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VCP, the mammalian homolog of cdc48, is tyrosine phosphorylated in response to T cell antigen receptor activation

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Activation of T cells through the T cell antigen receptor (TCR) results in the rapid tyrosine phosphorylation of a number of cellular proteins, one of the earliest being a 100 kDa protein. We have sought to identify this 100 kDa substrate by partially purifying the protein by antiphosphotyrosine (APT) affinity purification, in order to obtain amino acid sequence data and, using this information, to isolate the cDNA clone encoding the molecule. We report here that the amino acid sequence data showed pp100 to be the murine equivalent of porcine valosin containing protein (VCP), a finding confirmed from the cloning and sequencing of the murine pp100 cDNA. Sequence analysis has shown VCP to be a member of a family of ATP binding, homo-oligomeric proteins, and the mammalian homolog of *Saccharomyces cerevisiae* cdc48p, a protein essential to the completion of mitosis in yeast. We also provide proof that both endogenous and expressed murine VCP are tyrosine phosphorylated in response to T cell activation. Thus we have identified a novel component of the TCR mediated tyrosine kinase activation pathway that may provide a link between TCR ligation and cell cycle control.

Key words: cell cycle/phosphorylation/signal transduction/T-lymphocyte

Introduction

Signal transduction pathways mediated by protein tyrosine phosphorylation are central to the control of proliferation and differentiation in many biological systems. Ligand activation of receptors that either contain tyrosine kinase domains or are associated with tyrosine kinases, leads to the rapid phosphorylation of a number of protein substrates whose activities are presumably altered by this post-translational modification (reviewed by Ullrich and Schlessinger, 1990; Cantley *et al.*, 1991). The tyrosine kinase substrates that have been identified to date fall into a limited number of functional categories. The protein tyrosine kinases (PTK) themselves can be phosphorylated on tyrosine residues, resulting in regulation of kinase activity and association of additional signalling molecules. A number of substrates are enzymes, such as phospholipase Cγ, phosphatidylinositol-3-kinase and GTPase activating protein which regulate the production of intracellular second messengers or control distal enzymatic signalling pathways (Cantley *et al.*, 1991). The tyrosine phosphorylation of several cytoskeletal proteins such as ezrin, talin and vimentin may be involved in regulation of cell size, shape and motility (reviewed by Burridge *et al.*, 1988). Tyrosine phosphorylation and dephosphorylation of the p34^{cdc2} serine/threonine kinase is central to control of, and progression through, the cell cycle (Nurse, 1990).

Much of the characterization of tyrosine kinases and their substrates has focused on the study of fibroblast proliferation induced by growth factor tyrosine kinases such as the receptors for platelet derived growth factor and epidermal growth factor (Ullrich and Schlessinger, 1990; Cantley *et al.*, 1991). More recently, tyrosine kinase signalling pathways in T cells have become the subject of considerable investigation (reviewed by Klausner and Samelson, 1991). Engagement of the T cell antigen receptor (TCR) leads to the rapid tyrosine phosphorylation of multiple proteins before activation of a number of other intracellular signalling events (Hsi *et al.*, 1989; June *et al.*, 1990). These pathways lead ultimately to induction of expression of multiple cell surface molecules and secretion of lymphokines. At least three tyrosine kinases, p56^{lck}, via an association with CD4 and CD8 (Veillette *et al.*, 1989), p60^{fyn} (Samelson *et al.*, 1990b) and a kinase that associates with the activated antigen receptor (Chan *et al.*, 1991; Wange *et al.*, 1992) have been implicated in the induction of these phosphorylations.

Several substrates for tyrosine kinases activated by the TCR have been described. A subunit of the antigen receptor, the TCR ζ chain, was the first such substrate identified (Samelson *et al.*, 1986a; Baniyash *et al.*, 1988). Subsequently, several additional substrates have been described. The γ1 isozyme of phospholipase C is tyrosine phosphorylated on sites identical to those phosphorylated in fibroblasts following growth factor stimulation (Park *et al.*, 1991; Weiss *et al.*, 1991; Secrist *et al.*, 1991). Phosphorylation at these sites is required for activation of PLC (Kim *et al.*, 1991). Tyrosine phosphorylation of the p95^{vav} molecule also follows TCR engagement, as is observed in fibroblasts stimulated by growth factors (Margolis *et al.*, 1992; Bustelo *et al.*, 1992). Ezrin, a protein associated with the cytoskeleton (Bretscher, 1989), can also be tyrosine phosphorylated in T cells under certain conditions (Egerton *et al.*, 1992). The function of p95^{vav} and ezrin, and the effect of tyrosine phosphorylation on the activities of these proteins are not known.

The identity of most of the proteins that are tyrosine phosphorylated following TCR engagement remains to be determined. We have focused our attention on a 100 kDa substrate that is phosphorylated rapidly following TCR crosslinking (Samelson *et al.*, 1990a; June *et al.*, 1990). We isolated this protein using antiphosphotyrosine (APT) mAb affinity purification in order to obtain material for amino acid sequence analysis. Here we report the purification,

cDNA cloning, sequencing and expression of a 100 kDa cytosolic protein that is a PTK substrate. This protein is the murine equivalent of porcine valosin containing protein (VCP) (Koller and Brownstein, 1987), a protein of unknown function that has been reported to be the mammalian homolog of the *Saccharomyces cerevisiae* protein, cdc48p (Fröhlich *et al.*, 1991). Identification of this protein as a substrate suggests a means by which TCR engagement might effect cell cycle control.

Results

pp100 affinity purification and amino acid sequencing

Lymph node T cells from the MRL *lpr/lpr* (lpr) mouse were used to purify sufficient quantities of the pp100 substrate for amino acid sequencing. In these mice, loss of expression of the Fas antigen, a receptor involved in mediating cell apoptosis (Itoh *et al.*, 1991; Watanabe-Fukunaga *et al.*, 1992) results in a lymphoproliferative disorder (reviewed by Cohen and Eisenberg, 1991) such that as many as 10^{10} T cells can accumulate in the lymph nodes of an individual mouse. A feature of lpr T cells is that a subpopulation overexpress $p60^{fyn}$ PTK (Katagiri *et al.*, 1989) and display constitutively elevated tyrosine phosphorylation of a number of substrates (Samelson *et al.*, 1986b). In these experiments, we were able to further increase cellular phosphotyrosine levels by treating lpr T cells with a combination of the protein tyrosine phosphatase (PTPase) inhibitors phenylarsine oxide (PAO) and sodium orthovanadate (OV) (Garcia-Morales *et al.*, 1990), thereby improving our ability to purify these proteins partially by immunoaffinity purification with APT. Most of these substrates corresponded in molecular weight to those observed in TCR activated T cells (Egerton *et al.*, 1992). Figure 1 shows a number of Ponceau S stained proteins isolated by this procedure. We have recently demonstrated that the 81 kDa substrate is the cytoskeletal protein ezrin (Egerton *et al.*, 1992). The prominent 100 kDa protein was a candidate for the 100 kDa substrate of interest. We were unable to obtain N-terminal amino acid sequences, and therefore subjected the protein to partial proteolytic cleavage with lysylendopeptidase C (Lys C). Five resulting peptides were purified by HPLC and reliable amino acid sequences were obtained from the three listed in Table I. The amino acid sequences were compared to those in the GenBank protein database and were found to be identical to sequences obtained from a cDNA encoding a pig protein known as VCP (Koller and Brownstein, 1987).

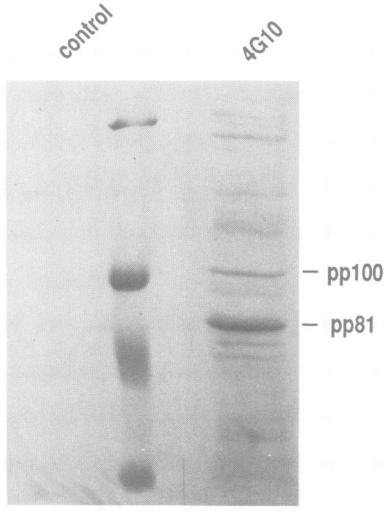

Fig. 1. Ponceau S stained nitrocellulose showing protein bands obtained after APT batch purification. Specifically bound proteins were eluted either from control (anti-2B4 preclear) or APT (4G10) beads with 40 mM phenyl phosphate and separated by 7.5% SDS–PAGE. This figure has been presented previously in a study describing the purification of the 81 kDa substrate, ezrin (Egerton *et al.*, 1992).

p100 cDNA cloning and sequencing

Since the pig and mouse amino acid sequences were identical, we used the published porcine VCP cDNA sequence encoding our peptide 1 to construct a 63 base non-degenerate oligonucleotide probe. The probe was used to screen a murine MRL *lpr/lpr* splenic T cell cDNA library constructed in λgt10, from which 11 positive plaques were obtained. All produced two fragments 1.2–1.6 kb in length after *Eco*RI digestion of the purified phage DNA. The cDNA from the clone with the longest combined fragment length (1.5 and 1.6 kb) was subcloned into pBluescript for double stranded DNA sequencing. Figure 2 shows the entire nucleotide sequence obtained after sequencing both strands of both DNA fragments. According to the translation initiation consensus sequence defined by Kozak (1991), the cDNA sequence contains a unique open reading frame starting at base 162 and extending through to base 2579. The sequence ends with a polyadenylated region at the extreme 3′ end.

Sequence analysis

The translated open reading frame sequence predicted an 806 amino acid, 89 232 Dalton protein (Figure 2). Comparison of our sequence with that of porcine VCP revealed that the two were 99.6% identical at the amino acid level and 88% identical at the nucleotide level, confirming that this cDNA encoded the murine equivalent of porcine VCP. There was no identifiable hydrophobic transmembrane domain or signal peptide leader sequence. There were no motifs suggesting that VCP was a PTK or PTPase, nor were SH2 or SH3 domains present. Dot matrix analysis of the predicted VCP

Table 1. Comparison of amino acid sequences from mouse T cell pp100 and porcine VCP

Peptide 1	* K N R P N R L I V D E A I N E D N S V V S
Porcine VCP (20–40)	K N R P N R L I V D E A I N E D N S V V S
Peptide 2	* K N A P A I I F I D E L D A I
Porcine VCP (295–309)	K N A P A I I F I D E L D A I
Peptide 3	* K K M D L I D L E D E T I D
Porcine VCP (425–438)	K K M D L I D L E D E T I D

The asterisk indicates that the lysine is inferred after Lys C digestion. VCP peptide sequences were obtained from the GenBank protein database.

amino acid sequence (data not shown) revealed a duplicated domain of ~220 amino acids containing a nucleotide binding site defined by the consensus sequence (G/A)X_4 (G/A)(H/K/R)X_{0-1}(T/S/K/R/H; Chin *et al.*, 1988). Analysis of the GenBank protein database indicated that this feature is shared by a number of other proteins, summarized in Table II. All family members display considerable sequence identity to VCP over this duplicated domain, although human TBP-1 (Nelbock *et al.*, 1990) contains only a single copy of this domain. It has been proposed that, by virtue of their 68% amino acid identity over their entire length, mammalian VCP and *S.cerevisiae* cdc48p are homologs (Fröhlich *et al.*, 1991).

Tyrosine phosphorylation of VCP

To characterize the murine VCP protein, antisera were raised by immunizing rabbits with a synthetic peptide corresponding to peptide 1 (Table I). These antisera specifically immunoprecipitated a [^{35}S]methionine labeled 100 kDa protein from both lpr and Jurkat T cell lysates, and also specifically immunoblotted a 100 kDa protein in Jurkat T cell lysates (Figure 3). VCP migrates with an apparent molecular weight much larger than predicted, as is the case for the related proteins cdc48p (Fröhlich *et al.*, 1991) and p97 ATPase (Peters *et al.*, 1990).

Additional experiments revealed that the anti-VCP antisera did not quantitatively immunodeplete VCP, presumably due to a low affinity for the protein. To determine whether VCP could be tyrosine phosphorylated, we immunoprecipitated the protein with APT mAb and used the anti-peptide sera for specific VCP detection. Using this approach, we demonstrated that the yield of an APT immunoprecipitable 100 kDa protein reactive with the anti-VCP antisera increased 5- to 10-fold either after TCR ligation (Jurkat cells)

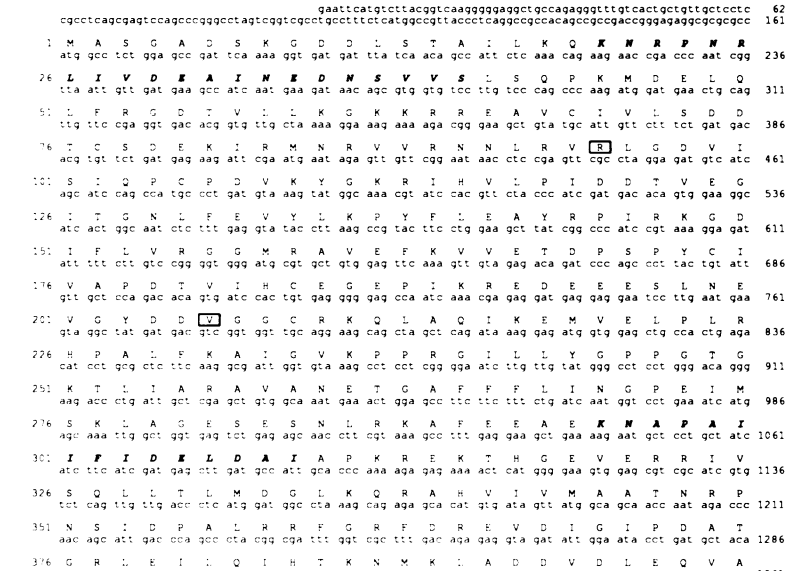

Fig. 2. Murine VCP cDNA nucleotide sequence and predicted ORF amino acid sequence. Letters in boxes indicate amino acid residue differences between mouse and pig sequences. The porcine sequence differs in the following residues: Arg95−His, Val206−Ile and Glu491−Asp. Amino acid sequences in bold italics correspond to those obtained by amino acid sequencing of partially purified pp100. The nucleotide sequence data of the murine VCP cDNA has been deposited in the EMBL sequence database under the accession number Z14044.

or in response to PTPase inhibition (lpr T cells) (Figure 4). APT immunoprecipitation of VCP protein was inhibited by 60 mM phenyl phosphate, indicating that this protein was immunoprecipitated specifically via phosphotyrosine residues. Comparison of the amount of APT immunoprecipitable VCP protein with that present in total cell lysates showed that 3–5% of total cellular VCP was APT immunoprecipitable from anti-CD3 stimulated Jurkat T cells, and 10–12% was APT immunoprecipitable from PAO + OV treated lpr T cells. These values provide an estimate of the fraction of VCP that is tyrosine phosphorylated in these cells, although they are most probably underestimated, as we could immunoprecipitate with APT mAb only 20–30% of VCP that could be immunoblotted with APT mAb. We have performed similar experiments in WEHI-231 B cells, RBL-2H3 basophils, PC-12 pheochromocytoma cells and NIH 3T3 fibroblasts (data not shown). Although VCP was detected in the lysates of all these cell types, only in B cells after surface immunoglobulin crosslinking and basophils after IgE receptor engagement could we immunoprecipitate VCP with APT. Nerve and epidermal growth factor treatment of fibroblasts failed to induce detectable VCP tyrosine phosphorylation. In both B cells and RBL cells the fraction of APT-immunoprecipitable VCP was 5- to 10-fold lower than in Jurkat T cells, and the increases in APT-immunoprecipitable VCP in response to activation were only of the order of 50–100%.

Table II. Summary of amino acid sequence similarity between murine VCP and other family members

Protein	Source	% Amino acid identity to murine VCP	Length of overlap (no. of amino acids)
VCP	pig	99.6	806
p97 ATPase	*X.laevis*	95.0	363
cdc48p	*S.cerevisiae*	68.4	806
TBP-1	human	41.8	232
pas1p	*S.cerevisiae*	33.9	304
sec18p	*S.cerevisiae*	31.9	395
NSF	hamster	30.6	536

All amino acid sequence comparisons were between murine VCP and sequences obtained from the GenBank protein database, except for cdc48p which was obtained from the Swiss database. Statistical analyses were performed using the FASTA sequence analysis program.

cDNA expression

To demonstrate conclusively that our cDNA encoded a 100 kDa tyrosine kinase substrate, we expressed the VCP cDNA in BW 5147 T cells, together with a selectable marker (neomycin resistance) encoded by pF Neo, and a chimeric receptor consisting of the extracellular and transmembrane domains of the human p55 IL-2Rα chain (TAC) linked to the cytoplasmic portion of CD3ε. This chimeric receptor is capable of activating T cells when cross-linked with anti-TAC mAb (Letourneur and Klausner, 1992), and also served as a surface marker for identification of successful transfectants. We added an epitope tag corresponding to amino acid sequence from human c-Myc (Evan *et al.*, 1985) to the C terminus of the VCP protein to allow us to immunoprecipitate the expressed protein, and to separate the expressed protein from endogenous VCP.

Stably transfected, neomycin resistant cells were screened for TTε expression by immunofluorescence. Cells expressing the chimeric receptor were cloned and then tested for transfected VCP protein. These clones expressed an ~95 kDa protein specifically immunoprecipitated by the anti-myc mAb 9E10 and reactive with anti-VCP antisera by immunoblotting (Figure 5A). The level of transfected VCP expression represented only 20% of total cellular VCP, and for unknown reasons, both endogenous and transfected VCP migrated with a slightly smaller apparent mol. wt (95 kDa

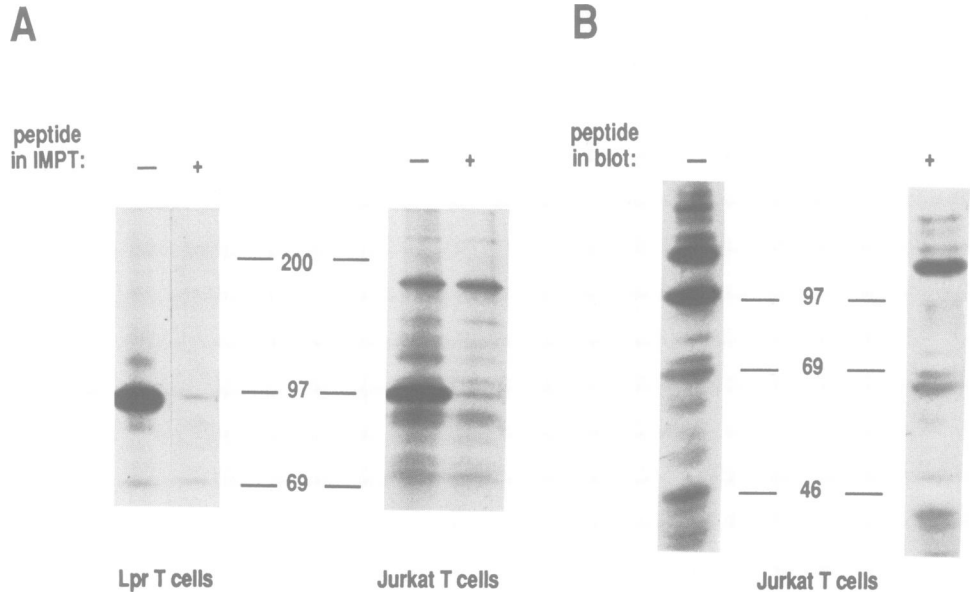

Fig. 3. Specificity of rabbit anti-VCP antisera. **(A)** Lpr and Jurkat T cells were labeled with [^{35}S]methionine/cysteine (5 × 10^6 cells/lane) as described in Materials and methods. The labelled cells were solubilized and the post-nuclear supernatants were subjected to immunoprecipitation using rabbit anti-VCP antibodies coupled to protein A–Sepharose in either the absence or presence of 25 μg immunizing peptide as a competitor. **(B)** Jurkat T cell lysates (2.5 × 10^6 cells) were subjected to electrophoresis and transfer to nitrocellulose. These membranes were then incubated with anti-VCP antibodies in either the absence or presence of 2.5 μg/ml immunizing peptide. Proteins were detected with [^{125}I]protein A. In both panels proteins were separated by 7.5% SDS–PAGE.

by SDS−PAGE) in BW 5147 than in other cell types (100 kDa as judged by SDS−PAGE).

The transfectant was activated via the TTε receptor with biotinylated anti-TAC mAb cross-linked with avidin (Figure 5B). Activation by this method resulted in the tyrosine phosphorylation of a prominent ~95 kDa protein specifically immunoprecipitated by anti-myc mAb. The level of phosphotyrosine in the VCP protein increased >10-fold, indicating that tyrosine phosphorylation of the expressed protein increased in response to cell activation.

Discussion

We have presented data that identify a 100 kDa tyrosine phosphorylated substrate observed in activated T cells as the murine equivalent of the VCP. The 100 kDa protein can be immunoprecipitated by APT mAb and immunoblotted with anti-VCP antisera. Moreover, the cloned murine VCP cDNA encodes a 100 kDa protein that when expressed can be tyrosine phosphorylated upon T cell activation.

VCP was first identified as a result of attempts to clone a putative peptide hormone called valosin. It was found that the cloned cDNA encoded a ubiquitously expressed 90 kDa cytosolic protein, termed VCP, which showed none of the characteristics of a peptide hormone precursor (Koller and Brownstein, 1987). Subsequently it was shown that the valosin peptide was generated as an artefact of extraction (Gill *et al.*, 1989). The function of VCP remained unknown, although amino acid sequence analysis has shown that VCP is a member of a family of proteins that share duplicated regions of sequence homology (~200 amino acids) each containing an ATP binding consensus sequence (Peters *et al.*, 1990; Erdmann *et al.*, 1991; Fröhlich *et al.*, 1991).

Several properties of these proteins have been identified.

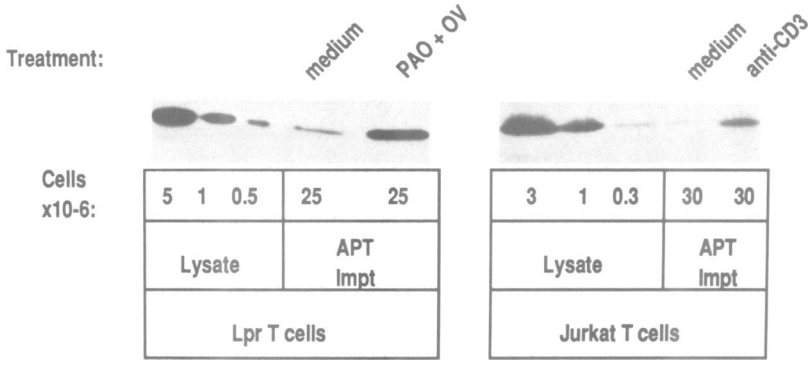

Fig. 4. VCP detection by immunoblotting T cell lysates and APT immunoprecipitates. T cells from lpr mice were incubated with medium or PAO plus OV and Jurkat T cells were incubated with medium or stimulatory anti-CD3 mAbs. Postnuclear supernatants from the medium or phosphatase treated lpr cells and from the medium or anti-CD3 treated Jurkat cells were subjected to immunoprecipitation with 4G10 monoclonal APT antibodies coupled to protein A−Sepharose. Proteins containing phosphotyrosine were specifically eluted with 40 mM phenyl phosphate. In addition to these immunoprecipitates, post-nuclear supernatants from the indicated number of lpr and Jurkat T cells were included on this gel ('Lysate'). The VCP protein was detected after electrophoresis on 7.5% SDS−PAGE and transfer to nitrocellulose with anti-VCP antibodies and [^{125}I]protein A.

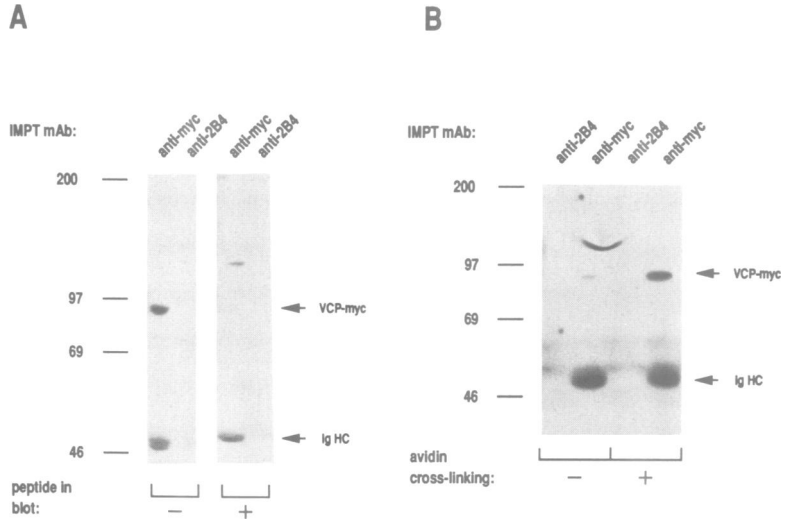

Fig. 5. VCP expression and VCP tyrosine phosphorylation after T cell activation. (**A**) BW 5147 cells stably co-transfected with the cloned murine VCP cDNA (including human c-myc epitope sequence at the 3′ end) and the TTε chimeric receptor construct were solubilized and subjected to immunoprecipitation with anti-myc or, as a control, anti-2B4 antibodies. The VCP-myc protein (arrow) was detected by immunoblotting with anti-VCP antibodies in either the absence or presence of the immunizing peptide. (**B**) These cells were stimulated for 5 min at 37°C by avidin cross-linking as described in Materials and methods. Tyrosine phosphorylated VCP was detected by immunoblotting with APT serum. Immunoprecipitations were performed using mAb coupled to protein G−Sepharose and lysates from either (a) 10^7 or (b) 9×10^6 cells, and proteins were separated by 7.5% SDS−PAGE.

VCP, cdc48p, *Xenopus* p97 ATPase and hamster NSF have been shown to exist as cytosolic homo-oligomeric proteins; VCP (data not shown), cdc48p (Fröhlich *et al.*, 1991) and p97 ATPase (Peters *et al.*, 1990, 1992) as hexamers, and NSF as a tetramer (Block *et al.*, 1988). The most extensive structural analysis has been performed on the *Xenopus laevis* p97 ATPase. Electron microscopy and analysis of two-dimensional crystals have shown that the protein exists as a hexamer. The hexamer itself can dimerize, resulting in a complex oligomer. The tetrameric structure of NSF has been shown to be ATP dependent and required for function (Block *et al.*, 1988). The magnesium dependent ATPase activity of *X.laeivs* p97 is found only when the protein is in the hexameric conformation (Peters *et al.*, 1992). Oligomeric structure, ATP binding properties and ATPase activity appear to be central to the function of members of this family of proteins.

Members of this multigene family have a critical role in cellular function. NSF/sec18p is required for membrane fusion, such as intracellular vesicular membrane transport between the endoplasmic reticulum and Golgi and between Golgi stacks (Eakle *et al.*, 1988; Block *et al.*, 1988; Wilson *et al.*, 1989). Pas1p is essential to yeast peroxisome biogenesis (Erdmann *et al.*, 1991) The cdc48 protein is required for progression through mitosis (Fröhlich *et al.*, 1991).

It has been proposed that, by virtue of their 68% amino acid identity, cdc48p and VCP are homologs, although it is not yet known whether the VCP gene can complement *cdc48*-1 mutants. The *cdc48* gene complements the cold-sensitive *S.cerevisiae* mutant strain *cdc48*-1 (Fröhlich *et al.*, 1991). In this strain, mitosis is arrested, with the unseparated mother and daughter cells joined by a 'neck' containing a single undivided nucleus. This phenotype is accompanied by an abnormal distribution of mitotic spindles and microtubules. Disruption of the endogenous *cdc48* gene in wild-type yeast by homologous recombination produces an identical phenotype. Tyrosine phosphorylation of cdc48p has not been reported, though analysis of tyrosine kinases and their substrates in *S.cerevisiae* is only a recent development. It is important to note, however, that studies of tyrosine phosphorylation in yeast have revealed that some cell cycle elements are regulated by this modification. Best studied is the regulation of $p34^{cdc2}$ in *Schizosaccharomyces pombe* (reviewed in Nurse, 1990). This critical serine kinase regulates passage through the cell cycle. The activity of this enzyme is subjected to multiple controls, one of which is tyrosine phosphorylation by $p107^{wee1}$ during S phase, and subsequent dephosphorylation by $p80^{cdc25}$, which allows progression from G_2 to mitosis (Dunphy and Kumagi, 1991; Featherstone and Russell, 1991; Gautier *et al.*, 1991). This pathway also exists in *S.cerevisiae*, although recent evidence indicates that tyrosine phosphorylation may not be as important a means of controlling p34 activity (Amon *et al.*, 1992; Sorger and Murray, 1992). A less well studied *S.cerevisiae* kinase is MCK1, which like $p107^{wee1}$ is a 'dual specificity' kinase capable of serine/threonine and tyrosine kinase activity (Neigeborn and Mitchell, 1991). It is required for early gene transcription during meiosis and for ascus maturation. In addition, it has a role in mitosis where it is required for kinetochore function. Cells lacking MCK1 are supersensitive to microtubule destabilizing agents. This phenotype is at least superficially similar to that observed in *cdc48*-1 mutants, raising the possibility that cdc48 may be a substrate for MCK1 in yeast.

If VCP does complement the *cdc48* mutation, then overexpression of mutant forms of VCP may allow us to determine whether VCP functions as a regulator of mitosis in mammalian cells. In particular, the role of tyrosine phosphorylation can be addressed in this manner. Our identification of VCP as a substrate for TCR-mediated tyrosine kinase activation is the first suggestion that this signalling pathway may be directly coupled to the regulation of the cell cycle.

Materials and methods

Mice

Female MRL *lpr/lpr* mice (3–6 months old) were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained at the NIH animal containment facility.

Antibodies

Anti-TCR stimulations were performed using either G19-4 (Ledbetter *et al.*, 1987) or OKT3 (Weiss *et al.*, 1986), mouse IgG_1 mAbs specific for human CD3ε chains. The rat anti-human p55 IL-2R α-chain mAb IOT-14 conjugated to biotin (AMAC, ME) was used for stimulation of BW 5147 thymoma cell transfectants. For APT immunoprecipitations, the mouse IgG_{2b} mAb 4G10 was used (Druker *et al.*, 1989). For anti-VCP (pp100) immunoprecipitations and immunoblotting, rabbit polyclonal antibodies were raised to a synthetic peptide corresponding to amino acid residues 20–40 of the murine VCP sequence. The mouse IgG_1 anti-human c-myc mAb 9E10 (Evan *et al.*, 1985) was used for anti-myc immunoprecipitations. The mouse IgG_{2a} mAb specific for the clonotypic TCR α-chain expressed by the murine T cell hybridoma 2B4 (Samelson *et al.*, 1986) was used as a control mAb for immunoprecipitations. For APT immunoblotting, antibodies generated against the abl PTK, which were affinity purified on phosphotyramine, were used as previously described (Samelson *et al.*, 1990a).

Cells

MRL *lpr/lpr* mice were killed by asphyxiation in a CO_2 chamber, the inguinal, mesenteric, axial and popliteal lymph nodes removed, and lymphoid cell suspensions prepared according to the method of von Boehmer and Shortman (1973). The human leukemic T cell line Jurkat E6.1 (Weiss *et al.*, 1986) and the murine thymoma BW 5147 (Hyman and Stallings, 1974) were maintained in R2E medium (Biofluids, MD) supplemented with 8% fetal calf serum (FCS), 2 mM glutamine and 100 μg/ml gentamicin sulfate in a 5% CO_2 incubator at 37°C. 2-Mercaptoethanol (30 μM) was included in BW 5147 culture medium.

Cell stimulation and PTPase inhibition

Jurkat T cells were stimulated with anti-CD3 antibodies as previously described (June *et al.*, 1990; Samelson *et al.*, 1990a). BW 5147 transfectants were stimulated by pre-binding 5 μg/ml biotinylated anti-TAC mAb to cells in DMEM for 30 min at 37°C, washing away unbound mAb and then cross-linking with 20 μg/ml avidin (Sigma, MO) in DMEM at 37°C (Letourneur and Klausner, 1992). All stimulations were stopped by the addition of 10 vol of ice-cold PBS with 100 μM Na_3VO_4 and 1 mM EDTA. To inhibit cellular PTPase, cells were suspended at $2-5 \times 10^7$ per ml RPMI/5% FCS with 10 μM PAO (Aldrich, MI) (Garcia-Morales *et al.*, 1990) and 100 μM Na_3VO_4 (OV; Sigma, MO), and incubated for 15 min at 37°C in a shaking water bath. Treatment was stopped by pelleting the cells and resuspending in ice-cold RPMI.

[^{35}S]Methionine labeling

Cells were metabolically labeled with 500 μCi/ml [^{35}S]methionine (Translabel, ICN, CA; 1 Ci = 37 GBq) in methionine- and cysteine-free medium with 10% FCS (dialysed against distilled H_2O) for 90 min at 37°C.

Cell solubilization

Cells were lysed at 10^8 per ml of ice-cold 0.5% Triton X-100 lysis buffer (LB) containing 150 mM NaCl, 50 mM Tris–HCl, pH 7.5, 1 mM OV, 5 mM EDTA, 10 μg/ml aprotinin and leupeptin and 25 μg/ml *p*-nitrophenyl-guanidinobenzoate for 40 min. Triton X-100 insoluble material was removed by centrifugation for 15 min at 12 000 *g*. Lysates not subjected to immunoprecipitation were boiled for 5 min in reduced SDS sample buffer.

Immunoprecipitation and APT affinity purification

Detailed descriptions of immunoprecipitation and APT batch purification have been published previously (Egerton *et al.*, 1992). In brief, cell lysates

were immunoprecipitated with antibodies coupled to either protein A or protein G Sepharose beads (Pharmacia, NJ), washed and eluted in SDS sample buffer. Phosphotyrosine containing proteins were batch purified from postnuclear cell lysates with the 4G10 mAb immobilized and dimethylpimelimidate (Pierce, IL) cross-linked to protein A–Sepharose beads. After extensive washing, proteins containing phosphotyrosine were specifically eluted in 40 mM phenyl phosphate (Sigma, MO) and boiled in SDS sample buffer.

Electrophoresis and Western blotting

Proteins were separated by SDS–PAGE, electrophoretically transferred to nitrocellulose and detected by immunoblotting as previously described (June *et al.*, 1990; Samelson *et al.*, 1990a). Proteins being prepared for amino acid sequencing were transferred to nitrocellulose and visualized by Ponceau S staining.

Peptide cleavage, purification and sequencing

A detailed description of these methods has been published (Aebersold *et al.*, 1987; Egerton *et al.*, 1992). Briefly, proteins were transferred to nitrocellulose membranes and stained with Ponceau S, and the bands were excised prior to limited proteolytic cleavage with Lys C. Peptides were eluted from the membrane, purified by reversed phase HPLC and subjected to automated Edman degradation and amino acid sequence analysis.

cDNA cloning and sequencing

A 63 base oligonucleotide probe derived from cDNA nucleotide sequence corresponding to peptide 1 (Table I) was synthesized with the sequence: 5′-AAG AAT CGT CCC AAT CGG TTA ATT GTT GAT GAA GCC ATC AAT GAG GAC AAC AGT GTG GTA TCT-3′. The oligonucleotide was ^{32}P end-labelled using T4 polynucleotide kinase (BRL, MD) to a specific activity $>10^8$ c.p.m./μg DNA, and used to probe a λgt10 MRL *lpr/lpr* splenic T cell cDNA library (kindly provided by Dr David Cohen, Laboratory of Immunoregulation, NIAID, NIH). 10^6 λgt10 plaques were transferred to duplicate nylon filters (Du Pont) then prehybridized at 50°C in 2 × SSC, 1 × Denhardt's, 1% SDS and 0.5% sodium pyrophosphate for 18 h prior to hybridization at 50°C for 18 h with 10 ng/ml labeled probe and 20 μg/ml denatured salmon sperm DNA. Filters were then washed according to the manufacturer's protocol, and positive plaques identified by autoradiography. Clones were isolated by two further rounds of plaque purification, and the cDNA inserts from each positive clone were checked by *Eco*RI digestion of the purified phage DNA and agarose gel electrophoresis. cDNA inserts were subcloned into the *Eco*RI site of pBluescript SK+ (Stratagene, CA) and both strands sequenced by the dideoxy nucleotide method using the Sequenase DNA sequencing method (United States Biochemical, OH) and synthetic oligonucleotide primers.

c-myc epitope tagging

Nucleotide sequence encoding a human c-myc epitope (recognized by the mAb 9E10; Evan *et al.*, 1985) was added to the extreme 3′ end of the VCP cDNA coding sequence as follows. A synthetic sense primer corresponding to VCP cDNA sequence including the unique *Bgl*II restriction site, and a synthetic antisense primer encoding the myc epitope sequence and the VCP unique *Sph*I restriction site, were used to PCR amplify a 660 bp *Bgl*II–*Sph*I fragment from the VCP cDNA template. This *Bgl*II–*Sph*I fragment containing the c-myc epitope sequence was then subcloned into the VCP cDNA *Bgl*II–*Sph*I sites in pBluescript SK+ and sequenced as already described.

cDNA expression

A 2.6 kb *Nco*I–*Apa*I p100 cDNA fragment was subcloned into the mammalian expression vector pCDLSRα (Takebe *et al.*, 1988). This construct was co-transfected together with the IL-2 receptor α-chain-CD3ϵ chimera TTϵ in pCDLSRα (Letourneur and Klausner, 1992) and the neomycin resistance plasmid pFNeo (Saito *et al.*, 1987) into BW 5147 thymoma cells by electroporation using a Gene Pulse apparatus (Bio-Rad, CA). DNA was introduced into cells suspended in phosphate-buffered saline, pH 7.4 (PBS) by a 290 V/250 pF electrical pulse at room temperature. Electroporated cells were grown in the presence of 1 mg/ml G418 (BRL, MD), and clonal cell lines expressing VCPmyc and surface TTϵ were obtained by limiting dilution.

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References

Aebersold,R.H., Leavitt,J., Saavedra,R.A., Hood,L. and Kent,S.B.H. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 6970–6974.

Amon,A., Surana,U., Muroff,I. and Nasmyth,K. (1992) *Nature*, **355**, 368–371.

Baniyash,M., Garcia-Morales,P., Luong,E., Samelson,L.E. and Klausner,R.D. (1988) *J. Biol. Chem.*, **263**, 18225–18230.

Block,M., Glick,B., Wilox,C.A., Wieland,F.T. and Rothman,J.E. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 7852–7856.

Bretscher,A. (1989) *J. Cell. Biol.*, **108**, 921–930.

Burridge,K., Fath,K., Kelly,T., Nuckolls,G. and Turner,C. (1988) *Annu. Rev. Cell Biol.*, **4**, 487–525.

Bustelo,X.R., Ledbetter,J.A. and Barbacid,M. (1992) *Nature*, **356**, 68–71.

Cantley,L.C., Auger,K.R., Carpenter,C., Duckworth,B., Graziani,A., Kapeller,R. and Soltoff,S. (1991) *Cell*, **64**, 281–302.

Chan,A.C., Irving,B.A., Fraser,J.D. and Weiss,A. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 9166–9170.

Chin,D.T., Goff,S.A., Webster,T., Smith,T. and Goldberg,A.L. (1988) *J. Biol. Chem.*, **263**, 11718–11728.

Cohen,P.L. and Eisenberg,R.A. (1991) *Annu. Rev. Immunol.*, **9**, 243–269.

Dunphy,W.G. and Kumagi,A. (1991) *Cell*, **67**, 189–196.

Druker,B.J., Mamon,H.J. and Roberts,T.M. (1989) *New Engl. J. Med.*, **321**, 1383–1391.

Eakle,K.A., Bernstein,M. and Emr,S. (1988) *Mol. Cell. Biol.*, **8**, 4098–4109.

Egerton,M., Burgess,W.H., Chen,D., Druker,B.J., Bretscher,A. and Samelson,L.E. (1992) *J. Immunol.*, in press.

Erdmann,R., Weibel,F.F., Flessau,A., Rytka,J., Beyer,A., Fröhlich,K.-U. and Kunau,W.-H. (1991) *Cell*, **64**, 499–510.

Evan,G.I., Leweis,G.K., Ramsay,G. and Bishop,J.M. (1985) *Mol. Cell. Biol.*, **5**, 3610–3616.

Featherstone,C. and Russell,P. (1991) *Nature*, **349**, 808–811.

Fröhlich,K.-U., Fries,H.-W., Rüdiger,M., Erdmann,R., Botstein,D. and Mecke,D. (1991) *J. Cell Biol.*, **114**, 443–453.

Garcia-Morales,P., Minami,Y., Luong,E., Klausner,R.D. and Samelson, L.E. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 9255–9259.

Gautier,J., Solomon,M.J., Booher,R.N., Bazan,J.F. and Kirschner,M.W. (1991) *Cell*, **67**, 197–211.

Gill,J.S., Ghatei,M.A., Domin,J. and Bloom,R. (1989) *Life Sci.*, **44**, 483–491.

Hsi,E.D., Siegel,J.N., Minami,Y., Luong,E.T., Klausner,R.D. and Samelson,L.E. (1989) *J. Biol. Chem.*, **264**, 10836–10842.

Hyman,R. and Stallings,V. (1974) *J. Natl. Cancer Inst.*, **52**, 429–457.

Itoh,N., Yonehara,S., Ishii,I., Yonehara,M., Mizushima,S.-I., Sameshima,M., Hase,A., Seto,Y. and Nagata,S. (1991) *Cell*, **66**, 233–243.

June,C.H., Fletcher,M.C., Ledbetter,J.A. and Samelson,L.E. (1990) *J. Immunol.*, **144**, 1591–1599.

Katagiri,T., Urakawa,K., Yamanashi,Y., Semba,K., Takahashi,T., Toyoshima,K., Tamamoto,T. and Kano,K. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 10064–10069.

Kim,H.K., Kim,J.W., Zilberstein,A., Margolis,B., Kim,J.G., Schlessinger, J. and Rhee,S.G. (1991) *Cell*, **65**, 435–441.

Klausner,R.D. and Samelson,L.E. (1991) *Cell*, **64**, 875–878.

Koller,K.J. and Brownstein,M.J. (1987) *Nature*, **325**, 542–545.

Kozak,M. (1991) *J. Biol. Chem.*, **266**, 19867–19870.

Ledbetter,J.A., Gentry,L.E., June,C.H., Rabinovitch,P.S. and Purchio,A.F. (1987) *Mol. Cell. Biol.*, **7**, 650–656.

Letourneur,F. and Klausner,R.D. (1992) *Science*, **255**, 79–82.

Margolis,B., Hu,P., Katzav,S., Li,W.,Oliver,J.M., Ullrich,A., Weiss,A. and Schlessinger,J. (1992) *Nature*, **356**, 71–74.

Neigeborn,L. and Mitchell,A.P. (1991) *Genes Dev.*, **5**, 533–548.

Nelbock,P., Dillon,P.J., Perkins,A. and Rosen,C.A. (1990) *Nature*, **248**, 1650–1653.

Nurse,P. (1990) *Nature*, **344**, 503–508.

Park,D.J., Rho,H.W. and Rhee,S.G. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 5453–5456.

Peters,J.-M., Walsh,M.J. and Franke,W.W. (1990) *EMBO J.*, **9**, 1757–1767.

Peters,J.-M., Harris,J.R., Lustig,A., Müller,S., Engel,A., Volker,S. and Franke,W.W. (1992) *J. Mol. Biol.*, **223**, 557–571.

Saito,T., Weiss,A., Miller,J., Norcross,M.A. and Germain,R.N. (1987) *Nature,* **325**, 125–128.
Samelson,L.E., Patel,M.D., Weissman,A.M., Harford,J.H. and Klausner,R.D. (1986a) *Cell,* **46**, 1083–1090.
Samelson,L.E., Davidson,W.F., Morse,H.C. and Klausner,R.D. (1986b) *Nature,* **324**, 674–676.
Samelson,L.E., Fletcher,M.C., Ledbetter,J.A. and June,C.H. (1990a) *J. Immunol.,* **145**, 2448–2454.
Samelson,L.E., Phillips,A.F., Luong,E.T. and Klausner,R.D. (1990b) *Proc. Natl. Acad. Sci. USA,* **87**, 4358–4362.
Secrist,J.P., Karnitz,L. and Abraham,R.T. (1991) *J. Biol. Chem.,* **266**, 12135–12139.
Sorger,P.K. and Murray,A. (1992) *Nature,* **355**, 365–368.
Takebe,Y., Seiki,M., Fujisawa,J.-I., Hoy,P., Yokota,K., Arai,K.-I., Yoshida,M. and Arai,N. (1988) *Mol. Cell. Biol.,* **8**, 466–472.
Ullrich,A. and Schlessinger,J. (1990) *Cell,* **61**, 203–212.
Veillette,A., Bookman,M.A., Horak,E.M., Samelson,L.E. and Bolen,J.B. (1989) *Nature,* **338**, 257–259.
von Boehmer,H. and Shorman,K. (1973) *J. Immunol. Methods,* **2**, 293–301.
Wange,R.L., Kong,A.-N. and Samelson,L.E. (1992) *J. Biol. Chem.,* **267**, 11685–11688.
Watanabe-Fukunaga,R., Brannan,C.I., Copeland,N., Jenkins,N.A. and Nagata,S. (1992) *Nature,* **356**, 314–317.
Weiss,A., Imboden,J., Hardy,K., Manger,B., Terhorst,C. and Stobo,J. (1986) *Annu. Rev. Immunol.,* **4**, 593–619.
Weiss,A., Koretzky,G., Schatzman,R.C. and Kadlecek,T. (1991) *Proc. Natl. Acad. Sci. USA,* **88**, 5484–5488.
Wilson,D.W., Wilcox,C.A., Flynn,G.C., Chen,E., Kuang,W.-J., Henzel,W.J., Block,M., Ullrich,A. and Rothman,J.E. (1989) *Nature,* **339**, 355–359.

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