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CD6 Regulates T-Cell Responses through Activation-Dependent Recruitment of the Positive Regulator SLP-76

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Deciphering the role of lymphocyte membrane proteins depends on dissecting the role of a protein in the steady state and on engagement with its ligand. We show that expression of CD6 in T cells limits their responsiveness but that engagement by the physiological ligand CD166 gives costimulation. This costimulatory effect of CD6 is mediated through phosphorylation-dependent binding of a specific tyrosine residue, 662Y, in its cytoplasmic region to the adaptor SLP-76. A direct interaction between SLP-76 and CD6 was shown by binding both to a phosphorylated peptide (equilibrium dissociation constant [K_D] = 0.5 μM at 37°C) and, using a novel approach, to native phosphorylated CD6. Evidence that CD6 and SLP-76 interact in cells was obtained in coprecipitation experiments with normal human T cells. Analysis of human CD6 mutants in a murine T-cell hybridoma model showed that both costimulation by CD6 and the interaction between CD6 and SLP-76 were dependent on 662Y. The results have implications for regulation by CD6 and the related T-cell surface protein, CD5.

Expression of T-cell surface proteins which are involved in regulating antigen-specific T-cell activation is coordinated by the T-cell receptor (TCR). During positive selection, both TCR and a number of T-cell surface proteins, including CD6, CD5, and CD2, are upregulated and remain expressed on mature T cells (36, 40). These proteins regulate antigen-specific responses through cell-cell contact and have substantial cytoplasmic regions which link to intracellular signaling machinery (Fig. 1A). Cell surface ligands for CD2 and CD6 have been identified (Fig. 1A), and there is evidence that CD5 mediates cell-cell contact (reference 11 and references therein). CD6 and CD5 are unusual in that their extracellular regions are comprised of scavenger receptor cysteine-rich domains instead of the more-common immunoglobulin (Ig)-like domains found in other T-cell surface proteins, such as CD2, CD4, CD8, etc. CD6 and CD5 are linked in the genome and have similar structures and expression patterns (3, 36, 38). CD6 binds the immunoglobulin superfamily protein CD166 (6) (Fig. 1A). CD6 is highly expressed on resting T cells, whereas CD166 is expressed on antigen-presenting cells, consistent with engagement of CD6 by CD166 being necessary for optimal antigen-specific T-cell activation (19). The functional role of another unidentified potential ligand for CD6 has not yet been described (34).

CD5 and CD6 both contain several tyrosine residues in their cytoplasmic regions, the tail of CD6 being remarkably long (244 amino acids), with nine tyrosine residues (7, 33). Initial studies with CD5 and CD6 monoclonal antibodies (mAbs) suggested a positive regulatory role for these proteins in regulating T-cell responses (1, 38). However, studies with CD5-deficient mice cast CD5 in the light of a negative regulator. This hypothesis was based on increased sensitivity to activation of cells deficient in CD5 (39). Negative effects were shown to be mediated by the cytoplasmic region (4, 30) and independently of extracellular engagement (4). These results are not necessarily contradictory, as the behavior of CD5 in a T cell independently of extracellular engagement is consistent with its presence raising the threshold of activation (30). Data showing that a CD5 mAb (5) or soluble CD5 inhibits cell activation (15) are consistent with a model in which recruitment of CD5 is necessary for an optimal immune response. Both CD5 and CD6 migrate to the interface between T cells and antigen-presenting cells (8, 15, 16). This interpretation of available data places CD5 and CD6 in a similar category of being positive regulators of T-cell activation.

Here we show that blocking of the CD6/CD166 interaction with a mAb specific for a particular epitope on domain 3 reduces antigen-specific interleukin-2 (IL-2) production, confirming conclusions drawn from blocking studies with soluble CD6 (19). We describe the first direct activation-dependent intracellular interaction of CD6 with the C-terminal tyrosine motif and the adaptor protein SLP-76, itself a positive regulator of T-cell activation (45). We describe the first quantitative measurements of interactions between SH2 domains and peptides at physiological temperatures. We have also developed a method to measure the binding of the SLP-76 SH2 domain directly to native CD6 at 37°C. We show that binding of SLP-76 to native phosphorylated CD6 is reduced by a single-point mutation of the C-terminal tyrosine residue, 662Y to 662F. This is the first time affinity measurements have been made on native receptors and at physiological temperatures. Evidence that the interaction between CD6 and SLP-76 could

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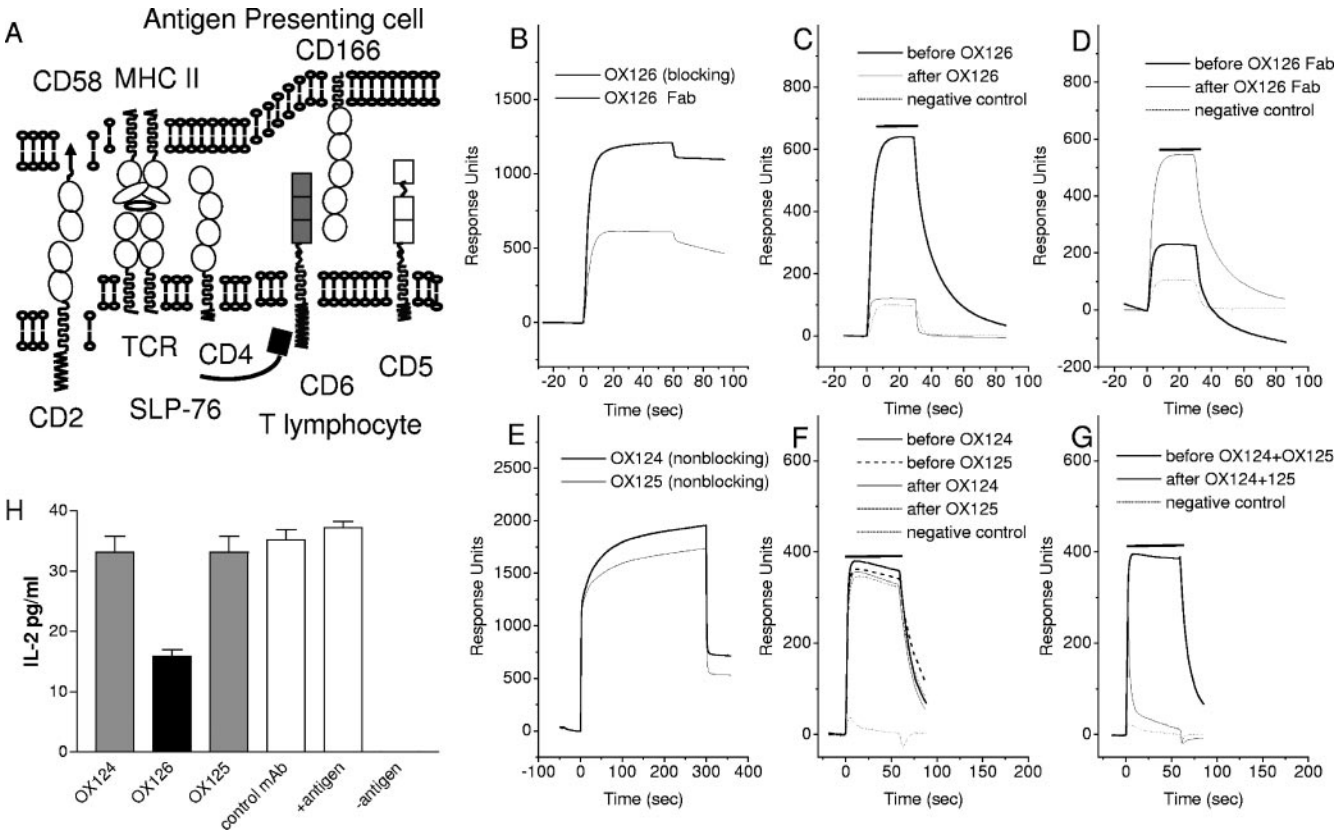

FIG. 1. (A) Diagrammatic representation of CD5 and CD6, showing the three scavenger receptor cysteine-rich domains (rectangles) in their extracellular regions and the large cytoplasmic region of CD6. The membrane proximal domain of CD6 binds the N-terminal Ig superfamily domain (oval) of CD166. The SH2 domain of SLP-76 (black rectangle) is shown binding to the CD6 cytoplasmic region (Fig. 2). (B to G) Blocking of the CD166/CD6 interaction with CD6d3 mAbs. Three CD6d3 mAbs, OX126, OX125, and OX124, were tested for binding to CD6d3CD4d3 + 4-biotin immobilized on a BIAcore chip and for blocking of CD166CD4d3 + 4 (1 μM) binding. CD166CD4d3 + 4 (1 μM) was injected simultaneously over CD6d3CD4d3 + 4-biotin and CD4d3 + 4-biotin. (B) OX126 and OX126 Fab bound CD6d3 and blocked CD166 binding (C and D). (E) OX124 and OX125 bound CD6d3 and did not block CD166 binding individually (F) but did block it when used together (G). OX126 Fab fragments blocked the CD166/CD6 interaction, but the dissociation rate was too high to observe blocking effects in cellular experiments at the concentrations tested (data not shown). The bars indicate the period of injection. (H) Antigen (tetanus toxoid)-specific proliferation of human T cells was inhibited by OX126 CD6 mAb but not OX124 or OX125 CD6 mAb or an isotype nonbinding control (OX122, IgG1) mAb, all at 10 μg/ml.

occur in cells and that it was dependent on 662Y was obtained by coprecipitation. As human CD6 bound human and mouse CD166 with the same affinity, costimulation by CD6 was analyzed in a murine T-cell hybridoma model with cells transduced with human CD6 and mutants. Antigen-specific responses were dependent on CD6/CD166 engagement and on the C-terminal tyrosine residue of CD6, 662Y. The results are consistent with SLP-76 having a critical role in activation-dependent interactions of CD6.

MATERIALS AND METHODS

Peptides. The following peptides from Sigma-Genosys were used: CD6(662)P, PDSTDNDD(pY)DDISAA; CD6(662), PDSTDNDDYDDISAA; CD5(487)P, PDNSSDSD(pY)DLHGAQRL; control, CD6(632), FQPPPQPPSEEQFGCPG SPSPQPD; moth cytochrome C (mcc), ANERADLIAYLKQATK (CRB, Cambridge, United Kingdom); and FcRγIIb(292)P, ADKVGAENTIT(pY) SLLMHPDA. The peptides were biotinylated at the N terminus. All peptides were purified by high-pressure liquid chromatography, were checked using matrix-assisted laser desorption ionization mass spectroscopy, and were at least 70% pure.

Peptide pulldown and immunoprecipitation. Jurkat cells were washed twice in phosphate-buffered saline (PBS), lysed at ~2.5×10^8 cells/ml in 10 mM Tris-HCl [pH 7.4] supplemented with 140 mM NaCl, 1 mM EDTA, 10% (vol/vol) glycerol, 0.2% (wt/vol) NaN_3, 1% (vol/vol) Brij-96, and 1 mM Na pervanadate; 1 mM phenylmethylsulfonyl fluoride; 20 μg/ml leupeptin; 1 μg/ml aprotinin; 1 μg/ml pepstatin; 50 mM benzamidine; 1 mM NaF; and 10 μg/ml DNase for 60 min at 4°C with rotation. The lysate was centrifuged at 13,000 rpm in a microfuge for 10 min at 4°C, and the supernatant was filtered (0.45-μm filter; Millipore Ltd., Watford, Herts, United Kingdom). Cell lysates (1 ml) were precleared with rotation for 30 min at 4°C, first with 25 μl streptavidin-coated Dynalbeads M-280 (Dynal, Oslo, Norway) and, second, with 20 μg irrelevant biotinylated protein bound to 25 μl streptavidin-coated Dynalbeads. The peptide (16 μg) was coupled to 25-μl beads at 4°C for 30 min. The beads were then washed twice with PBS and combined with the precleared cell lysate (~2.5×10^8 cells/ml lysate/pull-down) and incubated with rotation for 2 h at 4°C. These were then washed three times with 500 μl PBS, using the magnet to ensure that the beads were completely resuspended during washes, and were transferred into new 1.5-ml tubes after each wash. The beads were resuspended in 10 μl Novex sample buffer with 0.05 M dithiothreitol, incubated at 70°C for 10 min, and analyzed on a Novex precast gradient (4 to 12%) gel. Bands were isolated, digested with trypsin, and analyzed by matrix-assisted laser desorption ionization mass spectrometry (21).

For analysis of CD6 phosphorylation, cells were untreated or treated with pervanadate or precoated with saturating amounts of KT3 or OKT3 mAb, washed, and then cross-linked with rabbit anti-rat or anti-mouse IgG (20 μg/ml) for 5 min at 0°C before being incubated for 0, 2, or 5 min at 37°C, immediately spun, and resuspended in NP-40 (1% vol/vol) or Brij-96 lysis buffer at 4 × 10^7

cells/ml and human CD6 precipitated from 2×10^7 cells with CD6 mAb (MEM-98) coupled to Sepharose 4B. OKT11 (human CD2 [hCD2]) and Y2-178 (hCD5) mAbs coupled to Sepharose 4B were used for precipitation from human T cells. Precipitates were collected after 1.5 h of rotation at 4°C, washed four times with 1 ml lysis buffer, transferred to a new tube before sample buffer was added, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a Novex 4% to 12% gradient gel (Invitrogen) under reducing conditions, followed by Western blotting with a pTyr mAb. Lysates (5×10^5 cell equivalents) were analyzed under nonreducing conditions for Western blotting with a CD6 mAb (MEM-98). The blots were developed using ECL Plus (Amersham Biosciences).

Monoclonal antibodies. Three mAbs specific for human CD6 domain 3 (CD6d3), OX124 (IgG1), OX125 (IgG2b), and OX126 (IgG1), were raised by immunizing DA rats with a fusion protein consisting of human CD6d3 and rat CD4 domains 3 and 4 (CD6d3CD4d3 + 4) (19). Clones were initially selected by screening on 2B4 T-cell hybridoma cells expressing human CD6. Specificity for CD6d3 and capacity to block binding of CD166 to CD6 were tested using recombinant protein and a BIAcore (19) at 25°C. A rat anti-mouse CD6d3 mAb, OX129 (IgG2a), which did not cross-react with human CD6, was raised against soluble mouse CD6 and will be fully described elsewhere. Other mAbs used were mouse CD2 (RM2.1); mouse CD5 (53-7.3); mouse CD3 (KT3); human CD3 (OKT3); human CD6d1, MEM-98 (kindly provided by Vaclav Horesji and by Serotec), and 12.1 (ATCC); pTyr; biotinylated pTyr (Sigma-Aldrich); SLP-76 (Sigma-Aldrich); phycoerythrin-coupled anti-mouse TCRβ (Pharmingen); phycoerythrin- or fluorescein isothiocyanate-coupled secondary mAb (Serotec); and horseradish peroxidase-coupled secondary reagents, anti-mouse IgG (Bio-Rad), or streptavidin (Sigma-Aldrich). A CD6 mAb (MEM-98) was coupled at 2 mg/ml directly to CNBr-4B Sepharose (Pharmacia Biotech) as recommended by the manufacturer after purification by ammonium sulfate fractionation. OKT11 mAb (hCD2) and Y2-178 mAb (hCD5) Sepharose 4B beads were similarly prepared. For human cell culture experiments, possible lipopolysaccharide contamination was removed from mAbs, using Acticlean (Sterogene Bioseparations Inc., Carlsbad, Calif.).

Recombinant proteins. We designed the SLP-76 SH2 domain construct by comparing the sequence with that of the avian SRC SH2 domain, for which there is an X-ray crystallography structure (37). A fragment cut with BamHI and SalI encoding the SH2 domain of human SLP-76 (amino acids 418 to 533; cDNA was kindly provided by Gary Koretzky, Philadelphia, Pa.) was cloned into BamHI and XhoI sites in the pTrcHisA vector (Invitrogen, United Kingdom), and the SH2 domain of human SHIP (amino acids 1 to 110; construct provided by Gary Brooke, Oxford, United Kingdom) was expressed as N-terminally His-tagged fusion proteins and purified using Ni-agarose affinity chromatography. Extinction coefficients of the fusion proteins were calculated as follows: for SLP-76-SH2, 29,900 M^{-1} cm^{-1}, and for SHIP-SH2, 20,700 M^{-1} cm^{-1}. Proteins were subjected to gel filtration on Superdex 75 (Pharmacia) prior to BIAcore analysis.

To generate mutant forms of the human CD6 cytoplasmic region, fragments were generated by PCR from templates (kindly provided by Jane Parnes and Nora Singer), with a forward primer at the beginning of the transmembrane region and reverse primers at the 3′ end of CD6; the wild type (CD6), 662F (662Y mutated to F), 489F (489Y mutated to F), and the double mutant (489F/662F) were cut 5′ at an internal EcoRI site and 3′ with SalI and cloned into pBabe vectors (26). Two fragments encoding the extracellular region of CD6 (EcoRI-HindIII and HindIII-EcoRI) were excised from other constructs and simultaneously cloned into the EcoRI site of the pBabe vector containing the wild-type CD6 cytoplasmic region. The resultant EcoRI fragment was used to complete the 662F construct. Further constructs were made by inserting the mutated cytoplasmic region into pBabe engineered to contain a BamHI-EcoRI fragment encoding the extracellular region of CD6. Constructs were expressed by transfection into Eco Phoenix cells (www.stanford.edu/group/nolan) using FuGENE 6 (Roche, Indianapolis, Ind.), retroviral transduction in 2B4 hybridoma cells, and selection with 1 μg/ml puromycin. The extracellular regions of mouse CD6 and CD166 were produced as CD4d3 + 4 fusion proteins and biotinylated as necessary as previously described (19). Mouse CD6CD4d3 + 4 with the human CD5 leader (43) and mouse CD166CD4d3 + 4-biotin with its own leader and joined at the junction with CD4d3 + 4 had the sequences VKDS*STSIT* and NDQA*STSIT*, respectively, with the CD4 sequence shown in italics. Cell binding with mouse CD6CD4d3 + 4-biotin and CD4d3 + 4-biotin as a negative control was carried out as previously described (9).

BIAcore analyses. BIAcore analyses using a BIAcore 2000 were carried out essentially as previously described and referenced (21). Streptavidin and mAbs were directly coupled to CM5 chips by amine coupling for immobilization of biotinylated peptides and native receptors, respectively. Native receptors were captured from cell lysate (10^8 cell equivalents/ml) by passing the lysate (10 to 20 μl) over a mAb-coated flow cell at 1 μl/min at 37°C. Cell lysates were prepared from murine T-cell hybridoma cells expressing human CD6 or 662F that had been treated with pervanadate or not for 7 to 10 min at 37°C at 10^8 cell equivalents/ml, washed with PBS, and lysed as described for peptide pulldown experiments. The lysates were used immediately in BIAcore experiments.

Cellular techniques. Antigen-specific IL-2 production of human peripheral blood lymphocytes (PBL) was carried out as previously described (19). Human T blasts were produced from nonadherent PBL by stimulation with phytohemagglutinin (5 μg/ml) for 2 to 3 days, expanded in human IL-2 (50 to 100 U/ml), and allowed to quiesce. 2B4 hybridoma cells (10^5 cells/well of a round-bottomed microtiter plate) were stimulated with mcc peptide at the final concentration indicated and irradiated spleen cells (2,000 rads) from CBA mice antigen-presenting cells (APC) at ratios varying from 2.5:1 to 10:1 APC to T cells in triplicate at 37°C. The medium used contained 5% fetal calf serum, Dulbecco modified Eagle medium, 2×10^{-5} M β2-mercaptoethanol, and antibiotics with or without selection. No difference in results was noted when experiments were performed with or without selection antibiotics. The supernatants were assayed for IL-2 production by enzyme-linked immunosorbent assay at 24 h.

RESULTS

Blocking extracellular engagement of CD6 by CD166 with a CD6 mAb inhibits antigen-specific IL-2 production by normal human T cells. Our previous studies showed that blocking the CD6/CD166 interaction in an antigen-specific response by human T cells with soluble CD6 reduced IL-2 production (19). The argument that the effect was due to soluble CD6 primarily blocking CD6/CD166 interactions rather than the homophilic CD166 interaction was based on the relative affinities of the two interactions and distribution data. CD6 binds CD166 with ~100-fold-higher affinity than the CD166 homophilic interaction, and CD6 and CD166 were highly expressed on T cells and APC, respectively. To further differentiate between heterophilic CD6/CD166 and homophilic CD166/CD166 interactions, we produced three CD6 mAbs, OX124, OX125, and OX126, against the ligand binding domain of human CD6, domain 3 (Fig. 1B to G). The OX126 mAb blocked the interaction between CD6 and CD166, whereas OX124 and OX125 did not (Fig. 1B to G). The OX126-blocking mAb reduced antigen-specific IL-2 production by human T cells (Fig. 1H). The OX125 mAb did not have an effect, nor did the OX124 mAb. At the concentrations of mAb used in this experiment, cross-linking effects were not observed at the level of CD6 expression seen on the normal cells. Stimulatory cross-linking effects were seen at higher concentrations of mAb (not shown). Blocking effects by OX126 mAb support our previous conclusion that CD6 engagement by CD166 is necessary for an optimal antigen-specific immune response (19).

A C-terminal CD6 peptide bound SLP-76 in a T-cell-line lysate. A costimulatory role for CD6 suggests that positive regulators of T-cell activation will be recruited to the cytoplasmic region. The intracellular region of CD6 contains nine tyrosine residues, six of which conform to phosphorylation motifs, including 662Y and 489Y (NetPhos 2.0 Server, Technical University of Denmark), that could potentially mediate activation-dependent interactions. CD6 has been shown to be phosphorylated upon T-cell activation (22, 42). The first peptide we designed was based on the C-terminal tyrosine residue [CD6(662P)]. Results were compared with another CD6 cytoplasmic peptide, CD6 (632). Peptide pulldown experiments were performed with a phosphorylated form of CD6(662P) and Jurkat T-cell-line lysate. A single band marked in Fig. 2A was consistently pulled down with the C-terminal phosphorylated peptide CD6(662P) and not with a control CD6 peptide.

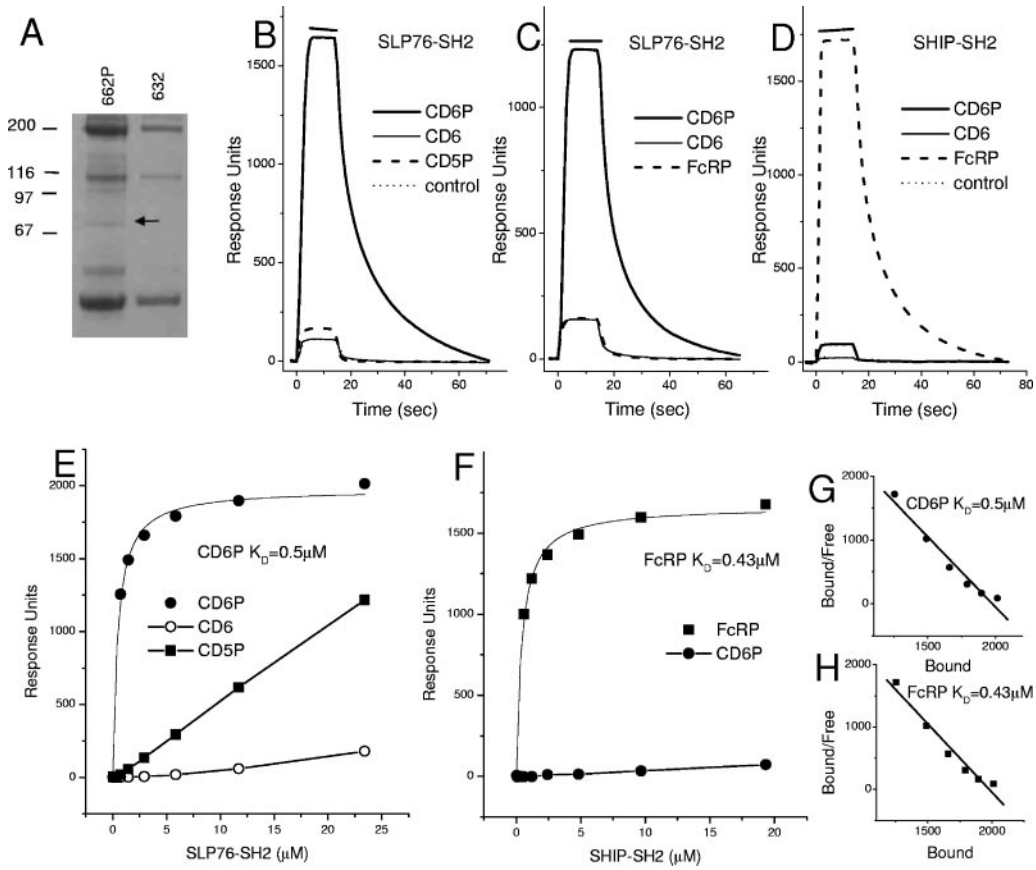

FIG. 2. The SH2 domain of SLP-76 binds specifically to the phosphorylated C-terminal CD6 peptide. (A) SLP-76 was isolated from Jurkat T-cell lysates in pulldown experiments using a peptide containing the phosphorylated C-terminal tyrosine of CD6, CD6(662)P. Coomassie blue-stained reducing gel shows that a single, specific band was isolated with CD6(662P) and not with a control CD6 peptide, CD6 (632). This band was shown to be SLP-76 by tryptic digestion and mass spectrometry (arrow). (B to D) SLP-76 SH2 (1.5 μM) or SHIP SH2 (19.3 μM), indicated by the bar above the trace, were injected (5 μl) over immobilized peptides at 37°C (amounts immobilized are given in response units [RU]). (B and C) CD6(662)P (CD6P, 220 RU); CD6 (662) (CD6, 231 RU); CD5(487)P (CD5P, 406 RU); and (streptavidin) control or FcRγIIb(292)P (FcRP, 244 RU). (D) CD6(662)P (CD6P, 341 RU); CD6 (662) (CD6, 329 RU); FcRγIIb(292)P (FcRP, 328 RU); and CD6 (632) (control, 488 RU). (E and F) Equilibrium binding data for a range of concentrations from the experiments for panels B and D are plotted, and the affinity for binding of SLP76-SH2 to CD6(662)P (CD6P) and SHIP-SH2 binding to FcRγIIb(292)P (FcR) were calculated from the fitted curve. Scatchard analyses of data are shown in panels G and H.

All other bands appeared with differing intensities in pulldowns with control peptides. The band specific to CD6(662P) was excised, digested with trypsin, and identified by mass spectrometry as SLP-76, an adaptor protein. The interaction was confirmed and shown to be phosphorylation dependent in pulldown experiments with phosphorylated CD6(662P) and the nonphosphorylated C-terminal peptide CD6 (662) and Western blotting with anti-SLP-76 (data not shown).

The SLP-76 SH2 domain is bound directly and specifically to the C-terminal CD6 phosphorylated peptide. Identification of SLP-76 as an interacting partner for CD6 was a consistent result, as SLP-76 is a hematopoietic adaptor protein containing a C-terminal SH2 domain and has a role in T-cell activation (45). Previously identified binding partners for the SH2 domain of SLP-76 are the adaptor ADAP (13, 27) and HPK1 kinase (35). The SLP-76 SH2 domain binding motif in these proteins, (E/D)(V/D)YDDV, is similar to that in human and mouse CD6, (E/D)DYDDI. The SLP-76 SH2 domain was prepared as a His-tagged protein rather than the commonly used glutathione-S-transferase (GST) fusion proteins. GST can form dimers (equilibrium dissociation constant [K_D] = 0.34 μM at 10°C) (41) which may lead to a higher apparent affinity. The purified monomeric SLP-76 SH2 domain was tested for direct binding to the phosphorylated C-terminal CD6 peptide by surface plasmon resonance using a BIAcore. As a specificity control, experiments were performed with the SHIP SH2 domain prepared in a similar way. Both proteins were subjected to gel filtration immediately prior to binding experiments to ensure that quantitative measurement of monomeric binding was being analyzed. Examples of binding data at 37°C are shown in Fig. 2B to D. The soluble SLP-76 SH2 domain was injected over immobilized C-terminal CD6 phosphorylated and nonphosphorylated peptide, phosphorylated C-terminal CD5 peptide, and a control peptide previously shown not to bind. The SLP-76 SH2 domain bound specifically to the C-terminal CD6 phosphorylated peptide, whereas the SHIP SH2 domain bound well to the phosphorylated FcRγIIb peptide (Fig. 2D), as expected for a previously established interaction

with functional consequences (29). The equilibrium dissociation constants for the interactions of the SLP-76 and SHIP SH2 domains were obtained from the equilibrium binding of a series of concentrations of the SH2 domains (Fig. 2E to F). Analysis of equilibrium binding data showed that the equilibrium dissociation constants for the SLP-76 SH2 domain binding to the phosphorylated CD6 peptide and the SHIP SH2 domain binding to the phosphorylated FcRγIIb peptide were comparable with a K_D of ~ 0.5 μM at 37°C (Fig. 2E to H). Binding of the SLP-76 SH2 domain to a C-terminal phosphorylated CD5 peptide was estimated to be two orders of magnitude weaker than binding to the phosphorylated CD6 peptide. Binding of the SLP-76 SH2 domain to unphosphorylated CD6 peptide and the SHIP SH2 domain to phosphorylated CD6 peptide were negligible (Fig. 2B to D).

The SLP-76 SH2 domain bound directly and specifically to native CD6. The data above clearly show binding of SLP-76 to a CD6 peptide, but other residues of the large cytoplasmic region could affect binding. We developed a method to test binding in a quantitative manner to intact membrane proteins rather than to short peptides by in situ purification of CD6 on the BIAcore chip. CD6 was captured directly from cell lysates on a BIAcore chip by a high-affinity CD6 mAb. The specificity of this capture was confirmed by the lack of binding of a CD5 mAb to immobilized CD6 and vice versa; the CD6 mAb did not bind significantly to CD5 immobilized in a similar manner (data not shown).

To test the importance of the C-terminal tyrosine motif in native CD6 for SLP-76 binding, wild-type human CD6 (CD6) and human CD6 with 662Y mutated to phenylalanine (662F) were expressed in a mouse T-cell hybridoma (see Fig. 5A). The human and mouse CD6 cytoplasmic regions are well conserved, including the SLP-76 binding tyrosine motif at the C terminus; thus, it was expected that human CD6 would engage functionally with the murine intracellular signaling machinery and be phosphorylated. Lysates were prepared from cells treated or not with pervanadate, and similar amounts of phosphorylated CD6 and 662F and unphosphorylated CD6 were immobilized. Unphosphorylated CD6 was used a negative control, as the SLP-76 SH2 domain did not bind to the unphosphorylated C-terminal CD6 peptide. Examples of the data for SLP-76 SH2 domain binding at 37°C are shown in Fig. 3A. Phosphorylation-dependent binding to CD6 was determined by subtracting equilibrium values for unphosphorylated CD6. Analyses of equilibrium binding data are shown in Fig. 3B. The SLP-76 SH2 domain bound CD6 with an affinity comparable to that found with the synthetic peptide.

Binding of SLP-76-SH2 to phosphorylated 662F was reduced (Fig. 3B). The estimated affinity of the remaining binding was a K_D of ~ 7 μM. Phosphorylated peptides representing all the other tyrosine residues, whether or not they were predicted to bind SH2 domains, were tested for binding to the SLP-76 SH2 domain. A peptide containing phosphorylated 489Y was the only peptide which showed a weak interaction with SLP-76 SH2. The affinity of the SLP-76 SH2 domain for this sequence was increased 10-fold to a K_D of ~ 7 μM at 37°C by phosphorylating both 486Y and 489Y in the same peptide (data not shown). It is uncertain whether the additional, weaker interaction between CD6 and SLP-76 is a physiologically relevant interaction for the CD6 cytoplasmic region. At higher concentrations, above 4 μM, the SLP-76-SH2 domain tended to aggregate and the specificity of binding was further diminished. The presence of phosphorylated CD6 captured on the chip was revealed using a phosphotyrosine mAb and a CD6 mAb against a different CD6 domain (Fig. 3C and D). Phosphorylation was reduced on 662F. Levels of phosphorylated CD6 were lower than those for the mutant and unphosphorylated CD6, increasing the relative specificity of phosphorylation-dependent binding by SLP-76 SH2. Immunoprecipitation and Western blotting confirmed the presence of phosphorylated CD6 in pervanadate-treated lysates (data not shown) (see Fig. 6B). Thus, 662Y is the major site for SLP-76 binding, as shown with both peptides and native CD6.

CD6 and SLP-76 interact in cells. It has been shown that CD6 is recruited to the contact sites in an antigen-specific interaction between a T cell and an APC (16). Thus, an interaction between CD6 and SLP-76 which has been well characterized as linking to T- cell activation machinery (45) in vivo is feasible. To obtain more direct evidence that the CD6/SLP-76 interaction could occur in cells, we carried out coprecipitation experiments to test whether complexes of CD6 and SLP-76 could be detected in cell lysates. As has been previously reported, CD6 was phosphorylated in response to CD3 mAb cross-linking of normal human T blasts (Fig. 3E). Among the phosphorylated bands coprecipitated with CD6, there was a faint band at the molecular weight expected for SLP-76. The level of phosphorylation of the band in the position of SLP-76 varied in experiments, presumably due to the activation status of T blasts from different donors. Binding of the CD6/SLP-76 interaction is independent of SLP-76 phosphorylation, as the binding studies with the isolated SH2 domain of SLP-76 showed. Blotting with a SLP-76 mAb confirmed the presence of SLP-76 specifically in CD6 immunoprecipitates and increased association correlated with phosphorylation of CD6 (Fig. 3F). Consistent with a complex containing SLP-76 being coprecipitated with CD6 is the presence of a highly phosphorylated band at the molecular weight expected for LAT (~36 kDa). These bands were not coprecipitated by CD2, which does not contain tyrosine residues in its cytoplasmic region. SLP-76 was not detected in CD2 or CD5 immunoprecipitates. There was overlap in the proteins coprecipitated by CD5 and CD6, consistent with previous reports describing a physical link between the two (12, 16), but there were also distinct differences. The major phosphorylated protein coprecipitated by the SLP-76 mAb comigrated with phosphorylated CD6, consistent with SLP-76 being precipitated by the SLP-76 mAb, despite the failure to detect it in these lanes by blotting with the SLP-76 mAb (data not shown). Coprecipitation results from several preparations of T blasts were consistent with a phosphorylation-dependent interaction between CD6 and SLP-76 occurring in cells.

CD6 costimulation of antigen-specific responses is dependent on 662Y. To address the functional consequences of mutating the C-terminal region of CD6, which is involved in phosphorylation-dependent recruitment of SLP-76, we tested 2B4 murine T-cell hybridoma cells transduced with human CD6 and mutants for differences in responses. The biochemical data above had shown that the C-terminal 662Y was the main site of binding for SLP-76. There was evidence for a weaker phosphorylation-dependent interaction with another region of the cytoplasmic tail. To test whether mutation of 489Y, which could bind weakly to SLP-76, had the same effect

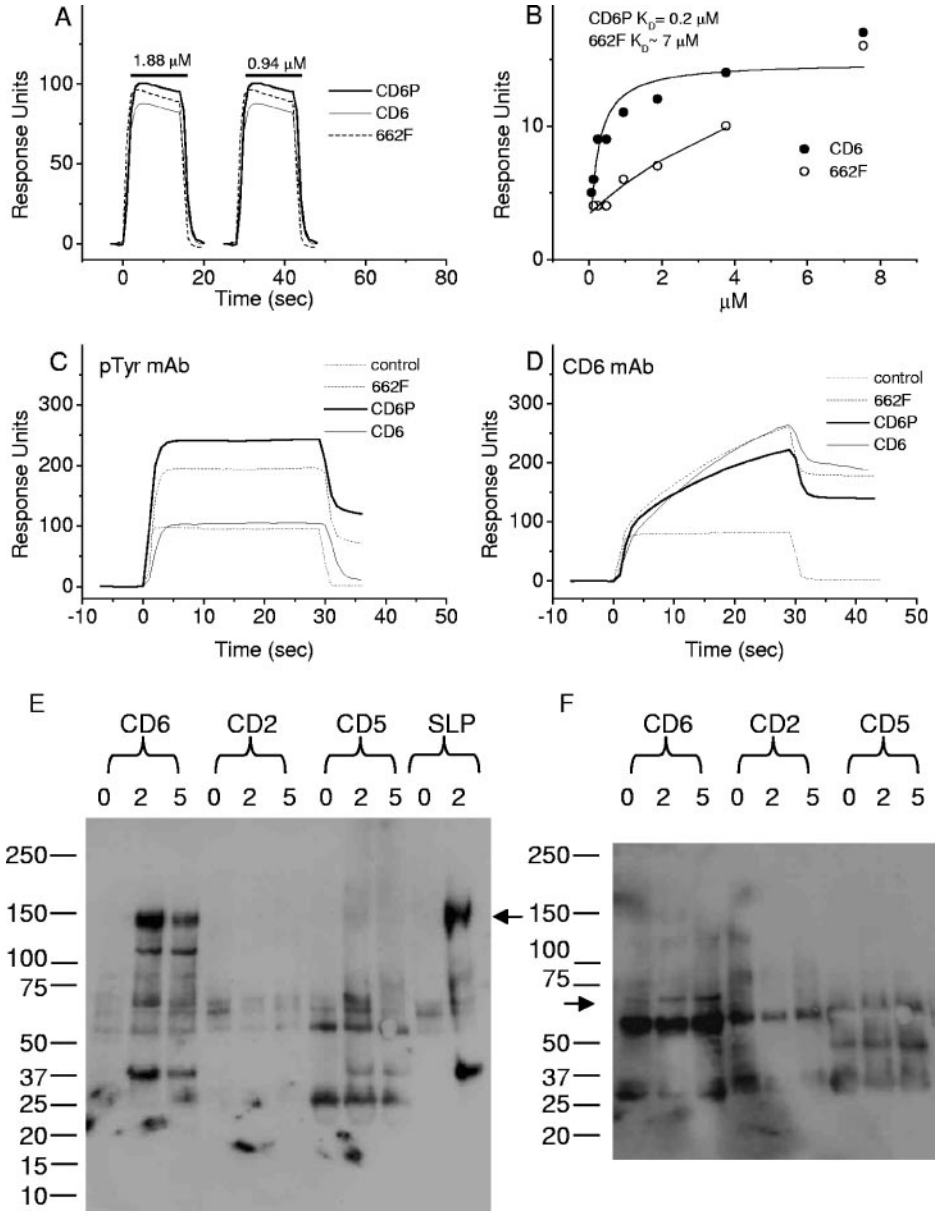

FIG. 3. The SH2 domain of SLP-76 binds native CD6. Similar amounts of the CD6 mAb MEM-98 were immobilized (~11,400 RU) on three BIAcore flow cells. Similar amounts of phosphorylated CD6 (CD6P; 1193 RU), phosphorylated 662F (662F;1664 RU), and unphosphorylated CD6 (CD6;1863 RU) were captured from hybridoma cell lysates. Increasing concentrations of SLP-76 were passed over CD6P, 662F, and CD6. (A) Data for two concentrations (μM) of the SLP-76 SH2 domain show binding to CD6P, weaker binding to 662F, and no binding to CD6, where only the bulk effect of the high-protein concentration is seen. (B) Specific equilibrium binding data for a range of concentrations from the experiment for panel A are plotted, and the affinity for binding of SLP-76SH2 to phosphorylated CD6 (CD6P) was calculated from the fitted curve. The affinity of SLP-76 SH2 for 662F was estimated by fixing the maximum binding at 19 RU to allow for there being more 662F on the chip (D) than CD6P (maximum, 14 RU). (C and D) The level of phosphorylation and amount of CD6 on the chip were quantitated by binding of phosphotyrosine and CD6 (OX124) mAbs. (E and F) Human CD6, CD2, CD5, or SLP-76 (SLP) were immunoprecipitated from Brij-96 lysates of human T blasts stimulated with CD3 mAb for 0, 2, or 5 min. Phosphorylated proteins (E) or SLP-76 (F) were detected by blotting with a pTyr mAb or a SLP-76 mAb, respectively. Arrows mark CD6 (~130 kDa) and SLP-76 (~68 kDa).

as 662Y, we produced cells expressing a 489F mutant and a double mutant in which both 662Y and 489Y were mutated to F, 489F/662F.

In this system, human CD6 would be engaged by mouse CD166 on APC. It has been shown that human CD6 binds mouse CD166 (43). To establish how effective this cross-species binding would be in the antigen-presentation experiments, we measured the affinity of human CD6 for mouse CD166 by surface plasmon resonance in the same way as we measured the interaction between human CD6 and human CD166 previously (19). A series of concentrations of human CD6 were passed over immobilized mouse CD166 (Fig. 4A). Calculation of the equilibrium-binding dissociation constants of human CD6 binding mouse and human CD166 at 37°C were the same

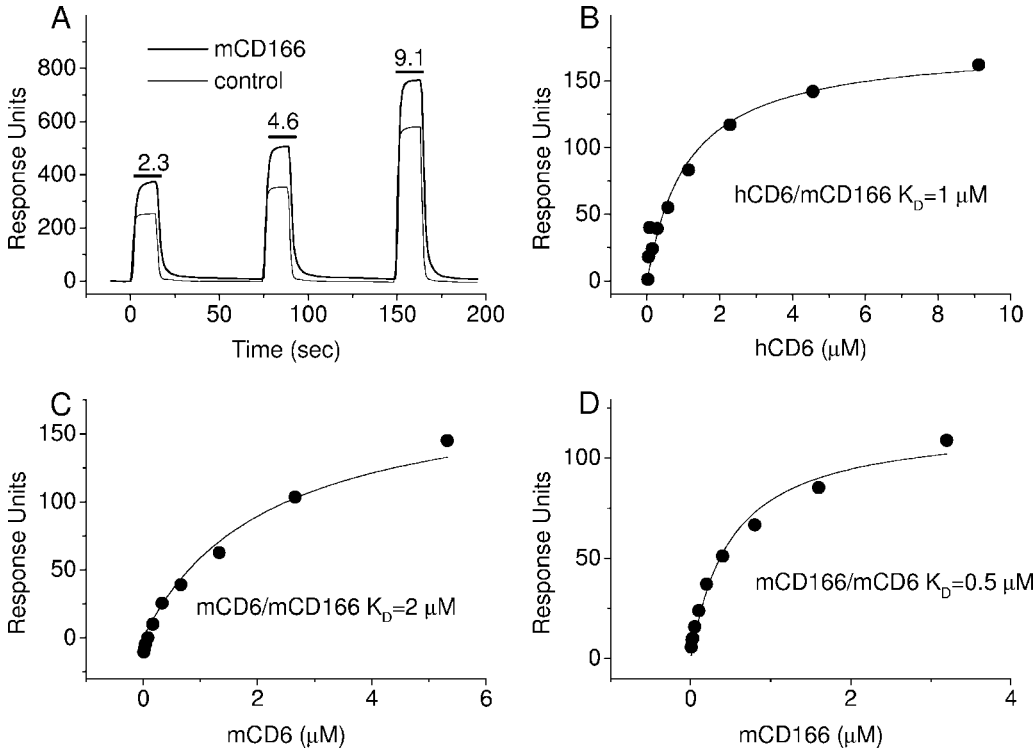

FIG. 4. Human CD6 (hCD6) and mouse CD6 (mCD6) bind mouse CD166 with the same affinity, a K_D of ~1 μM at 37°C. (A) Representative data for different concentrations (μM) of monomeric human CD6CD4d3 + 4 injected at 37°C over mouse CD166CD4d3 + 4-biotin, human CD166CD4d3 + 4-biotin (not shown), and a negative control, CD4d3 + 4-biotin, immobilized on a BIAcore chip. (B) Analysis of equilibrium binding from the experiment for panel A. (C and D) Data derived as for panels A and B for mouse CD6-CD4d3 + 4 injected over mouse CD166-CD4d3 + 4-biotin (C) and mouse CD166-CD4d3 + 4 over mouse CD6-CD4d3 + 4-biotin (D).

(Fig. 4B and data not shown). 2B4 cells express mouse CD6, so the consequences of engaging endogenous mouse CD6 could also be investigated in this model. Mouse CD6 was tested for binding to immobilized mouse CD166 (Fig. 4C) and vice versa (Fig. 4D). The mouse CD6/CD166 interaction had an affinity similar to that of the human CD6/CD166 interaction, a K_D of ~1 μM at 37°C (19).

Human CD6 and the mutants were expressed at similar levels on the hybridoma cells (Fig. 5A). The levels were estimated to be ~10-fold higher than that of mouse CD6 on the same cells by mAb staining (Fig. 5A) and mouse CD166 bead binding (data not shown). The levels of mouse CD3 were the same on the parental and the human CD6-expressing cells (Fig. 5A). Mouse CD166 was highly expressed on the APCs and not on the T-cell hybridoma cells (Fig. 5A). The ratio of irradiated spleen cells, the source of APC to T cells, was titrated (Fig. 5B). At a suboptimal APC-to-T-cell ratio, 2.5:1, cells expressing increased levels of wild-type human CD6 produced less IL-2 than the parental cells. At higher APC-to-T-cell ratios, IL-2 production by cells expressing wild-type human CD6 often exceeded that by the parental cells (Fig. 5C and data not shown). This would be consistent with the activation of cells overexpressing CD6 being dependent on a specialized APC present in low frequency in the APC preparation. With the higher APC-to-T-cell ratio, sensitivity to antigen was maintained, with cells expressing human CD6 producing as much as or more IL-2 than parental cells in response to a range of concentrations of antigen (Fig. 5C). IL-2 production showed the same trend at 12 h as at 24 h, with no evidence of IL-2 consumption until 48 h (data not shown).

Compared with the wild-type human CD6, cells expressing 662F showed a reduced response to antigen (Fig. 5B and C), consistent with CD6-dependent costimulation being compromised by mutation of 662Y to F, which would prevent phosphorylation-dependent recruitment of SLP-76 to the C-terminal region of CD6. Mutations of both 489Y and 662Y to F resulted in decreased IL-2 production, whereas the single mutation of 489Y to F did not (Fig. 5B and C), consistent with 489Y not recruiting a positive regulator such as SLP-76. The behavior of 662F, the double mutant 489F/662F, and 489F is consistent with the interaction between CD6 and SLP-76 being critical for costimulation by CD6. The double mutant consistently showed less of a decrease than 662Y, suggesting that 489Y may be important for a negative effect of CD6.

The effect of increased levels of CD6 in the hybridoma cells is comparable with the effect of upregulation of accessory T-cell proteins in T-cell development. Once thymocytes are positively selected and become mature T cells, the requirement for tight regulation of their proliferative capacity is increased. This correlates with increased expression of regulatory accessory proteins CD5 and CD2 (40). To test whether mouse CD6 is also upregulated in a similar manner, as has been shown for human CD6 (36), we compared expression of CD6, CD5, and CD2 on mouse thymocytes expressing low and high levels of TCR. As expected, increased expression of all three receptors correlated with TCR levels (Fig. 5D).

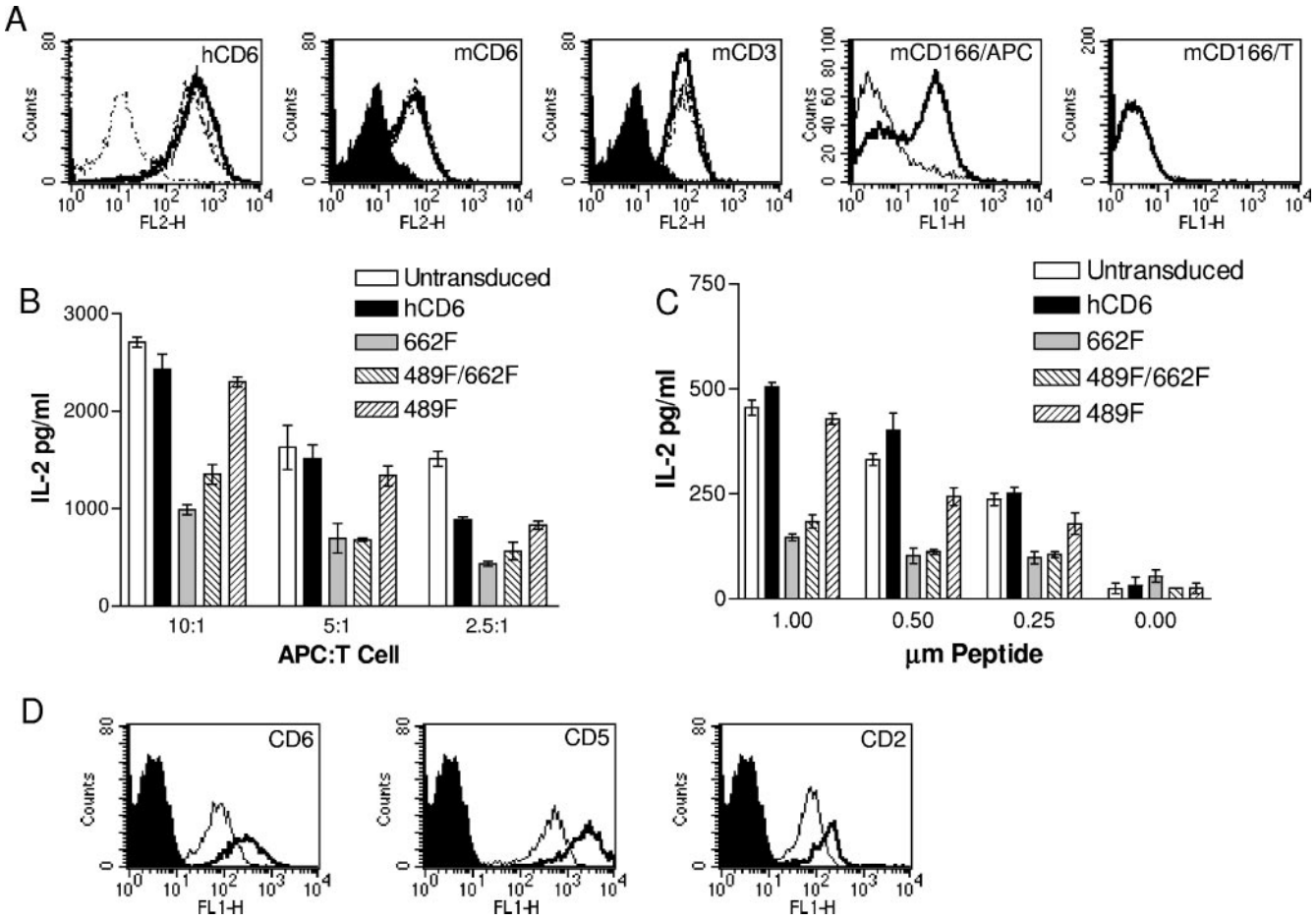

FIG. 5. Costimulation of T-cell responses by CD6 is dependent on 662Y. (A) Human CD6 (thick line) and mutants, 662F (thin line), 489F (dotted line), and 489F/662F (dashed line) (CD6 mAb, T.12) are expressed at equivalent levels in 2B4 T-cell hybridoma cells and not on the parental cells (dashed-dotted line). Staining of CD6 mAb on untransduced cells was superimposable with a negative control (data not shown). Murine CD6 and CD3 relative to a negative control mAb (filled histogram) were expressed at similar levels on CD6 and mutant hybridoma cells. CD166 as detected by binding of mouse CD6 beads was highly expressed on APC and not on the T-cell hybridoma cells. FL1-H and FL2-H denote fluorescence intensity. (B and C) Antigen-specific IL-2 production by 2B4 hybridoma cells untransduced or expressing human CD6 or mutants in response to 1 μM mcc peptide and varying ratios of APC to T cells (B) and the APC-to-T-cell ratio of 10:1 with varying mcc peptide concentrations (C). (D) Mouse thymocytes were double labeled with phycoerythrin-anti TCRβ and CD6, CD5, or CD2 mAbs. CD6, CD5, and CD2 expression was increased on TCR-high (thick line) compared with TCR-low (thin line) thymocytes.

CD6 costimulation is dependent on recruitment by CD166. Experiments with the hybridoma cells expressing human CD6 and mutants revealed a role for the cytoplasmic region of CD6 in regulating responses. Thus, costimulation by CD6 depends on both extracellular and intracellular engagement. To test the dependence of the response in this model on CD6/CD166 engagement, we compared responses when CD6 was available or not to bind CD166. As expected, antigen-specific IL-2 production by untransduced 2B4 hybridoma cells was sensitive to inhibition by soluble CD6 (Fig. 6A). The response of cells expressing the additional human CD6 was also sensitive to inhibition by soluble CD6, but to a lesser extent, reflecting the higher levels of CD6 at the cell surface. The reduced response by cells expressing 662F was not sensitive to inhibition by soluble CD6, showing that the decreased response is due to loss of signaling effects in 662F cells rather than extracellular engagement, which would be the same as for cells expressing wild-type human CD6.

CD6 costimulation is dependent on recruitment of SLP-76. The SLP-76/CD6 interaction requires CD6 to be phosphorylated. We had shown that human CD6 and the mutant 662F could be phosphorylated in the murine hybridoma cells in response to pervanadate treatment (Fig. 3). If the recruitment of SLP-76 to the C-terminal 662Y is critical for costimulation by CD6, which is not lost in 489F, it was important to know that 489F could be phosphorylated. We analyzed the phosphorylation status of CD6 and mutants in the hybridoma cells in response to pervanadate treatment. Phosphorylated human CD6 was immunoprecipitated specifically from NP40 lysates of pervanadate-treated cells expressing human CD6 and mutants but not from untransduced cells (Fig. 6B, upper panel). This experiment showed that CD6 can be phosphorylated on tyrosine residues other than 662Y and 489Y, suggesting that additional activation-dependent interactions of the CD6 cytoplasmic region can occur. A separate nonreduced gel showed that CD6 was expressed at similar levels in all

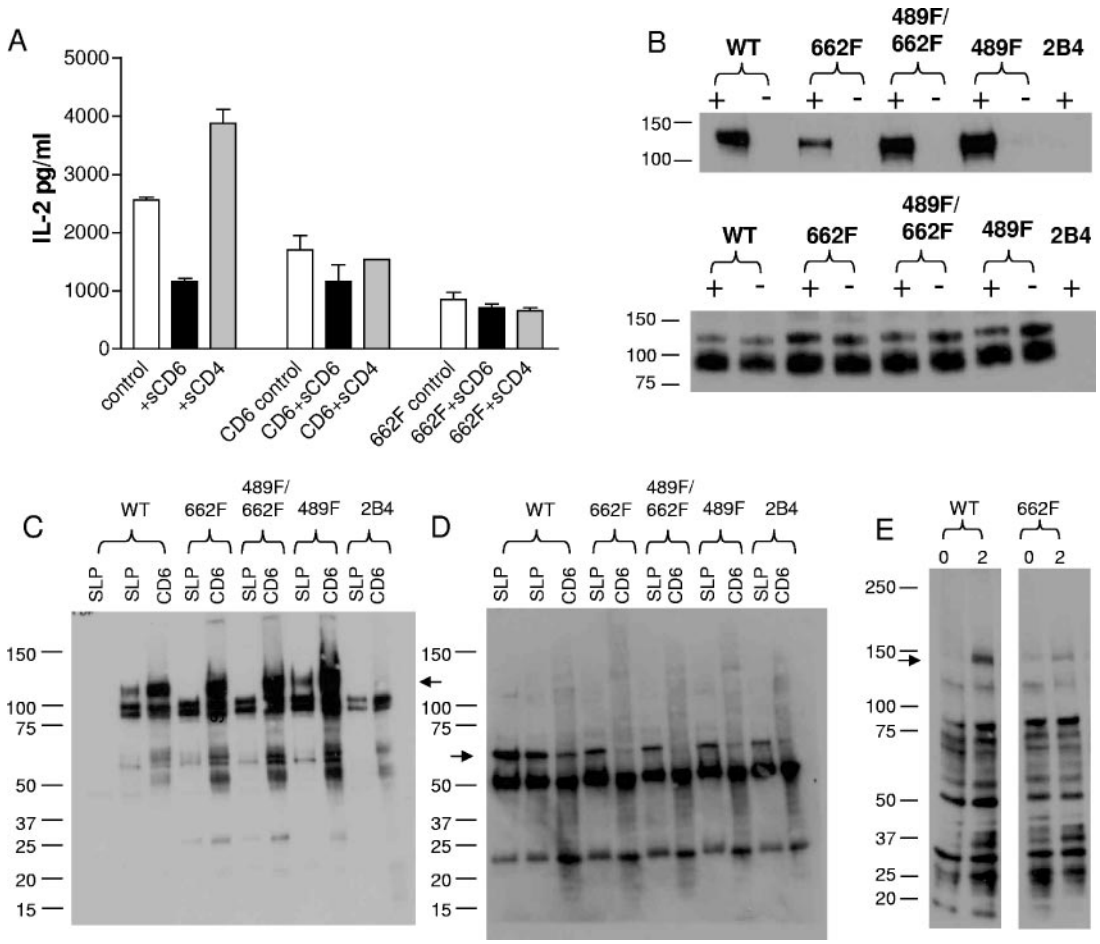

FIG. 6. CD6 costimulation is dependent on CD166/CD6 and CD6/SLP-76 interactions. Antigen-specific (1 μM peptide) IL-2 production by 2B4 hybridoma cells at 24 h at the APC-to-T-cell ratio of 10:1, untransduced or expressing hCD6 or 662F in the presence of soluble hCD6-CD4d3 + 4 (sCD6; 6 μM) (A) or control soluble rat CD4d3 + 4 (srCD4; 6 μM) (B). (Upper panel). Human CD6 and mutants can be phosphorylated in mouse T-cell hybridoma cells. Human CD6 was immunoprecipitated from NP-40 lysates of 2B4 hybridoma cells expressing wild-type (WT) human CD6 and mutants or untransduced (2B4) treated or not with pervanadate (indicated by + or −) and analyzed by Western blotting with a pTyr mAb. (Lower panel) Human CD6 was detected in total lysate by blotting with CD6 mAb under nonreducing conditions. The lower band most likely represents incompletely processed human CD6. (C and D) Coprecipitation of CD6 and SLP-76 is dependent on 662Y. Human CD6 was immunoprecipitated with SLP-76 mAb or CD6 mAb as indicated above each lane from Brij-96 lysates of 2B4 hybridoma cells expressing human CD6 and mutants or untransduced 2B4 cells treated or not (first lane) with pervanadate and analyzed by Western blotting with a pTyr mAb (C) or a SLP-76 mAb (D). (E) Receptor-mediated phosphorylation of CD6 was dependent on 662Y. Human CD6 was immunoprecipitated from NP-40 lysates of 2B4 hybridoma cells transduced with wild-type human CD6 or 662F stimulated with CD3 mAb for 0 or 2 min and analyzed by Western blotting with a pTyr mAb.

the cell lines (Fig. 6B, lower panel), corresponding to the cell surface analysis (Fig. 5A).

We tested the SLP-76 mAb for recognition of murine SLP-76. The SLP-76 mAb precipitated a phosphorylated band from cells corresponding to the molecular weight expected for CD6, ~130 kDa (Fig. 6C). Blotting with the SLP-76 mAb confirmed the presence of murine SLP-76 in the immunoprecipitate (Fig. 6D). As in human cells (Fig. 3E), SLP-76 immunoprecipitates from Brij-96 lysates of pervanadate-treated cells expressing human CD6 contained a band comigrating with CD6. By comparing SLP-76 and CD6 precipitates from cells expressing human CD6 and mutants, it was clear that a band comigrating with CD6 was coprecipitated by the SLP-76 mAb specifically from CD6 and 489F cells, precisely the forms of CD6 predicted to be able to bind SLP-76 (Fig. 6C). Blotting with SLP-76 confirmed the presence of SLP-76 in all the SLP-76 immunoprecipitates and that it was coprecipitated with CD6 and 489F but not 662F or 489F/662F.

It has been previously reported that truncation of CD6 at amino acid 661 resulted in reduced phosphorylation of CD6 in response to CD3 mAb cross-linking (22). We compared the effect of CD3 mAb cross-linking on human CD6 and 662F phosphorylation (Fig. 6E). Receptor-mediated phosphorylation of CD6 was reduced by the single-point mutation 662F. This confirms the importance of phosphorylation of 662Y in CD6 in response to CD3 stimulation.

DISCUSSION

Perturbation of the normal balance of interactions in an antigen-specific T-cell response by blocking a single interaction with a mAb specifically recognizing the CD166 ligand binding

domain of CD6 revealed the costimulatory effect of the CD6/CD166 interaction. This reinforced earlier experiments in which soluble monomeric CD6 effectively blocked IL-2 production (19). Blocking with a CD6d3 mAb shows that the CD166/CD6 interaction is important for costimulation by CD6. As expected from the conservation of CD6 structure and distribution, the CD6/CD166 interaction also regulated T-cell responses in a murine model. In an antigen-specific T-cell hybridoma model, the positive role of CD6 in T-cell activation was revealed. Increasing the level of expression of CD6 in a T cell made the cell less responsive unless it was optimally engaged by antigen-presenting cells expressing CD166. Sensitivity to antigen was retained once costimulation was optimal (Fig. 5). That the level of IL-2 production by cells expressing human CD6 did not greatly exceed that of parental cells may be due to domain 1 of human CD6 failing to mediate a species-specific interaction with a putative murine ligand. Interestingly a recent report describes a blocking effect of a CD6d1 mAb (49), providing further evidence for an additional ligand for CD6 domain 1. Interactions of CD5 domain 1 are species specific (2; A. Johnstone, N. J. Hassan, A. N. Barclay, and M. H. Brown, unpublished data).

The discovery of a positive regulator binding to the cytoplasmic region of CD6 is in keeping with CD6 recruitment being essential for an optimal immune response (15, 19). The interaction between CD6 and SLP-76 is the first direct activation-dependent interaction to be described for the large cytoplasmic tail of CD6 (Fig. 1) and provides a mechanism for linking CD6 engagement by CD166 or other ligands to T-cell regulation through the well-characterized adaptor SLP-76. SLP-76 was the only protein isolated in pulldown experiments from cell lysates with the peptide containing the DDYDDI motif at the extreme C terminus of CD6. This sequence fits with the motifs in ADAP and HPK1, which are the other proteins which bind the SH2 domain of SLP-76 with the consensus, (E/D)(D/V)YDD(V/I) (13, 27, 35). We compared the affinity of the SLP-76 SH2 domain for the phosphorylated CD6 peptide with the interaction between the SHIP SH2 domain and a phosphorylated FcRγIIb immunoreceptor tyrosine inhibitory motif peptide, a previously established interaction with functional consequences (29). A K_D of ~ 0.5 μM for both the SHIP/FcRγIIb and SLP-76/CD6 interactions was consistent with the latter being within the physiological range and indicative of a functionally relevant interaction. Together with our studies on CD200R (G. Brooke, S. Scheuringer, N. J. Hutchings, M. H. Brown, and A. N. Barclay, unpublished data) and CD244 (2B4) (N. G. Clarkson and M. H. Brown, unpublished data), these are the first measurements at physiological temperature for interactions between SH2 domains and phosphorylated peptides.

Affinity measurements have been reported for SH2 domains binding to phosphorylated peptides at 25°C and below (20, 23, 24, 31). The majority of values obtained were in the range of a K_D of ~ 0.1 to 1 μM. Although the difference in affinity between 25°C and 37°C is not always predictable, it is likely that there would be an increase in K_D if measurements were made at 37°C (46). The other common adaptor domain, SH3, also binds with a range of affinities (48). In the strongest interaction described, between an SH3 domain and a proline-rich motif, a peptide from SLP-76 bound the C-terminal SH3 domain of GADS at a K_D of 0.181 μM at 25°C (18), comparable with values discussed above for interactions between SH2 domains and phosphorylated peptides. An SH3 domain from CMS or CIN85 binds to a proline-rich motif from CD2 with a much lower affinity, a K_D of ~ 100 μM at 37°C, but in this case, other parts of CMS or CIN85 ensure these proteins are dimers, increasing the avidity (21).

Many proteins involved in signaling, such as SLP-76, bind a variety of proteins. Thus, quantitative analysis of each interaction is important to establish what interactions are likely to be functionally relevant. To assess the relevance of affinity measurements on short peptides to the interactions with native receptors, we developed a method using intact membrane protein by in situ purification of CD6 on the BIAcore chip and following binding of the SLP-76 SH2 domain to native CD6 at 37°C. Specific binding of SLP-76 SH2 to native CD6 was obtained with a K_D of ~ 0.2 μM at 37°C, comparable with measurements made on peptides. Binding by the SLP-76 SH2 domain to CD6 was dependent on phosphorylation of the C-terminal tyrosine motif of CD6. Coprecipitation data from normal cells provide evidence that CD6 directly links to the signaling complexes, which include SLP-76 and LAT. The coprecipitation data are also consistent with phosphorylated CD6 being a major binding partner for SLP-76 in T-cell activation. It is notable that SLP-76 is found at the periphery of the immunological synapse (10, 47), a region important for sustained signals (47). The CD166/CD6 interaction has also been shown to be important for sustaining T-cell proliferation (49). The predicted size of the CD166/CD6 complex (Fig. 1A) is consistent with a more peripheral position in the immunological synapse.

Mutation of 662Y compromised the costimulatory potential of CD6, and IL-2 production was reduced. This is consistent with phosphorylated 662Y recruiting SLP-76. Mutation of 489Y to F did not reduce IL-2 production under these conditions, consistent with weak binding of the phosphorylated 489Y peptide to SLP-76 not being physiologically relevant. 489F was able to bind SLP-76, consistent with SLP-76 recruitment being essential for the costimulatory effect of CD6. Similarly to published data for a mutant CD6 truncated at amino acid residue 661, phosphorylation of the single-point mutant 662F in response to CD3 mAb stimulation was reduced to below a level which would be expected for loss of one tyrosine phosphorylation site (22). SLP-76 recruitment appears to be important for phosphorylation of CD6. Thus, a failure to detect coprecipitation of SLP-76 with 662F in CD3 mAb-stimulated cells would not specifically be attributable to lack of binding to 662Y. However, coprecipitation of SLP-76 from pervanadate-treated cells revealed the potential of wild-type and 489F but not 662F or 489F/662F phosphorylated CD6 to bind SLP-76. It is not yet clear which tyrosine kinase phosphorylates CD6. ITK, which binds SLP-76 (45), was detected in CD6 immunoprecipitates (12).

In addition to binding SLP-76, an interaction between the C-terminal region of CD6 and syntenin, a scaffolding protein, has been reported (17). There is evidence that 662Y affects binding of syntenin, and it has been suggested that the interaction may be controlled by phosphorylation (17). It will be of interest to compare the binding properties of SLP-76 and syntenin for the C-terminal region of CD6 (17) and determine

whether a constitutive interaction with syntenin is important in the negative effects of CD6 in the absence of extracellular engagement. A model of activation-independent and -dependent interactions being important in the mechanism of costimulation by CD6 is applicable to other T-cell surface proteins expressed on resting cells. Constitutive or activation-independent interactions may generally act as scaffolds and/or link to the cytoskeleton. There is a constitutive interaction between CD2 and the cytoskeleton through the adaptor proteins CD2AP (called CMS in humans) and CIN85 through the acting capping protein, CAPZ. Kinetic analyses in living cells revealed that the link with actin is necessary for formation of clusters of CD2 but not for their maintenance (14). As with CD6, in the absence of extracellular engagement expression of CD5 (4) and CD2 (25, 28, 44) in cells reduces the level of activation in the absence of extracellular engagement. Increased positive selection in mice lacking CD5 (39) or CD2 (40) was accentuated in mice lacking both CD2 and CD5 (40). These effects can be explained by increased dysregulation due to the absence of constitutive activation-independent interactions of their cytoplasmic regions. Thymocytes from CD5-deficient mice were more readily stimulated than mature CD5-deficient T cells, consistent with an increased regulation of responses through accessory receptors which are upregulated during positive selection. Accessibility of a proline-rich motif in CD3ε, which binds the adaptor Nck, an adaptor protein, after activation (32), may be influenced by an adjacent NPXY motif. Release from cytoskeletal control of activation may increase the mobility of CD3ζ and/or aid in recruitment of signaling components at the site of cell-cell contact.

In conclusion, dissection of the functions of T-cell surface proteins needs to take account of the effects of extracellular and intracellular interactions as shown by these studies on CD6. The affinities of extracellular and intracellular interactions are in the micromolar range, providing a mechanism for dynamic interchange both at the cell surface and within the cell.

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AUTHOR'S CORRECTION

CD6 Regulates T-Cell Responses through Activation-Dependent Recruitment of the Positive Regulator SLP-76

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Volume 26, no. 17, p. 6727–6738, 2006. Page 6729, column 1, line 13: "DA rats" should read "BALB/c mice."

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AUTHOR CORRECTION

Second Correction for Hassan et al., "CD6 Regulates T-Cell Responses through Activation-Dependent Recruitment of the Positive Regulator SLP-76"

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Volume 26, no. 17, p. 6727–6738, 2006, https://doi.org/10.1128/MCB.00688-06. Recent data (R. F. Santos, L. Oliveira, M. H. Brown, and A. M. Carmo, unpublished data) have revealed that a labeling error caused the specificities of the OX124 and OX126 antibodies to be incorrectly defined; therefore, the changes listed below are needed.

"OX124" should read "OX126" and "OX126" should read "OX124" throughout.

Page 6728, Fig. 1 legend, lines 4 to 7: "CD6d3" should read "CD6" (six places).

Page 6729, column 1, lines 11 and 12: "domain 3 (CD6d3)" should be deleted.

Page 6729, column 1, lines 13 to 17: "CD6d3" should read "CD6" (four places). Note that a previous correction to line 13 (https://doi.org/10.1128/MCB.00499-09) changed "DA rats" to "BALB/c mice."

Page 6736, column 1, line 4: "CD6d3" should read "CD6."

These changes do not impact the overall conclusions of the paper.

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