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The truncation that generated the v-*cbl* oncogene reveals an ability for nuclear transport, DNA binding and acute transformation

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The v-*cbl* oncogene is the transforming gene of the murine Cas NS-1 retrovirus which induces pre-B cell lymphomas and myeloid leukaemias. Sequencing of c-*cbl* has revealed that v-*cbl* was generated by a large truncation that removed 60% of the C-terminus of the corresponding protein. In this study we prepared antibodies to cbl and found that c-*cbl* encodes a 120 kDa protein which is localized in the cytoplasm with a cytosolic and cytoskeletal distribution. Immunofluorescence studies show a striking pattern of brightly staining vesicles in mitotic cells similar to that observed with cytokeratin antibodies. In contrast to $p120^{c\text{-}cbl}$, which is exclusively cytoplasmic, the $p100^{gag\text{-}v\text{-}cbl}$ encoded by Cas NS-1 is localized in both the cytoplasm and the nucleus. This redistribution to the nucleus correlates with the ability of *cbl* to induce acute transformation. Furthermore the truncated protein encoded by v-*cbl* can bind DNA, unlike the full-length protein. These results suggest that the C-terminus of cbl is involved in the retention of $p120^{c\text{-}cbl}$ in the cytoplasm and the inhibition of DNA binding. The findings also suggest that a truncated protein encoded by c-*cbl* exists in the nucleus of normal cells.

Key words: cbl/DNA binding/NF-κB/nuclear localization/oncogene

Introduction

The study of oncogenes has been responsible for the elucidation of a wide range of genes that encode proteins with functions that are critical in regulating cell growth and development. Although the number of oncogenes is large, and their specific functions diverse, there presently appear to be only three biochemical mechanisms by which these genes act (Bishop, 1991): (i) the transmission of signals by GTPases, (ii) the phosphorylation of proteins with serine, threonine and tyrosine as substrates, and (iii) the regulation of gene transcription. Recent findings which could be considered additions to these categories are GDP-GTP exchange factors (Hart *et al.*, 1991) and a protein tyrosine phosphatase (Zheng *et al.*, 1992). Furthermore, the functions of some oncogenes remain unknown so it is possible that this list of mechanisms may be expanded in the future.

The biochemical mechanism by which the *cbl* oncogene functions has yet to be determined. This oncogene was discovered as the transforming gene of the Cas NS-1 murine leukaemia virus which arose from a recombination event between the ecotropic Cas-Br-M virus and the c-*cbl* oncogene (Langdon *et al.*, 1989). The Cas NS-1 virus induces pre-B cell lymphomas and myeloid leukaemias in mice inoculated at birth, and acute transformation of immortalized rodent fibroblast cell lines. 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 mouse and human cellular homologues of v-*cbl* have been identified and are localized on mouse chromosome 9 (Regnier *et al.*, 1989) and human chromosome 11q23.3 (Tunnacliffe and McGuire, 1990). In both species the c-*cbl* oncogene is closely linked to *Thy*-1, with the human genes residing on the same 600 kb *Nru*I fragment (Tunnacliffe and McGuire, 1990). The mapping of c-*cbl* to chromosome 11q23.3 is important since this is a region of translocation breakpoints in ~10% of acute myeloid leukaemias and 6% of acute lymphocytic leukaemias (Raimondi *et al.*, 1989). Mapping studies from a number of groups have shown that although c-*cbl* is translocated in these leukaemias the breakpoints are >300 kb from the coding region (Rowley *et al.*, 1990; Tunnacliffe and McGuire, 1990; Cotter *et al.*, 1991; Das *et al.*, 1991; Savage *et al.*, 1991). Thus, if c-*cbl* is involved in the generation of these leukaemias, the effect must be regulatory and from a large distance.

Expression studies have shown that the mouse and human c-*cbl* oncogenes are transcribed to produce an 11 kb mRNA which is present in a wide range of cell lineages (Langdon *et al.*, 1989). The cDNAs encompassing the coding regions of these mRNAs have recently been isolated, and sequencing has revealed that v-*cbl* is a markedly truncated form of c-*cbl* with encodes the 355 N-terminal amino acids of the 913 amino acids in the full-length protein (Blake *et al.*, 1991). This finding suggests that truncation is involved in activating *cbl*'s transformation potential. The predicted amino acid sequence also demonstrated a high level of conservation between the mouse and human genes with 98% identity within the N-terminal v-*cbl* region and 93% identity over the entire coding region. Searches of DNA and protein databases have not revealed any known gene 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 region encoded by v-*cbl* has a high proportion of basic amino acids (17%) and contains a possible nuclear localization sequence, KKTK, which fits the four residue consensus sequence (Chelsky *et al.*, 1989). The first 27 N-terminal amino acids comprise 13 glycine residues, although position number two is an alanine which indicates that the c-*cbl*-encoded protein is not myristoylated. The most notable features of the sequence that was lost in the generation of v-*cbl* are a stretch of 208 amino acids containing 23% proline and 19% serine/threonine residues, an acidic-rich domain of 150 amino acids immediately following the end of v-*cbl* and a putative leucine zipper at

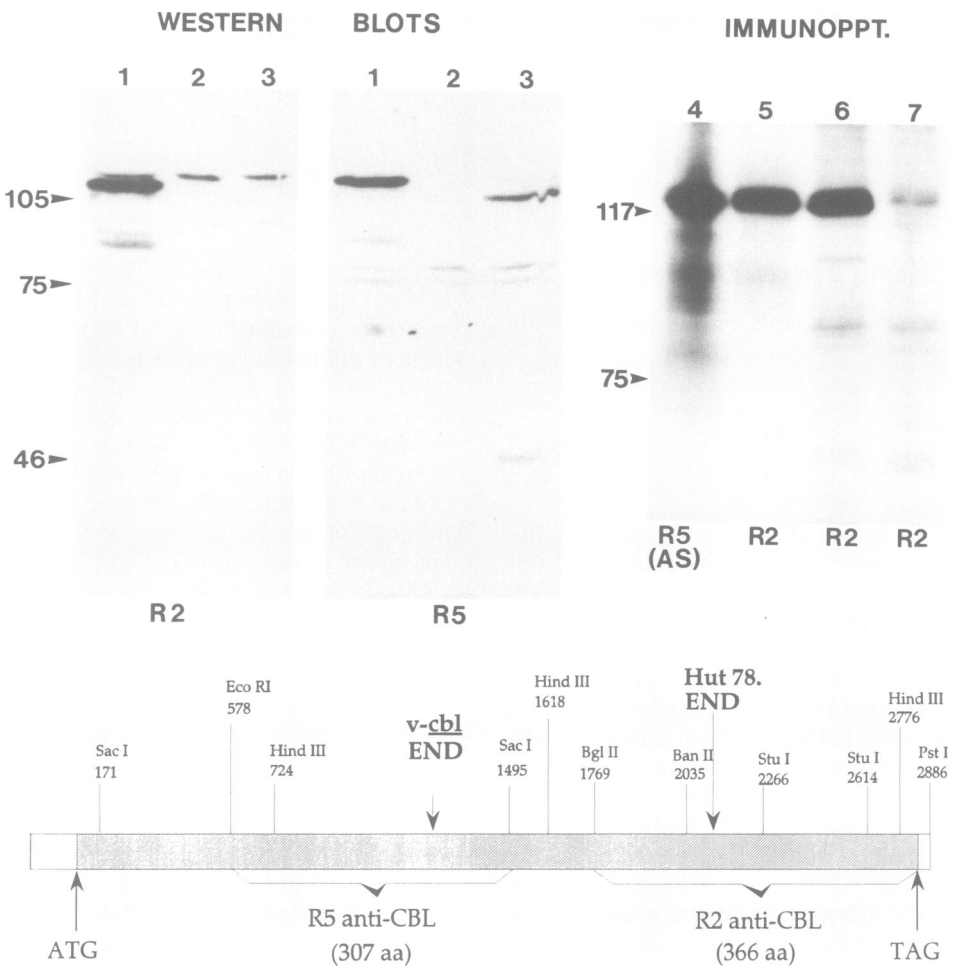

Fig. 1. Characterization of cbl antibodies. Antibodies to GST−cbl fusion proteins were raised in rabbits using the regions of human c-*cbl* shown below the gels (i.e. R2 and R5). The antibodies were examined by Western blotting (lanes 1−3) of total cell extracts from NIH 3T3 cells infected with the pJZenNeo retroviral vector containing the complete human c-*cbl* coding sequence (lane 1), normal NIH 3T3 cells (lane 2) or NIH 3T3 cells infected with the Cas NS-1 retrovirus (lane 3). The antibodies were also examined by immunoprecipitation of *in vitro* translated human c-*cbl* using R5 antiserum (AS) (lane 4) or affinity-purified R2 antibodies (lane 5). The R2 antibodies were also used to immunoprecipitate ^{35}S-labelled extracts from NIH 3T3 cells infected with the pJZenNeo-c-*cbl* retrovirus (lane 6) or normal NIH 3T3 cells (lane 7).

the C-terminus. To date there is no evidence that this sequence is involved in cbl homo- or hetero-dimerizations.

To aid our studies investigating the function of the c-*cbl* oncogene, and to determine the biological effects of the truncation that generated v-*cbl*, we have raised antibodies to both the region encoded by v-*cbl* and the C-terminal region of the human c-*cbl* product. In this study we show that these antibodies identify a 120 kDa protein which is encoded by c-*cbl* and has a cytoplasmic localization with a distribution between the cytosol and cytoskeleton. In contrast the p100$^{gag\text{-}v\text{-}cbl}$ of Cas NS-1 is distributed between the cytoplasm and the nucleus. This redistribution to the nucleus has a marked effect on *cbl*'s transformation potential and raises the question whether a naturally truncated form of a c-*cbl*-encoded protein exists in the nucleus.

Results

Characterization of cbl antibodies

The two regions of the human c-*cbl* oncogene which were used to raise antibodies (R2 and R5) are shown in Figure 1. These regions were expressed in *Escherichia coli* as glutathione-*S*-transferase (GST) fusion proteins, and rabbit antibodies were affinity purified on GST−cbl columns. Figure 1 shows experiments to characterize these antibodies by Western blotting (lanes 1−3) and immunoprecipitation (lanes 4−7). Lanes 1 of the Western blots contain total cell extracts from NIH 3T3 cells infected with a pJZenNeo retroviral vector containing the complete human c-*cbl* coding sequence. The overexpressed cbl protein of 120 kDa is recognized by both the R2 and R5 antibodies. Lanes 2 of the Western blots contain total cell extracts from normal NIH 3T3 cells, and show that the R2 antibodies detect the endogenous murine cbl protein whereas the R5 antibodies do not. With longer exposures the R5 antibodies are able to detect the cbl protein of NIH 3T3 cells (see Figure 6). A comparison of lanes 1 and 2 of the Western blot probed with the R2 antibodies shows that the human cbl protein is slightly more mobile than murine cbl. This could be because the murine gene encodes seven more amino acids, three of which are proline residues which may affect electrophoretic mobility. Lanes 3 of the Western blots contain cell extracts from NIH 3T3 cells infected with the Cas NS-1 retrovirus. The R2 antibodies, which do not encompass the v-cbl region of c-cbl, are unable to detect p100$^{gag\text{-}v\text{-}cbl}$. In contrast, the

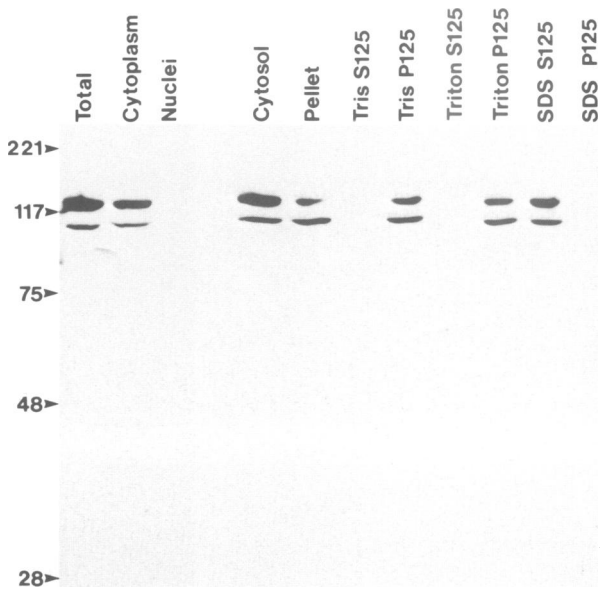

Fig. 2. Western blotting of subcellular fractions from Jurkat cells showing the localization of p120$^{c\text{-}cbl}$. Cells were initially fractionated into cytoplasmic and nuclear components which upon probing with R2 antibodies revealed the cytoplasmic localization of p120$^{c\text{-}cbl}$. The cytoplasm was further fractionated into a cytosolic component and a total membrane/cytoskeletal component (indicated as Pellet). This pellet was characterized by suspension in either 10 mM Tris buffer, 10 mM Tris buffer containing 1% Triton X-100 or 10 mM Tris buffer containing 0.1% SDS. Following centrifugation at 125 000 *g* the supernatants (S125) and pellets (P125) were examined by Western blotting using R2 antibodies. It is not known whether the smaller protein is encoded by c-*cbl*.

R5 antibodies, which were raised to a region of c-cbl that does encompass v-cbl, do recognize p100$^{gag\text{-}v\text{-}cbl}$.

Lanes 4 and 5 of Figure 1 show that *in vitro* translated human p120$^{c\text{-}cbl}$ is immunoprecipitated by R5 antiserum and by R2 affinity purified antibodies. For reasons that remain unclear, affinity purification of the R5 antiserum resulted in a dramatic loss of antibodies capable of immunoprecipitating cbl protein (data not shown), and as such they have been useful in neither immunoprecipitation nor immunofluorescence experiments. However, the unpurified R5 antiserum was informative in immunofluorescence studies (see Figure 6a and b). Lanes 6 and 7 show that the R2 antibodies immunoprecipitate cbl protein in [^{35}S]methionine-labelled extracts from NIH 3T3 cells infected with the pJZenNeo-c-*cbl* retrovirus (lane 6) and normal NIH 3T3 cells (lane 7). The immunoprecipitations also show that *in vitro* translated cbl protein has the same electrophoretic mobility as *in vivo* translated cbl. This is of interest since the deduced amino acid sequence predicts a cbl protein with a molecular weight of 100 kDa which is ~20 kDa less than that observed by SDS−PAGE. We originally thought that glycosylation might contribute to this large discrepancy; however, protein glycosylation does not occur in reticulocyte translations so other modifications or factors must be involved. Phosphorylation of proteins does occur in reticulocyte lysates but we have been unable to alter the mobility of the 120 kDa protein by phosphatase treatment (data not shown). A possible explanation for p120$^{c\text{-}cbl}$ migrating more slowly than predicted is the presence of the large proline-rich domain which could maintain secondary structures even under denaturing conditions.

p120$^{c\text{-}cbl}$ is exclusively localized in the cytoplasm

Fractionation of Jurkat cells into the cytoplasmic and nuclear components and examination by Western blotting with R2 antibodies shows that p120$^{c\text{-}cbl}$ is a cytoplasmic protein (Figure 2). Further fractionation of the cytoplasm into a soluble cytosolic component and an insoluble 125 000 *g* pellet shows that p120$^{c\text{-}cbl}$ is present in both fractions. The 125 000 *g* pellet comprises plasma and cytoplasmic membranes and the cytoskeleton, and these two components can be differentiated by treatment with non-ionic and ionic detergents. The pelletable p120$^{c\text{-}cbl}$ remains insoluble following treatment with Tris buffer containing 1% Triton X-100 but is completely solubilized in 0.1% SDS (Figure 2). These properties suggest an association with the cytoskeleton rather the membranes. This is indirectly supported by the c-*cbl* sequence which does not contain a signal sequence, a C-terminal KDEL sequence (Pelham, 1989) or a CAAX sequence for isoprenoid modification (reviewed by Der and Cox, 1991), the presence of which would indicate retention in the endoplasmic reticulum or membrane association. A protein slightly smaller than p120$^{c\text{-}cbl}$ is also recognized by the R2 antibodies (Figure 2). This protein shows the same localization and detergent solubility as p120$^{c\text{-}cbl}$ but whether it is encoded by c-*cbl* remains to be determined.

The cytoplasmic localization of the c-*cbl*-encoded protein was also shown by immunofluorescence. HeLa cells stained with affinity purified R2 antibodies show that the immunofluorescence is exclusively cytoplasmic with the suggestion of a reticular pattern consistent with cytoskeletal association (Figure 3a). This immunofluorescence could be completely blocked by absorption with GST-cbl protein but not with GST protein alone (data not shown). A cytoskeletal pattern is more obvious in a minority of cells where the staining is intense compared with the diffuse pattern seen in the majority of interphase cells (Figure 3b and c). A striking feature of the immunofluorescence is the intense vesicular staining of mitotic cells (Figure 3d and e). These brightly staining vesicles are first evident in the early prophase cells and remain throughout mitosis until they disperse at cytokinesis (data not shown). A similar pattern of mitotic cell staining was observed with antibodies to cytokeratin (Figure 3f) but not with antibodies to actin, vimentin, tubulin or the endoplasmic reticulum (data not shown). The similarity in staining between cbl and cytokeratin supports the finding that p120$^{c\text{-}cbl}$ associates with the cytoskeleton. It also raises the possibility that this association increases late in the cell cycle and is maintained during mitosis. Further investigations are required to determine whether quantitative and/or qualitative changes occur to cbl protein during the cell cycle.

Overexpression of p120$^{c\text{-}cbl}$ does not alter its subcellular localization

To test the possibility that overexpressing p120$^{c\text{-}cbl}$ may induce an altered subcellular distribution or permit its detection in compartments where its levels are possibly too low to be observed, the complete c-*cbl* coding region was inserted into the pJZenNeo retroviral vector. Virus supernatants from electroporated Ψ2 cells were used to infect NIH 3T3 cells which were examined for cbl protein by Western blotting. Figure 4 shows that the overexpressed p120$^{c\text{-}cbl}$ is only detectable in the cytoplasm, with a distribu-

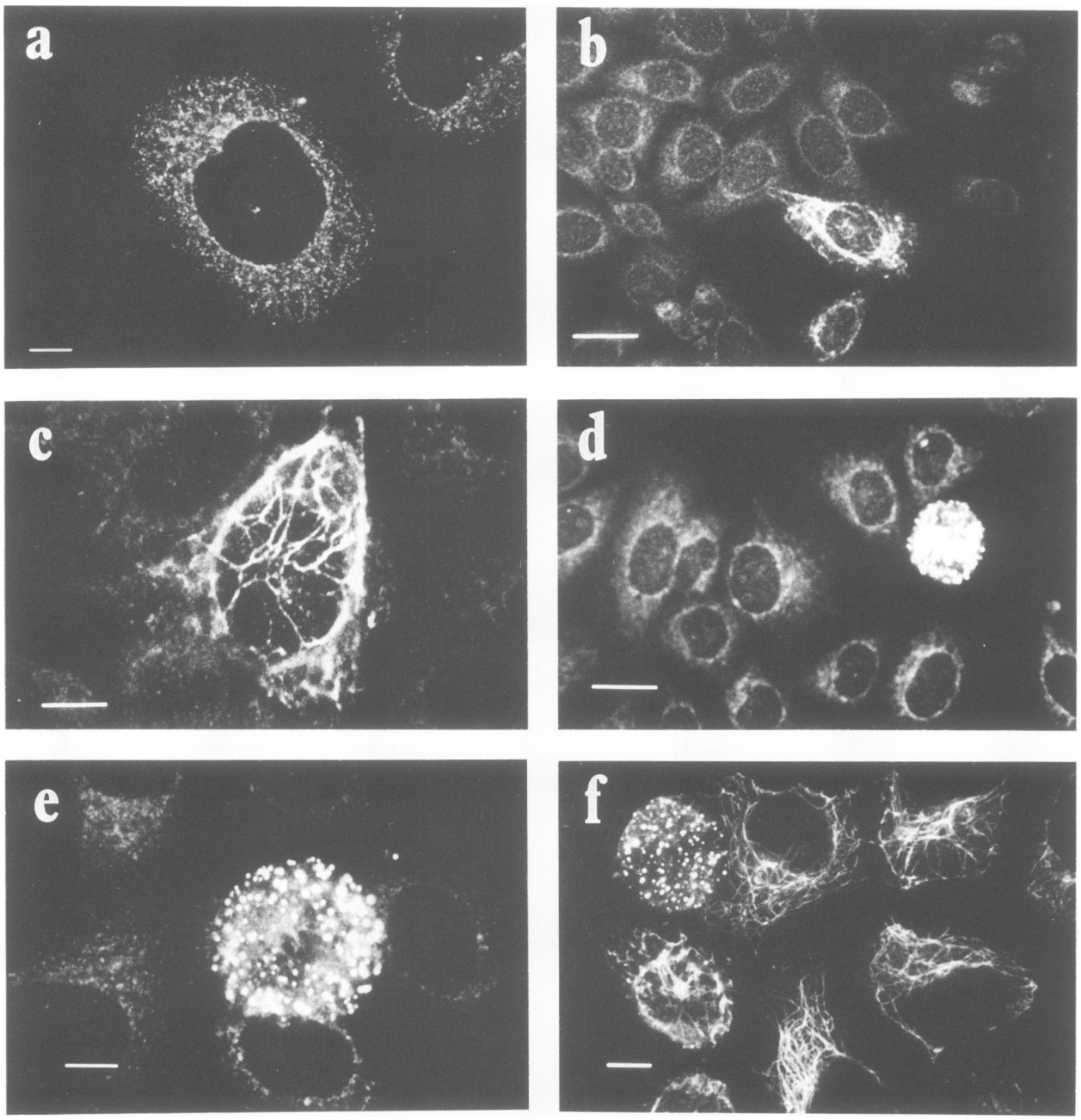

Fig. 3. Immunofluorescence of (a) HeLa cells or HEp-2 cells (b−f) following incubation with R2 antibodies (a−e) or mouse antibodies to human cytokeratin (f). Secondary antibodies were sheep anti-rabbit immunoglobulin or sheep anti-mouse immunoglobulin conjugated with FITC. The cells showing brightly staining vesicles in d, e and f are mitotic. All images were obtained by confocal laser microscopy and the scale bars indicate 10 μm in a, c, e and f and 25 μm in b and d.

tion between the cytosol and the insoluble 125 000 *g* pellet. This distribution is identical to that of endogenous $p120^{cbl}$ of Jurkat cells (Figure 2) indicating that overexpression does not alter the protein's subcellular localization. Furthermore, these results show that $p120^{c\text{-}cbl}$ is incapable of entering the nucleus. Immunofluorescence illustrates this observation where the intense cytoplasmic staining contrasts with the absence of fluorescence in the nucleus (Figure 5b).

The truncation that generated v-cbl allows nuclear localization of $p100^{gag\text{-}v\text{-}cbl}$

In contrast to full-length $p120^{c\text{-}cbl}$, the 100 kDa *gag*-v-*cbl*-encoded protein of Cas NS-1 is capable of being transported to the nucleus. The Western blot in Figure 6 shows that $p100^{gag\text{-}v\text{-}cbl}$ is present in the nucleus of Cas NS-1-infected NIH 3T3 cells whereas $p120^{c\text{-}cbl}$ is totally cytoplasmic in cells infected with the Zen-c-*cbl* retrovirus. Since proteins with molecular masses >40−60 kDa are unable to enter the nucleus and require active transport through the nuclear pores (Peters, 1986), the *gag*-v-*cbl*-encoded protein must utilize its putative nuclear localization sequence in order for this process to occur. Figure 6 also shows that an equivalent amount of $p100^{gag\text{-}v\text{-}cbl}$ is present in the cytoplasm of NIH 3T3 cells infected with Cas NS-1. Interestingly, cytoplasmic $p100^{gag\text{-}v\text{-}cbl}$ is associated with the 125 000 *g* pellet and none is detectable in the cytosol (data not shown).

This is presumed to be due to the myristoylation of gag which allows attachment to the plasma membrane (Rein *et al.*, 1986). It would appear that newly synthesized $p100^{gag\text{-}v\text{-}cbl}$ is either rapidly transported to the nucleus or bound to the plasma membrane, and that an apparent equilibrium of transport exists between these two sites. The Western blot of NIH 3T3 cells infected with Cas NS-1 also shows that the R5 antibodies detect endogenous $p120^{c\text{-}cbl}$. This provides a clear demonstration within these cells of the nuclear exclusion of $p120^{c\text{-}cbl}$ and the nuclear localization of $p100^{gag\text{-}v\text{-}cbl}$. Western blotting experiments were also carried out to examine the localization of the 40 kDa protein encoded by the cellular version of v-*cbl*, i.e. v-*cbl* with the *gag* sequences removed which we have termed c/v-*cbl*. This gene was inserted into the pJZenNeo retrovirus and NIH 3T3 cells infected. Subcellular fractionation demonstrated that the 40 kDa c/v-cbl protein is distributed between the cytoplasm and the nucleus (data not shown). Figure 6 also shows that a truncated form of cbl protein found in HUT78 T lymphoma cells (Blake and Langdon, 1992) is unable to enter the nucleus. This form of the cbl protein arose from a genetic alteration which produced a premature stop codon in the gene such that it encodes 655 N-terminal amino acids of the 906 amino acids in $p120^{c\text{-}cbl}$. The HUT c-*cbl* gene was also cloned into the pJZenNeo vector and expressed in NIH 3T3 cells. The cytoplasmic localization of the protein encoded by this gene indicates that this truncation is insufficient to allow nuclear transport, suggesting that the sequences between the end of v-cbl and HUT78 c-cbl may be involved in masking the nuclear localization sequence.

The localization of the *gag*-v-*cbl*-encoded protein was also examined by immunofluorescence. Figure 6 panel a shows normal NIH 3T3 cells and panel b shows NIH 3T3 cells infected with the Cas NS-1 retrovirus. Immunofluorescence using the R5 antiserum clearly shows the nuclear localization of $p100^{gag\text{-}v\text{-}cbl}$.

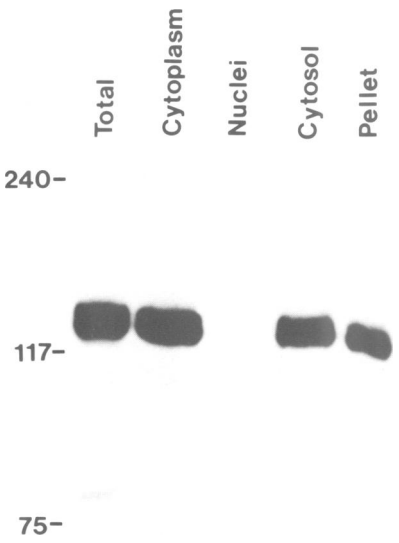

Fig. 4. Western blotting of subcellular fractions of NIH 3T3 cells infected with the pJZenNeo retroviral vector containing the human c-*cbl* oncogene. The cellular fractions examined are indicated above each lane. The cytoplasm was further fractionated into free cytosolic and particulate (i.e. Pellet) fractions. The Western blot was probed with affinity purified R2 antibodies.

The truncation that generated v-cbl allows binding to DNA

The three forms of cbl protein were translated *in vitro* and incubated with double-stranded DNA–cellulose to determine their binding properties (Figure 7). Both the full-length c-*cbl*- and HUT c-*cbl*-encoded proteins were unable to bind DNA since they were found to be totally or predominantly present in the flow-through fraction (lane 1). Although a small amount of the 80 kDa HUT cbl protein was retained on the DNA–cellulose afer the 50 mM and 500 mM salt washes (lane 3) its level was low compared with the unbound protein. In contrast, the 40 kDa protein encoded by the cellular version of v-*cbl* (i.e. c/v-*cbl*) could bind DNA. This protein was readily detectable in both the eluate from the 500 mM salt wash (c/v-*cbl*, lane 2) and the post-wash DNA–cellulose (c/v-*cbl*, lane 3). As a positive control for DNA binding the murine c-myb protein was included since it is a well characterized transcription factor. Although myb showed a greater resistance to removal from DNA with 500 mM salt than c/v-cbl, the proportions of each which bound DNA–cellulose appear comparable. These results do not demonstrate a specificity for DNA binding by the v-*cbl*-encoded protein but they do indicate a marked contrast

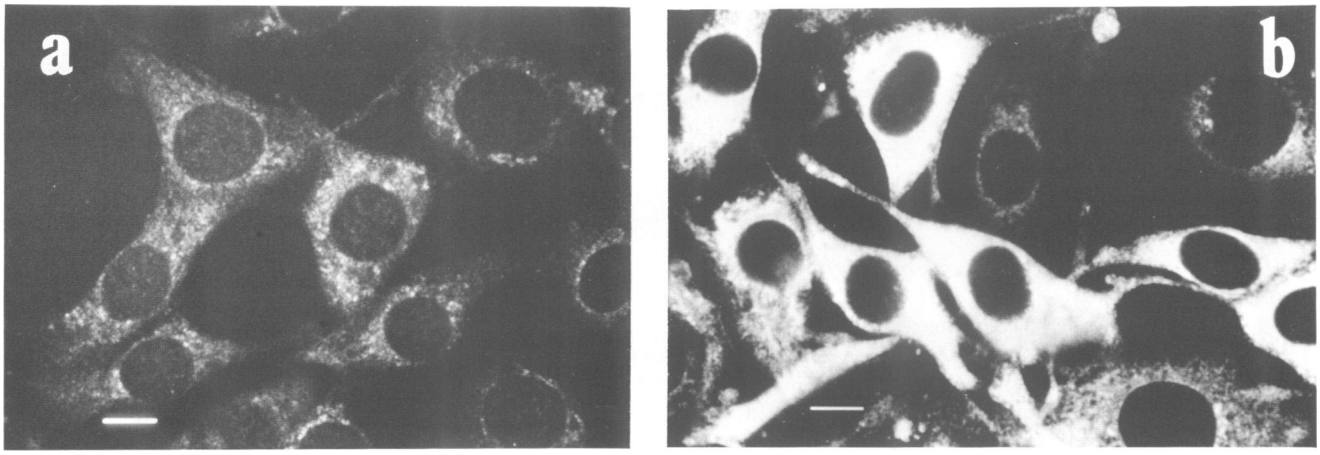

Fig. 5. Immunofluorescence of (**a**) NIH 3T3 cells and (**b**) NIH 3T3 cells infected with the pJZenNeo-c-*cbl* retrovirus. The primary antibody was affinity purified R2 anti-cbl, and sheep anti-rabbit immunoglobulin conjugated with FITC was used as the secondary antibody. The images were obtained by confocal laser microscopy and the scale bars indicate 10 μm.

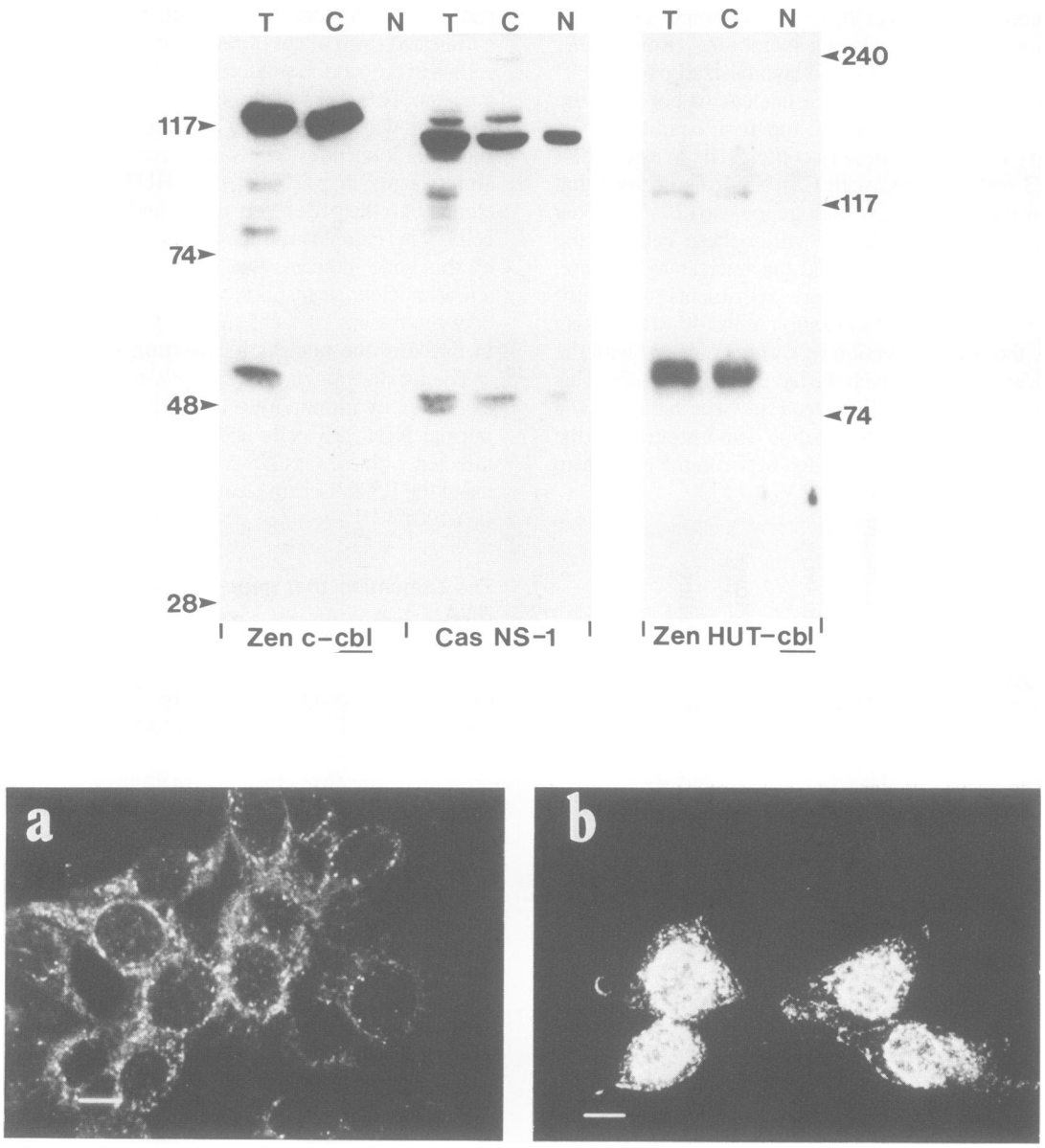

Fig. 6. Western blot of total cell extracts (T), cytoplasmic extracts (C) and nuclear extracts (N) from NIH 3T3 cells infected with the pJZenNeo-c-*cbl* retrovirus, the Cas NS-1 retrovirus or the pJZenNeo HUT-*cbl* retrovirus. The membrane was probed with affinity purified R5 antibodies. Immunofluorescence of (**a**) NIH 3T3 cells and (**b**) NIH 3T3 cells infected with the Cas NS-1 retrovirus was carried out using unpurified R5 antiserum followed by sheep anti-rabbit immunoglobulin conjugated with FITC. The images were obtained by confocal laser microscopy and the scale bars indicate 10 μm.

between v-*cbl* and c-*cbl*. Since basic charge clusters are often associated with the DNA binding domains of transcription factors (Brendel and Karlin, 1989) it is likely that the removal of C-terminal sequences exposes the basic regions in the N-terminus and allows interaction with DNA.

Correlation of transformation with nuclear localization and DNA binding

Table I shows that NIH 3T3 cells infected with the pJZenNeo retroviral vector containing either the human c-*cbl* or HUT c-*cbl* genes were unable to produce tumours in nude mice. Furthermore, these cells grew in culture as monolayers with a morphology that was indistinguishable from uninfected cells (Figure 8a and b). In contrast, NIH 3T3 cells infected with the Cas NS-1 virus or the pJZenNeo virus containing the c/v-*cbl* gene induced rapidly growing tumours (Table I) and grew in culture at high cell densities forming foci of refractile cells with a rounded morphology (Figure 8c and d). These results indicate a distinct correlation between nuclear localization, DNA binding and transformation. Interestingly, although the cells infected with vectors containing c-*cbl* and HUT c-*cbl* did not grow in nude mice and showed a flat morphology they did grow to slightly higher cell densities than NIH 3T3 cells infected with the pJZenNeo vector (Table I). This suggests that overexpression of c-*cbl* does affect the growth of NIH 3T3 cells but that this is insufficient to induce transformation.

R5-cbl antibodies recognize a nuclear protein

The results showing that the v-*cbl*-encoded protein localizes to the nucleus and binds DNA suggest that a similarly truncated form of cbl may exist endogenously in the nucleus.

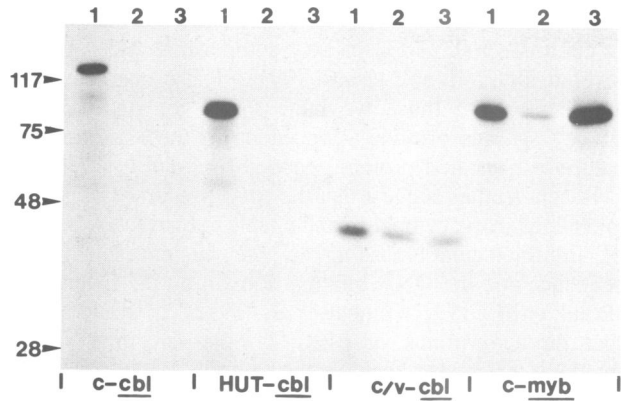

Fig. 7. DNA binding characteristics of *cbl*-encoded proteins. *In vitro* translated full-length human c-*cbl*, HUT-*cbl*, c/v-*cbl* and murine c-*myb* were incubated with double-stranded DNA bound to cellulose. Proteins unable to bind DNA were collected as the flow-through fraction and are shown in lane 1. Proteins recovered from the DNA by a 500 mM NaCl wash are shown in lane 2, and proteins which remained bound are shown in lane 3.

Table I. Tumour induction and growth characteristics of retrovirally infected NIH 3T3 cells

Virus	Tumours in nude mice[a]	Mean diameter of tumours (mm)	Saturation density (cells/cm^2)
ZenNeo	0/3	0	6.6×10^4
ZenNeo c-*cbl*	0/3	0	9.0×10^4
ZenNeo HUT-*cbl*	0/3	0	9.6×10^4
ZenNeo c/v-*cbl*	3/3	25	19.2×10^4
Cas NS-1	3/3	29	20.8×10^4

[a]Each BALB/c nude mouse was injected subcutaneously with 10^6 virus-infected NIH 3T3 cells. Tumours were measured on day 18.

However, NIH 3T3 cells overexpressing cbl showed no evidence of a nuclear protein even when examined with the R5 antibody which recognizes the v-cbl region of c-cbl (Figure 6). To test for the existence in other cells of nuclear protein(s) which may be recognized by the R5 antibody we prepared cytoplasmic and nuclear extracts from FDC-P1 cells (mouse myeloid progenitors), 70Z/3 cells (mouse pre-B lymphoid), AJ9 cells (mouse B lymphoid), BAMC1 cells (mouse pre-B lymphoid with myeloid potential), Jurkat cells (human T lymphoid), and HeLa cells (human epithelioid carcinoma). The Western blot examining these extracts (Figure 9) shows that a nuclear protein with an estimated molecular weight of 68 kDa is present in variable amounts in all but HeLa cells, with the highest levels found in BAMC1 and FDC-P1 cells. In other experiments we have been able to detect low levels of this protein in HeLa cells. HeLa cells also have an abundant nuclear protein of ~59 kDa which is not present in the other cells. Whether this protein and the 68 kDa protein are encoded by c-*cbl* requires further investigation but these proteins are within the size range that we would expect if a c-*cbl*-encoded protein was present in the nucleus, i.e. they are smaller than the 80 kDa protein encoded by HUT c-*cbl* which is localized in the cytoplasm.

Discussion

We have shown that the truncation which generated the v-*cbl* oncogene is the critical event in revealing the transforming potential of this gene. This truncation of c-*cbl*, by the Cas-Br-M retrovirus, removes almost two-thirds of the amino acids from the C-terminus of the corresponding protein, which is markedly altered. These alterations include an ability to enter the nucleus, bind DNA and induce acute

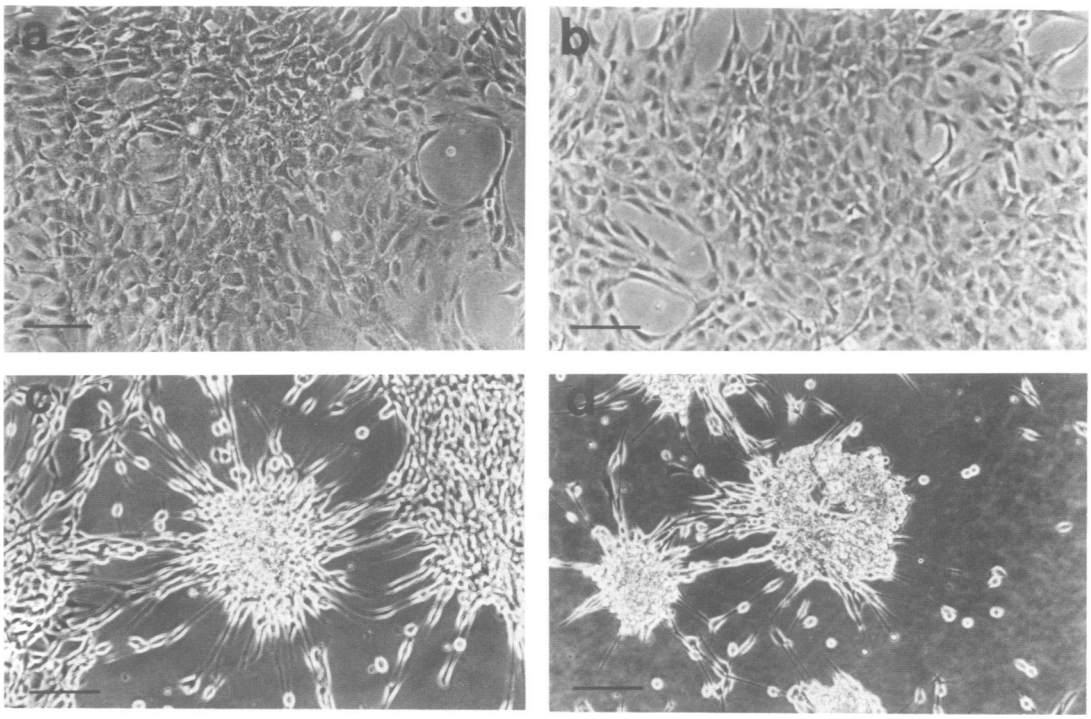

Fig. 8. The morphology of NIH 3T3 cells infected with (**a**) pJZenNeo-c-*cbl*, (**b**) pJZenNeo HUT-*cbl*, (**c**) pJZenNeo-c/v-*cbl* or (**d**) the Cas NS-1 retrovirus. The scale bar represents 140 μm.

transformation. In contrast, the full-length protein is unable to enter the nucleus or bind DNA and this correlates with an inability to induce transformation, even when overexpressed in a retroviral vector (for summary see Figure 10). These observations indicate that Cas NS-1-induced transformation is the result of inappropriate or unregulated nuclear localization and DNA binding by the v-*cbl*-encoded protein. From this it would appear that the v-*cbl* oncogene product functions as a transcription factor. Whether this function is by default or whether a naturally truncated and tightly regulated form of cbl protein exists in the nucleus of normal cells requires further investigations. The possibility of default, although theoretically not impossible, has no precedent nor does it seem likely that a truncation would reveal by chance two functions that are not normally attributed to a protein. The second possibility would seem more likely, i.e. that there is a c-*cbl*-encoded nuclear protein. We are now investigating this possibility and preliminary experiments show that cbl antibodies detect a 68 kDa protein in the nucleus of some cell lines (Figure 9).

Cytoplasmic retention and lack of DNA binding of p120$^{c\text{-}cbl}$ suggest similarities to the NF-κB precursor

The p50 subunit of the NF-κB transcription factor is synthesized as a 105 kDa precursor (Ghosh *et al.*, 1990; Kieran *et al.*, 1990). Processing of this molecule, p105, releases the N-terminal p50 which can enter the nucleus and bind DNA (Blank *et al.*, 1991; Fan and Maniatis, 1991). In contrast, p105 is exclusively cytoplasmic and is unable to bind DNA (Blank *et al.*, 1991). These characteristics are identical to those we have found for p120$^{c\text{-}cbl}$ and p100$^{gag\text{-}v\text{-}cbl}$ and provide a precedent for the existence of a naturally truncated protein encoded by c-*cbl*.

Recent studies suggest that the p105 precursor of NF-κB is retained in the cytoplasm and unable to bind DNA because of intramolecular masking of the nuclear localization sequence and the DNA binding domain by the C-terminus (Blank *et al.*, 1991; Henkel *et al.*, 1992). It would appear that the C-terminus of p120$^{c\text{-}cbl}$ functions in a similar manner. It has also been shown that DNA binding by the product of the c-*ets*-1 oncogene is masked by an intramolecular mechanism whereas the v-*ets* gene product, which has an altered C-terminus, is highly active in DNA binding and transformation (Lim *et al.*, 1992). Furthermore, the products of the v-*fos* and v-*jun* oncogenes have different nuclear localization patterns from their cellular homologues, and this appears to contribute to their tumorigenic potential (Roux *et al.*, 1990; Chida and Vogt, 1992). These observations suggest that transcription factors can be regulated by intramolecular modifications which control their capacity for nuclear transport and/or DNA binding. To study this control further we are currently preparing a range of truncated versions of c-*cbl* to determine the minimum C-terminal region required to mask the nuclear localization sequence and DNA binding domain. We are also targeting (by site-directed mutagenesis) the sequence between the end of v-*cbl* and HUT c-*cbl*, most notably a string of six proline and six acidic amino acids which may form secondary structures or interact with the N-terminus. It will also be interesting to determine whether a protein encoded by the C-terminal half of c-*cbl* can bind v-cbl and inhibit its nuclear localization and DNA binding. This would be informative in view of the recently described IκBγ which is identical to the C-terminal half of p105 (Inoue *et al.*, 1992; Liou *et al.*, 1992). An additional possibility is that the C-terminal region of cbl mediates interactions with one or more cytoplasmic proteins which may inhibit its nuclear transport. However, the observation that overexpressed p120$^{c\text{-}cbl}$ does not enter the nucleus may argue against this possibility since high levels of the protein could be expected to saturate these binding proteins and allow free p120$^{c\text{-}cbl}$ to enter the nucleus.

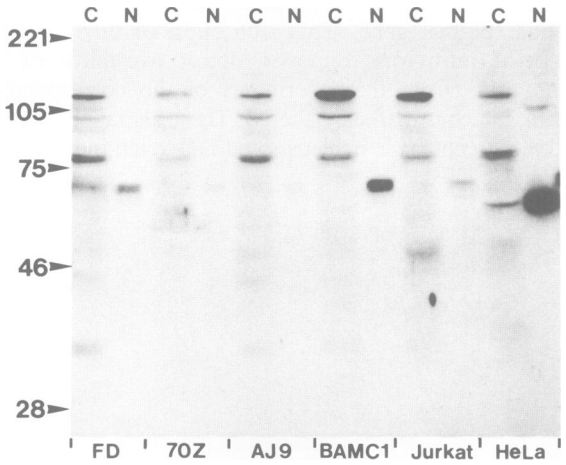

Fig. 9. Western blotting of cytoplasmic (C) and nuclear (N) extracts from a range of cell lines using affinity purified R5 antibodies. p120$^{c\text{-}cbl}$ is evident in the cytoplasm of all cell lines as the protein above the 105 kDa marker. Whether the other proteins recognized by the R5 antibodies are encoded by c-*cbl* is not known.

Cytosolic and cytoskeletal distribution of p120$^{c\text{-}cbl}$

The cytoplasmic retention of p120$^{c\text{-}cbl}$ is presumed to be due to intramolecular masking of the nuclear localization sequence. However, as mentioned above, the interaction with

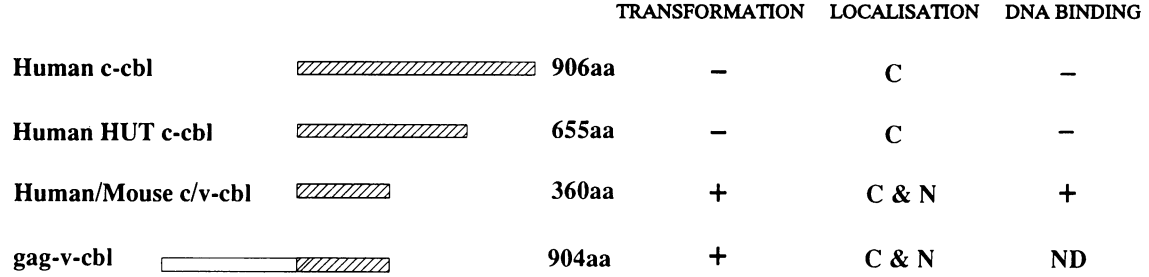

Fig. 10. Transformation, localization and DNA binding properties of various forms of the *cbl* oncogene. C, cytoplasmic; N, nuclear; and ND, not determined.

one or more cytoplasmic proteins that may mask the nuclear localization sequence cannot be ruled out. The retention of $p120^{c\text{-}cbl}$ in the cytoplasm does, however, appear to be regulated by a mechanism more complex than one solely dependent on nuclear localization sequence masking. This is based on the observation that $p120^{c\text{-}cbl}$ is distributed between the cytosol and cytoskeleton (Figure 2). The immunofluorescence studies support this observation and reveal some striking features of $p120^{c\text{-}cbl}$ in the cytoplasm (Figure 3): (i) the variability in staining of interphase cells in which rare cells show an intense pattern similar to that seen with cytoskeletal antibodies whereas most show a more diffuse pattern (Figure 3b and c). The other striking feature of the immunofluorescence is (ii) the intense vesicular staining of mitotic cells. These vesicles, which appear similar to those seen with antibodies to cytokeratin (Figure 3f), may indicate that $p120^{c\text{-}cbl}$ is regulated during the cell cycle by varying its distribution between the cytosol and cytoskeleton. It will be important to determine whether $p120^{c\text{-}cbl}$ levels change during the cell cycle or whether there is a redistribution of the cytosolic protein to the cytoskeleton late in the cell cycle. This cytoskeletal association could prevent the processing of $p120^{c\text{-}cbl}$ to its predicted nuclear form during stages of the cell cycle when its presence could be detrimental. In view of the acute transformation induced by v-*cbl* this could be an efficient regulatory safeguard for a protein with the ability to affect cell growth profoundly.

Materials and methods

Cells and cell culture

HeLa and Jurkat cells were obtained from the Division of Human Immunology within the Institute of Medical and Veterinary Science. NIH 3T3 cells were obtained from the American Type Culture Collection at passage number 127, and Ψ2 cells were obtained from the Walter and Eliza Hall Institute of Medical Research. The mouse hemopoietic cell lines 70Z/3, AJ9 and BAMC1 were provided by W.Davidson, National Institutes of Health, and FDC-P1 cells from T.Gonda, Hanson Centre for Cancer Research. The HeLa, NIH 3T3 cells and Ψ2 cells were maintained in Dulbecco's modified Eagle's medium and all haemopoietic cell lines in RPMI-1640 medium. Both media were supplemented with 10% heat inactivated fetal calf serum (FCS). FDC-P1 cells were grown in the presence of recombinant GM-CSF.

Antibodies

Fragments isolated from a cDNA clone spanning the complete coding region of human c-*cbl* were inserted into pGEX expression vectors and expressed in *E.coli* as glutathione-*S*-transferase (GST)−cbl fusion proteins (Smith and Johnson, 1988). The fragments encode amino acids 143−450 and 540−906, and these regions are denoted by R5 and R2 respectively as illustrated in Figure 1. The GST−cbl fusion proteins were purified from *E.coli* lysates by affinity chromatography on glutathione−agarose (Sigma) using the procedures of Smith and Johnson (1988). Rabbits were immunized with ~400 μg of GST−cbl fusion proteins emulsified in complete Freund's adjuvant and subsequently challenged with the protein in incomplete adjuvant. Antibodies were affinity purified on Affigel-10 agarose (Bio-Rad) coupled with 3 mg of fusion protein.

Mouse monoclonal antibodies to human smooth muscle α-actin, cytokeratin, vimentin and rough endoplasmic reticulum were obtained from the DAKO Corporation and mouse monoclonal anti-α and β tubulin from Amersham.

cbl retroviral constructs and NIH 3T3 transformation

The pJZenNeo retroviral vector (Johnson *et al.*, 1989) was used to express (i) the complete human c-*cbl* oncogene, (ii) a truncated form of c-*cbl* isolated from the cutaneous T-cell lymphoma line HUT78 (Blake and Langdon, 1992) and (iii) a cellular version of v-*cbl* in which the *gag* sequences have been removed and the human c-*cbl* initiation codon and the v-*cbl* stop codon are utilized. The HUT78 c-*cbl* gene encodes 655 N-terminal amino acids of c-*cbl*, and the cellular version of the v-*cbl* gene (i.e. c/v-*cbl*) encodes 360 N-terminal amino acids of c-*cbl*. The pJZenNeo clones were electroporated into the Ψ 2 packaging cell line (Mann *et al.*, 1983) and, following selection in Geneticin (G418, GIBCO Laboratories), the viral supernatants were used to infect NIH 3T3 cells at passage 133. Following selection in G418 the infected cells were examined for the presence of cbl protein (see below). Transformation of NIH 3T3 cells was tested by inoculating 8 week-old BALB/c *nu/nu* mice subcutaneously with 10^6 cells in 0.2 ml of medium. The mice were inoculated with cells 10 passages after virus infection.

Subcellular fractionation

Cytoplasmic and nuclear extracts were prepared by washing cells in PBS and resuspending in a PBS lysis buffer containing 1% Triton X-100, 10 μg/ml aprotinin, 1 μg/ml of chymostatin, leupeptin, antipain and pepstain, and 1 mM PMSF. The cells were incubated on ice for 30 min with occasional shaking at a concentration of 5×10^6 cells/ml after which the nuclei and cytoplasm were separated by centrifugation at 700 *g* for 5 min. Cytoplasmic proteins in the supernatant were removed and prepared for SDS−PAGE by adding the appropriate volume of 5 × sample buffer. Nuclei were washed once with lysis buffer before suspension in 1 × sample buffer (50 mM Tris, pH 6.8, 2% SDS, 4% β-mercaptoethanol, 10% glycerol and 0.02% bromophenol blue). To enable loading of the nuclear proteins the DNA was sheared by several passages through a 25G needle. Samples were heated at 100°C for 4 min before electrophoresis.

Additional fractionation of cytoplasmic proteins was carried out as described by Mann and Thorley-Lawson (1987). A particulate sedimentable fraction of cell lysate was prepared by suspending cells in 10 mM Tris pH 7.4, 0.5 mM $MgCl_2$, and the protease inhibitors described above, at 10^7 cells/ml and Dounce homogenizing ~40 times using a Wheaton Dounce homogenizer with a type A pestle. Nuclei were removed by centrifugation at 700 *g* for 5 min and the membrane and cytoskeletal components were pelleted at 125 000 *g* for 40 min at 4°C. The cytosolic proteins of the supernatant were removed and prepared for electrophoresis by the addition of 5 × sample buffer. The pellet was suspended in 10 mM Tris, pH 7.4, containing either 1% Triton X-100 or 0.1% SDS plus protease inhibitors to a volume equivalent to 10^7 cells/ml. Suspension was facilitated by vortexing and the samples were incubated at 4°C for 30 min. The detergent insoluble and detergent soluble components were separated by ultracentrifugation at 125 000 *g* for 60 min and both fractions were prepared for PAGE as described above.

Western blotting and immunoprecipitation

Following electrophoresis in 10% SDS−polyacrylamide gels the protein samples were transferred to Hybond ECL nitrocellulose membranes (Amersham) using standard procedures. The membranes were blocked in PBS containing 5% powdered milk and 0.1% Tween 20 (Blotto) for 1 h at room temperature before the addition of anti-GST-cbl antibody at 2 μg/ml in Blotto. After 1 h at room temperature the membranes were washed in Blotto then incubated with peroxidase-conjugated swine anti-rabbit immunoglobulins (DAKO Corporation) for 1 h at room temperature. Unbound antibody was washed from the membrane and bound antibodies were visualized by adding ECL detection solution (Amersham) and exposure to X-ray film.

For immunoprecipitation, proteins were labelled with [^{35}S]methionine by culturing cells in methionine-free medium containing 2% FCS and 20 μCi/ml of [^{35}S]methionine (NEN-DuPont) for 8 h. Cells were lysed in RIPA buffer (PBS containing 1% Triton X-100, 0.1% SDS, 2 mM EDTA, 1% deoxycholic acid and 1% aprotinin) at 5×10^6 cells/ml. The extracts were clarified by centrifugation at 100 000 *g* for 30 min and immunoprecipitated with anti-GST-cbl antibodies for 1 h at 4°C. Immunoprecipitates were recovered on protein A−Sepharose beads (Sigma), boiled in sample buffer and analysed by electrophoresis in 10% SDS−polyacrylamide gels and fluorography.

In vitro transcription and translation

DNA templates for *in vitro* transcription were cloned into the pGEM expression vector (Promega) and RNA was transcribed using the manufacturer's methods. The DNA templates included the full-length human c-*cbl* clone, the HUT78 truncated form of c-*cbl*, the cellular version of v-*cbl* and a full-length murine c-*myb* clone provided by T.Gonda. As described above the cellular version of v-*cbl* was constructed by exchanging the 5′ region of v-*cbl* with human c-*cbl* to remove *gag* sequences. The *in vitro* transcribed RNA was used to programme rabbit reticulocyte lysates using the protocol provided by the manufacturer (Promega) and translation was performed in the presence of 0.6 mCi/ml [^{35}S]methionine (NEN-DuPont, 1128 Ci/mmol).

DNA binding assay

Double-stranded calf thymus DNA bound to cellulose (Pharmacia) was washed in 50 mM NaCl, 10 mM Tris pH 8.0, 5 mM EDTA, 1 mM DTT,

1 mM PMSF to equilibrate and remove fines. To a 0.5 ml bed volume containing 0.5 ml of the above buffer was added 25 μl of *in vitro* translation mix which was incubated at 4°C for 2 h with mixing. The mix was loaded onto an Econo-Column (Bio-Rad) and the flow-through collected. The column was washed with 5 ml of the above buffer followed by a 2 ml wash with 500 mM NaCl, 10 mM Tris, pH 8.0, 5 mM EDTA, 1 mM DTT, 1 mM PMSF. Samples of flow-through, the 500 mM salt wash and the DNA–cellulose matrix were examined for the presence of *in vitro* translated protein by SDS–PAGE and fluorography.

Immunofluorescence

Cells were plated onto 8-well chamber slides (Nunc) at a density of $\sim 1-2 \times 10^4$ cells/cm^2 and grown overnight. Slides were washed with PBS and the cells fixed in absolute methanol for 5 min followed by acetone for 2 min at −20°C. Cells were then blocked in PBS containing 5% FCS for 1 h at room temperature. In some experiments slides containing acetone-fixed HEp-2 cells (Protrac Industries, San Antonio, TX) were used for immunofluorescence studies. Primary antibodies were used at 5–10 μg/ml in PBS containing 1% FCS and incubated for 30 min at room temperature. Following three 10 min washes in PBS the cells were incubated for 30 min with a 1:50 dilution of affinity purified sheep anti-rabbit or sheep anti-mouse immunoglobulin conjugated with fluorescein isothiocyanate (Silenus, Australia). After PBS washes the cells were mounted with a 50% glycerol solution containing 2 mM Tris pH 7.5, and 1.8% Dabco (1,4-diazabicyclo(2,2,2)octane, Sigma). Conventional epifluorescence microscopy was carried out using an Olympus fluorescence microscope and confocal laser scanning microscopy was performed on an MRC-600 system (Bio-Rad Corp.). Photography of confocal images was accomplished with a Polaroid Freeze-Frame Video Recorder using Kodak Ektachrome 35 mm EPW-100 colour film.

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