We have determined how the phosphorylation of the retinoblastoma family (pRb, p107, and p130) is governed in individual cell cycle phases of Daudi B-cells during cell cycle exit triggered by α-interferon (α-IFN). α-IFN causes dephosphorylation of pRb and loss of p130 phosphorylated Form 3. However, the change in p130 phosphorylation in response to α-IFN occurs before dephosphorylation of pRb is complete because loss of p130 Form 3 occurs throughout the cell cycle prior to complete arrest in G1, whereas pRb is dephosphorylated only in G1. In contrast, p107 is dephosphorylated and is then depleted from cells as they exit the cell cycle, p130, predominantly in Form 1, and hypophosphorylated pRb bind an E2F DNA binding site; p130 complexes E2F-4, whereas pRb binds both E2F-4 and E2F-1. The phosphorylated forms of E2F-4 that bind to the E2F DNA site are different from hyperphosphorylated E2F-4, which predominates in primary hemopoietic cells in G0. We conclude that although cell cycle arrest induced by α-IFN may be mediated in part by formation of a complex containing p130 and E2F-4, α-IFN does not induce hyperphosphorylation of E2F-4, which characterizes primary hemopoietic cells in G0.

The protein encoded by the RB gene, pRb, is a nuclear phosphoprotein that is involved in regulating progression through G1 into S phase (reviewed in Refs. 1 and 2). It is hypophosphorylated in G0 and is phosphorylated progressively as quiescent cells progress through G1, reaching a hyperphosphorylated form at the G1-S border. Hyperphosphorylation is thought to inactivate pRb, allowing cells to enter S phase. Microinjection of an excess of purified, hypophosphorylated pRb up to mid G1 results in reversible cell cycle arrest (1). Thus, pRb may only be required up to a point some time in mid- or late G1, and there is evidence that pRb forms part of the mechanism controlling the G1 restriction point (reviewed in Ref. 2).

pRb is thought to be phosphorylated by a succession of cyclin-dependent kinases (cdks), which are activated at different times during G1; for example, cdk6 and cdk4 are activated in early or mid-G1, followed by cdk2 in late G1 (reviewed in Ref. 3). pRb then remains hyperphosphorylated throughout the rest of the cell cycle and is dephosphorylated to a hypophosphorylated state during mitosis, possibly by a type 1 serine-threonine phosphatase (4). During continuous proliferation, cells in early G1 (G1A) contain partially phosphorylated pRb (5), which becomes hyperphosphorylated during late G1 (G1B) (5, 6). The hypophosphorylated form is present in cells that are quiescent or arrested in early G1 by the action of negative growth factors, such as α-interferon (α-IFN) (7, 8) or transforming growth factor-β (9–11) and in terminally differentiated cells (reviewed in Ref. 12).

The pRb protein shares regions of homology with two other proteins, p107 and p130, and there is evidence that all three can regulate cell proliferation (15–17). Like pRb, they are phosphoproteins that have consensus sites for phosphorylation by cdks. In T98G cells that are entering the cell cycle for the first time from G0, p130 is phosphorylated to Form 3 during mid G1, which coincides with the phosphorylation of pRb and the induction of cyclin D1 (13). Exit from the cell cycle caused by transforming growth factor-β results in the accumulation of p130 Form 1, and exit caused by serum starvation results in the accumulation of Form 2 (14). p107 is not phosphorylated as cells enter G1 from G0 but is phosphorylated from S phase onward (18). Cyclin A-cdk2 and cyclin E-cdk2 associate with both p107 and p130 in vitro (13, 16, 19–21), but it is not clear whether cdk2 phosphorylates either in vivo. In contrast, there is evidence that p107 can be phosphorylated in cells in culture by cyclin D1-cdk4 (18).

The hypophosphorylated form of pRb will bind transcription factors, such as E2F, which regulate genes encoding proteins that are required for cell cycle progression (reviewed in Ref. 22). It is thought that this is also how p130 and p107 function (reviewed in Ref. 23). The E2F transcription factor is a heterodimer of an E2F protein together with a member of the DP family (22). The major E2F complex in human primary, CD34+ hemopoietic progenitor cells (24) and in quiescent primary T-cells (17), B-cells, and monocytes is composed of E2F-4, together with DP-1 bound to p130 (25), but in cycling cells, E2F-4 binds both pRb and p107 (26) or p107 alone (24). Indeed, it has been suggested that the presence of the p130-E2F-4-DP-1 complex defines G0 (27), because this complex is not present in proliferating cells. Different members of the pRb family have some specificity for different E2Fs: E2F-1, -2, and -3 bind pRb rather than p107 (28, 29), whereas E2F-4 and -5 bind p130 and p107 in preference to pRb (17, 30–32). The functional consequences are that artificially elevating the level of E2F-1 preferentially overcomes cell cycle arrest by pRb, and E2F-4/DP-1 overcomes arrest caused by p130 (30, 33, 34). Furthermore, expression of E2F-1 (35, 36), E2F-2, or E2F-3 (37) alone or...
E2F-4 in combination with DP-1 (30, 34) causes quiescent cell lines to progress through G1 into S phase.

Different genes are induced preferentially by different E2Fs (37); for example, the genes for dihydrofolate reductase and thymidine kinase by E2F-2, for cyclin A and cdc2 by both E2F-1 and -2, and for cdk2 by E2F-3. Thus individual genes with specific E2F binding sites in their promoters are regulated by specific E2Fs, and so regulation of individual E2F-DP combinations by different pRb family members provides a mechanism for turning the transcription of specific genes on or off at specific times during entry into the cell cycle. Indeed, pRb, p107, and p130 have been shown to bind individual E2Fs at different stages during entry into the first cell cycle (Ref. 26 and references therein), and so transcription controlled by specific E2Fs could be repressed at different times. Such coordinated activation of transcription is required for cell proliferation because transfection of either pRb, p107, or p130 will prevent progression into the cell cycle, which can be overcome by increased expression of individual E2Fs (17, 38–40). However, suppression of cell proliferation by p107 and pRb are not the same. There are two domains in p107, either of which will prevent proliferation (41); one domain binds E2F, and in this respect the mechanism of growth inhibition may be similar to that of pRb; the second growth-inhibitory domain of p107 binds cyclin A or E-cdk2, and this domain does not have a counterpart in pRb. pRb and p107 may also differ in that the c-Myc protein, which is important in driving proliferation, is inhibited by binding to p107 (42). In addition to regulating E2F activity, pRb also regulates the activity of all three classes of RNA polymerases (reviewed in Ref. 43).

The balance between particular E2Fs and pRb or p130 is clearly important in determining whether a cell proliferates, remains quiescent, or is driven to apoptosis. Cells driven by E2F-1 enter the cell cycle and then die later by apoptosis (44–46). E2F-2 and E2F-3 also cause cells to enter S phase, but neither causes apoptosis (37). Thus, E2F-driven entry into S phase and the induction of apoptosis are not linked; rather, induction of apoptosis is unique to E2F-1. This has lead to the suggestion that E2F-1 but not E2F-2 or E2F-3 specifically induces genes that trigger apoptosis and that a proliferating cell is therefore primed for apoptosis unless rescued by the presence of anti-apoptotic proteins, such as pRb (48) or p53 (37).

From what has been described above, it is clear that regulation of cell cycle entry is complex and involves a coordinated series of phosphorylations of key cell cycle proteins and the activation of a number of genes. Cell cycle exit is also complex, and this is illustrated by the way α-IFN causes cell cycle arrest. α-IFN, which is naturally secreted as a component of the host defense mechanisms of the body, has marked antiviral properties, but α-IFN also has antiproliferative effects on certain cell types (49). α-IFN was thought to exert its antiproliferative effect in part via the pRb pathway (5, 7, 50, 51). However, the cytostatic effect of α-IFN may be mediated by a more complicated series of mechanisms that lead to orderly cell cycle arrest rather than triggering apoptosis. We know about some of the processes involved: cdk activity is decreased (51–55), which may be due in part to a decrease in cdc25A and cyclins D3, E, and H and an increase in p18INK4C in Daudi B-cells (54, 56) and induction of the cdk inhibitors p15INK4B, p16INK4A, p21Cip1, and p27Kip1 in other cell types (57–60). The initial phosphorylation of pRb during G1A is prevented (5), and free E2F binding to DNA is reduced (8, 62). The latter may be due to the induction by α-IFN of p202 (63), a protein that sequesters E2F in a form that does not bind DNA. Other transcription factors that are involved in controlling cell proliferation are also down-regulated. For example, the activity and abundance of the Oct-1 and Oct-2 transcription factors is decreased (64), and α-IFN also causes the depletion of the c-MYC protein (51, 62, 65), which is mediated in part by the double-stranded RNA-activated kinase, PKR (66).

Regulation of the expression and phosphorylation of pRb, p107, and p130 during the transition from G1 through G1 into S phase has been studied by a number of groups. However, the processes regulating entry into the cell cycle (G1→G1→S) may be different from those regulating cell cycle progression through G1 in actively cycling cells (M→G1→S) (67) and are poorly understood. This is particularly important because negative growth factors, such as α-IFN, exert their effects on actively cycling cells. We showed previously that α-IFN causes inhibition of pRb phosphorylation and that this only occurs in G1A (5, 7). In the study presented here, we investigated whether p130 and p107 are regulated by α-IFN and whether their regulation is also cell cycle-dependent. Our studies show that regulation of the phosphorylation of pRb and p107 is different from that of p130 during the cell cycle of actively proliferating cells and that each is regulated in a different manner by α-IFN as cells exit the cell cycle.

MATERIALS AND METHODS

Reagents—Chemicals were obtained from Sigma unless stated otherwise.

Cell Culture—Cell culture and T-cell isolation methods were described previously (5, 7, 52, 68), with additional details as described below. Primary T-cells were isolated from the peripheral blood of normal volunteers and were at least 80% pure, as determined by flow cytometry with CD3, CD4, CD8, CD14, CD19, and CD45. The purified T-cells were cultured in 1×10⁶ cells/ml in RPMI medium/10% FCS (Life Technologies, Inc.) and stimulated with 1μg/ml phytohaemagglutinin (PHA) (Glaxo-Welchcome) for the times shown. Cells were maintained in PHA for 60 h, Interleukin-2 (II-2) (Murex) was added to a final concentration of 20 ng/ml, and culture continued for 5–7 days. IL-2 was withdrawn by washing the cells three times in 50 ml of prewarmed RPMI medium/10% FCS per 2×10⁶ cells, and the cells were re-seeded in the same medium at 1×10⁶ cells/ml.

Human primary, CD34+ hemopoietic progenitor cells were isolated and cultured as described previously (24). Cells employed in this study were surplus to patient requirements and were used with ethical approval.

Proliferating Daudi cells (obtained from Dr. Ian Kerr, Imperial Cancer Research Fund, London, United Kingdom) were maintained at 2–10×10⁶ cells/ml in RPMI medium/10% FCS and were split to 2–4×10⁶ cells/ml at least 24 h before flow cytometric sorting. Where indicated, α-IFN (Roferon A, Hoffman-LaRoche) was added to a final concentration of 200 units/ml. HL60 cells (from Dr. Pamela Roberts, Dept. of Haematology, University College London Medical School) were cultured with or without 1μm all-trans-retinoic acid (RA) and their differentiation state was determined by the reduction of nitroblue tetrazolium (68).

Flow Cytometry—Two color flow cytometry of DNA (stained with propidium iodide (PI) and total cell protein (stained with fluorescein isothiocyanate (FITC)) (5, 69) was used routinely for cell cycle analysis. Analysis and electronic sorting was carried out using an Epics-Elite flow cytometer (Coulter Electronics). Doubles of cells in G1 can contamination be excluded as described previously (24). Cells employed in this study were surplus to patient requirements and were used with ethical approval.

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phosphate, Na₂VO₄,14H₂O, EGTA, phenylmethylsulfonyl fluoride, Na₂P₂O₇,10H₂O, aprotinin, pepstatin A, leupeptin; see Ref. 52). Either 1 or 2 × 10⁵ cells were loaded in each lane of Western blots.

**Immunoprecipitation, DNA Binding and Western Blotting—**Immunoprecipitations were carried out with 1 × 10⁵ Daudi cells lysed in 100 µl of modified immunoprecipitation buffer as described previously (52), except that the buffer contained 0.5% Nonidet P-40 rather than 1% Triton X-100. Solutions were kept on ice, and all manipulations were carried out in a 5 °C cold-room. The lysate was centrifuged for 2 min at 10,000 × g, the supernatant was transferred to a fresh tube, and the nuclear pellet was then extracted for 20 min with 80 µl of the same buffer containing 450 mM NaCl. After centrifugation for 2 min at 10,000 × g, the supernatants were pooled and preabsorbed for 1–2 h with 5 µg of rabbit IgG (DAKO Ltd., High Wycombe, UK) and 20 µl of protein A-agarose (Repligen, Cambridge, MA). Immunoprecipitation was carried out with 5 µg of anti-E2F-4 (C20) antibody and then with protein A-agarose or with 40 µg of anti-E2F-1 antibody (C20) coupled to Sepharose (Santa Cruz Biotechnology Inc.). One-third of each immunoprecipitate was electophoresed per gel. These procedures were sufficient to extract all of the p130, pRb, and p107 from Daudi cells, and none was detected when SDS lysates of the residual nuclear pellets were subjected to Western blotting (data not shown).

Lysates for DNA binding were prepared as described for immunoprecipitation, and all manipulations were at 5 °C. They were precleared by end-over-end agitation for 1 h with 10 µl of protein A-agarose. They were subjected to Western blotting (data not shown). p130, pRb, and p107 are very abundant in Daudi cells, so we were able to extract all of the p130, pRb, and p107 from Daudi cells, and none was detected when SDS lysates of the residual nuclear pellets were subjected to Western blotting (data not shown).

**RESULTS**

**p130 and p107 in Cells Arrested by α-IFN—**The pRb protein is dephosphorylated when Daudi cells are arrested by α-IFN (7), and this causes pRb to migrate faster on SDS gels. The form of p130 that migrates slowest (Form 3) is also lost in cells cultured with α-IFN and is replaced by Forms 1 and 2 (see, for example, Fig. 1A and B, lanes 1 and 2). In order to determine whether the change in migration of p130 caused by α-IFN can be accounted for by phosphorylation changes, we incubated whole cell extracts of proliferating Daudi cells in vitro with or without the dual-specificity λ-phosphatase. Phosphatase treatment (Fig. 1A) caused a shift from p130 Form 3 to a species that comigrated with Forms 1 and 2 (lanes 4–9), as well as a faster migrating form (Form 0) when a higher concentration of λ-phosphatase was used (lane 11). The shift was inhibited by the addition of phosphatase inhibitors (lanes 3 and 12). Thus, changes in the migration of p130 in response to α-IFN are consistent with its dephosphorylation.

We showed previously that dephosphorylation of pRb occurs exclusively in G₁ even after culturing Daudi cells for prolonged periods with α-IFN (5). We wished to determine whether dephosphorylation of p130 occurs in a similar manner, but before being able to do so, we first had to determine how its phosphorylation is regulated during the cell cycle of continuously cycling cells. Proliferating Daudi cells in different cell cycle phases were isolated by flow cytometric sorting, and the phosphorylation state of p130 was analyzed by Western blotting (Fig. 1B). The sorting protocol involves fixing the cells in 70% ethanol at −20 °C prior to staining for flow cytometry, but this did not alter the migration of p130 in SDS gels (Fig. 1B, compare lanes 1 and 2 (not fixed) with lanes 3 and 4 (fixed)). p130 was present predominantly as phosphorylated Form 3 in each cell cycle phase isolated from G₁ through to G₂/M (Fig.
1B, lanes 5–8), which indicates that the phosphorylation state of p130 does not change substantially as continuously cycling cells progress through the cell cycle. The migration of p130 Forms 1 and 2 can be seen in the sample of quiescent primary T-cells run in Fig. 1B, lane 11. It was difficult to isolate pure populations of cells in G2-M that were free of doubllets of cells in G1, and in most cases, sort purities were similar to the experiment shown. Because it has been suggested that p130 is depleted from cells in G2-M (14), the signal we observed from these cells could have been contributed solely by the G1 cell contaminants. In order to determine whether this is the case, we isolated a small number of ~95% pure cells in G2-M and compared the p130 signal with that obtained from the number of G1 cells (~99% pure) that contain the G2-M preparation (i.e. 5% of sorted cells). As shown in Fig. 1B, lanes 12 and 13, the contaminating G1 cells did not account for the p130 signal from cells in G2-M. The total cellular protein content increased as cells progressed from G1A through to G2-M, and the relative ratio for Daudi cells was approximately as follows: G1A = 1; G1B = 1.5; S = 1.7; G2-M = 2.2. Because the same number of cells were loaded on each lane of the gels shown in Fig. 1B, lanes 5–8, it is clear that the amount of p130 decreases during the cell cycle relative to total cell protein, but p130 is not depleted completely from Daudi cells in S and G2-M.

Phosphorylation of p130 and pRb during Cell Cycle Entry from G0—p130 is present in Forms 1 and 2 when Daudi cells cultured with 0.5% FCS exit the cell cycle (see Fig. 5B, lane 3). The two-color cell cycle profile of these cells coincides with proliferating cells in G1, and so they cannot be used to determine when p130 is phosphorylated to Form 3 as cells enter the cell cycle from G0. Human primary T-cells isolated from peripheral blood are in G0; they are small cells with 2N DNA content and low protein and RNA content, and both protein and RNA content increase as cells enter G1A from G0 (see Ref. 5). Therefore, we used these cells to determine when p130 and pRb are phosphorylated as cells enter G1 from G0. Two color flow cytometry showed that T-cells enter G1A at about 12–14 h after stimulation with PHA (Fig. 2A), at which time p130 Form 3 was detectable (Fig. 2B, lanes 4 and 5). Hyperphosphorylated pRb was also detectable by Western blotting at the same time (Fig. 2B, lanes 14 and 15). We have shown previously that p130 is also phosphorylated to Form 3 from Forms 1 and 2 as human primary CD34+ hematopoietic progenitor cells enter G1A from G0 (24). Thus, our data with two primary cell types are consistent with a model whereby p130 becomes inactivated at or before the G0 to G1A transition and thereafter remains inactive throughout the cell cycle of actively cycling cells.

Regulation of p130 and pRb by a-IFN—Next, we investigated how p130 is regulated during cell cycle arrest caused by a-IFN. At 24 h after the addition of a-IFN, p130 Form 3 is partially depleted and Forms 1 and 2 are detectable. Loss of Form 3 occurs only in G1 (Fig. 3A, lane 3) and not in S (lane 4) or G2-M phases (lane 5). This is similar to pRb, which we showed previously is only dephosphorylated to a hypophosphorylated form in G1 (5). After 36–48 h with a-IFN, p130 Form 3 is replaced completely by Forms 1 and 2 (Fig. 3B, lane 2). However, this occurs at a time when hyperphosphorylated pRb is still present (compare Fig. 3B, lanes 2 and 6; see also Fig. 2B, lanes 10 and 20). In order to determine whether p130 Form 3 is depleted in phases other than G1, we sorted Daudi cells that had been cultured with a-IFN for about 40 h. Two color flow cytometry of DNA versus protein content showed that at this time cell cycle arrest in G1 was not yet complete and that there was still a significant number of cells in mid to late S and G2-M (data not shown). The cells in G1 and S+G2-M were isolated by flow cytometric sorting and, as shown in Fig. 3B, p130 was present as Forms 1 and 2 in both S+G2-M, as well as in G1 (lanes 3 and 4), whereas pRb was hypophosphorylated in G1 and hyperphosphorylated in S+G2-M (lanes 7 and 8). In comparison, both p130 and pRb were fully phosphorylated in cycling cells in S+G2-M (Fig. 3B, lanes 1 and 5). Thus, in response to a-IFN, the initial dephosphorylation of pRb and p130 both occur in G1. However, thereafter, the phosphorylation state of p130 but not of pRb is regulated in S+G2-M.

Regulation of p107 by a-IFN—We fixed and sorted actively cycling Daudi cells into various cell cycle phases (Fig. 4A) and analyzed the phosphorylation state of p107 by Western blotting (18). The fixing and sorting procedures did not alter the migration of p107 (Fig. 4B, lanes 1 and 2 (fixed), lanes 3 and 4 (not fixed)), and in different cell cycle phases, p107 is hyperphosphorylated in G1A (Fig. 4B, lane 5), is present as hypo- and hyperphosphorylated forms in G1B (lane 6), and is hyperphosphorylated throughout the remainder of the cell cycle (lanes 7–9). These data are consistent with p107 being phosphorylated in late G1 in cycling cells, and the timing is the same for primary T-cells entering the cell cycle for the first time from G0 (data not shown). It has been suggested that there is a significant amount of hypophosphorylated p107 in S phase when fibroblasts are stimulated to enter the cell cycle for the first time after serum starvation (18). In order to determine whether p107 becomes hypophosphorylated in S phase in continuously cycling Daudi cells, we isolated proliferating cells in both early...
and late S phase (Fig. 4A), and in both cases, p107 was predominantly hyperphosphorylated (Fig. 4B, lanes 7 and 8). Thus, at least in proliferating Daudi cells, p107 only becomes hypophosphorylated in G_{1A} and is predominantly hypophosphorylated from G_{1B} throughout the remainder of the cell cycle.

In response to α-IFN, p107 becomes hypophosphorylated as cells begin to arrest in G_{1} (Fig. 4C, lanes 1, 3, and 5 (-α-IFN); lane 2 (24 h +α-IFN); lane 4 (48 h +α-IFN)) and is then depleted (Fig. 4C, lane 6 (72 h +α-IFN); tubulin controls are shown below lanes 5 and 6). Thus, the phosphorylation and abundance of p107 are regulated by α-IFN.

The Phosphorylation State of p130, pRb, and p107 during Cell Cycle Exit—We have shown above that the phosphorylation state of p130 and pRb are differentially regulated in response to α-IFN. In order to determine whether this is peculiar to α-IFN or whether the same occurs in response to other signals to exit the cell cycle, we analyzed p130 and pRb in proliferating primary T-cells after IL-2 withdrawal, in Daudi cells exposed to a suboptimal serum concentration, and in HL60 myeloid cells cultured with RA. The results are shown in Fig. 5, A, B, and C, respectively. We observed two responses: (i) p130 Form 3 is lost before pRb is dephosphorylated in T-cells and Daudi cells withdrawn from growth factors (Fig. 5, A and B, lane 3); and (ii) p130 Form 3 and hyperphosphorylated pRb are depleted coordinately in HL60 cells between days 3 and 4 after RA treatment (Fig. 5C, lanes 2 and 3), at a time when the cells arrest in G\(_{1}\). Therefore, α-IFN and growth factor withdrawal have the same effect, causing depletion of p130 Form 3 before dephosphorylation of pRb, whereas we have not detected depletion of p130 Form 3 before pRb in HL60 cells cultured with RA. As also shown in Fig. 5C, p107 is dephosphorylated (lane 2) and then depleted (lane 3) from HL60 cells between days 3 and 4 with RA, which is similar to the regulation of p107 in Daudi cells by α-IFN. p107, as well as p130 and pRb, is fully phosphorylated in proliferating cells cultured for 4 days without RA (Fig. 5C, lane 4).

E2F Complexes in α-IFN Arrested Cells—p130, pRb, and p107 each function at least in part by binding to E2F. The E2F proteins can be grouped into two classes, E2F-1, -2, and -3 and E2F-4 and -5, which have different specificities for binding members of the pRb family. In order to determine which forms of p130, pRb, and p107 associate with E2F during α-IFN-mediated cell cycle arrest, we carried out coimmunoprecipitations with antibodies against one member of each class of E2F, namely E2F-1 and E2F-4. As shown in Fig. 6, hypophosphorylated pRb and predominantly p130 Form 1 were coimmunoprecipitated with E2F-4 (Fig. 6, A and B, lane 7) from lysates of Daudi cells cultured with α-IFN for 24 h. We note that some of p130 Form 2 was also detectable on longer exposures but none of Form 3. Similar data were obtained for E2F-5 (not shown). Note that very little p130 or p107 and no detectable pRb were coimmunoprecipitated with E2F-4 from proliferating Daudi cells (Fig. 6, A and B, lane 6), and no p130, p107, or pRb was immunoprecipitated nonspecifically with rabbit IgG (Fig. 6, A and B, lanes 1 and 2). pRb coimmunoprecipitated with E2F-1 from α-IFN treated cells (Fig. 6A, lane 9), and a small amount

**Fig. 3.** Phosphorylation state of p130 during cell cycle arrest by α-IFN. A, Western blot of p130 in Daudi cells cultured for 24 h with α-IFN in G\(_{1}\) (lane 3; purity, 93%), S (lane 4; purity, 77%), G\(_{2}\)-M (lane 5; purity, 87%) phases of the cell cycle, or a total cell lysate (lane 2). Proliferating cell lysate is shown in lane 1. B, Western blots of p130 (lanes 1–4) and pRb (lanes 5–8) in Daudi cells cultured with α-IFN for 39 h are shown as follows: lanes 2 and 6, total lysate; lanes 3 and 7, G\(_{1}\) (purity, 98%); lanes 4 and 8, S+G\(_{2}\)-M (purity, 66%). Proliferating cells in S+G\(_{2}\)-M (lanes 1 and 5) are shown for comparison. (Note that p130 (lane 2) is the same as that shown in Fig. 1B, lane 12)

**Fig. 4.** p107 in proliferating and α-IFN-arrested cells. A, flow cytometric analysis of proliferating Daudi cells sorted into the cell cycle phases shown. The purity is indicated next to each profile. B, p107 Western blots of the sorted samples shown in A: G\(_{1A}\) (lane 5), G\(_{1B}\) (lane 6), early S (lane 7), late S+G\(_{2}\)-M (lane 8), and G\(_{2}\)-M (lane 9). Total lysates of Daudi cells cultured 39 h with α-IFN or without and lysed directly (lanes 3 and 4) or fixed in 70% EtOH prior to lysis (lanes 1 and 2) The positions of hyper- and hypophosphorylated p107 (p107/p) and p107, respectively) are shown C, p107 Western blots of total cell lysates of Daudi cells cultured with (lanes 2, 4, and 6) or without (lanes 1, 3, and 5) α-IFN for 24, 48, and 72 h. A tubulin blot of samples cultured for 72 h ±α-IFN is shown below the corresponding p107 blot.
was detected in proliferating cells (Fig. 6A, lane 8). However, no p130 or p107 was detected coimmunoprecipitating with E2F-1 (Fig. 6B, lanes 8 and 9). Thus, pRb coimmunoprecipitates with both E2F-1 and -4 form lysates of Daudi cells cultured with α-IFN, whereas p130 and p107 only bind E2F-4.

The coimmunoprecipitation data show the forms of p130, p107, and pRb, which can be isolated in complexes with different E2Fs from lysates of α-IFN treated cells. Formation of these complexes in response to α-IFN may depend not only on changes in the phosphorylation of the pRb family but also of the E2F. Because the functional forms of such complexes are thought to be those that are bound to an E2F DNA binding site, we determined the form(s) of E2F and pRb family members that bind DNA. Thus, cell lysates were incubated with a biotinylated E2F DNA binding site (E2FWT) bound to avidin-beads (see under “Materials and Methods”), and the forms of each
protein bound were determined by Western blotting. Note that in addition to the pRb family, the migration of E2F-4 in SDS-PAGE also changes as a result of differences in its phosphorylation state (17, 24). Because we had shown previously (24) that primary hemopoietic cells in G0, and during active proliferation contain different phosphorylated forms of E2F-4, we therefore used lysates of freshly isolated primary T-cells (in G0) and T-cells 40 h after stimulation with PHA (in late G1) to determine which forms of p130 and E2F-4 can bind an E2F DNA binding site. The data in Fig. 6C show that p130 Form 1 and 2 and hyperphosphorylated E2F-4 in lysates of cells in G0 bind the E2F\textsuperscript{WT} DNA (lanes 2–5), but not to a mutant E2F site (E2F\textsuperscript{MUT}, lane 1). After 40 h + PHA, p130 Form 3 predominates (lanes 6 and 12, total lysate), and as expected, this form does not bind DNA (lanes 8–11 and 14). Multiple, hyperphosphorylated forms of E2F-4 are present in these cells (lanes 6 and 12), which bind E2F\textsuperscript{WT} DNA (lanes 8–11 and 14) but not E2F\textsuperscript{MUT} (lanes 7 and 13).

The forms of each protein that bind the E2F DNA site from lysates of Daudi cells cultured with or without α-INF were then determined, and cell extracts within the linear range of the binding assay were used (as determined in Fig. 6C). Predominantly p130 Form 1 (with some Form 2 but no Form 3) (Fig. 6E, lane 2), hyperphosphorylated pRb (Fig. 6E, lane 2), and multiple hyperphosphorylated forms of E2F-4 (Fig. 6D, lane 6) bind E2F\textsuperscript{WT} DNA. Note that little or no protein binds the E2F\textsuperscript{MUT} site (Fig. 6, D (lane 7) and E (lane 1)). Hyperphosphorylated p107 also binds E2F\textsuperscript{WT} DNA from lysates of Daudi cells cultured for 24 h with α-INF (not shown). The forms of the pRb family that bind E2F\textsuperscript{WT} DNA are consistent with the forms that coimmunoprecipitate with E2F-4 (Fig. 6, A and B). The forms of E2F-4 present in α-INF-treated cells (Fig. 6D, lane 5) that bind E2F\textsuperscript{WT} DNA (lane 6) comigrate with the forms present in lysates of proliferating cells (Fig. 6D, lane 4), and even after prolonged incubations with α-INF, we detected little or no hyperphosphorylated E2F-4 (not shown). Lysates of CD34+ primary hemopoietic progenitor cells run on the same gel are shown for comparison because they contain hyperphosphorylated E2F-4 at t = 0 but not after 4 days in culture with SCF, IL-3, and IL-6 (24) (Fig. 6D, lanes 1–3). Thus, changes in the association of E2F-4 with different pRb family members in response to α-INF, detected either by coimmunoprecipitation or by E2F\textsuperscript{WT} DNA binding, are not due to gross changes in E2F-4 phosphorylation, and α-INF does not cause hyperphosphorylation of E2F-4 to that which is present in primary hemopoietic cells in G0.

**DISCUSSION**

Many proteins that are involved in controlling cell proliferation are regulated in susceptible cells by α-INF (reviewed in Ref. 70). The Daudi B-cell line has been used for a number of these studies because it is sensitive to growth arrest by α-INF. Proliferating Daudi cells cultured with α-INF arrest in G1, and this is accompanied by the dephosphorylation of pRb to a hypophosphorylated form (5, 7, 50). Recently (62), it was shown that this causes pRb to complex with E2F-1 and E2F-4, and it was suggested that this results in repression of E2F-containing promoters, such as those in the cdc2 and c-myc genes. We showed previously (5) that in response to α-INF, pRb is dephosphorylated only in the G1 phase of the cell cycle, and so it is only able to repress E2F activity once proliferating cells have entered G1. pRb is the first member of a family of proteins that also includes p130 and p107, each of which can bind and repress E2Fs (3, 23). In the study presented here, we investigated whether α-INF regulates p130 and p107 and to what extent their regulation is also cell cycle-dependent.

We determined first whether the phosphorylation states of pRb, p130, and p107 are regulated in different cell cycle phases during continuous proliferation. Second, we investigated whether dephosphorylation of each is regulated coordinately during α-INF-mediated cell cycle arrest. To answer these questions, we employed a rapid fixation method, followed by flow cytometric sorting and Western blotting, a protocol that we have used previously to analyze pRb (5). We have shown here that (i) in actively cycling Daudi cells, p130 is present predominantly as phosphorylated Form 3 in all cell cycle phases. p107 is hypophosphorylated in G\textsubscript{1A}, becomes hyperphosphorylated in G\textsubscript{1B}, and remains fully phosphorylated throughout the remainder of the cell cycle. Regulation of the phosphorylation state of p107 during G\textsubscript{1} is similar to pRb, except that pRb is partially phosphorylated in G\textsubscript{1A} (not hypophosphorylated) and is hyperphosphorylated from G\textsubscript{1B} onwards (5). (ii) In cells arrested with α-INF, p130 Form 3 is depleted, possibly by dephosphorylation, and principally Form 1, with some Form 2, predominates in every cell cycle phase. In contrast, pRb is dephosphorylated to a hypophosphorylated state only in G\textsubscript{1}, p107 is dephosphorylated and then depleted. p130 Form 1 and hyperphosphorylated p107 coimmunoprecipitate with E2F-4 from lysates of α-INF-treated Daudi cells. Hypophosphorylated pRb coimmunoprecipitates with both E2F-4 and E2F-1. These forms of p130 and pRb bind to an E2F\textsuperscript{WT} DNA binding site. The difference between the amounts of p130, p107, and pRb coimmunoprecipitated with E2F-4 from proliferating and α-INF-treated cells is most probably due to changes in the phosphorylation state of pRb, p130, and p107 rather than to gross changes in the phosphorylation state of E2F-4. Thus, α-INF may bring about cell cycle arrest not only through pRb but also by regulating the phosphorylation state of p130 and the abundance of p107.

Several studies have investigated the E2F complexes that are present as cells enter the cell cycle from a resting state (see Ref. 26 and references therein). Their function is presumed to be to induce the orderly transcription of specific genes, such as those encoding thymidine kinase and cdc2, which are required for progression into S phase and for controlling passage through the cell cycle (22). This may be true for entry into the first cell cycle. However, once in cycle the abundance of, for example, cdc2 changes only in line with the total amount of cellular protein (71–73). Therefore, regulation of E2F activity by the pRb family is likely to be different in cells entering the cell cycle for the first time and in those proliferating continuously. Cycling cells contained predominantly phosphorylated p130 Form 3 in all cell cycle phases from G\textsubscript{1A} through to G\textsubscript{2-M}. This form does not bind E2F complexes and was not isolated by binding to an E2F DNA binding site. Because p130 only binds E2Fs and DNA when it is in Forms 1 and 2, we conclude that p130 has no major role in controlling E2F function in Daudi cells during continuous proliferation. Our conclusions differ from those published recently that showed that a p130-E2F complex exists during G\textsubscript{1} (74). It is not clear whether there are differences because of the cell types or the methods used in the two studies. In addition, our data show that p130 is phosphorylated as quiescent primary CD34+ hemopoietic progenitors (24) and T-cells exit G\textsubscript{1} and enter G\textsubscript{1A}. It has been suggested that p130 maintains cells in G\textsubscript{0} (14, 17, 37), and inactivation of p130 by phosphorylation upon entry into G\textsubscript{1A} is most consistent with p130 playing a role in allowing the transition from G\textsubscript{0}↪G\textsubscript{1A} rather than controlling progression through G\textsubscript{1}.

In contrast to p130, both pRb and p107 in actively cycling cells are dephosphorylated during G\textsubscript{1} (pRb partially) and are fully phosphorylated from the G\textsubscript{1}-S border onwards. Thus, both

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3S. Tiwari and N. S. B. Thomas, unpublished data.
could have a role to play in G1. Recently, it was shown that p107-E2F complexes are present in the cytosol during G1, whereas pRb-E2F complexes are nuclear (74). Thus, p107 in actively cycling Daudi cells may bind E2F (E2F-4 and -5) in a cytosolic form during G1A, which is released as p107 becomes phosphorylated in G1B. In contrast to p107, pRb can only be hypophosphorylated for a brief period during G1A, because we detected multiple, partially phosphorylated forms in this subphase of the cell cycle. We have not detected these phosphorylated forms bound to E2F or to an E2FWT DNA site, and it is possible therefore that other proteins in the cell (23, 75) are regulated by these forms of pRb, but this has yet to be shown. Once cells have progressed through G1, the data presented here show that both p107 and pRb are predominantly hyperphosphorylated throughout S and G2-M. Our data on pRb are consistent with dissociation of pRb and E2F during the G1 to S phase transition reported previously (see Ref. 2). However, p107-E2F complexes have been detected by electrophoretic mobility shift assays in extracts of cells from late G1 into S phase (21, 74, 76), and p107 has also been reported to become dephosphorylated again late in S phase (18). From the data presented here and from hydroxyurea block/release experiments (not shown), we would suggest that because most of the p107 is hyperphosphorylated and hence inactivated from G1B through to G2-M, p107 may only have a major role in binding E2F in actively dividing cells during G1A.

In response to α-IFN, p130 Form 3 in Daudi cells was completely depleted at a time when hyperphosphorylated pRb was not. Flow cytometric sorting experiments showed that p130 Form 3 was depleted in all cell cycle phases after culturing for 40 h with α-IFN, whereas pRb was only dephosphorylated in G1. Thus there is a clear difference in the mechanisms regulating these proteins in different cell cycle phases. Our data show that there is an initial response to α-IFN during G1 that leads to the dephosphorylation of pRb and loss of p130 Form 3, but later, p130 Form 3 is depleted during S+G2-M. pRb may become dephosphorylated during mitosis (4) and, as certain cdks are inhibited by α-IFN (51, 52, 55), pRb would remain hypophosphorylated in G1. However, the effects of α-IFN on members of the cdk family, such as cdk4 and cdk6, which are active during early to mid G1 (3, 70) are as yet unknown. Such a mechanism does not explain why p130 Form 3 is depleted from all cell cycle phases. Our dephosphorylation experiments with λ-phosphatase would suggest that p130 Form 3 could be converted to Form 1 and/or Form 2 by dephosphorylation. However, we cannot rule out the possibility that p130 Form 3 could also be degraded. In continuously cycling cells, less p130 was detected in samples of cells sorted into S and G2-M as compared with G1, but it is not depleted in G2-M, as reported for other cell types (14). There is a precedent for two mechanisms regulating the activity of one protein. The amount of phosphorylated STAT1 is regulated both by the ubiquitin-proteasome pathway (77) and by dephosphorylation by a tyrosine phosphatase (47), and it will be interesting to determine whether p130 is regulated in a similar manner.

In order to determine whether loss of p130 Form 3 preceding the dephosphorylation of pRb is peculiar to cell cycle arrest caused by α-IFN, we analyzed what occurs during cell cycle exit for a number of hemopoietic cell types: by removing IL-2 from continuously proliferating primary T-cells, in Daudi cultured in low serum, and in HL60 cells during myeloid differentiation. In all cases, p130 Form 3 was depleted as cells arrested in G1, consistent with a role for p130 in promoting exit from the cell cycle. In HL60 cells cultured with retinoic acid, p130 and pRb were both dephosphorylated at the same time, in agreement with experiments that suggest that cell cycle arrest may be mediated by sequestration of E2F by pRb and p130 (61).

From previous work, it was suggested that α-IFN exerts its antiproliferative effects in part via the pRb pathway (5, 7, 50, 51). The cytostatic effect of α-IFN may occur by preventing the initial phosphorylation of pRb during G1 (5), by decreasing cdk activity (51, 52) and E2F binding (8, 62). Our data now show that α-IFN also causes the accumulation of p130 Form 1 and the depletion of p107. Finally, our coimmunoprecipitation experiments with lysates of Daudi cells cultured with and without α-IFN show that hypophosphorylated forms of pRb and p107, and p130 (principally Form 1) all associate with E2F-4, whereas E2F-1 only coimmunoprecipitates pRb. These data are in agreement with Ikeda et al. (61), who concluded that pRb may be a general regulator of E2Fs as cells exit the cell cycle, whereas p130 is more specific to E2F-4 and -5. Such complexes may also bind an E2FWT DNA site in vitro, but given that the subcellular localization of each complex may be different (74), it remains to be determined precisely how pRb, p130, and p107 in α-IFN-arrested cells control E2F: whether it is bound to a nuclear E2F DNA site or whether, for example, p130-E2F binding occurs in the cytosol. It has been shown by electrophoretic mobility shift assays that the principal E2F complex in extracts of primary hemopoietic cells in G0 contains p130-E2F-4-DP-1 (17, 24, 25), which we have shown here contains p130 Forms 1 and 2 together with a hyperphosphorylated form of E2F-4. Thus, if p130-E2F-4-DP-1 defines cells in G0 (61), our observations (Ref. 24 and this paper) would suggest that such a complex contains hyperphosphorylated E2F-4. In contrast, the p130-E2F-4 complex that exists in Daudi cells arrested by α-IFN contains hypophosphorylated forms of E2F-4. Thus, by this criterion, α-IFN causes Daudi cells to arrest in G1 rather than in G0, and this occurs in part by regulating the phosphorylation state of p130 rather than causing the hyperphosphorylation of E2F-4.

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