A Unique Transcription Factor for the Aα Fibrinogen Gene Is Related to the Mitochondrial Single-Stranded DNA Binding Protein P16†

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ABSTRACT: Although stimulation of hepatic cells with interleukin-6 induces the expression of fibrinogen, the molecular basis for this regulation remains largely uncharacterized. A recent examination of the Aα fibrinogen gene promoter identified a protein, termed the Aα-core protein, that bound constitutively to the IL-6 response element [Liu, Z. & Fuller, G. M. (1995) *J. Biol. Chem. 270,* 7580−7586]. This current study provides further characterization of this regulatory protein. The data presented show the following: (i) The Aα-core protein has a similar molecular weight and identical N-terminal sequence to that of the mitochondrial single-stranded DNA binding protein P16. (ii) The Aα-core protein and P16 have similar characteristics in terms of DNA binding preference and antigenic properties. (iii) Overexpression of P16 gene in the hepatoma cell lines Hep G2 and Hep 3B enhances the IL-6-induced expression of Aα fibrinogen. These results demonstrate that the Aα-core protein is closely related to P16 and involved in the IL-6-regulated transcription of Aα fibrinogen.

Fibrinogen is a hexamer, comprised of three pairs of nonidentical polypeptide subunits ($A\alpha_2$, $B\beta_2$, and γ_2) that are linked through a series of disulfide bridges (Doolittle, 1984; Henchen, 1993; Blomback, 1996). Each subunit is derived from a distinct gene (Chung et al., 1983; Kant & Crabtree, 1983; Rixon et al., 1983). Although expression of all three genes is tightly coordinated (Crabtree & Kant, 1982; Otto et al., 1987; Nesbitt & Fuller, 1991), each subunit is regulated by distinct combinations of cis-transcription elements (Chodosh et al., 1987; Morgan et al., 1988; Baumann et al., 1992; Dalmon et al., 1993; Hu et al., 1995; Liu & Fuller, 1995; Zhang et al., 1995).

The biosynthesis of fibrinogen takes place almost exclusively in hepatocytes. Its production increases 4−5-fold during acute inflammation and is a major acute phase reactant (APR)[1] (Fey & Fuller, 1987). Acute phase proteins are grouped into two classes (class I and class II) depending upon the regulatory signals to which they respond (Baumann et al., 1987, 1989, 1990; Dalmon et al., 1993). Fibrinogen belongs to class II, and its transcription is induced by the inflammatory cytokine interleukin-6 (IL-6). Glucocorticoids synergize with IL-6, enhancing the expression of class II APRs, although the precise mechanism for this synergism remains to be elucidated (Baumann et al., 1990; Hocke et al., 1992).

IL-6 stimulation of the signal transducing subunit (gp130) activates two signaling pathways. The first involves the Janus kinases (Jaks) that phosphorylate a group of latent cytoplasmic transcription factors known as Stats (signal transducers and activators of transcription) (Wegenka et al., 1993, Akira et al., 1994; Zhong et al., 1994; Heim, 1995; Stahl et al., 1995). The second pathway is the MAP (mitogen-activated protein) kinase signalling cascade and activates the C/EBP transcription factors that regulate expression of class I acute phase genes (Akira et al., 1990; Kinoshita et al., 1992). Both Stat 1α and Stat 3 are phosphorylated following IL-6 binding to its receptor, leading to the formation of cytosolic homo- and hetero-Stat dimers that translocate into nucleus and bind specifically to a Stat consensus palindrome [TT(A/C)(C/T)N(G/A)(G/T)AA] (Akira et al., 1994; Zhong et al., 1994). All class II APRs have a characterized IL-6 responsive element (IL-6RE: CTGGGA) (Crabtree et al., 1985; Huber et al., 1990; Anderson, 1993; Dalmon et al., 1993; Zhang et al., 1995) that partially matches the Stat consensus sequence. Whether this element within the APR promoter can form a palindrome structure depends on the flanking DNA sequences.

Examination of the Aα fibrinogen promoter shows that although the gene possesses two putative IL-6 RE, Stat 3 bound only one of these sites and had no effect on transcription (Hu et al., 1995; Z. Liu, and G. M. Fuller, unpublished data). The second site, instead of binding Stat 3 associates with an unidentified protein, termed the Aα-core protein. The Aα-core protein binds constitutively to the CTGGGA domain and the contiguous upstream hexanucleotide sequence GAATTT. Both of these sites are essential for the IL-6-mediated transcription of Aα fibrinogen (Liu & Fuller, 1995). In this study, we report the purification and characterization of the Aα-core protein. The Aα-core protein was found to be closely related to a mitochondrial single-stranded DNA binding protein (P16), and overexpression of P16 up-regulated the IL-6-induced transcription of Aα fibrinogen.

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[1] Abbreviations: IL, interleukin; bp, base pair(s); C/EBP, CAAT enhancer binding protein; Stat, signal transducers and activators of transcription; IL-6 RE, interleukin-6 response element; APR, acute phase reactant; MAP kinase, mitogen-activated protein kinase; EMSA, electrophoretic mobility shift assay; Mt, mitochondria.

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MATERIALS AND METHODS

Materials. Tissue culture media, antibiotics, and fetal bovine serum were purchased from GIBCO BRL (Gaithersburg, MD) and Hyclone (Logan, UT). The glutathione *S*-transferase (GST) fusion protein expression and purification system was obtained from Pharmacia (Piscataway, NJ). Radiolabeled deoxynucleotides were supplied by Amersham (Arlington Heights, IL). Chromatography resins were from Bio-Rad (Hercules, CA). All other chemicals were from Fisher Scientific (Pittsburgh, PA) or Sigma (St. Louis, MO).

Cell Culture. Rat primary hepatocytes were prepared and cultured as previously described (Fuller et al., 1988). The human hepatoma cell lines Hep G2 and Hep 3B (obtained from ATCC) were grown in Dulbecco's modification of Eagle's medium 1× (MOD), supplemented with 4.5 g/L glucose, 2 mM L-glutamine, ciprofloxacin hydrochloride (10 μg/mL), nonessential amino acids (0.1 mM), and 10% fetal bovine serum.

Preparation of Nuclear Extracts. Nuclear extracts from primary rat hepatocytes were prepared as previously described (Liu & Fuller, 1995). Briefly, cells were washed twice with cold phosphate-buffered saline (pH 7.4) and harvested. The cell pellet was resuspended in 10 mM HEPES (pH 7.6), 15 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 2 mM EDTA, 2.4 M sucrose, 0.5 mM DTT, 0.5 mM PMSF, and 1% Trasylol and homogenized. The cellular homogenate was overlaid on a cushion of 10 mM HEPES (pH 7.6), 15 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 2 mM EDTA, 2.0 M sucrose, 10% glycerol, 0.5 mM DTT, 0.5 mM PMSF, and 1% Trasylol and centrifuged at 80000*g* for 30 min. The nuclear pellet was resuspended in buffer containing 25% glycerol, 20 mM HEPES (pH 7.9), 420 mM NaCl, 1.2 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 2 mM benzamidine, and protease inhibitors (pepstatin, leupeptin, aprotinin (5 μg/mL each), and 0.5 mM PMSF]. The protein extracts were centrifuged at 70000*g* for 1 h to remove the chromatin. The concentration of protein in the supernatant was determined, and aliquots were stored at −80 °C.

Electrophoretic Mobility Shift Assay (EMSA). Double-stranded oligonucleotide probes with 5′ overhanging ends were labeled with [α-^{32}P]dNTP using the Klenow fragment of DNA polymerase I for use in EMSA. EMSA and antibody supershift assays were performed as previously described (Liu & Fuller, 1995). Briefly, a 0.2 ng (∼50 000 cpm) radiolabeled probe was mixed with 10 μg of protein extract, that had been preincubated with 2 μg of poly(dI-dC) and reaction buffer (10 mM Tris, pH 7.5, 1 mM DTT, 100 mM KCl, 1 mM EDTA, 0.2 mM PMSF, 1 mg/mL acetylated bovine serum albumin, and 5% glycerol) at 25 °C for 10 min. Following a 20 min incubation at room temperature, the samples were fractionated in a nondenaturing polyacrylamide gel containing 0.25 × TBE and 5% glycerol. For supershift assays, a given concentration of antibody (specified in the figure legend) was incubated with 10 μg of extract protein in binding buffer at 4 °C for 1 h prior to EMSA.

Preparation of a Sequence-Specific DNA Affinity Column. Using an established strategy (Rosenfeld & Kelly, 1986), a concatamer (32 repeats) of the following 30 bp DNA fragment containing the IL-6 RE of the Aα fibrinogen gene was constructed between the *Bgl*II and *Bam*HI sites of the pGL-2 plasmid vector. The concatamer fragment was isolated from the pGL-2 vector and coupled to Sepharose 2B as described (James et al., 1986).

5′-GATCCAAGAATTTCTGGGATGCCGTGGTTATA-3′

3′GTTCTTAAAGACCCTACGGCACCAATATCTAG-5′

Purification of the Aα-Core Protein. Four hundred and forty grams of rat liver was homogenized in buffer A [10 mM Tris (pH 7.5), 400 mM NaCl, 1 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, and 0.6% NP-40] and centrifuged at 10000*g* for 20 min at 4 °C. Ammonium sulfate was added to the supernatant to 40% saturation, and the slurry was centrifuged at 10000*g* for 15 min. The pellet was resuspended in buffer B [10 mM Tris (pH 7.5), 50 mM NaCl, 0.1 mM EDTA, 1.0 mM DTT, 0.2 mM PMSF, and 10% glycerol]. Aliquots of the pellet and supernatant were dialyzed against buffer B for use in EMSA. The pellet, containing the Aα-core protein, was then used as the starting material for further purification. A strong cation exchange resin (Macro-Prep High S, Bio-Rad) was used as the initial chromatographic step. A linear salt gradient (buffer B containing 0.1−1 M NaCl) was used to elute bound protein, and the presence of the Aα-core protein was determined by EMSA. Fractions containing the Aα-core protein were pooled, diluted 1:7 with buffer B containing 0.05 M NaCl, and reapplied to a Macro-Prep High S column. Fractions containing Aα-core protein were diluted 1:5 with buffer B containing 0.05 M NaCl and applied to a heparin−Sepharose affinity resin. Protein was eluted with a linear salt gradient (buffer B containing 0.1−1.0 M NaCl). To remove nonspecific DNA binding proteins, fractions from the heparin affinity column were passed through a resin coupled with double-stranded poly(dI/dC) (Pharmacia). The unbound fraction from this column was collected and reapplied to a second poly(dI/dC) column. Fractions containing the Aα-core protein were then loaded onto a specific DNA affinity resin and bound proteins eluted using buffer B containing 1 M NaCl. Aα-Core protein activity was then assayed by EMSA in the absence of poly(dI/dC). The purified Aα-core protein was then pooled and concentrated.

Preparation of Aα-Core Protein for N-Terminal Amino Acid Sequence Analysis. To determine the purity of the concentrated protein, samples were fractionated by SDS−PAGE under nonreducing condition without prior boiling. To determine protein bands corresponding to the Aα-core protein, 5 mm slices were cut from the top to the bottom of the gel. Proteins were eluted from these slices and subjected to a denaturation/renaturation process (Latchman, 1994). The renatured fractions were then tested by EMSA. A 46 kDa band caused a retardation pattern identical to that of the Aα-core protein. The purified protein was electrophoresed in a 10% SDS−polyacrylamide gel and transferred to a poly(vinylidene fluoride) (PVDF) membrane and stained with Coomassie Blue. The band corresponding to Aα-core protein was cut from the membrane and its amino acid sequence determined. Sequencing was performed at the Pfizer Pharm. Co., Groten, CT.

Expression and Purification of Recombinant P16 Protein. Two oligonucleotide primers were designed according to the rat P16 gene sequence (Pavco & Van Tuyle, 1985; Hoke et al., 1990; Tiranti et al., 1993; GenBank Accession No: M94557). The forward primer 5′- AGACAGGGATC-

Table 1: Enrichment of Each Purification Step

	protein	activity unit[a]	sp act. (units/mg)	yield (%)	purification (*x*-fold)
rat liver	440 g	–	–	–	–
crude extract (0−40%)	76 g	380000	5	100	1
Macro-prep high S support	2.3 g	260000	113	72	22.6
Macro-prep high S support	630 mg	202800	321	53	64.2
heparin sulfate−agarose	160 mg	121680	760	32	152
nonspecific DNA affinity	135 mg	109295	802	29	160
specific DNA affinity	0.008 mg	46250	5781250	12	1156250

[a] Activity unit: the minimum amount of protein for one gel shift assay is assigned as 1 activity unit.

CGAAGTAGCCAGCAGTTTGGTTC-3′ and the reverse primer 5′- CAAACAGAATTCAACGATGAATCATCCGTTC-3′ were used to PCR-amplify a DNA fragment corresponding to the mature P16 protein (residues 19−151) from a rat liver cDNA library (Stratagene). The amplified fragment was cloned into the GST fusion protein expression vector pGEX-2T (Pharmacia). Expression and purification of the GST−P16 fusion protein were performed using standard techniques, and P16 protein was obtained by digestion of the fusion protein with thrombin as described (Ausubel et al., 1989).

Function Expression of the P16 Gene. The complete encoding sequence (residues 1−151) of the P16 gene was amplified from a rat liver cDNA library by PCR using the following oligonucleotides as primers: 5′-TCAGGAAAAGCTTAAAGATTAGGTTATAAG-3′ and 5′-GATTTGCACTCGAGACTGTACCAAACAATG-3′. The amplified P16 gene was cloned into the *Hind*III and *Xho*I sites, between the human cytomegalovirus immediate-early gene enhancer promoter (Pcmv) and the SV 40 poly(A) site of the pCEP4 expression vector (Invitrogen). For expression of anti-sense P16, the P16 cDNA was first cloned into the *Hind*III and *Xho*I sites of pXP2. The DNA fragment was isolated from the vector and subcloned into pCEP4. The entire DNA fragment containing Pcmv, P16, and poly(A) was isolated from pCEP4 by *Sal*I digestion and subcloned into pGL-2(250) in which the promoter region (−250/+30) of the Aα fibrinogen gene was cloned upstream of a luciferase reporter gene (Liu & Fuller, 1995).

Transient Transfections of Hepatic Cells. Transfections were performed in 100 mm tissue culture plates using calcium phosphate-mediated transfection (Sambrook et al., 1989). Following transfection, the cells were trypsinized and seeded in 6-well microtiter plates. Cells were stimulated as indicated in the figure legend. Cell lysates were prepared, and luciferase activity was determined by luminometry using a luciferase assay kit (Promega).

RESULTS

Purification of Aα-Core Protein. Using well-defined methods for the purification of transcription factors (Ausubel et al., 1989), we developed a suitable protocol to purify the Aα-core protein from rat liver (see Materials and Methods). EMSAs were utilized to monitor Aα-core protein activity in each stage of the purification. The enrichment of the Aα-core protein following each purification step is outlined in the Table 1. Approximately 8 μg of the Aα-core protein was obtained from 440 g of rat liver, which represents a 10^6-fold enrichment.

The results of the EMSA using fractions obtained from the final purification step are shown in Figure 1. Only two

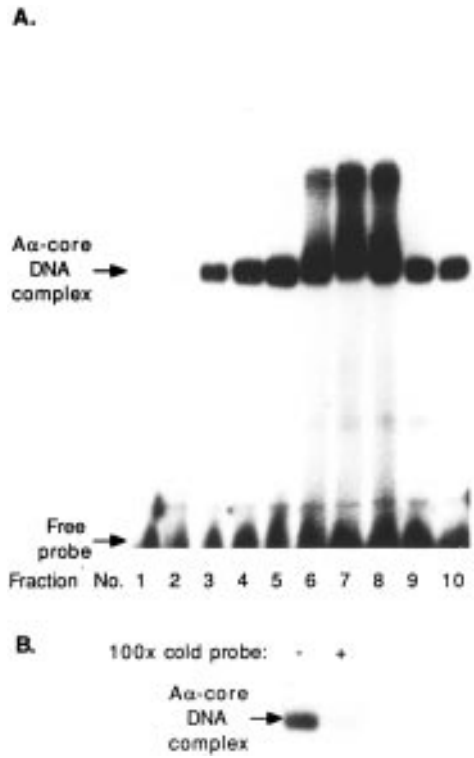

FIGURE 1: EMSA analysis of the fractions from the DNA affinity chromatography. A 30 bp fragment whose sequence was derived from the Aα fibrinogen gene promoter and including IL-6 RE was labeled with [α-^{32}P]dTTP. The fractions eluted from the DNA affinity resin were tested with EMSA *in the absence of* poly(dI/dC). The products of the reactions were examined in a 7% nondenaturing polyacrylamide gel. Aα probe:

5′-GAGCAAGAATTTCTGGGATGCCGTGGTT-3′

3′-CGTTCTTAAAGACCCTACGGCACCAATA-5′

The specificity of the Aα-core DNA complex was confirmed by including a 100-fold molar excess of nonlabeled Aα probe in a standard EMSA with fraction 9 (B).

dominant complexes appear after DNA affinity chromatography, indicating an enrichment and purification of the Aα-core protein in fractions 3−5 and 9−10. The specificity of the interaction between the protein and the oligonucleotide probe was confirmed by competition assays (Figure 1B**)**. A second complex, that migrated more slowly in EMSA, was also observed in fractions 6−8. We presumed that this slower migrating band was due to protein aggregation or additional copies of the Aα-core protein directly associating with the EMSA probe. To confirm this, we performed EMSAs using diluted and concentrated Aα-core protein (Figure 2). When concentrated protein was used, the intensity of the slower migrating band increased and the strength of the Aα-core protein−DNA complex decreased,

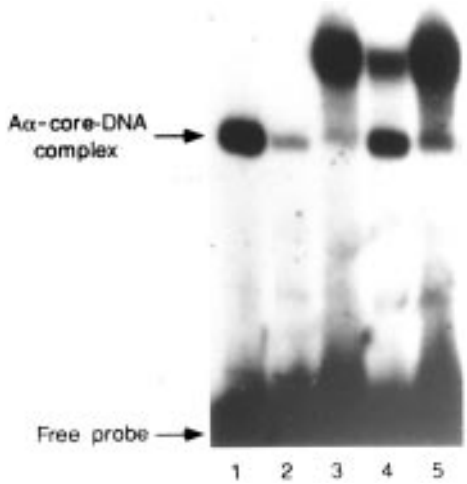

FIGURE 2: Characterization of the Aα-core protein/DNA complexes. Aα probe and purified Aα-core protein were used in EMSA. Lane 1, the pool of fractions 3−5 and 9−10 after the DNA affinity column; lanes 3 and 5, the concentrated pool (20-fold); lanes 2 and 4, 1:10 dilution of nonconcentrated and concentrated pools, respectively. In lane 5, twice the amount of radiolabeled probe was used. The samples were examined in a 7% nondenaturing polyacrylamide gel.

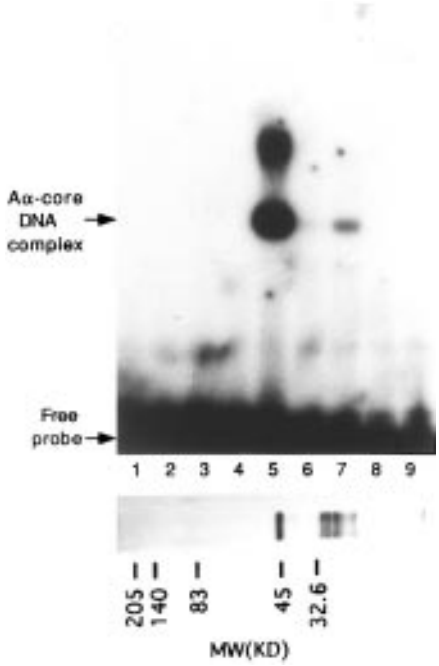

FIGURE 3: Identification of the Aα-core protein. The pools of protein fractions obtained following DNA affinity chromatography were combined with an equal volume of nonreducing sample buffer and immediately resolved in a 10% SDS−polyacrylamide gel without prior boiling. The protein bands were visualized by silver staining. Following protein fractionation, 5 mm slices of the gel were cut from top to bottom (corresponding to lanes 1−9). The proteins were eluted from the gel, subjected to denaturation/renaturation treatment, and then tested by EMSA using the Aα probe.

even when a 2-fold increase of ^{32}P-labeled Aα probe was used (lane 5). Accordingly, when the purified protein was diluted, its electrophoretic mobility increased. The two complexes that associate the Aα-probe are therefore not derived from different proteins.

To analyze the composition of this enriched (purified) fraction, proteins without prior boiling were fractionated by SDS−PAGE performed under nonreducing conditions (Figure 3). To determine which band corresponded to the Aα-core protein, 5 mm slices were cut from the gel (top to bottom), and the proteins were eluted and subjected to a denaturation/renaturation process (Latchman, 1994). The activity of the renatured fractions was then analyzed by EMSA. Figure 3 shows that the 46 kDa protein corresponded to the Aα-core protein. A trace amount of Aα-core protein was visible in fraction 7 (lane 7), corresponding to a protein of lower molecular mass. This suggests that the Aα-core protein may exist as a multiple subunit protein.

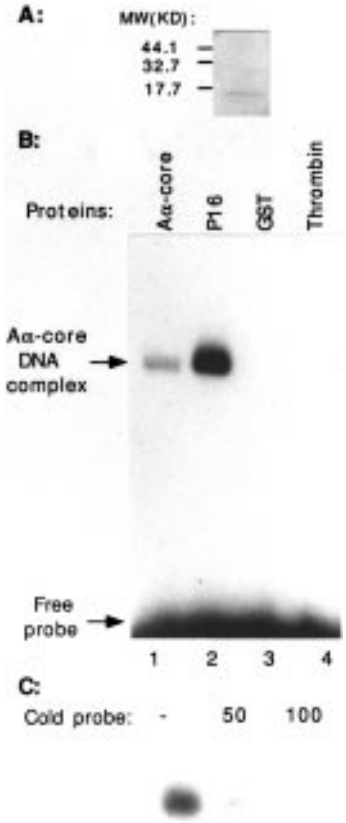

FIGURE 4: (A) Molecular mass of the Aα-core protein. The 46 kDa protein band corresponding to the Aα-core protein was eluted from the SDS−polyacrylamide gel as shown in (Figure 3) and then fractionated in a 15% SDS−polyacrylamide gel under reducing conditions. (B) Interaction of P16 with the IL-6 RE of the Aα fibrinogen gene. A 30 bp DNA fragment corresponding to IL-6 RE of the Aα fibrinogen gene was used as a probe (Aα probe). Nuclear extracts were prepared from nonstimulated primary hepatocytes (lane 1). 10 μg of nuclear extract, 20 ng of recombinant P16, 4 μg of GST, and 4 μg of thrombin were used in the EMSA as indicated. The products of the reactions were analyzed in a 7% nondenatured polyacrylamide gel. (C) The specificity of the P16−DNA complex was confirmed by competition assay as described for Figure 1B.

Amino Acid Analysis of the Aα*-Core Protein*. To obtain highly purified protein for amino acid sequence analysis, the enriched Aα-core protein was separated by electrophoresis, transferred to a PVDF membrane, and stained with Coomassie Blue. The band corresponding to the 46 kDa protein was cut from the membrane and its amino acid sequence determined. Five amino acid residues were obtained from the N-terminal sequence of three independent Aα-core protein preparations. The sequence obtained was E(V/S)-EVA. Using this sequence to search the protein database (GenBank), we found that a single-stranded DNA binding protein called P16 possesses the same N-terminal sequence (ESEVA) (Pavco & Van Tuyle, 1985; Hoke et al., 1990;

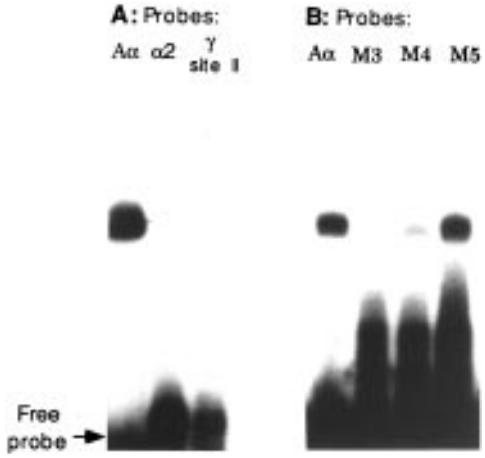

FIGURE 5: EMSA analysis of the specificity of interaction between P16 and IL-6 RE of the Aα fibrinogen gene. 20 ng of recombinant P16 was used in all assays. The sequences of the oligonucleotide probes used are listed below. The probes were labeled with the appropriate [α-^{32}P]dNTP. The Aα probe was as shown in the legend to Figure 1. α2 probe: Corresponding to the IL-6 responsive sequence of the α_2-macroglobulin gene

5′-GATCCTTCTGGGAATTCC-3′

3′-AGGAAGACCCTTAAGGAT-5′

γ-site II probe: derived from the site II IL-6 responsive element of the γ-fibrinogen gene

5′-gggCAAATCTGGGAATCCCTC-3′

3′-GTTTAGACCCTTAGGGAGggg-5′

M3 probe: mutated in the CTGGGA site of the Aα probe

5′-GAGCAAGAATTTAGTTTCTGCCGTGGTT-3′

3′-CGTTCTTAAATCAAAGACGGCACCAATA-5′

M4 probe: mutated in the hexanucleotides upstream of CTGGGA

5′-GAGCAATTCACTCTGGGATGCCGTGGTT-3′

3′-CGTTAAGTGAGACCCTACGGCACCAATA-5′

M5 probe: mutated in the hexanucleotides downstream of CTGGGA

5′-GAGCAAGAATTTCTGGGACGTACGGGTT-3′

3′-CGTTCTTAAAGACCCTGCATGCCCAATA-5′

All the mutation sites are underlined.

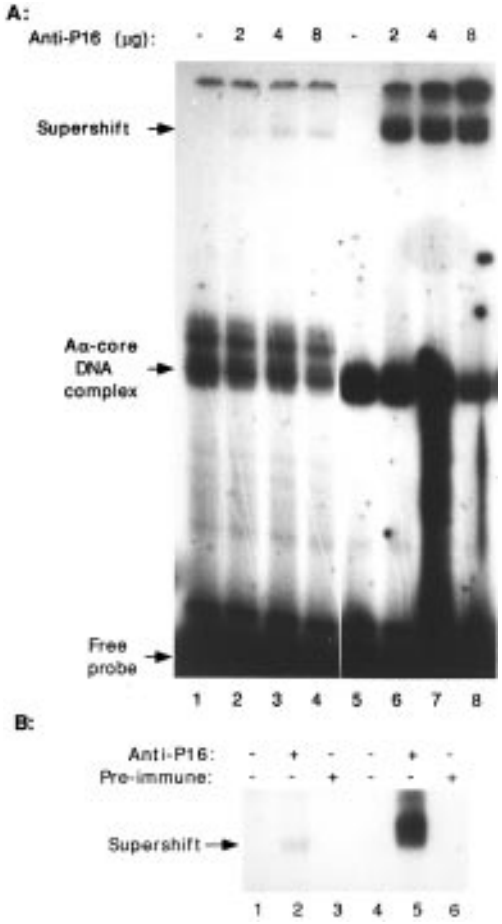

FIGURE 6: Supershift of the Aα-core protein with an anti-P16 specific antibody. (A) Ten microgram of nuclear extract from nonstimulated primary hepatocytes (lanes 1−4) and 20 ng recombinant P16 (lanes 5−8) were incubated with the indicated amount of anti-P16 antibody at 4 °C for 1 h and subjected to standard EMSA using the Aα probe. Products of EMSA were fractionated in a 7% nondenatured polyacrylamide gel. (B) Analysis of the specificity of the interaction between the Aα-core protein and anti-P16 antibodies. Three micrograms of antibodies from preimmune serum as a control and 3 μg of anti-P16 antibodies were used respectively in the supershift assays.

Tiranti et al., 1993). The molecular mass of P16 is 15.2 kDa (Hoke et al., 1990; Tiranti et al., 1993), and the native protein forms a tetramer (Hoke et al., 1990; Curth et al., 1994). The protein exists predominantly in the mitochondria; however, trace amounts have been detected in the cytoplasm and nuclei (Pavco & Van Tuyle, 1985).

To determine the relationship between P16 and the Aα-core protein, the molecular mass of the Aα-core protein was established. The 46 kDa Aα-core protein was eluted from a polyacrylamide gel, reduced with β-mercaptoethanol, and fractionated by SDS−PAGE. The results show a single protein band whose electrophoretic mobility corresponds to ∼16 kDa (Figure 4A). This indicates that the 46 kDa protein band, observed under nonreducing conditions, contains presumably three identical 16 kDa subunits. The Aα-core protein therefore appears homologous to the multisubunit mitochondrial protein P16.

Functional Comparison of the Aα-Core Protein with P16. To determine whether P16 and the Aα-core protein exhibit similar biological properties, recombinant P16 was generated, using a GST-fusion protein system (see Materials and Methods). By utilizing the recombinant P16 (Figure 4B, lane 2) and nuclear proteins from nonstimulated primary hepatocytes (Figure 4B, lane 1) in an EMSA, we found that P16 formed a complex with the IL-6 RE probe of the Aα fibrinogen gene. The molecular mass of this complex was similar to that formed by the Aα-core protein. We then determined whether the IL-6 RE found within the promoter region of α_2-macroglobulin and γ-fibrinogen could interact with P16, since it was previously shown that the Aα-core protein was unable to bind these IL-6 RE promoter sites (Liu & Fuller, 1995). In agreement with these findings, P16 was found not to interact with either probe (Figure 5A). Furthermore, it was known that the Aα-core protein binds constitutively to the expanded IL-6 RE (a typical IL-6 RE, CTGGGA, plus the upstream hexanucleotides, GAATTT) (Liu & Fuller, 1995). The purified recombinant P16 was therefore combined in an EMSA with the following mutant probes: M3 (mutated in all the CTGGGA), M4 (mutated in 5′ flanking hexanucleotides of CTGGGA), and M5 (mutated in hexanucleotides downstream of CTGGGA) (Figure 5B).

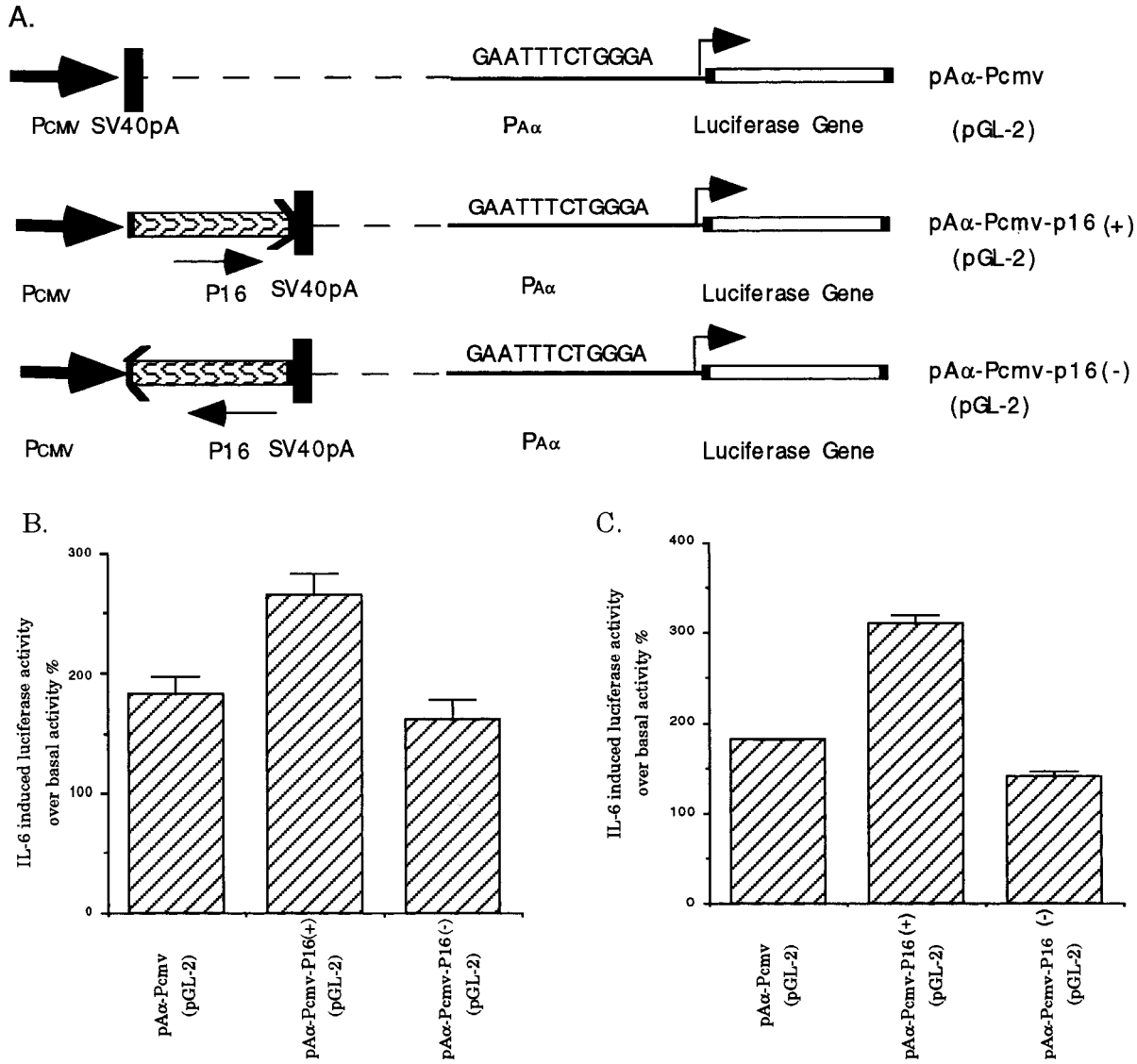

FIGURE 7: Regulation of IL-6-induced expression of Aα fibrinogen by P16. (A) Plasmid constructs used to measure the effect of P16 overexpression on the activity of Aα fibrinogen promoter. The pAα-Pcmv construct represents the empty vector. In pAα-Pcmv-P16(+), P16 was expressed under the control of Pcmv. In the pAα-Pcmv-P16(−) construct, P16 was inserted in the reverse orientation and results in overexpression of antisense P16 mRNA. The effect of P16 and antisense P16 on the Aα fibrinogen promoter in response to IL-6 is shown in sections B and C. Both Hep G2 (B) and Hep 3B (C) cells were transfected with the constructs and then treated with 300 ng/mL recombinant human IL-6 and 1 μM dexmethasone for 7 h. The luciferase activity of the cell lysate was measured.

The results show that mutations within M4 as well as the CTGGGA sequence (M3) inhibited P16 binding. Thus, the purified P16 displays identical DNA binding characteristics to those of the Aα-core protein. To confirm this relationship, a polyclonal anti-P16 antibody was raised against the recombinant protein. This antibody was added to the EMSA reaction mix in which either a nuclear extract from primary hepatocytes containing Aα-core protein or purified recombinant P16 was combined with the Aα probe (Figure 6). Anti-P16 antibody interacted with Aα-core protein in nuclear extracts as well as P16 to form a supershifted band. To confirm the specificity of the supershift complex, antibodies from preimmune serum were used as an appropriate control in the supershift assays. The result in Figure 6B shows that only anti-P16 antibodies can supershift the Aα-core protein−DNA complex as well as the P16 DNA complex. These data strongly indicate that the Aα-core protein is homologous to P16.

Function of the Overexpressed P16 Gene in the Regulation of IL-6-Induced Transcription of Aα Fibrinogen. To verify the function of this DNA binding protein, hepatoma cells (Hep G2 and Hep 3B) were transfected with either a sense or an antisense P16 cDNA (full length, residues 1−151). Plasmid constructs bearing a reporter gene for the detection of Aα promoter activity are shown in Figure 7A. The transfected cells were then stimulated with IL-6. The results (Figure 7B,C) show that overexpression of P16 enhances the IL-6-induced response. To determine whether this up-regulation was specific for Aα fibrinogen, further constructs were generated to determine the effect of P16 on the α_2-macroglobulin promoter. The results indicate that overexpression of the P16 gene could not enhance IL-6 activation of the α_2-macroglobulin promoter (data not shown). Taken together, these data show that the Aα-core protein is closely related to the mitochondrial P16 protein and is involved in the IL-6-regulated transcription of the Aα fibrinogen gene.

DISCUSSION

Previous examination of the Aα fibrinogen promoter showed that a novel DNA binding protein, termed the Aα-core protein, bound constitutively to a putative IL-6 RE site that was predicted to associate with Stat 3. As an initial step to explore how the Aα-core protein regulates the IL-6-induced expression of Aα fibrinogen, we have now purified and functionally characterized this regulatory protein.

Purification of the Aα-core protein yielded a protein with a molecular mass of ~16 kDa and whose NH_2-terminal amino acid composition was identical to that of the mitochondrial P16 protein. This mitochondrial DNA binding protein usually forms multimers (Curth et al., 1994), and both P16 and the purified Aα-core protein share similar DNA binding characteristics. Furthermore, studies showed that an anti-P16 antibody specifically recognized the Aα-core protein, while overexpression of P16 in hepatoma cell lines enhanced the IL-6-induced expression of Aα fibrinogen. These data suggest that P16 and the Aα-core protein are structurally and functionally related. It is therefore possible that they are encoded by the same gene, but are derived either through alternate mRNA splicing, through posttranslational modification, or from related ancestral genes.

P16 was originally detected in the mitochondria; however, trace amounts have been localized within the nucleus (Pavco & Van Tuyle, 1985). Using Western blot assay, we also confirm the presence of trace amounts of P16 in the nucleus (Z.L. and G.M.F, unpublished observation). Interestingly, an increasing number of proteins appear to play critical roles not only in the mitochondria but also in the nucleus. For example, the mitochondria element binding proteins, that are responsible for the control of mitochondrial gene expression and communication between the mitochondrian and the nucleus, have been identified in human, bovine, and rat cells (Suzuki et al., 1995). Futhermore, Trm 1p, a tRNA-processing enzyme, is utilized by both the mitochondria and the nucleus (Rose et al., 1995). It has also been reported that mitochondrial-derived reactive oxygen intermediates function as signal transducers of TNF-induced nuclear gene expression (Schulze-Osthoff et al., 1993; Duval et al., 1996). A recent publication shows that a 43 kDa protein, which is structurally related to the nuclear receptor of the thyroid hormone (c-Erbal), is located in the mitochondrial matrix and can regulate gene transcription (Wrutniak et al., 1995). The fact that the Aα-core protein is related to the mitochondrial P16 gene raises the intriguing question of whether the mitochondria may be somehow signaled during a cytokine-stimulated acute phase response. Accordingly, it has been proposed that specific mechanisms exist within eukaryotes, that regulate communication between the nucleus and mitochondria (Nagley, 1991).

A growing number of proteins have been shown to bind single-stranded DNA and exert regulatory events on downstream genes (Tada & Khalili, 1992; Jansen-Durr et al., 1992; Altiok & Groner, 1993; Smidt et al., 1995; Miller et al., 1996, 1997; Glucksmann-Kuiset et al., 1996; Ge & Roeder, 1994; Choi et al., 1995; Cho et al., 1995). For example, two single-stranded DNA binding proteins have been implicated in repression of the β-casein promoter (Altiok & Groner, 1993). Furthermore, experiments demonstrate that the actions of the apoVLDL II gene are mediated through a variety of DNA binding proteins among which is a protein that binds single-stranded DNA (Smidt et al., 1995). Taken together, these findings suggest that single-stranded DNA binding proteins serve various regulatory functions in nuclear gene expression. The molecular regulation of these single-stranded DNA binding proteins remains unclear. They may play a role in providing the appropriate DNA structure for RNA polymerase binding (Glucksmann-Kuiset al., 1996) and/or directly interacting with the general transcriptional machinery (Miller et al., 1997). P16 is a single-stranded DNA binding protein involved in the regulation of mitochondrial DNA replication. Here, we show that the Aα-core protein that is closely related to P16 binds to a specific double-stranded DNA sequence to up-regulate the IL-6-induced transcription of Aα fibrinogen.

This study shows that the IL-6 RE binding protein in the Aα fibrinogen gene, Aα-core protein, is closely related to a mitochondrial single-stranded DNA binding protein, P16. The observation that overexpression of P16 enhances the IL-6-induced expression of Aα fibrinogen clearly suggests that the Aα-core protein (P16) is regulated by some IL-6 signal transducer(s). The most likely IL-6 signal transducers are MAP kinase and the Stat family members. Whether there is interaction between the Aα-core protein and these signal transducers remains to be determined.

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