Regulation of ZAP-70 Intracellular Localization: Visualization with the Green Fluorescent Protein

By Joanne Sloan-Lancaster,* Weiguo Zhang,* John Presley,§ Brandi L. Williams,§ Robert T. Abraham,§ Jennifer Lippincott-Schwartz,* and Lawrence E. Samelson*

From *the Section on Lymphocyte Signaling and †the Unit of Organelle Biology, Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892; and ‡The Department of Immunology, Mayo Clinic Rochester, Rochester, Minnesota 55905

Summary

To investigate the cellular dynamics of ZAP-70, we have studied the distribution and regulation of its intracellular location using a ZAP-70 green fluorescent protein chimera. Initial experiments in epithelial cells indicated that ZAP-70 is diffusely located throughout the quiescent cell, and accumulates at the plasma membrane upon cellular activation, a phenotype enhanced by the coexpression of Lck and the initiation of ZAP-70 kinase activity. Subsequent studies in T cells confirmed this phenotype. Intriguingly, a large amount of ZAP-70, both chimeric and endogenous, resides in the nucleus of quiescent and activated cells. Nuclear ZAP-70 becomes tyrosine phosphorylated upon stimulation via the T cell receptor, indicating that it may have an important biologic function.

The TCR has a multisubunit structure composed of the polymorphic αβ heterodimer, responsible for antigen recognition, and the invariant CD3γ, δ, ε, and TCR-ζ subunits, required for receptor surface expression and signal transduction. Engagement of the TCR leads to a rapid rise in intracellular protein tyrosine phosphorylation, followed by a series of other biochemical events, eventually resulting in gene expression and effector function (for reviews see references 1, 2). The earliest biochemical event after TCR occupancy appears to be the activation of the Src family protein tyrosine kinases (PTKs)1 Lck and/or Fyn. These activated kinases phosphorylate the various immunoreceptor tyrosine-based activation motifs (ITAMs) of the TCR (3, 4), providing binding sites for molecules containing SH2 domains capable of binding phosphotyrosine in the proper context (5). One such molecule is ZAP-70, also a PTK, which binds via its tandem SH2 domains to the two phosphotyrosine residues of individual ITAMs (6, 7). Subsequent phosphorylation of ZAP-70 by Lck and/or Fyn induces its activation (3, 8, 9). Thereafter, a number of other signaling and adaptor molecules are phosphorylated and recruited to the activated TCR and ZAP-70, and may subsequently be activated (10). These include phospholipase Cγ1 (PLCγ1) (11, 12), the proto-oncogenes Vav and Cbl (13, 14), and SLP-76 and pp36 whose functions are still unknown (15). Thus, a multicomponent protein complex is formed at the TCR soon after activation, though the stoichiometry and kinetics of assembly of its components have remained largely unexplored.

A critical role for ZAP-70 in the initiation of T cell signaling has been demonstrated by several lines of evidence. For example, disruption of ZAP-70 recruitment to the activated TCR results in a loss of T cell signaling (16, 17). Moreover, a mutant Jurkat T cell line lacking ZAP-70, P116, shows no functional or biochemical evidence of TCR-mediated activation (B.L. Williams, manuscript in preparation) and patients with ZAP-70 mutations as well as ZAP-70 knockout mice display profound SCID phenotypes (18–21). Despite the information available regarding the function of ZAP-70 in T cells, little is known about its intracellular location or dynamics. Upon cellular activation, ZAP-70 is rapidly tyrosine phosphorylated and it transiently translocates to the TCR. However, subcellular fractionation experiments have indicated that most ZAP-70 remains in the cytosol, and only a fraction of the kinase becomes tyrosine phosphorylated when the cells are activated (W. Zhang, unpublished observations). Though interest has focused on the TCR-associated fraction of ZAP-70, clearly the function of the majority of ZAP-70 molecules has been insufficiently analyzed.

To further explore ZAP-70 intracellular localization and dynamics, we have used the green fluorescent protein (GFP) of the jellyfish Aequorea victoria. The fluorescent signal gen-
Placid, NY); anti–TCR BRL, Gaithersburg, MD) for maintenance of transgene expression. 1C2. All stably transfected lines were cultured in R10 complete medium was used for cell culture (8). The P116/ZAP-70 GFP subclones were adhered by aspirating 50 times using a 25-gauge needle and 1-ml syringe. and the percentage of broken cells was measured from this was subcloned into the corresponding sites of the pEGFP-N1 expression vector. A KD form of ZAP-70, created by site-directed mutagenesis of the lysine at position 369 to an alanine, replaced ZAP-70 GFP in the pEGFP/ZAP-10 vector.

Transfection. Cos 7 cells 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 at 20–26 h after transfection. P116 T cells (2 × 10^9) were electroporated using 20 μg DNA at 310 V and 500 μF. First stage subclones of P116/ZAP-70 GFP, 2G1, and 1C2, were selected from a bulk population by limiting dilution analysis, and these were further subcloned to produce C8, C11, H9 (from 2G1), and F4 (from 1C2), respectively.

Immunoprecipitation, Immunoblotting, Immune-complex Kinase Assays, Cytosol/Membrane and Nuclear Fraction Purification. Transfected Cos 7 cells were harvested and lysed in Brij 97 lysis buffer (8). The cells were stimulated for 10 min at room temperature (RT) in the presence or absence of pervanadate (PV) before lysis. Jurkat, P116, and ZAP-70 GFP subclones were left untreated or stimulated with F(ab')2 fragments of OKT3 for 2 min at 37°C before lysis. Post-nuclear lysates were analyzed by SDS-PAGE or were subjected to immunoprecipitation (IP) using the appropriate Ab adsorbed to protein A–Sepharose. An immune-complex kinase assay was performed using cf3 as an exogenous substrate (1 μg/l; reference 8).

For preparation of cytosolic/membrane and nuclear fractions, Jurkat cells (4 × 10⁷) were suspended in homogenization buffer (10 mM Tris, pH 7.6, 0.5 mM MgCl2, 1 mM PM SF, 10 μg/ml Aprotinin, 10 μg/ml Leupeptin, 5 mM EDTA, 1 mM NaVO₃), with (see Fig. 9 a) or without (see Fig. 9 b) 0.1% Triton X-100, and incubated on ice for 20 min. Swollen cells were then homogenized by aspirating 50 times using a 25-gauge needle and 1-ml syringe, and the percentage of broken cells was ≤95%. N-acetylcycteine (500 mM final) and Hepes (pH 7.5, 500 mM final) were added, and cells were centrifuged at 2,000 rpm for 5 min to pellet the nuclei. Supernatants were considered the cytosolic/membrane fraction. Nuclear pellets were washed three times with homogenization buffer with (Fig. 9 a) or without (Fig. 9 b) 0.1% Triton X-100, resuspended in the same buffer (400 μl), and sonicated (four times, 30 s each). Samples were boiled for 15 min, centrifuged at 15,000 rpm × 10 min, and supernatants collected as purified nuclear material. A portion of each cellular fraction was used to determine enrichment of nuclear material and percent contamination from cytosol. The remainder of each was immunoprecipitated with anti-ZAP-70 antiserum after adding Brij 97 to 1% final concentration.

A densitometer (Molecular Dynamics, Sunnyvale, CA) and ImageQuant software were used for quantitation analysis of autoradiographs.

Immunofluorescence Staining and Confocal Microscopy. Transfected Cos 7 cells were grown on sterile glass coverslips (10 or 25 mM diameter, N. o. 1 thickness). ZAP-70 GFP subclones were adhered to coverslips precoated with poly L-lysine (100 μg/ml; Sigma Aldrich, St. Louis, MO) by aspirating 50 times using a 25-gauge needle and 1-ml syringe. and the percentage of broken cells was measured from this was subcloned into the corresponding sites of the pEGFP-N1 expression vector. A KD form of ZAP-70, created by site-directed mutagenesis of the lysine at position 369 to an alanine, replaced ZAP-70 GFP in the pEGFP/ZAP-10 vector.
Chemical Co., St. Louis, MO (see Figs. 5 and 6) for 2 h at 37°C. Cells were stimulated directly on the coverslips (PV x 10 min for Cos 7 or F(ab')2 of OKT 3 x 2 min for 2G1), fixed in 2% formaldehyde in PBS for 15 min at RT, and examined directly or permeabilized and stained with the appropriate antibody. Antibody staining was performed with T cells in suspension in all other experiments, and cells were mounted onto coverslips immediately before microscopy analysis. The cells were fixed using 3.7% paraformaldehyde in PBS for 30 min at RT, 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 RT, washed (three times), and incubated for 45 min in PBS/FBS for pre-blocking. Cells were then incubated with first stage antibody (anti-ZAP-70, anti-GFP, or anti-Lck) in PBS/FCS for 45 min at RT, washed and incubated with second stage antibody (rhodamine-coupled goat anti-mouse or anti-rabbit IgG or (see Fig. 7) Cy3 donkey anti-rabbit IgG) for 45 min, followed by washing with PBS (three times). Cells were resuspended in Fluoromount G added (Southern Biotechnology) and pipetted onto microscope slides with a coverslip mounted on top. Hoechst stain (20 µg/ml) was included during the second antibody incubation (See Fig. 7, c and d). These cells were viewed using a Zeiss Axioskop microscope equipped with both UV and rhodamine optics and cells photographed directly. All other fixed cells were viewed as 0.5 µM dual color optical sections, or composites of a complete Z section analysis in (see Fig. 2) using a Zeiss laser scanning microscope 410 confocal microscope having a 100 x Zeiss planapo objective (numerical aperture 1.4) and optics for both fluorescein (GFP) and rhodamine (antibody stains). For experiments using live cells, the coverslips were affixed to a Leiden coverslip dish and mounted on a custom-made 37°C stage of the confocal microscope using the 100 x objective. The GFP molecule was excited with the 488 line of a krypton-argon laser and imaged using a 515–540-nm bandpass filter. Images were averaged 16 times to improve image quality. Two images of each cell were taken before addition of stimulant, and subsequent images were taken at 20-s intervals thereafter until 5 min after stimulation, with the same cell slice being viewed in each image.

Quantitation measurements (see Fig. 6, c and d) were determined using IP Lab Spectrum software. The middle section of a complete Z series (0.5-µM slices) was selected from a field of each subclone to ensure that a slice through the nucleus was being examined. Regions of interest (ROIs) were drawn around the entire cell and around the nucleus, and the sum of pixels in each ROI calculated. The following equation was used to determine the percent of ZAP-70 GFP in the nucleus: (sum of intensities of total pixels in nucleus)/(sum of intensities of total pixels in cell) x 100%. The mean fluorescence intensity for the entire cell is reported to compare the expression level of ZAP-70 GFP between individual cells.

Results

ZAP-70 GFP Chimera Retains the Antigenic and Kinase Properties of Native ZAP-70. The ZAP-70 GFP fusion protein was constructed in a mammalian expression vector fusing the GFP coding sequence to the COOH terminus of ZAP-70 kinase (Fig. 1a). To assess the enzymatic activity of the chimeric protein, Cos 7 cells were transfected with ZAP-70 GFP and Lck F505, left untreated, or stimulated with PV, and an in vitro kinase assay was performed on the anti-ZAP-70 IPs (Fig. 1b, top). Enzymatic activity was measured by detection of autophosphorylation and phosphorylation of cfb3, a substrate for ZAP-70. ZAP-70 GFP showed significant kinase activity, which was increased upon cellular stimulation, indicating that the fusion protein retained the enzymatic properties of the native ZAP-70 molecule. An anti-ZAP-70 immunoblot of the membrane demonstrated the amounts of protein in the lanes and confirmed that the antigenic properties of ZAP-70 had been retained (Fig. 1b, bottom). Anti-GFP antiserum also identified this single species (data not shown), whereas an irrelevant mAb failed to IP the chimeric protein (Fig. 1b).

ZAP-70 GFP Is Located throughout Cos 7 Cells, and Migrates to the Plasma Membrane upon Activation. Since Lck F505 induces ZAP-70 kinase activity (3, 8, 9), we next examined if simply coexpressing the two proteins resulted in more plasma membrane-associated ZAP-70. To compare cellular distribution of the two enzymes, transfected cells were immunostained with an anti-Lck antibody and a rhodamine-coupled secondary mAb. Thus, the green image represents ZAP-70, the red indicates Lck, and the yellow image indicates where the two molecules colocalize. Coexpression of active Lck had a dramatic influence on the distribution of ZAP-70. A significant amount was observed at the plasma membrane in the unstimulated cell, and further rapid membrane accumulation occurred as soon as 1 min after PV stimulation (Fig. 2, c and d). Lck colocalized at the plasma membrane with ZAP-70 in both instances.
Several intriguing observations were made from this initial experiment. ZAP-70 becomes closely associated with the plasma membrane specifically upon activation, apparently independent of the TCR, since Cos 7 cells do not express any TCR components. In addition, the presence of active Lck enhanced the membrane association of ZAP-70 GFP, suggesting that the phosphorylation and subsequent activation of ZAP-70 are prerequisites for its translocation to and association at the membrane. Furthermore, ZAP-70 was detected abundantly in the nucleus, a phenotype that was unaffected by cellular stimulation.

Active Lck Enhances Plasma Membrane Accumulation of ZAP-70. Although it seemed that F505 Lck coexpression enhanced ZAP-70 localization to the plasma membrane, these observations were made using fixed cells, preventing a comparison of the same cell at different stimulation time points. To more accurately study the role of F505 Lck on the quantitation and kinetics of ZAP-70 movement to the cell surface, we monitored individual live cells over time, both before and after stimulation. Transfected cells were treated as described in Materials and Methods, and monitored before and after PV stimulation, for a total of 5 min after stimulation, with images taken at 20-s intervals. Upon subsequent comparison of the images for ZAP-70 distribution, the kinetics of its movement to the plasma membrane in a single cell could be visualized.

A representative cell, expressing ZAP-70 GFP alone, was observed before and after stimulation with PV (Fig. 3, top). Consistent with the data acquired using fixed cells, the unstimulated cell displayed a diffuse pattern of ZAP-70 expression, with only a small amount of fluorescence at the plasma membrane (unstim). Upon pharmacologic stimulation, little change in ZAP-70 distribution was apparent during the first minute or so after stimulation (1 min PV). However, after 2 min, plasma membrane accumulation of ZAP-70 was evident (2 min PV, arrow highlights area of accu-
This redistribution to the cell surface steadily continued throughout the 5-min stimulation period (5 min PV).

A qualitatively different pattern was observed when cells cotransfected with ZAP-70 GFP and F505 Lck were monitored (Fig. 3, ZAP-70/F505). Unlike the cells transfected with ZAP-70 GFP alone, those coexpressing F505 Lck consistently showed a high concentration of perinuclear and intranuclear ZAP-70, and minimal diffuse cytosolic location. Moreover, a significant quantity of ZAP-70 was already at the cell surface in the resting cell (unstim). Further accumulation to the cell surface began immediately after PV addition (1 min PV), was rapidly and steadily enhanced with the entire cell surface outlined by ZAP-70 GFP as early as 3 min before stimulation, and increased until the conclusion of the experiment (5 min PV). Thus, active Lck has a dramatic positive influence on the kinetics and quantity of ZAP-70 redistribution to the plasma membrane.

Redistribution of KD ZAP-70 to the Plasma Membrane Is Highly Inefficient in Cos 7 Cells. Phosphorylation of specific tyrosine residues, and the subsequent activation of ZAP-70 kinase, result from interaction with active Lck or stimulation by PV (29). The phosphorylation and/or kinase activation may be responsible for ZAP-70 translocation to the plasma membrane. To determine whether an inactive form of ZAP-70 would demonstrate the same dynamics, a KD form of ZAP-70 (KD ZAP-70) was fused to GFP and the chimera used in live cell experiments as above. When expressed in Cos 7 cells alone, KD ZAP-70 GFP was abundantly expressed in the cytosol, but not at the cell surface (Fig. 3). Furthermore, PV stimulation had no effect on redistributing the protein at the early time points, in contrast to the wild-type chimera (compare ZAP-70 and KD ZAP-70, 1 min PV). At later time points, a small amount of KD ZAP-70 could be seen around the cell surface (2, 3 min PV, arrows indicate membrane accumulation), but this was not enhanced as the cells were stimulated further (4, 5 min PV). The addition of active Lck increased the redistribution significantly (Fig. 3, bottom). As with the wild-type chimera, some KD ZAP-70 was already at the cell surface in the resting cell, although the quantity was much less (unstim). Further movement to the membrane occurred after addition of PV, but again the total amount was less and the kinetics slower than with native ZAP-70. Thus, ZAP-70 kinase activity is required for optimal redistribution to the plasma membrane in TCR-negative epithelial cells.

Although the activation data suggest that Lck facilitates ZAP-70 redistribution by phosphorylating and ultimately activating it, a physical interaction between the two proteins is also possible. Membrane association of ZAP-70 could be mediated via this direct interaction since Lck associates...
with the plasma membrane via its NH₂-terminal myristylation site. Thus, we used a mutant form of F505 Lck in which the myristylation site had been destroyed (30), and determined its efficiency at facilitating ZAP-70 membrane association. Transition of ZAP-70 to the cell surface was similar regardless of whether Lck could associate with the lipid bilayer, supporting the thesis that Lck kinase activity is responsible for ZAP-70 movement (data not shown).

ZAP-70 GFP Reconstitutes Early Activation Events in P116 Cells Lacking ZAP-70. To study the intracellular location and redistribution of ZAP-70 in T cells, we reconstituted a mutant Jurkat T cell line, P116, which lacks ZAP-70 (Williams, B.L., and R.T. Abraham, manuscript submitted for publication). Individual subclones were selected from stable bulk cultures by limiting dilution analysis and two, 2G1 and 1C2, were used for further study. These lines were further subcloned to derive C8, C11, H9 (from 2G1), and F4 (from 1C2). Anti-ZAP-70 IP and Western blot analysis (Fig. 4 a) with subsequent densitometry measurements indicated that the relative expression of ZAP-70 GFP compared to endogenous ZAP-70 in Jurkat cells was as follows: 97% for C8, 143% for C11, 131% for H9, and 94% for F4. (b) Cells were lysed directly or first stimulated for 2 min with OKT3 F(ab')2, at 37°C, and antiphosphotyrosine Western blot analysis of whole cellular lysates (2 × 10⁶ cells/ lane) performed.
levels (Fig. 6 c). This was further verified when the H9 subclone was similarly assessed, whereas cells expressing much higher total cellular amounts of ZAP-70 GFP showed no significant increase in the amount residing in the nucleus (Fig. 6, b and d, cells 7, 8, and 9). Thus, nuclear ZAP-70 is not an artifact of overexpression since it exists at physiological protein levels. It should be noted that, although close to 50% of the material was nuclear in the slices quantitated, this may not reflect the percent nuclear ZAP-70 in the entire cell. To determine this value, the sum of values from all slices through an individual cell must be calculated for a population of cells.

Endogenous ZAP-70 Is Detected in the Nucleus of Jurkat Cells by Immunofluorescence. To rule out the possibility that nuclear ZAP-70 GFP was an artifact of the chimera, immunofluorescence (IF) staining was first performed on P116 cells reconstituted with ZAP-70 containing a COOH-terminal Myc epitope tag. As with ZAP-70 GFP, nuclear ZAP-70 was evident in about half of the cells (data not shown). Next, an anti–ZAP-70 antiserum was affinity purified against its immunizing epitope and then used to identify the location of endogenous ZAP-70. Jurkat cells were immunostained in suspension to maintain their three-dimensional structure and eliminate the possibility of misinterpreting cellular location due to cell flattening. Immediately after mounting, complete Z series analysis of immunostained cells was performed. An assessment of the immunostaining pattern in all cell slices verified that endogenous ZAP-70 is indeed present in the nucleus. Two slices (0.5 μM-thick) through the middle of a typical field of Jurkat cells, 1 μM apart, are displayed in Fig. 7 (a and b). The staining pattern clearly indicates that endogenous ZAP-70 is not only in the cytosol (note the intense peripheral staining), but also highly abundant in the nucleus, with a typical nucleolar exclusion pattern. The specificity of the staining pattern was verified when ZAP-70-negative P116 cells, treated in a similar manner, failed to show any IF staining (e), although cells were present in the field as seen under the reflector light (f). In addition, the secondary Ab alone did not significantly stain the Jurkat cells (data not shown). The nuclear location of endogenous ZAP-70 was further confirmed when Jurkat cells were costained with anti–ZAP-70 and a Hoechst DNA stain. Although only the Ab stained the cytosol, both anti–ZAP-70 and Hoechst showed a superimposable staining pattern in the nucleus (c and d). Thus, endogenous ZAP-70 is present in the nucleus of normal Jurkat T cells.

Efficiency of Ab Staining Is Hindered by the Nuclear Membrane. A recent study reported that ZAP-70 is found ex-
clusively in a cortical region of Jurkat cells, with no evidence of the protein in the nucleus (31). This is inconsistent with the findings presented here. However, a comparison of our results using Ab staining versus GFP to detect ZAP-70 revealed an interesting pattern that may explain this apparent discrepancy. Although the ZAP-70 GFP was present in the nucleus of all T cells examined, Ab staining using either anti-Myc or anti-ZAP-70 consistently detected nuclear ZAP-70 in only about half of the cells, indicating that some nuclear structure was acting as a physical barrier, preventing efficient access of the Abs to the nucleus (data not shown).

To formally test this hypothesis, Cos 7 cells expressing ZAP-70 GFP were immunostained with anti-GFP (a–d) or anti-ZAP-70 (d–f). Although GFP indicated a high concentration of the chimera in the nucleus (Fig. 8 a), this was completely unrecognized by the anti-GFP (Fig. 8 b). In contrast, expression of the protein elsewhere in the cell was detected comparably by either method (c, yellow indicates overlay of both signals). Although the anti-ZAP-70 appeared to be somewhat more accessible to the nucleus (e), some exclusion was apparent since the GFP signal was much stronger than the Ab signal (compare d, e, and overlay in f). The partial exclusion of anti-ZAP-70 is consistent with the detection of only ~50% of examined T cells displaying nuclear ZAP-70 using this Ab. Our studies demonstrate two strengths of the ZAP-70 GFP chimeric system. Intracellular locations of proteins, which are overlooked by conventional IF techniques, are efficiently detected. Also, accurate quantitative intracellular distribution measurements can be made.

ZAP-70 Can Be Isolated from Jurkat Nuclei and Is Tyrosine Phosphorylated after Anti-TCR Stimulation. To understand the role of nuclear ZAP-70 in T cell signaling, a biochemical cell fractionation procedure was used to yield highly purified cytosol/membrane and nuclear pools from Jurkat cells. ZAP-70 was abundant in both the cytosolic/membrane and nuclear fractions, as indicated by anti-ZAP-70 immunoblotting of whole lysate preparations (Fig. 9 b, top). About 35% of total cellular ZAP-70 was nuclear, but this value varied between experiments, a reflection of the inefficiency of nuclear material recovery (32). The membrane was then immunoblotted with anti–iron regulatory protein (IRP)-1 as a control for cytosolic contamination in the nuclear fraction (Fig. 9 b, bottom), since IRP-1 is purely cytosolic (27, 33). Densitometric analysis indicated that the ratio of nuclear/cytosolic ZAP-70 was 0.53, whereas that for IRP-1 was 0.1. Thus, ZAP-70 in the nucleus was enriched 5.3-fold as compared to IRP-1, and as much as 6.2-fold in other experiments, confirming that ZAP-70 truly resides in the cell nucleus. Similar results were found when nuclear material...
was isolated from Jurkat cells in the presence (Fig. 9 a) or absence (Fig. 9 b) of 0.1% mild detergent, and also in transfected Cos 7 cells using a sucrose gradient separation technique (data not shown). Moreover, the enrichment of nuclear material was confirmed by immunoblotting with an Ab against a nuclear pore complex protein (data not shown). Moreover, the enrichment of nuclear/cytoskeletal and nuclear matrix–associated proteins, showed little or no evidence of ZAP-70 presence, either by direct immunoblotting or by anti–ZAP-70 IP.

Discussion

Since the identification of ZAP-70 as a T cell–specific protein tyrosine kinase, a number of biochemical studies have focused on its activity, regulation, and molecular interactions (for reviews see references 29, 35). Although such research has been critical, a complementary cell biological study is necessary to fully understand ZAP-70 function. Here, we provide an analysis of ZAP-70 subcellular location and demonstrate its dynamics under different conditions. The visualization of ZAP-70 movement to the plasma membrane supports previous biochemical data. Our work also demonstrates that only a small fraction of total enzyme translocates to the membrane. That this membrane localization can be independent of TCR expression suggests that our knowledge of the sequence of events occurring after T cell stimulation may be incomplete. Moreover, the discovery that there is a large pool of ZAP-70 in the nucleus, which is activated upon cellular stimulation, suggests that the kinase has additional, heretofore unexpected functions.

Compelling evidence exists indicating that a physical interaction between ZAP-70 and TCR chains takes place soon after T cell stimulation. Early studies showed that ZAP-70 coprecipitates with TCR-ζ and the CD3 chains after activation (36, 37). This interaction depends on the highly specific binding of the tandem SH2 domains of ZAP-70 with tyrosine phosphorylated ITAMs of the TCR (6, 7). Our results, revealing plasma membrane translocation of ZAP-70 in the absence of any TCR chains, suggest that there are alternative mechanisms of ZAP-70 localization at the plasma membrane. Cos 7 cells may express a molecule at the surface with cytoplasmic tyrosine residues or ITAMs available for phosphorylation and SH2 binding. SH2-mediated binding could also be independent of tyrosine kinase as seen in the cases of Src family PTK SH2 domains binding to Raf and other proteins, Abl SH2 binding Bcr and Syk SH2 domains binding Cbl (38–40). However, the enhanced translocation of ZAP-70 to the membrane in response to pervanadate makes this possibility less likely. Thus, the kinase can likely be retained at the membrane by additional, non–SH2 mechanisms.

The tyrosine phosphorylation and subsequent activation of ZAP-70 by Lck are the critical parameters in the facilitation of its membrane localization. The observation that KD ZAP-70 could redistribute to the cell surface less efficiently than the native molecule suggests that ZAP-70 tyrosine phosphorylation or kinase activity is involved in the mechanism of translocation. Perhaps a phosphorylation-dependent conformational change in ZAP-70 occurs leading to its activation and/or redistribution, similar to that reported for other protein kinases (41–43). The activated kinase may then enhance the translocation either by increasing its own phosphotyrosine content by transphosphorylation or that of downstream substrates which may then serve as vehicles for ZAP-70 movement to the cell surface. We have found that only about half of phospho–ZAP-70 is retrieved from the membrane fraction of T cells. The rest is in the cytoplasm, indicating that the enzyme may indeed be activated before its accumulation at the cell surface (Zhang, W., unpublished observations).

The majority of known protein tyrosine kinases are involved in the transduction of extracellular signals, exerting...
their effects at or near the plasma membrane. The discovery that several nuclear proteins contain phosphotyrosine raised the possibility that some tyrosine kinases may reside and have additional functions in the nucleus (for review see reference 44). Indeed, several PTKs have a nuclear location, the best characterized of which is c-Abl (44). This PTK is found in both the cytoplasm and in the nucleus of cultured fibroblasts, and has a classical nuclear localization signal (NLS) and a DNA binding domain. Kinase activity and DNA binding of nuclear c-Abl is regulated during the cell cycle (45). The significance of Abl cellular localization is corroborated by the observation that oncogenic forms reside exclusively in the cytoplasm (45). Other nuclear PTKs include two members of the Src family of PTKs, which can be found in the nucleus as well as in the cytosol. The nuclear localization of Fgr appears to be constitutive, whereas that of Src is induced by Ca\textsuperscript{2+} ionophore (44). Two NLS sequences have been identified in the SH2 and tyrosine kinase domains of Fgr, but no analogous sites have yet been discovered in Src. The specific nuclear functions of these two kinases remains unknown (44).

Although unexpected, nuclear ZAP-70 was seen both as a chimeric protein overexpressed in endothelial cells or at physiological levels in T cells, and as the native endogenous protein by immunofluorescence and biochemical analyses. The advantages of using GFP chimeras to study cell localization were highlighted in the comparison of the GFP and immunofluorescence assay results. Ab detection of nuclear ZAP-70 was hindered by the nuclear membrane, leading to inaccurate results regarding the fraction of the molecule in different cellular compartments, both within a single cell and in the population as a whole. In contrast, the ZAP-70 GFP protein, produced within the cell, was detected efficiently in the nucleus of all cells, regardless of their expression levels. Moreover, the ability to quantify the amount of ZAP-70 GFP in individual cell fractions (46) facilitates the acquisition of accurate data regarding the percent of the protein in different compartments under several stimulation conditions. Preliminary biochemical results suggest that one third of the total cellular ZAP-70 resides within the nucleus of resting T cells (Fig. 6). Finally, the ability to visually study GFP-tagged proteins in living cells allows one to acquire precise and detailed kinetic measurements regarding protein redistribution in response to cellular stimulation, as shown by the results in Fig. 3.

The details of ZAP-70 nuclear localization remain to be defined. Because nuclear pores set a physical barrier to proteins above ~40 kDa, transit of ZAP-70 into the nucleus must be an active mechanism involving specific localization signals (47). Although no classical NLSs are obvious in the amino acid sequence, several candidate basic-rich regions can easily be highlighted (37). Moreover, the recent discovery of a novel receptor-mediated nuclear import pathway, using a nonclassical NLS, suggest other mechanisms for nuclear localization (48, 49). Interestingly, the lack of an NH\textsubscript{2}-terminal myristylation may facilitate nuclear localization of ZAP-70. Indeed, nuclear forms of Fgr lack the conventional NH\textsubscript{2}-terminal myristylation site, and addition of such a motif actually inhibits nuclear entry (45). The function of nuclear ZAP-70 is likewise uncertain. However, the clear demonstration of its increased phosphorylation content after anti-TCR stimulation (Fig. 9) strongly suggests that nuclear ZAP-70 is active. Indeed, many nuclear tyrosine phosphorylated proteins were apparent after stimulation (data not shown), suggesting that nuclear PTKs are active in the cells. Further investigation should reveal informative data identifying which of these phosphorylated proteins are substrates of nuclear ZAP-70. Finally, the mechanism of phosphorylation of nuclear ZAP-70 remains to be defined. The protein may be phosphorylated in the cytosol and subsequently shuttle into the nucleus, or alternatively be activated directly in the nuclear compartment. The mechanism of nuclear ZAP-70 phosphorylation and the identity of its nuclear substrates will require further investigation.

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Address correspondence to Lawrence E. Samelson, NICHD, CBMB, Bldg. 18T, Rm 101, Bethesda, MD 20892. Phone: 301-496-6368; FAX: 301-402-0078; E-mail: samelson@helix.nih.gov

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