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p56lck STABLY ASSOCIATES WITH A 115 kDa SUBSTRATE

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SUMMARY: Using antibodies directed against p56lck, we have identified a 115 kDa protein (p115) that is specifically immunoprecipitated with p56lck from whole cell lysates. The p56lck/p115 complex is stable in the presence of nonionic detergents. p115 becomes phosphorylated on tyrosine residues in p56lck immune-complex kinase assays. Treatment of whole cells with 12-*O*-tetradecanoyl phorbol-13-acetate decreases the subsequent tyrosine phosphorylation of p115 in immune-complex kinase assays.

Recently, several members of the src family of protein-tyrosine kinases have been found to be complexed to the cytoplasmic domains of transmembrane receptors found on the surface of immune cells (1). Of these signaling complexes, the T-lymphocyte specific p56lck/CD4 complex has been most extensively characterized (reviewed in 2,3). As a result of the extracellular cross-linking of the CD4 transmembrane receptor, the tyrosine kinase activity of p56lck becomes activated (4-6). The stimulation of p56lck activity is thought to be important for the activation of T-cells since T-cells containing CD4 mutants incapable of associating with p56lck, fail to activate (7). The p56lck/CD4 complex remains intact in the presence of nonionic detergents and antibodies to CD4 co-immunoprecipitate p56lck, while antibodies to p56lck co-immunoprecipitate CD4 (8,9). Using a similar approach, we asked whether antibodies to p56lck could co-immunoprecipitate specific substrates for p56lck. We have identified a 115 kDa protein that becomes phosphorylated on tyrosine residues when anti-p56lck immune complexes are phosphorylated in the presence of [γ-^{32}P]ATP. This protein is an attractive candidate for an *in vivo* substrate for p56lck.

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Abbreviations: TPA, 12-*O*-tetradecanoyl phorbol-13-acetate; EDTA, ethylenediaminetetraacetate; PNPP, p-nitrophenylphosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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MATERIALS AND METHODS

Antibodies to p56lck. Two anti-peptide rabbit polyclonal antisera directed against p56lck were separately prepared in rabbits. The N-terminal antisera was prepared against a peptide comprising amino acids 39-64 and the C-terminal antisera was prepared against a peptide containing amino acids 476-509. The preparation of these antibodies has been described previously (10).

Immune-complex kinase assays. Thymocytes were teased from 8-12g mice, washed in phosphate buffered saline and incubated on ice for 5 min before the addition of 0.45 ml/2.5 x 10^7 thymocytes of cold lysis buffer (50 mM Tris-HCl (pH, 7.6), 0.5% Triton X-100, 150 mM NaCl, 1mM EDTA, 10 µg/ml aprotinin, and 10 µg/ml leupeptin). After a 10 min incubation on ice, the lysates were centrifuged at 15,000xg, 4°C, for 6 min and the post-nuclear supernatants were incubated with 25 µl of Protein A-Sepharose (Sigma) that had been preincubated overnight in the presence of 5 µl of unfractionated anti-C-terminal peptide antiserum. Antibodies to the N-terminal peptide were affinity purified on peptide containing resin before use. The lysates were rotated in the presence of immunoglobulin-bound Protein A-Sepharose for 1 hr at 4°C. The resins were washed 3x in lysis buffer minus EDTA and phosphorylated in the presence of 10 mM $MnCl_2$, 5 mM PNPP, 2 µCi [γ-^{32}P]ATP (7000 Ci/mmol) in 25 mM Hepes (pH 7.5) at 30°C for 1 min. The reactions were stopped with SDS-sample buffer and analyzed by SDS-PAGE on 10% or 12% gels. For the peptide blocking experiment in Fig. 1A and B, immune-complexes were formed in the presence of 1 mM C-terminal peptide. We have determined that peptide concentrations as low as 25 µM completely blocked the immunoprecipitation of both p56lck and p115 using 5 µl of undiluted C-terminal antiserum (B. Aube and M.L. Harrison, unpublished observations).

Other Methods. In some experiments gels were transferred to PVDF membranes (Immobilon, Millipore) in buffer containing 0.05% SDS, 25 mM Tris-HCl, 0.2 M glycine and 10% methanol and subjected to base hydrolysis in the presence of 1N KOH for 1 hr at 55 °C.

Cells and Cell Lines. The human leukemic cell lines CEM and Jurkat as well as the murine cell line LSTRA were grown in RPMI 1640 medium supplemented with 8% heat-inactivated fetal calf serum, 6 mM Hepes, 50 µM 2-mercaptoethanol, 3g/L sodium bicarbonate, 5 mM L-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin G and 100 µg/ml streptomycin.

RESULTS

Exposure of anti-p56lck immune complexes from lysates of mouse thymocytes to [γ-^{32}P]ATP resulted not only in the phosphorylation of p56lck itself (transphosphorylation), but also of an additional protein of 115 kDa (p115), as shown in Fig. 1. The immunoprecipitation of both p56lck and p115 was blocked in the presence of the immunizing p56lck-peptide (Fig. 1A and B, lane 2). The phosphorylations were stable to treatment with base (Fig. 1B), indicating the presence of phosphotyrosine. Indeed, phosphoamino acid analysis of p115 confirmed that p115 was phosphorylated on tyrosine residues (data not shown). Although the antibodies used in this experiment were generated against the C-terminus of p56lck, a region of extensive identity in src family members (11), similar results were obtained with anti-p56lck antibodies generated against the unique N-terminus of p56lck (data not shown). These data indicate that a 115 kDa protein is specifically found in anti-p56lck immunoprecipitates

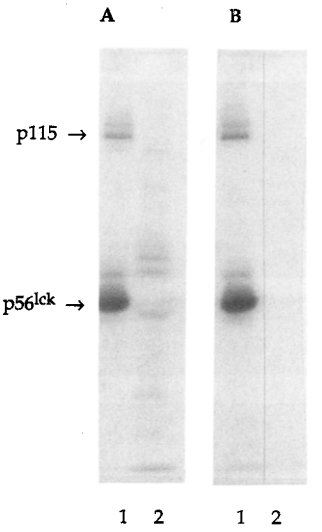

Figure 1. Anti-p56lck immune-complex kinase assays. p56lck in lysates from mouse thymocytes containing no (lanes 1) or 1 mM (lanes 2) C-terminal peptide was isolated in anti-C-terminal immune complexes and phosphorylated in the presence of [γ-^{32}P]ATP as described under Materials and Methods. Panel A is an autoradiogram of a 10% SDS-gel transferred to Immobilon and panel B is an autoradiogram of the phosphoproteins in panel A after KOH treatment. Autophosphorylating p56lck as well as the 115,000 Da (p115) substrate are indicated with arrows.

from detergent lysates, and that p115 is a substrate for a tyrosine kinase present in these complexes, most likely p56lck itself. In addition to mouse thymocytes, p115 was also found in anti-p56lck immune-complexes immunoprecipitated from the human T-cell-derived cell lines CEM and Jurkat (Fig. 2) and the murine derived cell line LSTRA (data not shown). Immunoprecipitates from WEHI 231 cells (a murine B-cell-derived cell line that expresses very low levels of p56lck) did not contain detectable p115 (data not shown). We have also observed that immune complexes formed with antibodies to p59fyn contain a 115 kDa protein that becomes tyrosine phosphorylated in the presence of [γ-^{32}P]ATP (data not shown). It therefore appears likely that other members of the src family of protein-tyrosine kinases may also associate with p115. Indeed, Samelson *et al.* (12) have recently shown that substrates with molecular weights of 130 kDa and 120 kDa respectively, are found in immunoprecipitates formed using antibodies to the fyn protein-tyrosine kinase.

TPA is a co-mitogenic agent for T-cells, augmenting the cellular proliferation induced by such mitogens as phytohemagglutinin (13). It has also been reported to cause the dissociation of p56lck from CD4 (14). In addition, TPA has been shown to stimulate N-terminal serine/threonine phosphorylation of p56lck, resulting in its retarded migration on SDS-polyacrylamide gels (15-19). Treatment of intact mouse thymocytes with TPA resulted in a dose-dependent decrease in the tyrosine phosphorylation of p115 in anti-p56lck immune-complex kinase assays (Fig. 3). As expected, more slowly migrating forms of p56lck were evident in samples from TPA treated cells (Fig. 3).

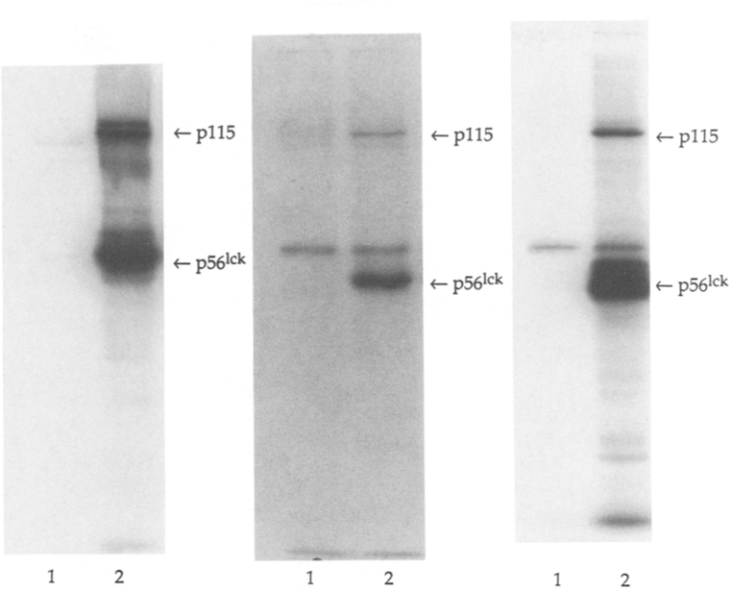

Figure 2. Anti-p56lck immune-complex kinases assays from T-cell lysates. p56lck in lysates from mouse thymocytes (left panel), CEM cells (center panel), and Jurkat cells (right panel) was isolated using pre-immune (lanes 1) or anti-p56lck (C-terminal) immune complexes and phosphorylated in the presence of [γ-^{32}P]ATP as described under Material and Methods. The panels are autoradiograms of 12% (left panel) or 10% (center and right panels) SDS-gels. Autophosphorylated p56lck and the p115 substrate are indicated by arrows.

DISCUSSION

Although p56lck appears to play a critical role in the activation of T-cells, the identification of relevant physiological substrates for p56lck has for the most part, remained difficult. Recently however, the potentially important signaling molecules

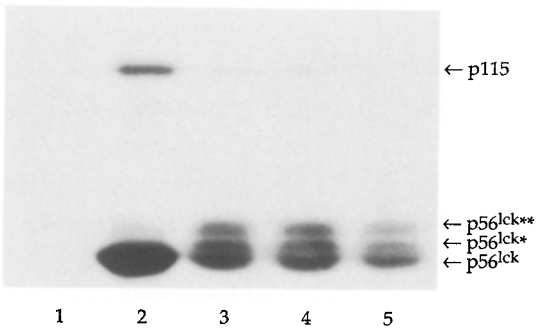

Figure 3. Anti-p56lck immune-complex assays from mouse thymocytes treated with TPA. Thymocytes were incubated in the absence (lane 1 and 2) or the presence of 5 ng/ml (lane 3), 50 ng/ml (lane 4) or 100 ng/ml (lane 5) TPA for 30 minutes before cell lysates were prepared and p56lck isolated in immune-complexes from preimmune (lane 1) or anti-p56lck C-terminal antisera (lanes 2-5). The figure is an autoradiogram of a 10% SDS-gel after KOH treatment. p56lck* and p56lck** represent the more slowly migrating forms of p56lck.

Raf-1 (20), the ζ chain of the T-cell antigen receptor (4) and the family of MAP kinases (21) have been shown to be tyrosine phosphorylated as a result of CD4 cross-linking, implicating p56lck in their phosphorylation. None of these proteins however, has a molecular weight similar to that of the 115 kDa substrate we have shown to be specifically associated with p56lck.

An important signaling molecule that does have a molecular weight similar to p115 is the 120 kDa GTPase-activating protein, GAP. GAP has been reported to be tyrosine phosphorylated in the p56lck overexpressing murine cell line LSTRA as well as in fibroblasts containing transfected p56lck (22). Moreover, GAP has been shown to be a substrate for p56lck *in vitro* (23). Preliminary re-immunoprecipitation studies indicated that anti-GAP antibodies immunoprecipitated a radioactive band migrating at approximately 120 kDa from anti-p56lck immune-complex kinase assays (data not shown). We are currently investigating whether this anti-GAP reactive band is p115.

The fact that TPA treatment caused a dose-dependent decrease in p115 tyrosine phosphorylation indicates that extracellular co-mitogenic signals influence the p56lck/p115 interaction. There are several interpretations of this result. The most straightforward is that less p115 is associated with p56lck in cells treated with TPA. This situation would be analagous to TPA causing the dissociation of p56lck from CD4 (14). Alternatively, TPA could have caused a serine/threonine phosphorylation of p115, rendering it a less efficient substrate for p56lck. We consider it unlikely that TPA treatment caused a significant decrease in p56lck activity since this has been difficult to demonstrate (19). Moreover, the addition of an exogenous substrate, myelin basic protein (24) to the p56lck immune-complex kinase assays showed no decrease in the activity of p56lck immunoprecipitated from TPA treated cells (P.R. Srinivas and M.L. Harrison, unpublished obervations). Alternatively, TPA could have caused an increase in the tyrosine phosphorylation of p115 resulting in less phosphate being incorporated from [γ-^{32}P]ATP in the immune-complex kinase assays. We consider this unlikely since the rapid exchange of unlabeled for labeled phosphate during autophosphorylation reactions has been demonstrated previously (25,26).

ACKNOWLEDGMENTS

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