Deficient Tyrosine Phosphorylation of c-Cbl and Associated Proteins in Phorbol Ester-resistant EL4 Mouse Thymoma Cells*

(Received for publication, December 3, 1996, and in revised form, January 30, 1997)

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Two tyrosine phosphoproteins in phorbol ester-sensitive EL4 (S-EL4) mouse thymoma cells have been identified as the p120 c-Cbl protooncogene product and the p85 subunit of phosphatidylinositol 3-kinase. Tyrosine phosphorylation of p120 and p85 increased rapidly after phorbol ester stimulation. Phorbol ester-resistant EL4 (R-EL4) cells expressed comparable amounts of c-Cbl and phosphatidylinositol 3-kinase protein but greatly diminished tyrosine phosphorylation. Co-immunoprecipitation experiments revealed complexes of c-Cbl with p85, and of p85 with the tyrosine kinase Lck in phorbol ester-stimulated S-EL4 but not in unstimulated S-EL4 or in R-EL4 cells. In vitro binding of c-Cbl with Lck SH2 or SH3 domains was detected in both S-EL4 and R-EL4 cells, suggesting that c-Cbl, p85, and Lck may form a ternary complex. In vitro kinase assays revealed phosphorylation of p85 by Lck only in phorbol ester-stimulated S-EL4 cells. Collectively, these results suggest that Cbl-p85 and Lck-p85 complexes may form in unstimulated S-EL4 and R-EL4 cells but were not detected due to absence of tyrosine phosphorylation of p85. Greatly decreased tyrosine phosphorylation of c-Cbl and p85 in the complexes may contribute to the failure of R-EL4 cells to respond to phorbol ester.

T lymphocyte activation is triggered by interaction between the T cell antigen receptor (TCR)† and its cognate antigen. One of the earliest signaling events following TCR stimulation is the rapid increase in tyrosine phosphorylation of a number of proteins (reviewed in Refs. 1 and 2). Unlike growth factor receptors that have intrinsic tyrosine kinase activity (reviewed in Ref. 3), the TCR components transduce their signals through noncovalently associated cytoplasmic tyrosine kinases (1, 2). Three tyrosine kinases, Lck, Fyn, and Zap-70, have been implicated in the function of the TCR. Identification of substrates for these tyrosine kinases has been an area of intense investigation. Several tyrosine kinase substrates in T cells have been identified in the past few years, including phospholipase C-γ (4, 5), the guanine nucleotide exchange factor Vav (6–8), an oligomeric ATPase valosin-containing protein (9, 10), the membrane-cytoskeleton linker protein ezrin (11, 12), and the ζ subunit of TCR (13). Recently, c-Cbl, a protooncogene protein of 120 kDa has been identified as another prominent tyrosine kinase substrate in T cells (14). Although it has been demonstrated that c-Cbl becomes rapidly tyrosine-phosphorylated upon TCR stimulation (14) and forms complexes with several signaling molecules (15–19), the function of c-Cbl in T cells is not clear.

T cell activation can be mimicked by the addition of tumor-promoting phorbol esters plus calcium ionophores, suggesting important roles for protein kinase C (PKC) and calcium (20). The role of PKC in T cell activation is further emphasized by studies showing that induction of the transcription factors AP-1, NF-κB, and nuclear factor of activated T cell for cytokine gene expression is regulated by PKC (21). PKC is a family of phospholipid-dependent serine/threonine kinases, most of which can be stimulated directly by phorbol ester treatment (reviewed in Refs. 22 and 23). Although a functional role for PKC in T cell activation is demonstrated, the signaling events mediated by PKC are not clear. Stimulation of T lymphocytes with phorbol esters induces a marked phosphorylation of Lck at N-terminal serine residues (24, 25). Whether this phorbol ester-stimulated serine phosphorylation of Lck has any functional significance is not well understood. Stimulation of some T cells with phorbol ester also induces accumulation of a Ras-GTP complex (26), suggesting that PKC might mediate Ras activation. Ras has been shown to interact directly with Raf-1 kinase in vitro and in vivo (27, 28). The evidence that PKC can phosphorylate and activate Raf-1 suggests that PKC may be an upstream regulator of this kinase (29, 30). Raf-1 can trigger a kinase cascade by phosphorylating and activating mitogen-activated protein (MAP) kinase kinase (MEK), which subsequently phosphorylates and activates MAP kinase (reviewed in Ref. 31). The demonstration that MAP kinase can translocate to the nucleus (32), where it can directly modulate transcriptional factors (33, 34), might provide a mechanism for PKC regulation of cytokine gene expression.

To study the PKC-mediated signaling events, we have employed an EL4 mouse thymoma cell line that requires only phorbol ester treatment to stimulate interleukin-2 (IL-2) production, growth inhibition, and adherence to the substrate (35, 36). Comparison with an EL4 variant that lacks all of these responses (36, 37) has allowed us to investigate signaling molecules that may be essential for these responses. Previous work has revealed deficient induction of c-Jun and Fra transcription factors (38) and deficient activation of MEK in phorbol ester-resistant EL4 (R-EL4) cells (39), suggesting potential functional roles for these signaling molecules. In addition, treatment of phorbol ester-sensitive EL4 (S-EL4) cells with tyrosine kinase inhibitors (genistein and herbimycin) blocks phorbol
ester-stimulated IL-2 mRNA production (40), which raised the possibility that tyrosine phosphorylation may be important for phorbol ester-stimulated IL-2 production. To examine the tyrosine phosphorylation events potentially important for phorbol ester-stimulated IL-2 production and other responses, we have compared tyrosine phosphoproteins between S-EL4 and R-EL4 cells. A tyrosine phosphoprotein, p85, was detected in S-EL4 but not R-EL4 cells, and phorbol ester stimulation enhanced its tyrosine phosphorylation only in sensitive cells (41). Here we report an additional tyrosine phosphoprotein, p120, that has a tyrosine phosphorylation response similar to that of p85. We have identified this p120 protein as the product of the c-Cbl protooncogene and further investigated its associated signaling proteins. Our studies have revealed greatly decreased tyrosine phosphorylation of c-Cbl in R-EL4 cells and some deficiencies in its associated signaling molecules in these cells.

**EXPERIMENTAL PROCEDURES**

**Reagents—**Anti-phosphotyrosine (anti-Tyr(P)) and anti-PI 3-kinase antibodies were obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Other antibodies, glutathione S-transferase (GST) fusion proteins, and glutathione-agarose beads were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Phorbol dibutyrate (PDB), aprotinin, sodium pyrophosphate, sodium orthovanadate, phenylmethanesulfonyl fluoride, and HEPES were purchased from Sigma. Protein A-agarose beads and enhanced chemiluminescence (ECL) reagents were supplied by Amersham Corp. Phorbol ester-sensitive and -resistant EL4 cells were purchased from ATCC (Rockville, MD), and fetal calf serum was obtained from Life Technologies, Inc. Luminescent stickers for marking were purchased from ATCC (Rockville, MD), and fetal calf serum was purchased from Amersham Corp. Phorbol ester-sensitive and -resistant EL4 cells were purchased from ATCC (Rockville, MD), and fetal calf serum was obtained from Life Technologies, Inc. Luminescent stickers for marking and aligning autoradiographs were obtained from Stratagene (La Jolla, CA).

Cell Culture and Phorbol Ester Treatment—EL4 cells were grown in RPMI 1640 medium supplemented with 5% heat-inactivated fetal calf serum and 2 mM glutamine to a density of approximately 1–2 × 10⁶/ml. Cells were treated with 150 mM PDB for the times indicated.

**Immunoprecipitation—**Cells (2 × 10⁷) were lysed in 1 ml of lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM EGTA) supplemented with protease and phosphatase inhibitors (1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml sodium pyrophosphatase). Particulate material was removed by centrifugation at 14,000 × g for 15 min, and supernatants were incubated with 2–5 μg of the indicated antibodies at 4 °C for 3 h, followed by incubation with 15 μl of protein A-agarose beads for 1 h. The immunoprecipitates were washed with lysis buffer five times and treated with 1 × Laemmlı sample buffer and then subjected to SDS-PAGE and transferred to nitrocellulose. Immunoblots were incubated with antibodies to c-Cbl, a prominent tyrosine kinase substrate of 120 kDa recently identified in T cells (14), revealed expression of c-Cbl in S-EL4 cells and equal or greater expression in R-EL4 cells (Fig. 1B). Identification of p120 as c-Cbl would suggest that tyrosine phosphorylation of c- Cbl was much higher in S- than in R-EL4 cells. To test this possibility, c-Cbl proteins were immunoprecipitated from both S- and R-EL4 cells, and the tyrosine phosphorylation of c-Cbl was determined by anti-Tyr(P) immunoblotting. C-Cbl did exhibit much greater tyrosine phosphorylation in S- than in R-EL4 cells (Fig. 2), consistent with the possibility that c-Cbl was p120. To further address the possible identity of p120 as c-Cbl, attempts to separate p120 from c-Cbl were made using two-dimensional gel electrophoresis. Lysates from cells that were stimulated with 150 nM PDB for 5 min were electrophoresed through isoelectric focusing gels followed by SDS-PAGE gels and then subjected to anti-Tyr(P) immunoblotting. The tyrosine phosphoproteins

**RESULTS**

**P120 Is the c-Cbl Protooncogene Protein—**Previous studies revealed a tyrosine phosphoprotein, p85, in phorbol ester-sensitive but not -resistant EL4 mouse thymoma cells and found that phorbol ester stimulation enhanced its tyrosine phosphorylation only in sensitive cells (41). As shown in Fig. 1A, anti-phosphotyrosine immunoblotting reveals an additional protein, p120, that exhibits a similar pattern. It could not be discerned from this experiment whether the proteins were deficient in the resistant cells or merely the tyrosine phosphorylation.

In attempt to identify these proteins, blots were probed with antisera to known tyrosine phosphoproteins of similar molecular weight that could have a role in T cell signaling. Probing with antibodies to c-Cbl, a prominent tyrosine kinase substrate of 120 kDa recently identified in T cells (14), revealed expression of c-Cbl in S-EL4 cells and equal or greater expression in R-EL4 cells (Fig. 1B). Identification of p120 as c-Cbl would suggest that tyrosine phosphorylation of c-Cbl was much higher in S- than in R-EL4 cells. To test this possibility, c-Cbl proteins were immunoprecipitated from both S- and R-EL4 cells, and the tyrosine phosphorylation of c-Cbl was determined by anti-Tyr(P) immunoblotting. C-Cbl did exhibit much greater tyrosine phosphorylation in S- than in R-EL4 cells (Fig. 2), consistent with the possibility that c-Cbl was p120. To further address the possible identity of p120 as c-Cbl, attempts to separate p120 from c-Cbl were made using two-dimensional gel electrophoresis. Lysates from cells that were stimulated with 150 nM PDB for 5 min were electrophoresed through isoelectric focusing gels followed by SDS-PAGE gels and then subjected to anti-Tyr(P) immunoblotting. The tyrosine phosphoproteins
were resolved into a number of distinct spots or arrays of spots, corresponding to major tyrosine phosphoproteins observed in one-dimensional gel analysis (Fig. 1A). A series of 120-kDa tyrosine phosphoproteins of varying isoelectric points was detected in S- but not in R-EL4 cells (Fig. 3, left panels). When the anti-Tyr(P) immunoblots were stripped and reprobed with anti-Cbl antibody, a set of anti-Cbl reactive proteins, also exhibiting several pI forms, was detected in both cell lines (Fig. 3, right panels). Overlaying the two films revealed that the p120 signal exactly overlapped with the c-Cbl signal, arguing strongly that p120 was c-Cbl.

Since phorbol ester stimulation of S-EL4 cells enhanced the tyrosine phosphorylation of p120, the time course of tyrosine phosphorylation after phorbol ester stimulation was examined. Lysates from cells stimulated with 150 nM PDB for various times up to 60 min were analyzed by anti-Tyr(P) immunoblotting. The tyrosine phosphorylation of p120 in S-EL4 cells began to increase about 1.5 min after PDB stimulation, remained elevated for 20 min, and decreased by about 30 min (Fig. 4A).

Although resistant cells exhibited a faint tyrosine phosphorylation of p85, no obvious p120 tyrosine phosphorylation was observed in R-EL4 cells (Fig. 4B). The blots were stripped and reprobed with anti-Cbl antibody to confirm that the amount of c-Cbl proteins did not change over the assay period (Fig. 4). c-Cbl, p85, and Lck Complex Formation in Phorbol Ester-stimulated Sensitive EL4 Cells—Since it has been reported that c-Cbl could form complexes with several signaling molecules (15–19), the possibility that c-Cbl may form a complex with tyrosine phosphoproteins in EL4 cells was investigated. c-Cbl proteins were immunoprecipitated from lysates of cells that had been unstimulated or treated with PDB for 2 min, and Cbl-associated tyrosine phosphoproteins were detected with an anti-Tyr(P) antibody. An 85-kDa tyrosine phosphoprotein was observed in phorbol ester-stimulated S-EL4 cells but not in unstimulated S-EL4 or in R-EL4 cells despite comparable immunoprecipitation of c-Cbl in both cells (Fig. 5). In addition to the similar pattern of expression, this 85-kDa tyrosine phosphoprotein co-migrated with the previously observed p85 detected by direct immunoblotting of lysates (data not shown), suggesting that they are the same protein. To our surprise, we did not observe enhanced tyrosine phosphorylation of c-Cbl in phorbol ester-stimulated S-EL4 cells. We suspect that some phosphotyrosines of c-Cbl in phorbol ester-stimulated S-EL4 cells may be lost during the longer time required for immunoprecipitation as opposed to direct immunoblotting of lysates.

Although p85 was detected by anti-Tyr(P) antibody in Cbl immunoprecipitates after phorbol ester stimulation of S-EL4 cells, it was not clear whether phorbol ester stimulation induced more tyrosine-phosphorylated p85 to be associated with c-Cbl in S-EL4 cells or whether phorbol ester stimulation induced the tyrosine phosphorylation of p85 that associated with c-Cbl in either phosphorylation state. To explore these possible mechanisms, we first tried to identify potential tyrosine kinases for p85. The lymphocyte tyrosine kinases Lck and Fyn were immunoprecipitated from lysates prepared after a 10-min treatment of cells with 150 nM PDB to determine whether p85 can be co-immunoprecipitated with these tyrosine kinases. Monoclonal antibodies to c-Fos and cyclin E were used as negative controls. The tyrosine kinase-associated tyrosine phosphoproteins were detected by anti-Tyr(P) antibody. A strong p85 signal was detected in Lck immunoprecipitates from S-EL4, not R-EL4, cells (Fig. 6A). A weak p85 signal also could be detected in Fyn immunoprecipitates from S-EL4 cells; however, this signal did not exceed that detected in control immunoprecipitates (Fig. 6A). Since p85 was observed to co-immunoprecipitate with Lck in phorbol ester-stimulated S-EL4 cells.
we next tried to determine whether phorbol ester stimulation had any effect on the co-immunoprecipitation of p85 with Lck. Lysates from S-EL4 cells that were unstimulated or treated with 150 nM PDB for 10 min were immunoprecipitated with anti-Lck antibody, and p85 was detected by anti-Tyr(P) antibody. A prominent p85 band was detected in Lck immunoprecipitates from stimulated S-EL4 cells, but signals in immunoprecipitates from unstimulated S-EL4 cells did not exceed those from control immunoprecipitates (Fig. 6B). The appearance of p85 in Lck immunoprecipitates after phorbol ester stimulation of S-EL4 cells was very rapid, being detectable after only 2 min of phorbol ester stimulation (data not shown).

To investigate which domains of Lck could bind p85, lysates from cells that were unstimulated or were treated with 150 nM PDB for 10 min were immunoprecipitated with anti-Lck antibody, and p85 was detected by anti-Tyr(P) antibody. A prominent p85 band was detected in Lck immunoprecipitates from stimulated S-EL4 cells, but signals in immunoprecipitates from unstimulated S-EL4 cells did not exceed those from control immunoprecipitates (Fig. 6B). The appearance of p85 in Lck immunoprecipitates after phorbol ester stimulation of S-EL4 cells was very rapid, being detectable after only 2 min of phorbol ester stimulation (data not shown).

That Western blotting with anti-Tyr(P) antibody provides a stronger p85 signal with the SH2 GST fusion protein than with the SH3. Collectively, these results suggested that Lck was a potential tyrosine kinase for p85.

Since we observed p85 in both c-Cbl and Lck immunoprecipi-
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| FIG. 7. In vitro association of p85 with the SH2 (A) or SH3 (B) domains of Lck. S-EL4 or R-EL4 cells (1.5 × 10⁷) were unstimulated or treated with 150 nM PDB for 10 (A) or 5 min (B). Cells were lysed in 1 ml of lysis buffer. Lysates were precleared by incubation with 40 µl of glutathione-agarose beads overnight and then incubated with 10 µg of GST, GST-Lck SH2 (A), or GST-Lck SH3 (B) fusion proteins conjugated with 15 µl of glutathione-agarose beads for 3 h. The bead-bound proteins were subjected to SDS-PAGE followed by anti-immunoblotting with anti-Tyr(P) antibody. Results are representative of three independent experiments. |
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| FIG. 8. Constitutive association of c-Cbl with SH3 (A) or SH2 (B) domains of Lck in S-EL4 and R-EL4 cells. S-EL4 or R-EL4 cells (1.5 × 10⁷) were unstimulated or treated with 150 nM PDB for 5 min. Lck proteins were immunoprecipitated from lysates prepared from sensitive or resistant EL4 cells that were unstimulated or treated with 150 nM PDB for 5 min and subjected to an in vitro kinase assay to detect ³²P transfer to any co-immunoprecipitated proteins. A ³²P-containing band of 56 kDa, probably reflecting autophosphorylation of Lck, was detected in all Lck immunoprecipitates, but an 85-kDa protein was phosphorylated in Lck immune complexes (Fig. 9, upper panel). Anti-Tyr(P) immunoblotting demonstrated its co-migration with p85 (Fig. 9, lower panel). The phosphorylation of p85 in Lck immune complex was very rapid, being detectable after only 2 min of phorbol ester stimulation (data not shown). These results supported the possibility that detection of p85 in Cbl-p85 and Lck-p85 complexes in phorbol ester-stimulated S-EL4 cells was due to the tyrosine phosphorylation of p85. But without identification of p85, we could not exclude the possibility that the detection of p85 in these complexes in phorbol ester-stimulated S-EL4 cells was due to association of more tyrosine-phosphorylated p85 with c-Cbl or Lck. |

| p85. We therefore tested whether p85 could be phosphorylated by Lck in vitro after phorbol ester stimulation of the cells. Lck proteins were immunoprecipitated from lysates prepared from unstimulated cells or from cells treated with 150 nM PDB for 5 min and subjected to an in vitro kinase assay to detect ³²P transfer to any co-immunoprecipitated proteins. A ³²P-containing band of 56 kDa, probably reflecting autophosphorylation of Lck, was detected in all Lck immunoprecipitates, but an 85-kDa protein was phosphorylated in Lck immune complex kinase assays only in phorbol ester-stimulated S-EL4 cells, not in unstimulated S-EL4 cells, in R-EL4 cells, or in control immune complexes (Fig. 9, upper panel). Anti-Tyr(P) immunoblotting demonstrated its co-migration with p85 (Fig. 9, lower panel). The phosphorylation of p85 in Lck immune complex was very rapid, being detectable after only 2 min of phorbol ester stimulation (data not shown). These results supported the possibility that detection of p85 in Cbl-p85 and Lck-p85 complexes in phorbol ester-stimulated S-EL4 cells was due to the tyrosine phosphorylation of p85. But without identification of p85, we could not exclude the possibility that the detection of p85 in these complexes in phorbol ester-stimulated S-EL4 cells was due to association of more tyrosine-phosphorylated p85 with c-Cbl or Lck. |

| Identification of p85 as the p85 Subunit of PI 3-Kinase— |
| Since it was reported that the p85 subunit of PI 3-kinase associated with c-Cbl (15, 16, 17) and bound the SH3 domains of Lck (42, 43), we speculated that p85 might be the p85 subunit of PI 3-kinase. To test this possibility, lysates from cells that were stimulated with 150 nM PDB for 5 min were subjected to two-dimensional gel electrophoresis, and immunoblots were probed successively with anti-Tyr(P) and anti-PI 3-kinase p85 antibodies. While a series of tyrosine-phosphoryl-|
ated p85 spots was detected in S- but not in R-EL4 cells (Fig. 10, left panels), a set of spots reactive with the antibodies to the p85 subunit of PI 3-kinase was observed in both S- and R-EL4 cells (Fig. 10, right panels). These PI 3-kinase spots exactly overlapped with the Tyr(P)-p85 spots in S-EL4 cells when the films were superimposed, arguing that p85 was the p85 subunit of PI 3-kinase.

To further confirm that p85 was the p85 subunit of PI 3-kinase, we examined whether Cbl- and Lck-associated p85 was recognized by the antibody to the p85 subunit of PI 3-kinase. c-Cbl proteins were immunoprecipitated from lysates prepared from cells that were unstimulated or treated with PDB and subjected to SDS-PAGE followed by anti-PI 3-kinase immunoblotting. In phorbol ester-stimulated S-EL4 cells, Cbl-associated p85 was recognized by the antibody to the p85 subunit of PI 3-kinase (Fig. 11, A). Furthermore, PI 3-kinase, like p85, also could be co-immunoprecipitated with Lck in phorbol ester-stimulated S-EL4 cells. In addition, binding between PI 3-kinase and Lck was observed in unstimulated S-EL4 and in R-EL4 cells, and phorbol ester stimulation slightly enhanced the association of PI 3-kinase with Lck in both cell lines (Fig. 11B). Compared with the strong tyrosine-phosphorylated p85 signal in Lck immunoprecipitates from phorbol ester-stimulated S-EL4 cells, the amount of PI 3-kinase p85 that associated with Lck seemed quite small. A potential explanation for this difference could be heavy tyrosine phosphorylation of the few Lck-associated PI 3-kinase molecules.

DISCUSSION

Two potentially important tyrosine phosphoproteins, p120 and p85, have been identified in phorbol ester-sensitive EL4 cells. These two proteins were detected by anti-phosphotyrosine antibody in S-EL4 but not in R-EL4 cells, and phorbol ester stimulation enhanced their tyrosine phosphorylation only in sensitive cells (Fig. 1). P120 was identified as the c-Cbl protooncogene product by anti-Cbl immunoblotting, by comparison of c-Cbl tyrosine phosphorylation between S- and R-EL4 cells, and by two-dimensional gel analysis (Figs. 1–3). Co-immunoprecipitation of c-Cbl with the p85 subunit of PI 3-kinase on two-dimensional gel analysis and reactivity of Cbl- and Lck-associated p85 with the antibody to the p85 subunit of PI 3-kinase (Figs. 10 and 11) strongly argue that p85 is the PI 3-kinase p85 subunit.

Association of Cbl with some signaling molecules has been observed in several cell lines (15–19) and also occurred in EL4 cells. Co-immunoprecipitation experiments revealed formation of complexes of c-Cbl with the p85 subunit of PI 3-kinase and of the p85 subunit of PI 3-kinase with Lck in both S-EL4 and R-EL4 cells (Fig. 11, A and B). Co-immunoprecipitation of c-Cbl with Lck was not observed. However, in vitro binding of c-Cbl with Lck SH2 or SH3 domains was detected in both cell lines (Fig. 8), suggesting that c-Cbl, Lck, and the p85 subunit of PI 3-kinase may form a ternary complex. Failure to detect associ-
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Fig. 11. PI 3-kinase co-immunoprecipitates with c-Cbl (A) and Lck (B) in S-EL4 and R-EL4 cells. S-EL4 or R-EL4 cells (2 × 10^6) were unstimulated or treated with 150 μM PDB for 2 min (A) or 10 min (B). Cells were lysed in 1 ml of lysis buffer, and lysates were immunoprecipitated with anti-Cbl antibody (A), anti-Lck antibody (B), or pre-immune serum (preimm.). The immunoprecipitates were subjected to SDS-PAGE followed by immunoblotting with anti-PI 3-kinase p85 subunit antibody. Results are representative of two independent experiments.

Experiments.

Subunit antibody. Results are representative of two independent SDS-PAGE followed by immunoblotting with anti-PI 3-kinase p85 immune serum (preimm). The immunoprecipitates were subjected to SDS-PAGE followed by immunoblotting with anti-PI 3-kinase p85 subunit antibody. Results are representative of two independent experiments.

While this might explain the lack of co-immunoprecipitation of c-Cbl with Lck and the weak Cbl-Lck SH2 association detected in vitro, the strong signal detected with the Lck-SH3 domain suggests some direct Cbl-Lck SH3 interaction.

Complexes of Cbl-PI 3-kinase p85 and of Lck-PI 3-kinase p85 have been observed also in other cells (16, 17). In Jurkat T cells, the p85 subunit of PI 3-kinase and PI 3-kinase activity were observed in c-Cbl immunoprecipitates. After TCR stimulation, the association of the p85 subunit of PI 3-kinase with c-Cbl and the PI 3-kinase activity in c-Cbl immunoprecipitates was further enhanced. In addition to binding to c-Cbl, the p85 subunit of PI 3-kinase also was observed in Lck immunoprecipitates in IL-2-dependent helper and cytolytic T cell clones (46). IL-2 stimulation not only enhanced the association of the p85 subunit of PI 3-kinase with Lck but also induced the tyrosine phosphorylation and activation of Lck-associated PI 3-kinase (46). The p85 subunit of PI 3-kinase can bind to Lck SH2 or SH3 domains dependent on the cell lines examined (44, 45). While the binding of PI 3-kinase p85 to Lck SH2 domains is largely phosphotyrosine-dependent, PI 3-kinase p85 binds to Lck SH3 domains through a proline-rich motif independent of phosphotyrosine (43). We also observed binding of the p85 subunit of PI 3-kinase to Lck SH2 or SH3 domains in EL4 cells (data not shown) in addition to the binding of tyrosine-phosphorylated p85 to these domains (Fig. 7).

Although S-EL4 and R-EL4 cells have comparable amounts of c-Cbl, PI 3-kinase, and Lck proteins and form complexes of Cbl-PI 3-kinase p85 and Lck-PI 3-kinase p85, the p85 subunit of PI 3-kinase in these complexes becomes tyrosine-phosphorylated only in phorbol ester-stimulated S-EL4 cells but not in unstimulated S-EL4 or in R-EL4 cells (Figs. 5 and 6), and tyrosine phosphorylation of c-Cbl is greatly diminished in R-EL4 cells (Fig. 2). Since phosphotyrosine residues can bind SH2 domains, promoting protein-protein interaction (42, 43), tyrosine phosphorylation of c-Cbl and of the PI 3-kinase p85 subunit that associates with c-Cbl and Lck may promote the downstream signal transduction that contributes to the phorbol ester-stimulated responses in S-EL4 cells. Since this tyrosine phosphorylation happens rapidly after phorbol ester stimulation of S-EL4 cells (2 min), it may contribute to rapid phorbol ester-stimulated responses such as adherence to the substrate, which occurs 30 min after phorbol ester stimulation, as well as to late responses such as IL-2 production or growth inhibition. Although treatment of osteoclast-like cells with c-Cbl antisense did not inhibit cell adherence (47), potential roles for c-Cbl in lymphocyte adherence or other responses remain to be tested.

Greatly diminished tyrosine phosphorylation of c-Cbl and the p85 subunit of PI 3-kinase that associates with c-Cbl or Lck in R-EL4 cells suggests that the tyrosine kinases for these proteins may be defective in R-EL4 cells. While the tyrosine kinases for c-Cbl have not been well examined, the fact that Lck can phosphorylate p85 in vitro only in phorbol ester-stimulated S-EL4 cells (Fig. 9) suggests that Lck may be responsible for the tyrosine phosphorylation of Cbl- and Lck-associated PI 3-kinase p85. However, we cannot exclude the possible involvement of other tyrosine kinases. Lck is expressed in both S-EL4 and R-EL4 cells and undergoes autophosphorylation in both cell lines (Fig. 9), suggesting that it is not inactive in R-EL4 cells. Determination of whether mutation of some tyrosine phosphorylation sites in c-Cbl or PI 3-kinase p85 contributes to the greatly decreased tyrosine phosphorylation of these proteins will require further investigation.

In addition to possible defects in tyrosine kinases or tyrosine phosphorylation sites, it is possible that some upstream regulators for c-Cbl, PI 3-kinase p85, or Lck are defective in R-EL4 cells, accounting for the greatly decreased tyrosine phosphorylation of these proteins. Several PKC isoforms (PKC-ε, -η, and -θ) exhibit greatly decreased expression in R-EL4 cells (48, 49), raising the possibility that these PKC isoforms may contribute to the regulation of c-Cbl, PI 3-kinase p85, or Lck by serine or threonine phosphorylation in S-EL4 cells and facilitate phorbol ester-stimulated tyrosine phosphorylation of these proteins.

In conclusion, these studies have identified two potentially important phosphotyrosine proteins in EL4 cells, p120 and p85, as the c-Cbl protooncogene product and the p85 subunit of PI 3-kinase. C-Cbl, Lck, and PI 3-kinase p85 proteins and complexes of Cbl-PI 3-kinase p85 and of Lck-PI 3-kinase p85 were observed in S-EL4 and R-EL4 cells; however, tyrosine phosphorylation of PI 3-kinase p85 in these complexes was detected only in phorbol ester-stimulated S-EL4 cells, and tyrosine phosphorylation of c-Cbl was much higher in S-EL4 than in R-EL4 cells, raising the possibility that these complexes and tyrosine phosphorylation of the proteins may be important for phorbol ester-stimulated responses in S-EL4 cells. Understanding how PKC regulates these signaling molecules and which PKC isoforms may be involved will be of special interest in the future.

Acknowledgments—We thank Moira Resnick for helpful advice and discussion throughout this work and Xuqiong Wu from the laboratories of A. P. and A. V. Somlyo at the University of Virginia for technical help with the two-dimensional gel analysis.
