ZAP-70 Association with T Cell Receptor ζ (TCRζ): Fluorescence Imaging of Dynamic Changes upon Cellular Stimulation

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Abstract. The nonreceptor protein tyrosine kinase ZAP-70 is a critical enzyme required for successful T lymphocyte activation. After antigenic stimulation, ZAP-70 rapidly associates with T cell receptor (TCR) subunits. The kinetics of its translocation to the cell surface, the properties of its specific interaction with the TCRζ chain expressed as a chimeric protein (TTζ and Tζζ), and its mobility in different intracellular compartments were studied in individual live HeLa cells, using ZAP-70 and Tζζ fused to green fluorescent protein (ZAP-70 GFP and Tζζ–GFP, respectively). Time-lapse imaging using confocal microscopy indicated that the activation-induced redistribution of ZAP-70 to the plasma membrane, after a delayed onset, is of long duration. The presence of the TCRζ chain is critical for the redistribution, which is enhanced when an active form of the protein tyrosine kinase Lck is coexpressed. Binding specificity to TTζ was indicated using mutant ZAP-70 GFPs and a truncated ζ chimera. Photobleaching techniques revealed that ZAP-70 GFP has decreased mobility at the plasma membrane, in contrast to its rapid mobility in the cytosol and nucleus. Tζζ–GFP is relatively immobile, while peripherally located ZAP-70 in stimulated cells is less mobile than cytosolic ZAP-70 in unstimulated cells, a phenotype confirmed by determining the respective diffusion constants. Examination of the specific molecular association of signaling proteins using these approaches has provided new insights into the TCRζ–ZAP-70 interaction and will be a powerful tool for continuing studies of lymphocyte activation.

Key words: ZAP-70 • TCRζ • protein tyrosine kinase • intracellular signaling • GFP

Engagement of the T cell receptor (TCR)[1] by antigenic ligand, in the form of a short linear peptide bound in the cleft of a major histocompatibility complex (MHC) class I or II molecule, is the critical binding event leading to T cell activation (Babbitt et al., 1985; Townsend et al., 1986; Bentley and Mariuzza, 1996; Garcia et al., 1996). The TCR is comprised of multiple integral membrane proteins (Jorgensen et al., 1992; Weiss, 1993; Weissman, 1994) and serves to initiate intracellular signaling, leading to new gene expression, protein synthesis, induction of multiple effector functions, and clonal expansion (Samelson and Klausner, 1992; Weiss and Littman, 1994). The αβ heterodimer binds to the antigen-MHC ligand, and the CD3 γ, δ, ϵ, and TCRζ chains translate this event into biochemical signals within the cell (Cantrell, 1996; Garcia et al., 1996; Wange and Samelson, 1996; Qian and Weiss, 1997). Since none of these molecules contains any intrinsic enzymatic activity, they recruit and bind signaling proteins via their conserved immunoreceptor tyrosine-based activation motifs (ITAMs), which are present as a single copy in each of the CD3 chains and in triplicate in TCRζ (Reth, 1989; Weiss and Littman, 1994; Wange and Samelson, 1996). The CD3 and TCRζ chains are phosphorylated on the tyrosines within their ITAMs within seconds of TCR engagement by the Src kinases Lck and Fyn (Iwashima et al., 1994; van Oers et al., 1996; Sloan-Lancaster and Samelson, 1998). The phospho-ITAMs are then able to bind SH2 domain–containing proteins, allow-

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1. *Abbreviations used in this paper*: FLIP, fluorescence loss in photobleaching; FRAP, fluorescence recovery after photobleaching; GFP, green fluorescent protein; ITAM, immunoreceptor tyrosine-based activation motif; MHC, major histocompatibility complex; PV, pervanadate; ROI, region of interest; TCR, T cell antigen receptor; TTζ, Tac Tac zeta; Tζζ, Tac zeta zeta.

ing a multiprotein complex to form under the membrane, which includes enzymes and adaptors responsible for triggering the various intracellular signaling pathways for successful T cell activation (Weiss and Littman, 1994; Wange and Samelson, 1996).

ZAP-70, a nonreceptor protein tyrosine kinase expressed exclusively in T cells, thymocytes, and natural killer cells, is a critical enzyme in early T cell signaling (Chan et al., 1992; Wange et al., 1992; Arpaia et al., 1994; Chan et al., 1994; Elder et al., 1994; Negishi et al., 1995). After binding via its tandem SH2 domains to the two phosphotyrosines of an individual ITAM during TCR engagement (Wange et al., 1993), ZAP-70 is phosphorylated by Lck and/or Fyn and is thus activated (Iwashima et al., 1994; Wange et al., 1995*a*; Kong et al., 1996). Subsequently, these kinases phosphorylate other specific substrates, resulting in the activation of the various intracellular signaling pathways required for T cell function. Although the absolute requirement of functional ZAP-70 for T cell activation has been clearly demonstrated both biochemically and genetically (Wange et al., 1995*b*; Qian et al., 1996; Williams et al., 1998), few studies have examined its intracellular localization and how this is affected by cellular stimulation. The primary structure predicts that ZAP-70 is a cytosolic protein (Chan et al., 1992), and biochemical data have shown that it rapidly translocates to the TCR upon activation (Wange et al., 1992; Chan et al., 1991). We have recently developed a cellular approach to examine the location and movement of ZAP-70 in single cells over real time, using a chimera of ZAP-70 fused to the green fluorescent protein (GFP) and time-lapse imaging confocal microscopy (Sloan-Lancaster et al., 1997). Our initial study revealed that ZAP-70 GFP was present not only throughout the cytosol but also in the nucleus, in both transiently transfected COS 7 cells and ZAP-70–deficient T cells stably reconstituted with the chimera. In COS 7 cells, ZAP-70 GFP rapidly moved from the cytosol to the cell surface in response to pharmacological stimulation. This was surprising since COS 7 cells do not express any TCR chains or other molecules known to contain ITAMs. We reasoned that another membrane-associated protein, which becomes tyrosine phosphorylated upon cellular stimulation, was able to bind ZAP-70 in order for this translocation and apparent binding to occur (Sloan-Lancaster et al., 1997).

Since the current model of T cell activation dictates that ZAP-70 is bound and concentrated at the region of activated TCR via a specific interaction with the phosphorylated ITAMs of TCR subunits, we wanted to refine our experimental system to study this association. This would enable us not only to assess the real time binding kinetics, but also to demonstrate the fine specificity of the molecular interaction in individual living cells. In addition, we wanted to measure the mobility of ZAP-70 in the different intracellular compartments to understand the mechanisms of retention at the plasma membrane. Here we report the stimulation-dependent translocation of ZAP-70 to the cell surface in HeLa cells is dependent on expression of a chimeric TCRζ chain. We describe the kinetics of this interaction and show that ZAP-70 translocation is enhanced by coexpressing active Lck. Moreover, we provide evidence that relocated ZAP-70 is specifically bound to the chimeric ζ chain, with properties that correspond precisely with the data generated biochemically (Wange et al., 1993; Koyasu et al., 1994). Using photobleaching techniques, we have revealed the highly mobile and freely diffusible nature of cytosolic and nuclear ZAP-70 and its conversion to a more static state accompanying its translocation to the cell periphery. Cell surface–located ZAP-70 is more diffusible than TCRζ, a transmembrane protein, a phenotype confirmed by calculating the diffusion constants for the individual proteins, which indicated that peripheral ZAP-70 diffuses 20-fold faster than TCRζ. Such observations suggest that the interaction between ZAP-70 and TCRζ upon cellular stimulation is dynamic.

Materials and Methods

Cells, Antibodies, and Reagents

HeLa cells were grown in complete D10 medium (DME containing 10% FBS, 2 mM glutamine, and 50 μg/ml gentamicin). All stably transfected lines were cultured in complete D10 medium supplemented with 1 mg/ml geneticin (G418; GIBCO BRL, Gaithersburg, MD) for maintenance of transgene expression. H/TTζ, H/Tζζ, and H/Tζζ truncated (H/Tζζ trunc) cells stably expressed the appropriate fusion protein as determined by frequent FACS® and immunoprecipitation analyses.

mAbs used include anti–IL-2 receptor α chain, 33B3.1 (Immunotech, Inc., Westbrook, ME), for FACS® analysis; rabbit anti–ZAP-70 antiserum (Wange et al., 1995*a*); mouse anti–human α-tubulin (Sigma Chemical Co., St. Louis, MO); rhodamine-coupled goat anti–mouse IgG; and fluorescein-coupled goat anti–rat IgG (KPL, Inc., Gaithersburg, MD).

Plasmids, Constructs, and Transfection

The generation of pSXSRα-Lck F505, pEGFP/ZAP-70, and pEGFP/kin.neg. (with deleted kinase domain) ZAP-70 have been described previously (Wange et al., 1995*a*; Sloan-Lancaster et al., 1997). pEGFP/double SH2 was made by ligating the NheI/XmnI fragment from pEGFP/ZAP-70, containing both SH2 domains and both interdomains, to the NheI/SmaI-digested pEGFP-N1 vector. For construction of pEGFP/ZAP-70 kin.dom. (expressing the kinase domain alone), the NheI/XmnI fragment was removed from pEGFP/ZAP-70, and the vector was religated using the oligos 5′ CTA GCA CCG GTG GAT CCT CTA GAA TGA AGC 3′ and 5′ GCT TCA TTC TAG AGG ATC CAC CGG TG 3′. For pEGFP/SH2(C) + kin.dom., the NheI/KpnI fragment of pEGFP/ZAP-70 was removed, and the vector religated using the oligos 5′ CTA GCG ATA TCA TGC CAG ACC CCG CGG CGC ACC TGC CCT GGT AC 3′ and 5′ CAG GGC AGG TGC GCC GCG GGG TCT GGC ATG ATA TCG 3′. pEGFP/SH2(N) + kin.dom. was made in two steps. First, an intermediate vector, ZAP 1 + 2, encoding the kinase domain alone with an inserted KpnI site, was derived by annealing the NheI/XmnI-digested pEGFP/ZAP-70 to the oligos 5′ CTA GCG ATA TCT GCA GGG TAC CTC GAG AAG C 3′ and 5′ GCT TCT CGA GGT ACC CTG CAG ATA TCG 3′. The NheI/KpnI fragment of pEGFP/ZAP-70, encoding the NH_2-terminal SH2 and interdomain 1, was then ligated to the NheI/KpnI-cut ZAP 1 + 2. pEGFP/Tζζ was constructed as follows: the EcoRI/BamHI fragment from pXSSRα/Tζζ (Letourneur and Klausner, 1991) was ligated to EcoRI/BamHI-digested pEGFP-N1 vector to create the intermediate plasmid pEGFP/Tζζ/BamHI. A PCR fragment from the BamHI site of Tζζ was created with an introduced COOH-terminal AgeI site, using the oligos 5′ GCA GGG ATC CAG AGA TGG GAG GC 3′ and 5′ GAC GAC CGG TGA GCG AGG GGC CAG GGT CTG 3′. Then BamHI/AgeI-digested PCR product and BamHI/AgeI-digested pEGFP/Tζζ/BamH1 vector were ligated together to produce the final construct, pEGFP/Tζζ. The construction of the TTζ, Tζζ, and Tζζ trunc chimeras have been described elsewhere (Letourneur and Klausner, 1991). All three contain the extracellular domain of the human IL-2 receptor α chain and the intracellular domain of TCRζ. TTζ includes the transmembrane region of the IL-2 receptor α chain, while Tζζ contains the transmembrane region of TCRζ. Tζζ trunc is a shortened form of the latter chimera, terminated after TCRζ amino acid residue 65, and thus lacks all three ITAMs (Letourneur and Klausner, 1991). HeLa cells, or their stably transfected counterparts, were electroporated using 15 μg of each DNA construct at

250 V and 500 μF using a Gene Pulser (Bio-Rad Labs., Hercules, CA) and used 20–24 h after transfection.

Immunofluorescence Staining

HeLa cells were grown overnight on sterile glass coverslips (10-mm diameter, No. 1 thickness). Cells, untreated or pretreated with nocodazole (33 μM, 30 min incubation at 4°C, followed by 30 min at 37°C), were then fixed in 3.7% paraformaldehyde in PBS for 30 min at room temperature, washed (three times) in PBS containing 10% fetal bovine serum (PBS/FBS), permeabilized using 0.1% Triton X-100 in PBS for 4 min at room temperature, washed (three times), and incubated for 45 min in PBS/FBS for preblocking. Cells were then incubated with a mouse anti–human tubulin Ab in PBS/FBS for 45 min at room temperature, washed, and incubated with rhodamine-coupled goat anti–mouse IgG for 45 min, followed by washing with PBS (three times). The coverslips were then mounted onto glass slides using Fluoromount G (Southern Biotechnology Associates, Inc., Birmingham, AL) and viewed using the 568-nm laser line of a confocal laser scanning microscope (model LSM 410; Carl Zeiss, Inc., Thornwood, NY) with a 100× planapochromat oil immersion objective (NA 1.4) and optics for rhodamine.

Fluorescence Microscopy, Time-Lapse Imaging, and Image Processing

Transfected cells were grown overnight in coverglass chambers (LabTek, Naperville, IL) in complete D10 medium. For time-lapse imaging experiments, the slides were mounted on a custom-made platform (of a confocal laser scanning microscope; Yona Microscope and Instrument Co., Rockville, MD) equipped with a triple line Kr/Ar laser, a 100× 1.4 NA Planapochromat oil immersion objective, a 25× 0.8 NA Neofluar immersion corrected objective, and a temperature-controlled stage. Time-lapse sequences were recorded with macros programmed with the Zeiss LSM software package that allow autofocusing on the coverslip surface in reflection mode before taking confocal fluorescence images. The media was replaced by PBS supplemented with magnesium and calcium salts before the start of imaging. In Fig. 4 *d*, cells were treated with nocodazole as above before beginning the time-lapse imaging. Two images of each cell were taken before addition of the pervanadate (PV) stimulant directly to the chambered coverglass, and subsequent images were taken at 30-s intervals thereafter until 15 min after stimulation, as previously described (Sloan-Lancaster et al., 1997).

Photobleaching Experiments

Fluorescence loss in photobleaching (FLIP) experiments were performed at room temperature on a custom-made stage of a confocal microscope (model LSM 410; Carl Zeiss, Inc.) using the 63× objective and the 488-nm line of a 400-mW Kr/Ar laser, which delivered 0.9 mW power (Cole et al., 1996). In brief, HeLa cells expressing ZAP-70 GFP alone, ZAP-70 GFP together with Lck F505, or Tζζ GFP were left untreated or stimulated with PV for 12 min before beginning the FLIP experiment, as indicated in figure legends. A small rectangular region defined by the boxed area was repeatedly illuminated with the laser at 100% power, 100% transmission. Between each intense illumination, the entire field of view was imaged at low-power laser light (20% power, 1% transmission) to assess the extent of loss of fluorescence outside the box as a consequence of photobleaching within the box. The time lapse between images was ~25 s. The possibility that regions on the edge of the illuminated box are progressively bleached by light leakage during FLIP was ruled out by repeating FLIP identically on fixed cells, which showed bleaching only in the area exposed to illumination. Furthermore, there was no significant photobleaching while imaging the recovering cell since control cells in the field did not lose any significant fluorescence intensity during the time followed.

Fluorescence recovery after photobleaching (FRAP; Edidin, 1994) was performed at room temperature on a confocal microscope (model LSM 410; Carl Zeiss, Inc.) essentially as described (Ellenberg et al., 1997). For the qualitative *D* measurements shown in Fig. 7, cells were stimulated for 12 min before commencement of photobleaching. The width of the rectangular regions of interest used were 2 μm (Tζζ GFP, some ZAP-70 GFP) or 4 μm (ZAP-70 GFP). Fluorescence within the strip was measured at low laser power (20% power, 1% transmission) before the bleach (prebleach intensity) and then photobleached with full laser power (100% power, 100% transmission) for 0.218 s (Tζζ GFP) or 0.436 s (ZAP-70 GFP) (which effectively reduced the fluorescence to background levels in fixed material). Recovery was followed after 2 s with low laser power at 2-s intervals for 200 s (Tζζ GFP) or 1-s intervals for 50 s (ZAP-70 GFP) and then at 10-s intervals until the recovered fluorescence intensity within the strip had reached a plateau. Zero of time *t*, taken as the midpoint of the bleach, was 2.399 s for Tζζ GFP and 2.513 s for ZAP-70 GFP. Numerical simulations were used to determine *D* using the prebleach intensity of entire cells (to assess the effects of geometry and nonuniform fluorescence density) and compared with experimentally derived *D* values, as described (Ellenberg et al., 1997; Sciaky et al., 1997).

Results

Redistribution of ZAP-70 to the Plasma Membrane in HeLa Cells Requires Both Cellular Activation and Coexpression of a TCR Chain

In a previous report, we made use of a ZAP-70 GFP chimera to study the intracellular location of this protein tyrosine kinase, and how it changed in response to cellular stimulation, using time-lapse imaging (Sloan-Lancaster et al., 1997). This approach revealed the very rapid redistribution of cytosolic ZAP-70 to the cell surface, with significant membrane accumulation detected as early as 1 min after stimulation. The phenotype was enhanced when an active form of Lck was coexpressed, which itself induced some ZAP-70 translocation. These results were somewhat surprising since the COS 7 cells, in which the chimeric ZAP-70 GFP was expressed, do not contain any TCR chains. We were therefore curious to determine whether the introduction of a TCR chain in this experimental system had any observable effect on the redistribution of ZAP-70.

To compare results in the presence or absence of a TCR chain, we made use of the HeLa cell line, which lacks any TCR chains, and its transfected derivative, H/TTζ, which stably expresses a chimeric form of TCRζ comprised of the extracellular and transmembrane portions of the human IL-2 receptor α chain, Tac, fused to the entire intracellular region of TCRζ (Letourneur and Klausner, 1991). The fusion protein is successfully expressed on the cell surface as an integral membrane protein independent of any other TCR component and provides an experimental system in which the contribution of the tandem ITAMs of TCRζ can be examined apart from the CD3 molecules (Letourneur and Klausner, 1991). In non–T cells, cross-linking TTζ at the cell surface does not induce cellular activation. Thus, to stimulate the cells, we used the pharmacological agent pervanadate (PV). PV inhibits intracellular phosphatases, thereby creating a steady state in which tyrosine residues are phosphorylated normally but not dephosphorylated and which is used as a surrogate for antigen or anti-TCR cross-linking (O'Shea et al., 1992; Secrist et al., 1993). Both HeLa and H/TTζ cells were transfected with ZAP-70 GFP, and time-lapse imaging was used to monitor the movement of the chimeric fluorescent molecule in response to pharmacological stimulation. Two images were taken before PV addition, with subsequent images taken at 30-s intervals thereafter.

Little if any ZAP-70 redistributed to the plasma membrane in HeLa cells with PV stimulation (Fig. 1, *top row*), while significant membrane accumulation was evident as early as 2 min after stimulation in COS 7 cells (Sloan-Lancaster et al., 1997). The lack of a similar phenotype in

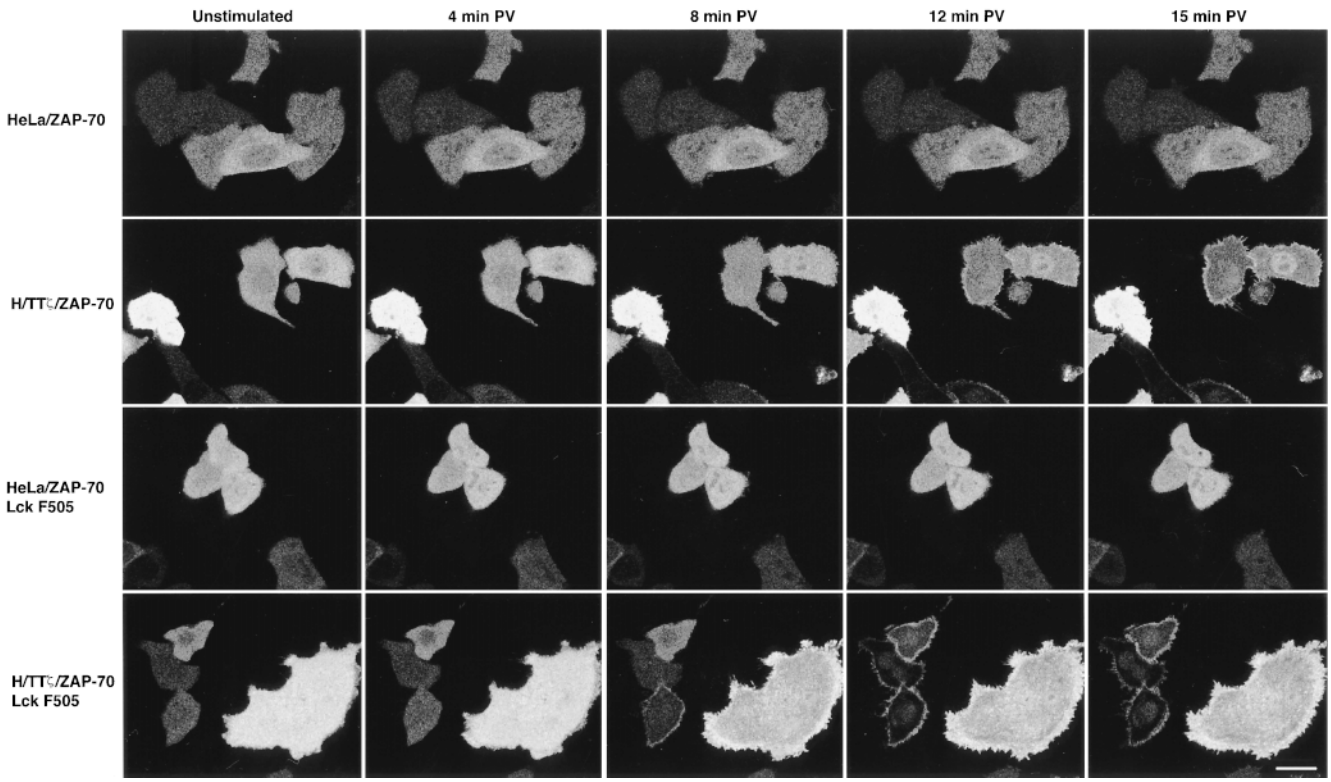


Figure 1. Activation-induced movement of ZAP-70 to the cell surface in TCRζ-expressing HeLa cells and its enhancement by Lck F505. Individual live HeLa or H/TTζ cells were monitored by time-lapse imaging confocal microscopy. Lck F505 was cotransfected with ZAP-70 GFP in the bottom two rows. Two images were taken before PV addition, with subsequent images collected every 30 s thereafter. One prestimulation image is shown for each experimental group, followed by those at 4, 8, 12, and 15 min after stimulation. Bar: (Rows *1*, *3*, and *4*) 21 μm; (row *2*) 27 μm.

HeLa cells suggests that no phosphotyrosine- or ITAM-containing proteins capable of binding ZAP-70 are expressed in these cells. Thus, in the absence of a TCR chain, pharmacological stimulation had little effect on ZAP-70 redistribution in HeLa cells. However, when H/TTζ cells were stimulated with PV, ZAP-70 dramatically redistributed to the cell surface in all cells examined (Fig. 1, *second row*). Unlike its rapid accumulation to the plasma membrane in COS 7, there was a significant delay in H/TTζ, with little detectable redistribution until 8–10 min after stimulation. At this time, movement to the plasma membrane, accompanied by cytosolic clearing, continued steadily around each cell until ~15 min after stimulation, when ZAP-70 was uniformly distributed over the inner surface. Results from a semiquantitative analysis of individual cells confirmed this phenotype, in which mean fluorescence intensity in a region of interest (ROI) over the center of each cell (cytosol and plasma membrane) was compared with an ROI at the edge (plasma membrane). These data indicated that there was, on average, a 1.1-fold increase in the surface to cytosolic fluorescence ratio 4 min after stimulation, which increased to 1.5-fold by 8 min and to 3.1-fold by 15 min after PV addition. The uniform distribution around the cell surface was consistent with ZAP-70 binding specifically to the chimeric TTζ molecule, which is localized throughout the plasma membrane under these conditions (data not shown).

Lck F505 Enhances ZAP-70 GFP Movement to the Cell Surface

The current model of physiological early T cell signaling suggests that ZAP-70 binds to the ITAMs of TCRζ only after they have been phosphorylated by Lck and/or Fyn (Iwashima et al., 1994; Wange and Samelson, 1996; Qian and Weiss, 1997). Our earlier studies in COS 7 suggested that expression of active Lck followed by PV stimulation enhances the redistribution of ZAP-70 to the cell surface over that induced by PV alone (Sloan-Lancaster et al., 1997). Thus, we performed time-lapse imaging in cells coexpressing the constitutively active Lck F505 to determine if this kinase influenced the recruitment of ZAP-70 and its binding to TCRζ. In HeLa cells, the coexpression of Lck F505 had no apparent effect on the location of ZAP-70 before or after PV stimulation, since ZAP-70 was not detected at the plasma membrane at any time point (Fig. 1, *third row*). This further confirmed the lack of other ZAP-70–binding proteins in HeLa cells, and thus the specificity of the interaction with the ITAMs in H/TTζ. With the expression of the chimeric TCRζ chain, Lck F505 enhanced the redistribution of ZAP-70 to the cell surface, and presumably its binding to TTζ (Fig. 1, *bottom row*). The time-lapse imaging results suggested that Lck F505 kinetically enhanced the accumulation of ZAP-70 at the cell surface. ZAP-70 accumulation was detected at the plasma mem-

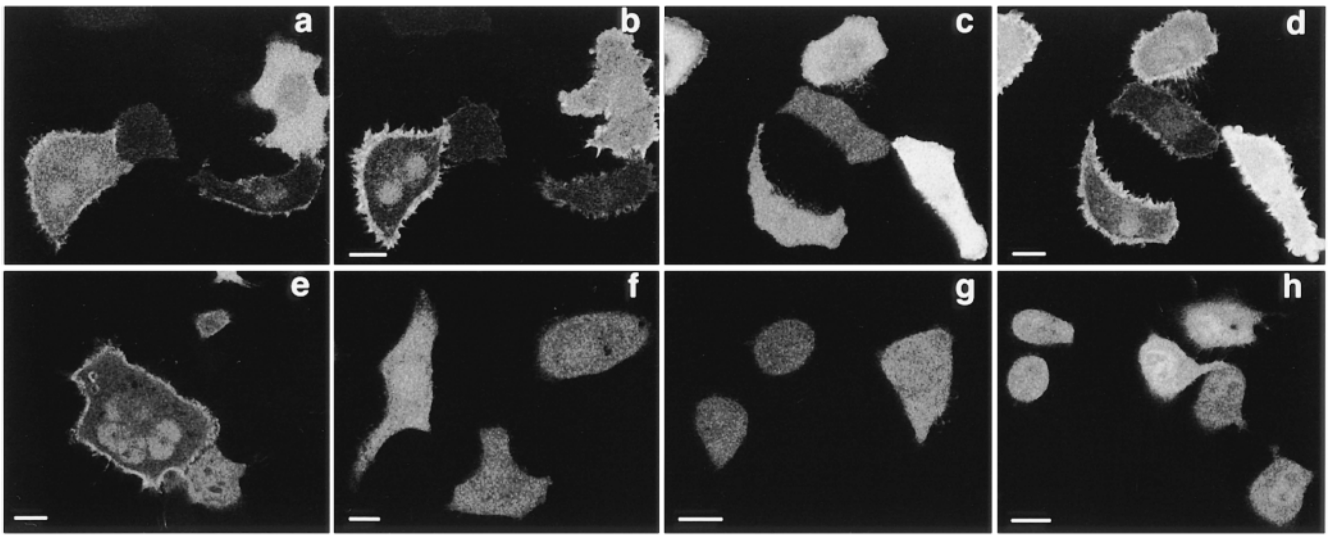


Figure 2. Analysis of the redistribution of ZAP-70 GFP mutants. H/TTζ cells expressing Lck F505 were cotransfected with the following ZAP-70 GFP constructs: wild-type (*a* and *b*), kinase-dead (*c* and *d*), tandem SH2 domains (*e*), kinase domain alone (*f*), SH2(N) + kinase (*g*), or SH2(C) + kinase (*h*). Protein movement in response to PV stimulation was followed by time-lapse imaging. Images are shown for unstimulated conditions (*a* and *c*) and 15 min after stimulation (*b* and *d–h*). Bars, 14 μm.

brane as early as 4–5 min after stimulation, reaching a plateau by 8 min (Fig. 1, *bottom row*). This was determined in several cells by comparing fluorescence intensities within ROIs over the center of the cell (cytoplasm and plasma membrane) and at the edge (plasma membrane only) with time. These data showed an increase in fluorescence intensity at the plasma membrane at a much earlier time after stimulation in cells coexpressing Lck F505. However, there was no apparent quantitative enhancement by Lck F505, since surface fluorescence increased threefold compared with cytosolic levels whether or not Lck F505 was coexpressed.

The Specificity of ZAP-70–TTζ Interaction

The above experiments indicated that, in the HeLa system, the specific molecular interaction of ZAP-70 and TTζ in response to cellular stimulation could be monitored at the single cell level. We next used this assay system to assess the basis of this interaction. To do so, we constructed various chimeras consisting of mutant ZAP-70 with GFP and used them to determine the contribution of individual protein domains in the translocation and binding of the kinase to TTζ (Fig. 2). Lck F505–expressing H/TTζ cells were cotransfected with the indicated ZAP-70 GFP mutant, and cells were monitored before and after addition of PV. Accumulation of ZAP-70 at the cell surface was monitored in response to PV stimulation in cells expressing wild-type ZAP-70 (Fig. 2 *a*, unstimulated, and *b*, 15 min PV). A similar pattern of redistribution was observed using a kinase-dead form of ZAP-70 (Fig. 2 *c*, unstimulated, and *d*, 15 min PV, and data not shown), and an analysis of multiple cells in several experiments indicated that the kinetics and amount of wild-type and kinase-dead ZAP-70 translocated to the cell surface did not differ significantly (data not shown). These data confirm that the kinase activity of ZAP-70 is not necessary for its binding to TCRζ (Wange et al., 1993; Hatada et al., 1995).

We next assessed the roles of the individual protein domains of ZAP-70 in its binding to TCRζ. While a mutant ZAP-70 GFP, containing the tandem SH2 domains without the kinase domain, redistributed to the cell surface with kinetics indistinguishable from those of the entire molecule (Fig. 2 *e*, 15 min PV, and data not shown), neither SH2 domain expressed alone with the kinase domain moved to the plasma membrane (Fig. 2 *g*, NH_2-terminal SH2, and *h*, COOH-terminal SH2, 15 min PV). Moreover, the kinase domain by itself could not bind to TTζ (Fig. 2 *f*, 15 min PV). These data agree with biochemical evidence showing that the tandem SH2 domains of ZAP-70, but not the kinase domain, are absolutely required for a stable interaction with any individual phosphorylated ITAM (Wange et al., 1993; Iwashima et al., 1994; Koyasu et al., 1994).

A similar in vivo analysis of the molecular properties of ζ required to bind to ZAP-70 was also undertaken. For this, two additional HeLa cell lines, which stably express distinct forms of the ζ chimera, were used. The first expresses Tζζ, which differs from TTζ in that its transmembrane domain is derived from TCRζ instead of from Tac. This ensures that Tζζ is expressed as a disulfide-linked homodimer on the cell surface. The second expresses a shorter form of Tζζ, called Tζζ trunc, terminated after ζ amino acid 65 and resulting in a homodimer with no ITAMs (Letourneur and Klausner, 1991). A schematic of the structures of these ζ chimeras is shown in Fig. 3 *A*. We tested the ability of these ζ chimeras to acquire phosphotyrosine, since only phospho-ITAMs can bind ZAP-70 biochemically (Bu et al., 1995; Isakov et al., 1995). The ζ chimeras were examined for phosphotyrosine content before

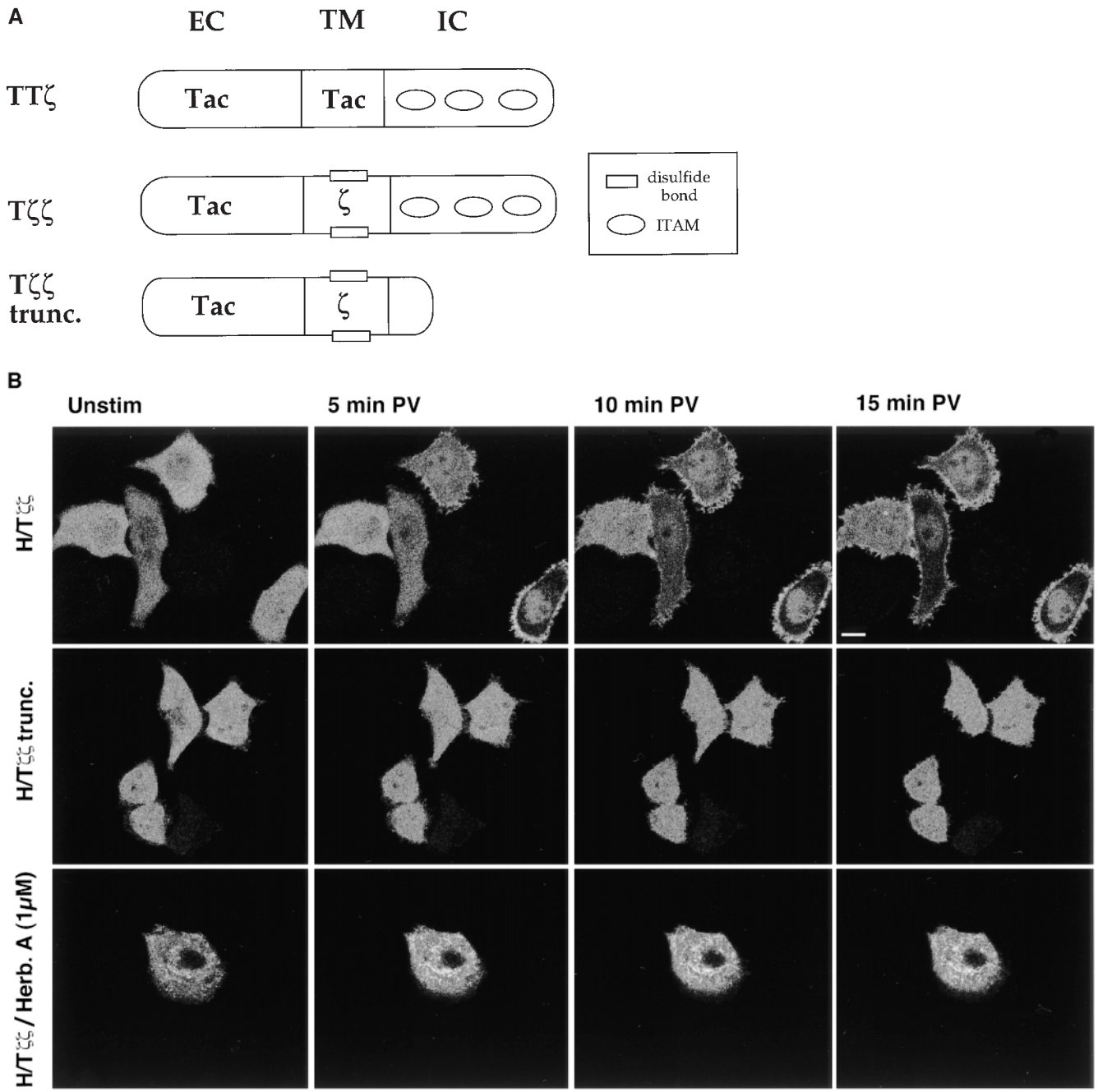


Figure 3. Requirements of TCRζ for ZAP-70 redistribution. (*A*) Schematic of the molecular domains of the various chimeric ζ molecules used. (*B*) H/Tζζ (*top* and *bottom rows*) or H/Tζζ trunc (*middle row*) cells, expressing Lck F505 and ZAP-70 GFP, were monitored by time-lapse imaging. Images were taken at 30-s intervals, and those at 5-min increments are shown. Bar, 13.3 μm.

or after PV stimulation of the respective cell lines. As expected, only H/TTζ and H/Tζζ, but not HeLa or H/Tζζ trunc, displayed a phosphoprotein at the apparent molecular weight for chimeric ζ, verifying the activation-dependent requirement of phosphorylation of chimeric ζ and the lack of intramolecular phosphate-binding sites in Tζζ trunc (data not shown). We also determined that the tyrosine kinase inhibitor, herbimycin A (1 μM), inhibited stimulation-dependent cellular phosphorylation (data not shown).

H/Tζζ (Fig. 3 *B*, *top* and *bottom rows*) and H/Tζζ trunc (*middle row*) cells, expressing ZAP-70 GFP and Lck F505 and including herbimycin A (1 μM, *bottom row*), were PV stimulated and monitored using digital imaging confocal microscopy. As expected, cells expressing full-length Tζζ were successful in recruiting ZAP-70 to the plasma membrane with kinetics similar to those defined earlier (Fig. 1). In contrast, H/Tζζ trunc cells showed no evidence of ZAP-70 redistribution to the cell surface even after 15 min PV stimulation (*middle row*), similarly to HeLa cells without a

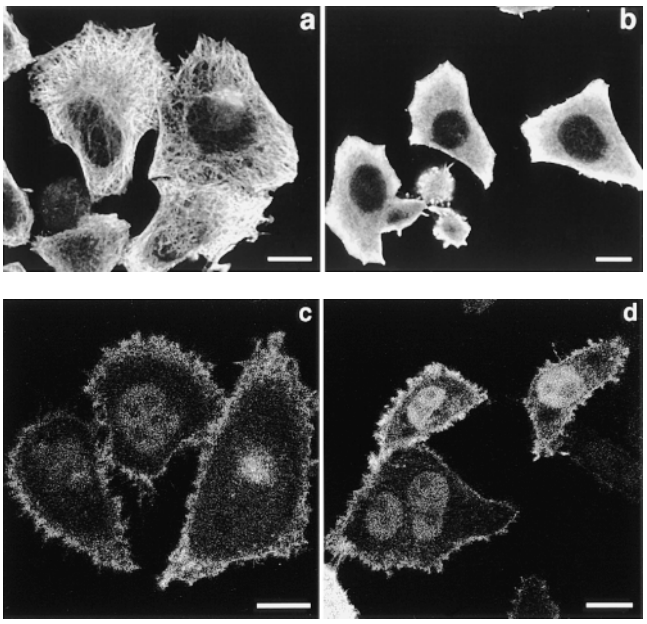


Figure 4. Testing microtubule involvement in ZAP-70's redistribution. H/TTζ cells, cotransfected with Lck F505 and ZAP-70 GFP, were left untreated (*a* and *c*) or pretreated with nocodazole (33 μM; *b* and *d*). In *a* and *b*, cells were fixed and immunostained with antitubulin mAb, followed by a rhodamine-coupled anti–mouse secondary mAb. In *c* and *d*, cells were stimulated with PV and monitored by time-lapse imaging. Time points 15 min after stimulation are shown. Bars, 15 μm.

TCR chain (Fig. 1, *first* and *third rows*). While herbimycin A pretreatment prevented ZAP-70 redistribution to the plasma membrane, there was some redistribution of the chimera under these conditions (Fig. 3, *bottom row*). This drug is likely causing several effects in the cells, many of which may be tyrosine kinase dependent. However, the lack of redistribution of ZAP-70 to the plasma membrane in the presence of herbimycin A is consistent with its inhibition of ITAM phosphorylation. Thus, the cytosolic tail of TCR containing ITAMs and tyrosine phosphorylation after activation were critical for movement of ZAP-70 to the membrane. TTζ and Tζζ appeared to perform equivalently for ZAP-70 recruitment, suggesting that TCRζ dimerization has no effect on the binding efficiency for ZAP-70 in this system (data not shown).

Intact Microtubules or Actin Cytoskeleton Are Not Required for ZAP-70 Translocation to the Cell Surface

Biochemical studies on ZAP-70 have not addressed how this kinase accumulates at the plasma membrane upon cellular stimulation. In fact, the model suggesting that it moves from the cytosol to the cell surface has only been an assumption. Our results confirm that translocation indeed occurs. To explore the mechanism of this translocation, we tested whether microtubules or the actin cytoskeleton were required. H/TTζ cells were treated with nocodazole, which leads to disassembly of the intracellular microtubule lattice. Antitubulin antibody staining of cells showed complete disassembly of the microtubule array in cells treated with nocodazole (33 mM, Fig. 4 *b*), but not in untreated cells (Fig. 4 *a*). Time-lapse imaging of ZAP-70 GFP– and Lck F505–transfected H/TTζ cells indicated that ZAP-70 translocated to the plasma membrane in response to cellular stimulation in both nocodazole-treated and untreated cells with similar kinetics (Fig. 4 *d*, nocodazole-treated, and *c*, untreated, both 15 min PV, and data not shown). Other cells from the population, treated identically, were stained with antitubulin mAb to confirm that nocodazole had disrupted the microtubule lattice. Moreover, treatment of cells with up to 100 μM nocodazole still did not impair ZAP-70 translocation to the cell surface. In addition, the breakdown of actin filaments by cytochalasin B had no effect on ZAP-70 redistribution (data not shown). These data indicate that the microtubule array and the actin cytoskeleton are not required for the intracellular translocation of ZAP-70.

Redistribution of ZAP-70 from the Cytosol to the Cell Surface Correlates with Its Conversion to a Less Mobile State

The ability to photobleach GFP chimeras makes them attractive tools for studying molecular dynamics in real time proteins (Cole et al., 1996). Using photobleaching techniques, we initially took a qualitative approach to determine whether there were any gross changes in ZAP-70 diffusibility after its redistribution within the cell. For this, we repetitively photobleached a small area within the cell and looked for fluorescence loss in the entire cellular compartment due to diffusional exchange of unbleached with bleached molecules (Cole et al., 1996; Ellenberg et al., 1997). The length of time required for fluorescence loss under these conditions depends on the diffusional mobility of the fluorescent protein and the extent of continuity of the cellular compartments. This approach, termed FLIP, was used to compare the dynamics of cytosolic and nuclear ZAP-70 in resting H/TTζ cells expressing ZAP-70 GFP (Fig. 5 *A*). Both pools of ZAP-70 were extremely mobile, with a rapid loss of fluorescence in cells repetitively bleached in the cytosol and in the nucleus. Fluorescence was significantly depleted after only two bleaches (<60 s), and a complete loss was apparent after five bleaches (Fig. 5 *A*, *bottom right cell*). In cells in which the bleached region encompassed only the cytosol, nuclear ZAP-70 GFP remained detectable for a longer time period (Fig. 5 *A*, *top right cell*). These data indicate that cytosolic and nuclear ZAP-70 are both highly mobile and that they are not freely interchangeable with each other. Also, the coexpression of Lck F505 had no effect on the mobility of ZAP-70 during the time frame of the experiment (Fig. 5, compare *B* to *A*).

The time-lapse imaging studies showed that ZAP-70 redistributed from the cytosol to a more peripheral location in TTζ-expressing cells in response to stimulation (Fig. 1). This phenotype was biochemically consistent with a specific molecular interaction between ZAP-70 and the ζ chimera (Figs. 2 and 3). We wanted to determine if this shift of ZAP-70 to the periphery correlated with a change in its diffusional properties. H/TTζ cells expressing ZAP-70 GFP and Lck F505 were stimulated with PV for 12 min before the commencement of FLIP to induce maximal ZAP-70 redistribution to the cell surface. After repetitive pho-

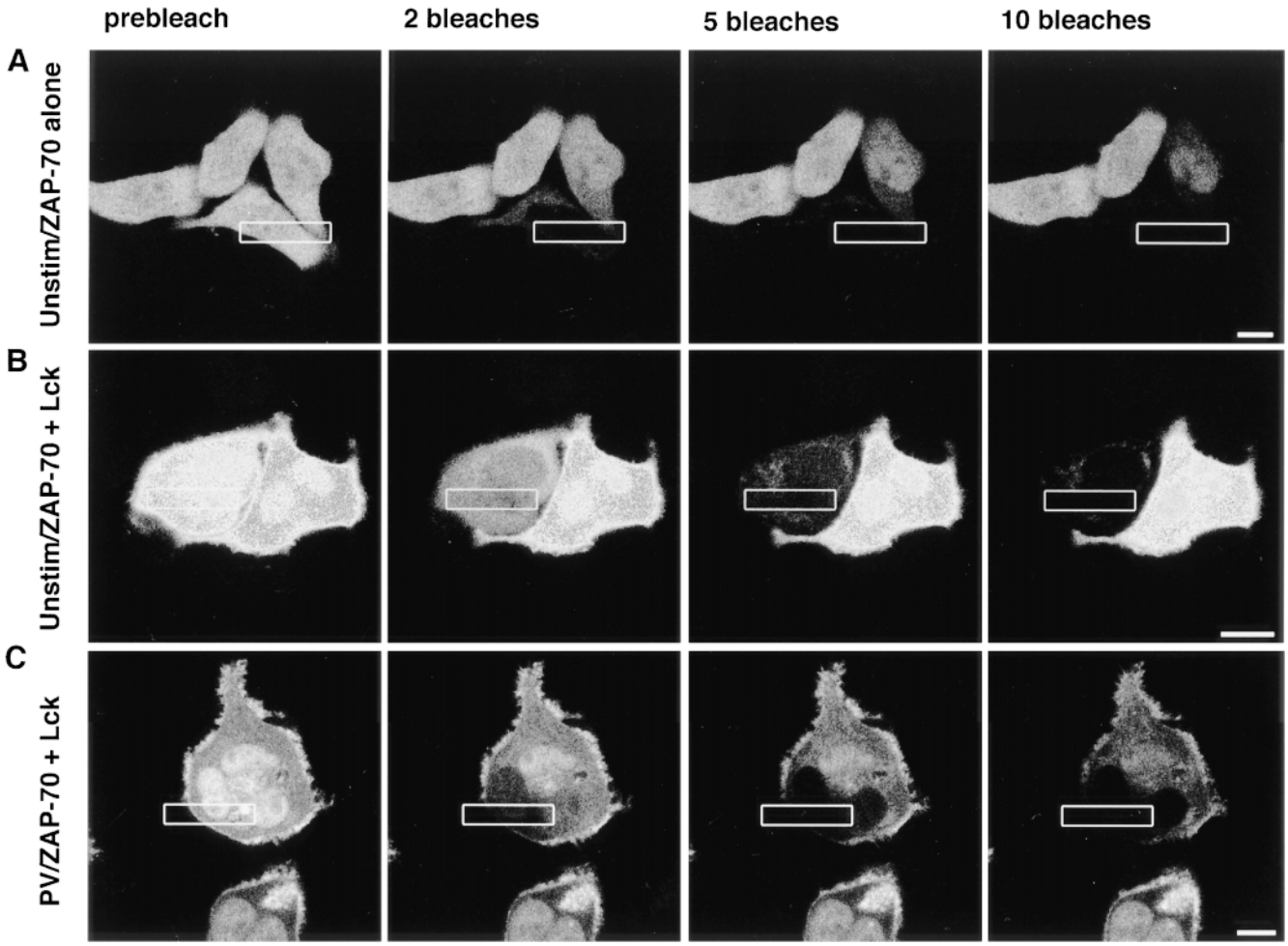


Figure 5. ZAP-70 becomes less mobile when it moves to the cell surface. H/TTζ cells expressing ZAP-70 GFP alone (*A*) or together with Lck F505 (*B* and *C*) were left unstimulated (*A* and *B*) or were stimulated with PV for 12 min before commencement of FLIP (*C*). FLIP was then carried out as described in Materials and Methods, with the boxed rectangle indicating the area being repetitively bleached. Images collected before bleaching and after 2, 5, and 10 bleaches are shown. Bars, 15 μm.

tobleaching of a region of the cell, it was obvious that the diffusional mobility of ZAP-70 had been altered (Fig. 5 *C*). Specifically, surface-localized ZAP-70 was less mobile and no longer able to exchange with the intracytoplasmic pool. A strong fluorescent signal was still apparent at the end of the bleaching sequence (~300 s). There are two possible explanations for this. Either all of the cytosolic ZAP-70 redistributes to the cell periphery, or some remains in the cytosol but converts to a less mobile phenotype, perhaps because of an activation-induced interaction with cytoskeletal proteins. The first hypothesis is the more likely since the conversion to the less mobile state only occurs after stimulation of TTζ-expressing cells, which is consistent with a molecular interaction between these two molecules (data not shown). Regardless, activation-dependent redistribution of ZAP-70 to the cell surface is accompanied by a decrease in its mobility.

Membrane-associated ZAP-70 Is More Mobile than TCRζ

Biochemical studies have indicated that cell surface–located ZAP-70 is physically bound to TCRζ, via the tandem SH2 domains of ZAP-70 and the phospho-ITAMs of TCRζ (Figs. 2 and 3) (Isakov et al., 1995; Bu et al., 1995). One prediction from these data would be that peripherally located ZAP-70 would acquire the same diffusion mobility as TCRζ. We therefore compared the diffusion mobility of the translocated ZAP-70 to that of the ζ chimera, Tζζ. A Tζζ–GFP fusion protein was constructed and expressed in HeLa cells and was expressed exclusively on the cell membrane, as expected for an integral membrane protein (Fig. 6 *C*). H/TTζ cells expressing ZAP-70 GFP alone or together with Lck F505 and HeLa cells expressing Tζζ–GFP were then stimulated with PV for 12 min, and FLIP was performed to compare the mobilities of the two proteins at the cell surface (Fig. 6). As before, peripheral ZAP-70 was relatively stable, showing a similar phenotype whether Lck F505 was coexpressed or not. However, when repetitive photobleaching inside the rectangular box was continued, complete loss of ZAP-70 GFP fluorescence outside the box occurred between 750 and 870 s (Fig. 6, *A* and *B*). In contrast, fluorescence of Tζζ–GFP remained prominently visible on the cell membrane after repetitive photobleaching for 30 min (Fig. 6 *C*, and data not shown). The mobility of Tζζ–GFP was not affected by PV stimulation since it

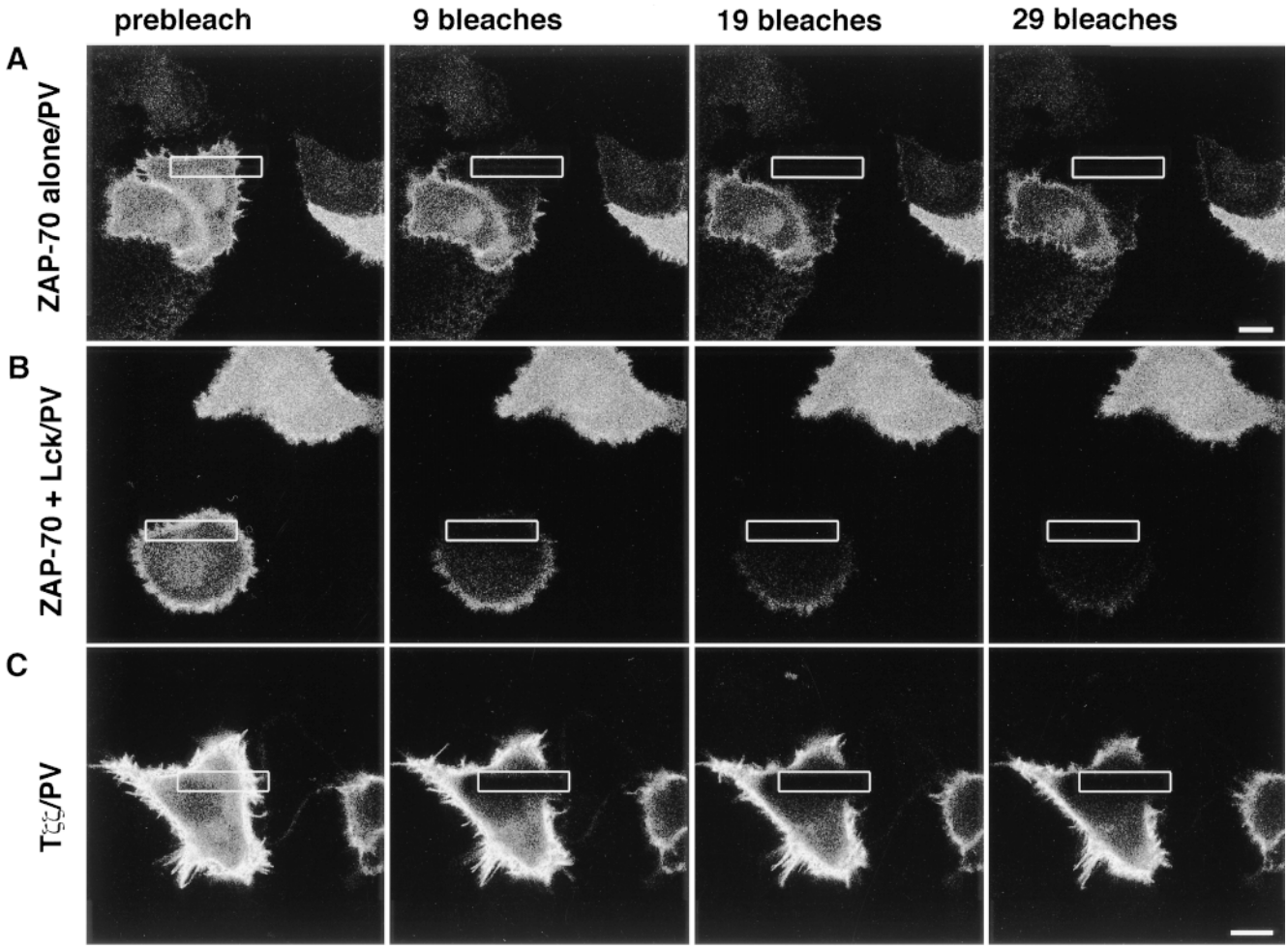
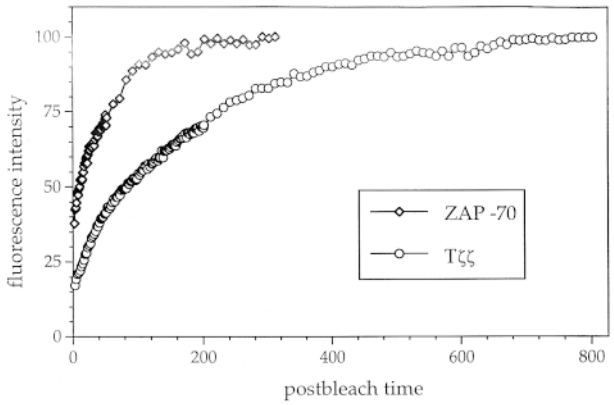


Figure 6. Membrane-associated ZAP-70 is more mobile than TCRζ. H/TTζ cells expressing ZAP-70 GFP alone (*A*) or together with Lck F505 (*B*), or HeLa cells expressing Tζζ–GFP (*C*), were stimulated for 12 min before commencement of FLIP. The area repetitively photobleached is indicated by the rectangle. Images collected before bleaching and after 9, 19, and 29 photobleaches are shown. Bars, 12 μm.

photobleached at a comparable rate in untreated cells. Furthermore, when a TTζ–GFP chimera was used, a similar phenotype was noted, indicating that ζ dimerization has no effect on its mobility in the plasma membrane (data not shown). Thus, it was apparent that the peripherally located ZAP-70 was more mobile than the integral membrane ζ chimera.

FLIP is limited to determining qualitative assessments since the rate at which a cell loses its fluorescence signal is a function of both the diffusion out of the surrounding areas to the bleach zone as well as the size of the bleach zone relative to the entire cell. To better understand the nature of the interaction between ZAP-70 and TCRζ, we wanted to be able to make quantitative measurements of protein diffusion. For this, we used the FRAP technique, in which fluorescence recovery into a bleached region of the cell after a single photobleach is monitored until recovery is complete (Edidin, 1994; Ellenberg et al., 1997; Lippincott-Schwartz, 1998). Experimental data are then plotted versus time and are fit to an empirical formula (Fig. 7 *c*) to determine the diffusion coefficient, *D*, for the protein. A representative plot of fluorescence recovery versus time is shown for both Tζζ–GFP and membrane-associated ZAP-70 GFP (Fig. 7 *a*). These data indicate that Tζζ moves slowly within the membrane, since a plateau of fluorescence recovery is not approached until >440 s for Tζζ compared with about 120 s for ZAP-70. Moreover, the *D* value derived for Tζζ indicated that it diffused slowly in the membrane and was similar to those derived for other plasma membrane proteins (Fig. 7 *b*; 0.011 ± 0.001 $\mu m^2/s$) (Pal et al., 1991). However, the *D* value derived for peripherally located ZAP-70 was >20-fold higher (Fig. 7 *b*; 0.234 ± 0.036 $\mu m^2/s$). These data thus provide strong evidence against the possibility that ZAP-70 irreversibly binds to TCR subunits at the plasma membrane and instead suggest that it interacts dynamically with TCRζ, continually exchanging on and off the plasma membrane. The extremely rapid motion of cytosolic ZAP-70 made it difficult to determine an accurate *D* value with our experimental setup, but it is greater than 1 $\mu m^2/s$.

A



B

$D_{T\zeta\zeta} = 0.011 \pm 0.001\ \mu m^2/sec$

$D_{ZAP\text{-}70} = 0.234 \pm 0.036\ \mu m^2/sec$

C Equation for determination of diffusion constants:

$$I_{(t)} = I_{(final)}\ (1 - (W^2\ (W^2 + 4\pi Dt)^{-1})^{1/2})$$

Figure 7. Quantitative FRAP experiments to determine diffusion constants for ZAP-70 GFP and Tζζ–GFP. (*A*) Fluorescence intensities in recovery after photobleaching, normalized to maximal levels (100%) for both ZAP-70 GFP and Tζζ–GFP, are plotted versus time. Data points were taken at 2-s intervals for 200 s (Tζζ–GFP) or at 1-s intervals for 50 s (ZAP-70 GFP) and then at 10-s intervals until they had reached a steady plateau. (*B*) Diffusion constants ± SD, expressed as a mean from five (Tζζ–GFP) or six (ZAP-70 GFP) independent experiments, are given. Simulated values were 0.016 and 0.09 $\mu m^2/s$ for Tζζ–GFP and ZAP-70 GFP, respectively. (*C*) Equation used to derive diffusion constants assuming one-dimensional recovery.

The equation used for FRAP *D* value measurements assumes one-dimensional recovery since the membranes are bleached all across their length and entire depth. To assess the effect of geometry, the calculated *D* values were checked against a numerical simulation that used the prebleach intensity of the entire cell as input to simulate diffusion recovery into the bleached strip (Ellenberg et al., 1997; Sciaky et al., 1997). For Tζζ GFP, the experimental *D* value correlated well with that derived from the simulated calculation, which was 0.016 $\mu m^2/s$ (compared with 0.011 $\mu m^2/s$). In contrast, the *D* value derived experimentally for ZAP-70 GFP (0.234 $\mu m^2/s$) was much faster than its simulated counterpart (0.090 $\mu m^2/s$). Moreover, the fits generated by the simulation for ZAP-70 GFP were poor and variable. These observations, together with the fact that ζ-associated ZAP-70 moves 20-fold faster than TCRζ, supports the hypothesis that the movement of ZAP-70 at the plasma membrane is more complex than simple diffusion and likely involves other dynamic parameters, such as exchange of ZAP-70 with the cytosolic pool.

Discussion

We have used GFP technology to study the movement, kinetics, and associations of key proteins used in signal transduction pathways coupled to the T cell antigen receptor. Biochemical data have suggested that ZAP-70 undergoes rapid intracellular translocation (Chan et al., 1991; Wange et al., 1992). Thus, we were curious to study its intracellular distribution and visualize its changes in response to cellular stimulation in live cells. Our earlier report provided preliminary data defining where ZAP-70 resides in resting cells and how it translocates to the cell surface upon cellular stimulation (Sloan-Lancaster et al., 1997). However, the specific nature of the translocation, and the subsequent binding to TCR subunits at the plasma membrane, could not be investigated. Here we analyze ZAP-70–TCRζ interaction in real time and the intramolecular properties required for this association. The kinetics of ZAP-70 translocation to the plasma membrane and its diffusional mobility in the different intracellular locations were measured.

The activation-induced movement of ZAP-70 to the cell surface in HeLa cells expressing TTζ, but not in HeLa, was a strong indication that we were monitoring the specific molecular interaction of two proteins over real time. This redistribution was much more dramatic than that in COS 7 cells, with an impressive clearing from the cytosol accompanying a uniform redistribution to the plasma membrane. Biochemically, the interaction of ZAP-70 and the ITAMs has been shown to require the tandem SH2 domains of ZAP-70 and a doubly phosphorylated ITAM (Wange et al., 1993; Iwashima et al., 1994; Koyasu et al., 1994). The crystal structure of the SH2 domains of ZAP-70 bound to a phospho-ITAM verified the molecular properties of this association (Hatada et al., 1995). Based on these data, we designed mutants of ZAP-70 and truncated Tζζ constructs to study the interaction of the two molecules in living cells. Our results validate the imaging approach taken, since they correlated exactly with the biochemical analyses. Moreover, they have allowed us to monitor for the first time the dynamics of these two proteins together in living cells. The tandem SH2 domains of ZAP-70 and an ITAM with phosphorylated tyrosines are clearly critical for any association to occur since there was no detectable redistribution at any time point for more than an hour after stimulation unless both of these criteria were met (Figs. 2 and 3 and data not shown).

How does ZAP-70 translocate to and remain at the plasma membrane? Because it is a cytosolic protein without any identified retention signal, it is likely freely diffusible within the cell, a phenotype supported by our FLIP data (Fig. 5). As the kinase moves randomly throughout the cytoplasm, it will be in a state in which some molecules are in close proximity to the cell surface at any time, and should therefore bind any available unoccupied phospho-ITAMs. After TCR engagement, when the CD3 and TCRζ ITAMs quickly become phosphorylated, the likelihood that ZAP-70 will bind to them and be retained at the

membrane increases tremendously. This should result in an accumulation of ZAP-70 at the cell surface and a reciprocal decrease in its cytosolic concentration, as seen in our time-lapse imaging studies (Fig. 1). Thus, the phosphorylation of the TCR ITAMs seems to be the only triggering event for ZAP-70 redistribution. Our data using mutant ZAP-70 GFPs indicate that the domains required for its redistribution and membrane retention parallel those required to bind TTζ (Fig. 2). Moreover, the enhancement of ZAP-70 redistribution accompanied by coexpression of Lck F505 indicates that simply increasing the level of ITAM phosphorylation results in more ZAP-70 at the plasma membrane (Fig. 1). Finally, neither the organized microtubular network nor the actin microfilaments are required for successful movement of ZAP-70 to the cell surface (Fig. 4).

The data reported by Huby et al. (1998) indicated that nocodazole treatment of T cells can prevent ZAP-70 activation, independently of the location of ZAP-70 at the cell surface. Moreover, in that study ZAP-70 appeared to be in a membrane proximal region in resting T cells, before cellular activation. The differences between this study and our data might reflect the presence of additional proteins in T cells that engage ZAP-70 and affect its dynamics and activation. Our studies in HeLa cells represent an early effort in studying these molecules in real time. The dynamic properties of ZAP-70 must now be analyzed with these methods in T cells.

The kinetics by which ZAP-70 relocated to the HeLa cell surface were much slower than would be predicted from the biochemical analyses in T cells, which indicate that ZAP-70 binds TCRζ within seconds of TCR cross-linking (Chan et al., 1991; Wange et al., 1992). Surprisingly, there was a significant delay between cellular activation and any detectable, redistributed ZAP-70 (Fig. 1). Perhaps the rate-limiting step is the tyrosine phosphorylation of proteins within the cells due to time required for PV, when delivered in the media, to be incorporated into the cells. Moreover, the live cell experiments were conducted at room temperature, and we anticipate that increasing the temperature to 37°C would also result in a faster translocation initiation time. Of course, the system employed here using HeLa cells and recombinant proteins is a simplification of the complexity of early T cell signaling events, in which multiple protein–protein interactions participate to initiate signal transduction. In the T cell environment, such interactions might affect the mobility of both ZAP-70 and TCRζ. However, in the HeLa system, once ZAP-70 began to translocate to the cell surface it quickly reached a steady state, without evidence of any reaccumulation in the cytosol even as long as several hours after PV addition (data not shown). Whether it moves back after the pharmacological or physiological stimulus ceases or is degraded at the plasma membrane is not known.

The data derived using the photobleaching techniques allowed us not only to qualitatively compare the movement of different pools of ZAP-70 with itself and with chimeric ζ, but also to calculate diffusion constants for these molecules. While a role for nuclear ZAP-70 has still not been defined, it clearly is not rapidly interchangeable with the cytosolic pool (Fig. 5 *A*). Both nuclear and cytosolic ZAP-70 are extremely mobile, so we could not determine a lower limit for their diffusion constants. This suggests that the protein is not associated with any anchoring molecules in these compartments. However, membrane-associated ZAP-70 in stimulated cells has dramatically different characteristics in that it is much less mobile. Clearly, membrane-associated ZAP-70 moves slowly relative to the cytosolic pool of unstimulated cells and is likely part of a large multiprotein lattice under the cell surface, containing many of the downstream molecules involved in intracellular signaling.

The peripherally located ZAP-70 had a faster diffusion rate than the chimeric ζ molecule. This was confirmed when the diffusion constants were determined, indicating that ZAP-70 moved ~20-fold faster than ζ (Fig. 7). This indicated that the binding between ζ and ZAP-70 is more complex than an irreversible and stationary interaction. The simulation data also confirmed that the movement of ZAP-70 at or near the membrane is not explained by a single diffusion constant. Instead, it seems that the SH2–phosphotyrosine interaction is dynamic, with specific on- and off-rates. Indeed, this dynamic relationship could explain how an immune response is regulated at the cellular level. Once initiated, T cell activation must eventually be turned off as antigen is cleared from the system. ITAM phosphorylation is a key initiating event of intracellular T cell activation, but dephosphorylation of these domains is critical for the disassembly of the activating lattice under the membrane. In fact, a proposed role of ZAP-70 is that it protects the phosphates of the TCR ITAMs by binding via its SH2 domains, thus maintaining the receptor in an "on" state (Iwashima et al., 1994). Only if ZAP-70 has a dynamic relationship with TCRζ will the phospho-ITAMs be exposed to phosphatases, which will then have an opportunity to dephosphorylate the tyrosine residues. As a result, the now dephosphorylated ITAMs will no longer be suitable targets for ZAP-70, which may eventually recycle to the cytosol or be degraded over time. As fewer active ZAP-70 molecules remain at the cell surface, all subsequent signaling events in the cell will also sequentially be turned off, until the cell returns to its quiescent state.

The ability to study intracellular signal transduction in real time now provides one with the tools to begin to answer many unaddressed questions. With the availability of several GFP variants that excite and emit at different wavelengths (Heim et al., 1994; Heim and Tsien, 1996; Ormo et al., 1996), the movements of several proteins have been successfully monitored simultaneously by time-lapse imaging (Rizzuto et al., 1995; Ellenberg et al., 1998). Moreover, the relationship of protein location and second messenger stimulation has also been studied (Miyawaki et al., 1997; Oancea et al., 1998; Stauffer et al., 1998). Fluorescence resonance energy transfer to assess protein–protein interactions will provide detailed information regarding how intracellular networks are established and maintained (Miyawaki et al., 1997; Romoser et al., 1997; Tsien and Miyawaki, 1998). As these techniques are refined and applied, more studies on how intracellular complexes form in many signaling systems should be performed.

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