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A c-Cbl Yeast Two Hybrid Screen Reveals Interactions with 14-3-3 Isoforms and Cytoskeletal Components

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The protein product of c-*cbl* proto-oncogene is known to interact with several proteins, including Grb2, Crk and PI3 kinase, and is thought to regulate signalling by many cell surface receptors. The precise function of c-Cbl in these pathways is not clear, although a genetic analysis in *Caenorhabditis elegans* suggests that c-Cbl is a negative regulator of the epidermal growth factor receptor. Here we describe a yeast two hybrid screen performed with c-Cbl in an attempt to further elucidate its role in signal transduction. The screen identified interactions involving c-Cbl and two 14-3-3 isoforms, cytokeratin 18, human unconventional myosin IC, and a recently identified SH3 domain containing protein, SH3 P17. We have used the yeast two hybrid assay to localise regions of c-Cbl required for its interaction with each of the proteins. Interaction with 14-3-3 is demonstrated in mammalian cell extracts.

The c-*cbl* (Casitas B-lineage lymphoma) gene was first identified as the cellular homologue of v-*cbl*, the transforming gene of a murine retrovirus (1, 2). The human c-Cbl protein consists of a phosphotyrosine binding domain, a RING finger motif, a proline rich region and a putative leucine zipper (3). A specific small internal deletion (4) or an extensive C-terminal truncation results in c-Cbl proteins which are potently transforming (3). The N-terminal half of c-Cbl is highly conserved among species and between c-Cbl and a related human protein, Cbl-b (5).

Recently, a series of reports have implicated c-Cbl in signalling by a wide range of cytokine receptors. c-Cbl is tyrosine phosphorylated in response to stimulation of the epidermal growth factor receptor (EGFR) (6-12), FCγ receptor (13), B-cell receptor (14, 15), T-cell receptor (16), c-Kit receptor (17), and others. c-Cbl also forms complexes with intracellular proteins, including the p85 subunit of PI3 kinase (18, 19), Crk (20-22), Nck (23) , CrkL, (24), Shc (14), cytoplasmic tyrosine kinases (14, 25) and Grb2 (26-29). Genetic studies suggest that Sli-1, the *C.elegans* c-Cbl homologue, acts as a negative regulator of the EGF receptor (30, 31).

To further characterise c-Cbl, we sought to identify additional protein partners for it using a yeast two hybrid screen (32, 33). We have also used the two-hybrid system to localise binding domains for each of these putative partners within c-Cbl, and to compare the strengths of these interactions with the known physiological interaction of c-Cbl and Grb2. Our biochemical analyses demonstrate an *in vivo* interaction between c-Cbl and 14-3-3 proteins in mammalian cells, and taken together the interactions uncovered in this screen suggest a possible role for c-Cbl.

MATERIALS AND METHODS

Plasmids. Restriction digestion was used to generate clones in pGBT9 or pGADGH which encoded, in frame, either full length c-Cbl (pGBT9-c-Cbl) or the fragments depicted in Figure 2. pGADGH-Grb2 is a full length cDNA cloned by restriction digest of a clone provided by Dr. T. Pawson. Negative control plasmids, pGBT9-Bcl-2 (human amino acids 1-239), pGBT9-ice (mouse, amino acids 1-402) and pGBT9-ced-4 (*C.elegans*, amino acids 1-549), were provided by Dr. D. Vaux. pGBT9-CD4 (human, amino acids 396-435) was from Dr. P. Bello. pGBT9-nt-mSos (mouse, amino acids 18-598) was from Dr. D. Bowtell and Dr Y. Hu. Positive control vectors, pPC86-Jun and pPC62-Fos, were from Dr. S. Demo. Full length Grb2 and 14-3-3β cDNA fragments, with appropriate 5′ and 3′ restriction sites, were generated by PCR and sub-cloned into pGEX2T (34). The complete sequence of constructs generated by PCR, and the sequences flanking cloning sites of constructs generated by restriction digests of existing plasmids, were determined using automated sequencing. Sequences were compiled and analysed using the GCG suite of programs maintained at the Australian National Genomic Information Service (ANGIS).

Yeast culture and transformation. The yeast stain YGH1 (*ura3-52, his-200, ade2-101, lys2-801, try1-901, leu2-3,* Can^r, *gal4 542, gal80-538, LYS::gal1$_{uas}$-gal1$_{tata}$-HIS3*) and the HeLa cell cDNA li-

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brary were provided by Dr. S. Fields. pGBT9 and pGADGH plasmids carry tryptophan and leucine selection markers, respectively. The yeast two hybrid screen was performed essentially as described (32, 33). Positive clones were tested for their ability to transactivate with the following non-specific partners: Bcl 2, ICE, Ced 4, CD4, and Sos. Liquid β-galactosidase assays were performed as described (35), except that 10ml cultures were concentrated to 1ml and varying amounts of cells were used according to strength of interactions (see also legend Fig. 1). The DNA sequences of positive clones were obtained by automated DNA sequencing on an ALF DNA sequencer (Pharmacia LKB) or a 377 DNA sequencer (ABI), according to the manufacturer's protocols.

Antisera and fine chemicals. Human EGF was generously provided by Dr. Ed Nice, Ludwig Institute for Cancer Research, Melbourne. Rabbit anti-human 14-3-3 polyclonal IgG was from Santa Cruz Biotechnology. Anti-c-Cbl antiserum R2 was as described previously (36). Rabbit polyclonal anti-HA antiserum was generated against a GST fused protein expressing a triple haemaglutinin epitope. Rabbit polyclonal GST antiserum was generated to purified GST protein. HRP-coupled goat anti-rabbit heavy IgG was from Bio-Rad. Aprotinin and leupeptin were from Boehringer Mannheim.

Cells and cell culture. Jurkat T-cells were maintained in RPMI (Trace Scientific, Melbourne) supplemented with 10% FCS, 500IU/mL penicillin and 50mg/mL streptomycin. Jurkat T-cells were treated with sodium orthovanadate (0.1mM vanadate in 0.2mM H_2O_2), as described previously (37).

Immunoprecipitation and affinity precipitation of c-Cbl, and Western blotting. Cells were grown to 10^6/ml and harvested by centrifugation, washed once with ice cold PBS, and lysed at 10^7/ml in 2ml PLC buffer (50mM HEPES, pH 7.5, 150mM NaCl, 10% glycerol, 1% Triton-X-100, 1mM EGTA, 1.5mM $MgCl_2$, 10mg/ml aprotinin, 10mg/ml leupeptin, 50mM β-glycerophosphate, 100uM sodium orthovanadate) for 30 min. at 4°C on a rotating wheel. Lysates were centrifuged at 15000g for 10 minutes at 4°C, and the supernatants collected. For antibody immunoprecipitations 3μl R2 antiserum was added and immunoprecipitates were collected on protein-A Sepharose (Pharmacia). For Ni-NTA precipitations 30μl Ni-NTA agarose (Qiagen) was added. For GST affinity precipitations 2.5mg glutathione agarose beads (Sigma) were added with purified GST fusion proteins as specified (see results). Immunoprecipitated proteins were separated on 7% SDS-PAGE gels for detection of c-Cbl, and 12% SDS-PAGE gels for detection of 14-3-3 proteins. Proteins were immunoblotted essentially as previously described (38).

RESULTS AND DISCUSSION

Novel Protein Partners for c-Cbl Identified in a Yeast Two Hybrid Screen

To identify proteins which interact with c-Cbl we took advantage of the highly sensitive yeast two hybrid protein-protein interaction assay to screen a HeLa cell cDNA library (32, 33). 4.4×10^6 transformants were screened for interaction with full length c-Cbl by their ability to grow on medium lacking histidine. Positive clones were tested for trans-activation of the *lacZ* gene by the appearance of blue colour in the presence of X-gal, and also for their inability to activate transcription when paired with a panel of irrelevant control bait proteins (see Materials and Methods). Twenty-one clones, including multiple isolates of several proteins, were obtained which interacted specifically with c-Cbl. Four of the proteins, 14-3-3β, 14-3-3ζ, cytokeratin 18 and human unconventional myosin IC (huncM IC), share the common feature of being associated with the cytoskeleton and with vesicles, consistent with subcellular localisation of a fraction of c-Cbl (36). This commonality, and the fact that several isolates of these genes were obtained (Fig. 1A), provided an initial indication that the proteins obtained represented physiologically relevant interactions. Interaction between c-Cbl and 14-3-3 has since been described elsewhere by Liu, *et al* (39).

A.

Identity	No. of isolates
huncM IC*	1
unknown	2
EST**	1
EST	1
14-3-3ζ	3
EST	1
14-3-3β	3
Keratin 18	3
SH3 P17	4

* human unconventional myosin IC
**exoressed sequence tag

B.

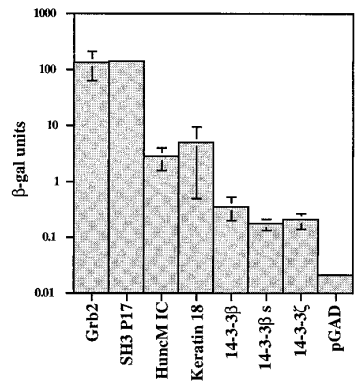

FIG. 1. (A) Multiple isolates of several interacting proteins were obtained from the yeast two hybrid screen. (B) Graph of β-gal assays, comparing relative strengths of interaction c-Cbl with proteins identified using the yeast two hybrid system. Results of three assays are averaged and graphed on a log scale. Grb2 and SH3 P17 interact most strongly. 14-3-3β s denotes the shorter 14-3-3 clone from the two hybrid screen, lacking the first 8 amino acids which encode part of the 14-3-3 dimerisation motif. β-galactosidase activity is given as $\Delta A_{420}.min^{-1}.ml^{-1}.A_{600}{}^{-1}$.

We performed quantitative β-galactosidase assays as an indicator of the strength of interaction between c-Cbl and the interacting proteins (Fig. 1B). These findings were compared to that seen with c-Cbl and Grb2. The SH3 domain containing proteins SH3 P17 and huncM IC interacted strongly with c-Cbl. Cytokeratin 18 also interacted strongly. In contrast, interaction of c-Cbl with 14-3-3 proteins was relatively weak. The two 14-3-3 isoforms differed in their strength of interaction, with 14-3-3β being reproducibly the stronger. Interestingly, a slightly N-terminally truncated 14-3-3β clone, missing part of the dimerisation motif, demonstrated reduced binding activity indicating that dimerisation of 14-3-3 proteins may be important for their interaction with c-Cbl.

Mapping of the Regions of c-Cbl Essential for Binding to Interacting Proteins

We next determined which regions of c-Cbl were required for interaction with the proteins identified in the yeast two hybrid screen. None of the proteins tested interacted with Cbl 1-480 (Fig. 2), indicating that all,

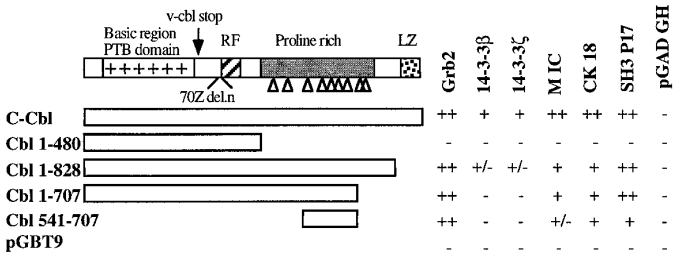

FIG. 2. The C-terminal region of c-Cbl is essential for protein-protein interaction. Schematic representation of c-Cbl deletion fragments. Triangles represent proline rich peptides. Plus symbols indicate degree of growth of transformants on selective media lacking histidine.

or essential parts, of their binding sites were located in the C-terminal region of c-Cbl. To further define binding sites in the C-terminus of c-Cbl we generated two C-terminal deletion constructs (Cbl 1-707, Cbl 1-828), together with a small internal fragment from within the proline rich region of c-Cbl (Cbl 541-707).

Binding of 14-3-3β and 14-3-3ζ was reduced by deletion of the C-terminal 78 amino acids of c-Cbl (Cbl 1-828, Fig. 2) and was completely abrogated by the deletion of a further 121 amino acids (Cbl 1-707, Fig. 2) This region contains many serine residues, consistent with 14-3-3 binding to phosphoserine. Liu et al. (40) report a different binding site for 14-3-3 proteins within 70Z Cbl. Our results suggest there is an additional site of interaction, or that the interaction is mediated by a different motif in 70Z Cbl.

Binding of CK 18 and huncM IC to Cbl 1-828 was reduced compared with the wild type protein, but not by a further small truncation of c-Cbl (compare Cbl 1-828 and Cbl 1-707; Fig. 2). In contrast, binding of Grb2 and SH3 P17 was undiminished by either C-terminal truncation in c-Cbl. Of all the interacting proteins tested, only Grb2 bound strongly to the small proline rich fragment (Cbl 541-707), with the other proteins showing either partial binding (CK 18, huncM IC, SH3 P17) or no detectable binding activity (14-3-3β and 14-3-3ζ).

These findings defined the C-terminal region as essential for the interaction of c-Cbl with each of the proteins identified in the yeast two hybrid screen and indicate that there are specific binding sites throughout the C-terminal half of c-Cbl which interact differentially with a range of proteins.

c-Cbl Interacts with 14-3-3 in Vivo

We used a GST-14-3-3β fused protein to affinity purify c-Cbl from Jurkat cell lysates. Precipitates were resolved on SDS-PAGE and immunoblotted for the presence of c-Cbl (Fig. 3A, top panel). Binding of c-Cbl to GST-14-3-3β, but not to an equivalent amount of native GST, was detected and this increased with additional GST-14-3-3β. The relatively strong interaction of the GST-Grb2 positive control was consistent with our quantitative analysis of the interaction of 14-3-3β and Grb2 with c-Cbl in yeast.

Next, c-Cbl proteins were purified using either specific antisera directed to the C-terminal third of c-Cbl, or by binding the naturally occurring poly-histidine peptide at the N-terminus of c-Cbl to Ni-NTA agarose. Although both methods recovered c-Cbl protein, substantially more was obtained with Ni-NTA agarose (Fig. 3B, top panel, lanes 1 and 2 respectively). Co-precipitation of 14-3-3 and c-Cbl was detected on Western blots of Ni-NTA agarose precipitates but not in

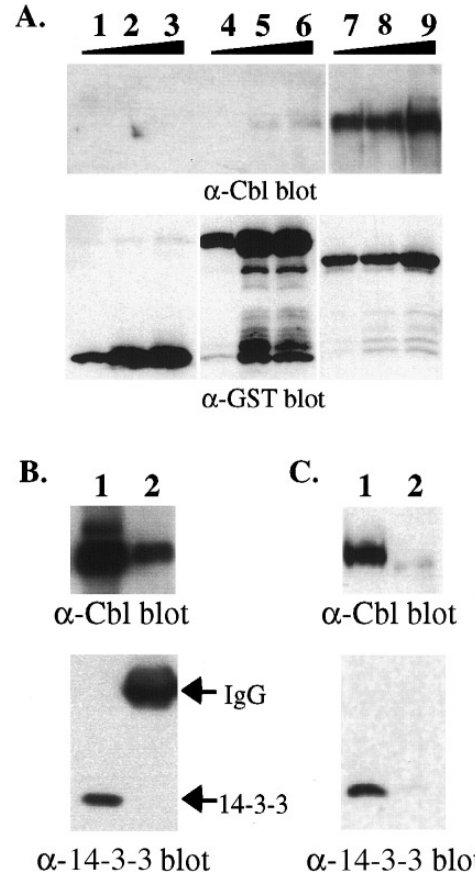

FIG. 3. c-Cbl interacts with 14-3-3 in whole cell extracts. (A) c-Cbl/14-3-3 complex is detected using GST-14-3-3 to precipitate c-Cbl from Jurkat cell extracts. Anti-c-Cbl blot: GST alone does not bind to c-Cbl (lanes 1-3), however, both GST-14-3-3 (lanes 4-6) and GST-Grb2 (lanes 7-9) bind to c-Cbl. Anti-GST blot: GST (lanes 1-3), GST-14-3-3 (lanes 4-6), and GST-Grb2 (lanes 7-9) were detected with an anti-GST antibody as a loading control. (B) c-Cbl/14-3-3 complex is detected using Ni-NTA agarose, but not the R2 antibody, to precipitate c-Cbl from Jurkat cell extracts. c-Cbl (lane 1, upper panel) and 14-3-3 (lane 1, lower panel) were detected in the precipitate by Western blot. No 14-3-3 was detected in complex with c-Cbl when the R2 antibody was used to immunoprecipitate cell extracts (lane 2). (C) A reduced amount of 14-3-3 was recovered when Ni-NTA agarose was used to precipitate Cbl from thymocytes of mice deficient for c-Cbl (lane 2) compared to wild type mice (lane 1).

anti-c-Cbl immunoprecipitates (Fig. 3B, bottom panel, lanes 1 and 2 respectively). This may be due to competition between 14-3-3 and the antibody for the same binding site on c-Cbl, as our mapping suggests that 14-3-3 interacts with c-Cbl within the R2 peptide to which the antibody was raised (Fig. 2 and (41)). We tested U937, 293 and BALB/c 3T3 cells for c-Cbl/14-3-3 interaction using Ni-NTA agarose. Each of these cell lines express significant levels of c-Cbl, and an interaction with 14-3-3 can be detected in all three using Ni-NTA (data not shown). Thus, the complex of c-Cbl and 14-3-3 proteins occurs in a range of cell types.

To determine whether the interaction with 14-3-3 detected using the Ni2+ was specific to c-Cbl we examined whether Ni2+ precipitates from thymocytes of mice homozygous for a targeted disruption of c-Cbl (Murphy and Bowtell, unpublished results) contain 14-3-3 protein. These mice express no wild type c-Cbl, and only trace amounts of an aberrant Cbl protein. Ni-NTA agarose precipitates from thymocyte lysates of mutant mice (Fig. 3C, lane 2, bottom panel) reveal a significantly reduced amount of co-precipitated 14-3-3 protein compared to wild type mice (Fig. 3C, lane 1, bottom panel). This reduction is consistent with the reduced abundance of Cbl protein (top panel), suggesting that the interaction we detect with the Ni-NTA agarose is largely specific for c-Cbl.

In the course of these studies Liu *et al* (1996) demonstrated an inducible interaction between c-Cbl and 14-3-3τ in TCR stimulated Jurkat cells, using an antibody directed against the C-terminal 15 amino acids of c-Cbl. In contrast, the complex we detected was constitutive and did not appear to increase or decrease in T-cells stimulated with pervanadate, or fibroblasts stimulated with EGF (data not shown). It is not clear why our findings differ to Liu *et al* (1996), but it may stem from the investigation of different isoforms of 14-3-3 with different antisera in the two studies. Despite these discrepancies, our findings support those of Liu *et al.* (1996) and demonstrate that 14-3-3 proteins are physiological partners for c-Cbl *in vivo.*

Possible Functional Significance of These Interactions with c-Cbl

C-Cbl has been previously shown to be exclusively cytoplasmic in Jurkat cells, where it is distributed equally between soluble and insoluble, cytoskeletal, fractions (36). Consistent with this, immunofluorescence studies in HeLa cells show a filamentous pattern of c-Cbl distribution, similar to the pattern of cytokeratin staining. In some cells c-Cbl staining was found in vesicles, similar to cytokeratin, 14-3-3 and huncM IC (42). Although the significance of this localisation is not known, 14-3-3 proteins are thought to be involved in vesicle transport, and, of particular interest, the *Saccharomyces cerevisiae* homologue of human unconventional myosin 1C, Myo 5, has recently been shown to be required for receptor endocytosis in clathrin coated vesicles (43). Thus, taken together, the interactions identified in our screen suggest a role for c-Cbl in receptor endocytosis. This is further supported by the fact that c-Cbl is transiently ubiquitinated in response to CSF-1 receptor stimulation (44), and may be targeting this receptor for degradation. This is consistent with c-Cbl's putative negative regulatory function.

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