Cell Cycle Regulation of Nuclear Factor p92 DNA-binding Activity by Novel Phase-specific Inhibitors

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The nuclear factor p92, originally discovered by its interaction with the human papillomavirus type 18 enhancer, is a cellular protein whose activity is restricted to S phase in human primary fibroblasts. The human papillomavirus type 18 p92 binding sequence confers enhancer activity on a heterologous promoter, suggesting that p92 acts as a transcription factor. We have identified a class of nuclear inhibitory proteins, I-92s, which noncovalently associate with p92 but not with other transcription factors such as AP1, E2F, or NF-kB. Different I-92s occur in G1, G2, and G0, while no I-92 is detectable in S phase. Phase-specific inhibitors, therefore, are responsible for the cell cycle dependence of p92 activity and provide a novel mechanism linking transcription factor regulation with the cell cycle.

We have previously identified a novel cell cycle-regulated DNA-binding protein termed p92, which interacts with multiple p92 recognition sites within the enhancer of human papillomavirus type 18 (HPV18) (1). HPV18 belongs to the group of human papillomaviruses with high oncogenic potential (2). p92 is a sequence-specific nuclear factor that specifically binds to the 5'-AATTGCTTGCATAA motif and other related motifs in the HPV18 enhancer (1, 3). p92 was originally identified by binding site blotting experiments (1), and retarded p92s containing DNA-protein complexes were identified by preparative electrophoretic mobility shift experiments and analysis of eluted proteins by binding site blotting (4). In addition, the 5'-AATTGCTTGCATAA motif is recognized by p92-related factors as was shown by UV cross-linking experiments (3), p92, however, is the most prominent retarded DNA-protein complex in electrophoretic mobility shift assays (4). The HPV18 p92 recognition sequence 5'-AATTGCTTGCATAA consists of two partially overlapping octamer-related motifs (1, 3). We have shown by electrophoretic mobility shift assays (EMSA) that in vitro translated Oct-1 protein interacts with the 5'-AATTGCTTGCATAA motif. p92, however, is not related to Oct-1, as was shown by immunoblotting using an Oct-1 antisera (1). In addition, the intracellular distribution of p92 is regulated by growth factors, because p92 was found in the cytoplasm of serum-starved nontumorigenic HeLa-fibroblast hybrid cells (444 cells) and was translocated to the nucleus upon serum stimulation at the beginning of S phase (1, 4). We have shown by centrifugal elution experiments that during the cell cycle p92 DNA binding activity is restricted to S phase of 444 cells that contain integrated human papillomavirus type 18 (4). In crude nuclear extracts, we have identified an uncharacterized activity, termed I-92, which inhibits DNA binding of p92 (4). In cell cycle populations of 444 cells, p92 DNA binding activity could not be detected in nuclear extracts from G0 phase and from G2 phase cells. It was, however, possible to detect p92 after treatment of these extracts with the detergent deoxycholate (4), suggesting that p92 was complexed to an inhibitor in G1 and in G2 phase of the cell cycle.

In the present communication we characterized I-92 biochemically and functionally. We have delineated a molecular mechanism underlying p92 cell cycle regulation and identified four nuclear inhibitors that link cell cycle regulation with p92 regulation. In normal diploid human fibroblasts (NHDFs), p92 DNA binding is restricted to S phase. In addition, we have determined that p92 may act as a transcription factor because a p92 binding site from the HPV18 upstream regulatory region conferred enhancer activity on a heterologous promoter. The p92/I-92 system, therefore, may represent a novel mechanism of phase-specific gene regulation.

EXPERIMENTAL PROCEDURES

Cell Culture, Cell Synchronization, and Preparation of Extracts—Normal human fibroblasts (PromoCell) and the nontumorigenic HeLa-fibroblast hybrid cell line 444 (5) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Life Technologies, Inc.). Nuclear extracts were prepared as described (6) with some modifications (1). In brief, cells were scraped from plates, washed with phosphate-buffered saline, and then lysed with 0.65% Nonidet P-40 (Sigma), and nuclei were prepared by low speed centrifugation at 4 °C. Nuclear proteins were eluted with 50 mM NaCl and slight agitation followed by dialysis against a buffer containing 50 mM glycerol, 50 mM NaCl, 10 mM Hepes (pH 7.9), 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM diithiothreitol. Lovastatin (a generous gift of Merck, Sharp and Dome) was used to prepare G0 phase 444 cells (7, 8) for the extraction of nuclear proteins and the analysis of I-92 during the cell cycle. Semiconfluent fibroblasts and 444 cells were cultivated for 24 h in the presence of 20 μM lovastatin and then processed for the extraction of nuclear proteins. The efficiencies of G0 phase synchronization were determined by nuclear staining with propidium iodide and fluorescence-activated cell sorting (FACS) measurements of the DNA content. FACS analysis showed that 70% of cells had a diploid DNA content (2n), S phase cells were 14%, and 4n cells were 15%. The drug aphidicolin blocks the activity of DNA polymerase α (9–11). We used this reagent to synchronize 444 in S phase of the cell cycle. In brief, we treated fibroblasts and 444 cells in culture for 24 h with 3 μM aphidicolin followed by a 3-h release in fresh medium. Under those conditions FACS analysis showed that 65% of cells were in S phase, 28% were G0, and 6% were G2N. We used an aphidicolin release strategy to obtain 444 cells in G2 phase of the cell cycle. We determined empirically that after a 24-h aphidicolin treatment (3 μM) and a 10-h release, 87% of the cells...
were 4n as was shown by FACS analysis. In addition we controlled this synchronization event by Western blot analysis using anti-cyclin B antibodies, because cyclin B expression is restricted to G2 phase of the cell cycle (12–14). We found strong expression of cyclin B in G2 phase-synchronized 444 cells, whereas no signal or very little signal was evident in G0, G1 phase and in S phase 444 cells. The latter result is another good indicator for phase synchronization of 444 as a target cell line for a read-out system. A nuclear extract of 444 cells was treated with deoxycholate and passed over heparin-Sepharose. p92 was eluted with 500 mM NaCl and dialyzed against EMS-DNA binding buffer, 10 mM Tris (pH 7.5), 50 mM NaCl, 50 mM dithiothreitol, 4% glycerol. In all electrophoretic mobility shift inhibition assays, 0.4 μg from the same p92 preparation was used. For EMSIA I-92, preparations were incubated with 0.4 μg of partially purified p92 at room temperature (20 min), and end-labeled RP3 double-stranded oligonucleotide (15,000 cpm) and 200 ng of poly(dI-dC) (di-dC) were added and incubated for 15 min. Subsequently DNA-protein complexes were analyzed on a 4% native polyacrylamide gel using 0.25% TBE (25) as a running buffer. Dried gels were exposed at −70 °C using Kodak X-Omat AR films.

Western Blot Analysis—SDS-polyacrylamide gel electrophoresis and Western immunoblot analysis were performed according to standard procedures (25). Samples (30 μg) were mixed with an equal volume of twice concentrated Laemmli sample buffer (26) and boiled for 5 min. Samples were subjected to SDS-PAGE (10% polyacrylamide), and the proteins were transferred onto nitrocellulose (Bio-Rad). The blots were blocked with 10% dried milk (Carnation) in TTBS buffer (25) for 2 h. Rabbit polyclonal antiserum directed against a peptide of the cyclin B protein was used at 1:200 dilution, and a monodonal antisera directed against the Ki67 antigen (Dianova) was used at 1:300 dilution. Detection of immunoreactive bands was by the enhanced chemiluminescence method (Amersham Corp.).

RESULTS

Our previous work suggested that the DNA binding activity of nuclear factor p92 is cell cycle-regulated by an inhibitor termed I-92 (4). It was the aim of this study to characterize I-92 and p92 biochemically and functionally.

Cell Cycle Regulation of p92 in Normal Human Diploid Fibroblasts—As shown previously, p92 DNA binding activity is cell cycle-regulated in 444 cells, which are immortalized, non-tumorigenic HeLa-fibroblast hybrid cells harboring integrated human papillomavirus type 18 DNA (1). First we wanted to address the question of p92 regulation in a nontransformed normal human cell population. We therefore analyzed p92 activity in NHDFs. NHDFs were synchronized in tissue culture using lovastatin for G1 phase cells, aphidicolin and a 7-h release for S phase cells, aphidicolin and a 7-h release for G2 phase cells, and nocodazole for mitotic cells, and serum starvation for G0 phase cells. From each synchronized cell population nuclear extracts were prepared and examined for p92 DNA binding activity by EMSAs. p92 was not detectable in serum-starved G0 phase cells (Fig. 1, lane 1), almost undetectable in lovastatin-treated G1 phase cells, and likewise almost undetectable in G2 phase cells (Fig. 1, lanes 2 and 4). In S phase cells, however, large amounts of p92 were present (Fig. 1, lane 3). In addition, p92 was almost undetectable in mitotic cells (Fig. 1, lane 5). We conclude from these results that p92 is a factor that operates in normal human diploid fibroblasts and that p92 activity is restricted to S phase of the cell cycle. These results were also confirmed by centrifugal elution of NHDF cells.

The p92 Recognition Motif Confers Activation of a Heterologous Promoter—One goal of this study was to characterize p92 functionally. In order to do so, we wanted to determine whether p92 might act as a transcription factor. We have previously characterized multiple p92 recognition sites in the enhancer of HPV18 (1) and presented evidence that the C-AATTGCTTG-AATT motif, consisting of two partially overlapping octamer-related sequences specifically interacted with p92 and other...
p92-related proteins, as was shown by UV cross-linking (3). EMSAs revealed, however, that in S phase the retarded RP3-p92 complex is always the most prominent DNA-protein complex (Fig. 1, lane 3) (4). The 5'-AATTGCTTGCATAA motif is part of the longer oligonucleotide RP3, which was previously identified as a p92 binding sequence element (1, 3). In oligonucleotide RP3-Δ the 5'-TTGCTTGCATAA sequence of the 5'-AATTGCTTGCATAA motif was deleted, and we showed that p92 no longer bound to the RP3-Δ mutant (3). In addition, we have shown previously that the deleted 5'-TTGCTTGCATAA sequence binds p92 in a sequence-specific manner (3). For functional analysis oligonucleotides RP3 and RP3-Δ were cloned in the enhancer tester pGL-2 and assayed in the nontumorigenic HeLa fibroblast hybrid cell line 444. RP3 mediated activation of the luciferase reporter gene to a level of 12.9% when compared with the cotransfected pSV-β-gal control, whereas the deletion mutant RP3-Δ did not confer significant luciferase gene activation (Fig. 2). Reporter gene assays were repeated 6 times with two different DNA preparations and were reproducible. The error bars in the control experiments pGL-2 and RP3-Δ are not visible due to low luciferase activities of these constructs. We have accomplished a biochemical and functional analysis of I-92 activities. In the following we summarize several features that candidate I-92 proteins must show in order to be considered as I-92. 1) The DNA binding activity of p92 should be inhibited by partially purified I-92. 2) I-92 inhibition of p92 DNA binding should be selective. 3) I-92 inhibition of p92 DNA binding should be cell cycle-regulated. 4) I-92 inhibition of p92 DNA binding should be reversible after treatment with the detergent DOC.

Selectivity of I-92 Inhibition—p92 binds to octamer-related sequences from the HPV18 enhancer (1). We have previously shown that “crude” I-92 did not inhibit DNA binding of the Oct-1 transcription factor (3). In order to prepare crude I-92, nuclear extracts of 444 cells were treated with the detergent DOC following a published procedure (4, 20, 21) and passed over heparin-Sepharose. The flow-through contains crude I-92. In order to further define selectivity of I-92 inhibition we chose to analyze other transcription factors whose DNA binding activities are regulated by specific inhibitors. We examined the transcription factor AP-1, which is regulated by IP-1 (27). In this experiment an AP-1 consensus oligonucleotide was used in EMSA. The identity of Fos-J un retarded complexes was verified by immunoshift analysis using a polyclonal rabbit anti-Fos antisera (Fig. 3, lanes 2 and 3). As a control an unrelated polyclonal rabbit antisera was used directed against the Gag protein of a human endogenous retrovirus HERV-K (Fig. 3, lanes 4 and 5). The addition of increasing amounts of crude I-92 did not affect AP-1 retarded complex formation, suggesting that AP-1 is not regulated by I-92 (Fig. 3, lanes 7, 8, and 9). The
DNAbinding of NF-1-92 unit definition was described (3). In addition we found that 11, 12, 13, and 14 (Fig. 4) were assayed for I-92 activity by EMSIA using a Sephacryl S300 chromatography. Individual Sephacryl fractions were assayed for I-92 activity by EMSIA (Fig. 4). In order to characterize I-92 further, we have analyzed crude I-92 by Mono Q chromatography. After loading crude I-92 onto the Mono Q column the flow-through fraction was collected. Subsequently, bound proteins were eluted with a NaCl step gradient, and from each salt step two 0.5-ml fractions were collected. All I-92 activities were retained on the column, because the flow-through fraction did not contain any detectable I-92 activity (Fig. 4C, lane FT). The Mono Q fractionation yielded four different salt fractions with I-92 activity: 100 mM NaCl, 200 mM NaCl, 700 mM NaCl, and 800 mM NaCl, as was shown by EMSIA (Fig. 4C, lanes 2, 4, 13, 14, 15, and 16). These results strongly support the observation that diverse I-92 proteins exist. Note that the faster migrating complexes in lanes 2, 3, 5, and 6 were derived from the crude I-92 preparation, because they could be detected by EMSIA without added p92, suggesting that they are not p92 degradation products that could have been generated during the electrophoretic mobility shift inhibition assay. Some of the fast moving complexes in fraction 2 of the 200 mM NaCl eluate, however, may have been generated, at least in part, during the electrophoretic mobility shift inhibition assay (see below). In contrast, none of these faster moving complexes could be detected either in SDS-PAGE eluates containing the four I-92s (Fig. 4F, lanes 2, 11, 12, 13, and 14) or in Sephacryl S300 fractions with I-92 activity (Fig. 4A).

Cell Cycle Regulation of I-92 Proteins—Next we analyzed whether the four different I-92 activities that were identified by Mono Q chromatography are subject to cell cycle regulation. The most important feature of I-92 candidate proteins is that their activities should be cell cycle-regulated. More specifically, they should be active either in G1 phase and/or in G2 phase but not in S phase. We have used biochemical cell synchronization approaches as outlined under "Experimental Procedures" in order to obtain sufficient amounts of crude I-92 for further characterization by Mono Q chromatography and EMSIA. The efficiencies of cell synchronization were controlled by propidium iodide staining of cellular DNA and FACS analysis. Western blot analysis using an antibody against the proliferation marker Ki67 (19) was used for the assessment of G1 phase synchronization, and cyclin B antibodies were used for the determination of the G2 phase synchronization and 444 cells (Fig. 5A). We conclude that our experiments were efficiently synchronizing G1, G2, G3, M, and G0 phase cell populations.

Identification of Two Different G1 Phase Inhibitors—444 cells were synchronized with lovastatin, and nuclear extracts were prepared. Crude I-92 was isolated after DOC treatment of the extract and passage over heparin-Sepharose columns. Subsequently I-92 was fractionated by Mono Q chromatography. I-92 was eluted with 100 mM NaCl, and two independent fractions were collected of each eluate. Each salt eluate was assayed for I-92 activity by EMSIA (Fig. 5B). We discovered here that I-92 was present in fraction 2 of a 100 mM NaCl eluate (Fig. 5B, lane 2) and in fractions 1 and 2 of a 700 mM NaCl eluate (Fig. 5B, lanes 13 and 14). For screening I-92 activities in Mono Q fractions, an arbitrary amount of each fraction was used (25 µl). Note that the 100 mM NaCl I-92 activity seems to be present in much lower concentrations than the 700 mM NaCl I-92 activity. It is, however, possible to titrate this inhibitory activity and obtain complete inhibition of p92 DNA binding in EMSIA (see also Fig. 6, lane 2). We conclude that the 100 mM and the 700 mM NaCl Mono Q eluates contain two forms of I-92 that are active in G1 phase of the cell cycle.

Fig. 3. Selectivity of I-92 inhibition. EMSA with a [γ-32P]ATP end-labeled AP-1 consensus recognition site (Santa Cruz) as a probe and a nuclear extract from 444 cells. Free AP-1 oligonucleotide and retarded AP-1 complexes are indicated by arrows (lane 1). As a specificity control for the identification of the retarded AP-1 DNA-protein complex a polydional anti-Fos antiserum (Medac), 1 and 5 µg was included in the binding reaction (lanes 2 and 3). Lanes 4 and 5 contained the same amounts of an unrelated rabbit polydional anti-Ganz antiserum directed at a human endogenous retrovirus antigen. In order to test whether I-92 affects DNA binding of AP-1 (lane 6), crude I-92 was included in the binding reaction prior to the addition of the probe: 0.5 units of crude I-92 (lane 7), 1 unit of crude I-92 (lane 8), and 2 units of crude I-92 (lane 9). EMSA was performed as described under "Experimental Procedures."

I-92 unit definition was described (3). In addition we found that DNA binding of neither NF-κB, which is regulated by I-κB (25), nor of E2F (23, 28), which is regulated by pRB (29), was regulated by I-92. These results demonstrate that I-92 inhibition is selective for p92.

Biochemical Characterization of I-92 Proteins—As a source of crude I-92 we used the nontumorigenic HeLa fibroblast hybrid cell line 444 (5), because the limited life span and slow growth rates of normal diploid human fibroblasts preclude large scale cell culture, which is required for I-92 preparations. Crude I-92 prepared from 444 cells was size-separated by Sephacryl S300 chromatography. Individual Sephacryl fractions were assayed for I-92 activity by a p92 EMSIA using a defined amount of partially purified p92 (0.4 µg) and the p92 recognition site RP3 as a probe. These experiments reproducibly revealed I-92 activities in fractions 46–53 of the S300 column, corresponding to a molecular mass range from 15 (slice 2) to 50 kDa (slice 14). In Fig. 4B eluates from slices 6–8 are not shown because they did not contain detectable I-92 activity. Given the fact that a reducing SDS-PAGE was used in this experiment we conclude that diverse I-92 proteins exist. In order to characterize I-92 further, we have analyzed crude I-92 by Mono Q chromatography. After loading crude I-92 onto the Mono Q column the flow-through fraction was collected. Subsequently, bound proteins were eluted with a NaCl step gradient, and from each salt step two 0.5-mL fractions were collected. All I-92 activities were retained on the column, because the flow-through fraction did not contain any detectable I-92 activity (Fig. 4C, lane FT). The Mono Q fractionation yielded four different salt fractions with I-92 activity: 100 mM NaCl, 200 mM NaCl, 700 mM NaCl, and 800 mM NaCl, as was shown by EMSIA (Fig. 4C, lanes 2, 4, 13, 14, 15, and 16). These results strongly support the observation that diverse I-92 proteins exist. Note that the faster migrating complexes in lanes 2, 3, 5, and 6 were derived from the crude I-92 preparation, because they could be detected by EMSIA without added p92, suggesting that they are not p92 degradation products that could have been generated during the electrophoretic mobility shift inhibition assay. Some of the fast moving complexes in fraction 2 of the 200 mM NaCl eluate, however, may have been generated, at least in part, during the electrophoretic mobility shift inhibition assay (see below). In contrast, none of these faster moving complexes could be detected either in SDS-PAGE eluates containing the four I-92s (Fig. 4F, lanes 2, 11, 12, 13, and 14) or in Sephacryl S300 fractions with I-92 activity (Fig. 4A).

Identification of Two Different G1 Phase Inhibitors—444 cells were synchronized with lovastatin, and nuclear extracts were prepared. Crude I-92 was isolated after DOC treatment of the extract and passage over heparin-Sepharose columns. Subsequently I-92 was fractionated by Mono Q chromatography. I-92 was eluted with 100 mM NaCl, and two independent fractions were collected of each eluate. Each salt eluate was assayed for I-92 activity by EMSIA (Fig. 5B). We discovered here that I-92 was present in fraction 2 of a 100 mM NaCl eluate (Fig. 5B, lane 2) and in fractions 1 and 2 of a 700 mM NaCl eluate (Fig. 5B, lanes 13 and 14). For screening I-92 activities in Mono Q fractions, an arbitrary amount of each fraction was used (25 µl). Note that the 100 mM NaCl I-92 activity seems to be present in much lower concentrations than the 700 mM NaCl I-92 activity. It is, however, possible to titrate this inhibitory activity and obtain complete inhibition of p92 DNA binding in EMSIA (see also Fig. 6, lane 2). We conclude that the 100 mM and the 700 mM NaCl Mono Q eluates contain two forms of I-92 that are active in G1 phase of the cell cycle.

3 E. Grinstein and H.-D. Royer, unpublished results.
We call I-92 from the 100 mM eluate G1 I-92a and from the 700 mM eluate G1 I-92b. Note that the 300 mM Mono Q eluate contains an activity that generates faster moving RP3-protein complexes (Fig. 5B, lane 6). This activity, however, was not further characterized and will be part of future work.

Identification of G2 Phase I-92—In order to obtain G2 phase 444 cells, we synchronized with aphidicolin treatment followed by a 10-h release, and subsequently crude I-92 was analyzed as described above. The efficiency of G2 phase synchronization was controlled by FACs analysis and Western blotting using a cyclin B antiserum (Fig. 5A, bottom right). Analysis of the Mono Q fractions by EMSIA demonstrated clearly that in G2 phase cells only a single I-92 activity was detectable in fraction 2 of the 200 mM NaCl eluate (Fig. 5D, lane 4). We call the G2 phase inhibitor G2 I-92. G2 I-92 was undetectable in the 200 mM Mono Q salt eluates of G1 phase and S phase cells (Fig. 5, panel B, lane 4, and panel C, lane 4), very low levels of G2 I-92 could be detected in 200 mM Mono Q salt eluates of M phase and G0 phase 444 cells (Fig. 5, panel E, lane 4, panel F, lane 4. Note that there are two faster moving complexes in EMSIA of the 200 mM NaCl fraction containing G2 I-92 (Fig. 5D, lane 4). These complexes were reproducibly detected by EMSIA experiments using Mono Q-separated G2 I-92 preparations. In contrast, G2 I-92 from Sephacryl S300 fractions did not contain similar complexes.3

I-92 Is Not Active in Mitotic 444 Cells—In NHDFs, p92 DNA binding was restricted to S phase of the cell cycle (Fig. 1), and p92 was undetectable in M phase. We noticed, however, that in mitotic 444 cells p92 was detectable by EMSA.3 In order to examine I-92 in M phase of 444 cells we have used nocodazole-treated 444 shake-off cells for I-92 analysis. An examination of a Mono Q fractionation of mitotic 444 cell nuclear extracts
**Fig. 5.** Cell cycle regulation of I-92: identification of G1, G2, and G0 phase inhibitors. A, assessment of cell synchrony of 444 cells: FACS analysis of propidium iodide-stained DNA from G1 phase cells that were treated with lovastatin (top, left). Shown are FACS analysis of propidium iodide-stained DNA from aphidicolin treated S phase cells (top, middle) and FACS analysis of G2 phase cells that were treated with aphidicolin and a 10-h release (top, right). The efficiency of G2 phase synchronization was verified by the detection of G2 phase cyclin B in nuclear extracts of G2 phase 444 cells (bottom, right). FACS analysis of propidium iodide-stained DNA from nocodazole-treated mitotic cells (bottom, middle) is shown. G0 synchronization (bottom, left), was verified by analysis of Ki67 expression in serum-starved 444 cells (lane G0) and from exponentially growing 444 cells (lane C, bottom left). As a detection system we used the ECL kit (Amersham). Details of the biochemical cell synchronization conditions are described under "Experimental Procedures." B, crude I-92 from lovastatin-treated 444 cells (G1 phase) was fractionated by Mono Q chromatography (as described in Fig. 4), and I-92 was monitored by EMSIA. Each salt elution was in two steps of 0.5 ml. p92 control (lane 1), 100 mM salt (lanes 2 and 3), 200 mM salt (lanes 4 and 5), 300 mM salt (lanes 6 and 7), 400 mM salt (lanes 8 and 9), 500 mM salt (lanes 10 and 11), 600 mM salt (lanes 12 and 13), 700 mM salt (lanes 14 and 15), 800 mM salt (lanes 16 and 17), and 900 mM salt (lanes 18) are shown. Free RP3 and the p92 retarded complex are indicated by arrows. C, crude I-92 from aphidicolin-treated 444 cells (S phase cells) was fractionated by Mono Q chromatography as above, and I-92 was monitored by EMSIA. Each salt elution was in two steps of 0.5 ml. p92 control (lane 1), 100 mM salt (lanes 2 and 3), 200 mM salt (lanes 4 and 5), 300 mM salt (lanes 6 and 7), 400 mM salt (lanes 8 and 9), 500 mM salt (lanes 10 and 11), 600 mM salt (lanes 12 and 13), 700 mM salt (lanes 14 and 15), 800 mM salt (lanes 16 and 17), and 900 mM salt (lanes 18) are shown. Free RP3 and the p92 retarded complex are indicated by arrows. D, 444 cells were treated with aphidicolin and a 10-h release. G2 phase was confirmed by a cyclin B Western blot and by FACS analysis of propidium iodide-stained cells. Crude I-92 was prepared from nuclear extracts of G2 phase 444 cells and analyzed by Mono Q chromatography as above, and I-92 was monitored by EMSIA. Each salt elution was in two steps of 0.5 ml. p92 control (lane 1), 100 mM salt (lanes 2 and 3), 200 mM salt (lanes 4 and 5), 300 mM salt (lanes 6 and 7), 400 mM salt (lanes 8 and 9), 500 mM salt (lanes 10 and 11), 600 mM salt (lanes 12 and 13), 700 mM salt (lanes 14 and 15), 800 mM salt (lanes 16 and 17), and 900 mM salt (lanes 18) are shown. Free RP3 and the p92 retarded complex are indicated by arrows. E, crude I-92 was prepared from nuclear extracts of serum-starved 444 cells. Crude I-92 was fractionated...
regulation in G0 phase of the cell cycle. 444 cells were serum- and a low amount of G2 I-92 was detected (Fig. 5).

We then tested whether “free” p92 could be recovered after DOC treatment. For this purpose, appropriate amounts of the 100 mM NaCl (G1 I-92a), 200 mM NaCl (G2 I-92), 700 mM NaCl (G1 I-92b), and 800 mM NaCl (G0 I-92 Mono Q eluates were used in order to inhibit DNA binding of partially purified p92 (0.4 μg) in the EMSIA (Fig. 6, lanes 2, 3, 4, and 5). We then tested whether “free” p92 could be recovered after DOC treatment (Fig. 6, lanes 7, 8, 9, and 10). We observed that G1 I-92a, G1 I-92b, G2 I-92, and G0 I-92 inhibited p92 binding in a reversible fashion, although subtle differences were observed. p92 DNA binding inhibition by G1 I-92 from the 700 mM eluate was 100% reversible (Fig. 6, lanes 4 and 9), whereas p92 DNA binding inhibitions by G1 I-92a (lanes 2 and 7) from the 100 mM eluate, by G2 I-92 from the 200 mM NaCl fraction (lanes 3 and 8) and by G0 I-92 from the 800 mM NaCl fraction (lanes 5 and 10) were reversible to a lesser extent. These results demonstrate that the four phase-specific I-92 forms were able to complex and inactivate p92 in a reversible manner.

**DISCUSSION**

p92 was previously identified in nontumorigenic HeLa fibroblast hybrid cells as a cell cycle-regulated DNA-binding protein interacting with the enhancer of human papillomavirus type 18 (1). We have established in this report that p92 is also present in normal human diploid fibroblasts and have shown by cell synchronization experiments that p92 DNA binding activity was restricted to S phase of the cell cycle. We have established in the present communication that a p92 recognition site conferred activation of the SV40 minimal promoter, suggesting that the proteins that bind to the 5′-ATTGCTTGCATAA motif act as transcription factors. We think, however, that p92 might be the responsible factor-mediating transcriptional activation because p92 is the major retarded complex detectable by EMSIA in 444 cells (4). This issue will have to be analyzed after regulated in vivo expression of p92 DNAs. We are currently engaged in sequencing p92 protein for cDNA cloning.

Diverse Phase-specific Nuclear Inhibitors Link Cell Cycle Regulation with Transcription Factor Regulation—The major finding of this report is the identification of a novel mechanism of phase-specific gene regulation due to the activity of phase-specific nuclear inhibitors, termed I-92s. The consequence of this regulation is that the DNA binding activity of the transcription factor p92 is restricted to S phase of the cell cycle. In nuclear extracts of 444 cells, we have identified two G1 phase inhibitors (G1 I-92a, and G1 I-92b), one G2 phase inhibitor (G2...
I-92) and a G0 phase inhibitor (G0 I-92). In S phase of the cell cycle I-92 was undetectable, demonstrating that the different I-92s regulate the DNA binding activity of the transcription factor p92 in a phase-specific manner, thereby linking cell cycle regulation with transcription factor regulation.

The existence of G1 I-92a, G1 I-92b, G1 I-92, and G0 I-92 was revealed by biochemical cell synchronization experiments of 444 cells and fractionation of crude I-92 preparations by Mono Q chromatography. The efficiency of cell synchronizations was assessed by measurements of the DNA content (FACS). Western blot analysis controlled for G0 and the G2 phase populations using the Ki67 antibody (G0) and an acyclin B antibody (G2). The latter was used to determine empirically the onset of G2 phase after an aphidicolin release. Chromatography of crude I-92 on Sephacryl S300 HR columns revealed I-92 activities in a molecular mass range from 15 kDa to 50 kDa. These results were confirmed by preparative, reducing SDS-polyacrylamide gel electrophoresis of crude I-92, subsequent elution of proteins from individual gel slices and renaturation of eluted proteins. I-92 was monitored functionally by an EMSIA. The identification of multiple I-92 activities with different molecular weights in this experiment supports the notion that diverse I-92s exist. A molecular weight determination of the G1 I-92a, G1 I-92b, G2 I-92, and G0 I-92 inhibitors from Mono Q fractions will be part of future experiments.

We have characterized I-92 functionally and determined that the inhibition of p92 DNA binding by the two G1 phase inhibitors, the G2 phase inhibitor, and the G0 phase inhibitor was reversible, because p92 could be released from inactive I-92-p92 complexes by treatment with the detergent deoxycholate.

The DNA binding activity of the G2 phase inhibitor G2 I-92 is present in the 200 mM NaCl Mono Q fraction of exponentially growing 444 cells and in G2 phase-synchronized 444 cells (Fig. 4C, lane 4 and Fig. 5D, lane 4), and low activities of this inhibitor could be detected in M phase and G0 cells (Fig. 5, lane 4, and panel F, lane 4). We noted that there were additional faster moving DNA-protein complexes present in the 200 mM NaCl Mono Q fractions that might have been generated during I-92 assays (Fig. 5D, lane 4), because they were undetectable in electrophoretic mobility shift inhibition assays without added p92. In contrast, fast moving complexes could not be detected in Sephacryl S300 fractions and in SDS-PAGE eluates (see Fig. 4, A and B). In addition, Sephacryl S300 fractions from normal human diploid fibroblasts likewise did not contain any detectable faster moving complexes. Furthermore, we have identified a cancer-derived cell line that contains only G2 I-92. G2 I-92 from this cell line was present in fraction 46 of a Sephacryl S300 column and in the 200 mM NaCl fraction of a Mono Q column. G2 I-92 assays from these cells revealed that this Mono Q fraction generated similar faster moving complexes as the one from 444 cells, whereas G2 I-92 from Sephacryl S300 fraction 46 did not contain any faster moving complexes. It is therefore possible that additional factors are present in the 200 mM NaCl Mono Q fraction that lead to a partial degradation of p92 in EMSIA. Partial degradation of p92 could also explain why in DOC release experiments lower amounts of p92 were recovered when compared with input levels (Fig. 6, lanes 3 and 8). Future experiments will be required to resolve this issue.

We have analyzed whether I-92 is selective in its ability to form complexes with p92. For this reason we selected three other transcription factors and asked whether they are targets for I-92. We could show that the DNA binding activities of AP-1 (Fig. 3). E2F, and NF-kB were not affected by one or two units of crude I-92, which completely inactivated a given amount of p92. These data demonstrate that I-92 is selective, and this result is further supported by the fact that the DNA binding activity of Oct-1 was not regulated by crude I-92 (3). A number of transcription factors are regulated by specific inhibitors, which modulate their DNA binding activities. AP-1 is regulated by IP-1 (27), the POU transcription factor Cfl-a is regulated by I-POU (30), and the helix-loop-helix protein MyoD is regulated by Id (31), and in these cases nonfunctional heterodimers are formed. I-92s negatively regulate p92 DNA-binding in G1, G2, and G0 phase by physical association with p92. During the process of p92 purification we have observed that other proteins exist that specifically bind to the p92 recognition site present in RP3 and have shown that the DNA binding activities of most of these were regulated by crude I-92 (3). These results show that I-92s selectively regulate, besides p92, the DNA binding activities of other related proteins, and this suggests that I-92s could link the activities of multiple factors with the cell cycle. It is conceivable that this regulatory principle could be a more general mechanism whereby multiple specific inhibitors for a variety of different transcription factors could link the regulation of many genes with the cell cycle.

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