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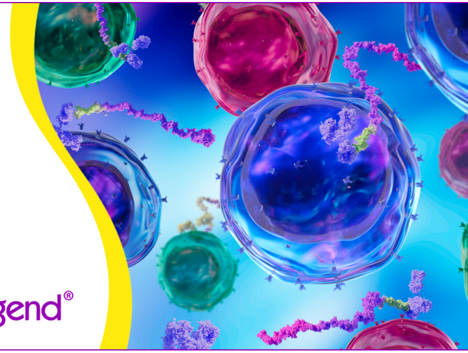

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Negative-Feedback Regulation of CD28 Costimulation by a Novel Mitogen-Activated Protein Kinase Phosphatase, MKP6[1,2]

Francesc Marti,* Anja Krause,[†] Nicholas H. Post,* Clay Lyddane,*[†‡] Bo Dupont,[‡§] Michel Sadelain,[†‡§] and Philip D. King[3]*[§]

TCR and CD28 costimulatory receptor-cooperative induction of T cell IL-2 secretion is dependent upon activation of mitogen-activated protein (MAP) kinases. Using yeast-hybrid technology, we cloned a novel CD28 cytoplasmic tail (CD28 CYT) interacting protein, MAP kinase phosphatase-6 (MKP6), which we demonstrate inactivates MAP kinases. Several lines of evidence indicate that MKP6 plays an important functional role in CD28 costimulatory signaling. First, in human peripheral blood T cells (PBT), expression of MKP6 is strongly up-regulated by CD28 costimulation. Second, transfer of dominant-negative MKP6 to PBT with the use of retroviruses primes PBT for the secretion of substantially larger quantities of IL-2, specifically in response to CD28 costimulation. A similar enhancement of IL-2 secretion is observed neither in response to TCR plus CD2 costimulatory receptor engagement nor in response to other mitogenic stimuli such as phorbol ester and ionomycin. Furthermore, this hypersensitivity to CD28 costimulation is associated with CD28-mediated hyperactivation of MAP kinases. Third, a retroviral transduced chimeric receptor with a CD28 CYT that is specifically unable to bind MKP6 costimulates considerably larger quantities of IL-2 from PBT than a similar transduced chimeric receptor that contains a wild-type CD28 CYT. Taken together, these results suggest that MKP6 functions as a novel negative-feedback regulator of CD28 costimulatory signaling that controls the activation of MAP kinases. ***The Journal of Immunology,*** **2001, 166: 197–206.**

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It is well established that to proliferate in response to Ag T cells must receive two signals (1). One of these signals is provided by TCR recognition of antigenic peptide/MHC complexes displayed on the surface of APC. The other signal is provided by T cell costimulatory receptor recognition of appropriate costimulatory ligands. One prominent T cell costimulatory receptor that has been studied extensively is the CD28 molecule (2). CD28 is found on the majority of mature T cells and recognizes B7-1 and B7-2 ligands that are either induced or are constitutively expressed on APC. In addition to delivering costimulatory signals, CD28 interaction with B7 provides T cells with important other signals that prevent T cells from entering into an anergic hyporesponsive state or from undergoing premature apoptotic cell death (3, 4).

As a costimulatory receptor, CD28 functions by inducing the secretion of multiple T cell cytokines, particularly IL-2, which following binding to newly synthesized IL-2 receptors, drives T cells through the cell cycle (5). Apparently, CD28 costimulation induces both de novo IL-2 gene transcription and stabilization of newly synthesized IL-2 mRNA (6, 7). Essential to TCR/CD28 induction of IL-2 is activation of the mitogen-activated protein (MAP)[4] kinases, extracellular signal-regulated kinase (ERK), and c-jun NH_2-terminal kinase (JNK) (8, 9). These serine/threonine kinases phosphorylate the c-fos and c-jun proteins, respectively, which assemble to form the AP-1 transcription factor (10). Together with NFAT and NF-κB transcription factors, AP-1 binds to and turns on the IL-2 promoter (11, 12). Additionally, JNK has been shown to play an important role in IL-2 mRNA stabilization (13). Both ERK and JNK are activated synergistically by the TCR and CD28 and thus represent early upstream points of signal integration in T cell costimulation (14, 15). Another type of MAP kinase that is activated synergistically by the TCR and CD28 is the p38 MAP kinase (16). However, in contrast to ERK and JNK, p38 activation is not necessary for the induction of IL-2 (17).

To understand further the mechanism of CD28 costimulation we used yeast-hybrid technology in an attempt to identify novel proteins that interact physically with the CD28 cytoplasmic tail (CD28 CYT). Previous studies have shown that the lipid/serine kinase, phosphatidylinositol 3-kinase (PI3-kinase), and the adaptor protein, Grb-2, bind CD28 CYT following phosphorylation of CD28 CYT on tyrosine residue 173, present in a YMNM motif (18). PI3-kinase and Grb-2 could potentially couple CD28 to MAP kinase and other intracellular signaling pathways (19, 20). However, the role of these signaling intermediates in CD28 costimulation is controversial, and other data indicate that distinct CD28 CYT interacting proteins may be involved in the relay of CD28 signals (21–23). Surprisingly, during our search for these proteins, one novel CD28 interactor that we identified was a previously undescribed member of the family of MAP kinase phosphatases

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[2] The sequence reported in this article is available in the Genbank database under accession number AF120032.

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[4] Abbreviations used in this paper: MAP, mitogen-activated protein; CYT, cytoplasmic tail; DAR-HRP, donkey anti-rabbit Ig-HRP; ERK, extracellular-signal regulated kinase; GAM, goat anti-mouse Ig; JNK, c-jun NH_2-terminal kinase; LNGFR, low affinity nerve growth factor receptor; MKP, MAP kinase phosphatase; PBT, peripheral blood T cell(s); PI3-kinase, phosphatidylinositol 3-kinase; *p*-NPP, *p*-nitrophenyl phosphate; URA, uracil.

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(MKPs). MKPs are thought to down-modulate cellular responses by dephosphorylating and inactivating MAP kinases. Rather than functioning as a positive signaling element, we provide evidence that this phosphatase acts as a physiologic attenuator of CD28 costimulation.

Materials and Methods

Yeast interaction traps

The yeast two-bait and modified two-bait interaction traps have been described (24, 25). Screening of cDNA libraries (contained in pJG4-5) was as before using TetR-fused human CD28 CYT as $bait_1$ (contained in pCWX200) and LCK as $bait_2$ (25). From the screen of a Hela cell library, clone 14 was characterized as promoting uracil (URA)-independent yeast growth regardless of LCK expression. Sequencing of the 1.1-kb cDNA insert revealed an open reading frame coding for a novel protein, MKP6, that by comparison with available sequences in the database of expressed sequence tags (Unigene Cluster Hs. 91448; Ref. 26) was missing the first 18 amino terminal amino acids. DNA covering the full-length coding region of human MKP6 was cloned by PCR from Jurkat cell cDNA using a forward primer based immediately upstream of the sequence shown in Fig. 1*A* and a reverse primer based immediately downstream of the MKP6 stop codon. Following sequence verification, the exact full-length MKP6 coding region was then subcloned into the *Eco*RI/*Xho*I sites of pJG4-5. Further analyses of the CD28-MKP6 interaction in yeast used the full-length form of MKP6 throughout. An additional prey protein used in these studies was the human VHR phosphatase (27). Additional bait proteins included the CYT of human TCRζ (28), p58 KIR clone 6 (25), CD2 (29), and Δ200, Δ199, and Y200F mutants of CD28 CYT (30). DNA encoding these proteins was generated by PCR and inserted into the *Eco*RI/*Xho*I sites of pJG4-5 or the *Eco*RI/*Bam*HI or *Eco*RI/*Xho*I sites of pCWX200, respectively. Assessment of bait-prey protein interaction by growth of transformed yeast on URA dropout plates was as before, in the absence of LCK.

GST fusion proteins

For expression of amino-terminal GST-tagged MKP6 in Jurkat cells, MKP6 DNA was subcloned into the *Bam*HI/*Cla*I sites of the eukaryotic GST expression vector, pEBG (a gift from Dr. B. J. Mayer, Children's Hospital, Boston, MA). For expression of bacterial GST fusion proteins, MKP6, JNK2, CD28 CYT, CD28Δ199 CYT, and p58 KIR clone 6 CYT DNAs were inserted into the *Bam*HI/*Eco*RI sites of the pGEX3X expression vector (Pharmacia, Piscataway, NJ). A pGEX3X-MKP6 (C111S) construct was produced by site-directed mutagenesis of pGEX3X-MKP6 using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). GST-ERK1 and GST-p38α bacterial expression constructs were gifts from Dr. Z. Luo (Boston Unversity) and Dr. S. Arkinstall (Serono Pharmaceuticals, Geneva, Switzerland) respectively. Bacterial GST fusion proteins were produced in protease-negative BL21(DE3)pLysS(Ion,ompT$^-$) bacteria (Novagen, Madison, WI) and purified from bacterial lysates with the use of glutathione-agarose (Sigma, St. Louis, MO). Purified GST-ERK2 and GST-p38–2 fusion proteins were obtained independently from Dr. M. Cobb (University of Texas Southwestern Medical Center, Dallas, TX).

MKP6 expression

Mutiple tissue Northern blots (Clontech Laboratories, Palo Alto, CA) were probed with ^{32}P-labeled MKP6 cDNA corresponding to the full-length MKP6 coding region. Blots were washed at high stringency before autoradiography.

PBMC were prepared by Ficoll separation of buffy coats from healthy volunteers and depleted of monocytes and B cells by negative selection using CD14 and CD19 Abs. Resultant T cells (>90% purity) were resuspended in complete medium (RPMI 1640 supplemented with 10% FCS and antibiotics) at 5×10^6 cells/ml and stimulated or not with OKT3 (CD3; Ortho Pharmaceuticals, Raritan, NJ) and CD28.2 (CD28; PharMingen, San Diego, CA) mAb (each at 0.3 μg/ml) plus goat anti-mouse Ig (GAM, 1.5 μg/ml; Sigma) for varying times as indicated. Total RNA was extracted from T cells with the use of TRIzol reagent (Life Technologies, Grand Island, NY) and subject to standard Northern analysis as above using the same MKP6 probe and a ^{32}P-labeled control human β actin probe.

To examine MKP6 protein expression, T cells (2×10^6 cells per condition) were lysed in Nonidet P-40 lysis buffer. Lysates were boiled in reducing SDS sample buffer, run on 10% SDS-PAGE gels, and transferred to polyvinylidene difluoride membranes (DuPont-NEN, Boston, MA). MKP6 protein was detected by enhanced chemiluminescence following the probing of membranes with a polyclonal MKP6 antiserum (1:1000 dilution) and donkey anti-rabbit Ig-HRP (DAR-HRP, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA). The MKP6 antiserum was raised by immunization of rabbits with an MKP6 peptide (^{72}EYVKVPLADMPHAPIGL YFDTVADK^{96}) coupled to keyhole limpet hemocyanin. The peptide sequence is unique among MKPs.

CD28-MKP6 association in vitro

MKP6 DNA was subcloned into the *Bam*HI/*Xba*I sites of pEF-FLAG (31; a gift of Dr. G. Koretzky, University of Iowa, Iowa City, IA) to produce a construct encoding for amino-terminal FLAG-tagged MKP6. COS-7 cells were transfected with pEF-FLAG-MKP6 (20 μg/5×10^6 cells) by electroporation (320 V/960 μF/0.4 cm gap cuvettes) and cultured in complete medium for 36 h. Cells were lysed in Nonidet P-40 lysis buffer, and 1 ml of lysate containing 0.5 mg of protein was incubated with 10 μl of packed glutathione-agarose beads coated with 4 μg of GST-CD28, CD28Δ199, or p58 KIR clone 6 CYT fusion proteins for 30 min. Beads were washed three times in Nonidet P-40 lysis buffer, and GST fusion proteins were eluted in 10 mM glutathione. Coeluted FLAG-MKP6 was detected by Western blotting as above using anti-FLAG M2 mAb (10 μg/ml; Sigma) and GAM-HRP (1:4000; Sigma).

CD28-MKP6 association in Jurkat cells

Jurkat TagC15 cells (32; a gift from Dr. J. Northrop, Affymax Research Institute, Palo Alto, CA) were transfected with pEBG-MKP6 or control pEBG by electroporation (20 μg/20×10^6 cells/condition as above) and cultured in complete medium for 36 h. Cells were washed and lysed in Nonidet P-40 lysis buffer, and lysates were precleared by rotation with Sephadex G-50 beads (Sigma) for 1 h. Precleared lysates (300 μl of 1 mg protein/ml/sample) were then incubated with 3 μg of 9.3 mAb (CD28, a gift from Dr. J. Ledbetter, Bristol-Myers Squibb, Seattle, WA) or control Leu 3a mAb (CD4; American Type Culture Collection, Manassas, VA) for 15 min, and immune complexes were rescued from lysates by rotation with 10 μl of packed protein A/G-coated agarose beads (Santa Cruz Biotechnology) for 1 h. Beads were washed five times in Nonidet P-40 lysis buffer and coimmunoprecipitated GST-MKP6 or GST alone was detected by Western blotting using a rabbit polyclonal anti-GST Ab (2 μg/ml; Santa Cruz Biotechnology) followed by protein A-HRP (1:10000; Zymed, San Francisco, CA).

In vitro phosphatase assays

Assays involving hydrolysis of *p*-nitrophenyl phosphate (*p*-NPP) were performed in 96-well plates. GST fusion proteins were incubated in 200-μl reaction volumes containing 20 mM *p*-NPP/50 mM imidazole (pH 7.5)/5 mM DTT for different times at 37°C. Hydrolysis of *p*-NPP was measured by absorbance at 405 nm.

For in vitro MKP assays, GST-MAP kinases were incubated with GST-MKP6 or MKP6 (C111S) or control GST alone in a 20-μl reaction volume containing 50 mM imidazole (pH 7.5)/5 mM DTT. After 1 h at 37°C, reactions were stopped and activated T-X-Y dual-phosphorylated GST-MAP kinases were detected by Western blotting using specific anti-phospho-ERK or phospho-JNK mAb or an anti-phospho-p38 rabbit polyclonal Ab (all 1:1000; New England Biolabs, Beverly, MA) plus GAM-HRP or protein A-HRP, respectively. To check that equivalent quantities of GST-MAP kinases were analyzed, blots were stripped and reprobed with anti-ERK or -JNK rabbit polyclonal Abs (2 μg/ml; Santa Cruz Biotechnology) or an anti-p38α rabbit polyclonal Ab (1:1000; New England Biolabs) followed by protein A-HRP.

Retroviral mediated gene transfer

DNA encoding FLAG-MKP6, FLAG-MKP6 (C111S), and human CD8-CD28 CYT or CD8-CD28Δ199 CYT chimeric proteins was subcloned into the *Nco*I/*Xho*I sites of pSFG (33). CD8-CD28 fusion constructs have been described previously (23). An internal ribosomal entry site of the encephalomyocarditis virus together with DNA encoding for neomycin phosphotransferase (G418^R) was inserted 3′ of FLAG-MKP6 and FLAG-MKP6 (C111S) and also 3′ of a control-inactive mutant human low affinity nerve growth factor receptor (LNGFR) gene, similarly cloned into pSFG (34, 35). Resulting constructs contain di-cistronic genes comprised of FLAG-MKP6 or FLAG-MKP6 (C111S) or LNGFR upstream of G418^R.

Recombinant virions were pseudotyped with the gibbon-ape leukemia virus envelope using the PG13 packaging cell line (36). Retroviral gene transfer to PHA-activated human peripheral blood T cells (PBT) was performed by overnight infection in the presence of polybrene as previously described (37), except that in experiments involving transfer of CD8 chimeric receptors, PHA blasts were predepleted of CD8$^+$ T cells by negative selection using the OKT8 mAb (CD8; American Type Culture Collection). For MKP6 gene transfer experiments, following infection, T cells were maintained in low dose IL-2 (10–20 U/ml; Chiron Therapeutics, Emeryville, CA) and selected in G418 (250 μg/ml; Sigma) for 5–7 days.

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Efficiency of CD8 chimera and LNGFR gene transfer was determined by flow cytometry using CD4-FITC- and CD8-PE-coupled mAb (Becton Dickinson, Mountain View, CA) or an LNGFR mAb as appropriate. For LNGFR, this was found to be in the range of 40–60% postselection. To confirm FLAG-MKP6 and FLAG-MKP6 (C111S) gene transfer, 1×10^7 transduced and selected T cells were lysed in 300 μl of Nonidet P-40 lysis buffer, and lysates were rotated with 2 μg of anti-FLAG-M2 mAb plus 10 μl of packed protein A/G-coated agarose beads. Beads were washed five times in Nonidet P-40 lysis buffer and precipitated FLAG-tagged proteins were detected by Western blotting.

IL-2 induction

Washed transduced T cells were stimulated in 96-well flat-bottom plates (1×10^5 cells/well in a 200 μl total volume) with combinations of OKT3 or OKT8 mAb (both plate-bound by preincubation of plates with 1 μg/ml Ab), soluble CD28.2 mAb (1 μg/ml), soluble 9.6 mAb (CD2, 1 μg/ml; a gift from Dr. J. Ledbetter, Bristol-Myers Squibb) and PMA (varying concentrations as indicated) plus ionomycin (1 μM) as described (38). After 24 h, supernatants were harvested and assayed for IL-2 content with the use of IL-2 ELISA kits (R&D Systems, Minneapolis, MN).

MAP kinase activation in vivo

LNGFR- and FLAG-MKP6 (C111S)-transduced T cells were washed, and 4×10^6 T cells per sample were stimulated or not with 0.2 μg each of OKT3 and CD28.2 plus 2 μg of GAM in a total volume of 100 μl of RPMI 1640 at 37°C for the indicated times. Cells were lysed, and activation of MAP kinases was determined by Western blotting of whole cell lysates as indicated in *In vitro phosphatase assays*, except that phospho-JNK was detected using an anti-phospho-JNK rabbit polyclonal Ab (Promega, Madison, WI), and DAR-HRP was used in place of protein A-HRP. Equivalent loading of MAP kinases was demonstrated with the use of the same anti-MAP kinase Abs and DAR-HRP.

Results

Identification of MKP6 as a novel CD28 CYT interacting protein in yeast

We used the yeast two-bait and modified two-bait interaction trap systems to identify novel interactors of the CD28 CYT (24, 25). From library screens we isolated both PI3-kinase and Grb-2. In addition, we identified one clone whose protein product bound CD28 CYT in yeast independent of CD28 CYT tyrosine phosphorylation. The 198 aa sequence of clone 14 is shown in Fig. 1*A*. Clone 14 has not been reported previously although it can be found in both the human and murine divisions of the database of expressed sequence tags, where it has been derived from a variety of tissue sources and cell types including T cells (26). A similarity search performed with the clone 14 sequence revealed significant homology to the family of MKPs (22–32% similarity) of which there are at least ten members (39). MKPs are dual specific phosphatases that dephosphorylate MAP kinases upon threonine and tyrosine residues present in T-X-Y sequences in the phosphorylation lip at the mouth of the MAP kinase catalytic site. As such, MKPs reverse MAP kinase phosphorylation mediated by upstream activating MEKs and inactivate MAP kinases. Homology of clone 14 to this family extends throughout most of the protein with the exception of the amino-terminal 20 and carboxyl-terminal 30 aa. Of note is a centrally located VHCXXGXSRSXTXXXAYLM catalytic core sequence that is conserved among MKPs. Therefore, taken together with enzymatic data (see below), we designate clone 14 as a novel member of the MKP family, MKP6.

The CD28 CYT-MKP6 interaction in yeast is specific and requires CD28 CYT residue tyrosine 200

The specificity of interaction between CD28 CYT and MKP6 in yeast cells is illustrated in Fig. 1*B*. MKP6 bound to the CD28 CYT but not to the CYT of a variety of other immunoreceptors including, as shown, TCRζ, p58 KIR clone 6, and CD2. Conversely, CD28 CYT failed to bind other MKPs apart from MKP6. Shown, for example, is the VHR MKP (27), which did not bind CD28 CYT.

To determine which residues of CD28 CYT are necessary for MKP6 binding we tested the effect of deleting increasing numbers of residues from the carboxyl-terminal end of the tail. Essential

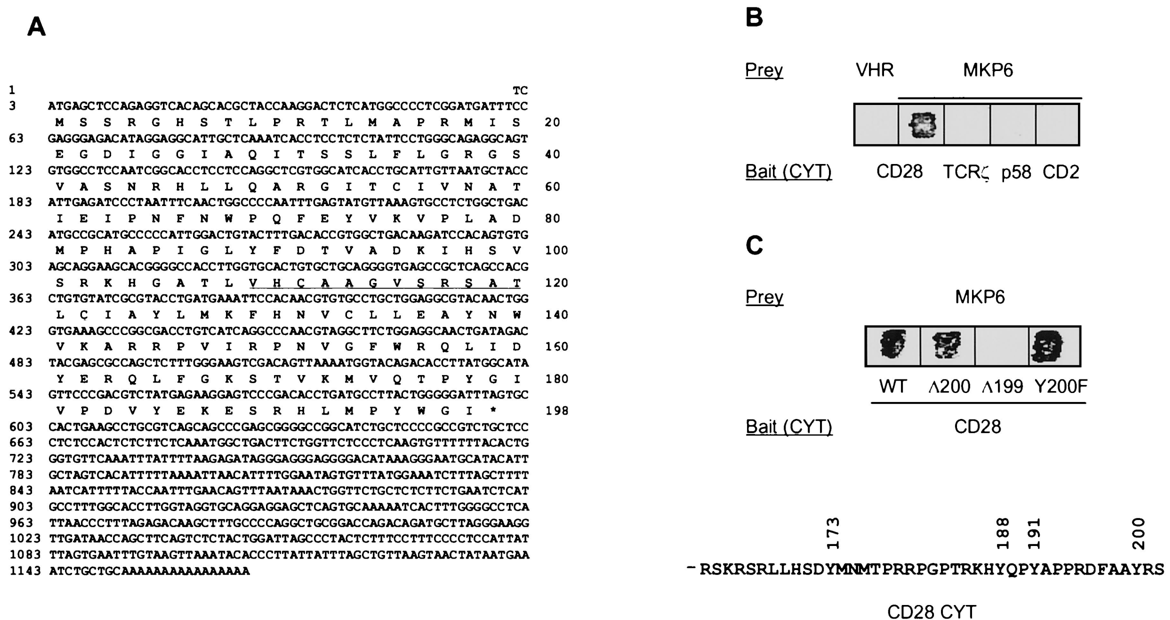

FIGURE 1. CD28 CYT interaction with MKP6 in yeast. *A*, Human MKP6 cDNA and translated protein sequence. A catalytic core sequence, conserved in MKPs, is underlined. *B*, Specificity of CD28 CYT-MKP6 binding. CWXY2 yeast were transformed with the indicated prey proteins and CYT bait proteins. Growth of yeast on URA dropout plates was assessed after 3-day culture. *C*, Mutational analysis of CD28 CYT-MKP6 binding. CWXY2 yeast were transformed with MKP6 and the indicated CD28 CYT baits. The human CD28 CYT sequence is shown below with numbered tyrosine residues. WT, wild type; Δ200 and Δ199, missing carboxyl-terminal residues RS and YRS, respectively; Y200F, mutation of tyrosine 200 to phenylalanine. Yeast growth was assessed as in (*B*).

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results are depicted in Fig. 1*C*, which shows that a CD28Δ200 CYT, missing carboxyl-terminal arginine 201 and serine 202, was able to interact with MKP6, in contrast to a CD28Δ199 CYT, additionally deleted of tyrosine 200, which was unable to mediate this interaction. These results show that arginine 201 and serine 202 are dispensable, whereas tyrosine 200 is required for CD28 CYT-MKP6 binding. However, it should be noted that tyrosine 200 can be mutated to phenylalanine (CD28 Y200F CYT) without loss of binding activity (Fig. 1*C*).

Expression of MKP6 in PBT is induced by CD28 costimulation

Combined Northern blot (Fig. 2*A*) and RNA dot blot (data not shown) analysis of different fetal and adult human tissues and a panel of hemopoietic and nonhemopoietic cell lines confirmed that MKP6 was expressed ubiquitously. Tissues with relatively strong MKP6 expression included adult trachea, placenta, liver, heart, and thyroid, and fetal thymus and lung. Somewhat lower levels of MKP6 were detected in other adult and fetal tissues. However, we wondered whether MKP6 was induced in T cells by CD28 costimulation. To address this we examined MKP6 mRNA expression in purified populations of PBT that had been stimulated with CD3 mAb (directed to the TCR complex) plus CD28 mAb for varying periods in vitro. As shown in Fig. 2*B*, the combination of CD3 and CD28 mAb induced strong expression of MKP6 mRNA in PBT. Induction was apparent by 2.5 h and persisted up to 24 h poststimulation. Little induction of MKP6 mRNA was noted when T cells were stimulated with CD3 or CD28 mAb alone (Fig. 2*B*). In parallel with the induction of MKP6 mRNA, CD3/CD28 stimulation of PBT also induced expression of MKP6 protein as detected by Western blotting using an MKP6-specific Ab (Fig. 2*C*). One other MKP, which has been shown to be induced in T cells by mitogens, is the PAC-1 phosphatase (40). However, PAC-1 and MKP6 differ in that PAC-1 is relatively restricted to the nucleus, whereas MKP6 is expressed in both nuclear and cytosolic compartments (as detected using green fluorescent protein-MKP6 fusion protein constructs; data not shown).

CD28 and MKP6 interact in vitro and in Jurkat T cells

We tested whether CD28 CYT and MKP6 interact in vitro and in T cells. For in vitro studies, bacterially expressed GST-CD28 CYT or control GST-p58 KIR clone 6 CYT or CD28Δ199 CYT fusion proteins were immobilized on glutathione-agarose beads and

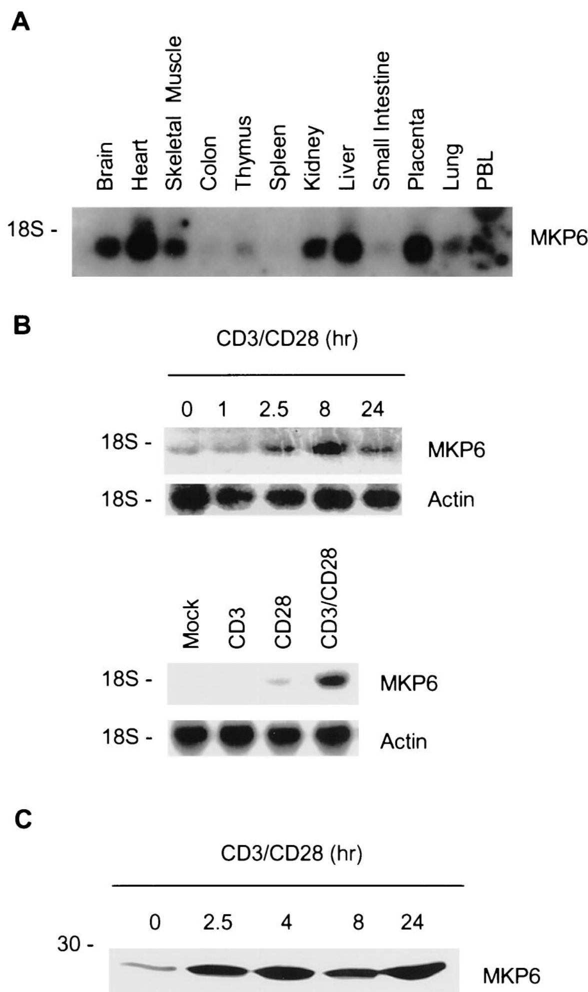

FIGURE 2. MKP6 expression. *A*, MKP6 mRNA expression in different tissues. Multiple tissue Northern blots (each lane contains 2 μg poly(A)+ RNA from the indicated adult human tissues) were probed with ^{32}P-labeled human MKP6 cDNA. The shown single hybridizing MKP6 mRNA species is ~1.8 kb. *B*, TCR/CD28 induction of MKP6 mRNA. Human PBT were stimulated or not with CD3 and/or CD28 mAb for different times (*top*) or for 2.5 h (*bottom*) as indicated. Ten micrograms of total cellular RNA was analyzed by Northern blotting as in (*A*). Blots were stripped and reprobed with ^{32}P-labeled actin cDNA to demonstrate equivalent loading of RNA. *C*, TCR/CD28 induction of MKP6 protein expression. Human T cells were stimulated as in (*B*). MKP6 protein expression was determined by Western blotting of whole cell lysates with an MKP6-specific Ab. The molecular mass of MKP6 is estimated at 25 kDa.

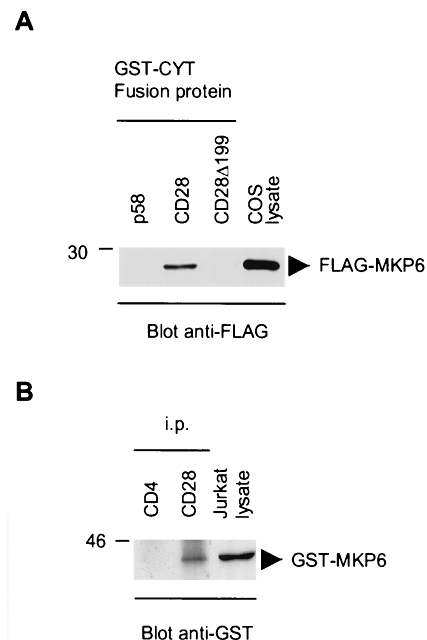

FIGURE 3. CD28-MKP6 interaction in vitro and in Jurkat T cells. *A*, CD28 CYT-MKP6 binding in vitro. Agarose beads coated with the indicated GST-CYT fusion proteins were incubated with a lysate of COS-7 cells transiently transfected with FLAG-MKP6. Beads were washed, GST fusion proteins were eluted from beads, and bound FLAG-MKP6 was detected by Western blotting using an anti-FLAG mAb. Equivalent elution of GST fusion proteins was shown by Coomassie staining of separate SDS-PAGE gels (data not shown). Molecular mass of FLAG-MKP6 is ~26 kDa. *B*, CD28-MKP6 binding in Jurkat cells. CD28 or control CD4 were immunoprecipitated from Jurkat TagC15 cells transiently transfected with GST-MKP6. Coimmunoprecipitated GST-MKP6 was detected by Western blotting using an anti-GST Ab. Molecular mass of GST-MKP6 is ~43 kDa. GST was not detected in CD28 immunoprecipitates from Jurkat TagC15 cells transfected with a control vector encoding GST alone (data not shown).

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bathed in lysates of COS-7 cells that had been transiently transfected with FLAG-tagged MKP6. Following bead washing, GST fusion proteins were eluted, and any bound MKP6 was detected by Western blotting using an anti-FLAG mAb. Fig. 3*A* shows that FLAG-MKP6 bound to GST-CD28 CYT but not to GST-p58 KIR clone 6 CYT or CD28Δ199 CYT. Therefore, CD28 CYT and MKP6 interact in vitro. Furthermore, as in yeast cells, this interaction is prevented by deletion of tyrosine 200 from the CD28 CYT.

To examine interaction in T cells, we transiently transfected Jurkat T leukemia cells with GST-tagged MKP6. Exponentially growing cells were then lysed, and either CD28 or control CD4 were immunoprecipitated from lysates using mAb. Coimmunoprecipitated MKP6 was detected by Western blotting using an anti-GST Ab. As shown in Fig. 3*B*, GST-MKP6 was detected in CD28 immunoprecipitates but not in CD4 immunoprecipitates. Similarly, GST-MKP6 was detected in CD28 immunoprecipitates from "rested" serum-starved Jurkat cells, and the amount of GST-MKP6 that was coimmunoprecipitated from these cells was not increased by prior mAb-mediated cross-linking of CD28 (data not shown). Therefore, in transfected Jurkat cells, CD28 and MKP6 interact specifically and constitutively.

We additionally examined whether endogenous MKP6 could be detected in CD28 mAb immunoprecipitates from PBT. However, we were unable to detect MKP6 by Western blotting using our MKP6-specific antiserum (data not shown). This could indicate that the physical interaction between CD28 and MKP6 is of low stoichiometry and/or that our MKP6-specific antiserum is of low affinity. A low stoichiometric interaction between CD28 and MKP6 is consistent with the observation that in in vitro binding experiments (Fig. 3*A*), we estimate that GST-CD28 CYT bound only one four-hundredth the total amount of FLAG-MKP6 that was available.

MKP6 behaves as a general MKP in vitro

To show that MKP6 could function as a MKP we performed two different types of in vitro assay. In the first assay we examined the ability of purified GST-MAP kinases to activate MKP6 phosphatase activity against the artificial substrate *p*-NPP. As shown in Fig. 4*A*, all tested GST-MAP kinases, including GST-ERK1, ERK2, JNK2, p38α, and p38–2, were able to induce the phosphatase activity of GST-MKP6. In contrast, control GST alone did not induce phosphatase activity. Previous studies have shown that the phosphatase activity of MKPs can be activated by MAP kinases in a substrate-specific manner (41). Therefore, these results imply that MKP6 can act as a general MKP in vitro.

In the second assay we examined directly the ability of GST-MKP6 to dephosphorylate different -phosphorylated GST-MAP

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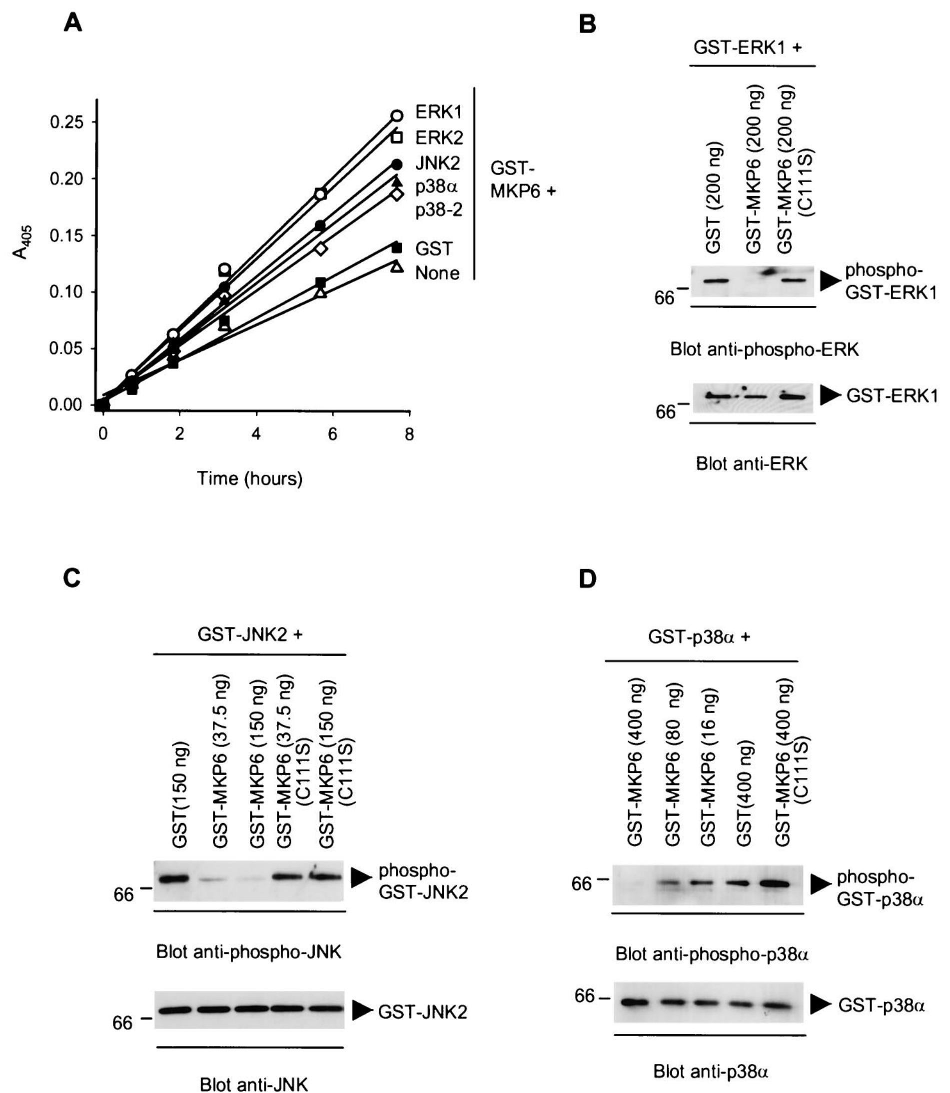

FIGURE 4. Characterization of MKP6 phosphatase activity in vitro. *A*, MAP kinase induction of MKP6 phosphatase activity. GST-MKP6 (0.7 μg) was incubated with 20 mM *p*-NPP in the presence or absence of GST-MAP kinases or GST alone (all 7 μg) for the indicated times at 37°C. Hydrolysis of *p*-NPP is presented as mean absorbance at 405 nm of triplicate determinations. SEs were <5% of the mean. *B–D*, MKP6 dephosphorylation of MAP kinases. GST-MAP kinases (100 ng) were incubated with GST-MKP6 or MKP6 (C111S) or GST alone for 1 h at 37°C. Dual phosphorylation of MAP kinases was assessed by Western blotting using anti-phospho-MAP kinase Abs that react specifically with respective dual phosphorylated T-X-Y motifs. To ascertain equal loading of GST-MAP kinases, blots were stripped and reprobed with appropriate anti-MAP kinase Abs.

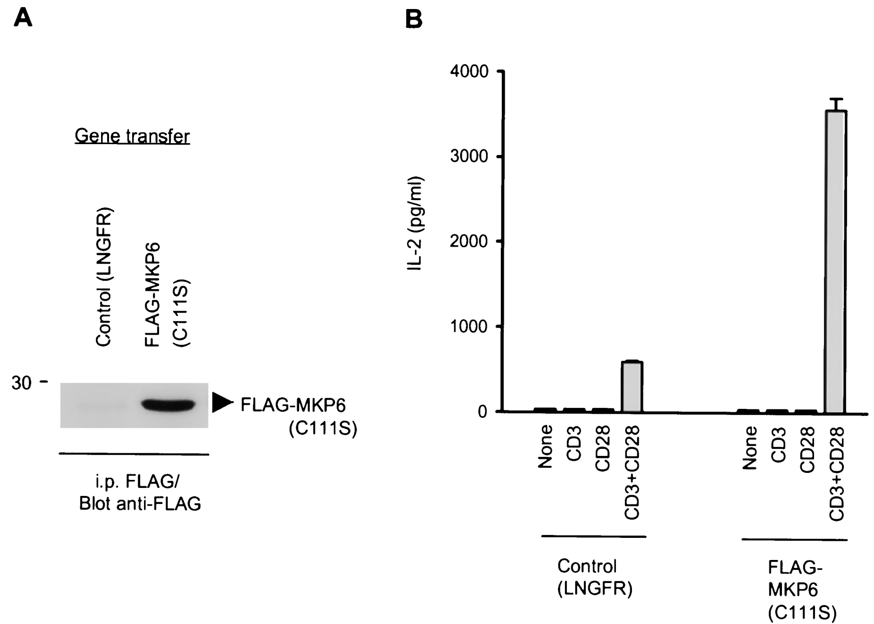

FIGURE 5. Influence of FLAG-MKP6 (C111S) upon CD28-induced IL-2 secretion from primary human PBT. *A,* Transfer of FLAG-MKP6 (C111S) to human PBT. PHA-activated PBT were infected with retroviruses encoding FLAG-MKP6 (C111S) or control LNGFR-G418^R di-cistronic genes. Following G418 selection, FLAG protein was immunoprecipitated from cell lysates and detected by Western blotting using an anti-FLAG mAb. *B*, TCR/CD28-induced IL-2 secretion in FLAG-MKP6 (C111S) transduced PBT. FLAG-MKP6 (C111S)- and control-transduced PBT were stimulated with CD3 and CD28 mAb, alone or in combination, for 24 h in 96-well plates. IL-2 concentrations in supernatants were determined by ELISA. Stimulations were performed in duplicate, and results are expressed as mean supernatant IL-2 concentration + 1 SEM.

kinases (Fig. 4, *B–D*). GST-MAP kinases were purified from bacteria in an already dual-phosphorylated state, obviating a requirement for incubation with MEKs in these experiments. Dual phosphorylation was monitored by immunoblotting using specific anti-phospho-MAP kinase Abs. As shown, GST-MKP6 was able to dephosphorylate each of GST-ERK1, JNK2, and p38α, thus confirming that MKP6 behaves as general MKP in vitro. As expected, conversion of the predicted nucleophilic cysteine residue to a serine residue (C111S) in the active site of MKP6 abrogated an ability to dephosphorylate these MAP kinases.

Retroviral transfer of dominant-negative MKP6 (C111S) to primary human PBT reveals the function of MKP6 as a negative regulator of CD28 costimulatory signaling

Because MAP kinase activation is necessary for TCR/CD28-induced T cell cytokine secretion we considered the possibility that MKP6 may function as a negative regulator of CD28 costimulatory signaling. To test this, we transferred phosphatase-inactive FLAG-MKP6 (C111S) (see above) to primary human PBT with the use of retroviruses. FLAG-MKP6 (C111S) might behave as a dominant-negative mutant by competitively inhibiting endogenous MKP6, thereby resulting in augmented CD28 costimulatory signaling.

Retroviruses were constructed with di-cistronic genes encoding FLAG-MKP6 (C111S) or a control null mutant LNGFR upstream of a G418 resistance gene. Following infection, T cells were then selected in G418 to maximize the percentage of cells expressing transgenes. To monitor transfer of FLAG-tagged proteins, G418-selected T cells were lysed, and lysates were analyzed by Western blotting using an anti-FLAG mAb (Fig. 5*A*). As shown, FLAG-MKP6 (C111S) was readily detected in infected T cells. No signals were detected in T cells infected with the LNGFR-encoding retrovirus.

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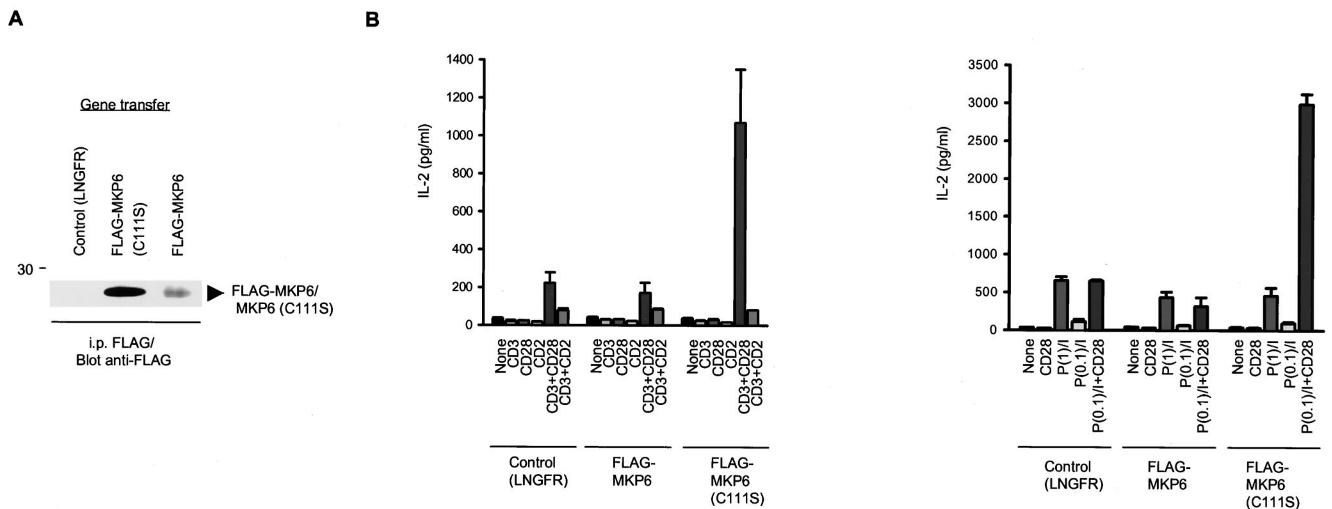

FIGURE 6. Dominant-negative FLAG MKP6 (C111S) enhances CD28-mediated IL-2 secretion specifically. *A*, Transfer of FLAG-MKP6 (C111S) and FLAG-MKP6 to PBT. Gene transfer of FLAG-MKP6 (C111S), FLAG-MKP6, and control LNGFR and subsequent Western blotting analysis of transferred proteins were conducted as in Fig. 5*A*. *B*, IL-2 secretion of transduced T cells in response to different stimuli. The indicated transduced PBT were stimulated with combinations of CD3, CD28, and CD2 mAb (*left*) or with combinations of PMA (varying concentrations shown in ng/ml) plus ionomycin (P/I) and CD28 mAb (*right*) for 24 h in 96-well plates. IL-2 secretion was assessed as in Fig. 5*B*.

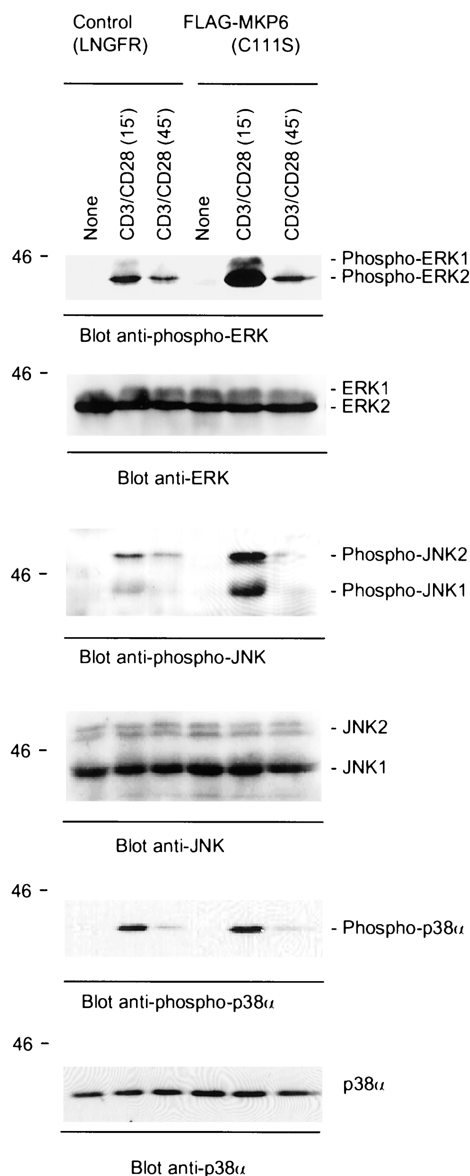

FIGURE 7. Effect of FLAG-MKP6 (C111S) upon TCR/CD28-mediated MAP kinase activation in PBT. LNGFR- and FLAG-MKP6 (C111S)-transduced human PBT were stimulated or not with CD3 and CD28 mAb for different times as indicated. Activation of MAP kinases was determined by Western blotting of whole cell lysates using anti-phospho-MAP kinase Abs. To determine that equivalent quantities of MAP kinases were analyzed, blots were stripped and reprobed with anti-MAP kinase Abs. All panels shown are from the same experiment.

We examined the influence of FLAG-MKP6 (C111S) upon CD3 plus CD28 mAb-induced T cell IL-2 secretion (Fig. 5*B*). In control LNGFR-transduced T cells, CD3 and CD28 mAb synergized to induce T cell IL-2 secretion. However, in FLAG-MKP6 (C111S) transduced T cells CD3 and CD28 mAb induced considerably larger quantities of IL-2 under identical stimulation conditions. We interpret these results as evidence that in primary human T cells, MKP6 functions as a negative regulator of CD28 costimulatory signaling.

Specific enhancement of CD28 costimulatory signaling by dominant-negative MKP6 (C111S) and influence of wild-type MKP6

In additional experiments, we examined whether the dominant-negative effect of FLAG-MKP6 (C111S) was specific to CD28 costimulation. Also, we examined the influence of FLAG-wild-type MKP6 upon CD28 costimulation, which might be expected to inhibit responses by principle of overexpression. Gene transfer of FLAG-MKP6 (C111S), FLAG-MKP6, and LNGFR to PBT was as before. However, although FLAG-MKP6 (C111S) was expressed well in transduced PBT, FLAG-MKP6 was expressed at ~10-fold lower levels (Fig. 6*A*).

As before, FLAG-MKP6 (C111S)-transduced T cells secreted severalfold more IL-2 in response to CD3 plus CD28 mAb stimulation compared with LNGFR-transduced control T cells (Fig. 6*B, left*). Conversely, less IL-2 was secreted by FLAG-MKP6-transduced T cells, although the inhibition was slight. Interestingly, the enhanced IL-2 secretion observed in FLAG-MKP6 (C111S)-transduced T cells was seen to be specific to CD28 costimulation. Thus, the same FLAG-MKP6 (C111S)-transduced T cells did not secrete enhanced quantities of IL-2 when stimulated with the combination of CD3 mAb plus mAb against another T cell costimulatory receptor, CD2 (42) (Fig. 6*B, left*).

We also examined IL-2 secretion in response to another control stimulus, i.e., PMA and ionomycin (Fig. 6*B, right*). Again, in contrast to results obtained with CD3 plus CD28 mAb, the three different populations of T cells secreted similar quantities of IL-2 in response to PMA (at 1 ng/ml) and ionomycin. However, when the combination of suboptimal PMA (0.1 ng/ml) and ionomycin plus CD28 mAb was used as a stimulus, FLAG-wild-type MKP6-transduced T cells secreted significantly less IL-2 (~50%) and FLAG-MKP6 (C111S)-transduced T cells again secreted severalfold more IL-2 than LNGFR controls (Fig. 6*B, right*). Altogether, these results provide strong evidence that MKP6 is involved specifically in negative regulation of CD28 costimulation in T cells. Presumably, that FLAG-MKP6 did not more strongly inhibit CD28 costimulatory signaling in these experiments can be accounted for on the basis that FLAG-MKP6 was only poorly expressed in the transduced T cells (Fig. 6*A*).

ERK and JNK but not p38 MAP kinases are hyperphosphorylated upon CD28 costimulation of dominant-negative MKP6 (C111S)-transduced PBT

Previous studies have shown that ERK and JNK rather than p38 MAP kinases are involved in TCR/CD28 induction of IL-2. Therefore, we asked whether ERK and JNK MAP kinases become hyperphosphorylated in dominant-negative MKP6 (C111S)-transduced primary T cells following TCR/CD28 stimulation, which could account for enhanced IL-2 secretion. Fig. 7 shows that in both LNGFR- and FLAG-MKP6 (C111S)-transduced T cells, ERK, JNK, and p38 MAP kinases became transiently dual-phosphorylated in response to stimulation with the combination of CD3 and CD28 mAb. However, the level of dual-phosphorylation of ERK and JNK was considerably larger in FLAG-MKP6 (C111S)-transduced T cells compared with LNGFR-transduced controls. Hyperphosphorylation of ERK and JNK in these preactivated T cells was evident by 15 min poststimulation. Unlike ERK and JNK, the degree of dual-phosphorylation of p38 was comparable between LNGFR- and FLAG-MKP6 (C111S)-transduced T cells. Thus, enhanced IL-2 secretion is associated with hyperphosphorylation of ERK and JNK but not p38 MAP kinases. Furthermore, these results demonstrate that, although in vitro MKP6 behaves as a general MKP, in T cells MKP6 controls specifically the activation of ERK and JNK during the course of CD28 costimulatory signaling.

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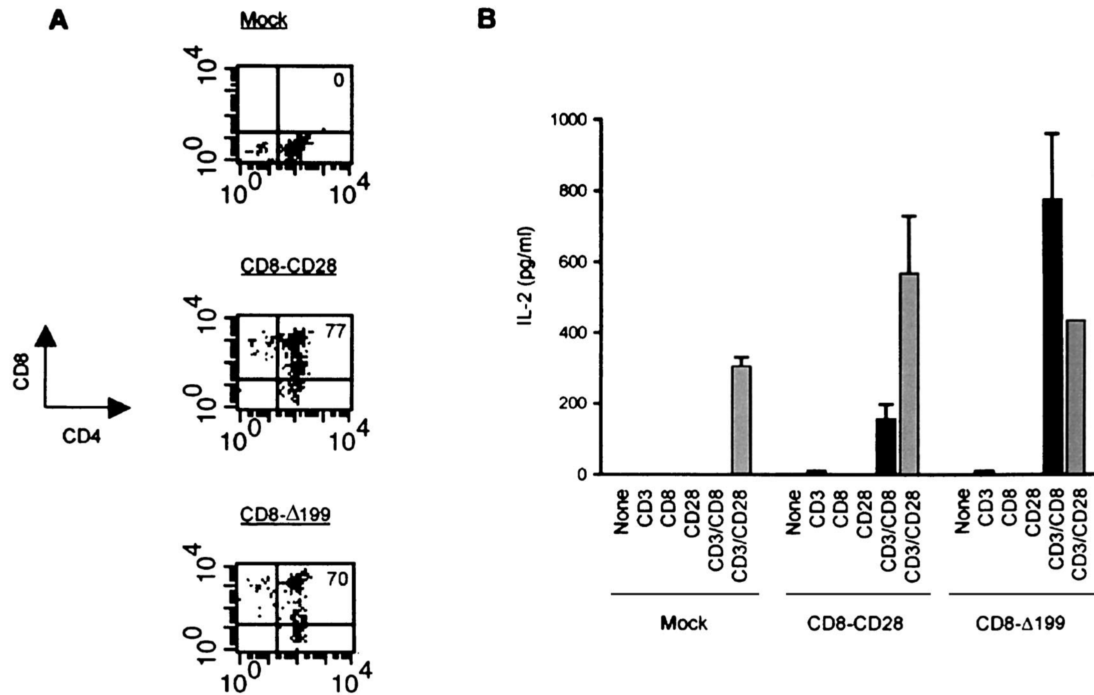

FIGURE 8. Hyperinduction of IL-2 from primary human PBT by an MKP6 binding-deficient CD8-CD28Δ199 CYT chimeric receptor. *A*, Transfer of chimeric receptors to human PBT. PHA-activated PBT were depleted of $CD8^+$ cells and were mock infected or infected with retroviruses encoding chimeric receptors comprised of the extracellular plus transmembrane domain of human CD8α fused to a wild-type CD28 CYT (CD8-CD28) or a CD28Δ199 CYT (CD8-Δ199). Expression of chimeric receptors was determined by flow cytometry using labeled CD4 and CD8 mAb. The percentage of CD4/CD8 double positive T cells is indicated. *B*, Chimeric receptor induction of IL-2 secretion in transduced PBT. Transduced PBT were stimulated or not with combinations of CD3, CD8, and CD28 mAb for 24 h in 96-well plates. IL-2 secretion was assessed as in Fig. 5.

A CD28Δ199 CYT hyperinduces IL-2 from PBT

Because a CD28-MKP6 physical association was difficult to detect in primary T cells, we examined whether such an association occurs functionally. We asked whether an MKP6 binding-deficient CD28 CYT would hypercostimulate IL-2 from primary T cells. To test this, we constructed chimeric receptors comprised of the extracellular plus transmembrane domains of human CD8α linked to either the wild-type CD28 CYT or a CD28Δ199 CYT that was unable to bind MKP6 in yeast and in vitro (Figs. 1 and 3). Chimeric receptors were then transferred to CD8-depleted primary human PBT, again using retroviruses. As detected by immunostaining with CD4 and CD8 mAb, CD8-CD28 CYT and CD8-CD28Δ199 CYT chimeric receptors were expressed at comparably high levels on the respective, predominantly $CD4^+$, transduced T cell populations (Fig. 8*A*). By contrast, mock transduced T cells were not reactive with CD8 mAb.

We examined IL-2 production in all three cell populations following stimulation with CD3 plus CD8 mAb (Fig. 8*B*). As expected, CD8-CD28 CYT-transduced T cells secreted IL-2 in response to CD3 plus CD8 mAb, thus confirming that the CD28 CYT is sufficient to transduce CD28 signals in primary T cells (43, 44). However, importantly, CD8-CD28Δ199 CYT-transduced T cells secreted severalfold more IL-2 upon challenge with CD3 and CD8 mAb. Mock transduced T cells did not produce any IL-2 when stimulated with CD3 and CD8 mAb (similarly, chimeric receptors, which contained only the first five amino acids of the CD28 tail or a CD28 tail with a Y173F mutation, induced no IL-2 or much reduced IL-2, respectively, as determined in separate experiments; data not shown). However, all three populations secreted comparable amounts of IL-2 in response to stimulation with CD3 plus CD28 mAb. As in experiments with dominant-negative FLAG-MKP6 (C111S), enhanced IL-2 production mediated by the CD8-CD28Δ199 CYT chimeric receptor was associated with hyperphosphorylation of MAP kinases (data not shown). Therefore, these results provide functional evidence that MKP6 interacts physically with CD28 in primary human T cells.

Discussion

We report here a novel MKP, MKP6, that we demonstrate dephosphorylates different MAP kinase substrates in vitro. MKP6 is expressed ubiquitously, although expression is stronger in certain cell types and tissues than in others. In T cells, MKP6 is initially expressed at low levels. However, expression in this cell type is rapidly and strongly induced in response to synergistic TCR/CD28 signaling. Because MKPs have previously been shown to downmodulate cellular responses that involve activation of MAP kinases (45), this suggested that MKP6 could function as an important negative-feedback regulator of CD28 costimulatory signaling in T cells. In support of this notion is the finding that primary human PBT that were transduced with a phosphatase-inactive dominant-negative MKP6 (C111S) secreted severalfold more IL-2 in response to CD28 costimulation. Furthermore, this enhanced IL-2 secretion was shown to be associated with hyperactivation of ERK and JNK MAP kinases, which have been previously implicated in the induction of this cytokine. This finding suggests that MKP6 acts as a negative regulator of CD28 costimulation in intact T cells by inactivating specifically these MAP kinases. However, one caveat to these conclusions is that because of the nature of dominant-negative experiments, we cannot at this point formally exclude an alternative possibility that some other MKP6-related MKP instead negatively regulates CD28 costimulation.

MKP6 was originally cloned by virtue of its ability to interact physically with the CD28 CYT in yeast. Moreover, this physical interaction can be demonstrated in vitro and in transfected Jurkat

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T leukemic cells. In these settings, MKP6 binding to CD28 is seen to be specific, independent of CD28 CYT tyrosine phosphorylation, but dependent upon tyrosine residue 200, located toward the distal end of the CD28 tail. Therefore, the mechanism of interaction with CD28 CYT is distinct from that seen with PI3-kinase and Grb-2, both of which bind CD28 CYT on phosphorylated tyrosine residue 173. However, it has been more difficult to demonstrate a physical association between endogenous MKP6 and CD28 in primary human PBT. Therefore, we sought independent functional evidence for this association in primary T cells. In this regard, a chimeric receptor with an MKP6 binding-deficient CD28 CYT (lacking the last three residues of CD28 CYT including tyrosine 200), in synergy with the TCR, induced severalfold more IL-2 from transduced primary human PBT than a similar receptor with a wild-type CD28 CYT. Excepting the possibility that the last three residues of CD28 CYT are involved in the activation of a distinct negative regulator of CD28 signaling, this finding is consistent with the idea that MKP6 and CD28 interact physically in primary T cells.

It is interesting to speculate as to why MKP6 might require binding to CD28 to execute its function. We have not observed that MKP6 becomes activated upon interaction with CD28. Therefore, we favor the possibility that MKP6 might bind to CD28 to become localized to plasma membrane MAP kinase substrates. In quiescent cells, MAP kinases are known to be concentrated at the plasma membrane, at which site, or at least in early endosome compartments, MAP kinase activation is initiated (46, 47). As such, MKP6 binding to CD28 could allow localization to recently activated MAP kinases and subsequent inactivation before MAP kinase nuclear translocation.

Alternatively, in binding to CD28, MKP6 could inactivate MAP kinases that function proximally. Although MAP kinases are traditionally thought to act in the nucleus, a growing body of evidence indicates that this family of kinases performs additional roles at nonnuclear locations (48, 49). Perhaps most pertinent to costimulation is the recent observation that, in T cells, the ERK1 MAP kinase functions at a plasma membrane location by controlling the amplitude of TCR-generated signals (50). Soon after TCR triggering, ERK1 associates physically with the TCR complex (50, 51). Ostensibly, in its activated state this recruited ERK1 prevents docking to the TCR of the SHP-1 tyrosine phosphatase, which acts to down-regulate TCR signals, probably by dephosphorylating the TCR-associated protein tyrosine kinase, ZAP-70 (52). Interestingly, during the course of T cell activation initiated by natural TCR and CD28 ligands, and in Ag-specific T cell-APC conjugates, CD28 is known to translocate to the region of the TCR that becomes concentrated in the "central TCR supramolecular activation cluster" (Refs. 53 and 54; and A. Kupfer, personal communication). Therefore, these findings suggest that MKP6, localized to the TCR by CD28, may function as a negative regulator by first inactivating TCR-associated ERK1, thereby resulting in more rapid SHP-1 recruitment. In turn, through the actions of SHP-1, MKP6 could ultimately down-regulate signals leading to the activation of other ERK isoforms and JNK MAP kinases. If true, then assumedly, at least some aspects of these signaling interactions are mimicked when CD3 plus CD28 mAb or the combination of PMA plus ionomycin and CD28 mAb are used to stimulate T cells in vitro.

Aside from the issue of physical interaction, one major and important finding in the current studies is that dominant-negative MKP6 (C111S)-enhanced CD28-mediated IL-2 secretion specifically. Similar enhancements of IL-2 secretion were not observed in response to PMA plus ionomycin alone or the combination of CD3 plus CD2 mAb. This indicates a specific involvement of MKP6 in CD28 costimulatory signaling. Furthermore, this finding raises the intriguing possibility that distinct MKPs may regulate different signaling pathways in T cells. With the exception of MKP6, described here, and PAC-1 (40), MKPs have been little studied in T cells. Like MKP6, PAC-1 expression is induced upon T cell activation. However, whether PAC-1 regulates specific signaling pathways has not been addressed. A full description of which MKPs are turned on in T cells and which types of signaling pathway each MKP regulates awaits.

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