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Protein kinase C-dependent up-regulation of CD5 surface expression on normal and lymphoblastoid T cells

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SUMMARY

As an approach to study the mechanisms regulating the surface expression of CD5 antigen on T cells, the effects of agents that activate different lymphocyte functions were examined. Active tumour promoter agents (TPA), such as phorbol ester analogues [phorbol 12-myristate 13-acetate (PMA), phorbol 12,13-dibutyrate (PBu_2)] and mezerein, were able to increase the expression of CD5 on the cell surface of all T-cell types studied (thymocytes, peripheral blood lymphocytes and some lymphoblastoid T-cell lines), as deduced from immunofluorescence and immunoprecipitation analysis. The TPA-induced CD5 up-regulation occurred in a dose- and time-dependent manner and was dependent on protein and RNA synthesis. From the other stimuli tested, the T-cell mitogens [phytohaemagglutinin (PHA) and concanavalin A (Con A)], as well as monoclonal antibodies (mAb) against the CD3 complex, also increased CD5 expression, although to a lesser degree. In all cases the increments were shown to be dependent on protein kinase C (PKC), as evidenced by their inhibition with staurosporine, a potent inhibitor of PKC activation. These data suggest that CD5 up-regulation on T cells can be a physiological event that depends on PKC activation.

INTRODUCTION

The CD5 (T1, Leu-1, Tp67 in human; Ly-1 in mouse) molecule is a 67,000 molecular weight (MW) surface differentiation antigen normally expressed on most thymocytes and peripheral T lymphocytes, but only on a small subset of peripheral B lymphocytes (Hardy & Hayakawa, 1986). Although its biological role is still poorly understood, several studies support a possible role for CD5 in delivering accessory signals in T-lymphocyte activation and proliferation (Ceuppens & Baroja, 1986; Ledbetter *et al.*, 1985; June, Ravinovitch & Ledbetter, 1987). However, the mechanisms by which CD5 expression is regulated on T lymphocytes are still unknown.

Active tumour promoter agents (TPA), such as phorbol ester analogues and mezerein, are able to induce the *in vivo* and *in vitro* translocation and activation of protein kinase C (PKC) (Nishizuka, 1984), and have been implicated in the regulation of the cell-surface expression and/or function of different lymphocyte receptors (Sibley *et al.*, 1987; Hoxie *et al.*, 1986; Cantrell, Davies & Crumpton, 1985). Regarding CD5, there is agreement in the induction of CD5 expression in human malignant and normal B cells by TPA (Miller & Gralow, 1984), as well as the down-regulation of this phenomenon by interleukin-4 (IL-4) (Freedman *et al.*, 1989). By contrast, the effect of TPA on CD5 expression of human T cells remains controversial. While some authors report no change in CD5 expression (Razak *et al.*, 1987; Triebel *et al.*, 1986), others state that CD5 can be up-regulated in T cells by TPA (Hardy & Hayakawa, 1986; Carrera *et al.*, 1989). Additionally, in the mouse, it has been shown that Ly-1 can be differentially regulated in thymocytes and spleen T lymphocytes (Lögdberg & Shevach, 1985).

In this work we have studied whether CD5 expression can be differentially regulated in distinct T-cell types by using TPA as well as other agents which activate some lymphocyte functions. Our results suggest that CD5 surface expression can be up-regulated by PKC activators in a RNA- and protein synthesis-dependent manner in all tested T cells. Furthermore, data are presented involving the CD3/T-cell receptor complex in the up-regulation of CD5 antigen, suggesting that this process could be important during physiological T-cell activation and/or proliferation.

Abbreviations: Ca^{2+}, calcium; Con A, concanavalin A; FITC-GAMIg, fluorescein-conjugated goat anti-mouse immunoglobulins; IFI, indirect immunofluorescence; mAb, monoclonal antibody (ies); PBMC, peripheral blood mononuclear cells; PBu_2, phorbol 12,13-dibutyrate; PE, phycoerythrin; PGE_2, prostaglandin E_2; PHA, phytohaemagglutinin; PKA, cyclic AMP-dependent kinases; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PWM, pokeweed mitogen; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TPA, tumour promoter agent.

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MATERIAL AND METHODS

Cells

Peripheral blood mononuclear cells (PBMC) were obtained from healthy volunteer donors by density gradient centrifuga-

tion on Ficoll–Hypaque (Pharmacia, Uppsala, Sweden) (density 1·077). Thymocytes were obtained from children undergoing cardiac surgery. The lymphoblastoid T-cell lines used in this work (Molt-4, HUT 78, Jurkat and CEM) were from the American Type Culture Collection (ATCC, Rockville, MD).

Chemicals and monoclonal antibodies

Phorbol 12-myristate 13-acetate (PMA), phorbol 12,13-dibutyrate (PBu_2), 4β-phorbol, mezerein, the Ca^{2+} ionophore A23187, prostaglandin E_2 (PGE_2), cycloheximide, phytohaemagglutinin (PHA), pokeweed mitogen (PWM) and concanavalin A (Con A) were purchased from Sigma (St Louis, MO). Staurosporine was purchased from Calbiochem (San Diego, CA), recombinant interferon-gamma (IFN-γ; activity 2000 UI/μg) from Genzyme Corporation (Boston, MA) and actinomycin D from Merck Sharp & Dohme (Rahway, NJ).

The Cris-7 (IgG2b; CD3), Edu-2 (IgG2a; CD4), Cris-1 (IgG2a; CD5) and 725D3 (IgG2a; CD45) monoclonal antibodies (mAb) were produced by Dr R. Vilella and have been assigned to the corresponding clusters in the International Workshops on Human Leukocyte Differentiation Antigens (Bernard *et al.*, 1984; Reinherz *et al.*, 1986; McMichael *et al.*, 1987). The mAb B67.6.1 (IgG; CD2) was provided by Dr Perussia (Wistar Institute, Philadelphia, PA). The D44 and D66 mAb were provided by Dr A. Bernard (Institut Gustave-Roussy, Villejuif, France). The Leu-1 mAb (IgG2a; CD5) was from Becton-Dickinson (Mountain View, CA).

Cell cultures

Cells were cultured at $1\text{–}2 \times 10^6$ cells/ml for PBMC and thymocytes and at $0{\cdot}5 \times 10^6$ cell/ml for T-cell lines in RPMI-1640 supplemented with 10% fetal calf serum (FCS) and antibiotics, and maintained for different periods of time at 37° in a humidified atmosphere containing 5% CO_2, in the presence or absence of stimuli. Experiments performed in the presence of staurosporine, cycloheximide or actinomycin D were carried out by a 45 min preincubation at 37° with these substances prior to the addition of stimuli, without washing. Cell viabilities after stimulation were found to be higher than 90%, as assessed by trypan blue exclusion.

Immunofluorescence assays and flow cytometry analysis

Indirect immunofluorescence (IIF) was done using a fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (FITC–GAMIg) (Sigma) as second antibody and analysed with a FACS analyser (Becton-Dickinson), as described elsewhere (Alberola-Ila *et al.*, 1989). Direct immunofluorescence was done using phycoerythrin (PE)-conjugated Leu-4 (CD3) and Leu-1 mAb (CD5), from Becton-Dickinson and with FITC-conjugated OKT11 mAb (CD2) from Ortho Diagnostics (Raritan, NJ) and analysed with a FACStar analyser (Becton-Dickinson). Fluorescence results were analysed with a Consort 30 data analysis system.

Radiolabelling and immunoprecipitation

Surface ^{125}I-labelling by the Iodogen (Pierce Chemical Co., Rockford, IL) method, immunoprecipitation and analysis by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) were performed as described elsewhere (Lozano *et al.*, 1989).

Table 1. Percentage and mean channel of log fluorescence intensity in control and PMA-treated PMBC after 24 hr of culture

	Control*	PMA*	*P*
Percentage positive	56·9 ± 15·4	61·1 ± 17·4	0·52 (NS)
Mean channel	17·2 ± 4·2	28·5 ± 6·9	< 0·0001

* Mean ± 1 SD for $n = 14$.
NS, not significant.

RESULTS

Enhancement of CD5 cell-surface expression by active TPA

PBMC cultured for 24 hr in the presence of PMA (10 ng/ml) showed increased CD5 surface expression, as deduced from a very significant increment in the mean channel of log fluorescence intensity ($P < 0{\cdot}0001$, $n = 14$), without concomitant increase in the percentage of $CD5^+$ cells (Table 1). As previously reported (Hoxie *et al.*, 1986; Cantrell *et al.*, 1985, 1988), we found that while PMA also up-regulated CD2 expression, it caused down-regulation of CD3 and CD4. No changes were detected in the CD45 expression. The results from a representative experiment can be seen in Fig. 1a. Similar enhancement of CD5 expression was obtained when the PMA effects on thymocytes and several lymphoblastoid T-cell lines (Molt-4, HUT 78, CEM and Jurkat) were studied (Fig. 1b).

Evidence for CD5 hyper-expression on cell surface was also obtained from semi-quantitative immunoprecipitation assays. PBMC were maintained in culture for 24 hr in the presence or absence of PMA (10 ng/ml), and then surface-labelled with ^{125}I. Samples of Nonidet P-40 lysates from stimulated and unstimulated PBMC were adjusted to contain identical acid-insoluble radioactivity, immunoprecipitated with Leu-1 mAb (CD5) and analysed by SDS–PAGE. As shown in Fig. 2, a several-fold more intense band was obtained in immunoprecipitates from PMA-stimulated PBMC, and this finding is consistent with the increase in CD5 fluorescence intensity.

In other experiments we compared the effects of an identical dose (10 ng/ml) of PMA, PBu_2 and mezerein on CD5 expression in all cell types mentioned above. While mezerein reproduced exactly the effect of PMA, the phorbol ester derivative PBu_2-induced increments in CD5 expression were less intense. By contrast, the inactive phorbol ester 4β-phorbol was unable to induce any change in CD5 surface expression, even at doses as high as 100 ng/ml (data not shown).

Dose–response and time–course of TPA-induced CD5 up-regulation

In all cell types studied (thymocytes, PBMC and Molt-4 cells), treatment for 24 hr with doses as low as 0·1 ng/ml of PMA was already able to induce increments in CD5 cell-surface expression. However, maximal increases were induced by 1 or 10 ng/ml doses, depending on individual variability. Higher PMA doses (50 and 100 ng/ml) did not increase the above mentioned effects. A representative dose–course obtained with thymocytes is shown in Fig. 3a. A similar PMA dose range was effective in regulating the surface expression of the CD2, CD3 and CD4 T-cell antigens (data not shown).

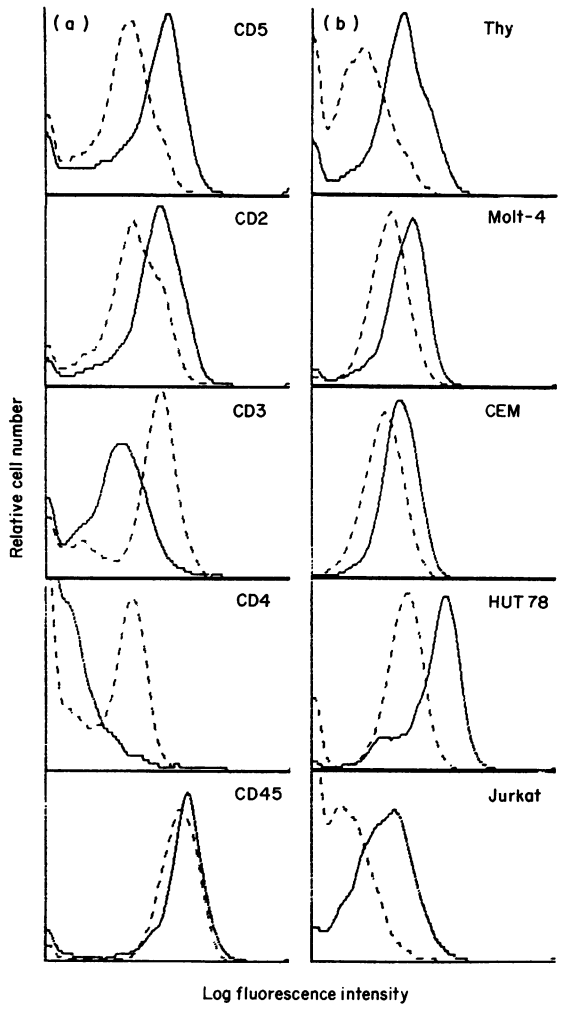

Figure 1. Effects of PMA on the surface expression of CD5 and other T-cell antigens. (a) Fluorescence histograms obtained by IIF with CD5 (Cris-1), CD2 (B67.6.1), CD3 (Cris-7), CD4 (Edu-2) and CD45 (725D3) mAb on PBMC. (b) Increase in CD5 expression as measured by IIF with the Cris-1 (CD5) mAb on thymocytes (Thy), Molt-4, CEM, HUT 78 and Jurkat cells. In all cases the broken line represents untreated cells and the solid line PMA-treated cells (10 ng/ml) cultured for 24 hr.

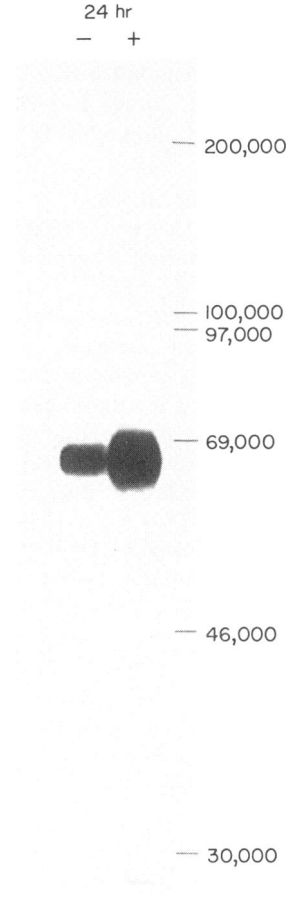

Figure 2. SDS-PAGE analysis (8% polyacrilamide gel) of Leu-1 mAb (CD5) immunoprecipitates from ^{125}I surface-labelled PBMC following stimulation with (+) or without (−) 10 ng/ml PMA for 24 hr.

To determine the kinetics of the PMA-induced CD5 up-regulation, thymocytes, Molt-4 cells and PBMC were also cultured in the presence of 10 ng/ml PMA for different periods of time (3, 6, 12, 24, 48 and 72 hr). Although CD5 fluorescence intensity was already slightly higher after 3 hr or 6 hr incubation, maximal increments were only reached after 72 hr incubation. In Fig. 3b a representative experiment on thymocytes is shown.

Effect of different lymphocyte activators on CD5 surface expression

We next analysed whether the T-cell mitogens PHA or Con A, known to cause synergistic Ca^{2+} mobilization and PKC activation (Nishizuka, 1984), and PWM were capable of inducing changes on the CD5 expression in thymocytes and PBMC after 24–48 hr culture. Whereas no increase was induced by PWM (5 μg/ml), clear increments were detected when PHA (1 μg/ml) and Con A (10 μg/ml) were used. However, these increments

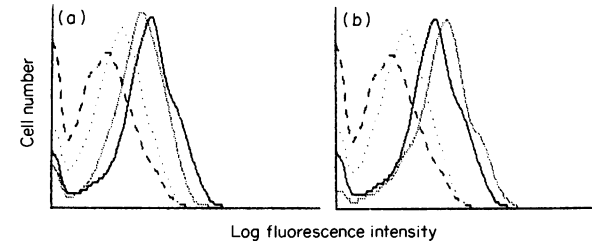

Figure 3. Dose-response and kinetics of CD5 up-regulation by PMA. (a) Fluorescence histograms obtained by IIF with the Cris-1 mAb (CD5) on thymocytes treated for 24 hr with 0 (broken line), 0·1 (dotted line), 1 (thin solid line) and 10 ng/ml (thick solid line) PMA. (b) CD5 expression on thymocytes treated with 10 ng/ml PMA for 0 (broken line), 6 (dotted line), 24 (thick solid line) and 72 hr (thin solid line).

were always inferior in magnitude to those induced by PMA, as can be seen in Fig. 4a,c. In a similar way to TPA, PHA was also able to induce the down-regulation of the CD3 antigen and the up-regulation of the CD2 antigen in PBMC (data not shown). No changes in the expression of any of the cell-surface antigens

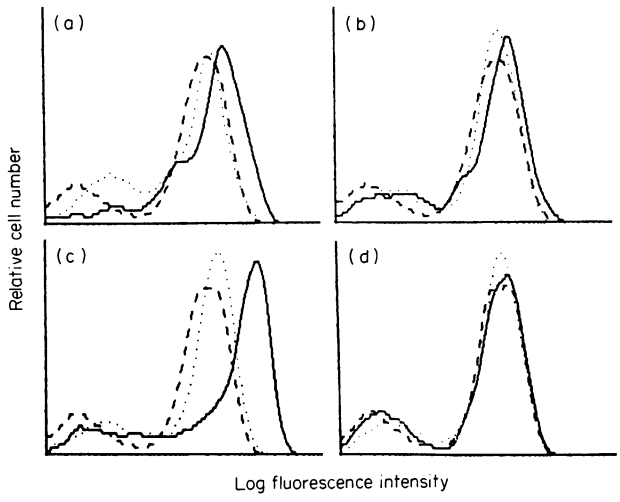

Figure 4. Up-regulation of CD5 surface expression by PHA and CD3 mAb. The fluorescence histograms were obtained by direct immunofluorescence with PE-conjugated Leu-1 mAb (CD5) on PBMC stimulated for 48 hr in the presence of 10 μg/ml PHA (a), 1/2500 ascites dilution of CD3 mAb (b), 10 ng/ml PMA (c) or 1/1000 supernatant dilution of D44 plus D66 mAb (d). In all histograms the broken line represents untreated cells, the solid line cells treated with the above mentioned stimuli and the dotted line cells pretreated with 100 nM staurosporine prior the addition of stimuli.

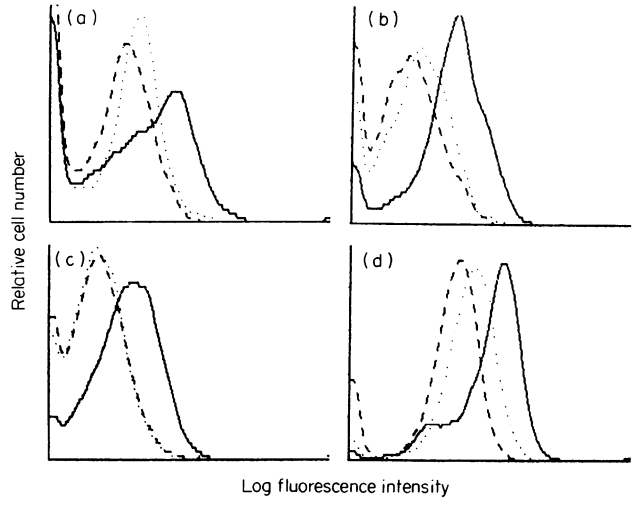

Figure 5. Effect of staurosporine on the PMA-induced CD5 up-regulation on PBMC (a), thymocytes (b) and the lymphoblastoid T-cell lines Jurkat (c) and Molt-4 (d). Shown are the fluorescence histograms obtained by IIF with the Cris-1 mAb (CD5) on 24-hr cultured cells, alone (broken line) or in the presence of 10 ng/ml PMA (solid line) or PMA plus 100 nM staurosporine (dotted line).

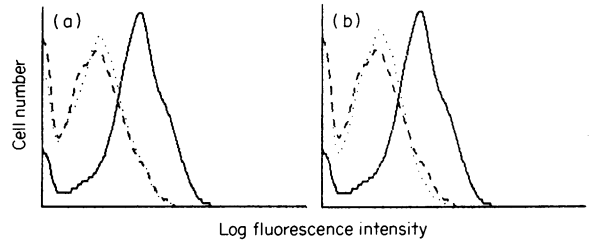

Figure 6. Effect of protein and RNA synthesis inhibitors on the PMA-induced CD5 up-regulation. Fluorescence histograms showing the Cris-1 mAb (CD5) reactivity by IIF on thymocytes cultured for 24 hr in the absence (broken line) or in the presence of 10 ng/ml PMA alone (solid line) or PMA plus 20 μg/ml cycloheximide (a) or 5 μg/ml actinomycin D (b) (dotted line).

studied were detected after incubation of cells with the calcium ionophore A23187 (500 ng/ml) or PGE_2 (10^{-6} M), both of which are capable of activating other kinases distinct from PKC (Sibley *et al.*, 1987; Cantrell *et al.*, 1989). As interferons can induce the transcriptional activation of genes from some surface antigens (Wallach, Fellows & Ravel, 1982), we also tested whether IFN-γ (500 UI/ml) could reproduce the TPA-induced changes in the surface expression of the above referred antigens without any success (data not shown). The effects of the physiological activation of lymphocytes through the CD3/T-cell receptor complex on the expression of some surface antigens was investigated by using mitogenic doses of soluble CD3 mAb (1/25000 ascites dilution). Up-regulation of the CD5 expression, although less intense than that induced by PHA, was clearly detected after 48 hr stimulation of PBMC with CD3 mAb (Fig. 4b). By contrast, the surface expression of CD2 was already up-regulated after 24 hr of culture (data not shown). No changes in the surface expression of CD5 were detected after 48 hr of culture with a mitogenic combination of CD2 mAb (D44 plus D66 mAb supernatants at 1/1000 dilution) (Fig. 4d).

Altogether, these results suggest that CD5 up-regulation can be accomplished not only by TPA but also by CD3 mAb and some T-cell mitogens (PHA and Con A), which have in common their ability to activate PKC.

CD5 up-regulation depends on PKC activation and RNA and protein synthesis

Further indirect evidence that CD5 up-regulation is mediated by PKC activation was obtained by using staurosporine, a highly effective inhibitor of PKC (Ward, Cantrell & Westwick, 1988). A 45 min preincubation of PBMC with 100 nM staurosporine before addition of 10 ng/ml of PMA or mezerein inhibited completely the CD5 up-regulation. Identical results were obtained when using thymocytes and the lymphoblastoid T-cell lines Molt-4, CEM, HUT 78 and Jurkat (Fig. 5). As can be seen in Fig. 4a,b, the up-regulation of the CD5 surface expression induced by PHA or CD3 mAb on PBMC was also clearly inhibited by the preincubation with staurosporine. In a similar way, staurosporine was also able to inhibit the CD2 up-regulation induced by these agents in PBMC (data not shown). In control experiments, staurosporine by itself had no effect on CD5 surface expression. Therefore, all these results suggest that the CD5 up-regulation can be dependent on PKC activation.

We also studied the effects of the RNA and protein synthesis inhibitors actinomycin D and cycloheximide, respectively, on the TPA-induced CD5 up-regulation. Preincubation of all cell types studied (thymocytes, T-cell lines and PBMC) with 20 μg/ml cycloheximide or 5 μg/ml actinomycin D for 45 min before the addition of 10 ng/ml PMA or mezerein, completely blocked the CD5 up-regulation at 24 hr without loss in cell viability. In Fig. 6, representative results obtained with thymocytes are shown. From these experiments, we conclude that RNA and

protein synthesis are required for CD5 to be up-regulated on the cell surface, suggesting a possible *de novo* synthesis.

DISCUSSION

In the present report, we show that CD5 antigen expression can be up-regulated on the cell surface in a PKC-dependent way in normal and lymphoblastoid T cells. The regulation of CD5 expression on human T cells by lymphocyte activators has, until now, been controversial. While some authors report no change (Razak *et al.*, 1987; Triebel *et al.*, 1986), others state that CD5 can be up-regulated in T cells (Hardy & Hayakawa, 1986; Carrera *et al.*, 1989). This controversy could be due to the different sources of T-cell populations used peripheral blood lymphocytes, purified peripheral or tonsil T cells, specific T-lymphocyte clones or leukaemia T-cell lines). In fact, in the mouse, PHA has been shown to increase the Ly-1 (the murine equivalent to human CD5) expression in thymocytes but not in spleen T lymphocytes (Lögdberg & Shevach, 1985). From our results, obtained by using TPA and T-cell mitogens, we can discard these apparent discrepancies by the finding that CD5 expression was up-regulated in all T-cell types studied (thymocytes, several lymphoblastoid T-cell lines and peripheral blood lymphocytes).

Interestingly all the agents able to up-regulate the CD5 expression (active phorbol ester analogues, mezerein, Con A, PHA and CD3 mAb) are also effective in translocating and activating PKC (Nishizuka, 1984; Bagnasco *et al.*, 1989), suggesting a possible PKC-dependent mechanism for CD5 up-regulation. Further evidence supporting the dependence on PKC activation was obtained from the inhibition of the CD5 up-regulation induced by TPA, PHA or CD3 mAb by pre-incubation of cells with staurosporine, a potent inhibitor of PKC. However, staurosporine not only inhibits PKC but also blocks intracellular Ca^{2+} increases and the activation of cyclic AMP-dependent kinases (PKA) (Ward *et al.*, 1988), and the contribution of both effects should be excluded. The fact that phorbol esters do not increase intracellular Ca^{2+} concentration but up-regulate CD5, and that PGE_2, an activator of PKA (Sibley *et al.*, 1987), is not able to up-regulate CD5, argue against a possible contribution of these other effects of staurosporine in the inhibition of the CD5 up-regulation.

Phosphorylation via PKC has been commonly proposed as a mechanism involved in down-regulating the surface expression of many cell receptors (Hoxie *et al.*, 1986; Sibley *et al.*, 1987; Cantrell *et al.*, 1985). However, CD5, which has been shown to undergo hyperphosphorylation after phorbol ester-induced PKC activation (Chatila & Geha, 1988; Lozano *et al.*, 1989), becomes up-regulated. Therefore, phosphorylation could be also implicated in the up-regulation of some antigens. Nevertheless, if this were the case, it would not be a general mechanism since CD2, which is not a substrate for PKC (Chatila & Geha, 1988), can be up-regulated by PKC activators (Cantrell *et al.*, 1988). Furthermore PMA-induced CD5 hyperphosphorylation is a rapid (already evident after 10 min) and transient (decreases after 6 hr, even in the continuous presence of PMA) event (F. Lozano, J. Alberola-Ila, L. Places, T. Gallart and J. Vives, manuscript submitted for publication), while up-regulation of CD5 follows a completely divergent kinetics (see the Results). All these data suggest that, despite the lack of direct evidence, CD5 up-regulation and hyperphosphorylation are not directly related events.

The fact that phorbol esters can induce expression of several genes (Nishizuka, 1986) caused us to consider that the up-regulation of CD5 by TPA could also be mediated by the induction of the CD5 gene expression. Our studies with protein and RNA synthesis inhibitors support the possibility that the TPA-induced CD5 up-regulation is not due to the translocation to the cell membrane of an internal pool of preformated molecules but to *de novo* synthesis of CD5 molecules, in agreement with previous results in the mouse (Hagiwara *et al.*, 1987) and human (Carrera *et al.*, 1989).

The biological relevance of CD5 up-regulation is unknown. However, the fact that PHA, which binds and acts mainly via CD3 (Valentine *et al.*, 1985), was able to up-regulate CD5 expression suggested that the physiological stimulation of T cells through the CD3 complex could reproduce such changes. In this respect, evidence is presented here showing that the direct stimulation of T cells through the CD3/T-cell receptor complex is able to up-regulate the CD5 expression, although in a delayed manner. By contrast, under our experimental conditions, stimulation via CD2 seems not to produce enhancements in CD5 expression. In any case, our results, together with the fact that CD5 is a surface molecule with a receptor-like structure which can provide intracellular positive signals, leads to the suggestion that its up-regulation during physiological T-cell activation could play a role in inducing or sustaining the activation/proliferative state.

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