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A novel nuclear inhibitor I-92 regulates DNA binding activity of octamer binding protein p92 during the cell cycle

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ABSTRACT

Nuclear DNA binding protein p92 is a sequence specific octamer binding protein with identical molecular weight as the ubiquitous octamer binding protein Oct-1. It binds to octamer related sequences from the enhancer of human papillomavirus type 18. The activity and intracellular distribution of p92 is regulated by extracellular signals. In serum starved Hela-fibroblast hybrid cells p92 is localized to the cytosol. Serum stimulation leads to nuclear import of p92. In fractions of asynchronously growing cells, which were separated according to cell cycle phases into G1, S, and G2 populations by centrifugal elutriation, p92 DNA binding is confined to S phase. In binding site blots however, p92 DNA binding activity is also present in G1 and G2. In G1 and G2 DNA binding activity of p92 is masked by a novel nuclear inhibitor I-92. The cyclic association of p92 with its inhibitor I-92 provides a new mechanism of regulating S phase dependent activity of a sequence specific DNA binding protein.

INTRODUCTION

In a study aimed at delineating human papillomavirus enhancer factors, we have discovered a novel octamer binding protein p92, which has an identical molecular weigth as the ubiquitous Oct-1 protein (33). The Oct-1 protein belongs to the homeodomain containing class of transcription factors, which have important functions during development and in controlling cell type specific gene expression. The octamer recognition sequence ATTTGCAT binds multiple proteins, these include the ubiquitous Oct-1 protein (8, 36, 37, 41) and the cell type specific Oct-2 protein, which is found in B lymphocytes (9, 35, 39). The DNA binding domain consists of the homeodomain and another conserved region, the POU specific box. The entire region is called the POU domain. The POU domain is found in a pituitary cell transcription factor Pit-1, the octamer binding protein Oct-1 and in Unc-86, a factor from the nematode *C. elegans* (13).

p92 binds to multiple octamer related sequences which are present in the enhancer of human papillomavirus type 18 (33). The intracellular distribution of this factor is regulated by extracellular signals, because in serum starved cells, or in cells at high saturation density p92 is found in the cytoplasm (33). Serum starvation or growth to high saturation density of cells in culture leads to cell cycle arrest at the G0/G1 boundary of the cell cycle (44). The control of intracellular distributions of sequence specific DNA binding proteins and transcription factors emerges as an important regulatory mechanism which controls the activities of these proteins (14). Proteins which are regulated by intracellular redistribution include heat shock proteins (4, 21, 43), proteins which are active in early embryonic development (6, 11, 31, 34, 40, 45), major nucleolar proteins (2), the catalytic subunit of the c-AMP dependent protein kinase II (25), the progesterone receptor (28), the transcription factor NF*K*B (1), the c-Fos protein (32) and c-MYC protein in early development (11).

Serum stimulation of quiescent fibroblasts which are in G0 of the cell cycle leads to new DNA synthesis and cell proliferation after 10–20 hours (30). Extracellular signals control the induction of DNA synthesis. After cells enter S phase they become independent of extracellular factors (27). Therefore, mechanisms which lead to DNA replication can influence cell cycle progression. The effect of serum stimulation of growth arrested fibroblasts is largely due to the presence of platelet derived growth factor in serum (30). In the present study we have examined the effects of serum stimulation on the intracellular p92 distribution in a Hela-fibroblast hybrid. The most important finding of this report is the discovery of a novel mechanism which confers cell cycle regulation of octamer binding protein p92 in the nucleus. A novel inhibitor I-92 associates with p92 in G1 and G2 of the cell cycle and prevents DNA binding. In S phase I-92 is inactive and therefore free p92 is able to bind to its recognition sequences.

MATERIALS AND METHODS

Procedures for the extraction of nuclear and cytoplasmic proteins, synthetic oligonucleotides, binding site blotting, and electrophoretic mobility shift analysis (EMSA) were described (33).

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Cell culture and cell lines

Nontumorigenic 444 Hela-fibroblast hybrid cell lines (38) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum. Hela-fibroblast hybrid cell line 444 was provided by Dr. Stanbridge. For serum starvation experiments, cells were grown subconfluent in DMEM containing 10% fetal calf serum, subsequently cells were held in DMEM containing 0.5% fetal calf serum for 40 hr. In serum stimulation experiments, cells were first serum starved for 40 hr and subsequently cells were grown in DMEM containing 10% fetal calf serum. For growth to high saturation density, cells at confluence were held in DMEM containing 10% fetal calf serum for 48 hr.

Preparative EMSA

For preparative EMSA 24 individual samples corresponding to 120 μg nuclear extract were electrophoresed as for analytical EMSA. After autoradiography of the polyacrylamide gel at 4°C over night, retarded complexes were excised and eluted from the gel in elution buffer: 50mM Tris HCl (pH 7.9), 0.1% SDS, 1mM DTT, 0.2mM EDTA, 0.1mM PMSF, 2.5% glycerol, for 12 hr at 4°C. Proteins from retarded complexes were precipitated with 9 volumes of acetone at minus 20°C over night in the presence of 20 μg bovine serum albumin, and recovered by centrifugation at 13000 rpm. For binding site blotting proteins were solubilized in SDS-PAGE loading buffer (16).

Sodium deoxycholate treatment of nuclear extracts and heparin Sepharose chromatography

Nuclear extract from 444 cells (1 mg) was treated with 0.4% sodium deoxycholate (DOC) for 15 min at room temperature in 2 ml of DNA binding buffer: 25mM Hepes (pH 7.9), 50mM NaCl, 10% glycerol, 0.05% NP40, 1mM DTT, 1mM EDTA. Before loading the DOC treated nuclear extract on a heparin Sepharose column, DOC was complexed by the addition of 1.2% NP40. After loading the column the flow through was passed over the column once more. The final flow through (2ml) was used as a source of the inhibitor. Bound proteins were eluted from the heparin Sepharose column after a G50 washing step, with G600 buffer, containing 600mM NaCl. Besides NaCl, G50 and G600 buffers contained 10mM Tris HCl (ph 7.5), 50mM NaCl, 1mM EDTA, 1mM DTT, 5% glycerol, 0.2% DOC, 0.2% NP40, 05% PMSF. The procedure followed published protocols (1).

Detection of I-92

Deoxycholate treated nuclear extracts from 444 cells were fractionated by heparin Sepharose. The flow through contained I-92. To assay for I-92 activity, S phase nuclear extracts (5 μg) from 444 cells were incubated with increasing amounts of flow through for 15 min. at room temperature. The reaction mix was analyzed by EMSA.

Separation of cells by centrifugal elutriaton

Cell monolayers which were no more than 80% confluent were harvested with trypsin, resuspended in medium containing serum and elutriated. For the elutriation, 10^7-10^8 cells were introduced into the JE-6B Beckman elutriation rotor connected with the Masterflex 7014 pump head. The elutriation was performed in Earle's balanced salt solution pH 7.4 buffered wit 10 mM Hepes. Nontumorigenic Hela-fibroblast hybrid (444) cells were elutriated at 1620 rpm after loading the cells at 5 ml/min. The flow rates for obtaining the fractions were 8−11 ml/min, 15−18 ml/min, 24−30 ml/min, and 34−38 ml/min. Samples of each fraction were ethanol fixed, dapi (4,6-diamidino-2-phenyl indole) stained, and analyzed for the relative DNA content by means of a fluorescence activated cell sorter.

RESULTS

Identification of octamer binding protein p92 in retarded protein: DNA complexes

We have previously described a novel nuclear octamer binding protein p92. p92 binds to DNA in a sequence specific manner, since various oligonucleotides devoid of octamer related sequences do not detect p92 in binding site blots (33, and unpublished data). The molecular weights of p92 and Oct-1 are both approximately 92 kD. p92 can be distinguished from Oct-1 by the following criteria: a polyclonal Oct-1 antiserum does not react with p92 in Western blots (33): Oct-1 elutes with 150−200 mM KCl from heparin Sepharose (41), in contrast p92 elutes with 500 mM KCl from heparin Sepharose (unpublished data): p92 is localized in the cytosol of nontumorigenic Hela-fibroblast hybrid cells (444) at high saturation density or under conditions of serum starvation, whereas in contrast, Oct-1 is found in the nuclear fraction of these cells (33).

In the present study we intended to use electrophoretic mobility shift analysis (EMSA) to detect p92 DNA binding activity, because EMSA is more sensitive than binding site blotting. The identification of p92 within retarded DNA-protein complexes was verified by binding site blotting. EMSA of cytosols from cells which were half confluent (Fig. 1A, lane 1) and from cells at confluence (lane 2) shows that at high saturation density (lane 3) additional retarded DNA-protein complexes are formed. Proteins which are present in retarded complex A and and in retarded complex B (Fig. 1A, lane 3) were isolated by preparative EMSA. After autoradiography, proteins from complexes A and

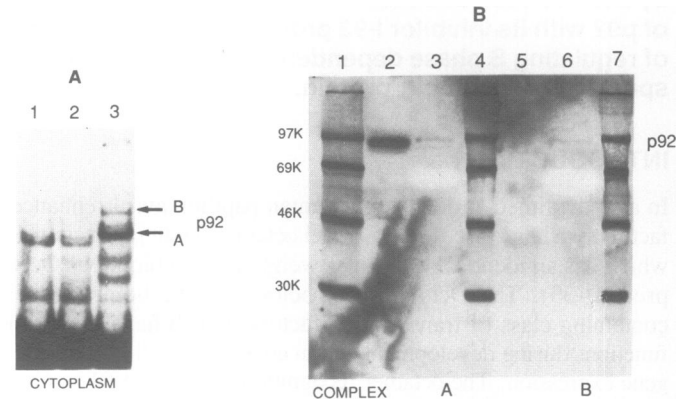

Figure 1. Identification of cytoplasmic octamer binding protein p92 by preparative EMSA and binding site blotting. (A) Cytoplasmic fractions of nontumorigenic Hela-fibroblast hybrid cells (444) at half confluence (lane 1), at confluence (lane 2) and 48 hours after reaching confluence (high density cells), (lane 3) were analyzed by EMSA. The positions of retarded DNA-protein complexes containing p92 are indicated by arrows. Human papillomavirus enhancer oligonucleotide RP3, containing p92 recognition sites was used in EMSA. (B) Retarded DNA-protein complexes designated p92A and p92B (Figure 1A, lane 3) were isolated by preparative EMSA, eluted and preciptated with 9 volumes acetone, before precipitation 20μg of bovine serum albumine was added as carrier. Binding site blot of eluted proteins. ^{14}C labeled proteins were used as size marker (lanes 1, 4 and 7). Cytoplasmic p92 (lane 2), p92 protein in retarded complex p92A (lane 3) and p92 protein in retarded complex p92B (lane 6), lane 5 contained sample buffer only. Oligonucleotide RP3 was used as a probe for p92.

B were eluted seperately, and precipitated with 9 volumes of acetone in the presence of 20 μg BSA at 4°C overnight. Proteins from retarded complexes were analyzed by binding site blotting. The retarded DNA-protein complexes A and B (Fig. 1A, lane 3) both contain p92 (Fig. 1B, lane 3= complex A, lane 6= complex B). As a control we have analyzed the cytoplasm containing complex A and B (Fig. 1A, lane 3) by binding site blotting for the presence of p92 (Fig. 1B, lane 2). The carrier protein bovine serum albumin did not contain any DNA binding activities (BSA control, not shown), lane 5 contained sample buffer only (16).

Nuclear import of octamer binding protein p92 is regulated by extracellular signals

Nontumorigenic Hela-fibroblast hybrid cells (444) were examined for the intracellular distribution of p92. p92 DNA binding activity is present in cytoplasm of serum starved 444 cells prior to serum stimulation. Except for the appearance of a novel retarded complex, which may be related to complex B, serum treatment does not increase cytoplasmic p92 DNA binding activity (Fig. 2A). After five hours, however, p92 concentration declines and is no longer detectable in the cytosol after nine hours (Fig. 2A). Disappearance of p92 from the cytoplasm is accompanied by the appearance of the binding activity in nuclear extracts (Fig. 2B). This result shows that nuclear import of p92 is regulated by extracellular serum factors in Hela-fibroblast hybrid cells. In addition, regulated p92 intracellular distribution was observed in various other cell lines (unpublished data).

DNA binding activity of nuclear p92 is cell cycle regulated

In asynchronously growing cells which have not yet reached confluence p92 is found in the nucleus but not in the cytoplasm. To study the fate and activity of nuclear p92 during the cell cycle, asynchronous populations of nontumorigenic Hela-fibroblast hybrid cells (444) were separated by centrifugal elutriation into fractions of G1-, S-, and G2-phase cells. The first fraction contained the majority of G1 cells, and the fourth fraction the majority of G2 cells, as demonstrated by the measurement of cellular DNA content (Fig. 3B), fractions two and three contained 20.8% and 51.9% S phase cells respectively. G1 cells from fraction one are diploid whereas G2 cells from fraction 4 are tetraploid. Nuclear extracts from these separated cell populations were analyzed by EMSA and binding site blotting for p92 DNA binding activity. Nuclear p92 DNA binding activity was only detected by EMSA in 444 cells in S phase (Fig. 3A, lanes 2 and 3). Two retarded complexes, A and B, were observed which may both contain p92 (as shown by by preparative EMSA and binding site blotting, see Fig. 1A and 1B). This result suggests that after nuclear import, p92 DNA binding activity persists in S phase and disappears in G2. In nuclear extracts from G1 cells p92 DNA binding activity cannot be detected and it seems that p92 DNA binding capacity is activated in S phase.

A nuclear inhibitor of p92 regulates S phase dependent p92 DNA binding activity

Nuclear and cytoplasmic fractions of nontumorigenic Hela-fibroblast hybrids which had been separated by centrifugal elutriation into G1-, S-, and G2-cells were also analyzed by binding site blotting. With this experiment we sought to control the results obtained by EMSA (Fig. 3A) and the same oligonucleotide was used. The binding site blot (Fig. 4) shows that equal amounts of p92 are present in nuclear extracts of cells in every cell cycle phase (lanes 2−5). In contrast, no p92 is found in the cytosol of G1, S or G2 cells (lanes 6−9). These data confirm our previous observation (33) that in cells grown asynchronously in culture p92 is located in the nucleus. In contrast to the results obtained by EMSA, the DNA binding activity of nuclear p92 revealed by binding site blotting is present in G1-

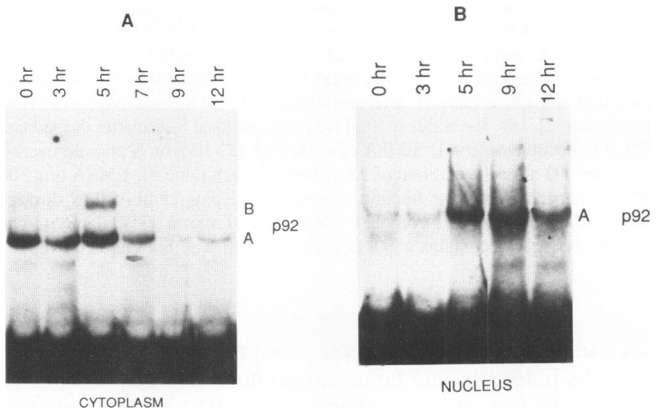

Figure 2. Extracellular signals induce nuclear import of p92 in serum starved 444 cells (nontumorigenic Hela-fibroblast hybrids). (A) EMSA of cytoplasmic fractions. 444 cells were serum starved for 40 hours. EMSA of cytoplasmic extract, 40 hours after serum starvation (0 hr), 3 hours after serum addition (3 hr), 5 hours after serum addition (5 hr), 7 hours after serum addition (7 hr) 9 hours after serum addition (9 hr) and twelve hours after serum addition (12 hr). (B) EMSA of nuclear extracts. 444 cells were serum starved for 40 hours (0 hr), 3 hours after serum addition (3 hr), five hours after serum addition (5 hr), nine hours after serum addition (9 hr) and twelve hours after serum addition (12 hr). Oligonucleotide RP3 was used in EMSA.

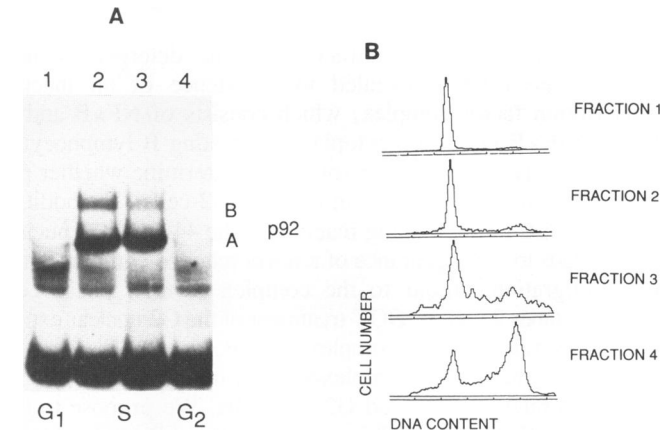

Figure 3. Cell cycle regulation of nuclear p92 DNA binding. (A) Asynchronously growing 444 cells (nontumorigenic Hela-fibroblast hybrids) were separated into cell cycle specific populations by centrifugal elutriation. EMSA of nuclear extracts of cells in the following stages are shown: G1: 79.2% (lane 1), S phase: 20.8% (lane 2), S phase: 51.9% (lane 3) and in G2: 66.9% (lane 4). The positions of retarded complexes containing p92 are indicated. The lower retarded complex contains a 50kD protein as was shown by preparative EMSA. Oligonucleotide RP3 was used in EMSA. (B) Analysis of 444 cells separated by elutriation into cell cycle specific populations. Cells for nuclear extracts (A) and DNA measurements (B) were from the same preparation and were separated by counterflow centrifugation into five fractions of increasing cell size. Fraction five contained aggregated cells and was not analyzed further. DNA content was measured by FACS analysis of dapi stained cells. In the first fraction (top curve) the majority of cells are in G1 with a 2n DNA content, in the fourth fraction (bottom curve) the majority of cells are in G2 with a 4n DNA content.

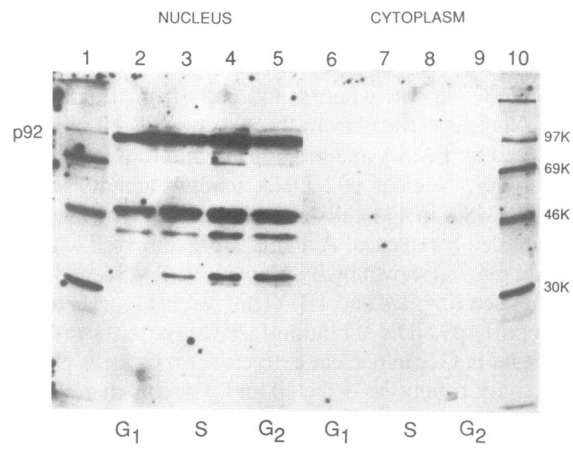

Figure 4. Binding site blotting reveals p92 DNA binding activity throughout the cell cycle. Binding site blot of nuclear and cytoplasmic 444 extracts from G1, S phase and G2 of the cell cycle, showing that p92 DNA binding activity is present in all phases of the cell cycle. p92 DNA binding activity in nuclear extracts of cells in G1: 79.2% (lane 2), in S phase: 20.8% (lane 3), in S phase: 51.9% (lane 4) and in G2: 66.9% (lane 5). The low molecular weight proteins have not been characterized. Cytoplasmic extracts from cells in G1 (lane 6), in S phase (lane 7), in S phase (lane 8) and in G2 (lane 9).

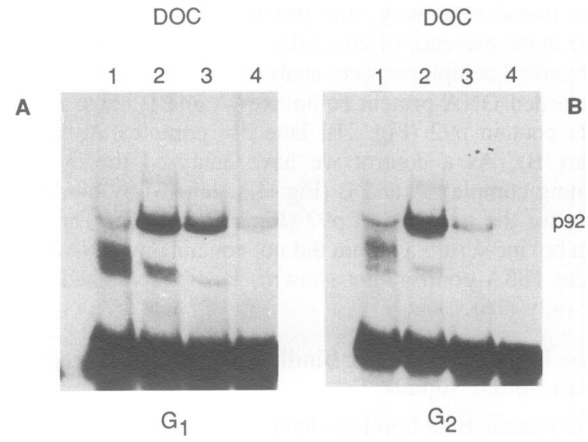

Figure 5. I-92 a novel nuclear inhibitor of p92 regulates S phase dependent p92 DNA binding activity. (A) EMSA of nuclear extracts from 444 cells in G1 (lane 1) shows that p92 DNA binding activity can be released by sodium deoxycholate treatment. 0.2% DOC (lane 2), 04% DOC (lane 3) and 0.6% DOC (lane 4). (B) EMSA of nuclear extracts. p92 DNA binding activity of cells in G2 (lane 1) can be induced by 0.2% DOC tratment (lane 2), nuclear extract after treatment with 0.4% DOC (lane 3) and after treatment with 0.6% DOC. The lower retarded complex is DOC sensitive and contains a 50kD protein as was shown by preparative EMSA. Oligonucleotide RP3 was used for EMSA.

and G2-cells, as well as S phase cells. This observation rules out that the DNA binding function of the p92 DNA binding domain is regulated by the cell cycle. One possibility to explain this cell cycle regulation of p92 DNA binding would be the existence of a nuclear inhibitor of p92, which is active only in G1 and G2 of the cell cycle. The association of p92 with the putative inhibitor in G1- and G2-cells could mask p92 DNA binding activity in EMSA but not in a denaturing procedure like binding site blotting.

Treatment of nuclear extracts with the detergent sodium deoxycholate (DOC) revealed the existence of an inactive transcription factor complex, which consists of NF$\varkappa$B and its inhibitor IE$\varkappa$B (1) in the cytoplasm of resting B lymphocytes. Similar experiments were performed to determine whether p92 exists in an inactive complex in G1- and G2-cells. The addition of 0.2% DOC to the binding reaction of the 444 cell G1 nuclear extract leads to the appearance of a novel retarded complex which has a migration similar to the complex A in S phase cells (Fig. 5A, lanes 2 and 3). DOC treatment of the G2 nuclear extract releases a similar retarded complex (Fig 5B, lane 2). These results demonstrate that p92 is complexed to an inhibitor of p92 DNA binding in nuclei of G1 and G2 444 cells. We propose to call this inhibitor I-92. The inhibitory activity is regulated by the cell cycle so that the mechanism which determines S phase dependent p92 DNA binding activity is the cyclic association of p92 with I-92 in G1 and G2 of the cell cycle. The association of p92 with I-92 is reversible, because active p92 can be released from the inactive complex by DOC treatment.

The inhibitor I-92 associates in vitro with p92 and prevents DNA binding

We attempted to separate I-92 from p92 by heparin Sepharose column chromatography. Nuclear extracts of nontumorigenic Hela-fibroblast hybrids (444) were treated with 0.2% DOC and subsequently passed over a heparin Sepharose column in 70mM NaCl. The flow through was collected. After washing the column

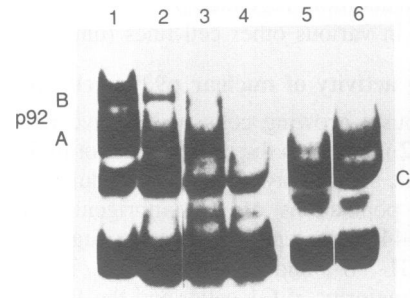

Figure 6. The inhibitor I-92 associates with p92 in vitro in a reversible way and inhibits p92 DNA binding. Nuclear extracts from confluent 444 cells were treated with 0.2% DOC and passed over a heparin Sepharose column. The flow through was used as a source of I-92. EMSA of nuclear extract from 444 cells in S phase, where I-92 is inactive (lane 1), after the addition of 5 μl heparin-Sepharose flow through (lane 2), after the addition 10 μl flow through (lane 3) and after the addition of 20 μl flow through (lane 4). EMSA of a 500 mM KCl heparin Sepharose fraction containing p92 after the addition of 25 μl flow through (lane 5), EMSA of a 500 mM KCl heparin Sepharose fraction containing p92 plus 25 μl of flow through after treatment with 0.2 deoxycholate (DOC) for 10 min at 37°C (lane 6). The retarded complex C contains a 50kD protein which was not characterized.

with loading buffer, bound proteins were eluted with 600 mM NaCl. As judged by the failure to produce retarded complexes in EMSA the flow through did not contain p92. p92 DNA binding activity was recovered in the 600 mM eluate (not shown). Nuclear extracts from 444 cells in S phase contain no detectable I-92 activity and were used to assay for the inhibitor by EMSA. Three retarded complexes are formed with oligonucleotide RP3 in S phase 444 cell nuclear extract. The top two correspond to p92:DNA complex A and B (Fig. 6, lane 1). The identity of the protein in complex C is currently under investigation. Isolation of complex C by preparative EMSA revealed that a 50kD protein is present in this complex (unpublished data). Increasing amounts

of flow through fraction were added to the DNA binding reaction of the S phase nuclear extract. The addition of 5 μl flow through reduces strongly complex B and to some extent complex A (Fig. 6, lane 2). The addition of 15 μl flow through prevents the formation of complex B completely (lane 3) and after addition of 20 μl of flow through neither complex B nor complex A is formed (lane 4). The formation of complex C is unaffected by the addition of flow through to the DNA binding reaction. The persistence of complex C after the addition of 20 μl flow through to the reaction shows that the association of I-92 with p92 in vitro is specific. The association of I-92 with p92 is reversible, because the addition of 0.2% DOC to a sample which contained 25 μl flow through and 7 μl of a heparin Sepharose 500 mM KCl fraction containing p92 (Fig 6, lane 5, released p92 from the inactive complex (lane 6). Treatment of flow through with trypsin abolished I-92 activity suggesting that I-92 is a protein (unpublished data).

DISCUSSION

p92 is an octamer binding factor that displays two interesting regulatory properties. Its concentration in the nucleus is controlled at the level of nuclear import. Secondly, its binding activity is regulated in a cell cycle dependent fashion by association with a nuclear inhibitor.

Serum stimulation brings about a cascade of regulatory events which is activated by growth factors present in serum. The induction of immediate early response genes or competence genes (30) characterized by e.g c-*fos* gene induction involves phosphorylation of serum response factor (SRF) (29) which may depend on the activity of casein kinase II (24). Growth factor induced transcription of the c-*fos* proto-oncogene is mediated by the serum response element (SRE) (42). The activation of c-*fos* transcription occurs within minutes after serum stimulation (15). Besides the c-*fos* gene, at least two other proto-oncogenes, the c-*myc* and the c-*jun* gene belong to this family.

The second class of genes which are induced by serum stimulation, are referred to as the progression genes (30). Genes of this class include the thymidine kinase gene, histone genes and the dihydrofolate reductase gene. Their expression is also regulated by the cell cycle, e.g. the histone H2b gene expression is increased in S phase. The S phase dependent gene activity is mediated by a subtype-specific consensus element containing an ATTTGCAT octamer sequence (17). p92 could be considered as a mediator of immediate early response. The kinetic of accumulation of the DNA binding activity in the nucleus (5−9 hours after serum stimulation) suggests that p92 may interact with control regions of progression genes such as the histone H2b gene. As was shown by EMSA, p92 binds to the subtype-specific consensus element of the human histone H2b gene containing an octamer sequence (unpublished data) and may therefore be involved in regulating S phase dependent H2b gene expression

Considering its size, nuclear import of p92 most likely requires an active transport. Molecules larger than 60 kD cannot pass nuclear pores by passive diffusion (18). In addition, specific recognition sequences are required for nuclear import (12). Regulated intracellular distribution is an important mechanism in the regulation of the activity of several sequence specific DNA binding proteins and transcription factors. The dorsal protein from Drosophila forms a nucleocytoplasmic gradient. Proceeding from the dorsal to the ventral side of the embryo an increasing amount of dorsal protein is found in the nucleus. It is believed that this nuclear gradient is responsible for dorsoventral morphogenesis (31, 34, 40). The transcription factor NF-*k*B is present in the ctoplasm in an inactive form in which it is complexed with the inhibitor I*k*B (1). NF-*k*B is activated upon induction by certain cytokines and a variety of other inducers. Here, I*k*B becomes phosphorylated, the complex dissociates, and free NF-*k*B can move into the nucleus, where it activates transcription (10). The product of the immediate early or competence gene c-*fos* is also regulated by translocation control (32). It was found that nuclear transport of the c-Fos protein in serum stimulated fibroblasts requires the continuous stimulation of cells by serum factors. When serum factors are omitted nuclear import is blocked by a labile inhibitor (32).

Cyclic activity of the inhibitor I-92 during the cell cycle is a novel regulatory principle for controlling DNA binding activity of octamer binding protein p92

Cyclic association of an inhibitor with a sequence specific DNA binding protein is a novel principle in determining cell cycle dependent activity of DNA binding. The inhibitor I-92 associates with p92 in G1 and G2 of the cell cycle, and leads to reversible inactivation of DNA binding activity of p92. In S phase cells I-92 is not active, therefore p92 is not complexed and available for binding to octamer sequences. Active p92 can be released experimentally from the inactive p92:I-92 complex by deoxycholate treatment implying that the association of p92 with I-92 is reversible. In addition, I-92 is able to reassociate with p92 in vitro to form an inactive complex. The formation of an inactive I-92:p92 complex with p92 from S phase cells in vitro, suggests that it is most likely the activity of the inhibitor that is regulated by the cell cycle. If the p92 protein were to be regulated by some kind of modification in its ability to associate with I-92, then p92 from S phase cells should not be inactivated by I-92 in vitro.

The activities of many proteins which are regulated during the cell cycle are modulated by phosphorylation. The product of the *cdc2* gene $p34^{cdc2}$, is the catalytic subunit of a protein complex responsible for driving the eukaryotic cell cycle. The $p34^{cdc2}$ protein is a kinase which is the central cell cycle regulator of species ranging from yeast to humans (7). The human homologue has been isolated (19), and the kinase is itself phosphorylated in response to extracellular signals (20). The product of the retinoblastoma susceptibility gene $p105^{RB}$ has properties of a cell cycle regulatory element (5) and its activity is regulated by phosphorylation (3, 22, 23). The active form of $p105^{RB}$ is underphosphorylated. The transforming protein large T antigen of SV40 virus associates with the underphosphorylated, active form of $p105^{RB}$ and inactivates it (22, 23). A similar mechanism may lead to the cyclic association of I-92 with p92, where differential phosphorylation of I-92 may regulate its activity during the cell cycle. $p105^{RB}$, large T antigen, human p53 protein and histone H1 are, besides many other proteins, substrate for the cdc2 kinase (7, 26). We are currently engaged in purifying I-92 and intend to determine whether the cyclic activity of I-92 regulated by phosphorylation, and whether it is a substrate for cdc2 kinase.

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