

Quantitative and Qualitative Signals Determine T-Cell Cycle Entry and Progression¹

Jaime F. Modiano,^{*2} Jocelyne Mayor,^{*} Carrie Ball,^{*} Carol G. Chitko-McKown,^{*3} Naoki Sakata,[†] Joanne Domenico-Hahn,[†] Joseph J. Lucas,[†] and Erwin W. Gelfand[†]

^{*}Department of Pathobiology, Texas A and M University, College Station, Texas; and [†]Division of Basic Sciences, Department of Pediatrics, National Jewish Medical and Research Center, Denver, Colorado

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Cell growth and proliferation as well as cell cycle arrest and apoptosis all play integral roles in the cellular immune response. The signals that lead to cytokine production by antigen- or mitogen-stimulated T cells have been studied in detail. However, it is not fully understood how these signals promote cell cycle entry and progression to DNA synthesis in T lymphocytes, especially in primary cells. We used a model distinguishing between competence and progression phases to examine quantitative and qualitative differences in signal transduction that resulted in cell cycle entry and G1 phase arrest or led to DNA synthesis in human T cells. Resting peripheral blood T cells were rendered competent by stimulation with submitogenic concentrations of phytohemagglutinin (PHA) or they were stimulated to proliferate using mitogenic concentrations of PHA. The competent state (that is, the capacity to proliferate in response to exogenous IL-2) was characterized by calcium mobilization, a protein kinase C-dependent internalization of CD3, increased mitogen-activated protein kinase (MAPK) activity, transient translocation of AP-1 transcription factors to the nucleus, expression of immediate early genes, activation of G1-phase cyclin-dependent kinases, and increased CD25 (IL-2R α) expression. However, all of these events were of lesser magnitude in T cells rendered competent than in T cells stimulated to proliferate. Furthermore, the mitogenic stimulus induced a different pattern of MAPK activation and sustained translocation of AP-1 to the nucleus with concomitant IL-2 production. The data indicate that quantitative and qualitative differences in early signaling events

distinguish the acquisition of the competent state or the induction of cytokine production with a commitment to T-cell proliferation. © 1999 Academic Press

Key Words: human; T lymphocytes; signal transduction; cellular activation.

INTRODUCTION

Cell cycle control, including cell growth and proliferation as well as cell cycle arrest and apoptosis, plays an integral role in the cellular immune response (1). Normal human peripheral blood T cells provide a population of cells in the G0 phase that can be manipulated to separate the signals that promote cell cycle entry from those that induce cell cycle progression, DNA synthesis, and proliferation (2–4). The signals that lead to cytokine production by antigen- or mitogen-stimulated T cells have been studied in detail (5, 6). It is known that the binding of antigen or mitogen to the T-cell receptor complex triggers a series of events that give rise to a number of interactive, nonlinear biochemical cascades (5, 6). Some of these events include receptor multimerization, activation and colocalization of protein tyrosine kinase (PTK) holoenzymes near their substrates, activation of the phosphoinositide second-messenger pathways, activation of the Ras and mitogen-activated protein kinase (MAPK) pathways, and, ultimately, alterations in the expression of genes that control cell growth and immune effector cell function. However, it is not fully understood how these signals promote cell cycle entry or how they interact to control cell cycle progression. The signaling events that follow T-cell activation can be divided into two stages: (1) induction of competence, which is defined as the acquisition by resting cells of the ability to respond to a second, exogenous growth factor (such as a lymphokine); and (2) progression to DNA synthesis. In the absence of a progression factor, competent cells become arrested at a point in the G1 phase that precedes the restriction point (R). The R point is defined as the stage

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² To whom correspondence should be addressed at present address: Division of Cancer Causation and Prevention, AMC Cancer Research Center, 1600 Pierce Street, Denver, CO 80214. Fax: 303-239-3560. E-mail: modianoj@amc.org.

³ Current address: Roman L. Hruska U.S. Meat Animal Research Center, P.O. Box 166, Clay Center, NE 68933.



at which cells are committed to undergo DNA synthesis in the absence of further stimulation by growth factors or synthesis of new proteins (4, 7).

Our aims were to assess quantitative and qualitative differences in signal transduction resulting in alternative outcomes of cell cycle entry and G1-phase arrest or progression to the S phase and proliferation. The data suggest that some of the biochemical events that occur following T-cell activation are directly proportional to the concentration of ligand (and presumably to the density of ligated receptors); however, the induction of other events appears to occur in an "all or none" fashion, and only when an activating threshold is surpassed.

MATERIALS AND METHODS

Chemicals and reagents. Tissue culture materials were obtained from Nalge Nunc (Naperville, IL); chemicals were obtained from Sigma (St. Louis, MO) unless otherwise specified. Phytohemagglutinin (PHA-P) was dissolved in sterile water; 12-*O*-tetradecanoylphorbol 13-acetate (TPA), phorbol 12,13-dibutyrate (PDB), ionomycin (Calbiochem, La Jolla, CA), herbimycin A (LC Laboratories, Woburn, MA), staurosporine (Calbiochem), calphostin-C (LC Laboratories), and wortmannin were dissolved in dimethyl sulfoxide and diluted at least 1000-fold in the cell cultures. Ha1004 (*N*-(2-guanadinoethyl)-5-isoquinolinesulfonamide hydrochloride) was obtained from Seikagaku America (Rockville, MD) and reconstituted to a concentration of 10 mM per the manufacturer's recommendation. The addition of 0.1% sterile water or dimethyl sulfoxide to T-cell cultures as controls did not affect any of the parameters measured. Human recombinant interleukin-2 (IL-2) was obtained from Hoffman-La Roche, Inc. (Nutley, NJ) through the Biologic Response Modifiers Program, Division of Cancer Treatment, National Cancer Institute (Frederick, MD).

Cell cultures. Peripheral blood T cells were purified from plateletpheresis residues by Ficoll-Hypaque (1.077 g/ml) density gradient centrifugation followed by depletion of adherent cells from the peripheral blood mononuclear cells and E-rosetting on neuraminidase-treated sheep erythrocytes as described (8, 9). The cellular population obtained by this method was routinely $\geq 96\%$ CD3⁺, $\leq 5\%$ CD25⁺ and DR⁺, $\leq 2\%$ CD16⁺, and $\leq 1\%$ CD14⁺ or CD20⁺, 98% viable, and did not proliferate in response to soluble anti-CD3 antibody. The proliferative response of T cells to mitogens was determined by the incorporation of [³H]thymidine (NEN Life Sciences, Boston, MA) into DNA 48 h after the onset of culture using a liquid scintillation counter (Beckman, Fullerton, CA). The data obtained in counts per minute (cpm) were normalized to disintegrations per minute

(dpm) based on the counting efficiency of calibration standards in the instrument.

Induction of competence. Peripheral blood T cells were rested overnight in RPMI 1640 culture medium (Gibco, Grand Island, NY) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 2 mM L-glutamine, 2 mM sodium pyruvate, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), heretofore referred to as complete medium. T cells were rendered competent by stimulation with a submitogenic concentration of phytohemagglutinin (PHA, 0.5 μ g/ml) for 1 h followed by extensive washing, or by a brief (20 min) stimulation by PDB (10 nM) and ionomycin (500 nM), also followed by extensive washing as described (10). In each experiment, an equal number of T cells as were rendered competent were allowed to remain unstimulated or were stimulated to proliferate by the addition of mitogenic concentrations of PHA (10 μ g/ml). To confirm the attainment of the competent state and the capacity of the competent T cells to respond to a progression signal, proliferation was examined in cultures with or without IL-2 (25 nM).

IL-2 production. IL-2 production by cultured T cells was examined using a CTLL-2 bioassay as described (11). The CTLL-2 clone used for these experiments did not proliferate in response to PHA-P, PDB or ionomycin alone or in combination, or IL-4.

Flow cytometry. DNA content and the expression of surface CD3 and CD25 (IL-2R α) in T cells were examined by flow cytometry using an Epics Profile flow cytometer (Coulter Corp., Hialeah, FL) or a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA) as described (11, 12). Briefly, for DNA content analysis, cultured T cells were fixed in 70% ethanol after 40 h, incubated with RNase A (25 μ g/ml, Boehringer Mannheim Biochemicals, Indianapolis, IN) in PBS for 30 min at 37°C, and stained with propidium iodide (25 μ g/ml, Sigma). CD3 expression and CD25 expression were quantified by direct immunofluorescence using an anti-CD3 monoclonal antibody (UCHT1, Dako, Carpinteria, CA) and an anti-CD25 antibody (Becton Dickinson) conjugated to phycoerythrin, respectively. Comparable results were obtained using indirect immunofluorescence with OKT3 antibody (as hybridoma supernatant) and anti-Tac antibody (a gift of Dr. T. Waldmann, Bethesda, MD) followed by a goat anti-mouse Ig antibody (Sigma) directly conjugated to fluorescein. Isotype controls for phycoerythrin-conjugated antibodies were obtained from Becton Dickinson; isotype controls for unconjugated antibodies were obtained from Sigma.

Intracellular calcium mobilization. Fluxes in intracellular Ca²⁺ were assessed by fluorimetry as described

(13). Briefly, cells were incubated with Indo-1 acetoxyethyl ester (1.5 μ M, Calbiochem) in serum-free Hanks' balanced salt solution for 1 h. At the end of the incubation period, the cells were washed and dye uptake was confirmed by evaluating changes in the emission of Ca^{2+} in cells stimulated by ionomycin. Cells were prestabilized in the fluorimeter chamber at 37°C for 3 min, after which time PHA was added directly to the cuvette at the indicated concentrations. Excitation was brought about at a wavelength of 331 nm, and emission was read at 410 nm. The concentration of Ca^{2+} was calculated by the formula $[\text{Ca}_i^{2+}](\text{nM}) = K_d(F - F_{\text{min}})/(F_{\text{max}} - F)$, where the K_d for the Ca^{2+} -Indo-1 complex was 250 nM, and F , F_{min} , and F_{max} represented experimental fluorescence output or fluorescence output obtained after lysis of the cells with digitonin in the presence or absence of 2.5 mM EDTA, respectively.

Immunoblotting. Immunoblotting was performed as described previously (11). Briefly, cultured T cells were lysed in a buffer containing 300 mM sodium chloride, 50 mM Tris, pH 7.6, 0.5% Triton X-100, 1 mM *N*-ethylmaleimide, 30 nM aprotinin, and 500 nM leupeptin. Insoluble material and nuclei were removed by centrifugation. Cellular proteins were separated by SDS-PAGE in 7–12% mini-gels and transferred to nitrocellulose membranes (Hybond, Amersham, Arlington Heights, IL). An antibody against phosphorylated p44/42 MAPK (New England Biolabs, Beverly, MA) was used to assess activation of the Erk1/2 MAPKs. The blots were reprobbed with an antibody that recognizes both phosphorylated and unphosphorylated Erk1/2 to ensure equivalent loading.

MAPK activity assays. MAPK activity was evaluated *in vitro* as described previously (14). Protein concentrations in whole cell lysates were determined using the bicinchoninic acid method (Pierce, Rockford, IL). Duplicate samples of whole cell lysates from unstimulated or stimulated T cells normalized to equivalent protein content were incubated at 30°C in a buffer containing 10 mM β -glycerophosphate, 50 μ M Na_3VO_4 , 10 mM MgCl_2 , 0.5 mM EGTA, 25 μ g/ml IP-20, 100 μ M ATP, 0.125 μ Ci [γ - ^{32}P]ATP (sp act 6000 Ci/mmol), and 0.33 μ g/ml myelin basic protein (MBP) as a substrate. After 15 min the reactions were stopped by adding EDTA to a concentration of 50 mM. The reaction mixtures were spotted onto 2-cm² Whatman P81 filters; the filters were washed four times in 75 mM phosphoric acid and dried, and radioactivity was quantified by liquid scintillation counting. Identical reactions lacking MBP were used as controls for phosphorylation of endogenous substrates; the presence of EGTA and IP-20 (Sigma) effectively inhibit calcium-dependent protein kinases (protein kinase C (PKC), calcium/calmodulin-dependent kinase) and cAMP-dependent kinases in this assay.

N-terminal Jun kinase assays. The activity of the N-terminal Jun kinases (Jnk) was examined by phosphorylation of a recombinant GST-Jun substrate in a solid-phase assay as described (15). Briefly, whole cell extracts were incubated at 4°C with a (GST)-c-Jun (1-223) fusion protein bound to agarose beads to immobilize Jnk proteins. The beads were washed and kinase reactions were performed for 0–60 min at 37°C. The reactions were stopped by boiling the samples in SDS sample buffer, and the proteins were separated by SDS-PAGE. Phosphorylated proteins were visualized by autoradiography.

Electromobility shift assays. For electromobility shift assays (EMSA), cells were washed in a buffer containing 10 mM Hepes, pH 7.8, 15 mM potassium chloride, 2 mM magnesium chloride, 1 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF, and lysed in the same buffer with 0.2% NP-40. The nuclei were separated by centrifugation and resuspended in a solution of 50 mM Hepes, pH 7.8, 50 mM potassium chloride, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, aprotinin, leupeptin, 10% (v/v) glycerol, and 0.3 M ammonium sulfate. Double-stranded oligonucleotides containing a consensus AP-1-binding sequence (cgcttgaTGAGTCagccggaa) were end labeled with [γ - ^{32}P]ATP using T4 polynucleotide kinase to generate probes. Binding reactions were performed in a buffer of 10 mM Tris, pH 7.5, 50 mM sodium chloride, 0.5 mM EDTA, 5% glycerol, and poly[d(I-C)] as a carrier. A 40-fold excess of unlabeled oligonucleotides was used for competition to determine specificity. Gel shifts were analyzed by separation in 6% TBE polyacrylamide gels.

Northern blotting. Cytosolic RNA was isolated from T cells as described (11). Ten micrograms of RNA was separated electrophoretically in 1% agarose, 5% formaldehyde denaturing gels and transferred to nylon 66 membranes (Gene Screen Plus, Du Pont-NEN, Boston, MA) by capillary blotting. Steady state levels of mRNA expression were assessed using human cDNAs for *c-jun*, *c-fos*, *c-myc*, IL-2, and IL-2R α . A cDNA encoding the human β_2 -microglobulin gene was used as a loading control. The cDNA probes were labeled by random primer extension and hybridized to the immobilized RNA as described (11). Autoradiography was performed at -80°C using a single amplifying screen and Reflection film (Dupont-NEN).

Cyclin-dependent kinase assays. The kinase activities of cyclin-dependent kinase 2 (Cdk2), Cdk4, and Cdk6 were determined as described (10, 16). Briefly, active cyclin-dependent kinase complexes were immunoprecipitated from cultured T cells, and immune complex kinase assays were performed using [γ - ^{32}P]ATP and p56/Rb (a truncated recombinant Rb protein) as a substrate. The reactions were terminated by addition of SDS sample buffer and separated by SDS-PAGE.

Incorporation of ^{32}P into the p56/Rb substrate was quantified by liquid scintigraphy after the phosphorylated bands were excised from the gels. One unit per minute of CDK-associated kinase activity was defined as the incorporation of 1 fmol of phosphate per minute into the substrate.

RESULTS

Induction of Competence by Stimulation of Resting T Cells with Submitogenic Concentrations of PHA

We analyzed the cellular DNA content, the incorporation of [^3H]thymidine into DNA, and the upregulation of CD25 expression to evaluate whether a submitogenic stimulus with PHA was sufficient to induce cell cycle entry and the competent state in T cells (defined by cytokine responsiveness). Figure 1 shows that >97% of resting, unstimulated T cells contained a G0/G1 DNA content and did not express significant levels of surface CD25 (IL-2R α). These cells exhibited negligible spontaneous proliferation after 40 h in culture in the absence of mitogenic stimulation (Fig. 1A). Addition of IL-2 (25 nM) did not induce proliferation of resting T cells and stimulated only a marginal increase in surface CD25 expression (Fig. 1B). PHA used at a submitogenic concentration (0.5 $\mu\text{g}/\text{ml}$) also did not induce proliferation of resting T cells, but in contrast to IL-2 alone, it stimulated the increased expression of surface CD25 (Fig. 1C). The increased expression of CD25 by the competent T cells was functionally significant, as the combination of PHA at the submitogenic dose with IL-2 induced progression of approximately 40% of the T cells to the S and G2/M phases of the cell cycle after 40 h and uniformly increased expression of surface CD25 (Fig. 1D). For comparison, PHA used at a mitogenic dose (10 $\mu\text{g}/\text{ml}$) induced progression of ~20% of the T cells to the S and G2/M phases of the cell cycle with similar levels of CD25 expression (Fig. 1E). The G1-phase arrest of the competent T cells (Fig. 1C) was at least partly due to the inability of the submitogenic stimulus to promote IL-2 production by these cells (<2 units/ml of IL-2 compared to >50 units/ml in T cells stimulated by PHA at a concentration of 10 $\mu\text{g}/\text{ml}$).

To determine the signaling components required to achieve the competent (cytokine-responsive) state, we examined how antagonists of PTKs or PKC affected CD25 expression in stimulated T cells. Figure 1 and Table 1 show that $\leq 3\%$ of resting, unstimulated T cells expressed surface CD25, and these cells did not proliferate in response to IL-2. Induction of competence increased the proportion of CD25 $^+$ T cells to 12–15% by 24 h, and these cells proliferated when stimulated by IL-2. Table 1 shows that the expression of surface CD25 by the competent T cells was markedly reduced

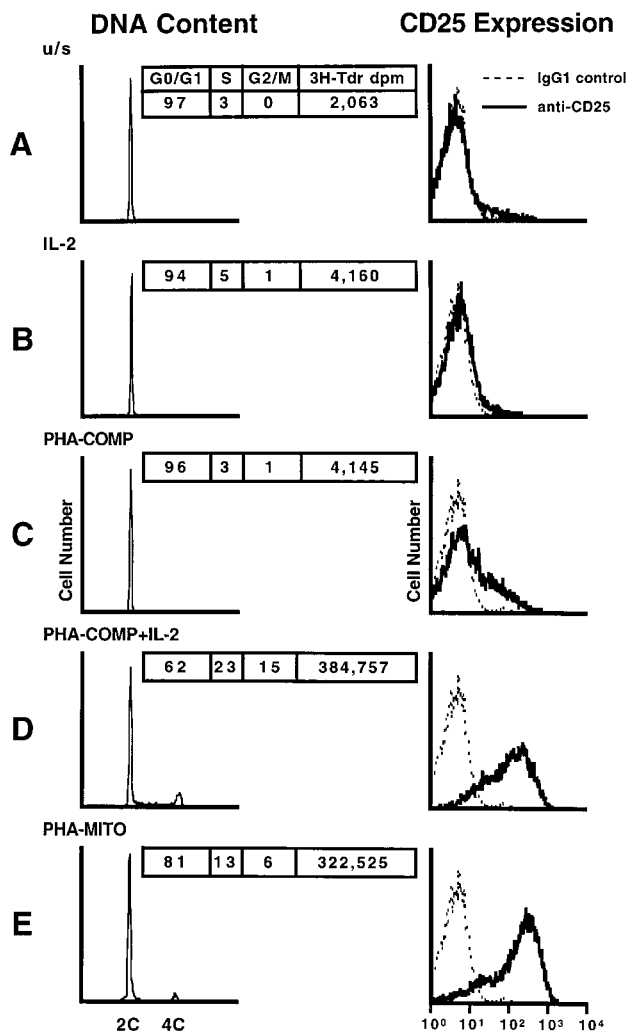


FIG. 1. Induction of competence in T cells with submitogenic doses of PHA. Resting peripheral blood T cells were purified and cultured as described under Materials and Methods for 40 h. Cells remained unstimulated (A) or were stimulated with 25 nM IL-2 (B) 0.5 $\mu\text{g}/\text{ml}$ PHA (C), 0.5 $\mu\text{g}/\text{ml}$ PHA + 25 nM IL-2 (D), or PHA at a mitogenic dose (10 $\mu\text{g}/\text{ml}$) (E). DNA content, shown on the left, was measured by flow cytometry in ethanol-permeabilized cells stained with propidium iodide. Diploid (2C) and tetraploid (4C) DNA contents, the percentages of cells in the G0/G1, S, and G2/M phases of the cell cycle, and the rate of DNA synthesis determined by incorporation of [^3H]thymidine to DNA ($^3\text{H-Tdr}$ dpm) are indicated. CD25 expression, shown on the right, was measured by direct flow cytometry with an anti-CD25 antibody directly conjugated to phycoerythrin (solid lines). The dashed lines represent background fluorescence using an irrelevant IgG1 antibody.

by preincubation with the PTK inhibitor herbimycin A (3 μM) or the PKC inhibitor staurosporine (10 nM). Consistent with this decrease in surface CD25 expression, herbimycin A and staurosporine inhibited the proliferation of competent T cells stimulated by IL-2 (Table 1). Moreover, the increase in CD25 expression seen in competent T cells was not inhibited by the protein kinase A inhibitor Ha1004 (11).

TABLE 1
CD25 (IL-2R α) Expression by Competent Human T Cells^a

	% CD25 ⁺ cells	Proliferation (dpm)	
		-IL-2	+IL-2
Unstimulated	3	1410	2076
PHA-COMP	12	876	96456
PHA-COMP + herbimycin A	4	627	780
PHA-COMP + staurosporine	4	1058	1598

^a Peripheral blood T cells remained unstimulated or were rendered competent (COMP) by a submitogenic stimulation with PHA (0.5 μ g/ml for 1 h followed by washing) after preincubation with or without herbimycin A (3 μ M) or staurosporine (10 nM) for 30 min. CD25 expression was measured in cells cultured for 24 h by flow cytometry using an anti-CD25 antibody directly conjugated to phycoerythrin. Proliferation was measured in cells cultured for 48 h by incorporation of [³H]thymidine to DNA.

Induction of T-Cell Competence Results in Activation of the Inositol Phospholipid Second Messenger Systems

The observed effects of T-cell competence induction on the expression of IL-2R α suggested stimulus-induced activation of PTKs and second messengers resulting from phospholipid hydrolysis. We compared the temporal progression of signal transmission in cells that were stimulated to acquire a competent state with suboptimal concentrations of PHA and in cells that received mitogenic concentrations of PHA. The competence-inducing signal was sufficient to stimulate mobilization of intracellular ionized calcium by normal T cells (Fig. 2), although this increase in intracellular calcium concentration was smaller in magnitude than that seen using mitogenic concentrations of PHA (Fig. 2). Both of these stimuli induced increases in intracellular calcium concentrations that were similar to those seen in anti-CD3-stimulated T cells (17, 18), and at both doses used, the PHA-stimulated calcium fluxes occurred more slowly and were smaller than those observed in T cells stimulated by the calcium ionophore ionomycin (500 nM, Fig. 2).

PHA has been shown to induce a PKC-dependent downmodulation of the CD3-T-cell receptor complex from the T-cell surface (11); therefore, we used the surface density of CD3 in T cells as an indirect measure of PKC activity. Unstimulated T cells expressed abundant CD3, which was uniformly distributed throughout the cell population (mean channel fluorescence, 87). Table 2 shows that after addition of PHA to the T cells, the mean channel fluorescence associated with CD3 expression decreased by $\geq 70\%$ within 30 min. The loss of surface CD3 in the competent T cells (43%) was less than that seen in cells stimulated by mitogenic concentrations of PHA (71% CD3 downmodulation) or cells stimulated by TPA (a PKC agonist; 61% CD3

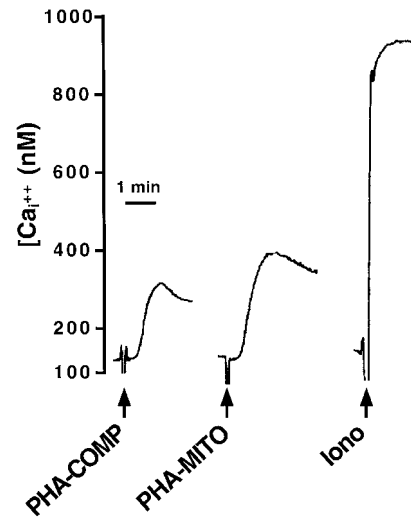


FIG. 2. Cytosolic calcium mobilization by competence-inducing signals in T cells. T cells were loaded with the Ca²⁺-selective fluorescent dye Indo-1 for 1 h, equilibrated in the fluorimeter chamber at 37°C, and stimulated by submitogenic concentrations of PHA (PHA-COMP) or mitogenic concentrations of PHA (PHA-MITO) at the indicated time points. Stimulation by ionomycin (Iono) was used as a positive control.

downmodulation). CD3 modulation in the PHA-stimulated competent cells was substantially inhibited by herbimycin A (16% CD3 downmodulation), staurosporine (no detectable CD3 downmodulation), or calphostin C (another PKC antagonist; 6% CD3 downmodulation). In contrast, the phosphatidylinositol-3-kinase (PI3K) antagonist wortmannin reduced CD3 modulation in response to PHA only partially (33%). Predictably, staurosporine and calphostin C prevented CD3 modulation in TPA-stimulated T cells (13 and 28% CD3

TABLE 2
Modulation of Surface CD3 by Mitogen-Stimulated Human T Cells^a

	MCF (% CD3 downmodulation)			
	Unstimulated	PHA-COMP	PHA-MITO	TPA
No inhibitor	87.0 (0)	49.6 (43)	25.2 (71)	34.0 (61)
Herbimycin A	96.6 (0)	73.1 (16)	94.0 (0)	33.0 (67)
Staurosporine	108.0 (0)	113.1 (0)	77.4 (11)	75.7 (13)
Calphostin C	101.0 (0)	81.8 (6)	80.9 (7)	62.6 (28)
Wortmannin	102.7 (0)	58.3 (33)	53.9 (38)	31.3 (64)

^a Peripheral blood T cells remained unstimulated or were stimulated using a submitogenic dose of PHA (0.5 μ g/ml, PHA-COMP), a mitogenic dose of PHA (10 μ g/ml, PHA-MITO), or TPA (10 nM) for 1 h after preincubation with or without herbimycin A (3 μ M), staurosporine (10 nM), calphostin C (50 nM), or wortmannin (100 nM) for 30 min. CD3 expression was measured by flow cytometry using an anti-CD3 antibody directly conjugated to phycoerythrin. The data represent the mean channel fluorescence (MCF) of unimodal CD3⁺ cell peaks. The percentage of CD3 complex downmodulation is shown parenthetically.

downmodulation, respectively), but herbimycin A and wortmannin did not (67 and 64% CD3 downmodulation, respectively; Table 2).

MAPK Activity in Competent T Cells

The specificity imparted to the downstream effectors of the T-cell activation cascades may result from the magnitude and duration of the signals transmitted through the antigen receptor. Various members of the MAPK family of proline-directed protein kinases are important signaling molecules that directly activate latent transcription factors in stimulated T cells (19, 20). Ample evidence suggests that the extracellular-regulated kinase 2 (Erk2 or p42 MAPK), but also Erk1 (p44 MAPK), Jnk, and p38 MAPK, participate in T cell activation (14, 20–24). We examined the induction of MAPK activity, and that which could be attributed to Erk1, Erk2, and Jnk, in unstimulated T cells, in T cells rendered competent, in T cells stimulated with mitogenic concentrations of PHA, and in T cells stimulated by phorbol esters (known MAPK activators). Induction of T-cell competence stimulated an approximately 3-fold increase in MAPK activity within 10 min, as determined by MBP phosphorylation in whole cell lysates (Fig. 3A), but this represented only approximately 70% of the activity seen in T cells stimulated with mitogenic concentrations of PHA and only 35% of that seen in T cells stimulated with phorbol ester (Fig. 3A). The MAPK activity detectable in competent T cells was unlikely to be due to activation of Erk1 or Erk2, as the phosphorylation state of these proteins was unchanged in unstimulated and competent T cells (Fig. 3B). In contrast, a 10-min stimulation by PHA at the mitogenic dose (10 μ g/ml) or by phorbol ester-induced phosphorylation of Erk2 but not Erk1 (Fig. 3B).

Jnk activity was not detectable in resting T cells (Fig. 3C). Stimulation of T cells by PDB in combination with ionomycin (P/I) induced Jnk activity, as measured by the phosphorylation of a GST–Jun substrate that was maximal at 20 min and progressively decreased thereafter (Fig. 3C). It is noteworthy that the removal of the PDB and ionomycin from the cells after 20 min (P/I–COMP, a condition that also results in the acquisition of the competent state without progression to DNA synthesis) stimulated a protracted increase in Jnk activity that remained for over 1 h (Fig. 3C). Consistent with previous reports (24), PHA did not stimulate the activation of Jnk in T cells.

Nuclear Translocation of AP-1 Transcription Factors in Competent T Cells

The sequence of events that determines the fate of stimulated T cells continues beyond the activation of MAPKs by modulating the activity of factors that promote or suppress expression of growth-associated genes. An important transcription factor that partici-

pates in the process of T-cell activation is activator protein-1 (AP-1). The nuclear translocation and the DNA-binding competence of AP-1 factors were examined by EMSA in unstimulated T cells, in T cells rendered competent by submitogenic stimulation with PHA, and in T cells stimulated to proliferate with mitogenic concentrations of PHA. Figure 4 shows the appearance of a single retarded band in nuclear extracts incubated with an oligonucleotide containing a consensus AP-1-binding sequence (cgcttgaTGAGT-CAGcggaa) that disappeared upon addition of a 40-fold molar excess of unlabeled oligonucleotide used as a competitor. This band may represent homodimers or heterodimers of Fos, Fra, or Jun family proteins (25). Nuclear AP-1 was undetectable in the unstimulated cells throughout the culture period. There was a transient increase in nuclear AP-1 in the competent T cells after 1 h of stimulation, which disappeared within 5 h. The cells that were induced to proliferate showed a progressive, sustained increase in AP-1 that was detectable after 1 h of stimulation, increased significantly after 5 h, and was maximal after 24 to 30 h. Similar results were seen when the nuclear translocation of NF κ B transcription factors was examined (data not shown).

Expression of Immediate Early Genes in Competent T Cells

Downstream effects of the activation of latent transcription factors include the coordinated expression of immediate early growth-associated genes such as *c-jun*, *c-fos*, and *c-myc* within the first 3 to 6 h following T-cell activation (5, 26). We examined the induction of gene expression for these three genes, along with those encoding IL-2 and the IL-2R α in unstimulated T cells, in T cells rendered competent by submitogenic stimulation with PHA and in T cells stimulated with mitogenic concentrations of PHA. Consistent with our findings evaluating the expression of surface CD25, the competence-inducing signals stimulated increased expression of the IL-2R α gene (Fig. 5). These signals also induced expression of *c-jun* and *c-fos*. Expression of the IL-2 and *c-myc* genes was not appreciably increased by the competence-inducing signal. Conversely, the mitogenic stimulus not only induced greater levels of IL-2R α , *c-jun*, and *c-fos* than the competence-inducing signal, but also resulted in increased expression of IL-2 mRNA and *c-myc* (Fig. 5).

Activation of G1-Phase Cyclin-Dependent Kinases in Competent T Cells

The ultimate fate of T cells in progressing through the G1 phase of the cell cycle may finally be determined by the activation of cyclin-dependent kinases and phosphorylation of the growth-suppressing Rb protein (10, 27, 28). We have shown previously that induction

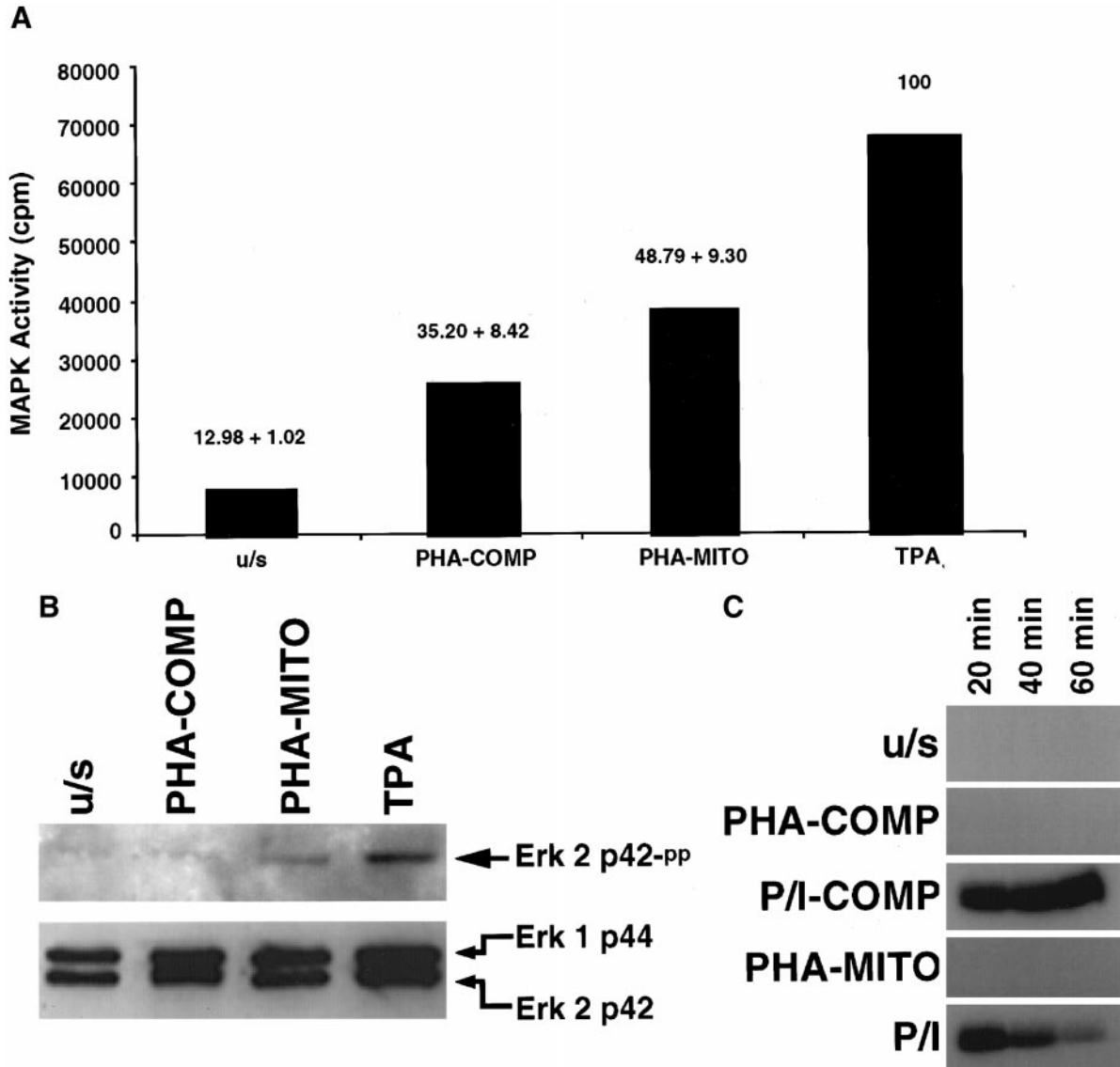


FIG. 3. Activation of MAPK in competent T cells. (A) MAPK activity was measured by an *in vitro* kinase assay using MBP as a substrate in unstimulated T cells (u/s), T cells rendered competent by a submitogenic stimulus with PHA (PHA-COMP), T cells stimulated with a mitogenic dose of PHA (PHA-MITO), or T cells stimulated with TPA for 10 min. MAPK activity shown is the cpm when MBP was included minus the cpm when MBP was omitted from the assay. The graph represents the MAPK activity in one experiment of three done. The numbers over the bars represent the means \pm SD normalized to the percentage MAPK activity in each sample from the three independent experiments, considering the activity seen upon stimulation of TPA to be equal to 100%. (B) Upper panel shows activation of Erk-1 and Erk-2 examined by immunoblotting with an antibody that specifically recognizes the biphosphorylated form of the proteins (Thr202/Tyr204). Lower panel shows blotting of the same membrane with an antibody that recognizes both the unphosphorylated and the phosphorylated forms of Erk1 and Erk2. Cells were stimulated as in (A). Note the appearance of a band representing biphosphorylated Erk2 in the cells stimulated using the mitogenic PHA dose or TPA. (C) N-terminal Jun kinase activity was measured by a solid-phase kinase assay. T cells remained unstimulated, were rendered competent by a submitogenic dose of PHA (PHA-COMP) or by a 20-min stimulation with PDB + ionomycin followed by washing (P/I-COMP), were stimulated by a mitogenic dose of PHA (PHA-MITO), or were stimulated with PDB + ionomycin present continuously in the culture (P/I). Cell aliquots were harvested at 20-min intervals and Jnk activity was assayed by the phosphorylation of an N-terminal Jun-GST fusion protein.

of T-cell competence using stimuli that bypass the T-cell receptor induces activation of the three G1-phase Cdks, Cdk2, Cdk4, and Cdk6 (10, 16). Similarly, induction of competence using a submitogenic concentration of PHA stimulated activation of these three G1 Cdks (Fig. 6), although the activity of each Cdk was smaller

in magnitude than that seen using mitogenic concentrations of PHA (Fig. 6; Cdk2, 33 to 51%; Cdk4, 53 to 67%; Cdk6, 38 to 69%). The difference in the activities of Cdk4 and Cdk6, but not of Cdk2, in resting vs competent T cells could be partly explained by reduced amounts of the proteins in the resting cells (10, 16;

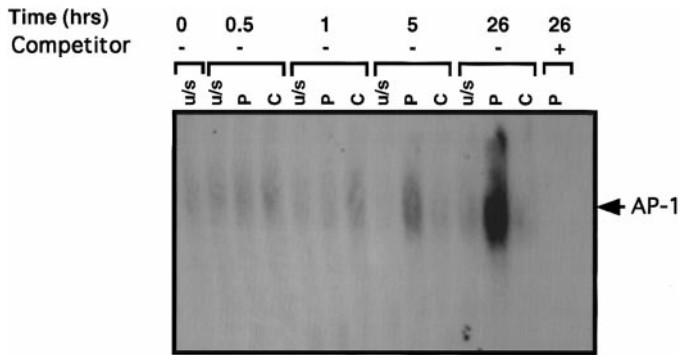


FIG. 4. Nuclear translocation of AP-1 transcription factors in competent T cells. Activation of AP-1 transcription factors was examined by EMSA assays in resting T cells (u/s), T cells stimulated with a mitogenic concentration of PHA (P), or T cells rendered competent with a suboptimal concentration of PHA (C) for the indicated time periods. The specificity of the binding reaction was ensured by adding a 40-fold molar excess of unlabeled oligonucleotide probe as a competitor. The data show that the competence-inducing signal stimulates a transient nuclear translocation of AP-1 of <5 h duration. In contrast, translocation of AP-1 by mitogenic signals appears to be slightly more delayed (cf. 1 h vs 5 h samples) and is sustained for at least the first 26 h of culture. The data for one representative donor are shown. Four independent experiments (four donors) showed similar results.

unpublished results). However, this may also have been due to changes in the amount and activity of the Cdk inhibitor p27/Kip-1 (28–30). Similarly, the additional increase in the activities of Cdk4, Cdk6, and Cdk2 in T cells stimulated using a mitogenic concentration of PHA could be attributed only in part to increased levels of these proteins, as regulation of Cdk activity is a complex, multifactorial process (3).

DISCUSSION

T-cell activation following engagement of the antigen receptor alone, or simultaneously with coreceptor molecules, can lead to distinct outcomes such as proliferation and differentiation, cell cycle entry with G1-phase arrest, anergy, or apoptosis (1, 31, 32). Both quantitative and qualitative signaling differences have been proposed to explain the fate of T cells to undergo proliferation, anergy, or apoptosis (1, 31). However, the signals that lead to cell cycle entry and growth arrest in a competent (cytokine-responsive) state have not been examined in detail.

Our results show that induction of competence in human T cells by stimulation with submitogenic doses of PHA, a lectin mitogen that binds to nonpolymorphic regions of the antigen receptor, CD2, CD3, and CD45 (33), resulted in calcium mobilization, PKC-dependent internalization of CD3, increased MAPK activity, transient translocation of AP-1 transcription factors to the nucleus, expression of immediate early genes, activation of G1-phase cyclin-dependent kinases, increased

CD25 (IL-2R α) expression, and the capacity to proliferate in response to exogenous IL-2. Yet these events were consistently of lesser magnitude in competent T cells than in T cells stimulated with the higher mitogenic concentration of PHA.

It has been shown that stimulation of T cells with high doses of anti-CD3 antibody promotes activation of the Ras/MAPK pathways and PI3K and ribosomal S6 kinases (14); that activation of MEK1, Erk1, and Erk2 is required for IL-2 production (20); and that stimuli which block the activation of Ras and the MAPKs result in T-cell anergy (19, 34). CD45 also plays a requisite role for tyrosine kinase-dependent signal transmission in T cells; co-stimulatory signals delivered through CD28 and CD4 may amplify some or all of these signals and may be absolutely necessary for activation of Jnk (24, 35, 36).

Our results suggest that activation of PTKs and PKC was required for acquisition of the competent state in T cells. In contrast, previous work using mitogenic doses of PHA showed that the inhibitory effects of PKC antagonists on lymphocyte proliferation were readily

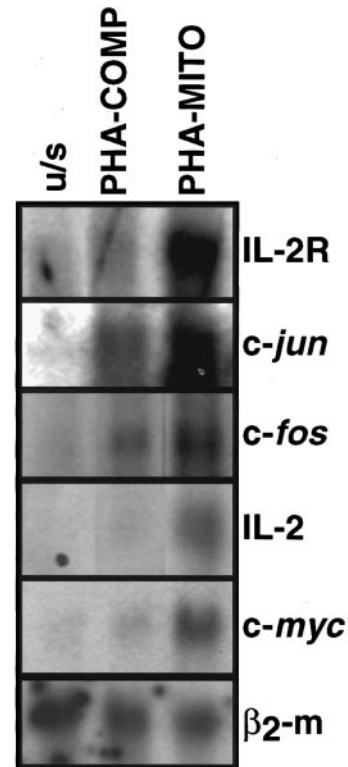


FIG. 5. Expression of growth-associated genes in competent T cells. RNA was isolated from resting T cells (u/s), from T cells rendered competent by stimulation with a submitogenic dose of PHA (PHA-COMP), or from T cells stimulated with a mitogenic dose of PHA (PHA-MITO). The expression of IL-2R, *c-jun*, *c-fos*, IL-2, and *c-myc* was evaluated by Northern blotting. Expression of *c-jun* and *c-fos* was assessed after 40 min of stimulation; *c-myc*, IL-2, and IL-2R after 4 h. β ₂-m expression was evaluated as a loading control. Similar results were obtained in three experiments for each gene analyzed.

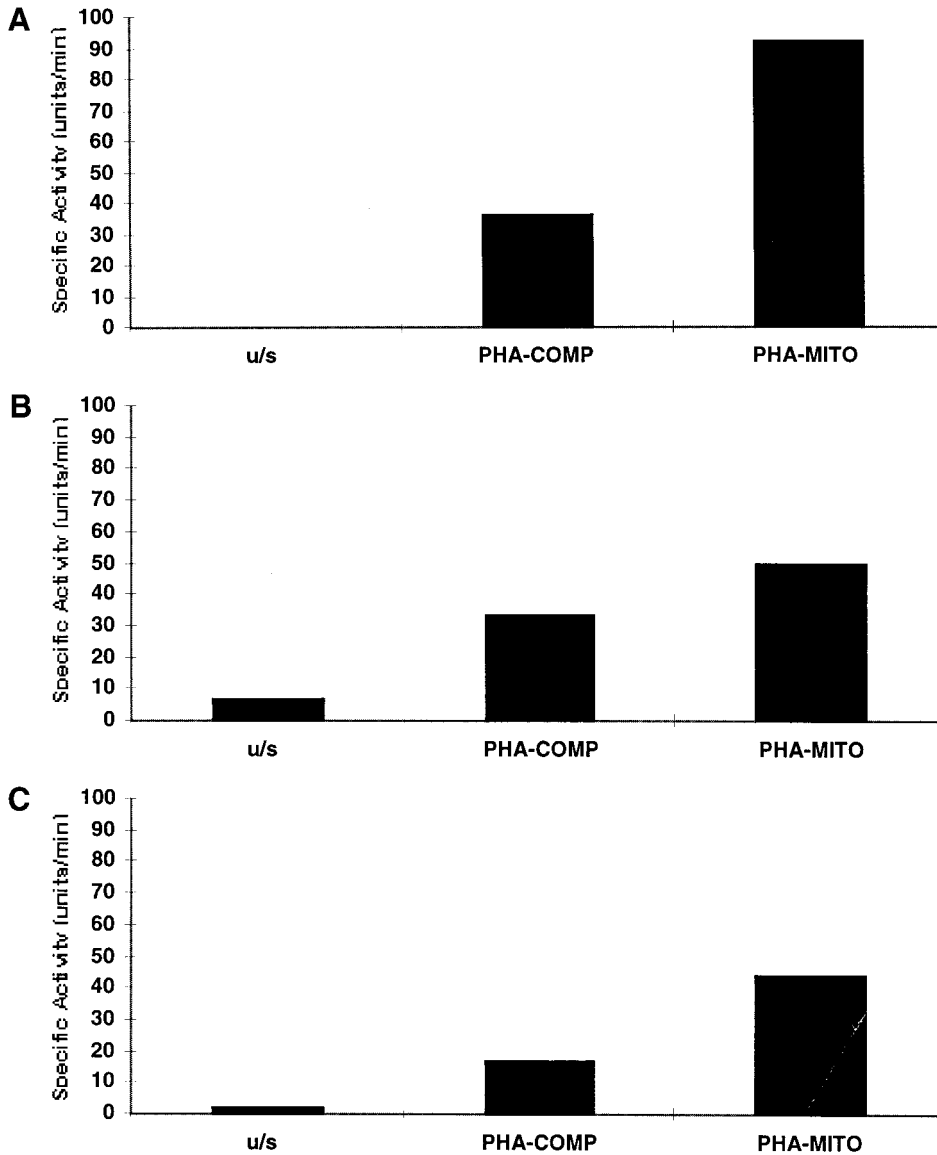


FIG. 6. Kinase activity of Cdk2, Cdk4, and Cdk6 in activated human T cells. The activities of (A) Cdk2, (B) Cdk4, and (C) Cdk6 were assessed in immunoprecipitates from resting T cells (u/s), from T cells rendered competent by suboptimal concentrations of PHA (PHA-COMP), or from T cells stimulated with a mitogenic dose of PHA (PHA-MITO) 15 h after the onset of culture using a recombinant, truncated form of the Rb protein (p56/Rb) as a substrate. 1 unit/min of specific activity was defined as the incorporation of 1 fmol of phosphate/min into the substrate. The data shown are representative of four experiments.

versed by exogenous IL-2 (11). Induction of T-cell competence also stimulated MAPK activity that appeared to be distinct from Erk1, Erk2, or Jnk. This activity could be attributed to p38, or another unidentified protein with MAPK activity, at least as defined by the phosphorylation of MBP. Conversely, the mitogenic stimulus induced activation of Erk2, sustained translocation of AP-1 to the nucleus (possibly as a result of *de novo* synthesis and assembly of Fos and/or Jun proteins), and ultimately led to IL-2 production. These differences in signaling observed in cells stimulated by the two concentrations of PHA could be directly proportional to antigen receptor multimerization, to the

recruitment of accessory co-stimulatory molecules into these multimeric receptor complexes, or to both. Yet we cannot entirely exclude the possibility that the kinetics of MAPK activation also may be affected by the stimulating dose of mitogen.

The data also show that the competent states induced in T cells using different experimental methods are not biochemically equivalent. In contrast to the results seen using PHA, induction of competence following a brief stimulation by PDB and ionomycin led to prolonged Jnk activation. This suggests that T cells may use various distinct pathways to achieve cytokine responsiveness or, alternatively, that primary human

T cells do not require Jnk activity to establish competence to proliferate. Finally, it has been shown that a mitogenic signal must be present for >2 h to effectively stimulate IL-2 production (37); thus, the absence of *c-myc* and IL-2 gene expression in the competent T cells could be partly due to the premature termination of the signal.

Studies using murine CD4⁺ T-cell clones have shown that the affinity of antigenic peptides as well as the quantity of antigenic peptides presented in the context of class II MHC molecules leads to distinct functional outcomes after T-cell activation (35, 38–40). In these experiments, altered peptide ligands that bound with low affinity to the T-cell receptor (dissociation constants 10- to 100-fold less than that of the wild-type peptide), or peptide concentrations 100 times below those required for production of interferon- γ in naïve CD4 cells, led to IL-4 production and differentiation to a Th2 phenotype. However, this functional dichotomy does not appear to occur in human competent T cells. Induction of competence stimulates neither IL-2 nor IL-4 production (41), but the cells can mount a proliferative response upon stimulation by either cytokine.

It is noteworthy that incomplete stimulation of human T cells did not prevent cell cycle entry or result in cytokine unresponsiveness despite the lack of Erk2 activity and IL-2 production. Cell cycle entry in competent cells was confirmed by the activation of the G1-phase Cdks, Cdk4, Cdk6, and Cdk2. It has been shown that activation of the G1 Cdks in competent T cells leads to transient phosphorylation of Rb (4), but this is insufficient to promote passage through the R point. One implication of the data is that activation of Erk2 may contribute to activation of the G1 Cdks and sustained phosphorylation of Rb. This is supported by the observation that Ras activation (which presumably would result in activation of Erk) is important for sustained phosphorylation of Rb in fibroblasts (42). Also, activation of MEK1 appears to contribute to the assembly and activation of D-type cyclins and Cdk4 (43). This not only promotes Cdk4-dependent phosphorylation of Rb, but also allows the Cdk4 complexes to serve as a sink for cyclin-dependent kinase inhibitors (CDKIs) that dissociate from Cdk2/cyclin complexes. The dissociation of CDKIs from Cdk2 in turn promotes cyclin E- and cyclin A-associated Cdk2 activity and S-phase entry. In fact, among the known effects of promotion factors such as serum and IL-2 are activation of the Ras/MAPK pathways (23, 44, 45), increased expression and activation of the G1 Cdks (10, 16), dissociation of p27/Kip-1 CDKI from Cdk2 complexes (29, 30), and reduction in p27/Kip-1 expression (28).

In summary, the data suggest that there are both quantitative and qualitative differences between competence-inducing signals and mitogenic signals that stimulate cell cycle entry, cytokine production, and

progression to the S phase in T cells. Since the T-cell competent state is lost within ~24 h of stimulation (9, 46), the competence and progression system of T-cell activation provide a relevant model for examining whether competent cells that do not receive a progression signal return to the G0 phase, become anergic, or undergo apoptosis.

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