Aiolos transcription factor controls cell death in T cells by regulating Bcl-2 expression and its cellular localization

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We searched for proteins that interact with Ras in interleukin (IL)-2-stimulated or IL-2-deprived cells, and found that the transcription factor Aiolos interacts with Ras. The Ras–Aiolos interaction was confirmed in vitro and in vivo by co-immunoprecipitation. Indirect immunofluorescence shows that IL-2 controls the cellular distribution of Aiolos and induces its tyrosine phosphorylation, required for dissociation from Ras. We also identified functional Aiolos-binding sites in the Bcl-2 promoter, which are able to activate the luciferase reporter gene. Mutation of Aiolos-binding sites within the Bcl-2 promoter inhibits transactivation of the reporter gene luciferase, suggesting direct control of Bcl-2 expression by Aiolos. Co-transfection experiments confirm that Aiolos induces Bcl-2 expression and prevents apoptosis in IL-2-deprived cells. We propose a model for the regulation of Bcl-2 expression via Aiolos.

Keywords: Aiolos/apoptosis/Bcl-2/phosphorylation

Introduction

The Aiolos transcription factor has been identified as a homologue of the Ikaros transcription factor, whose expression is restricted to the lymphoid lineage. Aiolos homodimers are potent transcriptional activators, whereas the transcriptional activity of Aiolos–Ikaros heterodimers ranges from low to undetectable. Aiolos was first described in committed lymphoid progenitors, and is strongly up-regulated as these progenitors become restricted into T- and B-lymphoid pathways (Morgan et al., 1997). Aiolos plays an important role as a regulator of B-cell differentiation, proliferation and maturation to an effector state (Wang et al., 1998). The interplay between these proteins in the regulation of gene expression is further complicated by additional Ikaros isoforms or by other proteins that can sequester either Ikaros or Aiolos in transcriptionally inert complexes (Morgan et al., 1997).

Ras proteins are involved in cell proliferation, differentiation and apoptosis. Their function is controlled by a GTP–GDP cycle that is regulated by at least two distinct classes of regulatory proteins (Boguski and McCormick, 1993; McCormick, 1998). The relevance of Ras proteins in cell proliferation is best exemplified by the high frequency of mutated Ras genes in neoplasia. Ras acts at several phases of the cell cycle and is required soon after the release of cells from quiescence (Dobrowski et al., 1994; Pan et al., 1994). There are two peaks of Ras activation before the S phase; the earlier one is growth-factor triggered, whereas the second, which is stronger than the first, is growth-factor independent (Taylor and Shalloway, 1996; Winston et al., 1996). Several reports have suggested that Ras activation can promote either proliferation or apoptosis (Ferrari and Greene, 1994; Gulbins et al., 1995; Gómez et al., 1996, 1997, 1998b; Wilson et al., 1996). Ras mediates the apoptotic effect triggered by deprivation of growth factors such as serum, interleukin (IL)-3 or IL-2 (Reed, 1994; Consiglio and Formisano, 1995; Wang et al., 1995; Gómez et al., 1997). According to the pro- or anti-apoptotic role of Ras, we can propose that two different Ras-mediated pathways may be triggered by an external stimulus, one involved in cell proliferation and the other in apoptosis. Alternatively, one Ras-mediated pathway may trigger either proliferation or apoptosis, depending on external signals and/or associated pathways.

Apoptotic cell death is a process ultimately leading to the activation of endogenous nucleases that promote internucleosomal DNA degradation (Wyllie, 1980). Apoptosis can be induced by growth-factor deprivation (Duke and Cohen, 1986; Nuñez et al., 1990; Williams et al., 1990), signalling via surface receptors (Smith et al., 1989; Baumon et al., 1990) and exposure to drugs (Zubiaga et al., 1992). Many components of the machinery that regulates and executes programmed cell death have been identified (Nagata, 1997). Numerous studies have demonstrated that Bel-2 is a positive regulator of cell survival, protecting various cell types from death induced by growth factor deprivation, heat shock and viral agents (Reed, 1994; Park and Hockembery, 1996; White, 1996; Gómez et al., 1997), but the biochemical mechanism by which this protein prevents apoptosis remains enigmatic (Yang and Korsmeyer, 1996). Bel-2 has been reported to interact with members of the Ras superfamily of proteins (Fernandez-Sarabia and Bischoff, 1993; Chen and Faller, 1996). IL-2-dependent T cells die by apoptosis after IL-2 withdrawal, and transfection of Bel-2 promotes survival in the absence of IL-2 (Broome et al., 1993; Minami and Taniguchi, 1995; Miyazaki et al., 1995; Gómez et al., 1996). Bel-2 is expressed in the IL-2-dependent murine T-cell line TS1/β, and its expression decreases when cells are deprived of IL-2. Overexpression of Bel-2 in these cells extends their survival time in the absence of IL-2 (Gómez et al., 1996). The Ras pathway induces expression...
of genes necessary for cell survival; accordingly, it has been demonstrated that the Ras signalling pathway induces and regulates Bcl-2 expression (Kinoshita et al., 1995).

We used the yeast two-hybrid method to identify proteins that interact with Ras in IL-2-stimulated or IL-2-deprived cells. The transcription factor Aiolos was identified as interacting with Ras in IL-2-deprived cells. The results were confirmed by a genetic approach, by co-immunoprecipitation and by indirect immunofluorescence. They suggest a physiological role for a Ras–Aiolos interaction in the control of Bcl-2 expression. They also indicate a mechanism by which Ras and Aiolos interact only in the absence of IL-2, and require reassessment of the meaning of the fact that Ras is inactive in its GDP-bound form. The relevance of these findings is discussed in the context of apoptosis.

Results
Isolation of Ras-interacting proteins in IL-2-stimulated or IL-2-deprived TS1αβ cells

We have described how Ras activation leads to cell proliferation or apoptotic cell death after IL-2 stimulation or IL-2 deprivation of the murine TS1αβ cell line, respectively (Gómez et al., 1997). We asked whether two different Ras-mediated pathways are triggered by an external stimulus, one involved in cell proliferation and the other in apoptosis using different effectors, or alternatively, whether one Ras-mediated pathway might trigger either cell proliferation or apoptosis.

To identify proteins that interact with Ras in IL-2-stimulated or IL-2-deprived cells, Ras wild-type was used as bait to screen, using the two-hybrid method, cDNA libraries made from IL-2-stimulated or IL-2-deprived TS1αβ cells. The nucleotide sequence of one specific clone (Figure 1A) isolated from the cDNA library of IL-2-deprived cells showed a 100% match to a partial cDNA encoding the proximal C-terminal region of the Aiolos murine transcription factor (Figure 1B). cDNA encoding full-length Aiolos was cloned in pLex10 and tested for interaction with Ras wild-type; both the full-length protein and the truncated form isolated during screening, interact with Ras. Aiolos was then tested for interaction with Ras mutants. Aiolos does not interact with N17 or V12 Ras mutants, suggesting that this protein probably requires the GDP form of Ras for interaction (Figure 1A). Other related members of the Ras family, such as Rac1, Rac2, Rap1A or RalB, do not interact with Aiolos (Figure 1A), suggesting that the Ras wild-type–Aiolos interaction is specific for Ras. Binding of Aiolos and Ras wild-type probably occurs through the effector domain of Ras, since the Ras effector domain mutant L35R37 does not interact with Aiolos.

Association of Ras wild-type and Aiolos in vivo and in vitro

To validate the results obtained using the yeast two-hybrid system, the interaction of wild-type Ras with Aiolos was studied in intact cells by co-immunoprecipitation using Aiolos- and Ras-specific antibodies. We performed reciprocal co-immunoprecipitation experiments of cytoplasmic proteins under IL-2 stimulation or deprivation conditions in TS1αβ cells (Figure 2A). High Ras levels were detected by Western blot in anti-Aiolos immunoprecipitates of cells deprived of IL-2 for 24 h. The amount of Ras detected in cytoplasm decreased slightly in 12 h IL-2-deprived cells, with minimum Ras levels detected in cytoplasmic Aiolos immunoprecipitates of IL-2-stimulated cells. Reprobing the membrane with anti-Aiolos antibody shows different amounts of Aiolos in the cytoplasm of IL-2-stimulated (low levels) or IL-2-deprived cells (high levels).

In reciprocal experiments, high Aiolos levels were detected by Western blot in anti-Aiolos immunoprecipitates of cells deprived of IL-2 for 24 h. The amount of Ras detected in cytoplasm decreased slightly in 12 h IL-2-deprived cells, with minimum Ras levels detected in cytoplasmic Aiolos immunoprecipitates of IL-2-stimulated cells. Reprobing the membrane with anti-Aiolos antibody shows different amounts of Aiolos in the cytoplasm of IL-2-stimulated (low levels) or IL-2-deprived cells (high levels).
in vivo and that this interaction is specific, since immunoprecipitation of cytoplasmic lysates with an irrelevant antibody (anti-IL-2) detects neither Aiolos nor Ras. Co-immunoprecipitation of cytoplasmic proteins isolated by digitonin lysis of the cells showed similar results (data not shown). These findings reveal that Ras complexed to Aiolos is recovered from cells, confirming the results obtained in the two-hybrid system. Total lysates from IL-2-stimulated or IL-2-deprived cells show the same level of Aiolos expression (Figure 2B), in contrast to the levels detected in the cytoplasmic fraction; this suggests a distinct cellular distribution of Aiolos rather than differences in the total protein level. We addressed this question by analysing whether Aiolos could be detected in the nuclei of IL-2-stimulated cells. Aiolos was detected by Western blot in nuclear extracts of IL-2-stimulated cells (Figure 2C). The amount of Aiolos decreased with the length of the IL-2 deprivation period, reaching minimum levels at 24 h of IL-2 deprivation. This deprivation period corresponds to the maximum Aiolos level detected in the cytoplasm of IL-2-deprived cells, suggesting the trafficking of Aiolos from the cytoplasm to the nucleus, depending on the presence or absence of IL-2. As internal control of protein fractionation, the blot was probed with pan-Ras (cytoplasmic marker) and anti-histone antibodies (nuclear marker).

The Ras–Aiolos interaction was also studied in in vitro binding experiments. Aiolos was produced as a glutathione S-transferase (GST) fusion protein and purified on glutathione–agarose beads. The Vav SH2 domain fused to GST was used as a negative control. Cytoplasmic lysates from IL-2-stimulated or IL-2-deprived cells were incubated with GST–Aiolos and with GST–Vav SH2 and, after several washing steps, proteins were resolved in SDS–PAGE and the blot developed with anti-Ras antibody. Figure 3A shows that Ras interacts with the GST–Aiolos fusion protein, whereas there is no interaction with GST alone or with GST–Vav. In reciprocal experiments, GST–Ras proteins were purified on glutathione–agarose beads, and the SH2/Vav construct was used as a negative control. The GST–Ras proteins were loaded with guanine nucleotides by incubation with GDP or GTP-γS. Aiolos interacts with GST–Ras/GDP in cytoplasmic lysates of IL-2-deprived or IL-2-stimulated cells, although in the latter case, the amount of Aiolos that interacts with GST–Ras/GDP is greatly reduced (Figure 3B). This observation is consistent with the two-hybrid results obtained using Ras alleles (Figure 1A), in which Aiolos interacts with the GDP form of Ras. To confirm Aiolos interaction with the GDP Ras form, GST–Ras proteins were loaded with the nonhydrolysable GTP analogue GTP-γS. Aiolos interacts weakly with Ras-GTP-γS in cytoplasmic lysates of IL-2-stimulated cells (Figure 3C), and we were unable to detect

Fig. 2. Reciprocal co-immunoprecipitation of wild-type Ras and Aiolos. (A) Cytoplasmic lysates from IL-2-stimulated (5 ng/ml) or IL-2-deprived cells (1×10⁷) were immunoprecipitated with anti-Aiolos or irrelevant antibody (anti-IL-2), washed, transferred to nitrocellulose and immunoblotted with a pan-Ras antibody. The blot was stripped and reprobed with anti-Aiolos antibody. Similarly, cytoplasmic lysates from IL-2-stimulated or IL-2-deprived cells were immunoprecipitated with pan-Ras, washed, transferred to nitrocellulose and immunoblotted with anti-Aiolos. The blot was reprobed with pan-Ras. Protein bands were detected using the ECL system. Molecular weight markers are indicated. Similar results were obtained in three independent experiments. (B) Total lysates from IL-2-stimulated (5 ng/ml) or IL-2-deprived cells (5×10⁷) were resolved in SDS–PAGE, transferred to nitrocellulose, probed with anti-Aiolos, and protein bands detected as above. Molecular weight markers are shown. Similar results were obtained in two independent experiments. (C) Nuclear proteins were isolated from IL-2-stimulated (5 ng/ml) or IL-2-deprived cells (2×10⁷). After quantification, 20 μg of protein were resolved in SDS–PAGE, transferred to nitrocellulose and immunoblotted sequentially with anti-Aiolos, pan-Ras (cytoplasmic marker) and anti-histone H1, H2A, H2B, H3 and H4 antibodies (nuclear marker). As internal control of cytoplasmic (C) and nuclear (N) fractionation, cytoplasmic proteins from IL-2-stimulated or IL-2-deprived cells were used and also blotted with anti-Aiolos, pan-Ras and anti-histone antibodies. Protein bands were detected as above. Molecular weight markers are indicated. Similar results were obtained in two independent experiments.
Fig. 3. Aiolos interacts with Ras in vitro. (A) Expression of the GST–Aiolos fusion protein was induced by IPTG addition and proteins were isolated from bacterial lysates by affinity chromatography with glutathione–agarose beads and incubated with cytoplasmic lysates from IL-2-stimulated or IL-2-deprived cells (7 \times 10^6). After several washing steps, eluted proteins were resolved in SDS–PAGE, transferred to nitrocellulose and immunoblotted with pan-Ras. Protein bands were detected using the ECL system. Molecular weight markers are indicated. Data are representative of two independent experiments.

Similarly, GST–Ras/GDP (B) or GST–Ras/GTP–γS (C) proteins were incubated with cytoplasmic lysates from IL-2-stimulated or IL-2-deprived cells (7 \times 10^6). Eluted proteins were immunoblotted with anti-Aiolos antibody, and protein bands detected using ECL or ECL Plus. Molecular weight markers are indicated. Similar results were obtained in two independent experiments.

this interaction in cytoplasmic lysates of IL-2-deprived cells. We did not observe Aiolos interaction with GST–SH2/Vav.

Aiolos mRNA expression is not modified in IL-2-stimulated or IL-2-deprived cells

It was of interest to determine whether IL-2 could modulate Aiolos mRNA levels. Total mRNA was isolated from IL-2-stimulated or IL-2-deprived cells, electrophoresed and hybridized with an Aiolos-specific probe that does not cross-react with Ikaros. The result shows that the presence or absence of IL-2 does not modulate the Aiolos mRNA level (Figure 4). The β-actin levels, as well as 28S and 18S mRNAs, were similar in both cases.

IL-2 controls the cellular localization of Aiolos

As nuclear and cytoplasmic fractionation procedures can lead to protein leakage or contamination from one compartment to another, Aiolos localization was studied in intact cells using indirect fluorescence microscopy. IL-2-stimulated or -deprived cells were attached to cover-slips, fixed, permeabilized with lysophosphatidylcholine and, after blocking of non-specific binding, were incubated with anti-Aiolos antibody followed by fluorescein isothiocyanate (FITC)-conjugated secondary antibody. Labelled cells were observed using fluorescence microscopy. IL-2-stimulated cells show strong punctate nuclear staining, whereas in the cytoplasm only very weak staining is observed (Figure 5). After 3 h of lymphokine deprivation, the punctate nuclear staining began to redistribute gradually. The picture after 6–9 h of IL-2 deprivation shows staining in the nucleus and in the cytoplasm. Staining distribution at 12 h of deprivation is localized progressively throughout the cytoplasm rather than in the nucleus and at 24 h after withdrawal the staining is mainly detected in the cell cytoplasm. This result suggests that IL-2 controls cellular Aiolos distribution.

Role of Aiolos transcription factor in the activation of the Bcl-2 promoter

IL-2 deprivation induces apoptosis and Bcl-2 down-regulation in TS1αβ cells (Gómez et al., 1998a). Given that one of the differences between IL-2-stimulated and IL-deprived cells is Bcl-2 expression, and that Aiolos is mainly detected associated with Ras in the cytoplasm of IL-2-deprived cells, we hypothesize that Bcl-2 transcription may be activated by Aiolos. Bcl-2 expression is driven by two promoters, P1 and P2 (Figure 6B). Transient transfection was therefore performed with plasmids in which luciferase expression is under the control of P1, P2
Ras and Aiolos interaction in IL-2-deprived cells

Fig. 5. Immunolocalization of Aiolos. IL-2-stimulated (5 ng/ml) cells or cells deprived of IL-2 for different periods (3–24 h) were attached to a glass slide, fixed, permeabilized with lysophosphatidylcholine and stained with anti-Aiolos antibody, followed by FITC-conjugated secondary antibody. After several washing steps, samples were mounted in the surface coating Mowiol with anti-fading agents, analysed using fluorescence microscopy and photographed using a phase-contrast fluorescence photomicroscope. Similar results were obtained in two independent experiments.

or P1 + P2 alone or in combination with plasmid expressing Aiolos. After transfection, cells were stimulated with IL-2 or deprived for 24 h and luciferase expression was analysed. Transfection of Aiolos with an irrelevant promoter (Δ7mp55 IL-2R) was used as a control.

Co-transfection experiments in TS1αβ cells revealed that Aiolos is capable of activating the P1 + P2 or P1 Bcl-2 promoter and, at lower levels, the P2 promoter in the absence of IL-2, compared with control cells transfected with luciferase reporter constructs only (Figure 6A). Cells co-transfected with the different Bcl-2 promoter constructs and an Aiolos dimerization domain mutant in the absence of IL-2 show no transactivation of the luciferase reporter (data not shown). Aiolos is able to activate the Bcl-2 promoter construct, even in IL-2-stimulated cells. IL-2-stimulated cells transfected only with Bcl-2 promoter reporter constructs show lower luciferase activity than those co-transfected with Aiolos (Figure 6A), indicating that Aiolos is able to activate the Bcl-2 promoter construct even in IL-2-stimulated cells. Aiolos had no effect on the activation of the murine Δ7mp55-IL-2R promoter, in either the presence or absence of IL-2. These results suggest that the enhanced reporter gene activation may be an effect of Aiolos on the Bcl-2 promoter. It is interesting to note that we detected Aiolos-binding sites on the Bcl-2 promoter identical to the Aiolos DNA-binding sequence described by Morgan et al. (1997) (Figure 6B).

To prove direct control of Bcl-2 expression by Aiolos, electrophoretic mobility shift assay (EMSA) and mutation of putative Aiolos-binding sites within the Bcl-2 promoter were performed. DNA-binding activity for Aiolos was detected in IL-2-stimulated cells. Inhibition of Aiolos DNA-binding activity was detected using mutated Aiolos-binding sites. The specific DNA–Aiolos interaction was confirmed by competition with unlabelled oligonucleotide (Figure 7). Cells co-transfected with different Bcl-2 promoter constructs containing mutated Aiolos-binding sites and Aiolos expression vector in the absence of IL-2 show no transactivation of the luciferase reporter gene compared with cells transfected with wild-type Bcl-2 promoter constructs (Figure 7). Taken together, these results suggest a direct effect of Aiolos on the Bcl-2 promoter.

Aiolos induces Bcl-2 expression and prevents apoptosis in the absence of IL-2

To characterize the extent to which Aiolos activates the Bcl-2 promoter, transiently transfected TS1αβ cells with the Aiolos expression vector were analysed for Bcl-2 expression (Figure 8). Cells maintained in IL-2 or IL-4 were used as positive and negative Bcl-2 expression controls, respectively.

Upon IL-2 stimulation, mock transfectants or cells transfected with Aiolos show Bcl-2 expression levels comparable with those of control IL-2-stimulated cells. Mock transfectants maintained in the absence of IL-2 show no Bcl-2 expression. Interestingly, when cells are transfected with Aiolos, weak Bcl-2 expression is induced without IL-2 addition. Unaltered Ras expression is shown.
under all transfection conditions as an internal protein loading control. Expression of transiently transfected Aiolos was confirmed by direct comparison of Aiolos protein levels in transfected cells and in mock-transfected controls. This suggests that Aiolos is able to induce Bcl-2 expression in the absence of IL-2 and confirms the previous result of Bcl-2 promoter activation.

As IL-2 deprivation in TS1αβ cells correlates with Bcl-2 downregulation and apoptosis, and Aiolos is able to induce Bcl-2 expression in the absence of IL-2, we hypothesized that Aiolos may prevent apoptosis. Mock transfectants or cells transfected with the Aiolos expression vector were selected from a mixed population of transfected and non-transfected cells. Cells transfected with Aiolos and deprived of IL-2 for 24 h show a strong reduction in the fraction of apoptotic cells compared with IL-2-deprived mock-transfected control cells (Figure 9A). The frequency of apoptotic cells remains similar in all transfected cells in the presence of IL-2. Figure 9A also shows the percentage of apoptosis in non-transfected cells alone or cultured in IL-2. Similar results were obtained when the percentage of apoptotic cells was estimated by annexin staining (Figure 9B).

**IL-2 induces tyrosine phosphorylation of Aiolos**

To further study the mechanism by which Ras and Aiolos interact in the cytoplasm of IL-2-deprived cells, we analysed whether IL-2 could induce Aiolos phosphorylation, which may be responsible for its dissociation. Tyrosine phosphorylated Aiolos was detected in Aiolos immunoprecipitates of IL-2-stimulated cell cytoplasmic lysates. The amount of cytoplasmic tyrosine phosphorylated Aiolos in IL-2-deprived immunoprecipitates decreases notably after 12 h of starvation, and is almost undetectable after 24 h of deprivation (Figure 10A). Cytoplasmic Aiolos immunoprecipitated from IL-2-stimulated or IL-2-deprived cells was detected by reprobing the blot with anti-Aiolos antibody, showing the highest levels at 24 h of starvation and confirming the results shown in Figure 2A.

Tyrosine-phosphorylated Aiolos was also detected in immunoprecipitates of nuclear proteins from IL-2-stimulated cells, decreasing strongly with the starvation period (Figure 10B). Nuclear Aiolos immunoprecipitated from IL-2-stimulated or IL-2-deprived cells was detected by reprobing the blot with anti-Aiolos antibody, with the highest Aiolos levels in IL-2-stimulated cells. The relative amount of tyrosine-phosphorylated Aiolos in cytoplasmic and nuclear extracts normalized to total Aiolos protein is shown in Figure 10C. This result suggests that IL-2 induces phosphorylation of Aiolos and may control its dissociation from Ras. Alternatively, IL-2 may influence the state of Ras and, consequently, its dissociation from Aiolos.

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**Fig. 6.** Effect of Aiolos on transactivation of P1, P2 or P1+P2 Bcl-2 promoter. (A) TS1αβ cells were transiently co-transfected by the DEAE-dextran procedure with P1–Luc, P2–Luc, P1+P2–Luc or an irrelevant promoter (Δ7 murine p55 IL-2R promoter), with or without Aiolos. After transfection, cells were IL-2 stimulated (5 ng/ml) or IL-2 deprived for 24 h, collected, washed and assayed for luciferase activity. The efficiency of transfection was monitored by the β-galactosidase activity. Relative light units (RLU) were normalized to protein concentration. Standard deviation (SD) is shown where n = 4. B, buffer; V, empty vector. (B) Schematic representation of murine Δ7mp55IL-2R and Bcl-2 promoters showing DNA-binding sites for some transcription factors. The Aiolos DNA-binding sequence and the mutated Aiolos-binding sites (Aiolos 1*, Aiolos 2* and Aiolos 3*) are shown. The schematic representation of P1, P2 and P1+P2-luciferase constructs is also shown.
transcription factor. (A) Nuclear proteins from IL-2-stimulated (5 ng/ml) cells were incubated with 32P-end-labelled oligonucleotide containing the wild-type (TGGGAA) or mutated (GACATG) Aiolos-binding site. For oligonucleotide sequence, see Materials and methods. Protein–DNA complexes were separated free oligonucleotide in 5% polyacrylamide gel, dried and exposed to X-ray film. Lane 1, mutated Aiolos-binding site; lane 2, Aiolos-binding site; lane 3, Aiolos-binding site competed by a 20-fold molar excess of unlabelled Aiolos-binding site. Data are representative of two independent experiments. (B) TS1/5β cells were transiently co-transfected with P1–Luc, P2–Luc, P1 + P2–Luc or P1–Luc, P2–Luc, P1 * + P2–Luc with Aiolos. After transfection, cells were IL-2-deprived for 24 h, collected, washed and analysed for luciferase activity. The efficiency of transfection was monitored by the β-galactosidase activity. Relative light units (RLU) were normalized to protein concentration. B, buffer; V, empty vector. P1 *, P2 * or P1 * + P2 * represent the fragments of Bcl-2 promoter with mutated Aiolos-binding sites. For oligonucleotide sequence, see Materials and methods and Figure 6B.

Discussion

Using the yeast two-hybrid approach (Fields and Song, 1989), we have shown that Ras wild-type interacts with the Aiolos transcription factor. The interaction of Ras with both C-terminal and full-length Aiolos probably requires the GDP form of Ras, since Ras V12 or N17 mutants, which exist in GTP-bound or nucleotide free forms, respectively (Lai et al., 1993), fail to interact with Aiolos. This result was confirmed by in vitro binding of purified fusion proteins containing Ras preincubated with GDP or GTP-γS. Aiolos interacts with Ras-GDP in IL-2-deprived cells, and only traces of Aiolos are detected in association with Ras-GDP in IL-2-stimulated cells. In addition, no Aiolos association with Ras-GTP is detected in IL-2-deprived cells. Traces of Aiolos were associated with Ras-GTP in IL-2-stimulated cells, although this interaction was not detected using the yeast two-hybrid approach. Binding probably occurs through the Ras effector domain, since a Ras L35R37 effector domain mutant does not interact with Aiolos in the yeast two-hybrid system. Binding of Ras wild-type to Aiolos appears to be Ras protein specific, since other Ras superfamily members such as Rac1, Rac2, Rap1A or RalB do not bind Aiolos in the yeast two-hybrid method.

The results obtained using the two-hybrid system were confirmed by in vivo experiments. In co-immunoprecipitation experiments, we recovered Aiolos or Ras from cytoplasmic lysates using Ras- or Aiolos-specific antibodies. It is of interest to note that Aiolos is detected in the nucleus of IL-2-stimulated cells, whereas it is mainly found in the cytoplasm of IL-2-deprived cells. This suggests a distinct cellular distribution of Aiolos depending on the presence or absence of IL-2. This differential Aiolos distribution was confirmed by Western blot of total extracts and by immunofluorescence of IL-2-stimulated or IL-2-deprived cells, which showed no differences in total protein, ruling out the possibility that the distribution difference is due to variation in the amounts of protein. Our results suggest that IL-2 controls the cellular distribution of Aiolos. Growth factor deprivation induces translocation of Aiolos to cytoplasm, coincident with induction of apoptosis and downregulation of Bcl-2 expression.

Whereas Bcl-2 is regulated in both a tissue- and time-specific manner, little is known about the regulatory mechanism governing its transcription. Two promoter regions have been identified in the 5′ regulatory region of the bcl-2 gene. P1 is a TATA-less, GC-rich region containing several SP1 and π1 recognition elements (Young and Korsmeyer, 1993; Chen and Boxer, 1995; Wilson et al., 1996). P2, located ~1.3 kb downstream of
Fig. 9. Cell-cycle analysis of TS1αβ cells transfected with Aiolos. (A) Cells were transfected with or without Aiolos and pHook3 using the DEAE-dextran method and maintained for 24 h after transfection with or without IL-2. Cells were washed, selected using the Capture-Tec kit from Invitrogen, permeabilized and stained with PI. Samples were analysed by flow cytometry. The most proximal region of the fluorescence scale represents the subG₁ region. The percentages of apoptotic cells in each sample are superimposed. Non-transfected cells maintained in the presence or absence of IL-2 for 24 h were also used as controls. Similar results were obtained in four independent experiments. Standard deviation is shown. (B) Cells were treated as in (A). After selection, samples were diluted in ice-cold binding buffer, stained with annexin and PI, then analysed by flow cytometry. Percentage of apoptotic cells is superimposed.

P1, contains a CCAAT box, an octamer motif and a TATA element. Although transcriptional regulators of Bcl-2 have been identified (Miyashita et al., 1994; Miyashita and Reed, 1994; Taylor et al., 1996; Frampton et al., 1996; Hewitt et al., 1996; Heckman et al., 1997), the identity of the transcription factors that regulate its expression in T cells remains uncharacterized. The data presented here identify Aiolos as the first transcription factor shown to regulate the Bcl-2 promoter in TS1αβ cells. We have identified putative Aiolos-binding sites in the Bcl-2 promoter identical to the Aiolos DNA-binding sites described previously. Here we show that Aiolos is indeed able to transactivate the Bcl-2 promoter. The Aiolos-binding sites mentioned above are probably involved in this effect, since mutation of binding sites inhibits transactivation of the reporter gene. We do not know whether other adjacent sequences or proteins are also necessary and we cannot exclude the possibility that Aiolos is also involved in the expression control of genes other than Bcl-2. It has recently been shown that Bcl-2 transcription from the proximal P2 promoter is activated in neuronal cells by the Brm3a family of transcription factors, which specifically regulate the proximal P2 promoter region, the predominant promoter in neuronal cells (Smith et al., 1998). Aiolos is also able to prevent apoptosis through the induction of Bcl-2 expression.

Aiolos is first detected at low levels in double negative (CD4⁻CD8⁻) thymocyte precursors and is greatly upregulated as these progress to double positive (CD4⁺CD8⁺) differentiation stages. Aiolos expression decreases in splenic T cells (Wang et al., 1998). In thymus, Bcl-2 is expressed in double negative (CD4⁺CD8⁻) cells and in a few double positives (CD4⁺CD8⁺), as well as in nearly all single-positive cells. In addition, Bcl-2 is expressed in mature T cells (Gratiot-Deans et al., 1993; Veis et al., 1993; Chao and Korsmeyer, 1998). These observations suggest that Aiolos and Bcl-2 expression occur in parallel throughout the lymphocyte differentiation stages. The physiological effects of lack of Aiolos are an increase in B-cell precursors, breakdown in B-cell tolerance and development of B-cell lymphomas. In addition, nonconventional peritoneal B cells and recirculating bone marrow B cells are depleted, suggesting that Aiolos plays distinct roles in signalling cascades that control differentiation or proliferation of nonconventional versus conventional B cells (Wang et al., 1998).

We propose that IL-2 starvation induces Ras–Aiolos association and, consequently, inhibition of Bcl-2 expression and the resulting apoptotic cell death. One of the functional consequences of the Ras–Aiolos interaction is the blockade of Aiolos translocation from the cytoplasm to the nucleus. In the absence of IL-2, dephosphorylated
Aiolos is sequestered in the cytoplasm by Ras, there is no Bcl-2 expression, and apoptosis is induced as a consequence. The suppression of Aiolos nuclear translocation is blocked by IL-2 addition, which in turn induces tyrosine phosphorylation of Aiolos and probably dissociation from Ras. Alternatively, IL-2 may influence the state of Ras and, consequently, its dissociation from Aiolos. Based on our results, we hypothesized that IL-2 plays a key role in the control of Ras–Aiolos interaction. IL-2-induced tyrosine phosphorylation of Aiolos probably diminishes the affinity of Aiolos for Ras, inducing its dissociation and translocation to the nucleus. In parallel, in IL-2-starved cells, Ras will be in a so-called inactive GDP-bound state and will therefore be able to bind Aiolos, working as a cytoplasmic trap for Aiolos. We cannot exclude the possibility that phosphorylated Aiolos increases its affinity for other partners that would allow its translocation to the nucleus. In addition, we do not know whether the pool of transcription factor Aiolos involved in Bcl-2 expression control is in a phosphorylated or unphosphorylated form.

Our data demonstrate a specific interaction between Aiolos and Ras in IL-2-deprived cells, and provide an explanation for the disruption of Bcl-2 expression due to the cytoplasmic localization of the Aiolos transcription factor. In addition, it is the first report describing the role of Aiolos in Bcl-2 promoter activation. We do not exclude the possibility that the Ras–Aiolos interaction could also be regulated by other proteins. The finding reported here suggests a novel role for Ras in IL-2-deprived cells as a blocker of Bcl-2 expression through the cytoplasmic sequestering of unphosphorylated Aiolos.

Materials and methods

Cells and cultures
TS1r6β is a murine T-cell line stably transfected with the α- and β-chains of the human IL-2 receptor (Pitton et al., 1993), and can be maintained independently in the presence of IL-2, IL-4 or IL-9. Cells were cultured in RPMI-1640 (BioWhittaker, Walkersville, MD) supplemented with 5% heat-inactivated fetal calf serum (FCS; Gibco-BRL, Gaithersburg, MD), 2 mM glutamine, 10 mM HEPES, 0.55 mM arginine, 0.24 mM asparagine, 50 μM 2-ME and 5 ng/ml of recombinant IL-2 (rIL-2) or 60 U/ml of IL-4.

Lymphokines, antibodies, reagents, plasmids and probes
Human rIL-2 was provided by Roussel Uclaf (Paris, France). Murine rIL-4 or supernatant of a HeLa subline transfected with pHKIRL4-neo was used as source of murine IL-4. Mouse pan-Ras was from Oncogene Science (Cambridge, MA), anti-mouse Bcl-2 monoclonal antibody (mAb) was from PharMingen (San Diego, CA), and anti-mouse Aiolos polyclonal antibody was generated in our laboratory. Peroxidase-conjugated (PO) goat anti-rabbit or anti-mouse Ig antibody was from Dako (Glostrup, Denmark). ECL or ECL Plus and γ-32P reagents were from Amersham (Amersham, UK), 2 mM glutamine, 10 mM HEPES, 0.55 mM arginine, 0.24 mM asparagine, 50 μM 2-ME and 5 ng/ml of recombinant IL-2 (rIL-2) or 60 U/ml of IL-4.

The mouse β-actin probe was a ClaI–BamHI 600 bp fragment. The Aiolos probe was a 330 bp fragment from the last exon of Aiolos, which does not cross-react with Ikaros. Probes were labelled...
with [32P]ATP. The p55-Luc IL-2R promoter was provided by Dr M.Nabholz (Lausanne, Switzerland). Anti–IL-2 antibody was provided by Dr A.Dautriche (Paris, France). pGEX-Vav-H2 was given by Dr S.Scher (Paris, France) and the mammalian L553R7 by Dr A.Vojtek (Ann Arbor, MI). The RapA construct was a gift of Dr J.de Gunzburg (Paris, France).

cDNA library and the two-hybrid screen

Two cDNA libraries from IL-2-stimulated or 16 h IL-2-deprived TS166 cells, derived from polyadenylated RNA, were constructed in fusion with Gal4 activation domain (AD) in pGAD10 (Bartel et al., 1993). Wild-type Ras cloned into the pLEx10 vector was used as bait to screen both cDNA libraries in the Saccharomyces cerevisiae libraries in the cells from polyadenylated RNA were constructed in fusion with Gal4 using standard procedures (Bartel and Field, 1997).

Sequence analysis

Sequencing of PCR fragments and cDNA inserts from positive clones of the two-hybrid screening was performed on both strands with an automatic sequencer (Applied Biosystems model 373A). Sequences were compared using the FASTA program.

Cloning of Aiolos

According to the published sequence, Aiolos was cloned by PCR using the following primers: 5'-ATGGAAAGATATACCAAGCG and 3'-GTGTCTCGGTAACACTCC. Amplified DNA of the correct size was confirmed by sequencing and was cloned in the pcDNA expression vector.

Immunoprecipitation and Western blotting

Cells (1×107) were IL-2 stimulated or IL-2 deprived, and lysed for 20 min at 4°C in lysis buffer (50 mM Tris–HCl pH 7.5, 1% NP-40, 150 mM NaCl, 5 mM EDTA and protease inhibitor cocktail). Lysates were immunoprecipitated with the appropriate antibody. Protein A–Sepharose was added for 1 h at 4°C and, after washing, immunoprecipitates were separated by SDS–PAGE. Alternatively, cells (1×106) were lysed in Laemmli sample buffer and protein extracts separated by SDS–PAGE, transferred to nitrocellulose, blocked with 5% non-fat dry milk in Tris-buffered saline (TBS; 20 mM Tris–HCl pH 7.5, 150 mM NaCl) and incubated with primary antibody in TBS/0.5% non-fat dry milk. Membranes were washed with 0.05% Tween-20 in TBS and incubated with the appropriate secondary antibody. Protein A–agarose beads were added for a 2-h incubation on ice, the suspension was supplemented with 25 mM MgCl2, 500 mM NaCl, 1% Triton X-100 and 5 mM EDTA. Cells were broken after induction, resuspended in phosphate-buffered saline (PBS) supplemented with 20 mM EDTA, 2 mg/ml lysozyme, 10 mM GDP or GTPγS and protease inhibitors. Following 10 min incubation on ice, the suspension was supplemented with 25 mM MgCl2, 500 mM NaCl, 1% Triton X-100 and 5 mM EDTA. Cells were broken by sonication, the supernatant removed after centrifugation and incubated at 4°C with a suspension of glutathione–agarose beads. The agarose was washed with washing buffer (Tris pH 7.5, 10% glycerol, 1 mM DTT, 5 mM MgCl2, 0.1 mM GDP or GTPγS, 100 mM NaCl and protease inhibitors).

RNA isolation

Total RNA was isolated following the method of Maniatis et al. (1989). For Northern blot analysis, 15 μg of RNA samples were electrophoresed in a 1% agarose gel in the presence of formaldehyde and transferred to a nitrocellulose filter. After hybridization with the probe, the filter was washed and exposed to X-ray film at –70°C with an intensifying screen. The filter was stripped and rehybridized with a cDNA probe encoding β-actin.

Luciferase assay

Cells were transiently co-transfected with or without the pcDNA Aiolos plasmid plus P1–Luc, P2–Luc or P1+P2–Luc. Alternatively, cells were transiently co-transfected with Aiolos plus P1–Luc, P2–Luc or P1+P2–Luc. In addition, cells were transiently co-transfected with pcDNA Aiolos and Δmp55IL-2R-Luc or empty vector pcDNA. After transfection, cells were unstimulated or stimulated with 5 ng/ml of rIL-2 for 24 h, then washed in cold PBS and lysed in Luc buffer (25 mM Tris–Phosphate pH 7.8, 8 mM MgCl2, 1 mM DTT, 1 mM EDTA, 1% Triton X-100, 1% BSA and 15% glycerol) at 4°C. The protein concentration was determined using the Bio-Rad protein assay. Extracts were diluted in Luc buffer and the reaction mixture prepared with 91 μl of 25 mM luciferin, 330 μl of 20 mM ATP and 4606 μl of Luc buffer. Luciferase activity in protein extracts was measured using a Berthold LB9501 luminometer.

Transient transfection

TS166 cells were transiently transfected using the DEAE-dextran method. Cells (10×106) in exponential growth were washed with TS buffer (25 mM Tris–HCl, 137 mM NaCl, 5 mM KCl, 0.8 mM CaCl2, 0.5 mM MgCl2 and 0.6 mM Na2HPO4 pH 7.4). Cells were co-transfected with the plasmid pHook-3 and pcDNA Aiolos or an irrelevant plasmid. pHook-3 vector expresses a hapten-specific single chain antibody (sFv) on the surface of transfected cells. A total of 5 μg of each plasmid, 375 μl of TS buffer and 375 μl of freshly prepared DEAE–dextran (1 mg/ml) in TS buffer were mixed successively. Cells were incubated (20 min, room temperature) and 13 ml of RPMI-1640/5% FCS were added. Cells were incubated (1 h, 37°C), centrifuged and resuspended in 12 ml of RPMI-1640/5% FCS alone or supplemented with 5 ng/ml of rIL-2. Cells expressing the sFv were isolated from the culture by binding to hapten-coated (pHOX) magnetic beads. Transfected cells were selected and analysed.

Cell-cycle analysis

A total of 2.5×106 cells were washed and resuspended in PBS, permeabilized with 0.1% NP-40 and stained with 50 μg/ml PI immediately before analysis. Samples were analysed using an EpicsXL flow cytometer (Coulter, Miami, FL). Apoptosis was measured as the percentage of cells in the subG1 region of the fluorescence scale having a hypodiploid DNA content. Alternatively, cells were diluted in ice-cold binding buffer, stained with annexin and PI, then analysed by flow cytometry.

Indirect immunofluorescence

IL-2-stimulated cells, or cells IL-2 deprived for different periods were harvested and centrifuged in a cytopsin ( Heraeus) for 10 min at 600 g on glass slides. Cells were methanol fixed for 2 min at –20°C and permeabilized with 50 μg/ml of lyso phosphatidylcholine in PBS for 2 min at room temperature in 1% PBS/BSA. Polyclonal anti-Aiolos antibody diluted in PBS/BSA was added to the slides under a cover-slip and incubated in a humidified chamber (1 h, room temperature). After washing once with PBS and twice with PBS/BSA, secondary fluorocentrated antibody was added and incubated for 45 min in a humidified...
Peptide synthesis and antibody production
A specific peptide of the Aiolos amino acid sequence 368–384 was synthesized on an automated multiple peptide synthesizer (AMS422, Abbico, Langford, Germany) using the solid phase procedure and standard Fmoc chemistry. Purity and peptide composition were confirmed in reverse-phase HPLC and by amino acid analysis in a Beckman 6300 amino acid analyser. The peptide, glutaraldehyde-coupled to KLH via N-terminal lysine, was injected into outbred New Zealand rabbits. Sera were collected 7–10 days after the last injection.

Site-directed mutagenesis
Site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit. The primers used for mutation were:

Aiolos 1*: 5'-GAACAGAAAGTCTGACCAATGTCAATCAAGATATCGTCAAC-3';
Aiolos 2*: 5'-TTTCTCTTCTTCAGGATTGACAT-3';
Aiolos 3*: 5'-TTTTCCTTCTTCAGGATTGACAT-3';

The putative Aiolos-binding site is underlined. Point mutations are shown in bold.

References
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Ras and Aiolos interaction in IL-2-deprived cells
Aiolos 3*: 5'-GAGACTGACGCTGGATCGATCTGGAATTCACGTA; Aiolos 5*: 5'-TTTCTCTTCTTCAGGATTGACATGCAT. The putative Aiolos-binding site is underlined. Point mutations are shown in bold.


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