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Tumour induction by activated abl involves tyrosine phosphorylation of the product of the cbl oncogene

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v-*cbl* is the transforming gene of a murine retrovirus which induces pre-B cell lymphomas and myelogenous leukaemias. It encodes 40 kDa of a gag fusion protein which is localized in the cytoplasm and nucleus of infected cells. The c-*cbl* oncogene encodes a 120 kDa cytoplasmic protein and its overexpression is not associated with tumorigenesis. The c-*cbl* sequence has shown that v-*cbl* was generated by a truncation that removed 60% of the C-terminus. In this study, we carried out experiments to identify the position within cbl where the transition occurs between non-tumorigenic and tumorigenic forms. These experiments focused attention on a region of 17 amino acids which is deleted from cbl in the 70Z/3 pre-B lymphoma due to a splice acceptor site mutation. This mutation activates cbl's tumorigenic potential and induces its tyrosine phosphorylation. We also show that the expression of the v-*abl* and *bcr–abl* oncogenes results in the induction of cbl tyrosine phosphorylation, and that abl and cbl associate *in vivo*. These findings demonstrate that tyrosine-phosphorylated cbl promotes tumorigenesis and that cbl is a downstream target of the bcr–abl and v-abl kinases.

Key words: bcr–abl/cbl/leukaemia/lymphoma/tyrosine kinase

Introduction

The activation of the oncogenic potential of normal cellular proteins has resulted in the identification of numerous genes whose products are part of the biochemical circuitry that determines the growth characteristics and phenotype of eukaryotic cells. Studies of the molecular changes that reveal this oncogenic potential have provided important clues to their cellular functions by identifying the pivotal regions that control these proteins. Hence, the study of oncogenes has provided an understanding of the underlying principles of the malignant state, and continues to enhance our knowledge about the function of eukaryotic genes that are critical in regulating cell growth and development.

In recent years, we have been studying a member of the oncogene family called *cbl* (for Casitas B-lineage lymphoma). This oncogene was identified as the transforming gene of the Cas NS-1 retrovirus which arose in a mouse by a recombination between the ecotropic Cas-Br-M virus and c-*cbl* sequences (Langdon *et al.*, 1989). The Cas NS-1 virus induces predominantly pre-B cell lymphomas and occasional myelogenous leukaemias in mice, and acute transformation of immortalized rodent fibroblasts. The transforming product of Cas NS-1 is a 100 kDa gag–v-cbl fusion protein, of which 40 kDa is encoded by v-*cbl*.

The cDNAs that encompass the complete coding region of c-*cbl* have been isolated from two human T cell lymphoma libraries (CEM and HUT 78) and two mouse B-lineage lymphoma libraries (70Z/3 and AJ9) (Blake *et al.*, 1991). The sequences revealed that v-*cbl* is a markedly truncated form of c-*cbl* which encodes 355 N-terminal amino acids of the 913 amino acids in the complete protein. This finding suggested that this large truncation was involved in activating cbl's transforming potential. Searches of DNA and protein databases have not revealed any known genes with significant similarities to c-*cbl*, and as such the sequence has not provided definitive clues for a possible function. The deduced amino acid sequence does, however, reveal some distinctive features. The v-*cbl* region has a high proportion of basic amino acids (17%) and contains a possible nuclear localization sequence (NLS) of KKTK which fits the four-residue consensus sequence (Chelsky *et al.*, 1989). The N-terminal region also contains seven consecutive histidine residues and searches of protein databases have revealed that the YY1 zinc finger-containing transcription factor (Shi *et al.*, 1991) and five homeobox-containing genes encode proteins with ≥6 consecutive histidine residues. Notable features of the sequence lost in the generation of v-*cbl* are a stretch of 208 amino acids containing 23% proline and 19% serine/threonine residues, two acidic-rich domains of ~150 amino acids and a putative leucine zipper at the C-terminus. Also of interest is a region of 45 amino acids lying C-terminal to the position of the v-*cbl* termination codon that contains a protein motif related to the zinc finger (Freemont *et al.*, 1991). This motif has been named the 'RING' finger and peptides spanning this region have been shown to bind DNA in a zinc-dependent manner (Lovering *et al.*, 1993). To date, the 'RING' finger motif has been found in 27 proteins, many of which localize to the nucleus and have putative DNA-binding functions.

These features of c-*cbl* are suggestive that it encodes a nuclear protein which may function as a transcription factor. However, we recently demonstrated that the 120 kDa protein encoded by c-*cbl* is localized in the cytoplasm (Blake *et al.*, 1993) and from this it would appear that cbl could not function as a transcription factor. In contrast, the 100 kDa gag–v-cbl protein is found in both the cytoplasm and the nucleus, indicating that it must possess a functional NLS (Blake *et al.*, 1993). Furthermore, the property of v-cbl of entering the nucleus correlates

with its ability to transform NIH 3T3 fibroblasts, since the cytoplasmic $p120^{c\text{-}cbl}$ is not oncogenic when overexpressed in a retroviral vector (Blake *et al.*, 1993). These findings suggested that the C-terminal half of cbl could be responsible for its cytoplasmic retention in a manner similar to the p105 cytoplasmic precursor of NF-κB which is processed to form the nuclear p50 subunit of this transcription factor (Ghosh *et al.*, 1990; Kieran *et al.*, 1990; Blank *et al.*, 1991). The characteristics of p105 and p50, therefore, provide a precedent for the existence of a naturally truncated cbl protein that could localize to the nucleus. To date, however, we have not identified such a protein in the nucleus, nor have we been able to induce nuclear transport of cbl by treating cells with a range of growth factors, mitogens or chemicals (unpublished observations).

The *abl* oncogene product is a member of the non-receptor class of tyrosine kinases. It is localized primarily in the nucleus and, like cbl, its overexpression does not result in cellular transformation (Franz *et al.*, 1989; Jackson and Baltimore, 1989; Van Etten *et al.*, 1989). Indeed, recent evidence indicates that overexpression of c-*abl* inhibits cell growth by causing cell cycle arrest (Sawyers *et al.*, 1994). However, alterations by retroviral transduction, or chromosomal translocations to the *bcr* gene in Philadelphia chromosome-positive human leukaemias, converts abl to an oncogenic and highly active cytoplasmic tyrosine kinase (reviewed by Kurzrock *et al.*, 1988; Rosenberg and Witte, 1988; Sawyers, 1992; Wang, 1993). Furthermore, a functional tyrosine kinase domain is essential for transformation, and a direct correlation has been shown between kinase activity and the oncogenic potency of bcr–abl and v-abl products (Lugo *et al.*, 1990). Studies have revealed that a limited number of proteins are tyrosine phosphorylated in response to activated abl (Naldini *et al.*, 1986; Lugo *et al.*, 1990; Muller *et al.*, 1991; Druker *et al.*, 1992) and it is likely that one or more of these have a role in promoting the tumorigenic process initiated by abl.

In this study, we carried out experiments to identify the position within cbl where the transition occurs between non-tumorigenic and tumorigenic forms by engineering a range of C-terminal truncations. These experiments focused attention on a region of 17 amino acids which is deleted from cbl in the 70Z/3 pre-B cell lymphoma as a result of a splice acceptor site mutation. We show that this mutation promotes cbl tumorigenesis by inducing its phosphorylation on tyrosine residues, and that this oncogenic form can be mediated by activated abl.

Results

A C-terminal truncation 31 amino acids from the v-cbl termination codon does not induce acute transformation

The large C-terminal truncation that generated v-cbl is responsible for revealing the tumorigenic potential of this oncogene (Blake *et al.*, 1991, 1993). A smaller C-terminal truncation found in the HUT 78 T cell lymphoma produces a protein of 655 amino acids which does not induce transformation of NIH 3T3 fibroblasts (Blake and Langdon, 1992; Blake *et al.*, 1993). A range of *cbl* mutants were therefore prepared that encoded proteins between 357 amino acids (i.e. v-cbl) and 655 amino acids to find the point of transition between non-tumorigenic and tumorigenic forms. These experiments initially examined *cbl* constructs encoding proteins of 563, 528 and 480 amino acids. Their expression in a retroviral vector did not induce transformation of NIH 3T3 fibroblasts (Table I) and raised the possibility that truncations disrupting the RING finger motif may be required to activate cbl's tumorigenic potential. The RING finger is localized from amino acids 380 to 425, and truncated proteins of 436, 421 and 388 amino acids were examined. Table I shows that none of these proteins induced vigorous tumour growth equivalent to v-cbl, even though all three lines produced high levels of protein (data not shown). These cells did exhibit small tumours in nude mice, but their morphology *in vitro* was indistinguishable from normal fibroblasts. This finding showed that a disruption of the RING finger is not involved in activating cbl's full tumorigenic potential and that the deletion of the 31 amino acids between 357 and 388 was the crucial event that revealed v-cbl's transforming ability.

Table I. Deletion of amino acids between 388 and 357 induces acute transformation

CBL retroviruses[a]	Tumours in BALB/c nude mice[b]	Mean diameter (mm)[c]
906 aa (c-cbl)	0/3	0
655 aa	0/3	0
563 aa	0/3	0
528 aa	0/3	0
480 aa	0/3	0
436 aa	3/3	4
421 aa	3/3	5
388 aa	3/3	4
357 aa (v-cbl)	3/3	27

[a]C-terminal truncations of cbl were expressed in NIH 3T3 cells using the pJZenNeo retroviral vector.
[b]Mice were injected with 10^6 NIH 3T3 cells.
[c]Tumours were measured 20 days after injection.

cbl's transforming potential is activated by a 17 amino acid deletion in 70Z/3 pre-B lymphoma cells

The results from Table I focused attention on a 17 amino acid deletion found in a cDNA clone isolated from the 70Z/3 pre-B cell lymphoma (Blake *et al.*, 1991). This deletion occurs between amino acids 366 and 382, and was not found in clones isolated from CEM, HUT 78 and AJ9 libraries. The 51 nucleotides encoding these amino acids were removed from human c-*cbl* by site-directed mutagenesis and expressed in NIH 3T3 fibroblasts using the pJZenNeo retroviral vector. Figure 1b shows that cells infected with this retrovirus exhibited a transformed phenotype, whereas cells overexpressing normal c-*cbl* were unaltered (Figure 1a). This finding demonstrated that the deletion of these 17 amino acids is sufficient for the activation of cbl's transforming potential and may have contributed to the development of the 70Z/3 lymphoma.

The 17 amino acid deletion in 70Z/3 cells was generated by a splice acceptor site mutation

The 70Z/3 pre-B cell lymphoma arose in a (DBA/2 ×C57BL/6)F_1 mouse following injection with methyl nitrosourea (Paige *et al.*, 1978), which suggested that the deletion in cbl may have been caused by a mutation. The

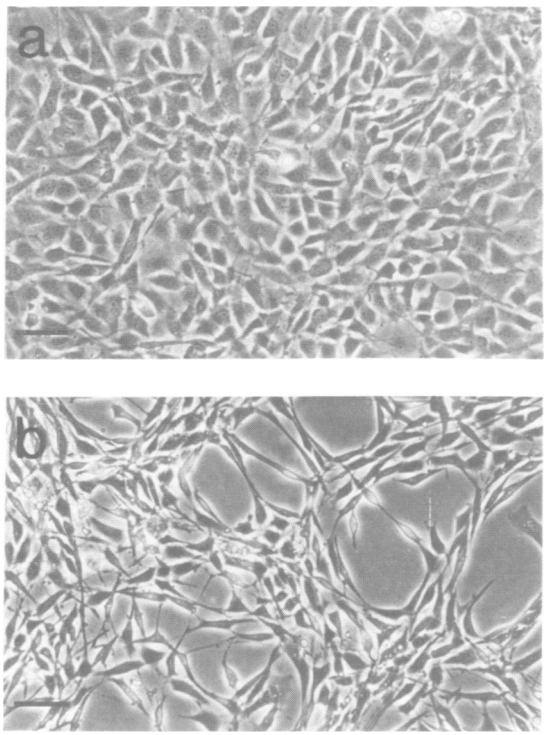

Fig. 1. Morphology of NIH 3T3 fibroblasts infected with the pJZenNeo retroviral vector expressing (**a**) the normal human c-*cbl* gene or (**b**) the human c-*cbl* gene with the 17 amino acid deletion found in 70Z/3 lymphoma cells. Scale bars indicate 100 μm.

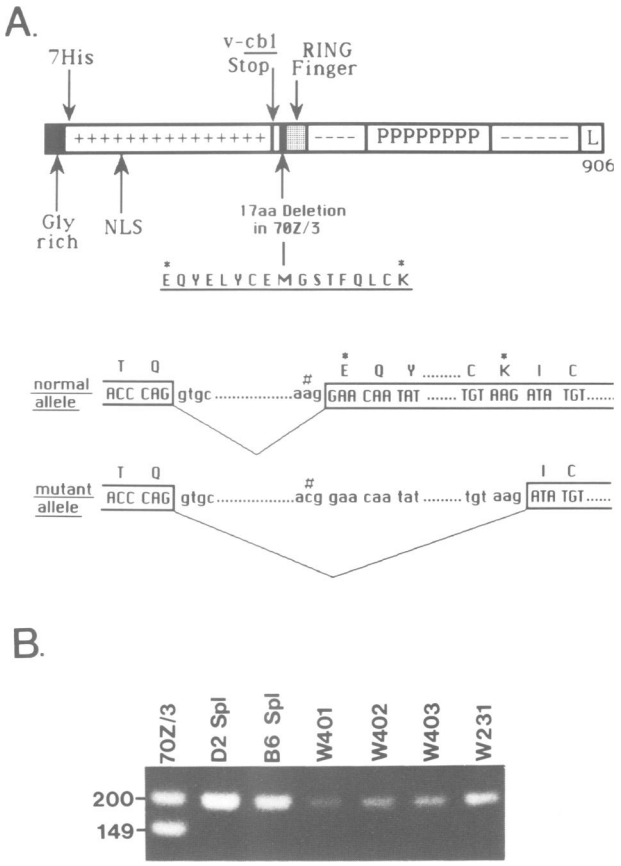

Fig. 2. The splice acceptor site mutation in 70Z/3 pre-B lymphoma cells. (**A**) Diagrammatic representation of the 17 amino acid deletion showing its position within cbl and the mechanism of the alternative splice generated by the AG→CG mutation (indicated by #). NLS: nuclear localization sequence; + or −: abundance of positively or negatively charged amino acids; P: proline-rich region; L: putative leucine zipper. (**B**) Amplification of cDNAs from 70Z/3 cells, DBA/2 and C57BL/6 spleens, and WEHI-401, -402, -403 and -231 pre-B and B cell lymphomas by the PCR showing the expected 200 bp DNA product from normal *cbl* and the 149 bp product generated by the alternative splice.

sequence of the 17 amino acid deletion is shown in Figure 2A. These amino acids are encoded by the first 51 nucleotides of an exon (M.Shapiro, personal communication). Sequencing of genomic DNAs from DBA/2 and C57BL/6 mice, and 70Z/3 cells across the intron/exon boundary, revealed that one allele from 70Z/3 had undergone an AG→CG mutation at the splice acceptor site (Figure 2A). This generated an alternative splice to the first AG within this exon which occurs at an AAG codon for lysine (amino acid 17 in this exon). The alternative splice therefore deletes codons for 17 amino acids, but does not result in a frameshift.

The alternative splice in 70Z/3 cells was also demonstrated by polymerase chain reaction (PCR) amplification of cDNA using primers that span the deletion and generate a product of 200 bp. The results in Figure 2B show that the predicted 200 bp product is present in 70Z/3 cells, DBA/2 and C57BL/6 spleen, WEHI-401, -402 and -403 pre-B cell lymphomas, and the WEHI-231 B cell lymphoma. However, an additional product consistent with the 51 bp deletion is only present in cDNA amplified from 70Z/3 cells.

The deletion of Y368 or Y371 activates cbl tumorigenesis

The region deleted from c-*cbl* in 70Z/3 cells encodes the amino acids EQYELYCEMGSTFQLCK, which are localized from 366 to 382 in human cbl. Further analysis of this region involved site-directed mutagenesis of human c-*cbl* to delete sequences encoding tyrosine 368, tyrosine 371 (i.e. Y368 and Y371) or amino acids 376–382 (i.e. STFQLCK), as well as substituting Y368 and Y371 with phenylalanine residues. Results from experiments examining the ability of these mutants to transform NIH 3T3 fibroblasts are outlined in Figure 3 and Table II. These show that the singular deletion of either Y368 or Y371 is sufficient to activate cbl to induce transformation. Cbl retroviruses expressing either of the tyrosine deletions or the 70Z/3 deletion, showed a high efficiency for colony formation in soft agarose and an ability to promote tumour growth in nude mice (Table II). The soft agarose assays indicate that the tyrosine deletions possess a greater oncogenic potency than v-cbl (Table II and Figure 3g−j). Furthermore, the first signs of morphological transformation of NIH 3T3 fibroblasts infected with retroviruses containing the deleted sequences preceded that of v-cbl-infected cells by 3 days (i.e. 5 days compared with 8 days). As previously shown, overexpression of normal cbl did not transform NIH 3T3 fibroblasts (Blake *et al.*, 1993).

These findings prompted the substitution of tyrosines 368 and 371 with phenylalanine residues (i.e. Y368F and Y371F). Table II shows that neither substitution activated cbl's ability to induce transformation to the extent of the tyrosine deletions. Therefore, the mechanism by which the tyrosine deletions convert cbl to its oncogenic form is

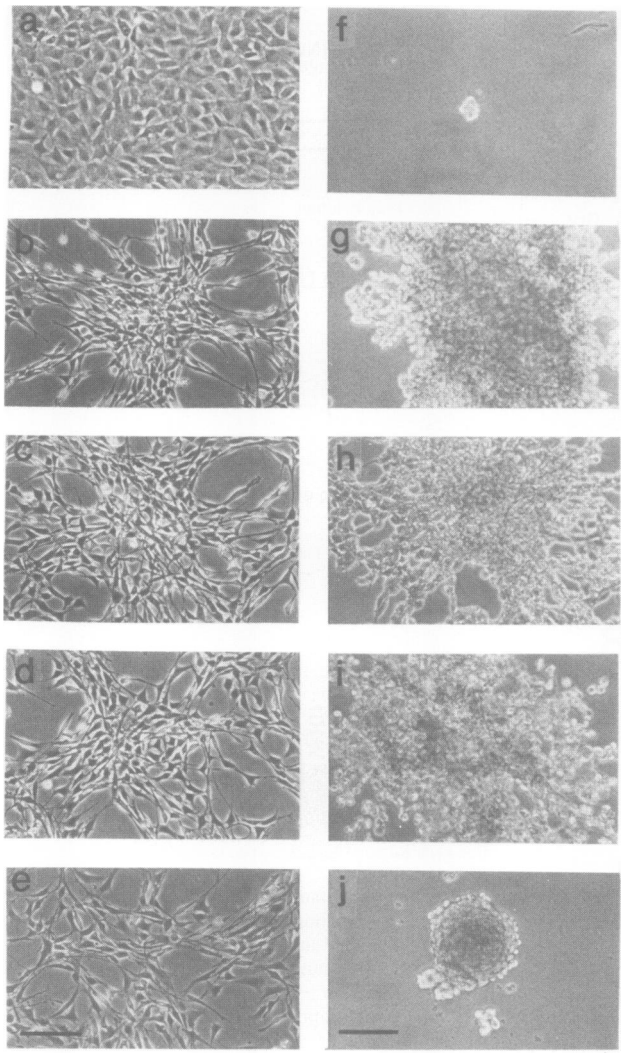

Fig. 3. NIH 3T3 fibroblast morphology (a–e) and anchorage-independent growth in soft agarose (f–j) following infection with the pJZenNeo retroviral vector expressing normal cbl (**a** and **f**), cbl with the 17 amino acid deletion in 70Z/3 cells (**b** and **g**), cbl with the deletion of tyrosine 368 (**c** and **h**), cbl with the deletion of tyrosine 371 (**d** and **i**), and v-cbl (**e** and **j**).

Table II. Transformation of NIH 3T3 cells by CBL retroviruses

CBL retroviruses[a]	Colony-forming efficiency in soft agarose[b] (%)	Tumours in BALB/c nude mice[c]	Mean diameter (mm)[d]
c-cbl	<0.001	0	0
c-cbl (70Z del)	29	3/3	24
c-cbl (Y368 del)	31	3/3	28
c-cbl (Y371 del)	25	3/3	24
c-cbl (Y368F)	2	1/3	2
c-cbl (Y371F)	4	0/3	0
c-cbl (376–382 del)	20	3/3	17
v-cbl	7	3/3	29

[a]cbl clones were expressed in NIH 3T3 cells using the pJZenNeo retroviral vector.
[b]Macroscopic colonies were counted 20 days after plating 1000 cells in 0.33% soft agarose.
[c]Mice were injected with 10^6 NIH 3T3 cells.
[d]Tumours were measured 20 days after injection.

not due to the removal of an ability of these residues to undergo phosphorylation, i.e. it is unlikely that phosphorylation of Y368 or Y371 has a role in maintaining cbl in an inactive state.

An additional mutation involved the deletion of amino acids 376–382. Expression of this form of cbl also induced transformation, but with a slightly reduced efficiency for colony formation in soft agarose and smaller tumour growth in nude mice compared with the tyrosine deletions (Table II). This region contains serine and threonine residues which are potential sites for phosphorylation, and it will be important to determine whether mutating these can promote transformation. This result indicates that tyrosines are not the sole residues of this region that have a crucial function in controlling cbl's potential to promote tumorigenesis.

The 70Z/3 mutant form of cbl is localized in the cytoplasm

The effect of the 17 amino acid deletion on cbl's ability to induce transformation is indicative of a marked alteration to the cbl protein. A possible consequence could be an altered subcellular localization, since we previously demonstrated that v-*cbl*-encoded protein has an ability for nuclear transport (Blake *et al.*, 1993). Figure 4A shows cytoplasmic and nuclear fractions of NIH 3T3 fibroblasts infected with a retrovirus expressing either normal cbl or the mutant 70Z/3 form of cbl. A Western blot of these fractions with a cbl antibody showed that cbl in both cell lines is exclusively cytoplasmic. The cytoplasmic localization of the 70Z/3 form of cbl was also evident by immunofluorescence (Figure 4C). These experiments indicate that the 70Z/3 mutation does not alter cbl's localization and that cbl-induced transformation can be mediated from the cytoplasm.

Oncogenic forms of cbl are tyrosine phosphorylated

The mutations in cbl that promote transformation were also found to induce tyrosine phosphorylation of cbl. Figure 5 shows that the 17 amino acid deletion, and the Y368 and Y371 deletions, have a marked effect on the amount of cbl protein which is tyrosine phosphorylated. This was demonstrated by immunoprecipitating cbl protein from lysates of cells infected with the pJZenNeo retroviral vector expressing normal cbl or the three deleted forms of cbl, followed by Western blotting with phosphotyrosine antibodies (Figure 5A). The relative amounts of cbl protein in each of the immunoprecipitates were shown by blotting with cbl antibodies (Figure 5A). This showed that only a small fraction of the overexpressed normal cbl protein is tyrosine phosphorylated, whereas up to 100% of the mutant proteins appear to have phosphorylated tyrosine residues. This effect was also observed by immunoprecipitation with phosphotyrosine antibodies and Western blotting with cbl antibodies (Figure 5B). These results show that mutations in this region induce tyrosine phosphorylation of cbl and that this correlates with an ability to promote tumorigenesis.

Tyrosine phosphorylation of cbl by abl

The findings described above indicate that a tyrosine kinase plays a role in cbl-induced tumorigenesis. The

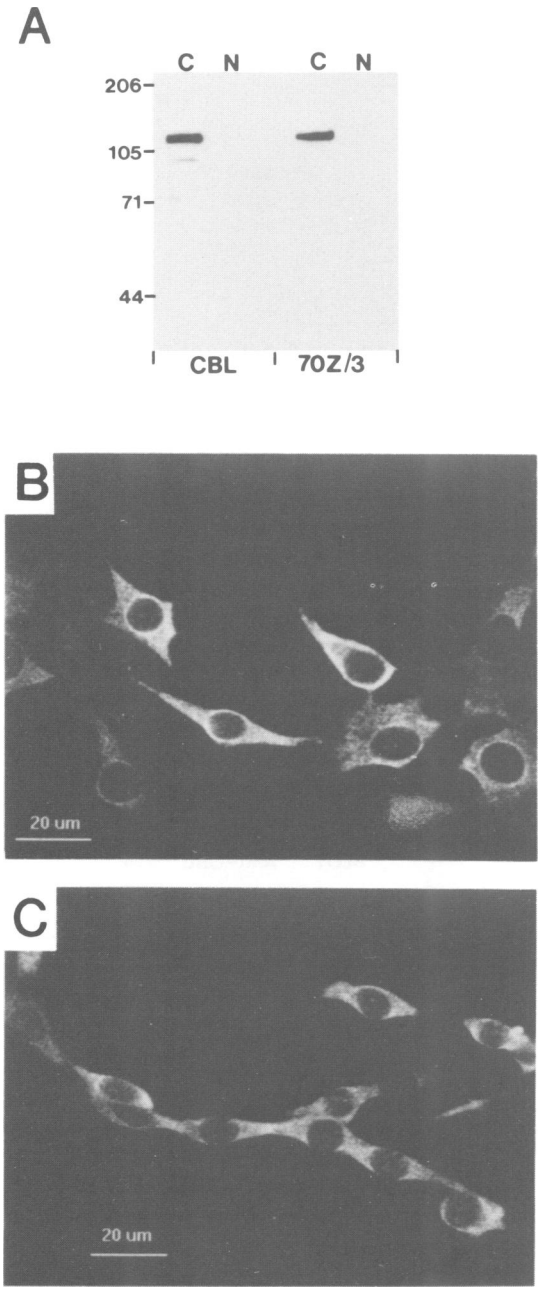

Fig. 4. Cytoplasmic localization of normal cbl and mutant 70Z/3 cbl. (**A**) Western blot of cytoplasmic and nuclear fractions from NIH 3T3 fibroblasts infected with the pJZenNeo retroviral vector expressing normal cbl or 70Z/3 mutant cbl. The membrane was probed with affinity-purified R2 cbl antibodies which recognize the C-terminal region of cbl (Blake *et al.*, 1993). The immunofluorescence shows NIH 3T3 fibroblasts infected with pJZenNeo expressing normal cbl (**B**) or 70Z/3 cbl (**C**). Cbl protein was detected using affinity-purified R2 cbl antibodies and sheep anti-rabbit immunoglobulin conjugated with FITC. The images were obtained by confocal laser microscopy.

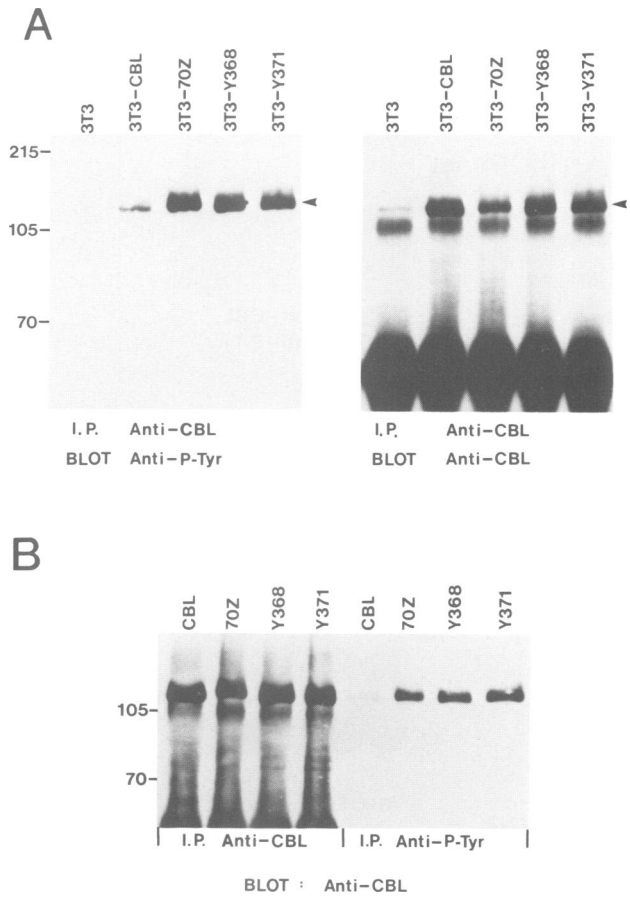

Fig. 5. Tyrosine phosphorylation of oncogenic forms of cbl. Lysates were prepared from NIH 3T3 fibroblasts (3T3) or NIH 3T3 fibroblasts infected with the pJZenNeo retrovirus expressing human c-cbl (CBL), human c-cbl with the 70Z/3 deletion (70Z), human c-cbl with tyrosine 368 deleted (Y368) or human c-cbl with tyrosine 371 deleted (Y371). The lysates were subjected to immunoprecipitation with rabbit anti-cbl antibodies (R2) or mouse anti-phosphotyrosine antibodies (4G10). Bound proteins were resolved by SDS–PAGE and analysed by Western blotting with either of the above antibodies as indicated. In (**A**), cbl immunoprecipitates were initially analysed by blotting with anti-phosphotyrosine antibodies. The membrane was subsequently reprobed with anti-cbl antibodies. In (**B**), lysates were divided equally and immunoprecipitated with either anti-cbl antibodies or anti-phosphotyrosine antibodies. Bound proteins were analysed by blotting with anti-cbl antibodies.

possibility that an activated abl tyrosine kinase could phosphorylate cbl was investigated since abl is similarly involved in the development of pre-B cell lymphomas and myelogenous leukaemias. This was tested by comparing tyrosine phosphorylation of cbl in normal NIH 3T3 fibroblasts and NIH 3T3 fibroblasts infected with the Abelson murine leukaemia virus (A-MuLV). Figure 6 shows that tyrosine phosphorylation of cbl in normal fibroblasts is not detectable (lane 1), whereas the A-MuLV-transformed fibroblasts have an abundant level of tyrosine-phosphorylated cbl (lane 2). This effect of the v-abl kinase was also demonstrated by A-MuLV infection of NIH 3T3 fibroblasts overexpressing cbl (lane 4). This showed that A-MuLV could induce tyrosine phosphorylation of cbl to a level equivalent to that observed with the deletion of Y368 (lane 5). In this experiment, the cells overexpressing normal cbl were infected with A-MuLV 2 days before phosphotyrosine analysis.

The relationship between tyrosine phosphorylation of cbl and the presence of an activated abl tyrosine kinase was further investigated by analysing a range of murine and human tumour lines. Figure 7 shows that the presence of v-abl in the murine ABLS-8 pre-B cell lymphoma, and bcr–abl in the human chronic myelogenous leukaemia cell line K562, corresponds with the tyrosine phosphorylation of cbl. In contrast, the murine BAMC1 pre-B cell lymphoma line which was induced by the v-*bas* oncogene

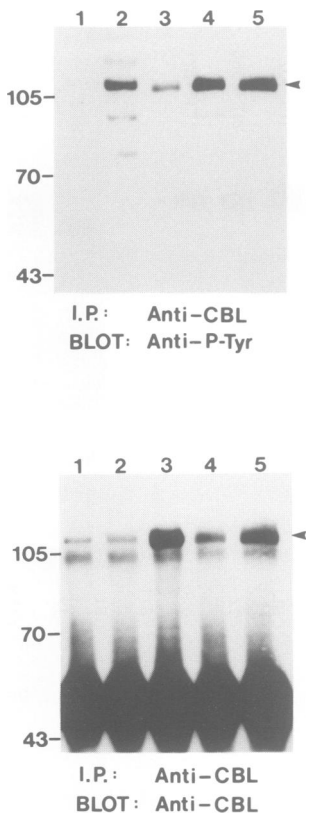

Fig. 6. Tyrosine phosphorylation of cbl induced by A-MuLV infection. Lysates prepared from NIH 3T3 cells (lane 1) or NIH 3T3 cells infected with A-MuLV (lane 2), pJZenNeo-c-cbl (lane 3), pJZenNeo-c-cbl and A-MuLV (lane 4), pJZenNeo expressing Y368-deleted c-cbl (lane 5) were subjected to immunoprecipitation with rabbit anti-cbl antibodies. Bound proteins were resolved by SDS−PAGE and analysed by Western blotting with anti-phosphotyrosine antibodies (upper panel) or anti-cbl antibodies (lower panel).

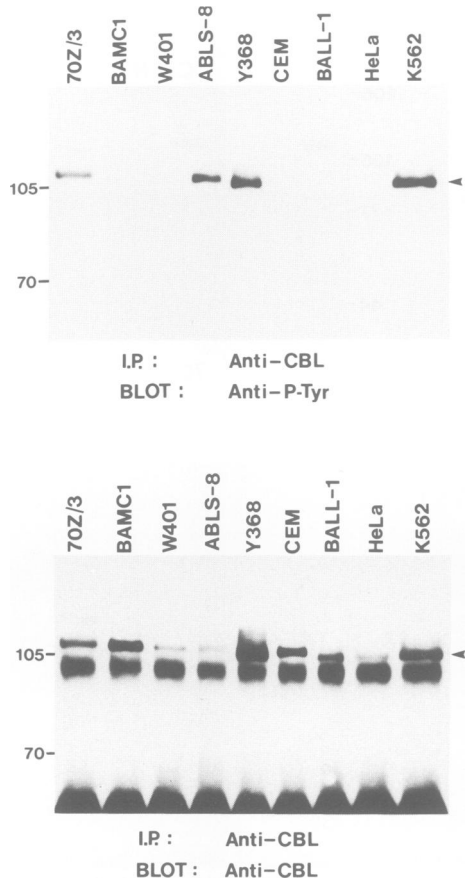

Fig. 7. Tyrosine phosphorylation of cbl in tumour cells expressing v-*abl*, *bcr−abl* or mutant *cbl*. Cell lysates were subjected to immunoprecipitation with rabbit anti-cbl antibodies, and bound proteins resolved by SDS−PAGE and analysed by Western blotting with anti-phosphotyrosine antibodies (upper panel) or anti-cbl antibodies (lower panel). The same membrane was used for both antibody blots. 70Z/3, BAMC1, WEHI-401 and ABLS-8 are murine pre-B cell lymphomas. BAMC1 was induced by the BALB sarcoma virus carrying the *ras*-related oncogene v-*bas*. WEHI-401 arose in an Eμ-*myc* transgenic mouse and ABLS-8 was induced by the A-MuLV. Y368 refers to NIH 3T3 fibroblasts infected with pJZenNeo expressing human cbl with tyrosine 368 deleted. CEM and BALL-1 are human T and B cell leukaemia lines, respectively, and HeLa cells are from a cervical carcinoma. K562 is a chronic myelogenous leukaemia line expressing $p210^{bcr-abl}$.

(Holmes *et al.*, 1986), and the WEHI-401 pre-B cell lymphoma from an Eμ-*myc* transgenic mouse (Adams *et al.*, 1985), showed no evidence of tyrosine phosphorylation of cbl. Similarly, the human T cell leukaemia line CEM (Foley *et al.*, 1966) and the B cell acute lymphocytic line BALL-1 (Hiraki *et al.*, 1977) exhibited undetectable levels of tyrosine-phosphorylated cbl. HeLa cells revealed a very low level of cbl tyrosine phosphorylation following a long exposure of the autoradiograph. The results in Figures 6 and 7 clearly show a relationship between the presence of an activated *abl* oncogene and the tyrosine phosphorylation of cbl. It is also noteworthy that the proportion of tyrosine-phosphorylated cbl in 70Z/3 pre-B cells appears to be less compared with A-MuLV-infected cells, K562 cells or NIH 3T3 fibroblasts overexpressing the Y368 deleted cbl. This is consistent with only half the cbl protein in 70Z/3 cells undergoing tyrosine phosphorylation, i.e. the cbl protein translated from the mutant allele.

Abl associates with cbl in vivo

The relationship between abl and cbl was investigated further by the immunoprecipitation of abl in ABLS-8, K562 and 70Z/3 cells, followed by Western blotting with cbl antibodies (Figure 8). This experiment clearly demonstrates an association between abl and cbl in these cells, thereby providing additional evidence that abl is involved in the tyrosine phosphorylation of cbl. An association was also detected in BAMC1 pre-B lymphoma cells where neither abl nor cbl are tyrosine phosphorylated (data not shown). This suggests that the tyrosine phosphorylation of abl and cbl is not required to effect their association, but mutations to either can act by deregulating their respective roles as a kinase and a kinase target. The identity of the additional bands detected by anti-cbl antibodies in the abl immunoprecipitates is unclear, but the presence of a faster migrating species suggests that a cleaved cbl protein is associating with abl.

Discussion

The findings from this study show that deregulated tyrosine phosphorylation of cbl promotes tumorigenesis and that this can be achieved by mutations to cbl or by the expression of activated forms of the abl tyrosine kinase.

The clue that led to these findings came from a mutant form of cbl in the 70Z/3 pre-B cell lymphoma. These

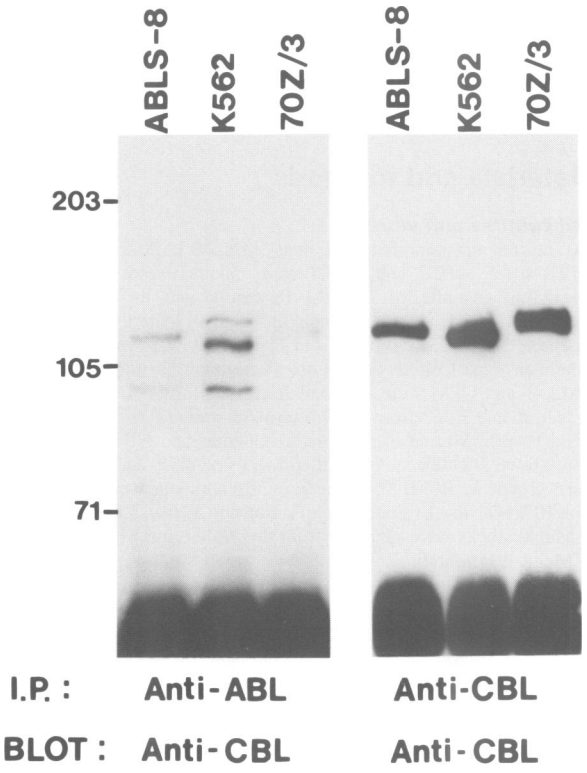

Fig. 8. Co-immunoprecipitation of abl with cbl. Cell lysates were subjected to immunoprecipitation with rabbit anti-abl antibodies or rabbit anti-cbl antibodies, and bound proteins were resolved by SDS−PAGE and analysed by Western blotting with rabbit anti-cbl antibodies. ABLS-8 and K562 express the v-*abl* and *bcr−abl* oncogenes, respectively, and 70Z/3 carries a mutant form of the *cbl* oncogene.

cells represent a prototypic pre-B cell line which has been invaluable for many years in the study of B-cell development. However, this lymphoma was induced by a chemical carcinogen and the genetic alterations that promoted tumour development have not been identified (Paige *et al.*, 1978). The results presented here demonstrate that a mutation in the *cbl* oncogene was generated at a splice acceptor site which deletes 17 amino acids to produce an acutely transforming cbl protein. This finding has, therefore, provided an insight into how the 70Z/3 lymphoma arose and revealed a mechanism for cbl activation.

The likely involvement of cbl in the generation of the 70Z/3 pre-B cell lymphoma is further evidence of its specificity for inducing tumours at this stage of B-cell development. The reason for this specificity is unknown, but recent evidence from A-MuLV-infected pre-B cells suggests that a direct block to immunoglobulin light-chain gene rearrangement is an important factor in holding these cells at this stage of development (Chen *et al.*, 1994; Klug *et al.*, 1994). It will be interesting to test whether cbl has the same effect on B cell differentiation. The broader question of why abl and cbl have a preference for transforming cells within the B and myeloid lineages remains to be resolved.

Tyrosine-phosphorylated cbl is oncogenic

The study of the oncogenic 70Z/3 form of cbl has revealed that the 17 amino acid deletion induces deregulated tyrosine phosphorylation. The results show that a high proportion of the mutated cbl protein is tyrosine phosphorylated compared with very low or undetectable levels of the normal protein (Figures 5A and B, 6 and 7). This marked effect demonstrated that a tyrosine kinase is involved in the activation of cbl's potential to participate in tumorigenesis and this could be due to structural changes that allow the kinase access to sites of phosphorylation. Thus, the control of tyrosine phosphorylation in normal cbl may involve intra- or intermolecular associations which are presumed to be disrupted in 70Z/3 cells. Whatever the mechanism, it is clear that this mutation has a profound effect on cbl and the key to future studies will be to determine the function of this activated form.

Results presented here also show that the deletion of either of two tyrosine residues (Y368 and Y371) can produce the same effect as deleting the complete 17 amino acid region. These amino acids were initially targeted in preference to others because of the link between tyrosine residues and the control of cell growth and tumorigenesis. It is conceivable, however, that the deletion of any single amino acid within this small region could cause a similar disruption to the cbl protein and thus permit deregulated tyrosine phosphorylation. Indeed, the deletion of amino acids from 376 to 382 was also effective in promoting tumorigenesis (Table II), which indicates that the tyrosine residues are not the sole controllers within this region. Furthermore, phosphorylation of tyrosines 368 and 371 does not appear to be a factor in the regulatory role of this region since the phenylalanine substitutions had a minimal effect in activating cbl transformation (Table II).

Cytoplasmic cbl can be oncogenic

As outlined previously, the *cbl* oncogene has features that suggest it may encode a nuclear protein (Blake *et al.*, 1991; Lovering *et al.*, 1993). Furthermore, it lacks a kinase domain, and Src homology 2 and 3 (SH2 and SH3) domains which are more commonly found in cytoplasmic rather than nuclear proteins involved in signal transduction (reviewed by Cantley *et al.*, 1991; Koch *et al.*, 1991). These features, and the nuclear localization of gag−v-cbl (Blake *et al.*, 1993), raised the possibility that tyrosine-phosphorylated cbl might localize to the nucleus. However, the results presented here show no evidence of cbl protein in the nucleus (Figure 4). This demonstrates that cbl can promote tumorigenesis by participating in a cytoplasmic signal transduction pathway. It also suggests that the mechanism of v-cbl transformation may differ from that of mutant cbl in 70Z/3 cells. Indeed, we have found no evidence of v-cbl tyrosine phosphorylation (C.Andoniou and W.Langdon, unpublished), suggesting it may bypass steps in the pathway activated by tyrosine-phosphorylated cbl. The absence of tyrosine phosphorylation is not surprising since v-cbl lacks 10 of 21 tyrosine residues and it is probable that it has lost sequences essential for tyrosine kinase binding, e.g. the proline-rich domain.

Therefore, although these findings have not resolved the issue of cbl protein in the nucleus, they have identified a form that should be investigated further following the treatment of cells with reagents that stimulate signal transduction pathways.

cbl is a target for tyrosine phosphorylation by abl

The results presented here have provided evidence that cbl is phosphorylated by the abl tyrosine kinase, and that this occurs in a deregulated manner when either cbl is mutated, e.g. in 70Z/3 cells, or when the abl kinase is activated by retroviral transduction or chromosomal translocation to the *bcr* gene. The initial evidence came from experiments showing that tyrosine phosphorylation of cbl is induced in tumour cells expressing v-*abl* or *bcr–abl*, but not in tumours where an activated *abl* oncogene is not present (Figures 6 and 7). The important connection with these observations is that the oncogenic form of cbl is tyrosine phosphorylated. These results have, therefore, identified a target for activated abl's capacity to induce tumorigenesis. To date, other abl targets that have been identified are rasGAP, the two GAP-associated proteins p190 and p62, and Shc (Ellis *et al.*, 1990; Druker *et al.*, 1992; Matsuguchi *et al.*, 1994, Puil *et al.*, 1994).

An additional connection that makes these findings intriguing is the similarities of the tumours induced by abl and cbl. Both oncogenes were discovered in acutely transforming retroviruses that induced pre-B cell lymphomas in mice (Abelson and Rabstein, 1970; Goff *et al.*, 1980; Langdon *et al.*, 1989). Furthermore, ~10% of the v-*cbl*-induced tumours in mice are myelogenous leukaemias that express macrophage surface markers and do not have rearranged immunoglobulin heavy-chain genes (Langdon *et al.*, 1989, H.C.Morse and W.Langdon, unpublished). This tumour type is similar to the chronic myelogenous leukaemias induced by the p210 encoded by *bcr–abl* (Daley *et al.*, 1990; Gishizky *et al.*, 1993).

More direct evidence for cbl phosphorylation by abl has come from their co-immunoprecipitation in lysates from ABLS-8, K562 and 70Z/3 cells (Figure 8). This suggests that cbl is a target for tyrosine phosphorylation by abl and that mutations to either activate a common pathway leading to tumorigenesis. It will be important to determine how activated forms of abl and cbl can overcome the regulatory controls that normally prevent the constitutive tyrosine phosphorylation of cbl. It is likely that this could occur by disrupting the function of an inhibitory protein which is predicted to interact through the region deleted from cbl in 70Z/3 cells. Further analysis of how this inhibition is overcome could have important implications for studies aimed at perturbing bcr–abl-mediated tumorigenesis.

The observation that abl and cbl can interact *in vivo* is interesting in the light of two independent investigations involving abl and cbl with the nck adaptor protein. Nck was isolated from a melanoma cDNA library (Lehmann *et al.*, 1990) and contains three SH3 domains and one SH2 domain, but no catalytic domain. Overexpression of nck results in the transformation of fibroblast cell lines (Chou *et al.*, 1992; Li *et al.*, 1992). It has recently been shown that nck associates with abl through one or more of its SH3 domains by an interaction with a proline-rich motif present in abl (Ren *et al.*, 1994). Nck SH3 domains have also been found to bind specifically to the C-terminal half of cbl which encompasses the proline-rich region (Rivero-Lezcano *et al.*, 1994). This interaction was discovered by screening an expression library with a glutathione-*S*-transferase fusion protein containing the SH3 domains of nck. It was also demonstrated that nck and cbl associate *in vivo*. These findings suggest that abl and cbl could interact through nck, and it will be important to determine if nck has a role in abl- and cbl-mediated tumorigenesis.

Materials and methods

Cell cultures and viruses

The murine haemopoietic cell lines ABLS-8.1, 70Z/3, WEHI-231.1, WEHI-401.1, -402.2 and -403.3 were obtained from the Walter and Eliza Hall Institute of Medical Research, and the BAMC1 pre-B lymphoma line from the National Institute of Allergy and Infectious Diseases. NIH 3T3 fibroblasts and HeLa cells were obtained from the American Type Culture Collection. The human haemopoietic cell lines BALL-1 and CEM were obtained from the Hanson Centre for Cancer Research, and K562 from the Western Australian Research Institute for Child Health. Murine haemopoietic cell lines and NIH 3T3 fibroblasts were grown in Dulbecco's modified Eagles medium and the human cells were grown in RPMI-1640 medium. Both media were supplemented with 10% heat-inactivated fetal calf serum and 50 mM 2-mercaptoethanol. A-MuLV stocks were obtained from the Walter and Eliza Hall Institute of Medical Research.

Antibodies

Rabbit anti-cbl antibodies (R2) have been described previously (Blake *et al.*, 1993). The mouse monoclonal anti-phosphotyrosine antibody (4G10) was purchased from UBI and the polyclonal anti-abl antibody (K12) from Santa Cruz. Peroxidase-conjugated and fluorescein isothiocyanate (FITC)-conjugated antibodies were obtained from Silenus, Australia.

Construction of cbl mutants and retroviral transformation of NIH 3T3 fibroblasts

All cbl mutations were prepared by oligonucleotide-directed mutagenesis using the pAlter plasmid (Promega). Mutations were confirmed by DNA sequencing (Sequenase, USB) and subcloned into the pJZenNeo retroviral vector (Johnson *et al.*, 1989). The retroviral DNAs were electroporated into Ψ2 packaging cells and the supernatants collected for the infection of NIH 3T3 fibroblasts using methods outlined previously (Blake *et al.*, 1993).

Infected NIH 3T3 cells were selected in geneticin (Gibco BRL) and tested for transformation by anchorage-independent growth in 0.33% soft agarose, and by s.c. inoculation of 8-week-old BALB/c *nu/nu* mice with 10^6 cells. The macroscopic colonies were counted 20 days after plating 10^3 cells in soft agarose. The diameter of tumours in the nude mice were measured 20 days after injection.

Immunoprecipitations

Cells (5×10^6) were washed in Tris-buffered saline [TBS; 150 mM NaCl, 10 mM Tris (pH 8.0)] and then lysed in 1 ml of ice-cold TBS containing 1% Triton X-100, 1 mM EDTA, 1 mM sodium orthovanadate, 10 μg/ml aprotinin and 1 μg/ml chymostatin, leupeptin, antipain and pepstatin. Cell debris and nuclei were removed by centrifugation at 10 000 r.p.m. in an Eppendorf centrifuge for 5 min at 4°C. The appropriate antibody was then added to the lysates and incubated for 2 h at 4°C, followed by incubation for 3 h with protein A–Sepharose (Pharmacia). The immune complexes were washed three times in lysis buffer before suspension and boiling in Laemmli sample buffer.

Western blot analysis

Immune complexes were electrophoretically separated on 10% SDS–polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked in TBS containing 5% powdered milk and 0.1% Tween 20 for 1 h before the addition of the primary antibody. For the phosphotyrosine antibody blots, the membranes were blocked overnight in TBS containing 10% powdered milk, 1% bovine serum albumin (BSA) and 0.1% Tween 20. The primary antibodies were incubated for 1 h at room temperature and the unbound antibodies were removed by four 15 min washes. Bound antibodies were visualized using horseradish peroxidase-conjugated antibodies and ECL reagents, as described by the manufacturer (Amersham).

Immunofluorescence

Cells were fixed in absolute methanol for 5 min and acetone for 2 min at −20°C. Blocking procedures and staining with cbl antibodies have been

described previously (Blake *et al.*, 1993). Conventional epifluorescence microscopy was carried out on a Nikon Diaphot 300 fluorescence microscope and confocal laser microscopy was performed on an MRC-1000 system (Bio-Rad Corp.). Confocal images were captured using a Focus Image Corder Plus and Ilford 100 delta black and white film.

Isolation of genomic DNA

Single-cell suspensions prepared from 70Z/3 cells and from DBA/2 and C57BL/6 mouse thymus were pelleted and resuspended in phosphate-buffered saline (PBS) (10^8 cells/ml). The cells and their nuclei were lysed in 10 vols of 6 M guanidine–HCl/0.1 M sodium acetate (pH 5.2) on a rotating wheel at 4°C for 2 h, and the resulting cell lysate layered under 2.5 vols of 100% ethanol at room temperature. Following gentle mixing, genomic DNA was isolated by spooling onto a glass rod, washed twice in 70% ethanol and dissolved overnight in 0.5 ml TE buffer at 4°C. The DNA was quantitated spectrophotometrically at 260 nm and an aliquot checked by electrophoresis through a 0.8% agarose gel.

cDNA synthesis

Total RNA was isolated from cultured cells or from DBA/2 and C57BL/6 mouse spleen by the acid guanidinium thiocyanate–phenol–chloroform extraction method described by Chomczynski and Sacchi (1987). One microgram of total RNA (8 µl) was heated to 65°C for 10 min with 2 µl random decanucleotide primers (Bresatec Gigaprime kit) and snap-chilled on ice. cDNA was synthesized in a 30 µl reaction by incubating the annealed RNA/primers in a buffered solution of 50 mM Tris (pH 8.3), 50 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 0.5 mM spermidine, 1 mM each dNTP, 25 U RNasin (Promega), 40 mM sodium pyrophosphate and 15 U AMV reverse transcriptase (Promega) at 42°C for 90 min, followed by heat inactivation of the enzyme at 65°C for 10 min.

Polymerase chain reaction

Approximately 100 ng of genomic DNA or 2 µl of the cDNA synthesis reaction were used as templates for amplification by the PCR. Reactions were carried out in 100 µl volumes containing 10 mM Tris (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 1 mg/ml BSA, 0.1% Triton X-100, 50 mM each dNTP and 50 pmol each of the forward and reverse primers (primers 1 and 3 for cDNA, primers 2 and 3 for genomic DNA). The tubes were heated to 94°C for 5 min, and 1 U of Taq DNA polymerase (Promega) added. The PCR was allowed to proceed for 30 cycles of denaturation (94°C, 30 s), annealing (55°C, 45 s) and extension (72°C, 1 min), with an extension time of 5 min for the final cycle. Primer sequences are as follows (introduced *Eco*RI restriction sites are in italics):

Primer 1: 5′ ACTGATTGATGGCTTCAGG 3′

Primer 2: 5′ AA*GAATTC*GGTTTATGTGAACCAACTCC 3′

Primer 3: 5′ AA*GAATTC*ACATCCTTATCATTCTCAGC 3′

For cloning, genomic PCR products were purified from 1% low-melting agarose gels by phenol/chloroform extraction and ethanol precipitation, digested with *Eco*RI and ligated into an *Eco*RI-digested pGEM-4Z vector (Promega). Resulting clones were sequenced using the Sequenase kit (USB).

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