in duplicated samples was examined as in Fig. 3*D* and presented as values relative to that of the cells expressing CD8:Igα^{F3} (set as 1). Shown is a representative of three times repeated experiments with essentially identical results.

(Fig. 3*C*), indicating that the 135–144 aa region is essential for the binding with H-Ras.

We next examined the functional significance of BLNK-Ras binding in BCR signal transduction using chicken B lymphoma cell line DT40. It had been reported that the induction of *in vitro* ERK-kinase activity shortly after BCR cross-linking is markedly attenuated, but not completely lost, in BLNK$^-$ DT40 cells (7). Here we assessed the ERK function *in vivo* by the established Elk1-mediated transcriptional activation system. The transcription factor Elk1 is known to be phosphorylated and activated by ERK. With this system we had previously shown that BCR cross-linking on DT40 cells induces a strong transcriptional activation of Elk1, which is completely abrogated by a MEK inhibitor U0126, verifying that BCR-mediated MEK-ERK signaling function can be assessed with this system (31). We transfected BLNK$^-$ DT40 cells with the constructs expressing wild type or various deletion mutants of BLNK together with the luciferase reporter constructs and treated the cells with anti-IgM antibody for 6 h, and Elk1 transcriptional activation was measured (Fig. 3*D*). No induction of the Elk1 activity was detected in the BLNK$^-$ DT40 cells ("vector"), but activity was restored by introduction of a full-length BLNK ("BLNK"). Therefore it is indicated that the BCR-induced modest ERK activation in BLNK$^-$ DT40 cells, which was demonstrated biochemically (7), is not enough to activate Elk1 *in vivo*. An N-terminal 62-aa region of BLNK was necessary for the Elk1 activation (Δ62), although not for Ras binding. This region includes a conserved leucine zipper that is necessary for plasma membrane localization of BLNK and its function in pre-B cell differentiation and therefore seems essential for BLNK function (8). The BLNK constructs lacking the Ras-binding domain (Δ134–158 and Δ135–144) failed to restore BCR-mediated Elk1 activation, whereas BLNK(Δ153–157) that retains Ras binding restored it, resulting in even stronger activation than wild type BLNK. This result indicates that Ras binding is necessary for BLNK to transmit the BCR signal to ERK so that it functions to activate Elk1.

Functional Activation of ERK through Igα-connected BLNK RBD—To test whether the identified Ras-binding domain (RBD) of BLNK (135–144 aa) is sufficient for binding to Ras, we prepared a construct (T7-Igα^{F3}RBD) in which the 131–147 aa sequence of chicken BLNK was fused at the C terminus of the T7-tagged cytoplasmic domain of the chicken Igα carrying a tyrosine-to-phenylalanine substitution in a putative BLNK SH2 domain-binding site (chIgα^{F3}) (11). The T7-Igα^{F3}RBD or the control protein without the RBD (T7-Igα^{F3}) was transiently co-expressed with FLAG-tagged H-Ras(61L) in Cos-7 cells, and their binding was examined by immunoprecipitation. As shown in Fig. 4*A*, the H-Ras(61L) was co-precipitated with the T7-Igα^{F3}RBD protein, but not with the T7-Igα^{F3} protein, indicating that the BLNK RBD is sufficient for the binding with H-Ras.

To understand the functional significance of the recruitment of H-Ras to the BLNK RBD, we prepared a fusion construct consisting of the extracellular and transmembrane domains of mouse CD8α and the chIgα^{F3}RBD (CD8α:Igα^{F3}RBD) or the chIgα^{F3} (CD8α:Igα^{F3}) (11). We transfected these constructs into the BLNK$^-$ DT40 cells and generated stable transfectants expressing the CD8α:Igα^{F3}RBD or CD8α:Igα^{F3} constructs on the cell surface at a similar level (Fig. 4*B*). Using these cells, ERK activation upon CD8α cross-linking was examined by Western blotting detecting an activation-associated phosphorylation. Cross-linking of the CD8α:Igα^{F3} resulted only in a transient phosphorylation of ERK, returning to a base line after 5 min. On the other hand, cross-linking of the CD8α:Igα^{F3}RBD resulted in a more prolonged ERK phosphorylation, not declining until at least 10 min (Fig. 4*C*). Next, ERK activation was assessed with the Elk1 transcriptional activation system as described above. As shown in Fig. 4*D*, cross-linking of the CD8α:Igα^{F3}RBD resulted in the significant activation of Elk1-mediated tran-

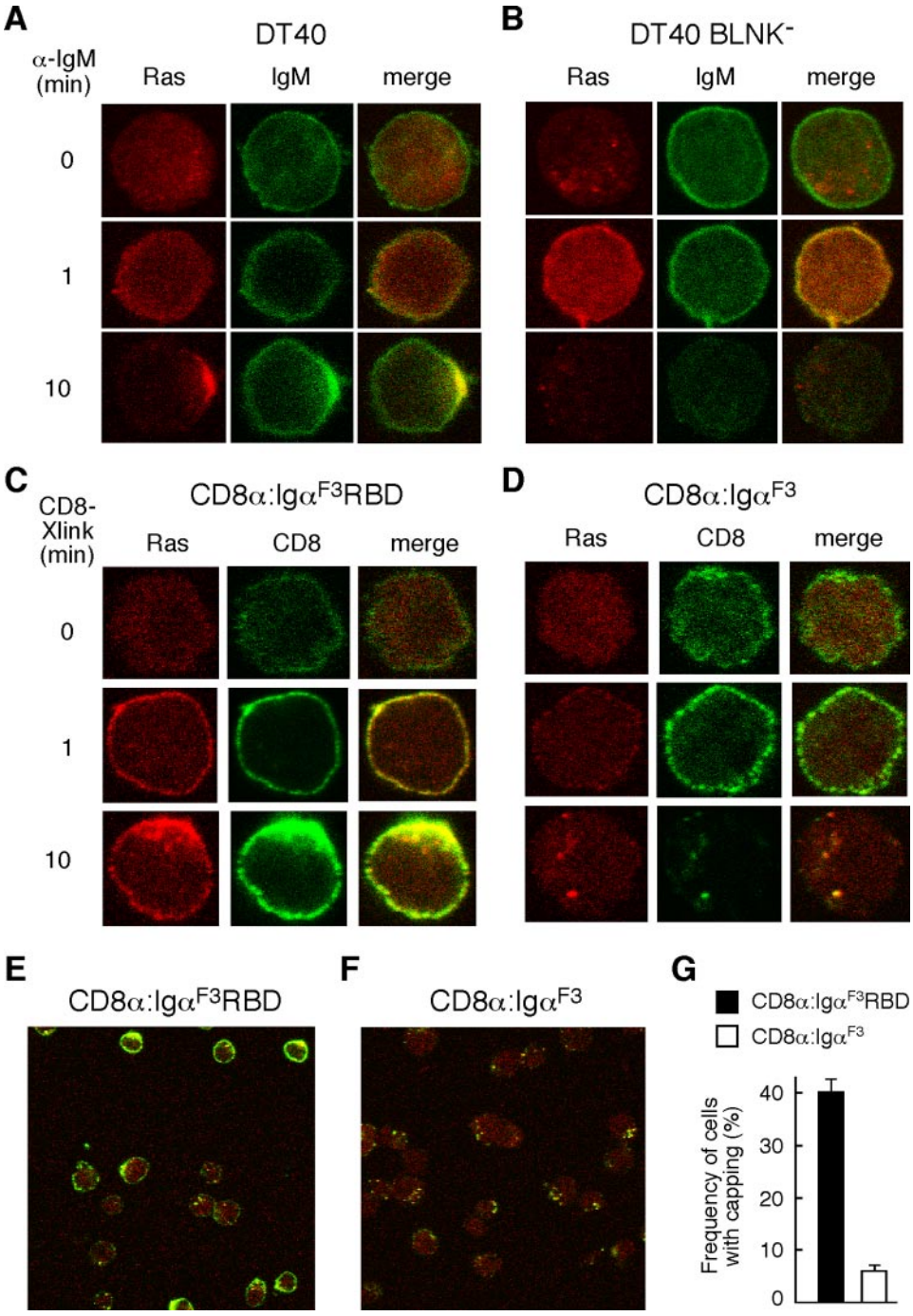

FIGURE 5. **BLNK RBD is necessary for the BCR capping.** *A–D*, DT40 (*A*) and BLNK$^-$ DT40 (*B*) cells were stimulated with anti-IgM (M4) and BLNK$^-$ DT40 cells stably expressing CD8α:Igα^{F3}RBD (*C*) or CD8α:Igα^{F3} (*D*) were stimulated with CD8α cross-linking (Xlink), for 1 or 10 min (1 and 10), or left on ice (0), and then the cells were stained and examined by CLSM, as described under "Experimental Procedures." TRITC fluorescence was digitally colored *red*, and FITC fluorescence is *green*. In the merged images (*merge*), *yellow* signals indicate the co-localization of both fluorescence signals. *E* and *F*, lower magnification merged images of the cells 10 min after stimulation shown in *C* and *D*. *G*, numbers of the cells with the cap were counted in 100 cells/trial. *Closed* and *open columns* indicate the average numbers with S.D. from two trials counting different areas of the visual field in each inspection represented in *E* and *F*, respectively.

scription, whereas that of the CD8α:Igα^{F3} did not. Cross-linking of BCR did not induce the Elk1 activation in these cells lacking BLNK. These data indicate that Ras recruitment to Igα through the BLNK RBD resulted in the prolonged ERK activation that is functional in activating downstream Elk1 *in vivo*.

BLNK RBD-dependent Formation of the Igα Cap Associated with Ras—It was shown that Ras co-caps with surface BCR upon its cross-linking (21). As shown in Fig. 5*A*, endogenous Ras rapidly accumulated at the plasma membrane 1 min after cross-linking of surface IgM on DT40 cells and redistributed together with surface IgM to form a typical cap after 10 min. In the BLNK$^-$ DT40 cells, Ras accumulated at the membrane after 1 min, but Ras and IgM did not form the cap and disappeared from the plasma membrane after 10 min (Fig. 5*B*), indicating that BLNK is necessary for co-capping of BCR and Ras and their persistence at the plasma membrane. We next tested whether the BLNK-mediated Ras recruitment to the BCR complex is involved in the cap formation by using the BLNK$^-$ DT40 cells stably expressing CD8α:Igα^{F3}RBD or CD8α:Igα^{F3}. Upon cross-linking of the surface CD8, both the endogenous Ras and the CD8α:Igα^{F3}RBD molecules accumulated at the plasma membrane after 1 min and co-capped after 10 min in many of the cells (Fig. 5, *C*, *E*, and *G*). In contrast, cross-linked CD8α:Igα^{F3} did not remain on the plasma membrane and barely formed the cap after 10 min (Fig. 5, *D*, *F*, and *G*). This result indicates that Ras recruitment to the Igα through the BLNK RBD is necessary for the capping that enables prolonged surface expression of BCR.

BLNK-RBD Is Responsible for Anti-apoptotic Signaling Transmitted from BLNK—To gain a biological insight into the BLNK-mediated Ras recruitment, we generated BLNK$^-$ DT40 cells stably reconstituted with full-length BLNK or BLNK(Δ135–158). The resultant transfectants express the surface IgM at almost equal levels to the parental BLNK$^-$ DT40 cells, whereas the latter express a little lower level than wild type (WT) DT40 cells (Fig. 6*A*), and they express the exogenous BLNK proteins at the similar levels as the endogenous BLNK in the WT DT40 cells (data not shown). As observed in the transient transfection assay (Fig. 3*D*), the BLNK$^-$ DT40 cells expressing BLNK(Δ135–158) protein hardly responded to

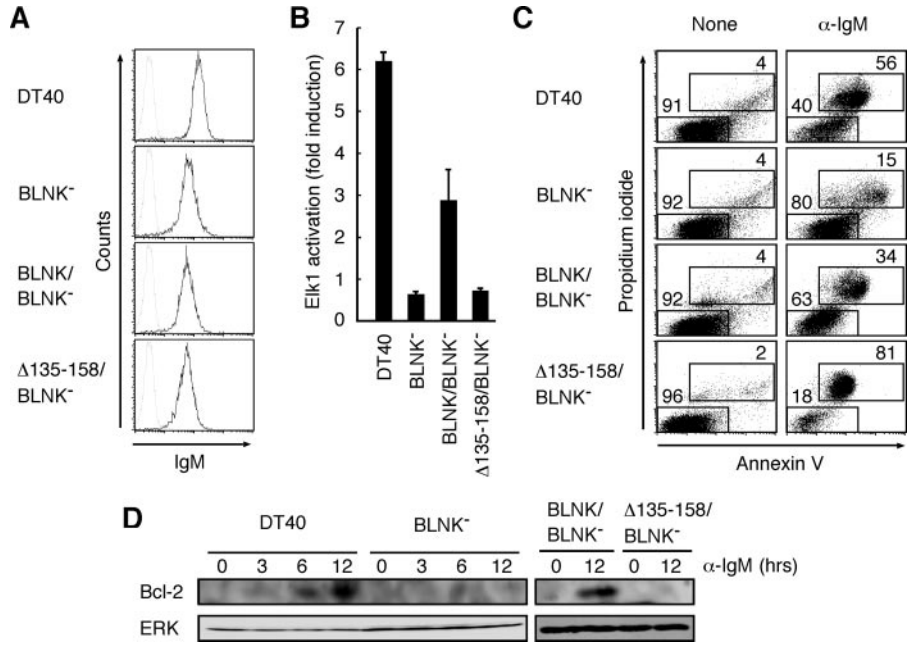

FIGURE 6. **BLNK RBD mediates anti-apoptotic signal from BCR.** *A*, surface IgM expression on the indicated cell lines as analyzed by flow cytometry. *B*, IgM cross-linking-induced Elk1 activation in the indicated cell lines was assessed and demonstrated as in Fig. 3*D*. Shown is a representative of experiments repeated twice with essentially identical results. *C*, BCR-induced apoptosis. The indicated cell lines were incubated with (α-IgM) or without (*None*) anti-IgM antibody for 48 h, stained with annexin V and propidium iodide, and analyzed by flow cytometry as described under "Experimental Procedures." Each number indicates the percentage of the cells falling in each neighbored window. *D*, BCR-induced Bcl-2 expression. The indicated cell lines were incubated with anti-IgM antibody for the indicated periods of time (h), and then the cells were lysed and analyzed by Western blotting with anti-Bcl-2 (*top panel*) or anti-ERK (*bottom panel*) antibodies.

the BCR cross-linking in Elk1 activation, unlike those expressing full-length BLNK (Fig. 6*B*).

DT40 is known to undergo apoptosis through BCR signaling that requires Btk and PLCγ2 (37, 38). BLNK is known to mediate the interaction of Btk and PLCγ2 and thus activation of PLCγ2. Accordingly, BLNK⁻ DT40 cells underwent much less apoptosis after BCR cross-linking compared with WT DT40 cells, and the BLNK-reconstituted cells significantly restored the apoptotic response (Fig. 6*C*). In DT40 cells, BLNK, Btk, and PLCγ2 also mediate the BCR-induced ERK activation (7, 39, 40), but the role of ERK in the apoptosis induction has not been studied. In mice, ERK rather serves as an anti-apoptotic and proliferative factor (41, 42). In the BLNK⁻ DT40 cells reconstituted with BLNK(Δ135–158) that failed to activate ERK function, the BCR-induced apoptosis was stronger than those with full-length BLNK or even WT DT40 cells (Fig. 6*C*). This result suggests that, in DT40 cells, BLNK transduces two opposite signals, pro-apoptotic and anti-apoptotic, simultaneously upon BCR cross-linking. Pro-apoptotic signal mediated through Btk and PLCγ2 is dominant and determines the apoptotic fate of the cells, whereas anti-apoptotic signal mediated through the RBD-Ras-ERK pathway attenuates the apoptosis. To support this hypothesis, expression of Bcl-2, a typical anti-apoptotic protein, was induced after BCR cross-linking in the only cells expressing BLNK with the intact RBD (Fig. 6*D*).

DISCUSSION

BLNK has been shown to be dispensable for the formation of GTP-bound active form of Ras but required for activation of ERK, in DT40 cells in response to BCR cross-linking (7), raising a question as to how BLNK is involved in ERK activation. In this study, we have demonstrated that BLNK directly binds an active form of Ras, and this binding is required for BCR-mediated activation of ERK leading to downstream Elk-1 activation *in vivo*. BLNK RBD-mediated Ras-recruitment to Igα was required for capping of the Igα-fused receptor, for prolonged ERK phosphorylation, and for Elk1 activation in the B cells lacking BLNK. The Elk1 activation by cross-linking of the Igα-BLNK RBD in BLNK⁻ DT40 cells was much weaker than that by BCR cross-linking in WT DT40 cells. This is likely due to the lack of JNK activation in the former cells (7), because we have found that JNK activity greatly contributes to the BCR-mediated Elk1 activation in DT40 cells (31).

These results indicate that, by binding both Igα and the active Ras, BLNK may recruit the active Ras to the BCR complex, which is likely to be critical for capping of BCR. Sustained cell surface expression of BCR in the form of the cap may enable sustained signal transduction through the associated Ras, resulting in the functional activation of ERK. This presumed function of BLNK is similar to that proposed for another Ras binding partner galectin-1, a cytoplasmic β-galactoside-binding protein. It was reported that galectin-1 selectively binds the active form of H-Ras, being independent of its sugar binding function, and the galectin-1 binding is required for stable plasma membrane localization and transformation activity of the Ras protein (43). Another similarity can be found in the function of Sur-8, a leucine-rich repeat protein that binds the active forms of Ras proteins and enhances epidermal growth factor-mediated ERK activation (44). Positive regulatory function of Sur-8 downstream of receptor tyrosine-kinase and Ras was first revealed through genetic studies in *Caenorhabditis elegans* (45, 46). Thus, it seems plausible that the GTP-bound form of Ras must be tethered to some scaffold molecule at the inner plasma membrane for the optimal activation of downstream ERK signaling that produces significant biological outcomes.

A small GTPase Rac and a Rac-guanyl nucleotide exchange factor Vav have been shown to be necessary for capping of T cell receptor (47–49), but no direct evidence for their requirement in BCR capping has been reported. It was also shown that activation-associated ERK phosphorylation upon T cell receptor cross-linking was abolished in T cells from mice lacking any Vav proteins (Vav1/2/3), but ERK phosphorylation upon BCR cross-linking was normal in B cells from the same mice (50). Recently Brezski and Monroe (51) have demonstrated that

Rac1 activity is not required for anti-BCR-induced BCR capping but required for cell spreading over anti-BCR-coated plates, another actin-dependent event. Thus, although it was reported that BLNK binds Vav to recruit it into the membrane raft upon BCR engagement and contributes to Rac activation (52), this function of BLNK does not play a major role in the cap formation and ERK activation.

The present study has revealed that BLNK RBD-mediated Ras recruitment to Igα is critical for capping of the receptor upon cross-linking. Currently we do not know the mechanism by which Ras is involved in the BCR cap formation that is dependent on the actin cytoskeleton (21). Ras is known to induce cortical actin rearrangement through phosphatidylinositol 3-kinase activation in nonlymphoid cells (53). Phosphatidylinositol 3-kinase is known to regulate the actin cytoskeleton through various proteins including Rho family GTPases (54). Another Ras downstream effector, Raf, has also been shown to regulate the actin cytoskeleton (55). We previously demonstrated that BLNK recruits PKCη to the plasma membrane in pre-B cells (56). Because PKC is known to phosphorylate and activate Raf (57), it is tempting to speculate that, under the cross-linked BCR, BLNK binds PKC and the active Ras, to which Raf is bound, and thus mediates prolonged activation of Raf by PKC.

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