Vol. 200, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1994 Pages 522-528

INTERFERON-α INHIBITS CYCLIN E- AND CYCLIN D1-DEPENDENT CDK-2 KINASE ACTIVITY ASSOCIATED WITH RB PROTEIN AND E2F IN DAUDI CELLS

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Received February 28, 1994

SUMMARY: The state of phosphorylation of retinoblastoma (RB) protein is regulated by CDK2 and CDC2 kinases. In the studies presented here, we have investigated the effect of interferon-α (IFN-α) on cyclin-dependent kinases in Daudi cells which were synchronized at different points of the cell cycle progression. We observed that the IFN-α enhances the expression of under-phosphorylated RB protein in Daudi cells released from the G1/S, and this was closely associated with the inhibition of CDK2 kinase and not CDC2 kinase activity. The observed IFN-α-sensitive CDK2 kinase activity was dependent on Cyclin E and cyclin D1 but not on cyclin A and was physically associated with transcriptional factors: RB and E2F. In addition, treatment of G1/S Daudi cells with IFN-α also inhibited the ability of CDK2 enzyme to phosphorylate the RB protein *in vitro*. These results suggest possible involvement of cell cycle kinases in IFN-α action.

Interferons (IFNs) are a family of secretory proteins with distinctive cellular effects including inhibition of cell growth. IFNs are thought to mediate their growth inhibitory actions by induction of IFN-induced gene products (1-3), down-regulation of oncogene expression (4), and modulation of expression and phosphorylation of retinoblastoma (RB) protein (5-8).

Tumor suppressor RB protein is a 105 kilodalton nuclear phosphoprotein with characteristics of transcriptional factor, cell cycle regulatory factor and growth suppressor (9). RB protein is phosphorylated in a cell cycle dependent manner, and exists as underphosphorylated form (RB) in Go/G1 phase, and highly phosphorylated form (RB^P) in G1/S and G2/M phases of cell cycle (10-12). Underphosphorylated RB protein has been shown to form a complex with transcriptional factors E2F and MYC protein, and inhibits the transition of cell from G1/S (13, 14). The state of phosphorylation of RB protein is regulated by the cell division cycle (CDC2)-related kinases (15). The RB protein contain multiple consensus sites for phosphorylation by CDC2, and can be phosphorylated by CDC2 *in-vitro* (16,17). The CDC2

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0006-291X/94 $5.00

kinase has been shown to be active during G1/S and G2/M cell cycle transitions in yeast (18). However, in higher eukaryotes, CDC2 kinase is believed to be active at G2/M phases (15), while RB protein become phosphorylated at the G1/S transition (15). The G1/S transition is regulated by cyclin-dependent kinase 2 (CDK2) that is homologous with, but distinct from, CDC2 (19-23). The activation of CDK2 and CDC2 kinases is regulated by their association with cyclins. Cyclin B forms complex with CDC2 at G2/M phases (15). Cyclin E, and cyclin A have been shown to form active kinase complex with CDK2 protein at late G1/early S phases (20, 21), and S/G2 phases (23), respectively. Recently cyclin D1 have been shown to be associated with the CDK protein (24). In addition to cyclins, the kinase activity of CDK2 is further regulated by its phosphorylation on tyrosine and threonine residues (inhibits kinase activity) and dephosphorylation by CDC25 phosphatase (stimulates kinase activity) (25).

We have previously shown that IFN-α enhances the expression of underphosphorylated RB protein in Daudi cells (8). In the experiments reported here, we have investigated the effect of IFN-α on cyclin-dependent kinases which modulate the RB protein phosphorylation, by using synchronized Daudi cells at different points of the cell cycle progression.

MATERIALS AND METHODS

Cell culture, interferon, and synchronization: Human Burkitt's lymphoma Daudi cells (31) were grown in RPMI-1640 medium containing 10% fetal bovine medium. Human recombinant IFN-α 2a (Specific activity, 5x 10^7 units/mg protein) was from the Hoffmann La Roche, Inc. Cells were synchronized in Go/G1 phase by serum starvation for 32 h. Cells were synchronized at G1/S phase by sequential treatment with 5 mM thymidine (Sigma) for 20 hr and 5μg/ml aphidicolin (Sigma) for 16 h (26). Cells were synchronized in G2/M phase by treating with 0.5 μg/ml nocodazole for 16 h (26). The synchrony of the cells was determined by FACS analysis of the DNA content (3). In general, cultures of 70% or greater synchronized cells were used.
Imunoprecipitation and immunoblotting: Cells were treated with IFN-α (500 U/ml) at a cell density of 2-4x10^5 cells per ml. Preparation of cell lysates and immunoprecipitation was as described (8,28). Protein 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 (27, 28).
Preparation of $P13^{Suc1}$-agarose beads: The bacterial strain BL21, which contains a plasmid in which expression of the suc1 DNA fragment from S. pombe is driven by the T7 promoter, was used to overexpress $P13^{Suc1}$ following induction of the T7 RNA polymerase with isopropyl-B-D-thiogalactoside as described (29). The isolation and purification of $P13^{Suc1}$ was essentially as described (30). The $P13^{Suc1}$ beads were prepared using Pharmacia's cyanogen sepharose.
Assay of CDC2 or CDK2 kinase using H-1 histone and RB protein as substrates: CDC2 or CDK2 kinase was extracted as described (31). CDC2 or CDK2 was isolated from 80 μg total protein by using either $P13^{Suc1}$-beads or C-terminal specific Abs as described (31). The $P13^{Suc1}$-CDC2 beads or the $P13^{Suc1}$-CDK2 beads were used as a source of CDC2/CDK2 kinases. When needed, CDC2 or CDK2 was eluted from the $P13^{Suc1}$ beads by using pure p13. Assay of CDC2/CDK2 kinase is based on the transfer of ^{32}P from labeled ATP by CDC2/CDK2 kinase to dephosphorylated H-1 histone. The reaction mixture for CDC2/CDK2 kinase assay contained 25 mM glycerophosphate pH 7.3, 10 mM EGTA, 10 mM $MgCl_2$, 1 mM DTT, 25 mM Hepes pH 7.3, assay 5 μg of H-1 histone, 100 pmole ATP and 0.5 μCi ^{32}P-ATP in a volume of 25 μl. Enzyme-$P13^{Suc1}$ beads or eluted enzyme solution was added into the 50 μl volume for 5 min at

30°C. The RB protein was immunoprecipitated from Daudi cells from the late Go to early G1 phases as described (8). The RB protein beads used as substrate for CDC2/CDK2 kinase assay.

RESULTS AND DISCUSSION

Effect of IFN-α on the expression of RB protein in different phases of the cell cycle. Since earlier we have shown that the IFN-α transiently enhances the expression of RB protein which predominantly exists as underphosphorylated RB protein in human Burkitt's lymphoma Daudi cells (8), and the fact that the status of RB protein phosphorylation changes in a cell cycle dependent manner (9), we investigated the possibility of inhibition of RB phosphorylation in IFN-α treated synchronized Daudi cells. In these experiments, cells were synchronized at different stages of the cell cycle progression (O time), and then released in the presence or absence of IFN-α for 6h. As shown in Fig.1, IFN-α enhanced the expression of faster migrating underphosphorylated forms (RB) of RB protein as compared to untreated cells in Daudi cells released from G1/S phase (similar results are shown in Fig. 3A). As expected, RB protein exits

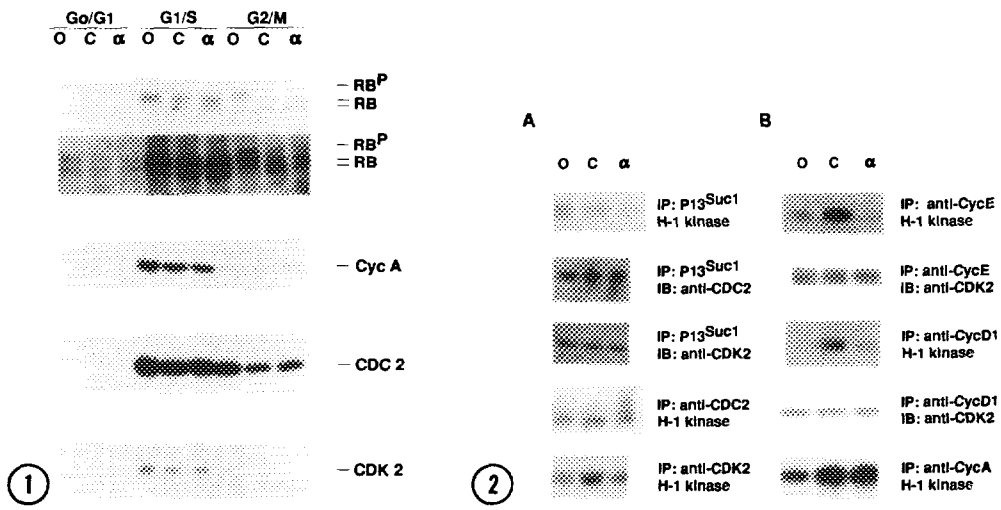

Fig.1. Effect of IFN-α on the expression of cell cycle regulatory proteins in Daudi cells synchronized at different phases of the cell cycle. Cells were synchronized at Go/G1, G1/S, and G2/M stages of cell cycle and then cultured for 6h with or without IFN-α. Cell extracts were made for assaying the expression of indicated proteins by immunoblotting. Relevant portion of the gel is shown here. Shorter and longer exposures of the same RB immunoblot are shown. O, cells at the time of release; C, control; α, IFN-α treated.

Fig.2. Effect of IFN-α on the cell cycle kinases in Daudi cells from G1/S phase. Cells synchronized at G1/S were treated for 6h with or without IFN-α. 80 μg total protein was immunoprecipitated with $P13^{Suc1}$ or anti-CDC2 mAb or anti-CDK2 Ab or indicated Abs. Immunoprecipitates were either immunoblotted with indicated antibodies or assayed for H-1 kinase activity. O, G1/S; C, untreated cells; α, IFN-α treated cells. IP, immunoprecipitation. IB, immunoblotting.

as underphosphorylated forms (RB) in Go/G1 phase and as highly phosphorylated form (RB^P) in G2/M phases. There was no effect of IFN-α on the modulation of expression of RB protein bands in cells released from Go/G1 and G2/M phases. Since the cell cycle progression from G1 through S phase is controlled by cyclin E, cyclin D1, and cyclin A which are produced and required at the G1/S (20-24), we examined the expression of cyclin A, and results indicated no effect of IFN-α on cyclin A expression. There was also no effect of IFN-α on the levels of cyclins E and D1 in the above samples (data not shown). Next, we examined the expression of proteins of CDC2 and CDK2 kinases which are believed to be activated by cyclins and regulate the state of phosphorylation of RB protein at different points throughout the cell cycle (15). As shown in Fig.1, there was appreciable expression of CDC2 in cells at G1/S and G2/M, and of CDK2 at G1/S, and IFN-α treatment had no effect on their expression. In brief, these results shows that IFN-α enhances the expression of underphosphorylated RB protein without affecting the levels of expression of cyclins A, D1 and E, and CDC2 and CDK2 kinase proteins in Daudi cells released from G1/S phase. All subsequent experiments were performed with G1/S cells.

IFN-α inhibits the cyclin E- and cyclin D1-dependent CDK-2 kinase activities in G1/S Daudi cells. In the next series of experiments, we examined the possible effect of IFN-α in preventing the phosphorylation of IFN-induced RB protein by examining the cell cycle kinases using H-1 histone as substrate. As $P13^{Suc1}$ has been shown to effectively bind to both CDC2 and CDK2 cell cycle kinases (15, 18), we prepared the cell cycle kinases by immunoprecipitation with P13-agarose beads followed by elution with purified P13. As shown in Fig.2A, the P13 eluted material(s) contains both CDC2 and CDK2 proteins, and IFN-α had no effect on their levels. However, IFN-α inhibited the H-1 kinase activity by 40%. Since P13 recognized H-1 kinase activity could result from CDC2 and/or CDK2, we determined the specific kinase activities of CDC2 and CDK2 enzymes, isolated by using specific non-cross reactive anti-CDC2 and anti-CDK2 Abs (10, 22). Results indicated that IFN-α inhibited the CDK-2 kinase activity but not CDC-2 kinase activity in G1/S synchronized cells. Since CDK2 protein exits as multiprotein complex and its kinase activity is regulated by cyclins: E, D1, and A, experiments were performed to determine the nature of the cyclins on which this IFN-α-sensitive CDK2 kinase activity depends (Fig. 2B). Results indicated that the IFN-α inhibited the H-1 kinase activity of cyclin E- and D1-associated CDK2 complex but not cyclin A-associated CDK2 kinase activity. There was no effect of IFN-α on the levels of CDK-2 proteins.

IFN-α-sensitive CDK2 kinase forms complex with RB protein, CDC25 and E2F. It is believed that the association of cyclins with CDK2 protein kinase allows the subsequent activation of the enzyme-complex which also consists of transcriptional factors RB protein and E2F which may act as substrates for the activated kinases (13). In the same vein, experiments were performed

to examine the possibility of physical association of IFN-α-sensitive CDK-2 kinase with the RB protein and E2F. As shown in Fig. 3, IFN-α induced underphosphorylated RB protein (shown by two lower arrows), and significantly reduced the associated H-1 kinase activity (50-80% in three different experiments) which apparently was due to associated CDK2 protein kinase (also see Fig.2). To examine the effect of IFN-α on the association of RB protein with CDK2 and the CDC25 [cell cycle phosphatase which regulates kinase activity of CDC2/CDK2 (25)], immunoprecipitated RB protein was immunoblotted with either CDK2 or CDC25 Abs. Results indicated that IFN-α treatment caused increased expression of phosphorylated forms of CDK2 and CDC25 proteins (Fig. 3A). Other experiments indicated that IFN-α increases the phosphotyrosine content of CDK2 protein (40%) as determined by immunoblotting with anti-phosphotyrosine (data not shown). Results in Fig. 3B demonstrate that the E2F is also a part of cyclin/CDK2/RB complex and IFN-α inhibited the E2F associated H-1 kinase (supposedly CDK2 kinase) activity, suggesting that the IFN-α may inhibits the CDK2 kinase activity by enhancing CDK2 tyrosine phosphorylation probably as a result of phosphorylation of CDC25 via an mechanism not delineated at the moment. In this regard, it is interesting to note that deactivation

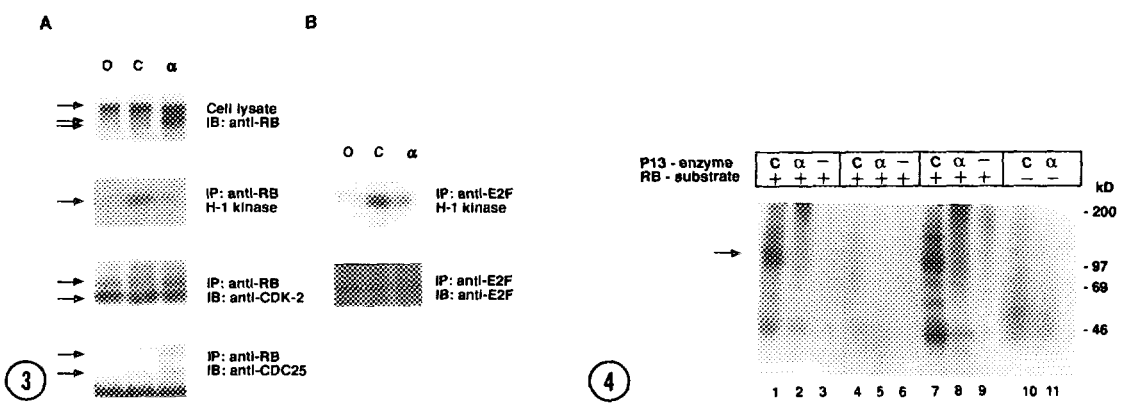

Fig.3. IFN-α inhibits the kinase activity associated with the RB protein in Daudi cells from G1/S phase. Cells were treated as in Fig. 2. (A) Cell lysates were either immunoblotted or immunoprecipitated with anti-RB mAb. RB Immunoprecipitates were assayed for H-1 kinase activity and immunoblotted with indicated antibodies. Upper arrows in the CDK2- and CDC25 blot indicate the slower moving phosphorylated forms. (B) Cell lysates were immunoprecipitated with anti-E2F mAb for determination of H-1 kinase activity and immunoblotting with anti-E2F mAb. Other details are same as in Fig. 2.

Fig.4. IFN-α inhibits the ability of cell cycle kinases to phosphorylate RB protein during *in vitro* kinase reaction. Daudi cells synchronized at G1/S were treated with or without IFN-α for 6h, and cell cycle kinases enzyme(s) was prepared by immunoprecipitation with $P13^{Suc1}$ agarose beads followed by elution of enzyme with free P13. RB protein was immunoprecipitated from Daudi cells synchronized at either G0/G1 (lanes 1-3) or G2/M (lanes 4-6) or cells 6 h release after G2/M block (lanes 7-11). Immunoadsorbed RB protein was phosphorylated *in vitro* by CDK2 enzyme from control (C) and IFN-α treated cells (α). No enzyme was added in lanes 3, 6 and 9. No RB-substrate was added in lanes 10 and 11.

of CDC25 is known to inhibit the CDK2 kinase activity as a result of increased CDK2 tyrosine phosphorylation (25). These results suggest that the IFN-α-induced underphosphorylated RB protein in G1/S synchronized Daudi cells can not be efficiently phosphorylated due to the inhibition of CDK2 kinase activity.

Direct evidence of inhibition of RB phosphorylation by P13 enzyme (CDK2) from IFN-α treated cells. Since CDK2 enzyme has been shown to phosphorylate the RB protein in *in-vitro* studies (22), next, we examined the ability of P13 (CDK2) enzyme from control and IFN-α treated G1/S synchronized Daudi cells to phosphorylate the underphosphorylated RB protein *in-vitro*. As shown in Fig. 4, the RB protein from G1/S and Go/G1 cells (not from G2/M cells) could be phosphorylated very efficiently by P13 enzyme from the control cells but not by enzyme from IFN-α treated cells (compare lanes 2 and 8 with 1 and 7). The differences in the extent of RB phosphorylation were due to the state of RB underphosphorylation as phosphorylation of RB continue to increased from Go/G1 - G1/S - G2/M phases. Immunoblot analysis of the immunoprecipitated RB protein indicated that the RB protein from Go/G1 cells was more underphosphorylated compared to protein from the G1/S cells, and was phosphorylated from the G2/M cells (data not shown). The observed RB protein phosphorylation was due to RB-substrate and P13-enzyme reaction as no phosphorylated RB band could be detected with either RB-substrate or P13-enzyme. On the basis of results discussed earlier in Figs. 2 and 3, we believe that the observed IFN-α mediated inhibition of the P13 enzyme activity was apparently due to CDK2 enzyme. Data in Fig. 4 also strengthen the notion that the RB protein exists as a multiprotein complex as a number of proteins were co-immunoprecipitated with RB protein [in the range of 40-130 kD which covers a number of known RB binding proteins: P130 and P107 (32) , E2F (13), Cyclin E (20)], and could act as substrates for CDK2 enzyme. The phosphorylation of co-immunoprecipitated proteins followed the pattern of RB protein phosphorylation. In summary, results presented here demonstrated that the IFN-α enhances the expression of underphosphorylated RB protein in Daudi cells released from the G1/S, and this was closely associated with the inhibition of cyclin E-and cyclin D1-dependent CDK2 kinase activity which was physically associated with CDC25, RB, and E2F. The observed relationship was not causal as treatment of G1/S Daudi cells with IFN-α also inhibited the ability of CDK2 enzyme to phosphorylate the RB protein *in-vitro*.

ACKNOWLEDGMENTS

We thank Jonathon Pines and Tony Hunter for providing cyclin A and cyclin B Abs, James M. Roberts for Cyclin E Ab, David Beach for CDC2 Ab, Tetsu Akiyama for CDK2 Ab,

Kristian Helin for E2F mAb, Peter C.L. John for P13 plasmid, and Neeta Sharma for expert technical support. This study was supported in part by grant CA56564 from the NIH.

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