0022-1767/90/1442-0647$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 144, 647–652, No. 2, January 15, 1990
Printed in U.S.A.

STIMULATION VIA CD3-Ti BUT NOT CD2 INDUCES RAPID TYROSINE PHOSPHORYLATION OF A 68-kDa PROTEIN IN THE HUMAN JURKAT T CELL LINE[1]

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Tyrosine phosphorylation is an early biochemical event associated with surface receptor triggering in many cellular systems. In T lymphocytes, Ag receptor (CD3-Ti) stimulation results in tyrosine phosphorylation of the CD3 ζ subunit. The tyrosine kinase responsible for this modification after CD3-Ti triggering has not been identified. Here we reported that a 68-kDa T cell membrane-associated protein (pp68) in human Jurkat T cells is phosphorylated on tyrosine residues within 1 min after anti-CD3 mAb addition. This induced tyrosine phosphorylation is detected either by in vivo [^{32}P]orthophosphate labeling of the Jurkat T cells or by in vitro [^{32}P]ATP labeling after immunoprecipitation by antiphosphotyrosine antibody. In contrast, mAb stimulation via CD2 and CD4 structures does not induce phosphorylation of pp68. These data are among the first to provide evidence that CD3-Ti and CD2 activation pathways are distinct. Furthermore, they imply that pp68 is itself a tyrosine kinase and/or is a rapidly phosphorylated substrate of a tyrosine kinase.

Activation of a given T lymphocyte is mediated by its Ag-specific, MHC-restricted TCR, which is composed of a clonotypic disulfide-linked α/β heterodimer (Ti) that is noncovalently associated with the invariant CD3 γ, δ, ϵ, ζ, and η subunits (for review see References 1, 2, and 3 to 5). In addition, human T lymphocytes can be activated through CD2 (T11) (6). Triggering of either set of structures by mAb or, in the case of CD3-Ti, by Ag and MHC, initiates a cascade of biochemical events consisting of phosphatidylinositol hydrolysis, increased intracellular-free calcium concentration, alterations in cellular pH, membrane translocation of proteins including kinase C, and stimulation of a variety of protein kinases (reviewed in References 1 and 2). These early signals are followed by activation of the genes for IL-2 and the IL-2R (7). The latter appears to be essential for subsequent T cell proliferation.

With regard to protein kinases, several studies have provided evidence for a role of protein kinase C in T cell activation and proliferation (8–11). Furthermore, one consequence of lymphocyte activation is the phosphorylation of CD3γ-chain on serine residues catalyzed by kinase C (12). More recently, it has been shown that TCR-mediated lymphocyte activation also stimulates a tyrosine-specific kinase activity that leads to tyrosine phosphorylation of CD3 ζ-chain and several other cellular substrates (13). Both kinase C and protein tyrosine kinase activities are, in turn, attenuated by intracellular c-AMP elevation (14). Such an interaction among these biochemical signals can block T cell activation as well as the associated phosphorylation of components of CD3-Ti complex (15).

The identity of the protein tyrosine kinase stimulated via the CD3-Ti receptor in T lymphocytes has not yet been defined. However, given the 1° structure of Ti α and β and CD3 γ, δ, ϵ, and ζ subunits deduced from their corresponding cDNA clones, it is apparent that a tyrosine kinase domain does not reside in the cytoplasmic region of any of these subunits. To begin to characterize the tyrosine-specific protein phosphorylation events triggered by CD3-Ti and CD2 stimulation, we have utilized an antiphosphotyrosine antibody in conjunction with in vitro and in vivo phosphorylation assays. Our results identify a 68-kDa protein (pp68) in the cellular membrane fraction, which is phosphorylated on tyrosine residues only after TCR stimulation. Given the rapidity with which this covalent modification occurs on pp68, we suggest that pp68 is itself a tyrosine kinase and/or an early substrate in the activation pathway via the TCR.

MATERIALS AND METHODS

Cells and antibodies. Variants of Jurkat clone 77-6.8 (kindly provided by Dr. K. Smith, Dartmouth Medical School, Hanover, NH), lacking CD3-Ti (31-13) were derived by mutagenesis, immunoselection and cell sorting as previously described (16). To obtain the reconstituted CD3-Ti$^+$ T cell clone (WT-6), the full-length Ti β cDNA from Jurkat was inserted into a derivative (pINK-2) of pT β-neo vector and transfected into 31-13 via protoplast fusion (17). mAb anti-T3 (IgM isotype; 2Ad2), anti-T11$_2$, and anti-T11$_3$ were produced and characterized as described elsewhere (6, 18). Antiphosphotyrosine antibody was prepared in rabbits by injection of *N*-bromoacetyl phosphotyramine conjugated to KLH. The serum was purified on a phosphotyramine-Sepharose column (19).

Cell stimulation and tyrosine phosphorylation analysis. Human Jurkat T cell was cultured in RPMI 1640 media (GIBCO, Grand Island, NY) with 10% FCS. A total of 10^7 Jurkat cells was washed twice by 20 ml of Dulbecco's PBS, resuspended in 1 ml of PBS, and prewarmed to 37°C. For stimulation, antibodies (1/200 dilution of ascites) and PMA (10 ng/ml) were added to the individual samples

Received for publication August 23, 1989.
Accepted for publication October 12, 1989.
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[1] This work was supported in part by NIH Grants AI19807 and AI21226. Y.-J. J. was supported by a postdoctoral fellowship from Cancer Research Institute.

and incubated at 37°C for 3 min. Subsequently, the sample was spun in a microfuge for 15 s to stop the stimulation, the supernatant removed, the cell pellet washed once with 1 ml of PBS and then once with 1 ml of washing buffer (150 mM NaCl, 20 mM Tris, pH 8). Next, the cells were lysed in 1 ml of lysis buffer (1% NP-40, 10% glycerol, 150 mM NaCl, 20 mM Tris (pH 8.0), 400 μM Orthovanadate, 1 mM PMSF, and 10 μg/ml aprotinin) at 4°C for 20 min with rotation. After microfuging at 4°C for 20 min, the cellular debris was removed and 2 μl (0.3 μg/ml) of affinity-purified polyclonal antiphosphotyrosine antibody was added to the 1-ml lysate, which was rotated at 4°C for 4 h. Then 10 μl of Sepharose-protein A beads (Pharmacia, Piscataway, NJ) were added and rotated for another 2 h. Subsequently, the protein A beads were washed once with solution A (0.5% NP-40 in PBS), twice with solution B (0.5 M LiCl, 0.1 M Tris (pH 7.4), and once with kinase buffer (10 mM $MgCl_2$, 20 mM Tris (pH 7.4). For the in vitro labeling, 2 μl of [γ-^{32}P]ATP (20 μCi) in 20 μl of kinase buffer were added into each sample and incubated at 25°C for 25 min with frequent shaking. After a brief spin (15 s in the microfuge), the supernatant was removed, the reaction was stopped by adding 30 μl of SDS-reducing sample buffer to the pelleted beads, and heated at 100°C for 5 min. The aqueous eluate was applied to 10% SDS-PAGE.

For in vitro labeling by [^{32}P]orthophosphate, 4×10^7 Jurkat T cells were washed twice by 10 ml of phosphate-free and serum-free RPMI 1640 media, then incubated for 3 h with 10 ml of phosphate-free RPMI 1640 medium containing 2% FCS and carrier-free [^{32}P]orthophosphate (0.5 mCi/ml). Thereafter, the cells were washed, stimulated, lysed, and immunoprecipitated as described above for the in vitro labeling. After immunoprecipitation, the protein A beads were directly added to the reducing SDS sample buffer, heated, and run in 10% SDS gel. After drying, the gel was autoradiographed.

Phosphoamino acid analysis. The in vitro labeled protein bands were excised from the gel according to the position of radiolabeled bands in the corresponding autoradiograph. Digestion of gel slices with trypsin and acid hydrolysis of phosphopeptides was carried out by a modification of the method of Hunter and Sefton (20), then separated by cellulose thin layer electrophoresis. The phosphoamino acid pattern was established by parallel analysis of standard samples.

Two dimensional gel electrophoresis (IEF/SDS-PAGE). The in vitro and in vivo labeled samples were prepared as described above. The proteins were dissociated from the beads by IEF sample buffer (9.8 M urea, 2% NP-40, 2% ampholyte (pH 7.9), 2% mercaptoethanol). The IEF dimension was run according to O'Farrell et al. (21) in a mini-tube gel (Bio-Rad) of 4% acrylamide and 2% NP-40 at 500 V for 10 min, then 1000 V for 2 h. The IEF gels were loaded on the top of the 10% mini-slab gel and run at 500 V for 30 min. pH gradient determinants were performed according to the procedure by O'Farrell.

Immunoprecipitation of fractionated cells. After washing by PBS, 100×10^6 J77 cells resting or anti-CD3 stimulated were homogenized in 2 ml solution of 10 mM $NaPO_4$, 1 mM EDTA, pH 7.4, in a Dounce homogenizer with 20 strokes. After removal of debris by centrifugation at 800 × *g* for 10 min, the crude membrane fraction was separated from the cytoplasmic fraction by centrifugation at 40,000 × g for 40 min. Then 0.5 ml of 5 × NP-40 lysis buffer was added to the cytoplasmic fraction and 2.5 ml of NP-40 lysis buffer was added to the membrane fraction. After 2-h rotation, all samples were centrifuged at 15,000 × *g* for 15 min. One milliliter of each supernatant was immunoprecipitated by antiphosphotyrosine antibody, in vitro labeled, and analyzed in SDS-PAGE.

RESULTS

The Jurkat 77 (J77) cell was selected for the present analysis because it represents a mature clonal T cell population expressing CD3-Ti and CD2 and can be stimulated by anti-CD3 and anti-CD2 mAb to produce IL-2 (16). To ascertain whether tyrosine phosphorylation is induced on proteins after CD3-Ti stimulation, Jurkat cells were stimulated by anti-CD3 mAb, lysed in 1% NP-40 solution, and after centrifugation, the soluble lysate was subjected to immunoprecipitations by affinity-purified antiphosphotyrosine antibody. Subsequently, in vitro phosphorylation was performed and labeled substrates were analyzed by SDS-PAGE. Autoradiograms of in vitro [^{32}P]ATP-labeled (Fig. 1*A*) samples reproducibly identified a band representing a 68-kDa protein, pp68, that is induced after T cell stimulation via the CD3-Ti complex but detected minimally or not at all in the uninduced state. The stimulation of pp68 phosphorylation is mediated only by anti-CD3 antibodies (*lane 4*) and not enhanced by PMA (*lane 5*). Similar induction was observed with anti-CD3 antibodies of both IgM (2Ad2) and IgG (RW28C8) isotypes. In contrast, the combination of two anti-CD2 antibodies, anti-$T11_2$ + anti-$T11_3$ mAb (*lane 3*), which is known to initiate IL-2 gene expression in T cells and Jurkat (16), did not induce the phosphorylation of this 68-kDa protein. Parallel stimulation of Jurkat cells with anti-CD3 + PMA or the combination of anti-$T11_2$ + anti-$T11_3$ resulted in both calcium flux and IL-2 production. Note the constitutive presence of ^{32}P-labeled bands at 56 and 60 kDa (*lane 2*).

To identify the amino acid residues of pp68 modified by phosphorylation, the gel band containing the phosphorylated product was excised and its phosphoamino acid composition determined. As shown in Figure 1*B*, >70% of the phosphate was linked to tyrosine residues. Nevertheless, ~30% of the phosphate was associated with serine or threonine phosphorylation, which could have been caused by an additional serine-threonine kinase activity in the lysate.

To further examine the phosphorylation of the 68-kDa band, Jurkat T cells were in vivo labeled with [^{32}P]orthophosphate, stimulated with specific anti-CD3 or anti-CD2 mAb, lysed and immunoprecipitated by antiphosphotyrosine heteroantisera. The autoradiogram of the SDS-PAGE analysis of the immunoprecipitate is shown in Figure 2. For comparison, in vitro labeled samples stimulated by anti-CD3 antibody were run in parallel (*lane 1*). After anti-CD3 stimulation and *in vivo* labeling, a 68-kDa band (*lane 3*) similar in size to that following in vitro labeling (lane 1) was observed. No increase in the 68-kDa phosphorylated band was detected with stimulation of cells by either anti-CD4 (*lane 4*) or anti-$T11_2$ + anti-$T11_3$ (*lane 5*). Taken together, both in vitro and in vivo experiments demonstrate that activation via the CD3-Ti complex and CD2 structures are not identical and that the pp68 phosphorylation is linked to CD3-Ti rather than to CD2 and CD4. As such, these data provide among the first direct evidence for differences in CD3-Ti and CD2 pathways of T cell stimulation.

Multiple experiments (Figs. 1*A*, 2, 3, and 4) comparing the intensity of the 68-kDa band in induced vs uninduced Jurkat showed that there was a 5 to 10-fold increase in phosphorylation as determined by densitometry scanning of the autoradiograph. To determine the time interval between TCR triggering and subsequent phosphorylation of pp68, a series of kinetic studies was performed. To this end, Jurkat cells were incubated with anti-CD3 antibody for varying times at 37°C before washing, then lysed, and in vitro phosphorylation of anti-P-Tyr immunoprecipitates performed. The autoradiographic and densitometry scanning results are shown in Figure 3. As indicated, phosphorylation of pp68 was detected within 20 s, reached a maximum at 1 to 3 min and decreased thereafter. Thus, the covalent modification of pp68 is extremely rapid, consistent with the temporal appearance of other rapidly occurring biologic responses such as Ca^{2+} flux, inositol turnover, etc. in T cells upon CD3-Ti stimulation (1). In contrast, Figure 3*B* demonstrates the absence of anti-$T11_2$ + anti-$T11_3$-induced pp68 phosphorylation at any time point tested (1 min to 30 min).

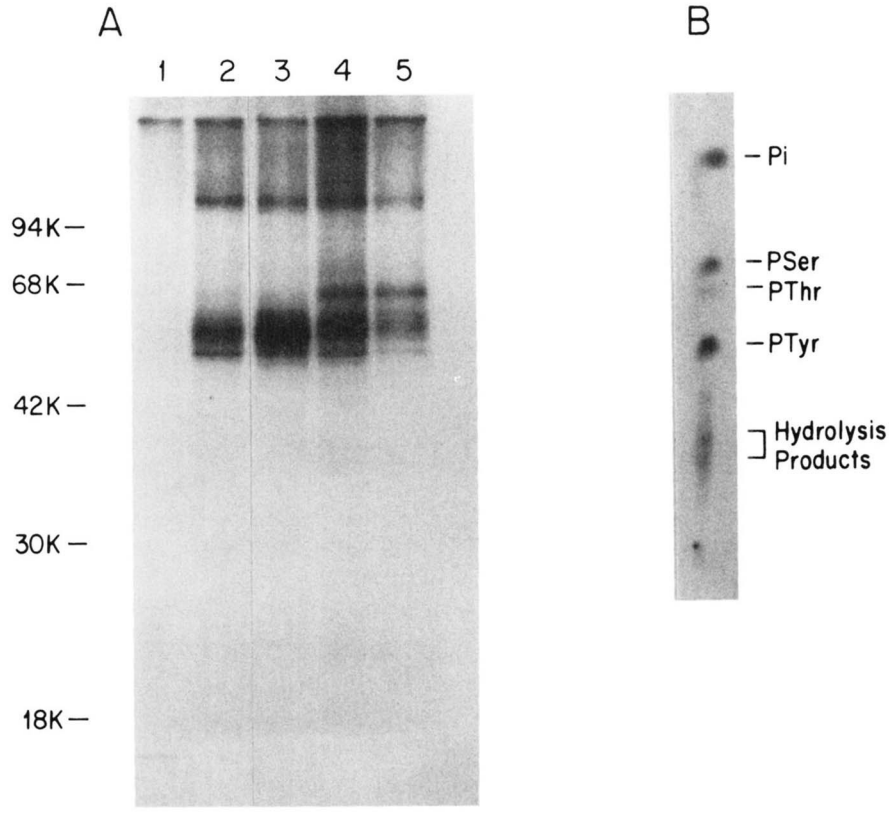

Figure 1. *A*, SDS-PAGE analysis of anti-CD3-induced tyrosine phosphorylation of pp68 from human Jurkat T cells labeled in vitro. *Lane 1*, negative control; no antiphosphotyrosine antibody was added to the lysate. All other lanes contain material precipitated by antiphosphotyrosine antibody. *Lane 2*, negative control; no stimulating antibody was added. *Lane 3*, anti-T11$_2$ + anti-T11$_3$ mAb was added to the cell suspension for stimulation. *Lane 4*, cells were stimulated by anti-CD3 antibody. *Lane 5*, cells were stimulated by anti-CD3 antibody + PMA. Molecular weight (×1000) markers are: 94K, phosphorylase B; 68K, BSA; 42K, OVA; 30K, carbonic anhydrase; 18K, lactoglobulin A. *B*, phosphoramino acid analysis of in vitro labeled pp68.

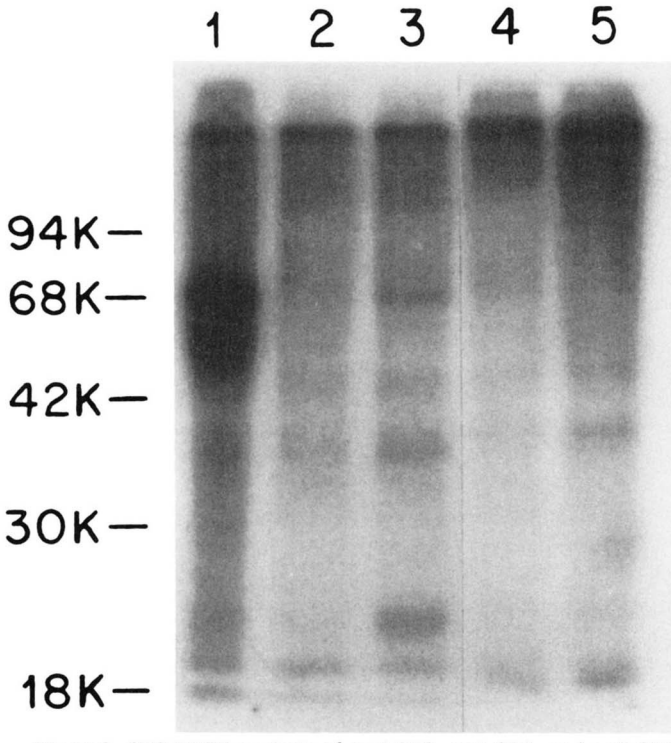

Figure 2. SDS-PAGE analysis of anti-CD3, anti-CD4, and anti-CD2 antibody-induced tyrosine phosphorylation of pp68 in vivo labeled from human Jurkat T cells. *Lane 1*, sample stimulated by anti-CD3, labeled in vitro for comparison; *lanes 2* to *5*, labeled in vivo; *lane 2*, negative control, no antibody stimulation; *lane 3*, stimulated by anti-CD3 antibody; *lane 4*, stimulated by anti-CD4 antibody; and *lane 5*, stimulated by anti-T11$_2$ + T11$_3$ antibody.

The presence of CD3-Ti receptor on the T cell surface is absolutely required to induce the phosphorylation of the pp68. As shown in Figure 4, when the Jurkat mutant 31-13, which lacks surface CD3-Ti as a consequence of a defective Ti β gene (16), is stimulated by anti-CD3 mAb, the basal phosphorylation of the pp68 is not affected (*lane 3*). In contrast, in the 31-13 Jurkat cell line reconstituted with the full length Ti β subunit (termed WT-6) and therefore expressing TCR (CD3-Ti$^+$), pp68 phosphorylation is induced (*lane 6*). Clearly, the binding of specific antibody to the surface of Jurkat cells and cross-linking of the CD3-Ti structures triggers the phosphorylation of pp68, thus ruling out any nonspecific artifacts resulting from the anti-CD3 antibody or the in vitro phosphorylation system.

To determine the cellular distribution of the pp68, the J77 cells were fractionated into cytoplasm and membrane components by centrifugation. The immunoprecipitates of antiphosphotyrosine antibody analyzed in SDS-PAGE show that the membrane fraction contains the pp68 protein (Fig. 5). By way of contrast, pp68 was not detected in surface radioiodinated cells by the lactoperoxidase (data not shown), indicating that the protein does not possess tyrosine residues that are exposed on the cell surface. Thus, this pp68 protein may be located on the cytoplasmic face of the plasma membrane as a consequence of a post-translational modification and/or associated with another structure.

To more precisely characterize the pp68 protein, two-dimensional gel electrophoresis in an O'Farrell system (21) was performed. As shown in Figure 6, both in vivo and in vitro labeled pp68 appears as a defined spot at an isoelectric point of 6.0 with some diffuse radiolabeled bands on both sides, probably indicating multiple sites of phosphorylation in the protein. Consistent with the 1-D SDS-PAGE analysis described above, Ag receptor activation via the CD3 molecules (*panel B*) resulted in a marked increase in intensity of the pp68 phosphorylated spot relative to the nonstimulated cells (*panel A*). That

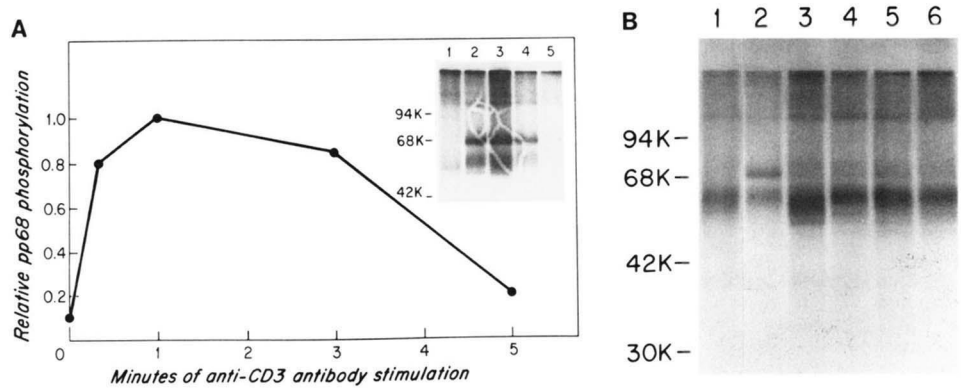

Figure 3. *A*, Time course of tyrosine phosphorylation of pp68 stimulated by anti-CD3 antibody. A total of 10^7 Jurkat T cells was stimulated by anti-CD3 antibody for different times at 37°C and in vitro labeled. The relative phosphorylation level was obtained from gel scanning (by LKB ultroscan XL Laser Densitometer). The inserted panel shows the SDS gel scanned for this analysis. *Lane 1*, no stimulation; *lane 2*, stimulated for ⅓ min; *lane 3*, stimulated for 1 min; *lane 4*, stimulated for 3 min; and *lane 5*, stimulated for 5 min. *B*, Time course of tyrosine phosphorylation of pp68 stimulated by anti-T11$_2$ + anti-T11$_3$ antibody. *Lane 1*, no stimulation; *lane 2*, stimulated by CD3 for 3 min; *lanes 3* to *6*, stimulated by anti-T11$_2$ + anti-T11$_3$; *lane 3*, 1 min; *lane 4*, 5 min; *lane 5*, 15 min; *lane 6*, 30 min.

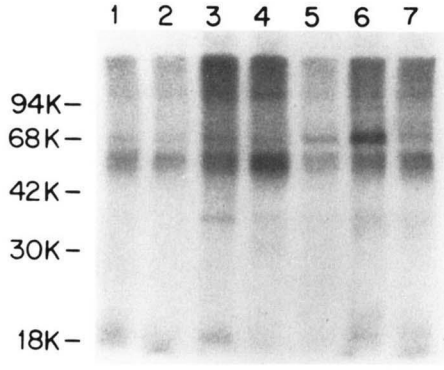

Figure 4. Tyrosine phosphorylation of pp68 in CD3$^-$ Jurkat mutant and CD3$^+$ reconstituted Jurkat T cell line stimulated by anti-CD3 antibody. *Lanes 1* to *4* show CD3$^-$ mutant T cells and lanes 5 to 7 show reconstituted Jurkat T cells. *Lanes 1* and *5*, no stimulation; *lanes 2* and *6*, stimulated by anti-CD3 (IgM isotype); *lane 3*, stimulated by anti-CD3 (IgG isotype); *lanes 4* and *7*, stimulated by anti-T11$_2$ + anti-T11$_3$.

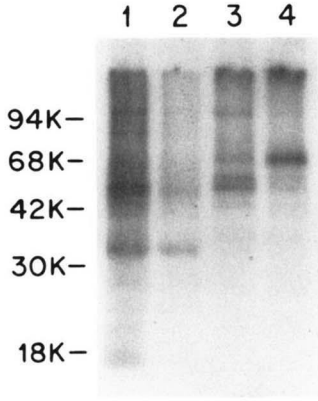

Figure 5. Cellular distribution of pp68 in human Jurkat T cells. *Lanes 1* and *2*, cytoplasmic fraction; *lanes 3* and *4*, crude membrane fraction; *lanes 1* and *3*, no stimulation; *lanes 2* and *4*, stimulated by anti-CD3 antibody. In vitro labeling was as performed in Figure 1.

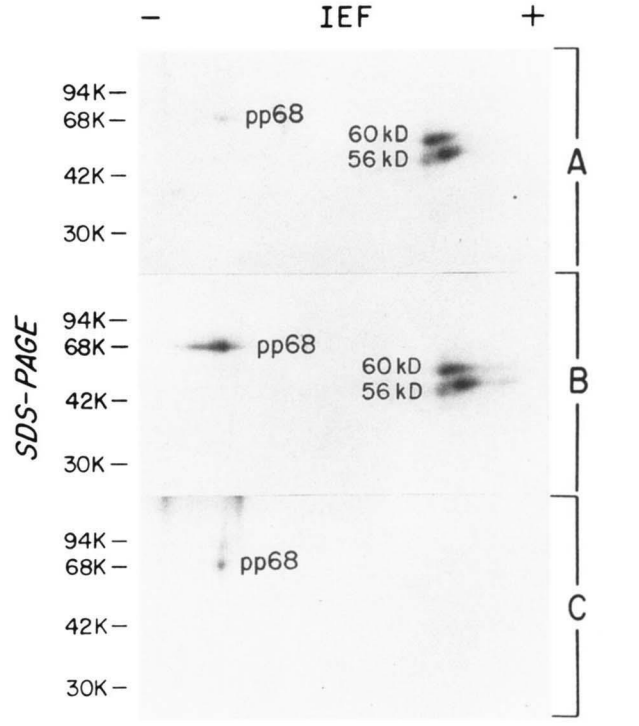

Figure 6. Two-dimensional gel electrophoresis (IEF/SDS-PAGE) of tyrosine phosphorylation of pp68 in Jurkat T cells. *A*, in vitro labeled without stimulation; *B*, in vitro labeled after stimulation by anti-CD3 antibody; *C*, in vivo labeled after stimulation by anti-CD3 antibody.

there is only one spot migrating into the 68-kDa region strongly suggests that the 68-kDa band previously seen in one-dimensional SDS-PAGE analysis is comprised of one major polypeptide (Figs. 1 and 3 to 5). Note that the two other major spots, pp60 and pp56, correspond to the 60-kDa band and the 56-kDa band in one dimensional SDS mini-gel separation (Figs. 4 and 5), which are likely to represent the known tyrosine kinase Lck in different phosphorylation states (22, 23). Perhaps more important, in vivo labeling (*panel C*) defines an identical isoelectric point for the pp68.

DISCUSSION

Our results demonstrated that a 68-kDa T cell membrane-associated protein (pp68) is phosphorylated on tyrosine and, to a lesser extent, serine and threonine residues after TCR cross-linking by anti-CD3 mAb in Jurkat cells. SDS-PAGE, IEF/SDS-PAGE, and analysis of Jurkat and CD3 variants all indicated that this induced tyrosine phosphorylation of pp68 is reproducible, rapid, and specific. This covalent modification is detected by both in vitro and in vivo labeling studies and may represent a biochemical signal in the regulation of T cell activation. Furthermore, these findings suggest that tyrosine phosphorylation events may be important in early signaling of T cell activation.

Human T lymphocytes are activated through either the antigen/MHC receptor complex (CD3-Ti) or the CD2 molecules to proliferate via an IL-2-dependent mechanism. The previous work from this laboratory indicated that these two activation pathways are interdependent and

the stimulation via CD3-Ti complex or CD2 is indistinguishable in terms of phosphoinositide turnover, calcium mobilization, and IL-2 production (16, 24). Indeed, CD2 stimulation requires CD3-Ti expression for the latter set of events to occur. Here we report that the tyrosine phosphorylation of a 68-kDa protein in the Jurkat T cell line can be activated via the CD3-Ti pathway but not the CD2 pathway. This observation is consistent with that of Weissman et al. (25) showing that anti-CD3, but not anti-CD2, activates a tyrosine-specific kinase to phosphorylate CD3-ζ and implying that both pp68 and CD3 proteins are substrates for the same kinase. These data together show that tyrosine phosphorylation is linked to the CD3 pathway. Similar observations appear to be the case for IL-2-dependent T cell clones (data not shown). Given that CD2 stimulation can result in IL-2 production, it would appear that the IL-2 may be activated in the absence of detectable tyrosine phosphorylation on pp68. One cannot exclude the possibility that the techniques used to demonstrate phosphotyrosine accumulation are insensitive.

Recent murine studies (26) have suggested that two TCR isoforms (CD3-Ti ζ-ζ and CD3-Ti ζ-η) accounting for 90% and 10% of TCR, respectively, exist on the surface of an individual T lymphocyte. The CD3-Ti ζ-ζ form is probably linked to a tyrosine kinase because mutant cells lacking η-chain can still be triggered via their TCR to activate a tyrosine kinase (27). In contrast, the TCR of these η mutant cells fail to link to phospholipase C, implying that the TCR-ζ-η isoform is associated with phospholipase C. One possibility is that CD2 is associated with the CD3-Ti ζ-η isoform and not the CD3-Ti ζ-ζ isoform.

Recently, it was reported that the src family member tyrosine specific kinase, lck, known to be expressed in T lymphocytes is associated with T cell surface CD4 and CD8 structures (28, 29). T cell activation via a variety of signals induced conversion of $p56^{lck}$ to a modified phosphorylated form termed $p60^{lck}$ (22, 23). Furthermore, the cross-linking of CD4 results in a rapid (within 30 s) and significant (three- to fivefold) increase in the tyrosine-specific protein kinase activity of $p56^{lck}$ (30). The p21 ζ-chain of CD3 is phosphorylated on tyrosine residues within minutes after lymphocyte stimulation (15). Because we observed that pp68 was rapidly (within 20 s) phosphorylated on tyrosine residues upon CD3-Ti stimulation after both in vivo and in vitro labeling, it may itself be a tyrosine kinase. Consistent with this possibility is the fact that tyrosine kinases are known to undergo autophosphorylation. The coprecipitation of pp68 with the 56-kDa and 60 kDa in vitro labeled phosphoproteins, in the antiphosphotyrosine immunoprecipitates, presumed to be lck isoforms, raises the possibility that pp68 might be a cellular substrate of lck such that, upon activation, increased tyrosine-specific kinase activity of lck catalyzes the tyrosine phosphorylation of pp68.

It will remain to be determined whether pp68 is a kinase or alternatively, a substrate of a kinase related or unrelated to lck. pp68 apparently is not the 68-kDa substrate found phosphorylated on threonine residues after IL-2 or TPA stimulation in lymphocytes (31). A 68-kDa Ca^{2+} binding protein whose 1° structure has been deduced by cDNA cloning was shown to be a substrate of protein tyrosine kinase (32). However, in an immunoblotting assay, our pp68 cannot be detected by antibody raised against 68-kDa Ca^{2+} binding protein (data not shown). Furthermore, unlike the former, pp68 is not a major protein component linked to the plasma membrane because silver staining of 2D gels of this fraction did not identify a 68-kDa protein with the same isoelectric point (data not shown). In addition, immunoblotting with specific anti-rat mAb proves that pp68 is distinct. Finally, a 72-kDa protein tyrosine kinase present in murine splenocytes and thymocytes may be related to the human 68-kDa protein described herein (33). In this respect, a weak 68-kDa radioactive band has been observed in in vitro labeling studies with human thymocytes, peripheral blood lymphocytes, and several IL-2 dependent T cell clones after anti-CD3 stimulation (data not shown). However, further biochemical and molecular characterization is now necessary to understand the physiologic function of pp68 in T cell activation, and the significance of tyrosine phosphorylation in TCR signal transduction.

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