Regulation of Phosphoinositide Kinases in T Cells

EVIDENCE THAT PHOSPHATIDYLINOSITOL 3-KINASE IS NOT A SUBSTRATE FOR T CELL ANTIGEN RECEPTOR-REGULATED TYROSINE KINASES*

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A phosphoinositide kinase that can phosphorylate phosphatidylinositol (PtdIns) is present in 4G10 monoclonal antibody (mAb)-activated T cells. These data suggest that this polypeptide is not immunoprecipitated by the 4G10 mAb from TCR-CD3-activated Jurkat cells. Moreover, immunoprecipitated PtdIns 3-kinase isolated from T cells with p85 antibodies is inhibited when PtdIns is presented in Nonidet P-40, whereas the PtdIns kinase activity present in 4G10 mAb phosphotyrosine immunoprecipitates is enhanced in the presence of Nonidet P-40. In vitro kinase assays of PtdIns 3-kinase immunoprecipitated with p85 antibodies from T cells indicate that it associates with a serine kinase that can phosphorylate a p85 polypeptide. However, no protein tyrosine kinase activity capable of tyrosine phosphorylating p85 in vitro associates with p85a immunoprecipitates in quiescent or TCR-CD3-activated Jurkat cells. Moreover, PTK activation precedes phosphoinositide breakdown in T cells (12) and is an obligate event for TCR-CD3-mediated tyrosine phosphorylation of several substrates following receptor ligation (12, 13). The mechanisms whereby the TCR-CD3 regulates PTKs in T cells are not fully understood, but seem to involve direct or indirect interactions of the TCR-CD3 with Src family PTKs p56lck and p56fos (12).

Tyrosine phosphorylation and its regulation are essential for TCR-CD3-mediated T cell activation (14, 17). Furthermore, PTK activation precedes phosphoinositide breakdown in T cells (12) and is an obligate event for TCR-CD3-mediated PLC activation. The mechanism that the TCR-CD3 uses to couple to PLC probably involves TCR-CD3-directed tyrosine phosphorylation of the PLCy1 isozyme, resulting in increased enzyme activity and hence elevated phosphoinositide metabolism (18-20). TCR-CD3-mediated PtdIns(4,5)P2 hydrolysis is a sustained response in T cells which requires a substantial increase in the net synthesis of PtdIns(4,5)P2 (21, 22). Analysis of cellular PtdIns(4,5)P2 levels during T cell activation supports such an increase occurs (21, 22) and indicate that it is regulated by the actions of ill-defined phosphoinositide lipid kinases, namely phosphatidylinositol (PtdIns) 4-kinase and phosphatidylinositol-(4)-monophosphate (PtdIns(4)P) 5-kinase (20). Little is known about the mechanisms that regulate the activity of the PtdIns 4-kinase, although there are reports that in fibroblasts this kinase is a substrate for PTKs and may therefore be regulated by tyrosine phosphorylation (23).

Recently, it has been shown that T cells express the lipid products of a novel PtdIns 3-kinase (24): PtdIns(3)P, PtdIns(3,4)P2, and PtdIns(3,4,5)P3 (21). The function of these D-3 (poly)phosphoinositides has not been determined, but they are not substrates for the known cellular PLCs and as such are not thought to be hydrolyzed during cellular activation (25, 26). During T cell activation via the TCR-CD3 complex, levels of PtdIns(3)P do not change, but there is an increase in cellular levels of PtdIns(3,4)P2 and PtdIns(3,4,5)P3 (21). It has also been described that stimulation of the IL-2R, which results in T cell growth, also increases in levels of D-3 (poly)phosphoinositides (27). Hence, these lipid prod-

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ucts may be involved in the signaling mechanisms that regulate both T-cell activation and growth.

The PtdIns 3-kinase comprises two major subunits of 85 and 110 kDa (24). The p85 polypeptide may function as the mediator for the interactions between the catalytic p110 subunit of PtdIns 3-kinase and activated receptor and nonreceptor PTKs (28-30). The p85 subunit contains Src homology (SH) regions (one SH3 and two SH2 regions) (28, 29) and associates with and is a substrate for many tyrosine kinases including p60
(30-32), the platelet-derived growth factor (PDGF) receptor (28-32), the colony-stimulating factor-1 (CSF-1) receptor (33, 34), the epidermal growth factor (EGF) receptor (35), and the insulin receptor (36). In T lymphocytes, recent studies have suggested that the PtdIns 3-kinase is a substrate for the Src family kinase p59
(37) and may also be tyrosine-phosphorylated by as yet unidentified IL-2R-controlled PTKs (37, 38). However, although these data implicate PTKs in the regulation of the PtdIns 3-kinase that occurs during IL-2R-directed T-cell growth, it is unknown whether it is a substrate for the TCR-CDC3-controlled PTKs that regulate T-cell activation.

It has been described that tyrosine phosphorylation of PtdIns 3-kinase correlates with its increased activity (30, 32, 36). Therefore, the object of the present study was to use the 4G10 monoclonal antibody (mAb) for phosphotyrosine and antibodies to the p85 subunit of the PtdIns 3-kinase to determine whether the subunits of the PtdIns 3-kinase are tyrosine-phosphorylated or associated with tyrosine kinases subsequent to triggering of the TCR-CDC3. The results of this study demonstrate that immunoprecipitates of tyrosine-phosphorylated proteins isolated from TCR-CDC3-stimulated T cells do contain a PtdIns kinase activity, but this is distinct from PtdIns 3-kinase. Moreover, PtdIns 3-kinase is not detectably phosphorylated on tyrosine in TCR-CDC3-activated T cells nor is there any evidence that it associates with tyrosine kinases during T-cell activation.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—The CD3 antibody UCHT1 and (Fab')
fragments of UCHT1 were generated as described (13). Polyclonal rabbit antisera to the 85 subunit of the PtdIns 3-kinase was described (28). Full purification details and characterization of the p85 recombinant protein are described elsewhere. The 4G10 phosphotyrosine antibody was kindly supplied by Dr. Brian Drucker and Dr. Tom Roberts (Harvard Medical School). The following were obtained from the sources indicated. Potassium oxalate and phosphoinositides (soybean PtdIns, bovine PtdIns(4)P, and PtdIns(4,5)P) were obtained from Sigma (Poole, Dorset, United Kingdom); [gamma-32P]ATP (5000 Ci/mmol) and [32P]ATP (30 mCi/ mg) were purchased from Pharmacia. [gamma-32P]ATP (25 mCi/M) and penicillin (50 units/ml) at 37°C. Jurkat cells were recovered from culture, washed three times in RPMI 1640, and resuspended at 2 x 10
cells/0.5 ml aliquots in RPMI 1640 containing 20 mM HEPS, pH 7.4, and stimulated as indicated. Reactions in Jurkat cells were terminated by the addition of equal volume of ice-cold 2 x cell lysis buffer (150 mM NaCl, 20 mM Tris, pH 7.5, 1 mM MgCl2, 1 mM DTT, 10% glycerol, 1% Nonidet P-40, 150 μM sodium orthovanadate, 100 μM leupeptin, and 200 μM PMSF when using the 4G10 mAb; 100 mM NaCl 20 mM Tris, pH 7.4, 10 mM NaF, 10 mM iodoacetamide, and 1% Nonidet P-40 or 1% Brij 96 when using p85 mAb). After 15 min on ice followed by centrifugation at 13,000 x g for 15 min, total Jurkat cell lysates were cells were either acetone-precipitated as described (13) or pre-cleared with protein A-Affi-Gel (Bio-Rad) followed by the addition of either the 4G10 mAb or the p85 polyclonal antisera which had been coupled to protein A-Affi-Gel beads as described (13). The immunoprecipitates were washed three times with lysis buffer (1 x), once with phosphate-buffered saline, twice with 0.5 M LiCl, 100 mM Tris, pH 7.6, once in H2O, and once in kinase buffer (5 mM MgCl2, 0.25 mM EDTA, 20 mM HEPS, pH 7.4). Immunoprecipitates were divided equally into two aliquots for analysis in PtdIns kinase assays and immunoblotting.

PtdIns Kinase Assay—PtdIns kinase activity was determined by the modified method of Whitman et al. (39). Briefly, the washed immunoprecipitates were resuspended in 30 ml cold buffer. A lipid mixture (50 μl of 0.1 mg/ml PtdIns and 0.1 mg/ml phosphatidylyserine, dispersed by sonication in 25 mM HEPS buffer, pH 7.4, and 1 mM EDTA) was added to the immunoprecipitates. The reaction was initiated by the addition of 20 μCi of [gamma-32P]ATP and 100 μM ATP and terminated after 15 min by the addition of 80 μl HCl (1 M) and 200 μl of chloroform:methanol (1:1). After vigorous mixing and centrifugation to separate the phases, the organic layer was removed, dried under N2, and resuspended in chloroform. The extracted phospholipids were then separated by TLC in 1-propanol:acetic acid (2 N:65:35 v/v/v) developing solvents (40) and visualized by exposure to ultraviolet light and autoradiography. Phospholipids were identified by comparison with nonlabeled standards.

Immunoblotting with 4G10 mAb and p85 Polyclonal Antibodies—Samples for immunoblotting were electrophoresed on 7-17% SDS-polyacrylamide gel and transferred by electroblotting onto PVDF filters (Immobilon P, Millipore, Bedford, MA). Blots were probed with the p85 antibody UCHT1 and (Fab')
fragments of UCHT1 were generated as described (13). The transfer buffer used was 192 mM glycine, 25 mM Tris, and blotting was carried out for 20-24 h at 0.2 A and 50 V. The blots were probed with either iodinated 4G10 for 2 h (13) or p85 polyclonal antisera overnight (28). Blots probed with the p85 antibodies were washed three times in blocking buffer, followed by incubation for 1 h at room temperature with [gamma-32P]-protein A (28). Blots were dried with Whatman 3MM paper and bands visualized by autoradiography of the filter at -70°C on XAR-5 film (Kodak).

In Vitro Protein Kinase Assays—Immunoprecipitates were prepared as above, and the immunoprecipitates were washed three times in kinase buffer prior to separation by SDS-PAGE. "P-Labeled proteins were visualized by autoradiography at -70°C on XAR-5 film (Kodak).

Phosphoamino Acid Analysis—After SDS-PAGE, labeled protein was electroblotted onto a PVDF filter as described (13), and the blot was then autoradiographed to determine the position of the labeled bands. Portions of the filter which contained labeled protein were excised, soaked in methanol, and then washed in distilled water. Bound protein was hydrolyzed in 6 M HCl (150-250 μl) for 1 h at 110°C. The hydrolysate was dried in a SpeediVac (Savant, Farmingdale, NY) and redissolved in 5 μl of 0.1 M acetic acid containing 3 μg each of unlabeled phosphothreonine, phosphoserine, and phosphoaspartic acid as standards. The phosphoamino acids were separated on a cellulose TLC (Polygram Cell, 300, Macherey-Nagel, Duren, Federal Republic of Germany) in acetic acid:pyridine:water (1:1:98, v/v/v). The labeled phosphoamino acids were visualized by autoradiography at -70°C.

RESULTS

The 4G10 Phosphotyrosine mAb Immunoprecipitates a PtdIns Kinase from Activated T Cells—Activation of the TCR-CDC3 complex with the CD3 mAb UCHT1 (41) induced tyrosine phosphorylation of a number of cellular substrates. The data in Fig. 1a show tyrosine-phosphorylated polyptides in 4G10 immunoprecipitates isolated from TCR-CDC3-
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FIG. 1. Kinetics of protein tyrosine phosphorylation and associated PtdIns kinase activity following TCR-CD3 activation. Jurkat cells were stimulated with 10 μg/ml UCHT1 (+) mAb for the times indicated (lanes 2, 4, 6, 8, 10). Control cells were treated with vehicle (−) and unstimulated (lanes 1, 3, 5, 7, 9). 4G10 mAb immunoprecipitates were prepared from Nonidet P-40 lysates, washed, and divided equally into two aliquots. a, one aliquot of immunoprecipitated phosphotyrosyl polypeptides was resolved by SDS-PAGE under reducing conditions, transferred to a PVDF membrane, detected by probing with iodinated P-Tyr, and visualized by autoradiography. b, the second aliquot of immunoprecipitated phosphotyrosyl polypeptides was assayed for associated PtdIns kinase activity using PtdInsP as a substrate. Extraction and separation of the PtdIns kinase products was performed as described under “Experimental Procedures.” The standard PtdInsP and PtdInsP2 were co-chromatographed with the samples and visualized by exposure to iodine vapor and autoradiography. Data are from a single experiment, representative of five others. Cell equivalents per lane are 2 × 10^7 cells.

Stimulated and control cells and confirm previous reports (13) that the induction of protein tyrosine phosphorylation after TCR-CD3 stimulation was maximal within 30 s of activating the cells and had returned to control levels by 30 min. To determine if PtdIns kinase(s) were tyrosine phosphorylated or associated with phosphotyrosyl polypeptides, a parallel set of 4G10 immunoprecipitates was assayed for PtdIns kinase activity. The data (Fig. 1b) show that a PtdIns kinase activity was present in 4G10 immunoprecipitates prepared from Jurkat cells after 1–10 min exposure to the TCR-CD3 agonist UCHT1 (10 μg/ml). The association of PtdIns kinase with 4G10 mAb was transient, since no PtdIns kinase was detectable in 4G10 mAb immunoprecipitates at 30 min post-TCR-CD3 ligand. 4G10 mAb immunoprecipitation of tyrosine phosphorylated polypeptides (Fig. 2a) was prevented by the addition of 50 mM phenyl phosphate (Fig. 2a, lanes 3 and 4) and 10 mM phosphotyrosine (Fig. 2a, lanes 5 and 6) during immunoprecipitation (Fig. 2a). Similarly, the association of PtdIns kinase activity with 4G10 mAb immunoprecipitates prepared from activated Jurkat cells was prevented by 50 mM phenyl phosphate (Fig. 2b, lanes 3 and 4) and 10 mM phosphotyrosine (Fig. 2b, lanes 5 and 6). Therefore, the association of PtdIns kinase activity with the 4G10 mAb appeared to be specific and dependent on the recognition of phosphotyrosine.

Phosphotyrosine and phenyl phosphate prevent recognition of phosphotyrosyl polypeptides and associated PtdIns kinase activity. Jurkat cells were stimulated with 10 μg/ml UCHT1 (lanes 2, 4, 6) for 2 min. Control cells were treated with vehicle and unstimulated (lanes 1, 3, 5). Cells were lysed as described under “Experimental Procedures,” and phosphotyrosine-containing proteins were immunoprecipitated from the Nonidet P-40 lysates using the 4G10 mAb alone (lanes 1 and 2), 4G10 mAb in the presence of 50 mM phenyl phosphate (lanes 3 and 4), and 4G10 mAb in the presence of 10 mM phosphotyrosine (lanes 5 and 6). The 4G10 immunoprecipitates were washed and divided equally into two aliquots, α, one aliquot of immunoprecipitated phosphotyrosyl polypeptides was resolved by SDS-PAGE under reducing conditions, transferred to a PVDF membrane, detected by probing with iodinated P-Tyr, and visualized by autoradiography. The migration of molecular mass calibration standards is indicated to the left of the autoradiograph in kilodaltons. b, the second aliquot of immunoprecipitated phosphotyrosyl polypeptides was assayed for associated PtdIns kinase activity using PtdInsP as a substrate. Extraction and separation of the PtdIns kinase products was performed as described under “Experimental Procedures.” The standard PtdInsP and PtdInsP2 were co-chromatographed with the samples and visualized by exposure to iodine vapor and autoradiography. Data are from a single experiment, representative of five others. Cell equivalents per lane are 2 × 10^7 cells. P-Tyr, phosphotyrosine.

P85 Subunit of PtdIns 3-Kinase Was Not Detectably Tyrosine-phosphorylated or Associated with PTKs in T Cells—In initial efforts to characterize the PtdIns kinase activity associated with the 4G10 mAb, we examined whether 4G10 immunoprecipitates isolated from TCR-CD3-activated Jurkat cells contained the p85 subunit of the PtdIns 3-kinase. At least two forms of p85 have been isolated, designated p85α and p85β (28). Initial Western blotting and immunoprecipitation studies with specific antisera to p85α and p85β established that Jurkat cells expressed p85α but not p85β (data not shown). In Western blotting studies with the polyclonal antisera to the p85α subunit, a single p85 polypeptide could be detected in 10^6 cell equivalents of total Nonidet P-40 cell lysates prepared from both TCR-CD3-activated and nonactivated Jurkat cells (Fig. 3a, lanes 2 and 3). These cell lysates also contained phosphotyrosyl polypeptides (Fig. 3b, lanes 2 and 3). In contrast, 4G10 immunoprecipitates from TCR-CD3-activated Jurkat cell lysates, which contained phospho-
tyrosyl proteins (Fig. 3b, lane 1), failed to Western blot with the p85α antisera, even though 2 × 10^5 cell equivalents of a 4G10 mAb immunoprecipitate were loaded onto these gels (Fig. 3a, lane 1). The p85α antisera can immunoprecipitate PtdIns 3-kinase activity from both activated and nonactivated Jurkat cells (Fig. 4a). However, p85α immunoprecipitates, when resolved by SDS-PAGE and transferred onto a PVDF filter, contained no detectable polypeptides reactive with the iodinated 4G10 mAb as determined by Western blotting analysis (Fig. 4b, lanes 3 and 4), whereas phosphotyrosyl polypeptides were present in parallel 4G10 mAb immunoprecipitates analyzed under identical Western blotting conditions (Fig. 4b, lanes 1 and 2).

4G10 immunoprecipitates derived from TCR-CD3-activated (10 µg/ml UCHT1 or 10 µg/ml UCHT1 (Fab')2 fragments) Jurkat cells have high levels of associated in vitro PTK activity (Fig. 5a) such that a number of the co-immunoprecipitated proteins were phosphorylated on tyrosine in vitro (Fig. 5b). The in vitro labeled substrates from the 4G10 mAb immunoprecipitates prepared from TCR-CD3-activated and nonactivated Jurkat cells were reprecipitated with the 4G10 mAb, confirming that they were phosphorylated on tyrosine residues (Fig. 6c, lanes 4–6). However, none of these proteins could be reprecipitated with the p85α polyclonal antiserum (Fig. 6b, lanes 4–6). As controls in this series of assays, parallel in vitro kinase protocols were carried out on p85α immunoprecipitates isolated from Jurkat cells and subjected to reprecipitation with either p85α or 4G10 antibodies. The data (Fig. 6a, lanes 1–3) show that p85α immunoprecipitates did contain associated in vitro protein kinase activity, which resulted in the phosphorylation of a p85 polypeptide. The in vitro kinase activity associated with p85α immunoprecipitates was present in TCR-CD3-activated and nonactivated Jurkat cells and could reflect the association of p85α with a PTK. However, the in vitro 32P-labeled p85 polypeptide was reprecipitated with the p85α antisera (Fig. 6b, lanes 1–3).

but not the 4G10 mAb (Fig. 6c, lanes 1–3). Moreover, phosphoamino acid analysis of in vitro 32P-labeled p85 that was immunoprecipitated by p85α antisera from TCR-CD3-activated and nonactivated Jurkat cells established that it was phosphorylated exclusively on serine residues (data not shown). In addition, p85α immunoprecipitates derived from either TCR-CD3-activated or nonactivated Jurkat cells did not contain a kinase activity that could phosphorylate the tyrosine kinase substrate Raytide” (42), although a serine kinase activity that resulted in the phosphorylation of a serine peptide substrate (Arg-Phe-Ala-Arg-Lys-Gly-Ser-Leu-Arg-Gln-Lys-Asn-Val) (43, 44) was observed (data not shown). These reciprocal Western blotting (Fig. 4b) and reprecipitation experiments (Fig. 6) with p85α and 4G10 antibodies indicate that p85α associated with a kinase but was not a substrate for PTKs and was not detectably associated with tyrosine kinases in Jurkat cells. The experiments in Figs. 4–6 were carried out following extraction in Brij 96 detergent. Brij 96 is a mild detergent which preserves loosely associated oligomeric peptides.3

Characterization of the PtdIns Kinase Activity Associated with the 4G10 mAb—Although 4G10 mAb immunoprecipitates did not apparently contain the p85 subunit of the PtdIns 3-kinase, they did contain PtdIns kinase(s) activity. This could reflect the presence of the p110 PtdIns 3-kinase catalytic subunit. The most direct way to address this issue is to

3 S. Ley and N. Osman, unpublished observations.
Jurkat cells were stimulated with "Experimental Procedures." The products were then separated by SDS-PAGE and were lysed with Brij 96, and the lysates were immunoprecipitated with antibodies from PDGF-activated fibroblasts (28-30). The migration of molecular mass calibration standards is indicated to the left of each autoradiograph in kilodaltons. Each autoradiograph is from a single experiment, representative of two others. 5-phosphoamino acid analysis of in vitro P-labeled polypeptides associated with the 4G10 mAb were separated by SDS-PAGE and then transferred to a PVDF filter. After autoradiography, the indicated phosphorylated bands were excised and bound protein hydrolyzed in HCl. Phosphoamino acids were resolved by TLC and compared with unlabeled standards. P-Tyr, phosphotyrosine; P-Ser, phosphoserine; P-Thr, phosphothreonine.

FIG. 5. 4G10 mAb associates with protein tyrosine kinases. Jurkat cells were stimulated with 10 μg/ml UCHT1 mAb for 2 min. Control cells were treated with vehicle and unstimulated. Jurkat cells were lysed with Brnj 96, and the lysates were immunoprecipitated with the 4G10 mAb. In vitro kinase reactions were performed on these immunoprecipitates as described under "Experimental Procedures." The products were then separated by SDS-PAGE (a). Polypeptides were visualized by autoradiography. The migration of molecular mass calibration standards is indicated to the left of each autoradiograph in kilodaltons. Each autoradiograph is from a single experiment, representative of two others. 5-phosphoamino acid analysis of in vitro P-labeled polypeptides associated with the 4G10 mAb were separated by SDS-PAGE and then transferred to a PVDF filter. After autoradiography, the indicated phosphorylated bands were excised and bound protein hydrolyzed in HCl. Phosphoamino acids were resolved by TLC and compared with unlabeled standards. P-Tyr, phosphotyrosine; P-Ser, phosphoserine; P-Thr, phosphothreonine.

analyze by anion exchange on HPLC the lipid products of the PtdIns kinase activity present in 4G10 mAb immunoprecipitates. However, it proved impossible to label these products sufficiently in vitro for such studies. Lipid products from PtdIns kinase assays of p85α immunoprecipitates could readily be analyzed by HPLC techniques, and such studies confirmed that the p85α antisera immunoprecipitated PtdIns 3-kinase activity (data not shown). However, the 4G10 mAb immunoprecipitated had less than 0.1% of the PtdIns kinase activity of the p85α immunoprecipitate (Fig. 7), which made it impossible to obtain sufficient labeled lipid products for quantitative HPLC analysis.

In an alternative approach, we examined the detergent and adenosine sensitivity of the PtdIns kinase activity present in 4G10 mAb immunoprecipitates. Previous work has shown that PtdIns 3-kinase is inhibited when PtdIns is presented in detergent but is resistant to adenosine (23, 24). In contrast, PtdIns 4-kinase can utilize PtdIns 4-kinase derived from TCR-CD3-activated Jurkat cells was enhanced by 0.5% Nonidet P-40 (Fig. 8a, lane 3) and was inhibited (approximately 30%) by 300 μM adenosine (Fig. 8a, lane 2). In marked contrast, the PtdIns 3-kinase activity immunoprecipitated by the p85α antibody from TCR-CD3-activated Jurkat cells (Fig. 8b, lane 1) was completely inhibited by Nonidet P-40 (Fig. 8b, lane 3) and unaffected by 300 μM adenosine (Fig. 8b, lane 2). Previous studies have established that PtdIns 3-kinase can be immunoprecipitated with phosphotyrosine antibodies from PDGF-activated fibroblasts (28-32). The data in Fig. 8c, lane 2, confirm increased levels of PtdIns kinase activity in 4G10 mAb immunoprecipitates isolated from PDGF-stimulated Swiss 3T3 cells compared with unstimulated quiescent cells (Fig. 8c, lane 1). The PtdIns kinase activity in 4G10 mAb immunoprecipitates derived from PDGF-stimulated fibroblast (Fig. 8c, lane 2) was inhibited by the detergent Nonidet P-40 (Fig. 8c, lane 4).

DISCUSSION

The present study demonstrates that activation of the Jurkat leukemic T-cell line via the TCR-CD3 induces tyrosine phosphorylation of a large number of cellular substrates and results in a rapid increase in the level of PtdIns kinase activity associated with phosphotyrosine antibodies. The increased levels of PtdIns kinase activity in 4G10 immunoprecipitates isolated from TCR-CD3 activated cells reflect either an increase in tyrosine phosphorylation of the enzyme or an associated protein. The present data also indicate that Jurkat cells express PtdIns 3-kinase which has been identified in previous studies to be associated with activated receptor and non-receptor PTKs in fibroblasts and hemopoietic cells (28-36).

Recent studies have shown that in T lymphocytes, triggering of the IL-2R results in an increase in the association of PtdIns 3-kinase with phosphotyrosine antibodies (27). However, several lines of evidence in this study indicate that the PtdIns kinase activity immunoprecipitated by the 4G10 mAb antibody from TCR-CD3-activated Jurkat cells is not PtdIns 3-kinase. First, in fibroblasts, it is the p85 subunit of the PtdIns 3-kinase that associates with PTKs (33, 35, 46), but specific immunoprecipitation and Western blotting studies failed to identify p85 in the 4G10 mAb immunoprecipitates derived from TCR-CD3-activated Jurkat cells. Second, a comparison of the detergent and adenosine sensitivity of the 4G10 phosphotyrosine mAb-associated PtdIns kinase activity and authentic PtdIns 3-kinase immunoprecipitated with p85α antibodies, isolated from TCR-CD3-activated Jurkat cells, revealed different biochemical properties of these lipid kinases. The PtdIns kinase activity immunoprecipitated with p85α antibodies from Jurkat cells or with 4G10 antibodies from PDGF-activated fibroblasts was sensitive to inhibition by the detergent Nonidet-P-40. Moreover, the p85α antibody-associated PtdIns kinase activity derived from Jurkat cells was resistant to adenosine inhibition. In contrast, the PtdIns kinase activity associated with 4G10 mAb immunoprecipitates from TCR-CD3-activated Jurkat cells was enhanced by Nonidet-P-40 and sensitive to inhibition by adenosine. These data indicate a PtdIns kinase activity that is tyrosine-phosphorylated or associated with phosphotyrosyl proteins in TCR-CD3-activated Jurkat cells and which is not identifiable as PtdIns 3-kinase. The 4G10 mAb-associated PtdIns kinase activity may be attributable to PtdIns 4-kinase or a PtdIns 5-kinase (23).

A PtdIns 4-kinase has been shown to associate with and be a substrate for ligand activated EGF receptors (23), although the functional significance of this phenomenon is unclear. Nevertheless, in T cells it has been demonstrated that T-cell activation is associated with an increase in the activity of PtdIns 4-kinase (22). This mechanism functions to replenish the PLC substrate PtdIns(4,5)P2 during a sustained period of TCR-CD3-induced PLC activation (21, 22). It is well established that tyrosine phosphorylation is essential for TCR-CD3 induction of PtdIns(4,5)P2 hydrolysis (14-17). This requirement is attributed to the role of PTKs in coupling the TCR-CD3 to PLC, since tyrosine phosphorylation of PLCγ1 is known to be induced by the TCR-CD3 and is thought to be essential for TCR-CD3 stimulation of this enzyme (18-20). The present observations of the tyrosine phosphorylation of a PtdIns kinase or an associated protein raises the possibility that PTKs may also be involved in TCR-CD3 regulation of PtdIns metabolism at the level of the replenishment of PtdIns(4,5)P2 during prolonged periods of TCR-CD3-induced PLC activation.

In fibroblasts, an increase in the activity of cellular PtdIns
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FIG. 6. In vitro kinase assays on 4G10 mAb and p85α antisera immunoprecipitates. Jurkat cells were stimulated with 10 µg/ml UCHT1 mAb or 10 µg/ml UCHT1 (Fab′)2 fragments for 2 min. Control cells were treated with vehicle and unstimulated. Jurkat cells were lysed with Brij 96, and the lysates were immunoprecipitated with the polyclonal antisera to p85α or the 4G10 mAb. In vitro kinase reactions were performed on these immunoprecipitates as described under “Experimental Procedures.” The products were then either separated by SDS-PAGE (a) or re-precipitated with p85α polyclonal antisera (b) or 4G10 mAb (c). The re-precipitated polypeptides were then separated by SDS-PAGE. All polypeptides (a-c) were visualized by autoradiography. The migration of molecular mass calibration standards is indicated to the left of each autoradiograph in kilodaltons. Each autoradiograph is from a single experiment, representative of two others.

FIG. 7. Comparison of the PtdIns kinase activity present in 4G10 mAb and p85α immunoprecipitates. Jurkat cells (2 x 10^7 cells/lane) were stimulated with 10 µg/ml UCHT1 (lanes 2 and 4) for 2 min. Control cells (lanes 1 and 3) were treated with vehicle and unstimulated. Cells were lysed as described under “Experimental Procedures” and the cell lysates immunoprecipitated with 4G10 mAb (a) or p85α polyclonal antisera (b). The washed immunoprecipitates were analyzed for PtdIns kinase activity using PtdIns as a substrate. Extraction and separation of the products was performed as described under “Experimental Procedures.” The standard PtdInsP and PtdInsP2 were co-chromatographed with the samples and visualized by exposure to iodine and autoradiography. The TLC is from a single experiment, representative of four others.

3-kinase and an increase in cellular levels of D-3 phosphoinositides (47) correlates with tyrosine phosphorylation of the p85 subunit of the PtdIns 3-kinase (28, 30-31). Similarly, IL-2R regulation of PtdIns 3-kinase activity correlates with tyrosine phosphorylation of the enzyme (27), although the requirement of tyrosine phosphorylated p85 subunit for PtdIns 3-kinase activity remains to be demonstrated. Ligation of the TCR-CD3 complex results in an increase in certain D-3 phosphoinositide lipids in intact cells (21), but data in this study indicate that p85α is not a detectable substrate for TCR-CD3-regulated PTKs. It would seem therefore that TCR-CD3 regulation of PtdIns 3-kinase is by an alternative mechanism that does not involve tyrosine phosphorylation of the p85α subunit. No increase in in vitro PtdIns 3-kinase activity immunoprecipitated by p85 antisera could be detected upon TCR-CD3 activation of Jurkat cells. Similarly, PDGF treatment of fibroblasts has been reported not to increase the in vitro PtdIns 3-kinase activity of p85 immunoprecipitates (35). Only 5% of the total PtdIns 3-kinase activity associates with the ligand-activated PDGF receptor in NIH 3T3 cells, suggesting that only a small percentage of the total PtdIns 3-kinase population is receptor-activated. Ligation of the TCR-CD3 complex may similarly activate a small percentage of the total cytosolic PtdIns 3-kinase, such that the remaining non-receptor-activated PtdIns 3-kinase, which is also immunoprecipitated by p85α antisera, may mask a rise in a receptor-coupled PtdIns 3-kinase activity in vitro following TCR-CD3 ligation.

The p85α subunit of PtdIns 3-kinase may be tyrosine-phosphorylated at low stoichiometry or be an exceptional substrate for tyrosine phosphatases. The in vitro immune complex kinase assays allow immunoprecipitation of PTKs and tyrosine kinase substrates which are then phosphorylated

* M. Fry and M. Waterfield, unpublished observations.
in vitro to label the substrates and allow their detection by reprecipitation with the 4G10 mAb. Under these conditions, it was still not possible to detect tyrosine-phosphorylated polypeptides in the p85α immunoprecipitates by reprecipitation with the 4G10 mAb. The p85 subunit of PtdIns 3-kinase is not only a substrate for PTKs in fibroblasts (28, 29) but is also known to associate with activated PTKs such as c-src (31–32) and v-src (48), as well as ligand activated PDGF, EGF, and CSF-1 receptors (28–33). These associations between p85 and PTKs may be mediated via SH2 domains (28, 29, 33, 49, 50, 51). The associations between p85 and PTKs may be important to the regulatory function of p85 by targeting the catalytic subunit of PtdIns to activated tyrosine kinases (38). Hence, the p85 subunit may associate with, but not act as a substrate for, TCR-CD3-regulated PTKs and suggests that any regulation of this relevant activated cell surface molecule with either p56lck or p59fyn. Thus, tyrosine phosphorylation of p85 during TCR-CD3 complex may be important to signal transduction mechanisms associated with the IL-2R and CD4 ligation but not those associated with the TCR-CD3 complex. Finally, the TCR-CD3 complex does induce the phosphotyrosyl polypeptide association of a PtdIns kinase distinct from the PtdIns 3-kinase, and the identity of this lipid kinase and its functional relevance to phosphoinositide metabolism in T cells remain to be resolved.

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