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A major cellular substrate for protein kinases, annexin II, is a DNA-binding protein

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Abstract

We have screened a human cDNA expression library in λgt11 for clones encoding Alu-binding proteins using direct binding of labeled Alu DNA to recombinant phage lysates fixed on a membrane, and isolated a clone 98% identical in sequence to the well-known substrate of protein kinases, annexin II, which was suggested earlier to play a role in transduction of mitogenic signals and DNA replication. A diagnostic property of annexins is their binding to phospholipids in the presence of calcium ions, and we have found that the interaction of proteins of human nuclear extracts with Alu subsequences is suppressed by Ca/phosphatidylserine liposomes, suggesting overlapping of Ca/phospholipid- and DNA-binding domains in annexin II.

Key words: Alu-binding protein; Annexin II; Mitogenic signal transduction; Phospholipid; Calcium ion

1. Introduction

Human nuclear extracts contain DNA-binding proteins interacting with ubiquitous dispersed repeats of the Alu family [1,2], which may be involved in regulation of transcription and cell proliferation [3–8]. One of the proteins preferentially binds to the conserved GC-rich Alu subsequence [2,5,9] located just upstream of the B box of the RNA polymerase III promoter, and several proteins bind to the Alu subregion located downstream from the B box [6,10]. In this study we have screened a cDNA expression library with a labeled Alu fragment and have isolated a clone which was found to be identical in sequence to the cDNA of the well-known cellular substrate for protein kinases, annexin II (also known as p36, lipocortin II, calpactin I heavy chain [11,12], which has been suggested to play a role in DNA replication [13–17] but was never recognized earlier as a DNA-binding protein.

2. Materials and methods

A human lymphocyte cDNA expression library in phage λgt11 was obtained from Clontech Co. (USA) and manipulated as described in [30]. Wet nitrocellulose replicas were blocked by shaking for 60 min at room temperature (RT) in 20 mM Tris-HCl, pH 8, 1 mM dithiothreitol, 1 mM EDTA (TDE buffer) with 5% low-fat dry milk, then for 30 min at RT in TDE buffer containing 10 ng/ml of ^{32}P-labeled BLUR8 Alu fragment (10^8 cpm/μg of DNA), 10 μg/ml of poly(dI)–(dC) plus 0.25% milk, and finally washed 3 times (30 min total) in TDE buffer plus 0.25% milk and exposed to X-ray film. The Alu fragment was excised from plasmid BLUR8 using *Bam*HI, purified by electrophoresis in a 4% polyacrylamide gel and end-labeled with [α-^{32}P]dATP using Klenow DNA polymerase.

Isolation of human nuclear extracts, and electrophoretic mobility shift assay (EMSA) with fully complementary oligonucleotides Alu B5 and Alu B6 [9] were done as already described [2,4]. EMSA with partially complementary Alu oligonucleotides 72 and 73 [6] was done as in [19] but without the glutaraldehyde fixation step. T7 polymerase dideoxy sequencing was performed using standard protocols [30]. Phosphatidylserine liposomes prepared by sonication [22] in 0.15 M NaCl, 1 mM dithiothreitol (10 mg/ml of L-α-phosphatidyl-L-serine from bovine brain; Sigma), were pre-incubated before EMSA for 30 min at RT with an equal volume of nuclear extract (10 mg of protein/ml) in the presence of 10 mM $CaCl_2$ or 10 mM EGTA. Southwestern blotting analysis of human nuclear proteins was done as described in [31] using the same ^{32}P-labeled Alu fragment which was used for Southwestern screening of the expression library.

3. Results

In three independent rounds of screening we have isolated two identical λ clones (r1 and w2) with 1.2 kb cDNA inserts containing an internal *Eco*RI–*Hin*dIII subfragment (about 0.5 kb) which was subcloned into pTZ18 vector and partially sequenced. A 220 nucleotide sequence read from the *Eco*RI site was found to be 98% identical to a subsequence of the HUMLIC file of GENBANK representing the cDNA of human lipocortin II [11,12]. We concluded, therefore, that the r1 clone contains an insert of annexin II cDNA and that recombinant annexin II expressed in *E. coli* has an Alu-binding activity. The molecular mass of annexin II predicted from the complete cDNA sequence is 38.4 kDA [11,12] and Southwestern blotting of human nuclear proteins probed for Alu-binding shows (Fig. 1A) a p39 band (B2), possibly representing annexin II, along with a p125 band (B1).

To detect DNA binding activity associated with annexin II in human nuclear extracts we have used EMSA with the Alu oligonucleotides B5/B6 forming a complete

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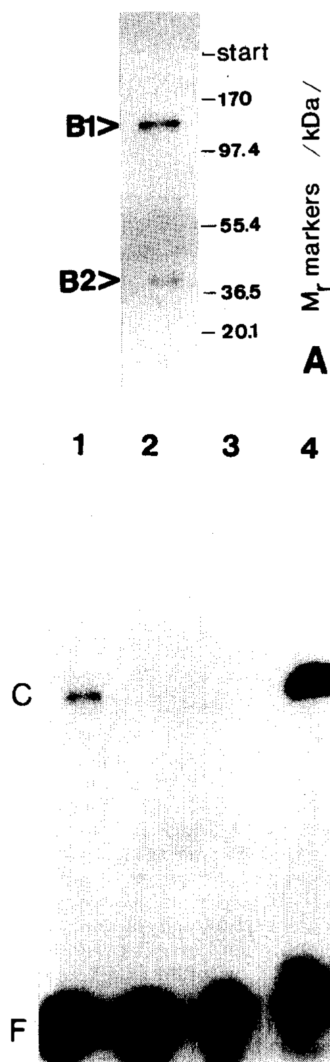

Fig. 1. (A) Southwestern blot of human nuclear extract (MOLT4 cells) analysed for binding of the ^{32}P-labeled Alu fragment. After separation in a 10% SDS-polyacrylamide gel, proteins (100 μg) were transferred to a nitrocellulose filter which was incubated with labeled Alu as described in section 2, and autographed. (B) Binding of primer-template junction oligonucleotide (PT substrate) 72/73 by human nuclear extract. EMSA was done as in [19] except that the glutaraldehyde fixation step was omitted. Lanes 1,2, without ATP; lanes 3,4, plus 1 mM ATP; in the absence of a competitor (lanes 1,4) or in the presence of 50 ng of calf thymus DNA (lanes 2,3).

duplex [9], as well as with partially complementary oligonucleotides 72/73 having two primer–template junctions in one partially duplex molecule (primer–template/PT/substrate). PT substrates were used since nuclear annexin II was reported to be a subunit of a primer-recognition protein [13,14], and some mammalian primer recognition complexes, e.g. replication factor C (RF-C), are known to be able to bind PT substrates [18–21]. With 72/73 substrate, a single complex was detected in EMSA (Fig. 1B, lane 4) but its stability was found to be much lower than that with the duplex substrate: the complex formation with the PT substrate 72/73 under conditions optimal for RF-C, including the presence of 1 mM ATP [19], was completely suppressed (Fig. 1B, lane 3) by the addition of 50 ng of non-specific competitor (duplex calf thymus DNA); complex formation with the duplex substrate Alu B5/B6 was only slightly inhibited by 800 ng of the competitor [9].

A diagnostic property of annexins is their binding to

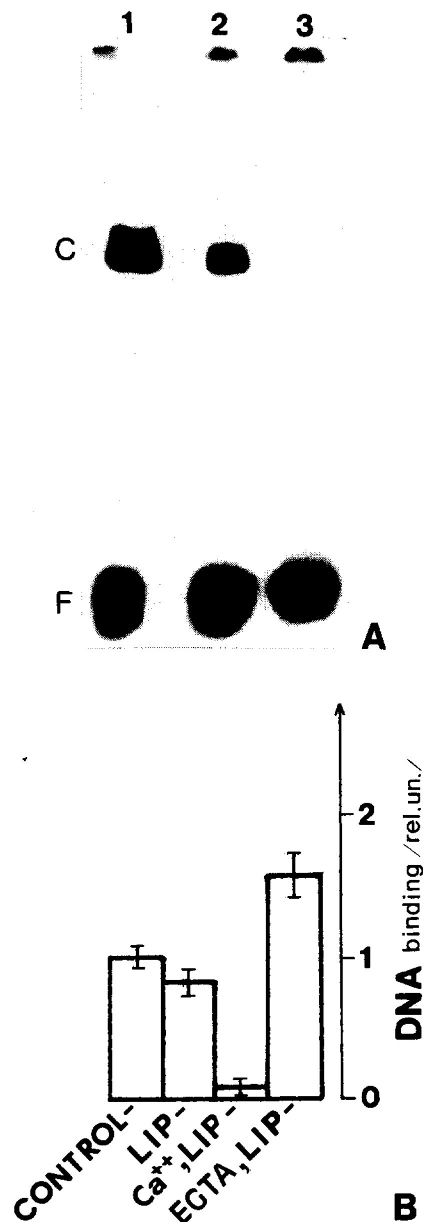

Fig. 2. Suppression of binding by human nuclear extract (from K562 cells) of the PT substrate 72/73 (A) and of the duplex substrate Alu B5/B6 (B) by calcium in the presence of phosphatidylserine liposomes. (A) EMSA patterns obtained in the control (lane 1, no Ca/phospholipid), after pre-incubation with liposomes in the absence (lane 2) or in the presence (lane 3) of 10 mM $CaCl_2$. C, complex of the PT substrate with proteins; F, free substrate. (B) Results of three EMSA experiments [9] with duplex substrate Alu B5/B6 (mean ± S.E.M.) are shown. DNA binding was quantitated by densitometry of radioautographs. LIP, liposomes. EGTA and Ca^{2+} ($CaCl_2$) were at 10 mM concentration.

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46    ^                                         ^        104
 IKTTGVDEVTIVNILTNRSNAQKQDIAFAYQRRTKKELASALKSALSGHLETVILGLL
                               +++++   ----------------
                                NLS   helix-turn-helix
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Fig. 3. Human annexin II N-terminal Ca/phospholipid-binding domain.^, shows residues suggested to be involved in the binding of Ca/phospholipids [25–27]; NLS, signature of nuclear localization signal [29].

phospholipids in the presence of calcium ions [22]. We have found that phosphatidylserine liposomes strongly inhibit the formation of a complex between the 72/72 PR substrate and nuclear proteins (Fig. 2A, lane 3). Liposomes alone also caused some inhibition (Fig. 2A, lane 2) but calcium and the Ca chelator, EGTA, without liposomes were inactive (not shown). It was also found that formation of a complex between the 72/73 PT substrate and nuclear proteins is strongly stimulated by ATP (Fig. 1B, lanes 1 and 4), as was observed earlier for RF-C [19], suggesting ATP involvement in the binding.

Ca/PL-mediated inhibition of DNA binding by nuclear extracts was also observed in experiments with the B5/B6 duplex DNA substrate (Fig. 2B) in which more stringent conditions of binding were used (200 ng of competitor calf thymus DNA was included). Again, liposomes alone were slightly inhibitory but liposomes in the presence of EGTA stimulated formation of the complex, suggesting that endogeneous bound calcium in the nuclear extract may cooperate with liposomes to inhibit formation of the EMSA complex. Nuclear phospholipids are probably inhibitory for DNA replication since their content was found to be decreased during S phase [23], and Ca ions (1 mM) were also reported to inhibit DNA replication in permeabilized human cells [24].

4. Discussion

Analysis of the 'annexin type' calcium-binding sites showed that they are formed by an inter-helical loop characterized by a conserved glycine residue and by an acidic amino acid (D or E) located 40 residues C-terminal to the glycine [25–27]. In annexin II these sites are present in domains 2, 3 and 4 but not in domain 1, where alanine is located 40 residues downstream of the conserved glycine [27]. It has been suggested that a second coordination site for the calcium ion in such 'defective' repeats can be provided by the negatively charged head group of phospholipids [26,27]. Since phospholipids inhibit annexin II's interaction with DNA in cooperation with calcium ions (Fig. 2) it seems likely that the region near the 'defective' coordination site in this protein is responsible for DNA binding in the absence of the lipids. Indeed, it contains (Fig. 3, underlined) a putative helix-turn-helix DNA-binding motif [28]. Interestingly, the region also contains (Fig. 3) a sequence (RRTKK) conserved in annexins II from different species which resembles casein kinase II-dependent nuclear localization signals [29]. ATP-stimulated binding of the annexin II-containing complex to PT substrate (Fig. 2B) is consistent with the suggested involvement of this protein in DNA replication [13–17], but the observed preference of the Ca/PL-sensitive DNA-binding activity to duplex DNA indicates that the protein may play a role in some other aspects of DNA metabolism, e.g. in regulation of transcription via binding to Alu [3–8] or to other regulatory sequences.

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