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CD43-mediated Signals Induce DNA Binding Activity of AP-1, NF-AT, and NFκB Transcription Factors in Human T Lymphocytes*

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Although numerous reports document a role for CD43 in T cell signaling, the direct participation of this molecule in cell activation has been questioned. In this study we show that CD43 ligation on human normal peripheral T cells was sufficient to induce interleukin-2, CD69, and CD40-L gene expression, without requiring signals provided by additional receptor molecules. This response was partially inhibited by cyclosporin A and staurosporine, suggesting the participation of both the Ca^{2+} and the protein kinase C pathways in CD43 signaling. Consistent with the transient CD43-dependent intracellular Ca^{2+} peaks reported by others, signals generated through the CD43 molecule resulted in the induction of NF-AT DNA binding activity. CD43-dependent signals resulted also in AP-1 and NFκB activation, probably as a result of protein kinase C involvement. AP-1 complexes bound to the AP-1 sequence contained c-Jun, and those bound to the NF-AT-AP-1 composite site contained c-Jun and Fos. NFκB complexes containing p65 could be found as early as 1 h after CD43 cross-linking, suggesting that CD43 participates in early events of T cell activation. The induction of the interleukin-2, CD69, and CD-40L genes and the participation of AP-1, NF-AT, and NFκB in the CD43-mediated signaling cascade implicate an important role for this molecule in the regulation of gene expression and cell function.

Successful activation of T lymphocytes requires at least two kinds of signals: those generated by the specific interaction of the TcR[1] with peptides presented by major histocompatibility complex molecules on the antigen presenting cells and those resulting from the interaction of other T cell surface molecules, known as co-receptors, with their ligands on antigen presenting cells (for reviews see Refs. 1 and 2). Thus, a cell senses not only the presence of an antigen but also its environment, and a particular cellular response will result from the integration of signals delivered through the antigen-specific receptor and the numerous co-receptors.

The CD43 co-receptor is very abundant on T cells surface; it comprises a highly glycosylated extracellular portion of 239 amino acids that protrudes 45 nm from the cellular membrane, a transmembrane region, and a cytoplasmic domain of 123 amino acids (3–5). On T cells, CD43 is differentially glycosylated in two major isoforms: a 113–123-kDa product, mainly present on resting $CD4^+$ T cells and a 125–135-kDa form expressed mostly on resting $CD8^+$ lymphocytes, which is up-regulated both on $CD4^+$ and $CD8^+$ cells, following cellular activation (6). The 125–135-kDa form has been shown to be down-regulated during positive selection of double positive cells in the thymus, involving CD43 in thymic selection events (7). Carbohydrate moieties have an important role in CD43 function, because alterations in the glycosylation patterns of the molecule are associated with severe immunodeficiency such as in the case of patients affected with the Wiskott Aldrich syndrome or infected with HIV (8–10). Although there is no direct evidence of how the interaction of CD43 with each of its three reported ligands intercellular adhesion molecule 1 (CD54), (galectin 1, or major histocompatibility complex I; Refs. 10–12) regulates T cell function, there is abundant experimental evidence to support the idea that CD43 participates in T cell adhesion and activation.

Because of its high content of sialic acid residues, resulting in a strong negative charge, it has been proposed that CD43 functions as an antiadhesive molecule, providing the cells with a repulsive barrier (13–15). However, CD43 ligation with specific mAbs has been shown to promote the formation of homotypic aggregates, some of which were only partially inhibited with mAbs against lymphocyte integrins, suggesting an active involvement of CD43 in regulating cellular adhesion (16, 17). A participation of CD43 in leukocyte rolling was suggested by experiments where an anti-CD43 mAb inhibited the interaction between T cells and endothelial cells (18). Evidence that CD43 participates in TcR-mediated T cell activation has been provided in different experimental models (for reviews see Refs. 19 and 20). Transfection of a cDNA encoding for human CD43 in a murine T cell hybridoma specific for human major histocompatibility complex II molecules resulted in enhanced IL-2 production upon stimulation with the appropriate antigen presenting cell only if cells expressed the wild type form of CD43, together with the specific major histocompatibility complex II molecules (21). Co-ligating CD43 with the TcR enhanced

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[1] The abbreviations used are: TcR, T cell receptor; mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; IL, interleukin; AP-1, activator protein-1; NFκB, nuclear factor κB; NF-AT, nuclear factor of activated T cells; PMA, phorbol myristate acetate; Iono, ionomycin; CsA, cyclosporin A; PKC, protein kinase C; MIF, mean fluorescence intensity; IL, interleukin; FACS, fluorescence-activated cell sorter; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CD40-L, CD40 ligand; EMSA, electrophoretic mobility shift assay.

T cell proliferation as compared with cross-linking the TcR alone, in normal mice[2] as well as in $CD28^{-/-}$ mice (22).

To date, the molecular mechanisms that transduce CD43-dependent signals from the cell surface to the nucleus are not fully understood. CD43-mediated activation promotes the generation of second messengers like diacylglycerol and inositol phosphates, Ca^{2+} mobilization, and protein kinase C activation (23). CD43 ligation was shown to induce the association of Fyn and Lck tyrosine kinases to the cytoplasmic domain of CD43 (24, 25) and to lead to the formation of a macromolecular complex which comprises Shc, Grb-2, SLP-76, and the guanine exchange factor Vav (26). Moreover, CD43-specific signals have been found to promote the nuclear translocation of ERK2, suggesting that the MAPKs pathway is involved in the CD43-dependent gene expression (26).

In this study, we provide evidence that CD43-mediated signals are sufficient to regulate gene expression. We show that CD43 ligation on human T cells induced IL-2 gene expression without requiring additional signals provided by other receptor molecules, in opposition to what was previously reported (27). CD43 signals leading to IL-2 mRNA synthesis were partially inhibited by the calcineurin inhibitor cyclosporin A and by the PKC inhibitor staurosporine, suggesting that both the PKC and the calcium pathways participate in the CD43-mediated control of IL-2 gene expression. We also show that CD43 ligation induced the DNA binding activity of the AP-1, NFκB, and NF-AT transcription factors. These data are consistent with a dual pathway of activation through the CD43 molecule because AP-1 and NFκB activation result from a transient high peak of calcium and signals coming from the MAPKs pathway (28–30), whereas NF-AT recruitment depends on a capacitative calcium entry into the cells (31, 32). Moreover, data provided here demonstrate that CD43 stimulates the expression of genes encoding for proteins necessary for T cell effector function and clonal expansion, such as CD69 as a result of activation of AP-1 and NFκB through the PKC pathway, and CD40-L because of NF-AT recruitment by the Ca^{2+} pathway (33–35). Data presented in this study considerably broaden the role of the CD43 molecule in the control of gene expression and cell function.

EXPERIMENTAL PROCEDURES

Reagents—Two monoclonal antibodies (both IgG1) that recognize different epitopes of the CD43 molecule were used: L10, which is specific for an epitope of the protein core (36), and MEM-59, which recognizes a neuraminidase-sensitive epitope (37). The 3D6 mAb was used as an isotype control for L10 (38). The anti-CD3 mAb OKT3 (IgG2) was originally obtained from the American Type Culture Collection. mAbs were used either as ascites or purified. Rabbit anti-mouse IgG was generated in our laboratory. Polyclonal rabbit antibodies specific for human p65, c-Fos, c-Jun, NF-ATc, and NF-ATp were from Santa Cruz Biotechnologies (Santa Cruz, CA). Anti-CD69 mAb (TP1/55) was a kind gift of Dr. Francisco Sanchez-Madrid (Hospital de la Princesa, Madrid, Spain), and anti-CD40-L mAb (5C9) was from the American Type Culture Collection. Ficoll-Hypaque, phorbol myristate acetate (PMA), ionomycin, cyclosporin A, a calcineurin inhibitor (39), and staurosporine, a PKC inhibitor (40), were from Sigma. Poly d(I-C) and dNTPs were from Roche Molecular Biochemicals, T4 polynucleotide kinase was from Promega (Madison, WI), murine mammary tumor virus reverse transcriptase and RNase inhibitor were from Life Technologies, Inc., Tfl DNA polymerase was from Epicenter Technologies (Madison, WI), and [γ-^{32}P]ATP was from NEN Life Science Products.

Cell Culture—Jurkat cells were cultured in RPMI 1640 (Hyclone, Logan, UT) supplemented with 5% fetal calf serum (Hyclone), 5% bovine iron-supplemented calf serum (Hyclone), 2 mM L-glutamine (Sigma), 50 units/ml penicillin, 50 μg/ml streptomycin and, 50 mM β-mercaptoethanol. Peripheral blood T cells were isolated from healthy adult donors by Ficoll-Hypaque gradient centrifugation. The mononuclear cells buffy coat was resuspended in supplemented RPMI before plating the cells overnight onto 100-mm Petri dishes (8×10^7 cells/plate) at 37 °C in 5% CO_2. Nonadherent cells were loaded on a nylon column pre-equilibrated with supplemented RPMI. The resultant purified cells were predominantly TcR^+ (>85% $OKT3^+$) and $CD43^+$ (>95% $L10^+$), as determined by FACS staining. Prior to experimentation, T cells were arrested for an additional 24 h in RPMI supplemented with 2% fetal calf serum. Subsequent manipulations were done in this medium.

T Cell Activation—Purified T cells or Jurkat cells (1×10^7 cells/ml) were incubated in 24-well plates at 37 °C and 5% CO_2 with the following antibodies, alone or in combination: L10, 1:500 dilution of ascites or 1 μg/ml of purified IgG; MEM-59, 1 μg/ml; OKT3, 1 μg/ml (optimal, OKT3o) or 10 ng/ml (suboptimal, OKT3s); equivalent amounts of the isotype control mAb, 3D6 were used for L10 or MEM-59. Additional cross-linking was achieved by adding rabbit anti-mouse IgG (1 μg/ml) to the wells. As positive controls, cells were activated with ionomycin (Iono; 1 μg/ml), phorbol myristate acetate (PMA; 50 ng/ml), or a combination of both. When indicated, cyclosporin A (CsA; 500 ng/ml), staurosporine (50 nM), or anisomycin (10 mM) were added to the cultures 15 min prior to activation. 6 h after the onset of the experiment, cells were collected by centrifugation, washed, and frozen, when used for IL-2 mRNA determination. Nuclear extracts were obtained immediately after cell harvest.

Reverse Transcription-PCR—Total RNA was obtained using *TRIzol* (Life Technologies, Inc.) following the manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA by standard reverse transcription conditions, using oligo(dT) as primer in a final volume of 25 μl. IL-2 mRNAs were amplified from 3 μl of cDNA, using the following oligonucleotides as primers: 3′ (5′-CGT TGA TAT TGC TGA TTA AGT CCC TC-3′) and 5′ (5′-CAT TGC ACT AAG TCT TGC ACT TGT CA-3′). PCR was accomplished by one denaturing cycle of 4 min at 94 °C followed by 30 cycles of amplification (1 min at 91 °C, 1 min at 60 °C, and 1 min at 72 °C) and one final extension cycle of 15 min at 72 °C to generate a product of 305 base pairs. As internal control, we evaluated the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in parallel PCR reactions, using 2 μl of the same cDNAs with the following oligonucleotides: 3′ (5′-TCC ACC ACC CTG TTG CTG TA-3′) and 5′ (5′-ACC ACA GTC CAT GCC ATC AC-3′). PCR conditions for GAPDH were: one denaturing cycle of 5 min at 95 °C, 30 cycles of amplification (1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C), and one final extension cycle of 15 min at 72 °C, resulting in a product of 452 base pairs. Amplified fragments from both reactions were resolved simultaneously in 4% agarose gels and visualized with ethidium bromide.

Nuclear Extract Preparation—Activated cells were washed with phosphate-buffered saline before obtaining nuclear extracts, basically as described (41) with the following modifications. Cells were lysed by incubating them for 5 min at 4 °C in a detergent-free, hypotonic buffer (10 mM Tris, pH 7.6, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM dithiothreitol, 1 μg/ml leupeptin, and 0.5 mM phenylmethylsulfonyl fluoride). Intact nuclei were washed with this lysis buffer, and nuclear extracts were obtained by incubating the nuclei in extraction buffer (20 mM Tris, pH 8.0, 450 mM KCl, 0.5 mM EDTA, 1 mM dithiothreitol, 1 μg/ml leupeptin, 5 mM spermidine, and 25% glycerol) for 45 min under constant mild agitation at 4 °C. DNA pellets were eliminated by centrifugation for 15 min at 13,000 × *g*; protein content of the extracts was determined by Bradford assay (42).

Electrophoretic Mobility Shift Assays—Experiments were conducted basically as described (43), using the following double stranded oligonucleotides: AP-1, 5′-CGC TTG ATG ACT CAG CCG GAA-3′; NF-AT, 5′-AAA GAA AGG AGG AAA AAC TGT TTC ATA CAG-3′; NFκB, 5′-AGT TGA GGG GAC TTT CCC AGG C-3′; and MCAAT (nonspecific competitor), 5′-CTC CTA TTG GCT TGA-3′.

Briefly, oligonucleotides were end-labeled with T4 polynucleotide kinase using 30 μCi of γ-^{32}P-labeled ATP/100 ng of oligonucleotide. Protein extracts (2.5 μg) from activated T cells (for 4 h, unless otherwise indicated) were incubated for 20 min at room temperature with the labeled oligonucleotide (1×10^5 cpm) in band shift buffer (25 mM Hepes, pH 7.9, 40 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol) containing 1 μg of poly(dI-dC) as a nonspecific competitor. DNA-protein complexes were resolved on nondenaturing 6% polyacrylamide gels and visualized by autoradiography.

For competition experiments, 50-fold molar excess of the cold oligonucleotides was added 5 min before adding the labeled probe, following which the assays were performed as above. For immune band shift assays, DNA-protein complexes were allowed to form prior to the addition of 0.1 μg of anti-p65 antibody or 1 μg of anti-c-Jun, anti-Fos, anti-NF-ATc, or anti-NF-ATp antibodies. Samples were incubated with the antibody for 5 h at 4 °C prior to resolving the DNA-protein com-

[2] Y. Rosenstein, unpublished data.

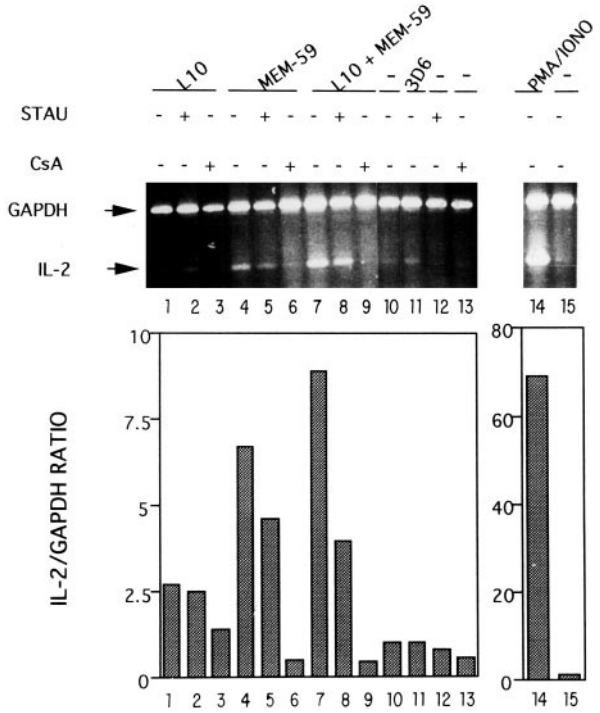

FIG. 1. **IL-2 mRNA expression induced by CD43 cross-linking is partially inhibited by cyclosporin A and staurosporine.** Human peripheral blood T cells were cultured for 6 h in the presence of 1 μg of two different anti-CD43 mAbs, L10 and MEM-59, alone or in combination. CsA or staurosporine (*STAU*) were added where indicated 15 min before the mAbs. As positive control, cells were treated with PMA and Iono. Specific IL-2 mRNA was detected by reverse transcription-PCR. As internal control, the expression of the housekeeping gene GAPDH was monitored. Ethidium bromide-stained gels of PCR products (*upper panel*) were scanned using computer-assisted densitometry (Fluor-S-Multi-imager, Bio-Rad) and the data (IL-2 cDNA/GAPDH cDNA signal) were plotted as the percentages of change (*lower panel*). Data shown are representative of four independent experiments.

plexes on 5% polyacrylamide gels. Equivalent amounts of an irrelevant antibody were used as control.

FACS Staining—T cells were activated by incubating them with the indicated amount of PMA in the presence or absence of anti-CD43 L10 mAb at 37 °C for 18 h. Cells were then collected and stained for FACS analysis. Briefly, cells (1×10^6) resuspended in 50 μl of phosphate-buffered saline containing 2% fetal calf serum and 1% sodium azide (FACS solution) were incubated with anti-CD43 (L10), anti-CD3 (OKT3), biotinylated anti-CD40-L, or anti-CD69 mAbs for 30 min at 4 °C. Cells were then washed by centrifugation at $300 \times g$ with FACS solution and incubated with second step reagent fluorescein isothiocyanate for 30 min at 4 °C and washed as above, following which cells were resuspended in FACS solution and fixed with 1% paraformaldehyde (final concentration). Cells were analyzed with a FACSort with the CELLQUEST program (Becton and Dickinson, San José, CA).

RESULTS

CD43-dependent IL-2 Gene Expression Involves the PKC and the Ca^{2+} Signaling Pathways—Given the importance of IL-2 in the proliferative and late stages of T cell activation and the fact that simultaneous signaling from CD43 and the TcR had been previously reported to result in IL-2 production (21, 27), we investigated the molecular mechanisms involved in the CD43-dependent IL-2 mRNA induction. As shown in Fig. 1, cross-linking CD43 on the surface of normal human peripheral blood T lymphocytes with two different mAbs, without co-ligating the TcR, was sufficient to generate signals that lead to IL-2 mRNA induction (*lanes 1* and *4*). When cells were incubated with both anti-CD43 mAbs simultaneously, the amount of IL-2 mRNA increased approximately 7-fold over control cells (compare *lanes 7*, *10*, and *11*), although to a lesser extent than when cells were treated with PMA and ionomycin (*lane 14*). Independent

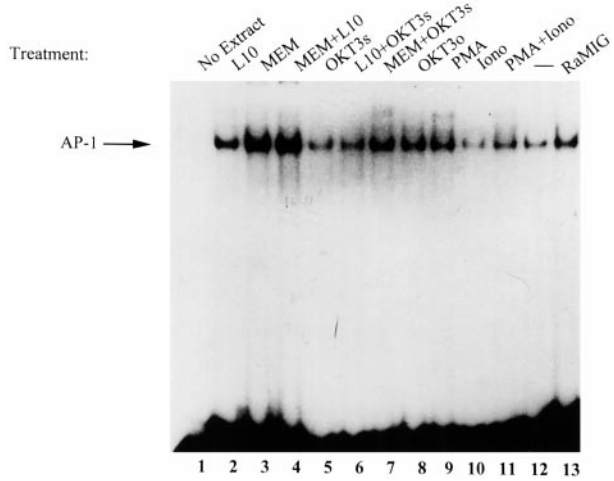

FIG. 2. **CD43 cross-linking induces AP-1 binding activity independently of TcR signaling.** Electrophoretic mobility shift assays (EMSAs) were performed with 2.5 μg of nuclear proteins from Jurkat cells stimulated with L10 and/or MEM-59 (1 μg/ml) and/or OKT3 at optimal (OKT3o, 1 μg/ml) or suboptimal (OKT3s, 10 ng/ml) amounts. Extracts treated with PMA, Iono, or both were included as positive controls. A ^{32}P-end-labeled probe containing the AP-1 site was used. The *arrow* indicates the specific DNA-protein complex. A representative of three independent experiments is shown.

of the anti-CD43 mAb that was used to stimulate the cells, signals resulting in the induction of IL-2 mRNA were blocked by CsA (*lanes 3*, *6*, and *9*) and partially blocked by staurosporine (*lanes 2*, *5*, and *8*). Cells treated only with CsA or staurosporine but no antibody (*lanes 12* and *13*), control antibody-treated (*lane 11*), and untreated cells (*lane 10*) had equivalent levels of IL-2 mRNA. The graph shown in the *lower panel* of Fig. 1 represents the percentages of change in the IL-2 cDNA/GAPDH cDNA signal. These data suggest that CD43-mediated signals can induce IL-2 gene expression, recruiting the PKC and the Ca^{2+} pathways.

CD43 Induces Binding of AP-1 Transcription Factor to DNA—To investigate the pathway leading to CD43-mediated regulation of gene expression, we looked for the induction of transcription factors following CD43 ligation, particularly for factors that bind to the IL-2 gene promoter such as AP-1, NF-AT, and NFκB (44). Cross-linking CD43 on the surface of Jurkat cells with either anti-CD43 mAb, L10, and/or MEM-59 promoted the formation of an AP-1 complex over levels of control cells (Fig. 2, *lanes 2*, *3*, *12*, and *13*); co-stimulation with both mAbs did not result in higher levels of AP-1 binding than those observed when cells were stimulated with MEM-59 alone (*lanes 2–4*). At this time point, CD43 ligation resulted in higher levels of AP-1 than ligation with optimal amounts of OKT3 (OKT3o) or stimulation with PMA (*lanes 8* and *9*), probably reflecting a difference in kinetics. As expected, ionomycin did not have any effect on the DNA binding capacity of AP-1 (*lanes 10* and *11*), because the AP-1 family of transcription factors is activated by the MAPK pathway and not by the sustained calcium plateau characteristic of ionomycin.

To determine the kinetics of the CD43-dependent AP-1 binding activity, human peripheral blood T lymphocytes were activated for different periods of time. Following CD43 cross-linking, AP-1 binding to DNA could be detected as early as 30 min and was maintained for at least 4 h (Fig. 3*A*, *lanes 1*, *4*, *7*, and *10*), whereas when cells were stimulated with PMA and ionomycin, the response was slower, as reflected by the fact that AP-1 induction could be observed only 1 h after the onset of the experiment (*lanes 2*, *5*, *8*, and *11*). When cells were treated with control mAb (3D6), AP-1 binding activity remained constant all through the experiment (*lanes 3*, *6*, *9*, and *12*).

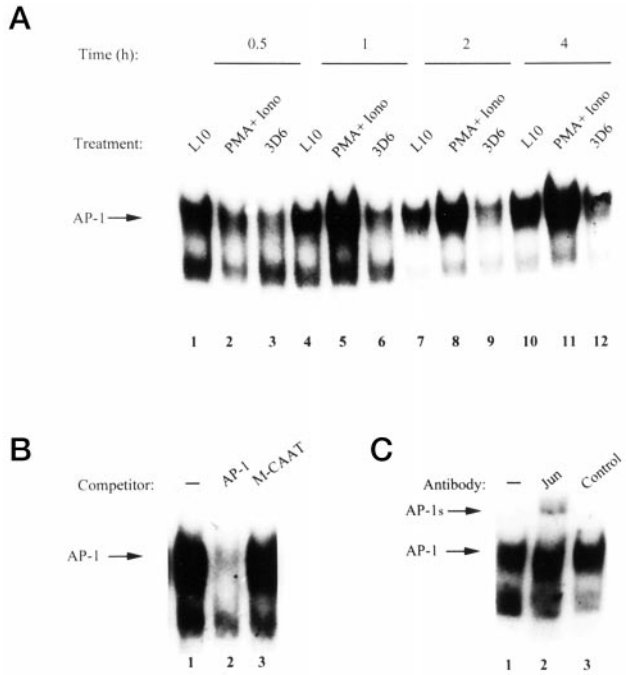

FIG. 3. **AP-1-DNA complexes induced by CD43 ligation contain c-Jun.** *A*, CD43 induces AP-1 binding activity in a time-dependent manner. Normal human peripheral T lymphocytes were incubated for the indicated times with mAb L10, PMA and Iono, or mAb 3D6, as described. Nuclear extracts were prepared and subjected to EMSAs with the AP-1 probe. The *arrow* indicates the position of the induced complex. Results were confirmed in three independent experiments. *B*, AP-1 complexes induced by CD43-mediated signals are specific. Human T lymphocytes were CD43-stimulated for 4 h, and nuclear extracts were used for competition experiments with 50-fold excess of the indicated cold oligonucleotides. The *arrow* indicates the position of the complex. *C*, AP-1 complexes induced by CD43 ligation contain c-Jun. Nuclear extracts from anti-CD43 mAb L10-treated normal human T lymphocytes were incubated with the AP-1 probe before adding anti-c-Jun or an irrelevant antibody (*Control*). The *lower arrow* indicates the position of the induced complex, and the *upper arrow* indicates that of the supershifted complex.

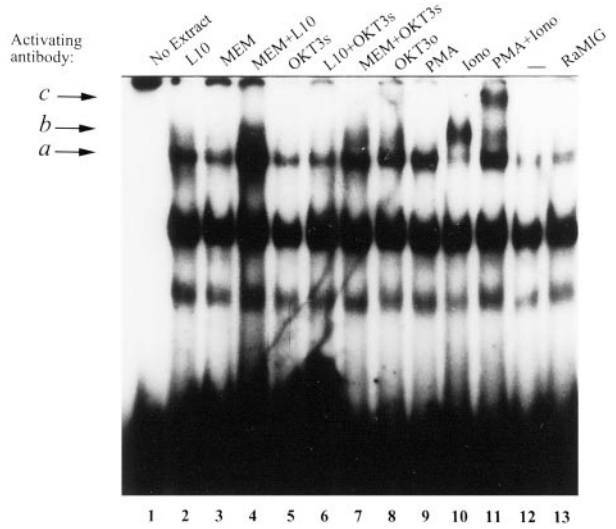

FIG. 4. **CD43 cross-linking induces binding activity to the distal NF-AT site of the IL-2 promoter.** Jurkat cells were treated with 1 μg/ml of L10, MEM-59, and/or OKT3 at optimal (1 μg/ml, OKT3o) or suboptimal (10 ng/ml, OKT3s) amounts for 4 h, following which nuclear extracts were obtained and used for EMSA, using a ^{32}P -labeled probe containing the distal NF-AT site of the IL-2 promoter. Extracts from cells treated with PMA, ionomycin, or both were included as positive controls; negative controls were provided by extracts from cells treated only with second antibody (rabbit anti-mouse IgG, *RaMIG*) or from unstimulated cells. The different inducible complexes were designated as *a* (AP-1), *b* (NF-AT), and *c* (AP-1/NF-AT). Data shown are representative of three independent experiments.

Specificity of the DNA-AP-1 complexes formed following CD43 ligation was demonstrated by competition experiments (Fig. 3*B*), where AP-1 binding to the labeled probe was competed with a 50-fold molar excess of cold AP-1 oligonucleotide (*lanes 1* and *2*), whereas similar amounts of a nonspecific oligonucleotide, M-CAAT, had no effect (*lane 3*). Because c-Jun is one of the most common members of AP-1 (45), we investigated whether it was present in the DNA-AP-1 complexes generated through CD43. As shown in Fig. 3*C*, when nucleoprotein complexes were incubated with anti-Jun antibodies, a partial supershift could be observed (*lane 2*), thus indicating the presence of c-Jun. Other members of the Jun family, however, might also be present, because the induced complexes did not disappear completely following supercomplex formation. Various members of the AP-1 family of proteins (c-Fos, Fos B, c-Jun, JunB, and Fra1) have been implicated in gene regulation upon T cell activation (46, 47). Altogether these data suggest that AP-1 participates in the CD43-specific signaling pathway leading to gene expression.

We had previously shown that simultaneous stimulation through the CD43 molecule and the TcR-CD3 complex resulted in enhanced IL-2 production (21). Here, we activated normal human peripheral blood T cells with saturating amounts of the anti-CD43 mAbs L10 or MEM-59 alone or in combination with suboptimal concentrations of OKT3 (OKT3s) for 4 h and evaluated the AP-1 binding activity. As shown in Fig. 2, integrating signals generated through the TcR with OKT3s and through CD43 with L10 or MEM-59 did not result in enhanced DNA binding activity of AP-1 as compared with cells activated either through the TcR alone or the CD43 molecule alone (*lanes 2*, *3*, *5*, *6*, and *7*). In fact, at this time point (4 h), an inhibitory effect was observed when OKT3s was used in combination with the anti-CD43 mAbs, suggesting an eventual cross-talk between the TcR and the CD43 signaling pathways.

CD43 Cross-linking Induced Two Complexes Recognizing a Composite NF-AT/AP-1 Site—We investigated next whether CD43 signaling would lead to NF-AT binding to DNA when Jurkat cells were activated for 4 h with L10, MEM-59, or both mAbs, with or without suboptimal amounts of OKT3 or with PMA and/or ionomycin. When cells were treated with PMA, a fast migrating complex was observed (Fig. 4, *lane 9*, *complex a*) that corresponds to AP-1 binding to the NF-AT site (48, 49). Ionomycin treatment induced the formation of a second DNA-protein complex (*complex b*, *lane 10*) that corresponds to NF-AT binding to its cognate site (48, 50). In addition to complexes *a* and *b*, treatment with PMA and ionomycin resulted in the formation of a third complex (*complex c*, *lane 11*) that contains AP-1 and NF-AT bound simultaneously to the composite NF-AT/AP-1 site (48, 51). Ligation of CD43 with either anti-CD43 mAb resulted in the formation of complex *a* (*lanes 2* and *3*). Simultaneous cross-linking of CD43 with MEM-59 and L10 induced the formation of complexes *a* and *b*, suggesting an additive effect of signals generated through each epitope (*lane 4*). Stimulation of the cells with OKT3o promoted also the formation of complexes *a* and *b* (*lane 8*), whereas OKT3s induced only AP-1 binding activity (*complex a*, *lane 5*). Co-ligation of CD43 through the MEM-59 mAb with OKT3s resulted in enhanced AP-1 DNA binding activity (*complex a*) as well as in the translocation of NF-AT (*complex b*) to the nucleus (*lane 7*); complex *b* contained NF-AT because immune band shift experiments showed that complex *b* was supershifted in the presence of anti-NF-ATc antibody (Fig. 5*C*) and because this complex was inhibited by cyclosporin A (Fig. 5*D*). None of the stimuli was capable of inducing the supercomplex formation that re-

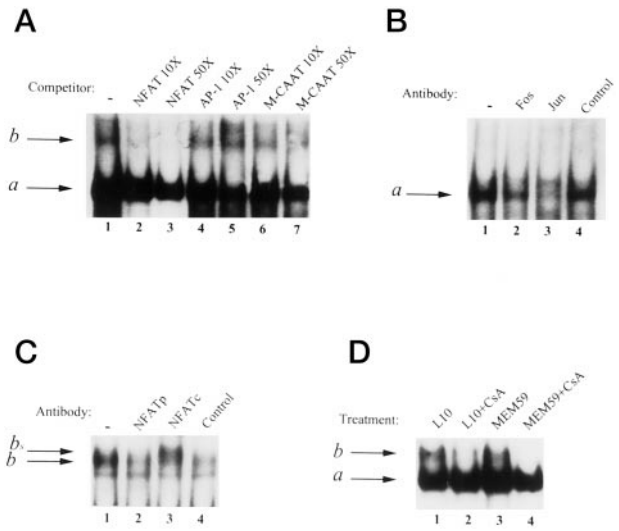

FIG. 5. **NF-AT binding activity induced by CD43 involves both AP-1 and NF-AT proteins.** *A*, CD43-induced NF-AT complexes are specific. Nuclear extracts (5 μg) from Jurkat cells co-stimulated with 1 μg/ml of each L10 and MEM-59 for 4 h were used for competition experiments in the presence of either 10- or 50-fold excess of the indicated cold oligonucleotides The *arrows* indicate the positions of induced complexes. *B*, within the NF-AT/AP-1 composite site, AP-1 complexes comprise c-Jun and c-Fos. Nuclear exctracts from MEM-59 stimulated Jurkat cells were incubated with the NF-AT probe as described before, following which DNA-protein complexes were incubated with anti-Jun, anti-Fos, or control antibody (anti-CDC42) and separated as described under "Experimental Procedures." *C*, complexes induced by CD43 contain NF-ATc. Nuclear extracts from ionomycin-treated Jurkat cells were incubated with the probe as described, prior to the addition of anti-NF-ATc, anti-NF-Atp, or control antibody (anti-CDC42). The *lower arrow* indicates the position of complex *b*, and the *upper arrow* indicates the position of the supershifted complex (b_s). *D*, CD43-induced NF-AT complexes formation is inhibited by CsA. Human peripheral blood T lymphocytes were treated with L10 or MEM-59 mAbs, in the presence or absence of CsA for 4 h. EMSA analysis was performed with 2.5 μg of nuclear extracts protein and ^{32}P-labeled NF-AT site containing probe. The *arrows* indicate the positions of the induced complexes.

sulted from PMA and ionomycin simultaneous addition (Fig. 4, *lane 11*). When the experiment was done with unstimulated cells or cells treated with the secondary antibody only, none of these complexes were induced (Fig. 4, *lanes 12* and *13*).

To investigate the nature of the two NF-AT recognizing complexes induced following CD43 cross-linking on Jurkat cells, competition experiments were performed with oligonucleotides containing the composite NF-AT/AP-1-binding site or the AP-1 consensus-binding site. In Fig. 5*A*, we show that complex *a* was partially competed with both oligonucleotides, confirming that this complex contained AP-1 (*lanes 1–5*), whereas addition of an unrelated oligonucleotide had no effect on complex *a* formation (*lanes 6* and *7*). A 10-fold molar excess of composite NF-AT oligonucleotide inhibited the formation of complex *b* (*lanes 1–3*), whereas comparable amounts of the AP-1 oligonucleotides had no effect (*lanes 4* and *5*), and MCAAT had only a slightly inhibitory effect (*lanes 6* and *7*).

To demonstrate that complex *a* induced by CD43 contained AP-1, DNA-protein complexes were allowed to react with anti-Fos, anti-Jun, or a nonspecific antibody (Fig. 5*B*). Addition of either anti-Fos or anti-c-Jun resulted in partial inhibition of complex *a* formation (*lanes 2* and *3*), whereas the nonspecific antibody had no effect (*lane 4*). The presence of NF-ATc in complex *b* was demonstrated by the fact that incubation of DNA-protein complexes from ionomycin-treated Jurkat cells with anti-NF-ATc antibody induced a supershift (Fig. 5*C*, *lane 3*), whereas anti-NF-ATp antibody had no effect (*lane 2*). Moreover, in normal human T lymphocytes, induction of complex *b* but not complex *a* was inhibited when cells were treated with cyclosporin A prior to CD43 cross-linking (Fig. 5*D*, *lanes 2* and *4*).

Co-ligation of CD43 with the L10 mAb and suboptimal amounts of anti-CD3 OKT3 mAb resulted also in reduction of the AP-1 binding activity (complex *a*) to both the composite NF-AT/AP-1 site (Fig. 4, *lanes 2* and *6*), and the AP-1 consensus site (Fig. 2, *lane 6*). On the contrary, co-ligation with the anti-CD43 mAb MEM-59 and suboptimal amounts of anti-CD3 OKT3 mAb resulted in higher AP-1 binding activity (Fig. 4, *lanes 3* and *7*). Under these conditions, NF-AT binding (*complex b*) was also induced.

Together, these results show that CD43-mediated signals induce AP-1 and NF-AT DNA binding activity to the distal NF-AT site of the IL-2 promoter, that different anti-CD43 mAb generate slightly different intracellular signals, and that, depending on the anti-CD43 mAb, TcR-mediated signals modulate the DNA-binding pattern to the composite NF-AT/AP-1 site of the IL-2 promoter.

CD43 Signaling Induces NFκB—The NFκB family of transcription factors controls the expression of numerous cytokine genes involved in T cell function (for review see Ref. 52). We investigated whether CD43 signaling would induce NFκB activity. As shown in Fig. 6, cross-linking CD43 on the surface of normal human T cells with the L10 mAb induced NFκB binding activity in a time-dependent manner. NFκB induction could be observed within 1 h, reaching a maximum at 2 h and remaining constant for up to 4 h after stimulation (*lanes 2*, *6*, *10*, and *14*). Interestingly, induction of NFκB DNA binding activity following CD43 ligation with equivalent amounts of MEM-59 mAb was detected only 4 h after stimulation (*lanes 3*, *7*, *11*, and *15*). The kinetics of NFκB binding activity induced by PMA and ionomycin treatment was faster, reaching a maximum as early as 30 min and decreasing slowly thereafter (*lanes 4*, *8*, *12*, and *16*). In all cases, the NFκB activity mediated through the CD43 molecule was weaker than that observed for PMA- and ionomycin-treated cells. Incubation with control mAb (3D6) did not result in induction of NFκB binding activity (*lanes 5*, *9*, *13*, and *17*).

Specificity of the NFκB-DNA complexes resulting of CD43 ligation was demonstrated by competition assays where the addition of 50-fold molar excess of cold NFκB abolished DNA binding (Fig. 7*A*, *lane 3*). No competition was observed when the same amount of the unspecific oligonucleotide MCAAT was added (*lane 2*). Additional proof that NFκB was present in those complexes came from immune band shift experiments. When the NFκB-DNA complexes induced after CD43 engagement (Fig. 7*B*) were treated with anti-p65 antibody, a supershift was observed correlating with the disappearance of the specific NFκB complex (*lane 3*), demonstrating that the CD43-mediated NFκB binding activity involves p65. Addition of a control antibody had no effect on the electrophoretic mobility of the DNA-protein complexes (*lane 2*). Altogether, these data show clearly that NFκB binding activity is induced by CD43-dependent signals.

CD43 Ligation Induces the Expression of CD69 and CD40-L in T Lymphocytes—The induction of AP-1, NF-AT, and NFκB by CD43-mediated signals implicated a potential role for this molecule in the expression of genes with recognition sites for these transcription factors in their promoters. To test this possibility, we investigated the effect of CD43 cross-linking on CD69 expression in human peripheral blood T cells. CD69 is an early marker of T cell activation whose expression is mediated by NFκB activation in response to tumor necrosis factor α (33) or by the proximal AP-1 site of the CD69 promoter region in response to TcR/CD3 cross-linking or PMA treatment (34). As shown in Fig. 8*A*, ligation of CD43 with the L10 or MEM 59

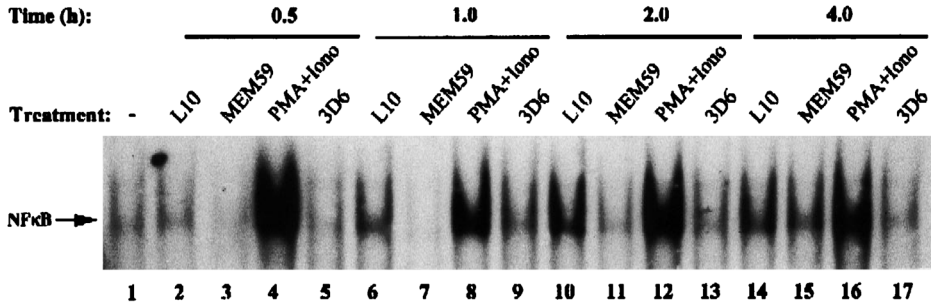

FIG. 6. **CD43 ligation induced specific NFκB binding activity in a time-dependent manner.** Human T lymphocytes were incubated with 1 μg/ml of L10, MEM-59, or 3D6 mAbs or with PMA and ionomycin for 0.5, 1, 2, or 4 h. Nuclear extracts were used for EMSA with a ^{32}P-labeled probe containing the consensus NFκB site. The *arrow* indicates the induced DNA-protein complex. A representative experiment out of three is shown.

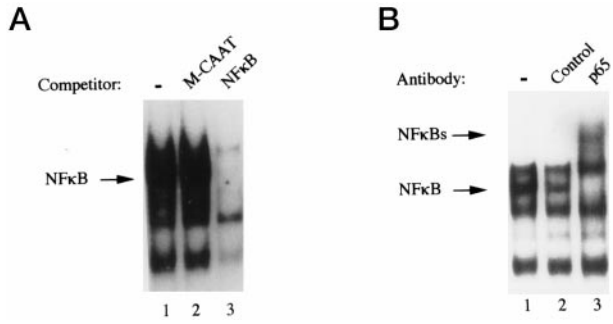

FIG. 7. **The CD43-dependent NFκB complex contains p65 (Rel A).** *A*, CD43-induced NFκB complexes are specific. Competition assays with 50-fold excess of cold NFκB or MCAAT oligonucleotides were done with nuclear extracts from human T lymphocytes isolated from peripheral blood, treated for 4 h with 1 μg/ml L10 mAb. The *arrow* indicates the position of the induced complex. *B*, CD43-induced NFκB complexes contain p65. Nuclear extracts from human T lymphocytes, stimulated for 2 h in the presence of mAb L10, were used for supershift assays with an anti-p65 antibody. The *lower arrow* indicates the induced complex, and the *upper arrow* indicates the p65 supershifted complex ($NF\kappa B_s$).

mAbs for 18 h resulted in a modest induction of CD69 expression with 5.3 ± 0.89 and 6.1 ± 2.0% $CD69^+$ cells, respectively, whereas when incubated with the isotype control (3D6) only 1% of the cells were $CD69^+$. As expected, TcR- and PMA-mediated activation resulted in higher percentages of $CD69^+$ cells with 24 ± 2.5 and 72 ± 14.5% $CD69^+$ cells, respectively. Because CD43 cross-linking resulted only in a weak induction of CD69 expression, we asked whether CD43 signals could enhance PMA-mediated CD69 expression. To test this possibility, human peripheral T cells were treated with different amounts of PMA alone or in combination with the anti-CD43 L10 mAb. As shown in Fig. 8*B*, although stimulation with 0.01 ng/ml of PMA did not induce CD69 expression, co-stimulation with the L10 mAb resulted in the same low levels of CD69 expression 4–5% (Fig. 8*A*) as observed with the L10 mAb alone. A higher dose of PMA (0.1 ng/ml) induced CD69 expression in 90 ± 5% of the cells, with a mean fluorescence intensity (MIF) of 61 ± 10. When cells were treated simultaneously with the L10 mAb and PMA (0.1 ng/ml), 100% of the cells were $CD69^+$ and the MIF almost doubled (MIF = 115 ± 7), compared with PMA alone. Stimulation with 50 ng/ml of PMA resulted in approximately three times higher CD69 expression (MIF = 196 ± 12), and co-stimulation by CD43 cross-linking further increased it (MIF = 258 ± 15). These data indicate that CD43 signaling induces CD69 expression and that CD43-dependent signals cooperate in an additive fashion with PMA signaling to enhance the expression of CD69, probably by an AP-1- and NFκB-dependent mechanism.

CD40 ligand (CD40-L) is transiently expressed on activated T cells and interacts with CD40, a constitutively expressed molecule present on antigen presenting cells. It was recently shown that deletion of a proximal composite NF-AT site of the CD40-L promoter reduced transcriptional activity of this gene to background levels, suggesting that the Ca^{2+} pathway plays an important role in CD40-L expression (35).[3] Because CD43 signaling involves Ca^{2+} mobilization and NF-AT DNA binding activity, we tested whether CD43 signaling induced CD40-L expression in human peripheral T cells. As shown in Fig. 8*C*, CD40-L expression in human peripheral T cells is clearly dependent on Ca^{2+} signaling, because 95 ± 8% of the cells were $CD40\text{-}L^+$ after 8 h treatment with ionomycin, whereas PMA treatment resulted only in 2.7 ± 0.7% of $CD40\text{-}L^+$ cells. When CD43 was cross-linked with L10 or MEM 59 mAbs, 12.86 ± 2 and 16.62 ± 1.5%, respectively, of the cells were $CD40\text{-}L^+$. Cells treated with 3D6 (isotype control) showed basal CD40-L expression (3.6%+0.8). When cells were activated through the TcR, 45 ± 3% of the cells were $CD40\text{-}L^+$. These results show that CD43 signaling induces the expression of CD40-L, most probably through Ca^{2+} mobilization and the induction of NF-AT binding activity to the proximal NF-AT site of CD40-L. All together the data provided here show that CD43 signaling can positively regulate the expression of different genes containing AP-1, NFκB, or NF-AT responsive elements in their regulatory regions.

DISCUSSION

Reports regarding the role of CD43 involve this co-receptor molecule in multiple aspects of cell function, yet the molecular basis of CD43 signaling is only partially understood. The participation of CD43 in T cell function (13–22) prompted us to search for the CD43 signaling pathway. The ideal way to evaluate the signaling capacity of a molecule would be to use natural ligands; however, in the case of CD43, this is particularly difficult because all the ligands that have been described so far are also ligands of other T cell surface molecules (10–12). To mimic the interaction with its ligands, CD43 was cross-linked on the cell surface with two different anti-CD43 monoclonal antibodies, L10 and MEM-59. Independent of the antibody used, CD43 signals were sufficient to induce IL-2 mRNA, and an additive effect was observed when both anti-CD43 mAbs were used simultaneously (Fig. 1). Consistent with previous reports showing that CD43 ligation resulted in both PKC activity and Ca^{2+} flux (23, 25), we found that CD43-mediated IL-2 mRNA induction was partially inhibited by cyclosporin A and staurosporine, regardless of whether the cells were activated with either mAb alone or in combination (Fig. 1). This dual pathway of activation was further confirmed by the CD43-dependent induction of CD69 and CD40-L (Fig. 8), whose expression depend on the MAPKs and the Ca^{2+} pathways, respectively (33–35).

Initial steps of T cell activation are orchestrated through the recruitment of several transcription factors, including NF-AT, AP-1 and NFκB (2, 44, 53). Cross-linking CD43 with either L10 or MEM-59 mAbs on Jurkat cells resulted in the induction of AP-1 DNA binding activity (Fig. 2). Complexes between the AP-1 probe and nuclear proteins were formed as early as 30 min following CD43 ligation and were still present after 4 h.

[3] H. Lindgren, personal communication.

These complexes were specific and contained c-Jun as evidenced by the supershift induced in the presence of anti-c-Jun antibody (Fig. 3) and the inhibition of the AP-1-NF-AT composite site by anti-Fos or anti-Jun antibodies. The differential behavior of anti-Jun antibody on the complex formation between c-Jun and the AP-1 or NF-AT/AP-1- sites could be due to the different affinity of c-Jun for either site. The fact that not all protein-DNA complexes supershifted when incubated with the anti-c-Jun antibody suggests that other members of the Jun family may be recruited in response to CD43 ligation. In T cells, AP-1 activity is tightly regulated by the MAPKs pathway at the transcriptional and posttranslational levels, increasing the amounts and enhancing the stability and binding activity of AP-1 proteins through phosphorylation (for review see Ref. 45). The CD43-mediated AP-1 activity reported here may result of signals generated through the MAPK pathway because we have shown that CD43 signaling leads to ERK2 activation and nuclear localization and to Fos-SRE-dependent transcription (26).[4]

Four different NF-AT proteins expressed in lymphoid cells as well as in other cell types have been described (54). Upon dephosphorylation by the calmodulin-dependent phosphatase calcineurin, NF-AT proteins become activated and translocate to the nucleus. The NF-AT transcription factors can bind to DNA directly or in combination with AP-1 family members (for review see Ref. 31). We report here that CD43-specific signals induced two specific NF-AT DNA-protein complexes (Fig. 4). One of the complexes, similar to that generated by incubating the cells with PMA, contained AP-1 proteins and was CsA-resistant. Interestingly, CD28 and PMA signaling have been shown to result in activation of the IL-2 promoter by NF-AT proteins in a CsA-resistant manner. The complex formed under such conditions was also competed by AP-1 sequences (49). This is particularly relevant considering the fact that CD43 has been shown to be a substitute for CD28 function in $CD28^{-/-}$ mice (22). Induction of the second CD43-mediated NF-AT complex, equivalent to that generated by ionomycin, required simultaneous cross-linking of CD43 with L10 and MEM-59 mAbs on Jurkat cells. However, in peripheral blood T lymphocytes, signals generated through either mAb were sufficient to induce that nucleoprotein complex, in a CsA-sensitive way.

Transient peaks of intracellular calcium result in AP-1 and NFκB activation (28–30). Data reported here show that the molecular events following CD43 cross-linking involved AP-1 and NFκB induction, correlating with the transient peak of intracellular calcium reported upon CD43 ligation on HPB-ALL or Jurkat cells with L10 or MEM-59, respectively (23, 25). Our results suggest that ligation with either mAb was not sufficient to induce NF-AT translocation to the nucleus in Jurkat cells but that the sustained elevated calcium concentrations required for NF-AT activation (31, 32) were presumably achieved when Jurkat cells were activated through simultaneous cross-linking of CD43 with L10 and MEM-59, because NF-AT DNA binding activity could be observed only under those conditions (Fig. 4). In peripheral blood T cells, however, the sustained Ca^{2+} fluxes that were reported following CD43 ligation with MEM-59 (25) correlate well with the NF-AT activity we report here with either mAb alone (Fig. 5*D*). The different responses we observed between peripheral blood normal T lymphocytes and Jurkat cells to CD43 ligation with different anti-CD43 mAbs could be due to a higher threshold for specific gene induction in actively dividing cells. Another possible explanation for the need of the combined effect of L10 and MEM-59 in Jurkat cells could be that particular epitopes of

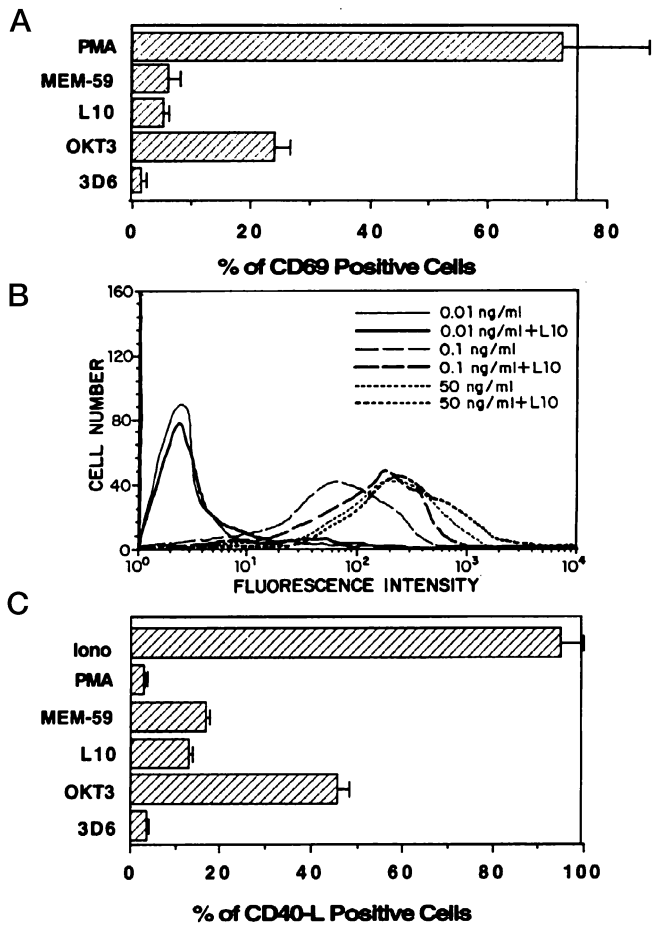

FIG. 8. **CD43 ligation enhances the expression of CD69 and CD40-L.** *A*, human peripheral T cells were stained for CD69 after 18 h stimulation with 1 μg/ml L10 or MEM-59 anti-CD43 mAbs, 1 μg/ml anti-CD3 mAb OKT3, PMA, or isotype control antibody 3D6. *B*, human peripheral T cells were stained for CD69 after 18 h co-stimulation with L10 and different amounts of PMA. *C*, to evaluate CD40-L expression, cells were stained after 8 h the indicated stimuli. Data shown represent the average of three independent experiments.

the CD43 molecule initiate different signaling events that eventually result in the additive induction of IL-2 mRNA we observed (Fig. 1).

The NFκB family proteins regulate the transcription of a wide range of genes, some of which play a central role in immunological processes. When inactive, these proteins reside in the cytoplasm, associated with specific IkB inhibitors that are degraded upon serine phosphorylation, allowing translocation of NFκB factors to the nucleus. MAPKs and PKC participate in the regulation of IkB kinases activity (29). We found that cross-linking CD43 on the surface of peripheral T lymphocytes resulted in a time-dependent induction of NFκB-DNA complexes containing p65, as determined by supershift formation in the presence of anti-p65 antibody (Figs. 6 and 7). Similar experiments performed with an anti-c-Rel antibody indicated that c-Rel was not present in those complexes (data not shown), consistent with the fact that the presence of c-Rel in NFkB complexes is a late response and that p65 recruitment precedes and is required for c-Rel expression. p65 translocation to the nucleus during inflammatory and immune responses is well documented (for review, see Refs. 55 and 56). Thus, our results suggest that CD43 participates actively in immune response modulation. However, not all CD43-mediated signals result in NFκB recruitment and T cell activation. In a recent report where Jurkat cells were activated through the CD43-specific mAb J393, cells were found to undergo apoptosis. The

[4] G. Pedraza-Alva, unpublished data.

molecular mechanisms underlying this event were associated with NFκB down-regulation (57), because this transcription factor has been linked with an anti-apoptotic function (58–60). Consistent with this, the CD43-dependent NFκB activity we report in this study did not correlate with apoptosis.[5] The fact that ligation of CD43 with two different monoclonal antibodies resulted in opposite effects on NFκB activity (this report and Ref. 57) supports the idea that different anti-CD43 monoclonal antibodies generate different signals and biological responses.

Altogether, data shown here, in combination with other reports where CD43 has been described as a signaling molecule (13–22), suggest that by regulating the activation of several transcription factors, CD43 has an important participation in cell signal and function. To further test that CD43-mediated induction of AP-1, NFκB, and NF-AT transcription factors could result in the induction of T cell activation genes, we tested the effect of CD43 ligation upon the induction of CD69, whose transcription depends upon AP-1 or NFκB activities (33, 34), and that of CD40-L, which is induced mainly by NF-AT ((Fig. 8). CD43 ligation slightly but significantly increased the amount of CD69 positive cells and cooperated with PMA signals by further increasing both the amount of cells that were $CD69^+$ and the density of CD69 molecules/cell. CD43 signals also resulted in an increased CD40-L expression, presumably through the activation of NF-AT transcription factors. The low percentage of $CD40\text{-}L^+$ and $CD69^+$ cells resulting of CD43 ligation suggested that a particular subset of cells was able to express these molecules. However, these cells did not correspond exclusively to a memory cell phenotype nor to the γδ lineage (data not shown). Considering that CD43 expression and signals might target specific subsets of memory and effector T cells (61), further experiments are needed to characterize these cells. In a recent report it was shown that CD43 ligation on the surface of dendritic cells induces the expression of IL-1β, tumor necrosis factor α, IL-6, IL-12, and IL-10, leading to macrophage activation (62). The promoters of IL-1β and tumor necrosis factor α are regulated by AP-1 (63, 64), functional roles for AP-1, CREB, NFκB, and NF-IL-6 have been described for the IL-6 promoter (65), and NFκB-mediated induction of IL-12 has been reported (66), further suggesting the participation of these transcription factors in the CD43-dependent gene expression.

T cell activation is the result of a series of intracellular events initiated through the specific interaction of multiple cell surface molecules with their counter-receptors. We have shown here that the AP-1 binding activity, either to the consensus site or to the AP-1 site of the NF-AT composite sequence upon ligating CD43 alone on the cell surface with the L10 mAb was diminished by minimal concomitant TcR signaling (Fig. 3). This down-modulation correlated with a reduction in IL-2 mRNA when cells were co-stimulated under the same experimental conditions for the same length of time (data not shown). On the other hand, co-stimulation with the anti-CD43 mAb MEM-59 rather than L10 (Fig. 5), resulted in inhibition of AP-1 binding activity to the consensus site but increased binding activity to the AP-1 site of the NF-AT composite sequence. Furthermore, co-ligation with this mAb also resulted in NF-AT translocation to the nucleus, probably because of a longer calcium flux and to the switch from a MAPK-dependent to a Ca^{2+}-dependent response (28). However, when cells where co-stimulated with either L10 or MEM-59 and OKT3 for 24 h, IL-2 mRNA production and IL-2 secretion to the medium were enhanced.[6] Thus, cross-linking CD43 with different mAbs results in different signals that in turn will be regulated differentially by concomitant TcR ligation. This cross-talk modulates the AP-1 binding activity to different sequences, suggesting that different sets of genes could be turned on or off, depending of the epitope of the CD43 molecule that is being recognized by a given ligand and whether the TcR is being activated or not. Additional examples of cross-talk between CD43 and other cell surface molecules have been described. Enhanced LFA-1-dependent homotypic aggregation was shown following up-regulation of CD43 expression by retinoic acids (67), correlating with previous reports where, upon activation with anti-CD43 mAbs, integrin-mediated cell adhesion and movement were up-regulated (68, 69). A mutual activation of CD43 and CD2 adhesive function has also been described (12), further documenting the cross-talk between co-receptors.

The CD43-dependent induction of IL-2 mRNA, CD69, and CD40-L expression and activation of DNA binding activity of AP-1, NF-AT, and NFκB implicate an important role for this molecule in the regulation of gene expression. Further work on CD43 signaling and control of gene expression in different scenarios will forward understanding of the multiple regulatory effects of CD43.

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