The BCL-6 POZ domain and other POZ domains interact with the co-repressors N-CoR and SMRT

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Virtually all diffuse large cell lymphomas and a significant fraction of follicular lymphomas contain translocations and/or point mutations in the 5' non-coding region of the putative oncogene BCL-6, that are presumed to deregulate its expression. BCL-6 encodes a Cys₂-His₂ zinc finger transcriptional repressor with a POZ domain at its amino-terminus. The POZ (or BTB) domain, a 120-amino-acid motif, mediates homomeric and, in some proteins, heteromeric POZ-POZ interactions. In addition, the POZ domain is required for transcriptional repression of several proteins, including BCL-6. Using a yeast two-hybrid screen, we identified N-CoR and SMRT as BCL-6 interacting proteins. Both N-CoR and SMRT, which were originally identified as co-repressors for the unliganded nuclear thyroid hormone and retinoic acid receptors, are components of large complexes containing histone deacetylases. We show that the interaction between BCL-6 and these co-repressors is also detected in the more physiologically relevant mammalian two-hybrid assay. The POZ domain is necessary and sufficient for interaction with these co-repressors. BCL-6 and N-CoR co-localize to punctate regions of the nucleus. Furthermore, when BCL-6 is bound to its consensus recognition sequence *in vivo*, it can interact with N-CoR and SMRT. We find, *in vitro*, that POZ domains from a variety of other POZ domain-containing proteins, including the transcriptional repressor PLZF, as well as ZID, GAGA and a vaccinia virus protein, SalF17R, also interact with varying affinities with N-CoR and SMRT. We find that BCL-6 POZ domain mutations that disrupt the interaction with N-CoR and SMRT no longer repress transcription. In addition, these mutations no longer self associate suggesting that self interaction is required for interaction with the co-repressors and for repression. More recently N-CoR has also been implicated in transcriptional repression by the Mad/Mxi proteins. Our demonstration that N-CoR and SMRT interact with the POZ domain containing proteins indicates that these co-repressors are likely involved in the mediation of repression by multiple classes of repressors and may explain, in part, how POZ domain containing repressors mediate transcriptional repression.

Keywords: POZ domain; BCL-6; transcriptional repression; N-CoR; SMRT

Introduction

The BCL-6 gene was identified because its 5' untranslated region is disrupted by chromosomal translocations associated with non-Hodgkin's lymphomas, in particular diffuse large cell and follicular lymphomas (Baron *et al.*, 1993; Deweindt *et al.*, 1993; Ye *et al.*, 1993; Lo Coco *et al.*, 1994; Miki *et al.*, 1994; Offit *et al.*, 1994; Ohno *et al.*, 1994; Otsuki *et al.*, 1995). These translocations, which juxtapose the intact BCL-6 coding sequences to one of at least 13 different loci, are believed to cause deregulated expression of the gene (Ye *et al.*, 1995; Chen *et al.*, 1998). In addition, point mutations in the 5' non-coding sequence of BCL-6 are found in a large proportion (40–76%) of these lymphomas (Migliazza *et al.*, 1995). When considered together, translocations and/or point mutations affecting the BCL-6 gene are found in almost all cases of diffuse large cell lymphoma and a large proportion of follicular lymphomas (Migliazza *et al.*, 1995). This supports a model whereby deregulated expression of intact BCL-6 plays a central role in the molecular pathogenesis of these lymphomas. BCL-6 normally is expressed in germinal centers in mature B cells and some CD4⁺ T cells (Cattoretti *et al.*, 1995; Flenghi *et al.*, 1995; Onizuka *et al.*, 1995; Pittaluga *et al.*, 1996). It is from these B cells that the BCL-6 associated lymphomas are thought to arise (Stein and Dallenbach 1992). Mice homozygous for a BCL-6 gene deletion have defects in T cell dependent antibody responses and do not form germinal centers (Dent *et al.*, 1997; Fukuda T *et al.*, 1997; Ye *et al.*, 1997).

BCL-6 is a Cys₂-His₂ zinc finger protein belonging to a subclass of zinc finger proteins which contain a POZ domain (also called the BTB or ZIN domain) at the N-terminus and zinc fingers at the C-terminus (Numoto *et al.*, 1993; Bardwell and Treisman 1994; Zollman *et al.*, 1994; Albagli *et al.*, 1995; Dhordain *et al.*, 1995). This 120 amino acid domain was named after the two main classes of proteins in which it can be found, POx virus and Zinc finger proteins (Bardwell and Treisman, 1994). There are over 20 POZ domain proteins characterized to date and numerous expressed sequence tag (est) sequences corresponding to POZ domain proteins. The function of the pox virus proteins, which lack a DNA binding domain, is unknown (Koonin *et al.*, 1992). Some of the zinc finger proteins such as BCL-6, PLZF, ZF5 and ttk have been shown to function as transcriptional repressors (Xiong *et al.*, 1993; Deweindt *et al.*, 1995; Albagli *et al.*, 1996; Chang *et al.*, 1996; Seyfert *et al.*, 1996; Kaplan and Calame, 1997; Li *et al.*, 1997a). The *Drosophila* protein

GAGA, which is also a zinc finger protein, is involved in chromatin remodeling (Tsukiyama *et al.*, 1994; Tsukiyama and Wu, 1995; Wall *et al.*, 1995). One other *Drosophila* gene, mod(mdg4) or (E(Var)3-93D), encodes several proteins with the same amino-terminal POZ domain but alternative non-zinc finger carboxy-terminal domains. This gene is an enhancer of position effect variegation (Dorn *et al.*, 1993; Gerasimova *et al.*, 1995).

Two properties have been ascribed to the POZ domain. First, the POZ domain can mediate homomeric as well as heteromeric POZ-POZ interactions (Bardwell and Treisman, 1994; Dhordain *et al.*, 1995; Seyfert *et al.*, 1996). The PLZF POZ domain, which has been studied in more detail, forms a very stable homodimer (Li *et al.*, 1997b). Second, the POZ domains of BCL-6, PLZF and ZF5 have been shown to mediate transcriptional repression (Deweindt *et al.*, 1995; Albagli *et al.*, 1996; Chang *et al.*, 1996; Seyfert *et al.*, 1996; Kaplan and Calame, 1997; Li *et al.*, 1997a).

To identify proteins that interact with BCL-6, and to better understand how BCL-6 mediates repression of its target genes, we have performed a yeast two-hybrid screen. We find that the POZ domain of BCL-6 and several other POZ domains interact with N-CoR and SMRT, which were identified as co-repressors of the nuclear hormone receptors (Chen and Evans, 1995; Horlein *et al.*, 1995; Sande and Privalsky, 1996). This suggests that these co-repressors are involved in mediating transcriptional repression by multiple classes of repressors.

Results

Identification of N-CoR and SMRT as proteins interacting with BCL-6 in a yeast two-hybrid screen

The yeast two-hybrid approach was used to screen Daudi cell activator-tagged cDNA libraries for proteins capable of interacting with BCL-6. (Daudi cells are a mature B-cell line that express BCL-6.) Two different bait constructs were made, containing BCL-6 amino acids 1–418 (POZ Long) or BCL-6 amino acids 74–508 ($\Delta 74$) fused to the GAL4 DNA binding domain (DBD). The latter bait is missing the first two-thirds of the POZ domain. Neither bait contains the BCL-6 DNA binding domain (Figure 1a). These bait proteins were used to screen a total of 11 million library transformants for co-activation of the GAL4 responsive His3 and LacZ reporter genes in the yeast strain Y153. We recovered 35 in-frame fusion clones, representing nine different genes, which re-tested positive with the original bait and did not interact with irrelevant test baits. None of these proteins contained a POZ domain (see Materials and methods). In this study we focused on two of these genes, the co-repressors N-CoR and SMRT (Chen and Evans, 1995; Horlein *et al.*, 1995; Sande and Privalsky, 1996), which were identified using the POZ Long bait. A clone containing human N-CoR, corresponding to amino acids 1019–2061 of murine N-CoR, was identified eight times and a clone containing human SMRT, amino acids 355–670 was identified once (Figure 1b). Neither of these clones contain regions

Figure 1 Identification of N-CoR and SMRT as proteins interacting with BCL-6 in a yeast two-hybrid screen. (a) Structures of the full length BCL-6 and the BCL-6 baits. ZnFs, 6 C₂H₂ zinc fingers; DBD, GAL4 DNA binding domain (1-147). (b) Structures of N-CoR and SMRT fragments identified from the two-hybrid screen in comparison to their full length versions. Depicted are the functional domains that have been previously characterized (Chen and Evans 1995; Horlein *et al.*, 1995; Sande and Privalsky, 1996; Seol *et al.*, 1996; Zamir *et al.*, 1996; Alland *et al.*, 1997; Heinzel *et al.*, 1997; Nagy *et al.*, 1997): RDI-III, N-CoR repression domains; SRDI and II, SMRT repression domains; NRI-III, nuclear receptor interaction domains; SID1 and 2, SIN3 interaction domains. Percent amino acid identity between the two proteins are indicated. Since we do not have a full length human N-CoR clone, amino acid numbers correspond to the murine sequence. The two proteins are highly conserved across this region with the exception of a deletion of murine amino acids 1731–1740 in the human clone. (c) Interactions of N-CoR and SMRT with BCL-6 require the POZ domain. *S. cerevisiae* Y153 strain (Trp⁺Leu⁺His⁺LacZ⁺) was sequentially transformed with the indicated combinations of bait and activator plasmids. The GAL4 activator-tagged N-CoR and SMRT proteins are encoded by the corresponding activator library plasmids identified from the screen. p53 and TRA-1 were used as negative controls. Results are in units of β -gal activity from liquid culture assays (protocol provided by Clontech) and are the average of a triplicate experiment. The standard deviations for those showing significant activity were less than 2%.

of N-CoR or SMRT that had been previously identified as being important for interaction with the nuclear hormone receptors (Figure 1b, NRI-III) (Chen and Evans, 1995; Horlein *et al.*, 1995; Sande and Privalsky, 1996; Seol *et al.*, 1996; Zamir *et al.*, 1996) indicating that the region(s) important for interaction with BCL-6 is distinct. The interaction in the yeast two-hybrid assay is specific in that N-CoR and SMRT do not interact with the GAL4 DNA binding domain alone, with the GAL4 baits containing p53(73-290), or with the N-terminal region of the zinc finger transcription factor TRA-1(1-220) (Zarkower and Hodgkin, 1992), (Figure 1c).

To test whether the interaction of BCL-6 and either of the co-repressors requires the POZ domain, we checked for interaction with the $\Delta 74$ bait. Neither co-repressor interacted with this fusion protein, suggesting that the POZ domain is required for interaction in yeast (Figure 1c).

BCL-6 POZ domain interacts with N-CoR and SMRT in the mammalian two-hybrid assay

To determine whether the interactions between BCL-6 and the co-repressors could take place in mammalian cells, we prepared mammalian expression constructs for bait and activator-tagged proteins and carried out a mammalian two-hybrid assay. Since the POZ domain of BCL-6 is required for transcriptional repression (Deweindt *et al.*, 1995; Albagli *et al.*, 1996; Chang *et al.*, 1996; Seyfert *et al.*, 1996), we also wanted to test whether the POZ domain alone was sufficient for the interaction with the co-repressors. Two of the bait constructs were similar to the yeast bait constructs, containing the GAL4 DNA binding domain fused to POZ Long or $\Delta 74$ BCL-6 fragments. In addition, a third construct containing GAL4 fused to BCL-6 amino acids 1–133 (POZ Short) was generated. The activator-tagged constructs contained the transcriptional activation domain from the herpes virus protein VP16 fused to the N-CoR and SMRT cDNA fragments identified in the yeast two-hybrid screen (Figure 2a). The mammalian two-hybrid assay was carried out by transiently transfecting EB3 cells, a Burkitt's lymphoma cell line which, in our hands, expresses BCL-6 (data not shown), with a GAL4 responsive luciferase reporter and pair-wise combinations of the appropriate bait and activator-tagged expression constructs. Transcription of the reporter gene was assessed by measuring luciferase activity in cell extracts from the transfected cells. Only the POZ-containing bait constructs in combination with VP16/N-CoR or VP16/SMRT construct resulted in significant, dose dependent, transcriptional activation. All other combinations of bait and activator constructs resulted in little or no activation of the reporter construct (Figure 2b). Similar POZ-dependent activation was also seen in HeLa cells and K562 cells (see below and data not shown), which express little or no endogenous BCL-6. From these results we conclude that the interaction between BCL-6 and N-CoR(1019-2061) or SMRT(355-670) can take place in mammalian cells and that the POZ domain, which is required for transcriptional repression (Deweindt *et al.*, 1995; Albagli *et al.*, 1996; Chang *et al.*, 1996; Seyfert *et al.*, 1996), is necessary and sufficient for the interaction.

Interaction of N-CoR and SMRT with BCL-6 bound to its DNA recognition site in vivo

We next wanted to test whether BCL-6, when bound to DNA, interacts with N-CoR and SMRT. This is an important test regarding specificity of the interaction since not all co-repressors interact with DNA-bound nuclear hormone receptors even though they show protein-protein interactions in solution (Zamir *et al.*, 1997). To test this, we needed to first determine the binding specificity of the zinc fingers of BCL-6. We used a binding site selection protocol (Pollock and

Figure 2 BCL-6 POZ domain interacts with N-CoR and SMRT in the mammalian two hybrid assay. (a) Structures of the luciferase reporter construct (pAH205), bait and activator proteins used in b. The bait proteins correspond to the indicated derivatives of BCL-6 fused to the GAL4 DBD. The activator-tagged proteins are VP16 fusions of the cloned SMRT and N-CoR fragments. (b) Luciferase and β -gal assays were conducted on cell extracts from EB3 cells which had been transiently transfected with 10 μ g of the GAL4 responsive reporter (pAH205), 10 μ g of the indicated bait expression constructs, either 0, 1.5 or 3 μ g of the activator-tagged expression constructs for the proteins diagram in a and 2 μ g of the pEFlacZ plasmid, as a control for transfection efficiency. Results represent the average of a triplicate experiment in which the luciferase activity was normalized to β -gal activity

Treisman, 1990) with *in vitro* translated Δ BCL-6. Forty-seven of fifty oligonucleotides selected in this assay contained variants of the consensus sequence (A/G/T)TTCCT(C/A)GAAAG or a more degenerate relaxed consensus (Figure 3). Because we sequenced a large number of selected oligonucleotides, after only three rounds of selection, the complexity of the derived matrix gives us a more accurate representation of the spectrum of sites to which the protein will bind than the similar but not identical BCL-6 recognition sites reported previously (Kawamata *et al.*, 1994; Baron *et al.*, 1995; Chang *et al.*, 1996; Seyfert *et al.*, 1996). We confirmed the specificity of the derived consensus site both *in vitro*, by competition with excess consensus or mutated oligonucleotides in the gel mobility shift assay, and *in vivo*, by testing BCL-6 binding to reporter constructs containing consensus BCL-6 binding sites or point mutated binding sites (data not shown).

Having determined the BCL-6 DNA recognition sequence, we were able to test *in vivo* whether BCL-6, when bound to its DNA recognition sequence, interacts selectively with N-CoR, SMRT or both. We first transfected EB3 cells, which contain endogenous BCL-6, with a reporter plasmid in which transcription of the chloramphenicol acetyltransferase (CAT) gene was controlled by four copies of the consensus binding site (WT reporter plasmid) or a mutated derivative binding site (MT1 reporter plasmid), in the absence of plasmids encoding effector proteins (Figure 4a). The levels of transcriptional activation from the WT reporter plasmid are lower than the MT1 reporter plasmid, presumably due to repression by endogenous BCL-6 (Figure 4b). We then tested for interaction between endogenous BCL-6 and the activator-tagged co-repressors. The WT reporter plasmid showed significant transcriptional activation upon co-transfection of VP16/N-CoR and VP16/SMRT (Figure 4b, left panel, compare column 1 versus 2–4), while the MT1 reporter plasmid shows no significant transcriptional activation (Figure 4b, right panel, compare column 1 versus 2–4). This suggests that endogenous BCL-6, when bound to DNA, is capable of interaction with VP16-tagged-N-CoR and SMRT.

We reasoned that if BCL-6 is limiting in these cells, then addition of extra exogenous BCL-6 should potentiate the transcriptional activation seen with a fixed amount of VP16/N-CoR or VP16/SMRT. We therefore transfected increasing amounts of a BCL-6 expression construct in the presence of the VP16/N-CoR or VP16/SMRT expression construct (Figure 4b). Strong potentiation of transcriptional activation was

seen upon addition of BCL-6. We note that with VP16/N-CoR and 2 μ g of transfected BCL-6 we begin to see squelching, presumably due to BCL-6 interacting with VP16/N-CoR off DNA. This is even more pronounced with higher amounts of BCL-6 (data not shown). The transcriptional activity seen upon the addition of VP16/N-CoR or VP16/SMRT is well above that seen with the MT1 reporter and therefore is not just due to relief of BCL-6 repression but rather represents further activation due to interaction of the VP16-tagged co-repressors with BCL-6. We conclude that BCL-6 when bound to its recognition sequence can interact with either N-CoR(1019–2061) or SMRT(355–670).

Subcellular distribution of N-CoR and BCL-6

Endogenous N-CoR, BCL-6 and some other POZ domain containing proteins are found in the nucleus in a diffuse microgranular fashion (Cattoretti *et al.*, 1995; Reid *et al.*, 1995; Soderstrom *et al.*, 1997). Overexpression of these proteins by transient transfection appears to enlarge the granules to produce punctate structures (Bardwell and Treisman, 1994; Dhordain *et al.*, 1995; Soderstrom *et al.*, 1997). To test whether BCL-6 and N-CoR are found in the same granular and punctate nuclear structures, we performed immunofluorescence on HeLa cells transiently transfected with BCL-6 and epitope-tagged N-CoR. We see a range of staining patterns for both proteins, from diffuse microgranular to discrete punctate structures. BCL-6 and N-CoR clearly co-localize to the small and large punctate structures (Figure 4c) suggesting that BCL-6 and N-CoR may function or be stored together in these structures.

Interaction of N-CoR and SMRT with BCL-6 *in vitro*

To determine whether full length N-CoR and SMRT interact with the BCL-6 POZ domain, and to determine if additional regions of BCL-6 contact N-CoR or SMRT, we tested for interaction between full length N-CoR and SMRT and subregions of BCL-6 *in vitro*. Glutathione S-transferase (GST) fusion proteins were generated which contained, in isolation, the POZ domain, the middle region, the zinc fingers, and amino acids 74–508 (Δ 74) of BCL-6 (Figure 5a). Approximately equal amounts of each of these fusion proteins as well as GST alone (Figure 5b) were used in GST pull down assays with *in vitro* translated full length N-CoR and SMRT (Figure 5c). For comparison, we also tested the co-repressor regions identified in the yeast two-hybrid screen, N-CoR(1019–2061) and SMRT(355–670). Each of these four proteins interacted most strongly with the POZ domain of BCL-6. None of the co-repressor proteins interacted with the middle region of BCL-6. There was minor but detectable interaction of the zinc finger-containing protein and the Δ 74 protein with all co-repressor proteins except SMRT(355–670). Interaction with the Δ 74 protein was not detected in the mammalian two hybrid assay (see above). The minor interaction with the zinc finger protein was also seen in the presence of 0.5 mg/ml ethidium bromide (data not shown) suggesting that the interaction is direct and not mediated by DNA. We noted that the intact N-CoR and SMRT interact with BCL-6 more strongly than the truncated fragments

Figure 3 Determination of BCL-6 DNA recognition site *in vitro*. Structure of the BCL-6 derivative used for the binding site selection. Sequences of 47 oligonucleotides recovered from the site selection were aligned with respect to the derived consensus BCL-6 DNA-binding site, and nucleotide frequency at each position is indicated. nC, not C; nT, not T

Figure 4 Interaction of N-CoR and SMRT with BCL-6 bound to its DNA recognition site *in vivo* and co-immunofluorescence of BCL-6 and N-CoR. (a) Structures of the chloramphenicol acetyl transferase (CAT) reporter construct and the proteins used in (b). The CAT reporter constructs are controlled by either four copies of the consensus (WT⁺⁺⁺⁺) or mutated (MT1⁺⁺⁺⁻) BCL-6 binding sites; pluses and minuses indicate the orientation of the sites with respect to the consensus sequence. (b) CAT and β -gal assays were performed on cell extracts from EB3 cells transiently transfected with 10 μ g of WT (left) or MT1 (right) reporter constructs along with 2 μ g pEFlacZ and combinations of the indicated amounts (in μ g) of plasmids encoding VP16-tagged co-repressors and full length BCL-6. Per cent conversion of chloramphenicol to its acetylated derivatives was quantitated by Phosphorimager scanning and normalized to β -gal activity. The

suggesting that an additional region of N-CoR and SMRT may also contact the BCL-6 POZ domain. We conclude that the major co-repressor interaction interface of BCL-6 is the POZ domain.

Interaction of N-CoR and SMRT with other POZ domains *in vitro*

To determine whether N-CoR and SMRT interact with POZ domains from other proteins, we again used the GST pull down assay. GST fusion proteins were generated with POZ domains from the additional zinc finger POZ proteins: PLZF (Chen *et al.*, 1993), ZID (Bardwell and Treisman, 1994) and GAGA (Soeller *et al.*, 1993), and from the non-zinc finger POZ proteins: SalF17R from vaccinia virus (Howard *et al.*, 1991), and Protein A, from the mod(mdg4) gene (Dorn *et al.*, 1993) (Figure 6a).

Approximately equal amounts of each of these fusion proteins (Figure 6b) as well as GST alone were used to test for interaction with *in vitro* translated intact N-CoR and SMRT. Both proteins interacted with the BCL-6 POZ domain most efficiently but

Figure 5 Interaction of N-CoR and SMRT with BCL-6 *in vitro*. (a) Structures of GST fusion proteins used in (b) and (c). (b) Coomassie staining of the GST fusion protein preparations used in (c). The middle and $\Delta 74$ fusions were susceptible to partial degradation. (c) GST pulldown experiment. GST protein alone (lane 2) or fused with the indicated BCL-6 derivatives (lanes 3–6), immobilized on beads, were used to 'pull-down' the indicated radiolabeled N-CoR and SMRT derivatives (listed to the right). The input (lane 1) was 1/20th of that used for each pulldown experiment. Equal volumes of GST fusion protein conjugated beads, which contained approximately 2–4 μ g of the indicated proteins, were used in the experiment

data displayed are from a single representative experiment. (c) Co-immunofluorescence of BCL-6 and N-CoR. Multiple Z series confocal microscopy images of the given fields were compiled for each image of individual BCL-6 and N-CoR immunofluorescence. The compiled images were merged to detect co-localization

detectable interaction was seen with all of the other POZ domains, except Protein A (Figure 6c). From these experiments we conclude that N-CoR and SMRT can interact *in vitro*, with varying avidity and selectivity, with a variety of POZ domains.

POZ domain point mutations eliminate repression

To more specifically correlate transcriptional repression of BCL-6 with interaction with the co-repressors, we set out to identify point mutations in the POZ domain that failed to interact with the co-repressors and also failed to repress transcription. We compared the sequences of the POZ domains of the proteins that interacted with the co-repressors with the one POZ domain, protein A, which did not interact with the co-repressors. We focused on positions that were generally conserved among POZ domains. We identified four amino acids in the amino-terminal region of the POZ domain of protein A that were significantly different from the amino acids found in the other POZ proteins at those positions (Figure 7a). Using site directed mutagenesis we generated three BCL-6 mutations, two single mutations (M1 position 19, L to S, M2 positions 23, N to H) and one double mutation (M3 positions 25 and 26, LR to SL), that code for the amino acid(s) found in protein A at these positions.

To test the ability of these mutations to interact with the co-repressors, we carried out the mammalian two-hybrid assay in HeLa cells. The reporter, the GAL4

DBD, the POZ Short bait and VP16-tagged co-repressor expression plasmids were identical to those used in Figure 2. In addition we used POZ short bait expression plasmids containing the mutations M1, M2, and M3. In this assay the bait protein M2 interacted with both VP16-tagged co-repressors with the same efficiency as the intact POZ Short bait. In contrast, M1 and M3 failed to interact with the co-repressors (Figure 7b). By Western analysis, using a GAL4 DBD antibody, all four POZ bait proteins were expressed at equal levels (data not shown).

To test the ability of these mutated POZ domains to repress transcriptions we co-transfected a SV40 enhancer driven, GAL4 responsive luciferase reporter together with each of the POZ Short baits used above. The intact POZ domain and M2 repress transcription in a dose dependent and DNA binding domain dependent manner. M1 and M3, the mutants that did not interact with the co-repressors, do not effectively repress transcription (Figure 7c).

Since the BCL-6 POZ domain can self associate we wanted to test whether these mutations also affect the POZ-POZ interaction. We used an immunoprecipitation assay, in which *myc*-epitope-tagged, POZ Short, and non-tagged, POZ Long, proteins were produced by co-translation and immunoprecipitated using the 9E10 *myc* epitope antibody. In this assay co-precipitation of the non-tagged protein is indicative of a stable interaction between the POZ domains (Bardwell and Treisman, 1994). Constructs encoding both POZ Short and POZ Long version of each of the three-point mutations, M1, M2 and M3, were generated. Using the co-immunoprecipitation assay, each mutation was tested for homomeric interaction and compared to the homomeric interaction of the intact BCL-6 POZ domain. Self association of the M2 POZ domain was equivalent to that of the intact POZ domain (Figure 7d, lanes 5 and 7). In contrast POZ domain mutants M1 and M3 failed to show significant self association (Figure 7d, lanes 6 and 8). The small amount of co-immunoprecipitated M1 and M3 POZ Long protein is likely to be non-specific binding since it is also seen in a mock co-immunoprecipitation in the absence of the POZ-tag protein (data not shown). In summary, those POZ domain mutants, M1 and M3, which fail to self associate, fail to interact with the co-repressors and fail to repress transcription.

Figure 6 Interaction of N-CoR and SMRT with other POZ domains *in vitro*. (a) Structures of GST fusion proteins used. (b) Coomassie staining of GST fusion preparations used in (c). GAGA fusion was highly susceptible to degradation. (c) GST pull-down experiment. GST alone (lane 2). GST fused with POZ domains of the indicated proteins (lanes 3–8). The input (lane 1) was 1/10th of that used for each pull-down experiment. Equal volumes of GST fusion protein conjugated beads, which contained approximately 2–4 μ g of the indicated proteins, were used in the experiment

Discussion

Five lines of evidence presented here support the conclusion that the co-repressors N-CoR and SMRT interact with BCL-6. First, a homologous subregion of both proteins was identified in a yeast two-hybrid screen as interacting specifically with BCL-6 (Figure 1). Second, the interaction was also detected in the more physiologically relevant mammalian two-hybrid assay, where we demonstrated that the POZ domain of BCL-6 is both necessary and sufficient for this interaction (Figure 2). Third, the interaction can take place *in vivo* when BCL-6 is bound to its DNA recognition site (Figure 4). Fourth, BCL-6 and N-CoR co-localized to punctate regions of the nucleus. Fifth, interaction was detected between purified BCL-6 derivatives and *in vitro* translated full length co-repressors (Figure 5).

A

B

C

D

Figure 7 POZ domain point mutations abrogate interaction with co-repressors, transcriptional repression and self association. (a) Alignment of the amino-terminal region of the POZ domains. The full 120 amino acid POZ domain is not shown. Arrows indicate the positions that were targeted for mutagenesis. Amino acid residues of BCL-6 at those positions were changed to those of Protein A. M1: L19S; M2: N23H; M3: L25S, R26L. (b) Interactions of POZ mutants in mammalian two-hybrid. Luciferase and β -gal assays were conducted on cell extracts from HeLa cells which had been transiently transfected with 500 ng of the GAL4 responsive reporter (pAH205), 50 ng of the bait expression plasmids [GAL4 DBD alone (DBD) or fusions with an intact POZ-Short domain (POZ) or POZ-Short domains with the indicated mutations (M1, M2, M3)], either 0, or 100 ng of the activator-tagged expression plasmids (VS and VN) and 25 ng of the pCMVlacZ plasmid, as a control for transfection efficiency. The reporter, the GAL4 DBD, the POZ bait (POZ-Short) and the VP16-tagged co-repressor expression plasmids, VS and VN were those used in Figure 2. Data displayed are representative of two independent experiments in which luciferase activity was normalized to β -gal activity. (c) Transcriptional repression activity by POZ mutants. Luciferase and β -gal assays were conducted on cell extracts from HeLa cells which had been transiently transfected with 50 ng of the indicated reporter plasmids, either 2.5 or 10 ng of the indicated GAL4 DBD fusions which were used as baits in b, and 25 ng of pCMVlacZ plasmid. Results represent the average of a triplicate experiment in which the luciferase activity was normalized to β -gal activity. (d) Co-immunoprecipitation of POZ mutants. Pairwise combinations of the 35 S-methionine-labeled nontagged (POZ-Long) and 9E10 epitope-tagged (POZ-Short-tag) proteins were produced by co-translation. Data shown is a 15% SDS-PAGE analysis of proteins recovered from immunoprecipitation using 9E10 antibody (lanes 5–8) and 20% of the input (lanes 1–4)

Very recently, others have reported interactions between PLZF and SMRT and N-CoR, and BCL-6 and SMRT (Dhordain *et al.*, 1997; Hong *et al.*, 1997; Grignani *et al.*, 1998; Guidez *et al.*, 1998; He *et al.*, 1998; Lin *et al.*, 1998). Here we show that BCL-6 and other POZ domain containing proteins interact with both SMRT and N-CoR suggesting that the interaction of POZ domains with N-CoR and SMRT is a general phenomenon (Figure 6).

POZ domains from several proteins have previously been shown to mediate transcriptional repression. Our identification of both co-repressors in the yeast two-hybrid screen and our demonstration that the POZ domain is necessary and sufficient for interaction with these co-repressors strongly suggests that these co-repressors are involved in BCL-6 transcriptional repression. In addition, we have identified point mutations in the BCL-6 POZ domain that fail to interact with the co-repressors, fail to repress transcription and fail to self associate. The simplest interpretation of these data is that the co-repressors interact with a dimerized POZ domain and that interaction with the co-repressor(s) is required for repression.

N-CoR and SMRT (also called TRAC-2) were originally identified as co-repressors for the unliganded thyroid hormone and retinoic acid receptors (Chen *et al.*, 1995; Horlein *et al.*, 1995; Sande *et al.*, 1996). N-CoR has also been implicated in transcriptional repression by Mad and Mxi (Alland *et al.*, 1997; Heinzel *et al.*, 1997). Our demonstration that these co-repressors can interact with the POZ domains of BCL-6, and other POZ domain proteins suggests these co-repressors are likely to be involved in repression by a variety of classes of transcriptional repressors. This is analogous to the situation with the co-activators CBP and p300, which are involved in transcriptional activation by several sets of transcriptional activators (Goldman *et al.*, 1997).

Recent studies have shown that N-CoR and SMRT can be found in multisubunit repressor complexes together with mSin3, a histone deacetylase, and a number of other proteins (Alland *et al.*, 1997; Heinzel *et al.*, 1997; Nagy *et al.*, 1997; Zhang *et al.*, 1997). The presence of deacetylase activity in these repressor complexes suggests a model in which repression by sequence-specific transcription factors can be mediated, at least in part, by recruitment of a deacetylase (Alland *et al.*, 1997; Heinzel *et al.*, 1997; Nagy *et al.*, 1997; Zhang *et al.*, 1997). We speculate that BCL-6 and other POZ-containing repressors also may modulate transcription in a similar manner. Indeed, very recently weak interaction between PLZF and Sin3A, Sin3B and HDAC1 have been detected by GST pull down (Guidez *et al.*, 1998; Lin *et al.*, 1998).

We have shown that the POZ domain of BCL-6 is the major interaction interface for both N-CoR and SMRT. Interaction of the BCL-6 POZ domain with the intact co-repressors was stronger than with the truncated co-repressors, suggesting that other regions of the co-repressors may stabilize binding to the POZ domain. In the case of PLZF, a second carboxy-terminal region of SMRT contributes to the interaction with that POZ-containing protein and regions outside of the POZ domain also contribute to interaction of PLZF with the co-repressors. (Hong *et al.*, 1997; Grignani *et al.*, 1998). Further deletional analyses will be necessary to specifically identify all regions of the

co-repressors that contribute to the interaction with the BCL-6 POZ domain.

In the GST pull down assay, the co-repressors interact more strongly with the BCL-6 POZ domain than with POZ domains from other proteins. This might reflect the relative strengths of the proteins as repressor. Alternatively, interaction *in vivo* may be stronger due to additional interacting domains or additional components of the co-repressor complexes. In support of this, Hong and coworkers also see weak *in vitro* PLZF–SMRT interaction but *in vivo*, in a two-hybrid analysis, the interaction is as strong as the RAR–SMRT interaction (Hong *et al.*, 1997).

The role the co-repressors play with other POZ domain containing proteins in other systems and species is not yet clear. GAGA, which can function as a transcriptional activator (Soeller *et al.*, 1993), is also capable of recruiting the NURF chromatin remodeling complex to the hsp70 gene (Tsukiyama and Wu, 1995; Wall *et al.*, 1995). Whether GAGA can recruit co-repressor complexes and NURF complexes under different circumstances remains to be investigated. The Protein A POZ domain is derived from the *Drosophila* mod(mdg4) gene which plays a role in position effect variegation (Dorn *et al.*, 1993; Gerasimova *et al.*, 1995). The lack of interaction of *Drosophila* protein A may reflect a subtle species-specific differences between human co-repressors and putative *Drosophila* co-repressors. If the interaction of POZ domains and co-repressors is universal, it is possible that recruitment of co-repressors and deacetylases may somehow be involved in position effect variegation. The function of the vaccinia virus protein SalF17R (Howard *et al.*, 1991) is unknown, so the relevance of the *in vitro* interaction is unclear.

Translocations involving PLZF and BCL-6 have been implicated in cancer. In rare cases of acute promyelocytic leukemia, PLZF is fused to the retinoic acid receptor (RAR), generating a protein that can interact with co-repressors via two domains, (Hong *et al.*, 1997) which may contribute to the oncogenic potential of this fusion protein. Indeed, while this paper was under review, several groups have demonstrated that the interaction of PLZF with N-CoR and SMRT is retinoic acid insensitive and this provides an explanation as to why patients with the PLZF–RAR fusion are not responsive to retinoic acid treatment (Grignani *et al.*, 1998; Guidez *et al.*, 1998; He *et al.*, 1998; Lin *et al.*, 1998). BCL-6 is presumed to be aberrantly expressed in diffuse large cell lymphomas because of the translocations and point mutations found in the 5' non-coding region of the gene in these lymphomas (Migliazza *et al.*, 1995; Ye *et al.*, 1995). Since the BCL-6 POZ domain shows the highest affinity of the POZ domains tested, it is conceivable that inappropriately expressed BCL-6 may sequester co-repressor complexes and therefore cause derepression of other key genes involved in growth control or B-cell maturation. Alternatively, the aberrant expression of BCL-6 may lead to inappropriate repression of such key genes, via recruitment of co-repressor complexes.

To date, only two potential BCL-6 responsive genes have been identified (Baron *et al.*, 1997; Dent *et al.*, 1997). However, we note that the BCL-6 binding site resembles that of the STAT proteins (Figure 3). In

addition, the WT but not the MT1 BCL-6 binding site can compete for gamma interferon induced protein complexes (which include STAT proteins) that bind to the IRF-1 GAS element and *in vitro* translated Δ BCL-6 can bind to the IRF-1 gas element (K Gustafson and V Bardwell, unpublished data). These findings suggest that BCL-6 may repress some genes regulated by the STAT proteins. Recently, Dent and coworkers have suggested a similar model and have shown that the STAT6 responsive gene, CD23, can be down regulated by BCL-6 (Dent *et al.*, 1997). The information derived from our comprehensive site selection and the similarity of the BCL-6 binding site to the STAT proteins' recognition sequence may help in the identification of other genes regulated by BCL-6 that may be involved in the generation of lymphomas.

Further elucidation of the molecular mechanism of transcriptional repression by BCL-6 with N-CoR and SMRT will help us to understand how BCL-6 functions normally and in the generation of cancer. We note that the central region of BCL-6, that is also found to be important for transcriptional repression (Albagli *et al.*, 1996; Chang *et al.*, 1996), may interact with some of the other candidate BCL-6 interacting proteins identified from our studies. Further analyses of the interactions of BCL-6 with these other candidates may reveal additional mechanisms through which BCL-6 acts to repress transcription.

Materials and methods

Plasmids

Construction was by standard methods; all plasmid structures were verified by appropriate restriction digest or sequencing. Details of plasmid constructions, beyond what is described below, are available on request.

Yeast two-hybrid BCL-6 baits: BCL-6 POZ Long, codons 1–418 were inserted into pGBT9 (Clontech); BCL-6 Δ 74, codons 74–508 were inserted into pAS1 (Durfee *et al.*, 1993) (a gift of S Elledge). Other baits: GAL4-p53 (Clontech) and GAL4-TRA-1 (a gift of D Zarkower). Activator Library: cDNAs were made from Daudi cell PolyA⁺ RNA using the Superscript Choice System (GibcoBRL) and inserted into pACTII, a derivative of pACT (Durfee *et al.*, 1993) in which the polylinker was modified (a gift of S Elledge). We call this the C-terminal library since the cDNAs are inserted Carboxy-terminal to the GAL4 activation domain in pACTII. We also made and used an N-terminal library, details of the construction of which are available upon request. Both SMRT and N-CoR clones were isolated from the C-terminal library screen.

Mammalian two-hybrid and transcriptional repression analyses Luciferase reporter plasmid for mammalian two-hybrid analyses (pAH205), containing 4 $\times$ GAL4 binding sites was kindly provided by D Bernlohr. This plasmid is a derivative of the pGL3-promoter plasmid (Promega). Luciferase reporter plasmid for repression analyses was constructed by excising a fragment containing five GAL4 binding sites from G5E1bLuc (Hsu *et al.*, 1994) (generously provided by R Baer) and inserting it into pGL3-Control plasmid (Promega). This plasmid contains the SV40 enhancer located downstream of the luciferase gene. Mammalian CMV expression vector containing GAL4 DNA binding domain (1–147) (Wilkinson and

Towle, 1997) was a gift from H Towle. BCL-6 derivatives were inserted into this vector to generate the various GAL4DBD–BCL-6 fusions. Expression construct containing herpes virus VP16 activation domain (AD) was made by inserting VP16 AD (codons 410–490) into a modified pEF-BOS (Mizushima and Nagata, 1990) vector, pEF-plink2 (a gift from R Marais). N-CoR(1019–2061) and SMRT(355–670) fragments, excised from their corresponding yeast activator library vectors were inserted into this vector to generate the VP16 AD fusions. pEF-lacZ and pCMVlacZ used as internal controls for transfection efficiency, were gifts of R Marais and H Towle respectively.

GST fusion proteins POZ domain cDNA sequences, derived from previously used plasmids (Bardwell and Triesman, 1994) or PLZF cDNA (a gift of A Zelent) were cloned into pGEX20T, a modified version of pGEX2T (Pharmacia) which contains the plink polylinker (Dalton and Treisman, 1992).

In vitro transcribed/translated proteins Expression plasmids for full length N-CoR (pCMX-N-CoR) (Horlein *et al.*, 1995) and SMRT (pCMX–SMRT) (Chen and Evans, 1995) were generously provided by M Rosenfeld and R Evans respectively. Expression constructs for N-CoR(1019–2061) and SMRT(355–670) were made by excising the fragments from their corresponding activator library vectors and cloning them into T7 β plink (Dalton and Treisman, 1992). Expression plasmids for BCL-6 derivatives were in T7 β plink and a modified version, containing a carboxy-terminal 9E10 Myc epitope-tag (Dalton and Treisman, 1992; Bardwell *et al.*, 1994), which was used for immunoprecipitation of Δ BCL-6 in the binding site selection and POZ-Short-tag in co-immunoprecipitation assays.

CAT assays CAT reporter plasmids containing four copies of either consensus or mutated BCL-6 binding sites were made by inserting the corresponding double-stranded oligonucleotides (WT: 5'-CTAGATTCCCTCGAAAGT-3'; MT1: 5'-CTAGATTCCTCCCTCAAGT-3', BCL-6 site is underlined) into pBLCAT5 vector (Boshart *et al.*, 1992). Full length BCL-6 (amino acids 1–706) was inserted into the expression vector pEFplink2.

Immunofluorescence analyses Full length BCL-6 expression plasmid and pCMX-N-CoR were described above. pCMX-N-CoR contains two copies of the 'FLAG' epitope-tag fused to the carboxy-terminal of the N-CoR cDNA.

In vitro protein-protein interaction assays

GST pull down experiments were modified from (Zamir *et al.*, 1996). *E. coli* strains harboring various GST fusion constructs were grown at 37°C to mid log phase and induced with 0.25 mM isopropylthiogalactopyranoside (IPTG) for 3 h. Cells were lysed in 1 $\times$ phosphate buffered saline (PBS) plus protease inhibitors (1 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin, 0.5 mM PMSF, and 1 mM benzamidine), by the French press method (@ 1000 p.s.i.). TritonX-100 was added to a final concentration of 1% (vol/vol) to the total lysate to aide in solubilization of the fusion proteins. The GST fusion proteins were purified by affinity chromatography on glutathione-agarose beads (Pharmacia) and retained as a 50% slurry in PBS plus protease inhibitors. Ten microliters of GST beads (pre-loaded with 2–4 μ g of GST fusion proteins) were incubated in 45 μ l binding buffer (20 mM HEPES pH 7.9, 100 mM KCl, 0.1% Nonidet P-40 (NP-40), 10% glycerol, 2 mM EDTA, 0.5% nonfat dry milk, 5 mM dithiotreitol (DTT), and protease inhibitors) with 5 μ l *in vitro* synthesized ³⁵S-methionine radiolabeled (TNT coupled

reticulocyte lysate system; Promega) SMRT or N-CoR or the truncated derivatives. After a 1 h incubation at room temperature, the beads were washed three times with binding buffer. The bound proteins were eluted by boiling beads in $2 \times$ SDS-gel loading buffer and fractionated on 8 or 12% SDS-polyacrylamide gels. Co-immunoprecipitation experiments were performed as described (Bardwell and Treisman, 1994) with the following modifications: *In vitro* synthesized ^{35}S -methionine labeled BCL-6 proteins were produced using the TNT coupled reticulocyte lysate system (Promega). Immunoprecipitates were washed twice with a modified RIPA buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 0.5% deoxycholate, 0.5% NP-40, 0.1% SDS, 10% glycerol, and 10 mM EGTA).

Yeast two-hybrid screen

To conduct the screens, *Saccharomyces cerevisiae* Y153 reporter strain (Trp⁻Leu⁻His⁻ LacZ⁻) was sequentially transformed with the bait constructs and the activator library and plated on minimal media lacking tryptophan, leucine and histidine, and containing 50 mM 3-aminotriazole. Yeast two-hybrid manipulations were carried out as described by Clontech. Positive colonies, His⁺ and LacZ⁺ (blue colonies in filter lift assays) were picked and cured of the bait plasmids by growth on SC medium lacking leucine. The activator plasmids were then isolated from yeast and transferred to *E. coli* for further manipulations using the Yeast DNA Isolation System (Stratagene). The screens were carried out on a pale blue background since the baits alone gave a low level of transcriptional activation of the integrated LacZ reporter. In addition to the 9 in-frame fusion proteins described in the results we also recovered two clones containing BCL-6 itself. In these clones the activation domain and BCL-6 were not in frame and were separated by the 5' untranslated region of BCL-6. We presume a low level of frame shift suppression occurred in this region giving rise to enough fusion protein capable of activating transcription through a homotypic POZ-POZ interaction.

Cell culture and transfections

EB3 and Hela cells were maintained in complete RPMI medium (containing 10% fetal calf serum and 0.1 mg/ml Penicillin G/Streptomycin). The EB3 and Daudi cells (both mature B cell lines derived from Burkitt's lymphomas) were from ATCC and a gift of T LeBien respectively. Transfections of EB3 cells were done by electroporation using the Electro Cell Manipulator 6000 (BTX) at the following setting: 210 V, 800 μF and 48 ohms. For each transfection, 1×10^7 cells were used along with 10 μg of reporter constructs, various amounts of effector constructs as indicated in the figure legends and 2 μg of pEFlacZ. Each transfection was adjusted to contain the same total amount of DNA by inclusion of the appropriate amount of empty parental expression vector. Transfections of Hela cells were done by the liposome-mediated method, using the Tfx-20TM reagent (Promega). Cells were plated one day prior to transfection. For immunofluorescence analyses, 4×10^5 cells were plated on coverslips in 60 mm plates. Each transfection contained 1 μg each of BCL-6 and N-CoR expression constructs along with 6 μl of Tfx-20TM reagent (yielding a 2:1 lipid to DNA ratio) in 1 ml of serum free media. After 1 h incubation at 37°C, 2 ml of complete media was

added. For mammalian two-hybrid and repression analyses, 2.5×10^5 cells were plated on 6-well plates (9.6 cm² well). Each transfection contained the amounts of reporter and effector constructs as indicated in the figure legends and 25 ng of pCMVlacZ, along with 3.6 μl of Tfx-20TM reagent (yielding a 2:1 lipid to DNA ratio) in 1 ml of serum free media. After 1 h incubation at 37°C, 2 ml of complete media was added. Each transfection was adjusted to contain the same total amount of DNA by inclusion of the appropriate amount of empty parental expression vector. For all transfections, cells were harvested 44–48 h post transfections.

Immunofluorescence analyses

Immunofluorescence analyses were done as described (Skinner *et al.*, 1997). The primary antibodies, rabbit polyclonal anti-BCL-6 (N-3, epitope corresponding to amino acids 3-484; Santa Cruz) and mouse monoclonal anti-flag M2 (Kodak) were used at 1:100 and 1:2000 dilution respectively. The secondary antibodies, goat anti-rabbit conjugated to lissamine rhodamine (Accurate Chemical and Scientific) and goat anti-mouse conjugated to Cy2 (Jackson ImmunoResearch) were both used at 1:200 dilution.

Other methods

Binding site was performed as described (Pollock and Treisman, 1990; Bardwell and Treisman 1994) with $\Delta\text{BCL-6}$ containing a carboxy-terminal myc epitope-tag. β -gal and CAT assays were conducted as described (Hill *et al.*, 1993). Site directed mutagenesis was carried out using GeneEditorTM *in vitro* Site-Directed Mutagenesis System (Promega) according to the manufacturer's instructions. Mutants were verified by sequencing. Luciferase assays were performed according to protocols provided by Promega.

Autoradiograms and SDS gels were scanned using Adobe Photoshop[®] 4.0 and the computer images inserted into the figures.

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