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Interaction of p59fyn Kinase with the Dynein Light Chain, Tctex-1, and Colocalization During Cytokinesis

Kerry S. Campbell, Suzanne Cooper, Mark Dessing, Sol Yates, and Annie Buder

The protein tyrosine kinase p59fyn (Fyn) plays important roles in both lymphocyte Ag receptor signaling and cytokinesis of proB cells. We utilized yeast two-hybrid cloning to identify the product of the tctex-1 gene as a protein that specifically interacts with Fyn, but not with other Src family kinases. Tctex-1 was recently identified as a component of the dynein cytoskeletal motor complex. The capacity of a Tctex-1-glutathione S-transferase fusion protein to effectively bind Fyn from cell lysates confirmed the authenticity of this interaction. Tctex-1 binding required the first 19 amino acids of Fyn and integrity of two lysine residues within this sequence that were previously shown to be important for Fyn interactions with the immunoreceptor tyrosine-based activation motifs (ITAMs) of lymphocyte Ag receptors. Expression of tctex-1 mRNA and protein was observed in all lymphoma lines analyzed, and immunofluorescence confocal microscopy localized the protein to the perinuclear region. Analysis of a T cell hybridoma revealed prominent colocalization of Tctex-1 and Fyn at the cleavage furrow and mitotic spindles in cells undergoing cytokinesis. Our results provide a unique insight into a mechanism by which Tctex-1 might mediate specific recruitment of Fyn to the dynein complex in lymphocytes, which may be a critical event in mediating the previously defined role of Fyn in cytokinesis. The Journal of Immunology, 1998, 161: 1728–1737.
unit that plays important roles in intracellular retrograde organelle transport, membrane trafficking, mitotic spindle localization, and centrosome separation during mitosis (38–40). Tctex-1 has also recently been shown to be a component in inner arm II of flagellar dynein (41). A human homologue of the tctex-1 gene was reported to encode 94% protein homology to the murine protein (42), and a related human gene, “candidate RP3,” encodes a protein with 55% amino acid identity to human Tctex-1 (43). A homologue of another mouse t complex-encoded protein, Tctex-2, which has limited sequence similarity to Tctex-1, was also recently identified as a light chain component of flagellar dynein in Chlamydomonas (44), and a Tctex-1 homologue in the same species exhibits 60% identity to the mouse protein (41).

To better define functionally important protein interactions with the unique N-terminal domain of Fyn, we have utilized the yeast two-hybrid technique. Using this system, we could not demonstrate direct Fyn interactions with the ITAM-containing B cell Ag receptor components, Ig-α or Ig-β. Upon screening a B cell cDNA library, however, Tctex-1 was recognized as a strong Fyn-binding protein in the yeast system, and this interaction was localized to the first 19 amino acids of Fyn. The validity of this protein-protein interaction was confirmed by the capacity of a Tctex-1 fusion protein to bind Fyn from cell lysates. Fyn and Tctex-1 were found to colocalize during cytokinesis in a T cell hybridoma, thereby suggesting that the interaction with Tctex-1 can selectively recruit Fyn to the dynein motor complex during mitosis of lymphocytes.

Materials and Methods

Mammalian cell lines

Cell lines and their sources were the murine proB cell line 38B9 (Dr. A. Rolink, Basel, Switzerland); cytotaxic T cell clone CTLL-2 (Dr. J. García-Sanz, Madrid, Spain); COS-7 cells (Dr. B. Imhof, Geneva, Switzerland); the murine B cell lymphomas K46, A20, and WEHI-231.7; and NIH 3T3 cells transfected with the Zip-Fyn plasmid to express high levels of p59ff protein (45) (all from Dr. J. Cambier, Denver, CO). The fyn-transfected murine T cell hybridoma N17 (10) was generously provided by Dr. T. Yamamoto and colleagues (University of Tokyo, Japan). Cells were cultured as previously described (46). Geneticin (G-418; Life Technologies, Gaithersburg, MD) was supplemented in cultures of COS-7 transfants (500 μg/ml) and N17 cells (770 μg/ml).

Antibodies

Anti-Tctex-1 polyclonal Abs (pAb) were generated in rabbits against purified full length murine Tctex-1, which was produced in Escherichia coli as a GST fusion protein (see below) and cleaved from the GST by thrombin (Sigma). The Tctex-1-specific Ab was affinity purified using the cleaved protein on CNBr-activated Sepharose (Pharmacia, Uppsala, Sweden), eluted with 3.5 M MgCl2 (pH 7.2), dialyzed against PBS, and stored at −70°C. Anti-Fyn Abs (both from Santa Cruz Biotechnology, Santa Cruz, CA) were a mAb (sc-434, mouse IgG1; agarose conjugate used for immunoprecipitation or unconjugated for intracellular staining) and a rabbit anti-Tctex-1 (10 μg/ml) or rabbit anti-Fyn pAb (1 μg/ml). Secondary reagents; this result can be avoided by using 125I-labeled protein A (0.5 μC/ml; Amersham), and proteins were visualized by autoradiography. It should be noted that detection of Tctex-1 can be obscured by a background band at 14 kDa in lymphocyte whole cell lysates when probed with horseradish peroxidase-conjugated secondary reagents; this result can be avoided by using 125I-labeled protein A.

Immunoblotting

Immunoprecipitates or whole cell lysates (lysed directly in Laemmli buffer) were separated on SDS-PAGE and electroblotter-transferred to Immobilon-P membranes (Millipore, Bedford, MA). Immunoblotting and stripping were performed as previously described (53). Abs were rabbit anti-Tctex-1 (10 μg/ml) or rabbit anti-Fyn pAb (1 μg/ml). Secondary reagent was 125I-labeled protein A (0.5 μC/ml; Amersham), and proteins were visualized by autoradiography. It should be noted that detection of Tctex-1 can be obscured by a background band at 14 kDa in lymphocyte whole cell lysates when probed with horseradish peroxidase-conjugated secondary reagents; this result can be avoided by using 125I-labeled protein A.

Metabolic labeling, protein precipitations, and in vitro kinase reactions

COS-7 cells were metabolically pulse-labeled in some studies for 15 to 45 min with 5 mCi of [35S]cysteine/methionine (Amersham) in 20 ml of cysteine/methionine-free Dulbecco’s modified Eagle’s medium and chased for 2 to 9 hr in normal medium. Cells were lysed for 30 min on ice in 1% digitonin buffer, 1% Triton X-100 (Surfact-Amps; Pierce, Rockford, IL) buffer, or RIPA buffer (1% Triton X-100, 0.1% sodium deoxycholate (Merck, Darmstadt, Germany)), and 0.1% SDS (Bio-Rad, Hercules, CA), each containing 75 mM NaCl, 10 mM Tris (pH 7.4), 10 mM NaF, 0.4 mM EDTA, 1 mM Pefabloc SC (Boehringer Mannheim), 2 mM sodium orthovanadate, and 1 μg/ml each of aprotinin, soybean trypsin inhibitor, and leupeptin (reagents were from Sigma unless otherwise noted). Lysates were microfuged at 14,000 rpm for 15 min and subjected to precipitation for 2 to 4 h with GST fusion proteins (10 μg/sample) or Abs (2–5 μg/sample)

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FIGURE 1. Tctex-1 can interact specifically with the N-terminal domain of Fyn. Yeast were transfected with various combinations of plasmid constructs encoding the indicated Gal4 DB fusion proteins and Gal4 TA fusion proteins. Transfectants were subsequently streaked on agarose media containing (+) or lacking (−) histidine. Yeast transfectants that grow without histidine demonstrate an interaction between the fusion protein partners that recruits the TA to the reporter gene and drives its transcription to permit growth. Fusion protein partners were Tctex-1, the N-terminal domains (NH) of Fyn, Blk, LynA, and LynB, and the cytoplasmic domains (cyto.) of Ig-α and Ig-β.

GST and epitope-tagged fusion proteins and protein expression

GST fusion protein constructs of the Fyn N-terminal domain (amino acids 1–85) and Tctex-1 (amino acids 1–113) were generated by excision of these cDNAs from pPC62 and pPC86 and ligation into pGEX-4T-2 plasmid (Pharmacia). Other GST fusion constructs were human platelet-derived growth factor receptor kinase insert region (amino acids 698–797 in pGEX-3X; provided by Dr. A. Kazlauskas, Harvard University) and cytoplasmic domains of murine Ig-α and Ig-β (3) (provided by Dr. J. Cambier). E. coli (Top10F) were transformed with these plasmids and induced for 2 to 4 h with 0.3 mM isopropyl β-D-thiogalactopyranoside as described (3). Cells were pelleted, and probe sonicated on ice in 10 ml of Tris-buffered saline (TBS; 10 mM Tris, pH 7.4, 150 mM NaCl) containing Complete protease inhibitor (Boehringer Mannheim; 1 tablet/50 ml TBS), quenched with 1% Triton X-100 buffer (as above), and secondarily immunoprecipitated with anti-Fyn mAb-coupled agarose as previously described (46). Phosphoamino acid analysis was performed as previously described (54).

Immunofluorescence confocal microscopy

Some cells were grown on chamber slides (see Fig. 7A) or allowed to adhere on poly-l-lysine coated slides (Fig. 7B) before staining. Other cells were stained in suspension after sorting and subsequently cytospun onto slides (Fig. 7C). The following staining protocol was utilized, since it resulted in optimal retention of β-tubulin structure in cells. Cells were washed in 37°C serum-free Iscove’s modified Dulbecco’s medium before fixing at 37°C for 10 min with 3% paraformaldehyde (in PBS, pH 7.0, and 5.4% glucose). Subsequent steps were performed at room temperature. After washing twice in PBS (pH 7.4), fixed cells were permeabilized for 30 min in PBS/saponin (PBS with 0.1% saponin and 1 mM HEPES) and washed again in the same. Cells were blocked for 30 min in PBS/saponin/BSA (PBS/saponin with 2% BSA) and washed once in PBS/saponin. Cells were then incubated for 30 to 45 min with optimal concentrations of primary Ab in PBS/saponin/BSA and washed three times with PBS/saponin. An optimal concentration of secondary Ab in PBS/saponin/BSA was added for 30 to 45 min, and cells were washed three times with PBS/saponin, followed by three washes with PBS. Some cells were fixed, stained with Hoechst 33342 (Molecular Probes), and sorted on a FACS Vantage into G0/G1, S, and G2/M stages (55) before intracellular Ab staining (Fig. 7C). Coverslips were mounted with Mowiol (Hoechst, Frankfurt, Germany) or Fluoromount-G (Southern Biotechnology) mounting solution and analyzed through a 63×/1.4 Zeiss Plan-Apochromat lens on a Zeiss Axioplan inverted microscope fitted with a Bio-Rad MRC 1024 laser scanning confocal imaging system. Images were acquired in accumulation mode, analyzed using LaserSharp software (Bio-Rad, version 2.1A), and processed with Adobe Photoshop (version 3.0, Adobe Systems, San Jose, CA). Compensations were rigidly controlled using single-stained cell samples and/or independent sequential excitation of each fluorescent dye to assure lack of bleedover between the green channel and the red channel. Pretreatment of the anti-Tctex-1 pAb preparation with recombinant Tctex-1 protein completely eliminated immunofluorescent reactivity, thereby indicating specificity of the staining.

Results

Identification of Tctex-1 as a Fyn-binding protein

Previous experiments with GST fusion proteins have suggested that the N-terminal domains of Fyn and LynA can interact directly with the cytoplasmic domains of the B cell Ag receptor chains, Ig-α and Ig-β (31). As demonstrated in Figure 1, we could not detect any direct protein interactions between these kinase N-terminal domains (or LynB; data not shown) and either Ig-α or Ig-β cytoplasmic domains in a Gal4-based yeast two-hybrid system in either orientation (DB or TA fusions), nor in a more sensitive LexA-based system (data not shown; sensitivity described in Ref. 46). All fusion proteins of appropriate size were produced in the yeast transfectants, however, as assessed by anti-GAL4 immunoblotting (data not shown). Although these results suggest that Fyn cannot directly interact with Ig-α or Ig-β, one must consider that many factors can contribute to a negative result in this assay, and
between Fyn and Ig-α protein in yeast two-hybrid experiments (data not shown). The majority of yeast colonies that scored positive in this screen contained the full length tctex-1 cDNA (top) and β-actin cDNA (bottom). The various murine cell lines tested are representative of proB cells (38B9), immature B cells (WEHI-231.7), mature B cells (K46), and mature T cells (CTLL-2).

Therefore, this is not proof that these interactions cannot occur in vivo.

To identify additional candidate proteins that interact efficiently with the Fyn N-terminal domain, we screened a cDNA library from the K46 B cell lymphoma using this domain as bait in the two-hybrid system. The majority of yeast colonies that scored positive in this screen contained the full length tctex-1 cDNA (34), which encodes a protein component of the ATP-dependent dynein motor complex (37). The interaction with Fyn was specific, as demonstrated in Figure 1, since the tctex-1 gene product did not interact with the N-terminal domains of LynA, LynB, Blk, or p56Lck; see Fig. 5) as measured by activation of either His3 (histidine-free growth selection) or lacZ (β-galactosidase; data not shown) reporters. In addition, the N-terminal domain of Fyn was not observed to form homotypic interactions (Fig. 1). Tctex-1 failed to interact with Ig-α or Ig-β cytoplasmic domains in this assay (data not shown), and we could not demonstrate any capacity of Tctex-1 to “couple” Ig-α to Fyn when expressed as a third protein in yeast two-hybrid experiments (data not shown). The interaction of Fyn with Tctex-1 is clearly a strong protein-protein interaction when compared with the lack of detectable interactions between Fyn and Ig-α or Ig-β.

Expression of Tctex-1 in lymphoid cells

The tctex-1 gene had been cloned from sperm cDNA and identified as a product of the t complex of mice (34). Previous analysis by Northern blotting had determined that the gene was strongly expressed in the testes and ovaries (34) and weakly in the thymus of wild-type mice, but a detailed analysis in hemopoietic tissues was lacking. As shown in Figure 2, Northern blot analysis demonstrated ample expression of tctex-1 mRNA in all tissues examined from BDF₁ mice. Thymic expression was more pronounced than that of spleen, while significant expression of tctex-1 mRNA was observed in both B and T lymphocyte lines (Fig. 2). Less abundant expression of tctex-1 was also evident in the brain, suggesting that the protein is also available for interaction with Fyn in neuronal cells (Fig. 2).

An affinity-purified pAb against recombinant Tctex-1 was prepared to test lymphocyte cell lines for protein expression. To characterize this pAb preparation, a C-terminal FLAG epitope-tagged version of Tctex-1 was stably expressed in COS-7 cells. As shown in Figure 3a, immunoprecipitation from a 35S metabolically labeled transfected (clone C20) with either anti-Tctex-1 or anti-FLAG Abs resulted in the purification of a specific band of about 15 kDa. This apparent mass is slightly higher than that predicted for Tctex-1 (~14 kDa) due to the epitope tag. This band was the major specific protein immunoprecipitated by the anti-Tctex-1 pAb preparation when compared with control immunoprecipitations (anti-human MHC class I and anti-tubulin mAbs in lanes 3 and 4 of Fig. 3a). In addition, the pAb preparation and anti-FLAG mAb reproducibly immunoprecipitated similar amounts of Tctex-1.

Several murine B and T cell lines were tested for Tctex-1 expression using the pAb preparation. Although Tctex-1 was difficult to detect by immunoblotting with the pAb in whole cell lysates of lymphocyte lines (one to two million cell equivalents per lane; data not shown), it could be readily immunoprecipitated with this Ab preparation from several lymphocyte cell lines and detected by immunoblotting with the same Ab. The protein was observed in immunoprecipitates from 50 to 70 million cell equivalents of the fyn-transfected T cell hybridoma, N17 (10), and the B cell lymphomas K46, A20, and WEHI 231.7 (Fig. 3b; data not shown). The specific reactive band was observed at 14 kDa, as predicted, and this migration corresponded exactly to that of recombinant Tctex-1 as shown (Fig. 3b). No significant improvement in the amount of Tctex-1 immunoprecipitated from lysates of K46 cells could be demonstrated upon lysis with the addition of other nonionic detergents, harsher RIPA buffer, 10 mM ATP, or nocodazole pretreatment of cells, and only slight improvement was achieved using a monoclonal anti-dynein intermediate chain Ab (data not shown). Therefore, both mRNA and the protein product of the tctex-1 gene were detectably expressed in hemopoietic tissues as well as T and B lymphocyte lines, but protein levels were routinely low in these cells.

Despite significant efforts, we could not detectably coimmunoprecipitate the two proteins from Fyn-transfectants of COS-7 cells, NIH 3T3 fibroblasts, or the T cell hybridoma N17 by immunoprecipitation of either protein or dynein intermediate chain (data not shown). Detergents tested in these studies included Triton X-100, digitonin, CHAPS (3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate), and RIPA, and detection methods employed were either 35S metabolic labeling, immunoblotting, or in vitro phosphorylation of immunoprecipitated kinase in the presence of [γ-32P]ATP. These results suggest that the protein interaction is either transient in these cells or the off rate of the interaction is high, thereby limiting detection after washing of immunoprecipitates.

Expression with Tctex-1-GST fusion protein in vitro

Due to the difficulties in demonstrating coimmunoprecipitation, we tested the capacity of recombinant Tctex-1-GST fusion protein to interact with Fyn in cell lysates. Digitonin lysates of Fyn-transfected NIH 3T3 cells (45) were adsorbed with various GST fusion proteins.
proteins, including Tctex-1, and precipitates were washed and subjected to in vitro \(^{32}\)PATP phosphorylation reactions to detect associated phosphoproteins. As presented in Figure 4a, GST fusion proteins of the cytoplasmic domains of Ig-\(\alpha\), and to a lesser extent, Ig-\(\beta\), were significantly phosphorylated in this assay, while Tctex-1-GST fusion protein was only weakly phosphorylated. The Tctex-1-GST fusion protein, however, selectively coprecipitated a phosphoprotein band of about 59 kDa, which comigrated with Tctex-1-GST was also found to be exclusively incorporated onto tyrosine phosphotyrosine, which further verified its identity as autophosphorylated Fyn (Fig. 4b). Although Tctex-1-GST did not provide a good substrate for the adsorbed Fyn in this assay, the minimal \(^{32}\)P labeling of Tctex-1-GST was also found to be exclusively incorporated onto tyrosine residues (Fig. 4b). These results suggest that Tctex-1 can be weakly tyrosine phosphorylated by Fyn kinase. Finally, Fyn binding to Tctex-1-GST was confirmed by immunoblotting with anti-Fyn Ab as demonstrated in Figure 4c. The Ig-\(\alpha\)-GST fusion protein was also able to adsorb Fyn in both of these assays as has previously been reported (3), while Ig-\(\beta\), PDGFR-KL, and the N-terminal domain of Fyn did not bind appreciable amounts of the enzyme. In summary, the Tctex-1-GST fusion protein can selectively bind Fyn from digitonin lysates in vitro, thereby confirming the yeast two-hybrid interaction.

**Truncation analysis defining interacting sequences in Fyn and Tctex-1**

To identify the specific interacting domains in both Fyn and Tctex-1, truncation analysis studies were undertaken in the yeast two-hybrid system. When truncations of the 85-amino acid N-terminal domain of Fyn were tested as demonstrated in Figure 5a, elimination of just the first 6 amino acids from the N terminus completely abrogated the interaction. The C-terminal amino acids of this domain, however, did not appear to contribute to the interaction with Tctex-1, yet amino acids 1 through 19 still exhibited strong interaction. On the other hand, further truncation to amino acids 1 through 10 completely abolished the interaction (see Fig. 5a). Previous studies by Timson Gauen et al. (32) have indicated that amino acids 1 through 10, and in particular the lysine residues at positions 7 and 9 of Fyn (Fig. 5b), are critical elements in the interaction of this domain with ITAM-containing sequences on lymphocyte Ag receptors. We also tested for involvement of lysines 7 and 9 in interaction with Tctex-1 by mutating them to alanines in the complete N-terminal domain. As shown in Figure 5c, this mutated fragment of Fyn did not interact with Tctex-1, indicating that these lysine residues are involved in interactions of the kinase with both ITAM-containing sequences and Tctex-1. Thus, Tctex-1 interacts with the first 19 amino acids of Fyn; lysine residues at positions 7 and 9 are critical elements in this interaction domain, although they are clearly not the only binding residues, as determined in the truncation analysis (Fig. 5a).

Tctex-1 was also truncated and tested for interaction with Fyn in the two-hybrid system. As shown in Figure 6, Tctex-1 was very sensitive to truncation from both ends, since truncation of amino acids 105 through 113 completely abolished the interaction with Fyn, and elimination of amino acids 1 through 12 significantly reduced the interaction. These results demonstrate that the protein requires integrity of both termini to form the Fyn-interacting structural domain.
Intracellular colocalization of Tctex-1 and Fyn proteins in T lymphocytes during cytokinesis

Confocal immunofluorescence analysis was performed to determine the intracellular localization of Tctex-1. Analysis of C-FLAG-Tctex-1-transfected COS-7 cells demonstrated that anti-FLAG mAb (Fig. 7A) and anti-Tctex-1 pAb (identical pattern; data not shown) diffusely stained the cytoplasm with the majority of staining concentrated in the perinuclear region. Alternatively, microtubules (stained with anti-β-tubulin) emanated from the perinuclear Tctex-1 stained region and extended to the periphery of the cell.

FIGURE 4. Fyn kinase binds to a GST-fusion protein of Tctex-1 in vitro. a, Fyn selectively binds to Tctex-1-GST fusion protein in vitro. Digitonin lysates of fyn-transfected fibroblasts (ZIP-Fyn cells; 2.5 × 10⁶/sample) were adsorbed with the indicated GST fusion proteins or anti-Fyn mAb and subjected to in vitro kinase reactions. Ten percent was retained to analyze primary precipitations (left panel), and the remainder was reimmunoprecipitated with anti-Fyn mAb (right panel). Fusion proteins were GST fused to cytoplasmic domains of Ig-α or Ig-β, kinase insert region of platelet-derived growth factor receptor (PDGFR KI), full length Tctex-1, or amino acids 1 through 85 of Fyn (Fyn-NH). Phosphoprotein bands corresponding to the Tctex-1-GST fusion protein (open arrow) and the p59fyn band reimmunoprecipitated from the Tctex-1-GST precipitation (closed arrow) are marked. b, GST-Tctex-1 and associated p59fyn are exclusively phosphorylated on tyrosine residues. The marked 32P-labeled phosphoprotein bands in panel a were subjected to phosphoamino acid analysis. c, Anti-Fyn immunoblotting confirmed Fyn binding to Tctex-1-GST. Digitonin lysates of ZIP-Fyn cells (14 × 10⁶/sample) were adsorbed with GST fusion proteins or anti-Fyn mAb. SDS-PAGE-separated samples were immunoblotted with anti-Fyn pAb.

FIGURE 5. Truncation mapping of the domain within the N terminus of Fyn that interacts with Tctex-1. a, The first 19 amino acids of Fyn interact with Tctex-1. Truncation mutants of the N terminus of Fyn were tested as Gal4 DB domain fusions for interaction with full length Tctex-1 fused to Gal4 TA domain. Tctex-1 fused to the Gal4 DB domain was also tested for interaction with the N-terminal domain of Lck kinase. Transfectants were tested for activation of a histidine-free growth reporter and a β-galactosidase reporter. Magnitudes of reporter activation are indicated (+++ = strong, ++ = none detected). b, Sequence of amino acids 1 through 19 of murine Fyn that interacts with Tctex-1. Lysines 7 and 9, myristoylated glycine 2 and palmitoylated cysteines 3 and 6 are marked. c, Mutation of lysines 7 and 9 of the N terminus (-NH) of Fyn abrogates interaction with Tctex-1. Mutant Fyn N-terminal domain (amino acids 1–85) with lysines 7 and 9 changed to alanines (KK/AA) was compared with wild-type Fyn domain (amino acids 1–85) for interaction with Tctex-1 or the N-terminal domain of Lck in the histidine-free growth assay.
Tctex-1 Truncations

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+ Histidine - Histidine

**FIGURE 6.** Truncation mapping of Tctex-1 domains that interact with Fyn. The N-terminal domain of Fyn (amino acids 1–85) was tested as a Gal4 TA fusion protein for interaction capacity with the truncation mutants of Tctex-1 fused to the Gal4 DB domain as assessed by growth on histidine-free medium. Magnitudes of reporter activation are indicated (++ = strong, + = weak, = none detected).

cells (Fig. 7A). Tctex-1 staining was not detected in the nucleus or plasma membrane.

Double-staining intracellular immunofluorescence studies were also performed to determine whether Tctex-1 and Fyn are colocalized in lymphocytes. The fyn-transfected murine T cell hybridoma, N17 (10), provided detectable levels of kinase for these studies. Elevated Fyn expression in N17 cells was clearly evident in immunoblots of whole cell lysates and immunofluorescent staining when compared with other lymphocyte cell lines (data not shown). Most N17 cells exhibited Fyn staining that was predominantly concentrated to the plasma membrane, although some cells exhibited significant foci of Fyn staining within the cytoplasm (Fig. 7B) as previously reported by Ley et al. (23). Although Tctex-1 staining was concentrated within the perinuclear/cytoplasmic region and generally distinct from Fyn-stained regions, a subset (~25%) of the T hybridoma cells demonstrated distinct cytoplasmic foci of Fyn, some of which were clearly colocalized with Tctex-1 (Fig. 7B). We surmised that this inconsistent colocalization of Fyn with Tctex-1 within the population of T hybridoma cells might represent colocalization only at a distinct stage(s) of the cell cycle. To subdivide the cells into distinct stages of cycle, the N17 cells were sorted by DNA content on FACS into G0/G1, S, and G2/M stages before intracellular Ab staining. Although no consistent colocalization was identified in the G0/G1 or S stage populations (data not shown), the G2/M stage population exhibited a unique overlap in staining that was observed in all dividing cells. As shown in Figure 7C, this population is enriched in cells undergoing cytokinesis, which exhibited a striking colocalization of Fyn and Tctex-1 at the developing cleavage furrow that forms as daughter cells begin to divide. In addition, colocalization was evident in these cells at the mitotic spindles. It is interesting to note that Tctex-1, but not Fyn or β-tubulin, seems to interdigitate through the segregated chromosomes and connects the mitotic spindles with the cleavage furrow via fibrous arrays. β-Tubulin staining, although a major component of the mitotic spindles, did not colocalize with Fyn and Tctex-1 at the center of the cleavage furrow in the cell cross-sections as presented in Figure 7C. Overall, this stage of cell division was the only interval during which we consistently observed intracellular colocalization of Fyn with Tctex-1. Tctex-1 staining was always perinuclear throughout all other stages of the cell cycle, and we never observed staining of the nucleus or plasma membrane. In conclusion, we observed highly reproducible colocalization of Fyn and Tctex-1 during cytokinesis at both the cleavage furrow and mitotic spindles in this T cell hybridoma.

**Discussion**

We have identified and characterized a novel protein interaction between the N-terminal domain of Fyn protein tyrosine kinase and Tctex-1, which has recently been described as a light chain component of the cytoplasmic ATP-dependent dynein motor complex. The interaction was confirmed in vitro using a Tctex-1-GST fusion protein. Tctex-1 can interact with the first 19 amino acids of Fyn, and lysines at positions 7 and 9 of this sequence are critical elements for the interaction. Immunofluorescent confocal microscopy showed that the two proteins consistently colocalize during cytokinesis at both the cleavage furrow and mitotic spindles of a T cell hybridoma, suggesting a role for this interaction in cell division. The ubiquitous expression of Tctex-1 suggests that its interaction with Fyn might also occur in other tissues in which Fyn is readily expressed, most notably neuronal tissues. In view of the limited proliferation of neuronal tissues, the interaction in such tissues would be less likely to play a role in the division of mature cells than in lymphocytes, suggesting additional functional roles for the interaction. Kai et al. (56) have also recently reported tctex-1 as one of several cDNAs that were cloned using a larger portion of Fyn as a bait in the yeast two-hybrid system, although they did not attempt to address the interaction biochemically or further define the interaction domains.

Amino acids 1 through 19 of Fyn (see Fig. 5b), which were identified as interacting with Tctex-1, encompass the domain that interacts with unphosphorylated ITAM motifs in lymphocyte Ag receptors (SH4, amino acids 1–10 (31, 32)) and contain three lipid modification sites (Fig. 5b). Cotranslational myristoylation of the glycine at position 2 is believed to be a permanent alteration, while posttranslational palmitoylation of cysteines at positions 3 and 6 is a reversible modification (24–29, 57). Interestingly, the two lysines at positions 7 and 9 within the Fyn interactive sequence are critical elements for interactions of Fyn with both Tctex-1 (Fig. 5c) and ITAM sequences (32). These basic residues have previously been implicated in enhancing plasma membrane association of Src family kinases by interacting with negatively charged phospholipid head groups (24). Our results and those of Timson Gauen et al. (32) indicate that these lysine residues and presumably other residues in the extreme N-terminal domain of Fyn are also important for protein-protein interactions. One attractive hypothesis would be that a lymphocyte activation or cell cycle-related event might promote depalmitoylation of Fyn and thereby release it from the membrane, exposing the domain that interacts with Tctex-1. It is unlikely that the Fyn fusion proteins are palmitoylated in our yeast experiments, since palmitoylation of this sequence is considered to be dependent upon nearby myristoylation in Src family kinases (24, 26, 28), which is not possible with the initiating methionine of Fyn fused to Gal4. Mutation of the two lysines of Fyn has been shown to alter its localization from the plasma membrane to the cytoplasm (32), although our mutational studies indicate that this localization is not due to Tctex-1 binding (Fig. 5c).

The colocalization of Fyn with Tctex-1 during cytokinesis is a particularly intriguing result in light of other recently published
reports. Of particular interest is a study by Yasunaga et al. (22), which determined that proB cells cultured from fyn-deficient mice grew essentially normally until transferred to defined serum-free conditions, at which point these cells arrested during cytokinesis at telophase. In striking contrast, proB cells from normal animals continued through the cell cycle in these serum-free conditions. Their results indicate that Fyn plays a critical role in cell division, although its requirement can be overcome by serum-derived growth factors that presumably bypass the Fyn deficiency block. The same report also demonstrated the localization of Fyn in the cleavage furrow at anaphase of normal proB cells, which we have reproduced in our studies of a T cell hybridoma. Taken together with this genetic evidence, our results suggest that Tctex-1 might provide the crucial scaffold link that tethers Fyn to cytoskeletal motor structures in lymphoid cells, where it functions during cytokinesis. Previous observations of decreased proliferative capacity of thymocytes from fyn-deficient mice (11, 12) might, in fact, be partially explained by this requirement and further reinforces the importance of this newly identified function for the kinase.

Ley et al. (23) have reported that Fyn is almost exclusively localized at the centrosome and mitotic spindles of interphase and mitotic T cells, respectively. Since dynein mediates retrograde transport toward these structures, the association of Fyn with Tctex-1 in the dynein complex could clearly mediate this localization of Fyn in lymphocytes. Although we have observed predominantly plasma membrane localization of Fyn in interphase cells using two different Abs in the murine T cell hybridoma, N17 (and other T lymphomas; data not shown), a subpopulation of cells demonstrated distinct cytoplasmic foci of Fyn staining, some of which colocalized with Tctex-1 (Fig. 7C). Ley et al. used a polyclonal Ab directed to the C-terminal domain of Fyn (23), while our Abs were N-terminal reactive, which may account for the predominantly plasma membrane staining pattern in our studies. Roche et al. (58) have demonstrated a requirement for Src kinases, including Fyn, at an earlier stage of the mitotic cell cycle. They reported increased activity of Fyn and other Src kinases in G2/M phase-blocked fibroblasts. In addition, fibroblasts were arrested before prophase, in the same report, by microinjection of an anti-Fyn/Src/Yes Ab (the same Ab used by Ley et al.) or a GST fusion protein of the Fyn SH2 domain. Finally, Marie-Cardine et al. (59) reported that T cell activation results in tyrosine phosphorylation of α-tubulin and that this phosphorylated α-tubulin can bind the Fyn SH2 domain. Katagiri et al. (60) have also noted tyrosine phosphorylation of tubulin during monocyte differentiation of HL-60 cells and concomitant association of Fyn and Lyn with tubulin.

FIGURE 7. Intracellular localization of Tctex-1 and colocalization with Fyn during cytokinesis. A, COS-7 cells were transfected with C-FLAG-Tctex-1 and intracellular staining was performed with anti-FLAG Ab to visualize Tctex-1 (FITC; green) and β-tubulin (Texas Red). B, Fyn and Tctex-1 colocalize in a subpopulation of N17 T cell hybridomas. The N17 cell line was double-stained with anti-Tctex-1 pAb (Oregon Green 488) and anti-Fyn mAb (Texas Red). The majority of cells demonstrate segregated plasma membrane staining of Fyn and perinuclear staining of Tctex-1, and colocalization of the two proteins was evident only in a subpopulation of cells as visualized in yellow (filled arrowheads), while some cells contained distinct cytoplasmic foci of Fyn at sites that are not enriched in Tctex-1 (red; outlined arrowheads). C, Fyn and Tctex-1 colocalize in the cleavage furrow and at the mitotic spindles of N17 T cell hybridomas undergoing cytokinesis. N17 cells in G2/M phase of the cell cycle were sorted by FACS and double-stained with either Oregon Green-labeled anti-Tctex-1 pAb or anti-Fyn pAb plus Texas Red-labeled anti-β-tubulin mAb or anti-Fyn mAb. Each horizontal series shows separate green and red channels of the indicated staining, and both channels merged in the same focal slice from individual cells. The bottom three cells demonstrate colocalization (yellow in the merged image) of Tctex-1 and Fyn at the cleavage furrow (open arrowheads) and mitotic spindles (closed arrowheads). These cells are characteristic of anaphase to early telophase, since they are all beginning to pinch into separate daughter cells. All cells found at this stage of cytokinesis exhibited this representative colocalization. The bar in each panel denotes 10 μm.
results taken together suggest potentially important roles for Fyn recruitment to and phosphorylation of microtubule cytoskeletal elements during cellular activation and mitosis and suggest that Tc-tex-1 might be an important mediator of the Fyn recruitment.

What is the role of this interaction during the cell cycle? Although we can only speculate, colocalization of Fyn with Tc-tex-1 at the cleavage furrow/mitotic spindles and fyn requirements for cytokinesis in proB cells (22) suggest specific roles for these proteins in the division of lymphocytes. Alternatively, the binding of Fyn to Tc-tex-1 may occur as a capture mechanism for specific cargo in dynein-mediated protein sorting during mitosis. Our understanding of the complex molecular events occurring at the cleavage furrow is only currently unfolding. The role of actin filaments and associated structures in this process is clear, but microtubules and even dynein appear also to play roles (38, 61). Cytoplasmic dynein has also previously been shown to play roles in centrosome separation, anaphase B spindle elongation, and positioning of mitotic spindles during mitosis (38–40). Recruitment of Fyn to dynein by Tc-tex-1 might affect some or all of these mitotic events.

Although Tc-tex-1 does not seem to serve as a major substrate for Fyn in our in vitro phosphorylation studies (Fig. 4a and direct mixing in in vitro phosphorylation reactions, data not shown), it was nevertheless detectably tyrosine phosphorylated. Other proteins within the dynein complex may, however, be more efficacious substrates once the kinase is recruited. Ley et al. (23) have described protein tyrosine phosphorylation surrounding the Fyn at microtubule organizing centers, and as previously mentioned, α-tubulin is a potential tyrosine phosphorylated substrate for Fyn at this location (59, 60). Phosphorylation events identified to date during cytokinesis have predominantly focused on the myosin chains, which undergo serine/threonine phosphorylation (reviewed in Ref. 61). Components of the cytoplasmic dynein complex have been reported to be phosphorylated during cell cycle and transport processes, but again, only serine and threonine phosphorylation has been identified (47, 62–64). Karki et al. (65) recently reported the association of casein kinase II with dynein, which appears to mediate some of this protein phosphorylation and thereby appears to affect function of the motor complex. Determination of the functional roles of recruited Fyn during cytokinesis in lymphocytes and the possibility of consequent tyrosine phosphorylation of the dynein complex and associated structures should allow for many enlightening future investigations.

In summary, accumulating evidence is implicating Fyn kinase as an important effector within the cytoskeleton of lymphocytes, particularly during mitosis. The identification of a direct interaction of the Fyn N-terminal domain with the cytoplasmic dynein motor complex light chain, Tc-tex-1, provides a novel mechanism for the recruitment of Fyn to this distinct intracellular location.

Note Added in Proof. Two potential Fyn substrates that might be important during cytokinesis are the cleavage furrow-associated protein PSTPIP (66) and the inositol 1,4,5-trisphosphate receptor (67).

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