The serine/threonine protein kinase Raf-1 is a part of a signaling cascade linking the extracellular stimulation of Ras to the activation of mitogen-activated protein kinases (or ERKs). Although this pathway has been most extensively studied in adherent cell lines such as fibroblasts, lymphocytes also express all members of the Ras-mediated signaling pathway (1). Not only is Raf-1 present in fibroblasts and HL-60 cells, Raf-1 becomes activated during mitosis. The mitotic activation of Raf-1 was not observed in the Lck-deficient cell line, J.CaM.1. During mitosis, Raf-1 was found to interact selectively with a mitotic form of Lck that migrated with a reduced electrophoretic mobility on SDS-polyacrylamide gels. We conclude that Raf-1 is activated during mitosis in T cells and that this mitotic activation of Raf-1 is dependent on the presence of Lck.

In addition to its well established role in mediating signals through growth factor receptors, it has been reported recently that in fibroblasts and HL-60 cells, Raf-1 becomes activated during mitosis (14,15). These findings implicate Raf-1 not only in the G2/M transition, but also in the mitotic phase of the cell cycle. We report here that in lymphocytes, Raf-1 also becomes activated at mitosis, that this activation is dependent upon the presence of the protein-tyrosine kinase Lck, and that at mitosis Raf-1 selectively associates with a mitotic form of Lck.

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**EXPERIMENTAL PROCEDURES**

**Antibodies**—Antibodies to human Raf-1 (residues 637–648) were purchased from Santa Cruz Biotechnology, Inc. Monoclonal antibody to Lck was the kind gift of Dr. Andrey Shaw. Monoclonal antibodies to phosphotyrosine, 4G10, were purchased from Upstate Biotechnology, Inc.

**Cells and Cell Treatments**—The murine cell line LSTRA, the human leukemia T cell line Jurkat (clone E6–1, from the American Type Culture Collection), and the Lck-deficient mutant of Jurkat (J.CaM.1, American Type Culture Collection) were grown and maintained as described (16). For cell cycle treatment experiments, cells (4 × 10⁷ cells/ml) maintained in the log phase of growth were either left untreated or incubated in the presence of nocodazole (1 μg/ml Sigma) for 10 h (LSTRA cells) or 14 h (Jurkat and J.CaM.1 cells) at 37 °C. Flow cytometric analysis of blocked cells indicated that after nocodazole treatment approximately 60% of the cells had undergone mitotic arrest. For treatment with phorbol ester, cells were incubated in the absence or presence of 8 ng/ml phorbol 12,13-dibutyrate (Sigma) for 30 min at 37 °C. Treated and control cells were equalized for cell number and lysed in ice-cold lysis buffer containing 0.5% Nonidet P-40, 25 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, and 20 μg/ml each of aprotinin and leupeptin at 4 °C for 15 min. Samples were centrifuged at 13,000 × g (microcentrifuge), and the supernatants were subjected to either immunoblotting or immunoprecipitation as indicated.

**Immunoblotting**—Cell lysates were analyzed by SDS-PAGE followed by transfer to Immobilon-P membranes. The membranes were blocked in TBST (15 mM Tris/HCl, pH 7.4, 0.9% NaCl, and 0.05% Tween 20) containing 2.5% bovine serum albumin (Sigma) and 2.5% non-fat dried milk (Carnation) for 1 h followed by incubation with antibodies to Raf-1. After extensive washings, the membranes were incubated for 1 h with goat anti-rabbit antibodies conjugated to horseradish peroxidase (Sigma) in TBST containing 5% goat serum, 1% bovine serum albumin, and 0.1% non-fat dry milk. The immunoblots were analyzed using the ECL detection system (Amersham). For immunoblotting anti-Raf-1 immunoprecipitations, proteins were separated by SDS-PAGE, transferred to Immobilon-P, and blocked in TBST containing 0.2% methylcellulose for 1 h followed by incubation with anti-Lck antibodies. After extensive washes, the membranes were incubated with goat-anti-mouse antibodies conjugated to horseradish peroxidase (Sigma). The immunoblots were analyzed using the ECL detection system.

**Immunoprecipitation and Immune Complex Kinase Assays**—Cell lysates were incubated at 4 °C with anti-Raf-1 antibodies bound to protein A-Sepharose (Sigma). The immune complexes were washed 3 times with ice-cold buffer containing 25 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM sodium orthovanadate, 0.5% Nonidet P-40, and 20 μg/ml each of protease inhibitors leupeptin and aprotinin. The complexes were assayed in a 50-μl volume containing 50 mM HEPES, pH 7.4, 10 mM MgCl₂, 5 mM α-nitrophenyl phosphate, 50 μM sodium orthovanadate, and 20 μg/ml each of aprotinin and leupeptin, 1 μM ATP, and 5 μCi of [γ-³²P]ATP (DuPont NEN, 6000 Ci/mmol). Reactions were carried out at 30 °C for 5 min. Where indicated, kinase-defective GST-MEK-1 (1 μg/ml) was added as an exogenous substrate, the ATP concentration was adjusted to 50 μM, and the reaction was carried out for 10 min at 30 °C. GST-kinase MEK-1 was purified by affinity chromatography on glutathione-agarose from lysates of Escherichia coli expressing human MEK-1 cDNA (gift of Dr. Kun-Liang Guan, University of Michigan) (17). Phosphoproteins were separated by SDS-PAGE, transferred to Immobilon-P, and detected by autoradiography.

**RESULTS AND DISCUSSION**

Raf-1 activation is accompanied by a hyperphosphorylation on serine residues, which often results in a form of Raf-1 that displays a retardation in its electrophoretic mobility on SDS-polyacrylamide gels (14, 15, 18). This retardation of Raf-1 mobility, however, poorly correlates with its activation in some systems (19), and its relationship to Raf-1 activation is presently unclear. Utilizing cell cycle-arrested T lymphocytes, we detected such a form of Raf-1 in cells that had been arrested in

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mitosis by treatment with nocodazole. Fig. 1 shows an anti-Raf-1 immunoblot analysis of lysates from cycling, unsynchronized (U) or nocodazole-treated (N) Jurkat (lanes 1 and 2), J.CaM.1 (lanes 3 and 4), and LSTRA (lanes 5 and 6) cells. Proteins were separated on an 8% polyacrylamide gel, transferred to Immobilon P, and probed with anti-Raf-1 antibodies using the ECL detection system. B, similar to A above except that cells were either untreated (U) or incubated in the presence of phorbol 12,13-dibutyrate (P) before lysis.

Lck is a member of the Src family of non-receptor tyrosine kinases. It is expressed primarily in T cells, where it functions in a Lck-dependent manner. A, detergent-solubilized lysates (2.5 × 10^6 cells/sample) were prepared from unsynchronized (U) or nocodazole-treated (N) Jurkat (lanes 1 and 2), J.CaM.1 (lanes 3 and 4), and LSTRA (lanes 5 and 6) cells. Proteins were separated on an 8% polyacrylamide gel, transferred to Immobilon P, and probed with anti-Raf-1 antibodies using the ECL detection system. B, similar to A above except that cells were either untreated (U) or incubated in the presence of phorbol 12,13-dibutyrate (P) before lysis.

The failure of Raf-1 to activate at mitosis in J.CaM.1 cells clearly implicated Lck in the mitotic activation of Raf-1. To test whether Raf-1 directly interacted with Lck in a mitosis-specific manner, anti-Raf-1 immunoprecipitations were immunoblotted with antibodies to Lck. As can be seen in Fig. 3, Lck was found to associate with Raf-1 in mitotic Jurkat cells (lane 6), but not in unsynchronized, cycling Jurkat cells (lane 5). As expected, no Lck was found associated with Raf-1 in J.CaM.1 cells (lanes 7 and 8). We have recently shown that at mitosis, a form of Lck is generated that migrates with a slower mobility on SDS-polyacrylamide gels (25). We attributed this mitosis-induced mobility shift of Lck to serine phosphorylation by Cdc2 and demonstrated that, in vitro, phosphorylation by Cdc2 resulted in a slowed mobility form of Lck. This mitosis-induced mobility shift of Lck is illustrated in Fig. 3. Cell lysates from unsynchronized Jurkat cells and Jurkat cells arrested during mitosis were immunoblotted with antibodies to Lck. As can be seen in lane 3, mitotic lysates contained an additional, more slowly migrating form of Lck. This mitotic form of Lck comigrated with the Lck found associated with Raf-1 at mitosis (compare lanes 3 and 6). We therefore conclude that Raf-1 was activated in the parental Jurkat cells, Raf-1 in the mutant Lck-deficient J.CaM.1 cells is capable of undergoing a shift in its electrophoretic mobility in response to the treatment of cells with an activator of Raf-1 activity.

To address the issue of whether this decreased mobility form of Raf-1 was indeed an activated form of the kinase, Raf-1 activity was directly assessed by measuring both autophosphorylation activity and phosphorylation of MEK-1, a physiological substrate for Raf-1. Raf-1 was immunoprecipitated from both cycling and mitotic Jurkat and J.CaM.1 cells and subjected to an autophosphorylation reaction in the presence of [γ-^32P]ATP. As demonstrated in Fig. 2A, only Raf-1 from mitotic Jurkat cells displayed detectable autophosphorylation activity, which was indicative of its activation in these cells. Similar results were obtained when inactive (kinase-defective) MEK-1 was added to the assay as an exogenous substrate, as illustrated in Fig. 2B. Anti-Raf-1 immunoblot analysis indicated that Raf-1 was indeed present in anti-Raf-1 immunoprecipitations from J.CaM.1 cells (data not shown). These results directly demonstrated the mitotic activation of Raf-1 in T cells and confirmed our earlier findings that this activation did not occur in the Lck-deficient J.CaM.1 cells.
Lck-dependent Activation of Raf-1

FIG. 3. Raf-1 selectively associates with the mitotic form of Lck. Detergent-solubilized lysates from unsynchronized (U) or nocodazole-treated (N) Jurkat or J.CaM.1 cells were analyzed directly (2.5 × 10^6 cells/sample, lanes 1–4) or immunoprecipitated with anti-Raf-1 antibodies (1 × 10^7 cells/sample, lanes 5–8). Proteins were resolved by SDS-PAGE on an 8% gel, transferred to Immobilon-P membranes, and immunoblotted with anti-Lck antibodies. Detection was by ECL. All eight lanes are from a single gel. The two panels originated when the intact membrane was exposed to two separate pieces of EDP paper to allow for a longer exposure time for the right-hand panel. This was necessary to visualize Lck in the immunoprecipitations. The panels were carefully realigned using the dye front as a reference.

selectively associates with a mitotic form of Lck that is generated as a result of phosphorylation by Cdc2.

The finding that Raf-1 is activated at mitosis in T cells and that this activation is absent in the Lck-deficient cell line J.CaM.1 implicates Lck in the mitotic activation of Raf-1. The finding that Raf-1 associates with Lck suggests that Raf-1 may serve as a substrate for Lck, as has been reported (26). Using anti-phosphotyrosine antibodies to immunoblot anti-Raf-1 immunoprecipitates, we were, however, unable to detect tyrosine phosphorylation of Raf-1 during mitosis (data not shown). Since none of the members of the Raf-1 signaling pathway were overexpressed in our system, it is clearly possible that mitosis-dependent tyrosine phosphorylation of Raf-1 does occur and it was simply below the limits of detection of our assay. Although the role of tyrosine phosphorylation of Raf-1 activation is presently unclear, Jelinek et al. (27) recently reported the phosphotyrosine-dependent activation of Raf-1 in NIH3T3 cells. Nonetheless, our data would strongly suggest that the mitotic activation of Raf-1 in T cells is dependent on Lck.

It is intriguing that Raf-1 selectively associates with a mitotic form of Lck that migrates with a slowed mobility on SDS-polyacrylamide gels. Thompson et al. (26) also reported a differential association of Raf-1 with Lck. In this case, Raf-1 was found only to associate with activated (CD4-associated) and not unactivated Lck. Our findings, along with those of Laird et al. (14) and Lovric et al. (15) implicate both Raf-1 and Lck in the regulation of mitosis. How these two traditional cell surface receptor-regulated signaling molecules participate in the mitotic process is currently unknown, but clearly either has the potential to serve as a substrate for the other. Although we have demonstrated that in T cells Raf-1 is activated at mitosis, we have no evidence that such is the case for Lck. However, at mitosis Lck does become hyperphosphorylated on serine residues, due at least in part to phosphorylation by Cdc2 (25). It is possible that in addition to Cdc2, Raf-1 also contributes to the mitotic serine phosphorylation of Lck. In any case, Raf-1 clearly associates with this hyperphosphorylated, mitotic form of Lck.

It will be important to determine the consequences of this interaction as well as to identify mitotic-specific substrates for both Raf-1 and Lck.

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Activation of T Cell Raf-1 at Mitosis Requires the Protein-tyrosine Kinase Lck
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