

Cloning of ACP33 as a Novel Intracellular Ligand of CD4*

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CD4 recruitment to T cell receptor (TCR)-peptide-major histocompatibility class II complexes is required for stabilization of low affinity antigen recognition by T lymphocytes. The cytoplasmic portion of CD4 is thought to amplify TCR-initiated signal transduction via its association with the protein tyrosine kinase p56^{lck}. Here we describe a novel functional determinant in the cytosolic tail of CD4 that inhibits TCR-induced T cell activation. Deletion of two conserved hydrophobic amino acids from the CD4 carboxyl terminus resulted in a pronounced enhancement of CD4-mediated T cell costimulation. This effect was observed in the presence or absence of p56^{lck}, implying involvement of alternative cytosolic ligands of CD4. A two-hybrid screen with the intracellular portion of CD4 identified a previously unknown 33-kDa protein, ACP33 (acidic cluster protein 33), as a novel intracellular binding partner of CD4. Since interaction with ACP33 is abolished by deletion of the hydrophobic CD4 C-terminal amino acids mediating repression of T cell activation, we propose that ACP33 modulates the stimulatory activity of CD4. Furthermore, we demonstrate that interaction with CD4 is mediated by the noncatalytic α/β hydrolase fold domain of ACP33. This suggests a previously unrecognized function for α/β hydrolase fold domains as a peptide binding module mediating protein-protein interactions.

The cell surface glycoprotein CD4 is expressed on subsets of thymocytes and mature T lymphocytes and, in humans, on monocytes and macrophages. Early clues to CD4 function came from a strong correlation between CD4 expression and MHC^I class II restricted T helper cell activity that corresponds to the MHC class I-mediated cytotoxic response of CD8-positive T cells. Subsequent studies using targeted gene disruption in mice and antigen-dependent *in vitro* T cell activation assays implicated an important role of CD4 in T helper cell development and activation (1, 2).

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¹ The abbreviations used are: MHC, major histocompatibility complex; TCR, T cell receptor; IL, interleukin; HIV, human immunodeficiency virus; IRES, internal ribosomal entry sequence; MBP, maltose-binding protein; DAMP, N-(3-((2,4-dinitrophenyl)amino)propyl)-N-(3-aminopropyl)methylamine dihydrochloride; LAT, linker for activation of T cells; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; cIg, cytoplasmic immunoglobulin; sIg, surface immunoglobulin.

The molecular mechanism of CD4 function is based on its direct interaction with invariant regions of MHC class II molecules on ACPs (3), hence stabilizing low affinity TCR-peptide-MHC complexes. To attain maximum T cell costimulation, CD4 must be specifically recruited to antigen-engaged TCR molecules (4). This selective recruitment is thought to be mediated by the cytoplasmic domain of CD4, which in turn is associated with the protein-tyrosine kinase p56^{lck} (5, 6). In this context, the phosphotyrosine-binding Src homology 2 domain of Lck has been shown to be sufficient for this interaction (7) and involves phosphorylated and TCR-associated ZAP-70 as a ligand (8). In addition to its adaptor function, p56^{lck} is thought to amplify TCR-dependent signal transduction by phosphorylation of phosphotyrosine adapter molecules, immunoreceptor tyrosine-based activation motifs of CD3 chain and the activation loop of ZAP-70 (9). Thus, both extracellular and cytosolic domains of CD4 are required for optimal coreceptor function. This bifunctional activity might account for the variable requirements for CD4 costimulation by different antigen-dependent T cell lines (10).

In contrast to its stimulatory role during antigen-dependent T cell activation, CD4 transmits inhibitory signals to T cells when engaged independently of the TCR complex, e.g. by the proposed natural CD4 ligands IL-16 (11) and gp17 (12). Preincubation with IL-16 has been shown to counteract mitogen- or TCR-induced activation of human peripheral T lymphocytes (13, 14). Since gp120 treatment of T lymphocytes leads to induction of anergy or apoptosis (15), binding of gp120 to CD4 is thought to contribute to the depletion of CD4-positive T cells in HIV-infected patients. Similarly, antibody-mediated CD4 clustering results in inhibitory signals that render T cells unresponsive to subsequent stimulation by TCR engagement (16–18). The latter finding is especially relevant for therapeutic approaches involving administration of anti-CD4 antibodies *in vivo* for the treatment of autoimmune syndromes, delayed type hypersensitivity reactions, and allograft rejection. The molecular basis for the inhibitory CD4 signaling is not known but has recently been shown to be, to some extent, independent of p56^{lck} under different conditions. For instance, anti-CD4 antibodies block ongoing antigen-induced T cell activation in cell lines expressing a CD4 mutant unable to associate with p56^{lck}, indicating that sequestration of Lck can only partially account for CD4-mediated inhibition of IL-2 production (19). Moreover, HIV gp120-induced apoptosis of a lymphoblastoid T cell line did not require p56^{lck} signaling (20), and in Lck-deficient cell lines HIV replication was nevertheless blocked by anti-CD4 antibodies directed against the CDR3-like region (21, 22). Therefore, it was proposed that inhibitory signal transduction by CD4 is mediated by alternative ligands of the CD4 cytoplasmic domain (23).

These results prompted us and others to investigate CD4-dependent signal transduction during T cell activation in more detail. Interestingly, the phosphotyrosine adaptor molecule

linker for activation of T cells (LAT) has recently been identified as an alternative CD4 cytoplasmic tail ligand and might contribute to CD4-mediated stimulation of TCR signaling (24). In the present study, we focused on the hydrophobic C terminus of CD4 and discovered that it contains a negative regulatory determinant of T cell activation. Moreover, we identified a novel CD4-interacting protein, ACP33, as a candidate molecule for mediating negative signaling by the hydrophobic C terminus of CD4.

MATERIALS AND METHODS

DNA Constructs—Full-length cDNA coding for ACP33 was isolated by a yeast two-hybrid screen using a transactivator fusion protein cDNA library in conjunction with a LEXA fusion of the cytoplasmic tail of human CD4 (amino acids 399–433), essentially as described before (25). The coding region for ACP33 was amplified by PCR and subcloned into the cIg fusion protein expression vector p5C7 (26). Mutagenesis of the cIg-ACP33 construct was performed by polymerase chain reaction to obtain a substitution of serine 109 by alanine.

Full-length cDNAs of human CD4 and p56^{lck} were obtained from Brian Seed (Harvard University) and subcloned into *Hind*III and *Not*I restriction sites of the mammalian expression plasmid pN1 (CLONTECH). Truncation mutants of CD4 and a double point mutation changing cysteines 420 and 422 to alanines were generated by replacing the *Bpu*1102I–*Not*I fragment of pCD4-N1 with polymerase chain reaction products.

For generation of stable transgenic cells, a new vector, pEF-IRES-puro, was constructed by inserting the CD4 cDNA, an internal ribosome entry site (IRES) (27) and a puromycin resistance gene into the pEF-BOS expression cassette (28).

Cell Lines and Antibodies—Jurkat E6, JCaM1.6, HUT 78, and HeLa cells were purchased from the American Type Culture Collection and maintained in RPMI containing 10% fetal calf serum and 10 µg/ml gentamicin. COS-7 cells were obtained from Brian Seed and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 10 µg/ml gentamicin. The antigen-dependent murine T cell line 171 was generously provided by Mark Hill and Dan Littman (New York University). Culture conditions for 171 and the respective FT7.1 antigen-presenting cells were as described (29).

Monoclonal antibodies directed against ACP33 were generated by immunizing Lou/C rats with a purified glutathione *S*-transferase fusion protein of full-length ACP33 expressed in bacteria. After an 8-week interval, a final boost was given 3 days before fusion of the rat spleen cells with the murine myeloma cell line P3X63-Ag8.653 (30). Hybridoma supernatants were tested in an enzyme-linked immunosorbent assay using bacterial extracts from *Escherichia coli* expressing a fusion protein of ACP33 and maltose-binding protein (MBP) or a control MBP fusion protein. Hybridoma supernatants reacting with ACP33-MBP but not with the control MBP fusion protein were analyzed by Western blots, and clone it1–2D5 (rat IgG2a) was selected for its reaction pattern.

Mouse monoclonal antibodies against p56^{lck} (Lck-01 and Lck-04) were generously provided by Vaclav Horejsi (Czech Academy of Science). Monoclonal anti-CD4 antibody MT-151 was donated by Peter Rieber (University of Dresden, Germany). Polyclonal anti-CD4 antiserum T4-4 (31) was provided by Raymond Sweet (SmithKline Beecham Pharmaceuticals), through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health. All secondary anti-IgG reagents were purchased from Jackson ImmunoResearch.

T Cell Activation Assays—Transgenic CD4 expressing 171 murine T cells were generated by electroporation of 10 µg of pCD4-EF-IRES-puro plasmid or mutant derivatives into 1 × 10⁷ cells. After a 48-h cultivation, transfected cells were selected for resistance to 2 µg/ml puromycin (Biomol) for 10–12 days. Expression levels of CD4 were monitored by immunofluorescence and Western blotting. T cell activation assays were performed essentially as described (29). Increasing concentrations of peptide antigen, an analog of hen egg lysozyme 74–88, were mixed with 10⁶ transgenic 171 T cells and 5 × 10⁵ FT7.1 ACPs in a final volume of 300 µl of Iscove's modified Dulbecco's medium. After 24 h, secreted levels of IL-2 were quantified by enzyme-linked immunosorbent assay (OptEIA-Set, BD Pharmingen).

Procedures for transient transfection of JCaM1.6 cells and measurement of luciferase activity were described before (26). Briefly, 10 µg of pIL2-GL2 reporter plasmid and 25 µg of pCD4-N1 or mutant derivatives were cotransfected by electroporation. After 20 h, cells were stimulated for 8 h with ionophore A23187 (0.5 µg/ml) or phorbol 12-myrist-

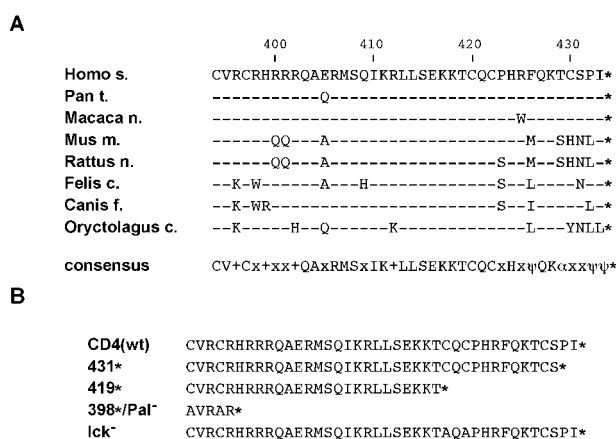


FIG. 1. Alignment and mutants of the CD4 cytoplasmic domain. A, amino acid alignment of mammalian CD4 cytoplasmic domains. Residues identical to human CD4 are dashed. In the consensus sequence, conserved positively charged residues are indicated by +, alcoholic residues by α, hydrophobic residues by ψ, and nonconserved residues by x. B, schematic representation of CD4 cytoplasmic domain mutants. *Homo s.*, *Homo sapiens*; *Pan t.*, troglodytes; *Macaca n.*, *Macaca nemestrina*; *Mus m.*, *Mus musculus*; *Rattus n.*, *Rattus norvegicus*; *Felis c.*, *Felis catus*; *Canis f.*, *Canis familiaris*; *Oryctolagus c.*, *Oryctolagus cuniculus*.

tate 13-acetate (50 ng/ml) or were left untreated, followed by the addition of reporter lysis buffer (Promega) and scintillation counting.

Subcellular Fractionation and Immunoprecipitation—HUT 78 cells were transiently transfected by electroporation using 20 µg of plasmid expressing cIg-ACP33 or a cIg control. Subcellular fractionation was performed as previously described (32). Briefly, cells were lysed by passing through a 26-gauge needle in hypotonic lysis buffer and centrifuged for 10 min at 20,000 × g. The supernatant corresponded to the cytoplasmic fraction and the soluble material from the pellet extracted by detergent lysis buffer, and further centrifugation was called the particulate fraction.

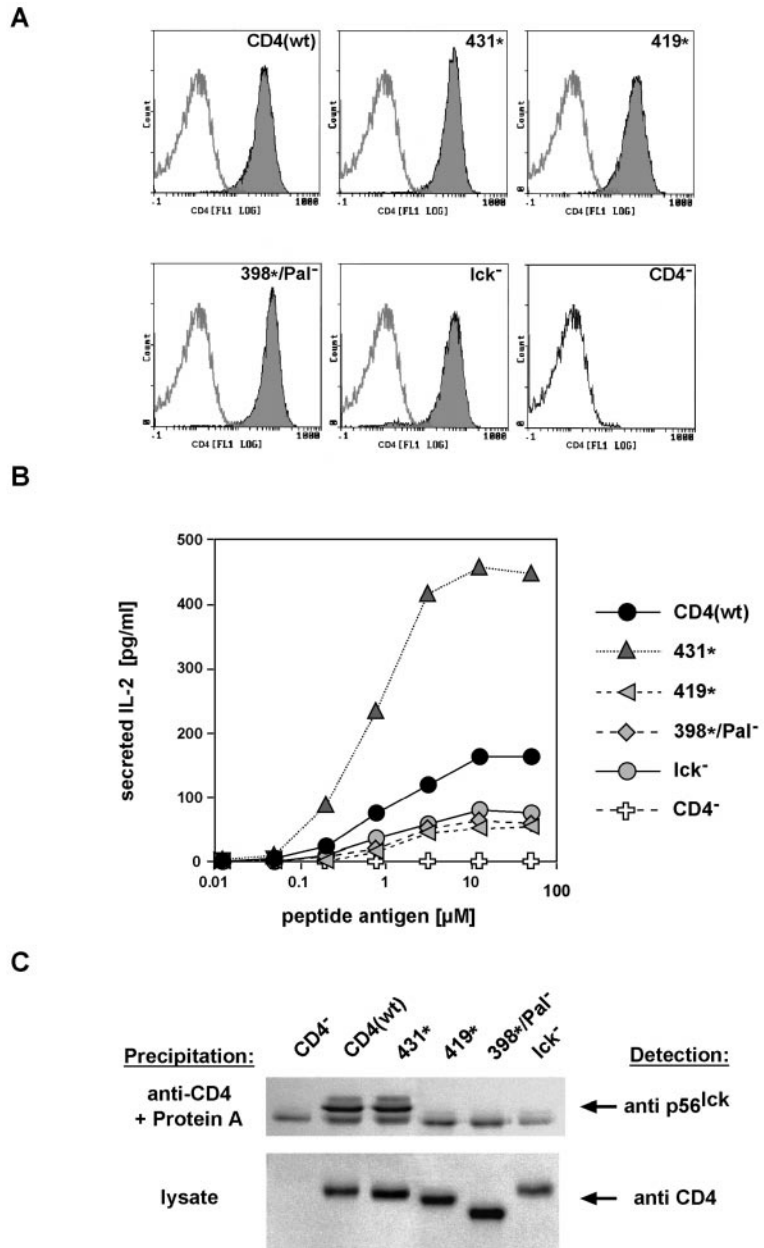
Immunoprecipitation was performed from lysates of 171 cells, electroporated HUT 78 cells, and COS-7 cells transfected by DEAE-dextran as described before (26). Lysis buffer contained 1% Nonidet P-40, 20 mM Tris (pH 7.5), 150 mM NaCl, 2 mM EDTA, 10 µg/ml leupeptin, 5 µg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride. After 30 min on ice, lysates were cleared at 20,000 × g and the supernatant was incubated for 1 h with 1 µl of antiserum for immunoprecipitation of CD4 or, in case of precipitation of cIg fusion proteins, was directly incubated for 30 min with protein A immobilized on Sepharose 6MB (Sigma). Beads were washed three times in lysis buffer, and bound proteins were released by boiling in SDS-loading buffer. Samples were resolved by SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting.

Immunofluorescence—To detect intracellular antigens, HUT 78 cells were placed on poly-L-lysine-coated coverslips in PBS for 30 min at 4 °C and fixed for 15 min with 2% (w/v) freshly prepared paraformaldehyde in PBS at 25 °C. Fixed cells were blocked by washing three times for 5 min with 2% (w/v) glycine in PBS and permeabilized for 15 min in 0.2% SDS, which was found to increase reactivity of anti-ACP33 mAb 2D5. Unspecific binding was blocked by washing three times for 5 min with 2% fetal calf serum in PBS. Endogenous ACP33 was stained by incubation with undiluted 2D5 hybridoma supernatant for 60 min and washing three times for 5 min with 2% fetal calf serum in PBS followed by detection with FITC-conjugated anti-rat IgG. An isotype-matched control antibody directed against a non-T cell protein did not result in significant fluorescence.

Where indicated, cells were incubated with rhodamine-conjugated wheat germ agglutinin, transferrin, or DAMP (all from Molecular Probes, Inc., Eugene, OR). Endogenous CD4 was detected using T4-4 antiserum and rhodamine-conjugated anti-rabbit IgG. Total rabbit IgG was used as a control. Staining of cIg fusion proteins was achieved using FITC-conjugated anti-human IgG. Microscopical imaging was done on a Leica TCS confocal laser microscope. FITC and rhodamine signals for a single confocal section were recorded separately and overlaid using Adobe Photoshop software.

For extracellular CD4 staining, cells were incubated with anti-CD4 mAb MT-151 and FITC-conjugated anti-mouse IgG and analyzed by flow cytometry (Coulter Epics XL).

FIG. 2. Functional analysis of CD4 mutants in antigen-dependent T cell activation. A, expression of CD4 mutants in transgenic pools of the CD4 negative murine T cell line 171 analyzed by flow cytometry. B, antigen-induced IL-2 production by 171 cell lines expressing mutated CD4 molecules. Indicated are average values of three independent experiments with S.D. less than 15%. Removal of two hydrophobic amino acids from the COOH terminus of CD4 (431*) results in a gain-of-function phenotype compared with native CD4 (*wt*), whereas further truncations impair IL-2 production. C, co-precipitation of p56^{lck} with CD4 mutants from stably transfected 171 cells. The indicated band represents p56^{lck}, and the lower band present in all lanes presumably results from cross reactivity with the precipitated anti-CD4 rabbit antiserum. Amounts of p56^{lck} coprecipitating with native CD4 or the 431* mutant are comparable.



RESULTS

Truncation of the Hydrophobic COOH Terminus of CD4 Results in a Gain-of-function Phenotype—The CD4 cytoplasmic domain is highly homologous among its mammalian orthologues (Fig. 1A). Conserved residues include the membrane-proximal cysteines 394 and 397, which can be palmitoylated (33), a short α -helix between amino acid 409 and 414, which mediates binding of the AP-2 and AP-1 adaptor protein complexes (34), and a double cysteine motif (position 420 and 422) required for interaction with p56^{lck} (35). We noticed an additional conserved motif consisting of two hydrophobic amino acids at the carboxyl terminus: either proline and isoleucine, leucine and isoleucine, or two leucines. Interestingly, hydrophobic COOH termini of transmembrane receptors have been implicated in mediating protein-protein interactions, e.g. with PDZ domain-containing proteins involved in signal transduction, multimerization, and subcellular sorting events (36, 37).

To analyze the functional role of the conserved hydrophobic C-terminal residues of CD4 with respect to antigen-dependent T cell activation, we used the CD4-negative murine T cell line 171, previously established as a model system for CD4 function

(7, 29). Oligoclonal pools of 171 cells expressing either native human CD4 or truncated mutant derivatives (Fig. 1B) were selected and found to display comparable amounts of CD4 on the cell surface (Fig. 2A). T cell activation was induced by adding increasing concentrations of peptide antigen, and secreted IL-2 was quantified by enzyme-linked immunosorbent assay. Consistent with earlier findings (29), expression of CD4 was required for measurable IL-2 production by 171 cells (Fig. 2B). Deletion mutants of CD4 clearly showed different influences on costimulatory function. Truncation of only two COOH-terminal amino acids (431*) led to a pronounced gain-of-function phenotype. However, further deletion (419*, 398*/Pal⁻) or double point mutations in the cysteine motif required for Lck binding (lck⁻) clearly resulted in reduced IL-2 secretion by the respective transfectants, confirming that p56^{lck} makes a significant contribution to stimulatory CD4 signal transduction (29). These results were highly reproducible; experiments with three independently derived oligoclonal cell populations yielded very similar functional data (not shown). In contrast to results obtained by Glaichenhaus *et al.* (29), expression of a truncation mutant removing the entire cytoplasmic domain of CD4 (398*/

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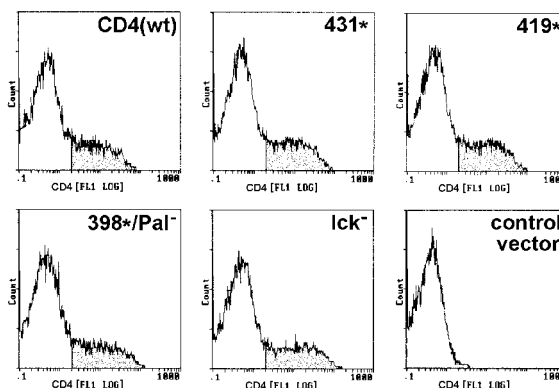
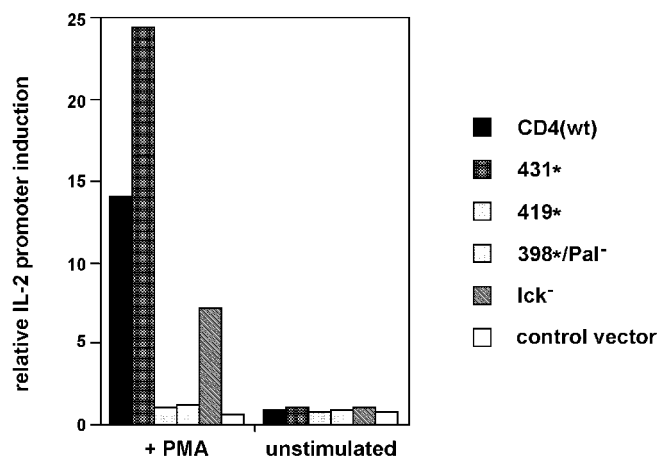


FIG. 3. Transient expression of CD4 induces stimulation of the IL-2 promoter in $p56^{lck}$ -deficient JCaM1 cells. A, CD4 surface expression in transiently transfected JCaM1 cells. B, IL-2 promoter induction in JCaM1 cells expressing CD4 was quantified by luciferase activity. Indicated are average luciferase activities relative to unstimulated, native CD4 expressing transfectants. Results are from triplicate experiments with S.D. values less than 20%. Expression of the truncation mutant 431* results in gain of function.

B



Pal⁻) resulted in measurable IL-2 secretion. This difference might be due to higher CD4 expression levels in our system. It has been shown that surface expression of tailless CD4 at high levels is able to circumvent the T cell development requirement for signal transduction by the cytoplasmic domain of CD4 (38).

Since gain-of-function mutants of CD4 have not been described before, we were interested in further characterizing this effect. One potential explanation for the 431* mutant phenotype implicates increased $p56^{lck}$ activity. However, association of $p56^{lck}$ with the CD4 mutant 431* was unaltered compared with native CD4 as analyzed by coimmunoprecipitation (Fig. 2C). As expected from previous reports (35), no anti- $p56^{lck}$ reactive band was detected in CD4 precipitates of 419*, 398*/Pal⁻, and Lck⁻-expressing cell lysates. Nevertheless, the gain-of-function phenotype of 431* could still be mediated by $p56^{lck}$ activity, so to exclude this possibility we analyzed CD4 function in the Lck-deficient Jurkat E6 derived cell line JCaM1 (39). Moreover, since 171 T cells are of murine origin, we also wanted to confirm the 431* phenotype in a human assay system.

The Phenotype of the 431* Truncation Mutant Is Independent of $p56^{lck}$ —The JCaM1 cells we used here to study Lck-independent CD4 function did not express detectable levels of $p56^{lck}$, in contrast to normal Jurkat cells (data not shown). Expression vectors for native CD4 or several CD4 mutants were transiently transfected into JCaM1 cells together with a

luciferase reporter plasmid that allowed quantification of IL-2 promoter activity. Expression levels were monitored by flow cytometry and found to be within a comparable range (Fig. 3A). To our surprise, transfection of native CD4 was sufficient to significantly induce IL-2 promoter activity in phorbol ester-costimulated JCaM1 cells without further cross-linking (Fig. 3B). The Lck⁻ CD4 mutant had a slightly reduced effect on phorbol 12-myristate 13-acetate-induced IL-2 promoter induction, whereas the 419* and 398*/Pal⁻ mutants were completely ineffective. These results indicate the existence of Lck-independent CD4 signal transduction in JCaM1 cells, presumably mediated by alternative ligands for the CD4 cytoplasmic domain, as discussed below. Strikingly, cotransfection of the 431* truncation mutant resulted in a moderate but consistent increase in IL-2 promoter activity compared with native CD4, resembling the gain-of-function phenotype in antigen-dependent T cell activation described above.

Taken together, removal of the hydrophobic amino acids proline and isoleucine from the COOH terminus of the CD4 cytoplasmic domain results in enhanced T cell costimulation in two independent assay systems. The presence of $p56^{lck}$ is neither required nor inhibitory for the gain-of-function phenotype of CD4 431*, suggesting the existence of an alternative mechanism involving novel mediators of CD4 function.

Cloning of ACP33 as a CD4-binding Protein—A yeast two-hybrid screen of a Jurkat T cell cDNA library was performed to

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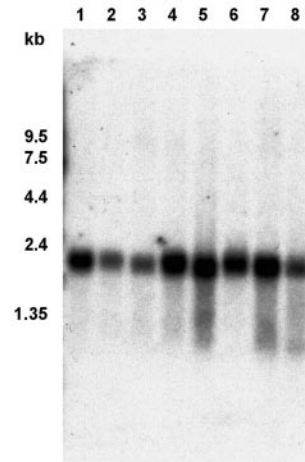
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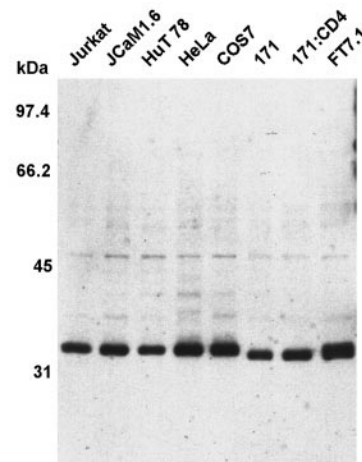
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FIG. 4. Sequence and expression pattern of ACP33. A, amino acid sequence of human ACP33. The acidic cluster at the N terminus is underlined. A predicted structural element conserved in α/β fold bacterial hydrolases surrounding serine 109 is highlighted. B, human multiple tissue Northern blot analysis of ACP33 mRNA expression. Lane 1, poly(A)⁺ RNA prepared from heart; lane 2, brain; lane 3, placenta; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; lane 8, pancreas. The amount of poly(A)⁺ RNA has been adjusted by the manufacturer (CLONTECH Laboratories) to obtain an equal β -actin signal in each lane. kb, kilobases. C, Western blot analysis of ACP33 protein expressed in various human and murine cell lines. For detection, hybridoma supernatant of the anti-ACP33 rat mAb 2D5 and a secondary goat anti-rat IgG peroxidase conjugate were used. Jurkat E6, JCaM1.6, and HUT 78 are human lymphoblastoid T cell lines, HeLa is a human epitheloid, COS-7 a simian fibroblastoid, 171 a murine T cell line, and FT7.1 a murine L cell fibroblast derivative.



C



identify alternative CD4 cytoplasmic tail binding partners. Several selected clones were analyzed, and all were found to contain an identical cDNA insert. To identify its translation initiation site, we isolated a further 250 nucleotides of this cDNA by polymerase chain reaction from a plasmid library. This revealed that the two-hybrid isolate contained the complete open reading frame for a protein of 308 amino acids. The deduced protein does not contain any known subcellular localization signals, leader sequence, or transmembrane helix. However, a cluster of four acidic amino acids is located close to the N terminus (Fig. 4A). Blast homology searches (40) of protein data banks revealed identity with the recent GenBankTM entry NP_057714, a hypothetical protein encoded by a bone marrow-derived mRNA. A very limited sequence similarity to bacterial enzymes containing an α/β hydrolase fold was also detected (41). Similarity is most significant within the region surrounding the catalytic nucleophilic residue of this class of hydrolases. In α/β fold hydrolases, the nucleophilic residue is part of a structural element known as the nucleophile elbow (42), which

comprises a β strand, a γ -like turn containing the nucleophilic residue, and an α helix. Secondary structure predictions for the region of similarity in the novel CD4-interacting protein indicate that a motif surrounding serine 109 might adopt a similar structural fold. Furthermore, homology searches in protein domain consensus data bases (e.g. Pfam, Prosite profiles, ESTHER) detected significant similarity between the newly identified protein and the family of α/β hydrolase fold proteins.

Multiple tissue Northern blot hybridization with a cDNA fragment from the CD4-binding protein detected a single mRNA species of 2.1 kilobases in length (Fig. 4B). All human tissues analyzed contained detectable and comparable amounts of this transcript. To address protein expression and localization of the novel CD4 binding factor, we generated monoclonal antibodies against the complete polypeptide expressed in *Escherichia coli*. One clone, mAb 2D5, specifically detected a protein of 33 kDa in immunoblotted detergent lysates of a variety of human and murine cell lines (Fig. 4C). Furthermore, the antibody reacts with several fusion proteins

of the novel CD4-binding protein, indicating that it specifically recognizes an epitope of this protein (data not shown). We propose the term ACP33 (acidic cluster protein of 33 kDa) to denote this new factor.

Subcellular Localization of ACP33—To test if subcellular distribution of ACP33 is compatible with a functional interaction with CD4, we analyzed the intracellular localization of the mAb 2D5 epitope in a CD4-positive T cell line, HUT 78. As an additional control, we expressed an ACP33 fusion protein (cIg-ACP33) by transient transfection, the two NH₂-terminal immunoglobulin domains from human IgG1 serving as an epitope for standard anti-human IgG reagents (Fig. 5A). Chimeric proteins containing Ig domains have been successfully used to study subcellular localization (43, 44), protein-protein interaction (26), and protein function (25). We employed a simple biochemical cell fractionation protocol with hypotonic cell lysates and analyzed ACP33 distribution in the soluble cytosol and the particulate fraction. The latter primarily consists of membrane- and cytoskeleton-associated proteins. As described previously (32), the cIg control protein is localized in the cytosolic fraction (Fig. 5B). In contrast, a membrane-resident derivative of the Ig fusion domain (sIg control) containing a leader sequence and transmembrane helix is totally confined to the particulate fraction. Both endogenous and transiently expressed cIg-ACP33 was evenly distributed with approximately equimolar amounts in both fractions, indicating that ACP33 is a cytosolic protein, which partially associates with particulate cellular structures.

To characterize these particulate structures, we studied the *in situ* subcellular localization of ACP33 by immunofluorescence in HUT 78 cells. Endogenous ACP33 was analyzed using mAb 2D5 and secondary anti-rat IgG fluorescein conjugates, while anti-human IgG reagents were used to detect cIg-ACP33 and cIg control proteins. Both endogenous and transfected cIg-ACP33 were partially localized in the cytosol but also accumulated on an intracellular vesicular compartment (Fig. 5C), whereas the control protein was uniformly distributed throughout the cell interior.

ACP33 Colocalizes with CD4 on the Endosomal/trans-Golgi Network—Using immunofluorescence, we compared localization of endogenous ACP33 relative to established cellular markers in HUT 78 cells. Wheat germ agglutinin is a lectin that preferentially stains the Golgi apparatus in most cell types (45). Overlay of the wheat germ agglutinin-rhodamine signal with the anti-ACP33 mAb 2D5 staining showed only very limited overlapping immunofluorescence (Fig. 6c). In contrast, the vesicular structures marked by ACP33 partially coincided with both rhodamine-labeled transferrin (Fig. 6f), which is confined to the early endosomal recycling pathway (46), and with acidic organelles (Fig. 6i) stained by the acidotropic dye DAMP (47). We therefore conclude that endogenous ACP33 is partitioned between the cytosol and vesicles of the endosomal/trans-Golgi network.

In unstimulated T cells, CD4 is distributed between the plasma membrane and the endosomal recycling pathway. T cell activation, however, induces rapid endocytosis and rerouting of CD4 to lysosomes. HUT 78 represents a partially activated T cell line expressing the activation molecules Ia and the IL-2 receptor (48). When analyzed by immunofluorescence using a highly specific antiserum against human CD4, the majority of CD4 molecules were localized to intracellular vesicles, and only a minor fraction resided in the plasma membrane (Fig. 6k). Importantly, the intracellular fraction of CD4 molecules colocalized to a large extent with endogenous ACP33 (Fig. 6l), supporting a potential physical and functional interaction of these molecules.

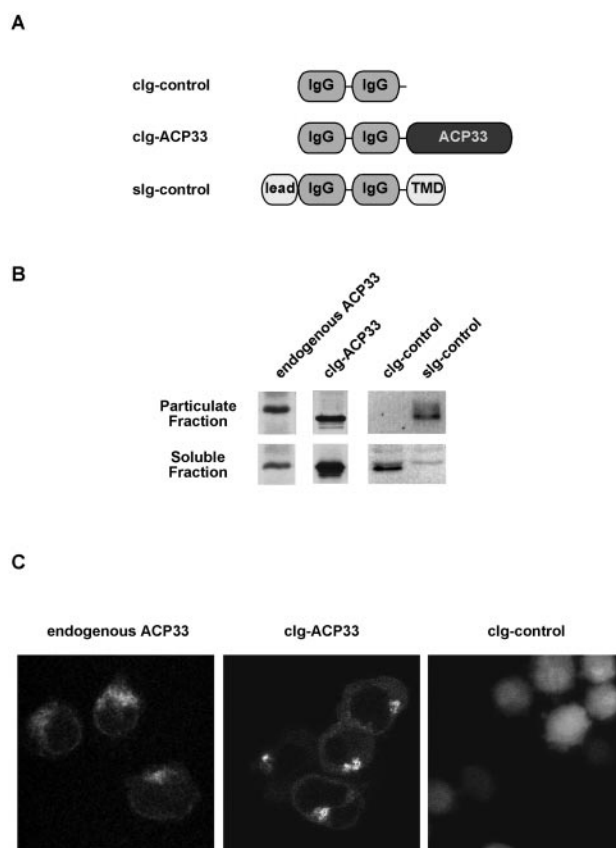


Fig. 5. Subcellular distribution of endogenous and transiently expressed chimeric ACP33 in HUT 78 T cells. A, schematic drawing of Ig fusion proteins. B, fractionation of hypotonic lysates of transfected HUT 78 cells into the soluble cytosol and the particulate (*i.e.* membrane and cytoskeletal) fraction. Endogenous and transfected cIg-ACP33 is distributed between both fractions. C, immunofluorescence detection of endogenous ACP33 (stained by mAb 2D5 and FITC anti-rat IgG), chimeric cIg-ACP33, and cIg control (detected by FITC anti-human IgG) in HUT 78 cells. ACP33 is detected in the cytosol and on intracellular vesicles, whereas cIg is evenly distributed throughout the cell.

CD4 Coprecipitates with cIg-ACP33—Since it was found that anti-ACP33 monoclonal antibody mAb 2D5 precipitates endogenous ACP33 poorly, the Ig fusion protein cIg-ACP33 was used for immunoprecipitations to analyze interactions with endogenous CD4. Ig fusion proteins were purified from detergent lysates of transfected HUT 78 cells and analyzed by immunoblotting using a polyclonal human CD4 antiserum. Endogenous CD4 specifically coprecipitated with cIg-ACP33 but not with cIg control protein (Fig. 7A). However, the stoichiometry of the interaction seems rather low with respect to the CD4 levels present in the total cell lysate (Fig. 7A, lane 3). This low stoichiometry might reflect physiological levels of a transiently formed complex or might be due to a high off-rate during precipitation.

Mutational Mapping of the CD4-ACP33 Complex—To determine the molecular requirements for CD4-ACP33 complex formation, we analyzed ACP33 binding to the various truncation mutants of CD4 described above. Moreover, we investigated the CD4 binding potential of a point mutant of ACP33 generated by replacing serine 109 by alanine (Fig. 7B). Serine 109 is likely to be part of the structural element resembling the nucleophile elbow of α/β fold hydrolases (42). Mutated proteins were expressed in transiently transfected COS-7 cells, and any physical interaction was determined by coprecipitation. Similar to the results obtained in T cells, native CD4 specifically coprecipitated with cIg-ACP33 but not the cIg control protein (Fig. 7C, lanes 1 and 3). Under these conditions, however, the

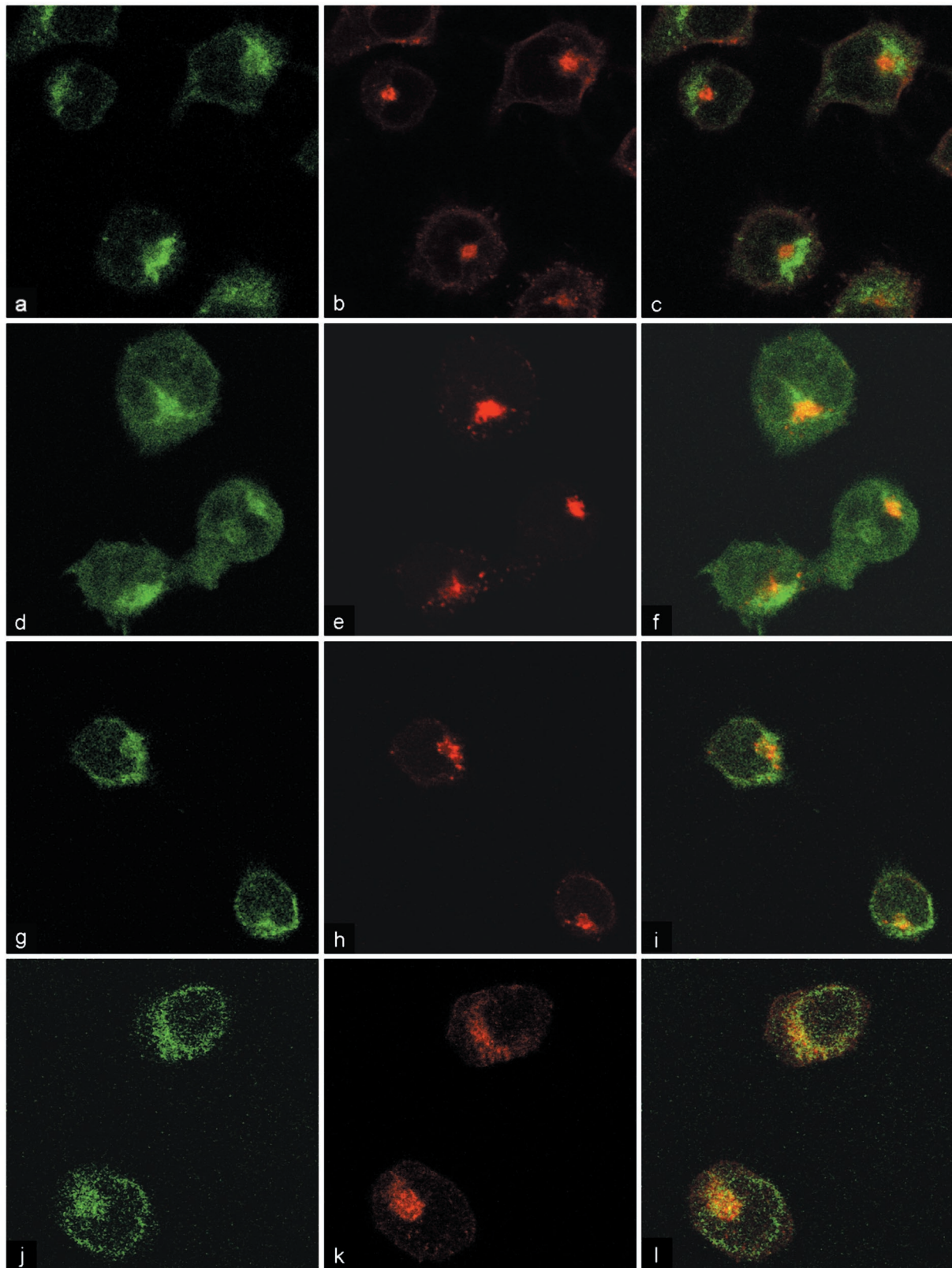


FIG. 6. ACP33 partially colocalizes with CD4 on TGN/endosomal vesicles. Endogenous ACP33 was detected by immunofluorescence in HUT 78 cells using mAb 2D5 and FITC anti-rat IgG (panels *a*, *d*, *g*, and *j*). Golgi vesicles were marked by wheat germ agglutinin-rhodamine (*b*), early endosomal vesicles by transferrin-rhodamine (*e*), late endosomal/lysosomal vesicles by DAMP and anti-DNP-rhodamine (*h*), and CD4 by anti-CD4 antiserum and rhodamine-conjugated anti-rabbit IgG (*k*). Overlays of the two preceding panels are shown in *c*, *f*, *i*, and *l*, respectively.

stoichiometry of the CD4 coprecipitation with cIg-ACP33 was markedly higher than in T cell lysates. Interestingly, the S109A point mutation in cIg-ACP33 completely abolished de-

tectable interaction with CD4 (lane 2). On the other hand, removal of the last two amino acids from the C terminus of CD4 (431*) was sufficient to prevent coprecipitation with cIg-ACP33

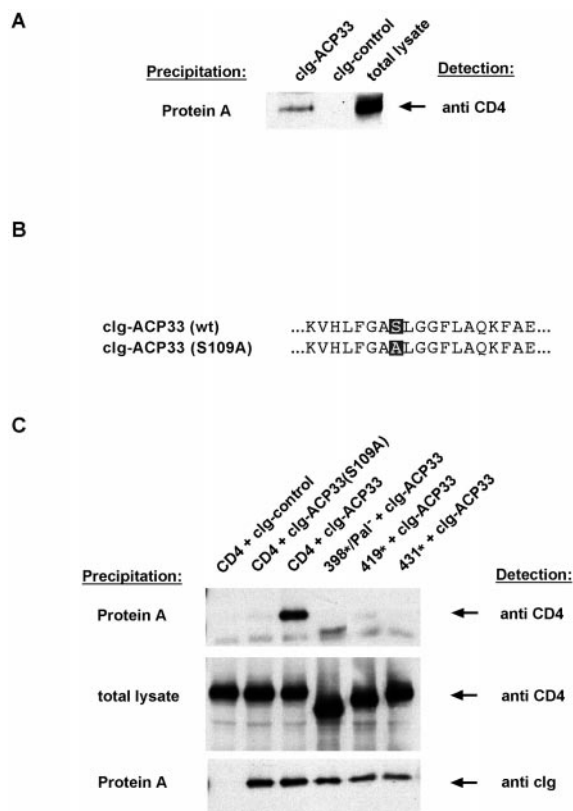


FIG. 7. Mutational analysis of CD4-ACP33 interaction by co-precipitation. A, endogenous CD4 coprecipitates with cIg-ACP33 but not with cIg control from lysates of transiently transfected HUT 78 cells. B, amino acid sequence surrounding serine 109 in cIg-ACP33 and cIg-ACP33 S109A. C, CD4 coprecipitates with cIg-ACP33 from lysates of transfected COS-7 cells. Coprecipitation is prevented by the S109A mutation in cIg-ACP33 and by truncation of two amino acids (431*) or more from the CD4 COOH terminus. In the *second panel*, expression of CD4 mutants is shown to be comparable; the *third panel* documents even expression levels of cIg-ACP33 and cIg-ACP33 S109A.

(lane 6). Logically, further C-terminal truncation mutants of CD4 also failed to interact with cIg-ACP33 (lanes 4 and 5). This indicates that the integrity of the hydrophobic amino acids proline and isoleucine at the COOH terminus of CD4 are essential for ACP33 binding. This interaction also requires a critical serine residue in ACP33. Therefore, these findings demonstrate a noncatalytic peptide-binding function for the α/β hydrolase fold domain of ACP33. A nonenzymatic protein recognition function has previously only been suggested for α/β hydrolase fold domains that are part of the extracellular portion of cell-cell-adhesion receptors, e.g. neuroligin (49–51).

Simultaneous Interaction of ACP33 and p56^{lck} with CD4—To determine whether ACP33 interferes with binding of p56^{lck} to the CD4 cytoplasmic tail or possibly interacts with p56^{lck} directly, we coexpressed CD4, p56^{lck}, and cIg-ACP33 in COS cells. Lck efficiently coprecipitated with ACP33 via the cIg-ACP33-CD4 complex (Fig. 8A, lane 1), but no direct interaction between p56^{lck} and cIg-ACP33 could be detected. As expected, neither p56^{lck} nor the truncated CD4 molecules were detected in cIg-ACP33 immunoprecipitates from cells expressing the CD4 431* C-terminal deletion mutant (lane 2). Equally, interaction with the p56^{lck}-CD4 complex was undetectable when cIg-ACP33 was replaced by the S109A substitution mutant (lane 4). Moreover, comparable amounts of CD4 coprecipitated with cIg-ACP33 in the presence or absence of p56^{lck} (lanes 1 and 3, *second panel*), suggesting that ACP33 and p56^{lck} can interact independently and simultaneously with CD4 (Fig. 8B). We therefore conclude that ACP33 is a likely candidate for

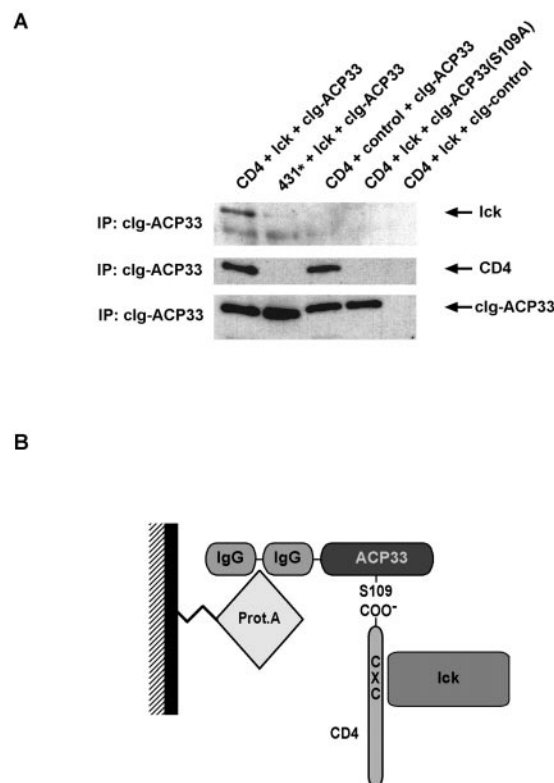


FIG. 8. cIg-ACP33 and p56^{lck} simultaneously interact with CD4. A, COS cells were cotransfected with expression vectors for cIg-ACP33, CD4, and p56^{lck}. Following immunoprecipitation (IP) of cIg-ACP33, p56^{lck} is detectable if coexpressed with native CD4, but not with the 431* CD4 truncation mutant unable to associate with ACP33. B, schematic drawing of the complex formed by cIg-ACP33, CD4, and p56^{lck}.

the observed modulation of CD4 function via an Lck-independent mechanism.

DISCUSSION

CD4 has been previously shown to exhibit a complex pattern of signal transduction, i.e. to mediate stimulatory or inhibitory signals after extracellular ligand binding (2). In this report, we identified a negative regulatory determinant in the cytoplasmic tail of CD4, which specifically interacts with a novel CD4-binding protein, ACP33. Using a well established antigen-dependent model system, we analyzed C-terminal truncation mutants of CD4 (Fig. 1) and showed that the negative regulatory determinant in the CD4 cytoplasmic tail depends on the integrity of two hydrophobic amino acids at its C-terminal end (Fig. 2). This finding was confirmed in an independent functional assay for CD4 employing the JCaM1 cell line, which is deficient in p56^{lck} expression.

Unexpectedly, CD4 exhibited a costimulatory effect on JCaM1 T cell activation in the absence of p56^{lck}, suggesting the involvement of a previously unknown positive regulatory determinant. This determinant resides between amino acids 431 and 419 of the CD4 cytoplasmic tail but is only partially dependent on cysteines 420 and 422, since the Lck⁻ mutant retained ~50% of native CD4 activity (Fig. 3). It has been previously suggested that CD4 associates with alternative binding partners in Lck-negative cell types, e.g. with other Src family kinases in monocytes (52). A recently described alternative ligand for the CD4 and CD8 cytoplasmic tails is the phosphotyrosine adaptor protein LAT, which competes with p56^{lck} for CD4 and CD8 binding in T cells (24). Intriguingly, substitution of the double cysteine motif in CD8 reduced but did not abrogate association of LAT, implicating LAT as a candidate

molecule responsible for the observed CD4-mediated signal transduction in JCaM1 cells.

Irrespective of the mode of CD4-mediated IL-2 promoter induction in these two functional assays, the stimulatory signal was negatively modulated by the hydrophobic carboxyl terminus of CD4. Therefore, its deletion resulted in both cases in a gain-of-function phenotype. Since the 431* mutant of CD4 completely lacks detectable association with ACP33 (Figs. 7 and 8), its gain-of-function phenotype clearly correlates with association of ACP33 but not with that of p56^{lck}. Moreover, ACP33 and CD4 colocalize on intracellular vesicles (Figs. 5 and 6), supporting the plausibility of a functional interaction.

As yet, we cannot discriminate between whether ACP33 actively transduces an independent negative regulatory signal or modulates a positive signal, *i.e.* by increasing CD4 internalization and degradation. In the latter case, deletion of the hydrophobic C-terminal amino acids would lead to a prolonged duration of signal transduction, resulting in the gain-of-function phenotype of the 431* mutant CD4. This possibility is supported by the subcellular localization of ACP33, the majority of which is recruited to the cytoplasmic face of endosomal/trans-Golgi vesicles (Fig. 6). CD4 internalization and/or degradation was closely monitored in phorbol 12-myristate 13-acetate-stimulated, stably transfected 171 cells, but so far we were unable to detect any difference between localization or half-life of CD4 and the 431* mutant. However, it is possible that binding of ACP33 is required for subtle changes in subcellular trafficking of CD4. Interestingly, HIV Nef-induced degradation of CD4 has recently been shown to depend on a diacidic motif in the Nef protein (53). Moreover, several other proteins contain clusters of acidic amino acids that are involved in protein sorting from endosomes to various cellular compartments, *e.g.* to lysosomes or to the plasma membrane. By interacting with the acidic clusters of furin and the cation-independent mannose 6-phosphate receptor, a family of cytosolic sorting proteins, termed PACS, mediate their cellular routing (54, 55). Since ACP33 contains a cluster of four aspartic acid residues, it might function by connecting CD4 to related cellular sorting factors, thus regulating the fate of internalized CD4. Endosomal CD4 is targeted to lysosomes (34), to glycolipid-enriched membrane compartments (56–58), or back to the plasma membrane, depending on the cellular activation status (59).

Interestingly, inhibition of T cell activation by CD4 antibodies (19) or HIV gp120 (60) was recently found to be p56^{lck}-independent, which implies that alternative CD4-associated proteins are actively transducing inhibitory signals under these conditions. We tried to analyze a potential inhibitory signaling function of ACP33 by overexpression studies, but we were unable to obtain cells that overexpress cIg-ACP33 more than 2-fold above the level of endogenous ACP33. Therefore, the mechanism of ACP33 mediated modulation of CD4 function could not be analyzed in detail.

Binding of ACP33 to CD4 depends on the ACP33 serine residue 109 (Fig. 7). This residue is predicted to be part of a structural element conserved in α/β fold hydrolases, suggesting that a protein-protein interaction module in ACP33 has evolved from an ancient enzymatic domain during molecular evolution. Examples for noncatalytic members of the α/β hydrolase fold-containing protein family have been described before (49). Neuroligin and related proteins, for example, contain a noncatalytic, acetylcholinesterase-like α/β hydrolase fold domain, which has been implicated in mediating heterophilic cell-cell adhesion (50, 51). However, our data demonstrate for the first time that this structural domain also serves as an intracellular protein-protein interaction module. Similar evo-

lutionary processes have been proposed for transcription factors (61) and phosphatase-like domains (62).

ACP33 is a ubiquitously expressed protein (Fig. 4), which implies that it interacts with alternative ligands in CD4-negative cell types. In the present study, however, we focused on a functional interaction of ACP33 and CD4 in T cells. We have evidence that the minimal interaction motif for ACP33 binding consists of certain combinations of paired hydrophobic, nonaromatic amino acid residues at the carboxyl terminus of polypeptides,² consistent with the motif conserved in mammalian CD4 cytoplasmic domains (Fig. 1).

Taken together, the presence of dissectable positive and negative regulatory determinants in the CD4 cytoplasmic tail confirms recently proposed models for T cell activation, in other words that integration of both stimulatory and repressive signals determine the outcome of T cell receptor stimulation (2, 10, 63). Since binding of ACP33 correlates with inhibition of CD4 function, we propose that ACP33 is a novel negative regulatory factor involved in CD4-dependent T cell activation.

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Cloning of ACP33 as a Novel Intracellular Ligand of CD4
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