Stimulation of the T cell antigen receptor (TCR) triggers a complex series of signaling events that culminate in T cell activation and proliferation. The complex structure of the TCR has hindered efforts to link specific signaling events induced by TCR cross-linkage to downstream activation responses, such as interleukin-2 (IL-2) gene transcription. Previous studies have shown that the polyomavirus-derived oncoprotein, middle T antigen (mT), transforms rodent fibroblasts by interacting with and activating several cytoplasmic signaling proteins (Src kinases, phospholipase C (PLC)-γ1, Shc, and phosphoinositide 3-kinase (PI3-K) implicated in cell growth control. In this study, we demonstrate that expression of mT activates Jurkat T cells, as measured by increases in IL-2 promoter- and NFAT (nuclear factor of activated T cells)-dependent reporter gene transcription. The transcriptional response provoked by mT was blocked by the immunosuppressive drug FK506, a potent inhibitor of TCR-mediated IL-2 gene expression. Mutations that disrupted the binding of mT to Src kinases or PLC-γ1 abrogated the ability of mT to deliver the signals needed for IL-2 promoter activation. In contrast, a mT mutant that failed to bind PI3-K induced a markedly elevated transcriptional response in Jurkat cells, whereas mutation of the Shc binding site in mT had little effect on the transactivating potential of this viral oncoprotein. Additional studies demonstrated that the association of mT with PLC-γ1 was necessary and sufficient to activate both Ca²⁺ and Ras-dependent signaling cascades in Jurkat cells. These results indicate that PLC-γ1 activation plays pivotal and pleiotropic roles in the stimulation of IL-2 gene expression, whereas activation of PI3-K negatively modulates this response in Jurkat T cells.

Engagement of the multi-subunit T cell antigen receptor (TCR) complex triggers a cascade of intracellular signaling events that culminate in the activation and proliferation of T lymphocytes (1, 2). The proximate biochemical response to TCR stimulation is the phosphorylation of several intracellular proteins on tyrosyl residues. A critical target for the TCR-activated protein-tyrosine kinases (PTKs) is the immunoreceptor tyrosine-based activation motif (ITAM), which is present in multiple copies in the ζ and CD3 subunits of the receptor itself (3–6). The current model posits that TCR engagement leads to the sequential activation of members of the Src and ZAP-70/Syk families of PTKs (1, 2). The Src family members, Lck and Fyn, initiate TCR signaling by phosphorylating tyrosine residues located within the ITAMs, which, in turn, serve as docking sites for the tandem SH2 domains of ZAP-70 or Syk. Although interaction with a phospho-ITAM may be sufficient for activation of Syk, ZAP-70 activation requires an additional tyrosine phosphorylation event catalyzed by Lck or Fyn (6–9). The clustered Src family and ZAP-70/Syk PTKs mediate the phosphorylation of multiple intracellular substrates, including the adapter proteins, SLP-76, Cbl, and LAT (1, 2, 10). Tyrosine phosphorylation of these adapter proteins provides additional binding sites for SH2 domain-containing proteins, effectively drawing them into the vicinity of the TCR-associated PTKs. Thus, as is the case for polypeptide growth factor receptors, TCR ligation triggers the assembly of multimolecular signaling complexes in T cells.

Steady progress has been made with respect to the identification of downstream substrates for the PTKs activated in response to TCR occupancy. A critical target for these PTKs is phospholipase C (PLC)-γ1, which itself becomes activated as a consequence of tyrosine phosphorylation (11–13). PLC-γ1 is responsible for the generation of second messengers that activate protein kinase C and mobilize intracellular calcium, both of which are crucial events for the transcriptional activation of the IL-2 and other cytokine genes in antigen-stimulated T cells. Furthermore, PLC-γ1 contains SH2, SH3, and pleckstrin homology domains (14), which suggests that this enzyme may also function as an adapter protein during TCR signaling.

Recent studies suggest that the components of the TCR-linked signaling machinery overlap extensively with those engaged by receptors for polypeptide growth factors. For example, the Ras-MAP kinase cascade, which has been widely implicated in the initiation of cell growth responses, is also activated during ligation of the TCR. Although the mechanism by which the TCR couples to Ras remains uncertain, the recruitment of kinase; NFAT, nuclear factor of activated T cells; PMA, phorbol 12-myristate 13-acetate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; GST, glutathione S-transferase; ITAM, immunoreceptor tyrosine-based activation motif; SH, Src homology; MAP, mitogen-activated protein; MEK, MAP kinase kinase; TBST, Tris-buffered saline with Tween 20.
a complex containing the adapter protein, Grb2, and the Ras-specific guanine nucleotide exchange factor, mSOS, to the plasma membrane may be an important step (15, 16). Another adapter protein, Shc, binds with low affinity to phospho-ITAMs, and it has been proposed that tyrosine phosphorylated Shc supplies a membrane docking site for the Grb2-mSOS complex during TCR stimulation (17). One well documented role of the Ras-MAP kinase pathway is the activation of AP1, which, in addition to its own transactivating functions, is a component of the nuclear factor of activated T cells (NFAT), a pivotal transcription factor for the induction of IL-2 gene expression in response to antigenic stimuli (18). A related signaling pathway induced by TCR ligation, as well as coligation of the TCR with CD28, involves the activation PI3-K (19–20).

Although PI3-K participates in mitogenic signaling through many growth factor receptors, its function with respect to TCR signaling is poorly understood.

The polyomavirus-derived oncprotein, middle T antigen (mT), offers a useful tool for understanding the roles of various signaling molecules in cell growth and transformation (21, 22). Like the TCR, mT is localized to the plasma membrane and lacks intrinsic protein kinase activity. The growth-promoting and oncogenic activities of mT are explained by its ability to nucleate a multimolecular signaling complex at the inner leaflet of the plasma membrane. Membrane-associated mT binds to and activates certain members of the Src kinase family, notably Src and Fyn. These PTKs, in turn, phosphorylate mT on several tyrosine residues, including Tyr250, Tyr315, and Tyr322. Mutagenesis studies indicate that tyrosine phosphorylation of mT generates binding sites for the SH2 domains of Shc, PI3-K, and PLC-γ1, respectively (23, 27). Association with the mT-Src PTK complex results in the tyrosine phosphorylation and functional activation of all three signaling proteins. A Tyr → Phe substitution at residues 250 or 315 severely impairs the transduction pathway induced by TCR ligation, as well as coligation of the TCR with CD28, involving the activation PI3-K (19–20). Although PI3-K participates in mitogenic signaling through many growth factor receptors, its function with respect to TCR signaling is poorly understood.

The strategy for the preparation of expression plasmids encoding wild-type PLC-γ1 (PLC-L) and the kinase-defective PLC-γ1 mutant (PLC-LI) will be described in detail elsewhere.3 The PLC-γ1 coding regions were appended at their 5′ termini with nucleotides encoding two tandem copies of the epitope tag sequence (single-letter amino acid code; DTTRYI) recognized by the AU1 monoclonal antibody (mAb), which was obtained from BabCo (Richmond, CA). All plasmid DNAs were double-doped on cesium chloride gradients before transfection into mammalian cells.

**Transfections**—Unless indicated otherwise, transient transfections were done by electroporation of 1 × 10^6 cells with a total of 30 μg of DNA. Prior to electroporation, the DNA was ethanol-precipitated and resuspended in 50 μl of RPMI 1640 medium containing 10 μM HEPES, pH 7.2. The dissolved DNA was mixed with 250 μl of cell suspension (4 × 10^6 cells/ml) in standard growth medium, and the mixture was incubated for 10 min at room temperature. The samples were transferred to 4-mm gap cuvettes, and electroporation was performed with an Electro Cell Manipulator model 600 system (BTX, San Diego, CA) at settings of 350 V and 960 microfarads. After electroporation, the cells were incubated for an additional 10 min at room temperature, and then diluted into 13 ml of standard growth medium. Two ml of the diluted cell suspension were then aliquoted into each well of a six-well tissue culture plate, and then 5 ml of growth medium were added to each well. The cultures were incubated for 6 h at 37 °C prior to the addition of pharmacologic agents.

The transfected cells were stimulated with 20 ng/ml phorbol myristate acetate (PMA; Sigma), diluted from a concentrated stock solution in dimethyl sulfoxide (Me2SO). The appropriate volumes of drug vehicle (maximum solvent concentration never exceeded 0.05%, v/v) only were added to unstimulated control samples. Where indicated, the cells were pretreated for 15 min with 100 nM wortmannin, added to the cells 10 min prior to stimulation with PMA.

Addition of stimuli or inhibitors, the transfected cells were cultured for an additional 18 h at 37 °C, at which time cell lysates were prepared for reporter gene assays and immunoblot analyses. The cells in each well were pelleted and lysed with 60 μl of 1% Triton X-100 culture lysis reagent (Promega) for 20 min at room temperature. The cell lysates were centrifuged to remove insoluble cellular debris, and 10 μl of the cleared extracts were assayed for luciferase activity with the luciferase assay reagent (Promega) and a Berthold LUMAT LB 9501 luminometer.

**Anti-mT mAb Immunoblotting**—To determine the levels of expression of mT proteins in transiently transfected cells, the cells from one culture tube (24, 25) were harvested and lysed for 10 min on ice in 300 μl of TNE buffer (20 μg/ml Tris-HCl, pH 7.4, 40 μg/ml NaCl, 5 μg/ml EDTA), containing 1% Triton X-100, 30 μg/ml Na2PO4, 50 μg/ml NaF, and a protease inhibitor mixture (10 μg/ml leupeptin, 5 μg/ml pepstatin A, 5 μg/ml aprotinin, and 2 μg phenylmethylsulfonyl fluoride). The cell lysates were cleared by centrifugation, and 30 μl of the extracts was mixed with 10 μl of 4× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (245 μg/ml Tris-HCl, pH 6.8, 40% glycerol, 9.2% SDS, 20% 2-mercaptoethanol, and 0.04% bromphenol blue).
blue). After heating for 5 min at 95 °C, the solubilized proteins were resolved by SDS-PAGE through a 10% gel, and were transferred for 1 h at 150 volts to an Immobilon-P membrane (Millipore, Bedford, MA). The membrane was blocked overnight at room temperature in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.2% Tween 20 (TBST) containing 2% bovine serum albumin (BSA). The solubilized proteins were probed with a 1:10 dilution in TBST plus 0.1% BSA of hybridoma-conditioned culture medium containing the mT-specific mAb, Pab 750 (34). After 1 h, the membrane was washed three times by agitation in TBST (10 min/wash cycle). The membrane was then incubated for 1 h with a 1:10,000 dilution of rabbit anti-mouse immunoglobulin G (IgG) (Pierce) in TBST plus 1% BSA. After three washes in TBST, the blot was incubated for 1 h with horseradish peroxidase-linked protein A (Amersham Pharmacia Biotech) at a 1:10,000 dilution in TBST plus 2% BSA. The blot was then washed as described above, and immunoreactive proteins were revealed with the enhanced chemiluminescence (ECL) detection system (Amerham Pharmacia Biotech).

**MEK Kinase Assay and Immunoblots.—** The MEK kinase assay was performed essentially as described (35) with minor modifications. Wild-type Jurkat cells were transfected with wild-type or mutant mT constructs, together with 10 µg of Myc-tagged MEK (mycMEK)-encoding reporter plasmid, as described above. After electroporation, the cells were diluted into 20 ml of standard growth medium and cultured for 18 h in 100-mm tissue culture dishes. The cells were harvested and lysed in 20 mM Tris-HCl, pH 7.4, 40 mM Na3P2O7, 50 mM NaF, 10 mM EGTA, 1% Triton X-100, 0.25% sodium deoxycholate, 1 mM Na3VO4, 20 µg/ml leupeptin, 20 µg/ml aprotinin, 10 µg/ml pepstatin A, 40 µg/ml microcystin, 600 µM phenylmethylsulfonyl fluoride, 50 mM para-nitrophenol phosphate, 100 mM β-glycerophosphate, pH 7.4, and 100 µM each of phosphoserine, phosphothreonine, and phosphotyrosine. The lysate was cleared by centrifugation, and 100 µl of the lysate were set aside for immunoblotting (see below). The remainder of the lysate was incubated for 30 min on ice with 15 µg of protein G-purified 9E10 mAb, which recognizes a Myc-derived peptide epitope (MEQKLISEEDL) (36). The resulting immune complexes were immunoprecipitated for 30 min at 4 °C on ice with 15 µg of rabbit anti-mouse IgG prebound to 15 µl of packed protein A-Sepharose beads. The immunoprecipitates were washed three times in MEK wash buffer (20 mM Tris-HCl, pH 7.4, 40 mM Na3P2O7, 50 mM NaF, 5 mM MgCl2, 10 mM EGTA, 1% Triton X-100, 0.25% sodium deoxycholate, 1 mM Na3VO4, 20 mM para-nitrophenol phosphate, 100 mM β-glycerophosphate, pH 7.4, and 0.1% SDS), two times in TE wash buffer (50 mM Tris-HCl, pH 7.4, 0.1 mM EGTA, 55 mM β-glycerophosphate, pH 7.4, 5 mM octyl-β-D-glucopyranoside, and 1 mM diethiothreitol), and once in kinase wash buffer (30 mM Tris acetate, pH 7.4, 0.1 mM EGTA, 10 mM magnesium acetate, 1 mM β-glycerophosphate, pH 7.4, 0.5 mM octyl-β-D-glucopyranoside, and 1 mM diethiothreitol). The substrate for the MEK kinase assays was a bacterially expressed and purified, catalytically inactive ERK1 mutant fused to glutathione S-transferase (GST-ERK1 kinase-inactive). The kinase reactions were initiated with 20 µl of reaction mixture (kinase wash buffer containing 10 µM adenosine 5’-triphosphate (ATP), 10 µM of [γ-32P]ATP, and 0.5 µg of GST-ERK1 kinase-inactive). The reactions were incubated for 10 min at 30 °C and terminated with 8 µl of 4X SDS-PAGE sample buffer. The samples were heated for 5 min at 95 °C, the beads were removed by centrifugation, and the solubilized proteins were resolved by SDS-PAGE in a 10% gel. The samples were transferred electrophoretically (50 V for 15 min, followed by 150 V for 1 h) to an Immobilon-P membrane (Millipore), and radiolabeled GST-ERK1-KD was visualized by autoradiography.

For immunoblot analysis, 100 µl of cell extract were mixed with 33 µl of 4X SDS-PAGE sample buffer and heated for 5 min at 95 °C, and the denatured proteins were resolved by SDS-PAGE in a 10% gel. The proteins were transferred to Immobilon-P as described in the preceding paragraph, and were immunoblotted with 9E10 mAb (1 µg/ml) as described above. The blot was then stripped for 10 min at room temperature with 7 M guanidine HCl, 10 mM diethiothreitol solution, and reprobed with anti-mT antibodies.

**Results**

**Activation of IL-2 Promoter-driven Transcription by mT—** Efforts to understand the intracellular signaling pathways engaged by growth factor receptors, such as the platelet-derived growth factor receptor, have been greatly facilitated by the availability of mutant receptors that fail to interact with defined cytoplasmic signaling proteins (37–40). Due to the structural complexity of the TCR, it has not been possible to apply a similar genetic approach to identify the roles of downstream signaling proteins in the induction of specific T cell activation responses though this receptor. However, we noted that the viral oncprotein, mT, contains defined binding sites for a number of intracellular proteins previously identified as components of the signaling machinery engaged by the ligand-stimulated TCR. In initial studies, we examined the possibility that ectopic expression of mT would deliver signals required for the transcriptional activation of the IL-2 gene in Jurkat T cells. Jurkat cells were transiently transfected with a plasmid encoding the wild-type mT protein, together with the pIL2-Luc reporter plasmid, which contains the luciferase coding sequence under the transcriptional control of the IL-2 promoter. Control cells were electroporated with the empty expression vector (pMH-Neo) and the pIL2-Luc reporter plasmid. As reported previously (41), activation of transcription from the IL-2 promoter in Jurkat cells requires two synergistic signals, one of which can be supplied by TCR cross-linkage, and the other by PMA (Fig. 1). Jurkat cells transfected with the mT expression vector displayed a strong increase in IL-2 promoter-mediated transcription, and this response was synergistically increased by addition of either PMA (Fig. 1) or ionomycin (data not shown) as a cotransactivator. These results demonstrate that expression of wild-type mT triggers the full complement of signaling events required for the stimulation of IL-2 gene transcription in Jurkat cells.

**Activation of Mutant T Cell Lines by mT—** To further characterize the mechanism of cellular activation by mT, we took advantage of a previously described panel of Jurkat T cell–derived somatic mutants that lack different components of the TCR-associated signaling machinery. A potential explanation for the activating effects of mT in Jurkat cells is that this protein induces the phosphorylation of ITAMs within the TCR by activating Fyn or Lck. This event might trigger the association of ZAP-70 with the ITAMs, which, in turn, could initiate the signaling cascade normally provoked by TCR ligation. To examine this possibility, we determined whether mT could activate the JRT3 subclone (42), which expresses no TCR complex, and hence no ITAMs, at the cell surface. Transient expression of mT in JRT-3 cells stimulated an increase in IL-2
promoter-driven transcription that was virtually indistinguishable from that observed in the TCR-positive, parental cell line (Fig. 2). These results indicate that the activating effects of mT in Jurkat cells are not simply due to a surreptitious interaction between mT and the CD3 and z subunits of the TCR at the inner leaflet of the plasma membrane.

Jurkat cell-derived somatic mutants that lack expression of either Lck or ZAP-70 exhibit severe defects in TCR signaling function. To determine whether these PTKs were similarly refractory to the activating stimulus provided by mT, transient transfection experiments were done with the Jurkat-derived J.CaM1 and P116 subclones, which are deficient in expression of Lck (43) and ZAP-70 (29), respectively. J.CaM1 cells displayed virtually no increase in IL-2 promoter-driven luciferase expression when cotransfected with mT (Fig. 2A). In contrast, mT expression provoked a significant increase in luciferase activity in P116 cells; however, the magnitude of the transcriptional response evoked by mT was clearly reduced in the ZAP-70-deficient cell line. The defective activation responses of J.CaM1 and P116 cells were not explained by the failure of these cells to express mT protein after transfection with the mT-encoding plasmid (Fig. 2B). Taken together, the results obtained with the mutant Jurkat T cell lines demonstrate that mT activates Jurkat cells in a TCR-independent fashion. Like the TCR, however, mT requires both Lck and ZAP-70 for optimal delivery of the signals required for IL-2 promoter-dependent transcription in these T cells.

Role of mT-associated Proteins in IL-2 Promoter Activation—
Tyrosine phosphorylation of mT by Src kinases creates binding sites for several SH2 domain-containing proteins, including PI3-K, Shc, and PLC-γ1 (21–23). To examine the roles of these signaling proteins in the activating effects of mT in Jurkat T cells, we transiently expressed a series of mT mutants containing the indicated Tyr (Y) → Phe (F) single amino acid substitutions. The cells were stimulated and luciferase activities were determined as described in the Fig. 1 legend. B, cellular extracts from the samples indicated in A were immunoblotted with anti-mT monoclonal antibody. C, partial reconstitution of reporter gene expression in mT(Y322F)-expressing cells by ionomycin plus PMA costimulation. Left panel, Jurkat cells were cotransfected with pIL2-Luc together with the indicated mT expression plasmids. Right panel, Jurkat cells were cotransfected with the pNFAT-Luc reporter and the indicated mT expression plasmids. The transfected cells were stimulated with ionomycin and/or PMA as indicated in the Fig. 1 legend. The lower panels show anti-mT immunoblots of extracts from the transfected cells.

PI3-K, Shc, and PLC-γ1 (21–23). To examine the roles of these signaling proteins in the activating effects of mT in Jurkat T cells, we transiently expressed a series of mT mutants containing Tyr (Y) → Phe (F) substitutions at the binding sites for each of these proteins (Fig. 3A). Previous studies have shown that these substitutions specifically block the interaction of mT with the appropriate target protein and alter the transforming activity of mT in rodent fibroblasts (21–27). As described above, Jurkat cells were cotransfected with the pIL2-Luc reporter plasmid, and luciferase activity was measured after cellular stimulation with PMA or with the drug vehicle only. The mT(Y250F) mutant fails to bind the Shc adapter protein, which couples certain receptors to the Ras signaling pathway (26, 27). Unexpectedly, expression of mT(Y250F) led to a moderate increase in IL-2 promoter-driven luciferase activity in Jurkat cells. An even more striking gain of activity was observed when
Jurkat cells were transfected with the mT(Y315F) mutant, which fails to bind or activate PI3-K (24). Relative to wild-type mT, expression of mT(Y315F) elevated luciferase activity by 4-fold in the absence of PMA, and by 6-fold when the cells were costimulated with PMA. Thus, interaction of mT with either PI3-K or Shc is not required for the stimulation of transcription from the IL-2 promoter. Rather, these results suggest that PI3-K activation actually delivers a net negative signal for IL-2 gene transcription in Jurkat T cells.

In contrast to the activating effects of Tyr → Phe substitutions at the Shc and PI3-K binding sites, a similar substitution at Tyr322 completely abolished the ability of mT to stimulate reporter gene expression in Jurkat cells (Fig. 3A). The failure of mT(Y322F) to activate Jurkat cells was not explained by the level of expression of the mT mutant in the transfected cells (Fig. 3B). These results indicate the association of mT with PLC-γ1 is essential for the activation of the IL-2 promoter in Jurkat cells. To determine whether the mT(Y322F) mutant was globally defective in terms of downstream signal propagation, we examined the ability of this mutant to deliver survival- and growth-promoting signals when expressed in the IL-3-dependent FDC-P1 myelomonocytic cell line. Interestingly, the activity of mT(Y322F) in this response system was virtually indistinguishable from that of wild-type mT, indicating that the PLC-γ1 binding site is dispensable for the mitogenic activity of mT in hematopoietic cells. Thus, loss of the PLC-γ1 binding site in mT selectively interferes with the transduction of signals needed for IL-2 gene transcription in Jurkat T cells.

An obvious explanation for the loss of function induced by the Tyr322 → Phe substitution is that this mT mutant fails to trigger the mobilization of intracellular Ca2+ due to its inability to activate PLC-γ1 in Jurkat cells. An increase in intracellular free Ca2+ is required for the activation of key transcription factors, including NFAT and the octamer-binding protein-associated factor, BOB1, in T cells (41, 44). If the transcriptional defect observed in mT(Y322F)-expressing cells is explained fully by the failure of the mutated mT to deliver a Ca2+-dependent activation signal, then costimulation of the cells with the Ca2+ ionophore, ionomycin, should elevate reporter gene expression to similar levels in wild-type mT and mT(Y322F)-transfected Jurkat cells. However, transcription of the IL-2 promoter-linked luciferase gene remained severely impaired in the mT(Y322F) transfectants stimulated with 2 μM ionomycin (Fig. 3C, left panel), which induces a maximal increase in intracellular Ca2+ in Jurkat cells (data not shown). These results indicate that the induction of an increase in intracellular free Ca2+ is not the only signal for IL-2 gene expression supplied by the interaction of PLC-γ1 with mT in T cells.

To focus more specifically on Ca2+-regulated transcription, we compared the stimulatory effects of wild-type mT and mT(Y322F) on NFAT-dependent luciferase expression using the pNFAT-Luc reporter plasmid. Wild-type mT alone strongly activated NFAT, and this response was synergistically increased by treatment of the transfected cells with ionomycin, but not with PMA (Fig. 3C, right panel). As expected, the mT(Y322F) mutant induced virtually no increase in NFAT-dependent reporter gene expression. However, the basal level of luciferase activity observed in the mT(Y322F)-expressing cells was elevated markedly by stimulation with ionomycin. The level of NFAT activation induced by ionomycin in the presence of mT(Y322F) was significantly higher than that observed in mock-transfected Jurkat cells. In contrast, PMA failed to synergize with either wild-type mT or mT(Y322F) in this assay. Thus, NFAT activation by mT(Y322F) is completely dependent on the Ca2+ signal supplied by ionomycin. Nonetheless, this mutated mT does supply an additional, synergistic signal(s) for NFAT-dependent transcription in the presence of the Ca2+ ionophore.

**Effect of Wortmannin on IL-2 Promoter Activation**—The striking increase in luciferase expression obtained with the mT(Y315F) mutant suggests that PI3-K delivers a negative signal during the assembly of the transactivating complex on the IL-2 promoter. To confirm this possibility, we examined the effect of wortmannin, a pharmacologic inhibitor of PI3-Ks (45), on transcriptional activation induced by wild-type mT. Jurkat cells were cotransfected with wild-type mT plus either the pIL2-Luc or the pNFAT-Luc reporter plasmid. The cells were treated with 100 nM wortmannin for 16 h prior to cell harvest for measurements of luciferase activity. At this drug concentration, wortmannin nearly abolishes intracellular p85-associ- ated PI3-K activity in T cells (46). Wortmannin treatment increased IL-2 promoter and NFAT-dependent luciferase activity in mT-transfected Jurkat cells by approximately 20- and 10-fold, respectively (Fig. 4). These pharmacologic results corroborate those obtained with the mT(Y315F) mutant, and support the conclusion that PI3-K activation negatively modulates a signaling pathway(s) involved in IL-2 gene expression in Jurkat T cells.

**Cellular Activation by mT Single, Double, and Triple Mutants**—The mechanism by which mT activates IL-2 gene transcription in Jurkat cells was explored further by studying a broader panel of mT mutants. Previous studies showed that several single amino acid substitutions in mT (Trp180 → Arg, Cys190 → Ala, and Cys190 → Tyr) disrupt the interaction of mT with Src family PTKs, and consequently abrogate the transforming activity of mT (23). In T cells, FynT is thought to be the principal Src family member that associates with mT (28, 47–49). To determine whether the association of mT with Src kinases is required for the activation of Jurkat cells, transient transfection experiments were performed with the mT(W180R), mT(C150A), and mT(C150Y) mutants (Fig. 5). These mT mutants uniformly failed to deliver the signals needed for IL-2 promoter-dependent transcription, indicating that the interaction of mT with FynT or Lck is required for initiation of the activation program in Jurkat T cells.

A representative set of results obtained with a series of

3 A. Sekulic, A. P. Kennedy, and R. T. Abraham, unpublished observations.
double and triple Tyr → Phe substitution mutants at the binding sites for PLC-γ1 (Tyr²²⁵), Shc (Tyr²³⁰), and PI3-K (Tyr²¹⁵) is also presented in Fig. 5. Because Shc and PI3-K may redundantly activate Ras-dependent signaling pathways (50–52), we were particularly interested in determining the impact of a Tyr²³⁰ → Phe/Tyr²¹⁵ → Phe double mutation on mT-induced IL-2 promoter activation in Jurkat cells. Expression of mT(Y250F/Y315F) in these cells yielded an activation phenotype similar to that observed with the mT(Y315F) single mutant; reporter gene expression was dramatically increased (approximately 10-fold) relative to that observed in cells transfected with wild-type mT. In contrast, all single, double, and triple mutants containing the Tyr²³² → Phe substitution were non-functional in this model system. Thus, the interaction of mT with PLC-γ1 and Src PTKs appears necessary and sufficient for the stimulation of IL-2 promoter-dependent transcriptional activation in Jurkat cells.

Sensitivity of TCR-dependent NFAT Activation to FK506—The immunosuppressive drug, FK506, interferes with TCR-dependent IL-2 production in T cells by inhibiting Ca²⁺-dependent activation of the serine-threonine phosphatase, calcineurin (53). A principal target of calcineurin during T cell activation is the cytoplasmic subunit of NFAT. To determine whether mT-induced NFAT activation was similarly dependent on calcineurin, Jurkat cells were cotransfected with wild-type mT or mT mutants and pNFAT-Luc, and parallel samples were treated with 10 nM FK506 or with drug vehicle only (Fig. 6). The activation of NFAT by mT, in the presence or absence of PMA, was uniformly sensitive to inhibition by treatment of the transfected cells with FK506. Thus, mT interacts with the signaling cascade leading to NFAT activation at a level upstream of calcineurin.

Inhibition of mT-induced IL-2 Promoter Activation by Dominant-negative PLC-γ1—The results presented above demonstrate that the phosphorylated Tyr³²² residue is crucial for the activation of Jurkat T cells by mT. Although Tyr³²² lies within the target binding site for PLC-γ1, it remained possible that this site binds an undefined signaling protein whose association with mT was essential for the activation of the IL-2 promoter. An alternative approach to determine the role of PLC-γ1 in mT-dependent transactivation is to overexpress a catalytically inactive form of PLC-γ1 in Jurkat cells transfected with the wild-type mT expression plasmid. The PLC-γ1(H335Q) mutant contains a single amino acid substitution in the "X" region of the catalytic domain. This mutation has been shown to ablate the lipase activity of PLC-γ1 (54).

The lipase-inactive form of PLC-γ1 (PLC-LI) blocked the transactivating effect of mT on the IL-2 promoter in Jurkat cells (Fig. 7). In contrast, transfection of these cells with a wild-type PLC-γ1 expression plasmid markedly increased the amount of reporter gene expression induced by mT. Similar results were obtained when anti-TCR antibodies, instead of mT, were used as the primary stimulus for IL-2 promoter activation. Interestningly, co-expression of PLC-LI also severely impaired the ability of mT to synergize with ionomycin in the stimulation of IL-2 promoter-driven transcription (Fig. 7). This result indicates that an increase in intracellular Ca²⁺ is not sufficient to replace the activating signals normally supplied by the association of PLC-γ1 with mT in Jurkat cells.

Role of PLC-γ1 in Activation of the MAP Kinase Cascade—In addition to supplying the triggering signal for the increase in intracellular free Ca²⁺ concentration, activation of PLC-γ1 may stimulate various components of the Ras to MAP kinase pathway in response to TCR ligation (40, 55). To determine whether PLC-γ1 carries out similar functions in mT-expressing Jurkat cells, transient transfections were performed with wild-type or the various mutant mTs, together with a reporter

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**Fig. 5.** Activation of IL-2 promoter-dependent transcription by mT single, double, or triple mutants. A, Jurkat cells were transfected with empty expression plasmid (pMH-Neo), or with plasmids encoding wild-type mT or mutated mT proteins bearing the indicated amino acid substitutions (residues indicated by single-letter code). The cells were cotransfected with the pIL2-Luc reporter. Luciferase activities were measured at 12 h post-transfection. The luciferase activity data are presented as fold increase in activity relative to that obtained in the pMH-Neo-transfected control sample. Numbers at the top of each bar indicate the actual fold increase over the pMH-Neo-transfected control. B, anti-mT immunoblot of extracts from the transfected cell populations shown in A.

**Fig. 6.** Sensitivity of mT-induced NFAT activation to FK506. Jurkat cells were cotransfected with the pNFAT-Luc reporter plasmid and the indicated wild-type or mutant mT expression vectors. At 6 h post-transfection, the cells were treated with drug vehicle alone or with 10 nM FK506. PMA was added to the indicated samples after an additional 30 min. The cells were harvested after an additional 6 h, and luciferase activities were measured as described in the Fig. 1 legend.
plasmid encoding a Myc epitope-tagged version of MEK (mMEK), the protein kinase that lies immediately upstream of MAP kinase (Fig. 8). Transient expression of wild-type mT strongly stimulated MEK activity in Jurkat cells, as did expression of the mT(Y250F) and mT(Y315F) mutants, indicating that binding of mT to Shc or PI3-K was not required for the activation of this downstream component of the Ras signaling pathway. In contrast, mT mutants that fail to interact with either a Src family kinase (mT(W180R)) or PLC-γ1 (mT(Y322F)) were clearly defective as MEK activators, indicating that both proteins critical roles in the activation of MEK and, in turn, MAP/Erk kinase in Jurkat T cells.

**DISCUSSION**

Cellular transformation by mT depends on the assembly of a multi-protein signaling complex at the inner leaflet of the plasma membrane. In this report, we demonstrate that the membrane-proximal signals generated as a consequence of mT expression elicit the full complement of nuclear responses needed for transcriptional activation of the IL-2 gene in Jurkat T cells. This finding led to the hypothesis that mT might serve as a surrogate TCR for genetic studies of the signaling machinery that controls IL-2 gene expression and other cellular responses normally associated with TCR ligation. The availability of a detailed map of the regions of mT that mediate association with specific signaling molecules permitted the use of a panel of mT mutants as probes for participation of these interacting proteins in the control of IL-2 gene transcription. The present results point toward a central role of PLC-γ1 in the orchestration of the cytoplasmic signaling events required for IL-2 gene expression in activated T cells.

The mechanism whereby ectopically expressed mT induces the activation of Jurkat cells bears a number of similarities to the activation response triggered by TCR stimulation. Both stimuli activate NFAT- and IL-2 promoter-dependent transcription in a PK506-sensitive fashion, indicating that both the TCR and mT transduce signals through the Ca<sup>2+</sup>-activated phosphatase, calcineurin. The signaling pathways engaged by both the TCR and mT synergize with those stimulated by the phorbol ester, PMA. Moreover, studies performed in Jurkat normal pathway of TCR signaling. Of particular interest was the mT(Y315F) mutant, which fails to associate with the reg-
ulation of the TCR with CD28 provokes a robust increase in PI3-K activity, although the function of this response is poorly understood (58). Nonetheless, based on the numerous parallels between TCR and growth factor receptor signaling, it seemed likely that PI3-K plays a positive role in the induction of the T cell activation program, and, consequently, that the mTY315F) mutant would fail to stimulate IL-2 promoter-dependent transcription. Instead, we found that disruption of the PI3-K binding site in mT led to a $4-6$-fold increase in the stimulation of both NFAT and IL-2 promoter-dependent transcription in Jurkat cells. Treatment of wild-type mT-expressing cells with the PI3-K inhibitor, wortmannin, provoked even more dramatic increases in reporter gene expression. Wortmannin treatment also enhanced the transcription of these reporter genes in untransfected Jurkat cells stimulated with anti-TCR antibodies plus PMA. Hence, it appears that PI3-K activation transmits a down-modulatory signal for the activation of NFAT and possibly other IL-2 promoter-linked transcription factors in Jurkat cells. The enhanced activity of the mTY315F) mutant in the Jurkat T cell activation model contrasted sharply with the dramatically impaired growth-promoting activity of this mT mutant in the FDC-P1 myelomonocytic progenitor cell line. 

While these studies were in progress, a report by Reif et al. (59) supplied more direct genetic evidence supporting a negative regulatory role for PI3-K activity during TCR signal transduction. These investigators transfected Jurkat cells with a constitutively active form of PI3-K, and observed a significant repression of the NFAT-dependent transcriptional response induced by anti-TCR antibody stimulation. Interestingly, two prominent targets of PI3-K, the protein serine-threonine kinase, Akt, and the small GTPase, Rac, were ruled out as downstream transducers of the negative signal delivered by PI3-K (59). Thus, the mechanism of negative signaling though PI3-K remains an important area for further investigation. The potential immunologic relevance of these findings is supported by the fact that CTLA4, an important inhibitory coreceptor on T cells, associates with PI3-K (58, 60). Although the outcome (positive or negative) of PI3-K activation will likely vary with the context in which the T cells are activated and the type of response under investigation, one function may be to down-regulate the transmission of stimulatory signals for the expression of IL-2 and other cytokine genes.

Like PI3-K, the adapter protein, Shc, participates in signaling pathways that promote cell survival and growth in cells stimulated with polypeptide growth factors. In cytokine-stimulated hematopoietic cells, Shc undergoes rapid phosphorylation on tyrosine residues and subsequently associates with the Ras-activating Grb2-SOS complex (61, 62). The notion that Shc positively regulates cell growth via Ras and/or other molecular interactions (62-67) is again supported by findings that the mTY250F) mutant is transformation-defective in rodent fibroblasts and non-mitogenic in hematopoietic cells (69). A recent report provided evidence that these growth-promoting activities of Shc are due to the mT-induced phosphorylation of Tyr299 and Tyr410 in Shc, which drives the recruitment of Grb2 into the mT signaling complex (69). Thus, the mTY250F) mutant should be defective in terms of coupling to the Ras-MAP kinase cascade. In the present study, we again obtained a paradoxical result when we examined the ability of mTY250F) to support an NFAT- or IL-2 promoter-dependent transcriptional response in Jurkat T cells. The disruption of the Shc binding site moderately increased the capacity of mT to stimulate the transcription of an IL-2 promoter-linked reporter gene in transiently transfected Jurkat cells. These results indicate that the formation of the Shc-Grb2-Sos complex is not essential for transcriptional activation of the IL-2 gene by mT, and, by inference, TCR ligation in these cells.

The impact of mutations in the binding sites for PI3-K and Shc on the activation of Jurkat cells by mT contrasts sharply with the drastic loss of function resulting from mutation of the binding site for PLC-γ1. Once again, this outcome would not have been anticipated based on the behavior of the mTY232F) mutant in fibroblast transformation assays. In culture medium containing 10% serum, mTY232F) transformed BALB/c3T3 fibroblasts as efficiently as wild-type mT. A transformation defect for mTY232F) only became apparent when focus formation was examined under low (3%) serum conditions (25). Similarly, we have found that mTY232F) is virtually indistinguishable from wild-type mT in terms of supporting the survival and growth of factor-dependent FDC-P1 cells cultured in optimal amounts of serum. Thus, whereas the activation of PLC-γ1 is, under normal conditions, dispensable for the generation of pro-mitogenic signals by mT, the interaction of mT with PLC-γ1 is absolutely essential for the induction of IL-2 gene transcription in Jurkat cells.

The studies performed with mT double and triple mutants support the conclusion that activation of PLC-γ1 is not only necessary, but may also be sufficient to activate the IL-2 promoter in Jurkat cells. In particular, the dramatic increase in reporter gene expression induced by the mTY250F315F) double mutant indicated that interaction of mT with a Src kinase and PLC-γ1 initiated the complete set of signaling events required for IL-2 promoter activation in these cells. Of course, our experiments do not rule out the possibility that other known (protein phosphatase 2A, 14-3-3 proteins; Ref. 22) or yet to be described mT-interacting proteins contribute to the observed transactivation response. However, a central role for PLC-γ1 in the activation process is underscored by the observation that coexpression of a lipase-inactive form of PLC-γ1 completely suppressed the activation of the IL-2 promoter by wild-type mT in Jurkat cells.

The multi-domain structure of PLC-γ1 raises the possibility that this protein functions as both an adapter and a lipid-metabolizing enzyme during T cell activation. The notion that PLC-γ1 carries out additional signaling functions is supported by our observations that ionomycin stimulation fails to reverse the defect in IL-2 promoter-dependent transcription caused by mutation of the PLC-γ1 binding site in mT. The nature of the additional signal(s) delivered by PLC-γ1 in T cells is not fully understood; however, one candidate is activation of the MAP/Erk kinases. In addition to intracellular Ca$^{2+}$ mobilization, PLC-γ1 activation triggers the production of the protein kinase C-activating second messenger, diacylglycerol. In T cells, protein kinase C may stimulate MAP/Erk kinases via activation of Ras, Raf-1, or MEK (55, 70-73). Whatever the actual mechanism, our results reinforce the possibility that, in addition to its role in the regulation of intracellular free Ca$^{2+}$, PLC-γ1 may serve as an upstream activator of the Ras to MAP kinase cascade in response to TCR engagement.

Previous studies have shown that the signaling machinery engaged by the TCR resembles in many respects that coupled to receptors for polypeptide growth factors. Given the complex structure of the TCR, the assignment of specific signaling pathways to the generation of nominal downstream responses remains a highly challenging undertakings. The use of mT mutants as molecular probes in Jurkat T cells allowed us to uncover some striking differences between the signaling requirements for T cell activation, as measured by IL-2 gene expression, and those for cell survival, growth, and transformation. Our results highlight the pleiotropic roles of PLC-γ1 as
a transducer of TCR-dependent activating signals in T cells. On the other hand, PI3-K activation may function to dampen TCR-mediated cytokine production in these cells. Further studies of mT may offer additional insights into the signaling pathways leading to both T cell activation and anergy.

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