The anti-apoptotic molecules Bcl-x_L and Bcl-w target protein phosphatase 1α to Bad

Verónica Ayllón[1], Xavier Cayla[2], Alphonse García[3], Aarne Fleischer[1] and Angelita Rebollo[1]

[1] Centro Nacional de Biotenología, Department of Immunology and Oncology, Campus de Cantoblanco, UAM, Madrid, Spain
[2] Laboratoire de Physiologie de la Réproduction, ESA CNRS 7080-INRA, Paris, France
[3] Institut Pasteur, Département d'Immunologie, Laboratoire de Signalisation Immunoparasitaire, Paris, France

Bcl-x_L and Bcl-w specifically interact with PP1α and Bad. A phosphatase activity sensitive to okadaic acid was detected in Bcl-x_L, Bcl-w and Bad immunoprecipitates. Serine phosphorylation of Bcl-x_L and Bcl-w correlates with the number of trimolecular complexes formed. Depletion of Bcl-x_L and Bcl-w decreases the remaining Bad-associated phosphatase activity and association of protein phosphatase 1 (PP1)α to Bad. Bcl-x_L and Bcl-w contain the R/K X V/I X F consensus motif shared by PP1 targeting subunits. This motif, in addition to F X X R X R motif, is involved in binding of Bcl-x_L and Bcl-w to PP1α. Disruption of Bcl-x_L/PP1α or Bcl-w/PP1α association strongly decreases Bad-associated phosphatase activity and stability of trimolecular complexes. These results suggest that Bcl-x_L and Bcl-w are PP1α targeting subunits and this trimolecular complex may be involved in the control of apoptosis.

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1 Introduction

The Bcl-2 family of proteins is divided into two functional groups: anti-apoptotic members such as Bcl-2, Bcl-x_L, Bcl-w, A1 and Mcl-1 and pro-apoptotic members such as Bax, Bak, Bcl-x_S as well as the BH3-only member Bad [1–3]. Balance between homo- and heterodimers of Bcl-2 family members may be critical to maintain cell proliferation or apoptosis [4–6]. Up- or down-regulation of these proteins may account for survival of some cell types, although it is also possible that survival factors use protein kinases or phosphatases to alter the ability of these proteins to promote cell survival or apoptosis. Anti-apoptotic Bcl-2 family members interact with other death agonist of the Bcl-2 family and with non-Bcl-2 family proteins, including R-Ras, H-Ras, Raf, caspases, calcineurin and the serine/threonine phosphatase PP1α [7, 8].

Bad shares identity only in the BH3 domain [9] and forms heterodimers with Bcl-2 and Bcl-x [10]. Upon stimulation of cells with IL-3, NGF and GM-CSF, Bad becomes serine phosphorylated [11, 12], resulting in association to the 14-3-3 protein and abolishing interaction with Bcl-x [13]. It has been recently shown that association of 14-3-3 protein to Bad is dependent on serine 155 phosphorylation of Bad [14, 15].

Bcl-w is a pro-survival protein bearing the four conserved Bcl-2-homology (BH) domains [16]. Enforced expression of Bcl-w, like Bcl-2, renders lymphoid and myeloid cell lines resistant to apoptosis induced by cytokine deprivation. The anti-apoptotic molecule Bcl-x_L also contains the four BH conserved domains [17]. A second Bcl-x isoform, Bcl-x_S, encodes a smaller protein of 170 amino acids that enhances apoptosis [18]. Bcl-x_L contains a hydrophobic segment at the C-terminal end that is believed to serve as a membrane anchor [19].

The serine/threonine phosphatases are classified as type 1 (PP1) or type 2 (PP2), depending on their substrate specificity and sensitivity to inhibitors. PP1 regulates cell cycle progression, proliferation, protein synthesis, cytokinesis and neuronal signaling [20]. PP1 is composed of a catalytic subunit and a wide variety of regulatory or anchoring proteins involved in targeting as well as in controlling phosphatase activity [21]. We have recently shown that Bcl-2 is a new targeting subunit of PP1α that controls its association to Bad in IL-2-stimulated cells [8]. In this study, we report that the anti-apoptotic members of the Bcl-2 family, Bcl-w and Bcl-x_L are also target-

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Abbreviations: OA: Okadaic acid **BH:** Bcl-2-homology **PP1/PP2:** Protein phosphatase of type 1or 2

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ing subunits of PP1α in IL-4-stimulated cells. The contribution of the trimolecular complex to the control of apoptosis is discussed.

2 Results

2.1 Identification of Bcl-w and Bcl-x_L as PP1α-interacting proteins

We have previously shown that anti-apoptotic molecule Bcl-2 is a targeting subunit of the serine/threonine phosphatase PP1α in IL-2-stimulated TS1αβ cells and that the sequence of Bcl-2 interacting with PP1α is the R/K X V/I X F motif [8]. Given that Bcl-x_L and Bcl-w also contain the well-conserved R/K X V/I X F motif observed in Bcl-2, we explored the possibility that anti-apoptotic molecules Bcl-x_L and Bcl-w may be as well associated to PP1α in IL-4-stimulated TS1αβ cells, which do not express Bcl-2 but express Bcl-x_L and Bcl-w. We performed reciprocal co-immunoprecipitation experiments of cytoplasmic proteins under IL-4-stimulation or -deprivation conditions using specific antibodies. PP1α and Bad were detected by Western blot in anti-Bcl-x_L immunoprecipitates of IL-4-stimulated cells, decreasing throughout the starvation period (Fig. 1A). Probing the membrane with anti-Bcl-x_L antibody showed similar levels in all conditions analyzed. PP1α and Bad were also detected in anti-Bcl-w immunoprecipitates of IL-4-stimulated cells, diminishing after lymphokine deprivation (Fig. 1A). Membrane was also probed with anti-Bcl-w antibody, showing similar levels. Immunoprecipitation for cytoplasmic lysates with an irrelevant antibody, anti-p55 IL-2R chain, was not able to detect those associations (Fig. 1B). Similarly, Bcl-x_L, Bcl-w and PP1α were detected in Bad immunoprecipitates and the interaction among these proteins was also observed by immunoprecipitation of detergent-free lysates as well as in cytoplasmic proteins isolated by digitonin lysis (data not shown). These associations were also observed in freshly isolated thymocytes (Fig. 1C). Given that the number of Bcl-x_L/PP1α/Bad and Bcl-w/PP1α/Bad complexes decreases after IL-4-deprivation, we analyzed whether down-regulation of the expression of any of the proteins involved in the formation of the complex may explain the result. We analyze total expression of Bcl-x_L, Bcl-w, PP1α and Bad in IL-4-stimulated or -deprived TS1αβ cells. All analyzed proteins were expressed in IL-4-stimulated or -deprived cells (Fig. 2A). As the number of aggregates decreases after IL-4-deprivation without modification of total expression of the proteins of the complex, we analyzed whether post-translational modifications of Bcl-x_L or Bcl-w may affect the formation of the trimolecular complex. We analyzed the status of serine phosphorylation of Bcl-x_L and Bcl-w. Cytoplasmic extracts from IL-4-stimulated

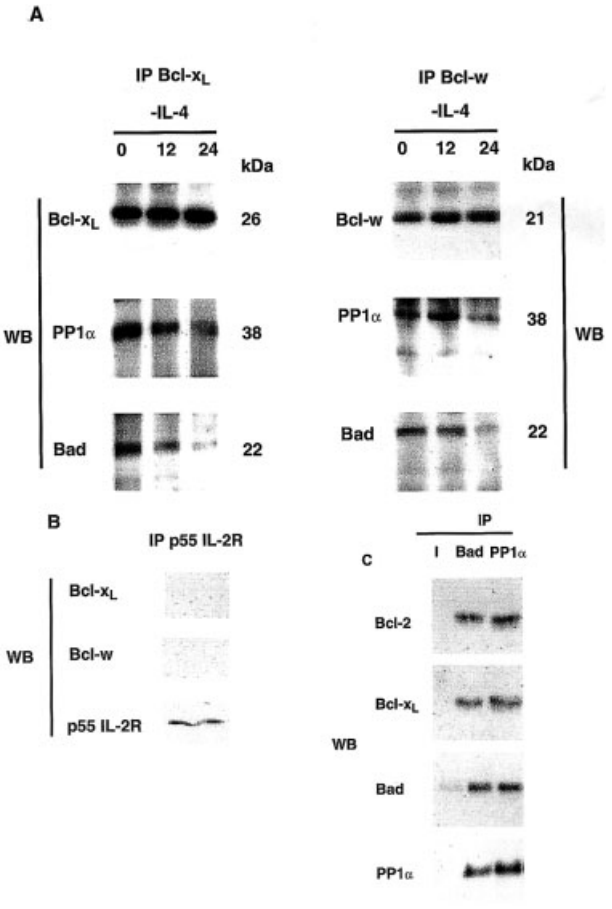

Fig. 1. Association of Bcl-x_L and Bcl-w to PP1α and Bad. (A) Cytoplasmic extracts from IL-4-stimulated (60 U/ml) or -deprived cells were immunoprecipitated with anti-Bcl-x_L or anti-Bcl-w antibody and blotted with anti-Bad, anti-PP1α, anti-Bcl-x_L and anti-Bcl-w antibodies. Similar results were obtained in three independent experiments. (B) Cytoplasmic extracts from IL-4-stimulated cells were immunoprecipitated with anti-p55 IL-2R chain antibody and blotted with anti-Bcl-w, Bcl-x_L or anti-p55 IL-2R antibodies. (C) Cytoplasmic extracts from freshly isolated thymocytes were immunoprecipitated with anti-Bad, anti-PP1α or an irrelevant serum and blotted with anti-Bcl-2, anti-Bcl-x_L, anti-Bad and anti-PP1α. Similar results were obtained in three independent experiments.

or -deprived cells were immunoprecipitated with anti-Bcl-x_L or anti-Bcl-w and blotted with anti-Pser, anti-PP1α, anti-Bad, anti-Bcl-w and anti-Bcl-x_L-specific antibodies (Fig. 2B). Serine phosphorylation of Bcl-x_L and Bcl-w was observed in control IL-4-stimulated cells, decreasing after IL-4 deprivation. This result suggests a correlation between serine phosphorylation of Bcl-x_L and Bcl-w and formation of trimolecular complexes.

We have recently shown that IL-2, as well as IL-3, induces serine phosphorylation of Bad [22]. Fig. 2C

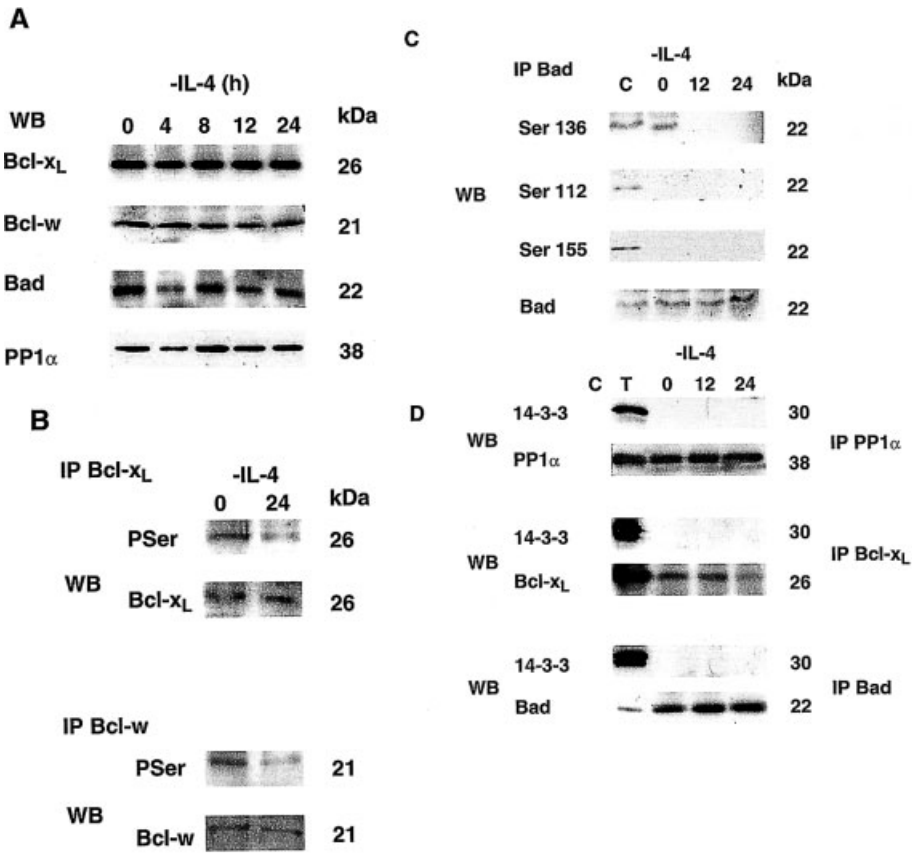

Fig. 2. Effect of IL-4 deprivation in serine phosphorylation of Bcl-x_L and Bcl-w and association to 14-3-3 protein. (A) TS1$\alpha\beta$ cells were IL-4 stimulated or deprived for the times indicated, then lysed. Proteins were probed with anti-Bcl-x_L, anti-Bcl-w, anti-Bad and anti-PP1α. antibodies. Similar results were obtained in two independent experiments. (B) Cytoplasmic extracts from IL-4-stimulated or 24 h-deprived cells were immunoprecipitated with anti-Bcl-x_L or anti-Bcl-w and blotted with anti-Pser and, as internal control, with anti-Bcl-x_L and anti-Bcl-w. Similar results were obtained in three independent experiments. (C) Cytoplasmic extracts from control, IL-4-stimulated or deprived cells (1×10^7) were immunoprecipitated with anti-Bad antibody and blotted with anti-Bad Ser 112, Ser 136 and Ser 155. Similar results were obtained in two independent experiments. Positive control for Ser 112 and 136 phosphorylation of Bad, IL-2- stimulated cells (lane C); positive control for Ser 155 phosphorylation of Bad, Bad-transfected COS cells (lane C). (D) Total extracts (T) or cytoplasmic lysates from IL-4-stimulated or -deprived cells (1×10^7) were immunoprecipitated with anti-PP1α, anti-Bcl-x_L or anti-Bad antibody and blotted with anti-14-3-3, anti-PP1α, anti-Bcl-x_L and anti-Bad antibodies. Similar results were obtained in two independent experiments.

shows that IL-4 induces Bad phosphorylation at serine 136 but not at serines 112 and 155. Moreover, IL-4 deprivation induces serine 136 dephosphorylation of Bad. IL-2-stimulated cells (C, Ser 112 and 136 phosphorylation) or COS (C, Ser 155 phosphorylation) cells overexpressing Bad were used as a positive controls. IL-3-induced serine phosphorylation of Bad results in its association to the 14-3-3 protein, abolishing interaction with Bcl-x [15]. Fig. 2D shows that serine phosphorylation of Bad in response to IL-4 does not result in binding to 14-3-3 protein. This protein was detected in total extracts from control IL-4-stimulated cells (lane T) and was neither observed in PP1α nor in Bcl-x_L or Bad immunoprecipitates of IL-4-stimulated or -deprived cells.

Fig. 3A shows phosphatase activity in Bcl-x_L, Bcl-w and Bad immunoprecipitates of IL-4-stimulated cells. The enzymatic activity in the immunoprecipitates was measured using ^{32}P-labeled phosphorylase *a* as substrate. It is interesting to notice that phosphatase activity detected in Bcl-x_L and Bcl-w immunoprecipitates nearly corresponds to the phosphatase activity observed in Bad immunoprecipitates. To confirm that this phosphatase activity was due to PP1α, enzymatic activity was estimated in Bad or Bcl-w immunoprecipitates from IL-4-stimulated cells in the presence of different okadaic acid (OA) concentrations (Fig. 3B). OA concentrations that inhibit type 2A activity (10^{-9} M) had no effect on Bad- or Bcl-w-associated phosphatase activity *in vitro*. Addi-

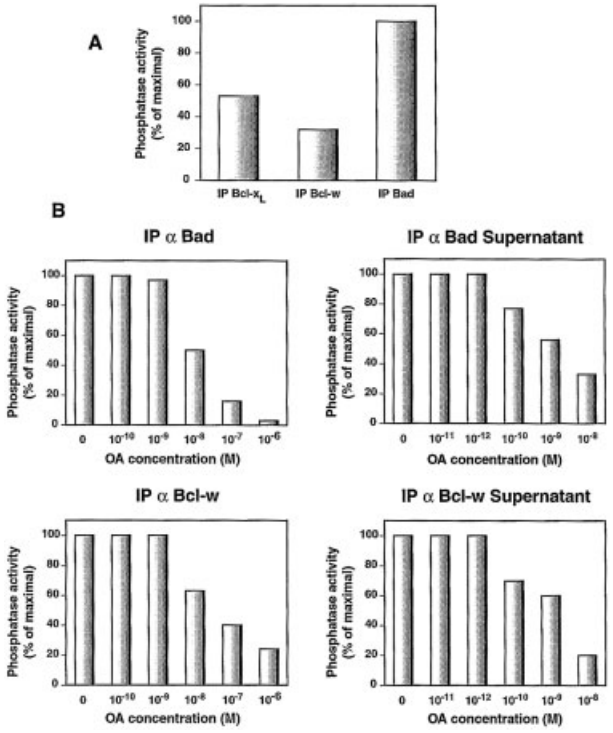

Fig. 3. Estimation of serine/threonine phosphatase activity in control or OA-treated Bcl-x, Bad and Bcl-w immunoprecipitates. (A) Phosphatase activity was estimated in Bad, Bcl-x_L and Bcl-w immunoprecipitates from IL-4-stimulated cells using [^{32}P]phosphorylase *a* as substrate. (B) OA at different concentrations was added to Bad or Bcl-w immunoprecipitates from IL-4-stimulated cells. Phosphatase activity was estimated as in (A). Similar results were obtained in three independent experiments. Phosphatase activity is represented as the percentage of maximal activity in untreated supernatants.

tion of 10^{-8} M of OA to Bcl-w or Bad immunoprecipitates results in ~50% inhibition of phosphatase activity, which is strongly reduced after addition of 10^{-6} M of OA (Fig. 3B). The effect of OA on phosphatase activity was also estimated in supernatants of Bad and Bcl-w immunoprecipitates. OA concentrations that had no effect on enzymatic activity in Bad and Bcl-w immunoprecipitates (10^{-9} M) show ~50% inhibition in the supernatant, as expected from an association of type 1 and type 2A activities (Fig. 3B). The selective effect of OA suggests that the phosphatase activity observed in Bad and Bcl-w immunoprecipitates is PP1α.

2.2 Bcl-w and Bcl-x_L are new targeting subunits of PP1α

We have recently shown that Bcl-2 is a targeting subunit of PP1α [8]. Given that Bcl-w and Bcl-x_L are also associated to PP1α and that sequence of binding site of Bcl-2 to PP1α is conserved in Bcl-x_L and Bcl-w, we hypothesized that these anti-apoptotic molecules may be new targeting subunits of PP1α. To test this hypothesis, we depleted Bcl-x_L and Bcl-w by sequential anti-Bcl-x_L+Bcl-w immunoprecipitation of cytoplasmic extracts of IL-4-stimulated cells. Supernatant from the fourth anti-Bcl-x_L+Bcl-w immunoprecipitation was immunoprecipitated with anti-Bad antibody (5th) and phosphatase activity estimated (Fig. 4A). Traces of Bad-associated phospha-

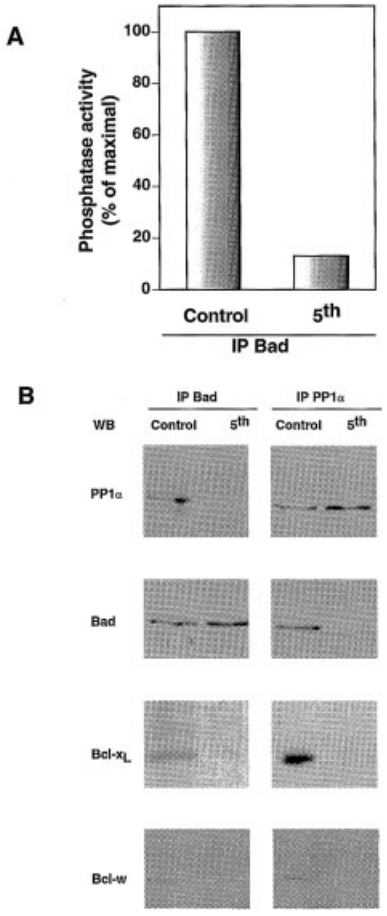

Fig. 4. Estimation of serine/threonine phosphatase activity after Bcl-x_L and Bcl-w depletion. (A) Bcl-x_L and Bcl-w were depleted from cytoplasmic lysates of IL-4-stimulated cells by four sequential immunoprecipitations. Phosphatase activity was estimated in Bad immunoprecipitates from control IL-4-stimulated cells or in Bad immunoprecipitates depleted of Bcl-x_L and Bcl-w. (B) The effect of Bcl-x_L and Bcl-w depletion in PP1α/Bad association was analyzed. Cytoplasmic extracts from control IL-4-stimulated cells or Bcl-x_L, and Bcl-w depleted cytoplasmic extracts were immunoprecipitated with anti-Bad or anti-PP1α antibody and blotted with anti-Bad, anti-Bcl-w, anti-Bcl-x_L and anti-PP1α. Similar results were obtained in three independent experiments.

tase activity were detected in Bcl-x_L and Bcl-w depleted extracts compared to the high level of activity observed in control anti-Bad immunoprecipitates of IL-4-stimulated cells. Given that in the absence of Bcl-x_L and Bcl-w we do not detect significant Bad-associated phosphatase activity, we explored the possibility that these anti-apoptotic molecules may control targeting of PP1α to Bad. For this purpose, anti-Bad immunoprecipitations were made in cytoplasmic extracts of IL-4-stimulated cells or in extracts depleted of Bcl-x_L and Bcl-w (5th). PP1α, Bcl-x_L and Bcl-w were detected in control anti-Bad immunoprecipitates and were not observed in anti-Bad immunoprecipitates from extracts depleted of Bcl-x_L and Bcl-w (Fig. 4B). In reciprocal experiment, Bad, Bcl-x_L and Bcl-w were detected in anti-PP1α immunoprecipitates of control cells and were not observed in PP1α immnunoprecipitates from extracts depleted of Bcl-x_L and Bcl-w (Fig. 4B). This result suggests that Bcl-x_L and Bcl-w are needed for association of PP1α to Bad.

2.3 Determination of Bcl-x_L and Bcl-w-binding site to PP1α

It has been described that R/K X V/I X F motif is shared by most of the PP1α targeting subunits [21, 23]. We have shown that PP1α targeting subunit Bcl-2 also shares this conserved motif [8]. Interestingly, Bcl-x_L and Bcl-w sequences also contain this motif (Fig. 5B). To analyze whether this sequence of Bcl-x_L and Bcl-w was involved in binding to PP1α, we generated nitrocellulose-immobilized peptides of Bcl-x_L and Bcl-w protein containing this motif. Membrane was incubated with purified PP1α protein. Fig. 5B shows the sequences interacting with PP1α. The R/K X V/I X F motif, present in Bcl-x_L and Bcl-w, interacts with PP1α and its mutation in critical V and F residues strongly reduces binding of Bcl-x_L and Bcl-w to PP1α (Fig. 5B). Analysis of the Bcl-2-binding sites to PP1α showed, in addition to R/K X V/I X F motif, two sequences (FSRRYR and FTARGR), that bind PP1α [8]. Interestingly, similar sequences were as well observed in Bcl-x_L and Bcl-w (Fig. 5A). FELRYR and FETRFR sequences of Bcl-x_L and Bcl-w, respectively, also interact with PP1α and their mutations inhibit binding to PP1α, although the affinity depends on the type of point mutation (Fig. 5B). The interacting consensus F X X R X R motif was determined by sequence comparison of Bcl-2, Bcl-x_L and Bcl-w.

To conclusively confirm that R/K X V/I X F and F X X R X R motifs are involved in binding of Bcl-x_L and Bcl-w to PP1α, we performed competition experiments in trimolecular complexes. Lysates from IL-4-stimulated cells were immunoprecipitated with anti-Bad antibody and the interaction Bcl-x_L/PP1α and Bcl-w/PP1α was competed using R* (NWG**RIAAA**FSF), R (NWG**RIVAF**FSF) or

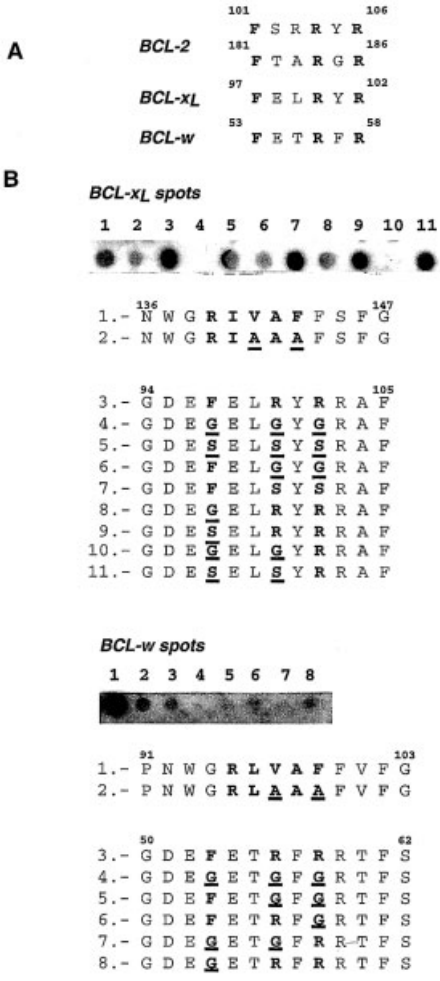

Fig. 5. PP1α binding assay on cellulose-bound Bcl-x_L or Bcl-w peptides. (A) Sequence of F X X R X R motif of Bcl-2, Bcl-x_L and Bcl-w. (B) Membrane with Bcl-x_L or Bcl-w peptides containing the R/K X V/I X F or F X X R X R motif, as well as peptides containing mutated motifs were incubated with purified PP1α, followed by anti-PP1α antibody and PO-conjugated secondary antibody. The R/K X V/I X F and F X X R X R motifs are in bold. Mutated amino acids into the motif are in bold and underlined. Similar results were obtained in two independent experiments. Peptide 1 corresponds to the PP1-binding motif of Bcl-x_L and Bcl-w. Peptide 2 corresponds to the mutated PP1-binding site where V and F residues were mutated to A. Similar results were obtained in three independent experiments.

F (GDE**FELRYR**RAF) peptides (Fig. 6A). Bcl-x_L, Bcl-w and PP1α were detected in control anti-Bad immunoprecipitates, as well as in anti-Bad immunoprecipitates treated with R* peptide. The amount of Bcl-x_L, Bcl-w and PP1α associated to Bad decreases after competition with F or R peptide, being almost undetectable upon competition of Bad immunoprecipitates with F+R peptides (Fig. 6A). Similar level of Bad is observed in control

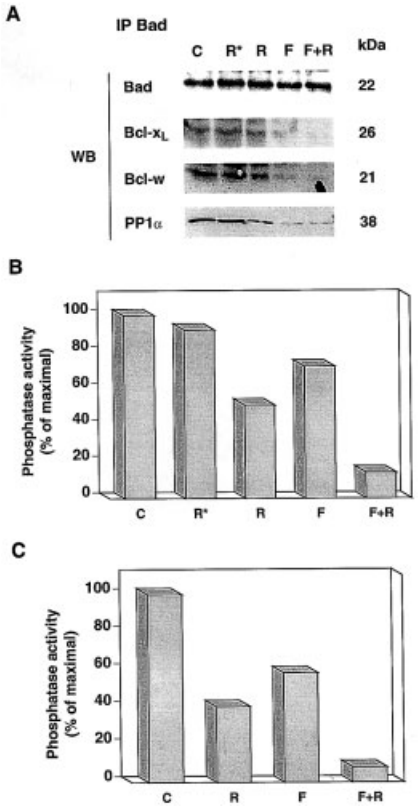

Fig. 6. Effect of R, R*, F and R+F peptides on the interaction Bcl-x_L/PP1α/Bad and Bcl-w/PP1α/Bad. (A) Cytoplasmic extracts from control IL-4-stimulated cells were immunoprecipitated with anti-Bad antibody. The interaction Bcl-x_L/PP1α/Bad and Bcl-w/PP1α/Bad was competed with 1.5 mM of R, R*, F or R+F peptides for 30 min at room temperature. Immunoprecipitates were blotted with anti-Bad, anti-PP1α, anti-Bcl-x_L and anti-Bcl-w. Similar results were obtained in two independent experiments. (B) Cytoplasmic lysates from IL-4-stimulated cells were immunoprecipitated with anti-Bad antibody. Immunoprecipitates were treated with 1.5 mM of R, R*, F or R+F peptides for 30 min at room temperature. Phosphatase activity in immunoprecipitates was estimated using [^{32}P] phosphorylase *a* as substrate. Similar results were obtained in two independent experiments. (C) Cytoplasmic lysates from IL-4-stimulated cells were immunoprecipitated with anti-Bad antibody and then treated with 1.5 mM of R+F peptide or 3 mM of R or F peptide (30 min, room temperature). Phosphatase activity estimated as in (B).

or peptide-treated anti-Bad immunoprecipitates. Finally, to confirm that Bcl-x_L and Bcl-w are targeting subunits of PP1α, we estimated phosphatase activity in control or peptide-treated Bad immunoprecipitates. Phosphatase activity was detected in control or R* peptide-treated immunoprecipitates (Fig. 6B), decreasing upon competition of the interaction with F or R peptides. Enzymatic activity was strongly decreased upon competition with R and F peptides. Fig. 6C shows the phosphatase activity in control or peptide-treated Bad immunoprecipitates. The concentration of F or R peptide used was twice the concentration used in F+R peptide-treated immunoprecipitates. As in Fig. 6B, phosphatase activity was drastically reduced upon treatment of Bad immunoprecipitates with F+R peptides. Taken together, these results suggest that Bcl-x_L and Bcl-w, as well as Bcl-2, are PP1α targeting subunits.

2.4 Inhibition of PP1α enzymatic activity blocks apoptosis

As IL-4 deprivation correlates with Bad dephosphorylation and apoptosis, we hypothesized that inhibition of phosphatase activity by OA treatment may prevent Bad dephosphorylation and apoptosis. Treatment of the cells with 1 μM of OA in the absence of IL-4 prevented Bad dephosphorylation at Ser 136 (Fig. 7A). No changes in total Bad expression were observed after OA treatment, suggesting that OA does not affect protein expression. In addition, IL-4-deprived cells treated with 1 μM of OA for 6 h showed significant reduction in the fraction of apoptotic cells compared with untreated cells (Fig. 7B). Finally, inhibition of Bcl-x_L and Bcl-w expression by antisense oligonucleotide treatment of cells also induces apoptosis in IL-4-stimulated cells (Fig. 7C). The inhibition of Bcl-x_L and Bcl-w expression upon antisense oligonucleotide treatment was estimated by Western blot.

3 Discussion

We have previously shown that Bcl-2 interacts with PP1α and Bad [8]. The interaction Bcl-2/PP1α requires the well-conserved R/K X V/I X F motif. This motif fits with the consensus sequence previously shown to mediate interaction of most of targeting subunits with PP1α. In this manuscript we show that Bcl-x_L and Bcl-w also interact with PP1α and Bad in TS1$\alpha\beta$ cells as well as in freshly isolated thymocytes. It is interesting to know that the level of trimolecular complexes is reduced in IL-4-deprived cells, as compared to control cells, although total expression of these proteins is not affected by IL-4 deprivation. Since targeting molecules Bcl-x_L or Bcl-w are not the limiting components in the formation of the trimolecular complex, our results suggest post-translational modifications of any of the molecules that may be involved in aggregates formation. Our data suggest a correlation between serine phosphorylation of Bcl-x_L and Bcl-w and formation of trimolecular complexes. In agreement, it has been shown that Bcl-2 is post-translationally modified by phosphorylation on a conserved serine (Ser 70) and that IL-3 induces and increases phosphorylation

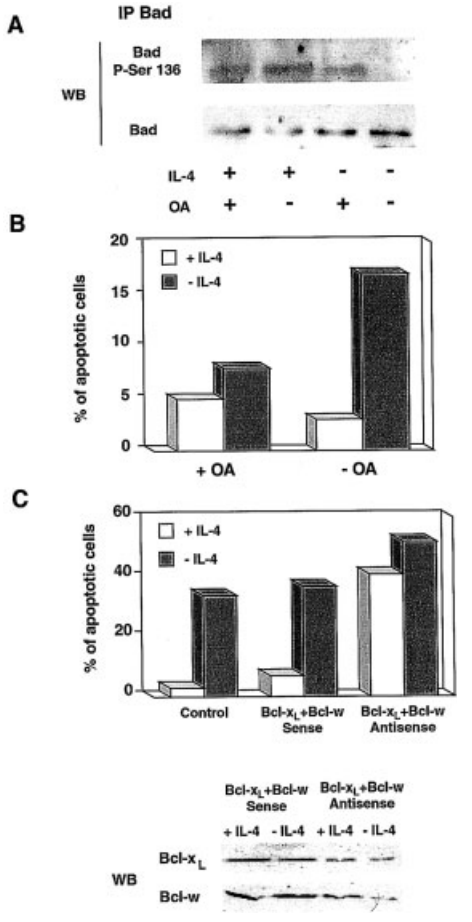

Fig. 7. Effect of OA and antisense oligonucleotides on apoptosis and Ser 136 phosphorylation of Bad. (A) Cells were treated with or without 1 μM OA in the presence or the absence of IL-4. Cytoplasmic lysates were immunoprecipitated with anti-Bad antibody and probed with phospho-Bad Ser 136 and anti-Bad, the latter to verify that OA treatment *in vivo* does not affect Bad expression. Similar results were obtained in three independent experiments. (B) Cells were treated for 6 h as in (A), then washed, stained with propidium iodide and analyzed by flow cytometry. Similar results were obtained in three independent experiments. (C) Cells were treated for 24 h with or without 15 M sense or antisense oligonucleotides in the presence or absence of IL-4. Oligonucleotides were added at 0, 12 and 18 h and then cells were treated as in (B). The expression of Bcl-x_L and Bcl-w upon sense and antisense treatment was analyzed by Western blot. Similar results were obtained in two independent experiments.

at this site [24], which then undergoes incomplete dephosphorylation [25]. This would presumably render Bcl-2 non-functional. According to recent structural analysis of Bcl-x_L, the corresponding Ser 70 would be located within a flexible loop domain and phosphorylation in this loop may change the affinity for partner proteins.

It has been published that serine phosphorylation of Bad in response to IL-3 results in binding to 14-3-3 protein, avoiding heterodimerization with Bcl-x [12]. We have shown that serine 112 and 136 phosphorylation of Bad is also induced by IL-2 [8]. Interestingly, IL-4 induces only serine 136 phosphorylation of Bad. Our results display that neither phosphorylated, nor dephosphorylated Bad are associated to 14-3-3 protein, showing that Bad can interact with Bcl-x_L or Bcl-w upon IL-4 stimulation or deprivation, as it is not sequestered by 14-3-3. These findings are in agreement with recent publications showing that association of Bad to 14-3-3 is dependent on serine 155 phosphorylation of Bad [14, 15].

The finding that anti-apoptotic molecules Bcl-x_L or Bcl-w form a complex with PP1α and Bad implies that recognition sites for binding are different. Binding of one of the anti-apoptotic molecules to PP1α excludes binding of the others, explaining the presence of two types of trimolecular complexes, Bcl-x_L/PP1α/Bad and Bcl-w/PP1α/Bad. It is tempting to speculate that these motifs are involved in binding and stabilization of the association Bcl-x_L/PP1α/Bad or Bcl-w/PP1α/Bad. Accordingly, competition of binding using only one of the peptides partially abolishes the association while competition of association using R+F peptides, which are on the BH1 and BH3 domain of the protein, entirely removes association and phosphatase activity. Mutation of V and F residues to A within R/K X V/I X F motif dramatically inhibits binding to PP1α. Our results are in agreement with previous publications showing that substitution of critical residues valine and phenylalanine of R/K X V/I X F motif to alanine disrupts binding of targeting subunits to PP1 [23]. Similarly, mutation of the conserved R residues to G within F X X R X R motif strongly blocks binding to PP1α, while mutation to S residues slightly inhibits binding.

The finding that R or F peptide only partially inhibit association of Bcl-x_L or Bcl-w to PP1α suggests that, at least, both motifs are critical for interaction with PP1α. We do not exclude that Bcl-x_L and Bcl-w may have other sites for interaction with PP1α. This is in agreement with recent results showing that some regulatory proteins have multiple sites of interaction with PP1 [26]. It has also been speculated that hormonal or growth factor regulation of PP1 may involve a control of the number and identity of interaction sites between PP1 and targeting subunits [21, 23].

Increasing evidence suggests that some phosphatases do not find their physiological substrates by simple diffusion and they are frequently directed to their substrates by interaction with different targeting subunits [27]. We have recently described that PP1α is targeted to Bad by Bcl-2 [8]. Our results show that the anti-apoptotic mole-

cules Bcl-x_L and Bcl-w are as well targeting subunits of PP1α in IL-4-stimulated cells, replacing Bcl-2 as targeting subunit of PP1α in IL-2-stimulated cells. It is known that most, if not all, targeting subunits have a PP1-binding motif. Bcl-2, as well as Bcl-x_L and Bcl-w contain this well-conserved PP1-binding motif described in other PP1 targeting subunits, in addition to F X X R X R motif, which is also conserved in some PP1 targeting subunits. PP1α enzymatic activity in Bad immunoprecipitates was inhibited using OA. Bad is dephosphorylated upon IL-4 deprivation, indicating the effect of a phosphatase. Inhibition of Bad dephosphorylation may occur through the inhibition of PP1α by OA. OA alone can prevent cell death and Bad dephosphorylation in IL-4-deprived cells, suggesting that, at least in part, Bad phosphorylation is important for cell survival. Inhibition of expression of the other components of the trimolecular complex, Bcl-x_L and Bcl-w, is also able to induce apoptosis. Taken together, our results show that Bcl-x_L and Bcl-w are PP1α targeting subunits.

4 Materials and methods

4.1 Cells and culture

TS1$\alpha\beta$ is a murine T cell line expressing α and β chains of the IL-2 receptor [28] that can be propagated independently in IL-2, IL-4 or IL-9. Cells were cultured in RPMI-1640 supplemented with 5% heat-inactivated fetal calf serum 2 mM glutamine, 10 mM Hepes, 0.55 mM arginine, 0.24 mM asparagine, 50 μM 2-ME and 60 U/ml of IL-4 or 5 ng/ ml of rIL-2.

4.2 Lymphokines, antibodies, reagents and plasmids

Murine rIL-4 or supernatant of a HeLa subline transfected with pKCRIL-4.neo was used as a source of murine IL-4. Anti-Bcl-x_L and anti-Bcl-w antibodies were from Calbiochem (La Jolla, CA), Transduction Laboratories (Lexington, KY) or StressGen Biotechnology (Victoria, Canada). Specific anti-PP1α was from UBI (Lake Placid, NY), Calbiochem or Transduction Laboratories. Anti-14-3-3 protein antibody was from UBI (Lake Placid, NY). Anti-Bad serine 112 and 136 were from New England BioLabs (Beverly, MA) and serine 155 was from Cell Signaling Technology (Beverly, MA). Anti-Pser and pan-Ras antibody were from Calbiochem. Recombinant PP1α protein was from Calbiochem.

4.3 Immunoprecipitation and Western blot

Cells (1×10^7) were IL-4-stimulated or -deprived and lysed for 20 min at 4°C in lysis buffer (50 mM Tris HCl pH 8, 1% NP-40, 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10% glycerol and protease inhibitors cocktail). Digitonine or detergent-free buffers were also used for immunoprecipitation. For phosphorylation analysis, the buffer was also supplemented with phosphatase inhibitors cocktail. Lysates were immunoprecipitated with the appropriate antibody and protein A Sepharose was added. Alternatively, cells were lysed in Laemmli sample buffer and protein extracts separated by SDS-PAGE, transferred to nitrocellulose, blocked and incubated with primary antibody. Membrane was washed and incubated with PO-conjugated secondary antibody. Proteins were developed using the ECL.

4.4 *In vitro* phosphatase assay

IL-4-stimulated cells (1×10^7) were lysed in lysis buffer, supernatants were immunoprecipitated with the corresponding antibody, followed by incubation with protein A Sepharose. Immunoprecipitates were washed with phosphatase buffer (50 mM Tris HCl, pH 7.5, 0.1% 2-ME, 0.1 mM EDTA and 1 mg/ml BSA) and mixed with the ^{32}P-labeled phosphorylase *a*, diluted in phosphatase buffer supplemented with caffeine. The reaction was incubated (40 min at 30°C), stopped with 200 μl 20% TCA and centrifuged. A total of 185 μl of the supernatant were used to estimate the generation of free phosphate liberated from the ^{32}P-labeled phosphorylase *a*.

4.5 Peptide synthesis

Peptides comprising the R/K X V/I X F (R) or F X X R X R (F) motif of Bcl-w and Bcl-x_L, as well as the mutated peptides (see Fig. 8A for sequence) were prepared by automated spot synthesis into an aminoderivatized cellulose membrane. Membrane was blocked, incubated with purified PP1α and, after several washing steps, incubated with anti-PP1α antibody, followed by PO-conjugated secondary antibody. Spots were developed using the ECL system.

R (NWG**RIVAF**FSF), F (GDE**FELRYR**RAF) or R* (NWG**RIAA-A**FSF) peptides were synthetized on an automated multiple peptide synthesizer using the solid-phase procedure and standard Fmoc chemistry. The purity and composition of the peptides was confirmed by reverse-phase high performance liquid chromatography and by amino acid analysis.

4.6 Protein-protein interaction competition

The interaction Bcl-w/PP1α and Bcl-x_L/PP1α was competed by the R, F, or R* peptides. Lysates from IL-4-stimulated cells were immunoprecipitated with anti-Bad antibody and protein A Sepharose was added. The interaction Bcl-w/PP1α and Bcl-x_L/PP1α was competed by incubation with R, F or R* peptides (30 min, room temperature). After washing, immunoprecipitates were either assayed for protein phosphatase activity or transferred to nitrocellulose and blotted with the corresponding antibody.

4.7 Sense and antisense oligonucleotides

The phosphothioate analogous of the oligonucleotides from Bcl-x_L and Bcl-w, including the ATG initiation codon were purchased from Isogen Bioscience. The sequences of the sense and antisense oligonucleotides are as follows: sense Bcl-x_L, ATG TCT CAG AGC AAC; antisense Bcl-x_L, GTT GCT CTG AGA CAT; sense Bcl-w, ATG GCG ACC CCA GCC; antisense Bcl-w, GGC TGG GGT CGC CAT.

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Correspondence: Angelita Rebollo, Laboratoire d'Immunologie Cellulaire et Tissulaire, INSERM U543, Bâtiment CERVI, Hôpital Pitié Salpêtrière, 83, Bd de l'Hôpìtal, F-75013 Paris, France
Fax: +33-1-4217-7490
e-mail: rebollo@chups.jussieu.fr