In vivo and in vitro phosphorylation of annexin II in T cells: potential regulation by annexin V

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In order to understand how signal transduction occurs during T cell activation, it is necessary to identify the key regulatory molecules whose function is influenced by phosphorylation. Annexins II (A-II) and V (A-V) belong to a large family of Ca^{2+} -dependent phospholipid-binding proteins. Among many putative functions, annexins may be involved in signal transduction during cellular proliferation and differentiation. In the present study we show that A-II is phosphorylated *in vivo* in the Jurkat human T cell line. Indeed, A-II is phosphorylated after stimulation by phorbol myristate acetate and on serine residues after

T cell antigen receptor (TcR) stimulation. In cytosol from Jurkat cells, A-II is phosphorylated only by Ca^{2+} /phospholipid-stimulated kinases such as Ca^{2+} -dependent protein kinases C (cPKCs). A-V inhibits the phosphorylation of A-II and other substrates of cPKCs and has no effect on kinases activated only by phospholipids. In conclusion, A-II is phosphorylated both *in vitro* and *in vivo* in Jurkat cells, and may play a role as a substrate during signal transduction in lymphocytes via the TcR through the PKC pathway. On the other hand, A-V could act as a potent modulator of cPKCs in Jurkat cells.

INTRODUCTION

Annexins are a group of at least 13 different but structurally related proteins [1–3]. They all have the capacity to bind Ca^{2+} as well as negatively charged phospholipids (PLs) in a Ca^{2+} -dependent manner. These proteins are characterized by a similar structure, with four or eight repeats of a 70-amino-acid conserved core, and differ by a variable N-terminal domain. Annexin cores share 40–60% amino acid sequence identity and contain the Ca^{2+} and PL binding sites. The N-terminal domain contains phosphorylation sites for different protein kinases such as protein kinase C (PKC) and tyrosine kinases, as well as protein–protein interaction domains. It is speculated that these unique domains are specific and responsible for the different functions of annexins. Annexins seem to be involved in various biological processes, but their precise functions *in vivo* have not yet been established.

PKCs belong to a family of 12 related serine/threonine protein kinases [4,5]. Enzymic studies and molecular cloning analysis have led to the classification of PKCs into three groups: the first group of conventional PKCs (cPKCs) includes the α -, β_1 -, β_{II} - and γ -PKC isoenzymes; the second group of novel PKCs (nPKCs) contains the δ -, ϵ -, η - and θ -PKC isoenzymes, and the third group of atypical PKCs (aPKCs) comprises the ζ -, μ -, ι - and λ -PKC isoenzymes. cPKC activation is dependent on Ca^{2+} , phosphatidylserine (PS) and diacylglycerol (DAG), whereas nPKCs and aPKCs are insensitive to Ca^{2+} for their activation.

PKCs play a central role in signal transduction initiated by a wide variety of agonists, including those which activate T cells following binding to the T cell antigen receptor (TcR) CD3 complex [6–8]. In T cells, the binding of antigen to its receptor induces an early activation of protein tyrosine kinases and the phosphorylation of phospholipase C- γ 1 (PLC- γ 1) [9,10]. Activation of PLC- γ 1 provokes the hydrolysis of phosphatidylinositol 4,5-bisphosphate, resulting in the production of two intracellular second messengers: inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 releases Ca^{2+} from intracellular stores,

inducing an increase in the cytosolic Ca^{2+} concentration and thereby activating Ca^{2+} -dependent proteins. DAG is the endogenous lipid activator of PKCs. This cascade of biochemical events leads to the expression of interleukin 2 (IL-2) and of the 55 kDa α -subunit of the IL-2 receptor, which results in autocrine stimulation of T cell proliferation.

Several PKC isoenzymes have been found to be expressed in T lymphocytes: cPKCs (α -, β_1 - and β_{II} -PKC), nPKCs (δ -, θ - and ϵ -PKC) and one aPKC (ζ -PKC) [6–8,11,12]. The critical targets of PKCs in T cells have not yet been determined, but a large number of proteins are phosphorylated on serine/threonine residues after PKC activation. These include the γ - and δ -subunits of the human CD3 complex [13], CD4 and CD8 [14,15], CD45 [16], p56^{lck} [17], PLC- γ 1 [18], c-Raf [19], mitogen-activated protein kinase [20], IL-2 receptor [21], HLA class I heavy chains [22], K^+ channel [23] and phosphatidylinositol 3-kinase [24].

Annexins and cPKCs possess the common characteristic of binding PL in a Ca^{2+} -dependent manner [25]. Our group and others have previously shown the presence of annexin I (A-I), A-II, A-V and A-VI in T lymphocytes, but their physiological functions are still unknown [26–28].

A-II exists in cells in a monomeric and a heterotetrameric form. In the latter case, A-II binds to p11, a member of the S-100 family, forming a complex termed calpactin I. A-II binds actin filaments and proteins of the spectrin family [29,30]. A-II is a substrate for the tyrosine kinase pp60^{v-src} [31,32] and for PKCs *in vitro* [27,33–35] and *in vivo* [33,36]. Moreover, a significant increase in A-II expression has been detected during differentiation of various cells, and A-II has been identified as the product of the growth-regulated gene 1B6 [37]. A-II synthesis is also induced in several cell lines which are able to differentiate *in vitro* [3]. The phosphorylation of A-II by PKCs and by protein tyrosine kinases, and its increased expression during cell growth and cell differentiation, indicate a possible role for A-II during these processes.

Among annexins, A-V appears to be the most ubiquitous.

Abbreviations used: A-II, annexin II; A-V, annexin V; DAG, diacylglycerol; GST, glutathione transferase; IL-2, interleukin 2; IP_3 , inositol 1,4,5-trisphosphate; TcR, T cell antigen receptor; PKC, protein kinase C; aPKC, atypical PKC; cPKC, conventional PKC; nPKC, novel PKC; PL, phospholipid; PLC- γ 1, phospholipase C- γ 1; PMA, 4 β -phorbol 12 β -myristate 13 α -acetate; PS, phosphatidylserine.

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Recently, purified A-V has been reported to inhibit PKC-dependent phosphorylation of purified A-I [38–40]. Thus A-V, which has never been demonstrated to be phosphorylated by PKCs [27,41], could be a potential inhibitor of these kinases.

The aim of this study was to analyse the phosphorylation of annexins following TcR and PKC activation in the Jurkat human T cell line. We report that A-II is phosphorylated after cell stimulation with either PMA or antibodies against CD3. A-II is phosphorylated *in vitro* only by Ca^{2+} /PL-stimulated kinases, suggesting that this protein is a preferential substrate for cPKCs. A-V inhibits the phosphorylation of A-II and other substrates of cPKCs and has no effect on kinases activated only by PL. In conclusion, A-II may play a role as a substrate in TcR-mediated signal transduction through the PKC pathway. On the other hand, A-V could act as a potent modulator of cPKCs in Jurkat cells.

MATERIALS AND METHODS

Materials

RPMI 1640 medium, penicillin/streptomycin, glutamine and PKC inhibitor peptide [PKC-(19–36)] were purchased from Gibco. L- α -Phosphatidyl-L-serine, 1,3-diolein, PMA, Protein A–Sepharose and BSA were all from Sigma, Nonidet P-40 was from Fluka and Pansorbin cells were from Calbiochem. Anti-CD3 antibodies (monoclonal antibody IoT3) were obtained from Immunotech. Polyvinylidene difluoride membranes were purchased from Millipore. Fetal calf serum was obtained from Boehringer-Mannheim, [^{32}P]P_i from Dupont de Nemours, and [γ - ^{32}P]ATP and hyperfilm autoradiographic films were from Amersham.

Cell culture

A Jurkat T lymphocyte cell line derived from clone J77-6.8 was used in all experiments [42]. Cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 50 units/ml penicillin/streptomycin and 2 mM glutamine.

Antibodies

Polyclonal antibodies against human A-II were raised in a rabbit by popliteal lymph node injection [43] of a mixture of native and denatured protein purified from human placenta [44]. These antibodies, A-II-Elie, were specific to A-II when tested against purified A-I, -II, -III, -IV, -V and -VI. Another anti-A-II antibody (A-II-Cava), kindly provided by J. C. Cavadore (Montpellier, France), was also used.

Immunoprecipitation of annexins from ^{32}P -labelled Jurkat cells

Cells (10^7 /ml) were incubated in phosphate-free RPMI medium supplemented with 5% dialysed fetal calf serum for 30 min and then labelled for 3 h with 500 $\mu\text{Ci}/\text{ml}$ [^{32}P]P_i (8500–9120 Ci/mmol). Cells were then incubated for the indicated times with anti-CD3 (IoT3; dilution 1:200) or with 4 β -phorbol 12 β -myristate 13 α -acetate (PMA; 10 ng/ml). Cells were washed with cold PBS (NaCl 8 g/l, KCl 0.2 g/l, Na_2HPO_4 0.9 g/l, KH_2PO_4 0.2 g/l) containing 1 mM sodium orthovanadate. Cells were then lysed in a lysis buffer containing 10 mM Tris/HCl (pH 7.4), 50 mM NaCl, 1%, Nonidet P-40, 0.5% sodium deoxycholate, 5 mM EGTA, 1 mg/ml BSA, 1 mM PMSF, 1000 units/ml aprotinin, 1 $\mu\text{g}/\text{ml}$ of each of antipain, leupeptin, pepstatin, 1 mM sodium orthovanadate, 25 mM NaF, 5 mM *p*-nitrophenyl phosphate and 10 mM sodium pyrophosphate. Lysates were precleared for 1 h at 4 °C with 1% (v/v) fixed *Staphylococcus*

aureus bacteria (Pansorbin cells). Preimmune serum or polyclonal antibodies direct against A-II were added (1:100 dilution) and the mixture was incubated overnight at 4 °C. Immune complexes were then recovered by addition of Protein A–Sepharose for 30 min at 4 °C. Immune complexes were washed three times with lysis buffer and three times with PBS. The phosphoproteins were analysed on 10% or 12.5% polyacrylamide gels in the presence of SDS and visualized by autoradiography. For the time course experiment, bands corresponding to phosphorylated A-II were excised from the gel and the radioactivity was quantified by Čerenkov counting in a scintillation counter.

Two-dimensional phosphoamino acid determination

The phosphoamino acid content of immunoprecipitated A-II was determined as described [45]. Briefly, [^{32}P]A-II was analysed by SDS/PAGE (10% gels) and transferred on to a polyvinylidene difluoride membrane (Immobilon-P) using transfer buffer (192 mM glycine, 25 mM Tris/HCl, pH 8.3, 0.01% SDS and 20% methanol). Radiolabelled proteins were visualized by autoradiography at –80 °C. Bands of interest were then excised, washed, resuspended in 5.7 M HCl and acid hydrolysis was performed at 110 °C for 1 h. Samples were then submitted to high voltage two-dimensional TLC as described [45a] using an HTLE-7000 chromatography apparatus (C.B.S. Scientific Co., Del Mar, CA, U.S.A.). ^{32}P -labelled phosphoamino acids were visualized by autoradiography at –80 °C with intensifying screens. Non-radiolabelled phosphoamino acid standards were visualized with ninhydrin solution (0.25% in acetone).

A-V purification from human placenta and from glutathione transferase (GST)–A-V fusion protein produced in *Escherichia coli*

A-V was purified as previously described [46]. Briefly, annexins were extracted from human placenta using Ca^{2+} precipitation. Each annexin was then separated by anion-exchange chromatography (FPLC system; Mono Q column; Pharmacia). A-V was purified using chromatofocusing chromatography (FPLC system; Mono P column; Pharmacia). A-V purity was confirmed by SDS/PAGE and by Western blot analysis. Protein concentration was determined using a method described by Bradford [47].

The same separation procedure was applied for recovering A-V from the GST–A-V fusion protein product in *E. coli*. Briefly, the GST–A-V fusion protein was cleaved by thrombin and A-V was recovered after passage through a column of glutathione. A-V was subsequently purified using Mono Q and Mono P columns.

In vitro PKC assays

Jurkat cells were washed with PBS and lysed in a buffer containing 20 mM Tris/HCl (pH 7.5), 250 mM sucrose, 10 mM EGTA, 2 mM EDTA, 1 mM PMSF and 20 $\mu\text{g}/\text{ml}$ leupeptin. Homogenates were centrifuged for 20 min at 1500 *g* at 4 °C. Supernatants were ultracentrifuged for 1 h at 200000 *g* at 4 °C. Cytosolic proteins were recovered in the supernatant and used for phosphorylation reactions. Phosphorylation of endogenous proteins was performed in conditions of Ca^{2+} -dependent and Ca^{2+} -independent PKC activation, respectively in the presence or in the absence of Ca^{2+} . The phosphorylation of cytosolic proteins by calcium-independent PKC activity was investigated in a final volume of 300 μl of a reaction mixture containing 100 μl of cytosol from 2.5×10^6 cells, 50 mM glycerol 2-phosphate, 0.3 mM EGTA, 2 mM theophyllin, 10 mM magnesium acetate in 1 mM potassium phosphate buffer (pH 7.4) and a mixture of PLs (26 μg of PS and 0.8 μg of diolein). CaCl_2 (0.2 mM, in excess)

was added when Ca^{2+} /PL-stimulated kinases were studied. Basal activity was measured in the absence of Ca^{2+} and PL. The effects of A-V on PKC activities were studied by adding purified A-V to the reaction. PKC-(19–36) (a peptide corresponding to the pseudosubstrate site of cPKCs, a specific inhibitor of these kinases) was also used in some cases at a final concentration of 100 μM . The reaction was initiated by adding $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ at a concentration of 50 μM and performed for 10 min at 30 °C. The reaction was stopped by adding 150 μl of a 3 $\times$ concentrated sample buffer (190 mM Tris/HCl, pH 6.8, 30 % glycerol, 15 % β -mercaptoethanol, 6 % SDS, 0.03 % Bromophenol Blue) [48]. Phosphoproteins were separated by SDS/PAGE (10 % gels) and visualized by autoradiography.

In some cases, an equivalent volume of lysis buffer (40 mM Tris/HCl, pH 7.4, 300 mM NaCl, 10 mM EDTA, 0.2 % BSA, 2 % Nonidet P-40, 6 mM orthovanadate, 50 mM NaF, 20 mM *p*-nitrophenyl phosphate, 1 mM PMSF, 1000 units/ml aprotinin and 1 $\mu\text{l}/\text{ml}$ of antipain, leupeptin and pepstatin) was added to the reaction. Each sample was then divided in two parts and incubated with either A-II antibodies or a preimmune serum. Incubation was performed for 2 h at 4 °C under agitation. Protein A-Sepharose was then added for 30 min at 4 °C. The immune complexes were separated from the cytosol after centrifugation at 10000 *g* for 10 min and were washed first with lysis buffer (diluted 1:1, v/v) supplemented with 0.1 % SDS, and then with diluted lysis buffer without detergents or BSA. Immune complexes were harvested in sample buffer and subjected to SDS/PAGE analysis.

RESULTS

A-II is phosphorylated *in vivo* in T cells after TcR activation and PMA treatment

In vivo phosphorylation of A-II was tested after TcR activation using anti-CD3 antibodies and after treatment with PMA, a well known activator of PKCs. Cells treated with anti-CD3 (Figure 1, lane a) or PMA (Figure 1, lanes c and e) showed A-II phosphorylation, as compared with control cells (Figure 1, lane b). A-II migrated as a doublet when phosphorylated (Figure

Figure 1 Phosphorylation of A-II in ^{32}P -labelled Jurkat cells

Jurkat cells were metabolically labelled with $^{32}\text{P}_i$ and were then incubated with IoT3 antibodies (lane a) or with PMA (lanes c, d and e), or in the absence of both activators (lane b), for 10 min at 37 °C. Cells were then lysed and immunoprecipitated with preimmune serum (lane d) or with anti-A-II antibodies (lanes a, b, c and e). ^{32}P A-II was then analysed by SDS/PAGE followed by autoradiography at -80 °C. Phosphorylated A-II is indicated by arrows and the positions of molecular mass standards are shown.

Figure 2 Phosphorylation of A-II after T cell stimulation

(a) Time course of A-II phosphorylation induced by anti-CD3 antibodies. The same protocol as in Figure 1 was used, except that cells were stimulated with IoT3 antibodies for the indicated times. After SDS/PAGE, bands corresponding to phosphorylated A-II were excised from the gel and the radioactivity was quantified by Čerenkov counting in a scintillation counter. (b) Two-dimensional phosphoamino acid analysis of phosphorylated A-II. The phosphoamino acid content of immunoprecipitated ^{32}P A-II was analysed at 0, 5 and 30 min after stimulation with anti-CD3 antibodies. The positions of standard phosphoamino acids are indicated as follows: S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine. Exposure time was 15 days at -80 °C with intensifying screens.

1, lanes a and c). Immunoprecipitation of PMA-treated cells with a preimmune serum did not reveal A-II phosphorylation, but additional non-specific radioactive bands were observed (Figure 1, lane d).

A-II phosphorylation after anti-CD3 stimulation reached a maximum after 30 min of incubation and was followed by a return to the basal level after 90 min (Figure 2a). The phosphoamino acid content of A-II after treatment of cells with anti-CD3 revealed that only serine residues were phosphorylated (Figure 2b). The incorporation of ^{32}P into these residues was increased with time (Figure 2b).

A-II is phosphorylated *in vitro* by Ca^{2+} /PL-stimulated kinases

The phosphorylation of A-II by PKCs was then studied in cytosol from Jurkat cells. Immunoprecipitation of cytosolic A-II with two different polyclonal antibodies against A-II (Figure 3, lanes 2 and 3) and with a preimmune serum (Figure 3, lane 1) showed that A-II was not phosphorylated under basal conditions in the absence of Ca^{2+} , diolefin and PS (Figure 3a). A-II was also not phosphorylated after addition of diolefin and PS to activate Ca^{2+} -independent PKCs (Figure 3b). However, when Ca^{2+} was added with diolefin and PS, A-II was phosphorylated (Figure 3c) by Ca^{2+} /PL-stimulated kinases. This phosphorylation was also associated with a doublet of A-II in SDS/PAGE autoradiography as just shown above in *in vivo* experiments.

A-V inhibits the *in vitro* phosphorylation of A-II

The effects of A-V on the *in vitro* phosphorylation of A-II were tested. A-II was phosphorylated in the presence of Ca^{2+} , diolefin

Figure 3 Phosphorylation of A-II by Ca^{2+} /PS-stimulated kinases

Cytosolic proteins were phosphorylated *in vitro* in the absence of Ca^{2+} and PL (a), in the presence of PL (b) or in the presence of Ca^{2+} and PL (c). After 10 min at 30 °C, A-II was immunoprecipitated as described in the Materials and methods section with a preimmune serum (lanes 1) or with two different polyclonal antibodies directed against A-II: A-II Elie (lanes 2) or A-II Cava (lanes 3). Phosphoproteins were analysed by SDS/PAGE, followed by autoradiography at -80 °C. The positions of molecular mass standards are shown and that of phosphorylated A-II is indicated.

Figure 4 Effects of A-V on the phosphorylation of A-II by Ca^{2+} /PS-stimulated kinases

Cytosolic proteins from Jurkat cells were phosphorylated in the presence of Ca^{2+} and PL. In (b) and (c), 1 μM purified placental (b) or recombinant (c) A-V was added. Phosphoproteins were analysed by immunoprecipitation with a preimmune serum as a negative control (lanes 1) or with anti-A-II antibodies (A-II Elie) (lanes 2) and SDS/PAGE before autoradiography. The positions of molecular mass standards are shown and that of A-II is indicated.

and PS (Figure 4a, lane 2) as compared with the control (Figure 4b, lane 1). After addition of placental (Figure 4b) or recombinant (Figure 4c) A-V at 1 μM , the amount of phosphorylated A-II (Figures 4b and 4c, lanes 2) decreased markedly.

Effects of A-V on PKCs

A-V inhibits cPKCs

The effects of A-V on the phosphorylation of substrates other than A-II by Ca^{2+} /PL-stimulated kinases were also tested. Activation of these kinases (Figure 5, lane b) revealed the phosphorylation of a large number of proteins (19–22, 28, 33–42, 50, 55–65 and 80 kDa, and several proteins of more than 95 kDa) as compared with the basal activity (Figure 5, lane a). However, the presence of A-V prevented the phosphorylation of some proteins such as p28 and several proteins between 33 kDa and 40 kDa (Figure 5, lane c). As expected, phosphorylation of A-II was also inhibited. Interestingly, the phosphorylation of a group of proteins around 50–60 kDa was increased by A-V (Figure 5, lane c). The other phosphoproteins were not affected by A-V. Similar results were observed with purified recombinant A-V

Figure 5 Effects of A-V on the phosphorylation of endogenous proteins by Ca^{2+} /PL-stimulated kinases

Cytosolic proteins from Jurkat cells were phosphorylated in the absence (lane a) or in the presence (lanes b and c) of Ca^{2+} and PL, and with (lane c) or without (lanes a and b) 1 μM exogenous purified placental A-V. Phosphorylated proteins were analysed by SDS/PAGE, followed by autoradiography at -80 °C. A-II is indicated on the left side. The positions of molecular mass standards are shown.

Figure 6 Dose-dependent effects of A-V on cPKC activities

Cytosolic proteins from Jurkat cells were phosphorylated in the absence (lane a) or in the presence (lanes b–g) of Ca^{2+} and PL. Purified placental A-V was added at different concentrations (0.1, 0.2, 0.5 and 1 μM in lanes c, d, e and f respectively). The inhibitor peptide cPKCs [PKC-(19–36)] was added in lane g at a final concentration of 20 μM . Phosphoproteins were analysed by SDS/PAGE followed by autoradiography at -80 °C. The positions of molecular mass standards are shown. Arrowheads on the left side indicate the phosphoproteins affected by A-V.

(results not shown). In conclusion, these data show that A-V had mainly inhibitory effects (for several, but not all, substrates) on kinases, such as cPKCs, activated by Ca^{2+} and PL.

The effects of A-V on the pattern of protein phosphorylation in the presence of Ca^{2+} and PL occurred in a dose-dependent manner (Figure 6; 0, 0.1, 0.2, 0.5 and 1 μM respectively in lanes b, c, d, e and f). The inhibitory effect of A-V was compared with that of a specific inhibitor of cPKCs, PKC-(19–36). This peptide inhibited the phosphorylation of all the proteins (including A-II) that occurred in the presence of both Ca^{2+} and PL (Figure 6, lane

Figure 7 Effects of A-V on the phosphorylation of endogenous proteins induced by Ca^{2+} -independent and PL-dependent kinases

Cytosolic proteins from Jurkat cells were phosphorylated in the absence (lane a) or in the presence (lanes b and c) of PL, and with (lane c) or without (lanes a and b) $1 \mu\text{M}$ purified placental A-V. Phosphoproteins were analysed by SDS/PAGE followed by autoradiography at -80°C . The positions of molecular mass standards are shown.

g). However, the protein phosphorylation which was inhibited by A-V was also affected by PKC-(19–36), demonstrating that A-V was an inhibitor of cPKCs. As shown in Figure 5 (lane c), A-V stimulated phosphorylation of proteins in the 50–60 kDa range in a dose-dependent manner (Figure 6).

A-V has no effect on PL-stimulated kinases such as aPKCs and nPKCs.

The effects of A-V on the pattern of protein phosphorylation induced by kinases activated only by dioleoin and PS were tested. Under these conditions, several cytosolic proteins were phosphorylated (17–20, 50, 55, 60, 80 and 110 kDa) (Figure 7, lane b) compared with basal conditions (Figure 7, lane a). When purified A-V was added, the same pattern of phosphoproteins was observed (Figure 7, lane c) as that observed in the absence of A-V (Figure 7, lane b). In addition, exogenous A-V was not phosphorylated under these conditions (Figure 7, lane c). In conclusion, these data showed that A-V had no effect on the activity of kinases activated by dioleoin and PS, such as nPKCs and aPKCs. Similar results were observed with recombinant purified A-V (results not shown).

DISCUSSION

Activation of protein kinases represents one of the earliest intracellular events following receptor-mediated activation of T lymphocytes. Activation of tyrosine kinases and PKCs has been shown to occur rapidly after T cell receptor stimulation. To understand how signal transduction occurs during T cell activation, it is necessary to identify the key regulatory molecules whose functions are influenced by phosphorylation.

In this paper, the possible role of A-II and A-V in T lymphocyte signal transduction has been investigated by studying their phosphorylation states after T cell activation. A-II and A-V are members of the annexin family of Ca^{2+} -binding proteins. Annexins have the property of binding PL in a Ca^{2+} -dependent manner. The exact role of annexins has not yet been elucidated, although different annexins have been shown to be involved in signal transduction during proliferation and/or differentiation processes.

We have shown the phosphorylation of A-II in Jurkat cells after stimulation of the TcR by anti-CD3 IoT3 antibody or after

PMA treatment. Among A-I, A-II and A-VI, only A-II was shown to be phosphorylated after cell stimulation (T. Dubois, J.-P. Oudinet, F. Russo-Marie and B. Rothhut, unpublished work). Time course data revealed that maximum phosphorylation was obtained after 30 min of stimulation with IoT3 and had returned to its basal level after 90 min; only serine residues were phosphorylated. It is well documented that PKCs are activated in T cells during the stimulation of the TcR by monoclonal antibodies [49,50]. Moreover, A-II was phosphorylated after stimulation by PMA, a phorbol ester that directly activates PKCs. These results suggested that A-II was probably phosphorylated by PKCs after TcR activation in Jurkat cells, and could play an important role in signal transduction in lymphocytes. However, the physiological relevance of this A-II phosphorylation in T cells is still unknown.

Phosphorylation of A-I and A-II by PKCs had been shown to decrease their lipid-binding characteristics [51–53]. Thus, when phosphorylated, A-II might have a modified physiological function dependent on its binding to PL. This phosphorylation could, for example, prevent inhibition of phospholipase A_2 as described for A-I [54]. On the other hand, A-II is known to be tightly associated with membranes at physiological concentrations of Ca^{2+} (in the micromolar range), and has been shown to play a role in exocytosis [55] and endocytosis [56,57]. A-II causes phospholipid vesicles and chromaffin granules to aggregate and fuse, and could be involved in the interactions of chromaffin granules with the plasma membrane, a process dependent on phosphorylation via PKCs. It is possible that A-II is involved in membrane trafficking by promoting membrane–membrane interactions. Therefore A-II could be implicated in the endocytosis and/or exocytosis of some components during T cell activation.

A-II was initially described as a substrate for the tyrosine kinase pp60^{v-src} [31,32]. In T cells, TcR stimulation induced an early activation of protein tyrosine kinases. However, we could not find any phosphorylation on tyrosine residues even at 1 min after TcR activation (results not shown). One explanation is that serine and tyrosine phosphorylation events appeared to be mutually exclusive in *in vitro* experiments, i.e. A-II phosphorylated on tyrosine was not phosphorylated on serine residues by PKCs, and vice versa [33].

As our *in vivo* results suggested strongly that A-II could be a substrate for PKCs in Jurkat cells, we then tested the ability of A-II to be phosphorylated by PKCs in cytosol from these cells. We showed that A-II was phosphorylated *in vitro* in the presence of both Ca^{2+} and PL, but not with PL alone. Indeed, A-II was not phosphorylated by Ca^{2+} -independent PKCs (nPKCs, aPKCs). However, as A-II was phosphorylated by Ca^{2+} /PL-stimulated kinases, and as the pattern of phosphoproteins was abolished in the presence of a specific inhibitor of cPKCs [PKC-(19–36)], we concluded that A-II was a substrate for cPKCs *in vitro* in cytosol from Jurkat cells and thus may be a substrate for one or more of the cPKCs found in Jurkat cells, i.e. PKC- α , PKC- β_1 and PKC- β_{II} .

Phosphorylated A-II migrated on SDS/PAGE as a doublet in 10% polyacrylamide gels in both *in vitro* and *in vivo* experiments. This phenomenon has also been described by others [33,36,58], and could be explained by the presence of several phosphorylated sites in the N-terminal domain of A-II. Indeed, serine-25 has been identified as the major PKC-dependent phosphorylation site [33], but additional minor sites could be present within the p11 binding site [58]. Therefore the slower-migrating species could represent the heterotetrameric form of A-II phosphorylated only on serine-25. On the other hand, the upper band could be the monomeric form of A-II phosphorylated both on serine-25 and on the minor sites. These latter sites have been shown to

inhibit the re-formation of the heterotetrameric form of A-II [58].

We then tested the role of A-V as an inhibitor of PKCs in Jurkat cells. The presence of proteins with PKC inhibitory activities has been demonstrated in different cell types [59]. In Jurkat cells, such an inhibitory activity for PKCs has been detected in the cytosol [60]. However, the nature of this inhibitor has not been identified. We have investigated the effect of purified A-V on PKC activities in cytosol from Jurkat cells. A-V had no effect on PL-dependent and Ca^{2+} -independent kinases such as nPKCs and aPKCs. In contrast, A-V inhibited the phosphorylation of several proteins, including A-II, by Ca^{2+} - and PL-dependent kinases. The phosphorylation of all of these proteins was also inhibited by PKC-(19–36), a specific inhibitor for cPKCs, demonstrating that A-V is indeed an inhibitor for cPKCs. However, the patterns of inhibition by A-V and PKC-(19–36) were not similar; the latter inhibited all cPKCs, whereas A-V might have an inhibitory effect on only one of the three isoforms of cPKC (α , β_1 and β_{11}) expressed in Jurkat cells. A-V may inhibit cPKCs by direct interaction between A-V and PKCs, as proposed by some authors [38]. Alternatively, A-V could bind to negatively charged PLs, masking the accessibility to PKCs [40].

Surprisingly, in addition to having an inhibitory effect on cPKCs, A-V was shown to activate the phosphorylation of a group of proteins around 50–60 kDa. The possibility that A-V inhibits a specific phosphatase activity could account for this observation.

We proposed as a hypothesis that A-II might translocate to the plasma membrane and be phosphorylated by cPKCs within 30 min after TcR stimulation. A-II has a higher affinity for Ca^{2+} -dependent binding to PLs than does A-V (in the micromolar range for A-II and about 10–50 fold lower for A-V). Thus, due to its lower affinity for PL binding compared with A-II, A-V might translocate to the plasma membrane later, inhibiting cPKCs at this time.

In conclusion, A-II was phosphorylated on serine residues after T cell activation with anti-CD3 antibodies as well as after PMA treatment; it was also phosphorylated *in vitro* by cPKCs. These results suggest that A-II could be a substrate for these kinases in T cells. On the other hand, A-V inhibited the cPKC-dependent phosphorylation of several substrates, including A-II. Therefore A-II could play a role, which has yet to be unravelled, in TcR/CD3 signal transduction through the PKC pathway in Jurkat cells. A-V, on the other hand, could act as a potent regulator of cPKCs in these cells.

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