

Dynamic Regulation of Tec Kinase Localization in Membrane-proximal Vesicles of a T Cell Clone Revealed by Total Internal Reflection Fluorescence and Confocal Microscopy*[§]

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Tec family tyrosine kinases are key regulators of lymphocyte activation and effector function. Several Tec family kinases (Tec, Itk, Rlk/Txk) are expressed in T cells, but it is still not clear to what degree these are redundant or have unique functions. We recently demonstrated that Tec alone, among the Tec kinase family members examined, can induce nuclear factor of activated T cell-dependent transcription. This unique functional characteristic correlated with a unique pattern of subcellular localization, as Tec (but not other family members) was found in small vesicles, the appearance of which requires signaling through the T cell receptor for antigen. Here we report on our studies of these Tec-containing structures in live T cells, using total internal reflection fluorescence microscopy. With this technique, we showed that, in live T cells, the Tec vesicles are located at the plasma membrane, the vesicles are unique to Tec (and not the related kinase Itk), and their formation and maintenance require T cell receptor signaling through Src family kinases and PI 3-kinase. Finally, we have imaged isolated T cell membranes by confocal microscopy, confirming the membrane-proximal location of Tec vesicles, as well as demonstrating overlap of these vesicles with the tyrosine kinase Lck, the Tec substrate PLC- γ 1, and the early endosomal antigen 1 marker EEA1.

The earliest detectable biochemical event after ligation of the T cell receptor (TCR)¹ for antigen is an increase in tyrosine kinase activity. Three classes of tyrosine kinase have been implicated in TCR signal transduction: those of the Src, Syk, and Tec families (1). The Src family kinases Lck and Fyn are responsible for the phosphorylation of the immunoreceptor tyrosine-based activation motifs in the CD3 and ζ chains, which are associated with the TCR. These phosphorylated tyrosines serve as docking sites for the tyrosine kinases ZAP-70 and Syk, which phosphorylate a number of downstream signaling inter-

mediates, including various adaptor proteins and enzymes. Finally, tyrosine kinases of the Tec family become activated; in T cells, these include Itk, Tec, and Rlk/Txk, whereas in B cells, the principal family member is Btk (1, 2).

In addition to a tyrosine kinase domain, Tec kinases also contain several protein-protein interaction and localization domains, including an Src homology 2 (SH2) domain that mediates interactions with phosphotyrosine moieties, an SH3 domain that mediates interactions with proline-rich sequences, and a domain that mediates membrane recruitment (3). In Tec, Btk, Itk, and BMX, the latter is a pleckstrin homology (PH) domain that binds to the PI 3-kinase products PI(3,4)P₂ or PI(3,4,5)P₃, whereas in Rlk, this function is carried out by a cysteine-rich string. Tec kinases also contain a proline-rich sequence that can bind to the SH3 domain of the same molecule, resulting in an intramolecular interaction that negatively regulates kinase activity (4). A major function of Tec family kinases is apparently to increase the efficiency of phospholipase C- γ activation, which results in the production of the second messengers inositol trisphosphate (IP₃) and diacylglycerol (5). The former causes release of intracellular calcium, which leads to capacitative calcium influx and activation of calcium-dependent enzymes such as calcineurin. The latter contributes to protein kinase C and Ras activation.

Unlike in the B cell lineage, where Btk is absolutely required for development and activation, Tec kinases appear to play a more modulatory role in T cell activation (1, 3, 5–7). Various knock-out studies have shown that ablation of one or more Tec kinases leads to moderate-to-severe impairment of T cell activation and effector function, although not as severe as the lack of Src or Syk family kinases (5, 7). One outstanding question regarding Tec family kinase function in T cells is the degree to which Itk, Tec, and Rlk/Txk are redundant or can perform unique functions. There is now evidence that, although Itk contributes preferentially to Th2 helper T cell differentiation, Rlk/Txk activation seems to bias T cells to adopt a Th1 phenotype (8–11).

We recently performed a series of structure/function analyses of several Tec kinases, comparing their ability to activate downstream signaling pathways in T cells, as well as their subcellular localization before and after T cell activation (12). We showed that Tec, unlike Itk or Btk, is a potent inducer of NFAT-dependent transcription. This was consistent with previous reports that Tec could activate NFAT and interleukin-2 reporters (13, 14). We also noted that the ability of Tec to activate NFAT correlated with a rather unique pattern of subcellular localization. Whereas Tec, Itk, and Btk all reside in the cytoplasm of resting T cells, activation by antigen results in different patterns of localization. Itk and Btk are both recruited to the plasma membrane, with especially high concentrations

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[§] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Movies 1–5.

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¹ The abbreviations used are: TCR, T cell receptor; SH, Src homology; PH, pleckstrin homology; GFP, green fluorescent protein; NFAT, nuclear factor of activated T cells; PLC- γ 1, phospholipase C- γ 1; LFA-1, lymphocyte function-associated antigen 1.

at the site of contact between the T cell and antigen-presenting cell. However, Tec is recruited into punctate structures that are oriented toward the antigen-presenting cell and appear to be at or near the plasma membrane.

Further evidence of a requirement for signaling in the formation of these structures came from examination of Tec localization in Jurkat T cells. Recent studies have shown that Jurkat cells have high constitutive levels of PIP₃ due to the lack of expression of two PIP₃ phosphatases, PTEN and SHIP (15, 16). When we examined Tec localization in these cells, it was found constitutively in punctate structures at or near the plasma membrane (17). Although a high percentage of Itk and Btk appeared to be at the plasma membrane in Jurkat cells, these kinases were not found in the same type of punctate structures.

We were interested in examining the Tec-containing structures in live cells, so we turned to a relatively novel technique for studying fluorescently tagged proteins near the cell surface, total internal reflection fluorescence (TIRF) microscopy. Using a highly sensitive TIRF system, we have followed the formation of the Tec-containing structures upon T cell contact with anti-CD3/CD28-coated coverslips. We have also studied the kinetics of their reversal after treatment with tyrosine kinase or PI 3-kinase inhibitors. Finally we have performed confocal microscopy of isolated T cell membrane patches. We show here that the Tec-containing vesicles: (1) form after encounter of T cells with a stimulatory (but not merely adhesive) surface; (2) require constant signaling for maintenance, because inhibition of tyrosine kinases or PI 3-kinase results in rapid loss; and (3) overlap with an early endosomal compartment, as well as with the important signaling molecules Lck and PLC- γ 1.

EXPERIMENTAL PROCEDURES

Cells—D10.G4.1 is a murine CD4⁺ Th2 clone that recognizes a fragment of chicken conalbumin presented by the Class II major histocompatibility complex molecule I-A^k (18). We previously reported a subline of the D10 clone that can be maintained for up to one month in medium supplemented with interleukin-2 after restimulation with antigen and that is also amenable to transient transfection (19).

Plasmids/Antibodies/Inhibitors—Plasmids encoding Tec-GFP and Itk-GFP have been described previously (17). Antibodies to murine CD3 and CD28 (2C11 and 37.51) were obtained from BD Biosciences. Anti-LFA-1 (anti-CD18) was obtained from Caltag (Burlingame, CA). PP2, LY294002, and LY303511 were obtained from Calbiochem. Anti-ZAP-70 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to PLC- γ 1 and EEA1 were obtained from Upstate Biotechnology (Lake Placid, NY). Cy3-conjugated anti-rabbit antibody was obtained from Jackson ImmunoResearch (West Grove, PA). Alexa555-conjugated anti-mouse antibody was obtained from Molecular Probes (Eugene, OR).

Transfections—D10 cells were transfected by electroporation essentially as described previously (19). Thus, 10 million D10 cells were resuspended in 0.4 ml of serum-free RPMI 1640 medium and added to a 0.4-cm gap electroporation cuvette containing 10 μ g of plasmid encoding Tec-GFP or Itk-GFP, plus 20 μ g of empty pCDNA3 vector as a carrier. Cuvettes were pulsed at 250 V, 959 microfarads in a GenePulser II electroporator (Bio-Rad). The cells were placed into culture with 10 ml of complete medium (RPMI 1640 medium + 10% fetal calf serum, penicillin, streptomycin, L-glutamine, β -mercaptoethanol, sodium pyruvate, non-essential amino acids, and HEPES buffer, pH 7.3) supplemented with 25 units/ml recombinant human interleukin-2 (Hoffmann-La Roche, Inc., Nutley, NJ; obtained through the National Institutes of Health AIDS Research and Reference Reagent Program). The next day, live cells were purified with Lympholyte-M (Cedarlane, Hornby, Ontario, Canada) and placed back into culture for 24 h before imaging.

Imaging—Glass bottom 35-mm cell culture dishes (MatTek, Ashland, MA) were pre-coated overnight at 4 °C with antibodies to CD3 and CD28 or LFA-1 and diluted to 10 μ g/ml in phosphate-buffered saline. The dishes were washed with phosphate-buffered saline, followed by distilled H₂O, and then left to dry. Transfected D10 cells were settled onto dishes held by a heating collar that maintained medium at a constant 37 °C. The cells were initially imaged as they settled and spread onto the glass coverslip, and then additional cells were imaged

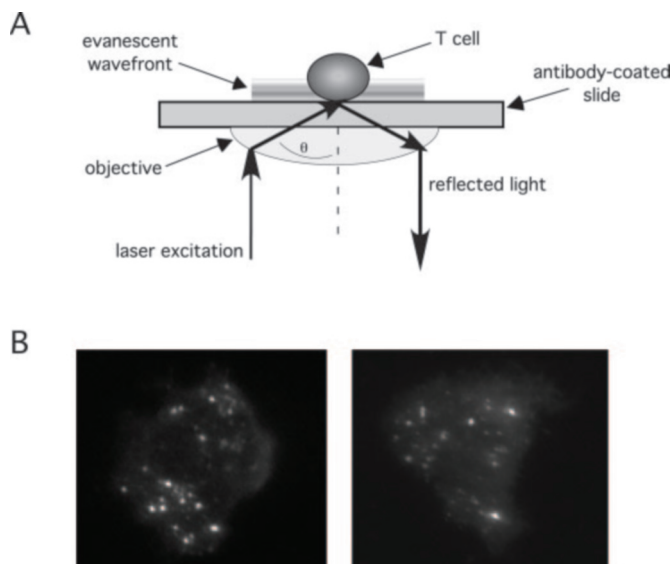


FIG. 1. Detection of Tec vesicles by TIRF microscopy. A, experimental design. D10 T cells were settled onto coated dishes and imaged by TIRF. GFP molecules in the T cells are not excited directly by laser light but by an evanescent wave that is generated as the result of laser reflection off the glass slide/culture medium interface at an incident angle (θ). This evanescent wave propagates through the medium and cells, decaying with the sixth power of the distance from the interface. See "Experimental Procedures" and "Results" for more details about the TIRF system. B, Tec-GFP imaged by TIRF. Representative TIRF images of D10 T cells expressing Tec-GFP, after allowing the cells to settle onto anti-CD3/CD28-coated dishes.

after establishing stable interactions with the antibody-coated coverslip (15–30 min after addition to the dish).

TIRF imaging was performed basically as described previously (20). Cells were imaged on a Nikon (Melville, NY) TIRF system using a 60 \times numerical aperture 1.45 oil immersion objective and a Prairie (Madison, WI) acousto-optic tunable filter controlled laser bench. All experiments used the 488 line on a 150-milliwatt argon source. Images were collected (1 frame/2 s) using Simple PCI software (Compix, Cranberry, PA) and a Q imaging Retiga EXI camera. Time-based series were collected and processed using Simple PCI. Further processing to generate scaled, false-color, images was performed with MetaMorph (Universal Imaging, Downingtown, PA). Grayscale still images were extracted from movies using QuickTime Pro, and all final figures were assembled in Canvas.

To prepare T cell membranes for confocal imaging, transfected D10 cells were allowed to settle for 30 min at 37 °C onto glass-bottomed dishes coated with anti-CD3 and anti-CD28 antibodies. The dishes were then washed vigorously three times with phosphate-buffered saline using a transfer pipette, leaving only tightly adhered T cell membranes attached to the dish, which were then fixed and stained as described previously (19). T cell membranes were imaged on an Olympus Fluoview 500 confocal microscope, using a 60 \times (numerical aperture 1.4) objective. Two-color imaging was performed sequentially to eliminate bleed-through between channels. TIFF images (24-bit) were imported into Canvas for presentation.

RESULTS

We employed a highly sensitive TIRF system to study the localization and dynamics of the Tec tyrosine kinase during T cell activation (Fig. 1A). TIRF imaging relies on an evanescent wave that is generated when light reflects off a surface at an incident angle, equivalent to or greater than the critical angle. The evanescent wave decays exponentially and effectively penetrates 100–150 nm beyond the coverslip into the cell. The method has a much higher signal:noise ratio than any other fluorescence imaging method, and as such, it allows very small numbers and concentrations of molecules to be excited.

D10 T cells transfected with a plasmid encoding Tec-GFP (17) were dropped onto anti-CD3/CD28-coated glass-bottomed culture dishes and imaged as shown in Fig. 1A. Tec-GFP-

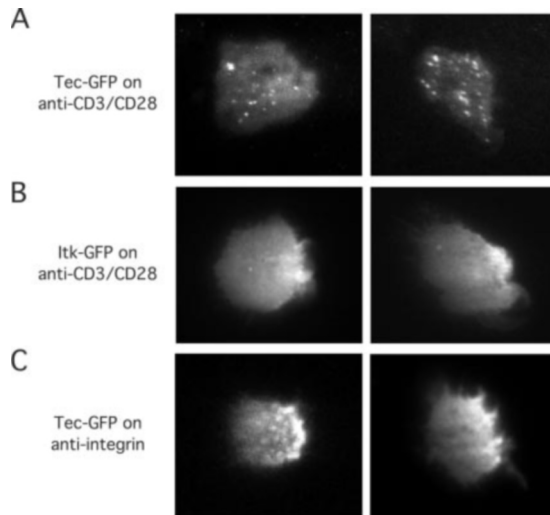


FIG. 2. TIRF imaging of Tec-GFP and Itk-GFP localization in D10 T cells. D10 T cells transfected with the indicated constructs were allowed to settle on anti-CD3/CD28- or anti-integrin (CD18)-coated dishes for 15 min at 37 °C. GFP fluorescence was then imaged in TIRF mode. *A*, cells transfected with Tec-GFP were settled onto anti-CD3/CD28-coated dishes. *B*, cells transfected with Itk-GFP were settled onto anti-CD3/CD28-coated dishes. *C*, cells transfected with Tec-GFP were settled onto anti-integrin (CD18)-coated dishes. Two representative images are shown for each condition. Each pattern was observed consistently over the course of 3–5 experiments in dozens of cells imaged.

expressing cells in contact with the dish (and therefore receiving stimulation through the TCR and CD28) exhibited a distinct pattern of punctate fluorescence that accounted for a significant fraction of Tec-GFP visible at the plasma membrane (Fig. 1*B*; see also supplemental Movie 1). These appear to be the same structures that we previously observed in Jurkat and D10 T cells by wide field immunofluorescence microscopy and deconvolution (17).

The localization of Tec in activated T cells was in marked contrast to that of the related kinase Itk. Again, as shown in Fig. 2*A*, Tec-GFP localized in small punctate structures upon contact with anti-CD3/CD28-coated dishes. However, Itk-GFP (Fig. 2*B*) adopted a more diffuse pattern of plasma membrane localization, with an increased concentration at what appeared to be a “leading edge,” similar to what is seen with an Akt PH domain-GFP fusion in chemotaxing cells (21). Indeed, this structure appeared very dynamic, with constant ruffling and apparent exchange of Itk-GFP into and of the leading edge (see supplemental Movie 2). A very similar pattern to Itk-GFP was observed when we imaged a GFP fusion with the PH domain of Akt in D10 cells by TIRF microscopy (data not shown).

We next applied T cells transfected with Tec-GFP to dishes coated with antibody specific for the integrin LFA-1 to determine whether the appearance of the Tec-containing vesicles is a specific result of signaling through the TCR-CD3 complex. As shown in Fig. 2*C* (and supplemental Movie 3), T cells settled onto dishes coated with anti-LFA-1 antibody and began to spread. Some areas of increased GFP fluorescence were noted, but they were of a much lower intensity than those seen when Tec-expressing cells were in contact with anti-CD3-coated dishes (Figs. 1*B* and 2*A*). Rather, it appeared that these represented membrane extensions, as they contacted the anti-LFA-1 antibody, whereas the structures seen with anti-CD3/CD28 stimulation appeared to be internal vesicles located at or near the plasma membrane. There also appeared to be a “leading edge” structure, similar to what was noted in anti-CD3-stimulated cells expressing Itk-GFP (Fig. 2*B*). Thus, adhesion through an integrin results in membrane translocation of Tec-GFP, which is not surprising, because inte-

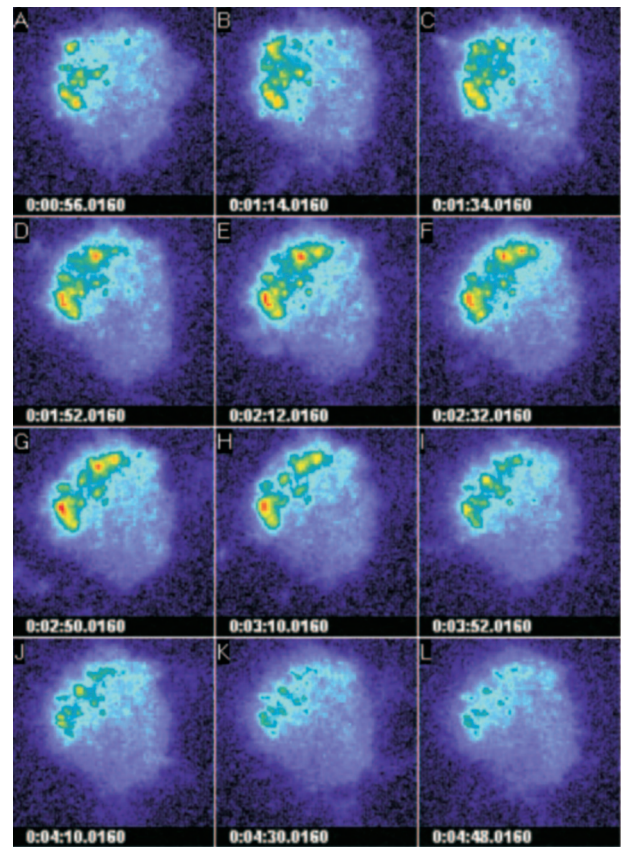


FIG. 3. Kinetics of loss of Tec-containing structures after addition of a PI 3-kinase inhibitor. Still images were extracted from supplemental Movie 4 and are displayed in scaled, false-color mode, with red representing areas of highest concentration and purple, the lowest. The PI 3-kinase inhibitor LY294002 was added just before the first frame, at a concentration of 10 μ M.

grins are known to activate PI 3-kinase and Tec family kinases (2). However, the distinct pattern of small vesicular fluorescence that is seen when cells encounter anti-CD3 antibody is not seen with an anti-integrin antibody.

Tec contains, at its N terminus, a PH domain that is critical for its activation and recruitment to the plasma membrane, as a result of binding to the PI 3-kinase product PIP₃ (3). As shown in Fig. 3, the addition of the PI 3-kinase inhibitor LY294002 resulted in gradual loss of punctate Tec-GFP fluorescence over the course of 3–5 min (see supplemental Movie 4). In some experiments, when the Tec-GFP structures were especially bright, we did not see complete loss until nearly 10 min (data not shown). We also employed a control compound, LY303511, that is structurally almost identical to LY294002 but has no detectable activity against PI 3-kinase (22). This compound did not cause any detectable loss of the punctate Tec-GFP-containing structures, even after 15 min (data not shown).

Somewhat different effects were noted when an Src family kinase inhibitor was added to T cells expressing Tec-GFP and stably interacting with anti-CD3/CD28 antibodies. As shown in Fig. 4 (and supplemental Movie 5), the addition of PP2 resulted in more rapid loss of the Tec-containing structures from the imaging field than with the PI 3-kinase inhibitor. We saw nearly complete loss of Tec-GFP puncta within 1 min after the addition of PP2, which is significantly faster than the kinetics noted with the PI 3-kinase inhibitor (Fig. 3). Therefore, it appears that loss of tyrosine kinase activity results in more rapid loss of Tec-containing structures from the plasma membrane, compared with loss of PI 3-kinase activity.

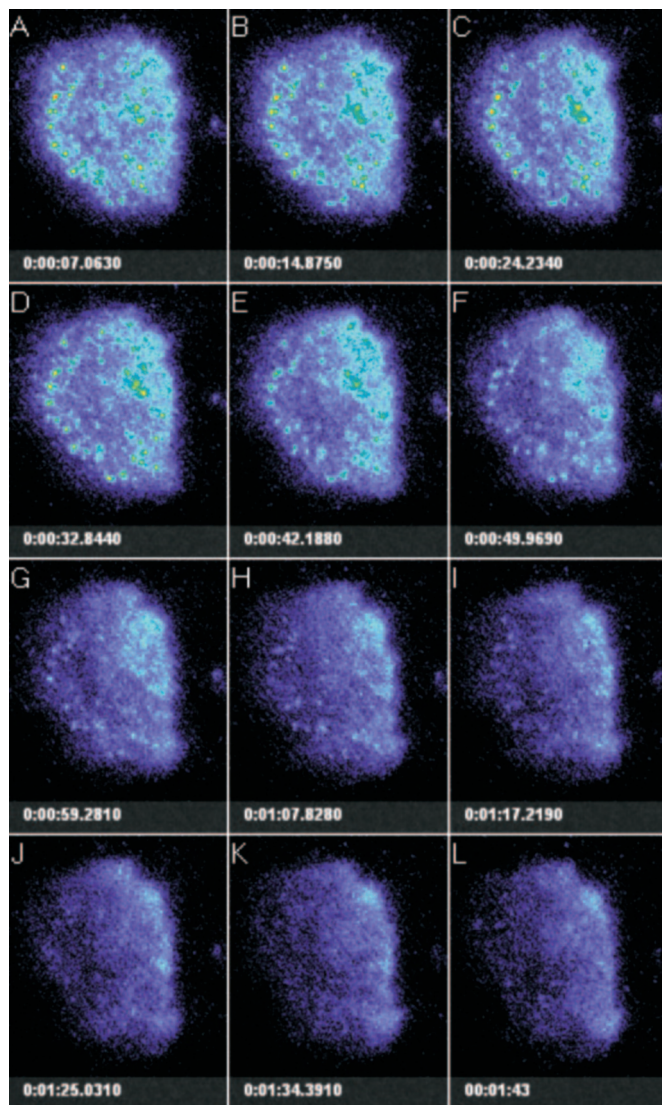


FIG. 4. Kinetics of loss of Tec-containing structures after addition of an Src family tyrosine kinase inhibitor. Still images were extracted from supplemental Movie 5 and are displayed as described in the legend for Fig. 3. The Src family tyrosine kinase inhibitor PP2 was added just before the first frame, at a concentration of 0.5 μ M.

We next sought to confirm our finding of membrane-proximal Tec vesicle localization with another technique; therefore, we prepared isolated T cell membranes and imaged them by standard confocal microscopy. This approach also allowed us to determine with a high degree of certainty what other proteins are contained within membrane-proximal Tec vesicles, because the planar nature of the membranes ensures definitive axial co-localization. The outline of this technique is shown in Fig. 5A. We took advantage of the fact that T cells will adhere very tightly to surfaces coated with antibodies to CD3 and CD28, which allowed us to strip away adhered cells, leaving behind membrane patches. These membrane preparations can be fixed and stained with antibodies for multicolor confocal analysis. As shown in Fig. 5B, *left panel*, Tec vesicles were observed in membrane patches from D10 T cells transfected with Tec-GFP, reminiscent of what was seen when intact cells were imaged by TIRF microscopy. As with the signaling complexes described by Bunnell *et al.* (23), it is still not clear in which intracellular compartment(s) the Tec vesicles reside. We noted that some Tec vesicles appeared to become internalized during stimulation (see supplemental Movie 1); therefore, we stained membrane preparations with an antibody to EEA1, a marker of

early endosomes (24). As shown in Fig. 5B, there was obvious, although not complete, overlap between Tec-GFP and EEA1, suggesting that Tec does transit through early endosomes. We have attempted to trace Tec further through the endosomal pathway but have so far failed to see detectable overlap between Tec and a late endosomal marker (data not shown).

Our previous study shows substantial overlap of Tec with phosphotyrosine staining, although not with a lipid raft marker (17). However, it is still not clear what other proteins are found in Tec vesicles after TCR stimulation. We therefore used the membrane patch technique to assess co-localization of Tec with other molecules involved in TCR signaling. Here we have compared Tec vesicle localization with a protein thought to lie immediately upstream of the Tec kinases (the Src family tyrosine kinase Lck) and another protein that is thought to be a direct target of Tec (PLC- γ 1) (3). As shown in Fig. 6, we observed substantial overlap of both Lck (Fig. 6A) and PLC- γ 1 (Fig. 6B) with Tec-GFP. Typically, \sim 75% of the Tec-containing vesicles overlapped with staining for either Lck or PLC- γ 1. By contrast, when Tec localization was compared with that of ZAP-70 (Fig. 6C), minimal overlap between the two was observed ($<20\%$). Thus, Tec vesicles also contain proteins that are known to function as its direct activators (Lck) and targets (PLC- γ 1).

DISCUSSION

These studies have extended the findings described in our previous report, where we showed for the first time that Tec, unique among the Tec family tyrosine kinases, organizes into punctate structures after antigen stimulation (17). We have now shown that the Tec structures appear in live T cells after TCR stimulation but not after cross-linking of the integrin LFA-1. Furthermore, based on imaging live cells with TIRF microscopy and membrane patches with confocal microscopy, we can conclude that the Tec-containing structures are at or near the plasma membrane (within \sim 100 nm). We have also shown that constant signaling is required for the maintenance of Tec in these structures, because inhibition of either tyrosine kinases or PI 3-kinase results in their disappearance.

Using confocal microscopy, Bunnell *et al.* (23) recently described novel structures, which they called "signaling assemblies," containing many proximal components of TCR signaling. These large macromolecular structures appeared after TCR signaling and included high concentrations of the TCR, ZAP-70, and the adaptors LAT, Grb-2, Gads, and SLP-76. Two differences between our study and that of Bunnell *et al.* are: 1) we have visualized signaling-dependent kinase localization with TIRF microscopy, which allows us to specifically focus on events at the plasma membrane, whereas their studies employed standard confocal microscopy; and 2) we have used the D10 T cell clone, a non-transformed T cell line, rather than the Jurkat leukemic T cell line. Nonetheless, there are similarities between our observations. First, the formation of Tec-containing structures required signaling through the TCR, as adherence to a non-stimulatory antibody is insufficient for their appearance. Second, the structures containing Tec appeared to be relatively stable once they are formed at the plasma membrane, similar to what was seen with most of the molecules examined by Bunnell *et al.*, including ZAP-70, LAT, and Grb-2. In contrast, they noted an interesting pattern of movement when they examined SLP-76-containing complexes. Thus, SLP-76 complexes appeared to be transported toward the center of the cell by an active process (23). Although there is evidence for interactions between SLP-76 and Tec family kinases (5, 25), we have never observed this type of directed movement by either Tec or Itk (see supplementary Movies).

Our live-cell TIRF imaging studies have shown that constant

FIG. 5. Analysis of Tec vesicle localization using confocal microscopy of T cell membrane patches. *A*, experimental design. D10 T cells were settled onto antibody-coated dishes and vigorously washed, leaving behind tightly adhered membrane patches. *B*, representative confocal image of a membrane patch from a D10 T cell transfected with Tec-GFP (*left panel*). The membrane was also stained with an antibody to the early endosome marker EEA1 (*middle panel*). Overlay of the two images demonstrates significant overlap between the Tec vesicles and early endosomes (*yellow color in right panel*).

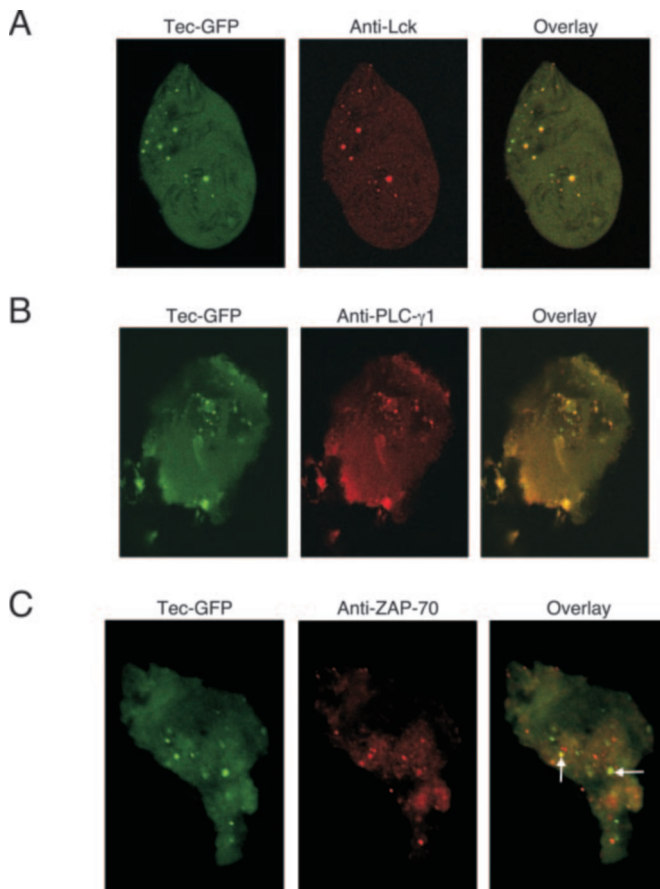
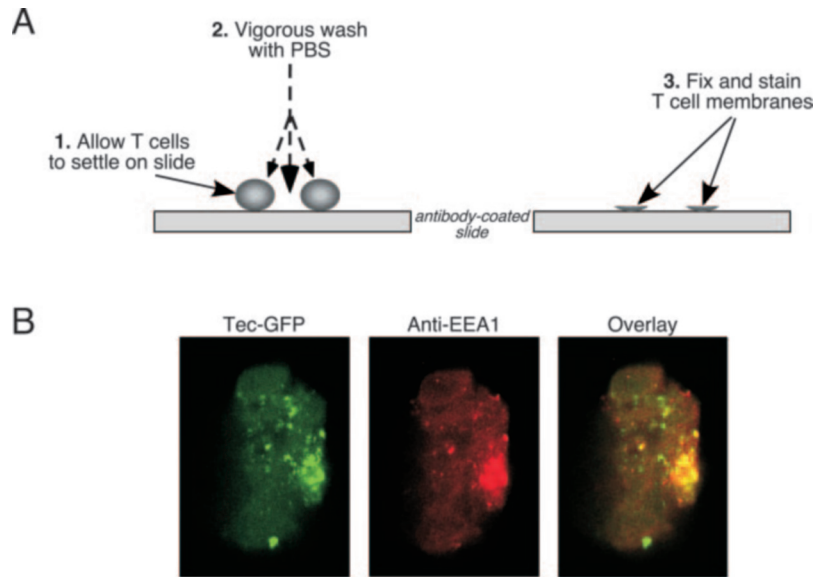


FIG. 6. Co-localization of Tec vesicles with signaling proteins. Membrane patches from D10 cells transfected with Tec-GFP (*left panels*) were fixed and co-stained with antibodies to the Src family tyrosine kinase Lck (*A*), the Tec target PLC- γ 1 (*B*), or the tyrosine kinase ZAP-70 (*C*) (*middle panels*). Overlays are shown in the *right panels*, with the color yellow indicating co-localization. The *arrows in C* indicate the few bright Tec-containing vesicles that overlap with ZAP-70 staining. Note the more extensive overlap of Tec with Lck (*B*) and PLC- γ 1 (*C*).

signaling is required for maintenance of the Tec-containing structures at the plasma membrane. Thus, the addition of the PI 3-kinase inhibitor LY294002 (Fig. 3) or the Src family kinase inhibitor PP2 (Fig. 4) resulted in loss of the Tec-containing structures from the T cell surface. However, we did note a

difference in the kinetics of the loss, depending on which inhibitor we used. Although inhibition of tyrosine kinases with PP2 resulted in the rather rapid loss of Tec-GFP fluorescence from the cell surface (<1 min), inhibition of PI 3-kinase led to a significantly slower loss (at least 3–5 min). It is unclear at this point what precisely accounts for this difference, but it could be the result of down-regulating phosphatases being recruited to the plasma membrane or to the Tec-containing structures at different rates. In the case of tyrosine kinase signaling, a prominent example is SHP-1 (26–28), whereas for the PI 3-kinase pathway the known phosphatases include PTEN and SHIP (29).

In our previous studies, we have used wide field fluorescence microscopy coupled with deconvolution to investigate the localization of various Tec family kinases in fixed T cells (17). We have noted that, in unstimulated D10 T cells, Tec is found diffusely throughout the cytoplasm, with the punctate pattern of Tec localization appearing after stimulation of cells with antigen-loaded antigen-presenting cell. We therefore believe that the Tec-containing “vesicles” are formed *de novo* after stimulation through the TCR, rather than being recruited to the cell surface. Likewise, after inhibition of tyrosine kinase or PI 3-kinase signaling (Figs. 3 and 4), not only did the Tec structures disappear from the cell surface, but they appeared to dissipate completely, because we could not find the Tec vesicles even in standard confocal mode after their loss from the TIRF field (data not shown).

We have reported here for the first time that Tec appears to traffic through an endosomal compartment after TCR stimulation. After cross-linking, the TCR-CD3 complex is known to be internalized and trafficks into endosomes (30). Thus far, we have not observed co-localization of Tec with the TCR after stimulation.² Recent studies have highlighted the apparent importance of endosomal compartments for initiating signal transduction by at least two distinct mechanisms. For instance, Alcover and colleagues (31) recently reported that internalization of TCRs into endosomes is required for their eventual localization to the developing immunological synapse. In addition, it has been known for some time that certain receptors, including the tumor necrosis factor receptor, actually require endocytosis for activation of downstream signaling pathways (32). The PI 3-kinase pathway, including its downstream PH domain targets, is a particularly good example of the intersec-

² L. P. Kane and S. C. Watkins, unpublished data.

tion between signal transduction and endocytosis (24, 33). It is therefore not very surprising that Tec might function within the endocytic pathway, although it is unclear at this point why other Tec kinase family members do not appear to localize similarly, despite the presence of a PH domain.

Nunes and colleagues (34, 35) have also recently investigated the localization and regulation of Tec during T cell activation. Consistent with previous reports (17, 34, 36), it has been found that the PH domain of Tec is critical for the activation of gene transcription in T cells. Nunes and colleagues (34, 35) have also noted that the PH domain of Tec is dispensable for recruitment of Tec to the plasma membrane, in agreement with our previous report (17) and, similar to our findings, observed that, although plasma membrane recruitment of Tec lacking a PH domain still occurs, Tec-containing vesicles do not form (17, 35). This is consistent with our finding here (Fig. 3) that PI 3-kinase activity was required for the maintenance of Tec in punctate structures. One point of divergence between our studies and previous reports concerns the question of whether the PH domain of Tec is *sufficient* for membrane recruitment. Although it has been reported that the PH domain is not sufficient (35), our data suggest that the Tec PH domain can mediate association with the plasma membrane on its own, in isolation from the remainder of the protein (17).³ The reasons for this discrepancy are not clear, because we observed this in both Jurkat and D10 cells and used a construct only one amino acid longer than that used in previous studies (35). Nonetheless, the Tec PH domain is *not* sufficient for localization to the vesicular structures seen with the full-length protein (17).

Apart from the PH domain, Tec contains a number of other domains that appear to regulate its function and localization. We and others (17, 36) have previously shown that the Tec SH2 domain is required for both proper localization and function. There is, however, conflicting evidence regarding the role of the SH3 domain, which has been variously reported to either negatively (37) or positively (34) regulate Tec, as well as to regulate its localization at the immunological synapse (34, 35). We have found that mutation of either the SH3 domain or proline-rich sequence (in the Tec homology domain) in Tec does not inhibit its vesicular localization after TCR stimulation.⁴ Taken together, our results indicate that localization of Tec to vesicles requires both its pleckstrin homology and SH2 domains but not the SH3 and proline-rich sequences.

We have now begun to identify other signaling proteins that co-localize with Tec in these vesicles, none of which coincide exactly with the distribution of Tec (Fig. 6). However, our findings thus far are consistent with previous biochemical and genetic studies that identified Src family kinases as direct activators of Tec family kinases and the enzyme PLC- γ 1 as a direct target, because localization of Lck and PLC- γ 1 do overlap considerably with Tec. On the other hand, we have observed minimal co-localization of ZAP-70 with Tec vesicles. Intriguingly, Bunnell *et al.* (23) reported that different TCR signaling intermediates dissociate from

signaling complexes with different kinetics, with ZAP-70 displaying the greatest stability. The ability of Tec to potentially activate NFAT correlates with its localization in the vesicles that we have described here and elsewhere (17). Identification of the full range of signaling molecules found in these vesicles should further clarify the role in T cell activation of, not only Tec, but also the endocytic pathway.

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³ M. G. Tomlinson and L. P. Kane, unpublished data.

⁴ L. P. Kane and S. C. Watkins, unpublished data.