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Stathmin phosphorylation patterns discriminate between distinct transduction pathways of human T lymphocyte activation through CD2 triggering

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CD2 triggering of human T lymphocyte activation has been associated with the activation of different interacting protein kinases, including protein kinase C (PKC). However the precise roles of its phosphorylated substrates are still unknown. We show here that PKC-dependent and -independent pathways are responsible for the CD2-induced phosphorylation of stathmin, a ubiquitous soluble phosphoprotein, most likely acting as a general intracellular relay integrating various second messenger pathways. The phosphorylated variants of stathmin provide a fingerprint reflecting the second messenger pathway(s) stimulated. The respective roles of both PKC and stathmin in the regulation of T lymphocyte proliferation are discussed.

Stathmin; T lymphocyte activation; CD2; Protein kinase C; Protein phosphorylation

1. INTRODUCTION

In recent years, studies on T lymphocyte activation have defined a number of specific surface molecules through which these cells can be stimulated. Beside the antigen-specific activation pathway mediated through the complex of the T cell receptor (CD3/TCR), proliferation and effector functions of human T cells can also be achieved by stimulating the CD2 antigen, a 55-kDa glycoprotein [1–3]. We have shown previously that CD2 triggering of a soluble-antigen-specific normal human $CD4^+$ T lymphocyte clone (P28D) [4] is associated with the activation of phospholipases C [5] and A2 [6], as well as the modulation of adenylate cyclase [7]. As in other cellular systems, the second messengers elicited are known to stimulate various protein kinases among which protein kinase C (PKC) has been proposed as a key enzyme in T lymphocyte activation (reviewed in [8]), although the precise roles of most of its phosphorylated substrates are still unknown.

We previously identified stathmin [9] as a ubiquitous soluble phosphoprotein most likely acting as a general intracellular relay integrating various second messenger pathways triggered by diverse extracellular signals (reviewed in [10]). Stathmin — also designated pp17 or prosolin [11], p18 [12], P19 [13], pp20, pp21 and pp23 [14] and 19 k [15] — is present in cells as at least two distinct isoforms, yielding several increasingly phosphorylated states ($M_r \approx 19\,000$–$23\,000$; pI ≈ 6.2–5.6) [16,17] most likely representing a molecular basis for signal integration [9,10]. As in various biological systems [10], expression and phosphorylation of stathmin in immune cells have been related to the regulation of cell proliferation, either of normal human proliferative peripheral lymphocytes [11,14] or of T leukemic lymphoblasts [18], as well as to the transformation of T leukemic lymphocytes [12].

The aim of the present study was to further investigate the intracellular transduction pathways involved in the CD2 activation process. The phosphorylated variants of stathmin provide a fingerprint of the second messenger pathway(s) stimulated. We show here that PKC-dependent and -independent pathways are responsible for the CD2-induced stathmin phosphorylation pattern in the P28D human normal $CD4^+$ T cell clone [4]. Thus, new insights are discussed on the respective roles of both PKC and stathmin in the regulation of T lymphocyte proliferation.

Abbreviations: DAG, diacylglycerol; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; PS, phosphatidylserine; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol-13-acetate

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2. MATERIALS AND METHODS

2.1. *Cell stimulation and preparation of subcellular fractions for PKC assay*

The soluble-antigen (diphteria)-specific LFA-3$^+$, CD2$^+$, CD3$^+$, CD4$^+$/CD8$^-$ T cell clone P28D was previously characterized [4].

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G_0/G_1 cells (10^8 cells) were stimulated either with TPA (100 ng/ml) (LC. Serv. Corp., Woburn, USA) or with the X11/D66 [19] mitogenic pair of anti-CD2 mAbs as described [5]. After stimulation, the cells were extensively washed and resuspended in 1.5 ml homogenization buffer (Buffer A: 20 mM Tris-HCl, pH 7.4, 2.5 mM EGTA, 2 mM EDTA, 50 mM 2-mercaptoethanol, 4 μg/ml leupeptin, 10 μg/ml aprotinin), and sonicated on ice for 30 s. Aliquots (1 ml) of cytosolic and particulate fractions, separated by centrifugation as described [20], were applied to 1 ml DE-52 cellulose columns equilibrated with Buffer A. After washing, the columns were eluted with 2 ml of Buffer A containing 0.12 M NaCl. The protein content of the eluate was determined by spot fluorometry [21].

2.2. *PKC assay*

PKC activity in cytosol and particulate fractions was assayed by measuring Ca^{2+}/phospholipid-dependent incorporation of ^{32}P from [γ-^{32}P]ATP (110 TBq/mmol) (Amersham) into histone Type III-S (Sigma) according to [20] with few modifications. The assay system contained 40 mM HEPES, pH 7.4, 8 mM Mg^{2+}-acetate, 5 μg cytosol- or particulate-protein, and 20 μg histone in 100 μl, with the addition of 8 μg/ml PS, 0.8 μg/ml DAG, and $CaCl_2$ to a final concentration of 250 μM. Reactions were initiated by the addition of 1 nmol [γ-^{32}P]ATP ($0.5–1 \times 10^6$ cpm), run for 3 min at 30°C and terminated by blotting the protein onto Whatman P81 paper. The PKC activity in the particulate fraction was measured as the stimulated activity (pmol ^{32}P transferred/min/mg protein in the presence of the three allosteric effectors Ca^{2+}, PS, DAG) minus basal activity (in the presence of Ca^{2+} + PS only).

2.3. *^{32}P-labelling and 2D-PAGE analysis of phosphorylated proteins*

$^{32}PO_4^{3-}$-labeling was performed by preincubating cells for 4 h with 5.55 MBq $^{32}PO_4^{3-}$ (CEA, France)/5×10^6 cells in 250 μl of phosphate-free medium, TPA or CD2 mAbs being added for the last 10 min. The labeling was stopped, and samples were analysed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) as described [22,23]. The isoelectric focusing gels contained 2% ampholines (LKB), pH 5–7 and 3–10 (4:1). The second dimension was run on 13% acrylamide gels. The same amount of trichloroacetic acid-precipitable radioactivity was used for each sample within a given experiment, allowing direct comparison of autoradiograms.

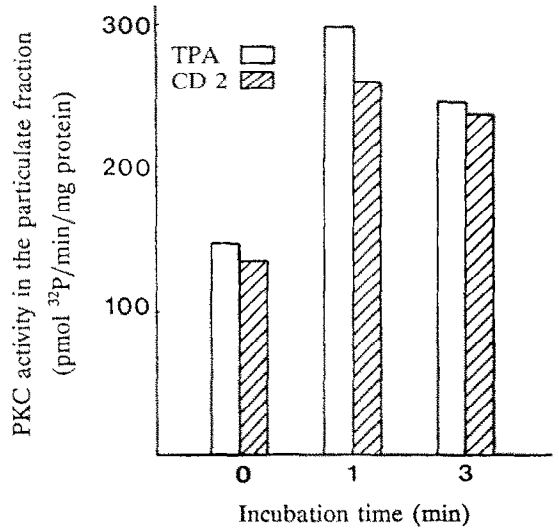

Fig. 1. CD2-stimulated translocation of PKC activity to the particulate fraction in P28D T cells. Intact P28D T cells were incubated for the indicated times at 37°C in the presence of either TPA (100 ng/ml) (open bars) or anti-CD2 mAbs (X11 + D66; 50 μg/ml each) (hatched bars). The PKC activity in the particulate fraction was measured as described in section 2. Values are the mean triplicate from one experiment out of three giving similar results, and correspond to the specific PKC activity in the presence of Ca^{2+}, PS and DAG, minus the activity in the absence of DAG.

3. RESULTS

3.1. *Translocation of protein kinase C activity in CD2-stimulated P28D cells*

To evaluate the involvement of PKC in the CD2-triggered activation of the P28D human normal $CD4^+$ T cell clone, we first examined the membrane translocation of the enzyme. As presented in Fig. 1, TPA (100 ng/ml), a known activator of PKC, caused a rapid increase of PKC activity in the particulate fraction within 1 min, this effect being sustained even after 60 min of treatment with TPA (not shown). Similarly, activation of P28D T cells by the mitogenic pair of anti-CD2 mAbs also induced a rapid rise of PKC activity in the particulate fraction. The involvement of PKC activation following the CD2-triggering of T lymphocytes is thus clearly confirmed in the P28D T cell clone.

3.2. *Regulation of stathmin phosphorylation by CD2-triggering of P28D T cells*

To further evaluate the relative contribution of PKC in the activation of T cells through the CD2 pathway, we analysed the phosphorylation pattern of stathmin, previously proposed to reflect the activation of diverse transduction pathways as well as their interactions [9,10,22,23].

Incubation of P28D T cells with $^{32}PO_4^{3-}$ resulted in the ^{32}P-labeling of the P1 and, to a lesser extent, P2 spots of stathmin ($M_r \approx 19\,000$), detected by 2D-PAGE autoradiography (Figs. 2 and 3) and corresponding to increasingly phosphorylated states of its alpha and beta isoforms [16].

Treatment of $^{32}PO_4^{3-}$-prelabeled P28D T cells with TPA (100 ng/ml, 10 min) stimulated the incorporation of ^{32}P mostly into P1 and P2, but also into the most basic spots of two additional sets ('16' and '17') of stathmin phosphorylated forms [17] (Fig. 3). Indeed a slight ^{32}P-labeling of spot 16 ($M_r \approx 21\,000$ (Fig. 3) was induced, as well as a very weak effect on spot 17 ($M_r \approx 23\,000$), detectable on the original autoradiograms. Beside these effects, TPA also strongly stimulated the phosphorylation of an 80-kDa phosphoprotein (Fig. 2) corresponding most likely to the protein also designated MARCKS, a major substrate for PKC in various cell systems [24].

When $^{32}PO_4^{3-}$-prelabeled P28D T cells were treated with CD2 mAbs for 10 min, the phosphorylation of several proteins detected on 2D-PAGE autoradiograms was stimulated, including the 80-kDa PKC-substrate (Fig. 2). CD2 triggering resulted also in an increased phosphorylation of stathmin into its forms yielding spots P1, P2, 16 and also 17 (Fig. 3). Incorporation into

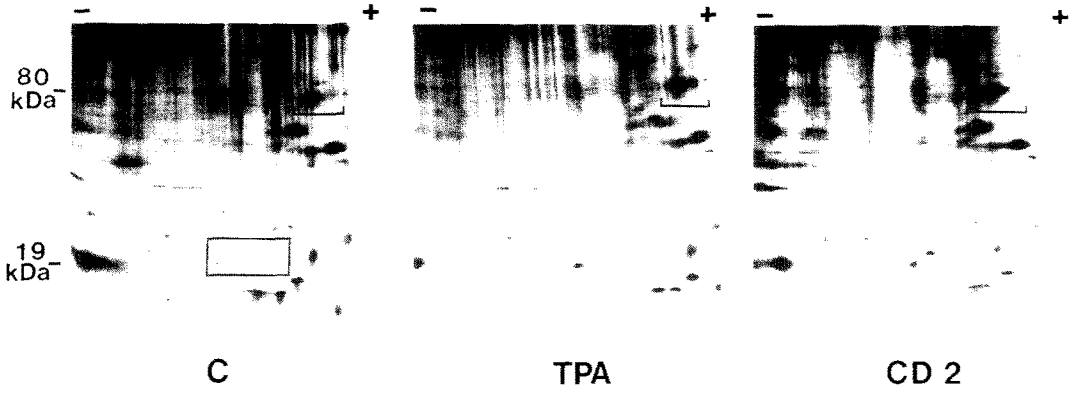

Fig. 2. TPA- and CD2-induced patterns of phosphorylation in intact P28D T lymphocytes. P28D T lymphocytes were labeled with $^{32}PO_4^{3-}$ for 4 h and then treated or not (C), with TPA (100 ng/ml) or anti-CD22 mAbs (X11 + D66, 50 μg/ml each) for 10 min. The box on the 2D-PAGE autoradiogram (left) indicates the area of stathmin spots shown in detail in Fig. 3. The open bar underlines the area of the major 80 kDa-MARCKS-substrate of PKC.

P1 and P2 was smaller than the effect obtained with TPA. On the other hand, phosphorylation of spot 16 was much more stimulated than in TPA-treated cells, and a more acidic, presumably more phosphorylated form of set '16' could also be detected on the autoradiograms. In addition, CD2 triggering induced a clear incorporation of ^{32}P also into spot 17.

4. DISCUSSION

We and others have recently associated the CD2-triggered T lymphocyte alternative pathway of activation with various signal transduction pathways [5–7,25,26]. However, the relative contribution of each pathway to the activation process is still a matter for investigation. Therefore, in the present work, CD2 activation was related to the regulation of stathmin phosphorylation, a protein present in the cells as at least two distinct isoforms [16] yielding several sets of phosphorylated forms whose pattern reflects the activation of diverse signal transduction pathways [10,22,23].

We first clearly demonstrated the involvement of PKC in the CD2-triggered activation of the normal human $CD4^+$ T lymphocyte P28D clone, as proved by the increase of PKC activity in the particulate fraction, and of the phosphorylation of the 80 kDa PKC-substrate MARCKS [24]. In our hands, high Ca^{2+} concentrations were found more effective to assay the PKC translocation. This result is slightly different from that reported in Jurkat cells [27]. However this could reflect the difference in the Ca^{2+}-dependence of PKC isoforms present in different cell types since the α-isoform is predominant in the transformed Jurkat cell line whereas the β-isoform of PKC is more expressed in normal T lymphoblasts [28] and thus most likely in P28D T cells.

CD2 stimulation of T lymphocytes has been shown to induce a rapid PKC-dependent phosphorylation of the γ-subunit and to a lesser extent of the δ-subunit of the CD3 antigen, as well as phosphorylation of CD4, CD8, CD45 and of class I molecules of the major histocompatibility complex (reviewed in [29]). Intracytosolic molecules are also rapidly phosphorylated during a CD2-activated response, including the 80 kDa-MARCKS protein. Finally CD2 triggering was related also to tyrosine phosphorylation [30–32]. Interestingly,

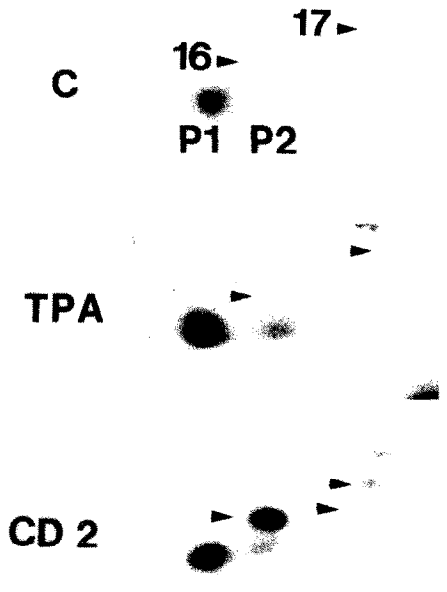

Fig. 3. TPA- and CD2-induced patterns of stathmin phosphorylation. Intact P28D T lymphocytes were labeled with $^{32}PO_4^{3-}$ for 4 h and then treated or not (C), with TPA (100 ng/ml) or anti-CD2 mAbs (X11 + D66, 50 μg/ml each) for 10 min. The areas of 2D-PAGE autoradiograms as boxed in Fig. 2 are presented. P1, P2 and P3 are the three increasingly phosphorylated 19 kDa forms of stathmin, whereas arrowheads numbered 16 and 17 point to the corresponding stathmin-derived phosphoprotein sets, at 21 and 23 kDa, respectively.

CD2 stimulation of T lymphocytes also induces the phosphorylation of stathmin, a ubiquitous cytosolic protein whose expression and/or phosphorylation have been related to the regulation of either proliferation, differentiation or of differentiated functions of various cell types [10]. The phosphorylated variants of stathmin most likely represent the molecular support for signal integration [10]. In this respect, we found that stathmin is also expressed in the P28D normal human $CD4^+$ T lymphocyte clone, in agreement with recent reports on resting peripheral T lymphocytes [11].

Comparison of stathmin phosphorylation patterns obtained after stimulation of cells with either TPA, a potent activator of PKC, or after CD2 triggering allowed to gain insight in the involvement of both PKC and stathmin in CD2-triggered T cell activation. Indeed both TPA and CD2 triggering of P28D T cells led to characteristic, partially overlapping but quantitatively and qualitatively distinct phosphorylation patterns of stathmin isoforms. As compared to the action of TPA, CD2 triggering induced a somewhat less intense stimulation of ^{32}P-incorporation into the 19 kDa forms of stathmin P1 and P2, and conversely a much stronger incorporation into the higher molecular weight sets '16' and '17'. In Jurkat cells phosphorylation of stathmin forms '16' and '17' has been related to the activation of a Ca^{2+}-dependent kinase [33], which might then also be involved in CD2 action. Thus the characteristic phosphorylation pattern of stathmin may indeed reflect the different transduction pathways involved in a considered activation signal. In this respect, our results clearly indicate that the activation via CD2 involves other kinase(s) beside PKC.

In CD2-stimulated P28D T cells, stathmin phosphorylation is related here to a signal positively associated with T cell activation. This is also the case in Jurkat cells [33] and in UW-4B, an IL2-dependent cell line where it is related to stimulation by IL-2 [34]. Conversely, in proliferating IL-2 dependent PBL as well as in several tumoral T cell lines, phosphorylation of stathmin in response to TPA alone or together with the Ca^{2+} ionophore A23187 has also been associated with down-regulation of DNA synthesis [11,18]. Thus, as in many non-immunological biological systems, phosphorylation of stathmin is taking place in response to activation of diverse intracellular pathways. Considered together, our data strongly support the proposed role of relay for stathmin, the final effect depending on the state of proliferation and/or differentiation of the cells considered [10].

The present analysis of stathmin phosphorylation in a normal human T cell clone gives further clues on the PKC-dependent and independent intracellular mechanisms involved in CD2-regulation of T lymphocyte proliferation. Further pharmacological analysis of the different kinases responsible for the various phosphorylation patterns of stathmin, and corresponding to physiological perturbations of T lymphocytes will give new insights on signal transduction pathways involved in T lymphocyte activation. In this respect the potential of stathmin as a new tool should be emphasized. Finally it also further documents the proposed ubiquitous role of stathmin as an intracellular relay for extracellular agents, integrating diverse second messenger pathways.

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