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REGULATION OF EXPRESSION OF CD27, A T CELL-SPECIFIC MEMBER OF A NOVEL FAMILY OF MEMBRANE RECEPTORS[1]

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CD27 belongs to a newly defined family of transmembrane R, including the nerve growth factor R, two distinct TNF R and CD40. The function of CD27 is unknown, but on the basis of structural and functional properties, we postulate that it plays a role in the events subsequent to T cell activation, possibly as a cytokine R. We have analyzed the mechanisms underlying the regulation of CD27 protein expression. Membrane expression of CD27 strongly increases after T cell activation via the TCR/CD3 complex or the CD2 molecule. In contrast, direct stimulation of protein kinase C by phorbol esters markedly down-regulates CD27 surface expression. This down-regulation most likely does not result from CD27 phosphorylation, because both anti-CD3 mAb and PMA induce hyperphosphorylation of CD27 on serine residues. Rather, membrane expression seems to be regulated primarily at the RNA level. Stimulation of T cells with anti-CD3 mAb strongly increases steady state CD27 mRNA levels, whereas PMA treatment greatly reduces these transcript levels. Dissection of the TCR/CD3-induced signaling pathways showed that cytoplasmic cAMP as well as Ca^{2+} concentrations contribute to the increase of CD27 expression. These data indicate that upon Ag-specific T cell stimulation, membrane expression of CD27 is regulated at the RNA level through the joint action of distinct TCR/CD3-associated signaling pathways.

CD27 is a T cell specific differentiation Ag expressed at low levels on about 80% of resting peripheral blood T cells. Both $CD4^+$ and $CD8^+$ T cell subsets contain $CD27^+$ and $CD27^-$ subpopulations (1, 2). The functional properties of $CD27^+$ and $CD27^-$ subpopulations within the $CD4^+$ subset differ in that Th cell activity for B cell differentiation can be provided by $CD27^+$ cells but not by $CD27^-$ T cells (3). However, the $CD27^+$ and $CD27^-$ subpopulations do not belong to separate T cell lineages but rather represent distinct T cell activation states (3).

On resting T cells, CD27 is present as a disulfide-linked homodimer with chains of 50 to 55 kDa (p55) (1, 2). Stimulation with the PK[3]C-activator PMA, causes a marked downregulation of CD27 at the plasma membrane (1). In contrast, activation of T cells via the TCR/CD3 complex results in a fivefold increase in CD27 membrane expression (1). Apart from the p55 form, a component with a M_r of 28 to 32 kDa (p32) can be isolated with anti-CD27 mAb from these stimulated cells (1, 4). This p32 CD27 form has extensive structural homology with p55 in at least the extracellular domain (4). Our recent observations indicate that p32 is released in the supernatant of activated T cells and can be found in both serum and urine.[4]

Recently a cDNA clone encoding the CD27 protein has been isolated (D. Camerini, G. Walz, W. A. M. Loenen, J. Borst, and B. Seed, manuscript in preparation). Nucleotide sequence determination showed that CD27 cDNA encodes a typical type I transmembrane molecule. The extracellular part contains a domain with a high number of cysteine residues, occuring in a repetitive pattern. The spacing of these cysteine residues places CD27 in a new R family, together with the nerve growth factor R (5), two distinct TNF R (6–8), the B cell Ag CD40 (9), the murine T cell Ag 4-1BB (10), and the rat T cell Ag OX40 (11). The homology between these proteins ranges around 25%. The cysteine-rich domain is thought to contain cytokine-binding capacity in the nerve growth factor R (5). The function of CD27 is unknown; however, its predicted protein structure, together with its strong upregulation subsequent to T cell activation and the observation that anti-CD27 mAb enhance T cell proliferation induced via the TCR/CD3 complex (1), would be in line with a possible role as a cytokine receptor.

Upon Ag recognition by T cells, different signal transduction pathways are activated, and therewith a number of different protein kinases: PKC (12, 13), PKA (14–16), and tyrosine kinase(s) (17, 18). Expression of membrane Ag may be directly affected through protein kinase-mediated phosphorylation of their cytoplasmic residues (19, 20). Kinase activity may also affect protein expression by modifying transcriptional activity, for instance, phos-

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[3] Abbreviations used in this paper: PK, protein kinase; DBcAMP, N-2-0-dibutyryladenosine 3′5′ cyclic monophosphate; CyA, cyclosporine A.

[4] **Hintzen, R. Q., R. de Jong, C. E. Hack, M. Chamuleau, E. F. R. de Vries, I. J. M. ten Berge, J. Borst, and R. A. W. van Lier.** A soluble form of the human T cell differentiation antigen CD27 is released after triggering of the T cell receptor/CD3 complex. *Submitted for publication.*

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phorylation may alter the DNA-binding properties of transactivating factors (21, 22). This study was undertaken to identify the control mechanisms that regulate CD27 membrane expression. We show that a number of second messenger systems interact to regulate CD27 membrane expression at the RNA level.

MATERIALS AND METHODS

mAb. The mAb CLB-T3/3 (IgG2a) and CLB-T3/4.E (IgE) directed against the CD3 molecule, CLB-T11.1/1 (IgG1) and CLB-T11.2/1 (IgG1; both anti-CD2), CLB-Il-2R (IgG2b; anti-CD25), CLB-CD27/2 (IgG2a; anti-CD27) and CLB-CD28/1 (IgG1; anti-CD28) were produced at the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service.

After purification from ascites fluids by either protein A-Sepharose column chromatography or HPLC, CLB-T11.1/1, CLB-IL-2R and CLB-CD27/2 were labeled with FITC according to the method described by The and Feltkamp (23). CLB-CD28/1 was biotine labeled following standard procedures.

Reagents. 1-0-Hexadecyl-2-0-methyl-glycerol (Bachem AG, Bubendorf, Switzerland), PMA (CMC Cancer Research, Katonah, NY), phorbol 12- 13-dibutyrate (Sigma Chemical Co., St. Louis, MO), ionomycine (Calbiochem, La Jolla, CA), A23187 (Sigma), forskolin (Sigma), and DBcAMP (Sigma) were prepared as stock solutions in DMSO, stored at −20°C and diluted appropriately before use. The final concentrations contained less than 0.001% dimethyl sulfoxide. CyA (a gift from Sandoz Pharmaceuticals, Basel, Switzerland) was dissolved in ethanol (10 mg/ml) and diluted with Iscove's modified Dulbecco's medium to a stock solution of 1 mg/ml, stored at −20°C. PHA was obtained from Wellcome (Beckinham, UK). rIL-2 was kindly provided by Sandoz (Vienna, Austria). Reagents were used at concentrations that were not toxic to lymphocytes or Jurkat cells; cell viability was more than 90% in all experiments.

Cells. PBMC were isolated from buffy coats derived from healthy blood bank donors by Ficoll-Isopaque (Pharmacia, Uppsala, Sweden) density centrifugation. Lymphocytes were obtained by counterflow centrifugation elutriation. The resulting lymphocyte fractions contained less than 1% monocytes as determined by flow microfluorimetry analysis.

The leukemic T cell line Jurkat was maintained in Iscove's modified Dulbecco's medium supplemented with 5% human pooled serum, 2-ME, and antibiotics.

Lymphocyte stimulation. Anti-CD3 mAb coated plates and flasks were prepared as previously described (24). Briefly, anti-CD3 mAb (CLB-T3/3) was diluted in PBS (5 μg/ml) and applied to 96-well flat-bottomed microtiter plates (100 μl/well) (Nunc, Roskilde, Denmark), 24-well culture plates (1 ml/well) (Nunc) or, for mRNA experiments, to 175 cm^2 ml culture flasks (40 ml/flask) (Nunc). After overnight incubation at 4°C, the wells/flasks were washed twice with PBS. PBMC, lymphocytes, or Jurkat cells (as indicated) were cultured in 24-well plates ($0.25.10^6$ cells/well) in 1 ml of Iscove's modified Dulbecco's medium supplemented with 10% FCS, 2-ME, and antibiotics (culture medium). Reagents and/or mAb were added as indicated in the legends of tables and figures. In parallel cultures, cells (50,000/well) were stimulated in 96-well flat-bottomed microtiter plates in 200 μl of culture medium to assay proliferation induction.

Immunofluorescence studies. Immunofluorescence studies were performed in PBS containing 0.5% BSA and 0.1% sodium azide at 4°C. After the stimulation period, cells were harvested, washed, and 2.10^5 cells were stained with saturating amounts (1 μg/ml) of directly labeled mAb for 30 min. Thereafter, the cells were washed three times. When biotine conjugated mAb were used, cells were subsequently incubated with streptavidin phycoerythrin (Becton Dickinson, Mountain View, CA) for 30 min and washed three times afterward. The 10^4 cells were analyzed on a FACScan (Becton Dickinson, Mountain View, CA). The percentage of reactive cells is presented. In addition, the mean fluorescence intensity of the positively stained cell population is given in arbitrary linear fluorescence units. In case of Jurkat cells, the mean fluorescence of the whole cell population is given in arbitrary linear fluorescence units.

T cell proliferation. Cells were cultured as described (see *Lymphocyte stimulation*) for 3 days. During the last 4 h of culture, 0.2 μCi (7.4 kBq) [^{3}H]-thymidine (2 Ci/mmol, Amersham, Buckinghamshire, UK) was added to measure proliferation. Results are shown as the mean cpm of triplicate cultures. Standard deviations between triplicate cultures were less than 10%.

Radiolabeling. For biosynthetic labeling with ^{32}P-orthophosphate, 200 to 300 × 10^6 PBMC were washed and suspended at 5 × 10^6 cells/ml in phosphate-free medium (RPMI 1640, GIBCO Ltd., Paisley, Scotland) containing 10% FCS (dialyzed against 10 mM Tris-HCl, pH 7.4, 150 mM NaCl) and cultured in the presence of ^{32}P-orthophosphate (Amersham) at 0.01 mCi/10^6 cells overnight, in case of resting PBMC, or for 3 h in case of anti-CD3 mAb-activated cells. For the experiment depicted in Figure 2, resting PBMC were subsequently stimulated either with coated anti-CD3 mAb, or with PMA at 60 ng/ml. For the experiment depicted in Figure 3, anti-CD3 mAb activated PBMC were exposed to PMA at 60 ng/ml. Samples were drawn at different time points, cells were washed and processed for immunoprecipitation.

Immunoprecipitation and gel electrophoresis. Cells were lysed in immunoprecipitation buffer, consisting of 0.01 M triethanolamine-HCl, pH 7.8, 5 mM EDTA, 1 mM PMSF, 1 mM *N-α-p*-tosyl-*l*-lysine chloromethyl ketone, 0.02 mg/ml ovomucoid trypsin inhibitor, 0.02 mg/ml leupeptin, 1% Nonidet P-40, 350 mM NaCl, 10 mM ATP, and 10 mM NaF, 0.4 mM Na_3VO_4, and 10 mM $Na_4P_2O_7.10H_2O$ as phosphatase inhibitors. Immunoprecipitation was performed as described (4) using CLB-CD27/1 mAb conjugated to protein A-CL4B sepharose. SDS-PAGE was performed on 10 to 15% gradient vertical slabgels. Samples were assayed under reducing conditions (5% 2-ME in SDS-sample buffer). Autoradiography took place at −70°C, in the presence of intensifier screens.

Phospho-amino acid analysis. Gel pieces containing proteins of interest were cut out of fixed and dried gels after autoradiography, acrylamide was finely ground and extracted for 5 min at 100°C and 2 h at 37°C in 0.05 M NH_4HCO_3, 0.1% SDS, and 5% 2-ME. Pancreatic ribonuclease (20 μg) was added as carrier protein and total protein was precipitated in 10% TCA, washed twice with ice-cold acetone, dissolved in 100 μl 6 N HCl and hydrolyzed for 1 h at 110°C. Phospho-amino acids were identified by electrophoresis on cellulose thin-layer plates (no. 5716, Merck, Darmstadt, FRG), at pH 1.9 in one dimension (horizontal in Figure 4) and at pH 3.5 in the other (vertical), as described (25). Plates were dried for 15 min at 65°C, unlabeled phospho-amino acids were visualized with 0.25% ninhydrin in acetone and labeled phospho-amino acids by autoradiography.

RNA analysis. Cells were harvested and frozen immediately. Pellets were resuspended in ice cold 3 M LiCl/8 M ureum (10 ml/200.10^6 cells), disrupted with a polytron and left overnight on ice. RNA was collected by centrifugation, resuspended in 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.1% SDS, extracted with phenol and precipitated with ethanol. Samples of 15 μg were run on formaldehyde gels, blotted onto nitrocellulose filters, and hybridized at 65°C under standard conditions (26). Filters were washed twice with 2× SSC/0.1% SDS and once with 0.1× SSC/0.1% SDS at 65°C. After exposure, label was removed from the blot by washing 1 h at 80°C in water before hybridization with another probe.

The complete cDNA clone encoding the CD27 p55 protein used as a probe, was a gift from D. Camerini and B. Seed (D. Camerini, G. Walz, W. A. M. Loenen, J. Borst, and B. Seed, manuscript in preparation). The CD25 probe used, was a 800-bp *Eco*RI fragment generated from the pIL-2R2 cDNA clone described by Leonard et al. (27). Probes were labeled by random priming.

RESULTS

Activation-related changes in CD27 protein expression. We previously reported that T cell triggering via the TCR/CD3 complex results in a marked increase of CD27 membrane expression (1). Similar to T cell activation with anti-CD3 mAb or PHA, stimulation with anti-CD2 mAb also resulted in a transient increase in CD27 membrane expression, peaking at day 3 with a fourfold enhanced expression level (Fig. 1). In contrast, after 2 days of stimulation with PMA, which directly activates PKC but does not cause a rise in $[Ca^{2+}]_i$ (13), a down-regulation of CD27 membrane expression was observed that persisted throughout the 11-day culture period (Fig. 1). In an additional experiment, we examined whether phorbol esters (PMA and phorbol 12-13-dibutyrate) also caused down-modulation of CD27 membrane expression on anti-CD3 mAb preactivated lymphocytes. This effect was indeed detectable within 18 h and complete within 72 h (Table I).

CD27 is a phosphoprotein that is hyperphosphorylated upon T cell activation. Inasmuch as protein phosphorylation has been described to serve as a direct signal for receptor internalization (19, 20), we examined

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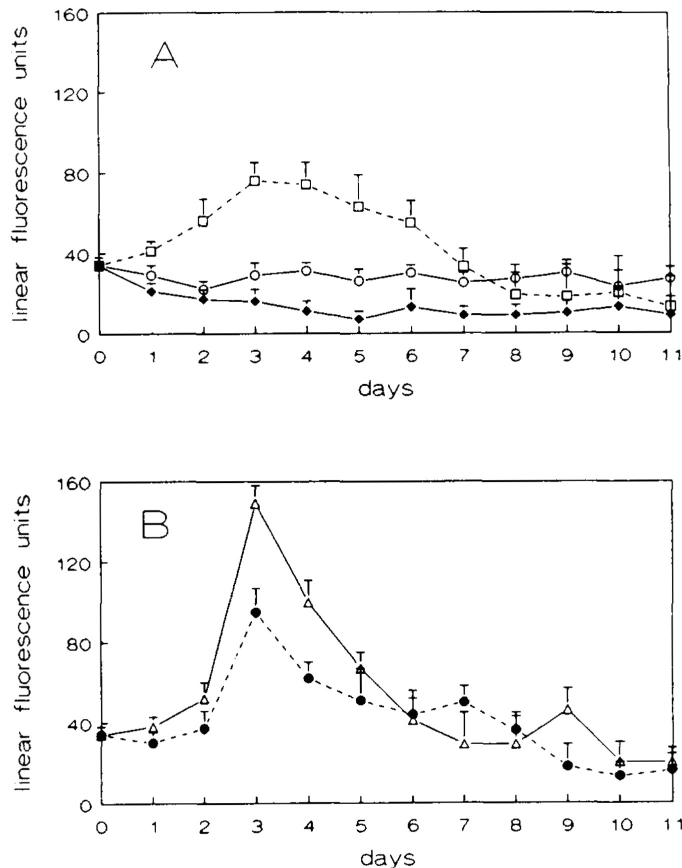

Figure 1. T cell activation via both the TCR/CD3 complex and CD2 molecules results in a transient increase in CD27 expression. Lymphocytes (0.25×10^6/ml) were cultured for 11 days in the presence of 5% autologous monocytes with (*A*) either PHA (1 μg/ml) (□), PMA (60 ng/ml) (◆), medium (○) or (*B*) with coated anti-CD3 mAb (CLB-T3/3, 5 μg/ml) (△) or a combination of the anti-CD2 mAb (CLB-T11.1/1 and CLB-T11.2/1 (●) used in a final ascites dilution of 1/1000). CD27 membrane expression was tested after various time intervals subsequent to T cell activation using directly FITC-conjugated anti-CD27 mAb. The statistical mean of the mean fluorescence of cell samples derived from five different donors is given in linear fluorescence units. Error bars indicate SEM. Cell viability was >95% after 1 to 6 days of culture and >85% after 7 to 11 days of culture.

whether PMA, but not anti-CD3 mAb, would induce CD27 phosphorylation. It appeared that CD27 is already phosphorylated on resting T cells (Fig. 2, *lane A*). Exposure of T cells to PMA considerably enhanced the level of CD27 phosphorylation over a 4-h period (Fig. 2, *lanes B* to *F*). Although less pronounced, T cell stimulation with anti-CD3 mAb also caused a significant increase in CD27 phosphorylation within this period (Fig. 2, *lanes G* to *K*). Phosphorylation of CD3 is shown as a control. In accordance with published data, there was no evidence for CD3 phosphorylation in resting cells (Fig. 2, *lane L*), but CD3 phosphorylation was induced by PMA (28). This increase followed similar kinetics as that of CD27 (Fig. 2, *lanes M* to *Q*).

Next, the effect of PMA on CD27 phosphorylation was examined in cells preactivated with anti-CD3 mAb. As shown in Table I, these conditions cause complete down-regulation of CD27 membrane expression within 72 h. For this experiment, activated lymphocytes were exposed to PMA for 30 min. CD27 was clearly phosphorylated on T cells activated with anti-CD3 mAb (Fig. 3, *lane B*), in contrast to the CD3 molecular complex (*lane D*). PMA treatment enhanced CD27 phosphorylation (Fig. 3, *lane C*), and induced CD3 phosphorylation (*lane E*). We have not observed the previously described activation specific p32 form of CD27 in these phosphorylation experiments. This may indicate that p32 does not contain phosphorylation sites.

Phospho-amino acid analyses indicated that both T cell stimulation with PMA and with anti-CD3 mAb induced phosphorylation of CD27 at serine residues (Fig. 4). Although TCR/CD3 triggering is known to activate tyrosine kinase activity as well (17, 18), no phosphorylation of CD27 at tyrosine residues was observed. Apparently, both PKC activation by phorbol esters and TCR/CD3 triggering enhance CD27 phosphorylation on serine residues, whereas opposite effects on membrane expression are observed. These data suggest that CD27 down-modulation is not a direct consequence of phosphorylation.

Activation related changes in CD27 mRNA expression. Next, we assessed to what extent the up- and down-regulation of CD27 membrane expression is controlled at the RNA level. Steady state CD27 mRNA levels were determined on Northern blots at different time points during activation with anti-CD3 mAb. Figure 5 shows the results of two such induction experiments using PBMC from different donors. Parallel with the changes in cell surface expression, TCR/CD3 triggering induced a very strong increase in CD27 mRNA levels. This increase was detectable within a 16-h period (Fig. 5*a*, *lane 2*), peaked between 48 and 72 h (Fig. 5*a*, *lanes 3* and *7*), and decreased within 5 days (Fig. 5*a*, *lane 8*). CD25 transcription showed different kinetics. Steady-state CD25 mRNA levels peaked within 16 to 48 h after activation, as expected (21) (Fig. 5*b*, *lanes 2, 3,* and *6*). Thus, the increase

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TABLE I

PMA causes down-modulation of CD27 on anti-CD3 mAb preactivated T cells

	Medium[a]		PMA[a]		PDBu[a]	
	Mean fl.	Percent	Mean fl.	Percent	Mean fl.	Percent
CD27 expression[b]						
Reculture period						
18 h	184 ± 22	95 ± 2	86 ± 30	92 ± 5	87 ± 29	91 ± 5
72 h	72 ± 9	94 ± 2	16 ± 2	46 ± 14	16 ± 3	52 ± 17
CD25 expression[b]						
Reculture period						
18 h	54 ± 4	92 ± 1	89 ± 6	98 ± 1	90 ± 5	99 ± 1
72 h	25 ± 1	40 ± 6	46 ± 1	87 ± 2	47 ± 1	86 ± 1

[a] Lymphocytes were activated with immobilized anti-CD3 mAb (CLB-T3/3) for 3 days. Cells were subsequently recultured for 18 or 72 h with either medium, PMA (10 ng/ml) or PDBu (10 ng/ml).

[b] Membrane expression was determined using directly conjugated mAb. The mean fluorescence intensity of the positively stained cell population in linear fluorescence units and the percentage reactive T cells are given. The results represent the statistical mean + SEM of three donors.

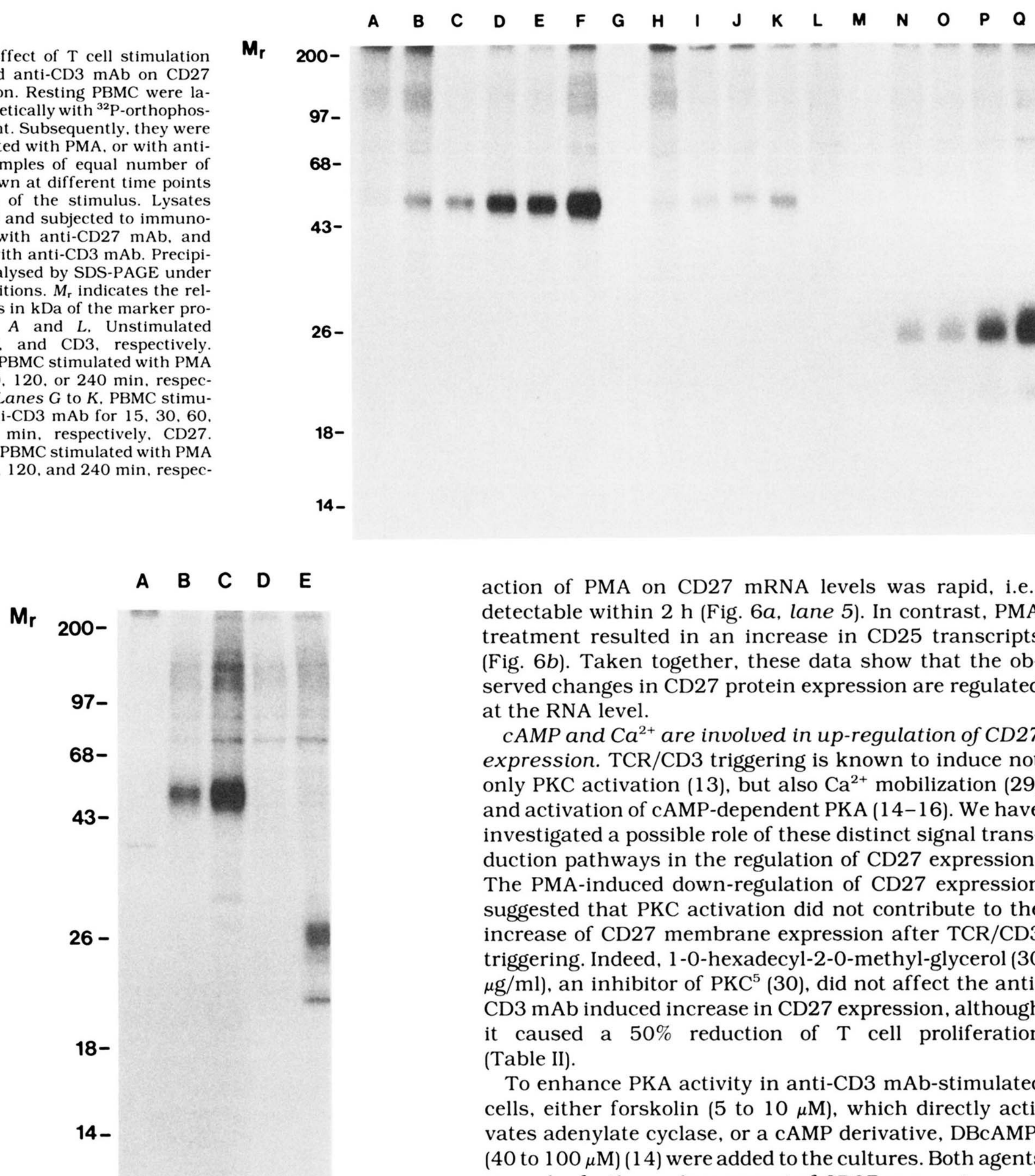

Figure 2. Effect of T cell stimulation with PMA and anti-CD3 mAb on CD27 phosphorylation. Resting PBMC were labeled biosynthetically with ^{32}P-orthophosphate overnight. Subsequently, they were either stimulated with PMA, or with anti-CD3 mAb. Samples of equal number of cells were drawn at different time points after addition of the stimulus. Lysates were prepared and subjected to immunoprecipitation with anti-CD27 mAb, and sequentially with anti-CD3 mAb. Precipitates were analysed by SDS-PAGE under reducing conditions. M_r indicates the relative mol mass in kDa of the marker proteins. *Lanes A* and *L*, Unstimulated PBMC, CD27, and CD3, respectively. *Lanes B* to *F*, PBMC stimulated with PMA for 15, 30, 60, 120, or 240 min, respectively, CD27. *Lanes G* to *K*, PBMC stimulated with anti-CD3 mAb for 15, 30, 60, 120, or 240 min, respectively, CD27. *Lanes M* to *Q*, PBMC stimulated with PMA for 15, 30, 60, 120, and 240 min, respectively, CD3.

Figure 3. PMA enhances phosphorylation of CD27 on anti-CD3 activated T cells. PBMC were activated with immobilized anti-CD3 mAb for 3 days, labeled with ^{32}P-orthophosphate for 3 h and taken directly from culture (*lanes A, B,* and *D*), or stimulated with PMA for 30 min (*lanes C* and *E*). Immunoprecipitation was performed sequentially with anti-CD27 (*lanes B* and *C*) and anti-CD3 mAb (*lanes D* and *E*). SDS-PAGE analysis was performed under reducing conditions. *Lane A*, Normal mouse Ig preclear.

in CD25 mRNA, resulting from TCR/CD3 triggering, occurs earlier in time than the increase in CD27 transcripts.

Inasmuch as CD27 mRNA was hardly detectable in resting T cells (Fig. 5*a*, *lanes 1* and *5*), anti-CD3 mAb preactivated lymphocytes were used to evaluate the effect of PMA on CD27 mRNA levels. PMA caused a striking decrease in steady state CD27 mRNA levels (Fig. 6*a*). Kinetic experiments indicated that the down-regulatory action of PMA on CD27 mRNA levels was rapid, i.e., detectable within 2 h (Fig. 6*a*, *lane 5*). In contrast, PMA treatment resulted in an increase in CD25 transcripts (Fig. 6*b*). Taken together, these data show that the observed changes in CD27 protein expression are regulated at the RNA level.

cAMP and Ca^{2+} are involved in up-regulation of CD27 expression. TCR/CD3 triggering is known to induce not only PKC activation (13), but also Ca^{2+} mobilization (29) and activation of cAMP-dependent PKA (14–16). We have investigated a possible role of these distinct signal transduction pathways in the regulation of CD27 expression. The PMA-induced down-regulation of CD27 expression suggested that PKC activation did not contribute to the increase of CD27 membrane expression after TCR/CD3 triggering. Indeed, 1-0-hexadecyl-2-0-methyl-glycerol (30 μg/ml), an inhibitor of PKC[5] (30), did not affect the anti-CD3 mAb induced increase in CD27 expression, although it caused a 50% reduction of T cell proliferation (Table II).

To enhance PKA activity in anti-CD3 mAb-stimulated cells, either forskolin (5 to 10 μM), which directly activates adenylate cyclase, or a cAMP derivative, DBcAMP, (40 to 100 μM) (14) were added to the cultures. Both agents caused a further enhancement of CD27 expression on T cells stimulated with anti-CD3 mAb, resulting in a eight- to ninefold increase in density of CD27 membrane molecules compared to resting cells (Table II). In comparison, no significant additional effect of PKA activation was observed on CD25 and CD2 expression, whereas CD28 expression and T cell proliferation were reduced (Table II). These data suggest that the cAMP-PKA pathway contributes to enhancement of CD27 expression. However, forskolin and DBcAMP were not effective in enhancing CD27 expression in the absence of anti-CD3 mAb (data not shown).

To analyze the role of Ca^{2+} mobilization, both T lymphocytes and cells of the leukemic T cell line Jurkat were

[5] **van Lier, R. A. W., M. Brouwer, E. de Groot, Y. Kramer, L. Aarden, and A. J. Verhoeven.** T cell receptor/CD3 and CD28 use distinct intracellular signalling pathways. *Submitted for publication.*

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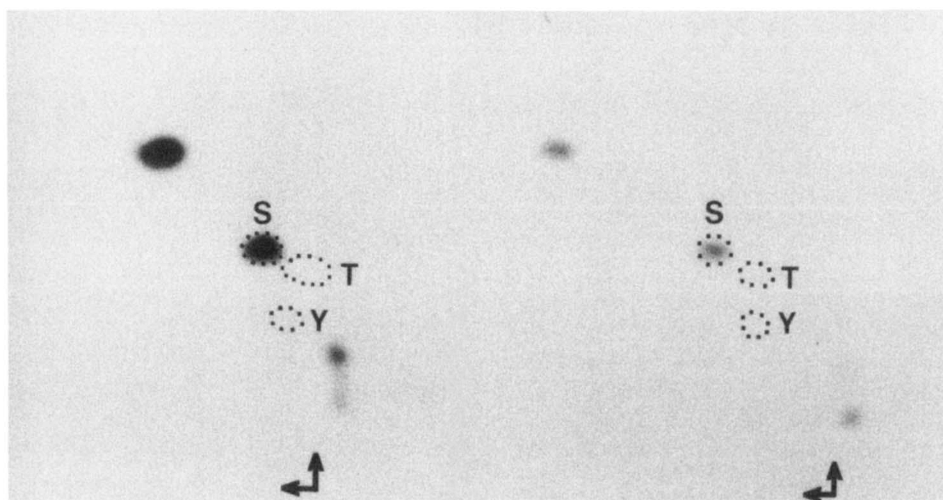
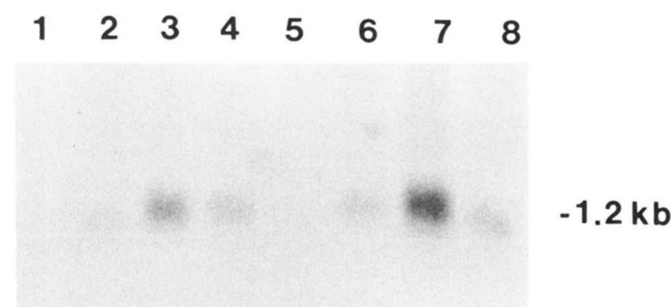


Figure 4. Phospho-amino acid analysis of phosphorylated CD27 molecular species. Samples equivalent to those analysed by SDS-PAGE in Figure 3, *lanes B* and *C*, were recovered from the gel and subjected to phospho-amino acid analysis by complete acid hydrolysis and separation of resulting amino acids by two-dimensional electrophoresis. *Right side*, CD27 sample recovered from cells stimulated for 3 days with anti-CD3 mAb. *Left side*, CD27 samples recovered from cells stimulated in addition to anti-CD3 mAb, with PMA for 30 min. The *dots* indicate the positions of the unlabeled phosphoamino acids used as markers. *S*, Serine; *T*, threonine; *Y*, tyrosine.



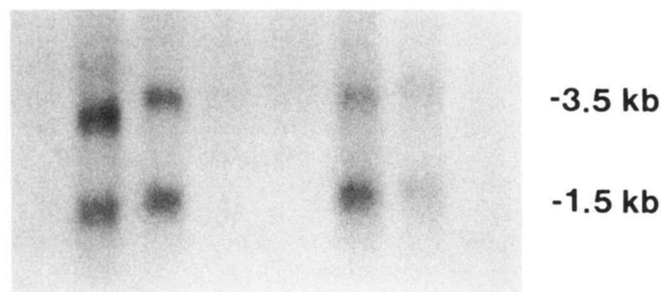
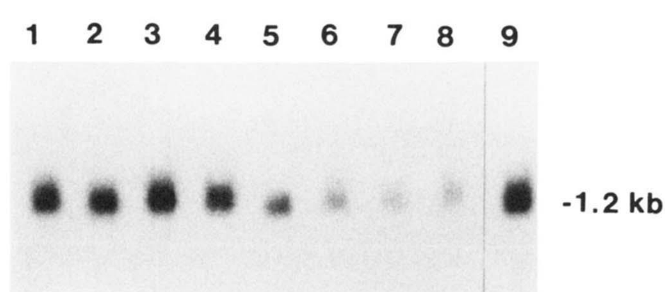


Figure 5. Time course of induction of CD27 mRNA in anti-CD3 mAb stimulated T cells (*a*). *b*, The induction of CD25 mRNA was used as a control for the activation state of the cells. PBMC of two different donors were stimulated with immobilized anti-CD3 mAb for various time periods. Samples of 100.10^6 cells were harvested at 0, 16, 48, and 72 h after stimulation (donor 1: *lanes 1, 2, 3,* and *4*) or at 0, 2, 3, and 5 days (donor 2: *lanes 5, 6, 7,* and *8*). The blot was probed sequentially for the presence of CD27 and CD25 mRNA, respectively. The size of the mRNA is indicated in kb.



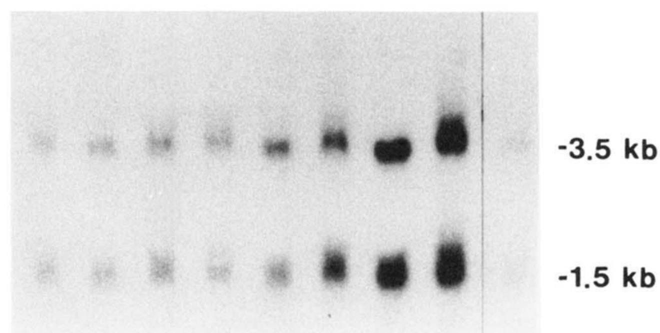


Figure 6. Effect of PMA on CD27 mRNA expression in activated T cells (*a*). The expression of CD25 mRNA was determined as a control (*b*). PBMC were activated with immobilized anti-CD3 mAb. At day 3 after activation, PMA (60 ng/ml) was added and samples of 50.10^6 cells were harvested and frozen after 15, 30, and 60 min; 2, 4, 8, and 12 h (*lanes 2* to *8*). Control samples derived from unstimulated cells cultured for 3 or 4 days are shown in *lanes 1* and *9*, respectively. The blot was probed sequentially for the presence of CD27 and CD25 mRNA, respectively.

activated with calcium ionophores (A23187 and ionomycine, both at 1 μM). Jurkat T cells do not express CD27 molecules, but stimulation with anti-CD3 mAb or calcium ionophores induced high levels of CD27 expression in a 3-day culture period (Table III). Addition of calcium ionophore caused an enhancement in CD27 membrane expression on peripheral blood T lymphocytes that varied between two- to fourfold (Table II). It should be noted that the enhancing effect of TCR/CD3 stimulation on CD27 expression exceeded that of stimulation with calcium ionophore. Addition of CyA, an inhibitor of Ca^{2+}-dependent intracellular events (31), to Jurkat cells stimulated with anti-CD3 mAb completely inhibited CD27 expression (Table III), whereas a further increase of CD28 expression was observed. Although less pronounced, CyA also reduced CD27 expression on peripheral blood T lymphocytes (Table II). The combined findings strongly suggest that both elevation of intracellular cAMP and $[Ca^{2+}]_i$ levels play a role in the up-regulation of CD27 expression, whereas on the other hand activation of PKC down-regulates CD27 expression.

DISCUSSION

In the present study, we have examined the mechanisms controlling the regulation of CD27 membrane expression subsequent to T cell activation. Stimulation

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TABLE II
Intracellular second messengers involved in regulation of CD27 membrane expression

Stimulus[a]	CD27[b]		CD25[b]		CD2[b]		CD28[b]		Proliferative Response
	Mean fl.	Percent	Mean fl.	Percent	Mean fl.	Percent	Mean fl.	Percent	
Expt. 1									
Unstimulated	42	66	NA	6	30	71	34	64	0[c]
aCD3	135	93	88	95	140	97	228	96	105
aCD3 + forskolin 5 μM	347	93	80	88	166	94	139	92	70
aCD3 + forskolin 10 μM	320	89	101	82	174	92	118	87	53
Expt. 2									
Unstimulated	28	68	NA	2	31	79	46	56	0
A23187 1 μM	106	81	44	44	125	91	104	74	1
aCD3	145	91	136	88	199	97	418	91	106
aCD3 + forskolin 5 μM	233	91	136	89	263	97	289	87	57
aCD3 + forskolin 10 μM	229	89	119	77	225	94	245	84	33
aCD3 + DBcAMP 40 μM	210	91	167	84	198	95	271	86	69
aCD3 + DBcAMP 100 μM	213	87	125	68	208	92	183	80	31
aCD3 + AMG 30 μg/ml	137	89	137	87	193	97	418	90	56
aCD3 + CyA 1 μg/ml	98	78	36	33	72	85	148	68	3

[a] Lymphocytes were cultured for 3 days with various stimuli/inhibitors as indicated.
[b] Membrane expression was determined using directly conjugated mAb in flow microfluorimetry analysis. Data represent percentage reactive cells. The mean fluorescence of the positively stained cell population is given in linear fluorescence units; NA, not applicable.
[c] cpm × 10^{-3} [^{3}H]thymidine incorporation at day 3. Mean of triplicate cultures.

TABLE III
Ca^{2+} mobilization is involved in up-regulation of CD27 expression

Stimulus[a]	CD27[b]	CD28[b]
	Mean fl.	Mean fl.
Expt. 1		
Unstimulated	11	137
Ionomycine 1 μM	78	461
aCD3	138	338
aCD3 + CyA 1 μg/ml	15	559
Expt. 2		
Unstimulated	13	281
aCD3	96	665
aCD3 + CyA 1 μg/ml	19	792

[a] The T cell line Jurkat was stimulated for 3 days as indicated.
[b] CD27 and CD28 membrane expression was determined with the use of directly conjugated mAb in FACS analysis. The mean fluorescence of Jurkat T cells is given in linear fluorescence units.

of T cells via the TCR/CD3 complex or CD2 molecule results in a marked increase in CD27 membrane expression. In contrast, direct activation of PKC, after phorbol ester treatment, causes down-regulation of CD27 expression. Martorell et al. (32) recently showed that CD27 expression can be induced on CD27$^-$ thymocytes by lectin stimulation, the effect being abolished by addition of CyA; PMA treatment, however, did not result in CD27 expression. These findings, together with our current data, point to similar control mechanisms for CD27 expression on thymocytes and peripheral blood T cells.

Treatment of T lymphocytes with PMA is known to cause down-modulation of CD3, CD4, and CD7 Ag (20, 28, 33–37). Several mechanisms have been put forward to account for this effect, including enhanced internalization (33), shedding of membrane molecules, or inhibition of gene transcription and/or destabilization of mRNA (34). In addition, PMA-induced down-modulation of CD3 and CD4 molecules has been suggested to be a direct consequence of PKC-mediated phosphorylation of these proteins (20, 28). Whereas the signaling pathways after direct PKC activation and those after TCR/CD3 triggering have opposite effects on CD27 expression, both lead to enhanced phosphorylation of CD27 on serine residues. It remains to be investigated whether TCR/CD3 triggering induces CD27 serine phosphorylation at different sites than PKC activation. However, in light of the RNA data, we prefer the option that CD27 down-regulation is not due to a direct effect of phosphorylation. Down-regulation of CD27 expression at the protein level could also involve enhanced shedding of the molecules from the plasma membrane. However, soluble CD27 levels detected in the supernatant of T cells activated with PMA and resting T cells were comparable (see footnote 4).

We have shown that CD27 down-modulation by PMA corresponded to a marked decrease in steady state CD27 mRNA levels. This decrease in CD27 transcripts was detected within 2 to 4 h after treatment with PMA, which suggests that PKC directly affects CD27 gene transcription or mRNA stability. Recently, Paillard et al. (34) found that, in case of the TCR, CD4, and CD8 molecules, T cell stimulation with PMA and anti-CD3 mAb caused a decrease in TCR, CD4, and CD8 mRNA due both to inhibition of transcription and reduction of the half-life of the mRNA. We are currently investigating whether CD27 mRNA levels are regulated at the level of gene transcription and/or mRNA stability.

TCR/CD3 triggering is known to induce not only PKC activation, but also Ca^{2+} mobilization (29), tyrosine kinase activity (17, 18), and activation of the cAMP-dependent PKA (15, 16). Inasmuch as PKC activation down-regulates CD27 expression, we have studied which signaling pathways, resulting from TCR/CD3 triggering, positively affected CD27 expression. Both Ca^{2+} mobilization and PKA activation were shown to be involved in the induction of CD27 expression. A possible role for tyrosine kinase activity remains to be investigated. It has been demonstrated in several reports that similar signaling pathways are activated after T cell stimulation via the CD2 molecule and the TCR/CD3 complex (38–40). Indeed, T cell activation via the CD2 molecule caused an increase in CD27 membrane expression, which followed similar kinetics as the TCR/CD3-mediated enhancement in CD27 expression.

The increase in CD27 mRNA levels after TCR/CD3 triggering is relatively late compared to the increase in CD25 mRNA levels. This may imply that elevation of cytoplasmic Ca^{2+} and cAMP levels does not directly affect CD27 gene transcription. Rather, they may induce the synthesis of (cytokine) receptors and/or their ligands,

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which in turn provide the signal for enhancement of CD27 transcription. As yet, we have not been able to demonstrate a regulatory role for rIl-1, rIl-2, rIl-4, rIl-6, rIFN-γ or rTNF-α in the up-regulation of CD27 membrane expression (data not shown). Moreover, we have not observed an effect of anti-CD27 mAb on CD27 membrane expression (data not shown), suggesting that the ligand of CD27 is not directly involved in the up-regulation of its receptor.

The activation related increase in CD27 expression occurs too late upon T cell activation to contribute to a more efficient interaction with APC during T cell activation. In our view, CD27 might rather function at a later stage of T cell activation, possibly as a receptor for a (yet undefined) T cell growth or differentiation factor.

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