Characterization of the mouse Cblc/Cbl3 gene

Frédéric Fiore, Vincent Ollendorff, Daniel Birnbaum

Biochemical and Biophysical Research Communications **280,** 182–187 (2001)
doi:10.1006/bbrc.2000.4116, available online at http://www.idealibrary.com on IDEAL®

Characterization of the Mouse *Cblc/Cbl3* Gene

Frédéric Fiore, Vincent Ollendorff, and Daniel Birnbaum
Laboratoire d'Oncologie Moléculaire, U.119 Inserm, IFR57, 27 Boulevard Leï Roure, 13009 Marseille, France

Received November 28, 2000

The mouse *Cblc/Cbl3* gene was cloned and characterized. It comprises 12 exons and encodes a putative protein of 496 amino acid residues which shares an overall 67% identity with its human ortholog; it also shares 70% of amino acid identity with mouse CBL over their conserved SH2 and Ring finger domains. Mouse *Cblc* mRNA is expressed in embryo and adult tissues and has a rather ubiquitous distribution.

Transmembrane receptors (RTKs) and cytoplasmic molecules (PTKs) endowed with tyrosine kinase activity are major components of cell communication processes. Their recycling and degradation is tightly regulated by a number of factors. The cytoplasmic CBL adaptor proteins play a role in the negative regulation and degradation of RTKs and PTKs. In mammals, there are three CBL related proteins known to date: CBL, CBLb, and CBLc/CBL3, best characterized in humans. They are encoded by paralogous genes (1). CBLs function as ubiquitin E3 ligase proteins (2); when bound to an RTK and phosphorylated, they recruit ubiquitin-activating and conjugating enzymes that target RTKs and other proteins to the proteasome degradation machinery (3–8). The primarily suppressive function of CBL proteins is examplified by the defects observed in null mutant mice obtained by gene targeting (9–13) or in non vertebrate animal models (14–16). CBL proteins are constituted of a N-terminus domain, a RING finger domain and a C-terminus extension of variable length containing proline-rich sequences. The N-terminus region is sometimes called the PTB domain; actually, upon characterization of its three-dimensional structure (17), it has been subdivided into three subregions that include a four helix bundle (4F), a calcium-binding EF-hand domain and an unusual SH2 domain.

The human *CBLc/CBL3* gene was recently characterized (1, 18, 19). To gather more information on the third member of the CBL family, as well as a to take necessary first step towards the use animal models for studying this molecule, we cloned the mouse *Cblc* gene and studied its structure and its expression in the adult and during development.

MATERIALS AND METHODS

cDNA and cosmid cloning and analysis. Mouse *Cblc* cDNA was obtained by PCR amplification of reverse transcribed RNA from several tissues [kidney, prostate, 17.5 days postconceptus (d.p.c.) embryos]. Specific primers designed from the mouse coding sequence were used: (5′-CGGCGGCAGCCCCGCAAGGGTG-3′ and 5′-ACAGCTTGGCCGGAAGATATA-3′). A 794-bp PCR product, named "troncus," was cloned. The "troncus" fragment was used to performed 3′ RACE-PCR (Marathon cDNA amplification Kit, Clontech) to isolate the full length cDNA.

Mouse cosmid clones were isolated from a RZPD library (129/ola library No. 121) using a human cDNA probe (1). Eight clones were obtained and characterized by restriction enzyme patterns and DNA sequence (Genome Express, Grenoble, France) analyses. These clones were used to identified the coding regions. In addition, we scanned the emerging mouse genome sequence (http://www.ncbi.nlm.nih.gov/blast/blast.cgi) with the full length *Cblc* cDNA. The mouse *Cblc* gene sequence is present in clone RP23-33H23 (AC073760). A draft genomic sequence was obtained which confirmed our data.

Alignment was done using NCBI software (http://www.ncbi.nlm.nih.gov/gorf/bl2.html).

Northern and dot blot hybridization analyses. The mouse *Cblc* cDNA probe "troncus" was used to hybridize the Clontech Northern (Mouse tissues Cat 7762-1 and Mouse Embryo Cat 7763-1) and Dot blot (Cat 7771-1) membranes. Northern blot membranes contain 2 μg of total RNA in each lane. They were first hybridized with the *Cblc* probe and second with a murine *Gapdh* or *β-actin* probes. The control dot blot images (using an *Hprt* probe) and adult Northern blot (using a *β-Actin* probe) were provided by the furnisher.

Nonradioactive in situ hybridization analysis. Dioxygenin-labeled sense or antisense riboprobes were synthesized in the presence of digoxygenin-dUTP (Boehringer Mannheim) by T3 sense or T7 antisense RNA polymerase from the "troncus" fragment of 5′ coding sequences of mouse *Cblc* cDNA, cloned into pBS-KS+ plasmid and digested by *Xba*I (sense) or *Xho*I (antisense) restriction enzymes.

The study was done on 14–16 μm fresh-frozen tissue sections of embryos of 12.5, 13.5, and 14.5 d.p.c. and on adult mouse tissues. The protocol described by Philippe *et al.* (20) was used. Detection was carried out by anti-digoxygenin antibody conjugated with alkaline-phosphatase (1:5000, Boehringer Mannheim). Colorimetric reaction with nitro-blue tetrazodium and 5-bromo-4-chloro-3-indolyl-1-phosphate (NBT/BCIP) was carried out at room temperature: 2 h for control samples and only 15 min for positive sections.

0006-291X/01 $35.00

B

```
                1              15              30              45              60
CblcMu MAAAAAPQGWQRGEPRALSRAVKLLQRLEEQCRDPRMVTGPPSLRDLLPRTAQLLGEVAK
       MA A AP G Q  E RAL RAV  LQRLEEQC DPR    PPSLRDLLPRTAQLL EVA
CBLcHu MALAVAPWGRQWEEARALGRAVRMLQRLEEQCVDPRLSVSPPSLRDLLPRTAQLLREVAH

CblcMu ARREARED-PEGPGGADDFLAIYLANLEVKGRQVAELLPPRGKKDVNQDVFREGSRFRRQ
        RR A    P GPGG  DFL IYLANLE K RQVA LLPPRG    N   FR GSR RRQ
CBLcHu SRRAAGGGGPGGPGGSGDFLLIYLANLEAKSRQVAALLPPRGRRSANDELFRAGSRLRRQ

CblcMu LAKLALIFSHMHAELSALFPAGKYCGHLYQLTKGSAHIFWRQNCGVRCVLPWAEFQSLLC
       LAKLA IFSHMHAEL ALFP GKYCGH YQLTK  AH FWR  CG RCVLPWAEF SLL
CBLcHu LAKLAIIFSHMHAELHALFPGGKYCGHMYQLTKAPAHTFWRESCGARCVLPWAEFESLLG

CblcMu ACHPVEPGPTMQALRSTLDLTCNGHVSVFEFDVFTRLFQPWPTLLRNWQLLAVNHPGYMA
        CHPVEPG T  ALR T DLTC GHVS FEFDVFTRLFQPWPTLL NWQLLAVNHPGYMA
CBLcHu TCHPVEPGCTALALRTTIDLTCSGHVSIFEFDVFTRLFQPWPTLLKNWQLLAVNHPGYMA

CblcMu FLTYDEVQTRLQAYRDKPGSYIFRPSCTRLGQWAIGYVSSDGSILQTIPLNKPLLQVLLK
       FLTYDEVQ RLQA RDKPGSYIFRPSCTRLGQWAIGYVSSDGSILQTIP NKPL QVLL
CBLcHu FLTYDEVQERLQACRDKPGSYIFRPSCTRLGQWAIGYVSSDGSILQTIPANKPLSQVLLE

CblcMu GQKDGIFLFPDGKKHNPDLTELCRVEPYQRIQVSEEQLLLYQAMNSTFQLCKICAERDKD
       GQKDG +L+PDGK HNPDLTEL + EP QRI VSEEQL LY AM+STF+LCKICAE +KD
CBLcHu GQKDGFYLYPDGKTHNPDLTELGQAEPQQRIHVSEEQLQLYWAMDSTFELCKICAESNKD

CblcMu VRIEPCGHLLCSCCLAAWQDSDSQTCPFCRCEIKGREAVSICQAQERPTEVRTAADGSRD
       V IEPCGHLLCSCCLAAWQ SDSQTCPFCRCEIKG EAVSI Q     T     A  S
CBLcHu VKIEPCGHLLCSCCLAAWQHSDSQTCPFCRCEIKGWEAVSIYQFYGQAT-----AEDSGN

CblcMu NCHQEAAEQKLGPVIPSAPSLLPEDQFPQGPQDKGWLTLAPLALPRLRPPLPLPKMASVL
          QE  E  LG V  SAP L P    P
CBLcHu SSDQEGRELELGQVPLSAPPLPPRPDLPPRK-PRNAQPKVRLLKGNSPPAALGPQDPAPA*

CblcMu WEVTSRPRAREEATESS*
```

C

	«PTB» Domain			
	4H	EF	SH2	Ring Finger
Mm-CBLc	100%	100%	100%	100%
Hs-CBLc	72%	78%	87%	90%
Mm-CBL	31%	52%	70%	69%
Hs-CBL	30%	52%	70%	72%
Hs-CBLb	28%	49%	69%	77%
Dm-CBL	30%	50%	69%	75%
Ce-SLI-1	27%	39%	58%	66%

FIG. 1. Mouse *Cblc* gene structure. (A) Structure of the mouse *Cblc* gene showing exon-intron organization. (B) Alignment of amino-acid sequences of mouse CBLc and human CBLc. Identical amino acid residues are shown in between the two sequences. According to "SMART" software (http://smart.embl-heidelberg.de/), the amino acid stretch shown in green is a potential SH2 domain, the ring finger (as defined in 25) is shown in blue and the proline-rich region is shown in red. Two other domains (as defined in 17) are indicated: the four helix bundle (in yellow) and the EF-hand fold (in brown). The exon-intron boundaries are schematized by red arrowheads. The "troncus" probe region is

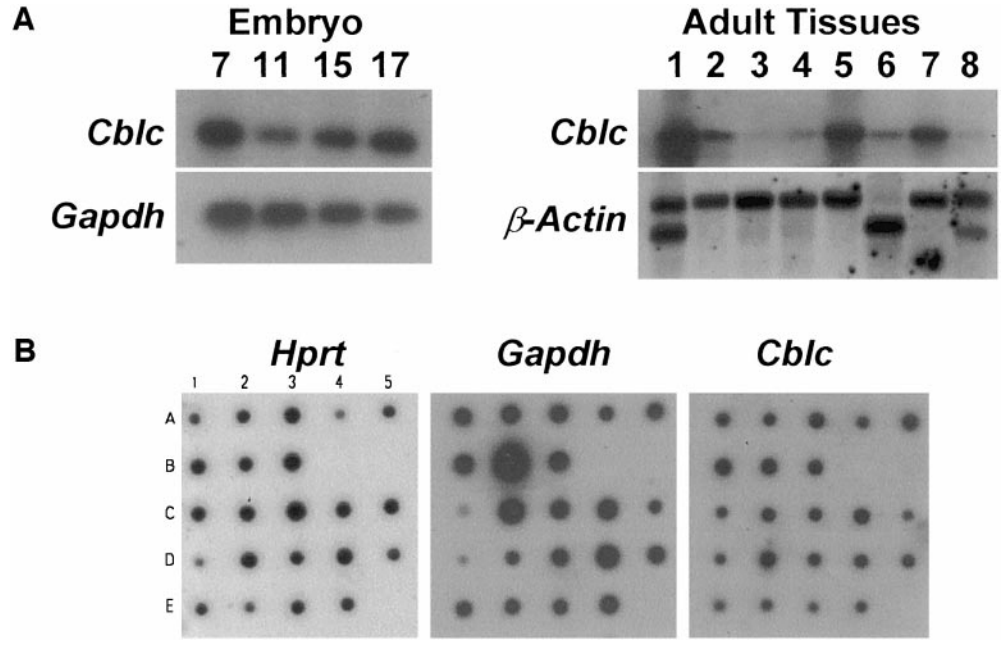

FIG. 2. Mouse *Cblc* mRNA expression. (A) Northern blot analysis of *Cblc* expression in adult mouse tissues and mouse embryos. Northern blot membranes containing polyA+ RNAs (2 μg per lane) from adult mouse tissues or embryos at different stages of gestation were hybridized with a 794-bp ^{32}P 5′ mouse *Cblc* fragment and *Gapdh* or *β-Actin* control probes. Mouse Embryo Northern blot: day 7; day 11; day 15; day 17 of gestation. Mouse multiple tissues Northern blot: heart (lane 1); brain (lane 2); spleen (lane 3); lung (lane 4); liver (lane 5); skeletal muscle (lane 6); kidney (lane 7); testis (lane 8). (B) Dot blot analysis of *Cblc* expression in adult mouse tissues and mouse embryos. Mouse RNA Master Blot containing polyA+ RNAs (100–500 ng per dot) was hybridized with the hypoxanthine guanine phosphoribosyl transferase (*Hprt*) ^{32}P-labeled probe and 5′ *Cblc* mouse probe. Brain (A1); eye (A2); liver (A3); lung (A4); kidney (A5); heart (B1); skeletal muscle (B2); smooth muscle (B3); pancreas (C1); thyroïd (C2); thymus (C3); submaxillary gland (C4); spleen (C5); testis (D1); ovary (D2); prostate (D3); epididymis (D4); uterus (D5); embryo day 7 (E1); embryo day 11 (E2); embryo day 15 (E3); embryo day 17 (E4).

RESULTS AND DISCUSSION

Mouse Cblc Gene Cloning

Mouse *Cblc* cDNA was obtained by PCR amplification of reverse transcribed RNA from several tissues. To characterize the 3′ end of *Cblc* cDNA, RACE-PCR was carried out on liver mRNA. The human *CBLc* cDNA probe col7, derived from EST zm28c07 (Accession No. AA112513) (1) was used to screen a mouse cosmid library from RZPD (http://www.tel.de/s/R/RZPD.htm). Eight positive cosmid clones were isolated. Cosmids B and F (Clone ID MPMGc121M0228Q4 and MPMGc121H07398Q3, respectively) were selected for further analysis. Mapping and sequencing of fragments reacting positively with the human *CBLc* probe identified exons 1 to 5 of the mouse *Cblc* gene. The structure of the mouse *Cblc* gene is shown in Fig. 1A. The gene comprises 12 exons which are distributed over 17 kb of genome.

Cloning and sequencing of the reconstituted cDNA yielded a deduced amino-acid sequence for the putative mouse CBLc/CBL3 protein. The mouse CBLc protein is 496 amino acids long and has a typical ring finger motif and a SH2 domain, two features shared by all CBL proteins. The alignment of mouse CBLc and human CBLc protein sequences is shown in Fig. 1B. Overall, mouse and human CBLc proteins share 67% amino-acid identity. As compared to the other CBL products, CBLc is shorter and lacks a C-terminus tail present in both CBL and CBLb. Mouse CBL and CBLc proteins share 70% amino acid identity over their conserved N-terminal portion (Fig. 1C). Whereas for CBL as well as CBLb, the C-terminus is well conserved between human and mouse, the C-terminal tail of murine CBLc differs greatly from that of human CBLc; however, the last proline-rich motif is preserved. This difference explains why the commercial antibodies, which are di-

underlined. The corresponding nucleotide sequence has the GenBank Accession No. AF319956. (C) Comparison of amino-acid sequence identities between different CBL proteins: the percentage of identity of amino acid residues between the various regions (including the so-called PTB region with 4H, four helix bundle; EF, EF-hand fold; and SH2, SH2 domain) of CBL proteins (Mm, Mus musculus; Hs, *Homo sapiens;* Dm, *Drosophila melanogaster;* Ce, *Caenorhabditis elegans*) is indicated below a schematic representation of the putative CBLc protein (the color code is the same as in B).

rected against the last amino acids of the human CBLc protein, are unable to recognize the mouse protein.

Cblc Expression

We first performed Northern blot analyses of *Cblc* expression in mouse tissues and embryos. Forward and reverse oligonucleotide primers were designed in exons 1 and 5, respectively, and used to amplify reverse-transcribed RNA from 17.5 d.p.c. embryo. This PCR product was used as probe in Northern blot analyses. *Cblc* was expressed in all tissues tested as a 1.7-kb transcript (Fig. 2A). Mouse embryos of 7, 11, 15, and

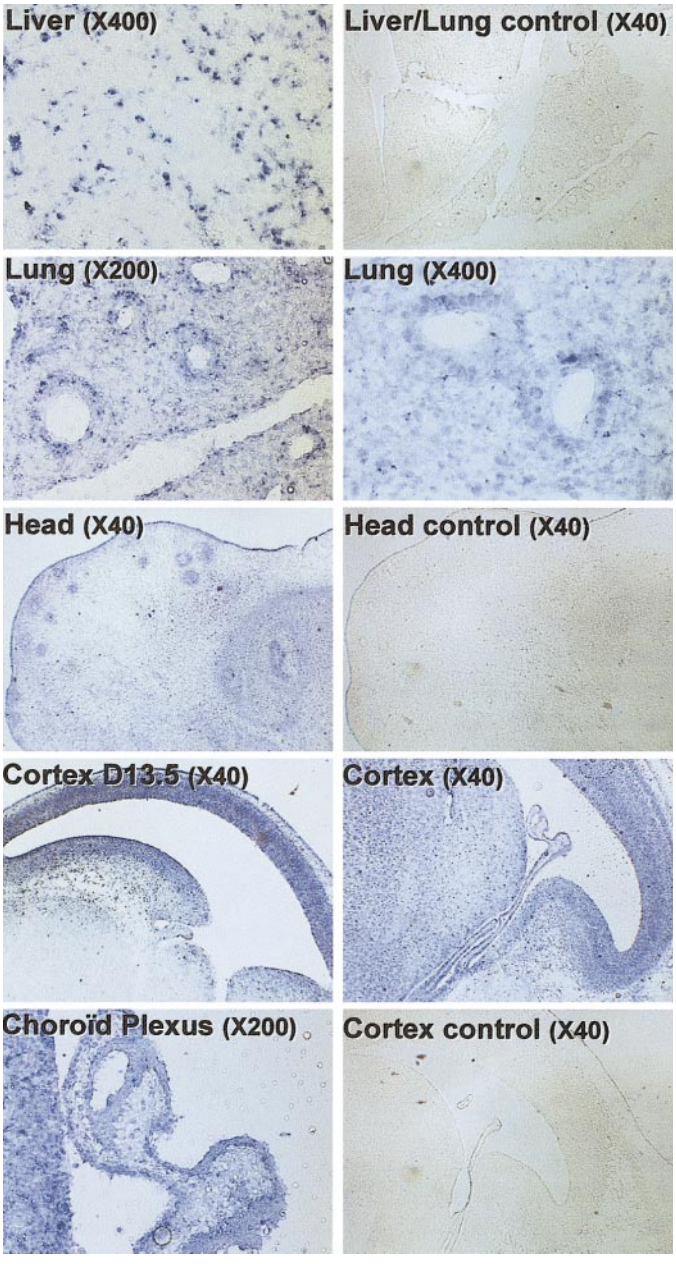

FIG. 3. *In situ* hybridization analysis of *Cblc* mRNA expression in mouse embryonic tissues. All organs express *Cblc* with various degrees and distribution. Details of liver, lung, head, and brain are shown. Control sense probe does not show any signal.

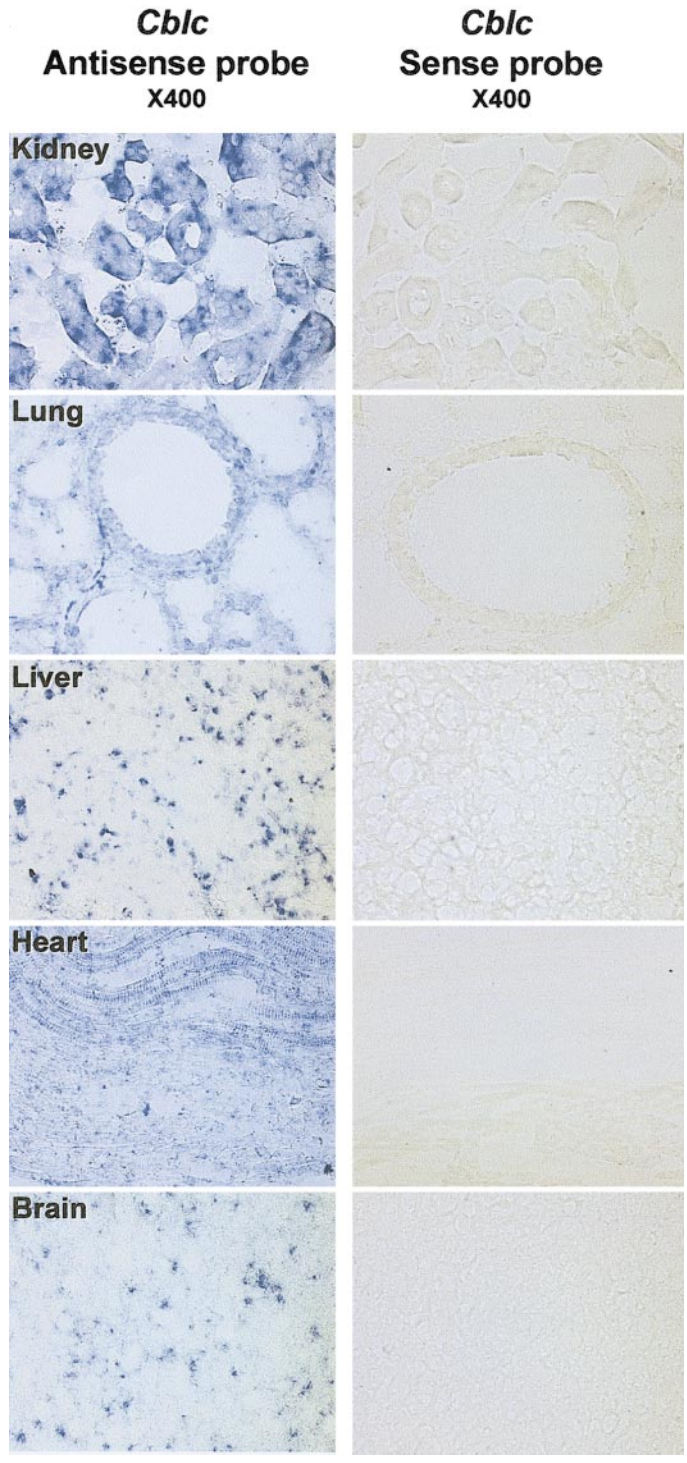

FIG. 4. *In situ* hybridization analysis of *Cblc* mRNA expression in adult mouse tissues. Several sections of adult tissues were analyzed by *in situ* hybridization. All tissues tested express *Cblc* mRNA. Details of kidney, lung, liver, heart, and brain are shown. Control sense probe does not show any signal.

17 d.p.c. express *Cblc*. No cross reacting signal with *Cbl* and *Cblb* mRNA was seen. To extend these data, we performed a dot blot expression study on mouse tissues. *Cblc* mRNA was found expressed in all tissues tested. However, expression was weaker in lymphohematopoietic organs such as thymus and spleen (Fig. 2B). As for mouse *Cblc*, analysis of the human *CBLc/CBL3* mRNA showed ubiquitous expression, with strong signals in endodermally-derived organs (18, 19). In contrast to human *CBLc*, mouse *Cblc* mRNA is also found in brain and in heart.

We next performed *in situ* hybridization on embryonic and adult tissues. *In situ* data correlated well with Northern blot results. *Cblc* is expressed in embryonic tissues. Figure 3 shows examples of *in situ* hybridization signals in the 14.5 d.p.c. fetal liver, with a punctuate perilobular labeling, lung where epithelial structures are prominently stained, head where the primordiae of follicles of vibrissae and eyes are strongly labeled, in the 13.5 and 14.5 d.p.c. fetal brain with a strong staining of cortex neurons and chroroïd plexus. *Cblc* mRNA was found in all adult tissues tested. Figure 4 shows examples of *in situ* hybridization signals: *Cblc* mRNA is present in the epithelial cells of the kidney nephron, the airway structures of the lung, the liver where the punctuate labeling is seen, as well as in myocardium and brain neurons.

The human *CBL* and *CBLb* genes are ubiquitously expressed but the mRNA levels of expression are variable according to tissues (21–23). Major transcripts of 11 and 4.4 kb are detected for *CBL* and *CBLb*, respectively. *CBL* and *CBLb* are specifically highly expressed, and play a role in lymphohematopoietic organs (8, 21–24). Accordingly, *Cbl* −/− (9) and *Cblb* −/− (12, 13) mice show immunological defects. In contrast, *Cblc* although also ubiquitously expressed, shows high expression in epithelial structures but its expression is lower in lymphohematopoietic tissues. It is possible that, depending on specific territories, differences in mRNA and/or protein levels are maintained between the three CBLs allowing differential regulation of signaling cascades. The CBL proteins play a major role in protein degradation via the ubiquitin pathway, but their respective spectrum and specificity of interaction with target proteins is not precisely known. Occupation of overlapping but slightly distinctive expression territories may be one way to exert their role in the degradation of different proteins.

ACKNOWLEDGMENTS

This work was supported by Inserm and Institut Paoli-Calmettes.

REFERENCES

1. Ollendorff, V., Mattei, M.-G., Fournier, E., Adélaïde, J., Lopez, M., Rosnet, O., and Birnbaum, D. (1998) A third human *CBL* gene is on chromosome 19. *Int. J. Oncol.* **13,** 1159–1161.
2. Jackson, P. K., Eldridge, A. C., Freed, E., Furstenthal, L., Hsu, J. Y., Kaiser, B. K., and Reimann, J. D. R. (2000) The lore of the RINGs: Substrate recognition and catalysis by ubiquitin ligases. *Trends Cell Biol.* **10,** 429–439.
3. Levkowitz, G., Waterman, H., Zamir, E., Kam, Z., Oved, S., Langdon, W. Y., Beguinot, L., Geiger, B., and Yarden, Y. (1998) C-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. *Genes Dev.* **12,** 3663–3674.
4. Miyake, S., Lupher, M. L., Druker, B., and Band, H. (1998) The tyrosine kinase regulator Cbl enhances the ubiquitination and degradation of the platelet-derived growth factor receptor alpha. *Proc. Natl. Acad. Sci. USA* **95,** 7927–7932.
5. Levkowitz, G., Waterman, H., Ettenberg, S. A., Katz, M., Tsygankov, A. Y., Alroy, I., Lavi, S., Iwai, K., Reiss, Y., Ciechanover, A., Lipkowitz, S., and Yarden, Y. (1999) Ubiquitin ligase activity and tyrosine phosphorylation underlie suppression of growth factor signaling by c-Cbl/Sli-1. *Mol. Cell* **4,** 1029–1040.
6. Joazeiro, C. A. P., Wing, S. S., Huang, H.-K., Leverson, J. D., Hunter, T., and Liu, Y.-C. (1999) The tyrosine kinase negative regulator c-Cbl as a RING-type, E2-dependent ubiquitin-protein ligase. *Science* **286,** 309–312.
7. Yokouchi, M., Kondo, T., Houghton, A., Bartkiewicz, M., Horne, W. C., Zhang, H., Yoshimura, A., and Baron, R. (1999) Ligand-induced ubiquitination of the epidermal growth factor receptor involves the interaction of the c-Cbl RING finger and UbcH7. *J. Biol. Chem.* **274,** 31707–31712.
8. Lupher, M. L., Rao, N., Eck, M. J., and Band, H. (1999) The Cbl protooncoprotein: A negative regulator of immune receptor signal transduction. *Immunol. Today* **20,** 375–382.
9. Murphy, M. A., Scnall, R. G., Venter, D. J., Barnett, L., Bertoncello, I., Thien, C. B., Langdon, W. Y., and Bowtell, D. D. (1998) Tissue hyperplasia and enhanced T-cell signaling via ZAP-70 in c-Cbl deficient mice. *Mol. Cell. Biol.* **18,** 4972–4882.
10. Naramura, M., Kole, H. K., Hu, R. J., and Gu, H. (1998) Altered thymic positive selection and intracellular signals in Cbl-deficient mice. *Proc. Natl. Acad. Sci. USA* **95,** 15547–15552.
11. Lee, P. S. W., Wang, Y., Dominguez, M. G., Yeung, Y.-G., Murphy, M. A., Bowtell, D. V. L., and Stanley, E. R. (1999) The Cbl protooncoprotein stimulates CSF-1 receptor multiubiquitination and endocytosis, and attenuates macrophage proliferation. *EMBO J.* **18,** 3616–3628.
12. Bachmeier, K., Krawczyk, C., Kozieradzki, I., Kong, Y.-Y., Sasaki, T., Oliveira-dos-Santos, A., Mariathasan, S., Bouchard, D., Wakeham, A., Itie, A., Le, J., Ohashi, P. S., Sarosi, I., Nishina, H., Lipkowitz, S., and Penninger, J. M. (2000) Negative regulation of lymphocyte activation and autoimmunity by the molecular adaptor Cbl-b. *Nature* **403,** 211–216.
13. Chiang, Y. J., Kole, H. K., Brown, K., Naramura, M., Fukuhara, S., Hu, R.-J., Jang, I. K., Gutkind, J. S., Shevach, E., and Gu, H. (2000) Cbl-b regulates the CD28 dependence of T-cell activation. *Nature* **403,** 218–220.
14. Pai, L-M., Barcelo, G., and Schüpbach, T. (2000) *D-cbl*, a negative regulator of the Egfr pathway, is required for dorsoventral patterning in *Drosophila* oogenesis. *Cell* **103,** 51–61.
15. Yoon, C. H., Lee, J., Jongeward, G. D., and Sternberg, P. W. (1995) Similarity of *sli-1*, a regulator of vulval development in *C. elegans*, to the mammalian proto-oncogene *c-cbl*. *Science* **269,** 1102–1105.
16. Yoon, C. H., Chang, C., Hopper, N. A., Lesa, G. M., and Sternberg, P. W. (2000) Requirements of multiple domains of SLI-1, a Caenorhabditis elegans homologue of c-cbl, and an inhibitory tyrosine in LET-23 in regulating vulval differentiation. *Mol. Cell. Biol.* **11,** 4019–4031.
17. Meng, W., Sawasdikoso, S., Burakoff, S. J., and Eck, M. J. (1999)

Structure of the amino-terminal domain of Cbl complexed to its binding site on ZAP-70 kinase. *Nature* **398,** 84–90.

18. Keane, M. M., Ettenberg, S. A., Nau, M. M., Cuello, M., Penninger, J., and Lipkowitz, S. (1999) Cbl-3: A new mammalian cbl family protein. *Oncogene* **18,** 3365–3375.
19. Kim, M., Tezuka, T., Suziki, Y., Sugano, S., Hirai, M., and Yamamoto, T. (1999) Molecular cloning and characterization of a novel cbl-family gene, cbl-c. *Gene* **239,** 145–154.
20. Philippe, J. M., and Pollock, R. A. (1998) Sequential in situ hybridization on tissue sections: A novel approach for analysing co-expression of different mRNAs in a single cell. Technical Tips on line (http://www.elsevier.com/locate/tto).
21. Langdon, W. Y., Hyland, C. D., Grumont, R. J., and Morse, H. C. (1989) The *c-cbl* proto-oncogene is preferentially expressed in thymus and testis tissue and encodes a nuclear protein. *J. Virol.* **63,** 5420–5424.
22. Keane, M. M., Rivero-Lezcano, O. M., Mitchelle, J. A., Robbins, K. C., and Lipkowitz, S. (1995) Cloning and characterization of cbl-b: A SH3 binding protein with homology to the c-cbl proto-oncogene. *Oncogene* **10,** 2367–2377.
23. Bustelo, X. R., Crespo, P., Lopez-Barahona, M., Gutkind, J. S., and Barbacid, M. (1997) Cbl-b, a member of the Sli-1/c-Cbl protein family, inhibits Vav-mediated c-Jun N-terminal kinase activation. *Oncogene* **15,** 2511–2520.
24. Kraczyk, C., Bachmaier, K., Sasaki, T., Jones, R. G., Snapper, S. B., Bouchard, D., Kozieradzki, I., Ohashi, P. S., Alt, F. W., and Penninger, J. M. (2000) Cbl-b is a negative regulator of receptor clustering and raft aggregation in T cells. *Immunity* **13,** 463–473.
25. Zheng, N., Wang, P., Jeffrey, P. D., and Pavletich, N. P. (2000) Structure of a c-CBL-UbcH7 complex: RING domain function in ubiquitin-protein ligases. *Cell* **102,** 533–539.