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Cyclosporin-A inhibits ERK phosphorylation in B cells by modulating the binding of Raf protein to Bcl2

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Abstract

Extracellular signal-related kinase (ERK) signaling is regulated by sequential phosphorylation of upstream kinases including Raf. We report herein that ERK phosphorylation is inhibited by a short incubation with Cyclosporin-A (CsA) in anti-IgM activated Daudi B cells. As Bcl2, through its BH4 domain, was previously shown to bind both Calcineurin (Can) and Raf proteins, we hypothesized that CsA inhibited Can binding to Bcl2 allowing the latter to bind more Raf at the mitochondria thereby diverting it from activating the ERK cascade. In support of this less Bcl2 coprecipitated with Can heterodimer in total lysates of cells treated with CsA as compared to controls. In parallel, Raf1 was augmented in both the mitochondrial fractions of cells treated with CsA and in Bcl2 immunoprecipitates under CsA. Finally, introduction of a Bcl2 BH4 domain into Daudi cells augmented ERK phosphorylation in unstimulated cells and this augmentation was unsensitive to CsA. We therefore suggest that CsA indirectly inhibited ERK activation through sequestration of Raf1, at the mitochondria.

Keywords: Cyclosporin-A; ERK; Raf1; Bcl2; Mitochondria; B-lymphocytes

In B-lymphocytes, the B-cell receptor (BCR) promotes mitogenesis and immune responses by initiating a branching biochemical cascade, which induces gene expression by translocating transcription factors such as NF-AT to the nucleus [1–3], and by activating MAP kinases such as ERK and JNK that phosphorylate preexisting transcription factors [4]. ERK is activated through upstream sequential phosphorylation events involving Ras, Raf, and MEK proteins [5–7]. Although the ERK pathway is Ca^{2+}-independent [8], we have observed an inhibition of ERK phosphorylation in B-cells, under conditions that inhibited B-cell receptor (BCR) activation-induced early signaling events including Ca^{2+} peak [9]. The potential role of a Ca^{2+}-dependent phosphatase was investigated, and the effect of Cyclosporin-A (CsA) was evaluated on B-cell lines. A major target of CsA is the Ca^{2+}-dependent serine/threonine phosphatase Calcineurin [1], however CsA is also able to block the activation of MAPK pathways in T-cells [10], suggesting functional connections between ERK and Calcineurin (Can). Cross talk between Can and MAP kinases was also evidenced by the ability of Can to dephosphorylate the transcription factor Elk1 [11,12], which is normally phosphorylated by ERK and p38 MAP Kinase. If however Can played a role in ERK dephosphorylation, adding CsA to our cells would have reversed this phenomenon. Instead, we made the intriguing observation that CsA inhibited ERK phosphorylation in anti-IgM-activated Daudi B-cells, making very unlikely that the Can phosphatase activity was at the origin of ERK dephosphorylation. Another hypothesis was explored: given that Can and Raf were previously shown

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to bind to Bcl2 [13,14], we investigated whether CsA disrupted the balance between these two proteins for binding to Bcl2. Our results suggested that CsA displaced endogenous Can from Bcl2 allowing the latter to bind more Raf thereby diverting Raf from the MAPK cascade. The potential implications in B-cell pathology are discussed.

Materials and methods

Plasmid constructs. A cDNA encoding the N-terminal 1–50 aa sequence of Bcl2 BH4 domain was amplified by PCR using primers sense: 5′AACTCGAG**ATG**GCGCACGCTGGGAGA, antisense: 5′AAGAATT CGCTG**CTA**GGAGAAGAT in which ser 50 was replaced by a stop codon, and inserted at *Xho*I and *Eco*RI into PCI vector.

Selection of B-cell lines. The human B-cell lymphoma Daudi cells were electroporated (260 V, 960 μF) with the BH4-Bcl2 construct or with empty vector (20 μg plasmid/5×10^6 cells) and selected with 1 mg/ml G-418 (Geneticin, Invitrogen).

Immunoblots. Cells cultured or not with CsA (Calbiochem) for 40 min were washed and resuspended in 10 mM Hepes, pH 7.2, RPMI medium for 10 min at 37 °C, before stimulation with 2 μg/ml affinipure $F(ab')_2$ Rabbit polyclonal anti-human IgM (Jackson Immuno Research) for 5 min. For ERK studies, proteins were extracted and analyzed as previously described [9], Western blots were done with anti-ERK-2 MAb (D-2, Santa Cruz, CA). After chemiluminescent detection (ECL kit, Amersham Biosciences), membranes were then exposed to film (Agfa, Mortsel, Belgium), for less than 1 min. The same membranes were blotted with anti-NFAT-c1 MAb (7A6, Santa Cruz). For Raf1 immunoprecipitations, lysates in 1% Brij 96 lysis buffer were incubated for 2 h at 4 °C with anti-Raf1 rabbit polyclonal Ab-coated protein G–Sepharose beads (Sigma) and revealed with anti-Bcl2 MAb (C-2, Santa Cruz) and anti-Raf1 Rabbit polyclonal IgG Ab (C-12, Santa Cruz). For Can immunoprecipitations, lysates in Brij from cells incubated or not with various CsA concentrations were incubated with a mixture of goat polyclonal Ab anti-CanA (PP2B-Aβ) and anti-CanB (PP2B-B1), both from Santa Cruz, for 2 h at 4 °C, then with protein G–Sepharose beads (Sigma), and revealed with anti-Bcl2 MAb, or with Rabbit polyclonal anti-Can Aβ and CanBα (see below).

Mitochondrial fractions. Cells (6×10^7/condition) were prepared as described [13]. Homogenized samples were centrifuged at 500*g* for 5 min to discard nuclei, the resulting supernatant was centrifuged at 10,000*g* for 30 min at 4 °C to obtain the mitochondrial fraction (pellet) and the cytosolic plus membrane fraction (supernatant). Pellets were washed and resuspended in 1% Brij 96 lysis buffer. Samples were analyzed by SDS–PAGE and blotted with anti-Bcl2 or anti-Raf1 Ab, Rabbit polyclonal anti-CanAβ isoform (Upstate Biotechnology), Rabbit polyclonal anti-CanBα isoform (Affinity BioReagents, Golden CO) and Rabbit polyclonal anti-apoptosis-inducing factor (AIF) that recognizes a 57 kDa mitochondrial flavoprotein (Sigma), Rabbit polyclonal anti-BLCAM (H-221) and anti-PI3-kinase (p85α) MAb both from Santa Cruz. Secondary Abs were Goat anti-Mouse-peroxidase and Goat anti-Rabbit-peroxidase (Bio-Rad).

Results

CsA inhibits ERK2 phosphorylation in Daudi B-cells

We investigated the role of CsA on ERK activation in human Burkitt lymphoma-derived Daudi B cells, and analyzed the ratio between phosphorylated and non-phosphorylated ERK2 (P-ERK/ERK) using a single anti-ERK2 MAb that revealed the heavier phosphorylated ERK2, and the lighter band of unphosphorylated ERK2 on the same blot. Membranes were rehybridized with anti-NF-ATc1 MAb to demonstrate the efficacy of CsA. Cells were incubated

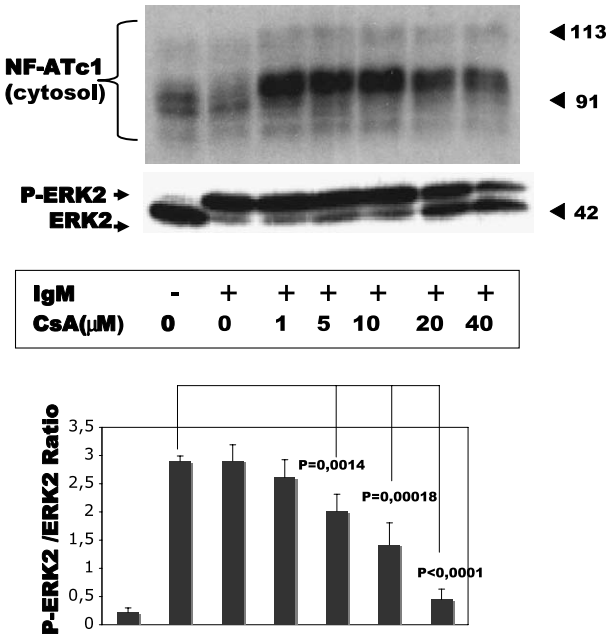

Fig. 1. Inhibition of ERK-2 phosphorylation in Daudi B cells by Cyclosporin A (CsA). Cells were cultured or not with graded concentrations of CsA for 40 min, washed, and stimulated or not with 2μg/ml $F(ab')_2$ Rabbit polyclonal anti-human IgM, for 5 min. Protein extracts (35μg) were immunoblotted with an anti-ERK2 MAb, revealing the 42 kDa unphosphorylated (ERK2) and the heavier, slow migrating phosphorylated form (P-ERK2) of ERK2 (arrows). The same membrane was also blotted with anti-NF-ATc1 MAb to evaluate the action of CsA on Calcineurin. Arrows: respective molecular weights. Histogram: means ± SD from five experiments. The ratio of phosphorylated (P-ERK2) over unphosphorylated (ERK2) was calculated by densitometric scanning of the bands (Scan Analysis Software Version 2.56 Biosoft). Student's *t* test was used to calculate *p* values by comparing the ratios of P-ERK2/ERK2 under a given CsA concentration with the same ratio under anti-IgM only without CsA (second histogram).

40 min with various concentrations of CsA before stimulation with anti-IgM. The inhibitory effect of CsA on anti-IgM-induced P-ERK2 started at 5 μM and was significant at 10 μM ($p = 0.0014$) and 20 μM CsA ($p = 0.00018$) (Fig. 1), whereas NF-AT dephosphorylation was already inhibited by much lower (1 μM) concentrations of CsA. The concomitant increase of NF-AT-c1 in the cytosolic extract from CsA-treated cells evidenced the retention of the phosphorylated form of NF-AT in the cytosol, and the efficacy of CsA as an inhibitor of the Can phosphatase activity. Of note, preincubation with CsA was non-toxic at doses up to 20 μM on Daudi cells as assessed by thymidine incorporation at day 3 of culture (data not shown). In summary, the inhibition of anti-IgM-induced ERK phosphorylation by CsA was dose dependent and was repeatedly superior to 50% at CsA concentrations of >20 μM in five independent experiments (histogram in Fig. 1).

CsA inhibits the association of Calcineurin heterodimer to Bcl2

Can phosphatase activity is not involved in the CsA effect on ERK since no effect is observed when Can is

practically completely inhibited (Fig. 1), pointing to an indirect effect of CsA on ERK. One possible explanation is that CsA displaced the Can heterodimer from Bcl2 allowing Raf1 to be sequestered by Bcl2 at the mitochondria [13]. Indeed, Bcl2 was previously shown to interact with both Raf1 and Can through its BH4 domain [13,14]. Cells were incubated or not with 2 or 20 μM CsA for 40 min as above, lysed and the Can heterodimer was immunoprecipitated using antibodies against catalytic A and regulatory B Can subunits. Immunoprecipitates were run on gels and blotted with distinct anti-Can Abs and with anti-Bcl2 Ab. As shown in Fig. 2, Bcl2 immunoprecipitated with Can as expected, however, the amounts of Bcl2 associated to Can were lower in the extracts from activated cells treated with CsA. Indeed, the association of Bcl2 to Can heterodimer was inversely related to the CsA concentration, as shown in a histogram representing the mean from

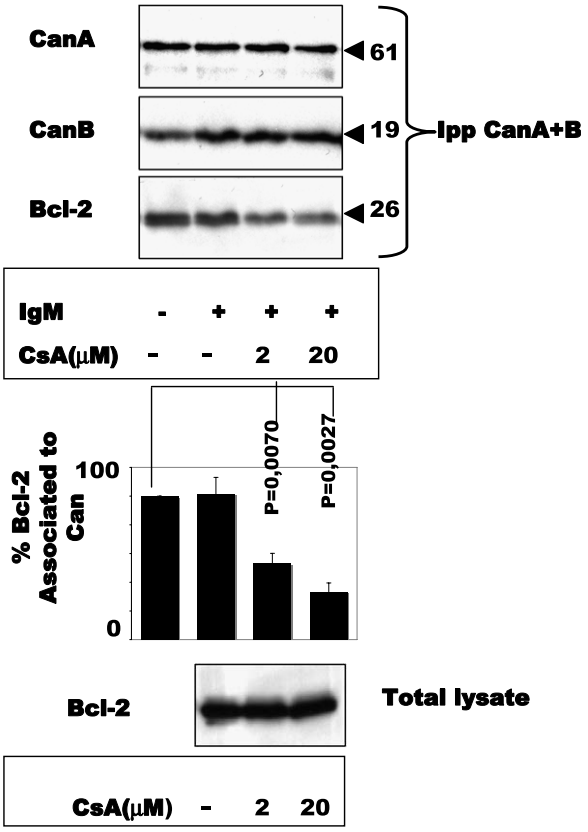

Fig. 2. CsA inhibits the association of Bcl2 to Calcineurin (CanAβBα) heterodimer. Daudi cells (15×10^6/condition) were incubated for 40 min with indicated concentrations of CsA with or without anti-IgM as in Fig. 1, and total proteins extracted and immunoprecipitated with a mixture of goat anti-CanAβ and Can-Bα Abs (ipp Can A + B) and submitted to electrophoresis. The gels were blotted and revealed with distinct Ab (from top to bottom: Rabbit anti-CanAβ, rabbit anti-CanBα, and mouse anti-Bcl2 Ab). Arrows: respective molecular weights. Histogram: means ± SD from three experiments. The percentage of Bcl2 associated to Can heterodimer was calculated by densitometric scanning, the unstimulated sample on the left considered arbitrary as 100%. (Bottom lane) Total lysates from Daudi cells incubated or not with CsA were electrophoresed, and blotted with anti-Bcl2 MAb, as controls of the Bcl2 content.

three distinct experiments. The pecentage of Bcl2 associated to Can was 55 ± 6.8 % ($p = 0.007$) and 34 ± 6.8 % ($p = 0.0027$) under 2 and 20 μM CsA, respectively. In contrast, the amounts of Can subunits were similar in all immunoprecipitates, and the amounts of Bcl2 were similar in control total cell lysates whether or not the cells have been incubated with CsA (Fig. 2, bottom). Thus, CsA specifically inhibited the association between Bcl2 and Can heterodimer in Daudi B-cells. We therefore investigated if, at variance with its effect on Can, CsA augmented the association of Raf to Bcl2.

CsA regulates ERK activation by modulating the mitochondrial localization of Raf1 and its association to Bcl2

We isolated the mitochondrial fractions from B cells and analyzed the expression of Bcl2, Can subunits and Raf1 proteins in these fractions. The purity of mitochondrial extracts was assessed by staining with anti-AIF (mitochondrial protein) antibody showing a positive and a negative pattern in mitochondrial and cytoplasmic fractions, respectively. Conversely, a contamination of mitochondrial extracts by proteins from membrane or cytosol was ruled out by respective hybridation with BLCAM (CD22) and PI3-kinase Abs (Fig. 3A). As expected, Bcl2 was highly enriched in the mitochondria as compared to cytosolic extracts and was not modified by anti-IgM or CsA (Fig. 3A). By contrast, Raf1 was undetectable in mitochondrial extracts from nonactivated cells, whereas it was detected at low level in anti-IgM- or CsA-treated cells. Raf1 was clearly augmented under CsA plus anti-IgM, whereas it was diminished in parallel in the cytosol, under the same condition. This suggested that Raf1 translocated from the cytosol to the mitochondria in activated cells preincubated with CsA. Note also that the mitochondria were highly enriched in CanAβ and CanBα (Fig. 3A). Finally the previously reported ability of Raf1 to bind directly Bcl2 [13] was confirmed in our cell system by immuno-precipitation of Raf1. As shown, Bcl2 was detected in Raf1 immunoprecipitates from Daudi cells, the amount of Bcl2 associated to Raf1 was very low in anti-IgM-stimulated cells but was strongly enhanced in extracts from CsA-treated cells whether or not the cells were stimulated with anti-IgM (Fig. 3B).

The role of Bcl2 in the regulation of ERK phosphorylation

Our results suggested that Bcl2 is central in the regulation of ERK phosphorylation by CsA. To further demonstrate the role of Bcl2 in ERK phosphorylation, a cDNA encoding the 1–50 aa N-terminal sequence of Bcl2, encompassing the BH4 domain, was transfected into Daudi cells in the hope that it would compete with endogenous Bcl2 for binding to Can and Raf1. Indeed, it has previously been demonstrated that the BH4 domain of Bcl2 binds to Raf1 and Can [13,14]. Three distinct clones were derived from our BH4 cell line and analyzed separately. The results were similar in the three

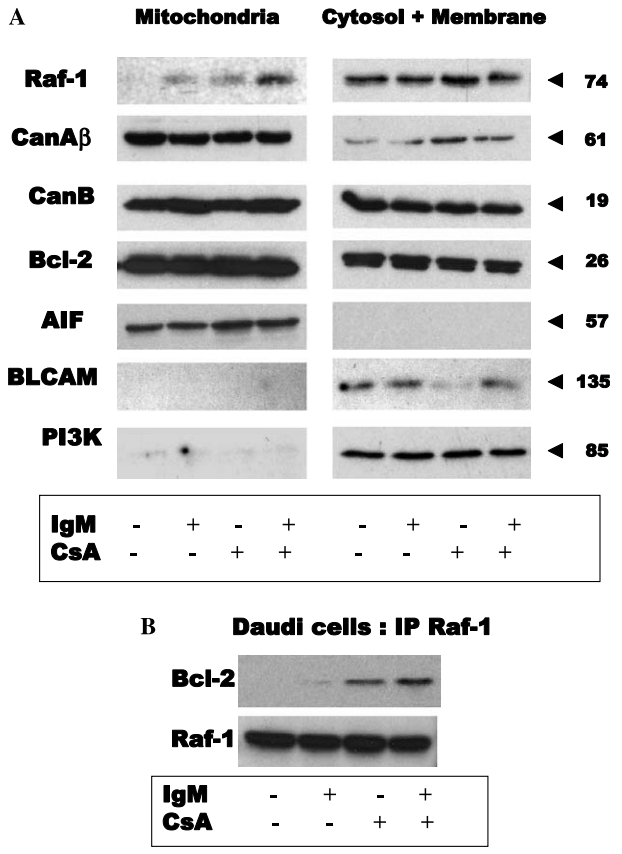

Fig. 3. CsA regulates the MAP kinase pathway by modulating Raf1 localization to the mitochondria. (A) CsA induces the translocation of Raf1 to the mitochondria. Cells were treated with or without anti-IgM after preincubation or not with 20 μM CsA, and the mitochondrial and cytosolic fractions were extracted. Both fractions (50 μg protein extract/sample) were run in parallel, hybridized together and after hybridization, membranes were exposed to film for the same time. For Western blots, membrane was sequentially incubated with anti-AIF (control mitochondrial marker), anti-Raf1, anti-Bcl2, anti-CanAβ, anti-CanBα, anti-BLCAM/CD22 (membrane marker), and anti-PI3-kinase (cytosolic marker) antibodies. (Left panel) Mitochondrial extracts (right panel) cytosolic plus membrane extracts. Arrows: respective molecular weights. (B) Raf1 coprecipitated with Bcl2 in CsA-treated, anti-IgM-activated cells. Extracts from cell lysates (15×10^6 cells/condition) were immunoprecipitated with anti-Raf1 Ab, submitted to electrophoresis, and revealed by anti-Raf1 and anti-Bcl2 Abs. Representative from three experiments.

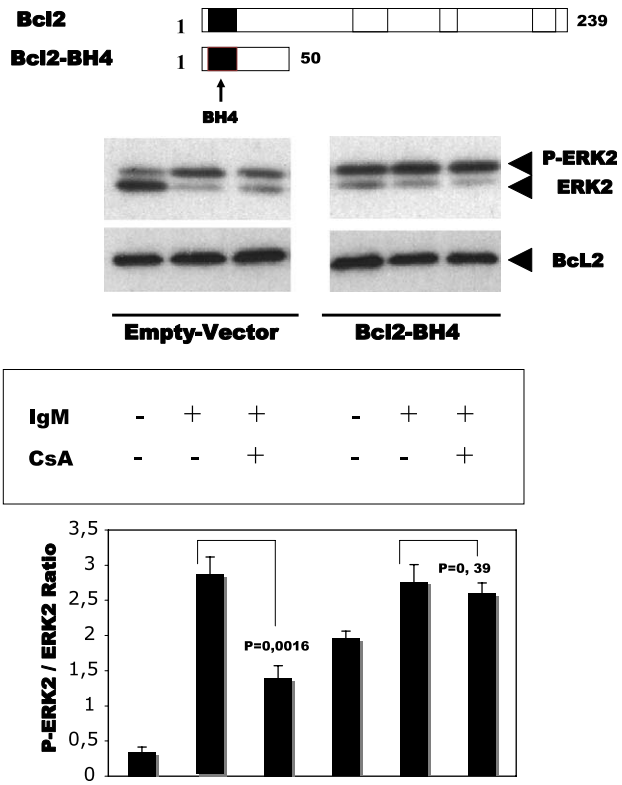

Fig. 4. Bcl2 regulates ERK phosphorylation. Daudi cells were transfected with empty vector or with the 1–50 amino acid sequence containing the BH4 domain of Bcl2, selected with geneticin, and cloned. Cells from each clone were incubated or not with 20 μM CsA, stimulated or not with anti-IgM, and analyzed for ERK activation. Shown is a representative Western blot with anti-Bcl2 and anti-ERK2 Abs. The ratio of phosphorylated (P-ERK2) over unphosphorylated (ERK2) was calculated as in Fig. 1. Representative of experiments from three clones. Histogram: mean ± SD of P-ERK/ERK ratios from one independent experiment with each clone.

clones, and the means ± SD from P-ERK/ERK are shown in Fig. 4. ERK2 was spontaneously augmented prior to any stimulation with anti-IgM in BH4-transfected cells in contrast to empty vector-transfected cells. P-ERK was strongly induced by anti-IgM in vector-transfected cells but only moderately in BH4-transfected cells. In parallel, CsA was inefficient on BH4-transfected cells as it was unable to inhibit ERK-phosphorylation, whereas it did inhibit P-ERK in vector-transfected cells, as expected from our previous experiments.

Discussion

We have demonstrated herein the ability of CsA to regulate the MAP kinase pathway in B cells in vivo and thereby provided a molecular explanation for this mechanism. The most likely primary event responsible for CsA-mediated ERK inhibition in Daudi cells is the sequestration of Raf1 at the mitochondria. It implies that mitochondrial Raf1 is unable to phosphorylate MEK and ERK. This is not totally surprising since although an active form of Raf1 was able to phosphorylate Bcl2 partners such as BAD when targeted to the mitochondria, it was unable to phosphorylate ERK unless targeted to the plasma membrane [13]. In support of this, Raf1, which is cytosolic, relocalized in part at the mitochondria upon treatment with CsA (Fig. 3A), which could not be attributed to contamination of mitochondrial preparations by cytosol or membrane as inferred from hybridization with anti-PI3 kinase and CD22 control Abs. In parallel, we have shown by immuno-precipitation of total cell extracts that Bcl2 associates to Raf1 only in CsA-treated cells (Fig. 3B). Our data therefore suggest that Raf1 associated (at least partly) to Bcl2 at the mitochondria after a short incubation with CsA, that was sufficient to inhibit ERK phosphorylation (Fig. 1). The essential role of Bcl2 in the regulation of ERK phosphorylation was also evidenced by the ability of a mini BH4-Bcl2 protein to render Daudi cells

unsensitive to CsA and to augment ERK activation in Daudi cells (Fig. 4). The amount of intracellular Bcl2 is probably central in CsA-mediated regulation of ERK activation, implying that cells expressing low Bcl2 levels should be insensitive to CsA in this respect. Indirectly supporting this is the report that CsA did not modulate ERK activation in splenic murine B cells [15], and our finding that CsA was unable to inhibit ERK activation in human blood B cells which express much less Bcl2 than Daudi cells (unpublished data).

Our results show that CsA inhibited the association of Bcl2 to Can heterodimer (Fig. 2) while augmenting the association of Raf1 to Bcl2 (Fig. 3) and inhibiting ERK phosphorylation (Fig. 1). The mechanism by which CsA enhanced the association of Bcl2 to Raf1 is more speculative. However, because Can is a natural target of CsA and given that Bcl2 binds Can and Raf1 through the same BH4 domain [13,14], one explanation could be that Can heterodimer and Raf1 compete for binding to Bcl2, and that CsA displaced endogenous Can from Bcl2 thus generating more binding sites for Raf1. Indirectly supporting our hypothesis is the finding that CsA modulated the association of Can heterodimer to Bcl2 in total cellular extracts (Fig. 2). It could be argued that there is no clear displacement of Can subunits from the mitochondria towards the cytoplasm under CsA (Fig. 3A). We think that due to the huge amount of mitochondrial Can, small variations cannot be visualized. In contrast, since the total amount of Raf1 is limiting, as compared to Can and Bcl2, as shown by us and others [13] it may be very sensitive to small variations of Bcl2-bound endogenous Can.

A 5- to 10-fold CsA was needed to inhibit ERK, than to inhibit NFAT dephosphorylation in Daudi B-cells (Fig. 1), suggests that Can has a greater affinity for Bcl2 than for NFAT. This may explain that the sequestration of Can by Bcl2 [14] resulted in the downregulation of NF-AT turn over and synthesis in Bcl2-transgenic mice [16]. Alternatively the mitochondria may be uneasy of access to CsA. Finally, the subcellular localization of Can subunit isoforms and the combinations of Can heterodimers [17] may also be important. We have studied CanAβ which is mostly mitochondrial in Daudi cells. However, Daudi cells also expressed another, CanAα isoform which is essentially cytosolic (data not shown). Thus, we cannot exclude that CsA is unable to regulate ERK in cells expressing predominantly cytosolic Can dimers.

What might be the relevance of this model to physiology? Sequestration of Can by Bcl2 was previously demonstrated in a T-cell line [19] but its effects of ERK were not evaluated. Interestingly, enforced expression of Bcl2 in B cells [18] elicited autoimmune disorders in autoimmune disease-prone mice [20]. Since on the other hand, a continuous signaling to ERK is needed to protect B cells from CpG DNA-induced autoimmunity [21], a diminished ERK signaling together with an augmented B cell survival may possibly contribute to autoimmunity in these Bcl2 transgenic mice. Finally, although CsA-mediated autoimmune disorders have long been attributed to the induction of imbalance within T-cell subpopulations [22,23], we suggest that a direct effect of CsA on B-cells might play a role in autoimmunity.

Acknowledgments

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