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The Tec Family Kinase Itk Exists as a Folded Monomer *in Vivo*[*] [S]

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Tec family tyrosine kinases transduce signals from antigen and other receptors. In particular, Itk plays an important role in T-cell development and activation. Itk has an N-terminal pleckstrin homology domain, a Tec Homology domain with a proline-rich region, SH3 and SH2 domains and a kinase domain, the structure each of which has been determined. However, the full structure of Itk and other Tec kinases remain elusive. Models of Itk suggest either a head to tail dimer, with the SH2 domain interacting with the SH3 domain, or a folded monomer with the SH3 domain interacting with the proline-rich region. We show here that *in vivo* Itk exists as a monomer, with the pleckstrin homology domain less than 80 Å from the C terminus. Zn^{2+} coordinating residues in the Tec Homology domain, not the proline-rich region, are critical for this intramolecular interaction. These data have implications for our understanding of Tec family kinase structure.

The Tec family of non-receptor tyrosine kinases, including Itk,[2] is the second largest family of non-receptor tyrosine kinases (1). They regulate signals emanating from multiple receptors, most prominently the TcR and BcR (1–6). Itk in particular has been shown to regulate TcR signals leading to increases in intracellular calcium, ERK (extracellular signal-regulated kinase)/mitogen-activated protein kinase, and activation of transcription factors NFAT and AP-1 (7, 8). More recently, it has been determined that Itk regulates the secretion of Th2 cytokines (9–11). In addition, Itk has been shown to be involved in the development of conventional or naïve phenotype $CD8^+$ T cells, $CD4^+$ T cells and NKT cells (12–16).

Itk is structurally organized into five domains, an N-terminal pleckstrin homology (PH) domain, followed by a TH domain, which contains a Zn^{2+}-binding BH motif and one PRR, SH3 and SH2 domains, and a C-terminal kinase domain. During stimulation of the TcR, phosphatidylinositol 3-kinase is activated, resulting in the formation of cell membrane phosphoinositides, to which the PH domain of Itk binds. Itk also forms dimers specifically at the plasma membrane in the vicinity of receptors that activate phosphatidylinositol 3-kinase (17). Once Itk is recruited to the membrane, it is phosphorylated by Src family kinases (18, 19). Upon activation, Itk is enriched in membrane rafts and interact with other signaling proteins through its SH2, SH3, and TH domain. Subsequently, Itk activates several downstream signaling components, including phospholipase Cγ1, and regulates the Ca^{2+} signaling pathway (9).

Although the structure of each of the individual domains of Itk is known, that of the full-length protein is unknown. A number of studies have suggested that the conformation of protein-tyrosine kinases is controlled by the self-interaction of domains, thus retaining them in the inactive state (20, 21). Src family tyrosine kinases, which have similar overall structures to Tec kinases with the exception of the TH and PH domains, are folded via intramolecular interactions between C-terminal negative regulatory phosphotyrosine and the SH2 domains that keep the kinase in the inactive state prior to receptor stimulation (20). Although Itk lacks the conserved C-terminal negative regulatory tyrosine phosphorylation site, Itk may also be regulated by intramolecular and/or intermolecular interactions among its domains. Indeed, two types of inter- and intra-domain interactions in Itk have been suggested. An intramolecular interaction between the SH3 and PRR domains of Itk has been suggested, which may act to maintain Itk in a folded state and thus prevent the binding of each domain to its respective ligands (22). A second type of intermolecular interaction has been suggested where the Itk SH2 domain interacts with the SH3 domain of a second Itk molecule, thus dimerizing Itk in a head-to-tail configuration (23). This model also suggests that a proline-dependent conformational switch exists in the SH2 domain of Itk, which directs a *cis* or *trans* conformer of the SH2 domain. The *cis*-conformer favors the dimerization of Itk via reciprocal SH2-SH3 interactions and may inhibit Itk kinase activity, while the *trans*-conformer favors the binding of phos-

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We dedicate this work to our great mentor Hidesaburo Hanafusa, professor emeritus of the Rockefeller University, who passed away on March 15, 2009 at the age of 79. He devoted his life to science and in particular to creating the oncogene research field, to teaching, and to providing profound affection to his students and postdocs. All of the alumni of Saburo's laboratory pride themselves in having been his apprentices, and we would like to hereby express our deeply felt gratitude to Saburo.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.

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[2] The abbreviations used are: Itk, inducible T cell kinase; PH, pleckstrin homology domain; BcR, B cell receptor; BH, Btk homology domain; CypA, cyclophilin A; ECFP, enhanced cyan fluorescent protein; GFP, green fluorescent protein; PRR, proline-rich region; TcR, T cell receptor; TH, Tec homology domain; SH2, Src homology domain 2; SH3, Src homology domain 3; YFP, yellow fluorescent protein; Y1I, YFP1 fragment tagged to Itk; Y2I, YFP2 fragment tagged to Itk; FRET, fluorescence resonance energy transfer; WT, wild type.

photyrosine containing ligands such as phosphorylated residues of SLP-76 (24, 25). This model is based on interpretations of NMR studies of isolated SH3 and SH2 domains; however, because the crystal structure of full-length Itk is not available, the exact conformation of Itk in the inactive state is still not clear.

We have examined the existence of Itk superstructures using fluorescence complementation assays (26). Our data suggest that Itk exists predominantly in an intramolecular folded conformation in the inactive state, where the PH domain is within 80 Å of the C terminus. Furthermore, our data indicate that the TH domain of Itk is critical for maintaining the intramolecular fold. We therefore propose that in cells Itk exists as a monomer, folded intramolecularly in a similar fashion to the Src and Abl non-receptor tyrosine kinases (27, 28).

EXPERIMENTAL PROCEDURES

Plasmids and Reagents—Antibodies against Itk were from Cell Signaling. Anti-GFP antibody was from Roche Applied Science. The pCDNA3.1-YFP1-zipper, pCDNA3.1-YFP2-zipper were a kind gift from Dr. Stephen Michnick (University of Montreal, Montreal, Canada) (26). 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) (29). YFP1-Itk and YFP2-Itk, as well as Itk mutants lacking the PRR within the TH domain, SH3, or SH2 domains were previously described or generated as previously described (17, 30). Other mutants in the TH domain included ΔTH+10N (Δ amino acids 145–156) and ΔTH+15C (Δ amino acids 155–171) were generated by standard molecular biology approaches. The caspase reporter SCAT used as a positive control for the FRET experiments was a kind gift of Dr. Masayuki Miura (University of Tokyo, Japan) (31). The ECFP and Venus variant of EYFP fluorescent proteins used in the SCAT construct were transferred to R29C Itk as indicated in Fig. 6.

Cell Transfection—HEK 293T cells and Jurkat T cells were cultured and transfected as previously reported (17, 30). In most cases Jurkat T cells were transduced using lentiviral infection. Human PBL T cells were expanded as previously described, then infected with lentiviral vectors carrying the indicated Itk cDNAs (29). The reagent, CypA$^{-/-}$ Jurkat T cells, was obtained from Jeremy Luban (through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health).

Flow Cytometric Assay—Cells were analyzed for fluorescence as previously described (17). Quantification of YFP fluorescence is described in the next section below.

Quantification of Fluorescence Complementation—Cells were analyzed for fluorescence as previously described (17). YFP fluorescence was corrected for expression by analysis of Itk expression either by Western blot, or in most experiments, by analysis of YFP or Itk expression using an antibody against GFP or Itk and flow cytometry. These values were then compared with control cells transfected with YFP1-Itk or YFP2-Itk alone, which was set at 1 (when analyzing YFP1-Itk-YFP2 and mutants, see examples in Fig. 3). In addition, controls for fluorescence complementation included the following: co-expression of YFP-1 zipper plus zipper-YFP2 (referred to as zip-YFP), which served as a positive control for fluorescence complementation (26), or expression of one of these two proteins along with YFP-Itk or Itk-YFP2 (when analyzing YFP1-Itk plus Itk-YFP2 and mutants, see Fig. 3). Signals from cells expressing the latter control was subtracted as background for co-expression experiments and the normalized fluorescence set at 1. Alternatively, normalized fluorescence of YFP1-R29CItk-YFP2 was set at 100%.

We and others have shown that mutants in the SH2, SH3, and ΔTH domains are stably expressed and in some cases have the predicted function of the remaining intact domains (32, 33). We have shown that the ΔTH mutant can be recruited to the membrane and phosphorylate the CD28 receptor (33). However, in all cases, protein expression was verified by Western blotting with specific Itk antibodies and/or anti-GFP.

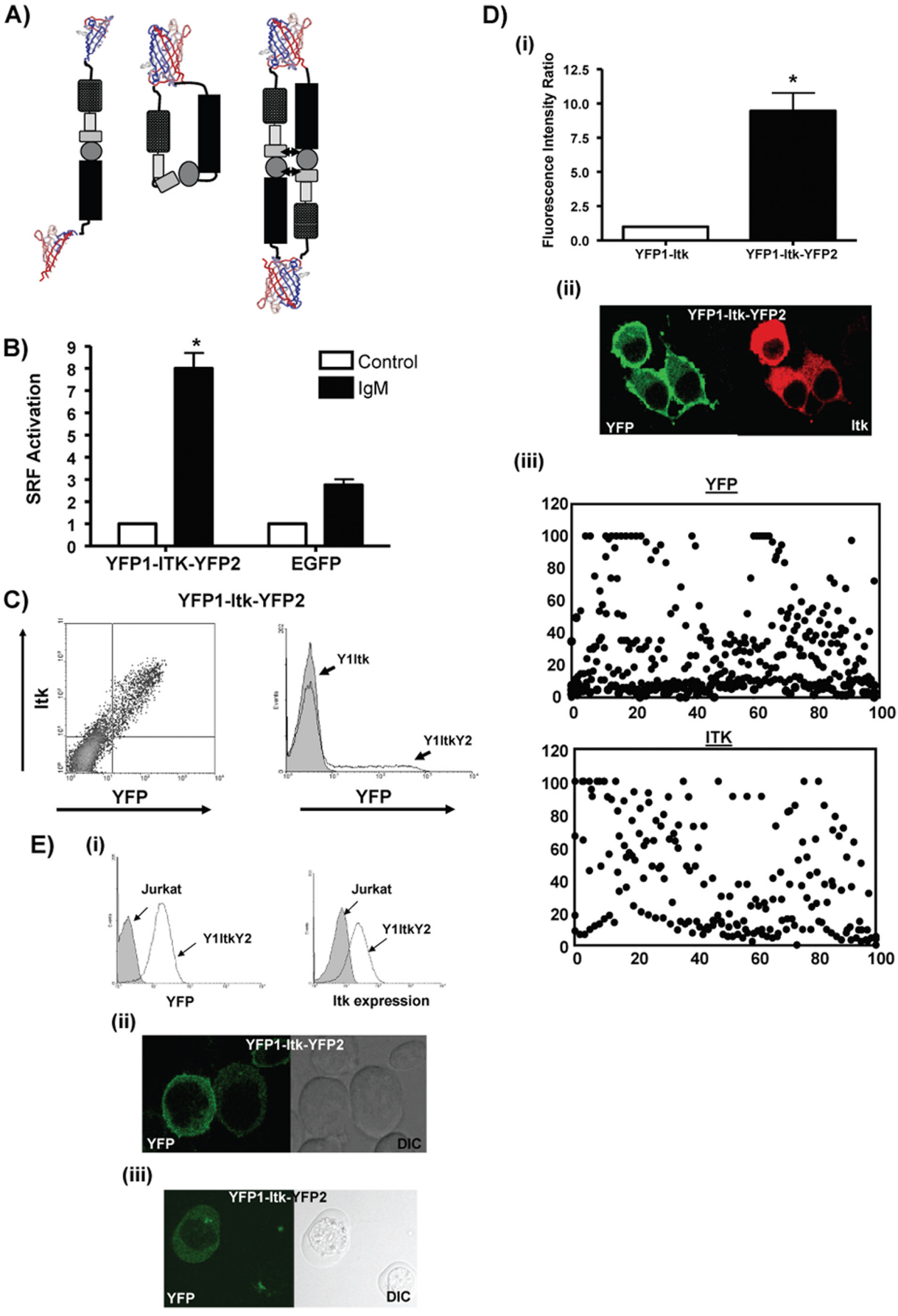
Confocal Microscopy, Cellular Localization Analysis, and FRET—Cells were analyzed by confocal microscopy as previously described (17). Subcellular localization of Itk and mutants were determined as previously described (17). In brief, transfected cells were fixed using 4% paraformaldehyde, then incubated with anti-Itk antibody in blocking buffer (2% fetal bovine serum and 0.1% Triton in phosphate-buffered saline). Following three washes, Itk was detected using an Alexa 663-conjugated secondary antibody. The cells were then analyzed by confocal microscopy. Given the varying size, shape, and level of expression of Itk in the transfected cells, we used the algorithm described in a previous study (17) 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). In brief, this algorithm included the following steps: 1) drawing a line across the cells to exclude the nucleus, collecting the fluorescence intensity, and mapping the data points for each cell; 2) setting as a percentage of the cell, the points along the line, from one end of the line to the other (0–100%); 3) calculation of the fluorescence at each point as a percentage of the maximum fluorescence observed for that cell (set at 100); and 4) plotting these values for each cell as a % maximum fluorescence (*y* axis) *versus* % 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%) (17).

For FRET analysis, cells were imaged 36 h following transfection into 293T cells, using an Olympus Fluoview confocal microscope. The normalized FRET was calculated using the FRET software package from Olympus.

Statistical Analysis—Data were analyzed by Student's *t* test, using Microsoft Excel and GraphPad Prism. A probability value of $p < 0.05$ was considered statistically significant.

RESULTS

Itk Exists as an Intramolecular Folded Monomer in the Inactive State in Vivo—We utilized fluorescence complementation assays using the split YFP system to examine the conformation of Itk *in vivo* (26). The N-terminal YFP1 fragment was fused to the N terminus of Itk and the C-terminal YFP2 fragment was fused to the C terminus of the same Itk molecule, thus constructing YFP1-Itk-YFP2 (Fig. 1*A*). Itk tagged with full-length GFP at the N or C termini have been demonstrated to maintain function and behavior (34, 35). However, we verified that this


A)
B)
SRF Activation
Control
IgM
YFP1-ITK-YFP2
EGFP
C)
YFP1-Itk-YFP2
Itk
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Y1Itk
Y1ItkY2
D)
(i)
Fluorescence Intensity Ratio
YFP1-Itk
YFP1-Itk-YFP2
(ii)
YFP1-Itk-YFP2
YFP
Itk
(iii)
YFP
ITK
E)
(i)
Jurkat
Y1ItkY2
YFP
Itk expression
(ii)
YFP1-Itk-YFP2
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(iii)
YFP1-Itk-YFP2
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modified YFP1-Itk-YFP2 was able to rescue antigen receptor-mediated activation of the SRF transcription factor in DT40 cells lacking Tec kinases ($BTK^{-/-}$ DT40 cells) (30, 36) (Fig. 1*B*).

Both structural models proposed for Itk (intramolecularly folded monomer and head to tail dimer) support the view that the N and C termini of Itk should be in close proximity (see Fig. 1*A*, and see supplemental Fig. S1*a* for schematic models). Using the YFP fusions described in Fig. 1*A*, this should result in complementation of the YFP fragments and the generation of fluorescence. As expected, when YFP1-Itk-YFP2 was expressed in cells, strong fluorescence was detected by flow cytometry (Fig. 1*C*, quantified in Fig. 1*D*(*i*)). The fluorescence of YFP1-Itk-YFP2 was located at both the cell membrane and cytoplasm, which was similar to the distribution pattern of Itk when analyzed by anti-Itk antibodies (Fig. 1*D*(*iii*)). Furthermore, expression of this protein in the Jurkat T cell line, as well as human peripheral blood T cells, also resulted in fluorescence complementation (Fig. 1*E*). These data suggest that the N and C termini are in close proximity (within 80 Å based on the length of the linkers used (17)) in the cytoplasm of these cells, suggesting that one or both models of Itk exist *in vivo* (see supplemental Fig. S1*a* for schematic models).

Because the YFP molecule will be complemented (and thus be fluorescent) regardless of whether Itk molecules fold intramolecularly as a monomer or intermolecularly as a dimer, we next determined which of these two models were more likely by analyzing Itk fused only to YFP1 at the N terminus (YFP1-Itk) expressed along with YFP2 fused to the C terminus (Itk-YFP2). If the intermolecular dimer model is more likely, then co-expression of YFP1-Itk and Itk-YFP2 should result in fluorescence complementation. However, if the intramolecular monomer is more likely, then this combination may result in little fluorescence complementation (see supplemental Fig. S1*a* for schematic models)). Analysis of cells expressing Itk tagged at either end by flow cytometry also revealed similar complementation of the YFP (Fig. 2*A*, expression confirmed in Fig. 2*B*). However, the reconstituted YFP fluorescence was only found at the cell membrane although Itk was localized at the cell membrane and in the cytoplasm, suggesting that Itk only forms dimers or higher order clusters at the cell membrane (Fig. 2*C*, (*i*) and (*iii*)) (17). Similar results were found in Jurkat T cells (Fig. 2*C*(*ii*)). Because Itk is only activated after it has been recruited to the cell membrane, these data suggest that these clusters of Itk are active (18, 19). In contrast, our data suggest that when Itk is in the inactive state in the cytoplasm, it is likely in an intramolecularly folded conformation.

To test this interpretation, we generated an Itk mutant, R29C, which disrupts the binding of the PH domain to lipids at the cell membrane, thus keeping Itk in the cytoplasm and in the inactive state (see Ref. 17, and see supplemental Fig. S1, *b* and *c*, for schematic model). Analysis of cells expressing N-terminally tagged YFP1-Itk^{R29C} and C-terminally tagged Itk^{R29C}-YFP2 indicated that Itk is unlikely to form head-to-tail homodimers when it cannot be recruited to the plasma membrane and is entirely cytoplasmic (Fig. 3*A*, with expression confirmed in Fig. 3*A*, (*iii*) and (*iv*)). Similar experiments performed where WT Itk fused to YFP1 was co-expressed with the Itk^{R29C}-YFP2 mutant revealed that co-expression of the WT Itk could not induce the formation of dimers at any location in the cell (membrane or cytoplasm) suggesting that Itk is unlikely to form dimers prior to being recruited to the plasma membrane (data not shown).

In contrast, R29C Itk tagged at both ends with the split YFP showed high fluorescence in both HEK-293T cells and Jurkat T cells, with complete cytoplasmic localization (Fig. 3, *B* and *C*). Together, these data further confirm that Itk most likely exists as an intramolecularly folded monomer in the inactive state in cells (schematic model in supplemental Fig. S1*d*). However, because the YFP fluorescence is only complemented when the N and C termini of Itk are within ~80 Å, we cannot exclude the possibility that Itk could also form homodimers with N- and C termini further apart or that a homodimer represents a small percentage of the total species of molecules in these cells.

Binding Via the SH2 and SH3 Domains Is Not Required to Maintain the Folded Inactive Monomer of Itk—Because Itk forms clusters in the cell membrane, which complicates the analysis, we used the R29C mutation as a base to analyze specific domain mutants of Itk (Figs. 1–3) (17). The dimer model proposes that the SH2 domain of Itk exists in both a *cis*- and *trans*-conformation, around Pro-287, the conformers of which are regulated by cyclophilin A. In experiments using isolated SH2 and SH3 domains of Itk, the *cis* conformer of SH2 domain has been shown to interact with the SH3 domain of Itk in *trans* via Pro-287. These experiments also suggest that the isolated SH2 domain mutant, P287G, primarily exists in the *trans*-conformation and does not participate in SH2–SH3 domain interactions, favoring instead a monomer (and open) form of Itk (24, 25). This should result in a change in the percentage of molecules that fold in the intramolecular state, thus altering the fluorescence complementation in our system (see supplemental Fig. S1*e*(*i*) for schematic model). We introduced the P287G mutation into the cytoplasmic YFP1-Itk^{R29C}-YFP2, and the fluorescence intensity was determined by flow cytometry. The results show that this mutant had similar fluorescence comple-

FIGURE 1. **Itk interacts with itself in cells.** *A*, model of split YFP fusions to Itk. *B*, split YFP-tagged Itk rescues Tec kinase function in Tec null cells. $BTK^{-/-}$ DT40 B cells were transfected with SRF-luciferase along with YFP1-Itk-YFP2 or GFP. Cells were then stimulated with anti-IgM antibodies, or left unstimulated, then harvested 8 h later. Cells were lysed and luciferase determined. *, $p < 0.05$ compared with cells transfected with GFP. *C*, 293T cells were transfected with Itk fused to YFP1 at the N terminus and YFP2 at the C terminus. Cells were then fixed and permeabilized, and Itk expression was determined by staining with an anti-Itk antibody (which recognizes these YFP-tagged proteins), followed by a PE-tagged anti-mouse IgG. The cells were then analyzed by flow cytometry (*left two-color plot*). We also determined in parallel the YFP fluorescence in live cells to quantify the YFP fluorescence complementation due to reduction in YFP fluorescence in fixed cells (*right histogram*). In the histogram, the control cells were transfected with Itk fused to YFP1 at the N terminus. *D*: (*i*), quantification of YFP fluorescence as under "Experimental Procedures" and in *C* ($n = 3$, *, $p < 0.05$). (*ii*), confocal analysis of cells expressing YFP1-Itk-YFP2 (*green*, YFP fluorescence; *red*, Itk expression as detected using anti-Itk). (*iii*), Itk localization was analyzed as described under "Experimental Procedures." Combined scans from at least five cells are shown analyzed for YFP localization (*top panel*) and for Itk localization (*bottom panel*). *E*: (*i*), flow cytometry plots of Jurkat T cells transduced with lentiviruses expressing YFP1-Itk-YFP2, YFP fluorescence (*left panel*), Itk expression as detected by anti-GFP antibodies (*right panel*). (*ii*), confocal analysis of Jurkat T cells expressing YFP1-Itk-YFP2 (*green*, YFP fluorescence). (*iii*), confocal analysis of peripheral blood T cells expressing YFP1-Itk-YFP2 (*green*, YFP fluorescence).

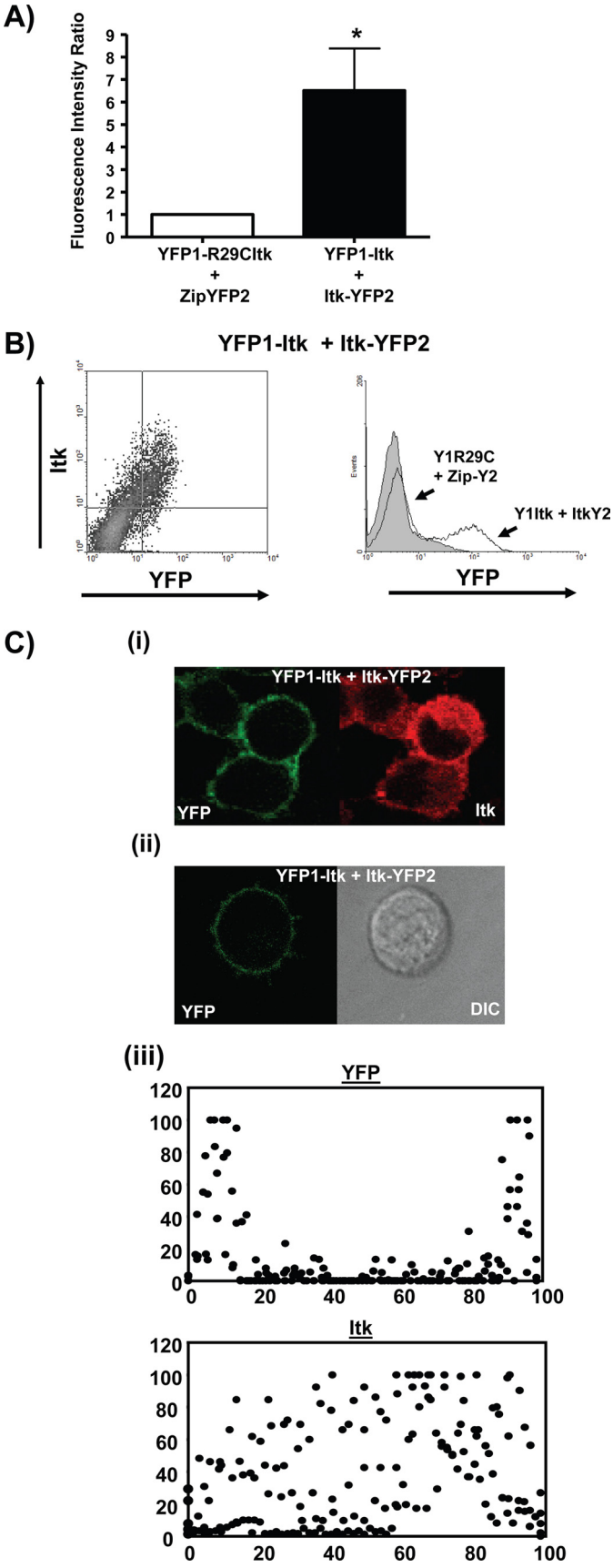


FIGURE 2. **Itk does not form dimers in the cytoplasm of cells.** *A*, 293T cells were transfected with Itk fused to YFP1 at the N terminus along with Itk fused to YFP2 at the C terminus and YFP fluorescence quantified ($n = 3$, *, $p < 0.05$). *B*, 293T cells transfected with Itk fused to YFP1 at the N terminus and along with Itk fused to YFP2 at the C terminus were analyzed as in Fig. 1*C* for Itk and YFP (*left two-color plot*), for YFP (*right histogram*). *C*: (*i*), confocal analysis of 293T cells expressing YFP1-Itk along with Itk-YFP2 (*green*, YFP fluorescence; Itk expression as detected using anti-Itk antibodies, Red). (*ii*) Confocal analysis of Jurkat T cells transfected with YFP1-Itk along with Itk-YFP2 (*green*, YFP fluorescence). (*iii*) Combined scans from at least 5 cells are shown analyzed for YFP localization (*top panel*) and for Itk localization (*bottom panel*).

mentation to the "WT" R29C mutant (Fig. 4, *A–C*). These results suggest that if a head-to-tail homodimer of Itk exists, it does not contribute to the YFP fluorescence in WT R29C, further confirming that the intramolecular folded monomer is the likely conformation of Itk in the inactive state. These data also suggest that R29C/P287G mutant has a similar structure as the WT R29C "cytoplasmically trapped" inactive Itk.

To further probe the potential *cis* conformer of Itk, we also utilized Jurkat T cells lacking CypA (37), where Itk should exist in a predominantly *trans* (and thus open) conformation around the Pro-287 in the SH2 domain. Expression of the R29C PH mutant of Itk tagged at both ends with the split YFP in these cells resulted in similar fluorescent complementation to that seen in WT Jurkat cells, arguing that CypA does not alter the overall structure of Itk in cells, and that *cis-trans* isomerization of Itk around Pro-287 may not affect the structure of Itk (Fig. 4*C*). However, we cannot rule out that in the absence of CypA, other prolyl isomerases can act on Itk, although treating YFP1-R29CItk-YFP2-expressing cells with cyclosporine A results in no change in fluorescence complementation (data not shown). Together, these data further confirm that an intramolecular folded conformer of Itk exists *in vivo* and indicate that the *cis*/*trans* conformation of the Itk SH2 domain is not important for conformation. Expression of these mutants was confirmed by flow cytometry and Western blotting (Fig. 4*B*(*iii*)).

The residue Trp-208 is centrally located and solvent-exposed in the ligand-binding pocket of Itk SH3 domain. Mutation of this conserved Trp to Lys prevents the SH3 domain from mediating protein-protein interactions (23). A W208K mutant should therefore eliminate any SH3 interactions with the *cis*-conformer of the SH2 domain, thus disrupting any dimer. In addition, this mutant should also disrupt any SH3-PRR interactions if they exist (see supplemental Fig. S1*e*(*ii*) for schematic model). However, introducing this mutant into YFP1-R29C-YFP2 (*i.e.* YFP1-Itk$^{R29C/W208K}$-YFP2) did not change fluorescence complementation, indicating that SH3 domain interactions, at least via the classic binding site, may not alter the intramolecular folded conformation of Itk (Fig. 4*D*, with expression confirmed by Western blotting in Fig. 4*D*(*iv*)).

PRR mutants P158A/P159A in the TH domain, which disrupt SH3 domain binding to the PRR region, should also destroy any SH3-TH interaction (see supplemental Fig. S1*e*(*iii*) for schematic model (38)). However, this mutation did not change YFP fluorescence complementation of the R29CItk, further confirming that the SH3-PRR interaction is not critical for maintaining this conformation of Itk (Fig. 4*D*, with expression confirmed by Western blotting in Fig. 4*D*(*iv*)). The folded monomer model proposes that specific domains of Itk interact with an N-terminal PRR in the TH domain of Itk to maintain the monomer (24, 25). However, the fact that we can alter both the

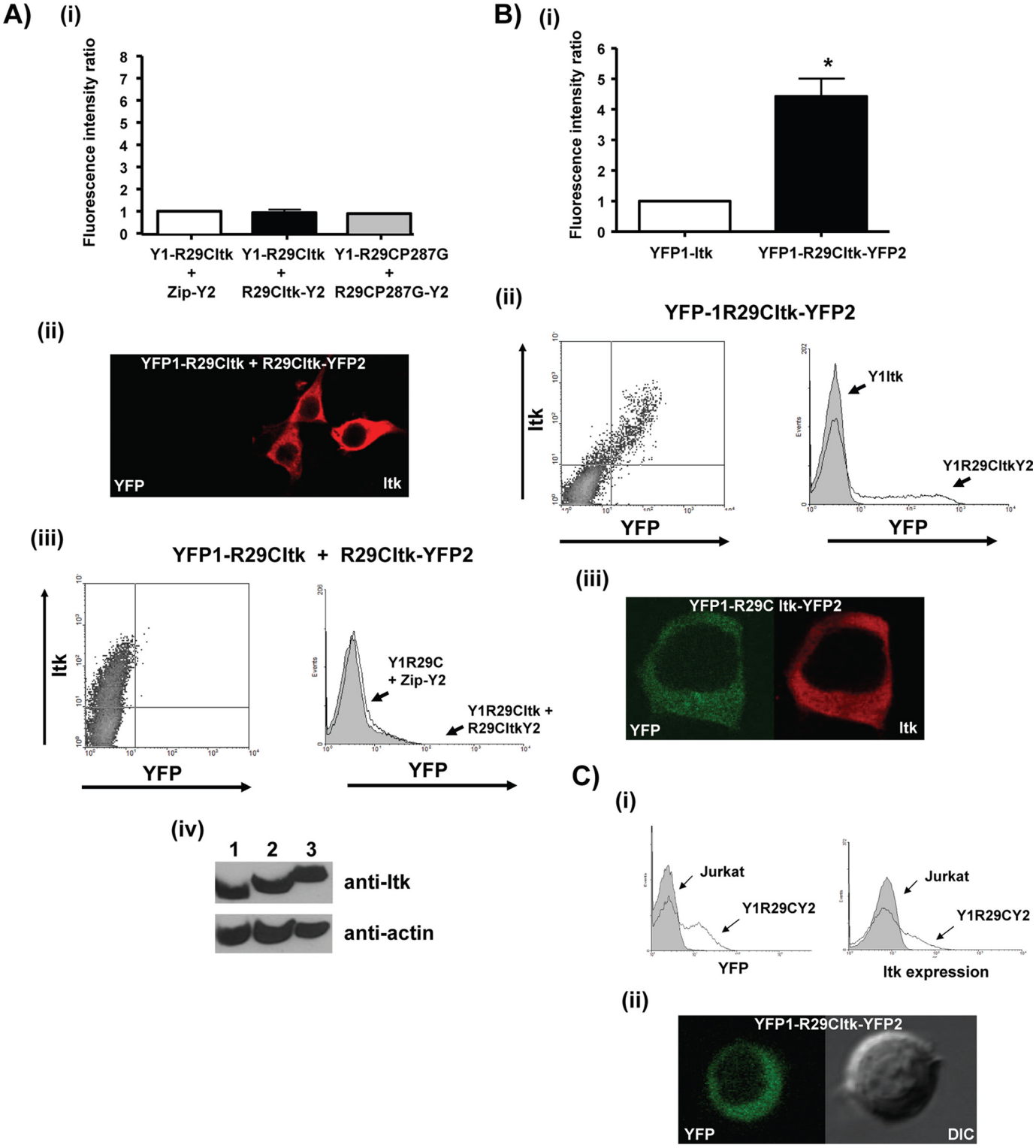
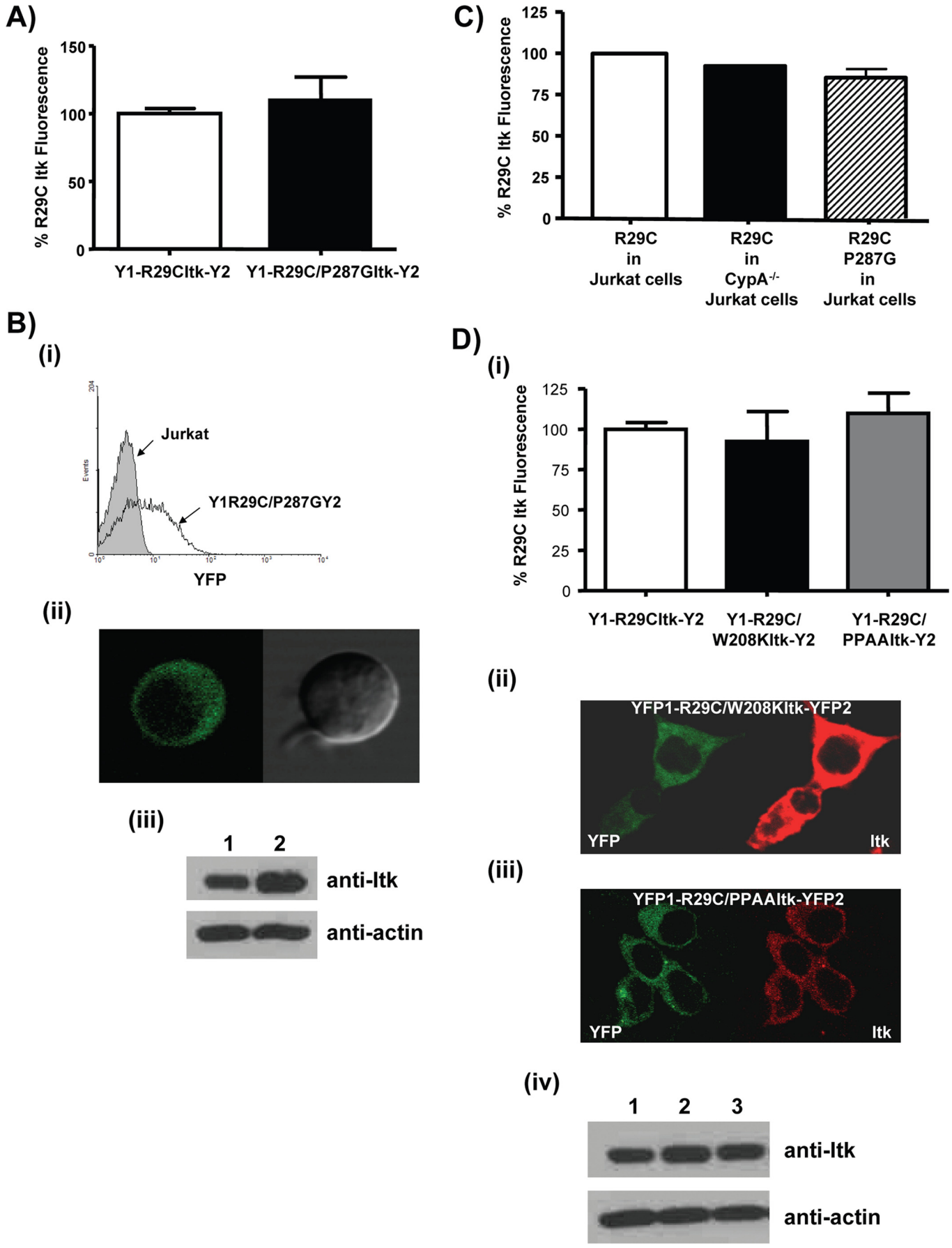


FIGURE 3. **Cytoplasmically localized Itk exists as a head to tail folded monomer in cells.** *A*: (*i*) 293T cells were transfected with R29CItk fused to YFP1 at the N terminus along with R29CItk fused to YFP2 at the C terminus and YFP fluorescence quantified. (*ii*) Confocal analysis of cells expressing YFP1-R29CItk along with R29CItk-YFP2 (*green*, YFP fluorescence; *red*, Itk expression as detected using anti-Itk). (*iii*), analysis of 293T cells transfected with R29CItk fused to YFP1 at the N terminus along with R29CItk fused to YFP2 at the C terminus as in Fig. 1*C* for Itk and YFP (*left two-color plot*), for YFP (*right histogram*). (*iv*), Western blot analysis of whole cell lysates from 293T cells expressing: *lane 1*, YFP1-R29CItk; *lane 2*, R29CItk-YFP2; and *lane 3*, YFP1-R29CItk-YFP2. *Top panel*, anti-Itk; *bottom panel*, anti-actin. *B*: (*i*), 293T cells were transfected with R29CItk fused to YFP1 at the N terminus and YFP2 at the C terminus and YFP fluorescence quantified ($n = 3$, *, $p < 0.05$). (*ii*), flow cytometric analysis of cells in *B*(*i*) for Itk and YFP (*left two-color plot*), for YFP (*right histogram*). (*iii*), confocal analysis of cells expressing YFP1-R29CItk-YFP2 (*green*, YFP fluorescence; *red*, Itk expression as detected using anti-Itk). *C*: (*i*), flow cytometry plots of Jurkat T cells transfected with YFP1-R29CItk-YFP2. YFP fluorescence (*left panel*), Itk expression as detected by anti-GFP antibodies (*right panel*). (*ii*), confocal analysis of Jurkat T cells expressing YFP1-R29CItk-YFP2 (*green*, YFP fluorescence).


A)
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anti-actin

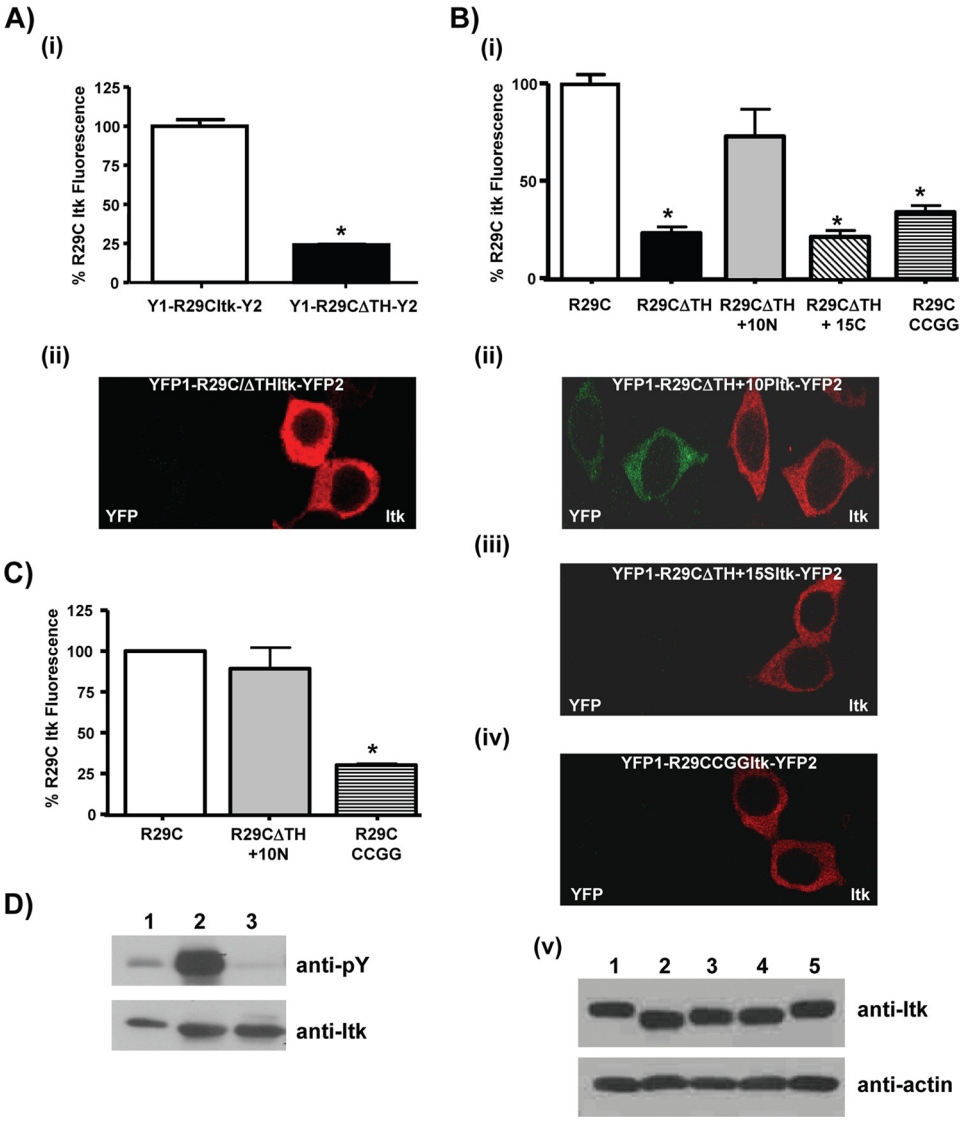


FIGURE 5. **The Zn^{2+} binding region in the TH domain of Itk is required to maintain the head to tail folded monomer in cells.** *A*: (*i*), 293T cells were transfected with YFP1-R29C/ΔTHItk-YFP2 followed by quantification of YFP fluorescence. (*ii*), confocal analysis of cells expressing YFP1-R29C/ΔTHItk-YFP2 (*green*, YFP fluorescence; *red*, Itk expression as detected using anti-Itk). *B*: (*i*), 293T cells were transfected with YFP1-R29C/ΔTHItk-YFP2, YFP1-R29C/ΔTHItk+10N-YFP2, YFP1-R29C/ΔTHItk+15C-YFP2, or YFP1-R29C/C132GC133GItk-YFP2, followed by quantification of YFP fluorescence. (*ii*), confocal analysis of cells expressing YFP1-R29C/ΔTHItk+10N-YFP2 (*green*, YFP fluorescence; *red*, Itk expression as detected using anti-Itk). (*iii*), confocal analysis of cells expressing YFP1-R29C/ΔTHItk+15C-YFP2 (*green*, YFP fluorescence; *red*, Itk expression as detected using anti-Itk). (*iv*), confocal analysis of cells expressing YFP1-R29C/C132GC133GItk-YFP2 (*green*, YFP fluorescence; *red*, Itk expression as detected using anti-Itk). (*v*), Western blot analysis of whole cell lysates from 293T cells expressing YFP1-R29CItk-YFP2 (*lane 1*), YFP1-R29C/ΔTHItk-YFP2 (*lane 2*), YFP1-R29C/ΔTHItk+10N-YFP2 (*lane 3*), YFP1-R29C/ΔTHItk+15C-YFP2 (*lane 4*), or YFP1-R29C/C132GC133GItk-YFP2 (*lane 5*). Anti-Itk (*top panel*) and anti-actin (*bottom panel*) are shown. *C*, Jurkat T cells were transduced with lentiviruses expressing the indicated Itk mutants tagged at the N terminus with YFP1 and the C terminus with YFP2, followed by quantification of YFP fluorescence. *, $p < 0.05$. *D*, 293T cells were transfected with Lck, along with WT Itk (*lane 1*), the C132G/C133G mutant of Itk (*lane 2*), or kinase-inactive Itk (*lane 3*). Itk was immunoprecipitated and analyzed for phosphotyrosine (*top panel*) or Itk (*bottom panel*).

SH3 binding pocket as well as proline residues in the PRR suggest that, although this mode of interaction may exist *in vivo*, it may not be necessary for the maintenance of a folded monomer.

The Zn^{2+} Binding Region in the TH Domain Is Critical for Maintenance of the Folded Inactive Monomer of ITK—Because disrupting interactions between the two predicted domains (*cis*-SH2 interaction with SH3 or SH3 interaction with PRR) proposed by the existing models does not alter the intramolecular folded monomer conformer of Itk, we deleted the PRR region and part of Zn^{2+}-binding Btk homology (BH) motif in the R29C mutant of Itk (resulting in a mutant referred to as ΔTH, see supplemental Fig. S1*f*(*i*) for schematic model), and determined its ability to induce complementation of YFP fluorescence (32). Note that we have previously shown that this ΔTH mutant is functional when the mutation is on a WT Itk background (32). Strikingly, we found that this mutant had dramatically decreased YFP fluorescence complementation (Fig. 5*A*, with expression confirmed by Western blotting in Fig. 5*B*(*v*)). When the PRR was restored, including key amino acids required for the proposed interaction between PRR region and SH3 domain giving the mutant ΔTH15C, YFP fluorescence was not recovered (Fig. 5*B*). However, when amino acids N-terminal to the PRR were restored (giving mutant ΔTH10N), fluorescence was largely recovered (Fig. 5, *B* and *C*). Because the deletion variant ΔTH15C missing only a portion of the BH motif had reduced complementation, whereas the mutant

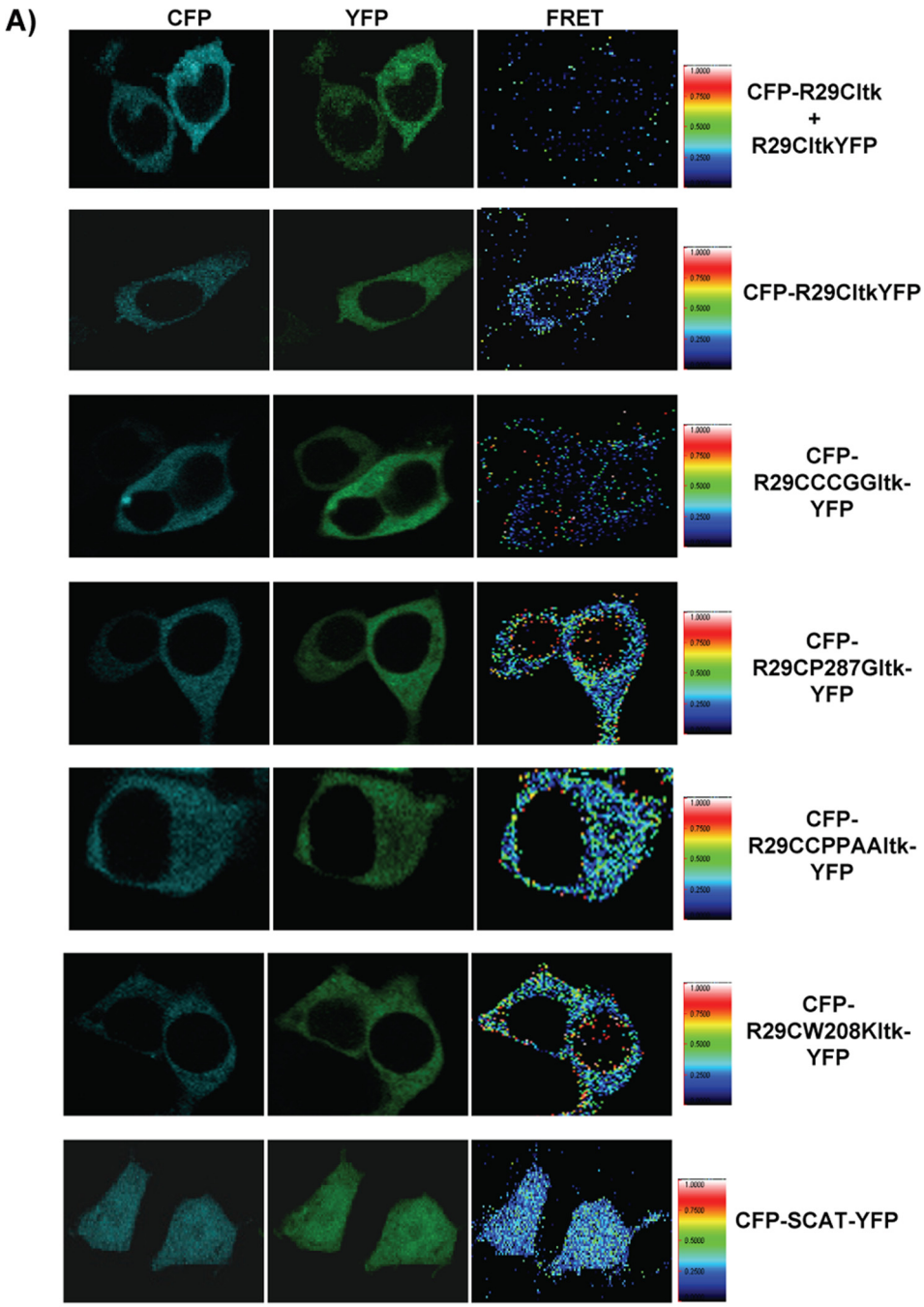
FIGURE 4. **Interactions via *cis*/*trans* conformation, the PRR, SH3, or cyclophilin A are not required for the formation of the head to tail folded monomer of Itk.** *A*, 293T cells were transfected with YFP1-R29CItk-YFP2 or YFP1-R29C/P287GItk-YFP2 and YFP fluorescence quantified. *B*: (*i*), flow cytometry plot of Jurkat T cells transfected with YFP1-R29C/P287GItk-YFP2 (YFP fluorescence). (*ii*), confocal analysis of Jurkat T cells expressing YFP1-R29C/P287Gtk-YFP2 (*green*, YFP fluorescence). (*iii*), Western blot analysis of whole cell lysates from 293T cells expressing (*1*) YFP1-R29CItk-YFP2 and (*2*) YFP1-R29C/P287GItk-YFP2. Anti-Itk (*top panel*), anti-actin (*bottom panel*). *C*, WT or CypA$^{-/-}$ Jurkat T cells were transduced with lentivirus expressing YFP1-R29CItk-YFP2. Similarly, WT Jurkat T cells were transduced with lentivirus expressing YFP1-R29C/P287GItk-YFP2 followed by quantification of YFP fluorescence. *D*: (*i*), 293T cells were transfected with YFP1-R29C/W208KGItk-YFP2 or YFP1-R29C/P158AP159AItk-YFP2, followed by quantification of YFP fluorescence. (*ii*), confocal analysis of cells expressing YFP1-R29C/W208KItk-YFP2 (*green*, YFP fluorescence; *red*, Itk expression as detected using anti-Itk). (*iii*), confocal analysis of cells expressing YFP1-R29C/PPAAItk-YFP2 (*green*, YFP fluorescence; *red*, Itk expression as detected using anti-Itk). (*iv*), Western blot analysis of whole cell lysates from 293T cells expressing YFP1-R29CItk-YFP2 (*lane 1*), YFP1-R29C/W208KGItk-YFP2 (*lane 2*), or YFP1-R29C/P158AP159AItk-YFP2 (*lane 3*). Anti-Itk (*top panel*) and anti-actin (*bottom panel*) are shown.

ΔTH10N that included the BH domain maintained fluorescence, these data suggest that the BH motif is important for maintaining the intramolecular folded conformation of Itk (see supplemental Fig. S1*f*, (*ii*) and (*iii*), for schematic models).

The BH motif of Tec family kinases is conserved, with amino acids involved in coordinating a molecule of Zn^{2+} in identical positions (39, 40). Cys-155 is one of the conserved Zn^{2+} ligands and when mutated to Gly in BTK, has been reported to disrupt the Btk motif (39). To further test the role of the BH motif in the conformation of ITK, Cys-132 and Cys-133, two corresponding conserved amino acids in Itk, were mutated to Gly in YFP1-Itk^{R29C}-YFP2 (to generate YFP1-$Itk^{R29C/C132G/C133G}$-YFP2), and this mutant was tested. The data show that this Zn^{2+}-binding mutant of Itk had significantly decreased fluorescence complementation, suggesting that the folding was disrupted (Fig. 5, *B* and *C*; see supplemental Fig. S1*f*(*iv*) for schematic model).

The data suggest that this TH domain mutant is in an open conformation. This would suggest that it would be easier to activate than WT Itk. To test this, we expressed this mutant (minus the YFP fusions) along with Lck in cells, and determined whether it had been activated, as evidenced by increased phosphotyrosine content. We found that the C132G/C133G-Itk had increased levels of phosphotyrosine compared with WT Itk (Fig. 5*D*, note that the blot is underexposed relative to WT Itk, which has increased phosphorylation over the kinase inactive Itk when expressed along with Lck).

To confirm that the fluorescence complementation assay was able to faithfully report the structure of Itk in cells, we also used FRET analysis to examine the interactions between the N and C termini of Itk. Itk was tagged with ECFP at the N terminus and Venus at the C terminus, transfected into cells, and



imaged for FRET analysis. These data (Fig. 6, *A* and *B*), show that FRET only occurred when Itk was tagged at the N and C termini with ECFP and Venus, but not when Itk molecules separately tagged with ECFP or Venus were expressed together in cells. In addition, mutation of P287G (CypA binding site), W208K (SH3 binding site), and PPAA (PRR) in Itk did not change the FRET. However, the C132G/C133G mutant of Itk had similar FRET to the separately tagged Itk (Fig. 6, *A* and *B*). Expression of the SCAT molecule, consisting of ECFP and Venus separated by an 18-amino acid peptide (~72 Å apart) served as a positive control and gave similar robust FRET (Fig. 6, *A* and *B*) (31). Thus the data support the conclusion that the N and C termini of Itk are in close proximity to each other and that Itk forms an intramolecular monomer in the cytoplasm of cells. Taken together, our data provide support for a model of Itk where the inactive protein in the cytoplasm exists as a folded monomer, with the N terminus within ~80 Å from the C terminus. Our data also suggest that the Zn^{2+} binding region of the BH motif of Itk is important for its ability to maintain a monomeric intramolecularly folded conformation.

DISCUSSION

We show here that Itk exists in an intramolecular folded monomeric conformation in the inactive state *in vivo*. This monomer is not dependent on the integrity of the SH3 binding site, nor is it dependent on the proline-rich region as previously suggested (22). Instead, we show that the folded monomer of Itk is dependent on residues within the BTK motif, responsible for coordinating zinc, within the TH domain.

Luban and colleagues have previously demonstrated that Itk may form dimers in cells using a co-immunoprecipitation assay (24, 25). We have also detected a dimer of Itk, however this was only found with WT Itk at the cell membrane (26). Because Luban *et al.* only examined WT Itk, it is possible that they detected the membrane-directed dimer of Itk in their co-immunoprecipitation assays. We should note that our approach of using fluorescence complementation of YFP is not without pitfalls. This approach, while relatively simple to use and score, may reduce the requirement for high affinity interactions and amplify those low affinity interactions. This may result in our finding that the SH3 and PRR are not required to maintain the folded monomer. However, this is also a potential strength because we may be able to detect interactions that may be too weak or not stable enough to detect via FRET or other approaches. However, we have confirmed our experiments using FRET, indicating that both approaches report an intramolecularly folded monomer for Itk in cells.

Although our data do not support a head to tail homodimer conformer for inactive Itk, such dimers may exist. One possibility is that such dimers only represent a small percentage of the total species of molecules in cells. The other possibility is that the distance between the N and C termini of a potential homodimer of Itk is greater than 80 Å such that our system could not detect it. However, the fact that we can detect homodimers of WT Itk at the plasma membrane when these molecules are tagged at either end would tend to argue that our system can detect dimers, if they exist (17).

Although the crystal structure of a full-length Tec family tyrosine kinase has not yet been reported, Márquez *et al.* have predicted that Btk likely exists as a monomer with little interaction between its domains (41). This prediction was based on x-ray synchrotron radiation scattering and rigid body modeling, using purified protein from bacteria. Our experiments were performed in mammalian cells, including T cells, and it is possible that the cellular environment provides the proper requirements for the folding patterns that we detect. It is also possible that Btk has a different conformation from Itk. We should note that the BH motif within the TH domain has been previously suggested to regulate the activity of Btk. Huang and colleagues have reported that the $G\alpha_q$ protein interacts with Btk via the TH motif, and that this interaction is involved in the ability of this G-protein to regulate the activity of Btk (42–44). It is possible that recruitment of Itk to the membrane via its PH domain allows $G\alpha_q$ to interact with the TH motif, thus opening the kinase for activation. It was not possible for us to test this model using our approaches as discussed below, but membrane localization (and dimerization) of Itk is (are) not dependent on the TH domain (17). We cannot rule out that mutation of Cys-132 and Cys-133 in Itk disrupts the integrity of the TH domain, which may result in an "open" conformation. However, our analysis of expression and activity suggest that this mutant is functional by other criteria. In addition, if the integrity of the TH domain is disrupted, this would be similar to other approaches that generate temperature-sensitive mutants in the SH3 domains of proteins to determine their function (45).

Crystallographic studies of other non-receptor tyrosine kinases of the Src, Abl, and Syk family have all shown that, in the inactive state, these kinases fold as monomers with the N terminus near the C terminus of the protein (27, 28). However, the mechanisms involved in maintenance of these structures are varied. In the case of Src family kinases, the SH2 domains interact with a C-terminal phosphorylated tyrosine, while the SH3 domain forms contacts with pseudo-proline regions to maintain the inactive structure. Similar analysis of c-Abl reveals an analogous structure, except that the SH2 and SH3 domains make other contributions to the maintenance of the inactive protein. More recently the structure of the Syk kinase was solved and revealed a similar folded inactive conformer (46).

Our work here supports the view that non-receptor tyrosine kinases Src, Abl, and Tec have similar modes of regulation from the perspective of folding. The data also suggest that active Itk may be in an open configuration, allowing enhanced kinase activity. We were only able to determine the "folded" confor-

FIGURE 6. **FRET evidence for intramolecular folded conformation for inactive Itk in cells.** *A*, 293T cells were transfected with either ECFP along with Venus alone (not shown), ECFP-R29CItk along with R29CItk-Venus, ECFP-R29CItk-Venus, ECFP-R29C/C132GC133GItk-Venus, ECFP-R29C/P287GItk-Venus, ECFP-R29C/PPAAItk-Venus, or ECFP-R29C/W208KItk-Venus, or ECFP-SCAT-Venus (as a positive control), followed by analysis by confocal microscopy for ECFP fluorescence (*left panels*), Venus fluorescence (*middle panels*), or normalized FRET (*right panels*). *B*, quantification of FRET efficiency (max = 1) for the above experiments (*, $p < 0.05$).

mation of Itk using a PH mutant that is cytoplasmically localized, avoiding membrane-associated dimers or higher order clusters. However, we were able to determine that the Zn^{2+}-binding mutant (Cys-132 and Cys-133) of Itk with the proposed open conformation is easier to activate when co-expressed along with the Src family kinase Lck. While we cannot rule out that small pools of Itk may exist in a head to tail dimer as previously suggested; our data support a model where the majority of Itk molecules exist in a folded monomer, with the N and C termini in close proximity, a conformation that requires the Zn^{2+} binding region of the BH motif in the TH domain. Our data also suggest that the reported CypA-Itk interaction may not significantly affect the structure of Itk in cells.

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