Brief Report

Caspase Recruitment Domain (CARD)-dependent Cytoplasmic Filaments Mediate bcl10-induced NF-\(\kappa\)B Activation

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Abstract. Mucosa-associated lymphoid tissue (MALT) lymphomas are associated with overexpression and constitutive activity of bcl10, a caspase recruitment domain (CARD)-containing protein that activates NF-\(\kappa\)B. Here, we show that arrangement of overexpressed bcl10 protein in cytoplasmic filaments is essential for recruitment of signal transducer molecules-involved NF-\(\kappa\)B activation. We also show that cytoskeleton elements regulate bcl10 signaling. Thus, organized assemblage of proteins in ordered structures linked to the cytoskeleton network may represent a general mechanism for intracellular signaling.

Key words: bcl10 • filaments • mucosa-associated lymphoid tissue • NF-\(\kappa\)B • scaffold

Introduction

Mucosa-associated lymphoid tissue (MALT) lymphomas with t(1;14)(p22;q32) display a recurrent breakpoint upstream of bcl10 that juxtaposes the gene under the control of the Ig heavy chain enhancer (Willis et al., 1999; Zhang et al., 1999). As a consequence of this translocation, bcl10 cDNA's from MALT lymphomas are overexpressed and contain a variety of mutations, mostly consisting of either nucleotide deletion or insertion, which result in truncations or distal to the CARD (Willis et al., 1999; Zhang et al., 1999). Thus, overexpression of truncated forms of bcl10, which retain constitutive NF-\(\kappa\)B-inducing activity, transform primary rat embryonic fibroblasts (Willis et al., 1999). However, the mechanism by which deregulated expression of bcl10 leads to cellular oncogenesis is currently not known.

Here, we show that bcl10 protein assembles in cytoplasmic filaments that serve as scaffold for recruitment of NF-\(\kappa\)B-activating signal transduction molecules. Cytochalasin D, a potent inhibitor of actin filament function, disassembles bcl10 filaments and specifically inhibits bcl10-induced NF-\(\kappa\)B activation. Thus, assemblage of bcl10 in filaments connected to the cytoskeleton network is essential for bcl10-mediated NF-\(\kappa\)B induction.

Materials and Methods

Cell Culture, Reagents and Luciferase Assay

HeLa, HEK 293, and Rat-2 cells were cultured in DME 10% FCS. HeLa and Rat-2 cells were transfected using lipofectamine (GIBCO BRL); 293 cells were transfected by calcium phosphate precipitation. To assess NF-\(\kappa\)B activation, HeLa and Rat-2 cells were transfected with 2 \(\mu\)g of the indicated cDNA's, together with pNF-\(\kappa\)B-luc in 12-well plates. Cells were then lysed and luciferase activity was determined with the Luciferase Assay System (Promega). A plasmid expressing \(\beta\)-galactosidase was added to the transfection mixture for normalization of the efficiency of transfection.

Cytochalasin D was obtained from Sigma Chemical Co.

Plasmids Construction and Reagents

PCR-based random mutagenesis of bcl10 was carried out in the following reaction buffer: 10 mM Tris-HCl, pH 8, 50 mM KCl, 0.5 mM MnCl\(_2\), 125 \(\mu\)M dNTPs. Reaction was performed using 2.5 U standard Taq polymerase, 200 ng of plasmidic template, and the oligos 5'-AAGAATTC-CATGGAGCCCACCGCACC (forward) and 5'-AACTCGAGTCATGGAAAAGGTTCACAACTGCT (reverse). PCR products were purified and cloned in pcDNA3 expression vector (Invitrogen) provided with an HA epitope. The vectors pNF-\(\kappa\)B-luc and pCMV-\(\beta\)-gal were from Clontech.

Immunofluorescence

HeLa cells were grown and transfected in chamber slides. 16 h after trans-
Infection, cells were fixed in 4% paraformaldehyde for 15 min at room temperature and then permeabilized in PBS/0.1% Triton X-100. Primary antibodies were incubated for 30 min in 5% FCS–PBS, followed by several washes with 5% FCS–PBS, and then incubating for 30 min with secondary antibody in 5% FCS–PBS. All steps were done at room temperature. Sources of antibodies and reagents for immunofluorescence were: anti-HA (Roche Molecular Biochemicals); anti-FLAG and anti-α-actinin (Sigma Chemical Co.); anti-TRADD and anti-RIP (Santa Cruz).

Two-Hybrid Screen and β-Gal Assays

The two-hybrid screening was conducted using the Matchmaker system (Clontech) according to the manufacturer’s instructions. In brief, yeast strain H7Fc, expressing GAL4-DR4 fused protein, was transformed with a human peripheral blood leukocyte cDNA library cloned in the pGAD 10 vector (Clontech) by lithium acetate/PEG 4000 procedure. 2 × 10⁶ clones were analyzed. Transformed yeast were selected on SD agar plates lacking leucine, tryptophane, and histidine for 5 d at 30°C. Selected colonies were blotted on filter paper, permeabilized in liquid nitrogen, and placed on another filter soaked in Z buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, and 37.5 mM β-mercaptoethanol) containing 1 mM 5-bromo-4-chloro-3-indolyl-β-D-galactoside. Colonies that developed color were restreaked on selective plates to allow plasmid segregation and tested again for β-galactosidase activity. The liquid β-galactosidase assay was performed according to the manufacturer’s instructions using CPRG as substrate.

Results and Discussion

To determine the cellular localization of oncogenic bcl10, we transfected HeLa cells with an HA-tagged vector encoding the full-length bcl10, and the expressed protein was detected using a monoclonal anti-HA antibody. The results in Fig. 1 show that bcl10 exhibits a clear pattern of discrete and interconnecting cytoplasmic filaments resembling the death-effector filaments (Perez and White, 1998; Siegel et al., 1998). Similar filaments were observed when bcl10 was visualized using specific affinity-purified rabbit antisera raised against a bcl10-derived polypeptide (Fig. 1).
The filaments were not an artifact of the staining procedure, since competition with the immunogenic peptide abolished the immunofluorescence (data not shown). Double-staining experiments indicated that bcl10 filaments partially overlap other cellular filaments, such as tubulin, keratin, and actin (data not shown).

The NH₂-terminal region of bcl10 contains a CARD motif (Costanzo et al., 1999; Koseki et al., 1999; Srinivasula et al., 1999; Thome et al., 1999; Willis et al., 1999; Yan et al., 1999; Zhang et al., 1999), which is both necessary and sufficient for NF-κB induction (Costanzo et al., 1999; Koseki et al., 1999; Willis et al., 1999; Zhang et al., 1999).

To verify whether the CARD was involved in determining the filamentous arrangement of bcl10, we tested deleted versions of bcl10 for filament formation. A truncated bcl10 polypeptide (bcl10128-233) that lacks the CARD region showed a nonfilamentous diffuse distribution, whereas the full-length protein (bcl101-127) formed filaments identical to the full-length protein (Fig. 2A). Since the CARD of bcl10 retains the NF-κB–inducing activity, these experiments suggested a correlation between filament formation and bcl10-mediated NF-κB activation. To further explore this possibility, we generated the point mutants bcl10(L41Q) and bcl10(G78R), because mutations of corresponding

Figure 2. Mutations in the CARD abolish filament formation and NF-κB activation. A, Fluorescence confocal micrographs of bcl10 mutants. HeLa cells expressing bcl10128-233, bcl101-127, bcl10(L41Q), and bcl10(G78R) were stained with anti-HA mAb followed by FITC-conjugated anti-mouse IgG. B, HeLa cells were cotransfected with pNF-κB-luc, pCMV-β-gal, and an equal amount of the indicated bcl10 mutant. Data represent relative luciferase activity normalized for β-galactosidase expression and is representative of three independent triplicate experiments. C, HeLa cells expressing bcl10 together with dominant negative versions of IκB (right) or NIK (left) were stained with anti-HA mAb, followed by FITC-conjugated anti-mouse IgG.

bcl10+IκB dom. neg.  bcl10+NIK dom. neg.
residues in the CARD of the cell death-inducing proteins, CED-3 and RAIDD, abrogate their functional activity (Shaham and Horvitz, 1996; Duan and Dixit, 1997). Although expressed at levels comparable to the wild-type protein (data not shown), both mutants, bcl10(L41Q) and bcl10(G78R), did not form filaments and failed in activating NF-κB (Fig. 2 B).

bcl10 filament formation is not an effect of NF-κB activ-
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because when bcl10 was cotransfected with dominant negative forms of IκB and NIK, which abrogate bcl10-induced NF-κB activation (Costanzo et al., 1999; Koseki et al., 1999; Srinivasula et al., 1999), bcl10 filaments were formed (Fig. 2 C).

To determine whether bcl10 filaments are unequivocally linked to NF-κB activation, we generated a panel of random mutations in the CARD region of the protein. Microscopy and functional analysis of these mutants indeed revealed that only bcl10 mutants still able to form filaments could activate NF-κB (Fig. 3).

Previously, we and others have shown that bcl10 acti-

Figure 4. Recruitment of TRADD and RIP into bcl10 filaments. A, HeLa cells expressing bcl10 and FLAG-tagged TRADD were co-stained with anti-bcl10, followed by FITC-conjugated anti-rabbit IgG (green fluorescence) and anti-FLAG followed by rhodamine-conjugated anti-mouse IgG (red fluorescence). Overlay of the two channels generate a yellow overlapping fluorescence. B, HeLa cells untreated (left) or transfected with bcl10 (right), were stained with anti-TRADD antibody followed by FITC-conjugated anti-rabbit IgG. C, HeLa cells transfected with HA-tagged bcl10 were co-stained with anti-bcl10, followed by FITC-conjugated anti-mouse IgG (green fluorescence) and anti-Tradd, followed by rhodamine-conjugated anti-rabbit IgG (red fluorescence). Overlay of the two channels generate a yellow overlapping fluorescence. D, HeLa cells transiently transfected with bcl10 were stained with anti-RIP antibody, followed by FITC-conjugated anti-rabbit IgG.
vates NF-κB through a molecular pathway involved in tumor necrosis factor receptor 1 (TNF-R1) signaling (Costanzo et al., 1999; Koseki et al., 1999; Srinivasula et al., 1999). To determine whether bcl10 filaments served as scaffolds for recruitment of signal transduction molecules, we performed double-staining experiments of bcl10 together with antibodies against proteins involved in TNF-R1 signaling. Fig. 4A shows that TRADD, an adapter molecule that is recruited to TNF-R1 complex and signals NF-κB activation (Hsu et al., 1995), localizes on bcl10 filaments. To determine whether TRADD could be actively recruited on bcl10 filaments, HeLa cells were left untreated or transfected with bcl10 and localization of endogenous TRADD was detected with anti-TRADD antibody by confocal microscopy. The results in Fig. 4, B and C, show that whereas in untransfected cells, endogenous TRADD displays a diffuse cytoplasmic and nuclear distribution, expression of bcl10 results in translocation of cytoplasmic TRADD to bcl10 filaments. This observation is consistent with coprecipitation of TRADD with bcl10 (Costanzo et al., 1999), and implies that only filamentous organization of bcl10 allows recruitment of TRADD. The death domain-containing serine/threonine kinase RIP is essential for TNF-induced NF-κB signaling (Kelliher et al., 1998) and is recruited to TNF-R1 complex via interaction with TRADD (Hsu et al., 1996). Similarly to TRADD, whereas in untransfected cells endogenous RIP displays a diffuse cytoplasmic localization (data not shown), expression of bcl10 results in translocation of RIP to bcl10 filaments (Fig. 4D; and data not shown).

To assess whether cytoskeleton dynamics influence bcl10 activity, HeLa cells expressing bcl10 were treated with cytochalasin D. Figure 5. Cytochalasin D disassembles bcl10 filament and bcl10-induced NF-κB activation. HeLa cells were cultured in presence of cytochalasin D (20 μM) for the indicated time and bcl10 distribution (A) and NF-κB activation (B) were determined as described in Materials and Methods. Data presented in B is representative of three independent triplicate experiments.
Table I. Specificity of the Interaction between bcl10 and Cytoskeleton Proteins

| Protein fused to GAL4 domain | DNA-binding | Activating | β-Galactosidase filter assay | Liquid assay Miller units |
|-----------------------------|-------------|-----------|-----------------------------|--------------------------|
|                            |             |           |                             |                          |
| –                           | α-Actinin   | White     | 0.04 ± 0.02                 |                          |
| Vector                      | α-Actinin   | White     | 0.03 ± 0.01                 |                          |
| pLAM5                       | α-Actinin   | White     | 0.03 ± 0.01                 |                          |
| bc110                       | α-Actinin   | Blue      | 0.40 ± 0.03                 |                          |
| DR4                         | α-Actinin   | White     | 0.01 ± 0.01                 |                          |
| Flice                       | α-Actinin   | White     | 0.02 ± 0.02                 |                          |
| Fadd                        | α-Actinin   | White     | 0.01 ± 0.00                 |                          |
| bc110L1A1Q                  | α-Actinin   | Blue      | 0.32 ± 0.04                 |                          |
| bc110G78R                   | α-Actinin   | Blue      | 0.44 ± 0.02                 |                          |
| mut 6                       | α-Actinin   | Blue      | 0.36 ± 0.02                 |                          |
| mut 9                       | α-Actinin   | Blue      | 0.40 ± 0.80                 |                          |
| mut 11                      | α-Actinin   | Blue      | 0.24 ± 0.03                 |                          |
| bc110                       | –           | White     | 0.01 ± 0.00                 |                          |

HF7c cells were cotransformed with vectors encoding for the indicated polypeptides fused to GAL4 DNA-binding domain and GAL4 transcriptional activation domain. Filter and liquid assays for β-galactosidase activity are shown. Yeast colonies were scored as positive when a bright color developed within 2–5 h, a negative colony was scored when color failed to develop within 12 h. Assays were done for 5–10 independent transformants.

with cytochalasin D, a potent inhibitor of actin filament function, and bc110 localization was determined. As shown in Fig. 5, cytochalasin D disassembles bc110 filaments and specifically inhibits bc110-induced NF-κB activation, whereas NF-κB activation induced by TNF stimulation or NIK overexpression is not affected. Thus, assemblage of bc110 in filaments is essential for bc110-mediated NF-κB induction.

bc110 filament formation could either reflect an intrinsic property of that protein or be ordered via interactions with preexisting filamentous proteins. The observation that cytochalasin D disassembles bc110 filaments prompted us to explore the possibility these filaments might be organized by CARD-mediated association with cytoskeletal components. To test for this, we performed a two-hybrid screen fusing the C A R D of bc110 to the DNA binding domain of GAL4 and searched a plasmid library of fusion between the GAL4 transcription activation domain and cDNA s from peripheral blood leukocytes. 13 independent clones were isolated that activated the β-galactosidase reporter gene when ~2 × 10^6 transformants were analyzed. Restriction mapping and partial sequencing of these 13 cDNA s revealed that six positive clones had the same ~2-kb cDNA insert encoding for the polypeptide Pro^{355–Leu^{892}} of α-actinin, a member of the actin-binding proteins superfamily that is thought to cross-link actin filaments. As summarized in Table I, a library plasmid encoding for Pro^{355–Leu^{892}} of α-actinin did not activate β-galactosidase by itself, when coexpressed with the empty GAL4BD vector, or with unrelated control plasmids. Conversely, it strongly interacted with the C A R D of bc110. However, both wild-type and mutant forms of bc110 bind similarly to α-actinin in yeast and in vitro (Table I and Fig. 6 A). Together with cytochalasin D experiments, the two-hybrid data suggest that interaction of bc110 with cytoskeleton components is necessary, but not sufficient for filament formation.

Next, we tested whether filament formation could result from bc110 multimerization. Immunoprecipitation analysis shown in Fig. 6 B revealed that wild-type bc110 self-associates via a C A R D-mediated homophilic interaction, however, both mutants bc110(L41Q) and bc110(G 78R) did not dimerize, suggesting that bc110 filaments result from self-assembly. Thus, both dimerization of bc110 and binding to α-actinin are necessary for filament formation and NF-κB activation.

bc110 is expressed in many normal tissues and overexpression of the gene causes cellular transformation (Willis et al., 1999; Zhang et al., 1999). Therefore, we tested whether filamentous organization of bc110 and deregulated NF-κB activation both derive from deregulated level of expression of the protein. Indeed, in R a t-2 cells, endog-
Figure 7. Cellular localization of endogenous bcl10. A. Immunoblot analysis of protein lysate from Rat-2 cells. 10 μg of total cell lysate was resolved by SDS-PAGE and transferred onto nitrocellulose membrane probed with anti-bcl10 antisera, followed by HRP-conjugated secondary mAb b. B. Left, Confocal image of Rat-2 cells stained with anti-bcl10 antisera (Ab-1), followed by FITC-conjugated anti-rabbit IgG. Right, Rat-2 cells transfected with bcl101-127 were stained with anti-bcl10 (A b-1), followed by FITC-conjugated anti-rabbit IgG. C. Rat-2 cells were cotransfected with pNF-κB-luc, together with bcl101-127 or empty plasmid. Data represent relative luciferase activity and is representative of two independent triplicate experiments.

Cytoskeletal-like shape of bcl10 filaments and direct interaction of bcl10 with cytoskeletal proteins suggest that cytoskeleton dynamics may regulate bcl10 signaling. Indeed, activation of the NF-κB transcription factors is directly influenced by changes in the cytoskeleton network (Rosette and Karin, 1995). bcl10 filaments resemble the death-effector filaments, cytoplasmic filaments that sequester and activate procaspase-2 (Perez and White, 1998; Siegel et al., 1998), and similar structures are formed when the CARD of caspase-2 is overexpressed (Colussi et al., 1998). Generation of death-effector filaments is essential for activation of a signaling cascade leading to cellular apoptosis, and inhibition of filament formation prevents cell death. Thus, formation of ordered assembly of protein may represent a general mechanism for receptor-independent activation of specific signal transduction pathways.

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