The Tec family of tyrosine kinases transduces signals from antigen and other receptors in cells of the hematopoietic system. In particular, interleukin-2 inducible T cell kinase (Itk) plays an important role in modulating T cell development and activation. Itk is activated by receptors via a phosphatidylinositol 3-kinase-mediated pathway, which results in recruitment of Itk to the plasma membrane via its pleckstrin homology domain. We show here that membrane localization of Itk results in the formation of clusters of at least two molecules within 80 Å of each other, which is dependent on the integrity of its pleckstrin homology domain. By contrast, the proline-rich region within the Tec homology domain, SH3 or SH2 domains, or kinase activity were not required for this event. More importantly, these clusters of Itk molecules form in distinct regions of the plasma membrane as only receptors that recruit phosphatidylinositol 3-kinase reside in the same membrane vicinity as the recruited Itk. Our results indicate that Itk forms dimers in the membrane and that receptors that recruit Itk do so to specific membrane regions.

The Tec family of non-receptor tyrosine kinases is the second largest family of non-receptor tyrosine kinases (1). This family, which includes Itk,2 is involved in transducing signals from a number of receptors, including the T cell receptor, B cell receptor, FcεR, c-Kit, CD28, CD2, CD32, and the erythropoietin receptor (1–6). These kinases play important roles in regulating cytokine and immune receptor signals that regulate the immune response (7, 8). Itk in particular is involved in early T cell receptor signaling and regulates increases in intracellular calcium and activation of the nuclear factor of activated T cells family of transcription factors (9, 10). In addition, Itk regulates the development of Th2 cells and their subsequent cytokine secretion, thereby modulating the immune response (10–13). A common feature of these kinases is that they require the activation of PI3 kinase, as well as Src kinase activation for their activity (14–17). These kinases, including Itk, have a PH domain that allows them to be recruited to the plasma membrane by an activated PI3 kinase (1). Thus, membrane recruitment is a critical component of their activation, and Itk can be found at the plasma membrane of cells, although other events are required for its full activity (17–19). It is, however, not clear whether this is general membrane localization of the protein in anticipation of activation or a specific localization in certain regions of the plasma membrane.

The structure of Itk in cells is not known, although it has been suggested to form dimers in its inactive state, with a resultant monomerization upon activation (20, 21). We have examined these issues using a split YFP system (22) and find that Itk forms dimers or higher order clusters; however, it does so only at the plasma membrane and only in the vicinity of a receptor that can recruit PI3 kinase. Our data shed new light on the regulation, structure, and localization of this family of kinases in cells.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Reagents**—Antibodies against Itk were a gift from Dr. Gordon Mills (M. D. Anderson Cancer Center) (19). The pCDNA3.1-YFP1 zipper and pCDNA3.1-YFP2 zipper were a kind gift from Dr. Stephen Michnick (University of Montreal, Montreal, Canada) (22). The lentivirus vector FUGW and packaging plasmids pΔ8–9 and pVSVG were a kind gift from Dr. David Baltimore (California Institute of Technology, Pasadena, CA) (23). The pCDNA3.1-YFP1-Itk was generated by deleting the zipper sequence of pCDNA3.1-YFP1-zipper and replacing it with the human Itk cDNA generated by PCR and sequenced to confirm the integrity. pCDNA3.1-YFP2-Itk plasmids was generated by deleting the YFP1 sequence of pCDNA3.1-YFP1-Itk and replacing it with the human Itk cDNA generated by PCR and sequenced to confirm the integrity. pCDNA3.1-YFP2-Itk plasmids was generated by deleting the YFP1 sequence of pCDNA3.1-YFP1-Itk and replacing it with the YFP2 fragment. Itk mutants lacking the PRR within the TH, SH3, or SH2 domain or carrying a K391M mutation in the kinase domain that significantly reduce kinase activity were previously described (19, 24). The pG-ICOS-YFP1 and pMex-CD8a-YFP1 were generated by subcloning linker-YFP1 to the C terminus of pG-ICOS or pMex-CD8 fragment lacking the cytoplasmic tail (25), respectively. Point mutations were introduced by using the QuikChange II site-directed mutagenesis kit (Stratagene). The FUGW-YFP1-Itk and FUGW-YFP2-Itk plasmids were generated by substituting the...
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GFP fragment for YFP1-Itk or YFP2-Itk in the FUGW vector, respectively. The pGFP-Itk was generated by inserting GFP fragment to the N terminus of HA-Itk (24).

Cell Transfection—HEK 293T cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. The plasmids were transfected into HEK-293T cells by using TransIT-293T (Mirus). Jurkat E6-1 T cells were cultured in RPMI supplemented with 5% fetal bovine serum and were transfected by electroporation. Cells were infected with lentiviruses generated as described (23).

Flow Cytometric Assay—24 h after transfection, the cells were incubated at 30 °C overnight to promote fluorophore maturation. The cells were cultured, washed twice with PBS, and resuspended in PBS followed by flow cytometric analysis. YFP fluorescence was corrected for expression by analysis of Itk expression either by western blot, or in most experiments, by analysis of YFP expression using an antibody against GFP and flow cytometry. These values were then compared with control transfections of YFP1-Itk or YFP2-Itk alone, which was set at 1.

Confocal Microscopy—HEK 293T cells were grown on glass slides. 24 h after transfection, the cells were incubated at 30 °C overnight, and the fluorescence image was analyzed by confocal microscopy. For immunofluorescence microscopy, cells were fixed using 4% paraformaldehyde for 20–30 min at room temperature. The fixed cells were then incubated with anti-Itk antibody in blocking buffer (2% fetal bovine serum and 0.1% Triton in PBS) for 1 h. After washing three times with PBS, cells were then stained with Alexa Fluor 663-conjugated secondary antibody for 30 min in the dark. The unbound antibody was removed by washing three times with PBS. The cells were then incubated in PBS and analyzed by confocal microscopy. Since each cell is of a different size and may express varying levels of protein, an algorithm was developed to compare the localization of Itk molecules in cells of different sizes and varying levels of expression using the program CCDi Image (Stellar Image Software) as follows. 1) A line was drawn across the cells, excluding the nucleus, and the fluorescence intensity and map data points were collected for each cell; 2) points along the line were set as a percentage of the cell from one end of the line to the other (0–100%); 3) the fluorescence at each point was calculated as a percentage of the maximum fluorescence observed for that cell; and 4) these values were then plotted for each cell as the percentage of maximum fluorescence (y axis) versus the percentage of cell (x axis) for each cell. In general, the cell membrane represented that 15% of the cell at the edges (0–15% and 85–100%).

RESULTS AND DISCUSSION

Itk is recruited to cell membrane after receptor stimulation (3, 4, 26–28). However, whether the localization of Itk results in specific clustering or a more dispersed distribution is not clear. To answer this question, we utilized the split YFP system to probe the interaction between molecules of Itk (22). Either YFP1 or YFP2 was fused to the N terminus of Itk using a linker peptide of 10 amino acids, thus constructing the protein designated as YFP1-Itk or YFP2-Itk, respectively (Fig. 1a). Since the average distance of each amino acid is ~4 Å, the fluorescence can be detected when Itk molecules fused to the two fragments of YFP are within an average distance of 80 Å. These constructs were expressed in 293T cells, and fluorescence was analyzed by flow cytometry and confocal microscopy.

The expression of either YFP1-Itk or YFP2-Itk alone in cells did not show any detectable fluorescence when compared with non-transfected cells (data not shown), suggesting that expression of individual YFP fragment does not exhibit fluorescence, similar to what has been previously determined for other fusions (Fig. 1b) (22). However, when YFP1-Itk and YFP2-Itk were coexpressed, strong fluorescence was detected (Fig. 1b). Since the YFP fluorescence can be detected when Itk molecules are in close proximity (~<80 Å), these data indicate that Itk proteins formed specific dimers or higher order clusters in living cells that are within this range. To determine the subcellular sites of Itk clustering, we analyzed the localization of YFP fluorescence in these cells by using confocal microscopy. The YFP fluorescence was found predominantly at the cell membrane. In comparison, analysis of cells with an anti-Itk antibody revealed that Itk was distributed in both cytoplasm and cell membrane (Fig. 1c, control non-transfected 293T cells are negative for Itk staining by western and immunofluorescence, data not shown; see also Fig. 2b). In addition, GFP-tagged Itk also showed the same pattern of subcellular localization as that seen with the anti-Itk antibody (Fig. 1c). Quantification of the localization of these proteins dramatically illustrated the point that the YFP1 Itk and the YFP2 Itk only interact at the membrane, and thus, fluorescence is only detected at that location, whereas GFP Itk is found in both the cytoplasm and the membrane (Fig. 1d). Together, these results suggested that although localized both in the cytoplasm and at the cell membrane, Itk only forms dimers or higher order clusters as detected by complementation of YFP1 and YFP2 and subsequent fluorescence emission at the cell membrane.

To further test the possibility that interaction between Itk molecules in the cytoplasm could occur, we generated an Itk mutant, R29C, which disrupts the ability of the PH domain to interact with lipids, rendering this mutant unable to be recruited to the membrane (29, 30). Thus, this mutant is totally constrained to the cytoplasm. The expression level of this mutant fused to YFP1 and YFP2 was determined by western blot (data not shown), and although expressed at a similar level as wild-type Itk, the R29C PH mutants did not show any detectable fluorescence when compared with untransfected 293T cells (Fig. 2a and b). Specific anti-Itk antibody analysis of Itk, as well as YFP fusion to this Itk mutant, confirmed that it is localized only in the cytoplasm (Figs. 1, c and d, and 2b). More importantly, even when wild-type YFP1-Itk was coexpressed with the mutant YFP2-R29CItk, no interaction was detected as measured by YFP fluorescence and confocal microscopy (Fig. 2b). Thus, we conclude that the dimers and perhaps higher order clusters of Itk form only at the cell membrane, and thus, YFP fluorescence of YFP1-Itk plus YFP2-Itk can only be detected at the cell membrane but not in the cytoplasm. This experiment also indicates that the interaction between Itk molecules at the membrane is specific.

Itk has several domains that can act as protein-protein interaction domains. We therefore disrupted the integrity of each of
these domains to determine whether they were required for this dimerization. The PRR within the unique TH domain of Itk has been suggested to interact with the SH3 domain of Itk in an intramolecular interaction (31). However, we found that Itk mutants carrying a deletion in the PRR of the TH domain could still form dimers with the WT Itk (Fig. 3a). In addition to potential interactions with the PRR of the TH domain, the SH3 domain of Itk has been suggested to interact with a proline residue in the SH2 domain of Itk in an intermolecular fashion (20, 21). The SH2 domain can interact with other proteins upon their tyrosine phosphorylation. Nevertheless, we found that Itk mutants lacking SH3 or SH2 domains could still form dimers with WT Itk (Fig. 3b, and c). Finally, we could show that the kinase activity of Itk was not required for this event since a mutant of Itk lacking kinase activity still formed dimers with WT Itk (Fig. 3d). Since molecules of WT Itk cannot transport a

FIGURE 1. Itk forms dimers specifically at plasma membrane of cells. a, a model of split YFP fusions to Itk. Y1I, YFP1 fragment tagged to Itk; Y2I, YFP2 fragment tagged to Itk. b, 293T cells were transfected with Itk fused to YFP1 along with Itk fused to YFP2, both at the N terminus. Cells were then analyzed by flow cytometry for YFP fluorescence (left) or quantified as in fig. 1 (right). Bottom, fluorescence of cells transfected with YFP1 fused to Itk along with R29CItk fused to YFP2. Y1I, YFP1 fragment tagged to Itk. b, panel i, cells transfected with R29CItk fused to YFP1 along with R29CItk fused to YFP2. Top panel, YFP fluorescence. Bottom panel, cells stained with anti-Itk antibody. Panel ii, cells transfected with Itk fused to YFP1 along with R29CItk fused to YFP2. Top panel, YFP fluorescence. Bottom panel, cells stained with anti-Itk antibody. Cells were analyzed by confocal microscopy.

FIGURE 2. The PH domain is required for the formation of dimers of Itk at the plasma membrane. a, 293T cells were transfected with R29CItk fused to anti-Itk along with R29CItk fused to YFP2. Cells were then analyzed by flow cytometry for YFP fluorescence (left) or quantified as in fig. 1 (right). Bottom, fluorescence of cells transfected with YFP1 fused to Itk along with R29CItk fused to YFP2. Y1I, YFP1 fragment tagged to Itk. b, panel i, cells transfected with R29CItk fused to YFP1 along with R29CItk fused to YFP2. Top panel, YFP fluorescence. Bottom panel, cells stained with anti-Itk antibody. Panel ii, cells transfected with Itk fused to YFP1 along with R29CItk fused to YFP2. Top panel, YFP fluorescence. Bottom panel, cells stained with anti-Itk antibody. Cells were analyzed by confocal microscopy.
PH domain mutant of Itk to the membrane and form dimers (Fig. 2), we conclude that the ability of these mutants to form dimers with WT Itk indicates that these domains or activities are not required for dimer formation. These data also indicate that the PRR or SH3 and SH2 domains are not critical for the formation of dimers or higher order clusters of Itk at the plasma membrane and that membrane localization is the critical event that brings two molecules of Itk together and allows the formation of dimers or higher order clusters.

To investigate the specificity of Itk dimerization/clustering at the cell membrane, we determined whether Itk could interact with a transmembrane protein that does not normally interact with Itk. We deleted the cytoplasmic tail of the human CD8α receptor and fused it to YFP1 at the C terminus. CD8α is randomly distributed in the cell membrane, and if Itk was also randomly distributed, then the two proteins should be able to interact. Flow cytometric analysis demonstrated that coexpression of CD8α-YFP1 and YFP2-Itk did not result in the emission of any YFP fluorescence when compared with non-transfected 293T cells (Fig. 4a), suggesting that there was no interaction between these two proteins at the plasma membrane. The expression of the CD8α-YFP1 was confirmed by flow cytometry for YFP fluorescence (left panel). Alternatively, cells were stained for expression of CD8α using a specific antibody (right panel). Y11, YFP1 fragment tagged to Itk; Y2I, YFP2 fragment tagged to Itk. b, cells were transfected with full-length ICOS fused to YFP1 at its C terminus, along with Itk fused to YFP2. Cells were then analyzed by flow cytometry for YFP fluorescence (left panel). Alternatively, cells were stained for expression of ICOS using a specific antibody (right panel). Bottom panel, fluorescence images of ICOS-YFP2 and YFP1-Itk. c, cells were transfected with full-length ICOS fused to YFP1 at its C terminus, along with R29CItk fused to YFP2. Cells were then analyzed by flow cytometry for YFP fluorescence (left panel). Alternatively, cells were stained for expression of ICOS using a specific antibody (right panel).
recruitment of Itk to the membrane by PI3 kinase as a R29C PH mutant of Itk could not interact with ICOS (Fig. 4c). Together, these data indicate that Itk forms specific dimers/clusters at specific regions of the cell membrane, around the vicinity of receptors capable of activating PI3 kinase.

Since Itk is predominantly expressed in T cells, we analyzed the ability to form dimers/clusters at the plasma membrane in Jurkat T cells. Expression of YFP1-Itk and YFP2-ITK in Jurkat T cells demonstrated that Itk forms similar dimers/clusters in these cells at the cell membrane (Fig. 5a). In addition, similar to what we found in 293T cells with CD8α and ICOS, Itk only interacted with ICOS, but not with CD8α, in Jurkat T cells (Fig. 5, b and c). All together, our data suggest that Itk is recruited to specific regions of the plasma membrane, where it can form dimers/clusters, and this occurs in the vicinity of receptors that can recruit and activate PI3-kinase.

We and others have shown that Itk is recruited to the plasma membrane in a PI3-kinase- and PH domain-dependent manner; however, whether this recruitment results in clustering/dimerization of Itk was not known. Our data demonstrate that at least dimers of Itk form specifically at the plasma membrane of cells. In addition, receptors capable of recruiting this kinase specifically interact with Itk, although we note that they do so less efficiently than the formation of Itk dimers. This could indicate that all the receptors are not capable at any one time of interacting with Itk, and thus, the efficiency is reduced. Although we cannot distinguish between the formation of dimers or higher order clusters, the data suggest that at least dimers are formed. In addition, our data also indicate that these dimers are at least 80 Å apart and that the interaction between Itk and ICOS is also at least the same distance.

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FIGURE 5. Formation of dimers of Itk in T cells. a, Jurkat T cells were infected with lentiviruses carrying YFP1-Itk and YFP2-Itk. Cells were then analyzed by flow cytometry for YFP fluorescence (left panel) or by confocal microscopy (right). Y11, YFP1 fragment tagged to Itk; Y21, YFP2 fragment tagged to Itk. b, cells were transfected with full-length ICOS fused to YFP1 at its C terminus, along with Itk fused to YFP2. Cells were then analyzed by flow cytometry for YFP fluorescence (left panel). Alternatively, cells were stained for expression of ICOS using a specific antibody (right panel). c, cells were transfected with cytoplasmic tail deleted CD8α fused to YFP1 at its C terminus, along with Itk fused to YFP2. Cells were then analyzed by flow cytometry for YFP fluorescence (left panel). Alternatively, cells were stained for expression of CD8α using a specific antibody (right panel).

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