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Serine 16 of Stathmin as a Cytosolic Target for Ca\(^{2+}\)/Calmodulin-Dependent Kinase II After CD2 Triggering of Human T Lymphocytes\(^1\)

Sabine le Gouvello\(^2\), Valérie Manceau, and André Sobel\(^3\)

We investigated specific signaling events initiated after T cell triggering through the costimulatory surface receptors CD2 and CD28 as compared with activation via the Ag receptor (TCR/CD3). We therefore followed the phosphorylation of stathmin, a ubiquitous cytoplasmic phosphoprotein proposed as a general relay integrating diverse intracellular signaling pathways through the combinatorial phosphorylation of serines 16, 25, 38, and 63, the likely physiologic substrates for Ca\(^{2+}\)/calmodulin (CaM)-dependent kinases, mitogen-activated protein (MAP) kinase, cyclin-dependent kinases (cdk)s, and protein kinase A, respectively. We addressed the specific protein kinase systems involved in the CD2 pathway of T cell activation through the analysis of stathmin phosphorylation patterns in exponentially growing Jurkat T cells, as revealed by phosphopeptide mapping. Stimulation via CD2 activated multiple signal transduction pathways, resulting in phosphorylation of distinct sites of stathmin, the combination of which only partially overlaps the CD3- and CD28-induced patterns. The partial redundancy of the three T cell activation pathways was evidenced by the phosphorylation of Ser\(^{16}\) and Ser\(^{38}\), substrates of MAP kinases and of the cdk family kinases(s), respectively. Conversely, the phosphorylation of Ser\(^{16}\) of stathmin was observed in response to both CD2 and CD28 triggering, but not CD3 triggering, with a kinetics compatible with the lasting activation of CaM kinase II in response to CD2 triggering. In vitro, Ser\(^{16}\) of recombinant human stathmin was phosphorylated also by purified CaM kinase II, and in vivo, CaM kinase II activity was indeed stimulated in CD2-triggered Jurkat cells. Altogether, our results favor an association of CaM kinase II activity with costimulatory signals of T lymphocyte activation and phosphorylation of stathmin on Ser\(^{16}\). The Journal of Immunology, 1998, 161: 1113–1122.

**T** lymphocyte activation is a semiotic process in which the lymphocyte interprets microenvironmental signals delivered by its Ag receptor that include features of the Ag itself, as well as signals delivered by costimulatory molecules and cytokines. Moreover, the interpretation of signals may be influenced by the state of differentiation of the responding cells (1). In addition to the Ag-specific recognition pathway involving the TCR/CD3 complex (2), at least one additional signal delivered by APCs is required to allow T cell proliferation or effector function (this second signal is referred to as costimulation). Various costimulatory receptors on the surface of resting T cells have been defined, among which are the CD2 and CD28 molecules (for a review see Refs. 3 and 4). However full T-PBL proliferation/activation via the CD3, CD2, or CD28 pathways can only be achieved either in the presence of accessory cells (4–6) or, if complementatory activation signals are delivered, via the stimulation of other T cell surface molecules such as CD4, CD5, CD8, CD40L, CD44, or CD45 (for review see Ref. 7), which suggests that these distinct receptors, although interdependent, are functionally distinct and deliver critical regulatory transducing signals in the control of T cell proliferation.

In this respect, the function of CD2 is still a debated question. The hypothesis that CD2 and CD3 share a common activation pathway was supported by the observation that both CD2 and CD3 molecules apparently triggered the same cascade of some of the earliest biochemical events (8, 9). In particular, phosphorylation on tyrosine occurred mostly on the same set of proteins after stimulation of both CD3 and CD2 receptors (10), including phospholipase C\(^{γ}\)-1 (9, 11), in which phosphorylation correlates with the activation of phosphoinositide turnover (12, 13).

Alternatively, we and others have proposed that CD2 induces distinct signal transduction events that would, at least in part, differ from those initiated by the TCR/MHC complex engagement (14–19). In support of this postulate, we showed previously (20) that the stimulation of a CD4\(^{+}\) T lymphocyte clone via CD2 was associated with a specific phosphorylation pattern of stathmin (21), differing from that induced in response to PMA, an activator of protein kinase C (PKC).\(^4\)

Stathmin (22), also referred to as p19 (23), prosolin (24), p18 (25), pp20 (26), and Op18 (27), was identified in several cellular systems as a ubiquitous, conserved cytosolic phosphoprotein in which expression and phosphorylation are highly regulated in relation to cell proliferation and differentiation (for review see Ref. \(^4\)).

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\(^4\) Abbreviations used in this paper: PKC, protein kinase C; PKA, cAMP-dependent protein kinase A; MAP kinase, mitogen-activated protein kinase; CaM kinase II, Ca\(^{2+}\)/calmodulin-dependent kinase II; OA, okadaic acid; cdk, cyclin-dependent kinase.
After CD2 triggering of human T lymphocytes

Therefore, it appeared to be an intracellular proteic relay integrating diverse intracellular signaling pathways (21). It was recently proposed that it might function by controlling the mitotic microtubule dynamics (28–32).

Stathmin is highly expressed in activated T-PBL as well as in transformed T lymphocytes, including Jurkat cells (24, 25). In intact cells, stathmin is a target for both cell cycle and cell surface receptor-regulated phosphorylation events that are initiated by the activation of diverse protein kinase systems such as the cAMP-dependent kinase (PKA), PKC, Ca2+-dependent kinases, cyclin-dependent kinases (cdks), mitogen-activated protein (MAP) kinases, and tyrosine kinases (reviewed in Ref. 21) (33–39). Site mapping studies performed in intact cells and in vitro revealed four phosphorylation sites, namely Ser16, Ser38, Ser76, and Ser110. PKA catalyzes the phosphorylation of stathmin on Ser16 and Ser110, being the major target of this kinase (36, 40); MAP kinase and cdc2 kinase both induce phosphorylation on Ser38 and Ser110, respectively, but with an opposite site preference (Ser38 for MAP kinase, and Ser110 for cdc2) (36, 39). Moreover, phosphorylation of only these four sites and their specific combinations account for all the phosphoforms of stathmin identified so far in vivo in diverse biologic systems (36, 41). Thus, analysis of the phosphorylation site patterns of stathmin might give clues for the identification of intracellular pathways involved in various cell regulatory processes. Site-mapping studies of stathmin revealed that CD3 stimulation of Jurkat T cells resulted in an apparently PKC-independent activation of both the MAP kinase and the Ca2+/calmodulin-dependent kinase IV (Gr) (37, 38).

In the present study, to address specific protein kinase systems involved in the CD2 pathway of T cell activation, we analyzed the site-specific phosphorylation of stathmin in response to the stimulation of the CD2 receptor in the Jurkat T cell line. Our data show that stimulation of CD2 activated multiple signal transduction pathways, resulting in phosphorylation of distinct sites of stathmin, the combination of which only partially overlaps the CD3- and CD28-induced patterns. Indeed, in intact cells, prolonged CD2 stimulation induced a major phosphorylation of Ser16 without phosphorylation of Ser76, indicating the involvement of a kinase distinct from PKA. Furthermore, site-mapping studies show that purified Ca2+/calmodulin-dependent protein kinase II (CaM kinase II) was able to phosphorylate Ser16 of recombinant stathmin in vitro. Studies performed in intact cells with CD2 and A23187 indicate that CaM kinase II might be responsible for the CD2-induced phosphorylation of stathmin on Ser16 in vivo.

**Materials and Methods**

**Materials**

Forskolin, PMA, calmodulin, aprotinin, pepstatin, leupeptin, DTT, temed, trizma base, and ammonium persulfate were from Sigma Chemical Co. (St. Louis, MO). TPCK (tosylamide-2-phenylethyl chloromethyl ketone)-treated trypsin was from Worthington (Freehold, NJ); A23187 from Boehringer (Mannheim, Germany); okadaic acid (OA) and thermolysin from Calbiochem (La Jolla, CA); [γ-32P]ATP from Amersham (Amersham, U.K.); TLC sheets and cellulose plates from Kodak (New Haven, RI); acrylamide and bisacrylamide (Acrylogel 2.6) from Merck (Darmstadt, Germany); SDS from Serva (Heidelberg, Germany); amphotolines from Pharmacia LKB (Uppsala, Sweden); FCS from Life Technologies (Grand Island, NY); and phosphate-free Eagle’s MEM from Flow Laboratories (Irvine, U.K.).

**Methods**

**Monoclonal Abs.** ag-specific recognition can be artificially triggered after binding of the appropriate anti-CD3 or anti-TCR mAbs (2). The mAb UCHT1 (IgG1), specific for CD3ε chain, kindly provided by Dr. P. C. L. Beverley (Imperial Research Cancer Fund, London, U.K.) (42), was used for stimulation experiments at a 1:500 dilution of an ascitic fluid. Several distinct natural ligands have been described for the CD2 molecule (43), and artificial activation via CD2 requires the appropriate combination of two mAbs directed against distinct epitopes of the CD2 molecule. Anti-CD2 mAbs X11 (IgG1) and D66 (IgG2b) were previously described: mAb X11 recognizes the T11.1 epitope of the CD2 molecule, whereas mAb D66, specific for a cryptic epitope on resting T cells, is unmasked after X11 mAb binding (44). Thus, for inducing mitogenic stimulation, these mAbs must be used in combination, and the pair X11 + D66 will therefore be designated as anti-CD2 mAbs throughout the text. For all stimulation experiments, both mAbs were thus added together at saturating concentrations (50 μg/ml). YTH655.5, a mAb (kindly provided by Drs. J. P. Revillard, Institut National de la Santé et de la Recherche Médicale U80, Lyon, France and H. Waldmann, University of Oxford, Oxford, U.K.) directed against another cryptic epitope of the CD2 molecule expressed only on activated T cells (45), was also tested in combination with the X11 mAb. Anti-CD28 mAb (IOT28; Immunechn, Marseille, France) was used at 20 μg/ml.

**Cells.** The leukemic CD2+CD3+ Jurkat T cell line, clone E-6.1, kindly given by Dr. A. Alcover (Institut Pasteur, Paris, France), was maintained in RPMI 1640 medium (Flow Laboratories) supplemented with 10% FCS, penicillin (50 U/ml), streptomycin (50 μg/ml), l-glutamine (2 mM), and sodium pyruvate (1 mM). To stimulate them when they are exponentially growing, cells were always diluted at a density of 0.5 × 106 cells/ml 16 h before stimulation.

**Protein preparations.** Recombinant stathmin was expressed in Escherichia coli in its unphosphorylated form and purified as described (40). Briefly, the NcoI-BamHII fragment of the cDNA containing the entire sequence coding for human stathmin (46) was cloned into the expression vector pET8c, and transfected into E. coli BL21 (DE3). Upon induction with IPTG (isopropyl-thio-β-D-galactoside), stathmin was purified to homogeneity by a two-step procedure involving chromatography on DEAE-Sepharose CL-6B and gel-filtration on Superose 12 (Pharmacia LKB). CaM kinase II, purified from rat brain, was a generous gift from Dr. J. A. Girault (Institut National de la Santé et de la Recherche Médicale U114, Paris, France).

**Phosphorylation of stathmin in vitro by CaM kinase II.** Recombinant stathmin (1 μg) was incubated at 30°C with 1 U CaM kinase II (1 U is the amount of enzyme that catalyzes the phosphorylation in 1 min of 1 pmol of synapsin I), and 370 kBq of [γ-32P]ATP (8.11 GBq/mmol), in 100 μl of phosphorylation buffer: 50 mM Tris/HCl, pH 7.6, 100 μM EGTA, 10 mM CaCl2, and calmodulin (0.1 mg/ml). The reactions were initiated by adding 20 μl of 250-μm radioactive ATP (sp. act., 3 MBq/ mmol), after preincubitation of the other components for 1 min at 30°C. After 5 min at 30°C, the reactions were stopped by adding 50 μl of 3 × STOP solution (Tris·HCl (200 mM), pH 6.8; 7% SDS; 33% glycerol; 3% β-mercaptoethanol), and the samples were boiled and further submitted to two-dimensional PAGE as described below. All of the relevant [32P]-labeled stathmin spots were excised from the gels and pooled. After Cerenkov counting, the gel pieces were rehydrated and the samples were prepared for [32P]Phosphate mapping analysis as described further below.

**Radioactive labeling and pharmacologic treatments.** 32PO4- labeling was performed by preincubating 5 × 106 cells in 250 μl of phosphate-free medium with 5.55 MBq 32PO4-. (DuPont-New England Nuclear Research Products, Nenours, France), for 4 h. Test agents were added directly to the radioactive medium for the last 30 min, except for OA, which was added at the very beginning of the radioactive incubation. The labeling was stopped as described previously (47), preparing the samples for two-dimensional electrophoresis. The same amount of TCA-precipitable radioactivity was used for each sample within a given experiment, allowing direct comparison of autoradiograms.

**Polyacrylamide gel electrophoresis.** Two-dimensional PAGE was performed as described previously (47). The isoelectric focusing gels contained 2% total amphotolines, pH 4–6, 5–7, and 3–10, in the proportion 2:2:1. The second dimension was run on 13% acrylamide gels. The fixed gels were dried and exposed for autoradiography with Kodak XR-5 film. Quantification of stathmin phosphorylation was obtained by analysis of the gel and direct counting of the radioactivity in each relevant [32P]-labeled spot with an Instant Imager apparatus (Packard Instrument, Meriden, CT).

**Phosphopeptide mapping.** Two-dimensional thin layer phosphopeptide mapping of stathmin was conducted as described (48) with modifications (36): protein hydrolysis was performed with trypsin at 75 μg/ml overnight followed with thermolysin at a concentration of 100 μg/ml, and the radioactive material was spotted in the middle, 4 cm from the bottom of the TLC sheet. Autoradiography was performed using Kodak XAR-5 film at ~70°C with Kodak Quanta III intensifying screens. Instant Image analysis of radioactive spots was used for quantification of phosphopeptides.

**CaM kinase II assay.** CaM kinase II activity was assayed in cell lysates using CaM kinase II assay kits (Upstate Biotechnology, Lake Placid, NY) and using autocamtide 2 (KKALRRQETVDAL) as a peptide substrate.
with relative selectivity for CaM kinase II. Exponentially growing Jurkat T cells, stimulated or not, were lysed in assay dilution buffer (20 mM MOPS, pH 7.2, 25 mM β-glycerol phosphate, 1 mM sodium orthovanadate, 1 mM DTT, 1 mM CaCl₂). Cell lysates were centrifuged 15 min at 12,500 rpm at 4°C. The concentration of protein was determined in the resulting supernatant by the Bradford assay (Bio-Rad, Hercules, CA). The reaction mixture contained, following the manufacturer’s instructions, 10 μl of the sample extract, 10 μl of substrate mixture, 10 μl of a mixture containing inhibitors of other serine/threonine kinases such as PKA and PKC, and 10 μl of Mg²⁺/ATP mixture containing [γ-32P]ATP. The mixture was incubated at 30°C for 10 min, and the phosphorylated substrate was separated from the residual [γ-32P]ATP using p81 phosphocellulose paper. The papers were washed with five rinses of 0.75% phosphoric acid, then washed in acetone for 2 min, and the bound radioactivity was quantified with a scintillation counter. Blanks to correct for nonspecific binding of [γ-32P]ATP and its breakdown products to the phosphocellulose paper and controls for phosphorylation of endogenous proteins in the sample were performed, and CaM kinase II activity was expressed as pmol/min/mg protein.

Results

Increased phosphorylation of stathmin in response to CD2, CD3, or CD28 activation

We have previously shown that PKC-dependent and -independent pathways are responsible for the stathmin phosphorylation pattern observed in the P28D CD4⁺ T cell clone stimulated via the alternative pathway of T lymphocyte activation, which is triggered via the CD2 molecule. Indeed, when these T cells were treated with the phorbol ester PMA, the resulting pattern of the various phosphorylated forms of stathmin only partially mimicked the pattern observed after treatment with anti-CD2 mAbs (20). Moreover, we have previously identified specific phosphorylation sites of stathmin as hallmarks of specific kinase activities in vitro as well as in intact cells (36, 39).

To further characterize intracellular signaling pathways associated with the stimulation via CD2 and to distinguish them from those related to CD3 or CD28 triggering, Jurkat T cells were used in the present study to identify stathmin phosphorylated sites in response to treatment with either anti-CD2, anti-CD3, or anti-CD28 mAbs. Exponentially growing Jurkat cells, which express high levels of stathmin (24), were prelabeled with [32P]orthophosphate to achieve isotopic equilibrium and then submitted to treatments with the various agonists. Following two-dimensional PAGE separation, the radioactive phosphoproteins were revealed by autoradiography (see Fig. 2), and the radioactivity incorporated in relevant spots was quantified directly on the gels (see Materials and Methods). Figure 1 shows that stimulation of [32P]-labeled Jurkat cells via CD2, CD3, or CD28 resulted in a similar increase in phosphorylation of stathmin. The D66 mAb by itself did not stimulate stathmin phosphorylation, although it was shown to trigger a tyrosine kinase independently of the CD3-dependent signaling pathway (18). However, this mAb has no mitogenic effect by itself, i.e., without the cooperative action of the XI1 mAb. Treatments with pharmacologic agents known to partially stimulate T cell activation, such as PMA, the Ca²⁺ ionophore A23187, or OA, also increased the total amount of radioactive phosphate incorporated in stathmin. The increased incorporation of [32P] into stathmin in response to the various stimuli corresponded to major changes in the actual phosphorylation state of stathmin rather than only to an increased phosphate turnover, as it was previously shown in Jurkat cells by direct protein detection of the unphosphorylated and phosphorylated forms of stathmin on silver-stained two-dimensional gels (41).

We further characterized the incorporation of [32P] in the diverse forms of stathmin separated by two-dimensional PAGE and reflecting its phosphorylation on one or several of its identified phosphorylation sites (36) (Fig. 2). Basal incubation of Jurkat T cells with [32P]PO₄³⁻ resulted in the clear labeling of spots P1 and P2 of stathmin (molecular mass = 19 kDa), together with a slight labeling of spots 16₁, the less acidic form of set 16 (molecular mass = 21 kDa), and 17₁, the less acidic spot of set 17 (molecular mass = 23 kDa). This observation is in agreement with our previous results in the normal P28D T cell clone (20). However, it is clear from the results shown in Figure 2 that the various types of treatment differed qualitatively according to the stathmin phosphoforms induced. When [32P]-prelabeled Jurkat cells were treated with anti-CD2 mAbs for 30 min, the labeling of spots P1, P2, 16₁, and also 17₁ became more intense, indicating that the phosphorylation of stathmin was strongly increased into its forms yielding these four spots. Enhancement of the labeling of the same four spots was much less pronounced after CD3 triggering. Moreover, spot 16₁ appeared even more intense than spot P2 in CD2-stimulated cells; the relative intensity of spot 16₁ compared with that of spot P2 appeared also somewhat increased in CD28-treated cells. Conversely, in CD3-stimulated cells, no relative increase in the labeling of spot 16₁ was apparent. Furthermore, CD2 triggering induced also a clear enhancement of the labeling of the more acidic, thus more phosphorylated forms of set 16 and set 17, namely 16₂ and 17₂.

These results illustrate that the various forms of stathmin might be used as hallmarks of the activation as well as of the interaction of distinct intracellular signaling pathways involved in the various stimulating modes of T cell activation. They further document the fact that CD2 and CD3 or CD28 activate intracellular pathways that are at least partially nonoverlapping.

Site-specific phosphorylation of stathmin in response to CD2 stimulation

We further investigated the CD2-dependent alternative pathway of T cell activation and the related intracellular kinase pathways on the basis of the kinase specificity of the four distinct phosphorylation sites of stathmin accounting for all of the forms encountered
in numerous biologic systems and in response to diverse regulatory signals (36). Experiments were performed to identify the specific sites of stathmin involved and to quantify their phosphorylation in response to CD2 stimulation. In control and in CD2-stimulated Jurkat cells, the various phosphoforms of stathmin were resolved by two-dimensional PAGE, and the corresponding spots were excised and analyzed by two-dimensional phosphopeptide mapping (see Materials and Methods). The excised spots from each gel were either pooled (Fig. 3) or each spot was processed individually (Fig. 4), according to a procedure yielding characteristic migration patterns for each of the four previously identified phosphorylation sites of the protein (36). The sites of stathmin phosphorylated after CD2 activation of T cells (Fig. 3) were the same four sites (Ser16, Ser25, Ser38, and Ser63) for which phosphorylation was described in other cellular systems. As compared with the control, CD2-stimulation induced a strong phosphorylation of Ser16, Ser25, and Ser38, and only a very slight one of Ser63. This comprehensive method of analysis of stathmin phosphoforms was coupled with the quantitative evaluation of the level of phosphate in each serine phosphorylated in each phosphoform (Fig. 4). In control cells, stathmin was mostly phosphorylated on single sites yielding spot P1 on two-dimensional gels, or on two sites yielding spot P2 and low levels of spot 161 (see also Fig. 2B). In control cells, the kinase showing the highest basal activity was the kinase responsible for the phosphorylation of Ser38 (Figs. 3 and 4). CD2 treatment resulted in the incorporation of [32P] into spots P1 and P2, as well as into both forms of each slowly migrating set 16 and 17 resulting from the concurrent phosphorylation on Ser16 and Ser25 (Figs. 2 and 4). Accordingly, Ser16 and Ser25 appeared overall severalfold more phosphorylated than in control cells (Figs. 3 and 4). Phosphorylation on Ser38 was also stimulated after CD2 triggering, yielding some P2 but mostly 17, and 17 spots, whereas phosphorylation on Ser63 was undetectable in control and minimal in CD2-treated cells.

Altogether, these phosphate incorporation experiments can be interpreted as follows: 1) the increased phosphate incorporation

FIGURE 2. Differential patterns of stathmin phosphoforms after Ag-specific triggering (CD3) and cosignaling molecule stimulation (CD2; CD28). A. Intact Jurkat T cells were labeled with [32P]PO4 for 4 h, as indicated under Materials and Methods, and stimulated for the last 30 min with one of the following: solvent (control), 50 μg/ml of two anti-CD2 mAbs (X11 + D66) (CD2), 1:500 dilution of an ascitic fluid of the anti-CD3 mAb UCHT1 (CD3), or 20 μg/ml of the anti-CD28 mAb IOT28 (CD28). Radioactive phosphoproteins were then analyzed by two-dimensional PAGE autoradiography. The box on the two-dimensional PAGE autoradiogram (left) indicates the area of stathmin spots shown in detail after the different treatments (right). P1, P2, and P3 are the three increasingly phosphorylated 19-kDa forms of stathmin, whereas open bars numbered “16” and “17” indicate the corresponding stathmin-derived phosphoprotein sets, at 21 and 23 kDa, respectively. B. Scheme representing the diverse forms of stathmin generated by phosphorylation on various combinations of the four sites identified. This scheme accounts for all the stathmin forms and two-dimensional spots identified so far in numerous biologic systems and conditions (36).
into spot P2 (Fig. 1) resulted from the net phosphorylation of stathmin on Ser 16 and Ser 38 , yielding double-phosphorylated forms on Ser 38 and Ser 16 , or on Ser 38 and Ser 25 (originally phosphorylated in control cells); 2) on the other hand, the analysis of spot 16 showed, in agreement with our previous reports (36, 40), that its observed electrophoretic retardation due to the conformational changes of the protein resulted from the combined phosphorylation of Ser 16 and Ser 25; 3) moreover, the increased phosphate incorporation into spots of set 17 is due, as expected (36, 40), to the combined phosphorylation on Ser 16, Ser 25, and Ser 38, resulting either from the phosphorylation on Ser 16 and Ser 25 of stathmin molecules already phosphorylated on Ser 38 in untreated cells or from the further phosphorylation on Ser 16 or Ser 25 of P2 isoforms basally phosphorylated on Ser 25 and Ser 38, or on Ser 16 and Ser 38 (refer to Fig. 2B).

Altogether, since increased phosphorylation of Ser 16 and Ser 25 appeared predominant as compared with that of Ser 38 in CD2-treated cells, it can be concluded from our results that the preferential kinase activities stimulated by CD2 treatment of Jurkat T cells displayed a preference for phosphorylation of stathmin on Ser 16 and Ser 25.

Differential regulation of phosphorylation of serines 16, 25, and 38 of stathmin in CD2-, CD3-, or CD28-stimulated Jurkat T cells

To characterize the protein kinase(s) responsible for stathmin phosphorylation specifically in response to CD2 treatment, we compared the phosphorylation of stathmin after stimulation of proliferating Jurkat T cells via CD2, CD3, or CD28. Actually, it has been proposed that Ser 16, Ser 25, Ser 38, and Ser 63 of stathmin may be respective physiologic substrates for either the CaM kinase Gr (38), MAP kinase (37, 39), members of the cdc2 kinase family (36,
CD2 triggering, the phosphorylation increase of Ser16 was of similar magnitude in CD2- and CD3-stimulated T cells. After stimulation, Ser25 and, to a lesser extent, Ser16, but poorly stimulated phosphorylation of Ser38, whereas the relative phosphorylation of Ser38 was in about the same range as in CD2-stimulated cells, whereas phosphorylation of Ser16 was much more enhanced after CD2-stimulation.

The CD2 values were calculated from the data presented in Figure 4. Corresponding control values have been subtracted.

Characterization of the `serine 16-kinase` activity

Our results (Fig. 5) indicated that phosphorylation of Ser16 corresponds to a pathway activated particularly in Jurkat T cells in response to CD2 as compared with CD3 treatment. Phosphorylation of Ser16 of stathmin has been previously correlated with Ca2+-regulated kinase pathways (26, 33, 38). Indeed A23187 treatment of Jurkat cells resulted in phosphorylation of Ser16 (Fig. 5). The amino acid sequence context of Ser16 fits the minimal consensus Arg-X-X-Ser/Thr. Both kinases have been proposed as being involved in T lymphocyte activation (54–56). Recently, stathmin was proposed as an early cytosolic target for CaM kinase IV activity was shown to be a good substrate for PKA (34, 36); in vitro experiments, Ser25 has been shown to be a good substrate for PKA (34, 36).

Our results (Fig. 5) indicated that phosphorylation of Ser16 corresponds to a pathway activated particularly in Jurkat T cells in response to CD2 as compared with CD3 treatment. Phosphorylation of Ser16 of stathmin has been previously correlated with Ca2+-regulated kinase pathways (26, 33, 38). Indeed A23187 treatment of Jurkat cells resulted in phosphorylation of Ser16 (Fig. 5). The amino acid sequence context of Ser16 fits the minimal consensus Arg-X-X-Ser/Thr. Both kinases have been proposed as being involved in T lymphocyte activation (54–56). Recently, stathmin was proposed as an early cytosolic target for CaM kinase IV activity was shown to be a good substrate for PKA (34, 36). In vitro experiments, Ser25 has been shown to be a good substrate for PKA (34, 36).

Recent findings in various cellular systems show that, although key regulatory phosphoproteins may boost early cellular activation when they are held in a partially phosphorylated state, overphosphorylation could be associated also with the blockade of cellular activation, cell cycle progression thus requiring further dephosphorylation (51). OA, a polyether fatty acid that inhibits protein phosphatase activities PP1 and PP2A (52), has been shown to activate T cell proliferation. Therefore, we investigated the effects of the phosphatase inhibitor OA on stathmin phosphorylation in Jurkat cells with those of CD2 mAbs. We have shown in Figure 1 that a 4-h OA treatment of Jurkat cells increased the total amount of radioactive phosphate incorporated into stathmin. The results of quantitative phosphopeptide analysis showed that OA treatment induced the phosphorylation of Ser63, in addition to that of Ser16, Ser25, and Ser38. In vitro experiments, Ser43 has been shown to be a good substrate for PKA (34, 36). In the T lymphocyte cellular system, the PKA pathway has been inhibitory rather than activating regulations (53). However, in response to OA treatment, the three other sites of stathmin, associated with T cell activating signals, are also phosphorylated, which might result in an overall activating signal rather than inhibitory one like with PKA activation alone.

Therefore, our results suggest that specific combinations of relative phosphorylation levels of the same four specific serine sites of stathmin, resulting in the integration of different kinase/phosphatase activities, might correlate with different functional states of T lymphocytes.

### Materials and Methods

Jurkat T cells were labeled with [32P]PO4 and either treated for the last 30 min with 50 μg/ml of two anti-CD2 mAbs (X11 + D66) (CD2), 1:500 dilution of an ascitic fluid of the anti-CD3 mAb UCHT1 (CD3), 20 μg/ml of the anti-CD28 mAb IOT28 (CD28), 0.5 μM of A23187, or 100 ng/ml of PMA or treated for the entire 4 h with 0.5 μM of OA. Phosphorylated proteins were separated by two-dimensional PAGE, and gel pieces corresponding to the individual stathmin spots were excised, pooled, and then digested by trypsin and thermolysin, as described in Materials and Methods. The resulting phosphopeptides were analyzed by two-dimensional peptide mapping, and the relevant spots were analyzed and further quantified with an Instant Imager.

The results of quantitative phosphopeptide analysis showed that OA treatment induced the phosphorylation of Ser63, in addition to that of Ser16, Ser25, and Ser38. In vitro experiments, Ser43 has been shown to be a good substrate for PKA (34, 36). In the T lymphocyte cellular system, the PKA pathway has been inhibitory rather than activating regulations (53). However, in response to OA treatment, the three other sites of stathmin, associated with T cell activating signals, are also phosphorylated, which might result in an overall activating signal rather than inhibitory one like with PKA activation alone.
Furthermore, we found that recombinant stathmin was a substrate for CaM kinase II in vitro (Fig. 7) in standard Ca\textsuperscript{2+}, calmodulin, and Mg\textsuperscript{2+} conditions for CaM kinase II activation (57). Phosphopeptide mapping of the resulting phosphorylated stathmin revealed a pattern essentially characteristic of the phosphorylation of Ser\textsuperscript{16} (Fig. 7A), with two additional, less labeled and uncharacterized phosphopeptides, “w” and “y,” which have never been observed after phosphorylation in vivo. The kinetics of the phosphorylation reaction clearly showed that Ser\textsuperscript{16} of stathmin was the first and predominant target of CaM kinase II in vitro (Fig. 7B) and that phosphopeptides w and y correspond, most likely, to non-physiologic, secondary targets for this enzyme.

To test the biologic relevance of the phosphorylation of the CaM kinase II-specific site of stathmin, we examined whether CaM kinase II activity was indeed stimulated in response to CD2 triggering of Jurkat cells. A cell extract from CD2-stimulated cells did display an enhanced kinase activity on the relatively specific CaM kinase II substrate, autocamtide 2 (58, 59), in a manner similar to an extract from cells treated with the A23187 Ca\textsuperscript{2+} ionophore (Fig. 8). The combination of the X11 mAb with the YTH655.5 (45) instead of the D66 mAb yielded a similar increase in autocamtide II phosphorylation, further confirming the CD2 specificity of the CaM kinase II activation observed (data not shown).

Altogether, these results clearly demonstrate an increase in CaM kinase II activity concomitant with Ser\textsuperscript{16} phosphorylation, suggesting that CaM kinase II is a good candidate for the kinase responsible for Ser\textsuperscript{16} phosphorylation of stathmin after CD2 triggering of Jurkat T cells.

Discussion

The major molecular supports of cross-talks between several second messenger-dependent enzymatic cascades activated independently are the phosphorylation-dephosphorylation substrates of the corresponding signaling pathways and their spatial and temporal coordinations. In this respect, previous studies have suggested a role of stathmin in signal transduction as a relay integrating diverse intracellular regulatory pathways in various cellular systems (21, 22), its action on various target proteins (60), including tubulin and the control of microtubule dynamics (28–32), being a function of its combined phosphorylation state (21, 36). Moreover, sites of stathmin have been identified as substrates of specific kinase activities (36, 37, 39). Therefore, to distinguish the specific kinase systems involved in T cell activation stimulated via the CD2-dependent alternative pathway, we compared the site-specific phosphorylation of stathmin in the Jurkat T cell line in response either...
to stimulation via CD2 or via the Ag receptor-associated CD3 complex or to the triggering of another costimulatory receptor, CD28. The partial redundancy of the three activation pathways was evidenced by the phosphorylation of Ser25 and Ser38, substrates of MAP kinases and of kinase(s) of the cdk family, respectively. Conversely, the phosphorylation of Ser16 of stathmin was observed in response to both CD2 and CD28 but not CD3 triggering of Jurkat T cells. Furthermore, we were able to show that Ser16 of stathmin is phosphorylated by CaM kinase II in vitro and that CaM kinase II activity is enhanced in vivo in response to CD2 stimulation of Jurkat cells. Altogether, these results favor the hypothesis of an association of CaM kinase II activity with the CD2 stimulatory signal of T lymphocyte activation.

Redundancy between CD3-dependent and costimulatory pathways: activation of MAP kinase and cdc2 kinase families

In the present study, Jurkat T cells were examined in exponential growth phase. Therefore, the basal levels of phosphorylation of Ser25 and Ser38 in unstimulated cells (control) are in agreement with previous data on the enhanced phosphorylation of stathmin during both the S phase and the mitotic phase of the cell cycle, which have been attributed to the activity of members of the cdk2 kinase family (61). All of the stathmin phosphorylation modifications observed in response to CD3 stimulation may be explained by the increased phosphorylation of Ser25 and Ser38. The p21ras/MAP kinase enzymatic cascade (62) has already been correlated by the increased phosphorylation of Ser25 and Ser38. The p21ras/MAP kinase enzymatic cascade (62) has already been correlated with the previously described activation of MAP kinase by these two pharmacologic agents in T cells (65, 66).

Although it has been shown that Ca2+-sensitive signals do not regulate MAP kinase activity in Jurkat T cells (62), phosphorylation of Ser25 of stathmin in response to A23187 treatment of Jurkat T cells could be due to stimulation of one of the MAP kinase-like parallel pathways (67). Alternatively, it might be speculated that, like the phosphorylation of Ser38, the phosphorylation of Ser25 in response to A23187 treatment results, at least partially, from stimulated cdc2 or cdk2 kinase activity, as these enzymes are known to phosphorylate Ser25 of stathmin, even if with an approximately fivefold lower efficacy than on Ser38 (36, 39).

Stimulation of CaM kinase II is specific to costimulatory pathways

T cell responses to the CD3 vs CD2 activation pathway, depending on the in vitro experimental systems examined, were most often found to be identical in terms of early responses (e.g., calcium mobilization (68), CD3 phosphorylation (69), tyrosine phosphorylation (9–11)), whereas they were found to be rather distinct when focusing on later responses to costimulatory second signals (e.g., cytokine responsiveness (70), pp19 dephosphorylation (17, 71), p67 phosphorylation (19)). We therefore chose to examine potential differences between CD3- and costimulation-induced signals at treatment times that revealed phosphorylation events distant from those occurring during very early responses.

Our previous studies showed that CD2 stimulation generated preferentially the less acidic spots, 16, and 17, of the 21- and 23-kDa phosphoisoforms of stathmin in the normal P2SD CD4+ T cell clone (20). Here, we show that a strong increase of stathmin phosphorylation on Ser16 occurred in response to a 30-min treatment of proliferating Jurkat cells with anti-CD2 mAbs, and to a lesser extent with anti-CD28 mAb, but not with anti-CD3 mAb. Indeed, in CD2-stimulated Jurkat T cells, the same major spots, 16, and 17, most likely resulted from the stimulated phosphorylation on Ser16 of the P1 phosphoform basally phosphorylated on Ser25 in control cells and of the P2 phosphoform basally phosphorylated on Ser25 and Ser38 in control cells, respectively.

We therefore also investigated the kinase activity responsible for the phosphorylation of Ser16 of stathmin in response to costimulatory signals. PKA was not a good candidate, as we have previously shown that Ser16 of stathmin was not a good in vitro substrate for PKA although it is within a consensus site for this enzyme in the stathmin sequence (36). CaM kinase IV was recently proposed as the kinase responsible for the early transient phosphorylation of Ser16 of stathmin in response to stimulation of the CD3 Ag (37, 38). We found that Ser16 is also an efficient substrate for CaM kinase II in vitro, in agreement also with the fact that CaM kinase II and CaM kinase IV have been shown to share several substrates in vitro (72). Moreover, CaM kinase II activity was enhanced in vivo following CD2 triggering of Jurkat cells. These observations suggest that CaM kinase II is likely to contribute to the phosphorylation of stathmin, in particular in response to the activation of costimulatory pathways.

The proposed involvement of CaM kinase IV (37, 38) is in agreement with previous data showing a peak at 1 min, followed by a rapid decline in CaM kinase IV autophosphorylation and both autonomous (Ca2+-independent) and Ca2+/CaM-dependent kinase activities, despite the persistence of elevated [Ca2+]i for at least 10 min following TCR-CD3 signaling (54). In line with these results is the lack of phosphorylation of Ser16 after 30 min of treatment with anti-CD3 mAb. Therefore, the fact that phosphorylation of Ser16 was high at 10 min and maintained for over 30 min after treatments with anti-CD2 mAbs, anti-CD28 mAb, or A23187, as opposed to stimulation via CD3, made us favor the hypothesis of the involvement of CaM kinase II, another potential target for Ca2+-signaling in lymphocytes (73), in the phosphorylation of Ser16 of stathmin in response to CD2 and CD28 treatment. Moreover, in the present report, the observed phosphorylation of Ser16 in unstimulated Jurkat cells might also not be attributed to minimal autologous or Ca2+/CaM-dependent catalytic activity of CaM kinase IV, as it has been shown that CaM kinase IV molecules isolated from unstimulated Jurkat cells exhibited negligible autonomous or Ca2+/CaM-dependent catalytic activity (54). Finally, phosphorylation of Ser16 after treatment of unstimulated Jurkat cells with OA also favors the hypothesis of CaM kinase II involvement. Indeed, previous studies have demonstrated that CaM kinase II activity was up-regulated by autophosphorylation and inhibited by the Mg2+-independent phosphatases 1 and 2A, whereas CaM kinase IV autophosphorylation and subsequent inhibitory effects on enzyme activity were strictly Mg2+ dependent, which raised the possibility that phosphatases 1 and 2A do not act on this enzyme (54, 55). Thus, OA, an inhibitor of phosphatases 1 and 2A, cannot stimulate phosphorylation processes via the stimulation of basal catalytic activity of CaM kinase IV by preserving autophosphorylation of the enzyme.

Enhanced and prolonged CaM kinase II activation at later times of cell stimulation has been associated with increased cytosolic availability of calmodulin due to its release from calmodulin-binding proteins after their PKC-dependent phosphorylation or after treatment with high doses of Ca2+-ionophore (74). This process could thus account for the phosphorylation of stathmin Ser16 in response to both PMA and ionophore in Jurkat cells. Moreover, this mode of regulation of CaM kinase II activation could also account for the contrasting results on Ser16 phosphorylation after CD3 triggering and via CD2 or CD28 stimulation. Indeed, arachidonic acid...
metabolites have been shown to be responsible for sustained activation of some PKC isotype(s) (75). We have previously reported that CD2 and not CD3 stimulation of the P82D T cell clone generated lipid messenger molecules due to a phospholipase A₂ activity (56). Interestingly, CD28 has recently also been shown to transduce the activation signal through phospholipase A₂ and S-lipoxygenase activation (76). Therefore, in CD2- and CD28-stimulated cells, prolonged CaM kinase II activity might be due, indirectly, to sustained activation of certain PKC isotype(s) by arachidonic acid metabolites. In contrast, the transient PKC activity observed after CD3 stimulation (20) could not positively regulate CaM kinase II activity.

The CD2-dependent pathway has been shown, according to the activation state of peripheral blood T cells, to direct them either toward proliferation or toward apoptosis (56, 77). In Jurkat T cells, CaM kinase II has been shown to induce an IL-2 transcriptional block, independently of the Ca²⁺/calmodulin-responsive phosphatase, calcineurin (55). Accordingly, cyclosporin A, a calcineurin inhibitor, had no effect on early (15 min) phosphorylation of stathmin in OKT3-induced activation of freshly isolated PBL (78). Phosphorylation of stathmin has been associated with both activation and association with phospholipase Cgamma1. Accordingly, cyclosporin A, a calcineurin inhibitor, had no effect on early (15 min) phosphorylation of stathmin in OKT3-induced activation of freshly isolated PBL (78).


