Genome Therapeutics Corporation Web site at www.genomecorp.com. Sequencing of *Clostridium acetobutylicum* was accomplished with support from the U.S. Department of Energy.
18. The standard SOD assay involves steady-state generation of superoxide by means of bovine xanthine oxidase plus xanthine modified from the following: L. Flohé, R. Becker, R. Brigelius, E. Lengfelder, F. Ötting, in *CRC Handbook of Free Radicals and Antioxidants in Biomedicine*, J. Miquel, Ed. (CRC Press, Boca Raton, FL, 1988), vol. 3, p. 287. Reaction conditions were 50 mM potassium phosphate (pH 7.8), 0.2 mM xanthine, and 20 μM horse heart cytochrome c at 25°C. Superoxide directly reduces cytochrome c as measured by the increase in absorption (at 550 nm) due to the reduced cytochrome. SOD (bovine, Sigma) inhibits superoxide-dependent reduction of cytochrome c; one unit of activity is the amount of protein necessary to inhibit the rate by 50%.
19. G. Fiala and K. O. Stetter, *Arch. Microbiol.* **145**, 56 (1986).
20. F. E. Jenney Jr. and M. W. W. Adams, in preparation. The Genbank accession number for SOR from *P. furiosus* is AF156097. Iron concentration was determined with a Thermo Jarrell-Ash 965 inductively coupled argon plasma spectrometer (ICAP) at the Chemical Analysis Laboratory of the University of Georgia.
21. P. R. Blake *et al.*, *Biochemistry* **30**, 10885 (1991).
22. A. V. Coelho *et al.*, *J. Biol. Inorg. Chem.* **2**, 680 (1997).
23. L. Chen *et al.*, *Eur. J. Biochem.* **226**, 613 (1994); N. V. Shenvi and D. M. Kurtz Jr., GenBank direct submission, accession number AF034965 (1997).
24. G. Silva *et al.*, *Eur. J. Biochem.* **259**, 235 (1999); C. V. Romão *et al.*, *ibid.* **261**, 438 (1999).
25. Inhibition of pyrogallol auto-oxidation, epinephrine auto-oxidation, and nitroblue tetrazolium reduction were measured as described in the following references, respectively: S. L. Marklund, in *CRC Handbook of Methods for Oxygen Radical Research*, R. A. Greenwald, Ed. (CRC Press, Boca Raton, FL, 1985), p. 243; D. G. Searcy, J. P. Whitehead, M. J. Maroney, *Arch. Biochem. Biophys.* **318**, 251 (1995); C. Beauchamp and I. Fridovich, *Anal. Biochem.* **44**, 276 (1971).
26. A. Azzi, C. Montecucco, C. Richter, *Biochem. Biophys. Res. Comm.* **65**, 597 (1975).
27. C. M. Fraser *et al.*, *Science* **281**, 375 (1998).
28. The only exception is the methanogen *Methanobacterium thermoautotrophicum*, but it possesses a desulfoferrodoxin homolog (as well as a classical SOD gene) [D. R. Smith *et al.*, *J. Bacteriol.* **179**, 7135 (1997)]. Anaerobic eukaryotes also appear to have mechanisms to detoxify oxygen in the absence of detectable SOD and catalase [F. D. Marvin-Sikkema, T. M. Pedro Gomes, J.-P. Grivet, J. C. Gottschal, R. A. Prins, *Arch. Microbiol.* **160**, 388 (1993)].
29. An amino acid sequence alignment of the nine available SORs is available as supplementary material at www.sciencemag.org/feature/data/1042812.shl.
30. T. Kaneko *et al.*, *DNA Res.* **3**, 109 (1996).
31. F. R. Blattner *et al.*, *Science* **277**, 1453 (1997).
32. S. I. Liochev and I. Fridovich, *J. Biol. Chem.* **272**, 25573 (1997); M. J. Pianzzola, M. Soubes, D. Touati, *J. Bacteriol.* **178**, 6736 (1996).
33. G. R. Moore and G. W. Pettigrew, *Cytochromes c: Evolutionary, Structural and Physicochemical Aspects* (Springer-Verlag, Berlin, 1990), p. 328.
34. R. Bau *et al.*, *J. Bioinorg. Chem.* 3, 484 (1998).
35. M. W. W. Adams, *Adv. Inorg. Chem.* **38**, 341 (1992).
36. L. Chen *et al.*, *FEBS Lett.* **216**, 443 (1993).
37. K. Ma and M. W. W. Adams, *J. Bacteriol.*, **181**, 5530 (1999).
38. Catalase activity was assayed as in H. Aebi, *Methods Enzymol.* **105**, 121 (1984).
39. The anaerobe genomes (*12–16*, *27*, *28*) contain several genes whose products could be involved in peroxide detoxification. All have open reading frames (ORFs) that are highly similar to mammalian 1-cys peroxiredoxin {the *P. furiosus* ORF [R. Weiss *et al.*, available at http://combdna.umbi.umd.edu/bags.html (1999)] has 32% identity with the gene in *Mus musculus* [T. H. Lee *et al.*, GenBank direct submission, accession number AAD03716 (1998); S. W. Kang, I. C. Baines, S. G. Rhee, *J. Biol. Chem.* **273**, 6303 (1998)]}. Many also contain genes encoding one or more putative NAD(P)H-dependent peroxidases. *Pyrococcus furiosus* contains an ORF that is 48% similar to NADH peroxidase from a mesophilic facultative aerobe [R. P. Ross and A. Claiborne, *J. Mol. Biol.* **277**, 658 (1992); T. C. Mallett and A. Claiborne, *Biochemistry* **37**, 8790 (1998); T. Stehle, S. A. Ahmed, A. Claiborne, G. E. Schulz, *J. Mol. Biol.* **221**, 1325 (1991)].
40. L. B. Poole and H. R. Ellis, *Biochemistry* **35**, 56 (1996); L. A. Tartaglia, G. Storz, M. H. Brodsky, A. Lai, B. N. Ames, *J. Biol. Chem.* **265**, 10535 (1990).
41. D. M. Kurtz Jr., *J. Biol. Inorg. Chem.* **2**, 159 (1997).
42. Y. Lehmann, L. Meile, M. Teuber, *J. Bacteriol.* **178**, 7152 (1996); F. Bonomi, D. M. Kurtz Jr., X. Cui, *J. Biol. Inorg. Chem.* **1**, 67 (1996); P. S. Alban, D. L. Popham, K. E. Rippere, N. R. Krieg, *J. Appl. Microbiol.* **85**, 875 (1998).
43. M. W. W. Adams and A. Kletzin, *Adv. Prot. Chem.* **48**, 101 (1996).
44. Y. Fouquet *et al.*, *Econ. Geol.* **88**, 2018 (1993); M. K. Tivey, in *Seafloor Hydrothermal Systems: Physical, Chemical, Biological, and Geological Interactions*, S. E. Humphris, R. A. Zierenberg, L. S. Mullineaux, R. E. Thomson, Eds. (American Geophysical Union, Washington, DC, 1995), pp. 158–177.
45. R. Huber, P. Stoffers, J. L. Cheminee, H. H. Richnow, K. O. Stetter, *Nature* **345**, 179 (1990); M. Summit and J. A. Baross, *Deep-Sea Res. Part II* **45**, 2751 (1998).
46. Supported by grants from the American Cancer Society (PF-4254), the Oliver S. and Jennie R. Donaldson Charitable Trust, the Department of Energy (FG05-95ER20175), and NSF (MCB 9809060).

18 June 1999; accepted 23 August 1999

The Tyrosine Kinase Negative Regulator c-Cbl as a RING-Type, E2-Dependent Ubiquitin-Protein Ligase

Claudio A. P. Joazeiro,[1] Simon S. Wing,[2] Han-kuei Huang,[1] Joel D. Leverson,[1] Tony Hunter,[1]* Yun-Cai Liu[3]

Ubiquitination of receptor protein-tyrosine kinases (RPTKs) terminates signaling by marking active receptors for degradation. c-Cbl, an adapter protein for RPTKs, positively regulates RPTK ubiquitination in a manner dependent on its variant SRC homology 2 (SH2) and RING finger domains. Ubiquitin-protein ligases (or E3s) are the components of ubiquitination pathways that recognize target substrates and promote their ligation to ubiquitin. The c-Cbl protein acted as an E3 that can recognize tyrosine-phosphorylated substrates, such as the activated platelet-derived growth factor receptor, through its SH2 domain and that recruits and allosterically activates an E2 ubiquitin-conjugating enzyme through its RING domain. These results reveal an SH2-containing protein that functions as a ubiquitin-protein ligase and thus provide a distinct mechanism for substrate targeting in the ubiquitin system.

Specific and precise signaling by RPTKs requires that both the intensity and the duration of the elicited intracellular signals be tightly regulated. To terminate signaling, ubiquitination can mark receptors for degradation (*1*). Free ubiquitin (Ub) is recruited to ubiquitination pathways by the E1 Ub-activating enzyme through the formation of a thioester between a cysteine in E1 and the COOH-terminus of Ub (*2*). Ub is subsequently transferred, also as a thioester, to members of the E2 Ub-conjugating (Ubc) enzyme superfamily. Ub-protein ligases, or E3s, are defined as the pathway components responsible for substrate recognition and for promoting Ub ligation to substrate (*2*). HECT domain–containing E3s (*h*omologous to *E6*-AP *C*OOH-*t*erminus) accept Ub from E2s in the form of a thioester and then catalyze the formation of stable Ub-substrate conjugates (*2*). E3s such as the Skp1/cullin/F box (SCF) and anaphase-promoting complexes (APC) (*2*) do not appear to form thioesters with Ub (*3*). Rather, these complexes act by bringing E2~Ub and substrate together and by allosterically activating E2 (*3*, *4*). The efficiency of poly-Ub chain synthesis may be increased by other factors, such as the E4 proteins (*5*).

c-Cbl, a 120-kD protein that contains a variant SH2 domain, a RING finger, a proline-rich region, and a Ub-associated domain, binds to, and stimulates the ubiquitination of, active platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and colony-stimulating factor–1 receptors (*6–8*). Moreover, the c-Cbl homolog in *Caenorhabditis elegans*, SLI-1, has been genetically defined as a negative regulator of RPTK signaling (*9*). The fact that c-Cbl can function as an adapter protein in tyrosine phosphorylation–dependent signaling raises the possibility that it recruits E3 or E4

[1]The Salk Institute, Molecular Biology and Virology Laboratory, La Jolla, CA 92037, USA. [2]Department of Medicine, McGill University, Montreal, Quebec H3A 2B2, Canada. [3]La Jolla Institute for Allergy and Immunology, San Diego, CA 92121, USA.

*To whom correspondence should be addressed. E-mail: hunter@salk.edu

proteins to ubiquitinate active receptors. A critical role for the RING finger in regulating this process is suggested by the observation that c-*cbl* alleles that are defective in promoting RPTK ubiquitination contain either an entire deletion (v-*cbl*), a small deletion (70Z/3 *cbl*), or a point mutation (Cys^{381}→Ala) in the RING domain (*8*, *9*). Because RING finger subunits are essential components of SCF and APC ligases (*3*, *4*, *10*) and, together with cullins, these RING proteins recruit and activate E2s (*3*, *4*), we tested whether c-Cbl might itself be an E3 ligase for RPTKs.

To determine whether the c-Cbl RING finger has intrinsic E3 activity, we generated glutathione-S-transferase (GST) fusion proteins encompassing residues 359 to 447 of wild-type (WT) human c-Cbl (*11*). This sequence includes the entire RING domain and 10 to 20 residues on either end. A similar construct was generated containing the 17–amino acid deletion (residues 366 to 382) found in the ubiquitination-deficient 70Z/3 Cbl, that includes the first cysteine in the RING consensus, Cys^{381}. GST fusion proteins purified from *Escherichia coli* were assayed for the ability to promote substrate-independent Ub-protein ligation in the presence of human E1 and the E2 Ubc4 (both also purified from *E. coli*), adenosine 5′-triphosphate (ATP), and ^{125}I-labeled Ub (*12*).

The WT c-Cbl RING activated the ligation of ^{125}I -Ub to proteins in the reaction (Fig. 1A), whereas no ubiquitinated products were observed in the presence of GST or of GST-70Z/3 RING, even after prolonged incubation, at times when the reaction with the WT RING domain reached a plateau.

The products of substrate-independent ubiquitination reactions with the Cbl RING finger were also analyzed by immunobloting with an antibody to GST (anti-GST) (Fig. 1B) (*12*). Incubation of the WT, but not the 70Z/3 RING-GST fusion protein, with E1, Ubc4, ATP, and Ub led to the formation of slower migrating proteins that were recognized by anti-GST (*13*). Therefore, in the absence of a substrate, the GST-RING fusion protein itself becomes poly-ubiquitinated on the GST or RING moiety, or both. Because the RING component of E3s does not appear to form a thioester with Ub (*3*), the products of the reactions shown in Fig. 1, A and B, presumably arise from a direct transfer of Ub from an E2~Ub thioester to GST-RING lysyl residues. We conclude that the c-Cbl RING has E3 activity.

Two components of SCF ligases, the Hrt1/Roc1/Rbx1 RING finger protein and cullin/Cdc53, form a complex that allosterically stimulates the basal rate of auto-ubiquitination of the E2 Cdc34 (*3*, *4*). No auto-ubiquitination of the E2 Ubc4 was detected in the presence of E1, Ub, and ATP (Fig. 1C), but addition of GST-RING WT, but not of GST-RING 70Z/3, caused ubiquitination of Ubc4 (*14*). This result suggests that the c-Cbl

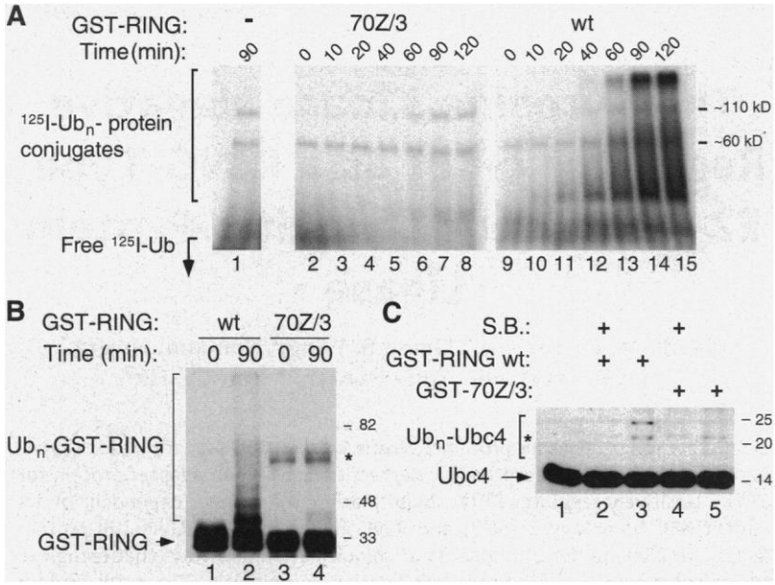

Fig. 1. Intrinsic Ub ligase activity of the c-Cbl RING finger. (**A**) Ubiquitination reactions were performed, as indicated, with GST or GST fusion proteins with c-Cbl and 70Z/3 Cbl RING fingers, E1, Ubc4, ^{125}I-Ub, and ATP for the indicated time. Ub-protein conjugates were resolved by reducing SDS-PAGE and detected by autoradiography. The indicated 60-kD ^{125}I-labeled protein is present in unreacted samples. Free Ub was run off the gel. (**B**) Ubiquitination reactions were performed as in (A), except that unlabeled Ub was used and the reaction was developed by immunoblot with antibody to GST. SDS gel sample buffer (2×) was added to the reactions corresponding to lanes 1 and 3 before proteins. The positions of GST-RING and poly-ubiquitinated (Ub_n) GST-RING are indicated. The asterisk indicates cross-reacting proteins that are present in certain unreacted GST preparations (*27*). Numbers at the right are molecular sizes (in kilodaltons). (**C**) Reactions performed as in (B) developed by immunoblot with antibody to His tag to detect His-Ubc4. S.B. refers to the addition of SDS gel sample buffer to the reaction before proteins. The positions of Ubc4 and ubiquitinated Ubc4 (Ub_n-Ubc4) are indicated. The asterisk indicates the position of a weakly staining band present in both unreacted (lane 4) and reacted (lane 5) 70Z/3 samples that comigrates with a band present only in the reacted WT sample (lane 3).

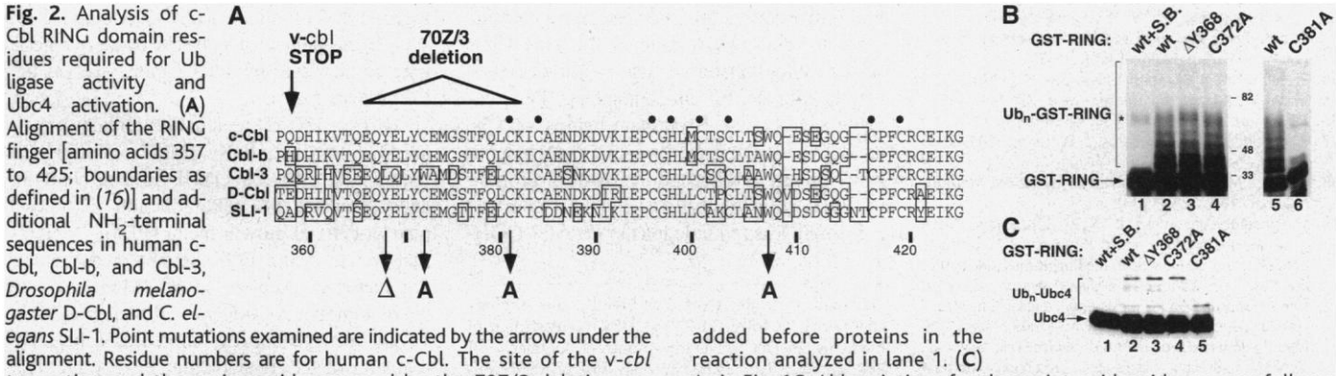

Fig. 2. Analysis of c-Cbl RING domain residues required for Ub ligase activity and Ubc4 activation. (**A**) Alignment of the RING finger [amino acids 357 to 425; boundaries as defined in (*16*)] and additional NH_2-terminal sequences in human c-Cbl, Cbl-b, and Cbl-3, *Drosophila melanogaster* D-Cbl, and *C. elegans* SLI-1. Point mutations examined are indicated by the arrows under the alignment. Residue numbers are for human c-Cbl. The site of the v-*cbl* truncation and the amino acids removed by the 70Z/3 deletion are indicated at the top. The Cys and His residues that fit the RING consensus for zinc binding are indicated by dots. Nonconserved residues are boxed. (**B**) As in Fig. 1B. SDS sample buffer (S.B.) was added before proteins in the reaction analyzed in lane 1. (**C**) As in Fig. 1C. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

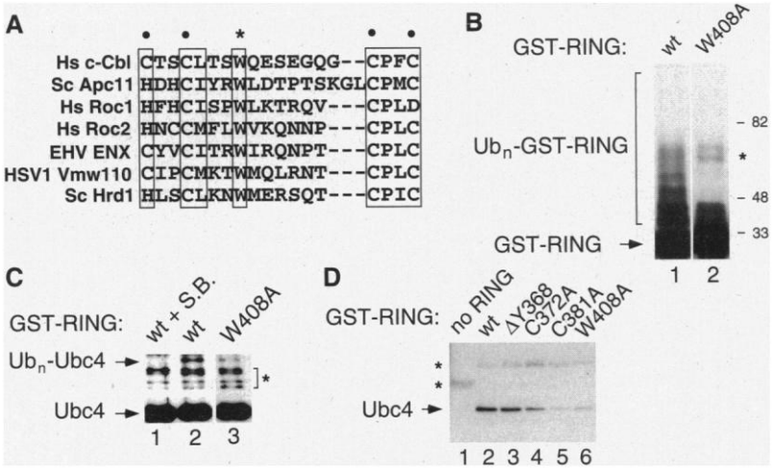

Fig. 3. Requirement of a tryptophan residue for the Ub ligase function of c-Cbl. (**A**) Alignment of the sequences between the third and fourth pairs of zinc-chelating residues (indicated by dots) of human c-Cbl, *S. cerevisiae* Apc11, human Roc1 and Roc2, equine herpes virus ENX, herpes simplex virus Vmw110, and *S. cerevisiae* Hrd1. Conserved residues and conservative substitutions are boxed. Cbl Trp^{408} is indicated by an asterisk. (**B**) As in Fig. 1B. (**C**) As in Fig. 1C. The asterisk indicates antibody cross-reacting proteins that were also present in the unreacted samples shown in lane 1. (**D**) GST pulldown assay. The association between Ubc4 and the indicated GST fusion proteins was measured as described (*20*). The asterisks indicate cross-reacting GST and GST-RING fusion proteins (*28*).

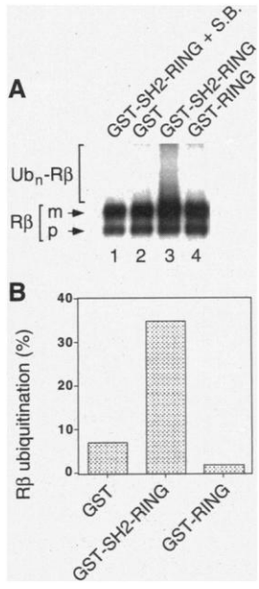

Fig. 4. Ubiquitination of the PDGF β-receptor (Rβ) by the c-Cbl RING in an SH2-dependent manner. (**A**) Immunoprecipitated Rβ was labeled with [γ-^{32}P]ATP in an in vitro kinase reaction, extensively washed, and subsequently incubated with the indicated GST fusion proteins in ubiquitination reactions (*22*). The position of polyubiquitinated (Ub_n) Rβ is indicated at the left, as well as the 190-kD mature (m) and 160-kD precursor (p) forms of Rβ (*29*). Lane 1 represents the unreacted control, in which SDS sample buffer was added to the reaction before proteins. (**B**) Results in (A) were quantified and normalized as described in (*30*). In a separate experiment with smaller amounts of proteins, receptor poly-ubiquitination was 9% with GST-SH2-RING and 0% with GST alone (*21*).

RING can allosterically activate the E2 Ubc4.

We examined the effect of point mutations in three residues that lie in the region deleted in 70Z/3 Cbl (Fig. 2A) on c-Cbl RING Ub ligase activity: Cys^{381} fits the RING consensus and its mutation impairs c-Cbl's ability to stimulate ubiquitination of EGF receptor (EGF-R) in vivo (*8*); Cys^{372} might participate in metal binding, although it does not fit the RING consensus and is not conserved in Cbl-3; and Tyr^{368}, whose deletion renders Cbl oncogenic (*15*). The $Cys^{381}\rightarrow$Ala mutant was defective in both ubiquitination and Ubc4 activation assays, whereas $Cys^{372}\rightarrow$Ala and ΔTyr^{368} were as active as the WT RING (Fig. 2, B and C). Therefore, loss of a RING finger consensus cysteine (Cys^{381}) can account for the ubiquitination defect of 70Z/3 Cbl. In contrast, the nearly WT Ub ligase activity of ΔTyr^{368} RING does not correlate with the oncogenic potential of ΔTyr^{368} Cbl. Thus, the ΔTyr^{368} mutation may indirectly affect RING function in the context of full-length c-Cbl in vivo.

Only the few residues thought to maintain the RING structural scaffold are conserved among RING proteins (*16*). A subset of these proteins, that either possess Ub ligase activity or are implicated in protein degradation, have a conserved tryptophan at a position where other hydrophobic residues are otherwise found (*3*, *4*, *10*, *16–18*) (Fig. 3A). The sequence COOH-terminal to Trp^{408}, Trp-Gln-Glu-Ser-Glu-Gly, also bears resemblance to a small portion of a domain (Trp-Gln-Ser-Asp-Gly) present in the Apc10 subunit of APC (*19*). c-Cbl RING Trp^{408} is required for E3 ligase activity, because its replacement by alanine reduced the ability of the RING to promote Ub ligation and Ubc4 activation (Fig. 3, B and C).

Binding of c-Cbl RING-GST fusion protein to Ubc4 was examined (Fig. 3D) (*20*). The GST-RING bound to Ubc4, whereas GST did not. We also determined the effect of c-Cbl RING mutations on Ubc4 binding (Fig. 3D). The ΔTyr^{368} mutant was almost as efficient as the WT RING domain in Ubc4 binding. The $Cys^{381}\rightarrow$Ala mutant bound Ubc4 much less efficiently than the WT protein. $Trp^{408}\rightarrow$Ala showed similarly weak binding to Ubc4. Therefore, the reduction in $Cys^{381}\rightarrow$Ala and $Trp^{408}\rightarrow$Ala binding to Ubc4 can account for their defects in ubiquitination and E2 activation. The $Cys^{372}\rightarrow$Ala mutant was also less efficient than the WT RING domain in Ubc4 binding. However, $Cys^{372}\rightarrow$Ala was indistinguishable from the WT protein in Ub ligase activity and Ubc4 activation (Figs. 2, B and C) even under lower Ubc4 concentrations and shorter reaction times (*21*). Possibly, Ala^{372} weakens the association with Ubc4, but makes activation of Ubc4 more efficient.

To determine whether the PDGF β-receptor (PDGF-Rβ) can serve as a direct substrate for ubiquitination by c-Cbl, GST-RING or a GST fusion protein with the c-Cbl 480 NH_2-terminal residues, containing both the variant SH2 and the RING domains (GST-SH2-RING), were tested for the ability to ubiquitinate PDGF-Rβ. In vitro ^{32}P-phosphorylated receptor was incubated with E1, Ubc4, Ub, ATP, and GST fusion proteins (*22*). Cbl·RING·Ubc4 complexes did not cause ubiquitination of the receptor (Fig. 4, A and B). In contrast, efficient PDGF-Rβ ubiquitination was observed in the presence of GST-SH2-RING·Ubc4 (Fig. 4, A and B), suggesting that the c-Cbl variant SH2 domain is required for substrate recognition (*23*).

The small RING components of E3 ligases that consist mostly of the RING domain require an accessory cullin subunit to activate E2s and additional subunits for substrate recognition (*3*, *4*, *10*). However, cullins are not required for the Ub ligase activity of Cbl (*24*) or Ubr1p (*25*), and these larger proteins can also recognize substrate.

The results of this work suggest how phosphorylated tyrosine can serve as a targeting signal for the Ub pathway in vivo: by mediating interactions either with SH2 domain–containing E3s such as c-Cbl or with SH2-containing proteins that in turn recruit E3s, as may occur with Grb2, a major Cbl-associated protein (*23*, *26*).

References and Notes

1. L. Hicke, *FASEB J.* **11**, 1215 (1997); C.-H. Heldin, A. Östman, L. Rönnstrand, *Biochim. Biophys. Acta* **1378**, F79 (1998).
2. A. Hershko and A. Ciechanover, *Annu. Rev. Biochem.* **67**, 425 (1998); A. L. Haas and T. J. Siepmann, *FASEB J.* **11**, 1257 (1997).
3. J. H. Seol *et al.*, *Genes Dev.* **13**, 1614 (1999).
4. D. Skowyra *et al.*, *Science* **284**, 662 (1999).
5. M. Koegl *et al.*, *Cell* **96**, 635 (1999).
6. P. S. Lee *et al.*, *EMBO J.* **18**, 3616 (1999).
7. S. Miyake, M. L. Lupher Jr., B. Druker, H. Band, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 7927 (1998); S. Miyake,

K. P. Mullane-Robinson, N. L. Lill, P. Douillard, H. Band, *J. Biol. Chem.* **274**, 16619 (1999).
8. G. Levkowitz *et al.*, *Genes Dev.* **12**, 3663 (1998); H. Waterman, G. Levkowitz, I. Alroy, Y. Yarden, *J. Biol. Chem.* **274**, 22151 (1999).
9. C. H. Yoon, J. Lee, G. D. Jongeward, P. W. Sternberg, *Science* **269**, 1102 (1995).
10. P. Tan *et al.*, *Mol. Cell* **3**, 527 (1999); T. Ohta, J. J. Michel, A. J. Schottelius, Y. Xiong, *ibid.*, p. 535; T. Kamura *et al.*, *Science* **284**, 657 (1999); W. Zachariae *et al.*, *ibid.* **279**, 1216 (1998).
11. DNA fragments encoding the RING finger were amplified by polymerase chain reaction (PCR) of c-Cbl WT and 70Z/3 cDNA with primers CAAGACCATATCAAAGTGACCCAG and CTATGCTCCTTGCCTCAACAG. PCR products were subcloned first into pCRII, verified by sequencing, and then subcloned into the Eco RI site of pGEX-4T2. Point mutations were generated with QuikChange (Stratagene) with pGEX-4T2-RING as template. pGEX-cbl 1-480 (encoding GST-SH2-RING) was a gift from L. Samelson. GST fusion proteins were produced in *E. coli* BL21 (DE3) by induction with 0.4 mM isopropyl-β-D-thiogalactopyranoside for 3 hours at 30°C. Cells were lysed in 50 mM tris-HCl (pH 8), 120 mM NaCl, 1 mM dithiothreitol (DTT), and protease inhibitors. Proteins bound to glutathione-Sepharose beads were eluted with lysis buffer containing 20 mM glutathione and dialyzed against lysis buffer containing 50 mM NaCl and 10% glycerol.
12. His-tagged E1 (a gift of F. Yamao) and Ubc4 [a gift of M. Nakao; T. Anan *et al.*, *Genes Cells* **3**, 1 (1998)] were produced as in (*11*), except that Talon beads (Clontech) and 100 mM imidazole were used for His-tagged protein binding and elution, respectively. Iodination of Ub was as described [R. Oughtred, N. Bedard, A. Vrielink, S. S. Wing, *J. Biol. Chem.* **273**, 18435 (1998)]. Ubiquitination and Ubc4 activation assays: Reactions (10 to 15 μl) contained His-E1 (50 to 500 nM), His-Ubc4 (0.5 to 5 μM), GST fusion proteins (10 μg), ubiquitin (5 μM), and ATP (2 mM) in Ub buffer [50 mM tris-HCl (pH 7.5), 2.5 mM $MgCl_2$, and 0.5 mM DTT]. Incubation was at 23°C for 90 min, unless otherwise indicated. Reactions were stopped with 2× SDS sample buffer, containing 4% SDS and 5.8 M β-mercaptoethanol. Ub-protein ligation was dependent on the presence of both E1 and Ubc4 (*21*). Ubc4 was chosen for these experiments because it had been shown to support EGF-R ubiquitination in fractionated cell extracts [S. Mori *et al.*, *Eur. J. Biochem.* **247**, 1190 (1997)]. Monoclonal antibodies used for protein immunoblots were anti–Tetra-His (Qiagen) and anti-GST (Santa Cruz Biotechnology). Prestained molecular weight markers were used to determine approximate protein sizes.
13. The difference between the distribution of Ub adducts in Fig. 1, A and B, is likely due to the fact that any given poly-ubiquitination product of GST-RING has only one site for anti-GST binding, but multiple labeled Ub moieties.
14. The species indicated in Figs. 1C, 2C, and 3C as Ub_n-Ubc4 are resistant to cleavage by 2.9 M β-mercaptoethanol and boiling and therefore do not represent E2~Ub thioesters. The inefficiency of the Ubc4 activation reaction is likely due either to a lower propensity of Ubc4 to auto-ubiquitinate [as compared with Cdc34; A. Banerjee, L. Gregori, Y. Xu, V. Chau, *J. Biol. Chem.* **268**, 5668 (1993)] or to the competing reaction, ubiquitination of GST-RING.
15. C. E. Andoniou, C. B. Thien, W. Y. Langdon, *EMBO J.* **13**, 4515 (1994).
16. P. S. Freemont, *Ann. N. Y. Acad. Sci.* **684**, 174 (1993).
17. R. Y. Hampton, R. G. Gardner, J. Rine, *Mol. Biol. Cell* **7**, 2029 (1996).
18. J. Parkinson, S. P. Lees-Miller, R. D. Everett, *J. Virol.* **73**, 650 (1998).
19. R. Grossberger *et al.*, *J. Biol. Chem.* **274**, 14500 (1999).
20. GST pulldown assay: GST fusion protein (5 μg) and His-Ubc4 (1 μg) were incubated for 2 hours at 4°C in 1 ml of binding buffer [20 mM tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% NP-40, bovine serum albumin (25 μg/ml)], and then with 30 μl of glutathione-Sepharose beads (Pharmacia) for 30 min. Precipitates were washed four times with binding buffer and subjected to 12% SDS–polyacrylamide gel electrophoresis (PAGE) and immunoblotting with anti-Ubc4 [V. Rajapurohitam *et al.*, *Dev. Biol.* **212**, 217 (1999)].
21. C. A. P. Joazeiro and T. Hunter, unpublished data.
22. PDGF-Rβ cDNA in the pcDNA3 expression vector was transfected into 293T human embryonic kidney cells. Cells were kept in 0.5% serum for 48 hours before lysis with 10 mM tris-HCl (pH 7.5), 1% Triton X-100, 5 mM EDTA, 50 mM NaCl, and protease inhibitors. PDGF-Rβ was immunoprecipitated from cleared lysates with PR7212 monoclonal antibody (a gift of D. Bowen-Pope). Beads were washed three times with lysis buffer, and twice with 20 mM Hepes (pH 7.4), 100 mM NaCl, and aprotinin (20 μg/ml). An in vitro kinase reaction with [γ-^{32}P]ATP was done to label the receptor and to provide phosphotyrosine binding sites for the c-Cbl SH2 domain: Beads were washed twice and resuspended with kinase reaction buffer [20 mM Hepes (pH 7.4), 10 mM $MnCl_2$, aprotinin (20 μg/ml), and 20 μM Na_3VO_4], to which 20 μCi of [γ-^{32}P]ATP was added for 45 min at room temperature. The reaction was chased with 1 mM ATP for 20 min. Beads were washed once with phosphate-buffered saline containing 0.5 mM EDTA, and four times with Ub buffer, and samples were taken for ubiquitination reactions (*12*).
23. The tyrosyl residues in RPTKs that mediate direct interaction with the Cbl SH2 domain are unknown. The association of the Cbl SH2 and COOH-terminal domains with RPTKs in vivo has been discussed [L. Smit and J. Borst, *Crit. Rev. Oncog.* **8**, 359 (1997)].
24. To test whether cullins interact with c-Cbl in vivo to regulate its activity, hemagglutinin A (HA)–tagged c-Cbl or Roc1 and Myc-tagged cullins were overexpressed in 293T cells. Whereas HA-tagged Roc1 efficiently coimmunoprecipitated with cullins 1, 2, 3, 4A, and 5 (*3*, *4*, *10*) (gifts of Z.-Q. Pan, J. Michel, and Y. Xiong), c-Cbl, 70Z/3 Cbl, and Cbl 1-480 (L. Samelson) failed to do so. In addition, neither EGF stimulation nor v-Src coexpression promoted c-Cbl-cullin association (*21*).
25. F. Ou and A. Varshavsky, personal communication.
26. S. Miyake *et al.*, *Crit. Rev. Oncog.* **8**, 189 (1997); C. B. Thien and W. Y. Langdon, *Immunol. Cell Biol.* 76, 473 (1998); Y.-C. Liu and A. Altmann, *Cell Signal.* **10**, 377 (1998).
27. The intensity of these bands does not correlate with the Ub ligase activity of the protein preparation. In addition, mixing of ubiquitination-defective c-Cbl RING mutants with the WT protein did not reduce the WT Ub ligase activity (*21*).
28. Ponceau S staining and antibody cross-reaction with GST revealed similar relative amounts of GST fusion proteins.
29. L. Claesson-Welsh *et al.*, *Mol. Cell. Biol.* **8**, 3476 (1988).
30. The efficiency of the reactions in Fig. 4A was determined as follows: Total counts in each lane (Ub_n-Rβ + Rβ) were normalized to those in lane 1. Counts in the Ub_n-Rβ or Rβ regions were then divided by the respective normalization factor. Counts in the Ub_n-Rβ portion of each lane were further corrected by subtracting from the background in lane 1. The values in Fig. 4B were calculated as (Ub_n-Rβ)/(Rβ + Ub_n-Rβ). This measure is an underestimate, because oligoubiquitinated precursor Rβ (160 kD) cannot be resolved from unmodified mature Rβ (190 kD), and oligoubiquitinated and unmodified mature Rβ bands are not resolved in 7.5% acrylamide gels.
31. We thank M. Nakao, L. Samelson, F. Yamao, and A. Kumar for reagents; D. Koepp for advice; A. Altman, W. Langdon, G. A. Kassavetis, J. Noel, M. Verdecia, and the Hunter laboratory for discussions; E. P. Geiduschek for comments on the manuscript; C. Elly, J. Meisenhelder, S. Simon, and A. Young for help, and F. Orzalesi for encouragement. This work was supported by Fellowship 2-41-98 from the American Cancer Society, California Division (C.A.P.J.), by Medical Research Council of Canada grant MT12121 (S.S.W.), by Fellowship DRG1531 from the Damon Runyon–Walter Winchell Foundation (H.-k.H.), by American Cancer Society grant PF9922801CCG and NIH grant T32CA09523 (J.D.L.), by grant R01 DK56558 from NIH (Y.-C.L.), and by U.S. Public Health Service grants CA39780 and CA82683 from the National Cancer Institute (T.H.). S.S.W. is a recipient of a Clinician-Scientist Award from the Medical Research Council of Canada; T.H. is a Frank and Else Schilling American Cancer Society Professor.

19 August 1999; accepted 9 September 1999

Congenital Nephrotic Syndrome in Mice Lacking CD2-Associated Protein

Neng-Yao Shih,[1]* Jun Li,[1]* Vladimir Karpitskii,[1] Ancho Nguyen,[1] Michael L. Dustin,[1] Osami Kanagawa,[1] Jeffrey H. Miner,[2] Andrey S. Shaw[1]†

CD2-associated protein (CD2AP) is an 80-kilodalton protein that is critical for stabilizing contacts between T cells and antigen-presenting cells. In CD2AP-deficient mice, immune function was compromised, but the mice died at 6 to 7 weeks of age from renal failure. In the kidney, CD2AP was expressed primarily in glomerular epithelial cells. Knockout mice exhibited defects in epithelial cell foot processes, accompanied by mesangial cell hyperplasia and extracellular matrix deposition. Supporting a role for CD2AP in the specialized cell junction known as the slit diaphragm, CD2AP associated with nephrin, the primary component of the slit diaphragm.

CD2AP is an adapter protein that interacts with the cytoplasmic domain of CD2 (*1*). CD2, a T cell and natural killer cell membrane protein, facilitates T cell adhesion to antigen-presenting cells. CD2AP enhances CD2 clustering and anchors CD2 at sites of cell contact (*1*). As CD2AP is widely expressed, it may play roles in other tissues.

Mice lacking CD2AP were generated by replacing the exon encoding the first SRC homology 3 (SH3) domain of CD2AP with a neomycin-resistance gene (Fig. 1A) (*2*). Two independent homologous recombinant clones were injected into blastocysts to generate chi-

References and Notes

[4] **Reconstitution of G₁ Cyclin Ubiquitination with Complexes Containing SCF ^{Grr1} and Rbx1**

Dorota Skowyra; Deanna M. Koepp; Takumi Kamura; Michael N. Conrad; Ronald C. Conaway; Joan Weliky Conaway; Stephen J. Elledge; J. Wade Harper

Science, New Series, Vol. 284, No. 5414. (Apr. 23, 1999), pp. 662-665.

Stable URL:

http://links.jstor.org/sici?sici=0036-8075%2819990423%293%3A284%3A5414%3C662%3AROGCUW%3E2.0.CO%3B2-O

[7] **The Tyrosine Kinase Regulator Cbl Enhances the Ubiquitination and Degradation of the Platelet-Derived Growth Factor Receptor &#945**

Sachiko Miyake; Mark L. Lupher; Brian Druker; Hamid Band

Proceedings of the National Academy of Sciences of the United States of America, Vol. 95, No. 14. (Jul. 7, 1998), pp. 7927-7932.

Stable URL:

http://links.jstor.org/sici?sici=0027-8424%2819980707%2995%3A14%3C7927%3ATTKRCE%3E2.0.CO%3B2-M

[9] **Similarity of sli-1, a Regulator of Vulval Development in C. elegans, to the Mammalian Proto-Oncogene c-cbl**

Charles H. Yoon; Junho Lee; Gregg D. Jongeward; Paul W. Sternberg

Science, New Series, Vol. 269, No. 5227. (Aug. 25, 1995), pp. 1102-1105.

Stable URL:

http://links.jstor.org/sici?sici=0036-8075%2819950825%293%3A269%3A5227%3C1102%3ASOSARO%3E2.0.CO%3B2-2

LINKED CITATIONS

[10] **Rbx1, a Component of the VHL Tumor Suppressor Complex and SCF Ubiquitin Ligase**

T. Kamura; D. M. Koepp; M. N. Conrad; D. Skowyra; R. J. Moreland; O. Iliopoulos; W. S. Lane; W. G. Kaelin Jr.; S. J. Elledge; R. C. Conaway; J. W. Harper; J. W. Conaway

Science, New Series, Vol. 284, No. 5414. (Apr. 23, 1999), pp. 657-661.

Stable URL:

http://links.jstor.org/sici?sici=0036-8075%2819990423%293%3A284%3A5414%3C657%3ARACOTV%3E2.0.CO%3B2-%23

[10] **Mass Spectrometric Analysis of the Anaphase-Promoting Complex from Yeast: Identification of a Subunit Related to Cullins**

Wolfgang Zachariae; Andrej Shevchenko; Paul D. Andrews; Rafael Ciosk; Marta Galova; Michael J. R. Stark; Matthias Mann; Kim Nasmyth

Science, New Series, Vol. 279, No. 5354. (Feb. 20, 1998), pp. 1216-1219.

Stable URL:

http://links.jstor.org/sici?sici=0036-8075%2819980220%293%3A279%3A5354%3C1216%3AMSAOTA%3E2.0.CO%3B2-C