The potential role of the cytoskeleton in signaling via the T cell antigen receptor (TCR) was investigated using pharmacological agents. In Jurkat T cells, disruption of the actin-based cytoskeleton with cytochalasin D or disruption of the microtubules with colchicine did not affect TCR induction of proximal signaling events triggered by CD3 mAbs. Polymerized actin and tubulin, therefore, were not required for TCR-mediated signal transduction. Nocodazole, however, was found to inhibit dramatically TCR signaling, independently of its ability to depolymerize microtubules. This effect was TCR-specific, because signaling via the human muscarinic acetylcholine receptor 1 in the same cells was unaffected. A mechanism for the inhibition of TCR signaling by nocodazole was suggested by in vitro assays, which revealed that the drug inhibited the kinase activity of LCK and, to a lesser extent, FYN. The kinase activity of ZAP-70 in vitro, however, was unaffected. These results, therefore, suggested that nocodazole prevented initial phosphorylation of the TCR by LCK after stimulation, and as a result, blocked activation of downstream signaling pathways. Immunofluorescence analyses also revealed that nocodazole and the specific SRC-family kinase inhibitor PP1 delocalized ZAP-70 from its constitutive site at the cell cortex. These effects did not require the SH2 domains of ZAP-70. The localization of ZAP-70 to the cell cortex is, therefore, regulated by the activity of SRC-family kinases, independently of their ability to phosphorylate immunoreceptor tyrosine-based activation motifs of the TCR.

The αβ disulfide-linked heterodimer of the T cell antigen receptor (TCR) interacts with an antigenic peptide bound to a major histocompatibility complex molecule on the surface of an antigen-presenting cell (1). Together with signals from accessory surface receptors, such as CD28 (2), this interaction promotes the proliferation and differentiation of resting T cells into effector T cells. The signaling function of the TCR is mediated by the CD3 complex of polypeptides (γδε) and disulfide-linked ζ homodimers, which are both noncovalently associated with αβ heterodimers (3) and required for efficient surface expression of the intact TCR (4).

The earliest detectable biochemical event following stimulation of the TCR is the induction of protein tyrosine kinase (PTK) activity, which is essential to couple it to downstream signaling pathways (5). However, none of the component subunits of the TCR contain any intrinsic tyrosine kinase domains. Rather, the TCR activates intracellular protein tyrosine phosphorylation by interacting sequentially with two different types of cytoplasmic PTKs (6). Following TCR oligomerization, the cytoplasmic domains of the CD3 complex subunits (7–9) and ζ homodimers (10) become rapidly phosphorylated within 16 amino acid motifs (YXXLX$_{16}$–YXXL), termed immunoreceptor tyrosine-based activation motifs (ITAMs) (3, 11). ITAM phosphorylation is mediated by members of the SRC-family PTKs, primarily LCK (12–14) and, under some circumstances, FYN (15, 16).

Tyrosine phosphorylation of ITAMs by LCK (or FYN) recruits a second family of cytoplasmic PTKs to the TCR, which comprises ZAP-70 and SYK (17, 18). Both ZAP-70 and SYK are present in T cells, although only the former is critical for T cell development and function, and also is more abundant (19–21). These PTKs bind to the TCR via binding of their two N-terminal SH2 domains with the doubly phosphorylated ITAMs (9, 17, 22, 23). The association of ZAP-70 and SYK with the TCR facilitates their phosphorylation and subsequent activation. For ZAP-70, phosphorylation is thought to be mediated, in part, by LCK (24, 25), with which it associates following engagement of the TCR (26). In contrast, binding to the doubly phosphorylated ITAM appears to activate SYK directly (27, 28). Once activated, ZAP-70 and SYK autophosphorylate multiple tyrosines, generating SH2 binding sites for other signaling proteins, including Vav (29, 30), Cbl (31), Ras-GAP, abl (32), and FakB (33). In this way, ZAP-70 and SYK may act as scaffolding proteins, recruiting other signaling molecules to the activated TCR and into close proximity with their upstream regulators and downstream targets (11). Subsequent to ZAP-70 and SYK activation, downstream effector functions are triggered, which include the activation of Ras and consequent activation of ERK1/2 MAP kinases (34, 35) and the mobilization of intracellular Ca$^{2+}$ (6).

Two recent reports have indicated that a small percentage of the ζ subunit of the TCR is associated with a Triton X-100 detergent-insoluble fraction in T cells, and this increases after TCR stimulation with CD3 mAbs (36, 37). Association between ζ and the insoluble fraction is broken by treatment of T cells with the actin-disrupting drugs, cytochalasins D and B, suggesting that the ζ subunit might interact directly with the actin cytoskeleton. Based on these data, it has been suggested that the actin cytoskeleton may play a role in signal transduction.
and then transferred to 25 ml of RPMI medium supplemented with 5% serum, 2 mML -glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were maintained in a rapid growth phase prior to use in experiments. Transient transfection was carried out by electroporation, as described previously (42). Briefly, cells were washed three times in serum-free RPMI medium and resuspended at 1 × 10⁶ cells/ml. 250 µl were transferred into a Bio-Rad gene pulser cuvette, and 5 µg of the appropriate plasmid were added. Cells were pulsed at 960 microfarads and 250 V (Gene pulser, Bio-Rad laboratories), left for 10 min at RT, and appropriate plasmid were added. Cells were pulsed at 960 microfarads and 250 V (Gene pulser, Bio-Rad laboratories), left for 10 min at RT, and then transferred to 25 ml of RPMI medium supplemented with 5% fetal calf serum and cultured for 14 h before harvesting. All drugs were made up as stocks in Me₂SO. Control cells were treated with equivalent volumes of Me₂SO alone. Nocodazole was obtained from Sigma-Aldrich and was determined to be over 99% pure by the manufacturers (data not shown). Nocodazole repurified by reverse phase high performance liquid chromatography was found to have inhibitory effects on TCR signaling identical to those of the starting material (data not shown). Thus, the inhibitory effects of nocodazole were not due to a low level of contaminating material in preparations of the drug. Nocodazole stocks were prepared at 20 mM. Colchicine (Fluka Biochemicals; 20 mM stock solution), cytochalasin D (Sigma-Aldrich; 5 µg/ml stock solution), and Taxol (Calbiochem; 20 mM stock solution) were also over 99% pure, as determined by the manufacturers. The SRC-family kinase inhibitor, PP1, was a gift of Yajun Xu and Rainer Munshaw (BASF Bioresearch Corp., Worcester, MA). The concentrations of pre-treatment used are indicated in the figures and their legends. Cells were pretreated with nocodazole for either 5 or 30 min before stimulation in biochemical experiments. No difference was detected between these two different times of incubation with the drug. The maximal inhibitory effect on TCR signaling was detected within 1 min of pretreatment with nocodazole (data not shown).

Antibodies and DNA Constructs—The generation of ZAP-4 and LCK-1 antisera has been described previously (29, 43). Anti-FYN mAb was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Immunofluorescence staining of hemagglutinin (HA) epitope-tagged ZAP-70 was carried out using 12CA5 mAb (44) and fluorescein isothiocyanate-conjugated goat anti-mouse Ig (Jackson Laboratories, Bar Harbor, ME). Tyrosine-phosphorylated proteins were detected using 4G10 (from Brian Drucker, Oregon Health Sciences University, Portland, OR), the rat anti-α-tubulin mAb, YOL34, was obtained from Serotec. The TCR γ chain was recognized in Western blotting using N39 antisemur (8), kindly provided by Jaime Sancho (Granada, Spain). To stimulate T cells via their TCR, Fab, fragments of the OKT3 mAb (American Type Culture collection, Rockville, MD) were used to avoid binding of the stimulating antibody to the protein A-Sepharose used for precipitation. This antibody preparation was kindly provided by M. Glennie and A. Tutt (Tenovus, Southampton, United Kingdom). For Western blotting, horseradish peroxidase-coupled antibodies, and protein A were obtained from Amersham Pharmacia Biotech. The wild-type and ΔSH2 (amino acids 6–251 deleted) HA epitope-tagged ZAP-70 constructs in the pcDNA3neo vector have been described previously (17, 40).

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and 405 nm, representing free versus Ca\textsuperscript{2+}-associated Indo-1, respectively, to give an absorbance ratio.

RESULTS

Nocodazole Inhibits TCR-induced Tyrosine Phosphorylation of ZAP-70—As discussed under “Introduction,” the initial event that occurs after TCR cross-linking is phosphorylation of CD3 and \( \zeta \) ITAMs and their association with ZAP-70, which then itself becomes tyrosine-phosphorylated and activated (6). To investigate whether intact microtubules were important in this proximal signaling pathway, Jurkat T cells were preincubated with Me\textsubscript{2}SO control (which was used as the vehicle to solubilize all of the drugs tested), nocodazole, or colchicine for 30 min. The cells were then stimulated for 5 min with F\textsubscript{Ab}\textsubscript{2} fragments of the OKT3 CD3 mAb, lysed, and ZAP-70 immunoprecipitated/Western blotted, and probed for PTyr. Blots were stripped and reprobed with ZAP4 to confirm equal loading of ZAP-70. Duplicate cultures were pretreated with the same drugs and lysed in the microtubule-stabilizing buffer PM2G. The insoluble fraction, which corresponded to polymerized microtubules, was then Western blotted for \( \alpha \)-tubulin. Similar results were obtained in three separate experiments. B. anti-PTyr immunoprecipitates of lysates, prepared as in A, were Western blotted for PTyr. C. Jurkat T cells were pretreated for 5 min at 37 °C with the indicated concentrations of nocodazole and then stimulated with OKT3 CD3 mAb for a further 5 min (+) or left unstimulated (−). ZAP-70 was immunoprecipitated from cell lysates and then Western blotted for PTyr. The PTyr blot was densitometrically scanned to quantify the effect of nocodazole on ZAP-70 and associated \( \zeta \) phosphorylation in stimulated and unstimulated cells. Data are presented graphically as arbitrary units. Reprobing the blot with ZAP4 confirmed that equal amounts of ZAP-70 were present in each immunoprecipitate (data not shown). Essentially identical dose-response curves were obtained when cells were pretreated with nocodazole for 30 min (data not shown).

Pretreatment of the cells with cytochalasin D, which disrupted the actin cytoskeleton as determined by phalloidin staining and fluorescence microscopy (data not shown) or Taxol, which promoted microtubule polymerization (see Fig. 1B), also had no effect on TCR-stimulated ZAP-70 and associated \( \zeta \)-chain tyrosine phosphorylation.

Because tyrosine phosphorylation of ZAP-70 is involved in its activation (24), the inhibition by nocodazole of TCR-induced phosphorylation of ZAP-70 suggested that the drug might also inhibit the tyrosine phosphorylation of downstream intracellular proteins. To investigate this possibility, PTyr proteins were immunoprecipitated with an anti-PTyr mAb from lysates of cells pretreated with the panel of drugs and Western blotted with the same antibody. In Fig. 1B, it can be seen that nocodazole, but not colchicine, cytochalasin D, or Taxol, dramatically inhibited both basal and OKT3-induced tyrosine phosphorylation of multiple intracellular proteins. Taken together, the data in Fig. 1 suggested that polymerization of the microtubules (result of colchicine) or actin cytoskeleton (result of cytochalasin D) was not essential for TCR induction of ZAP-70 and associated \( \zeta \)-chain tyrosine phosphorylation.

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In Fig. 1C, Jurkat T cells were pretreated with a range of nocodazole concentrations, and ZAP-70 tyrosine phosphorylation...
Nocodazole Inhibits TCR Signaling

Inhibitory Effect of Nocodazole on ZAP-70 Phosphorylation Is Not Reversed by Taxol—The observation that ZAP-70 phosphorylation was inhibited by nocodazole but not by colchicine suggested that the former drug might have inhibited TCR signaling independently of its effects on microtubule cytoskeleton polymerization. To address this question directly, Jurkat T cells were treated with nocodazole, Taxol, or a combination of these drugs for 30 min. The cells were then stimulated for 5 min with CD3 mAb and lysed, and ZAP-70 immunoprecipitates were Western blotted for PTyr. In a parallel experiment, the effect of these drug treatments was tested on the polymerization of tubulin using PM2G buffer extraction. In Fig. 2B, it can be seen that Taxol completely blocked the depolymerization of microtubules by nocodazole and that the amount of polymerized α-tubulin was actually increased relative to control (MeSO4-treated) cells. However, the inhibition of ZAP-70 phosphorylation by nocodazole was unaffected by the simultaneous addition of Taxol (Fig. 2A). These data, therefore, demonstrated that nocodazole mediated its effects on TCR-induced ZAP-70 phospho-

phorylation independently of its ability to depolymerize microtubules.

Signaling via the HM1 Receptor Expressed on Jurkat T Cells Is Unaffected by Nocodazole—It was important to determine whether the inhibitory effect of nocodazole on TCR signaling was due to some pleiotropic effect on cell metabolism or was affecting a specific component of signal transduction machinery of the TCR. Trypan blue dye exclusion ruled out the trivial possibility that nocodazole was simply killing the Jurkat T cells (data not shown). Furthermore, the inhibitory effect of nocodazole was reversed by extensive washing of pretreated cells with control medium (data not shown). To investigate this question further, the effect of nocodazole was determined on signaling via a G protein-coupled receptor, the human muscarinic acetylcholine receptor 1 (HM1), expressed on stably transfected Jurkat T cells (41). The HM1 receptor activates both increases in intracellular free Ca2+ and ERK MAP kinase but does not require LCK or ZAP-70 to activate these signaling pathways, unlike the TCR (13, 47).

As in the E6.1 parental Jurkat cell line, nocodazole inhibited TCR-stimulated tyrosine phosphorylation in HM1-Jurkat T cells (data not shown). The induction of intracellular signaling events downstream from activation of the TCR were also inhibited. Thus, nocodazole effectively inhibited induction of intracellular Ca2+ fluxing (Fig. 3A) and phosphorylation of ERK MAP kinase (Fig. 3B), which normally occur following TCR stimulation. Nocodazole had no effect on the fluxing of calcium following treatment with the calcium ionophore ionomycin (data not shown), confirming that the cells were correctly loaded with Indo-1. Pretreatment of cells with colchicine or cytoskeleton D had no effect on TCR stimulation of intracellular Ca2+ fluxing and ERK MAP kinase phosphorylation (data not shown), consistent with their effects on TCR stimulation of tyrosine phosphorylation (Fig. 1).

Stimulation of the HM-1 receptor with carbachol induced increases in intracellular free Ca2+ (Fig. 3A) and ERK phosphorylation (Fig. 3B), as expected. In contrast to stimulation via the TCR, however, nocodazole had no effect on the stimulation of either of these signaling pathways. These data supported the hypothesis that nocodazole was able to inhibit proximal signaling events specifically associated with the TCR but not the G protein-coupled HM-1 receptor.

Nocodazole Inhibits LCK and FYN Kinase Activity—The HM-1 Jurkat experiments (Fig. 3) suggested that nocodazole specifically inhibited a proximal component of the TCR signaling machinery, involved in the induction of cytoplasmic PTyr proteins. The level of tyrosine-phosphorylated ζ-chain is governed by its phosphorylation by LCK and its dephosphorylation by undefined protein tyrosine phosphatases. Although ZAP-70 is unable to phosphorylate the ζ chain directly (39) it may nevertheless promote the accumulation of phospho-ζ by binding to phospho-ITAMs and preventing their dephosphorylation by constitutively active protein tyrosine phosphatases (12, 48). Inhibition of ζ phosphorylation by nocodazole after TCR stimulation, therefore, might have resulted either directly, from preventing the action of LCK, or indirectly, from preventing ZAP-70 binding to phospho-ITAMs.

Initial experiments investigated whether nocodazole affected the activity of LCK and FYN in vitro. These kinases were individually immunoprecipitated from Jurkat T cell lysates and then tested for their ability to phosphorylate a synthetic peptide substrate, which is the optimal substrate for SRC kinases (43, 49), in the presence of a range of concentrations of nocodazole. The kinase activity of LCK was found to be profoundly inhibited by nocodazole (Fig. 4). FYN kinase activity was also inhibited, although to a lesser extent. The IC50 for...
LCK was 2.5–5 μM (determined in four separate experiments), whereas that of FYN was 12.5–25 μM (determined in three separate experiments). At 100 μM, LCK kinase activity was inhibited by 90%, whereas that of FYN was inhibited by 65%. The inhibitory effect of nocodazole on SRC kinase activity was apparently specific, because the in vitro kinase activity of ZAP-70, tested by its ability to phosphorylate a synthetic peptide substrate (Fig. 4) or to autophosphorylate (data not shown), was completely unaffected by nocodazole. These data suggested that the inhibitory effect of nocodazole on TCR signaling primarily resulted from its ability to prevent LCK phosphorylation of ITAMs, thereby blocking the activation of all signaling pathways downstream of the TCR.

**Nocodazole Delocalizes ZAP-70 from the T Cell Cortex**—As discussed above, it was possible that decreased TCR-induced ζ and ZAP-70 phosphorylation, after nocodazole pretreatment of Jurkat T cells, was due to inhibition of the interaction of ZAP-70 with the TCR after TCR stimulation, such that phospho-ζ became dephosphorylated. Our laboratories have recently shown that ZAP-70 is constitutively targeted to the T cell cortex in a diffuse band beneath the plasma membrane (40). Thus, ZAP-70 is held close to the TCR, with which it must interact to become activated. During the course of these studies, therefore, the effect of nocodazole on ZAP-70 localization was also assessed to investigate whether alteration of ZAP-70 localization might also contribute to the inhibitory effects of the drug.

To investigate this possibility, Jurkat T cells were transiently transfected with a C-terminally HA epitope-tagged ZAP-70 cDNA, cultured for 14 h, pretreated with control Me2SO or 100 μM nocodazole for 1 h, fixed, and then immunofluorescently stained with an anti-HA mAb. ZAP-70 was found to be delocalized in the majority of cells and redistributed throughout the cytoplasm in cells pretreated with nocodazole (Fig. 5A). Treatment with colchicine, however, had no effect on ZAP-70 localization (data not shown), suggesting that delocalization was not mediated by the ability of nocodazole to disrupt microtubules. Consistent with this hypothesis, simultaneous addition of Taxol did not prevent delocalization of ZAP-70 by nocodazole (data not shown). In order to quantify the effect of nocodazole on ZAP-70 localization, cells were treated with a range of nocodazole concentrations, and ZAP-70-HA in transfected cells was scored as either localized (discrete cortical rim) or delocalized (cortical structure lost). The resulting titration curve for the effect of nocodazole on ZAP-70 localization indicated an IC50 of approximately 10 μM (Fig. 5C). Kinetic experiments indicated that a detectable effect on ZAP-70 localization was evident after pretreatment of cells with nocodazole for 15–30 min (data not shown).

To determine whether ZAP-70 delocalization was mediated by inhibition of SRC-family kinase activity, the effect of nocoda-
zole was compared with that of PP1, a specific SRC-family kinase inhibitor (50). PP1, like nocodazole, was able to delocalize ZAP-70 from the cell cortex (Fig. 5A), with an IC_{50} of approximately 0.3 μM (Fig. 5B), comparable to that for its inhibition of TCR-induced tyrosine phosphorylation in vivo (data not shown). Unlike nocodazole, PP1 had no effect on microtubule polymerization (data not shown). Taken together, these data suggested that SRC-family kinase activity is required for the efficient localization of ZAP-70 in the cell cortex. However, even high concentrations of PP1 or nocodazole failed to completely delocalize ZAP-70 in all cells, suggesting that SRC-family kinase activity is not an absolute requirement for ZAP-70 cortical localization.

ZAP-70 is localized to the cell cortex independently of its SH2 domains (40). However, it remained possible that the SH2 domains could be involved in the active delocalization of ZAP-70 following inhibition of SRC-family kinase activity, because this would inhibit any low levels of constitutive phosphorylation of ITAM, to which the tandem SH2 domains could be involved in the active delocalization of ZAP-70 (38). Taken together, these data raised the possibility that microtubules might be important for signaling via the TCR, which requires ZAP-70 function (56–58). However, experiments in this study using colchicine to disrupt the microtubules failed to reveal an effect on TCR stimulation of tyrosine phosphorylation (Fig. 1), increases in intracellular free Ca^{2+}, or activation of ERK MAP kinases (data not shown). Thus, intact microtubules did not appear to be important for TCR activation of these proximal signaling events. The association of ZAP-70 and SYK with tubulin may nevertheless be important for other signaling events triggered by the TCR, perhaps directly influencing microtubule polymerization (59).

Although the experiments in this study failed to reveal a role for the microtubules in TCR signaling, a novel biological effect of nocodazole was demonstrated: it was found to inhibit significantly TCR-induced ZAP-70 phosphorylation and activation of downstream signaling pathways (Fig. 1). This effect persisted when microtubule integrity was maintained by the simultaneous addition of Taxol (Fig. 2), suggesting that an unrelated biological activity had been revealed, probably mediated by binding to a target molecule distinct from β-tubulin (60). Nocodazole inhibited Ca^{2+} mobilization and ERK phosphorylation stimulated by the TCR but not by the G protein-coupled HM-1 receptor in the same Jurkat T cell subline (Fig. 3). Thus, nocodazole appeared to be affecting a component of the TCR signaling machinery that was not involved in HM-1 receptor activation of these signaling pathways.

The ability of nocodazole to inhibit ζ-chain phosphorylation after TCR ligation (Fig. 1) raised the possibility that it mediated its inhibitory effects on TCR signaling by preventing LCK from phosphorylating the TCR. Consistent with this hypothesis, nocodazole was found to profoundly inhibit the in vitro kinase activity of LCK, assayed using an exogenous peptide substrate (Fig. 4). LCK autophosphorylation in vitro was also suppressed by nocodazole, although this was found to be a less robust assay for LCK specific activity (data not shown). The kinase activity of FYN was inhibited to a lesser extent, whereas ZAP-70 kinase activity was completely insensitive to the drug (both assayed using exogenous peptide substrates (Fig. 4)). Because TCR signaling in Jurkat T cells is completely depend-
ent on functional LCK (13), these data provided a plausible mechanism to explain how nocodazole blocked TCR signaling and also identified a second target molecule for the drug. Experiments with purified recombinant LCK protein produced in baculovirus confirmed that the inhibitory effect of the nocodazole on LCK activity is mediated directly. The potency of nocodazole in vivo (measuring ZAP-70 phosphorylation; IC50 = 12.5–25 μM) was reduced relative to kinase inhibition of immunoprecipitated LCK in vitro (IC50 = 2.5–5 μM). This difference may be attributed to permeability of the compound and its distribution within cells relative to LCK or to metabolism of the drug in vivo.

Previously, our laboratories have shown that ZAP-70 is constitutively targeted to the cell cortex and that this localization requires its active kinase domain (40). Although nocodazole did not affect the kinase activity of ZAP-70 (Fig. 4), it was found to delocalize ZAP-70 from the cell cortex in the majority of the transfected cells (Fig. 5). This effect could be attributed to inhibition of SRC-kinase activity, because PP1, which is a specific inhibitor of this class of PTKs (50), also delocalized ZAP-70. These data indicate that the efficient localization of ZAP-70 to the cell cortex requires SRC kinase activity. However, because the ΔSH2-ZAP-70 mutant was also delocalized by both nocodazole and PP1 (Fig. 5), this effect did not result from inhibition of the low level of constitutive phosphorylation of ITAMs by LCK in Jurkat T cells. Delocalization of ZAP-70 from the cell cortex would be expected to significantly reduce the efficiency with which it could bind to phospho-ITAMs to become phosphorylated and activated by LCK. However, given the slow kinetics of delocalization after nocodazole or PP1 treatment (data not shown), these effects may not contribute significantly to ability of these drugs to inhibit TCR signaling, which are mediated very rapidly (data not shown).

LCK phosphorylates ZAP-70 after TCR stimulation, thereby activating its kinase activity (11). It was possible, therefore, that nocodazole inhibition of LCK kinase activity might indirectly affect the kinase activity of ZAP-70 and thereby its localization. However, this possibility is unlikely, because it has been previously been shown, by analysis of point mutants, that the major sites of phosphorylation on ZAP-70 are not required for cortical localization (40). Furthermore, the ΔSH2-ZAP-70 mutant, which is cortically localized, is not detectably tyrosine-phosphorylated (data not shown). Localization of ZAP-70 through a direct interaction with LCK is also unlikely, because expression of a delocalized C3A/C5A-LCK mutant (43) does not affect the cortical targeting of co-expressed ZAP-70 (data not shown). Furthermore, when ZAP-70 is expressed in the Jurkat subline JCam 1.6 (data not shown) or in 3T3 cells (40), neither of which express LCK, ZAP-70 is localized to the cell cortex in this majority of cells. Thus, LCK is not absolutely required for ZAP-70 targeting. Presumably, in these cells, other SRC-family members can substitute for LCK to regulate ZAP-70 localization. Consistent with this hypothesis, ZAP-70 is delocalized by both nocodazole and PP1 in transfected 3T3 fibroblasts (data not shown).

Our laboratories have speculated that the specific cortical targeting of ZAP-70 implies the existence of an anchoring molecule, which tethers its in this location (40). Thus, an attractive hypothesis for the effects of nocodazole and PP1 on ZAP-70 localization is that the anchor protein requires phosphorylation by a SRC kinase for efficient ZAP-70 binding. Clearly, the identification of the anchor protein for ZAP-70 will be important to determine whether this model is correct.

The benzimidazole drug nocodazole was originally generated synthetically and was identified by virtue of its ability to inhibit the growth of a panel of model tumor cell lines (61, 62). Subsequently, it was found that the drug’s antitumor activity resulted from its ability to bind to tubulin and inhibit its polymerization (63, 64). In this study, a novel biological activity of nocodazole has been revealed that is unrelated to its effects on the microtubule cytoskeleton. Thus, nocodazole was found to inhibit the activity of the SRC-family kinases LCK and FYN, thereby inhibiting signal transduction via the TCR, which depends on LCK activity (13). Nocodazole is structurally distinct from PP1, a specific SRC-family kinase inhibitor (59). Unlike, PP1, nocodazole shows specificity within the SRC-family. Thus, LCK is approximately 7-fold more sensitive to nocodazole than FYN (Fig. 4). LYN, a SRC-family kinase that is essential for signaling via the B cell antigen receptor (60), is also significantly less sensitive to nocodazole inhibition than LCK in vitro (data not shown). In the future, it may be possible to generate nocodazole analogs that inhibit SRC-family kinases without disrupting the microtubules. If such analogs can be generated that are completely selective for LCK, they might be useful clinically to suppress T cell function in disease.

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REFERENCES

1. Clevers, H., Alarcon, B., Wileman, T., and Terhorst, C. (1988) Annu. Rev. Immunol. 6, 629–662
2. Rudd, C. E. (1996) Immunity 4, 527–534
3. Weiss, A. (1993) Cell 73, 209–212
4. Weiss, A. (1991) Annu. Rev. Genet. 25, 487–510
5. Klausner, R. D., and Samelson, L. E. (1991) Cell 64, 875–878
6. Weiss, A., and Littman, D. R. (1994) Cell 76, 263–274
7. Qian, D., Grieswd-Prenner, I., Rosner, M., R., and Fitch, P. F. (1993) J. Biol. Chem. 268, 4488–4493
8. Sancho, J., Franco, R., Chatila, T., Hall, C., and Terhorst, C. (1993) Eur. J. Immunol. 23, 1636–1642
9. Stranz, D. B., and Weiss, A. (1993) J. Exp. Med. 178, 1523–1530
10. Banisash, M., Garcia-Morales, P., Luong, E., Samelson, L. E., and Klausner, R. D. (1988) J. Cell Biol. 104, 1825–1823
11. Chan, A. C., and Shaw, A. S. (1996) Curr. Opin. Immunol. 8, 394–401
12. Iwashima, M., Irving, B. R., van Oers, N. S. C., Chan, A. C., and Weiss, A. (1994) Science 263, 1136–1139
13. Stranz, D. B., and Weiss, A. (1992) Cell 70, 585–593
14. van Oers, N. S. C., Kiiroen, N., and Weiss, A. (1996) J. Exp. Med. 183, 1053–1062
15. Groves, T., Smiley, P., Cooke, M. F., Forbrush, K., Permutt, M. R., and Guidos, C. J. (1996) Immunology 5, 417–428
16. van Oers, N. S. C., Lowin-Kropf, F., Finlay, D., Connolly, K., and Weiss, A. (1996) Immunology 5, 429–436
17. Chan, A. C., Iwashima, M., Turek, C. W., and Weiss, A. (1992) Cell 71, 649–662
18. Taniguchi, T., Kobayashi, T., Kondo, J., Takahashi, K., Nakamura, H., Suzuki, J., Nagai, K., Yamada, T., Nakamura, S., and Yamamura, H. (1991) J. Biol. Chem. 266, 15790–15796
19. Negishi, I., Motoyama, N., Nakayama, K.-I., Nakayama, K., Senju, S., Hatakeyama, S., Zhang, Q., Chan, A. C., and Loh, D. Y. (1995) Nature 376, 435–438
20. Hivroz, C., and Fischer, A. (1994) Curr. Biol. 4, 731–733
21. Turner, M., Mee, P. J., Costello, P. S., Williams, O., Price, A. A., Duddy, L. P., Furlong, M. T., Geahlen, R. L., and Tybulewicz, V. L. J. (1995) Nature 376, 259–262
22. Chan, A. C., van Oers, N. S. C., Tran, A., Turka, L., Law, C. L., Ryan, J. C., Clark, E. A., and Weiss, A. (1994) J. Immunol. 152, 4758–66
23. Wange, R. L., Malek, S. N., Desiderio, S., and Samelson, L. E. (1993) J. Biol. Chem. 268, 19797–19801
24. Chan, A. C., Dalton, M., Johnson, R., Kong, G.-H., Wang, T., Thomas, D., and Kurysinski, T. (1995) EMBO J. 14, 2499–2509
25. Wange, R. L., Guittain, R., Isakov, N., Watts, J. D., Aebberold, R., and Samelson, L. E. (1995) J. Biol. Chem. 270, 18730–18733
26. Duplay, P., Thome, M., Herve, F., and Acuto, O. (1994) J. Exp. Med. 179, 1163–1172
27. Rowley, R. B., Burkardt, A. L., Chao, H.-G., Matsueda, G. R., and Bolen, J. B. (1995) J. Biol. Chem. 270, 11590–11594
28. Zhou, S., Zoller, M. J., and Brugge, J. S. (1995) J. Biol. Chem. 270, 10968–10972
29. Huby, R. D. J., Carlile, G. W., and Ley, S. C. (1995) J. Biol. Chem. 270, 30241–30244
30. Kataev, S., Sutherland, M., Packham, G., Yi, T., and Weiss, A. (1994) J. Biol. Chem.
31. Fournel, M., Davidson, D., Veil, R., and Veillette, A. (1996) J. Exp. Med. 183, 301–306
32. Neumeister, E. N., Zhu, Y., Richard, S., Terhorst, C., Chan, A. C., and Shaw, A. S. (1995) Mol. Cell. Biol. 15, 3171–3178
33. Kanner, S. B., Aruffo, A., and Chan, P. Y. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 10484–10487
34. Marshall, C. J. (1995) Cell 80, 179–185
35. Izquierdo Pastor, M., Reif, K., and Cantrell, D. (1995) Immunity 16, 159–164
36. Rozdzial, M. M., Malissen, B., and Finkel, T. H. (1995) Immunity 3, 623–633
37. Salmeron, A., Ahmad, T. B., Carlile, G. W., Pappin, D., Narsimhan, R. P., and Ley, S. C. (1996) EMBO J. 15, 817–826
38. Kabouridis, P. S., Magee, A. I., and Ley, S. C. (1998) EMBO J. 17, 6411–6419
39. Solomon, F. (1986) Methods Enzymol. 120, 139–147
40. Schneidere, C., Newman, R. A., Sutherland, D. R., Aszer, U., and Greaves, M. F. (1982) J. Biol. Chem. 257, 10766–10769
41. Mege, D., Di Bartolo, V., Germain, V., Tuost, L., Michel, F., and Acuto, O. (1996) J. Biol. Chem. 271, 32644–32652
42. Songyang, Z., and Cantley, L. C. (1995) Trends Biochem. Sci. 20, 470–475
43. Marshall, C. J. (1995) Cell 80, 179–185
44. Hanke, J. H., Gardner, J. P., Dey, R. L., Changelis, P. S., Brissette, W. H., Wieringer, E. J., Polio, B. A., and Connell, P. A. (1996) J. Biol. Chem. 271, 695–701
45. Valitutti, S., Dissing, M., Aktories, K., Gallati, H., and Lanzavecchia, A. (1995) J. Exp. Med. 181, 577–584
46. Stowers, L., Yelon, D., Berg, L. J., and Chant, J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 5027–5031
47. Dietrich, J., Hou, X., Wegener, A. M., and Geisler, C. (1994) EMBO J. 13, 2156–2166
48. Hsi, E. D., Siegel, J. N., Minami, Y., Luong, E. T., Klausner, R. D., and Samelson, L. E. (1989) J. Biol. Chem. 264, 10836–10842
49. Schneider, C., Newman, R. A., Sutherland, D. R., Aszer, U., and Greaves, M. F. (1982) J. Biol. Chem. 257, 10766–10769
50. Atassi, G., Schaus, C., and Tagzon, H. J. R. (1975) Eur. J. Cancer 11, 609–614
51. Atassi, G., Schaus, C., and Tagzon, H. J. R. (1975) Eur. J. Cancer 11, 599–607