The Ca^{2+}/Calmodulin-dependent Kinase Type IV Is Involved in the CD5-mediated Signaling Pathway in Human T Lymphocytes*

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The CD5 receptor on T lymphocytes is involved in T cell activation and T-B cell interactions. In the present study, we have characterized the signaling pathways induced by anti-CD5 stimulation in human T lymphocytes. In T lymphocytes, anti-CD5 co-stimulation enhances the phorothemagglutinin/anti-CD28-induced interleukin-2 (IL-2) mRNA accumulation 1.6-fold and IL-2 protein secretion 2.2-fold, whereby the up-regulation is mediated at both the transcriptional and post-transcriptional level. The CD5 signaling pathway up-regulates the IL-2 gene expression by increasing the DNA binding and transactivation activity of activator protein 1 but affects none of the other transcription factors like nuclear factor of activated T cells, nuclear factor kappa B, Oct, and CD28-responsive complex/nuclear factor of mitogen-activated T cells involved in the regulation of the IL-2 promoter activity. The CD5-induced increase of the activator protein 1 activity is mediated through the activation of calcium/calmodulin-dependent (CaM) kinase type IV, and is independent of the activation of mitogen-activated protein kinases Jun N-terminal kinase, extracellular signal-regulated kinase, and p38/Mpk2, and calcium/calmodulin-dependent kinase type II. The expression of a dominant negative mutant of CaM kinase IV in T lymphocytes transfected with an IL-2 promoter-driven reporter construct completely abrogates the response to CD5 stimulation, indicating that CaM kinase IV is essential to the CD5 signaling pathway. In addition, it is demonstrated that calcium/calmodulin-dependent kinase type IV is also involved in the stabilization of the IL-2 transcripts, which is observed after co-stimulation of phorothemagglutinin/anti-CD28 activated T lymphocytes with anti-CD5.

The CD5 receptor, expressed on the surface of T lymphocytes as well as on a subset of B lymphocytes, is a 67-kDa monomeric transmembrane glycoprotein that belongs to the scavenger receptor cysteine-rich family of extracellular domain-like structures (1–3). CD5 is associated with a receptor complex on the surface of T lymphocytes comprising the T cell receptor (TCR),

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¶ The abbreviations used are: TCR, T cell receptor; IL-2, interleukin 2; NFAT, nuclear factor of activated T cells; NFkB, nuclear factor kappa B; AP-1, activator protein 1; CD28RC, CD28-responsive complex; NF-MAT, nuclear factor of mitogen-activated T cells; MAP, mitogen-activated protein; JNK, Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; CaM, calcium/calmodulin-dependent; CaMK, CD3 and associated protein tyrosine kinases p56^{ck} and p59^{yn} and depends on this physical association for its functional activity (4–8). The counter-receptor of CD5 has been identified as CD72, a dimeric receptor consisting of a 42-kDa glycoprotein, which is commonly expressed on B lymphocytes (9). Recently, a novel inducible cell surface ligand of CD5, distinct from CD72, termed CD5L, has been identified on activated splenic B cells (10). A role has been proposed for CD5 in the regulation of the immune response through its involvement in the interactions between T and B lymphocytes and between different subsets of B lymphocytes (1, 10–13).

Co-stimulation of T lymphocytes through anti-CD5 antibodies has been shown to augment the intracellular calcium and cGMP levels (8, 14) and subsequently the interleukin-2 (IL-2) secretion and interleukin-2 receptor expression (2, 8, 15). The signal transduction routes used by CD5 to induce these elevations remain largely unknown. The cytoplasmic domain of CD5 possesses no intrinsic enzymatic activities; however, it does contain a potential tyrosine kinase phosphorylation motif (Y{X}{Y}{Y}) also present in the TCR z and CD3 chains (4, 11). This motif contains two tyrosine residues that can serve as docking sites for Src homology 2 domain containing proteins once they have been phosphorylated (11, 16). Upon engagement of the TCR/CD3 complex by ligand, the cytoplasmic domain of CD5 becomes rapidly phosphorylated on tyrosine residues similar to the TCR z chain (5, 11, 17). The protein tyrosine kinase p56^{ck}, which is associated with CD4 or CD8 (18), seems to be responsible for this phosphorylation. It has also been demonstrated that p56^{ck} binds to the cytoplasmic domain of CD5 through its Src homology 2 domain and becomes fully activated once bound to CD5, probably by autophosphorylation (11). Because the targets of p56^{ck} are unknown, it remains unclear how CD5 generates the increase of intracellular Ca^{2+}. It seems likely that p56^{ck} directly or indirectly activates an effector protein that will induce the activation of Ca^{2+}-specific ion channels in the membrane, resulting in the influx of Ca^{2+}, similar to the epidermal growth factor- and platelet-deriviated growth factor-induced Ca^{2+} influx, which is mediated through the small Ras-related GTPase Rac1 (19, 20).

To elucidate the signaling pathways activated by CD5, we analyzed the regulation of the IL-2 gene. The expression of the IL-2 gene is tightly controlled by the 300-base pair promoter (21), which contains several well defined binding sites for both calcium/calmodulin-independent kinase; PHA, phorothemagglutinin; MEK, MAPK/ERK kinase; CAT, chloramphenical acetyltransferase; GST, glutathione S-transferase; CREB, cAMP-responsive element binding protein; SRF, serum response factor; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; SB203580, 4-(4-fluoro-phenyl)-2-(4-methyl-sulfinylphenyl)-5-(4-pyridyl)-imidazole; KN-62, N,N'-bis-(5-isouquinolinesulfonyl)-N-methyl-t-tyrosyl-4-phenyl-piperazine; DTT, dithiothreitol; MOPS, 4-morpholinepropanesulfonic acid.
ubiquitous and T cell-specific transcription factors, including NFAT, NFκB, AP-1, Oct, and CD28RC/NF-MAT (22–24).

AP-1, which is a heterodimer composed of different fos and jun family members can bind to the IL-2 promoter alone or complexed with NFAT1 (25–27). The AP-1 activity is regulated both at the level of fos and jun gene transcription and by post-translational modifications of the fos and jun proteins. The MAP kinases Jun N-terminal kinase (JNK) and Fos-regulating kinase stimulate the transcriptional activity of cJun and cFos, respectively, by phosphorylation of the transcriptional activation domains (28–30). JNK is also involved in the transcriptional activation of the fos and jun genes, like several other kinases, including two other MAP kinases, extracellular signal-regulated kinase (ERK), and p38/MKp2, several members of the Janus kinase family, CaM (Ca\(^2+\)/calmodulin-dependent) kinases, and protein kinase A (31–38).

The AP-1 proteins are also involved in the regulation of the IL-2 promoter through the proximal Oct site. At this site, fos and jun family members cooperate functionally with different octamer proteins, like the ubiquitous factor Oct-1 and the lymphoid-specific factor Oct-2. It has been shown that the binding of fos and jun proteins to this site is induced via a signal transduction route involving the Ca\(^{2+}\)/calmodulin-dependent, cyclosporin A-sensitive phosphatase calcineurin (39–41). Our results in the present study demonstrate that CD5 signaling regulates the IL-2 gene expression through the AP-1 binding site in the IL-2 promoter and through stabilization of the IL-2 mRNA. Most interestingly, we found that the CD5 signaling pathway involves the activation of Ca\(^{2+}\)/calmodulin-dependent kinase type IV, which mediates the effects of the CD5 signal at the transcriptional level as well as at the post-transcriptional level.

**EXPERIMENTAL PROCEDURES**

**T Lymphocyte Isolation**—Human peripheral blood cell suspensions were obtained from healthy volunteer platelet donors, and mononuclear cell suspensions were prepared by Ficoll-Hypaque (Lymphoprep; Nycomed, Oslo, Norway) density gradient centrifugation. T lymphocytes were isolated by 2-aminothiazolium bromide-treated sheep red blood cell rosetting. The sheep red blood cells were lysed with 155 mM NH\(_4\)Cl, 10 mM KHCO\(_3\), 0.1 mM EDTA according to standard procedures. The remaining cell preparations contained more than 98% T lymphocytes as assessed by flow cytometry analysis after staining with a CD3 monoclonal antibody (Becton Dickinson, Mountain View, CA) and leucine-enkephalin-positive cells (Becton Dickinson). After isolation, T lymphocytes were kept overnight at 37 °C in RPMI 1640 medium (BioWhittaker, Verviers, Belgium) containing 2% fetal calf serum (FCS), HyClone, Logan, UT) supplemented with 2 mM l-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 6 μg/ml colistin.

**Stimulation**—Human T lymphocytes (5 × 10\(^6\)/ml) were incubated for various time periods with 2 μg/ml phytohemagglutinin (PHA; Sigma), in combination with a monoclonal antibody against CD19 (22) (a gift from Dr. R. van Lier, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) used at a final concentration of 5% hybridized immunoblasts or and a monoclonal antibody against CD5 (83-P2E6; MCA Development, Groningen, The Netherlands) also used at 5% hybridized calcium control supernatant and or a monoclonal antibody against CD5 (83-P2E6; MCA Development, Groningen, The Netherlands) used at a final concentration of 5% hybridized calcium control supernatant and/or a monoclonal antibody against CD5 (83-P2E6; MCA Development, Groningen, The Netherlands) also used at 5% hybridized calcium control supernatant. Anti-CD5 (WT32; Division of Clinical Immunology, University of Groningen, the Netherlands) was also used at a final concentration of 5% hybridized calcium control supernatant. Various inhibitors were added 30–60 min before stimulation: PD98059 (New England Biolabs, Beverly, MA), an inhibitor of MEK1, was used at a final concentration of 10 μM; SB203580 (a gift from Dr. J.C. Lee, SmithKline Beecham Pharmaceuticals, King of Prussia, PA), a p38/MKp2 inhibitor, was used at a final concentration of 1 μM; and KN-62 (Alexis Corporation, Laufelfingen, Switzerland), an inhibitor of CaM kinases, was used at a final concentration of 10 μM.

**Measurement of Secreted IL-2 Protein**—Human T lymphocytes (3 × 10\(^6\)/ml) were stimulated with PHA alone or PHA in combination with anti-CD28 for 24 h in the presence or the absence of anti-CD5 were also performed. Inhibitors were added 30 min before stimulation. Secreted IL-2 protein was quantified in cell-free supernatants using a human IL-2 ELISA kit (R & D Systems, Minneapolis, MN) as recommended by the manufacturer.

**RNA Preparation and Northern Blot Analysis**—Total cellular RNA from 2.5 × 10\(^7\) human T lymphocytes stimulated with PHA alone or PHA in combination with anti-CD28 in the presence or the absence of anti-CD5 for 6 h was isolated using the guanidium thiocyanate/cesium chloride method (42). KN-62 was added 1 h prior to stimulation. To determine the mRNA stability, half-life studies were performed by adding actinomycin D (Boehringer Mannheim) to a final concentration of 10 μg/ml after 6 h of stimulation of PHA and anti-CD28 in the presence of anti-CD5. 0, 30, 60, 90, and 120 min after the addition of actinomycin D, total cellular RNA of the T lymphocytes was isolated. 20-μg samples of total cellular RNA were size fractionated on 1.1% agarose gels with 2.2 M formaldehyde and blotted onto nylon membranes (Qiagen Nylon Plus, Qiagen, Chatsworth, CA) (43). cDNA probes were labeled with [α-32P]dCTP (3000 Ci/mmol, Amersham) using the oligolabeling kit (Pharmacia Biotech, Inc.) as recommended by the manufacturer. The following cDNA probes were used: 1) the 0.8-kilobase pair PouII insert of human IL-2 cDNA purified from the pGEM-T plasmid (a gift from Dr. E. G. E. de Vries, Division of Medical Oncology, University of Groningen, The Netherlands) and 2) the EcoRI-linearized pBR322 plasmid containing a 7.8-kilobase pair human 28 S cDNA insert. Hybridization was performed at 65 °C for 18 h in 0.1× Na\(_2\)HPO\(_4\), 7% SDS. Membranes were washed once in 2 × SSC, 0.1% SDS; once in 1 × SSC, 0.1% SDS; and finally in 0.3 × SSC, 0.1% SDS for 30 min at 65 °C. mRNA levels were quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and the ImageQuant software (Molecular Dynamics). mRNA levels were normalized with respect to the 28 S signal.

**Intracellular Calcium Measurements**—5 × 10\(^6\) T lymphocytes were incubated for 15 min with 2.5 μM Fluo-3/AM (Calbiochem, La Jolla, CA) and subsequently stimulated with either PHA plus anti-CD28 or PHA with anti-CD28 plus anti-CD5 in the presence or the absence of KN-62. KN-62 was added 1 h prior to stimulation. The fluorescence of Flu-3 was measured in time with a AMINCOBOWMAN® Series 2 Luminescence spectrometer (SILM-Amino, Urbana, IL) (excitation wavelength = 480 nm; emission wavelength = 526 nm). The stimuli were added at time 0. The fluorescence of Flu-3 is a measure for the intracellular calcium concentrations.

**Nuclear Extract Preparation**—Nuclear extracts of stimulated T lymphocytes were prepared by a modification of the method described by Park et al. (44). 12 × 10\(^9\) T lymphocytes were stimulated with PHA in combination with anti-CD28 in the presence or the absence of anti-CD5 for 6 h was isolated using the guanidium isothiocyanate/guanidinium thiocyanate/cesium chloride method (42). Northern blot analysis of the human IL-2 promoter was performed in time with a AMINCOBOWMAN® Series 2 Luminescence spectrometer (SILM-Amino, Urbana, IL) (excitation wavelength = 480 nm; emission wavelength = 526 nm). The stimuli were added at time 0. The fluorescence of Flu-3 is a measure for the intracellular calcium concentrations.

**Electrophoretic Mobility Shift Assays**—The sequences of the synthetic oligonucleotides containing the binding sequences of the human IL-2 promoter were incubated in the gel shift assays were as follows: NFAT, 5'-GGAGAAAACGTGTTCATACAGGCGT-3' (corresponding to positions -286 to -257 in the human IL-2 promoter); AP-1, 5'-AAATCCAAGGATCATAAGGACGCT-3' (positions -160 to -140); Oct-5, 5'-TTGGTATAATT-GTTGAATATGGAAACACAT-3' (positions -97 to -69); NFκB, 5'-AA-CAAGGATCATAAGGACGCT-3' (positions -213 to -190); and CD28 response element, 5'-ggatggTGAATGAAATACTTAA-3' (positions -168 to -151) (40, 47–49).

50 ng of high pressure liquid chromatography purified single-stranded oligonucleotides (EuroGentec, Seraing, Belgium) were end-labeled with T4 polynucleotide kinase (Promega Corporation, Madison, WI) and [γ-32P]ATP (3000 Ci/mmol, Amersham), separated from non-incorporated radiolabel by Sephadex G-50 chromatography, ethanol
precipitated, dried, and dissolved in 20 μl of annealing buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM MgCl₂, 1 mM EDTA, 1 mM DTT) with a 4-fold excess of the opposite strand. Annealing of the two strands was performed by heating the mixture for 2 min at 90 °C and slow cooling to room temperature. The DNA extract was incubated with 0.1–0.2 ng of double-stranded labeled oligonucleotide in 15 μl of binding buffer (20 mM HEPES, pH 7.9, 60 mM KCl, 0.06 mM EDTA, 0.6 mM DTT, 2 μM spermidine, 10% glycerol) supplemented with 2 μg (NFAT, AP-1, Oct and NFκB) or 5 μg of poly(dI-dC) (CD28 response element). The binding reaction was performed for 20 min at 28 °C. In competition experiments, a 100-fold molar excess of unlabeled competitor oligonucleotide was preincubated with the nuclear extract for 10 min on ice prior to the addition of the labeled oligonucleotide. The samples were loaded onto a 4% nondenaturing polyacrylamide gel in 0.5 × Tris borate-EDTA and run for 1 h at 140 V. Quantification of binding protein was performed using a PhosphorImager and the ImageQuant software (Molecular Dynamics).

Expression and Purification of GST-c-jun-1–135—The GST-c-jun expression plasmid pGEX-2T-c-jun-1–135 (a gift from Dr. J. Borst, The Netherlands Cancer Institute, Amsterdam, The Netherlands) was transformed into the electrocompetent strain DH5α and expression of GST-c-jun-1–135 protein was induced with 1 mM isopropyl-β-thiogalactopyranoside (Boehringer Mannheim). GST-c-jun-1–135 protein was purified with glutathione-Sepharose beads (Pharmacia) and eluted from the beads with 5 mM reduced glutathione as recommended by the manufacturer (Pharmacia) with minor modifications. Protein concentration of the purified proteins was determined by the Bradford assay (46).

JNK Kinase Activity Assay—Total cell lysates were prepared from stimulated human T lymphocytes after 15 min of stimulation for measurement of the JNK kinase activity. 5 × 10⁶ T lymphocytes were left unstimulated or stimulated with PHA and anti-CD28 in the presence or the absence of anti-CD5. Cells were harvested and washed once with phosphate-buffered saline, resuspended in 400 μl of lysis buffer (20 mM HEPES, pH 7.4, 2 mM EGTA, 1 mM DTT, 1% Triton X-100, 10% glycerol) supplemented with protease inhibitors (10 μg/ml leupeptin, 10 μg/ml aprotonin, and 0.4 mM phenylmethylsulfonyl fluoride, all from Sigma) and phosphatase inhibitors (50 mM β-glycerophosphate and 1 mM Na₃VO₄), and incubated on ice for 20 min. Insoluble debris was spun down at 10,000 × g for 10 min by 4 °C. The protein concentration of the supernatants was determined by the Bradford assay (46). JNK was immunoprecipitated from 400 μg of cell lysate by incubating with 400 ng of anti-JNK1 antibody (sc-474, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at 4 °C while rotating and incubating for an additional 18 h after the addition of 25 μl of protein A-Sepharose beads (50% slurry, Pharmacia Biotech). The immunoprecipitates were spun down in a microcentrifuge for 20 s at 4 °C, washed three times with lysis buffer, washed twice with LiCl buffer (500 mM LiCl, 100 mM Tris-HCl, pH 7.6, 1 mM DTT, 0.1% Triton X-100), and finally washed three times with assay buffer (20 mM MOPS, pH 7.2, 2 mM EGTA, 10 mM MgCl₂, 1 mM DTT, 0.1% Triton X-100).

The JNK immunoprecipitates were assayed for kinase activity for 20 min at 30 °C in 30 μl of assay mix containing 20 mM MOPS, pH 7.2, 2 mM EGTA, 30 mM MgCl₂, 1 mM DTT, 0.1% Triton X-100, 25 μM ATP, and 5 μCi of [γ-32P]ATP (3000 Ci/mmol, Amersham) using 7 μg of bacterially produced GST-c-jun-1–135 as substrate. The reaction was terminated by the addition of 5 × SDS-polyacrylamide gel electrophoresis sample buffer and boiling. Proteins were separated by SDS-polyacrylamide gel electrophoresis on a 12.5% gel, using Rainbow™ colored protein molecular weight markers (Amersham) as a reference. Quantification of phosphorylated GST-c-jun-1–135 substrate was performed using PhosphorImager and the ImageQuant software (Molecular Dynamics).

Discussion—The CaaHindIII fragments of the pIL2CAT and p22.6CAT plasmids (both gifts from Dr. C. Verweij, Department of Rheumatology, Academic Hospital Leiden, Leiden, The Netherlands) respectively containing the IL-2 promoter from positions ~319 to +47 (50) and three copies of the distal AP-1 site in front of the promoter (51) were subcloned in the ClaI/HinII fragments of the pME18s (52–54). The pIL2CAT and p22.6CAT expression plasmids pGEX-2T/c-jun-1–135 (a gift from Dr. J. Borst, The Netherlands Cancer Institute, Amsterdam, The Netherlands) was transformed into the electrocompetent strain DH5α and expression of GST-c-jun-1–135 protein was induced with 1 mM isopropyl-β-thiogalactopyranoside (Boehringer Mannheim). GST-c-jun-1–135 protein was purified with glutathione-Sepharose beads (Pharmacia) and eluted from the beads with 5 mM reduced glutathione as recommended by the manufacturer (Pharmacia) with minor modifications. Protein concentration of the purified proteins was determined by the Bradford assay (46). JNK was immunoprecipitated from 400 μg of cell lysate by incubating with 400 ng of anti-JNK1 antibody (sc-474, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at 4 °C while rotating and incubating for an additional 18 h after the addition of 25 μl of protein A-Sepharose beads (50% slurry, Pharmacia Biotech). The immunoprecipitates were spun down in a microcentrifuge for 20 s at 4 °C, washed three times with lysis buffer, washed twice with LiCl buffer (500 mM LiCl, 100 mM Tris-HCl, pH 7.6, 1 mM DTT, 0.1% Triton X-100), and finally washed three times with assay buffer (20 mM MOPS, pH 7.2, 2 mM EGTA, 10 mM MgCl₂, 1 mM DTT, 0.1% Triton X-100).

Maturation of the antibodies was performed by use of a prestimulation method (55). Purified human T lymphocytes were cultured in RPMI 1640 medium containing 10% FCS, 2 mM l-glutamine, and antibiotics, supplemented with PHA at 1 μg/ml and recombinant human IL-2 (Cetus, Emeryville, CA) at 100 units/ml. After 2 days of culture, the nonadherent cells were harvested, washed once with phosphate-buffered saline, and resuspended in RPMI 1640 medium containing 10% FCS, 2 mM l-glutamine, and antibiotics, supplemented with only recombinant human IL-2 at 100 units/ml. The T cells were incubated for 2 more days, washed with phosphate-buffered saline, and used for transient transfection assays. 15 × 10⁶ T lymphocytes were resuspended in 400 μl of RPMI 1640 containing 10% FCS, 2 mM l-glutamine, and antibiotics, 100 units/ml IL-2, and either 20 μg (1 μg/ml) of reporter plasmid DNA or 15 μg of reporter plasmid DNA plus 15 μg of expression plasmid DNA was added. After a 10-min incubation on ice, cells were electroporated using a Bio-Rad Gene Pulser (Bio-Rad) at 400 V, 960 microfarad. After an additional 10-min incubation period on ice, cells were transferred to RPMI 1640 containing 10% FCS, 2 mM l-glutamine, antibiotics, and 100 units/ml IL-2. 1 h after electroporation, cells were either left unstimulated or stimulated with PHA and anti-CD28 or PHA and anti-CD28 plus anti-CD5. KN-62 was added 1 h after electroporation and 30 min prior to stimulation. After 24 h cells were harvested and resuspended in 150 μl of 250 μl Tris-HCl, pH 7.8. Total cell extracts were prepared by five repeated freeze-thaw cycles.

CAT ELISA—CAT concentrations in total cell extracts were measured by the CAT ELISA kit (Boehringer Mannheim) as recommended by the manufacturer. The protein concentration of the cell extracts was determined by the Bradford assay (46), and results from the CAT ELISA were normalized by calculating the CAT concentration per μg of protein in the total cell extract.

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(57) Dynamics).

Expression of phosphorylated GST-c-jun-1–135 substrate was performed using PhosphorImager and the ImageQuant software (Molecular Dynamics).

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2 G. A. Wayman and T. H. Soderling, unpublished observations.
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RESULTS

CD5 Co-stimulation Enhances the IL-2 mRNA Accumulation and Subsequent Protein Secretion by Activated T Lymphocytes—To determine the effect of CD5 co-stimulation on activated T lymphocytes, purified T cells were stimulated with either PHA or anti-CD3 plus anti-CD28 in the presence or the absence of anti-CD5. Unstimulated T lymphocytes or T lymphocytes stimulated with PHA or anti-CD3 in the presence or the absence of anti-CD5 secreted only very low levels of IL-2 (Fig. 1). T lymphocytes stimulated with PHA plus anti-CD28 secreted high levels of IL-2 protein: 4516 ± 953 pg/ml (mean ± S.E.; n = 6). Co-stimulation of T lymphocytes with anti-CD5 resulted in a 2.2-fold higher IL-2 secretion: 9736 ± 1178 pg/ml (mean ± S.E.; n = 4; p = 0.008). When anti-CD3 plus anti-CD28 was used as a stimulus, the up-regulation of the IL-2 secretion induced by co-stimulation with anti-CD5 was even 5.7-fold: 1390 ± 226 pg/ml IL-2 secretion versus 7915 ± 1157 pg/ml IL-2 (mean ± S.E.; n = 4; p = 0.006).

The up-regulation in response to CD5 stimulation was also observed at the mRNA level (Fig. 2A). The addition of anti-CD5 resulted in an 1.6-fold enhancement (n = 8) of the IL-2 mRNA accumulation compared with the level of IL-2 mRNA accumulation in PHA-anti-CD5-stimulated T lymphocytes (Fig. 2B).

CD5 Co-stimulation Acts at the Post-transcriptional Level—To examine whether the effect of the CD5 signal on the IL-2 gene expression is mediated at the post-transcriptional level, we determined the effect of CD5 co-stimulation on the IL-2 mRNA stability. T lymphocytes were stimulated with either PHA plus anti-CD28 or PHA and anti-CD28 plus anti-CD5 for 6 h, after which the transcription was blocked by the addition of actinomycin D (Fig. 3A). In T lymphocytes stimulated with PHA and anti-CD28, the IL-2 mRNA decayed with a half-life of 60 min (n = 4). In T lymphocytes co-stimulated with PHA and anti-CD28 plus anti-CD5, this half-life was prolonged to 105 min (n = 4), indicating that the stability of the IL-2 mRNA was increased with almost a factor 1.8 (Fig. 3B).

The CD5 Signaling Pathway Affects the Activity of AP-1—To investigate the molecular mechanisms underlying the up-regulation of the IL-2 gene transcription by the CD5 signal, we performed electrophoretic mobility shift assays to identify the transcription factors involved in the regulation of the IL-2 promoter that are the targets for the CD5 signal. Nuclear extracts were prepared from unstimulated T cells or T cells stimulated for 2 h with PHA plus anti-CD28 or PHA plus anti-CD28 and anti-CD5. In unstimulated T lymphocytes no DNA binding of AP-1, NFAT, NFkB, and CD28RC/NF-MAT could be detected, whereas a constitutive DNA binding activity was observed to the proximal Oct site. Upon stimulation of the T cells with PHA and anti-CD28, the DNA binding activities of AP-1, NFAT, NFkB, and CD28RC/NF-MAT were induced, and the Oct DNA binding activity was increased. All the nuclear complexes bound specifically because they could be competed for by a 100-fold molar excess of unlabeled double-stranded oligonucleotides containing the same respective sites and not by negative controls (data not shown). Co-stimulation of the T lymphocytes with PHA, anti-CD28, and anti-CD5 resulted in an up-regulation of the DNA binding activity of AP-1 (Fig. 4). The AP-1 DNA binding activity was enhanced almost 1.6-fold ± 0.04 (mean ± S.E.; n = 6; p = 0.019). Co-stimulation of the CD5 signaling pathway had no effect on the DNA binding activities of NFAT, NFkB (Fig. 4), CD28RC/NF-MAT, and Oct (data not shown).
To ascertain that this CD5-induced enhancement of the AP-1 DNA binding to the IL-2 promoter is functional and can account for the increase of the IL-2 gene transcription rate, we performed transient transfection experiments with CAT reporter constructs driven by either the complete IL-2 promoter, three copies of the AP-1 binding site, or three copies of the distal NFAT binding site. Because resting T lymphocytes are refractory to conventional transfection methods, it was necessary to use the prestimulation method as described by Park et al. (55). Purified T cells were first cultured for 48 h in PHA plus IL-2, washed, and then incubated for another 48 h in the presence of IL-2 alone. The prestimulated T lymphocytes were then transfected with the various constructs or the empty pCAT3 enhancer plasmid as a negative control. No CAT protein could be detected in cells transfected with the empty pCAT3 enhancer plasmid in any of the experiments. Transfection of the CAT construct driven by the complete IL-2 promoter resulted in a significant CAT expression by the transfected T cells after stimulation with PHA plus anti-CD28. The CAT expression increased by a factor 1.8 (mean ± S.E.; n = 4; p = 0.045) when anti-CD5 was added as a co-stimulus (Fig. 5A). In T lymphocytes that were transfected with the CAT construct driven by the 3 AP-1 binding sites, we observed an 1.5-fold ± 0.09 (mean ± S.E.; n = 4; p = 0.011) enhancement of the CAT expression when stimulated with PHA plus anti-CD28 and anti-CD5 compared with stimulation with PHA and anti-CD28 alone (Fig. 5B), indicating that the transcriptional activity of AP-1 was increased by the CD5 signaling pathway. T lymphocytes that were transfected with the CAT construct driven by the three NFAT binding sites showed no response to CD5 co-stimulation; the level of CAT protein expressed by the T cells stimulated with PHA plus anti-CD28 was not changed when anti-CD5 was added as a co-stimulus (Fig. 5C).

The MAP Kinases ERK, p38/Mpk2, and JNK Are Not Involved in the Up-regulation of the AP-1 Activity through the CD5 Signaling Pathway—Because we identified AP-1 as an important target of the CD5 signal transduction route, we set out to elucidate the mechanisms leading to the up-regulation of the AP-1 DNA binding and transcriptional activity. The fos and jun proteins that form the AP-1 complex are regulated at the post-translational and transcriptional level by members of the MAP kinase family. JNK and Fos-regulating kinase up-regulate the transactivation activity of c-jun and c-fos (28, 30), whereas JNK, ERK, and p38/Mpk2 regulate the activity of the c-jun and c-fos promoters through phosphorylation of Elk-1, c-jun, and ATF-2 (31–33, 57–59). To determine whether ERK or p38/Mpk2 are involved in the up-regulation of the AP-1 activity induced by the CD5 co-stimulation signal, we performed secretion experiments using inhibitors specific for these signaling pathways.

PD98059 specifically inhibits the kinase activity of MEK1 (MAPK/ERK kinase 1), which is responsible for the phosphorylation and activation of ERK1 and ERK2 (60, 61). Co-stimulation with anti-CD5 enhanced the IL-2 secretion 2-fold in T lymphocytes stimulated with PHA plus anti-CD28: 4854 ± 1179 pg/ml IL-2 (mean ± S.E.; n = 4) versus 9863 ± 1639 pg/ml IL-2 (mean ± S.E.; n = 4; p = 0.006; Fig. 6). Incubation of
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**FIG. 5.** CD5 co-stimulation enhances the transactivation activity of AP-1 but not NFAT. Human T cells pre-stimulated as described under “Experimental Procedures” were transfected with 20 \(\mu\)g of CAT reporter constructs driven by the complete IL-2 promoter, pCAT3e-IL-2(-319/+47) (A), three copies of the proximal AP-1 site, pCAT3-3xAP-1/IL-2 (B), or three copies of the distal NFAT site, pCAT3e-3xNFAT/IL-2 (C). Transfected cells were left alone for 1 h, divided into three groups, and subsequently left unstimulated or stimulated with PHA plus anti-CD28 (αCD28) or PHA plus αCD28 and anti-CD5 (αCD5) for 24 h. CAT expression was measured as described under “Experimental Procedures.” The results are expressed as the relative CAT expression compared with the PHA/CD28-induced CAT expression, which was set at 1. The mean values ± S.E. found for the relative CAT expression in four independent experiments are shown.

PHA/anti-CD28-stimulated T cells with PD98059 reduced the level of secreted IL-2 more than 5-fold to 957 ± 105 pg/ml (mean ± S.E.; \(n = 4\)). The addition of anti-CD5 resulted in a 2.1-fold enhancement of the IL-2 secretion: 2035 ± 577 pg/ml IL-2 (mean ± S.E.; \(n = 4\); \(p = 0.036\)), suggesting that the ERK pathway is not involved in the CD5 signaling pathway.

SB203580 inhibits another MAP kinase signaling pathway by specifically blocking the kinase activity of p38/Mpk2 (62). The addition of SB203580 to T lymphocytes stimulated with PHA and anti-CD28 reduced the IL-2 secretion 3.5-fold to 1377 ± 144 pg/ml IL-2 (mean ± S.E.; \(n = 4\); Fig. 6). Similar to co-stimulation with the MEK1 inhibitor PD98059, co-stimulation with anti-CD5 resulted again in a 1.9-fold up-regulation of the IL-2 expression: 2662 ± 168 pg/ml IL-2 (mean ± S.E.; \(n = 4\); \(p = 0.045\)), suggesting that the p38/Mpk2 is not involved in the CD5 signaling pathway.

To examine the involvement of the third MAP kinase pathway in the CD5-induced up-regulation of AP-1, we performed a JNK-specific kinase assay. JNK1 was immunoprecipitated from T lymphocytes that were left unstimulated or stimulated with either PHA plus anti-CD28 or PHA and anti-CD28 plus anti-CD5. The specific JNK1 kinase activity was determined by measuring the phosphorylation of its substrate, GST-c-Jun.

Only a basal kinase activity of JNK1 was present in unstimulated T cells, and this activity was enhanced 1.7-fold ± 0.07 (mean ± S.E.; \(n = 3\); \(p = 0.010\)) in PHA/anti-CD28-stimulated T cells. Co-stimulation with anti-CD5 did not further increase the JNK1 kinase activity (\(n = 3\); Fig. 7).

The *CaM Kinase Inhibitor KN-62 Specifically Blocks the CD5 Signal—CD5 signaling modulates the intracellular Ca\(^{2+}\) levels (8, 14). Several Ca\(^{2+}\)-mediated signal transduction routes are known that regulate the AP-1 activity. CaM kinases are involved in the up-regulation of the transcriptional activity of the c-fos promoter through phosphorylation of the transcription factors CAMP-responsive element binding protein (CREB) and serum response factor (SRF) (63–65). To determine whether CaM kinases are involved in the CD5 signaling pathway, we performed secretion experiments using an inhibitor specific for CaM kinases, KN-62 (66, 67). Co-stimulation with anti-CD5 up-regulated the IL-2 secretion by PHA/anti-CD28-stimulated T lymphocytes more than 1.8-fold: 5767 ± 903 pg/ml IL-2 versus 10,000 ± 1394 pg/ml IL-2 (mean ± S.E.; \(n = 4\); \(p = 0.017\)). The addition of KN-62 did not affect the IL-2 secretion by PHA/anti-CD28-stimulated T cells: 5521 ± 794 pg/ml IL-2 (mean ± S.E.; \(n = 4\); Fig. 8). However, the up-regulation of the IL-2 secretion due to co-stimulation with anti-CD5 was completely blocked in the presence of KN-62. After the addition of KN-62, PHA/anti-CD28 plus anti-CD5-stimulated T lymphocytes secreted only 6236 ± 45 pg/ml IL-2 (mean ± S.E.; \(n = 4\)). To ascertain that KN-62 indeed blocks the activity of CaM
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kinases in T lymphocytes and not the Ca\(^{2+}\) influx as has been reported for some cell types (68, 69), we performed intracellular Ca\(^{2+}\) measurements using the fluorescent dye Fluo-3. As shown in Fig. 9, KN-62 has no effect on the TCR- or CD5-induced Ca\(^{2+}\) influx in T lymphocytes. The complete inhibition of the CD5 signaling pathway by KN-62 indicates that CaM kinases constitute a major part of this pathway.

**The CD5-induced Up-regulation of the AP-1 Activity Involves a CaM Kinase-dependent Pathway**—To establish that CaM kinases are involved in the up-regulation of the AP-1 activity by the CD5 signaling pathway, we first investigated the effect of KN-62 on the DNA binding of AP-1. Nuclear extracts of T lymphocytes stimulated with PHA and anti-CD28 in the presence of the CaM kinase inhibitor KN-62 contained the same amount of AP-1 DNA binding activity as T lymphocytes stimulated with PHA and anti-CD28 alone (n = 6; Fig. 10). Co-stimulation of PHA/anti-CD28-stimulated T cells with anti-CD5 resulted in an 1.6-fold ± 0.04 (mean ± S.E.; n = 6; p = 0.019) up-regulation of the AP-1 DNA binding activity. This CD5-induced up-regulation of the AP-1 DNA binding activity was completely abolished by the addition of KN-62, indicating that CD5 signaling is indeed mediated by a CaM kinase-dependent pathway.

Transient transfection experiments confirmed that the CD5

signal regulates the IL-2 transcription and AP-1 activity through a CaM kinase-dependent pathway. Stimulation of pCAT3e-IL2((-319/+47) transfected T lymphocytes with PHA plus anti-CD28 resulted in a CAT expression that is insensitive to KN-62 (n = 4), whereas the 1.8-fold ± 0.17 (mean ± S.E.; n = 4; p = 0.045) enhancement of the CAT expression induced by co-stimulation of the transfected cells with PHA/anti-CD28 plus anti-CD5 was completely blocked by the addition of KN-62 (n = 4; Fig. 11A). Likewise, in T lymphocytes transfected with the AP-1-driven reporter construct, the 1.5-fold ± 0.09 (mean ± S.E.; n = 4; p = 0.011) up-regulation of the CAT expression induced by co-stimulation with PHA anti-CD28 plus anti-CD5 was completely blocked in the presence of KN-62 (n = 4; Fig. 11B). The NFAT-driven CAT expression in transfected T lymphocytes was not affected by co-stimulation with CD5 and was therefore not influenced by the addition of KN-62 (n = 4; Fig. 11C).

CaM Kinases Are Also Involved in the CD5-induced Stabilization of the IL-2 mRNA—Because we showed that CaM kinases are involved in the up-regulation of the IL-2 gene transcription rate by enhancing the activity of the transcription factor AP-1, we were interested to know whether these kinases were also involved in the observed stabilization of the IL-2 mRNA by the CaD signal (Fig. 3). To this end, we determined the effect of the CaM kinase inhibitor KN-62 on the IL-2 mRNA stability (Fig. 12A). We found that in T lymphocytes stimulated with PHA anti-CD28 plus anti-CD5 in the presence of KN-62 the IL-2 mRNA decayed with a half-life of approximately 55 min (n = 4; Fig. 12B), which is similar to the half-life of the IL-2 transcripts isolated from T lymphocytes stimulated with PHA and anti-CD28 alone and significantly shorter than the IL-2 mRNA half-life (105 min) in T lymphocytes stimulated with PHA/anti-CD28 and anti-CD5 (Fig. 12D).

**CaM Kinase Type IV but Not Type II Is Involved in the CD5 Signal Transduction Route**—Both CaM kinase type II (CaMK II) and type IV (CaMK IV) have been reported to be involved in the regulation of c-fos gene transcription (63, 64, 70). To identify the Ca\(^{2+}\)/calmodulin-dependent kinase that is involved in the CD5 signaling pathway, we performed kinase-specific activity assays. Both CaMK II and CaMK IV showed a basal activity in unstimulated T lymphocytes. The basal activity of

**Fig. 7.** CD5 signaling is independent of JNK1 activation. T cells were left unstimulated or stimulated with PHA plus anti-CD28 (αCD28) in the presence or the absence of anti-CD5 (αCD5) for 15 min. Cells were lysed, and immunoprecipitated JNK1 was assayed for kinase activity using GST-c-Jun-1–135 as a substrate. The kinase activity is determined by quantification of phosphorylated GST-c-Jun using a PhosphorImager. The kinase assay shown is representative of three independent experiments.

**Fig. 8.** The CD5-induced IL-2 secretion is completely blocked by KN-62, an inhibitor of CaM kinases. T cells were stimulated with either PHA plus anti-CD28 (αCD28) plus anti-CD5 (αCD5) in the presence or the absence of 10 μM KN-62, an inhibitor of Ca\(^{2+}\)/calmodulin-dependent kinases. Cell-free supernatants were harvested after 24 h and analyzed for secreted IL-2 protein. The mean values ± S.E. for the IL-2 secretion found in four independent experiments are shown.
CaMK II was up-regulated 1.8-fold \( \pm 0.13 \) (mean \( \pm \) S.E.; \( n = 3; p = 0.026 \)) by stimulation with PHA plus anti-CD28, but no further enhancement was observed when the T cells were co-stimulated with PHA/anti-CD28 plus anti-CD5 (\( n = 3; p = 0.035 \)) in T lymphocytes stimulated with PHA plus anti-CD28 (Fig. 13B). In contrast with CaMK II, the addition of anti-CD5 induced a further enhancement of the CaMK IV activity, which reached a peak after 5 min of stimulation. At this time point the induction was 1.8-fold \( \pm 0.11 \) (mean \( \pm \) S.E.; \( n = 3; p = 0.033 \); Fig. 13C) compared with the CaMK IV activity in PHA/anti-CD28-stimulated T lymphocytes. After 5 min, the kinase activity of CaMK IV decreased again slowly but still remained higher after 15 min compared with the activity after 3 min (Fig. 13C).

\( \text{Ca}^{2+}/\text{Calmodulin-dependent Kinase Type IV Is Indispensable for the CD5 Signaling Pathway Leading to the IL-2 Gene Expression—} \)

To establish whether CaM kinase type IV is involved in the CD5 signaling pathway leading to the up-regulation of the IL-2 gene expression, we co-transfected T cells with the IL-2 promoter-driven CAT construct and either an expression plasmid encoding a dominant negative mutant of CaMK IV or the empty expression plasmid as a control. The CAT expression of PHA plus anti-CD28-stimulated control T cells was enhanced 1.9-fold \( \pm 0.08 \) (mean \( \pm \) S.E.; \( n = 3; p = 0.007 \)) after co-stimulation with anti-CD5. The expression of dominant negative CaM kinase IV inhibited 19 \( \pm \) 2\% (mean \( \pm \) S.E.; \( n = 3; p = 0.009 \)) of the PHA plus anti-CD28-induced CAT expression, whereas the co-stimulatory effect of anti-CD5 was completely abrogated by the expression of the dominant negative CaM kinase IV mutant (\( n = 3; p = 0.002 \); Fig. 14), indicating that \( \text{Ca}^{2+}/\text{calmodulin-dependent kinase type IV plays a vital role in the CD5 signaling pathway leading to the enhanced IL-2 expression in human T lymphocytes.} \)

**DISCUSSION**

CD5 acts as a co-receptor on T lymphocytes and plays an important role in T cell signaling and T-B cell interactions; however, limited information is available regarding the signaling events induced by ligation of the CD5 receptor (2, 10, 12). The activation of the protein tyrosine kinase p56\( ^{6} \)-lck has been reported to be an early event in the CD5-induced signal trans...
FIG. 11. KN-62 completely inhibits the CD5-induced transactivation activity of AP-1. Human T cells prestimulated as described under “Experimental Procedures” were transfected with 20 μg of CAT reporter constructs driven by the complete IL-2 promoter, pCAT3e-IL-2(-319/+47) (A), three copies of the proximal AP-1 site, pCAT3xAP1-IL-2 (B), or three copies of the distal NFAT site, pCAT3xNFAT/IL-2 (C). Transfected cells were left alone for 1 h, divided into five groups, and subsequently left unstimulated or stimulated with PHA plus anti-CD28 (αCD28) in the presence or the absence of anti-CD5 (αCD5) for 24 h. KN-62 was added as indicated 1 h prior to stimulation. CAT expression was measured as described under “Experimental Procedures.” The results are expressed as the relative CAT expression compared with the PHA/αCD28-induced CAT expression, which was set at 1. The mean values ± S.E. found for the relative CAT expression in four independent experiments are shown.

Table 1: CAT expression

| Condition          | Relative CAT expression |
|--------------------|-------------------------|
| unstimulated       | 1.0                     |
| PHA, αCD28         | 0.8                     |
| PHA, αCD28, αCD5   | 0.6                     |
| PHA, αCD28 + KN-62 | 0.4                     |
| PHA, αCD28, αCD5 + KN-62 | 0.2                   |

The inhibition of the ERK and p38/Mpk2 pathways with the specific inhibitors PD98059 (60) and SB203580 (62) has no effect on the CD5 signaling pathway. In addition, the JNK-specific kinase assays show that co-stimulation of T lymphocytes with PHA anti-CD28 plus anti-CD5 does not induce JNK activity compared with T lymphocytes stimulated with PHA and anti-CD28 alone.

CD5 signaling has been shown to modulate the intracellular Ca²⁺ levels (8, 14). The c-fos promoter is regulated through several Ca²⁺-dependent pathways, whereby some of these pathways eventually result in the activation of MAP kinases (72, 73). In the present study, we demonstrate that Ca²⁺/calmodulin-dependent kinases play a major role in the CD5 signaling pathway. The use of the CaM kinase-specific inhibitor KN-62 (66) completely blocks the enhanced AP-1 DNA binding and transactivation activity in response to anti-CD5 and subsequently also the CD5-induced up-regulation of the IL-2 secretion. CaM kinases induce the expression of the c-fos gene (63–65) and in this way contribute directly to the induction of the AP-1 DNA binding activity, because it is known that fos/jun heterodimers have a higher affinity for DNA than jun/jun homodimers (26).

CaM kinases regulate the activity of the c-fos promoter through activation of the transcription factors CREB and SRF. Both CaM kinase type II and type IV have been implicated in the activation of CREB and SRF (37, 63, 64, 70, 74, 75). CaMK IV that exists as a monomer has only been detected in a limited number of tissues, including brain, testis, spleen, thymus, and T lymphocytes (76–80). Recently, a CaM kinase kinase has been identified that is responsible for the activation of CaM kinase type IV and also of CaM kinase type I (53, 81). This CaM KK has a similar tissue distribution as CaM kinase type II (53) and seems to activate CaMK IV through phosphorylation of Thr196, a Thr residue that is present in both CaMK IV and CaMK I but not in CaMK II (54, 82). It has also been shown that a constitutively active form of CaMK IV localizes to the nucleus in neurons, where it might mediate CREB- and SRF-dependent...
transcription (83). CaMK IV up-regulates the transactivation activities of CREB and SRF through phosphorylation of Ser133 and Ser 103, respectively (63, 64). The more ubiquitously expressed CaMK II consists of 6–12 subunits, depending on the isoform and tissue distribution (84). Activation of CaMK II is achieved through elevation of the intracellular Ca\(^{2+}\) levels, which induces the autophosphorylation of Thr286 (\(\alpha\) isoform) or Thr287 (\(\beta\) and \(\gamma\) isoforms) (85). Several groups have demonstrated that CaMK II down-regulates the transactivation activity of CREB by phosphorylation of Ser142, although it can also phosphorylate Ser133, which will up-regulate the transactivation activity of CREB (64, 65). The regulation of the transactivation activity of SRF by CaMK II seems to involve phosphorylation of the same Ser residue that is phosphorylated by CaMK IV (64). In the present study, we demonstrate that CaMK IV but not CaMK II is involved in the CD5-induced up-regulation of the AP-1 activity and the IL-2 gene expression.

Although Enslen et al. (86) recently reported that the CaMKK/CaMK IV cascade can also activate the MAP kinases JNK1, ERK2, and p38/Mpk2 and subsequently stimulate transcription dependent on phosphorylation of c-jun, Elk-1, and ATF-2, we conclude that the CD5 activity induced in T lymphocytes through stimulation of the CD5 receptor directly up-regulates the c-fos expression through phosphorylation of either CREB or SRF and is independent of the activation of MAP kinases. Further experiments are necessary to clarify the exact role of CREB and SRF activation in the CD5 signaling pathway.

Besides affecting the IL-2 gene transcription through AP-1, the effect of the CD5 signal on the IL-2 expression is also mediated at the post-transcriptional level, because KN-62 inhibits the CD5-mediated IL-2 mRNA stabilization. Further studies are required to elucidate the mechanisms involved in this process. The results presented in this study provide evidence that ligation of the CD5 receptor on T lymphocytes induces a specific signaling pathway involving the Ca\(^{2+}\)/calmodulin-dependent kinase type IV, which regulates the IL-2 gene expression both at the transcriptional and post-transcriptional level.

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