Nature **398,** 77–80; 1999

Cytotoxic T-cell immunity to virus-infected non-haematopoietic cells requires presentation of exogenous antigen

Luis J. Sigal*, Shane Crotty†, Raul Andino† & Kenneth L. Rock*

* *Department of Pathology, University of Massachusetts Medical Center, Worcester, Massachusetts 01655-0118, USA*
† *Department of Microbiology and Immunology, University of California, Box 0414, San Francisco, California 94143-0414, USA*

Cytotoxic T lymphocytes (CTLs) are thought to detect viral infections by monitoring the surface of all cells for the presence of viral peptides bound to major histocompatibility complex (MHC) class I molecules. In most cells, peptides presented by MHC class I molecules are derived exclusively from proteins synthesized by the antigen-bearing cells[1]. Macrophages and dendritic cells also have an alternative MHC class I pathway that can present peptides derived from extracellular antigens; however, the physiological role of this process is unclear[2]. Here we show that virally infected non-haematopoietic cells are unable to stimulate primary CTL-mediated immunity directly. Instead, bone-marrow-derived cells are required as antigen-presenting cells (APCs) to initiate anti-viral CTL responses. In these APCs, the alternative (exogenous) MHC class I pathway is the obligatory mechanism for the initiation of CTL responses to viruses that infect only non-haematopoietic cells.

The 'classical' MHC class I antigen-presentation pathway is thought to be the major mechanism used by the immune system to detect viral infections in all cells. In this pathway, proteins synthesized by a cell are degraded in the cytoplasm into oligopeptides, a fraction of which are transported into the endoplasmic reticulum (ER) by the transporter associated with antigen presentation (TAP). In the ER these peptides bind to new MHC class I molecules and the resulting complexes are transported to the cell surface. As MHC class I molecules must bind peptides in order to be transported to the plasma membrane, TAP is required for normal MHC class I expression at the cell surface and for antigen presentation[1].

To determine whether non-haematopoietic cells can function as APCs to initiate CTL responses to viruses, we constructed bone-marrow chimaeras by lethally irradiating C57Bl/6 mice (B6 mice; MHC class I haplotype H-2^b) and reconstituting them with bone marrow from $TAP^{0/0}$ mice[3] (also H-2^b; all bone-marrow chimaeras will be referred to as bone-marrow donor → irradiated recipient). As bone-marrow-derived cells in $TAP^{0/0}$ → B6 mice cannot transfer peptides from the cytosol to the ER, they are unable to use the classical MHC class I pathway[3,4].

The chimaeric mice were assayed for the generation of CTL responses following infection with wild-type vaccinia virus or with vaccinia–OVA, a recombinant vaccinia virus carrying chicken ovalbumin (OVA) as a full-length protein[5]. Although they have intact MHC class I antigen presentation in non-haematopoietic tissues, $TAP^{0/0}$ → B6 mice did not generate CTL responses to vaccinia-viral antigens (Fig. 1a) or to OVA (Fig. 1b). In contrast, robust CTL responses to these antigens were detected in control B6 → B6 mice. These results indicate that the generation of CTL responses to vaccinia virus requires bone-marrow-derived cells with functional TAP molecules.

Next, we determined whether the inability of $TAP^{0/0}$ → B6 mice to generate CTL responses was due to a defect in $CD8^+$ T cells or to a failure in antigen presentation by bone-marrow-derived APCs. $TAP^{0/0}$ (non-chimaeric) mice almost completely lack $CD8^+$ T cells

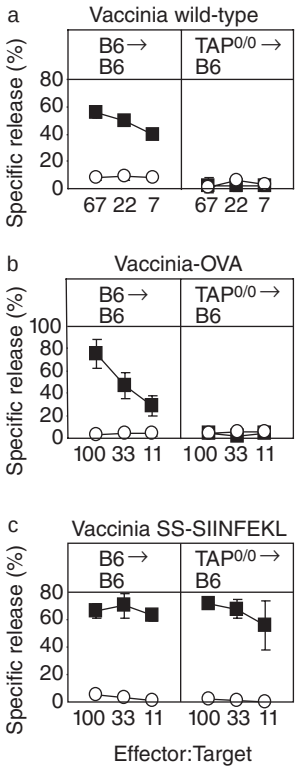

Figure 1 CTL responses to wild-type vaccinia virus and OVA in recombinant vaccinia virus requires a bone-marrow-derived APC. **a**, CTL response to wild-type vaccinia. The indicated bone-marrow chimaeras were infected with 2×10^7 p.f.u. of wild-type vaccinia virus. One week later, mice were killed and freshly explanted spleen cells were assayed in ^{51}Cr-release assays on vaccinia-infected MC57G cells (filled squares) or uninfected MC57G cells as controls (open circles). The $TAP^{0/0}$ → B6 mice did not generate a response to the vaccinia-viral antigens. **b**, CTL response to OVA in vaccinia–OVA protein. The indicated bone-marrow chimaeras were infected with 2×10^7 p.f.u. of vaccinia–OVA. One week later, mice were killed and their spleen cells were cultured in the presence of mitomycin-C (Sigma)-treated EG7 cells[29] (an EL-4-derived cell line stably transfected with OVA). After five days cells were collected and tested in ^{51}Cr-release assays on EG7 targets (filled squares) on EL-4 targets as controls (open circles). **c**, CTL response to vaccinia–SS-SIINFEKL. The experiment was performed as in **b** except that mice were infected with vaccinia–SS-SIINFEKL. The *x*-axis shows the ratio of effector cells to target cells.

because expression of MHC class I molecules in epithelial thymic cells is necessary for the positive selection of $CD8^+$ T cells in the thymus[3]. However, as shown by flow-cytometry analysis, $TAP^{0/0}$ → B6 mice had normal numbers of $CD8^+$ T cells in peripheral blood and spleen (ref. 4 and data not shown) as a consequence of positive selection on wild-type thymic epithelial cells (which are radioresistant and non-haematopoietic). These $CD8^+$ T cells were fully functional. $TAP^{0/0}$ → B6 mice and control B6 → B6 mice generated a similar CTL response to vaccinia–SS-SIINFEKL[5] (Fig. 1c), a recombinant vaccinia virus expressing the antigenic peptide of OVA (SIINFEKL)[6] preceded by a signal sequence that delivers the peptide directly into the ER, thus bypassing the need for TAP. $TAP^{0/0}$ → B6 chimaeras and control mice also generated anti-SIINFEKL CTL when injected intravenously with a graded number of B6 dendritic cells that had been preincubated with a single concentration of synthetic SIINFEKL (not shown) or when injected with 5×10^5 dendritic cells that had been incubated with graded concentrations of SIINFEKL (Fig. 2). In these latter experiments, cells incubated with 10 nM SIINFEKL and cells infected *in vitro* with vaccinia–OVA and polio–OVA (see below) stimulated a T-cell

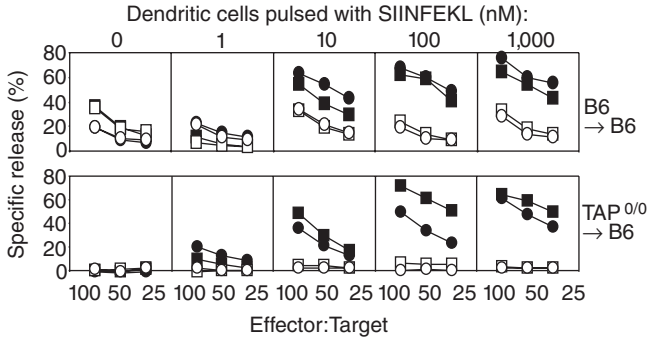

Figure 2 TAP$^{0/0}$ → B6 mice can generate CTL responses comparable to those in B6 → B6 mice when immunized with APCs loaded with antigenic peptide. The indicated chimaeric mice were immunized intravenously with 5×10^5 B6-derived, *in vitro*-cultured dendritic cells that had been incubated with SIINFEKL peptide at the indicated concentrations. One week later mice were killed and their spleen cells restimulated *in vitro* for 4 days with mitomycin-C-treated EL-4 cells that had been incubated with SIINFEKL. For each panel, squares represent one mouse and circles represent another. Targets were EL-4 cells that had been preincubated with SIINFEKL (filled symbols) or EL-4 cells that had not been incubated with peptide as controls (open symbols).

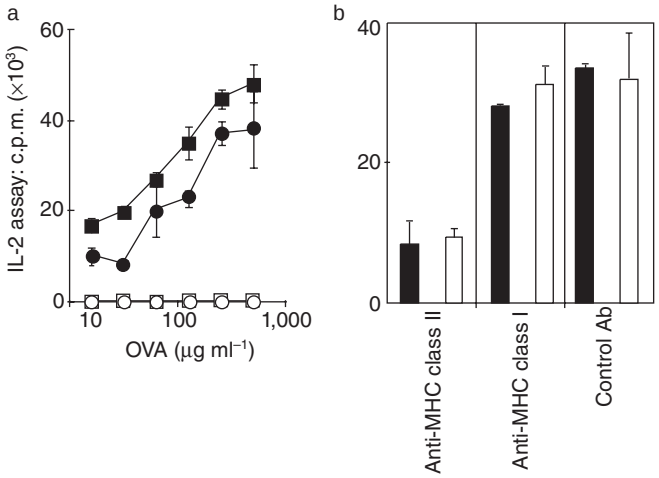

Figure 3 TAP$^{0/0}$ → B6 mice can generate MHC class II-restricted responses comparable to those in B6 → B6 mice. **a**, Production of IL-2 by lymph-node cells of immunized chimaeras in response to different concentrations of OVA. Cells were from OVA-immunized TAP$^{0/0}$ → B6 mice (filled squares) and B6 → B6 mice (filled circles) or from unimmunized controls (open squares and open circles respectively). **b**, The same cells as those used in **a** were incubated with 0.5 mg ml^{-1} OVA in the presence of the indicated antibody-containing supernatants. Only the results for immunized TAP$^{0/0}$ → B6 mice (filled columns) or B6 → B6 mice (open columns) are shown.

hybrid specific for the peptide–MHC complex SIINFEKL–K^b at roughly similar levels (data not shown).

These results indicate that the inability of TAP$^{0/0}$ → B6 mice to generate a CTL response to vaccinia virus is due to a failure of antigen presentation. This failure to present antigens did not result from an inability of the TAP$^{0/0}$ → B6 chimaeras to reconstitute bone-marrow-derived APCs, because these mice generated MHC class II-restricted T-cell responses to OVA that were at least as strong as those generated by B6 → B6 animals (Fig. 3a, b). Together these data show that bone-marrow-derived professional APCs, possessing a functional TAP, are required to initiate CTL responses to vaccinia, and that non-haematopoietic tissues infected with vaccinia cannot prime CTLs, despite the ability of vaccinia to infect many different tissues, including respiratory organs, liver, kidney, spleen, ovaries and the central nervous system[7–9]. There may be several explanations for the inability of non-haematopoietic cells to stimulate a CTL response. Although non-haematopoietic cells are able to present antigenic peptides bound to MHC class I, they express low levels of these molecules in the absence of inflammation. Moreover, they do not express MHC class II molecules, which are essential for the stimulation of CD4$^+$ helper T cells, and they lack adhesion and co-stimulatory molecules that might be required to stimulate naive T cells. Non-immune cells also lack the ability to migrate to lymphoid organs, where many immune responses are initiated[10]. On the other hand, some bone-marrow-derived cells, such as macrophages and dendritic cells, express high levels of MHC class I and class II molecules and several adhesion and co-stimulatory molecules, including B7.1 and B7.2, and can migrate to central lymphoid organs. However, after naive CTLs are stimulated to become effectors, they no longer require co-stimulation or T-cell help and can recognize lower levels of peptide–MHC complexes. Therefore, once stimulated by professional APCs, the effector CTLs acquire the ability to migrate out of the lymphoid organs to clear viral infections in all tissues.

In the experimental model described above, bone-marrow-derived APCs might acquire the viral antigens by becoming infected themselves[11]. In fact, SIINFEKL was presented by vaccinia-OVA infected dendritic cells and macrophages *in vitro* (not shown). However, it seems unlikely that professional APCs would become infected by all viruses and therefore this mechanism would be unavailable to detect infections by many tissue-specific viruses. Alternatively, the bone-marrow-derived APCs might acquire vaccinia-viral antigens exogenously from other antigen-bearing cells and this mechanism could operate in all infections, as proposed previously[2,12]. To determine whether the presentation of exogenous antigen is important in viral immunity, we developed a model in which bone-marrow-derived cells could not be infected with a virus.

Poliovirus (polio) is a positive-strand RNA virus with a host range that includes humans but not mice. This host-range restriction is determined by the expression of a suitable poliovirus receptor (PVR) on host cells[13]. Cells from wild-type mice can not be infected with polio, but mouse cells transfected with human PVR can be infected (ref. 14 and data not shown). In this study we used two human PVR-transgenic mice. We constructed a transgenic mouse (cPVR, in an ICR background) expressing a full-length PVR complementary DNA, driven by the β-actin promoter, in all tissues studied. Such mice are susceptible to polio infection and die with poliomyelitis following injection with wild-type polio (not shown). Following intraperitoneal infection of cPVR and control (B6) mice with the wild-type strain of polio, we found much higher titres of virus (expressed as plaque-forming units per tissue) in the skeletal muscle (12,000), brain (1,600) and spinal cord (19,000), and slightly higher titres in the kidney (1.3) and liver (0.64), of the cPVR mice (B6-mouse titres: 0.70, <0.60, <0.30, <0.10 and <0.20, respectively). We mated cPVR mice with B6 mice, and their progeny, (cPVR × B6) F_1 mice (referred to here as cPVR mice), were used here. Another transgenic mouse strain (referred to here as gPVR) possesses the human PVR genomic locus, including the endogenous human promoter, backcrossed onto the B6 background. It also supports viral replication in skeletal muscle and central nervous system[13,15].

To examine the role of the exogenous MHC class I pathway in the initiation of CTL responses, we generated a series of bone-marrow-chimaeric mice, including two sets (B6 → cPVR and B6 → gPVR) that allowed infection of only non-bone-marrow-derived cells. In a previous study[14], we constructed a polio virus recombinant (polio–OVA) expressing the carboxy-terminal half of OVA (which includes the SIINFEKL epitope). In this construct, the OVA fragment is synthesized as part of the viral polyprotein and is released in the cytosol by viral proteinases. We showed that polio–OVA can induce

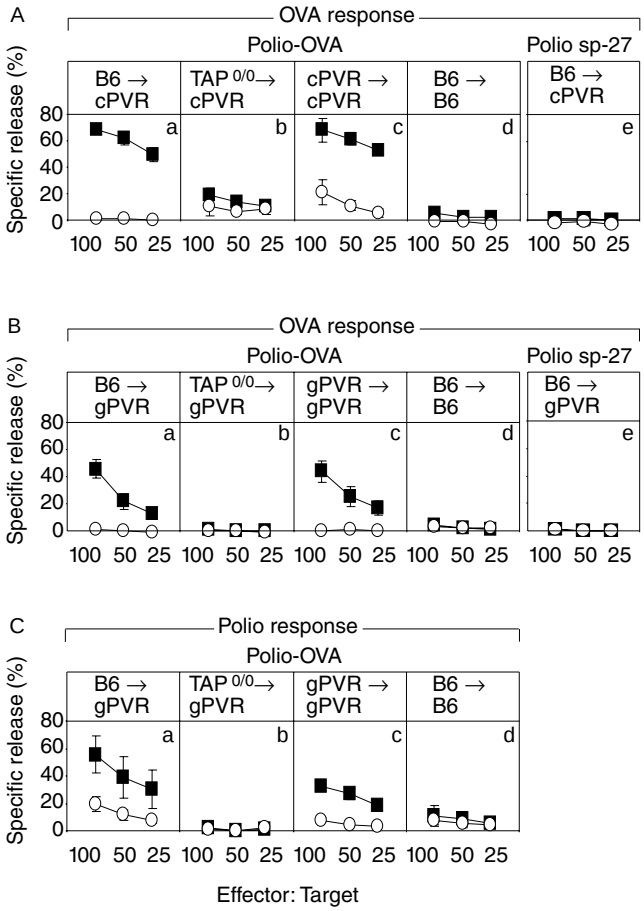

Figure 4 Initiation of the CTL response to polio–OVA requires the presence of bone-marrow-derived APCs, but not their infection. Each panel corresponds to a single experiment. **A**, **B**, Bone-marrow chimaeras (**Aa**–**e**, cPVR recipients and B6 control recipients; **Ba**–**e**, gPVR recipients and B6 control recipients) were infected with polio–OVA or polio–sp27 as indicated. Mice were killed 3 weeks later and their spleen cells were co-cultured for 5 days with mitomycin-C-treated EG7 cells and used in ^{51}Cr-release assays. Filled squares, EG7 targets; open circles, EL-4 targets as controls. **Ca**–**d**, Bone-marrow chimaeras obtained using gPVR recipients and B6 control recipients were infected with polio–OVA. Three weeks later, mice were killed and their spleen cells were cultured in the presence of mitomycin-C-treated EL-4 cells that had been preincubated with 2 μg ml^{-1} of the D^b-binding polio-derived peptide P22. After 5 days, cells were collected and tested in ^{51}Cr-release assays. Targets were EL-4 cells preincubated with P22 (filled squares) or EL-4 cells without peptide as controls (open circles).

anti-OVA CTL responses in gPVR mice and cPVR but not in B6 mice (ref. 14 and data not shown). Consistent with this result, cPVR → cPVR mice infected with polio–OVA generated anti-OVA CTLs (Fig. 4Ac), but B6 → B6 mice did not (Fig. 4Ad). B6 → cPVR chimaeric mice, which have bone-marrow-derived cells that cannot be infected by polio (PVR-negative cells), generated strong anti-OVA CTL responses when infected with polio–OVA (Fig. 4Aa) but not when infected with a recombinant polio (polio–sp27) expressing an irrelevant protein (Fig. 4Ae). Identical results were obtained when using gPVR recipients (Fig. 4Ba, c and e). Therefore, either the infected non-haematopoietic cells are stimulating CTL responses, or bone-marrow-derived cells are acquiring the polio-expressed OVA from exogenous sources.

To distinguish between these two possibilities, we constructed chimaeric mice by using TAP$^{0/0}$ mice as bone-marrow donors and PVR$^+$ transgenic mice as recipients. Remarkably, TAP$^{0/0}$ → cPVR (Fig. 4Ab) and TAP$^{0/0}$ → gPVR (Fig. 4Bb) mice did not generate anti-OVA CTL responses when infected with polio–OVA. As expected, their CTLs were functional and generated a strong response to vaccinia–SS-SIINFEKL (data not shown). In contrast to the control B6 → gPVR mice, these TAP$^{0/0}$ chimaeric mice also failed to generate CTL responses to a D^b-presented epitope from the polio VP0 protein (Fig. 4C). As shown above with vaccinia virus, these data indicate that non-haematopoietic cells are unable to stimulate CTL immunity to another virus, indicating that this may be a general rule. These results also indicate that the bone-marrow-derived cells in B6 → c/gPVR mice acquired antigen from exogenous sources. That TAP$^{0/0}$ → c/gPVR mice did not respond to either antigen also indicates that the response in B6 → c/gPVR mice was not due to residual PVR$^+$ bone-marrow-derived cells that survived irradiation.

Until now, the physiological function of the exogenous MHC class I pathway has been unclear. Stimulation of CTLs by this route has been shown to occur in several situations, such as transplantation ('crosspriming' for minor histocompatibility antigens)[16] and injection of particulate antigens[17], but it has been thought to make a minor contribution to overall responses. Two situations in which this pathway has been shown to be important are in the generation of CTL responses to a tumour[4] and in the homing and development of tolerance of adoptively transferred T cells specific for a transgenic antigen expressed in pancreatic β-cells[18,19]. However, in these cases it was unclear whether the exogenous pathway might be dominant only because of the lack of inflammation (which stimulates antigen presentation and provides an adjuvant effect) and/or because these cells might be poor stimulators. It has been suggested that the exogenous MHC class I pathway is inefficient and unlikely to play an important part in most physiological situations[20]. Our experiments with B6 → c/g PVR mice contradict this view and indicate that the exogenous MHC class I pathway is essential for the initiation of CTL

responses to viral infection that is confined to non-haematopoietic tissues. In fact, if this pathway did not exist, viruses could escape immune surveillance by using receptors that are not expressed on the critical, bone-marrow-derived APCs. Our results indicate that the presentation of exogenous antigen is a major pathway *in vivo* and may contribute to the stimulation of CTL responses even in situations in which viruses may infect bone-marrow-derived cells.

How do bone-marrow-derived cells acquire viral exogenous antigens? When infected cells die *in vivo*, they are rapidly cleared by bone-marrow-derived phagocytes, which will import the viral antigens into the exogenous MHC class I and class II pathways[21]. Interestingly, antigens from apoptotic cells are avidly presented on class I molecules of dendritic cells[22]. Although our results do not specifically establish the identity of the bone-marrow-derived APCs responsible for initiating CTL responses through the exogenous pathway, macrophages and/or dendritic cells are again the likely candidates, because they can present antigen through the exogenous MHC class I pathway *in vitro*[17,23]. These cells can also ingest dying cells and cellular debris by phagocytosis and can thereby import viral antigens into the exogenous MHC class I pathway. Moreover, their migratory nature allows them to acquire antigen at a site of infection and then travel to the lymphoid tissues.

Two routes for the exogenous MHC class I pathway *in vitro* have been described, a TAP-independent pathway, in which antigen is probably hydrolysed in endosomes[24,25], and a phagosome-to-cytosol pathway[26] that is TAP-dependent. Our data provide indirect evidence that, *in vivo*, vaccinia–OVA and polio–OVA antigens may follow the TAP-dependent exogenous MHC class I pathway.

Our results show a strict requirement for professional APCs in the generation of anti-viral CTL immunity, and demonstrate that the exogenous pathway plays a key part in the immune surveillance of non-haematopoietic tissues. These findings have implications for vaccine delivery and gene therapy as well as for immune evasion by viruses. The results indicate that, to stimulate strong immunity, viral vectors or naked DNA must be expressed in professional APCs or delivered in a manner that will promote exogenous antigen presentation. Moreover, these mechanisms may limit the ability of viruses to block the generation of CTLs by downregulating MHC class I expression on infected cells[27], because this is unlikely to affect the exogenous pathway in uninfected professional APCs. □

Methods

TAP$^{0/0}$ (B6,129-Tap$^{1tp\ 1Arp}$; Jackson Laboratory) and B6 (Taconic) mice were obtained at 6–8 weeks of age. cPVR mice were made by standard transgenic techniques using the plasmid pVR-9, which has already been described[14]. gPVR mice (a gift from Cynamid) were bred at UMMC animal facilities. To prepare chimaeras, bone-marrow cells from 1–3-month-old donor mice were treated with anti-Thy1 antibody (M5/49.4.1; ATCC) and complement to eliminate mature T cells, washed twice and resuspended in PBS. Recipients were irradiated with 650 rad and then with 450 rad 4 h later. Irradiated mice were reconstituted by intravenous inoculation of 4–6 $\times$ 10^6 bone-marrow cells from the different donors. To avoid rejection of donor MHC class I-negative TAP$^{0/0}$ cells by host natural-killer cells, chimaeras also received an intraperitoneal injection of 10 µl rabbit anti-asialo GM1 gammaglobulin (Wako Chemicals) on the day of the transplant, and a second injection 3 days later. Bone-marrow chimaeras were rested for 4–6 months following reconstitution to allow for complete elimination of host-derived professional APCs. Mice were inoculated with virus and CTL killing was measured from fresh spleen cells (wild-type vaccinia), or from cultures restimulated with antigen for 5 days, using a ^{51}Cr-release assay as described[28]. For MHC class II-restricted responses, mice were injected at the base of the tail with 100 µg OVA (Sigma) emulsified in complete Freund's adjuvant (Gibco) in a final volume of 50 µl. Ten days later mice were killed and 2 $\times$ 10^5 cells from pooled para-aortic lymph nodes were incubated for 48 h in triplicate wells of microtitre plates in the presence of OVA. Supernatants were assayed for the presence of interleukin (IL)-2 by measuring the incorporation of ^{3}H-thymidine by the IL-2-dependent cell line CTLL. When indicated, antibodies were added to culture as a 1:8 dilution of hybridoma culture supernatants. The antibodies M5/114, Y-3 and BBM.1 (ATCC) were used as anti-MHC class II, anti-MHC class I and control antibodies, respectively. All experiments were performed at least three times. Data points in all figures except Fig. 2 represent averages ± s.e.m. for three mice (all experimental groups) or two mice (vaccinia–SS-SIINFEKL; polio–sp27 controls). In Fig. 2, two mice were used per group and results from these mice are shown individually. All mice used in these experiments were housed at the UMMC animal facilities and experiments were conducted in compliance with NIH and institutional guidelines.

Viruses were produced and used as described[14,28] except that inoculation of mice with polio was performed intravenously rather than intraperitoneally. For the generation of CTL responses to all viruses, the inoculation dose was 2 $\times$ 10^7 plaque-forming units (p.f.u.) per mouse diluted in 0.5 ml PBS. For the recovery of infectious virus from organs, mice were inoculated intraperitoneally with 2 $\times$ 10^8 p.f.u. of the poliovirus wild-type Mahoney 1 strain. Six paralysed cPVR mice were killed at days 4.5–6.5 after infection. Four control B6 mice were killed at days 4.5–5.5. Tissue samples were homogenized, and poliovirus titres in each tissue were determined by plaque assay.

P22 is a D^b-binding peptide corresponding to amino acids 22–30 of the poliovirus polyprotein that we have recently identified (L.J.S. and K.L.R., manuscript in preparation). Both SIINFEKL and P22 were synthesized at the peptide core facility at UMMC.

All cell lines used in this study have been described and were cultured as previously[28]. To obtain dendritic cells, bone-marrow cells obtained from B6 mice were incubated overnight in RPMI media (Irvine Scientific) supplemented with 10% fetal calf serum (Atlanta Biologicals), 5 $\times$ 10^{-5} M 2-mercaptolethanol (Sigma) and 2 mM L-glutamine, antibiotics (Fungi-Bact), 0.01 M HEPES buffer and non-essential amino acids (all from Irvine Scientific). Non-adherent cells were collected and grown in the same media supplemented with 10 ng ml^{-1} granulocyte/macrophage colony-stimulating factor and 5 ng ml^{-1} IL-4 (Pharmingen) for 5–6 days with further addition of cytokines every other day. Most cells in these cultures were dendritic cells, as judged by morphology and analysis expression of specific markers by flow cytometry. Before being injected into mice, 3 $\times$ 10^6 dendritic cells were thoroughly washed in PBS, resuspended in 1 ml PBS containing the indicated concentrations of SIINFEKL and 10 µg human β_2-microglobin (Calbiochem) and incubated at 37 °C for 1 h. Following incubation the cells were washed in PBS and injected intravenously into mice.

Received 23 July 1998; accepted 18 January 1999.

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Acknowledgements

We thank R. O. Donis and the members of the Rock laboratory for helpful discussions; R. Welsh, L. Berg, G. Soldevila and N. Kisaiti for critical reading of the manuscript; W. Yong Zang and L. Rothstein for technical assistance; and M. Bevan for EG7 cells, P. Doherty for MC57G cells, and J. Yewdell for vaccinia-OVA and vaccinia SS-SIINFEKL. This work was supported by NIH grants to K.L.R. and R.A. L.J.S. was supported by an NIH Research Training Grant.

Correspondence and requests for materials should be addressed to K.L.R. (e-mail: Kenneth.Rock@banyan.ummed.edu).

Nature **398,** 84–90; 1999

Structure of the amino-terminal domain of Cbl complexed to its binding site on ZAP-70 kinase

Wuyi Meng*, Sansana Sawasdikosol†, Steven J. Burakoff† & Michael J. Eck*

*Departments of *Biological Chemistry and Molecular Pharmacology and †Pediatrics, Harvard Medical School, and Departments of *Cancer Biology and †Pediatric Oncology, Dana-Farber Cancer Institute, 44 Binney Street, Boston, Massachusetts 02115, USA*

Cbl is an adaptor protein that functions as a negative regulator of many signalling pathways that start from receptors at the cell surface[1–4]. The evolutionarily conserved amino-terminal region of Cbl (Cbl-N) binds to phosphorylated tyrosine residues and has cell-transforming activity. Point mutations in Cbl that disrupt its recognition of phosphotyrosine also interfere with its negative regulatory function and, in the case of v-*cbl*, with its oncogenic potential[5]. In T cells, Cbl-N binds to the tyrosine-phosphorylated inhibitory site of the protein tyrosine kinase ZAP-70[6]. Here we describe the crystal structure of Cbl-N, both alone and in complex with a phosphopeptide that represents its binding site in ZAP-70. The structures show that Cbl-N is composed of three interacting domains: a four-helix bundle (4H), an EF-hand[7] calcium-binding domain, and a divergent SH2 domain[8] that was not recognizable from the amino-acid sequence of the protein. The calcium-bound EF hand wedges between the 4H and SH2 domains and roughly determines their relative orientation. In the ligand-occupied structure, the 4H domain packs against the SH2 domain and completes its phosphotyrosine-recognition pocket. Disruption of this binding to ZAP-70 as a result of structure-based mutations in the 4H, EF-hand and SH2 domains confirms that the three domains together form an integrated phosphoprotein-recognition module.

Cbl becomes tyrosine-phosphorylated upon engagement of several cell-surface receptors, including the multichain immune receptors, and growth-factor and cytokine receptors[1–4]. The amino-acid sequence of Cbl reveals a RING-finger[9] domain adjacent to the phosphotyrosine-binding Cbl-N segment, and a carboxy-terminal region with numerous docking sites for SH3- and SH2-containing proteins (Fig. 1). The oncogenic v-Cbl includes only the first 357 residues of Cbl and is a potent transforming protein[10]. The highly conserved Cbl-N region binds to the receptor for epidermal growth factor (EGFR)[11], Syk[12] and the negative-regulatory phosphorylation site in ZAP-70[6]. Genetic studies in *Caenorhabditis elegans* revealed that the Cbl homologue Sli-1 inhibits vulval induction by the EGFR[13]. Expression of *D*-Cbl, a *Drosophila melanogaster* Cbl homologue, disrupts EGFR-regulated development of the R7 photoreceptor[14]. Cbl also diminishes FcεRI-mediated degranulation in mast cells by inhibiting the tyrosine kinase Syk[15]. Mutational analysis demonstrates that Cbl-N is central to these functions. To understand better the diverse recognition and regulatory functions of Cbl-N, we have determined its three-dimensional structure.

As shown in Fig. 1, Cbl-N comprises three interacting domains: an N-terminal four-helix bundle (4H), a calcium-binding domain with the EF-hand fold, and an unusual SH2 domain. None of these folding motifs were previously recognized in the amino-acid sequence of Cbl. In spite of the structural and functional similarity of this unusual SH2 domain with other SH2 domains, it shares very little sequence identity (~11%) with them.

The N-terminal 4H domain contains four long α-helices. Structural comparisons with the DALI[16] server show that it has a topology and overall structure similar to many functionally unrelated four-helical proteins, including cytochrome *c*, interleukin-5 and apolipophorin III. The C and D helices in this domain pack against the adjacent EF-hand domain, and a highly conserved loop connecting the A and B helices contacts the SH2 domain.

Table 1 Data collection, phasing and refinement statistics

	Cbl-N	Cbl-N MeHg derivative	Cbl-N / ZAP70-292 complex
Resolution (Å)	15–2.20	15–2.24	20–2.10
Space group	*C*2	*C*2	P6
Unit cell (Å)	*a*=159.96 *b*=105.48 *c*=84.92 *β*=92.06°	*a*=160.04 *b*=106.73 *c*=84.84 *β*=92.30°	*a*= 122.3 *c*=55.65
Molecules/a.s.u.	3	3	1
R_{sym}(%)	6.7	5.5	7.9
Reflections (total/unique)	178,845/70,918	186,473/67,069	35,952/20,620
Completeness (%)	99.3	98.1	76.9 (91% to 2.5Å)
R_{iso}/R_{anom} (%)		35.1 / 6.5	
Phasing power (centric/acentric)		1.11 / 1.51	
R_{cullis} (centric/acentric/anomalous %)		0.68 / 0.74 / 0.90	
FOM		0.41	
Number of sites		15	
Refinement statistics			
Resolution range (Å)	15–2.20		20–2.10
Non-hydrogen atoms	8,092		2,919
Water molecules	669		358
R_{cryst} / R_{free} (%)	22.1 / 28.7		17.3 / 24.6
R.m.s.d. bond lengths / angles	0.018Å / 2.1°		0.013Å / 1.84°