Oncogene (1998) 16, 217–225

Interferon-induces expression of cyclin-dependent kinase-inhibitors $p21^{WAF1}$ and $p27^{Kip1}$ that prevent activation of cyclin-dependent kinase by CDK-activating kinase (CAK)

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To understand the mechanism of interferon (IFN)-mediated suppression of cell cycle progression, we have earlier shown that IFN-α enhances the expression of underphosphorylated retinoblastoma protein by inhibiting the cyclin-dependent kinase-2 (CDK-2) activity (Kumar and Atlas, *Proc. Natl. Acad. Sci.* 89, 6599–6603, 1992; Zhang and Kumar, *Biochem. Biophysi. Res. Comm.*, 200, 522–528, 1994). In the studies presented here, we investigated the mechanism of inhibition of CDKs in IFN-treated cells by delineating the potential role(s) of CDK-inhibitors (CKIs) and CDK-activating kinase (CAK). We report that IFN-α inhibits the H-1 kinase activity associated with CDK-4 or CDK-2 due to induction of expression of CDK-inhibitor $p21^{WAF1}$ (but not $p27^{Kip1}$) as its immunodepletion from IFN-treated extracts restored the CDK-associated H-1 kinase activity. In addition, we also show that IFN-γ induces expression of CDK-inhibitors $p21^{WAF1}$ and $p27^{Kip1}$ and inhibited the H-1 kinase activity associated with CDK-2 or CDK-4. The observed IFN-γ-mediated inhibition of CDK-2 and CDK-4 kinase activity was due to enhanced interactions with $p21^{WAF1}$ and $p27^{Kip1}$, respectively. We also demonstrated that IFN-induced CKIs prevent CAK from activating the CDK-2 as immunodepletion of induced CKIs from the inhibitory extracts resulted in the restoration of CAK-mediated activation of CDK-2.

Keywords: interferons; cell cycle; CDK; CDK-inhibitors; CDK-activating kinase

Introduction

Regulation of cell proliferation is a complex process involving the regulated expression and/or modification of discrete gene products. There is a growing list of evidence to support the involvement of paracrine growth inhibitors and intracellular growth suppressors in the regulation of cell growth. Representatives of these classes of molecules are secretory proteins like interferons (IFN), and cell cycle inhibitors like $p21^{WAF1}$ and $p27^{Kip1}$ that inhibit the cyclin-dependent kinases (CDKs). An obvious question is whether the growth inhibition by IFN is mediated through the pathway(s) that control the activation of CDKs.

Interferons (IFNs) are a family of hormone-like secretory proteins which interact with neighboring cells and bring about many phenotypical changes in these cells. Like most cytokines, IFNs must bind to specific high affinity cellular receptors to exert biological effects. These effects include regulation of expression of specific genes, antiviral properties, and inhibition of cell growth and proliferation (Sokawa, 1977; Pestka *et al*., 1987; Kumar and Atlas, 1992; Kumar *et al*., 1994; Yamada *et al*., 1994; Tiefenbrum *et al*., 1996). The inhibition of cell proliferation by IFNs is an active process, and generally involves arrest of cells in the G0/G1-phase of the cell cycle. IFN-α and IFN-β have also been considered to have a natural negative regulatory role in the autocrine regulation of cell proliferation, as several growth factors induce the formation of IFN in the cells they stimulate (Zullo *et al*., 1985; Moore *et al*., 1984), and this possibly could prevent uncontrolled cell proliferation. The mechanisms underlying growth inhibitory effects by IFNs are not well established.

The regulation of cell cycle is controlled by families of essential proteins which control transition between different stages of the cell cycle. These proteins either facilitate cell cycle progression or serve to break the progression. The cell cycle progression through the specific phases of the cell cycle is regulated by the sequential formation, activation and inactivation of a family of serine/threonine kinases, the CDKs (reviewed in Reed, 1992; Sherr, 1993; Hunter and Pine, 1994). The CDKs are a family of enzymes that are inactive as monomers, and require association with cyclins for activation. The activities of CDK-4 and CDK-2 appear to be most critical to the restriction point transitions, particularly, passage through the G1-phase and entry into the S-phase (Hunter and Pine, 1994). CDK-complexes also associate with proliferating cell nuclear antigen (PCNA) to form a catalytically active kinase complex that phosphorylates retinoblastoma protein (pRB) (abrogate growth-inhibitory function of pRB). This allows release of transcription factor E2F, which activates the expression of target genes required in DNA synthesis (Reed, 1991; Dulic *et al*., 1992; Xiong *et al*., 1993). The activity of CDKs is negatively controlled by CDK-inhibitors (CKIs) such as $p21^{WAF1}$ and $p27^{Kip1}$. Both $p21^{WAF1}$ and $p27^{Kip1}$ have been shown to inhibit the activity of CDK-2- and CDK4-cyclin complex's *in vitro* (Xiong *et al*., 1993; Harper *et al*., 1993; Kato *et al*., 1994; Hall *et al*., 1995). The $p21^{WAF1}$ has been shown to also bind to PCNA and block DNA replication (Waga *et al*., 1994). It is believed that CKIs bind to cyclin/CDK complexes and set stoichiometric inhibitory thresholds of CDKs kinase activity, which prevents premature or inappropriate progression of the cell cycle by impeding their ability to phosphorylate target substrates such as pRB family proteins.

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Received 7 May 1997; revised 29 August 1997; accepted 29 August 1997

In addition to CKIs, the activity of CDKs is regulated by cycles of phosphorylation and dephosphorylation (reviewed in Clarke, 1995; Morgan, 1995). One of the key phosphorylation event controlling CDKs activity is the activation of phosphorylation on a residue in various CDKs, corresponding to threonine (Thr) 161 in human prototypic CDK-1. Phosphorylation of this residue is positively required for function of both human and yeast CDK-1 and recent reports have shown that an analogous phosphorylation step is essential for activities of CDK-2 and CDK-4 (Poon *et al*., 1993; Matsuoka *et al*., 1994). A kinase responsible for phosphorylating the Thr^{161} of CDK-1 and the corresponding residue in other CDKs (T^{172} in CDK-4, T^{160} in CDK-2) has been identified in various organisms and designated CAK, for CDK-activating kinase (also known as CDK-7). The catalytic subunit of CDK-7 requires binding of a regulating cyclin H subunit to become active (Fisher and Morgan, 1994).

To understand the mechanism of inhibitory effect of IFN in the G0/G1-phase of the cell cycle, we and others have earlier shown that IFN-α enhances the expression of underphosphorylated RB protein (pRB) by inhibiting the pRB phosphorylation in human Burkitt's lymphoma Daudi cells (Burke *et al*., 1992; Kumar and Atlas, 1992; Resnitzky *et al*., 1992). We have also demonstrated that IFN-γ inhibits the growth of human epidermoid A-431 cells in the G0/G1-phase of the cell cycle (Kumar and Mendelsohn, 1989). Recently, Harvat and Jetten (1996) have shown that IFN-γ, like IFN-α, also enhances the levels of the underphosphorylated pRB form. The inhibition of pRB phosphorylation in IFN-α-treated Daudi cells has been shown to be related to the inhibition of H-1 kinase activity associated with cyclin D1- and E-associated CDK complexes which contain pRB and E2F (Zhang and Kumar, 1994), and suppression of DNA-binding activity of E2F (Melamed *et al*., 1993). Using prolonged (48–72 h) IFN-α-treatment of Daudi cells, Yamada *et al*. have shown the downregulation of cyclin H (Yamada *et al*., 1995). While this study was in-progress, recent reports have shown a correlation between the induction of $p21^{WAF1}$ and inhibition of CDK-2 activity in IFN-α-treated cells (Sangfelt *et al*., 1997; Hobeika *et al*., 1997). Taken together, inspite of our increased understanding of the effect of IFN on the cell cycle during the last few years, the precise mechanism of inhibition of CDKs (CDK-2 and CDK-4) including the potential roles of CKI ($p21^{WAF1}$ and $p27^{Kip1}$) and CAK in the growth inhibition by IFNs, remains still unclear.

In the present study, we have investigated the possible roles of CKIs and CAK in the regulation of CDKs in IFN-treated cells. Here, we report that IFN-α inhibits the H-1 kinase activity associated with CDK2 or CDK-4 without any change in its expression. The inhibitory effects of IFN-α on CDKs activities were in-part due to induction of expression of $p21^{WAF1}$ (but not $p27^{Kip1}$) as its immunodepletion restored CDK-associated H-1 kinase activity. In addition, we have now shown that IFN-γ induces expression of both $p21^{WAF1}$ and $p27^{Kip1}$, and inhibited the kinase activity associated with CDKs. It was observed that IFN-γ inhibited CDK-2 and CDK-4 kinase activity due to enhanced interaction with $p21^{WAF1}$ and $p27^{Kip1}$, respectively. We also show that immunodepletion of induced CKIs from the inhibitory extracts resulted in the restoration of CAK-mediated activation of CDK-2. This indicates that IFN-induced CKIs prevent the CAK from activating the CDK-2 in IFN-treated cells.

Results

IFN-α inhibits RB protein phosphorylation and CDK-associated H-1 kinase activity

To understand the mechanism of IFN-mediated growth-inhibition of cells in the G0/G1-phase of the cell-cycle, we and others have earlier shown that IFN-α-treatment of Daudi cells leads to enhanced expression of under-phosphorylated form of pRB (Kumar and Atlas, 1992; Resnitzky *et al*., 1992). To illustrate this effect, Figure 1 shows the kinetics of inhibition of pRB phosphorylation (Figure 1a) and inhibition of CDK-2 associated H-1 kinase activity without any effect on CDK-2 expression (Figure 1b). Results in Figure 1a show that in exponentially growing cells, we can resolve three different electrophoretically migrating forms and IFN-α predominantly inhibits the expression of slowest migrating form (Figure 1a, lane 1, band number 1) and enhances the expression of intermediate migrating band (band number 2) as early as 6 h post-treatment. However, at 24 h post-treatment, there was

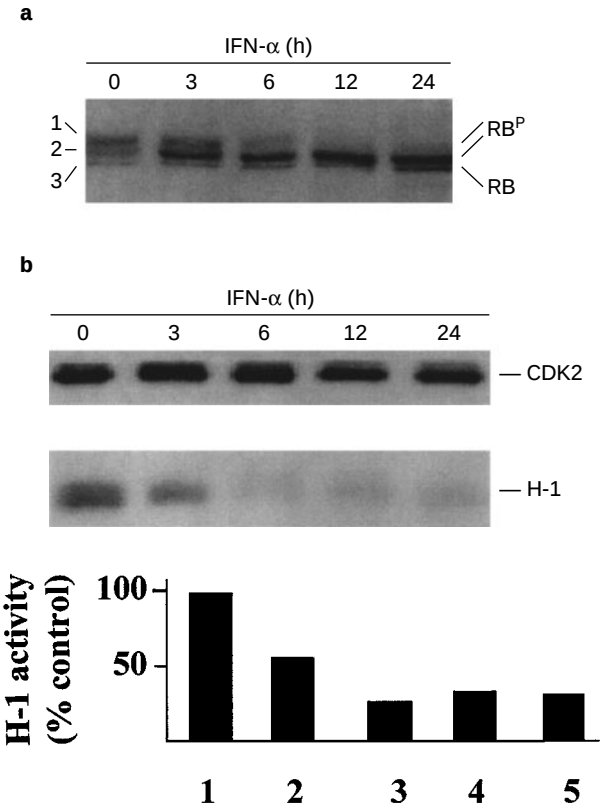

Figure 1 IFN-α inhibits pRB phosphorylation and CDK-2 kinase activity. (**a** and **b**) Daudi cells were treated with IFN-α (500 U/ml). Cell extracts (30 μg protein) were blotted with anti-RB mAb or anti-CDK-2 mAb. Extracts (50 μg protein) were also immunoprecipitated (IP) with anti-CDK-2 Ab, and assayed for H-1 kinase activity

also enhancement of expression of slowest migrating pRB band (Figure 1a, 24 h, band number 3). In brief, these results suggested that IFN-α may have differential effect on the expression of different forms of RB protein, presumably by targeting different CDKs complexes at different stages of cell cycle.

Induction of expression of CDK-inhibitor p21^{WAF1} and inhibition of CDK-associated H-1 kinase activity in IFN-α treated cells

Understand the possible basis of IFN-α mediated inhibition of CDK kinase activity in Daudi cells, we investigated the possibility of regulation of CKIs inhibitors-p21^{WAF1} and p27^{Kip1} by IFNs. Figure 2a shows that IFN-α induces the expression of p21^{WAF1} (lower band, marked by *) starting at 6 h post-treatment. The levels of induced p21^{WAF1} protein persisted up to 48 h after IFN-α-treatment of Daudi cells. There was no effect of IFN-α on p27^{Kip1} expression in Daudi cells (data not shown). Since p21^{WAF1} is known to inhibit the H-1 kinase activity associated with CDK-2 or CDK-4 (Clarke, 1995), we examined the effect of IFN-α on the inhibition of both CDK-2 and CDK-4 kinase activity, and investigated whether depletion of induced p21^{WAF1} would have any effect on CDK-2- and CDK-4-associated kinase activity using H-1 as a substrate. Results in Figure 2 also show that IFN-α differentially inhibited the H-1 kinase activity associated with CDKs as CDK-2- and CDK-4-associated H-1 kinase activities were inhibited by 3 h and 6 h (Figure 2b, lanes 1–5), respectively. In addition, data in Figure 2b (lanes 6–10) also illustrate that the depletion of p21^{WAF1} before immunoprecipitation (IP) with CDKs could partially restore the inhibition of CDKs kinase activity. The observed IFN-α-mediated early inhibition (6–24 h treatment) of CDK-4-associated H-1 kinase activity was not due to the inhibitory effect of CDK-4 expression, as IFN-α-treatment did not inhibit the expression of CDK-4 protein upto 24 h treatment (Figure 2c). Results in Figure 2d show that the induced p21^{WAF1} also interacts with CDK-4, in addition to the reported interaction with CDK-2 (Poon *et al*., 1993; Matsuoka *et al*., 1994). Since the kinetics of restoration (due to depletion of p21^{WAF1}) of H-1 activity associated with CDK-2 or

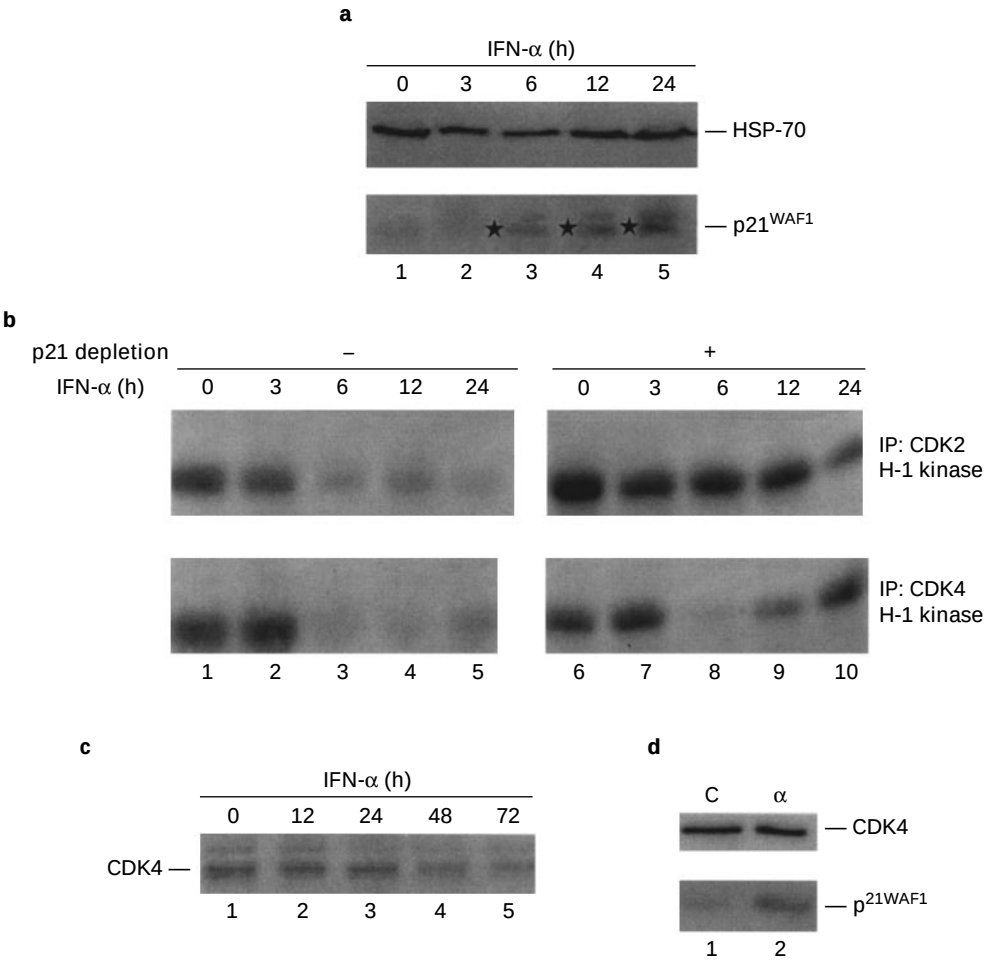

Figure 2 IFN-α induces the expression of p21^{WAF1} in Daudi cells. (**a**) Induction of p21^{WAF1}. Daudi cells were treated with IFN-α, and cell extracts (30 μg protein) were immunoblotted with a anti-p21^{WAF1} mAb (**a**). The position of p21^{WAF1} is shown by *, lower band. As an internal control, the upper part of the same blot was probed with an unrelated anti-heat shock protein (HSP)-70 mAb. (**b**) Effect of immunodepletion of p21^{WAF1} on H-1 activity associated with CDK-2 or CDK-4. Extracts (50 μg) from **a** were IP with anti-CDK-2 or anti-CDK-4 mAb. Samples in lanes 6–10 were first IP with a anti-p21^{WAF1} mAb before IP with anti-CDK mAbs. Precipitated CDKs were subjected to H-1 kinase assay. (**c**) Effect of IFN-α on the expression of CDK-4. Daudi cells were treated with IFN-α for different times, and extracts (30 μg) were blotted with a anti-CDK-4 mAb. (**d**) Enhanced interaction between p21^{WAF1} and CDK-4. Daudi cells treated with IFN-α (24 h) were immunoprecipitated with CDK-4 and blotted with an anti-p21^{WAF1} mAb (lower panel) or anti-CDK-4 mAb (upper panel)

CDK-4 was in-parallel with the kinetics of $p21^{WAF1}$ induction, these results suggested that the observed inhibition of CDK-2 or CDK-4 kinase activity in IFN-α-treated Daudi cells may be due to conditional induction of CDK-inhibitor $p21^{WAF1}$ by IFN-α.

Expression of CDK-inhibitors $p21^{WAF1}$ and $p27^{Kip1}$ in IFN-γ treated cells

We have previously reported (Kumar and Mendelsohn, 1989) that IFN-γ inhibits the growth of human epidermoid A-431 cells by arresting the cells in the G0/G1-phase of the cell cycle. To investigate the role of CDK inhibitors in IFN-γ-mediated growth-inhibition, we examined the expression of $p21^{WAF1}$ and $p27^{Kip1}$ in A-431 cells treated with IFN-γ for different lengths of time. Results in Figure 3 indicated that IFN-γ inhibits pRB phosphorylation (Figure 3a), and also induces the expression of $p21^{WAF1}$ and $p27^{Kip1}$ (Figure 3b). As an internal control, the upper part of the $p27^{Kip1}$ blot was blotted with an unrelated HSP-70 mAb. To examine whether the expression of $p27^{Kip1}$ can be also induced by IFN-α, we examined the effect of IFN-α on $p27^{Kip1}$ in A-431 cells and there was no effect (Figure 3c, lane 4). To examine the generality of IFN-γ-mediated expression of 27^{Kip}, we examined another IFN-γ growth-sensitive cell line NB-4 cells (Korutla and Kumar, 1996). Results indicated that IFN-γ-mediated inhibition of pRB phosphorylation (Figure 3d, lanes 1–3) was also associated with the induction of $p27^{Kip1}$ (Figure 3d, lanes 4–6). There was no effect of IFN-α on $p27^{Kip1}$ in NB-4 cells (data not shown). In brief, these results demonstrated that IFN-γ induces the expression of both $p27^{Kip1}$ and $p21^{WAF1}$.

Effect on IFN-γ on CDKs kinase activity

Since treatment with IFN-γ was associated with induction of expression of CKI inhibitors, we next examined the effect of IFN-γ on H-1 kinase activity associated with CDKs in A-431 cells. As shown in Figure 4a, IFN-γ treatment of A-431 cells resulted in the inhibition of both CDK-2 and CDK-4 kinase activity in a time-dependent manner starting at 12 h post-treatment. Since pRB is the preferred *in vivo* substrate for CDK-4, we also examined the effect of IFN-α on CDK-4-associated kinase activity, using pRB as a substrate (Choubey and Lengyel, 1995). Treatment with IFN-α inhibited the ability of CDK-4 to phosphorylate pRB *in vitro* (Figure 4b). Since IFN-γ induced the expression of both $p21^{WAF1}$ and $p27^{Kip1}$, we examined the possible contribution of induced CDK-inhibitors in the observed inhibition of H-1 kinase activity associated with CDKs by prior immunodepletion of CDK-inhibitor before IP with CDK. Results in Figure 4c demonstrated that immunodepletion of $p21^{WAF1}$ but not $p27^{Kip1}$ resulted in partial restoration of CDK-2 kinase activity. In contact, immunodepletion of $p27^{Kip1}$ but not $p21^{WAF1}$ led to partial restoration of CDK-4 kinase activity. Figure 4d show the increased association of $p21^{WAF1}$ with CDK-2, and $p27^{Kip1}$ with CDK-4 in IFN-γ-treated A-431 cells as compared to control untreated cells. Taken together, these observations suggested that IFN-γ-induced $p21^{WAF1}$ and $p27^{Kip1}$ may have preferential interaction with CDK-2 and CDK-4, respectively.

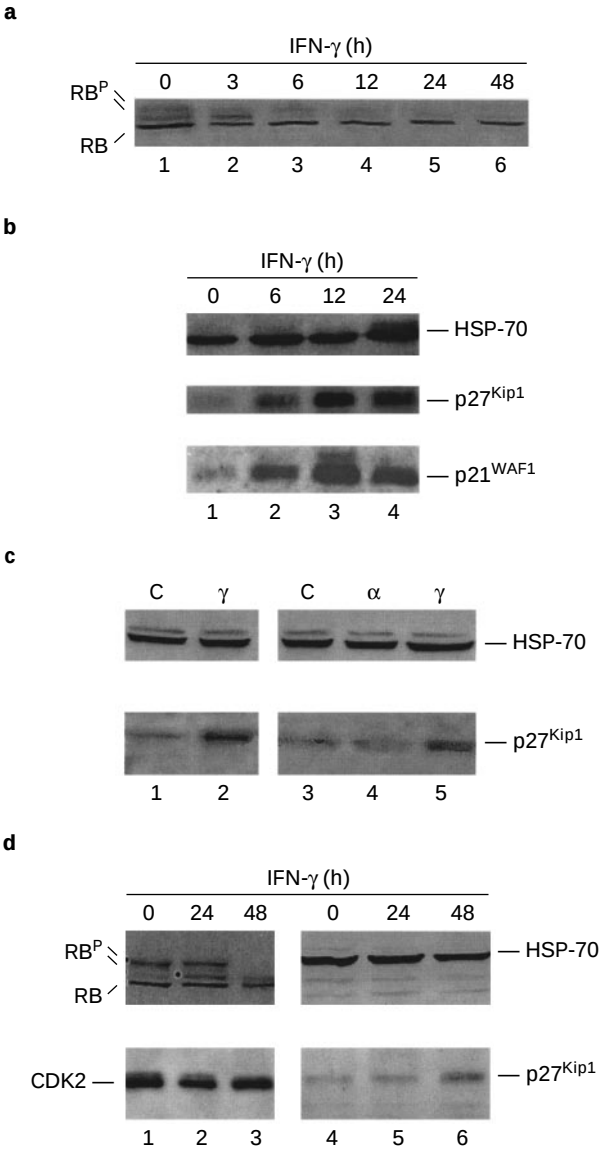

Figure 3 Induction of $p21^{WAF1}$ and $p27^{Kip1}$ by IFN-γ. (**a**) A-431 cells were treated with IFN-γ (500 U/ml), and cell extracts were immunoblotted with an anti-RB mAb (**a**) or anti-$p21^{WAF1}$ mAb or anti-$p27^{Kip1}$ mAb (**b**). As an internal control, upper portion of the p27 blot was blotted with an unrelated HSP-70 mAb. (**c**) A-431 cells were treated with IFN-γ (lanes 2 and 5) of IFN-α (lane 4) for 24 h. Cell extracts (30 μg) were blotted with anti-$p27^{Kip1}$ mAb or HSP-70 mAb. (**d**) NB-4 cells were treated with IFN-γ for 24 or 48 h. Cell extracts (30 μg) were blotted with the indicated mAbs. In lanes 4–6, both HSP-70 and $p27^{Kip1}$ were from the same blot

Effect of IFNs on the expression of CDK-7

In addition to CKIs, the activities of CDKs have been shown to be influenced by phosphorylation by CDK-7 (see Introduction). Since we were not able to completely restore the CDK2 H-1 kinase activity by immunodepletion of CKI, we examined the possibility whether CDK-7 could be a potential IFN target using a well-characterized anti-CDK-7 mAb (Tassan *et al*., 1994). Figure 5 shows that IFN-α had no inhibitory effect on CDK-7 expression except that we noticed the appearance of a fused band (not resolved fully in this gel, shown by an arrow) of a slightly faster moving band at 24 h post IFN-α-treatment (Figure 5a, lane 5).

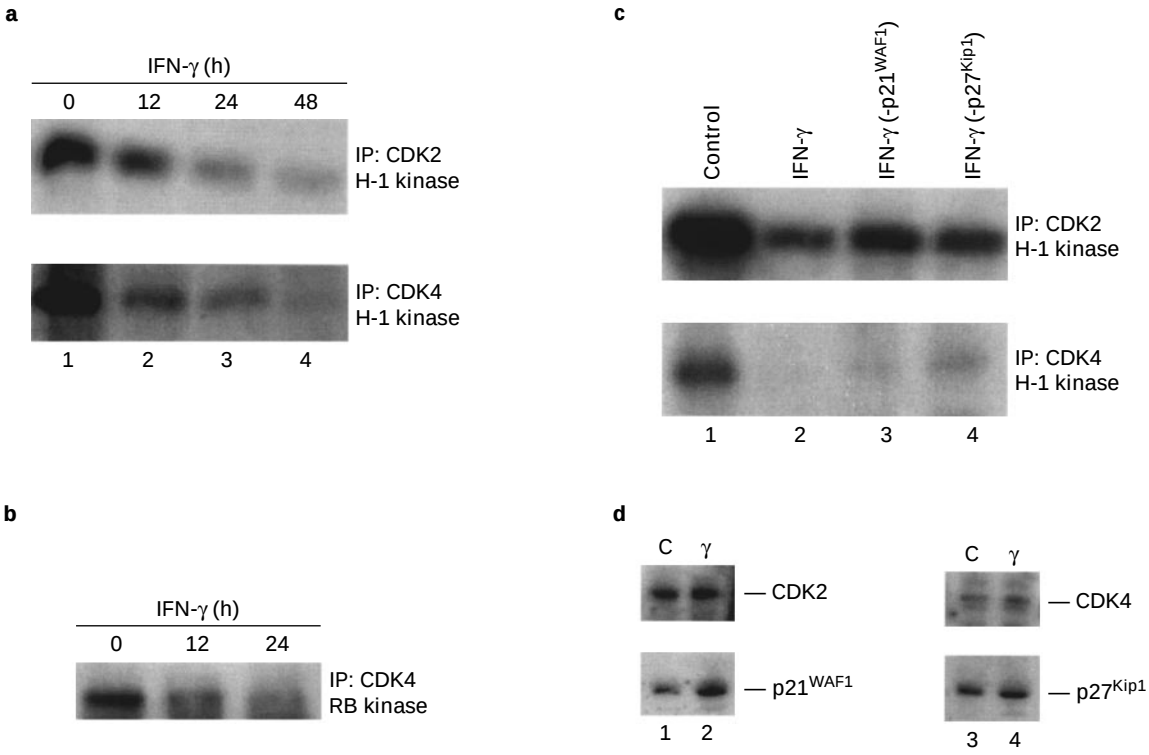

Figure 4 Effect of IFN-γ on CDK activity in A-431 cells. (**a**) Cells were treated with IFN-γ for different times, and extracts (100 μg) were IP with either CDK-2 or CDK-4 mAbs and assayed for the H-1 kinase activity. (**b**) Cell extracts (100 μg) were IP with CDK-4 mAbs and assayed for the pRB kinase activity. (**c**) A-431 cells were treated with IFN-γ (24 h) and extracts (100 μg) were IP with CDK-2 or CDK-4 and assayed for H-1 kinase activity. In lanes 3 and 4, samples in were first IP with an anti-$p21^{WAF1}$ mAb (lane 3) or $p27^{Kip1}$ mAb (lane 4) before IP with anti-CDK mAbs. (**d**) Interaction between CDKs and CKIs. A-431 cells were treated with IFN-γ (24 h), and extracts (100 μg) were IP with CDK-2 or CDK-4 and immunoblotted with an anti-$p21^{WAF1}$ mAb (lanes 2 and 3) or $p27^{Kip1}$ mAb (lanes 3 and 4). The blot was stripped and reprobed with the indicated CDK mAbs

Additional experiments indicated that 24 h or longer treatment of Daudi cells with IFN-α is accompanied by appearance of a distinct faster migrating band of CDK-7 as determined by direct blotting (IB) (Figure 5a, compare lane 7 with 6) or immunoprecipitation (IP) followed by blotting (Figure 5b, lane 2). Since the activation of CDK-7 has been shown to require binding of a regulatory cyclin H, we examined whether IFN-α-treatment have any effect on the ability of CDK-7 to interact with cyclin H. Sequential blotting of above blot (Figure 5b, lanes 1 and 2) with cyclin H Ab indicated that there was no effect of IFN-α on the CDK-7 interaction with cyclin H (Figure 5b, lanes 1 and 2). However, when cell extracts were blotted with CDK-7 after IP with cyclin H, the amount of CDK-7 bound with cyclin H was decreased in IFN-α-treated Daudi cells (Figure 5b, lanes 3 and 4). It is possible that the differences between CDK-7 mAb immunoprecipitates (lanes 1 and 2) and cyclin H immunoprecipitates (lanes 3 and 4) may be due to the fact that CDK-7 mAb immunoprecipitated both free and cyclin H-bound CDK-7, but cyclin H Ab immunoprecipitated only CDK-7 that was bound with cyclin H. Direct blotting of cell extracts with anti-cyclin H Ab demonstrated that IFN-treatment up to 24 h had no effect on cyclin H expression (Figure 5c, lanes 2 and 3). However, prolonged 72 h treatment was associated with inhibition of cyclin H (Figure 5c, lane 4), as shown earlier by Yamada *et al*. (1995). It was interesting to note that in lanes 2 and 4 (Figure 5b, IFN-α-treated extracts), the appearance of the faster migrating band was at the expense of a slower migrating band in control cell extracts (lanes 1 and 3). We next examined the effect of IFN-γ on the expression of CAK in A-431 and NB-4 cells. Results in Figure 5d and e show that treatment of A-431 and NB-4 cells respectively with IFN-γ resulted in the appearance of expression of faster migrating form of CDK-7 (shown by an arrow).

IFN-induced CDK-inhibitors prevent CAK from activating CDK-2

Though there was no early effect of IFN on CDK-7 expression, in an attempt to investigate the possible basis of the inhibition of H-1 kinase in IFN-treated A-431 cells, we examined the possibility whether CDK-activating kinase (CAK) is a direct and/or indirect target of IFN-γ. CAK was immunoprecipitated from the control and IFN-γ-treated A-431 cell extracts and assayed for its ability to activate recombinant GST-CDK2 which in-turn was assayed for histone H-1 kinase activity (CAK assay). Results in Figure 6a (left panel, immunecomplex CAK assay) show that there was no significant change in the levels of CAK activity present in IFN-γ-treated cells compared to untreated A-431 cells, suggesting that IFN-γ may not have a direct effect on CAK activity associated with CDK-7. Results of other experiments indicated that there was no co-IP of IFN-induced CDK-inhibitor $p21^{WAF1}$ with

CDK-7 (data not shown). In addition to the immunecomplex CAK assay, we also assayed CAK activity by in-solution method that allows other components of cell lysate to influence the activation of GST-CDK2 by CAK. In contrast to the results from the immunecomplex CAK assay, results in Figure 6a (right panel) indicated that IFN-γ treatment was associated with the inhibition CAK activity. As control, GST-beads were treated in an identical manner and there was no effect (data not shown). Since IFN-γ induces expression of CDK-inhibitors, we reasoned that the observed inhibition of in-solution CAK activity in IFN-treated A-431 cell extracts may be due to induced expression of CDK-inhibitors which may prevent CAK from phosphorylating GST-CDK2. Data in Figure 6b show that immunedepletion of $p21^{WAF1}$ (lane 4) or $p27^{Kip1}$ (lane 5) from the IFN-γ-treated (24 h) inhibitory cell extract (lane 3) resulted in partial restoration of CAK activity, suggesting that IFN-induced CDK-inhibitors prevents the activation of GST-CDK2 by CAK.

Discussion

IFNs are potent regulators of cellular proliferation. The growth inhibitory action of IFN is an active process, and involves suppression of the cell cycle progression. Both IFN-α and IFN-γ have been shown to arrest human cells in the G0/G1 phase of the cell cycle. Earlier we and others have shown that IFN inhibits pRB protein phosphorylation by inhibiting CDK-2 kinase activity. We have undertaken the present investigation to further delineate the basis of IFN-mediated inhibition of CDK activity by examining the role(s) of pathways such as CDK-inhibitors and

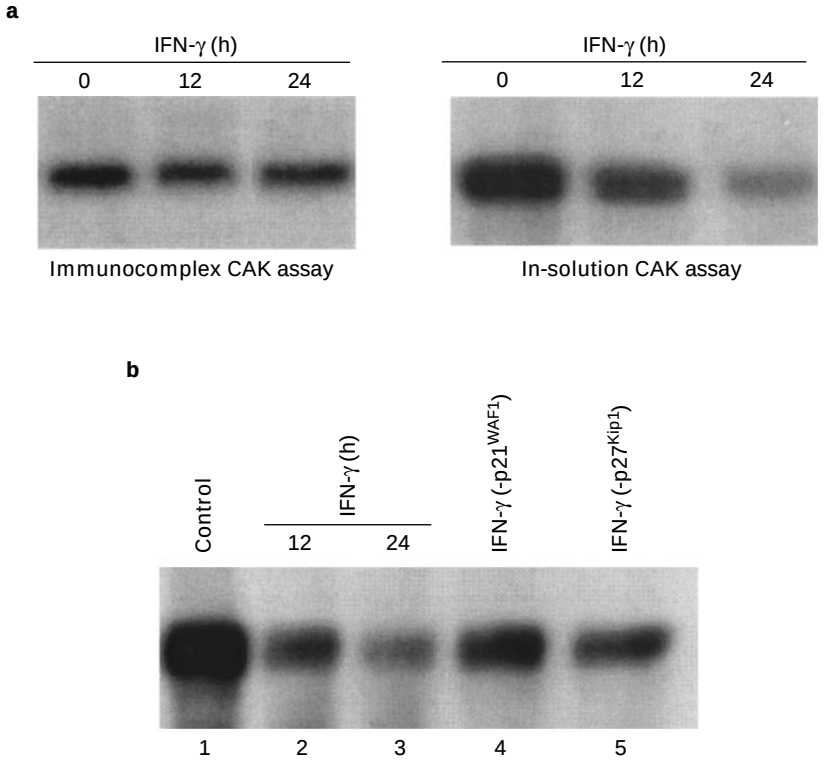

Figure 6 Effect of IFN-γ on CAK activity in A-431 cells. (**a**) Cells were treated with IFN-γ for different times, and extracts (80 μg) were assayed by either immunocomplex (left panel) or in-solution (right) CAK assay as described in Materials and methods. (**b**) Cells were treated with IFN-γ and cell extracts (80 μg) were assayed by in-solution CAK assay as described above. In lanes 4 and 5, samples were first IP with an anti-p21^{WAF1} mAb (lane 4) or p27^{Kip1} mAb (lane 5) before CAK assay

CDK activating kinase that control the function of CDKs.

Results presented here demonstrated that treatment of Daudi cells with IFN-α significantly inhibited both CDK2- and CDK4-associated H-1 kinase activity without any effect on its expression, and this was accompanied by in-parallel induction of expression of CDK-inhibitor p21^{WAF1} but not p27^{Kip1}. Our this view is supported by recent reports showing a correlation between the induction of p21^{WAF1} and inhibition of CDK-2 kinase activity in IFN-α-treated cells (Sangfelt *et al*., 1997; Hobeika *et al*., 1997; reported their results while this study was in progress). In the past, Chin *et al*. have shown the induction of p21^{WAF1} mRNA in IFN-γ-treated A-431 cells (Chin *et al*., 1996). We have also demonstrated that the observed relationship between the induction of p21^{WAF1} and inhibition of CDK kinase activity was not causal as immunodepletion of p21^{WAF1} from the inhibitory IFN-α-treated extracts restored in-part the H-1 activity associated with CDK-2 or CDK-4. Since one of the well-accepted function of CDKs is to phosphorylate different substrates such as pRB, IFN should not inhibit the growth of cells that are defective in pRB. However, this view may not always be correct as Hobeika *et al*. (1997) have recently demonstrated both the induction of p21^{WAF1} and inhibition of CDK-2 kinase in IFN-α-treated human prostate carcinoma DU-145 cells that are defective in pRB. Possible significance of IFN-α-induced expression of p21^{WAF1} in a pRB minus cell line remains to be investigated. It is possible that IFN-α may inhibit the phosphorylation of other members of pRB family such as p130 which is functional in DU-145 cells (Peng *et al*., 1996) and/or IFN-α-induced p21^{WAF1} could bind to PCNA and block DNA replication.

The finding that IFN-α induces the expression of p21^{WAF1} while IFN-γ induces the expression of both p21^{WAF1} and p27^{Kip1}, raises a possibility that p21^{WAF1} may be regulated by both IFN-α and IFN-γ generated signals but p27^{Kip1} can be only induced by IFN-γ signal(s). It will be important to further explore the possible molecular basis of differential regulation of CKIs by IFNs, and such efforts are underway. We have also demonstrated that the induced expression of CKIs may have a functional role in IFN-γ-treated cells as immunodepletion of induced CKIs from IFN-treated extracts could partially restore the H-1 kinase activity associated with CDKs. We have also provided evidence to suggest that IFN-γ-mediated inhibition of CDK2 and CDK4 was due to preferential enhanced interactions with p21^{WAF1} and p27^{Kip1}, respectively. In brief, our results suggested a regulatory role of IFN-induced CKIs in the observed inhibition of CDKs in IFN-treated cells. However, due to the partial nature of restoration of CDKs kinase activities by immunodepletion of CKIs, our results raise the possibility of involvement of pathway(s) other than CKIs as potential target(s) in IFN-treated cells.

Our observation that treatment of a variety of cell-types with IFN was accompanied by the late appearance of the faster migrating band of CDK-7, is important as it suggests that the modified CDK-7 in IFN-treated cells may affect its ability to interact with other proteins in multiprotein complexes such as p36/MAT-1 (Adamczewski *et al*., 1996). Since CAK has

been shown to be responsible for the C-terminal domain (CTD) kinase activity associated with a multiprotein transcription factor complex TFIIH (Feaver *et al*., 1994), it is possible that IFN-mediated appearance of the faster migrating CAK form may influence the CTD kinase activity due to possible modulation of interactions between components of CAK (CDK-7, cyclin H and p36/MAT1) of TFIIH (p89 and p62). (Devault *et al*., 1995). Further investigations are required to examine these possibilities.

Another notable finding of this study was the indirect inhibitory effect of IFNs on CAK activity. Although recent studies have demonstrated the induction of $p21^{WAF1}$ by IFN-α but the mechanism through which CDK-inhibitors inhibit CDK in IFN-treated cells remains poorly understood. Since activation of CDKs requires an essential phosphorylation step by CAK, we therefore examined the hypothesis that IFN-induced CDK-inhibitors may interfere with the activation of CDK by CAK. In agreement with this view, our results have shown that the presence of a CAK inhibitor(s) in IFN-treated extracts, and immunodepletion of $p21^{WAF1}$ or $p27^{Kip1}$ from the inhibitory IFN-treated extracts resulted in part restoration of CAK-mediated activation of CDK-2. Taken together, these results suggested that IFN-induced CDK-inhibitors prevent CAK from activating the CDK holoenzyme.

Materials and methods

Cell culture

Human Burkitt's lymphoma Daudi cells (Kumar and Atlas, 1992) and acute promyelocytic leukemia NB-4 cells (Korutla and Kumar, 1996) were grown in RPMI-1640 medium containing 10% fetal bovine medium. Human epidermoid carcinoma A-431 cells (Kumar and Mendelsohn, 1989) were maintained in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Human recombinant IFN-α 2a (Specific activity, 5×10^7 IU/mg protein) and recombinant IFN-γ (Specific activity 4×10^6 IU/mg protein) was obtained from the Hoffman La Roche Inc. and Genentech Inc., respectively.

Cell extracts and immunoblotting

All experiments were performed with cells in logarithmic phase by controlling the plating density. Cells' extracts were prepared as described (Kumar and Atlas, 1992). Cell lysates containing equal amount of total protein (15–30 μg) were resolved on a 7% (for RB protein) or 10% (for other proteins) SDS–PAGE, followed by immunoblotting using alkaline phosphatase or ^{125}I-protein A or ECL method (Mandal *et al*., 1996). As an internal control, the same blot was reprobed with an unrelated antibody. Low-molecular-mass colored markers (Amersham Corp.) were used as molecular weight standards. The following antibodies were used purchased from the Neomarkers, Inc: anti-RB mAb (clone IF8/Rb-1), anti-p21 mAb (clone DCS 60.2), anti-p27 mAb (clone DCS 72.F6), anti-CDK-7 mAb (clone MO 1-1), and anti-HSP70 mAb (clone BMR 22). Anti-CDK2 mAb (clone 55) and anti-CDK4 mAb (clone 97) were from the Transduction Laboratories. Antibody against cyclin H (SC-609) was purchased from the Santa Cruz, Inc. Quantitation of specific protein bands was performed by using protein databases scanner (Molecular Dynamics).

Histone H-1 kinase assay

Specific CDK was immunoprecipitated from 100 μg total protein by using a specific Ab as described (Zhang and Kumar, 1994). Assay of CDK kinase is based on the transfer of ^{32}P from labeled ATP by CDK kinase to dephosphorylated H-1 histone. The kinase reaction mixture contained 25 mM glycerophosphate pH 7.3, 10 mM EGTA, 10 mM $MgCl_2$, 1 mM DTT, 25 mM HEPES pH 7.3, 5 μg of H-1 histone, 100 pmole ATP and 5 μCi [^{32}P]-ATP in a volume of 25 μl. Reaction product was resolved onto a gel, transferred on a nitrocellulose and subjected to autoradiography. Subsequently the blot was immunoblotted with a specific CDK Ab to evaluate the immunoprecipitation efficiency.

Expression and purification of recombinant proteins

GST-CDK2 and Histidine-tagged Protein A-cyclin A (PA-cyclin A) were generous gifts from Randy Poon and described previously (Poon *et al*., 1993). GST-CDK2 was expressed in *E. coli*, cultures were grown up to log phase and expression of recombinant GST-CDK2 was induced with isopropyl-b-D-thiogalactopyranoside (0.1 mM) for 6 h. Cells were lysed, sonicated and centrifuged at 10 000 *g* for 30 min as described (Poon *et al*., 1993). GST-CDK2 was purified from the supernatant using glutathione-Sepharose 4B protein purification kit from the Pharmacia Fine Chemicals as per manufacturer's instructions. Histidine-tagged Protein A-cyclin A (PA-cyclin A) was also expressed in *E. coli* and purified by NTA-Ni^{2+} agarose affinity chromatography following a kit from the Qiagen.

CAK Assay

Immumne-complex CAK assay Cell lysates are IP with anti-CDK-7 mAb, immunoprecipitated complexes (source of CDK-7) were washed with 3 × lysis buffer and 3 × with CAK kinase buffer, resuspended in 50 μl of CAK buffer (Poon *et al*., 1993), and 0.2 μg of bacterially produced cyclin A and GST-CDK2 in CAK buffer containing 1 mM ATP. The mixture was incubated at 22°C for 1 h (CAK-mediated activation step). Activated cyclin-CDK complexes in the supernatant were recovered onto glutathione-Sepharose beads, washed with kinase buffer, and assayed for H-1 kinase activity.

Soluble CAK assay Cell extracts (100 μg protein, source of CDK-7) were mixed with 0.2 μg of bacterially produced cyclin A and GST-CDK-2 in CAK buffer containing 1 mM ATP. The mixture was incubated at 22°C for 1 h (CAK-mediated activation step). Activated cyclin-CDK complexes were recovered onto glutathione-Sepharose beads, washed with kinase buffer, and assay for H-1 kinase activity.

Acknowledgements
We thank Randy Poon for a generous gift of recombinant PA-cyclin A and GST-CDK2 plasmids, and helpful suggestions to establish the CAK assay in our laboratory. We also thank Divakar Choubey for providing us with the purified recombinant pRB. This work was supported in part by National Institutes of Health grant CA56564.

References

Adamczewski JP, Rossignol M, Tassan JP, Nigg EA, Moncollin V and Egly JM. (1996). *EMBO J*., **15,** 1877–1884.

Burke LC, Bybee A and Thomas NS. (1992). *Oncogene*, **7,** 783–788.

Chin YE, Kitagawa M, Su W-S, You Z-H, Iwamoto Y and Fu XY. (1996) *Science*, **272,** 719–722.

Clarke PR. (1995). *Current Biol*., **5,** 40–42.

Choubey D and Lengyel P. (1995). *J. Biol. Chem*., **270,** 6134–6140.

Devault A, Martinez AC, Fesquet D, Labbe JC *et al*. (1995). *EMBO J*., **15,** 5027–5036.

Dulic V, Lees E and Reed SI. (1992). *Science*, **257,** 1958–1961.

Feaver WJ, Svejstrup JQ, Henry NL and Kornberg RD. (1994). *Cell*, **79,** 1103–1109.

Fisher RP and Morgan DO. (1994). *Cell*, **78,** 713–724.

Hall M, Bates S and Peters G. (1995). *Oncogene*, **11,** 1581–1588.

Harper JW, Adami GR, Wei N, Keyomarsi K and Elledge SJ. (1993). *Cell*, **75,** 805–816.

Hobeika AC, Subramaniam PS and Johnson HM. (1997). *Oncogene*, **14,** 1165–1170.

Harvet BL and Jetten AM. (1996). *Cell Growth & Diff*., **7,** 289–300.

Hunter T and Pines J. (1994). *Cell*, **79,** 573–582.

Kato JY, Matsuoka M, Polyak K, Massague J, Sherr CJ. (1994). *Cell*, **79,** 487–496.

Korutla L and Kumar R. (1996). *Anticancer Res*., **16,** 2789–2796.

Kumar R and Mendelsohn J. (1989). *Cancer Res*., **49,** 5180–5184.

Kumar R and Atlas I. (1992). *Proc. Natl. Acad. Sci. USA*, **89,** 6599–6603.

Kumar R, Korutla L and Zhang K. (1994). *J. Biol. Chem*., **269,** 25437–25441.

Mandal M, Maggirwar SB, Sharma N, Kaufmann SH, Sun SC and Kumar R. (1996). *J. Biol. Chem*., **271,** 30354–30359.

Matsuoka M, Kato J, Fisher RP, Morgan DO and Sherr CJ. (1994). *Mol. Cell. Biol*., **14,** 7265–7275.

Melamed DN, Tiefenbrun A, Yarden A and Kimchi A. (1993). *Mol. Cell. Biol*., **13,** 5255–5265.

Moore RN, Larsen HS, Horohov DW and Rouse BT. (1984). *Science*, **223,** 178–180.

Morgan DO. (1995). *Nature*, **374,** 131–134.

Pestka S, Langer JA, Zoon KC and Samuel CE. (1987). *Ann. Rev. Biochem*., **56,** 727–777.

Peng D, Fan X, Lu Y, Deblasio T, Scher H and Mendelsohn J. (1996). *Can. Res*., **56,** 3666–3669.

Poon RY, Yamashita K, Adamczewski JP, Hunt T and Shuttleworth J. (1993). *EMBO J*., **12,** 3123–3132.

Reed SI. (1991). *Trends Genet*., **7,** 95–99.

Reed SI. (1992). *Ann. Rev. Cell. Biol*., **8,** 529–561.

Resnitzky D, Tiefenbrun N, Berissi H and Kimchi A. (1992). *Proc. Natl. Acad. Sci. USA*, **89,** 402–406.

Sangfelt O, Erickson S, Einhorn S and Grander D. (1997). *Oncogene*, **14,** 415–423.

Seroz T, Hwang JR, Moncollin V and Egly JM. (1995). *Curr. Opin. Genet. Dev*., **5,** 217–221.

Sherr CJ. (1993). *Cell*, **73,** 1059–1065.

Sokawa Y, Watanabe Y, Watanabe Y and Yawada Y. (1977). *Nature*, **268,** 236–238.

Tassan JP, Schultz SJ, Bartek J and Nigg EA. (1994). *J. Cell Biol*., **127,** 467–478.

Tiefenbrun N, Malamed D, Levy N, Resnitzky D, Hoffmann I, Reed SI and Kimchi A. (1996). *Mol. Cell. Biol*., **16,** 3934–3944.

Waga S, Hannon GJ, Beach D and Stillman B. (1994). *Nature*, **369,** 574–578.

Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R and Beach D. (1993). *Nature*, **366,** 701–704.

Yamada H, Ochi K, Nakada S, Memot T and Horiguchi YJ. (1994). *Mol. Cellular Biochem*., **136,** 117–123.

Yamada H, Ochi K, Nakada S, Takahara S, Nemoto T, Sekikawwa T and Horiguchi YJ. (1995). *Mol. Cellular Biochem*., **152,** 149–158.

Zhang K and Kumar R. (1994). *Biochem. Biophysi. Res. Comm*., **200,** 522–528.

Zullo J, Cochran BH, Huang A and Stiles CD. (1985). *Cell*, **43,** 793–800.