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Purification of intercalator-released p67, a polypeptide that interacts specifically with the c-fos serum response element

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ABSTRACT

Incubation of intact nuclei in buffers containing the DNA intercalating drug chloroquine leads to release of proteins that interact with DNA (1). We demonstrate here that a protein which binds to a motif within the human c-fos promoter, identified as the serum response element (SRE), is quantitatively released from HeLa nuclei, whereas nuclear factor 1 (NF 1) is not. Purification of the SRE binding protein by affinity chromatography to greater than 95% homogeneity allowed us to identify it as a polypeptide of approximately 67,000 daltons. The DNA contacts made by p67, as identified by methylation interference experiments, are indistinguishable from those of the serum response factor described previously (2).

INTRODUCTION

The transcriptional activity of eukaryotic genes may be regulated in a temporal or tissue-specific manner and may also be subject to extracellular inducing signals (for recent review see ref. 3). At the molecular level, transcriptional control involves the sequence-specific binding of trans-acting transcription factors to DNA cis-elements of promoter upstream regions (3). Recent advances in techniques of DNA-affinity chromatography (4,5,6) have allowed the identification and purification of several such factors, including SP1 (7), AP-1 (8), AP-2 (9) and NF1 (4, 10; Rupp and Sippel, submitted).

Success in purification of low-abundance transcription factors requires their efficient extraction out of cells as the first step in the purification protocol. We have demonstrated earlier (1) that incubation of nuclei with DNA intercalating drugs (e.g. ethidium bromide, chloroquine) can cause the highly selective release of DNA-binding proteins. The specific conditions of the intercalator treatment influence the profile of

released proteins. Here we describe conditions for the generation of HeLa nuclear intercalation extracts which result in the selective release of sequence-specific DNA-binding proteins. The use of chloroquine (CQ) in the nuclear intercalation protocol enabled us to extract quantitatively a polypeptide which binds sequence-specifically to the promoter of the human c-fos proto-oncogene, an efficient first step in its purification to homogeneity.

The cellular c-fos proto-oncogene is stimulated in its transcriptional activity by a variety of extracellular signals, including serum, individual peptide growth factors, ion flux or tumour-promoting phorbol esters (for reviews: 11,12). Within the c-fos promoter, the cis-acting serum response element (SRE) was identified as a site of specific DNA-protein interaction (2,13,16) and was shown to be essential for stimulation of c-fos transcription by serum (2,13,14,16). In the present communication we report the purification to virtual homogeneity of a polypeptide of approximately 67,000 D (p67) which specifically interacts with the SRE. The binding specificity of p67 is confirmed with renatured, electrophoretically pure material. Contacts between SRE and p67 were analysed by methylation interference experiments and shown to be indistinguishable from those reported earlier for the serum response factor (SRF) (2).

MATERIALS AND METHODS

Buffers.

buffer A: 10mM HEPES (pH 7.0), 1mM spermidine, 0.3mM spermine, 1mM EDTA, 0.5mM EGTA, 10% glycerol

buffer B: 20mM HEPES, 2mM $MgCl_2$, 0.2mM EDTA, 20% glycerol, 20mM KCl, (adjusted with KOH to pH 7.9)

buffer C: 20mM HEPES (pH 7.9), 0.1% NP40, 0.2mM EDTA, 20% glycerol.

Buffers A to C contain 1mM DTT and 0.1mM PMSF.

TBE: 89mM Tris-HCl, 89mM boric acid, 2.5mM EDTA.

Generation of nuclear extracts.

HeLa cells were grown as spinner cultures in Joklik's medium supplemented with 10% newborn calf serum. Nuclei were prepared according to (17). Salt extracts were prepared as described

(17) with slight modifications (18). Chloroquine intercalation extracts were prepared with modifications according to (1). Nuclei from 10g of HeLa cells were washed and resuspended in extraction buffer A to a total volume of 15ml. To this, an equal volume of extraction buffer A containing 20mM chloroquine was added and the mixture incubated on ice with gentle shaking for 30 min. Nuclei were then pelleted and the supernatant clarified by centrifugation in the cold for 15 min. at 15,000 g. Proteins present in the resulting supernatant were precipitated by $(NH_4)_2SO_4$ added as salt to a final concentration of 77% saturation. The pH was maintained at 7.0 by addition of NaOH.

After precipitation for three hours, the protein pellet was collected by sedimentation (30 min. at 15,000 g), resuspended in 1ml of buffer B and dialysed against buffer B. The dialysed sample was clarified by centrifugation (15 min., 13,000 g) to give a protein concentration of around 6mg/ml.

DNA-affinity chromatography.

The DNA-affinity matrix was prepared according to the method of Kadonaga and Tjian (5). The oligonucleotides shown in figure 1C were synthesized by M. Nassal (ZMBH) with an Applied Biosystems synthesizer. The purified complementary oligonucleotides were first phosphorylated, then annealed and ligated to multimeric forms as described (5). DNA was phenol extracted and ethanol precipitated out of the ligation reaction in the presence of 2M $(NH_4)OAc$ and 10mM $MgCl_2$ to remove ATP. The ligation products were covalently linked to cyanogen bromide activated Sepharose 4B (Pharmacia) (5). Chloroquine extract (3ml), supplemented with 360µg poly d(I-C) as unspecific competitor DNA, NP40 (to 0.1%), EDTA (to 5mM), DTT (to 1mM) and PMSF (to 0.1mM), was passed twice through a 1ml column of SRE affinity matrix. The column was first washed with 10ml of buffer C containing 0.3M KCl and the bound protein material eluted with 3 column volumes of buffer C containing 1M KCl. For additional cycles of affinity chromatography the eluate was diluted 4 fold, supplemented with additional competitor DNA and reapplied to the column.

Renaturation of purified p67. Purified polypeptide p67 was prepared for gel electrophoresis, subsequent elution from the

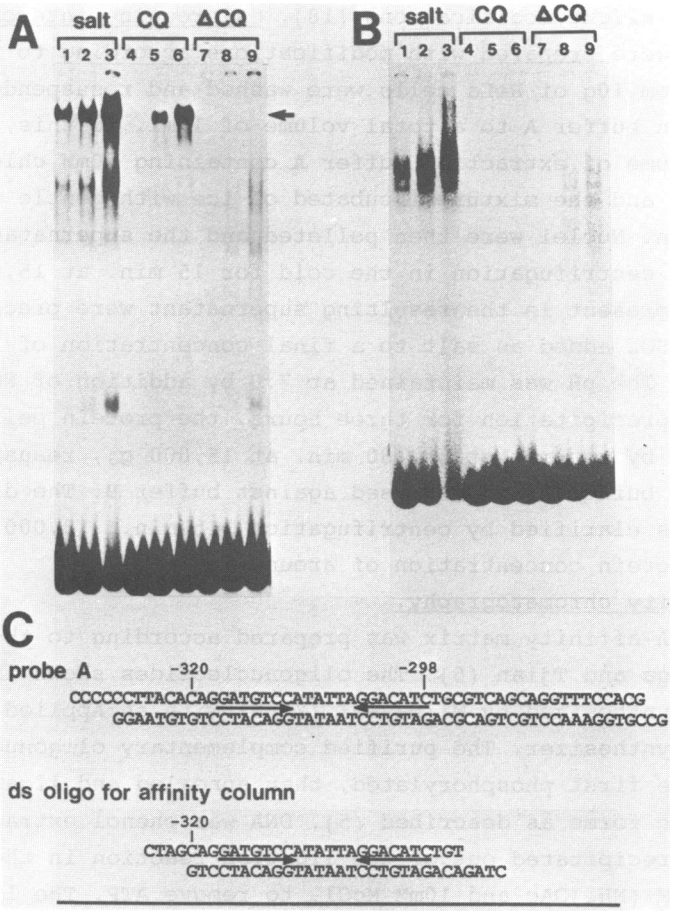

Figure 1: Selective release of c-fos promoter binding proteins upon chloroquine intercalation of HeLa nuclei.

Gel retardation assays were performed with HeLa nuclear extracts generated by incubation of nuclei with salt (lanes 1-3), chloroquine (lanes 4-6), or salt subsequent to chloroquine (lanes 7-9). The double-stranded, radiolabelled oligonucleotide probes used were probe A (A) and probe B (B). The sequence of probe A is shown in (C); the sequence of probe B covers human c-fos promoter positions -209 to -160. (C) also shows the double-stranded oligonucleotide used to generate the SRE DNA-affinity column; the c-fos promoter positions -320 to -295 are encompassed by this oligo.

gel and renaturation according to (19). Affinity purified p67 was supplemented with 20µg BSA and 4µg myoglobin and precipitated with 2% TCA overnight on ice. The precipitate was sub-

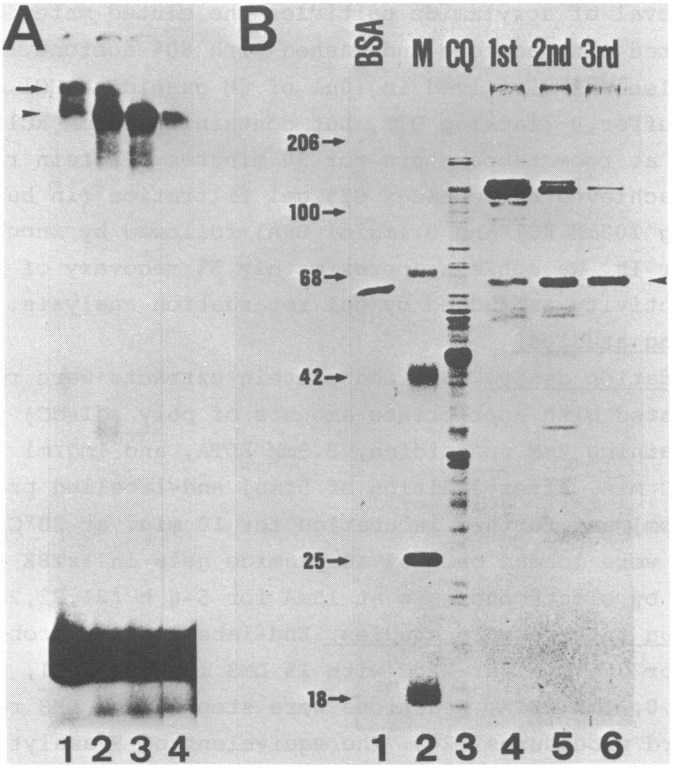

Figure 2: Purification of the SRE binding protein.
(A) Gel retardation assay using protein preparations from different purification stages. The radiolabelled DNA fragment was probe A (see figure 1C). Protein preparations were from the chloroquine (CQ) extract (lane 1), first passage over SRE affinity column (lane 2), and third passage over SRE affinity column (lane 3). With the unfractionated CQ extract two complexes were generated, a slower (arrow) and a faster (arrowhead) migrating form. The latter form is obtained with affinity purified material. Lane 4, electrophoretically pure, renatured p67.
(B) SDS-gel electrophoresis of protein composition at different stages of purification. Lanes 1 and 2 represent bovine serum albumine (BSA) and size markers, respectively, as standards. Lane 3, chloroquine extract; lanes 4-6, protein eluates from the SRE affinity column after first, second and third passages over the column, respectively.

jected to SDS-gel electrophoresis (20) with 50µM thioglycolate in the upper electrophoresis buffer. The desired gel regions were cut out, crushed, and incubated in 300µl elution buffer (19) for 60 min. at room temperature with occasional agitation.

After removal of acrylamide particles the eluted material was precipitated with acetone and washed with 80% acetone. The dried pellet was dissolved in 10µl of 6M guanidine-HCl and 0.1% NP40 in buffer B (lacking DTT, but containing 100mM KCl) and incubated at room temperature for 30 minutes. Protein renaturation was achieved by Sephadex G25 gel filtration (in buffer B, containing 100mM KCl and 0.1mg/ml BSA) followed by incubation on ice for 1h. We achieve approximately 5% recovery of p67 binding activity as judged by gel retardation analysis.

DNA binding studies.

Gel retardation assay. Purified protein extracts were routinely pre-incubated with appropriate amounts of poly (dI-dC) in buffer B containing 2mM spermidine, 2.5mM EDTA, and 1mg/ml BSA on ice for 20 min. After addition of 5fmol end-labelled probe (30,000 cpm) and further incubation for 10 min. at 20°C, the reactions were loaded onto 5% acrylamide gels in 1xTBE and separated by electrophoresis at 15mA for 5-6 h (21,22,23).

Methylation interference studies. End-labelled DNA probe A was treated for 3.5 min. at 22°C with 1% DMS in 60mM NaCl, 10mM Tris pH 8.0, 1mM EDTA. Reactions were stopped and DMS removed by standard procedures (24). The equivalent of 9 analytical gel retardation reactions was taken for each preparative experiment. Bound and free DNA were separated in 5% acrylamide gels and visualised by autoradiography. Material was electrotransferred from the gel directly onto strips of NA45 paper (Schleicher and Schuell) and re-eluted in 1M NaCl, 20mM Tris pH 8, 1mM EDTA by incubation for 1h at 70°C. After phenol/chloroform extractions and addition of carrier, DNA was recovered by precipitation in isopropanol and washed in 70% ethanol. Samples were subsequently cleaved with piperidine and exhaustively lyophilised. Thereafter equal counts were taken from each, re-lyophilised out of 100µl H_2O and run over 8% sequencing gels.

RESULTS

Quantitative elution of a c-fos promoter binding activity from HeLa nuclei by the DNA intercalating drug chloroquine.

Our previous work demonstrated that a selective and virtually quantitative release of the high mobility group proteins HMG

14 and HMG 17 can be achieved upon incubation of chicken erythrocyte nuclei with the DNA intercalating drug chloroquine (1). HMG proteins are known to interact with DNA in a largely sequence-independent manner. We were now interested to find intercalation conditions that instead might selectively release sequence-specific DNA-binding proteins. We were especially interested in the identification of nuclear proteins that interact with promoter elements of the human c-fos proto-oncogene.

For comparative purposes we generated nuclear extracts from HeLa cells by either salt extraction (17) or incubation of nuclei with the intercalator chloroquine (CQ) at pH=7.0 (1). These extracts were tested for the presence of sequence-specific DNA binding proteins by gel retardation assays (21-23) with end-labelled, double-stranded oligonucleotide probes derived from different regions of the human c-fos promoter.

In the presence of appropriate amounts of poly d(I-C) as non-specific competitor DNA, distinct complexes were generated in nuclear salt extracts by both probe A and probe B, which span human c-fos sequences -327 to -278 and -209 to -160, respectively (figure 1A, lanes 1-3; figure 1B, lanes 1-3; see figure 1C for nucleotide sequence of probe A). Chloroquine intercalation extracts, which contain a protein spectrum of reduced complexity compared with salt extracts (figure 2B, lane 3, and data not shown), form a specific complex with probe A (figure 1, lanes 4-6) but not with probe B (figure 1B, lanes 4-6). Upon re-extraction of chloroquine treated nuclei with salt, no additional binding activity to probe A is obtained (figure 1A, lanes 7-9) whereas binding activity to probe B can be recovered (figure 1B, lanes 7-9). We calculate that this quantitative chloroquine release of binding activity to probe A represents a 20 fold purification over nuclear salt extraction. The binding activity shown in figure 1B not to be released by chloroquine has been identified as a CTF/NF1-related transcription factor (Shaw and Nordheim, unpublished).

To characterise further the complex formed with probe A (figure 1A, lanes 4-6), gel retardation competition experiments were performed. We observed that oligonucleotide competitor DNAs containing transversion mutations within the centre of the

symmetrical sequence of the SRE (around position -307) fail to compete for complex formation (data not shown). The DNA binding protein efficiently released upon nuclear CQ extraction therefore appeared to be the SRE binding protein (2,13). This prompted us to examine the activity released by chloroquine from cells subjected to serum induction. No difference between the binding activity present in extracts derived from nuclei of normally growing and serum-induced HeLa cells could be detected.

Purification of the SRE binding protein.

The significant degree of initial enrichment for the SRE binding activity in our nuclear chloroquine extracts allowed us to attempt purification of this activity from the chloroquine extract directly by DNA-affinity chromatography. To generate a DNA-affinity matrix, oligonucleotides encompassing the SRE sequence (figure 1C) were synthesized, annealed, multimerized by ligation and covalently cross-linked to an agarose support as described (5). CQ extracts were concentrated and separated from CQ by ammonium sulphate precipitation, resuspended and applied directly to the affinity column in the presence of excess competitor DNA. After an extensive 0.3M KCl wash, the column-bound material was eluted with 1.0M KCl and reapplied to the affinity column after dilution and incubation with additional unspecific competitor DNA. Each affinity chromatography step was monitored by gel retardation analysis with probe A (figure 2A, lanes 1-3) and the protein composition of the eluted material assayed by SDS-gel electrophoresis (figure 2B). Figure 2A shows that the binding activity present in the starting CQ extract gives rise to two complexes (lane 1), of which only the faster migrating form is generated with purified material (lanes 2,3). Gel electrophoretic analysis of the protein fractions obtained during the course of purification demonstrates the steady enrichment of a polypeptide with an apparent molecular weight of approximately 67,000 daltons (figure 2B, lanes 4-6). We refer to this protein as polypeptide p67.

An apparently unspecifically bound protein of approximately 120,000 daltons (see also references (8), (9)) can be competed

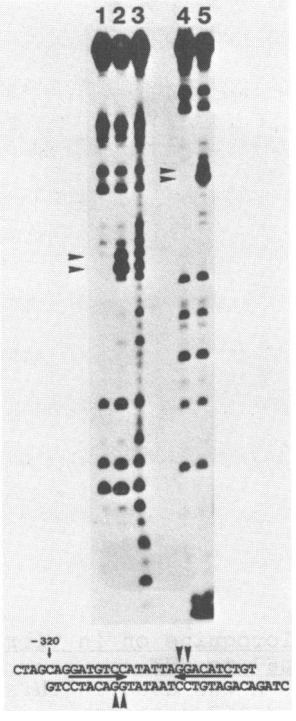

Figure 3: Methylation interference analysis of the purified p67 protein.
The protein material used is identical to the material shown in figure 2B, lane 6. The methylation-sensitive G-residues found on both the upper (lanes 1, 2) and lower (lanes 4, 5) DNA strands of probe A are indicated by arrowheads. Lane 3 shows a G+A reaction on the upper strand. Lanes 1 and 4 represent p67-bound material and lanes 2 and 5 show free DNA. The extent of the symmetrical SRE region, as defined by (13), is indicated.

away following its initial enrichment in the first round of DNA-affinity chromatography.

Characterisation of purified p67 SRE binding protein.

To obtain further confirmation that the p67 polypeptide is a bona fide SRE binding protein, we excised a p67 protein band from an acrylamide gel, eluted and renatured the protein material and were able to demonstrate that the eluted material formed a specific DNA-protein complex with probe A as assayed by gel retardation analysis (figure 2A, lane 4). In addition,

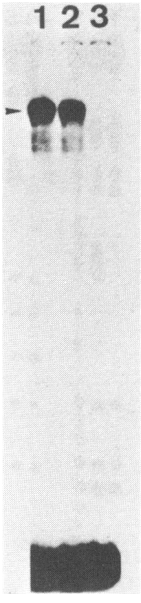

Figure 4: Effect of chloroquine on in vitro binding of polypeptide p67 to SRE.
Radiolabelled DNA probe A was used in this gel retardation experiment with purified p67 protein.
Chloroquine concentrations in the binding reactions were:
lane 1, 0mM; lane 2, 0.1mM; lane 3, 1.0mM.

the complex formed by the purified protein material was characterised by methylation interference experiments. Such an analysis identifies G-residues within the DNA binding site, which, when N7-methylated, interfere with protein recognition (25). Figure 3 displays the methylation interference pattern characteristic for the interaction between p67 and SRE. This pattern, obtained with our purified p67 protein, is indistinguishable from the one described earlier for the serum response factor (SRF) present in a crude HeLa cell nuclear extract (2).

The effect of chloroquine on the binding of p67 to SRE sequences.

As shown in figure 1A we obtain efficient release of p67 binding activity upon nuclear intercalation by chloroquine. If this release were primarily due to DNA intercalation in chromatin we would predict that binding of purified p67 protein to the SRE *in vitro* would be affected by CQ. This is indeed the

case as shown in figure 4, lanes 1-3. The presence of 1mM CQ in the binding reaction efficiently inhibits complex formation between p67 and SRE, whereas 0.1mM CQ is still tolerated.

DISCUSSION

The technique of elutive intercalation (1) employs DNA intercalating drugs to induce selective release of DNA-binding proteins from isolated nuclei. The elution conditions chosen previously permitted preferential extraction from chicken erythrocyte nuclei of the abundant, non-histone high-mobility group proteins HMG14 and HMG17 (1). HMG proteins are known to bind DNA in a sequence independent manner.

Upon extending this work we show here for the first time that DNA intercalators can also be used to induce quantitative release from nuclei of low-abundance DNA-binding proteins that interact with DNA in a sequence specific fashion. This is demonstrated for a protein, p67, which binds to the SRE of the human c-fos promoter. The extraction conditions used display a high degree of selectivity in protein release since we can show that another DNA-binding protein, nuclear factor 1 (NF1), is not extracted. The spectrum of proteins released can be influenced, however, by subtle changes in ionic strength, pH or the choice of intercalator (1). Although the biochemical basis for the intercalator-induced nuclear release of DNA-binding proteins and its selectivity is not understood, we show in figure 4 that DNA intercalation by chloroquine abolishes the DNA-p67 protein interaction. This is consistent with our idea that nuclear DNA intercalation releases a spectrum of DNA-bound proteins, one of which is p67. The quantitative extraction of p67 in this manner would therefore indicate its presence inside the nucleus in a chromatin bound state, even in uninduced cells.

In comparison to nuclear salt extracts, intercalator extracts contain a much reduced total protein concentration with greatly diminished complexity in protein composition. We estimate that the intercalator extraction technique yields at least a 20 fold purification over salt extraction as a first step in the purification of this protein.

The high degree of enrichment for SRE binding activity in

the chloroquine extract permitted subsequent purification of this protein by subjecting the CQ extract directly to DNA-affinity chromatography. We report the identification of the c-fos SRE binding protein as a single polypeptide of apparent molecular weight 67,000 D. The combination of elutive intercalation and DNA-affinity chromatography permitted our purification of this protein, p67, to greater than 95% homogeneity.

Several lines of evidence indicate that p67 is indeed the bona fide SRE binding protein. First, p67 exhibits high affinity to the DNA-affinity column that contains the SRE binding sequence. Second, gel retardation experiments with purified p67 generate band shifts found with SRE binding activity from crude extracts. Third, identical methylation interference patterns are obtained with p67 when present either in its purified form or in a crude extract. Fourth, purified p67 protein can be extracted from protein gels and be shown to generate correct band shifts following protein renaturation. We note that two specific types of SRE-protein complex are observed with the unfractionated CQ extract in gel retardation experiments (figure 2A, lane 1) but only the faster migrating form is generated by purified p67 (figure 2A, lane 3). Detailed characterisation of the slower migrating complex is in progress.

The methylation interference pattern that we observe for the complex between the symmetrical SRE and purified p67 is indistinguishable from the pattern reported previously for SRF (2). We therefore suggest that p67 is identical to SRF. Indeed, while this manuscript was in preparation, R. Treisman reported the purification of SRF and determined its molecular weight as 67,000 D (26).

The binding site of p67, SRE, has been shown to be necessary for transcriptional activation of the c-fos gene in response to serum stimulation of resting fibroblasts (13) and the functional participation of the SRE binding protein in serum induction has been proposed (2,14,15). At the present time it is unclear which molecular events take place during c-fos gene activation and which role is played by p67. Post-transcriptional modification has been proposed to modulate the activity of some transcription factors which may, in turn, influence the conforma-

tion of transcription complexes by affecting DNA-protein as well as protein-protein interactions. We observe no significant quantitative or qualitative difference between chloroquine-released p67 activity from nuclei of normally growing and induced HeLa cells. This suggests that p67 is bound to the DNA in both induced and normally growing HeLa cells.

The availability of p67 in purified form will now permit a detailed biochemical and functional characterisation of the protein and will thereby lead to insights into the mechanisms of gene activation in response to extracellular signalling.

Acknowledgement

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Note Added in Proof: Prywes and Roeder (Mol. Cell. Biol. 7, 3482-3499, 1987) recently described the purification of a protein of approximately 62,000 daltons m.w. with similar DNA sequence binding specificity.