

Distinct Intracellular Localization of Lck and Fyn Protein Tyrosine Kinases in Human T Lymphocytes

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Abstract. Two src family kinases, lck and fyn, participate in the activation of T lymphocytes. Both of these protein tyrosine kinases are thought to function via their interaction with cell surface receptors. Thus, lck is associated with CD4, CD8, and Thy-1, whereas fyn is associated with the T cell antigen receptor and Thy-1. In this study, the intracellular localization of these two protein tyrosine kinases in T cells was analyzed by immunofluorescence and confocal microscopy. Lck was present at the plasma membrane, consistent with its proposed role in transmembrane signalling, and was also associated with pericentrosomal vesicles which co-localized with the cation-independent mannose 6-phosphate receptor. Surprisingly, fyn was not detected at the plasma membrane in either Jurkat T cells or T lymphoblasts but was

closely associated with the centrosome and to microtubule bundles radiating from the centrosome. In mitotic cells, fyn co-localized with the mitotic spindle and poles. The essentially non-overlapping intracellular distributions of lck and fyn suggest that these kinases may be accessible to distinct regulatory proteins and substrates and, therefore, may regulate different aspects of T cell activation. Anti-phosphotyrosine antibody staining at the plasma membrane increases dramatically after CD3 cross-linking of Jurkat T cells. The localization of lck to the plasma membrane suggests that it may participate in mediating this increase in tyrosine phosphorylation, rather than fyn. Furthermore, the distribution of fyn in mitotic cells raises the possibility that it functions at the M phase of the cell cycle.

THE T cell antigen receptor (TcR)¹ comprises the products of six genes ($\alpha\beta\gamma\delta\epsilon\zeta_2$; Clevers et al., 1988). The $\alpha\beta$ heterodimer recognizes an antigenic peptide bound to a major histocompatibility complex (MHC) molecule on the surface of an antigen-presenting cell. The CD3 complex ($\gamma\delta\epsilon$) and ζ chains are involved in the signal transduction function of the TcR. Analysis of chimeric polypeptides containing the cytoplasmic tails of the ζ chain or of the CD3 ϵ chain has characterized a sequence motif which is responsible for the signal transduction capability of these chains (Weiss, 1993). This motif is triplicated in the ζ chain and present as a single copy in each of the CD3 chains and couples the TcR to downstream signalling pathways.

Stimulation of the TcR induces the rapid tyrosine phosphorylation of multiple proteins on tyrosine (Hsi et al.,

1989; Ley et al., 1991). Pharmacologic experiments (June et al., 1990; Mustelin et al., 1990) and genetic experiments (described below) have established that the induction of protein tyrosine kinase (PTK) activity is essential for the signal transduction function of the TcR. However, the primary sequences of the identified components of the TcR complex contain no recognizable kinase domains (Klausner and Samelson, 1991). Rather, it appears that the signalling motifs present in the cytoplasmic tails of the CD3 chains and the ζ chain couple directly or indirectly to non-receptor PTKs (Weiss, 1993). Two members of the src family of non-receptor PTKs have been implicated in signal transduction via the TcR: lck and fyn (Veillette and Davidson, 1992). In addition, a novel T cell specific PTK, ZAP 70, has recently been identified which is rapidly induced to associate with tyrosine-phosphorylated ζ chains, via its two SH2 domains, after CD3 cross-linking (Chan et al., 1992; Irving et al., 1993).

Lck is non-covalently associated with the cytoplasmic tails of CD4 or CD8 (Veillette and Davidson, 1992). The extracellular domains of CD4 and CD8 bind to MHC class II and class I molecules, respectively (Bierer et al., 1989). Mutant forms of CD4 or CD8 that lack cytoplasmic tails, or contain cytoplasmic tails to which lck cannot bind, are considerably debilitated in their ability to augment T cell responsiveness

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1. *Abbreviations used in this paper:* CI-MPR, cation-independent mannose 6-phosphate receptor; GPI, glycosylphosphatidylinositol; MHC, major histocompatibility complex; PTK, protein tyrosine kinase; PTyr, phosphotyrosine; TCR, T cell antigen receptor.

to antigen in T cell lines (Zamoyska et al., 1989; Glaichenhaus et al., 1991). The association of *lck* with CD4 and CD8, therefore, appears to be important for the signalling function of these receptors. Physiologically, binding of the TcR clonotypic chains to an antigen-MHC complex brings the cytoplasmic domains of the TcR into close proximity with either a CD4 or CD8 molecule that can independently bind to the same MHC molecule (class II or I, respectively). This provides a mechanism by which *lck* could regulate signalling via the TcR. Consistent with this possibility, cross-linking of the TcR with CD4 enhances the induction of tyrosine phosphorylation as well as later events associated with T cell activation (Ledbetter et al., 1988). Furthermore, TcR cross-linking of a mutant leukemic T cell lacking functional *lck* fails to activate PTK activity or to stimulate an increase in intracellular Ca^{2+} (Straus and Weiss, 1992). *Lck*, therefore, appears to be necessary for effective signalling via the TcR. Mice containing a disrupted *lck* gene show a profound decrease in the number of thymocytes and in mature peripheral T cells indicating that this kinase also plays an important role in thymic development (Molina et al., 1992).

Fyn is expressed in T cells as a uniquely spliced form of the gene (*fyn-T*; Cooke and Perlmutter, 1989). Sensitive *in vitro* kinase assays can detect *fyn* in TcR immunoprecipitates (Samelson et al., 1990) and a function for this kinase in TcR signalling has been suggested by genetic studies. Thus, overexpression of *fyn-T* increases antigen-induced IL 2 production in a T cell hybridoma (Davidson et al., 1992). Similarly, thymocytes from transgenic mice which express high levels of *fyn-T* in developing T lineage cells are hyper-responsive to stimulation via the TcR (Cooke et al., 1991). However, disruption of the *fyn* gene in mice by homologous recombination does not substantially affect T cell development (Appleby et al., 1992). The TcR is still capable of signal transduction in immature (CD4+CD8+) thymocytes and in peripheral T cells from these mice, although signalling is defective in single positive (CD4+ or CD8+) thymocytes. Thus, a function for *fyn-T* in TcR signalling may be restricted to a subpopulation of thymocytes at a particular stage of development.

Lck and *fyn* may also be associated with glycoposphatidylinositol (GPI)-anchored molecules on the surface of T cells. Thus both *lck* and *fyn* have been shown to coprecipitate with Thy-1 (Stefanova et al., 1991; Thomas and Samelson, 1992). GPI-linked proteins appear to cluster in membrane microdomains that are enriched in glycolipids (Bohuslav et al., 1993) and the interaction of PTKs with GPI-linked molecules such as Thy-1 may be due to the colocalization of these molecules to discrete areas within the plasma membrane. Functionally, the association of Thy-1 with *lck* and *fyn* may provide a mechanism by which Thy-1 cross-linking leads to tyrosine phosphorylation and T cell activation. Consistent with this hypothesis, thymocytes from transgenic mice that overexpress *fyn* are more sensitive than wild type cells to stimulation via Thy-1 (Cooke et al., 1991).

In this study, the localization of the *src*-family kinases, *lck* and *fyn*, was investigated in human T lymphocytes by immunofluorescence and confocal microscopy. Only *lck* could be easily detected at the plasma membrane whereas the majority of detectable *fyn* was intracellular. *Lck* was also detected in intracellular vesicles but these were distinct from the intracellular structures containing *fyn*. The implications

of these results for the functions of these two PTKs in T cells are discussed.

Materials and Methods

Cells and Antibodies

The J6 subline of the Jurkat T leukemic cell line (Robb et al., 1981) was passaged in RPMI 1640 medium containing 5% FCS, penicillin (100 U/ml), and streptomycin (50 U/ml). Cells were maintained in a rapid growth phase in Falcon tissue culture flasks for use in analytical experiments. T lymphoblasts were prepared as described previously (Monostori et al., 1990) and were kindly provided by S. Lucas and D. Cantrell (ICRF, London, UK). The J-108 cell line was produced by transfection of the J6 cell line with a cDNA encoding human *fyn-T* (Slocombe, P., Celltech, unpublished data) in the expression vector pEE6hCMV (Bebbington, 1991). The J-GPT control cell line was produced by transfection of J6 cells with the same vector lacking an insert cDNA.

The CD3 antibody, OKT3, was obtained from the American Type Tissue Collection (Rockville, MD). For cell stimulations, this antibody was used as (Fab')₂ fragments, kindly provided by A. Tutt and M. Glennie (Tenovus, Southampton, UK). The UCHT-1 CD3 antibody was the generous gift of Peter Beverley (University College, London, UK) and was used for capping of the TcR (Beverley and Callard, 1981). The murine anti-phosphotyrosine (PTyr) antibody, 4G10 (Kanakura et al., 1990), was used as a purified Ig and was the generous gift of B. Druker and T. Roberts (Dana-Farber Cancer Institute, Boston, MA). The rat anti- α tubulin antibody, YOL 34, was obtained from Serotec. The affinity purified, anti-*fyn*-1 peptide antibody was the kind gift of S. Courtneidge (Kypta et al., 1988). The affinity purified, anti-*lck* peptide antibody, KERP, has been described previously (Pelchen-Matthews et al., 1992). The endoplasmic reticulum was localized using the rat monoclonal antibody, MAC 259 (Napier et al., 1992) which recognizes the ER retention sequence KDEL. This antibody was kindly provided by Richard Napier (Horticulture Research International, Kent, UK). The rat monoclonal antibody, 23C, which recognizes β -COP (Harrison-Lavoie et al., 1993) was used to localize the Golgi apparatus (the kind gift of K. Willison, Chester-Beatty Research Laboratories, London, UK). The lysosomes were localized using a rabbit polyclonal antiserum, 52K, directed against mature, human Cathepsin D (kindly provided by B. Wesley, Newcastle University, Newcastle, UK). A rabbit polyclonal antiserum specific for the cation-independent mannose 6-phosphate receptor (CI-MPR) was kindly provided by Dr. W. J. Brown (Cornell University, Ithaca, NY) and has previously been described (Brown and Farquhar, 1984).

The fluorochrome-labeled antibodies used for immunofluorescent staining of cells were obtained from a variety of suppliers. Control experiments confirmed that these species-specific antibodies did not cross-react when used in double-labeling experiments. An anti-mouse IgG2b antibody coupled to FITC (Southern Biotechnology Birmingham, AL) was used for labeling with the Anti-PTyr antibody, 4G10. For tubulin staining with the YOL 34 antibody, an anti-rat IgG antibody coupled to Texas red (Amersham International, Amersham, UK) was used. Two different second stage antibodies were used to visualize rabbit antibodies depending on which fluorochrome was required (i.e., FITC or Texas red). Both of these antibodies were obtained from the Jackson ImmunoResearch Laboratories (Avondale, PA).

Immunofluorescence Staining

Cells were washed twice in warm RPMI 1640 medium by centrifugation (500 g for 3 min) and then resuspended to 5×10^6 cells per ml in the same medium maintained at 37°C. 2 ml of cell suspension was then activated by addition of OKT3 (Fab')₂ antibody to a final concentration of 0.5 μ g/ml and incubated for 1 min or left unstimulated. At the end of the incubation period, cells were immediately centrifuged at 500 g for 30 s and then resuspended in 4 ml of 3.7% paraformaldehyde in PBS. Paraformaldehyde, which was prepared as a 20% stock and frozen to -20°C, was diluted immediately prior to use and filtered to remove any particulate matter. Vanadate was added to 10 mM to inhibit protein tyrosine phosphatase activity. For optimal staining with anti-PTyr antibody, fixation was carried out at room temperature for 30 min. For localization of *fyn*, fixation was carried out at 37°C for 30 min which was found to maintain the integrity of the microtubule cytoskeleton more effectively than fixation at room temperature. After fixation, cells were pelleted by centrifugation and then resuspended in 1 ml of PBS supplemented with 10 mM vanadate (PBS-V).

Cells were immobilized by settling onto coverslips that had been coated with "chromic gelatin." To prepare the chromic gelatin solution, pig skin gelatin (Sigma Chemical Co., St. Louis, MO) was dissolved in water at 100°C and then allowed to cool to room temperature. This solution was then mixed with an equal volume of a 0.1% solution of chromic potassium sulphate and 200 μ l of formalin was added as preservative. The chromic gelatin solution was stored at room temperature for 2 to 3 wk before use. 150 μ l of chromic gelatin was pipetted onto the surface of a 1-cm coverslip and then excess liquid was removed with a tissue. The coated coverslip was then dried in an oven at 65°C for 30 min. The suspension of fixed cells (150 μ l) was pipetted onto the surface of the coated coverslip and left at 4°C for 2 h to allow the cells to settle. The coverglass was washed once with 2 ml of PBS-V and then incubated for 4 min in 2 ml of PBS-V supplemented with 0.1% Triton X-100 solution to permeabilize the cells. After two washes with PBS-V, non-specific staining was blocked by incubation in 2 ml of 0.5% fish skin gelatin (Sigma Chemical Co.) in PBS-V for 10 min at room temperature. Excess liquid was removed with a tissue and 50 μ l of primary antibody diluted in 0.5% gelatin in PBS-V was then pipetted onto the cells. Cells were incubated with the first stage antibody for 45 min at room temperature and then washed three times in PBS-V. 50 μ l of the second stage, fluorescently labeled antibody, was then pipetted onto the cells (diluted 1/50–1/100 in 0.5% gelatin in PBS-V) and incubated for 30 min at room temperature. In experiments in which cells were labeled with two antibodies, the first stage antibodies were added sequentially with three washes in-between each incubation. The two second stage antibodies were added together at the appropriate dilution. Cells were finally washed three times in PBS-V and then mounted upside down onto a glass slide in 5 μ l of glycerol/PBS solution (Citifluor). The coverslips were sealed with nail varnish to prevent evaporation and stored for up to 5 d at 4°C before imaging.

To study the effect of capping the TcR on the intracellular distribution of lck and fyn, T lymphoblasts were incubated at 37°C for 40 min with the CD3 antibody, UCHT-1. After fixing with paraformaldehyde and permeabilizing with Triton X-100, the cells were stained with lck or fyn antibody and then the appropriate second stage antibodies.

Confocal Microscopy

Confocal imaging was performed using a laser scanning head (MRC-600; Bio-Rad Laboratories, Cambridge, MA) fitted onto a Nikon Optiphot microscope. A 60 \times NA/1.4 planapochromat oil immersion lens (Nikon) was used for all imaging. Fluorescein and Texas red fluorochromes were excited at 488 and 568 nm, respectively, using a Krypton/Argon mixed gas laser (Bio-Rad Laboratories). Two filter blocks were used, K1 and K2. K1 is a double dichroic filter enabling excitation at 488 and 568 nm, whereas the K2 filter is a 560 nm dichroic combined with 522 nm green emission and 585 nm red emission filters. Images were collected using the Kalman filter. Bleed-through from the Texas red channel to the fluorescein channel was negligible. Care was taken to ensure that the Texas red signal was sufficiently bright relative to the fluorescein signal to minimize the contribution of bleed through from the green channel into the red channel (\sim 10%). Correction of images for bleed through and other processing was carried out using the COMOS and SOM programs (Bio-Rad Laboratories) run on a Compaq Deskpro 66M 486 computer (66 MHz). For Z-series, optical sections were recorded at 0.5- or 1.0- μ m intervals. Data are presented both as projections of sequential optical sections or individual optical sections as indicated in the figure legends. Final images were photographed directly from the VDU screen.

Results

Localization of Phosphotyrosine in Jurkat T Cells

Previous immunoblotting experiments demonstrated that CD3 cross-linking induced the rapid and transient tyrosine phosphorylation of multiple polypeptides (Ley et al., 1991). Consistent with these biochemical experiments, the level of immunofluorescent staining with anti-PTyr antibody was rapidly increased following CD3 antibody stimulation (Fig. 1 A) and this returned to baseline levels by 30 min. Determination of the mean pixel intensity per cell indicated approximately a 10-fold increase in anti-PTyr antibody staining after CD3 cross-linking. The addition of 50 mM phenyl-

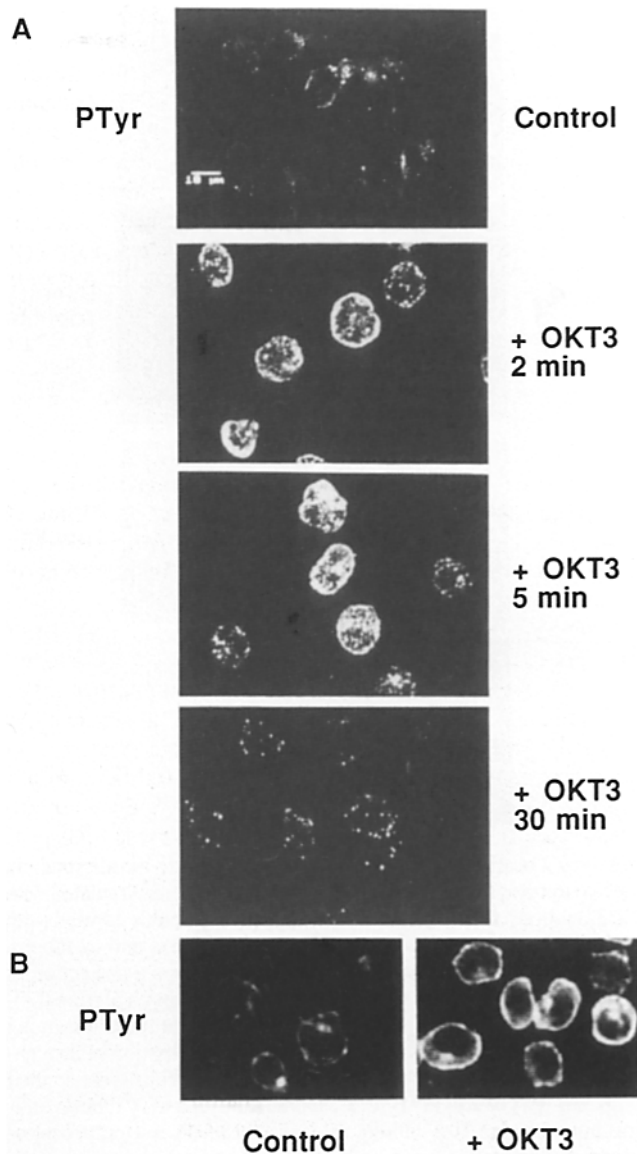


Figure 1. Time course of phosphotyrosine staining after stimulation with CD3 antibody. (A) Jurkat T cells were stimulated with the CD3 antibody, OKT3, for the times indicated or left unstimulated. At each time point the cells were fixed, permeabilized and then stained with anti-PTyr antibody. A complete Z series of the cells, comprising 21 optical sections, was performed using 0.5- μ m increments. The optical sections were then projected to produce the composite image shown. (B) Unstimulated and CD3 antibody-stimulated Jurkat T cells were stained with anti-PTyr antibody. The image shows a single optical section through a field of cells to indicate the relative distribution of phosphotyrosine between plasma membrane and intracellular structures. Bar, 10 μ m.

phosphate, an analog of phosphotyrosine, to unstimulated or OKT3-stimulated cells during the incubation of anti-PTyr antibody reduced staining to background levels, confirming the specificity of the signals obtained (data not shown).

In Fig. 1 B, an optical section is shown of Jurkat T cells stimulated for 1 min with CD3 antibody, or left unstimulated, and then stained with anti-PTyr antibody. In the stimulated cell population, phosphotyrosine could be detected at the

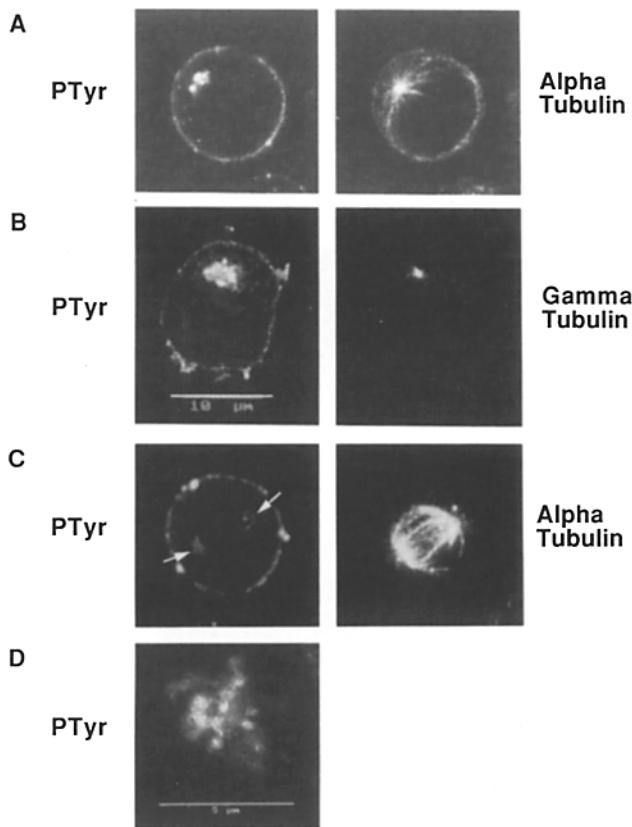


Figure 2. Localization of phosphotyrosine relative to the microtubule cytoskeleton in Jurkat T cells. (A) Jurkat T cells were stimulated with CD3 antibody for 1 min and after fixing were double labeled with anti-PTyr antibody (green channel) and with a rat anti- α tubulin antibody, YOL 34 (red channel). The image shows a projection of three sequential optical sections of a Z series through a typical interphase cell, using 0.5- μ m increments. The bright tubulin staining on the upper left side of the image identifies the position of the centrosome. (B) CD3 antibody stimulated Jurkat T cells were stained with anti-PTyr antibody (green channel) and an anti- γ tubulin antiserum (red channel). γ tubulin is localized exclusively to the centrosome. A single optical section is shown of a typical interphase cell. The magnification is the same for images a to c. (C) A Z series was performed, using 0.5- μ m increments, on a mitotic Jurkat cell manipulated as described in a. Optical sections 8 to 14 were projected to produce the image shown. The cell has been counterstained with an anti- α tubulin antibody (red channel) to reveal the position of the mitotic spindle and poles. The arrows on the left hand image indicate the weak anti-PTyr antibody staining present at the poles. (D) High power image of the peri-centrosomal region of a CD3 antibody stimulated Jurkat T cell stained with anti-PTyr antibody. The image was generated from a single optical section. Bars: (A, B, and C) 10 μ m; (D) 5 μ m.

plasma membrane and in some cells in internal structures. These structures were adjacent to the nucleus as demonstrated by propidium iodide staining (data not shown). Comparison with unstimulated Jurkat T cells indicated that a major increase in phosphotyrosine staining occurred at the plasma membrane following CD3 antibody stimulation. Little change in anti-PTyr antibody staining of the juxta-nuclear structure was detected following cross-linking of the TcR.

Counterstaining with an anti- α tubulin antibody suggested that the juxta-nuclear phosphotyrosine was localized around

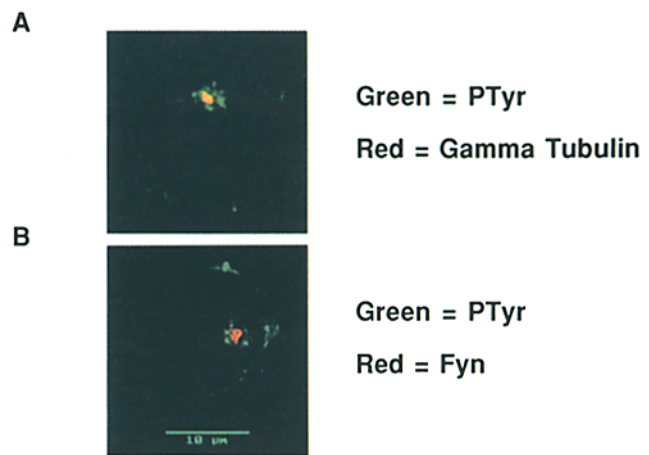


Figure 3. Localization of phosphotyrosine and fyn relative to the centrosome. In A, the images from Fig. 2 b have been merged to show more clearly the relative distributions of phosphotyrosine (green channel) and γ tubulin (red channel). B shows the merged images of Fig. 6 D and clearly demonstrates that the bright punctate phosphotyrosine staining at the centrosome was not coincident with fyn staining. Bar, 10 μ m.

a convergence of microtubules (Fig. 2 A) characteristic of the microtubule organizing centre or centrosome (Kalt and Schliwa, 1993). This localization was confirmed in Fig. 2 B in which cells were double stained for PTyr and γ tubulin, which is only present at the centrosome (Zheng et al., 1991; Stearns et al., 1991). This experiment revealed that the bright intracellular phosphotyrosine staining formed a "shell" around the centrosome, which is demonstrated most clearly in the merged image of Fig. 2 B (Fig. 3 A). Very weak phosphotyrosine staining was also detected at the centrosome itself. In CD3 antibody-stimulated Jurkat cells that were in mitosis, phosphotyrosine was detected at the plasma membrane and there was also very weak staining at the spindle poles (Fig. 3 c).

Characterization of the Peri-centrosomal Structures Containing Phosphotyrosine

At high magnification, it can be seen that the anti-PTyr antibody staining in the peri-centrosomal region was punctate (Fig. 2 D) and in some cell preparations produced a staining pattern which was characteristic of vesicles (e.g., Fig. 4 B). A series of specific antibodies was used to investigate whether the peri-centrosomal phosphotyrosine was coincident with any defined intracellular membrane-bound organelles in double-labeling experiments (Fig. 4). From this, it can be seen that the peri-centrosomal phosphotyrosine was distinct from the endoplasmic reticulum, the lysosomes and the Golgi complex (Fig. 4, A-C). In contrast, anti-PTyr antibody staining was essentially coincident with that obtained with the CI-MPR-specific antibody (Brown and Farquhar, 1984; Fig. 4 D). These data suggested that tyrosine phosphorylated proteins around the centrosome were associated with vesicles containing the CI-MPR, which may correspond to late endosomes (Matteoni and Kreis, 1987). However, the CI-MPR is also detected in the *trans*-Golgi network in some cell types (Griffiths et al., 1993) and definitive identification of these structures will require analysis by electron microscopy.

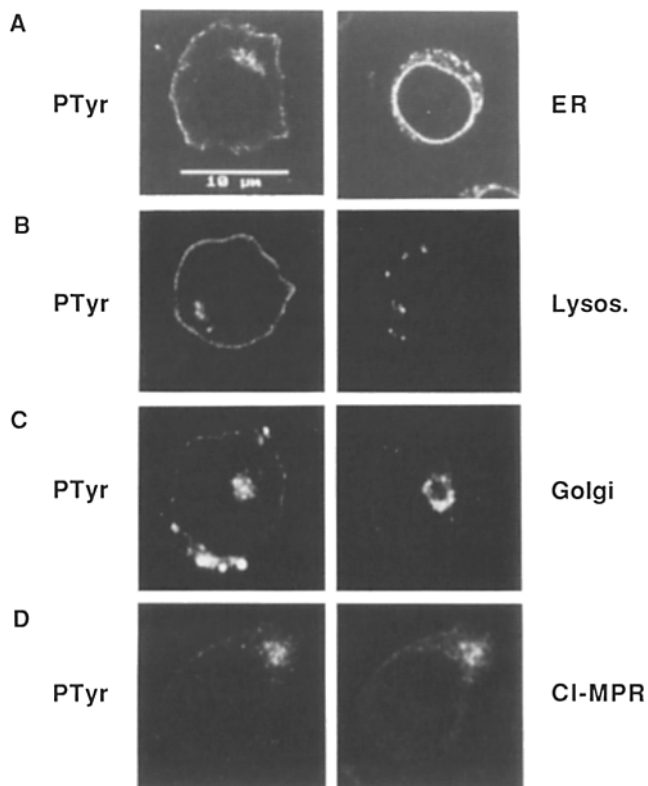


Figure 4. Localization of phosphotyrosine relative to intracellular vesicular organelles. Jurkat T cells were stimulated for 1 min with the CD3 antibody, OKT3, fixed, and permeabilized. Cells were then stained with anti-PTyr antibody (green channel) and counterstained with one of the following specific antibodies to identify intracellular vesicular organelles (red channel): (A) the KDEL-specific antibody, 259, which localized the endoplasmic reticulum. (B) A rabbit anti-Cathepsin D antiserum, 52K, was used to localize the lysosomes; (C) the rat monoclonal antibody, 23C, which is specific for β -COP, a 102-kD protein specifically localized to the Golgi; and (D) a rabbit polyclonal antibody specific for the CI-MPR, a marker for the late endosomes and *trans*-Golgi network. Images A, B, and D are single optical sections from cells which stained strongly with anti-PTyr antibody in the peri-centrosomal region. Image C is a projection of three sequential optical sections of a 0.5- μ m increment Z series. (The same magnification was used for images A to D.) Bar, 10 μ m.

Localization of lck Protein Tyrosine Kinase in Jurkat T Cells

Two members of the src family of PTKs, lck and fyn, have been implicated in the activation of T lymphocytes. In this and the following section, the localization of these two PTKs in Jurkat T cells was determined by immunofluorescence. In Fig. 5 A, it can be seen that lck was detected at the plasma membrane in all of the stained cells. As lck is a cytoplasmic PTK, it is presumably associated with the inner face of the plasma membrane (Veillette and Davidson, 1992). In the majority of cells, lck was also present in spherical structures inside the cells.

The KERP peptide antibody which was used for localization of lck recognizes the COOH terminus of the kinase. Transient transfection experiments in Cos cells indicated that this antibody did not recognize fyn (Huby, R., and Ley, S., unpub-

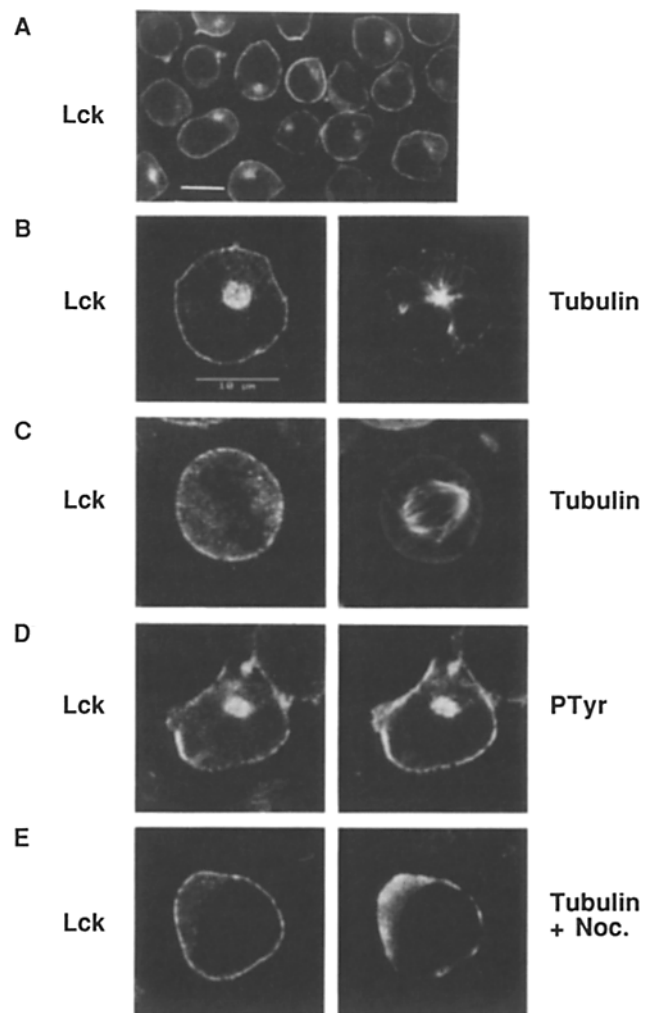


Figure 5. Localization of the protein tyrosine kinase, lck, in Jurkat T cells. In A, unstimulated Jurkat T cells were stained with a rabbit antibody specific for lck. The image is a single optical section through a field of cells to indicate the relative distribution of lck between the plasma membrane and intracellular structures. The majority of cells contained intracellular lck but, in some of the cells shown, the intracellular staining was above or below the plane of the optical section. In B and C, unstimulated Jurkat T cells were double labeled with anti-lck antibody (green channel) and an anti- α tubulin antibody (red channel). A Z series of optical sections was generated using 0.5- μ m increments and three sequential optical sections were projected to produce the images of: (B) an interphase cell demonstrating intracellular lck; and (C) a cell in mitosis. The same magnification was used for images B to E. In D, Jurkat cells have been stimulated with CD3 antibody for 1 min and then double labeled for lck (red channel) and phosphotyrosine (green channel). The image shown was generated from a single optical section. In E, unstimulated Jurkat cells were incubated with 20 μ M nocodazole for 30 min to depolymerize the microtubules *in vivo*. Cells were then double labeled for lck (green channel) and α tubulin (red channel). The image was generated from a single optical section. The homogenous cytoplasmic staining with anti- α tubulin antibody indicates that the microtubules had been effectively depolymerized *in vivo*. Bars, 10 μ m.

lished observations). The specificity of the KERP immunofluorescence staining was confirmed by competition with the immunizing peptide which reduced the signal to background levels (data not shown). In addition, no staining with KERP antibody was detected in the lck negative variant of Jurkat, JCam-1 (Straus and Weiss, 1992; Ahmad, T., and Ley, S., unpublished observations) but these cells stained brightly for lck after transient transfection with lck cDNA. Interestingly, three different antibodies directed against the NH₂-terminal unique region of lck did not produce immunofluorescent signals (data not shown). This suggests that the NH₂-terminal unique region of lck is inaccessible to antibodies, perhaps due to interaction with other proteins.

Counterstaining with an anti- α tubulin antibody localized the internal lck structures to the peri-centrosomal region of the cell. In mitotic Jurkat cells, lck was detected at the plasma membrane but was not obviously associated with the mitotic apparatus or any other intracellular structures (Fig. 5 C). Double staining cells with anti-lck antibody and anti-PTyr antibody revealed a similar distribution for lck and the major phosphotyrosine proteins in Jurkat T cells stimulated with CD3 antibody (Fig. 5 D). From the results of the previous section, these data implied that the peri-centrosomal lck was associated with vesicles containing the CI-MPR. This conclusion is supported by recent data indicating the presence of lck on endocytic vesicles (Marie-Cardine et al., 1992). Interestingly, it has also been reported that c-src is localized to late endocytic vesicles in 3T3 fibroblasts (Kaplan et al., 1992). No change in the distribution of lck was detected following CD3 antibody stimulation of Jurkat cells (data not shown). In Fig. 5 E, it can be seen that the peri-centrosomal localization of lck was lost following depolymerization of the microtubules in vivo with nocodazole (DeBrabander et al., 1986). In contrast, the presence of lck at the plasma membrane was unaffected by microtubule depolymerization.

Localization of fyn Protein Tyrosine Kinase in Jurkat T Cells

Staining of interphase Jurkat T cells with an anti-fyn-1 peptide antibody produced a pattern that was strikingly different to that seen for lck. In contrast to lck, no fyn staining could be detected at the plasma membrane but was concentrated inside the cell to a structure adjacent to the nucleus (Fig. 6 A). However, in some cells fyn staining was also detected radiating from this central structure to produce a stellate pattern. Counterstaining with an anti- α tubulin antibody revealed that the central structure labeled with anti-fyn antibody colocalized with the centrosome (Fig. 6 B). The stellate fyn staining co-distributed with microtubule bundles radiating from the centrosome. In mitotic Jurkat cells, fyn staining co-distributed with the mitotic spindle and poles identified with the anti- α tubulin antibody (Fig. 6 C).

In Fig. 6 D, it can be seen that the region of the centrosome that stained with anti-fyn antibody was distinct from the vesicular structures that stained with anti-PTyr antibody. Fig. 3 B, produced by merging the two images in Fig. 6 D, indicated that the phosphotyrosine-containing vesicles formed a "shell" around the anti-fyn antibody staining area (Fig. 3 B). This was very similar to the distribution of phosphotyrosine staining relative to γ tubulin (Fig. 3 a) and supported the notion that fyn was closely associated with the

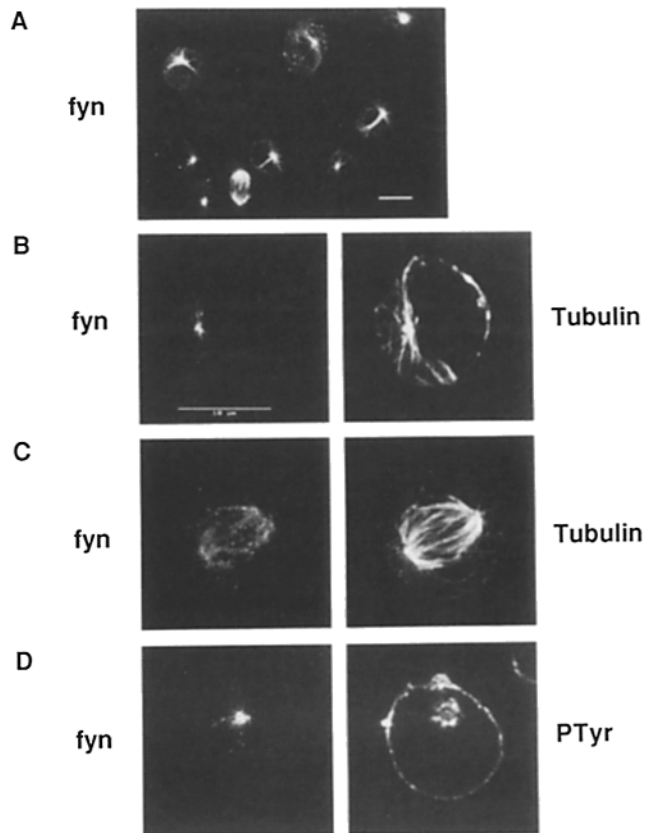


Figure 6. Localization of the protein tyrosine kinase, fyn, in Jurkat T cells. In *A*, unstimulated Jurkat T cells were stained with a rabbit antibody specific for the PTK, fyn. A Z series of optical sections was performed using 0.5- μ m increments and the image shown is a projection of images 1 to 22. In *B* and *C*, unstimulated cells were double labeled with anti-fyn peptide antibody (green channel) and the anti- α tubulin antibody, YOL 34 (red channel). Images *B*, *C*, and *D* are at the same magnification. Image *B* was generated from a single optical section. The image in *C* was generated by projecting images 1 to 36 of a Z-series using 0.5 μ m increments. In *D*, Jurkat T cells were stimulated for 1 min with a CD3 antibody and then double labeled with antibodies specific for fyn (red channel) and phosphotyrosine (green channel). A Z series of optical sections was generated using 0.5- μ m increments and sections 3 to 5 were projected to produce the image shown. Bars, 10 μ m.

centrosome itself. These data implied that although both lck and fyn were concentrated to the centrosomal region of the cell, they were in distinct locations. In mitotic Jurkat cells, weak anti-PTyr antibody staining was detected at the spindle poles which was coincident with anti-fyn antibody staining (data not shown). No change in the intracellular localization of fyn was detected after CD3 antibody stimulation of Jurkat T cells (data not shown).

The specificity of staining with the anti-fyn-1 peptide antibody (Kypta et al., 1988) was tested in two ways. Firstly, it was demonstrated that specific peptide (fyn-1), but not an irrelevant peptide (fyn-2), reduced staining to background levels (Fig. 7, *A* and *B*). A homology search of the data bank did not reveal any significant homology between the fyn-1 peptide and either tubulin isoforms (α , β , or γ) or any known microtubule associated proteins (MAPs). Secondly, staining

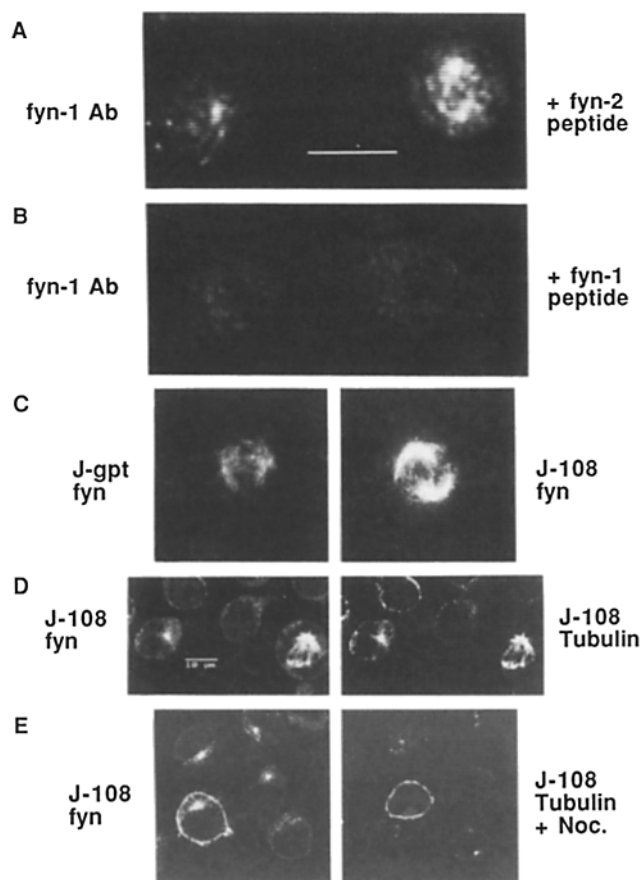


Figure 7. Specificity of staining with anti-fyn-1 peptide antibody. In *A* and *B*, unstimulated Jurkat T cells were stained with anti-fyn-1 peptide antibody in the presence of the irrelevant peptide, fyn-2, or specific peptide, fyn-1. Bound anti-fyn-1 antibody was visualized with FITC-labeled anti-rabbit IgG. The images were produced by performing a complete Z series of the stained cells using 1.0- μm increments and then projecting these optical sections as a composite image. In *C*, two transfected Jurkat cell lines have been stained with anti-fyn-1 antibody. The J-GPT cell line is transfected with control plasmid and the J-108 cell line is transfected with a vector encoding human fyn-T. The J-108 cell line expressed approximately 10-fold higher levels of fyn PTK compared with J-GPT. A complete Z series of a mitotic cell from each cell line was performed using 0.5- μm increments. These sections were then projected to produce the composite images shown and are at the same magnification as *A*. Determination of the mean pixel intensities for the two images indicated that the mitotic J-108 cells stained fivefold more intensely with anti-fyn antibody than did the control J-GPT cells. In *D*, unstimulated J108 cells were double labeled for fyn (green channel) and α -tubulin (red channel). A Z series of optical sections was generated of a field of cells using 0.5- μm increments and the images shown are a projection of five sequential sections. In *E*, J-108 cells were incubated with 20 μM nocodazole for 30 min to depolymerize the microtubule cytoskeleton *in vivo* or incubated in control medium. Cells were then stained for fyn PTK. The images are single optical sections through groups of nocodazole treated and control untreated cells and are at the same magnification as in *D*. Staining with an anti- α tubulin antibody verified that depolymerization of the microtubule cytoskeleton was effective (data not shown). Bars, 10 μm .

with the anti-fyn-1 peptide antibody was compared between "J-GTP" Jurkat T cells, which had been transfected with a control vector, and "J-108" cells, which had been transfected with a vector encoding human fyn-T, the haematopoietic isoform of the PTK. The J-108 cells expressed 10 fold higher levels of fyn PTK than the J-GPT cells (data not shown). The J-108 cells consistently stained more brightly with the anti-fyn-1 peptide antibody than the J-GPT cells and this was particularly evident for cells in mitosis in which a five fold difference in fluorescence intensity could be detected (Fig. 7 *C*). Taken together, these data strongly supported the contention that staining with the anti-fyn-1 peptide antibody reflected the distribution of fyn PTK in Jurkat T cells rather than that of a cross-reactive protein.

Significantly, the distribution of fyn in the J-108 cells was very similar to that in J6 cells and was concentrated at the centrosome in interphase cells and to the mitotic apparatus in mitotic cells (Fig 7 *D*). In the majority of J-108 cells, no fyn was detected at the plasma membrane. However, it was possible to detect fyn at the plasma membrane (Fig. 7 *E*) in a small fraction ($\sim 5\%$) of the J-108 cells in some experiments. Similar to *lck*, the localization of plasma membrane fyn in the J108 cells was unaffected by depolymerization of the microtubules *in vivo* with nocodazole, whereas the intracellular localization to the centrosome was dependent on an intact microtubule cytoskeleton (Fig. 7 *E*). It should be noted that γ tubulin remains associated with the centrosome when microtubules are depolymerized (Stearns et al., 1991) distinguishing its localization from fyn to this intracellular structure.

Localization of *lck* and *fyn* in Human T Lymphoblasts

In the experiments shown in Fig. 8, the distributions of *lck* and *fyn* were analyzed in human T lymphoblasts, which more closely represented normal T cells than the leukaemic T cell line, Jurkat. In Fig. 8 *A*, it can be seen that *lck* was present at the plasma membrane in these polyclonal T cell populations. However, in contrast to the Jurkat T cells, no *lck* was detected in the peri-centrosomal region. The distribution of *fyn* in the T lymphoblasts was very similar to that seen in Jurkat T cells. Thus, *fyn* was localized to the centrosome and also along microtubule bundles emanating from the centrosome in interphase T lymphoblasts (Fig. 8 *B*). No *fyn* was detected at the plasma membrane. In mitotic cells, *fyn* staining was very similar to α tubulin staining and co-distributed with the mitotic spindle and poles (Fig. 8 *C*).

When the TcR is induced to cap, any *fyn* that is associated with the receptor (Samelson et al., 1990) should also be localized to the cap, thereby increasing its local concentration at the inner face of the plasma membrane. The detection by immunofluorescence of any *fyn* at this location, therefore, should be facilitated. To test the effect of capping of the TcR, CD3 antibody was added to T lymphoblasts and the cells were then incubated at 37°C. After fixing and permeabilization, the cells were then counterstained with anti-fyn-1 antibody. From Fig. 9 *A*, it can be seen that *fyn* was not detected at the plasma membrane coincident with the CD3 caps but was clearly evident at the centrosome. These data imply that even when *fyn* was concentrated to one area of the plasma membrane, the level of PTK at this site was still too low to

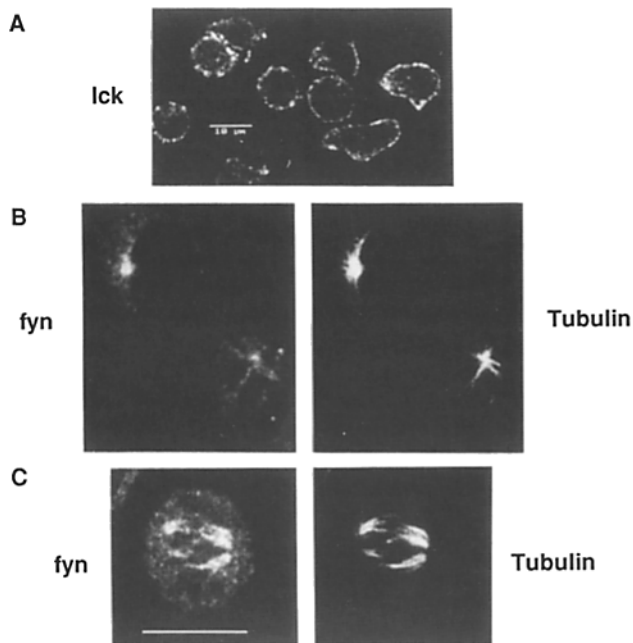


Figure 8. Localization of lck and fyn in human T lymphoblasts. In *A*, T lymphoblasts were labeled with anti-lck peptide antibody. The image of a field of cells shows a projection of three sequential optical sections of a Z-series, using 0.5- μ m increments. In panels *B* and *C*, T lymphoblasts were double labeled with anti-fyn peptide antibody (green channel) and anti- α tubulin antibody (red channel). In *B*, the image was produced from a single optical section. In *C*, a Z series of optical sections of a mitotic cell was generated using 0.5- μ m increments and the image was generated by projecting five sequential sections. Bars, 10 μ m.

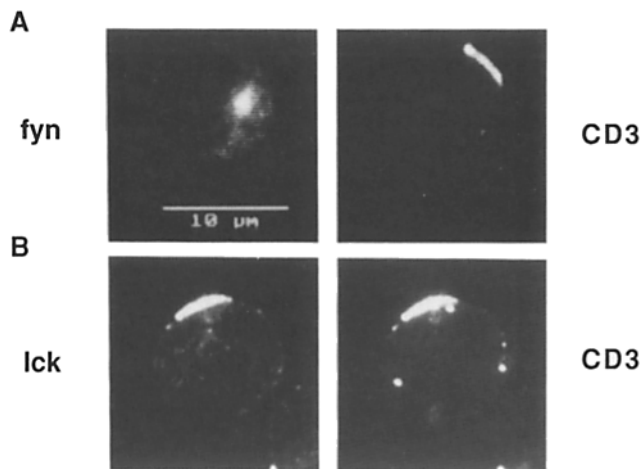


Figure 9. Effect of CD3 capping on the intracellular distribution of fyn and lck. T lymphoblasts were incubated at 37°C with the CD3 antibody, UCHT-1, to induce the formation of caps. The cells were then fixed, permeabilized, and counterstained for lck or fyn. In *A*, the capped cell was stained for fyn (red channel) and CD3 (green channel). The image was generated from a single optical section. *B* shows the image of a capped cell, double stained for lck (red channel) and CD3 (green channel), generated from a single optical section. Bar, 10 μ m.

detect by immunofluorescence. Interestingly, when the TcR was induced to cap with CD3 antibody, essentially all detectable lck at the plasma membrane was found to co-distribute with the caps (Fig. 9 *B*).

Discussion

The results presented in this study show that two members of the src family of protein tyrosine kinases in human T lymphocytes have distinct intracellular localizations. In interphase cells, lck was located at the plasma membrane and was also associated with vesicular structures containing the CIMPR, which surrounded the centrosome. These may correspond to late endosomes or the trans-Golgi network (Griffiths et al., 1993). In CD3 antibody-stimulated Jurkat T cells, the localization of lck was essentially coincident with the major anti-PTyr antibody staining pattern. Fyn was closely associated with the centrosome and also with microtubule bundles radiating from the centrosome in interphase cells. In mitotic cells, fyn, but not lck, co-localized with the mitotic spindle and poles. Fyn co-distributed with very weak anti-PTyr antibody staining at the centrosome in interphase cells and at the spindle poles in mitotic cells.

50–90% of cellular lck is stably bound to CD4 and 10–25% to CD8 in CD4+ and CD8+ T cells, respectively (Veillette and Davidson, 1992). Lck is also complexed to the β subunit of the IL 2 receptor but only at low stoichiometry (Hatakeyama et al., 1991). J6 cells do not express CD4, CD8, or the IL 2 receptor β chain as judged by FACS analysis (results not shown). In the majority of these cells, lck was detected on peri-centrosomal vesicles and at the plasma membrane. These data indicate that a fraction of lck can target to the inner face of the plasma membrane independently of its association with CD4, CD8, or the IL 2 receptor. However, it is possible that lck is associated with GPI-anchored cell surface molecules in these cells (Stefanova et al., 1991). In T lymphoblasts, which express CD4, CD8, and the IL 2 receptor β chain at their surface (Boyer et al., 1993), lck was only detected at the plasma membrane. Therefore, the localization of lck to peri-centrosomal vesicles may result from its failure to associate with these cell surface molecules and be targeted to the plasma membrane.

Fyn was not detected at the plasma membrane in wild type Jurkat cells or in T lymphoblasts. Furthermore, when CD3 was induced to form caps in T lymphoblasts and any fyn associated with the TcR would be expected to concentrate to one region of the plasma membrane, fyn was not detected at the caps. These data were somewhat surprising in view of the reported co-precipitation of fyn with the TcR (Samelson et al., 1990; Gauen et al., 1992), although the stoichiometry of this interaction is very low. One possible explanation for the immunofluorescence results is that the fyn-1 epitope in the NH₂-terminal unique region of the PTK is masked at the plasma membrane by an associated protein. However, the CST-1 antibody (Courtneidge and Smith, 1984), which recognizes the COOH terminus of fyn, produced the same staining pattern as the fyn-1 antibody, making this possibility unlikely (Jordan, P., and S. Ley, unpublished data). Rather, the inability to detect fyn at the plasma membrane by immunofluorescence is probably a reflection of the lower sensitivity of this assay relative to in vitro kinase assays. Consistent with this

notion, fyn was detected at the plasma membrane of a small fraction of Jurkat T cells that had been transfected to overexpress this PTK. Taken together, these results are consistent with a very low level of fyn, which is undetectable by immunofluorescence in wild type T lymphocytes, being targeted to the plasma membrane, where it may be associated with the antigen receptor. However, immunofluorescence analyses indicate that the majority of fyn is localized to the microtubule cytoskeleton. The specificity of the anti-fyn-1 peptide antibody staining of microtubular structures is strongly supported by several lines of evidence. Firstly, staining was ablated by addition of specific peptide but not an irrelevant peptide during the incubation with the anti-fyn-1 peptide antibody. Secondly, Jurkat T cells transfected to overexpress fyn stained more brightly with anti-fyn-1 peptide antibody than control cells. Lastly, Cos cells, which contain very low levels of fyn by *in vitro* kinase assay (Koegel et al., manuscript submitted for publication), did not stain detectably with anti-fyn-1 peptide antibody by immunofluorescence (Jordon, P., and S. Ley, unpublished data).

It has been reported previously that fyn co-localizes with the TcR at the plasma membrane of a human T lymphocyte clone, derived from peripheral blood mononuclear leukocytes, after capping of CD3 (Gassmann et al., 1992; Mustelin et al., 1992). It is possible that the intracellular distribution of fyn may vary in different types of T cells and that a large fraction of cellular fyn may be localized to the plasma membrane in specific T cell clones. However, as fyn was found to be predominantly associated with the microtubule cytoskeleton in Jurkat T cells, in HPB-ALL leukemic T cells (unpublished data) and in polyclonal T lymphoblast populations, it is unlikely that this distribution is restricted to a subset of T cells but rather is the normal location of this PTK in human T cells. It is unclear why the previous studies failed to detect any fyn associated with the microtubule cytoskeleton in the T cell clone analyzed.

Several integral membrane proteins which are localized to the plasma membrane are phosphorylated on tyrosine following activation of T lymphocytes. These include the ζ chain of the TcR (Baniyash et al., 1988), the CD3 complex (Qian et al., 1993), CD5 (Davies et al., 1992), CD6 (Wee et al., 1993), and CD45 (Stover et al., 1991). In addition, ZAP 70 (Chan et al., 1992) and PLC γ 1 (Park et al., 1991; Weiss et al., 1991) are thought to translocate to the plasma membrane following activation (Rhee, 1991; Weiss, 1993). Consistent with the location of these substrates, a dramatic increase in the level of phosphotyrosine was detected at the plasma membrane by immunofluorescence, after activation of Jurkat T cells with CD3 antibody. Lck colocalized with phosphotyrosine staining at the plasma membrane. However, fyn was present at undetectable levels at the plasma membrane in Jurkat T cells. The relative distributions, therefore, suggest that lck, rather than fyn, may mediate the increase in tyrosine phosphorylation at the plasma membrane after TcR cross-linking. Consistent with this hypothesis, CD3 cross-linking does not induce tyrosine phosphorylation in a mutant of Jurkat that does not express functional lck, but has normal levels of fyn (Straus and Weiss, 1992). Lck, therefore, appears to be necessary for the induction of tyrosine phosphorylation by the TcR, although the involvement of other PTKs, such as ZAP 70 (Chan et al., 1992), is not excluded. The co-distribution of lck with CD3 following

capping suggests that the TcR may be physically linked with lck. This may provide a direct mechanism by which lck could be regulated by the TcR. In support of this hypothesis, lck is found to co-immunoprecipitate with CD3 antibodies (Beyers et al., 1992; Burgess et al., 1992). However, it is not excluded that the association between the TcR and lck may be an indirect one mediated via CD4 (Mittler et al., 1989).

Genetic experiments in cell lines and in mice indicate a role for fyn in the early signaling events triggered by the TcR. However, this may be restricted to a particular stage of T cell development (Cooke et al., 1991; Appleby et al., 1992; Davidson et al., 1992). The co-precipitation with the TcR (Samelson et al., 1990) suggests that fyn may function in T cell signaling via a direct interaction with the antigen receptor, although this may involve only a small fraction of total cellular fyn. The activation of fyn following CD3 cross-linking supports this hypothesis (Tsygankov et al., 1992). However, it is possible that the microtubule-associated fyn could be functionally important in the activation process. It is not presently clear whether the effects on signaling attributed to fyn in genetic experiments are due to the small fraction of the PTK that is associated with the TcR, or alternatively to the population of kinase that is concentrated to the centrosome and along microtubule bundles. The generation of mutants of fyn, deficient in their ability to bind to the TcR or to target to the microtubule cytoskeleton, may help to resolve which of these possibilities is correct.

Src PTKs contain a conserved site of tyrosine phosphorylation near their carboxy terminus (Cooper, 1990) and phosphorylation of this site negatively regulates PTK activity (Amrein and Sefton, 1988; Cartwright et al., 1987; Piwnicka-Worms et al., 1987). Surface expression of the protein tyrosine phosphatase, CD45, is necessary for antibody-mediated coupling of the TcR to PTKs (Koretzky et al., 1990; Koretzky et al., 1991). The requirement for CD45 in T cell activation may be to control the activity of src family kinases by dephosphorylation of the negative regulatory COOH-terminal tyrosine. Consistent with this hypothesis, lack of CD45 expression increases the regulatory COOH-terminal phosphorylation of lck and fyn. However, in the majority of CD45-deficient T cell lines, the phosphorylation of lck is affected to a greater extent than fyn (Ostergaard et al., 1989; Sefton and Trowbridge, 1989; Shiroo et al., 1992; Hurley et al., 1993; McFarland et al., 1993; Sieh et al., 1993). This has raised the possibility that fyn may not be as efficient a substrate as lck for CD45 *in vivo*, although CD45 can dephosphorylate both fyn and lck *in vitro* (Mustelin and Altman, 1990; Mustelin et al., 1992). However, the confocal data suggests that it is the localization of lck and fyn relative to CD45 that is important. Thus, lck is present at the plasma membrane where it can interact with CD45 and be dephosphorylated. Consistent with this hypothesis, lck co-precipitates with CD45 after mild detergent extraction of human T cells (Schraven et al., 1991; Koretzky et al., 1993) and also lck co-distributes with CD45 in co-capping experiments (Guttinger et al., 1992). In contrast, the majority of fyn is unavailable for interaction at the plasma membrane and its COOH-terminal phosphorylation is, therefore, relatively insensitive to CD45 expression.

The kinase activity of c-src increases at mitosis (Chachalaparampil and Shalloway, 1988) as a result of dephosphorylation of its COOH-terminal tyrosine 527 (Bagrodia et al.,

1991). This process is regulated by p34^{cdc-2} which phosphorylates c-src at three terminal serine and threonine residues at mitosis (Shenoy et al., 1989; Morgan et al., 1989; Shenoy et al., 1992). Thus, there may be a signalling pathway from the M phase cell cycle regulator, p34^{cdc-2}, to c-src. Consistent with this notion, c-src is localized to the spindle poles in mitotic 3T3 fibroblasts (David-Pfeuty and Nouvian-Dooghe, 1990; Kaplan et al., 1992). The striking association of fyn with the mitotic apparatus implies that this PTK may also be involved in regulating events that occur at M phase. This possibility is currently being investigated.

In conclusion, the data in this paper demonstrate that two members of the src family of PTKs, lck and fyn, have essentially non-overlapping intracellular distributions in T lymphocytes. In vitro, src-family kinases demonstrate little specificity for phosphorylation of substrates (Cooper, 1990). However, as a consequence of their localizations, lck and fyn may be accessible to distinct regulatory proteins and substrates. Therefore, the intracellular targeting of lck and fyn could be an important contributory factor in determining their function. This raises the possibility that these two PTKs regulate different aspects of T cell activation or development. Indeed, studies of both mutant T cell lines and mutant mice have indicated that lck and fyn are not functionally identical which supports this hypothesis (Appleby et al., 1992; Molina et al., 1992; Straus and Weiss, 1992).

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