Surface membrane CD4 turnover in phorbol ester stimulated T-lymphocytes

Evidence of degradation and increased synthesis

B.K. Møller[1], B.S. Andresen[1], E. Ilsø Christensen[2] and C. Munck Petersen[1]

[1]*Department of Clinical Immunology, University Hospital of Aarhus* and [2]*Department of Cell Biology, Institute of Anatomy, University of Aarhus, Aarhus, Denmark*

Received 3 October 1990

Down-regulation of surface membrane CD4 (smCD4) in phorbol ester stimulated T-cells resulted from internalization. Internalization ($T_{1/2}$ = 15 min at 50 ng PMA/ml) was followed by degradation of CD4-bound antibodies. Degradation in unstimulated T-cells was comparatively insignificant. Release of degradation products was PMA dose-dependent and could be inhibited by methylamine. Uptake and degradation continued after maximal down-regulation of surface membrane CD4, and methylamine did not inhibit reappearance of smCD4 antigens. Metabolic labelling of T-cells further showed that ongoing synthesis rather than recycling contributed to an accelerated smCD4 turnover in activated cells.

CD4 modulation; Phorbol ester; Human T-lymphocyte

1. INTRODUCTION

CD45 is a 55 kDa membrane glycoprotein, which is expressed in monocytes and approximately 60% of CD3 positive T-lymphocytes. Surface membrane CD4 (smCD4) is known to participate in T-cell antigen recognition by complex-formation with monomorphic MHC class II determinants on antigen presenting cells [1]. Apart from this adhesive function, recent findings have suggested that the CD4 molecule may play an active role in the primary T-cell response. Thus, binding of anti-CD4 antibody induces transmission of specific intracellular signals, possibly regulating the cellular response to T-cell antigen receptor/CD3 complex triggering [2]. Moreover, activation of T-cells by mito- or antigens, and in particular by phorbol esters modulates the surface membrane density of CD4 [3-5]. It follows that an interconnection between T-cell responsiveness and the smCD4 density is likely.

In that context the cellular handling of CD4 expression could hold important information. Several studies have described the phosphorylation and pronounced down-regulation of smCD4 resulting from phorbol ester stimulation. Recent evidence has strongly indicated an endocytotic mechanism [6], but shedding of CD4 has been proposed as well [7]. The fact remains, however, that little is known with respect to CD4 regulation in normal T-cells.

The present study was undertaken to describe the CD4 turnover in unstimulated human T-cells, as well as the fate and new-synthesis of smCD4 during activation.

Correspondence address: B. Møller, Dept. of Clinical Immunology, University Hospital of Aarhus, Skejby, DK-8200 Aarhus N, Denmark

2. MATERIALS AND METHODS

2.1. *Cell cultures*

Human mononuclear cells (PBM) and T-cells were prepared from defibrinated peripheral blood [8] and cultured in RPMI 1640 (Gibco, Paisley, UK) supplemented with glutamine, antibiotics and heat-inactivated human serum pool (s-RPMI). Phorbol 12-myristate 13-acetate (PMA, Sigma Chemical Co., St. Louis, MO, USA) was stored in dimethylsulphoxide (1 mg/ml at −70°C) and added to cell cultures at 2-50 ng/ml.

2.2. *Antibodies*

Unlabelled monoclonal anti-CD4 antibodies (mAb1) were generously supplied by DakoPatts (Copenhagen, Denmark). The mAb1 was radioiodinated by the iodogen-technique to a specific activity of 800 Ci/mmol (^{125}I-mAb1). ^{125}Iodine (1600 Ci/mmol) was from Amersham Int. (Bucks., UK). FITC conjugated monoclonal antibodies anti-Leu-3A + 3B and anti-Leu-4A and phycoerythrin conjugated anti-Leu-2A were from Becton-Dickinson (Meylan Cédex, France). FITC conjugated anti-CD4 and rabbit anti-mouse Ig were from DakoPatts.

2.3. *^{125}I-mAb1 binding assay*

For experiments PBM and T-cells (2×10^6/ml) were incubated at 37°C in s-RPMI at 50 pmol/l ^{125}I-mAb1. Alternatively, the cells were preincubated at 4°C in a 1% bovine serum albumin (BSA) buffer (pH 7.4) containing 50 pmol/l ^{125}I-mAb1 prior to washing (×3) and resuspension in s-RPMI at 37°C. Incubations were stopped by centrifugation on oil [9] and the amount of radioactivity associated with the cell pellet or found in the medium on trichloroacetic acid (TCA)-precipitable or -soluble form was determined in a gamma-counter [9]. For experiments involving methylamine (MeA), the cells were preincubated (30 min) in 5 mmol/l MeA prior to addition of PMA.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

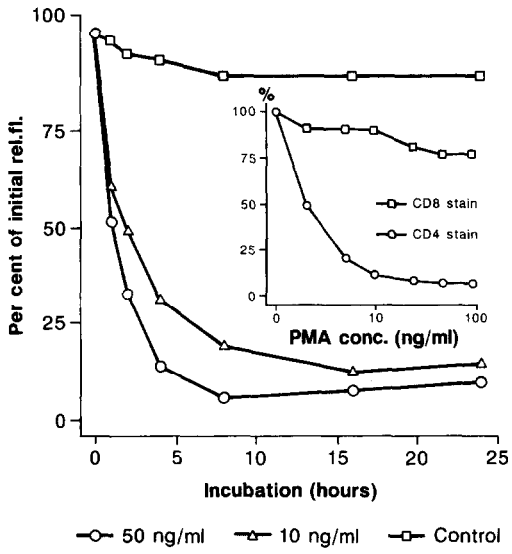


Fig. 1. Dose- and time-dependency of smCD4 modulation. Rel.fl. of PMA stimulated 50 ng/ml (○), 10 ng/ml (△) and control T-cells (□) at different times during incubation. Inset shows rel.fl. of CD8 positive (□), and CD4 positive cells (○) after 24 h of incubation at 37°C with PMA at concentrations indicated.

2.4. *Flow-cytometric analysis*

Cells were stained with fluorescent antibodies as suggested by the manufacturers. Relative fluorescence (rel.fl.) was determined according to the equation:

$$\text{rel.fl.} = 10^{(\text{mean channel}/256)}$$

The flow-cytometer (FACScan, Becton-Dickinson, USA) was used in logarithmic mode of 1024 channels at a maximum reading of 10^4 units. Internalized CD4 was defined by the following procedure: T-cells were incubated with 5 nmol/l unlabelled mAb1 (4°C, 10 h) and subsequently washed 3 times in cold buffer. At zero time the cells were resuspended in warm (37°C) s-RPMI with or without 50 ng PMA/ml, and at given times the reaction was stopped by addition of a surplus of cold buffer. The cells were washed (×3), labelled with FITC anti-mouse Ig (DakoPatts), and the amount of anti-CD4 Ig on cell surfaces was determined by flor-cytometry. The difference between fluorescence on unactivated cells and that on PMA activated cells, that is the amount of CD4 which could no longer be detected in activated cells, was defined as internalized antigen. Intracellular CD4 was detected in cells made permeable for FITC-mAb1 by treatment with digitonin. Digitonin (Sigma) was purified as described elsewhere [10] and used at an optimal concentration of 0.4 mg/ml.

2.5. *Metabolic labelling*

T-cells (4×10^6/500 μl) were incubated with or without 50 ng PMA/ml in methionine-free RPMI 1640 supplemented with 0.05% dialysed fetal calf serum (FCS) and 200 μCi [^{35}S]methionine (Amersham). After 6 h of incubation the cells were washed (×3, 4°C) and lysed in 1% Triton X-100 (Boehringer, Mannheim, FRG) lysis buffer (pH 8.0) in the presence of 2 mmol/l PMSF (Sigma). Labelled protein was precipitated by incubation (4°C) with mAb1 fixed on immunomagnetic beads (DynaBeads, Dynal, Oslo, Norway). After extensive washing the beads were pelleted, resuspended in 100 μl reducing sample buffer (5% v/v mercaptoethanol, 4% SDS) and boiled for 1 min. The samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in a Laemmli system using a 4% stacking and 15% separation gel. Molecular weight markers were from Sigma. PPO-fluorographies were examined by densitometry (Dual wavelength TLC scanner, Shimadzu).

3. RESULTS

3.1. *PMA induced down-regulation of smCD4*

Activation of separated and unseparated T-cells by PMA rapidly reduced CD4 presentation. Down-regulation was dose- and time-dependent (Fig. 1), and elapsed with a half-time of approximately 60 min at 50 ng PMA/ml. In 8 h smCD4 density had reached an appearent minimum at 8.2 ± 1.7% ($n=3$) of that in unstimulated controls. Similar findings resulted from estimates based on ^{125}I-mAb1 binding (at 4°C) to PMA-blasts (not shown). Activation also affected the surface membrane density of CD8 antigens in CD8+ T-cells although the effect was less pronounced (inset Fig. 1). PMA-induced down-regulation of smCD4 in MeA treated T-cells did not differ from that in untreated cells. Yet, >80% of prebound (at 4°C) ^{125}I-mAb1 remained associated to MeA treated cells even after 4 h with PMA (Table I), indicating the involvement of an endocytotic process. FACS analysis of activated cells also indicated internalization of CD4 antigens (Fig. 2). Thus, the reduction in smCD4 fluorescence was partly compensated for by an increase (>25% at 60 min) in fluorescence following digitonin treatment. Detection of CD4 and CD8 in control T-cells was unaltered by digitonin, indicating low intracellular pools of CD4 and CD8 antigens in unactivated cells. After 16 h of PMA activation digitonin-treated cells did not express detectable intracellular CD4 reactivity, suggesting a dramatic reduction in total cellular CD4.

Table I

Cells were preincubated with or without 50 pmol/l ^{125}I-mAb1 at 4°C for 16 h prior to washing (×3). Cells were resuspended at 37°C with or without PMA (50 ng/ml). PMA stimulated cells were incubated with or without MeA

	^{125}I-mAb1 in pellet			smCD4 (rel.fl.)			TCA-soluble ^{125}I		
	Contr.	PMA	PMA+MeA	Contr.	PMA	PMA+MeA	Contr.	PMA	PMA+MeA
(1)	71.0%	7.2%	84.4%	100%	6.9%	9.6%	13.4%	68.3%	8.8%
(2)	54.5%	1.6%	40.6%	99.1%	21.3%	18.8%	-	-	-

(1) After 5 hours of incubation cell-associated radioactivity (^{125}I-mAb1 in pellet) and TCA-soluble radioactivity of the medium was determined (mean of triplicate samples in percent of prebound radioactivity). Parallel cultures were analysed after FITC-anti-CD4 staining, and rel.fl. was determined (smCD4) (2) All cultures were washed at 4°C (×3) and reincubated without PMA, but with or without MeA as indicated. The cells were cultured for 12 h at 37°C to allow reexpression of smCD4.

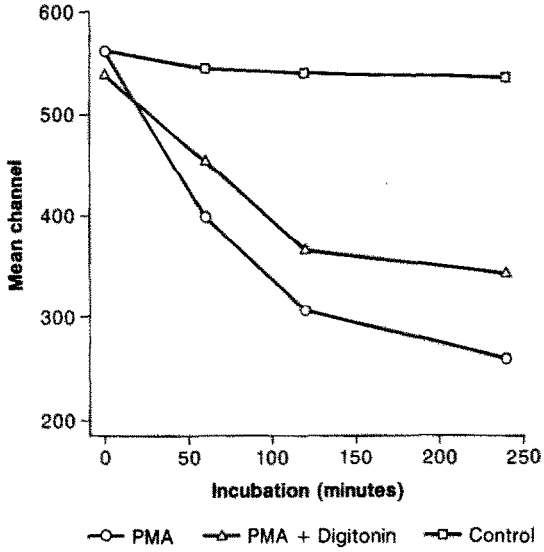


Fig. 2. Detection of intracellular CD4 antigens during PMA stimulation. Anti-CD4 fluorescence (mean channel) of T-cells incubated with or without (□) 50 ng PMA/ml. PMA stimulated cells were Leu-3a+3b (FITC) stained in the absence (○) or presence (△) of 0.4 mg digitonin/ml.

3.2. *Internalisation and degradation*

As demonstrated in Fig. 3A internalization followed first-order kinetics with an estimated half-time of 15 min. Internalization was faster than CD4 surface density down-regulation, indicating PMA induced recycling or, more likely, new-synthesis of CD4 antigens (Table I). Fig. 3A shows the results of an experiment in which T-cells were incubated (at 4°C) with ^{125}I-mAb1 and subsequently stimulated with 50 ng PMA/ml at 37°C. After a lag time of about 30 min TCA-soluble radioactivity (degraded products) appeared in the medium following a sigmoidal curve, and activity in the cell pellet decreased accordingly. Fig. 3B shows the PMA dose-dependency of ^{125}I-mAb1 degradation. Degradation rate constant of controls was 0.4×10^{-3} min^{-1} versus 8.4×10^{-3} min^{-1} at 50 ng PMA/ml.

3.3. *Uptake and turn-over*

At 37°C ^{125}I-mAb1 was taken up by activated T-cells and degradation products appeared in the medium (Fig. 4A). In spite of a high tracer uptake, release of TCA-soluble products was much lower in unactivated cells. Cells stimulated for 36 or 96 h prior to incubation with tracer released radioactive degradation products at a constant rate, which, in spite of low smCD4 levels, exceeded that in control cells (not shown). TCA-soluble radioactivity was insignificant upon 24 h of incubation with CD4 negative cells (K562). These experiments clearly demonstrate that CD4 molecules constantly reappear on the cell surface either due to recycling or new-synthesis.

3.4. *Synthesis*

Metabolic labelling of T-cell cultures in the absence or presence of PMA indicated enhanced CD4 synthesis in PMA-blasts. Thus, the amount of immunoprecipitable, CD4-incorporated [^{35}S]methionine was higher in PMA-blast lysates than in lysed unstimulated cells. It follows that activated cells contained at least as much newly synthesized CD4 as unstimulated cells, in spite of the reduced CD4 half-life (Fig. 4B).

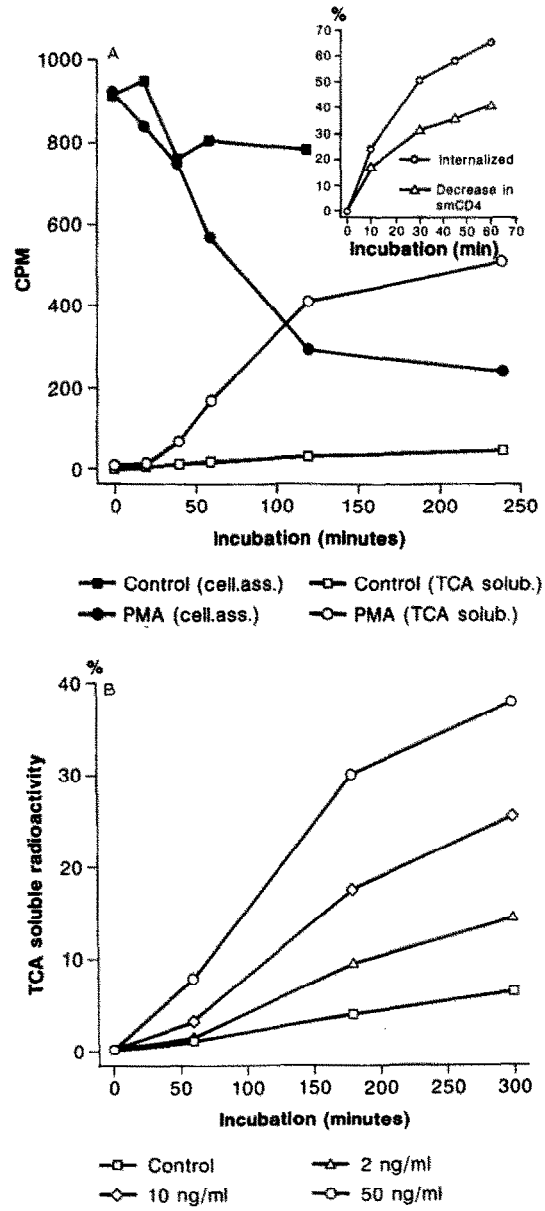
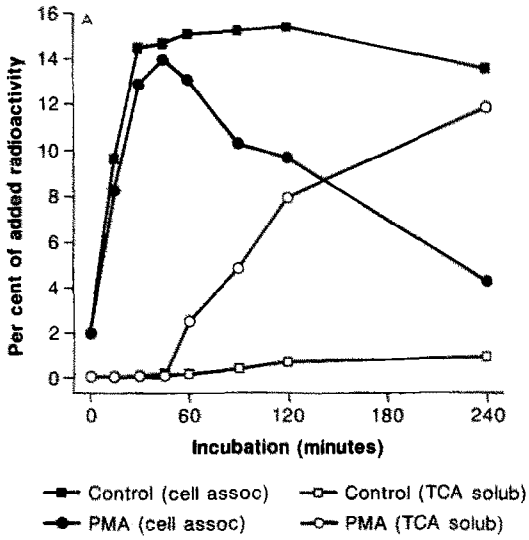


Fig. 3. (A) PMA-induced degradation of prebound ^{125}I-anti-CD4. Cells were incubated with ^{125}I-mAb1 at 4°C, washed and resuspended at 37°C. Cell-associated radioactivity (filled symbols) and TCA-soluble radioactivity released in the medium (open symbols) by control (□) and PMA stimulated T-cells (○) were determined at times indicated. (Inset) Internalized CD4 and total smCD4 at given times during PMA (50 ng/ml) stimulation of T-cells. (B) PMA dose-dependency of anti-CD4 antibody degradation. TCA-soluble radioactivity in percent of prebound ^{125}I-mAb1 (at 4°C). Release from T-cells (2×10^6/ml) in response to different concentrations of PMA is shown as a function of time of incubation.



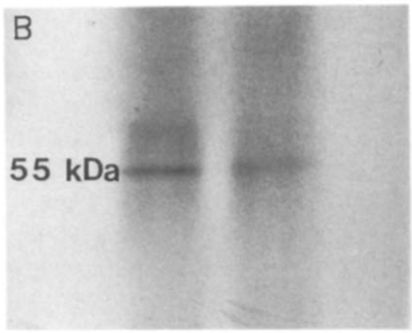


Fig. 4. (A) Uptake and degradation of anti-CD4 antibody. ^{125}I-mAb1 was added at zero time to PMA stimulated (○) and control (□) T-cells. Cell associated (filled symbols) and TCA-soluble radioactivity (open symbols) at different times are indicated. (B) New-synthesis of CD4 antigens in PMA activated and control T-cell. Biolabelled CD4 antigens immunoprecipitated from cultures of PMA-activated (50 ng/ml) and control human T-cells.

4. DISCUSSION

Recent findings have established that CD4 antigens in phorbol ester and antigen stimulated cells are phosphorylated via protein kinase C and subsequently down-regulated [11,12]. CD4-down-regulation has been ascribed to shedding [7], impaired new-synthesis [13], and internalization [6,11] but evidence concerning post-activation events are scarce. The present study demonstrates that smCD4 is in fact down-regulated by way of internalisation. Moreover, experiments involving labelled ligand (mAb1) indicate that the total pool of CD4 in activated cells is reduced by rapid degradation of internalized antigen. CD4 is also internalized and degraded in unstimulated T cells, but at a much lower rate. The data indicates that activation by PMA (50 ng/ml) increased the degradation rate constant by 20-fold. A minimum smCD4 density of 5–10% was detected in activated cells. Evidently recycling of CD4 antigens cannot be excluded, but in contrast to Richie et al. [13] our findings strongly suggest that an unchanced or increased synthesis contribute to the continued presentation of smCD4 in PMA activated cells. Thus, continued synthesis of CD4 antigens may contribute to the 'recovery' of cells from PMA induced CD4 down-regulation, which has been ascribed to down-regulation of phorbol ester receptors [6]. In conclusion, smCD4 in resting T-cells is characterized by a slow turnover and a half-life exceeding 24 h. Upon activation, catabolism (possibly by lysosomal degradation) is drastically increased with internalisation as the rate limiting step. A partly compensating increase in new-synthesis contributes to an accelerated turnover at a new steady state and a lower smCD4 density. Infection of CD4+ cells by HIV does not require internalisation of CD4 antigens [11,14]; in fact, the decreased infectivity of phorbol ester stimulated cells might reflect the down-regulation of HIV attachment [15,16], and perhaps even degradation of CD4-associated virus.

REFERENCES

[1] Doyle, C., Shin, J., Dunbrack Jr, R.L. and Strominger, J.L. (1989) Immunol. Rev. 109, 17–37.
[2] Neudorf, S.M.L., Jones, M.M., McCarthy, B.M., Harmony, J.A.K. and Choi, E.M. (1990) Cell. Immunol. 125, 310–314.
[3] Acres, R.B., Conlon, P.J., Mochizuki, D.Y. and Gallis, B. (1986) J. Biol. Chem. 261, 16210–16214.
[4] Weyand, C.M., Goronsky, J. and Fathman, C.G. (1987) J. Immunol. 138, 1351–1354.
[5] Blue, M.-L., Hafler, D.A., Kimberly, A.C., Levine, H. and Schlossman, S.F. (1987) J. Immunol. 139, 3949–3954.
[6] Hoxie, J.A., Matthews, D.M., Callahan, K.J., Cassel, D.L. and Cooper, R.A. (1986) J. Immunol. 137, 1194–1201.
[7] Solbach, W. (1982) J. Exp. Med. 156, 1250–1255.
[8] Munck Petersen, C., Davidsen, O., Moestrup, S.K., Sonne, O., Nykjær, A. and Møller, B.K. (1990) Eur. J. Clin. Invest. 20, 377–384.
[9] Munck Petersen, C., Nykjær, A., Christiansen, B.S., Heickendorff, L., Mogensen, S.C. and Møller, B. (1989) Eur. J. Immunol. 19, 1887–1894.
[10] Sonne, O. (1988) J. Biochem. 103, 348–353.
[11] Hoxie, J.A., Rackowski, J.L., Haggarty, B.S. and Gaulton, G.N. (1988) J. Immunol. 140, 786–795.
[12] DiSanto, J.P., Klein, J.S. and Flomenberg, N. (1989) Immunogenetics 30, 494–501.
[13] Richie, E.R., McEntire, B., Phillips, J. and Allison, J.P. (1988) J. Immunol. 140, 4115–4122.
[14] Bedinger, P., Moriarty, A., von Borstel II, R.C., Donovan, N.J., Steimer, K.S. and Littman, D.R. (1988) Nature 334, 162–165.
[15] Clapham, P.R., Weiss, R.A., Dalgleish, A.G., Exley, M., Whitby D. and Hogg, N. (1987) Virology 158, 44–51.
[16] Firestein, G.S., Reifler, D., Richman, D. and Gruber, H.E. (1988) Cell. Immunol. 113, 63–69.