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Interleukin-2-dependent phosphorylation of the retinoblastoma-susceptibility-gene product p110–115RB in human T-cells

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The state of phosphorylation of the retinoblastoma-susceptibility gene product, p110–115RB, is thought to have fundamental importance in controlling the progression of the cell through the cell cycle. We have studied RB phosphorylation in human T-cells in the context of T-cell activation, stimulated by phytohaemagglutinin (PHA) and interleukin-2 (IL-2). We show that, of the signals associated with T-cell activation, only signals that directly lead to movement into S phase of the cell cycle are capable of stimulating RB phosphorylation. Cyclosporin A (CsA), a potent inhibitor of IL-2 synthesis and cellular proliferation, blocked RB phosphorylation, and this was recovered with exogenous IL-2, indicating a direct involvement of IL-2 in controlling RB phosphorylation. We found that PHA did not stimulate RB phosphorylation within 10 h of treatment, but IL-2 could effectively stimulate RB phosphorylation within 2 h, and this approached a maximum within 8–10 h of IL-2 treatment. Further, by using actinomycin D to inhibit new gene transcription following IL-2 stimulation, we found that early-cell-cycle phosphorylation of RB required IL-2-stimulated gene transcription. From these data we conclude that, in human T-cells, RB phosphorylation is not directly associated with T-cell receptor-mediated events, but requires the interaction of IL-2 and new gene transcription following IL-2 stimulation.

INTRODUCTION

The understanding of events which are necessary for progression through the eukaryotic cell cycle has been the focus of much research. One event that is thought to have fundamental importance is the phosphorylation of a nuclear protein, the product of the retinoblastoma-susceptibility gene, p110–115RB. The RB gene was one of the first to be characterized as an anti-oncogene, with the wild-type gene functioning as a suppressor of cell cycle progression [1,2]. Studies have shown that deletions or mutations of the RB gene are associated with retinoblastomas, osteosarcomas, small-cell lung carcinomas and some breast cancers [3]. In addition, the transforming proteins of adenovirus (E1A), polyomavirus (SV40) and papillomavirus (E7) all form stable complexes with the RB protein, and mutations in these proteins which eliminate RB complex formation also eliminate their normal transforming ability [4–6]. Thus the elimination of functional RB protein by either deletion/mutation or direct binding to certain viral proteins leads to uncontrolled cell growth. The RB protein has also been shown to undergo cell-cycle-dependent phosphorylation, with the hypophosphorylated form existing in the G_0/G_1 phase [7–10]. As the cell progresses through the cell cycle, RB is heavily phosphorylated on serine residues, with maximal phosphorylation occurring in S phase. Because the SV40 large T antigen binds only to the hypophosphorylated form of RB [5] and because the activity of large T antigen is associated with cellular transformation, it is this hypophosphorylated form of RB which is thought to act as a suppressor of cellular proliferation. This suggests that RB phosphorylation may be an event which releases the cell from a progressive block imposed by the hypophosphorylated protein, thus allowing progression through the cell cycle.

Because of the role that the RB protein may have in controlling cell growth, it is important to understand how mitogens and growth factors affect RB phosphorylation during the early phases of the cell cycle. T-cells provide a unique model for the study of factors affecting cellular proliferation. Generally speaking, there are two clearly separable events controlling T-cell growth: the generation of T-cell competence, stimulated by signal transduction through the T-cell receptor, and cell cycle progression, stimulated by interleukin-2 (IL-2). The first step, called T-cell activation, is a Ca^{2+}-dependent event resulting in a state of competence characterized by the increased transcription of a number of genes which are mandatory for subsequent cell cycle progression [11]. Among those genes which are important for T-cell growth are the principal T-cell growth factor IL-2 and the IL-2 receptor α subunit (IL-2Rα) [12,13]. Up-regulation of IL-2/IL-2Rα and generation of the high-affinity IL-2 receptor occurs in G_0/G_1, and progression through the cell cycle (G_1 to S) is highly dependent on the signal transduced by the IL-2 and IL-2R interaction [11]. In this report we examine the effect of mitogen signal transduction on the phosphorylation of RB in human T-cells, in the context of mitogen-induced activation of T-cells as well as cell cycle progression regulated by IL-2.

MATERIALS AND METHODS

Tissue culture media and reagents

Normal RPMI-1640 medium, 100 × penicillin/streptomycin, and 100 × glutamine were purchased from Mediatech, Washington, DC, U.S.A. Phosphate-free RPMI-1640 medium was purchased from Advanced Biotechnologies, Columbia, MD, U.S.A. Fetal calf serum was purchased from Gibco Laboratories, Life Technologies Ind., Grand Island, NY, U.S.A. Cyclosporin A

Abbreviations used: CsA, cyclosporin A; IL-2, interleukin-2; mAb, monoclonal antibody; PAS, Protein A–Sepharose; PHA, phytohaemagglutinin; PMA, phorbol 12-myristate 13-acetate; RB, retinoblastoma-susceptibility gene product; SV40, simian virus.

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(CsA) was purchased from Sandoz, East Hanover, NJ, U.S.A. Ionomycin was purchased from Calbiochem, La Jolla, CA, U.S.A. Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Phytohaemagglutinin (PHA) was purchased from Wellcome Diagnostics, Temple Hill, Dartford, U.K. Recombinant human IL-2 was purchased from Hoffmann–LaRoche, Nutley, NJ, U.S.A. All other chemicals and reagents were from standard vendors.

Lymphocyte preparation and cell culture

Human peripheral blood mononuclear cells were obtained by leukapheresis of normal volunteers at the blood bank of the National Institutes of Health. After density sedimentation of the mononuclear cells with lymphocyte separation medium (Organon Teknika, Durham, NC, U.S.A.), the lymphocytes were purified by counterflow centrifugal elutriation as previously described [14], except that pyrogen-free phosphate-buffered saline was used in the elutriation procedure. The purified lymphocytes were 75–85% T-cells, as determined by a positive reaction towards an antibody to the CD3 component of the T-cell receptor ($CD3^+$). The remaining cells consisted of 5–15% B-cells and 5–7% null cells [14]. These lymphocyte preparations contained $< 1\%$ monocytes, as assessed by non-specific esterase staining.

T-cells were cultured in the presence of PHA, or activated with PHA and cultured in the presence of IL-2. Lymphocytes were cultured for up to 4 days in the presence of PHA (1 μg/ml) at a density of 1×10^6 cells/ml in RPMI-1640 medium supplemented with 10% fetal calf serum, glutamine and antibiotics, and analysed as described below. To prepare activated lymphocytes for stimulation with IL-2, newly isolated T-cells were cultured with PHA for 48 h. Cells were then recovered by centrifugation (1000 g, 10 min) and washed twice with low-pH RPMI-1640 medium by bubbling CO_2 through the cell suspension. Cells were resuspended in RPMI-1640 medium supplemented with 1% fetal calf serum, glutamine and antibiotics, and cultured for 24 h to establish a G_1-enriched population. Activated T-cells were recovered by centrifugation (1000 g, 10 min), washed twice with RPMI-1640 medium plus CO_2 and once with RPMI 1640 medium alone. T-cells were resuspended at 1×10^6 cells/ml in RPMI-1640 medium supplemented with 10% fetal calf serum, glutamine and antibiotics, and cultured in the presence of recombinant purified IL-2 (0.01–1 μg/ml) for 1–72 h and analysed as described below.

CsA, ionomycin and PMA treatments were performed on newly isolated T-cells suspended at 1×10^6/ml in RPMI-1640 medium supplemented with 10% fetal calf serum, glutamine and antibiotics. Cells were treated with CsA (1 μg/ml) or ionomycin (0.5 μM) for 30 min at 37 °C, followed by the addition of PHA (1 μg/ml) or PMA (100 ng/ml). T-cells were then cultured for 72 h at 37 °C and analysed as described below. Actinomycin D treatments were performed on PHA-activated and G_1-enriched T-cells by preincubation for 30 min at 37 °C with 1 μg of actinomycin D/ml followed by IL-2 stimulation, cell labelling, lysis and immunoprecipitation as described below.

For all experiments described above, cell proliferation was monitored by [^{3}H]thymidine incorporation [15].

Cell labelling, lysis and immunoprecipitation

Cultured T-cells were washed twice with phosphate-free RPMI-1640 medium and resuspended at 35×10^6 cells/ml in phosphate-free RPMI-1640 medium supplemented with 5% dialysed fetal calf serum, glutamine and antibiotics, and containing 0.5 mCi of [^{32}P]P_i/ml (Dupont/New England Nuclear, Boston, MA, U.S.A.). Labelling was carried out for 90 min at 37 °C. Labelled cells (35×10^6) were recovered by centrifugation at 12000 g for 1 min and lysed with 1 ml of extraction buffer (10 mM-Tris, pH 7.6, 1% Triton X-100, 100 mM-NaCl, 5 mM-EDTA, 30 mM-sodium pyrophosphate, 50 mM-NaF, 50 μM-sodium vanadate, 1 mM-phenylmethanesulphonyl fluoride and 0.1% BSA) at 4 °C for 1 h. Lysates were clarified by centrifugation at 12000 g for 20 min at 4 °C. A 1 ml sample of clarified lysate (approx. 250 μg of protein, as determined by the method of Bradford [16]), was incubated with 2 μg of a specific monoclonal antibody, MAb245, against RB protein (Bethesda Research Laboratories, Life Technologies Inc., Gaithersburg, MD, U.S.A.) for 1 h at 4 °C. Then, 100 μl of 20% Protein A-conjugated Sepharose (PAS; Pharmacia LKB Biotechnologies, Uppsala, Sweden) in extraction buffer was mixed with 5 μg of anti-(mouse IgG) polyclonal rabbit sera (Kirkegaard & Perry, Gaithersburg, MD, U.S.A.) and incubated for 1 h at 4 °C to give anti-(mouse IgG)–PAS. Anti-(mouse IgG)–PAS was mixed with antibody-containing lysate and incubated for 1 h at 4 °C. Immunoprecipitates were washed three times with extraction buffer and three times with extraction buffer without BSA. Immunoprecipitated proteins were eluted from the Sepharose pellet with 50 μl of $2 \times$ SDS loading buffer and subjected to SDS/PAGE [17] followed by autoradiography or immunoblotting. Quantification of autoradiographs was performed using an LKB Ultrascan Laser Densitometer.

RESULTS

RB phosphorylation in activated T-cells

Several investigators have demonstrated that RB is phosphorylated in a cell-cycle-dependent manner. However, little has been done to examine the effect of mitogens on RB phosphorylation, especially in the context of cellular activation towards the proliferative state and the effect of promoters of cell cycle progression on RB phosphorylation in early G_1 phase. In order to more fully examine the effect that mitogen stimulation of T-cells has on the modulation of RB phosphorylation, we treated T-cells with PHA or IL-2, labelled the cells with [^{32}P]P_i and examined the phosphorylation state of the RB protein using immunoprecipitation and electrophoretic analysis. Analysis of cell lysates in this manner with MAb245 and comparison with control antibody preparations substantiated the specificity of MAb245 for Rb (results not shown). As can be seen (Fig. 1), RB phosphorylation was stimulated by PHA, peaking at 48 h after treatment with PHA, during the S phase of the cell cycle. RB phosphorylation at 48 h was increased 4-fold over that seen at 24 h, and was approx. 2-fold greater than that seen at 72 or 96 h (Fig. 1*a* and densitometric analysis in Fig. 1*c*). This is shown by increased ^{32}P incorporation as well as by increases in the slower-migrating P115RB species (most easily seen in Fig. 4*b*), which is indicative of RB phosphorylation [9,18]. Analysis of the levels of RB protein in these T-cell preparations by immunoblotting revealed little change in the amount of P110–115RB in the 96 h after treatment with PHA (results not shown). Phosphorylation of RB species occurred in two distinct stages, with the first detectable phosphorylation occurring on the P112–115 species at 24 h after PHA stimulation (Fig. 1*a*). Later, as the level of RB phosphorylation increased as the cells progressed through S phase, more complete phosphorylation of p110–115RB could be seen (48 h, Fig. 1*a*). The molecular mass shift of phosphorylated RB from 110 kDa to 115 kDa, as well as the appearance of phosphorylated p110RB, can be most easily seen in Figs. 2(*a*) and Fig. 4(*b*). This is indicative of multiple phosphorylation sites and has been substantiated elsewhere [19]. These results support the observations of others reporting PHA-stimulated phosphorylation of RB in T-cells [9,18].

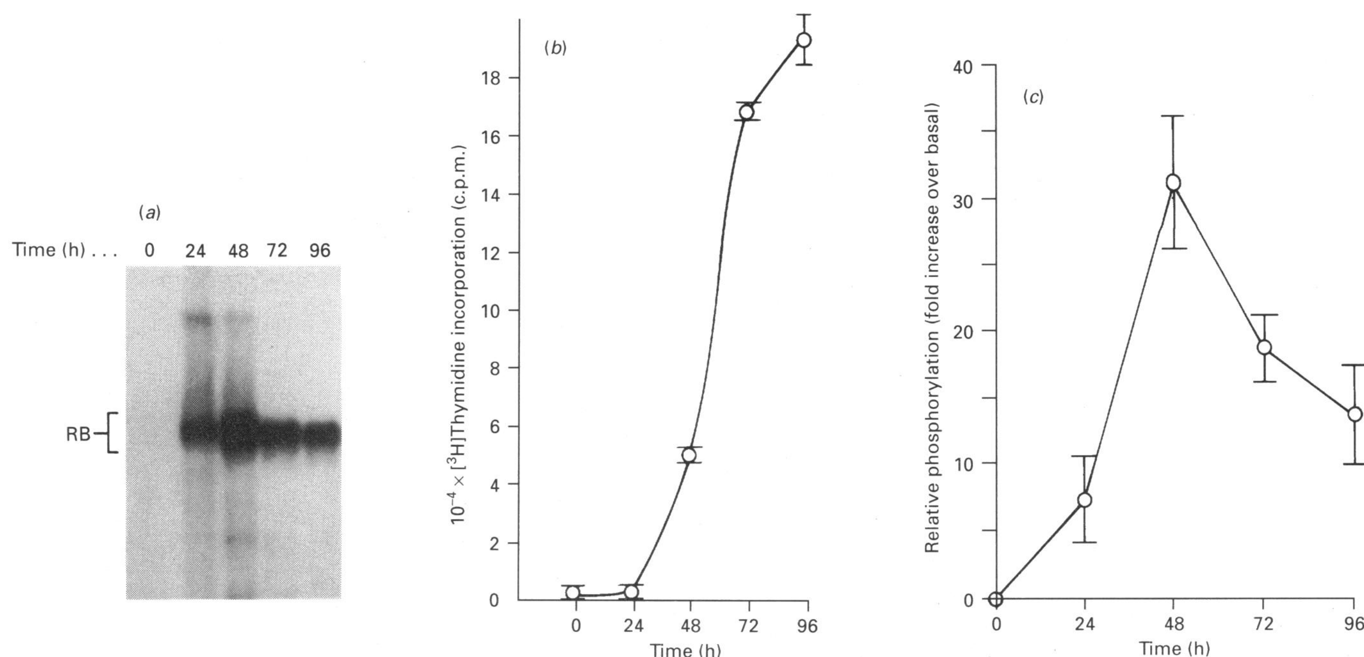

Fig. 1. Anti-RB immunoprecipitates from T-cells treated with PHA

(*a*) Mitogen-treated cells were labelled with [^{32}P]P$_i$ and lysed, and equal amounts of radioactivity were immunoprecipitated with anti-RB antibody, electrophoresed and subjected to autoradiography. Lanes are labelled with time (h) after treatment with PHA. (*b*) [^{3}H]Thymidine incorporation of T-cell preparations used in (*a*). (*c*) Densitometric analysis of PHA-stimulated RB phosphorylation. Basal phosphorylation is the minimum level of RB phosphorylation observed in untreated cells. Data presented for [^{3}H]thymidine incorporation and the relative phosphorylation of RB as determined by densitometric analysis are expressed as the means ± S.D. of three independent experiments.

Interaction of lectins with the T-cell receptor in the presence of accessory cells produces a series of cellular signals which eventually lead to cell growth. The earliest signals resulting in the development of T-cell competence involve the activation of members of the protein kinase C family of protein serine kinases, as well as Ca^{2+} influx [20,21]. We therefore analysed the effect that modulators of these activities have on controlling the cellular levels of phosphorylated RB in quiescent T-cells treated with PHA for 72 h. To mimic T-cell activation signals we used ionomycin, a Ca^{2+} ionophore, and PMA, an activator of protein kinase C. Ionomycin alone did not promote a proliferative response, as judged by thymidine uptake (Fig. 2*b*), and stimulated RB phosphorylation only slightly above that in untreated cells (less than 1% of maximum phosphorylation seen in PHA-treated cells; Fig. 2*c*). Ionomycin in combination with PMA resulted in approx. 50% of maximum PHA-stimulated RB phosphorylation and approx. 50% of maximum thymidine uptake, and in combination with PHA resulted in RB phosphorylation and thymidine uptake equal to that seen in cells treated with PHA alone (Figs. 2*b* and 2*c*). PMA alone resulted in 20% of maximum PHA-stimulated RB phosphorylation and 18% of maximum thymidine uptake (Figs. 2*b* and 2*c*). When PHA or ionomycin was added in combination with PMA, RB phosphorylation and thymidine uptake were restored to approx. 50% of the PHA-stimulated maximum (Figs. 2*b* and 2*c*). Taken together, these data demonstrate that the degree of RB phosphorylation is directly correlated with the proliferative response of these cells to agents which mimic the effects of T-cell receptor activation. Of these signals it is clear that the most effective is PHA, which stimulates T-cell receptor signalling directly. Ca^{2+} influx alone has little effect, based on the results seen with ionomycin alone; however, if added in combination with PMA in an attempt to reconstitute competence development by protein kinase C activation and Ca^{2+} influx, an almost 10-fold increase in RB phosphorylation and thymidine uptake was seen (Figs. 2*a* and 2*c*) compared with ionomycin alone. The PMA/ionomycin combination was also twice as effective at stimulating RB phosphorylation and three times more effective at promoting thymidine uptake than PMA alone. The addition of PHA to PMA-treated cells reconstituted the Ca^{2+} influx and produced results similar to the PMA+ionomycin treatment (Fig. 2). The addition of PHA to ionomycin+PMA-treated cells did not promote any further increase in RB phosphorylation or thymidine uptake, suggesting that only these two signal-transducing pathways are necessary to promote RB phosphorylation and cellular proliferation. In all experiments, the addition of PMA inhibited the ability of PHA to stimulate RB phosphorylation or thymidine uptake to the levels seen with PHA alone. From our experience and that of others [22,23], when working with human T-cells, extensive incubation with the very slowly degraded phorbol ester (PMA) results in the depletion of cellular protein kinase C levels. In the experiments described, this depletion will have resulted in an inability to respond to PHA through the protein kinase C pathway, and will express itself as a less than maximal thymidine incorporation. This is evident in Fig. 2, in that PHA+PMA or PHA+PMA/ionomycin did not result in RB phosphorylation or thymidine uptake equal to that seen with PHA alone or PHA+ionomycin respectively. In this experiment the addition of PHA will promote Ca^{2+} influx or protein kinase C activation that has not been stimulated by the addition of ionomycin or PMA. In this regard, PHA stimulated Ca^{2+} influx in the PMA+PHA-treated cells, and protein kinase C activation in the ionomycin+PHA-treated cells, giving results which were equivalent to those obtained by stimulation of both pathways by ionomycin and PMA. Taken together, these results suggest that RB phosphorylation, and therefore cellular proliferation, is optimal when both the Ca^{2+} influx and protein kinase C signalling pathways are activated upon T-cell competence development.

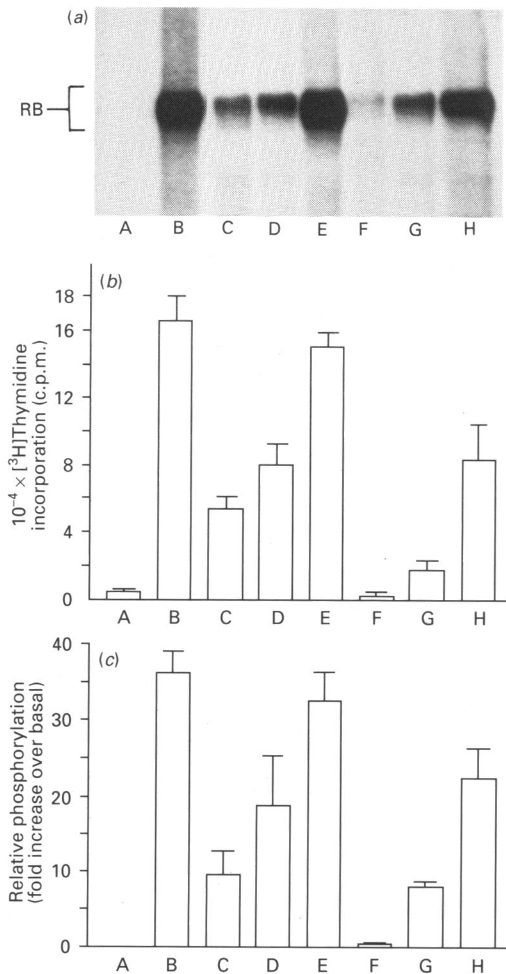

Fig. 2. Effects of PHA, ionomycin and PMA on RB phosphorylation associated with the generation of T-cell competence

(*a*) Cells were treated as described below, cultured for 72 h, labelled with [^{32}P]P$_i$ and lysed, and equal amounts of radioactivity were immunoprecipitated with anti-RB antibody, electrophoresed and subjected to autoradiography. (*b*) [^{3}H]Thymidine incorporation of preparations in (*a*). (*c*) Densitometric analysis of the relative level of RB phosphorylation in cells treated as described in (*a*). Basal phosphorylation is the minimum level of RB phosphorylation observed in untreated cells. Data presented for [^{3}H]thymidine incorporation and the relative phosphorylation of RB as determined by densitometric analysis are expressed as means ± S.D. of three independent experiments. Treatments used: A, control; B, PHA; C, ionomycin + PMA + PHA; D, ionomycin + PMA; E, ionomycin + PHA; F, ionomycin; G, PMA; H, PMA + PHA.

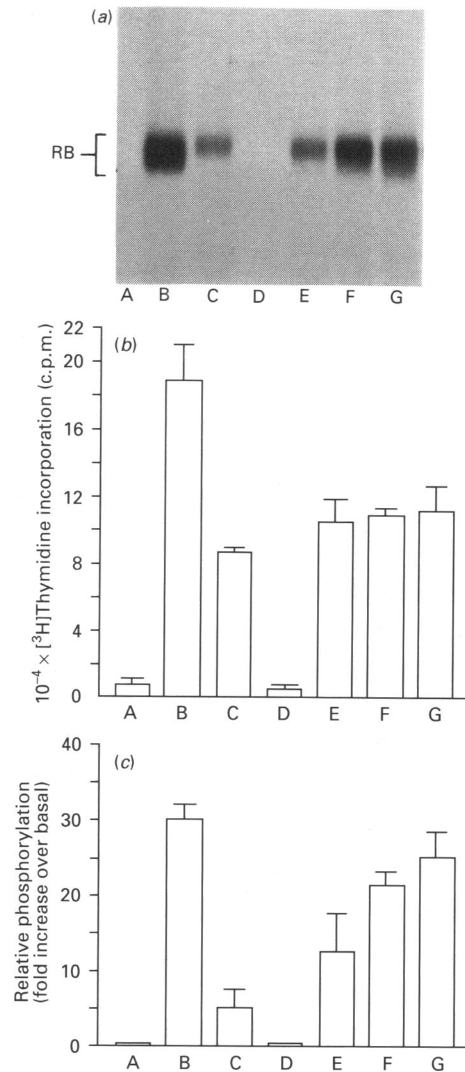

Fig. 3. Inhibition of RB phosphorylation in PHA-treated T-cells by CsA and recovery of phosphorylation with exogenous IL-2

(*a*) Cells were treated as described below, cultured for 72 h, labelled with [^{32}P]P$_i$ and lysed, and equal amounts of radioactivity were immunoprecipitated with anti-RB antibody, electrophoresed and subjected to autoradiography. (*b*) [^{3}H]Thymidine incorporation of preparations in (*a*). (*c*) Densitometric analysis of the relative level of RB phosphorylation observed in cells treated as described in (*a*). Basal phosphorylation is the minimum level of RB phosphorylation observed in untreated cells. Data presented for [^{3}H]thymidine incorporation and the relative phosphorylation of RB as determined by densitometric analysis are expressed as means ± S.D. of four independent experiments. Treatments used: A, control; B, PHA; C, CsA + PHA; D, CsA; E, CsA + PHA + IL-2 (10 units/ml); F, CsA + PHA + IL-2 (100 units/ml); G, CsA + PHA + IL-2 (1000 units/ml).

Effect of CsA on phosphorylation of RB

Analysis of the proliferative state of T-cell preparations treated with ionomycin, PMA or PHA (Fig. 2) showed that the phosphorylation of RB was directly correlated with movement into the S phase of the cell cycle (Figs. 2*b* and 2*c*). Mitogen-induced proliferation of human T-cells is highly dependent on the synthesis and secretion of IL-2, the generation of high-affinity IL-2 receptors and the subsequent signal transduced by the IL-2–IL-2 receptor interaction [11–13]. CsA has been shown to inhibit the T-cell mitogenic response in a complex manner which involves, among other things, the transcriptional inhibition of IL-2 synthesis [24]. In order to observe the effect that exogenous IL-2 may have on RB phosphorylation in PHA-activated T-cells, we used CsA to inhibit autocrine IL-2 production, and then observed whether CsA could inhibit PHA-induced RB phosphorylation, and whether this was due to inhibition of IL-2 synthesis (and therefore could be stimulated by exogenous IL-2). RB phosphorylation was inhibited by over 90% upon addition of CsA, and stimulated in a dose-dependent manner by adding exogenous IL-2 (Figs. 3*a* and 3*c*). Inhibition of RB phosphorylation by CsA was directly correlated with a decreased proliferative response, as determined by [^{3}H]thymidine uptake. The addition of exogenous IL-2, while directly stimulating RB phosphorylation, was not sufficient to overcome the complex proliferative block imposed by CsA (Figs. 3*b* and 3*c*). These data suggest that IL-2 is directly responsible for stimulating RB

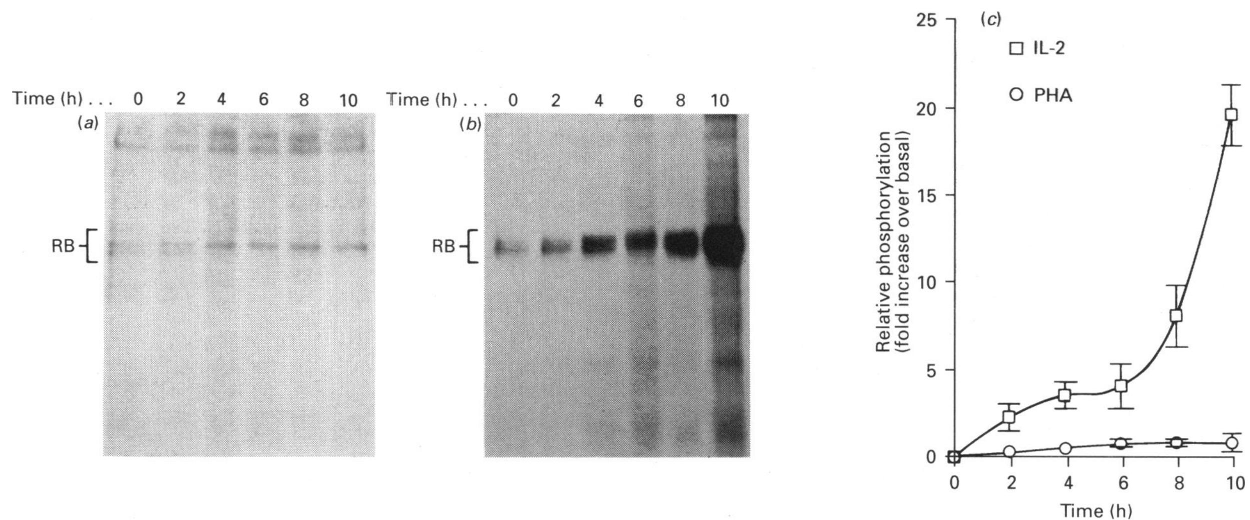

Fig. 4. Short-term phosphorylation of RB in the presence of PHA or IL-2

(*a*) Resting T-cells were treated with or without PHA (1 μg/ml) and then radiolabelled, and equal amounts of radioactivity of total cell lysate were assayed for phosphorylated RB protein as described. (*b*) PHA-activated T-cells were G_1-enriched and treated with or without IL-2 (1 μg/ml), radiolabelled and equal amounts of radioactivity of cell lysate were assayed for RB phosphorylation as previously described. The concentration of PHA and IL-2 was maintained during labelling and the times given for both (*a*) and (*b*) are inclusive of the labelling period. (*c*) Densitometric analysis of the relative level of RB phosphorylation observed in cells treated with PHA (○) as described in (*a*) and with IL-2 (□) as described in (*b*). Basal phosphorylation is the minimum level of RB phosphorylation observed in untreated cells. Data presented for densitometric analysis are expressed as means ± S.D. of three independent experiments.

phosphorylation and that this phosphorylation is not simply the result of attaining a state of cellular growth, as IL-2 stimulated RB phosphorylation without significant stimulation of cellular proliferation in the presence of CsA.

Effect of actinomycin D on IL-2 stimulated RB phosphorylation

In order to fully substantiate the direct involvement of IL-2 in stimulating RB phosphorylation, we analysed the short-term phosphorylation state of RB in response to PHA or IL-2. As expected, PHA was not capable of significantly stimulating RB phosphorylation within 10 h (Figs. 4*a* and 4*c*) in resting T-cells. IL-2, however, stimulated phosphorylation of the RB protein by 2-fold within 2 h, and this progressed until maximum RB phosphorylation (approx. 20-fold over that at time zero) was seen within 10 h of IL-2 treatment in preactivated quiescent T-cells (Figs. 4*b* and 4*c*). IL-2 stimulation of RB phosphorylation was further substantiated by an increase in the slower-migrating forms of RB upon IL-2 treatment (Fig. 4*b*), which is indicative of phosphorylated RB and is not seen in cells treated with PHA (Fig. 4*a*). Immunoblot analysis of PHA- or IL-2-treated samples revealed little change in total RB protein production within 10 h of addition of PHA or IL-2 (results not shown).

Fig. 3 showed that IL-2 was capable of restoring RB phosphorylation in the presence of CsA, and Fig. 4 showed direct IL-2 stimulation of RB phosphorylation in preactivated T-cells. These data clearly indicate that IL-2 is directly responsible for stimulating RB phosphorylation in T-cells as these cells progress through the cell cycle. IL-2 has been shown to stimulate the increased transcription of a number of gene products [11]. In an attempt to determine whether new gene transcription is necessary in order to stimulate RB phosphorylation after IL-2 treatment, we preincubated T-cells with or without actinomycin D, a potent RNA polymerase inhibitor, incubated the cells with or without IL-2 (1 μg/ml) for 2 h and then labelled these cells for 2 h in the presence or absence of IL-2. Densitometric quantification of autoradiographs of immunoprecipitated phosphorylated RB from these cells showed that actinomycin D inhibited IL-2-stimulated RB phosphorylation by 80% at 4 h (Table 1). IL-2 alone more than doubled RB phosphorylation within 4 h (Table 1). Three separate experiments were performed and each gave equivalent results. The addition of actinomycin D blocked IL-2-stimulated RB phosphorylation (Table 1). This suggests that mitogen stimulation of RB phosphorylation in the early G_1 phase of the cell cycle requires new gene transcription and is not directly modulated by early IL-2-receptor-mediated events.

Table 1. Effect of actinomycin D on IL-2-stimulated RB phosphorylation

Activated T-cell samples were pretreated for 30 min with or without actinomycin D (1 μg/ml) then incubated with or without IL-2 (1 μg/ml) for 2 h, followed by radiolabelling with $[^{32}P]P_i$ for 2 h in the presence or absence of IL-2, resulting in a total incubation time of 4 h. Samples were lysed and equal amounts of radioactivity of total cell lysates were immunoprecipitated with anti-RB antibody and analysed as described in the Materials and methods section. The data represent densitometric measurements of RB protein autoradiographs [absorbance units (a.u./mm)], and are also expressed as percentage stimulation of RB phosphorylation above basal level by IL-2 in 4 h. The experiment was repeated three times with equivalent results.

Treatment	Relative phosphorylation (a.u./mm)	Stimulation by IL-2 (%)
Control	0.473	–
IL-2	1.259	166
Actinomycin D	0.370	–
Actinomycin D-IL-2	0.499	35

DISCUSSION

The use of human T-cells allowed us to study early G_1 phase phosphorylation of RB in the context of competence development (activation) and subsequent cell cycle progression. Thus we have established that RB phosphorylation is directly associated with the development of a proliferative state, and is dependent on

lymphokine stimulation. From the data presented it is clear that in T-cells the stimulation of RB phosphorylation is dependent on the action of IL-2, can be observed within 2 h of treatment and appears to require new gene products for its modulation. It may be argued that RB phosphorylation is simply the result of attaining a proliferative state and is not responsible for driving the cell towards that state. However, studies on the proliferation of T-cells induced by IL-2 have shown that a requirement for progression into S phase is persistent receptor occupancy (G. A. Evans, L. M. Wahl & W. L. Farrar, unpublished work). Removal of IL-2 even after 4 h of receptor stimulation will result in regression back to early G_1 phase and elimination of cellular proliferation. Thus it seems unlikely that RB phosphorylation is simply the result of moving into a state of cellular proliferation, as stimulation can be seen very early in G_1 (Fig. 4), before the cell is committed to cell cycle progression towards mitosis. Furthermore, IL-2-driven RB phosphorylation can be seen while the cell is held in a proliferative block imposed by CsA (Fig. 3), indicating that phosphorylation precedes and is not dependent on the G_1 to S phase transition.

The control of RB phosphorylation may involve a number of mechanisms. It has been shown that p34CDC2 kinase complex can phosphorylate RB *in vitro* on the same sites which are phosphorylated *in vivo* [19]. It is apparent that certain proteins when complexed with p34CDC2 affect its substrate specificity. Two general complexes have been described: a mitotic complex consisting of p34CDC2 and p62/cyclin, and a complex which is most active during interphase containing p34CDC2 and p60/cyclin [25]. Because the activation of p34CDC2 kinase activity is associated with the production of cyclin proteins and cyclin association [25–27], a limiting factor in RB phosphorylation may be p60/cyclin production during G_1 phase. This would suggest a distinct function for an active p34CDC2 kinase complex in early G_1 phase of the cell cycle in addition to its described mitotic histone H1 kinase activity. Because cyclin proteins are produced and degraded in a cell-cycle-dependent manner, the requirement for new cyclin production in order to activate p34CDC2 in G_1 phase may explain the need for new gene transcription in order to observe mitogen-stimulated RB phosphorylation.

An attractive hypothesis for the role of RB phosphorylation resulting from work with SV40 large T antigen suggests that the hypophosphorylated RB protein functions as a suppressor of cell cycle progression [5,28]. In this regard, early cell cycle phosphorylation of RB would be a requirement for cellular proliferation. This is supported by the work presented here, with the observation of lymphokine-directed phosphorylation of RB within 2 h of treatment and its association with the development of a cellular proliferative state. By understanding the complex series of events which govern the control of RB phosphorylation in early G_1 phase of the cell cycle, we may gain a better understanding of the mechanisms which control mitogen-stimulated cell growth. This must centre on identifying the kinases and phosphatases which undoubtedly directly alter RB phosphate content.

Understanding the direct role that RB protein has in controlling cell cycle progression is an important goal. However, the exact role of RB in this process will not be determined until a specific cellular function for hypo- or hyper-phosphorylated RB can be identified. This will be dependent on the identification and determination of the function of complex associated proteins and on deducing the exact mechanisms which work to control the state of RB protein phosphorylation.

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