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Identification of a Type 6 Protein Ser/Thr Phosphatase Regulated by Interleukin-2 Stimulation

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Abstract We have identified a 36 kD phosphoprotein that forms a complex with spliceosomal small nuclear ribonucleoproteins in lymphocyte extracts. This 36 kD protein is differentially phosphorylated in transformed human lymphoid cell lines and is regulated by IL-2 in peripheral blood T cells. We purified the 36 kD protein from human lymphocytes by employing a combination of immuno-affinity chromatography and preparative two-dimensional gel electrophoresis. Internal amino acid sequence analysis of the purified protein yielded two peptides that had perfect matches with sequences in the human protein serine/threonine phosphatase 6 (PP6). Using degenerate primers corresponding to the peptides, we obtained from a human T lymphocyte cDNA library a DNA fragment whose sequence is homologous to an EST cDNA clone (R05547). The predicted amino acid sequence of this clone showed over 98% sequence identity to human PP6. The identification of an IL-2 regulated type 6 protein serine/threonine phosphatase in lymphocytes was further substantiated by immunoblotting with anti-peptide antibodies. These findings suggest that PP6 is a component of a signaling pathway regulating cell cycle progression in response to IL-2 receptor stimulation. J. Cell. Biochem. 73:153–163, 1999.

Key words: Protein ser/thr phosphatase; IL2 receptor; cellular activation; cell cycle regulation

Recent studies on the regulation of signal transduction by protein phosphorylation has opened a new field for studying cell growth and differentiation. The reversible phosphorylation of serine/threonine residues on proteins is a mechanism utilized by eucaryotic cells to regulate several cellular processes including metabolism, signal transduction, and cell growth and differentiation [Cohen, 1989; Cohen et al., 1990; Shenolikar et al., 1994 and references therein]. Recent crystallographic and X-ray studies have provided important information about the structure and catalytic mechanism of protein serine/threonine phosphatase (PP). Although ser/thr phosphatase and protein tyrosine phosphatases both catalyze phosphodiester hydrolysis, they have completely different structures and distinct catalytic mechanism [Goldberg et al., 1995; Denu et al., 1996].

Protein serine/threonine phosphatase are among the most highly conserved proteins in evolution. These enzymes have been classified conventionally in four subtypes [PP1, PP2A, PP2B, and PP2C] based on their sensitivity to inhibitors and divalent cation requirements [Ingebristen and Cohen, 1983; Cohen, 1989]. Several of these enzymes consist of catalytic subunits coupled with variable regulatory subunits. The functional diversity of these enzymes appear to arise from the association of different catalytic subunits with one or more regulatory subunits. The regulatory subunits control enzyme activity, confer substrate specificity, and target the enzyme to specific cellular locations [Shenolikar et al., 1994 and refer-

Abbreviations used: NEPHGE, two-dimensional non-equilibrium pH gradient gel electropheresis PHA, phytohemagglutinin; PP, protein ser/thr phosphatase; PP6, protein ser/thr phosphatase type 6; SDS-polyacrylamide gel electrophoresis, SDS-PAGE; PVDF, polyvinylidene difluoride membrane; 2D-PAGE, two-dimensional isoelectric focusing gel electropheresis.

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ences therein; Denu et al., 1996]. The catalytic subunits of PP exist as multigene families, cDNAs encoding the catalytic subunits of these enzymes have been classified into two structurally distinct families, the PP1/2A/2B and PP2C family [Cohen et al., 1990, Shenolikar et al., 1994 and references therein]. cDNA cloning led to the identification of different isoforms of PP1, PP2B, and PP2A, isoforms of these PP demonstrate 80 to 97% degree of homology [Stone et al., 1987; Arino et al., 1988]. In addition, four structurally related phosphatases designated PP4 [Da cruz e silva and Cohen, 1989; Brewis et al., 1993], PP5 [Chen et al., 1994a; Becker et al, 1994; Chinkers et al., 1994], rat PPV [Becker et al., 1994]; PP6 [Bastians et al., 1996] and PP7 [Huang et al., 1998] have been identified. Based on comparison of their sequences, PP4, rat PPV, and PP6 have been placed into the PP2A-like family group and it was proposed that they have evolved from the same gene as PP2A [Cohen et al., 1990].

The PP2A holoenzyme is a heterotrimer composed of a 36-kD catalytic (C) subunit, a 63-kD structural (A) subunit, and a third subunit termed B or phosphatase regulatory subunit. There are at least five distinct families of proteins that interact with and regulate the PP2A core enzyme including the B, B', B" families of regulatory subunits and the small and middle tumor antigens of DNA tumor viruses [Li et al., 1996; Ogris et al., 1997]. Genetic studies in lower eukaryotic cells have begun to be exploited to gain new insight into the physiological functions of these enzymes. PP2A has been implicated in the control of cell cycle, growth and proliferation, DNA replication, viral transformation, morphogenetic events, and the regulation of metabolism [Clarke et al., 1993; Shenolikar et al., 1994 and references therein]. Activation of growth factor receptor tyrosine kinases leads to transient phosphorylation and inhibition of PP2A regulating the mitogenic response to growth factors [reviewed in Shenolikar, 1994].

Another example of how PP may regulate cell growth is represented by PP5 which was shown to be necessary for mammalian cell proliferation. PP5 is thought to act upstream of the tumor supressor protein p53 to regulate the induction of $p21^{WAF/Cip1}$ protein and to mediate growth arrest [Zuo et al., 1998]. The ability of p53 to induce the expression of $p21^{WAF/Cip1}$ in combination with the inhibitory activity of $p21^{WAF/Cip1}$ against the cyclin-dependent kinases suggest that the regulation of $p21^{WAF/Cip1}$ expression is important aspect of mammalian cell cycle growth control [Zuo et al., 1998]. Interestingly the PP5 and PP6 open reading frames are closely similar, except for the presence of tetratricopeptide repeat (TPR) sequences in PP5. It was suggested that the presence of TPR sequences in PP5 implies that PP5 interacts with other TPR-containing proteins, many of which are involved in the regulation of cell cycle progression [Chen et al., 1996 and references therein]. However, little information is available about the biochemical characterization of PP6 and its function. A possible role of PP6 in the regulation of the cell cycle was implied from complementation studies in fission yeast. The Drosophila PPV and human PP6 were shown to rescue the mutant growth arrest (Sit4) in yeast, a protein phosphatase required for the G1 to S transition of the cell cycle [Arndt et al., 1989; Shimanuki et al., 1993]. It was suggested that PPV and PP6 may have a similar function in the cell cycle [Matsmuto and Beach, 1993; Bastians et al., 1996].

The role of PP in the regulation of T cell growth remains poorly understood. T cell activation proceeds in a sequence of complex events that comprise two major phases. The first one, which ensues triggering of the T cell receptor and co-stimulatory molecules results in the transcriptional activation of several immediate/early genes including lymphokine genes, and leads from a quiescent (G0) to a competent (G1) state of the T cells. The second phase involves the response of such competent T cells to secreted growth-promoting cytokines such as IL-2 in an autocrine or paracrine fashion, and drives their entry into the proliferation cycle through G1/S phase progression [Dumont et al., 1996 and references therein]. Recent studies indicated that multiple signaling pathways cooperate in mediating IL-2 receptor-induced stimulation of early proto-oncogene expression [Myazaki et al., 1995]. Subsequently, IL-2 stimulation rapidly induces the expression and the enzymatic activity of several kinases including Raf-1 [Zmuidzinas et al., 1991], PI-3 kinase, and p70 S6 kinase [Dumont et al., 1996]. Interestingly, recent data indicated that PP2A forms a complex with p70 S6 kinase suggesting that this complex may be involved in intracellular signaling [Westphall et al., in press]. However, the role of PP in regulating IL-2 receptor signal-

ing pathways remains unknown. Ultimately, IL-2 regulates the expression of genes that are involved in the regulation of cell cycle progression such as the cylcins and the cyclin-dependent protein kinases [Dumont et al., 1996]. The exact role of PP in the regulation of mammalian cell cycle is not yet understood [reviewed in Shenolikar, 1994]. Recent data indicated that PP5 regulates mammalian cell cycle progression acting upstream of the tumor supressor protein p53 suggesting an important role for this new family of PP in the regulation of mammalian cell growth [Zuo et al., 1998].

In the present study, we report the identification and purification of a type 6 protein Serine/Threonine phosphatase from lymphocytes. We provide evidence that PP6 protein phosphorylation and expression is regulated by IL-2 stimulation. The potential role of PP6 in regulating cell cycle progression of lymphocytes is discussed.

MATERIALS AND METHODS

Cell Culture and ^{32}P-Labeling of Cells

The human T-leukemic Jurkat cell line (J32) were cultured in RPMI 1640 medium (Life Technologies, Grand Island, NY), supplemented with 10% (v/v) prescreened, heat inactivated Fetal Calf Serum, and 2 mM L-glutamine. Peripheral mononuclear blood cells were purified from healthy volunteer blood donors using a ficoll-hypaque density gradient centrifugation. T cells were purified by negative selection using magnetic beads (Dynal, Inc., Fort Lee, NJ) and a cocktail of monoclonal antibodies specific to B cells, monocytes, and NK cells. The purity of T cells was higher than 99% as assessed by immunofluorescence analysis using PE-conjugated mAb anti-CD3 and flow cytometry (FACS Calibur, Becton Dickinson, San Jose, CA). The purity the T cell preparation was checked by the inability of cells to proliferate to stimulation by phytohemagglutinin (PHA) alone. For labeling with [^{32}P]-phosphorus (Amersham, Arlington Heights, IL), cells were cultured for 30 min in a T75 flask in a phosphate free RPMI 1640 medium containing 5% of dyalized FCS at 37°C. The medium was discarded and replaced with fresh medium containing 50 µCi/ml of ^{32}P-(Phosphorus). After 3 h of labeling, the cells were washed once with RPMI and once with PBS.

Monoclonal and Anti-Serum Antibodies

Monoclonal antibody (mAb) 3E10 was raised against a synthetic peptide containing the PPP-GHRSQ sequence motif [Filali et al., in press]. The anti-CD2 antibody 9.6 was used as a control. The polyclonal antibody (U811) was raised in rabbit that were immunized the peptide QITQVYGFYDECQT free and conjugated to KLH (Cocalico Biologicals Inc., Reamstown, PA). Anti-PP2Ac (Calbiochem, San Diego CA) is affinity-purified antibody that recognizes the 36 kD catalytic subunit of mammalian PP2A. This antibody was raised against a synthetic peptide corresponding to residues 298–309 of PP2Ac (PHVTAATPDYFL) coupled to KLH. Anti-PP6 is an affinity-purified antibody that recognizes the catalytic subunit of mammalian PP6 (Dr. Brian Wadzinski, unpublished communications).

Immunoprecipitation and Immunoblotting

Cells were resuspended in a lysis buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X100, 0.5% NP40, 5 mM EDTA, 1% aprotinin, 200 µM phenylmethyl sulfonyl fluoride, 100 mM NaF, 100 mM sodium pyrophosphate, 2 µg/ml of leupeptine, and 200 µM sodium orthovanadate). Cell lysates were cleared by centrifugation at 12,000 rpm for 20 min, and the supernatants were immunoprecipitated as previously described [Filali et al., in press]. The proteins in the immunoprecipitates were eluted from the beads either with boiling them in 2× Laemmli -SDS buffer or vortexing the final pellet in the urea loading buffer (ULB) [9.5 M Urea, 2% NP-40 and 4% Ampholytes pH 3–10 [Oxford Glycosystems Inc., Bedford, MA]. The resulting samples were then analyzed by SDS-polyacrylamide electrophoresis (SDS-PAGE) or two-dimensional gel-poly-acrylamide electrophoresis (2D-PAGE).

For immunoblotting, the protein content of the samples were determined with Bradford microassay (Bio-Rad, La Jolla, CA). Approximately 50 µg of the total proteins from the cell lysate were run on a 10 to 12% poly-acrylamide containing 0.2% SDS and then transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) in 0.5 × Towbin buffer. The membranes were blocked in 3% BSA/PBS for 1 h at room temperature or at +4°C overnight. The membranes were washed four times with PBS containing 0.1% Tween 20, then incubated for

at least 1 h with 12 ml of appropriately diluted in 1.5% BSA/PBS/0.1% Tween 20. After washing, antigen-antibody complexes were detected by incubating the membranes under agitation for 1 h with 0.2 µCi/ml of ^{125}I-protein A. The membranes were then washed as previously, partially dried, and exposed to an X-ray film at -70°C for 1 to 3 days.

Two-Dimensional Gel Electrophoresis

Proteins were separated in the first dimension gel by either isoelectric focusing or NEPHGE as previously described [Filali et al., in press]. Rod gels were stored in the Petri dishes at -20 or -70°C. Just before SDS-PAGE, the IEF or NEPHGE were thawed in the same SDS buffer for 3 to 15 min at 36°C. For the second dimension, the rod gels were transferred onto the top of the stacking gel, the resolving gel is 10 to 12.5% acrylamide slab gel (1.5 mm thick). To improve the reliability and reproducibility of the 2D-PAGE patterns, all the gels to be compared were run at the same time. Only proteins that were reproducibly detected in the gel were considered for data analysis.

Purification of the 36 kD Phosphoprotein

A large scale preparation from the soluble protein fraction of J32 cells was applied for immunopurification using a single step affinity antibody column with mAb 3E10 followed by a preparative 2D-PAGE. Soluble protein lysate was prepared from a large scale culture of J32 cells (5 x 10^{10}) by sonication in a 100 ml of lysis buffer containing a standard mixture of protease inhibitors without adding detergents. The lysate was clarified by centrifugation at 47,000 rpm for 2 h in a 50.2 Ti rotor (Beckman Instruments, Fullerton, CA). The supernatants were precleared and transferred to a new tube containing 3 ml of protein G-Sepharose beads that was covalently cross-linked to 9 mg of affinity-purified mAb 3E10 using dimethyl pimelimidate as previously described [Filali et al., in press]. The antibody column was washed twice in a buffer containing 10 mM Tris HCl pH 8.0, 140 mM NaCl, 0.5% Triton X-100, 0.5% NP-40, and 2% NaN3, followed by two washes in a buffer containing 20 mM Tris HCl pH 8.0, 0.1% Triton X-100, 500 mM NaCl. Proteins were eluted three times with 1.5 ml of a buffer containing 50 mM Tri-ethanolamine, 500 mM NaCl, 0.1% Triton X-100. The eluted fractions were neutralized with 0.2 volumes of 1 mM Tris HCl pH 6.7 then dialyzed against ULB diluted 1:10. Samples were concentrated 10 times in a Speed Vac, then mixed and equilibrated with ampholytes at room temperature before loading (300 µg) on the IEF tube.

For a precise identification of the 36 kD phosphoprotein, eluted proteins were mixed with a 3E10 immunoprecipitate from ^{32}P-labeled phosphorylated proteins. The protein mixture was separated by two-dimensional gel electrophoresis and were then electroblotted on a high retention PVDF membrane (Bio-Rad) in Towbin 0.5× transfer buffer containing 10% MeOH, and the membrane was stained using 0.5% p/v Amido Black. The membranes were air dried and then exposed to the an X-ray film at -70°C. After careful analysis of the stained proteins on the membrane, approximately 2 µg of the 36 kD protein spot corresponding to a specific signal on the X-ray film was localized. The protein spot was eluted from the membrane, cleaved and peptides were resolved by reverse-phase high pressure liquid chromatography (HPLC). Selected HPLC peaks were analyzed by Mass spectrometry and two peptides (91 and 66) were chosen for microsequencing using a gas phase sequencer (Applied Biosystems model 470, Foster City, CA). Peptide purification and microsequencing was performed at the Wistar Institute Protein Microchemistry Laboratory (Wistar Institute, Philadelphia, PA).

Isolation and Characterization of cDNA Clones

Three cDNA templates, two from human lymphocytes and one from human NK cells were used for PCR. The following pair of degenerate primers were used. The sequence of the forward primer 5′-TG(A/C)CA(C/T)TC(A/G)TA(A/G)-AA(A/G/C/T)CC-3′ corresponds to the peptide NYIFMGD; the sequence of the reverse primer 5′-TG(AC)CA(CT)TC(AG)TC(AG)TA-(AG)AA(AGCT)CC-3′ corresponds to the peptide YGFYDEC. Amplification were performed as follow (1 min, 94°C; 1 min, 55°C; and 1 min, 72°C) for 28 cycles using Taq polymerase (BMB, Germany). The amplification product of about 170 bp band was cloned in *Eco*RI site of the PCR II vector (Invitrogen, La Jolla, CA) and sequenced. All DNA sequencing was performed using an automatic sequencer at the DNA Se-

quencing Shared facility of the University of Pennsylvania Cancer Center.

RESULTS AND DISCUSSION

Identification of the 36 kD Phosphoprotein in Human Lymphocytes

In a separate study, we have described a mAb, designated 3E10, against a polyproline motif that recognizes spliceosomal Sm B/B' proteins [Filali et al., in press]. Here we show that mAb 3E10 specifically immunoprecipitates a 36 kD phosphoprotein from lymphocyte total cell extracts. The reactivity of mAb 3E10 was analyzed by immunoprecipitation from ^{32}P-labeled J32 total cell lysate and SDS-PAGE. As shown in Figure 1A, mAb 3E10 specifically immunoprecipitated a prominent phosphoprotein migrating at an apparent molecular weight of 36 kD. This protein was not seen with the control antibody 9.6 (anti-CD2). Interestingly, the intensity of the 36 kD phosphoprotein band in the 3E10 immunoprecipitate from various ^{32}P-labeled lymphoid cell lines varied significantly and was most prominent in the Molt-4 leukemic T cell line [Fig. 1B] suggesting a differential level of protein expression and/or phosphorylation in some leukemic T cell lines. The presence of the 36 kD phosphoprotein in the 3E10 immune complex is quite stable since it is resistant to a high salt up to 500 mM NaCl and to buffers containing 1% SDS (data not shown). To further characterize the 36 kD phosphopro-

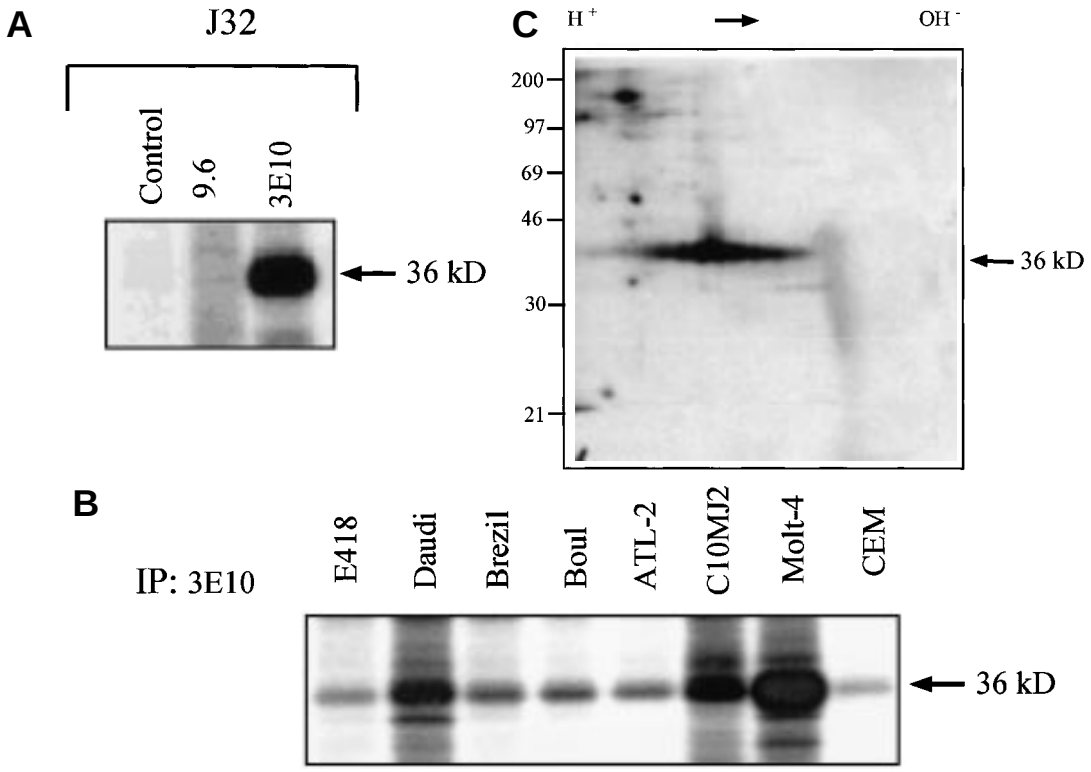

Fig. 1. Identification of a 36 Kd phosphoprotein with mAb 3E10. **A**: Immunoprecipitation with mAb 3E10 from [^{32}P]-labeled J32 total cell extract. J32 cells were labeled with ^{32}P-orthophosphate, and equal amounts of labeled proteins for each immunoprecipitate were analyzed by 12.5% SDS-PAGE. 9.6 (anti-CD2) and an isotype matched antibody were used as a control. Protein bands were visualized by fluorography. **B**: Immunoprecipitation with mAb 3E10 from [^{32}P]-labeled human lymphoid cell line extracts. E418 (B lymphoid cell line); Daudi (B cell line derived from a Burkitt Lymphoma); Brezil (HTLV-1 T cell line), Boul (HTLV-1 T cell line); ATL-2 (Acute T-cell leukemia) C10MJ2 (HTLV-1 T cell line); MOLT-4 (T cell leukemia), and CEM (T cell leukemia) were labeled with ^{32}P-orthophosphate as described in Materials and Methods. An equal amount of labeled proteins for each immunoprecipitate were analyzed by 12.5% SDS-PAGE. Protein bands were visualized by fluorography. **C**: Two-dimensional non-equilibrium pH gradient gel electrophoresis (NEPHGE) of the 36 kD phosphoprotein identified by immunoprecipitation with 3E10. J32 cells were phosphorylated as in A and the cell lysate was immunoprecipitated with mAb 3E10. Immunoprecipitated protein were then resolved on two-dimensional non-equilibrium pH gradient gel electrophoresis as a first dimension. The molecular weight markers are presented on the left side of the gel box. The letters H^+ and OH^- designate the positive and the negative poles of the NEPHGE gel. The top arrow indicates the direction of protein migration in the first dimension.

tein, we used two-dimensional non-equilibrium pH gradient gel electrophoresis [NEPHGE]. Figure 1C shows that the 36 kD is the major phosphorylated protein found in the 3E10 immunoprecipitate and its migration was reproducible when compared in several experiments (not shown).

Regulation of the 36 kD Phosphoprotein by IL-2 Stimulation

We used purified human peripheral blood T cells to examine the changes in the amount of the 36 kD phosphoprotein in the 3E10 immunoprecipitate in response to stimulation by the growth factor IL-2. Purified peripheral blood T cells were stimulated with PHA for 0, 4, 18 and with PHA and IL-2 for 48 h cells were then labeled with ^{32}P-orthophosphate and 3E10 immunoprecipitates from each cell lysate were analyzed by SDS-PAGE. As shown in Figure 2A, a very small amount of the 36 kD phosphoprotein band is seen in the 3E10 immunoprecipitate from resting T cells or from cells stimulated with PHA alone for 4 or 18 h. In contrast, a prominent 36-kD phosphoprotein band representing more then 10-fold increase in radio labeled proteins can be seen in the 3E10 immunoprecipitate from cells that were stimulated with PHA plus IL-2 for 48 h. Based on the kinetics of the IL-2 receptor (CD25) expression in PHA-stimulated peripheral blood T cells [Reed et al., 1985], this experiment suggests that the changes in the amount of radiolabeled 36 kD protein in the 3E10 immunoprecipitate is secondary to IL-2 stimulation. This conclusion was further substantiated using the IL-2 dependent murine CTLL-2 cell line, a model of IL-2 driven T cell proliferation. As shown in Figure 2B, removal of IL-2 for 3 h significantly decreased the amount of ^{32}P-labeled 36 kD protein in the 3E10 immunoprecipitate. This decrease was more profound if the IL-2 removal was prolonged to 18 h which coincides with a cell cycle arrest (Fig. 2B). Taken together, these findings indicate that the 36 kD protein is regulated by the growth factor IL-2. These observed changes may be due to an increase in protein phosphorylation as well as protein expression.

Immuno-Purification and Identification of the 36 kD Phosphoprotein From the Human T Leukemic Cell Line J32

In order to identify the 36 kD phosphoprotein, we purified this protein in sufficient quantities for peptide microsequencing. The protein purification strategy was based on our observation that this protein is present in the cytosolic fraction of J32 cell extracts. A large scale preparation from the soluble protein fraction was used for immunopurification in a single step affinity-antibody column made of protein G-Sepharose beads covalently cross-linked to mAb 3E10. For precise identification of the 36 kD phosphoprotein, eluted proteins were mixed with the 3E10 immunoprecipitate from ^{32}P-labeled phosphorylated proteins. The protein mixture was separated by two-dimensional gel electrophoresis and then electroblotted on a high retention PVDF membrane. A single protein spot that perfectly coincided with a major radiolabeled 36 kD protein was selected for

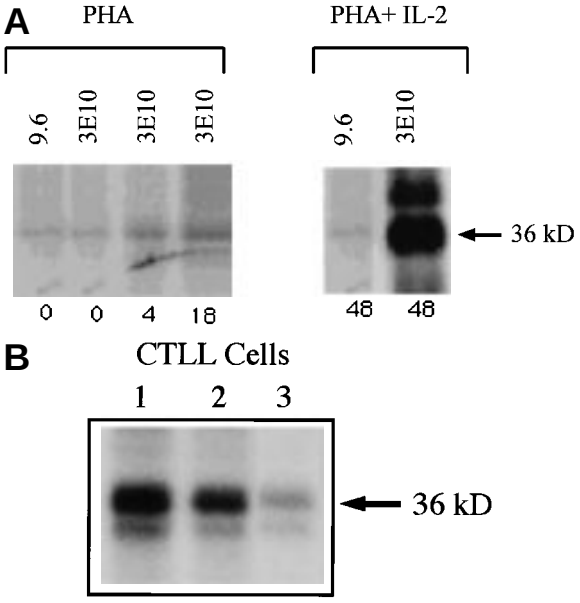

Fig. 2. Regulation of the 36 kD phosphoprotein by stimulation with IL-2. **A**: Immunoprecipitation with mAb 3E10 from [^{32}P]-labeled resting and IL-2-stimulated peripheral blood T cells. Peripheral blood T-cells from a healthy volunteer blood donor were as described in Materials and Methods. Purified T-cells were then stimulated with PHA for 0, 4 h, 18 h, or with PHA+IL-2 for 48 h. Cells were then labeled with ^{32}P-orthophosphate and cell lysates were immunoprecipitated with 3E10 or with the antibody control 9.6 (anti-CD2) as described in Materials and Methods. Proteins in immunoprecipitates were analyzed by 12.5% SDS-PAGE and protein bands were visualized by fluorography. **B**: Immunoprecipitation with mAb 3E10 from [^{32}P]-labeled CTLL-2 cells. CTLL-2 cells were growth in IL-2-free RPMI medium for 1 h (**lane 1**), 3 h (**lane 2**), or 18 h (**lane 3**). Cells were then labeled with ^{32}P-orthophosphate and cell lysates were immunoprecipitated with 3E10 as described in Materials and Methods section. Proteins in immunoprecipitates were analyzed by 12.5% SDS-PAGE and protein bands were visualized by fluorography.

microsequencing. The amino acid sequence from two peptides (91 and 66) were determined and sequence homology was performed using the BLAST search program of NCBI protein data base [Atshull et al., 1990]. As shown in Figure 3, peptide 91 and 66 have a perfect match with two sequences found in rat PPV, a homologue of the Drosophila serine/threonine phosphoprotein phosphatase PPV [Mann et al., 1993; Becker et al., 1994] and human PP6 [Bastians et al., 1996]. In addition, Figure 3 shows that these peptides have a close homology with amino acid sequences of human PPX [Brewis et al., 1993], human PP2A [Arino et al., 1988; Stone et al., 1989], and ppe1 from S. pombe [Bastians et al., 1996 and references therein]. The perfect sequence matching of the two peptides derived from the 36 kD with amino acid sequences found in PP6 suggested that the 36 KD is identical or very closely related to PP6 which has a calculated molecular mass of 35.2 kD.

Characterization of an EST cDNA Clone (R05547) Encoding Human PP6

Using the amino acid sequence of peptide 91 and 66 from the purified 36 kD phosphoprotein, we designed degenerate oligonucletide primers and amplified with PCR DNA fragments from three human lymphocyte cDNA libraries. Electrophoretic analysis of PCR products yielded an apparent single prominent band of 170 bp. These DNA fragments were eluted from the agarose gel and subcloned in TA plasmid (Invitrogen). Sixty clones were isolated, sequenced, and analyzed using the Multiple Sequence Alignment program. These results showed that about 70% of all subclones had a perfect match with PPX (PP4) and about 30% matched the human PP1α subunit. One single PCR clone, designated PEER-26, showed a strong homology to human PP6 (data not shown). Thus, it is likely that PP6 cDNA is under represented in these cDNA libraries, which may indicate that the expression of PP6 is low in lymphoid cells. This observation is consistent with previous data showing that PP6 transcripts are present in low abundance in Hela cells [Bastians et al., 1996].

Search of the GenBank Expression Sequence Tags (EST) data base [Atshull et al., 1990] showed that PEER-26 clone had a very high homology with an EST clone (GenBank accession #R05547). Both strands of this 1500 bp long cDNA clone were sequenced and the complete sequence of this clone was submitted to

Peptide (91)	(1-20)	TGGQVPDTNYIFMGDFVDRG
HPP6	(67-86)	TGGQVPDTNYIFMGDFVDRG
RPPV	(67-86)	TGGQVPDTNYIFMGDFVDRG
HPP4	(69-72)	**V**GG**D**VP**ER**NY**L**FMGDFVDRG
PP2A	(71-90)	**I**GG**K**SPDTNY**L**FMGD**Y**VDRG

Peptide (66)	(1-15)	QITQVYGFYDECQTK
HPP6	(118-132)	QITQVYGFYDECQTK
RPPV	(118-132)	QITQVYGFYDECQTK
HPP4	(102-116)	Q**L**TQVYGFYDECQ**R**K
PP2A	(102-116)	QITQVYGFYDEC**LR**K

Fig. 3. Amino acid alignment of peptide sequences 91 and 66 of 36 kD phosphoprotein and regions of homology in Ser/Thr protein phosphatases. Human PP6 gi:3183123 (HPP6); rat PPV gi: 1071868 (RPPV); human PP4 gi: 417523 (HPP4), and human PP2A gi: 323045. gi, denotes GenBank identification number. Bold residues indicate mismatches.

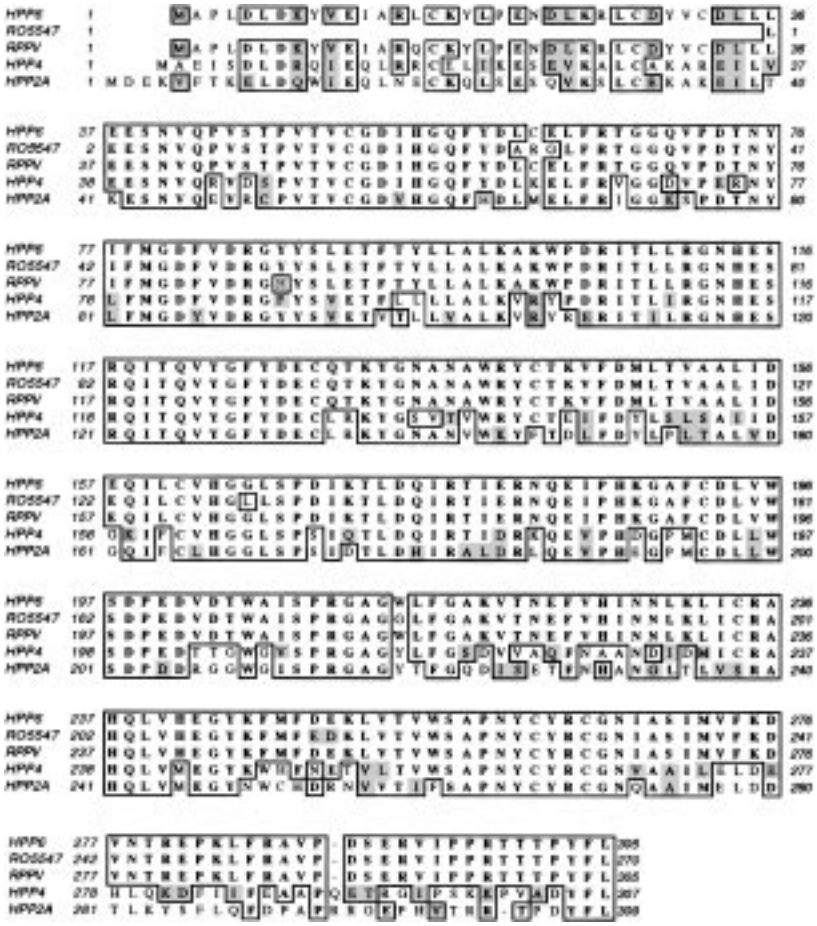

Fig. 4. Comparison of amino acid sequences from the EST clone R05547 and closely related serine/threonine protein phosphatases human PP6 (HPP6), Rat PPV (RPPV), human PP4 (HPP4), and human PP2Ac (HPP2A). The boxes indicate identical amino acids and the borderless gray boxes indicate similar amino acids. Amino acids are abbreviated using single letter-code.

the GenBank (accession #AF035158). This sequence revealed that R05547 clone has an incomplete 5′ end, and a novel 3′-untranslated region containing a polyadenylation signal. As shown in Figure 4, the predicted amino acid sequence of R05547 cDNA showed up to 97% sequence identity to rat PPV and human PP6. In contrast, the homology with the closely related PP4 did not exceed 65%. Conceptional translation of this cDNA clone revealed that 42 amino acid residues are invariant among all the known PPases [Barton et al., 1994]. These 42 residues are essential for the catalytic activity [Goldberg et al., 1995]. In addition, PP6 contains a carboxy-terminal sequence (-YFL) highly conserved among PP2A-like family, which was shown to be subject for tyrosine phosphorylation [Chen et al., 1992] and carboxymethylation [Xie and Clarke et al., 1994] in PP2A. Taken together, this data indicate that RO5547 cDNA is likely to represent a human PP6 clone or a closely related isoform.

Characterization of PP6 Expression in Lymphocytes

To further demonstrate that the 36 kD phosphoprotein immunopurified from J32 cells is PP6, we raised antibodies (U811) by immunizing rabbits with a synthetic peptide (CQITQVYGFYDECQTKY, conjugated to KLH) which was derived from the amino acid sequence of peptide 66 of purified 36-kD phosphoprotein. Selection of this amino acid sequence was based on a hydrophilicity plot and antigenic index analysis. This amino acid sequence maps also to an area of a homology with PP4 and to a lesser extent to the PP2Ac. However, the sequence of the peptide 66 is quite distinct from PP1, and PP2B sequences. This sequence would predict

an alpha helix in the common motif of the central β-α-β-α-β scaffold at the active site based on the three-dimensional structure of PP1. This scaffold of the catalytic domain is expected to be closely preserved in PP2A and PP2A-like protein phosphatases [Goldberg et al., 1995].

The reactivity of affinity-purified U811 antibodies with total J32 cell extract was analyzed by 2D-PAGE and immunoblotting. As shown in Figure 5A, U811 antibodies reacted with two sets of protein spots, one set of protein appears to migrate at 36 kD with a pI ranging from 5.2 to 5.4; and the second set appears to migrate at 35.5 kD with a pI ranging from 5.3 to 5.8. Immunoblotting experiments with U811, antibodies specific to PP2Ac, and antibodies specific to PP6 revealed that the upper set of protein spots resolved by 2D-PAGE and recognized by U811 antibodies (Fig. 5A) exactly correspond to the migration of PP2Ac, while the more acidic and a slightly lower molecular weight protein spots corresponded to PP6 (data not shown). The relative migration of PP2Ac and PP6 shown in Figure 5A is consistent with the respective molecular mass (35.6 and 35.2) and the pI (5.13 and 5.19) calculated for these two proteins. Furthermore, the U811 immunoprecipitate from ^{32}P-labeled J32 cell lysate revealed a major specific 36 kD phosphoprotein band. The migration of this phosphoprotein band on 2D-PAGE was very similar to that obtained with the 3E10 immunoprecipitate (Fig. 5B). Taken together, these findings confirm that the 36 kD phosphoprotein purified with the 3E10 mAb from lymphocyte extract was PP6 or a new isoform of this phosphatase.

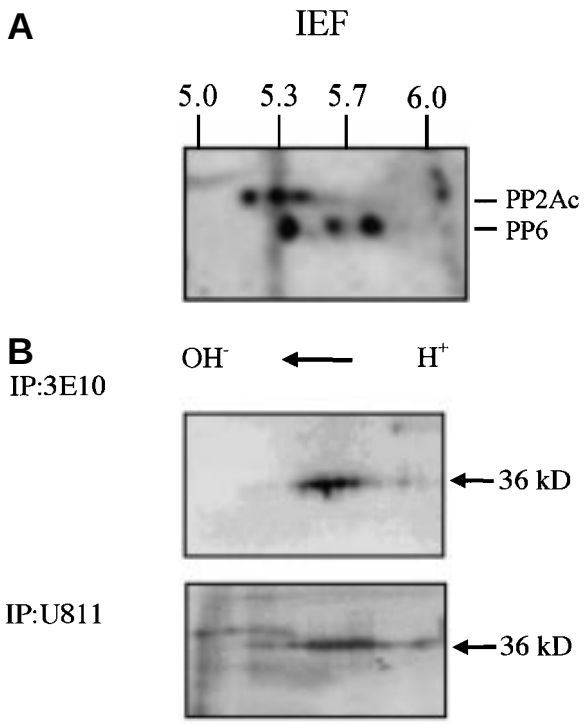

Fig. 5. Identification of PP6 with U811 antibodies. **A**: Immunodetection of PP6 in human lymphocytes by two-dimensional equilibrium pH gel electrophoresis (2D-PAGE-IEF) and immunoblotting with U811 antibodies. Proteins from J32 total cell extract were separated by IEF in the first dimension then subjected to SDS-PAGE in the second dimension. After migration, proteins were transferred to a PVDF membrane which was immunoblotted with U811 antibodies as described in Materials and Methods. The pI values indicated at the top of the figure were measured from the pH gradient of a gel that was run in parallel with loading buffer only. **B**: Immunoprecipitation with U811 antibodies from [^{32}P]-labeled J32 cells. J32 cells were phosphorylated as described in Materials and Methods and cell lysates were immunoprecipitated with 3E10 mAb or with U811 antibodies. Immunoprecipitated proteins were then resolved on two-dimensional non-equilibrium pH gradient gel electrophoresis as in Figure 1C. The letters H^+ and OH^- designate the positive and the negative poles of the IEF gel. The top arrow indicates the direction of protein migration in the first dimension.

Our findings shown in Figure 2 clearly indicated that the phosphorylation and/or expression of the phosphatase in the 3E10 immunoprecipitate is regulated by IL-2 stimulation. This conclusion was further substantiated by immunoblotting with U811 antibodies. Human peripheral blood T cells were stimulated in a time course fashion with PHA alone to induce IL-2 receptor expression, or with PHA and IL-2. U811 antibodies reacted very weakly with the PP6 protein band in resting T cells but, this reactivity increased rapidly after 18 h of stimulation of T cells with PHA and IL-2 but not with PHA alone (data not shown). To our knowledge, this provides the first evidence that the expression of a type 6 protein phosphatase is regulated by IL-2 stimulation. Our finding of a low protein expression of PP6 in resting human T cells is consistent with recent studies of human PP6 mRNA expression by Northern blotting which indicated that the highest amount of PP6 mRNA were in testis, heart and skeletal muscles and no detectable amounts was observed in lymphoid tissue [Bastians et al., 1996].

In addition to change in PP6 protein expression induced by IL-2 stimulation discussed above, data shown in Figure 2B suggested that IL-2 may induce post-translational changes by protein phosphorylation. This observation is of considerable interest because, previous studies indicated that both tyrosine and threonine phos-

phorylation inhibit the catalytic activity of PP2A in vitro. It was suggested that this inhibition can potentially be mediated by the occupation of the active site by a phosphorylated carboxy-terminus (on either tyrosine or threonine) [Guo and Damuni, 1993; Chen et al., 1992]. Furthermore, some data suggest that these or similar modifications may occur in vivo in response to transformation or growth stimulation [Chen et al., 1994b]. Similarly, post-translational changes by phosphorylation of PP6 in response to IL-2 may play an important role in the regulation of its function. Molecular genetic approaches should help elucidate the structure-function relationship of PP6. The exact function of PP6 in human cells will require the purification of the PP6 holoenzyme and further information on its regulatory or inhibitory subunits.

In this study we have used a reverse genetics approach to identify an unknown growth regulated phosphoprotein as PP6. To our knowledge, this represents the first study describing the identification and protein purification of PP6 by 2D-PAGE from human lymphocytes. Our study strongly suggests that PP6 protein phosphorylation and expression is regulated by IL-2 stimulation. Thus, PP6 may play an important role in regulating cell cycle progression in response to IL-2 receptor stimulation.

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REFERENCES

Arino J, Woon CW, Brautigan DL, Miller TB, Johnson GL. 1988. Human liver PP2A. cDNA and amino acid sequence of two catalytic subunit isotypes of PP2A. Proc Nat Acad Sci USA 85:4252–4256.

Arndt KT, Styles CA, Fink GR. 1989. A suppressor of $His4^{+}$ transcriptional defect encodes for a protein with homology to the catalytic subunit of a protein phosphatase 2A. Cell 56:527–537.

Atshull SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tools. J Mol Biol 215:403–410.

Barton Gj, Cohen PTW, Bradford D. 1994. Conservation analysis and structure prediction of the serine/threonine protein phosphatases. Sequence similarity with diadenosine tetraphosphate from Escherichia coli suggests homology to the protein phosphatase. Euro J Biochem 220:225–237.

Bastians H, Ponsting LH. 1996. The novel human protein serine/threonie phosphatase 6 is a functional homologue of budding yeast Sit4 and fission yeats ppe1, which are involved in cell cycle regulation. J Cell Sci 109:2865–2874.

Becker W, Kentrup H, Klump S, Shultz EJ, Jooost HG. 1994. Molecular cloning of a serine/threonine phosphatase containing a putative regulatory tetratricopeptide repeat domain. J Biol Chem 9:22586–22592.

Brewis ND, Street AJ, Prescot AR, Cohen PTW. 1993. PPX, a novel protein serine/threonine phosphatase localized to centrosome. EMBO J 3:987–996.

Chen J, Martin BL, Brautigan DL. 1992. Regulation of Protein serine/threonine phopshatase type-2A by tyrosine phosphorylation. Science 257:1261–1264.

Chen MX, McPartlin AE, Brown L, Chen YH, Barker HM, Cohen PTW. 1994a. A novel human protein serine/threonine phosphatase which possesses a four tetratricopeptide repeat motif and localized to the nucleus. EMBO J 13:4278–4290.

Chen J, Parson S, Brautigan DL. 1994b. Tyrosine phosphorylation of protein phosphatase 2A in response to grwoth stimulation and V-src transformation of fibroblasts. J Biol Chem 269:7957–7962.

Chen MS, Silverstein AM, Pratt WB, Chinkers M. 1996. The tetratricopeptide repeat domain of protein phosphatase 5 mediates bindings to glucocorticoid receptor heterocomplexes and acts as a dominant negative mutant. J Biol Chem 271:32315–32320.

Chinkers M. 1994. Targeting of a distinctive protein-serine phosphatase to the protein kinase-like domain of the atrial natriuretic peptide receptor. Proc Nat Acad Sci USA 91(23):11075–11079.

Clarke PR, Hoffman I, Draetta G, Karsenti E. 1993. Dephosphorylation of cdc25-C by a type-2A protein phosphatase: Specific regulation during the cell cycle in Xenopus egg extracts. Mol Biol Cell 4:397–411.

Cohen P. 1989. The structure and regulations of protein phosphatases. Annu Rev Biochem 58:453–508.

Cohen PTW, Brewis ND, Hughes V, Mann DJ. 1990. Protein serine/threonine phosphatases; An expanding family. FEBS Lett 268:355–359.

Da Cruz e Silva EF, Cohen PTW. 1989. Isolation of a cDNA likely to encodes for a Ca2+dependent/calmodulin-stimulated protein phosphatase. Biochem Biophys Acta 1009:293–296.

Denu MJ, Stuckey JE, Saper AM, Dixon JE. 1996. Form and function of protein dephopshorylation. Cell 87:361–364.

Dombradi V, Axton JM, Glover DM, Cohen PTW. 1989. Molecular cloning and chromosomal localization a novel Drosophila protein phosphatase. FEBS Lett 247:391–395.

Dumont FJ, QingXiang S. 1996. Mechanism of action of immunosuppressant rapamycin. Minirev Life Sci 58:373–395.

Goldberg J, Huang HB, Kwon YG, Greengard P, Nairn AC, Kuryan J. 1995. Three-dimensional structure of the catalytic subunit of the serine/threonine PP1. Nature 367:745–756.

Filali M, Qiu J, Awasthi S, Fischer U, Monos D, Kamoun M. In press. Monoclonal antibody specific to a subclass of polyproline-Arg motif provides evidence for the presence of an snRNA-free spliceosomal Sm protein complex in vivo: Implications for molecular interactions involving proline-rich sequences of Sm B/B' proteins. J Cell Biochem.

Guo H, Damuni Z. 1993. Autophosphorylation activated protein kinase phosphorylates and inactivates protein phosphatase 2A. Proc Nat Acad Sci USA 90:25000–52004.

Huang X, Honkanen RE. 1998. Molecular cloning, expression, and characterization of a novel human serine/threonine PP7. J Biol Chem 273:1462–1468.

Ingebristen TS, Cohen P. 1983. Protein phosphatases: Properties and role in cellular regulation. Science 221:331–337.

Li M, Makkinje A, Damuni Z. 1996. The myeloid leukemia-associated protein SET is a potent inhibitor of protein phophatase 2A. J Biol Chem 271:11059–11062.

Mann DJ, Dombradi V, Cohen TWP. 1993. Drosophila protein phosphatase V functionally complemente SIT4 mutant in sacharomyces cervisiea and its amini-terminal region can confer this complementation to heterologous phosphatase catalytic domain. EMBO J 12:4833–8442.

Matsumoto T, Beach D. 1993. Interaction of pim/spi 1 mitotic checkpoint with other protein phosphatase. Mol Cell Biol 4:337–345.

Miyazaki T, Liu ZJ, Kawahara A, Yasuhiro M, Yamada K, Yoshihide T, Barsoumian EL, Perlmutter RM, Tanagushi T. 1995. Three distinct IL-2 signaling pathways mediated by bcl-2, c-myc, and lck cooperate in hematopoietic cell proliferation. Cell 81:223–231.

Ogris E, Gibson DM, Pallas DC. 1997. Protein phosphatase 2A subunit assembly: The catalytic subunit carboxy terminus is important for binding cellular subunit but not polyomavirus middle tumor antigen. Oncogene 15:911–917.

Reed JC, Tadmori W, Kamoun M, Koretzky G, Nowell PC. 1985. Suppression of interleukin-2 receptor acquisition by monoclonal antibodies recognizing the 50 kd protein associated with the sheep erythrocyte receptor on human T lymphocytes. J Immunol 134:1631–1639.

Shenolikar S. 1994. Protein serine/threonine phosphatases, new avenue for cell regulations. Ann Rev Cell Biol 10:55–86.

Shimanuki M, Kinoshita N, Ohkura H, Yoshita T, Yanagida M. 1993. Isolation and characterization of the fission Yeast protein phsphatase ppe1 involved in the cell shape and the control of mitosis. Mol Biol Cell 4:303–313.

Stone SR, Wernet W, Mayer R, Maurer F, Hofsteenge J, Hemmings BA. 1989. The nucleotide sequence of the cDNA encoding the human protein PP2A catalytic subunit. Nucl Acids Res 16:11365.

Waldmann TA. 1989. The multi-subunit interleukin-2 receptor. Annu Rev Biochem 58:875–911.

Westphal RS, Coffee RL, Moretta A, Pelech SL, Wadzinski BE. In press. Identification of kinase-phosphatase signaling modules comprising p70 S6 kinase-protein phosphatase 2A (PP2A) and P21-activated kinase-PP2A. J Biol Chem.

Xie HY, Clarke S. 1994. Protein phosphatase 2A is reversibly modified by methyl esterification at its c-termianl leucine residue in bovine brain. J Biol Chem 269:1981–1984.

Zmuidzinas A, Mamon HJ, Roberts TM, Smith KA. 1991. Interleukin-2-triggered Raf-1 expression, phosphorylation, and associated kinase activity increase through G_1 and S phase in CD3-stimulated primary human T cells. Mol Cell Biol 11:2794–2799.

Zuo Z, Dean NM, Honkanen RE. 1998. Serine/threonine phosphatase type 5 acts upstream of p53 to regulate the induction of $p21^{WAF/Cip1}$ and mediate growth arrest. J Biol Chem 20:1250–1258.