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Interleukin-2-Triggered Raf-1 Expression, Phosphorylation, and Associated Kinase Activity Increase through G_1 and S in CD3-Stimulated Primary Human T Cells

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To gain further insight into the role of Raf-1 in normal cell growth, c-*raf*-1 mRNA expression, Raf-1 protein production, and Raf-1-associated kinase activity in normal human T cells were analyzed. In contrast to the constitutive expression of Raf-1 in continuously proliferating cell lines, c-*raf*-1 mRNA and Raf-1 protein levels were barely detectable in freshly isolated G_0 T lymphocytes. Previous work with fibroblasts has suggested that Raf-1 plays a signaling role in the G_0-G_1 phase transition. In T cells, triggering via the T-cell antigen receptor (TCR)-CD3 complex (TCR/CD3) resulted in an approximately fourfold increase in c-*raf*-1 mRNA. In addition, the promotion of G_1 progression by interleukin 2 (IL-2) was associated with a 5- to 10-fold immediate/early induction of c-*raf*-1 mRNA, resulting in up to a 12-fold increase in Raf-1 protein expression. TCR/CD3 activation did not alter the phosphorylation state of Raf-1, whereas interleukin 2 receptor stimulation resulted in a rapid increase in the phosphorylation state of a subpopulation of Raf-1 molecules progressively increasing throughout G_1. These findings were complemented by assays for Raf-1-associated kinase activity which revealed a gradual accumulation of serine and threonine autokinase activity in Raf-1 immunoprecipitates during G_1, which remained elevated throughout DNA replication.

c-*raf*-1 is the normal cellular homolog of v-*raf*, a transforming gene originally discovered as an insert in a murine Moloney sarcoma virus isolate (49). The c-*raf*-1 gene encodes a 70- to 75-kDa cytoplasmic phosphoprotein, Raf-1, that has associated serine- and threonine-specific kinase activity (7, 8, 40). On the basis of sequence analysis, the Raf-1 protein can be divided into two domains of approximately equal sizes, an amino N-terminal regulatory domain and a carboxy C-terminal catalytic kinase unit (48). The N-terminal domain is thought to exert negative regulatory control over the C-terminal kinase domain, in that all oncogenic forms of Raf-1 identified thus far contain deletions or other mutations of the N-terminal half of the molecule (8). Moreover, experimental mutations of c-*raf*-1 by the construction of 5′ gene deletions reveal that virtually the entire N-terminal regulatory domain of Raf-1 must be removed before the kinase activity becomes fully constitutive and before transforming activity for murine embryonic fibroblasts (3T3 cells) becomes manifest (25, 61, 64).

Since v-*raf* transforms murine 3T3 cells and since c-*raf*-1 5′ deletion mutants can induce DNA replication in serum-starved 3T3 cells (60), Raf-1 kinase activity is thought to play a key role in signaling cell cycle progression and DNA replication. Work to date has centered on very early events in this process, and the regulated activation of the Raf-1 kinase by platelet-derived growth factor (PDGF) has recently been the focus of considerable interest. Experiments show that the Raf-1 molecule is induced to associate with the PDGF-β receptor upon PDGF-receptor binding (41). Subsequently, there is an increase in Raf-1 phosphorylation and its associated kinase activity (42).

In order to gain further information regarding the role of Raf-1 in cell growth regulation, we focused on normal human T lymphocytes (T cells). T cells are an ideal system for growth regulation studies, because they exist normally in a noncycling (G_0) state yet can be made to enter the cell cycle semisynchronously simply by activating the T-cell antigen receptor (TCR)-CD3 complex (TCR/CD3) (58). A series of experiments has now established that T-cell cycle progression occurs as a result of the sequential expression of a series of genes. In particular, the TCR/CD3 complex initiates G_0 to G_1 transition, whereby the cells are made competent to proliferate by the expression of the genes encoding the receptor for the T-cell growth factor, interleukin 2 (IL-2) (12, 14, 51, 58, 65). Simultaneously, the IL-2 gene itself is transcribed and the IL-2 receptor (IL-2-R) interaction promotes G_1 progression to DNA replication.

Because T-cell cycle progression depends on two distinct and well-characterized cell surface receptors, we sought to determine whether Raf-1 is involved in T-cell growth control by coupling to either or both of these receptor systems and their associated signaling pathways. The TCR/CD3 complex stimulates tyrosine-specific phosphorylation (3, 27, 43) and activates the phosphatidyl-inositol pathway, raising free intracellular calcium and activating protein kinase C (PKC) (67). By comparison, the IL-2-R interaction stimulates tyrosine phosphorylation (1, 35, 54, 55) but does not activate phosphatidyl-inositol hydrolysis (36–38).

Thus far, c-*raf*-1 mRNA expression has been found to be constitutive and to remain invariant throughout the cell cycle (13, 63, 68). However, most studies to date have utilized established cell lines, all of which have various alterations of growth regulation (45). Upon examination of freshly isolated, resting T cells, we found c-*raf*-1 message and protein expression to be barely detectable. Activation of the TCR/CD3 complex resulted in an ~4-fold increase in c-*raf*-1 mRNA expression, and IL-2-R stimulation led to a further 5-

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to 10-fold immediate/early induction of c-*raf*-1 mRNA expression. The combined effect of inducing c-*raf*-1 mRNA resulted in up to a 12-fold increase in Raf-1 protein levels which accumulate throughout G_1. However, signaling via the two systems differed, in that TCR activation did not alter the phosphorylation state of Raf-1, whereas IL-2-R stimulation was associated with Raf-1 hyperphosphorylation and an increase in associated kinase activity. These changes in Raf-1 accumulate during G_1 and remain elevated throughout DNA synthesis, implicating Raf-1 as functioning in later stages of the cell cycle, a concept which has not previously been considered.

MATERIALS AND METHODS

Reagents. Cyclosporin A (CsA) (Sandoz, Hanover, N.J.) was dissolved in ethanol-Tween 80 and stored as a 1-mg/ml stock as recommended by the manufacturers. 4-β-12-Myristate-13-acetate (PMA), phorbol-12-13-dibutyrate (PdBU), N^6,$O^{2'}$-dibutyryl cyclic AMP (db-cAMP), and cycloheximide (CHX) were obtained from Sigma Chemical Co. (St. Louis, Mo.). PMA and PdBU were solubilized in dimethyl sulfoxide to yield stock concentrations of 5 mg/ml. db-cAMP and CHX were solubilized in RPMI 1640 medium to yield stock concentrations of 10 mg/ml. Stock solutions of all reagents were diluted to the appropriate final concentrations in RPMI 1640 medium prior to each incubation.

Homogeneous human recombinant IL-2 was provided by Takeda Chemical Industries (Osaka, Japan) as a 1.0-mg/ml solution in ammonium acetate buffer (pH 5.0).

Cell culture. Venous whole blood was drawn from healthy human donors, and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque discontinuous gradient centrifugation and cultured at 10^6 cells per ml in complete media consisting of RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% heat-inactivated (56°C for 30 min) fetal bovine serum (Sterile Systems, Logan, Utah), L-glutamine (200 μg/ml), penicillin G (50 U/ml), and gentamicin (50 μg/ml) (GIBCO). Cells were stimulated for 72 h by monoclonal antibodies reactive to the T3 (CD3) component of the T-cell receptor complex (OKT3, 1:10,000 dilution; Ortho Pharmaceuticals). The cells were washed to remove α-CD3 and any IL-2 produced in situ and then were recultured in IL-2-free medium for 48 h or maintained for 10 to 12 days at concentrations between 2×10^5 and 1×10^6 cells per ml by supplementation with recombinant IL-2 (500 pM) (62). After 13 to 15 days, the cells (>99% T cells, represented by $CD4^+$ and $CD8^+$ subpopulations) were washed and cultured without IL-2 for 36 to 48 h to ensure their reaccumulation in the early G_1 phase of the cell cycle (23). IL-2-R expression was achieved by exposure to 50 ng of PdBU per ml for 10 to 12 h, after which the cells were extensively washed and kept in complete medium without IL-2 for 12 to 14 h. The expanded, IL-2-R-positive T cells were kept in IL-2-free medium or stimulated into G_1 cell cycle progression by the addition of IL-2 (1 nM). High-affinity IL-2-R expression was monitored by binding of radioiodinated IL-2 as described previously (66).

An enriched primary human T-cell population was obtained from PBMCs by using a modification of the method previously described (10). Monocytes and natural killer and B cells were removed while T cells were positively selected with α-CD3 monoclonal antibodies. PBMCs (5×10^6 cells per ml) were cultured in serum-free medium at 22°C for 60 min in the presence of a lysosomotropic compound, 5 mM L-leucine methyl ester hydrochloride (Sigma). This treatment selectively lyses monocytes and natural killer cells. Cells were washed and then incubated with 5 μg of α-CD3 monoclonal antibody (64.1 [immunoglobulin G2a], a generous gift of E. Vitetta, University of Texas Health Science Center at Dallas) per ml for 60 min at 4°C. After the removal of unbound 64.1, the cells were allowed to adhere to goat anti-mouse Fc (Organon Teknika Corp., West Chester, Pa.)-coated petri dishes for 60 min at 4°C. Unbound CD3(−) cells were removed by gentle aspiration and washed three times with cold media. This cell separation procedure typically yielded a population of T cells which was >98% $T11^+$ as determined by flow cytometric analysis. Adherent, resting T cells were immediately harvested or subsequently incubated at 37°C in the presence or absence of various additives to initiate T-cell activation.

[^{3}H]thymidine ([^{3}H]TdR; 2.0 μCi/ml; Schwartz/Mann Division, Becton Dickinson) incorporation was monitored at 2- to 4-h intervals by liquid scintillation counting (11).

RNA isolation and analysis. Total cellular RNA was extracted with guanidine isothiocyanate and purified by centrifugation through 5.7 M CsCl as previously described (10). Samples (15 μg) were subjected to 1% agarose formaldehyde gel electrophoresis and blotted onto nitrocellulose. Filters were vacuum baked at 80°C for 90 min. Prehybridizations were carried out for 8 to 24 h at 42°C in a solution containing 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0]), 5× Denhardt's solution, 0.2% sodium dodecyl sulfate (SDS), and 100 μg of heat-denatured sheared salmon sperm DNA per ml. Hybridizations were carried out for 48 to 72 h in identical conditions except for the addition of 1.5×10^6 cpm of heat-denatured ^{32}P-labeled probes (specific activity, $>1 \times 10^9$ cpm/μg of DNA) per ml of hybridization solution (19). Filters were washed for 10 min at room temperature with 1× SSC and 0.1% SDS, for 30 min at 42°C with 0.1× SSC and 0.1% SDS, and for 30 min at 55 to 65°C with 0.2× SSC and 0.1% SDS. Specific hybridization signals were visualized by autoradiographic exposure to Kodak XAR-5 film with Dupont Cronex intensifying screens. The autoradiographic bands were quantitated by scanning with an EC densitometer (EC Apparatus Corp., St. Petersburg, Fla.). Radiolabeled probes were removed from nitrocellulose filters by treatment with 0.1× SSC and 0.1% SDS at 100°C for 20 min before subsequent hybridizations.

Probes. Plasmids containing cDNA inserts, which were used as probes in Northern (RNA) blot analysis, were generously provided by the following individuals: human c-*raf*-1, U. Rapp (8); human p75 subunit of IL-2-R, W. Leonard (24); human p55 subunit of IL-2-R, T. Honjo (44); human IL-2, G. Crabtree (26); and rat β-actin, B. M. Paterson (32).

Western immunoblot analysis. PBMCs or primary or expanded T cells (10×10^6 to 50×10^6) were spun down at 650 × *g* for 5 min, washed once with cold normal saline containing 0.5 mM EDTA (pH 8.0) and 100 μM orthovanadate, respun, and lysed as previously described (42) in 0.4 ml of 1% Triton X-100 lysis buffer containing 20 mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 2 mM EDTA (pH 8.0), 2 mM phenylmethylsulfonyl fluoride, aprotinin (0.15 trypsin-inhibiting U/ml), 20 μM leupeptin, 1 mM sodium vanadate, 5 mM sodium fluoride, and 1 mM dithiothreitol at 4°C for 10 min. Insoluble material was removed by centrifugation at 4°C for 15 min at 10,000 × *g*. Cell lysates were normalized for protein content by an adaptation of the bicinchoninic acid protein assay (Pierce Chemical Company, Rockford, Ill.) as previously described (50) before electrophoretic separation by SDS–7.5% polyacrylamide gel electrophoresis (PAGE)

and transferred to nitrocellulose filters as previously described (42). Protein blots were probed with an anti-Raf-1 antibody, SP63, directed against the carboxy 12 residues of Raf-1 (56), and immunological reactions were identified by using an alkaline phosphatase detection system (Promega). Protein band intensities were quantified by scanning the blots into a Macintosh IIfx computer with a ScanJet Plus flatbed scanner (Hewlett-Packard). Densitometry was performed on the scanned image by using Scan Analysis (Biosoft, Cambridge, United Kingdom) and a customized version of Enhance (Microfrontiers, Des Moines, Iowa). Serial dilutions of cell lysates prepared from c-*rafA* cells, a Raf-1-overproducing murine fibroblast cell line (42), were blotted and scanned in order to determine that Raf-1 peak tracings fell within a linear range of detection.

In vitro immune complex kinase assays. PBMCs or expanded T cells (25×10^6 to 50×10^6) were lysed in Triton lysis buffer in the presence of protease and phosphatase inhibitors as described for Western blot analysis. Polyclonal anti-Raf-1 serum was prepared and affinity purified as previously described (56). Affinity-purified antibody was coupled to protein A-Sepharose beads by being incubated in 1.0 ml of lysis buffer for 60 min at 4°C. Lysate, prepared from equivalent cell numbers, was then added to the bead-antibody complex and incubated for 3 to 6 h at 4°C. Immunoprecipitates were washed once in lysis buffer, twice in 0.5 M LiCl–0.1 M Tris (pH 7.4), and once in kinase buffer (25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.4], 10 mM $MgCl_2$). Kinase assays were performed by adding 40 μl of kinase buffer (made 1 mM for dithiothreitol and 15 μM for ATP) and 10 μCi of [γ-^{32}P]ATP per reaction for 20 min at 25°C. Reactions were terminated by diluting into 1.0 ml of phosphate-buffered saline, washing away the unincorporated [γ-^{32}P]ATP, and boiling the samples in electrophoresis buffer prior to SDS-PAGE.

RESULTS

Raf-1 expression is potentiated during T-cell cycle progression. In previous studies we established that after activation of the TCR, T-cell cycle progression occurs asynchronously because individual cells within the population express IL-2-Rs asynchronously (12). Thus, even though the initiation of cell cycle progression results from the instantaneous activation via the TCR, the cells respond to the signal heterogeneously. As shown in Fig. 1A, IL-2 mRNA transcripts are expressed transiently, peak by 6 h, and then decline to undetectable levels by 36 to 48 h. The levels of IL-2-R p55 chain also become evident by 6 h after α-CD3 stimulation but do not peak until 24 to 36 h. The IL-2-R p75 mRNA transcripts also peak at 24 to 36 h after activation (10).

Determination of c-*raf*-1 mRNA expression after α-CD3 stimulation revealed a time course remarkably similar to the expression of the IL-2-R. Steady-state c-*raf*-1 transcripts were detectable before stimulation and increased gradually after stimulation, reaching peak levels of 8- to 10-fold 12 to 36 h after activation (Fig. 1A and B).

The increased c-*raf*-1 mRNA expression was paralleled by augmented Raf-1 protein levels detected by Western blot analysis, as shown in Fig. 1B. Maximal levels occurred between 48 and 72 h after activation, increasing 8- to 12-fold over the level present in unstimulated cells. Although maximum levels of c-*raf*-1 mRNA and Raf-1 protein occurred at least 24 h after CD3 stimulation, a small increase in levels was evident as early as at 3 h of activation, suggesting a biphasic mode of induction. c-*raf*-1 mRNA and Raf-1 protein levels in control, unactivated PBMCs remained at basal (t = 0) levels even after 120 h in culture (data not shown).

Also shown in Fig. 1D is a shift in the electrophoretic mobility of Raf-1 molecules, such that at least two more slowly migrating forms (from 72 to 76 kDa) were detectable between 6 and 72 h after α-CD3 activation. Previous studies have established that this mobility shift is indicative of an increase in Raf-1 phosphorylation and is usually associated with enhanced serine and threonine kinase activity in Raf-1 immunoprecipitates (2, 6, 30, 41, 42, 57).

The kinetics of Raf-1 induction after activation via α-CD3 were of interest in relationship to the transition from G_1 to S phase of the cell cycle, monitored by tritiated thymidine ([^{3}H]TdR) incorporation. As shown in Fig. 1E, increased Raf-1 protein expression preceded the onset of DNA replication by at least 12 h. Thereafter, Raf-1 levels remained elevated and were in a highly phosphorylated state as long as the cells continued to proliferate. When the cell population was washed free of α-CD3 and any IL-2 produced in situ after 72 h of culture, cell cycle progression gradually declined; by 120 h, [^{3}H]-TdR incorporation had decreased to t = 0 levels. In parallel, c-*raf*-1 mRNA and protein levels fell gradually; by 120 h they had decreased considerably compared with peak levels between 48 and 72 h. Subsequently, in noncycling cells there was also a dramatic decrease in the extent of Raf-1 phosphorylation; by 120 h only one lower migrating form of Raf-1 was evident (Fig. 1D).

Induction of c-*raf*-1 message expression by the TCR versus that of the IL-2-R. Because IL-2 and IL-2-R expression occur within 6 h of TCR/CD3 activation, it was impossible to ascertain which receptor system was responsible for the effect on c-*raf*-1 and Raf-1 levels, which peaked much later. Therefore, we capitalized on the observation that CsA selectively prevents IL-2 production without impairing IL-2-R expression after α-CD3 triggering (18, 53). As shown in Table 1, CsA inhibited T-cell cycle progression by ~94% as monitored by [^{3}H]TdR incorporation, yet it had no effect on the four- to fivefold increase in c-*raf*-1 mRNA measured 6 h after α-CD3 triggering. Moreover, the increase in c-*raf*-1 transcripts at 6 h was not affected by treatment with CHX, thereby indicating an immediate/early induction of c-*raf*-1 mRNA expression via the TCR/CD3 complex. By comparison, CsA suppressed the later augmentation of c-*raf*-1 mRNA determined at 48 h; control cells underwent a 15-fold increase in c-*raf*-1 mRNA, but cells treated with CsA expressed about the same levels of mRNA at 48 h that they had after only 6 h. This later accumulation of c-*raf*-1 mRNA was in response to IL-2 treatment, since IL-2 supplementation largely overcame the suppressive effect of CsA.

IL-2-induced c-*raf*-1 mRNA expression. The results from the CsA experiments indicated that both the TCR and the IL-2-R stimulated the expression of c-*raf*-1 mRNA. To further investigate the relative effects of the IL-2-R from TCR triggering, we utilized our previous observations by using expanded G_1-synchronized T-cell cultures (11, 62). T cells were expanded selectively in IL-2 for 12 to 14 days and then cultured for 48 h without IL-2 to allow their reaccumulation into a quiescent, early G_1 state. Subsequently, a brief exposure to PdBU resulted in the rapid reexpression of high-affinity IL-2-Rs (from <100 to ~1,500 to 2,000 sites per cell) without stimulating TCR triggering or IL-2 production (11, 62). No increment in c-*raf*-1 mRNA expression occurred after PdBU stimulation monitored after 1, 6, and 11 h of PdBU treatment (data not shown). As depicted in Fig. 2, the addition of human recombinant IL-2 promoted G_1 progres-

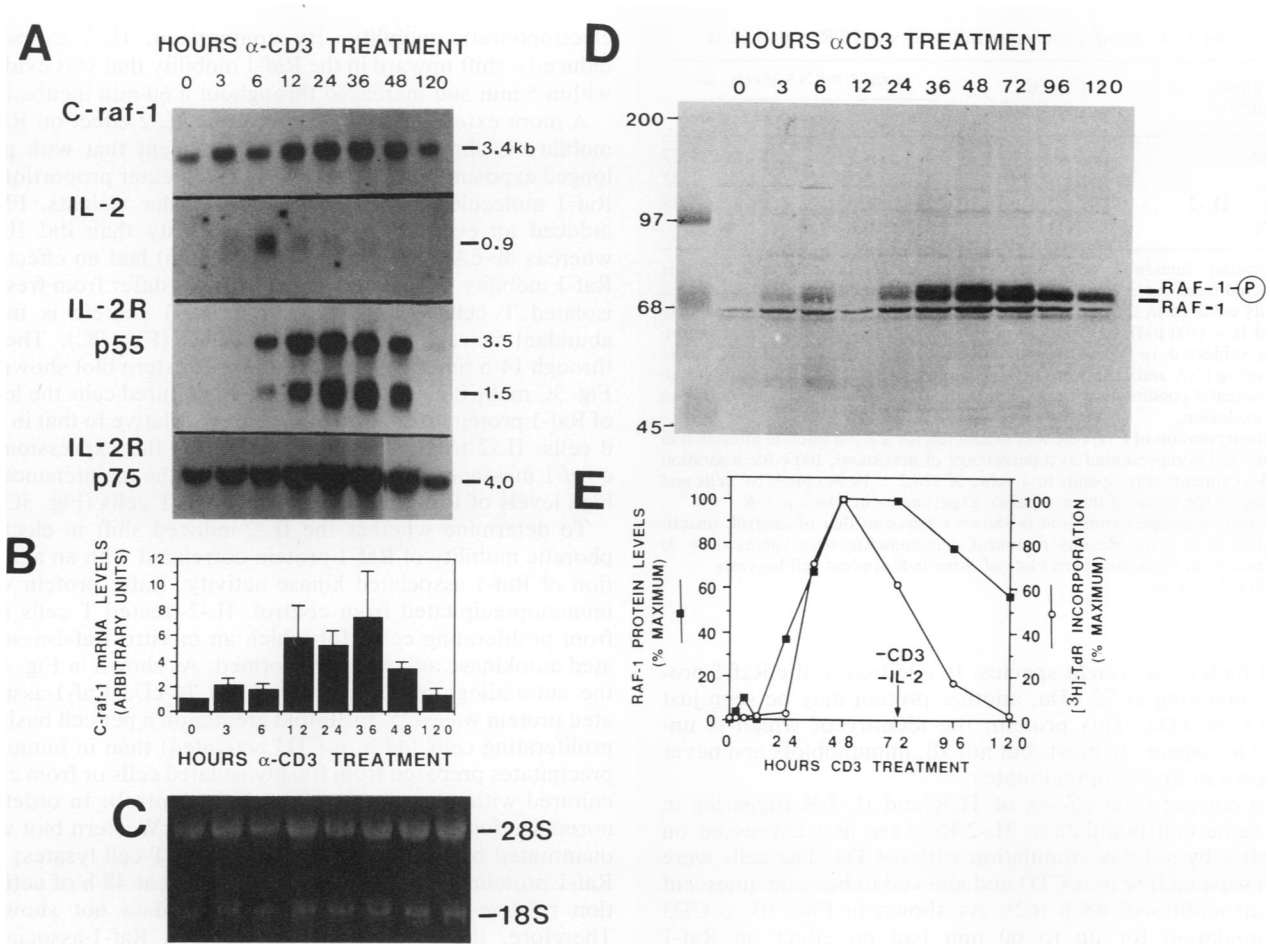

FIG. 1. c-*raf*-1 message and Raf-1 protein are increased following α-CD3 activation. (A) Autoradiographs of Northern blots showing the time course of c-*raf*-1, IL-2, and IL-2-R (p55 and p75) expression in 15 μg of total cellular RNA isolated from PBMCs before or at various times subsequent to α-CD3 activation. (B) Relative intensity of c-*raf*-1 signal with respect to resting (t = 0) PBMCs was measured by densitometry. Error bars represent the standard deviation of values determined from scans of autoradiographs obtained from at least three Northern blots of RNA isolated from three independent cell harvests. (C) The 28S and 18S ribosomal bands were visualized by ethidium bromide staining used to affirm the integrity and equivalent loading of RNA in gels. (D) Immunoblot analysis showing Raf-1 protein expression and changes in Raf-1 phosphorylation in α-CD3-activated PBMCs. Cell equivalents 2.5 × 10^6 were analyzed by SDS-PAGE. Protein blots were probed with an anti-Raf-1 antibody and visualized by an alkaline phosphatase detection system. Molecular size markers are shown to the left (in kilodaltons). (E) Raf-1 protein induction coincides with cell entry into DNA synthesis. Quantitation of Raf-1 protein expression in the Western blot shown in panel D was determined by scanning and densitometry as described in Materials and Methods. Results are representative of several experiments. A value of 100% corresponds to the maximal Raf-1 expression after 48 h of α-CD3 stimulation. [^{3}H]TdR incorporation was monitored at 2-h intervals by liquid scintillation counting at various times following α-CD3 treatment. Results are expressed as percent maximum incorporation, where 100% corresponds to a value of 1,225 cpm/h/10^4 cells. Standard error bars are not indicated for reasons of clarity but were always <5% of the mean.

sion in a synchronous fashion, with S-phase transition first becoming detectable via [^{3}H]TdR incorporation after 10 to 12 h. The IL-2-R interaction resulted in a rapid induction of c-*raf*-1 transcripts, which was first evident within 1 to 2 h and continued to increase through G_1, reaching 8- to 10-fold-greater levels within 10 to 12 h, just prior to the onset of DNA synthesis (Fig. 2). Although not shown, CHX treatment of T cells did not inhibit the IL-2-induced c-*raf*-1 message accumulation.

Effects of the TCR and IL-2-R on Raf-1 protein phosphorylation and associated kinase activation. Given the ~5-fold increase in c-*raf*-1 message expression in response to stimulation of the TCR and the further increase to ~15-fold following stimulation of the IL-2-R, we sought to determine whether stimulation of either of these receptors led to a modification of the c-*raf*-1 gene product.

Previous experiments have revealed that phosphorylation of Raf-1 alters its electrophoretic mobility on SDS-PAGE analysis (2, 6, 30, 41, 42). Phosphorylation occurs predominantly on serine and threonine residues and to a much lesser extent on tyrosine (12a, 29, 42, 64a). Since the TCR is known to activate tyrosine kinase activity in addition to serine- and threonine-specific phosphorylation via activation of PKC (43, 67), it was of interest to determine the effects of TCR triggering on Raf-1 gel mobility. A representative experiment in which Western blot analysis was used to detect Raf-1 is shown in Fig. 3A. Before stimulation, freshly isolated resting T cells contain Raf-1 protein that migrates as a single 72-kDa band. No change in the electrophoretic mobility of the Raf-1 protein was observed after α-CD3 stimulation for 30 min, whereas treatment with PMA at 50 ng/ml resulted in a dramatic shift of the entire Raf-1 population of molecules to

TABLE 1. c-*raf*-1 message induced via TCR and IL-2-R

Culture additive[a]	% [^{3}H]TdR incorporation[b]	c-*raf*-1 mRNA levels[c] at:	
		6 h	48 h
None	100.0	4.5 ± 1.95	15.8 ± 4.27
CsA	6.0 ± 3.45	4.2 ± 2.38	4.8 ± 1.42
CsA + IL-2	78.6 ± 13.00	6.3 ± 2.38	13.8 ± 3.08
CHX	ND[d]	6.2 ± 0.64	ND

[a] Primary human T cells were isolated, and total cellular RNA was extracted from control unactivated T cells (t = 0) and from α-CD3-activated T cells cultured in the presence or absence of CsA (1 μg/ml), exogenously added IL-2 (500 pM), or CHX (10 μg/ml) for various amounts of time before being subjected to Northern blot analysis. In long-term cultures, media containing CsA and IL-2 were replenished every 12 h. The T cells under all experimental conditions were greater than 90% viable as assayed by trypan blue exclusion.

[b] Incorporation of [^{3}H]TdR was monitored for a 2.5-h interval after 48 h in culture and is represented as a percentage of maximum; 100% incorporation (CD3 treatment) corresponds to a value of 926.2 ± 18.94 cpm/h/10^4 cells and represents the mean of three separate experiments in which n = 6.

[c] c-*raf*-1 message expression is shown relative to that of control, unactivated (t = 0) cells. Results represent densitometric scan values (n = 3) obtained from three Northern blots of three independent cell harvests.

[d] ND, Not done.

high-molecular-weight species. In addition to the Raf-1 protein migrating at 72 kDa, another protein may be seen just below 68 kDa. This protein, the identity of which is unknown, appears in most, but not all, immunoblots and never appears in immunoprecipitates.

To compare the effects of TCR and IL-2-R triggering in the same cell population, IL-2-Rs were first expressed on PBMCs by a 3-day stimulation with α-CD3. The cells were then washed free of α-CD3 and allowed to become quiescent for an additional 48 h (62). As shown in Fig. 3B, α-CD3 restimulation for up to 60 min had no effect on Raf-1 electrophoretic mobility. By comparison, IL-2 exposure induced a shift upward in the Raf-1 mobility that was evident within 5 min and increased throughout a 60-min incubation.

A more extensive time course of the IL-2 effect on Raf-1 mobility is shown in Fig. 3C. It is evident that with prolonged exposure, i.e., 6, 10, and 14 h, a greater proportion of Raf-1 molecules migrate at high molecular weights. PMA induced an even greater shift in mobility than did IL-2, whereas db-cAMP treatment (5 to 60 min) had no effect on Raf-1 mobility. Expanded T-cell cultures differ from freshly isolated T cells (Fig. 1D) in that Raf-1 protein is more abundant in resting, expanded T cells (Fig. 3C). The 6- through 14-h times represented in the Western blot shown in Fig. 3C make it clear that in untreated cultured cells the level of Raf-1 protein decreased dramatically relative to that in t = 0 cells. IL-2 treatment, which enhances the expression of c-*raf*-1 message (Fig. 2), correlates with the maintenance of high levels of Raf-1 protein in expanded T cells (Fig. 3C).

To determine whether the IL-2-induced shift in electrophoretic mobility of Raf-1 protein correlated with an activation of Raf-1-associated kinase activity, Raf-1 protein was immunoprecipitated from control, IL-2-treated T cells and from proliferating cells, for which an in vitro Raf-1-associated autokinase assay was performed. As shown in Fig. 4A, the autoradiographic intensity of the 74-kDa Raf-1-associated protein was ~25- to 30-fold greater on a per cell basis in proliferating cells (48 h, α-CD3 activated) than in immunoprecipitates prepared from freshly isolated cells or from cells cultured without activation (t = 48 h, control). In order to normalize for Raf-1 protein expression, a Western blot was quantitated by using a parallel aliquot of T-cell lysates; the Raf-1 protein was four- to fivefold greater at 48 h of activation relative to control cells (t = 0) (data not shown). Therefore, there was a net increase in Raf-1-associated

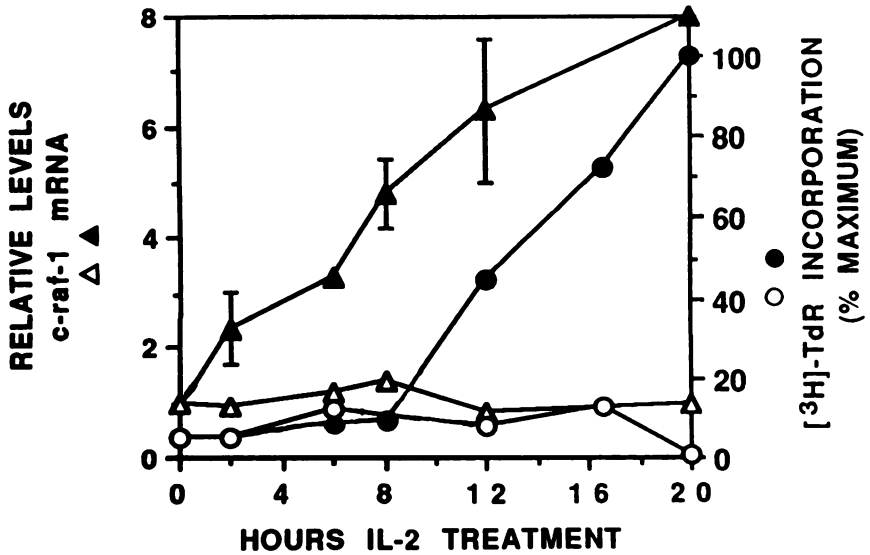

FIG. 2. Kinetics of c-*raf*-1 mRNA accumulation during IL-2-induced T-cell cycle progression. IL-2-R-positive quiescent T cells (t = 0) were stimulated into G_1 cell cycle progression by the addition of IL-2 (1 nM) or kept in IL-2-free media as described in Materials and Methods. Total cellular RNA was isolated at various times, and c-*raf*-1 message expression was evaluated by Northern blot analysis. The relative intensity of the c-*raf*-1 signal with respect to resting (t = 0) cells without IL-2 (△) or with IL-2 (▲) was measured by densitometry. Standard error bars represent the compiled results from three to four independent Northern blots and cell harvests. The incorporation of [^{3}H]TdR without IL-2 (○) and with IL-2 (●) was monitored at 2- to 4-h intervals as indicated. Results are expressed as percent maximum incorporation, where 100% equals a value of 331.3 ± 88.56 cpm/h/10^4 cells, and are means of four representative experiments in which n = 6. In control cell cultures lacking IL-2, [^{3}H]TdR incorporation was <15 cpm/h/10^4 cells.

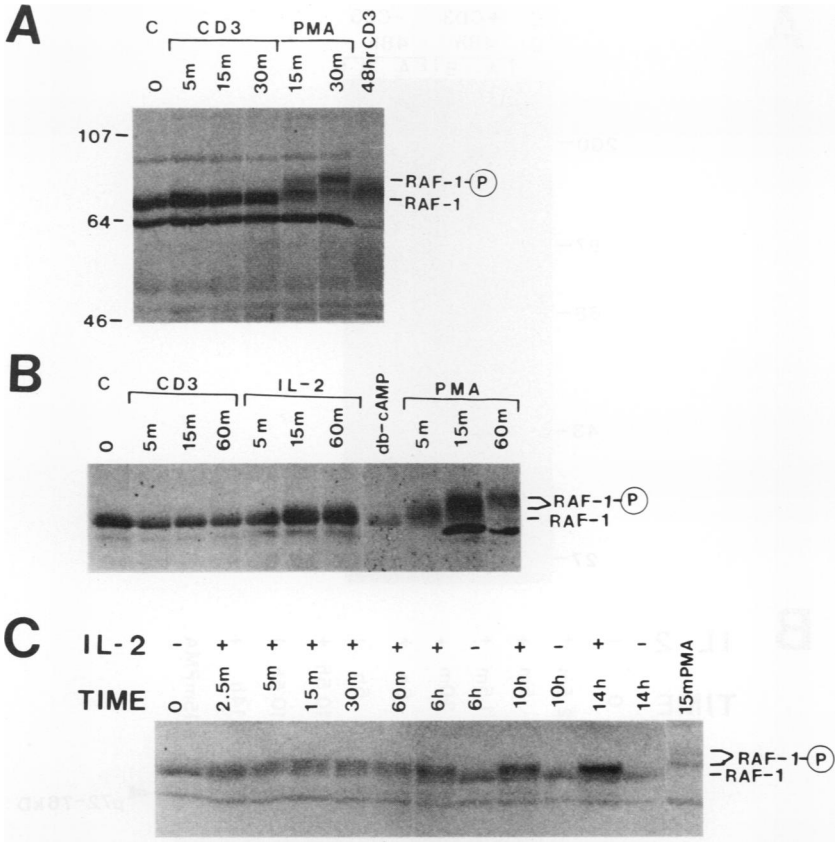

FIG. 3. Effects of α-CD3, IL-2, db-cAMP, and PMA treatment on Raf-1 electrophoretic mobility. Raf-1 protein was detected by immunoblot analysis and visualized by an alkaline phosphatase detection system. (A) Freshly isolated primary human T cells were harvested (lane C) or activated with immobilized α-CD3 antibodies for various times. Parallel α-CD3-activated T-cell cultures were supplemented with PMA (50 ng/ml) for 15 to 30 min prior to cell lysis. Molecular size standards (in kilodaltons) are indicated on the left. (B) Quiescent early G_1 human T cells, which express both T-cell antigen and IL-2-Rs, were isolated (Materials and Methods). The resting T cells (lane C) were harvested or treated with soluble CD3 (1:10,000 dilution), IL-2 (500 pM), PMA (50 ng/ml), or db-cAMP (0.5 mM) (60-min treatment shown) for 5 to 60 min prior to Raf-1 immunoblot analysis. (C) Early G_1-synchronized IL-2-R-positive expanded human T cells were isolated as described in Materials and Methods. The cells were kept in IL-2-free media or stimulated into G_1 cell cycle progression by the addition of saturating concentrations of IL-2 (1 nM) and were incubated at 37°C. Parallel early G_1 cell cultures were treated with PMA (50 ng/ml) for 15 min. Cell lysates were prepared, normalized to per cell equivalents, and subjected to Raf-1 immunoblot analysis. These data are representative of at least four independent cell harvests and Western blots.

kinase activity of five- to sixfold. The Raf-1 molecule displayed decreases in its electrophoretic migration upon activation, as previously illustrated in Fig. 1D.

As shown in Fig. 4B, the autoradiographic intensity of the 74-kDa Raf-1-associated protein after 5 min of IL-2 treatment was enhanced approximately threefold relative to that of control T cells ($t = 0$). The band intensity increased with prolonged IL-2 exposure, reaching maximum levels of four- to sixfold relative to those of controls between 6 and 10.5 h. By comparison, PMA treatment caused a dramatic increase in the electrophoretic shift of the related 74-kDa band and revealed a concomitant ~10-fold increase in its autoradiographic intensity. Even so, as illustrated in Fig. 3A, TCR/CD3 stimulation of primary T cells did not affect the electrophoretic mobility of the Raf-1 protein. In addition, we have not been able to detect an induction of Raf-1-associated autokinase activity from Raf-1 immunoprecipitates after TCR/CD3 triggering of primary T cells (data not shown).

A prominent protein of 112 kDa was also observed in Raf-1 immunoprecipitates isolated from T cells whose autoradiographic intensity was moderately (1.4- to 3.0-fold) affected by α-CD3 activation and IL-2 treatment (Fig. 4). Characterization of this protein is in progress.

DISCUSSION

Although the cellular proto-oncogene c-*raf*-1 has been implicated as functioning during cell cycle progression, its precise role in this process has remained obscure. Most of the work to date has dealt with early cellular activation events associated with the G_0/G_1 transition in established cell lines. By comparison, the findings with freshly isolated T cells described in this report are noteworthy, since they reveal that c-*raf*-1 message, Raf-1 protein expression, and Raf-1-associated kinase activity are all stimulated markedly through G_1, reaching maximal levels just prior to the peak in DNA replication.

In contrast to the findings reported here, c-*raf*-1 mRNA levels are reported not to vary appreciably in a wide variety of established mammalian cell lines, particularly at different phases of the cell cycle. For example, mitogen treatment of NIH 3T3 cells, IL-3-dependent cell lines, and IL-2-depen-

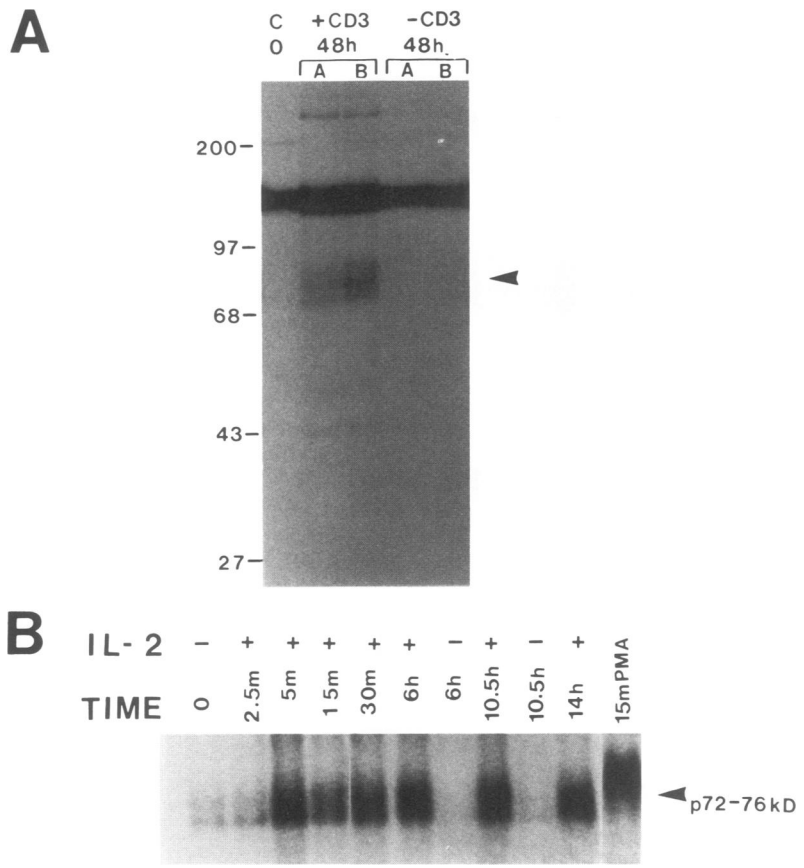

FIG. 4. Raf-1-associated autokinase activity is elevated in proliferating T cells and after IL-2 stimulation. (A) Raf-1 was immunoprecipitated from lysates of freshly isolated (t = 0) human PBMCs or from 48-h α-CD3-stimulated and 48-h unstimulated cells (harvested in duplicate, lanes A and B). Raf-1-immune complexes, prepared from equivalent cell numbers (5×10^6), were washed, and Raf-1-associated kinase activity was assayed by incubation with [γ-^{32}P]ATP. Samples were analyzed on SDS–7.5% PAGE, and phosphoproteins were detected by autoradiography. (B) Expanded human T cells were synchronized into early G_1 by IL-2 deprivation and were then treated with IL-2 (1 nM) or kept in IL-2-free media for 0 to 14 h. Parallel cultures were treated with PMA for 15 min. Cell lysates were prepared and Raf-1-associated autokinase activity was assayed (from 5×10^6 cell equivalents) as described above for panel A.

dent cell lines does not affect Raf-1 mRNA expression (63). We too have found that IL-2 stimulation of G_1-synchronized murine IL-2-dependent CTLL-2 cells does not influence the level of c-*raf*-1 mRNA transcripts (69). By comparison, the expression of mRNA transcripts for several other proto-oncogenes (e.g., c-*fos*, c-*myc*, and *pim-1*) is markedly enhanced by IL-2 in CTLL-2 cells (15). It should be emphasized that many established cell lines cannot be induced to enter a true G_0 resting stage; they can only be retarded from progressing from one phase of the active cell cycle to the next (45). In contrast, human peripheral T cells exist physiologically in a true metabolic and biochemical G_0 state. Only upon activation of the TCR/CD3 complex do they leave G_0 and enter early G_1 (14). Therefore, it is notable that Raf-1 protein is barely detectable in freshly isolated T cells and only becomes readily manifest after triggering of the TCR/CD3 complex.

Consistent with the proposed ubiquitous expression of c-*raf*-1 message, the 5′ regulatory promoter region of the c-*raf*-1 gene is GC rich, lacks TATA and CAAT boxes, and contains four potential binding sites (GC boxes) for the transcription factor SP1, all of which are characteristic features of housekeeping genes (4). However, the basal levels of c-*raf*-1 mRNA vary among cell types and are elevated under deregulated proliferative states, which suggests that tissue-specific factors, as well as growth factors, may influence the positive or negative expression of c-*raf*-1 (33, 63). The c-*raf*-1 promoter also contains an octamer enhancer element, ATGCAAAT (4), which may bind the octamer series (Oct-1) transcription factors (47, 48). Human *pim-1* and c-*myb*, two genes which are known to be transcriptionally activated by IL-2, share identical housekeeping motifs and, as well, contain the SP1- and Oct-1-binding motifs in their regulatory promoter regions (5, 34). Therefore, the interactions of specific DNA-binding proteins to these sequences may compose a common mechanism by which IL-2 regulates gene expression.

Many proteins whose expression has been shown to increase are important molecules for signal transduction and play a regulatory role in T-cell activation (14). Even though the TCR/CD3 complex and the IL-2-R activate distinct biochemical pathways, which result in altogether different cellular changes, both of these cellular receptor systems induce the rapid expression of c-*raf*-1 mRNA, even when protein synthesis is prevented. In this regard, several genes, including c-*fos*, c-*myc*, and the IL-2-R p55 chain, are induced by both receptor systems, whereas other genes are activated specifically by one receptor system but not the

other (46, 52). The timing of c-*raf*-1 expression (which is significantly delayed relative to that of c-*myc* and c-*fos*, for example) is more consistent with a regulated change in expression than with a general increase in cellular metabolism (14).

The TCR/CD3 complex triggers phosphatidyl-inositol turnover with a resultant activation of PKC and a sustained increase in the intracellular free calcium concentration (i.e., from 100 nM to 0.5 to 1.0 μM) (67). However, phorbol esters (PMA and PdBU) failed to mimic the TCR and IL-2-R effect of stimulating c-*raf*-1 mRNA expression (data not shown). Moreover, IL-2 does not stimulate a rise in intracellular free calcium (36), yet it clearly causes immediate/early c-*raf*-1 mRNA expression. Therefore, calcium-dependent pathways, particularly calcium-dependent kinases, are probably not involved in either TCR or IL-2-R stimulation of c-*raf*-1 mRNA expression. Recent experimental evidence indicates that tyrosine-specific protein kinase activation is an early biochemical change common to both receptor signaling pathways (55). Therefore, a reasonable working hypothesis includes receptor-stimulated tyrosine phosphorylation of molecules composing an immediate signaling pathway, leading to expression of a common set of genes.

Siegel and coworkers recently reported that activation of murine T-cell hybridomas results in Raf-1 phosphorylation and enhancement of Raf-1-associated kinase activity via a PKC-dependent pathway (57). In support of their findings and those of others, we have found that phorbol esters, which activate PKC, promote a rapid mobility shift of the entire Raf-1 population as well as enhanced Raf-1-associated autokinase activity (42). However, stimulation of primary human T cells with the α-CD3 monoclonal antibody 64.1 (22) did not affect the electrophoretic mobility or the associated autokinase activity of Raf-1. It is difficult to reconcile these conflicting results at this time, but since IL-2 stimulates phosphorylation and kinase activity associated with Raf-1 by a PKC-independent mechanism, it appears that both PKC-dependent and -independent pathways may be operative.

The combined effect of TCR and IL-2-R stimulation of c-*raf*-1 mRNA results in up to a 12-fold increase in Raf-1 protein expression that accumulates throughout G_1. These findings are consistent with the attainment of a crucial Raf-1 concentration during the latter half of G_1 that is important either for late G_1 progression or for G_1/S-phase transition. In addition, because the IL-2-R interaction results in an immediate phosphorylation of Raf-1 molecules, as detected by a shift in electrophoretic mobility, and a concurrent activation of Raf-1-associated kinase activity, our results suggest that Raf-1 activity may also increase markedly throughout G_1. Therefore, the IL-2-R interaction serves two functions: to increase the concentration of Raf-1 protein and to activate the Raf-1-associated kinase activity.

The four- to sixfold increase in Raf-1-associated kinase activity that accumulates during G_1 is reminiscent of the IL-2-R interaction threshold, which is crucial for IL-2-induced G_1 progression to DNA replication. The commitment on the part of the T cell to enter S phase and subsequent mitosis is a quantal event that depends upon ~10,000 IL-2-R interactions requiring at least 5 to 6 h of continuous IL-2 stimulation (12). Since fully activated primary human T cells express only ~1,000 high-affinity IL-2-R per cell, this time interval depends upon the rate of new receptor synthesis, which we have calculated to be ~35 receptors per cell per min (59). Although the earliest biochemical events in IL-2-R signaling still remain obscure, recent reports have suggested that tyrosine-specific kinase activity may be involved. The p75 subunit of the IL-2-R (1) and intracellular proteins of ~130,000, 100,000, 80,000, and 69,000 Da are rapidly phosphorylated on tyrosine residues after IL-2 binding (35, 54). Therefore, the Raf-1 kinase may be phosphorylated and activated by this signaling pathway, either directly or indirectly soon after IL-2-R binding, by analogy to the phosphorylation and activation of Raf-1 by the PDGF-β receptor (41, 42).

We have not assessed tyrosine phosphorylation of Raf-1 in response to stimulation by IL-2 in primary human T cells. However, tyrosine phosphorylation of Raf-1 in lymphoid and myeloid cells has been examined by several groups. Tyrosine phosphorylation of Raf-1 was not detected following colony-stimulating factor 1 stimulation of a macrophage line (2). More germane to our study, IL-2 stimulation of the murine T-cell line, CTLL-2, was reported to induce phosphorylation of Raf-1, with 50% of the new phosphate occurring on tyrosine (64a). Similarly, IL-3 and granulocyte macrophage colony-stimulating factor have been reported to induce a significant level of tyrosine phosphorylation in murine myeloid cell lines (12a). In a human myeloid line, however, less than 2% of the increase in phosphorylation in response to IL-3 and granulocyte macrophage colony-stimulating factor was on tyrosine (29). Taken together, these results suggest that at least some of the increased phosphorylation of Raf-1 in response to stimulation of the IL-2/IL-3 family of receptors (28) is attributable to phosphotyrosine.

From the results described, further experiments exploring the role of Raf-1 in IL-2-promoted G_1 progression, G_1/S-phase transition, as well as in DNA synthesis, are now warranted. A search for Raf-1 substrates important for cell cycle progression should be undertaken. Obvious candidates include the retinoblastoma gene product, Rb (9, 16, 20), and the *cdc2* catalytic serine- and threonine-specific kinase, p34 (17, 21, 31), proteins which are known to be phosphorylated during G_1 and at the G_1/S-phase boundary.

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