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Carcinogenic heavy metals replace Ca^{2+} for DNA binding and annealing activities of mono-ubiquitinated annexin A1 homodimer

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ABSTRACT

Mono-ubiquitinated annexin A1 was purified from rat liver nuclei. The homodimer form of mono-ubiquitinated annexin A1 was able to unwind dsDNA in a Mg^{2+}- and ATP-dependent manner, and to anneal ssDNA in a Ca^{2+}-dependent manner. Phospholipids decreased the concentration of Ca^{2+} required for maximal annealing activity. Heavy metals such as As^{3+}, Cr^{6+}, Pb^{2+} and Cd^{2+} substituted for Ca^{2+} in the ssDNA binding and annealing activities of annexin A1. While these metals inhibited the unwinding of dsDNA by nuclear annexin A1 in the presence of Mg^{2+} and ATP, they enhanced dsDNA-dependent ATPase activity of annexin A1. Heavy metals may have produced dsDNA, a substrate for the DNA unwinding reaction, via the DNA annealing reaction. DNA synthesomes were isolated from L5178Y *tk*(+/−) mouse lymphoma cells in exponential growth, and were found to contain helicase activities. The As^{3+}- or Cr^{6+}-induced increases in ssDNA binding activity of DNA synthesomes were reduced by a mono-specific anti-annexin A1 antibody, but not by anti-Ig antibody. Anti-annexin A1 antibody also blocked the inhibitory and stimulatory effects of As^{3+} or Cr^{6+} towards DNA unwinding and annealing activities of DNA synthesomes. Based on these observations, it can be concluded that the effects of heavy metals on DNA annealing and unwinding activities are mediated, at least in substantial part, through actions of the mono-ubiquitinated annexin A1 homodimer.

Introduction

Annexin A1, a 37-kDa protein previously termed lipocortin I or lipomodulin, is a member of the protein family that binds to phospholipids, e.g. biomembranes, in a Ca^{2+}-dependent manner (Gerke and Moss, 2002; Hirata, 1998). Its N-terminus contains a SH2-like sequence, making it a major substrate of oncogenic tyrosine kinases associated with or intrinsic to growth factors receptors (Gerke and Moss, 2002; Hirata, 1998). Since we observed that the tyrosine phosphorylation of lipomodulin (annexin A1) in murine thymocytes stimulated by Con A is closely associated with lymphocyte proliferation (Hirata et al., 1984), we proposed that the tyrosine phosphorylation of lipomodulin was involved in signal transduction for cell proliferation. This proposal was substantiated by later findings that treatment with anti-sense oligonucleotides directed against lipocortin I reduced the synthesis and subsequent phosphorylation of lipocortin I, resulting in inhibition of proliferation of A489 cells stimulated with HGF (Skouteris and Schröder, 1996). However, mechanisms of signal transduction *via* tyrosine phosphorylation of annexin A1 in cell proliferation remain poorly understood.

Annexins including annexin A1 are present in cytosol, membranes, and nuclei. Their translocation from the cytosol to the nuclei apparently requires tyrosine phosphorylation and Ca^{2+} signals, and is associated with the S phase of the cell cycle (Mohiti et al., 1997; Patte and Blanquet, 1992). Nuclear translocation of annexin A1 is also stimulated by the carcinogenic metal, As^{3+}, and by oxidative stress (Rhee et al., 2000; Sacre and Moss, 2002). It is nuclear annexin A1 rather than cytosolic and/membrane annexin A1 that is now thought to be closely associated with cell transformation and/or hyperproliferative states of various cell types (Lin et al., 2008; Liu et al., 2003). Therefore, focusing investigation on the function of nuclear annexin A1 should yield a greater understanding of cell proliferation and/or cell transformation mechanisms related to this protein.

We have recently discovered that purified nuclear annexin A1 with a molecular weight of around 94 kDa is able to bind DNA and RNA in a Ca^{2+}- and Mg^{2+}-dependent manner (Hirata and Hirata, 1999). This form of nuclear annexin A1 is now understood to be a homodimer of mono-ubiquitinated annexin A1 (Hirata et al., in press). Our studies have also demonstrated that nuclear annexin A1 unwinds dsDNA in a Mg^{2+}- and ATP-dependent manner. Annealing of ssDNA by annexin A1 requires Ca^{2+} and phospholipids (Hirata and Hirata, 2002). Helicases activity is essential for the initiation of DNA transcription and replication, and annexin A1 helicase activity may play an important role in cell proliferation and/or cell transformation.

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Heavy metals are reported to be tumor promotors (Rhee et al., 2000; Wu et al., 1999). They are thought to promote the mutagenic effects of DNA damaging agents, while alone may not themselves be mutagenic (Goyer, 1996: Maier et al., 2002). The non EF-hand Ca^{2+}-binding sites of annexin A1 appear to bind metals other than Ca^{2+} as measured by phospholipid aggregation (Mel'gunov et al., 2000). To investigate the function of annexin A1 helicase in cell proliferation and/or transformation, we evaluated the effects of carcinogenic heavy metals As^{3+} and Cr^{6+} on DNA binding, DNA annealing and DNA unwinding activities of nuclear annexin A1 helicase. We report here that As^{3+} and Cr^{6+} stimulate the ssDNA binding and DNA annealing activities of annexin A1 helicase by substituting for Ca^{2+}, while they act as functional antagonists against Mg^{2+} in DNA unwinding by annexin A1 helicase.

Methods and experimental procedures

Materials. Anti-peptide polyclonal antibodies against annexin A1 (lipocortin I/calpactin II) and annexin A2 (lipocortin II/calpactin I) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Ig antibody was the product of Miles (Tarrytown, NY). Anti-ubiquitin antibody was purchased from Invitrogen (Carlsbad, CA).

Purification of nuclear annexin A1. Nuclear annexin A1was purified from rat liver according to the modified method described previously (Hirata and Hirata, 1999). Briefly, the membrane fraction of rat liver homogenate in the presence of 5 mM $CaCl_2$ was extracted with 50 mM Tris–HCl buffer, pH 7.4 containing 10 mM EDTA, 5 mM β-mercaptoethanol and 50 times diluted protease inhibitor cocktails (Sigma-Aldrich Chemicals). Extracts were applied to a DEAE-sepharose column, which was washed with 50 mM Tris–HCl buffer, pH 7.4, containing 50 mM NaCl and 5 mM β-mercaptoethanol, and eluated with 50 mM Tris–HCl buffer, pH 7.4, containing 300 mM NaCl and 5 mM β-mercaptoethanol. Eluent was concentrated and combined with 1 M $(NH_4)_2SO_4$. Extracts were subjected to FLPC Hi Prep 16/10 Octyl FF column chromatography with a gradient from 50 mM Tris–HCl, pH 7.6 containing 1 M $(NH_4)_2SO_4$ to 50 mM Tris–HCl, pH 7.6. Fractions exhibiting ssDNA binding activity were combined, dialyzed against 50 mM Tris–HCl, pH 7.6, and subjected to FLPC MonoQ 4.6/100 PE column chromatography with a gradient of 0 to 1 M NaCl in 50 mM Tris–HCl, pH 7.6. Fractions demonstrating ssDNA binding activity were concentrated, and applied to a FLPC Superrose 6 10/300 column with 10 mM Tris–HCl, pH 7.4, containing 40 mM NaCl. Thus, the final preparation of purified nuclear annexin A1 was in 10 mM Tris–HCl, pH 7.4, containing 40 mM NaCl. Poly$(dC)_{20}$ binding and phospholipases A_2 inhibitory activities were employed to measure annexin A1 activity as described previously (Hirata and Hirata, 1999). Purified nuclear annexin A1 exhibited a protein band with an apparent molecular weight of 92,000 ± 2000 Daltons on SDS-PAGE and 45,000 Daltons under reducing conditions. Protein was detected by staining with Coomassie blue, and by Western blots analysis with anti-annexin A1. The 45,000 Dalton protein was stained by anti-ubiquitin antibody and anti-annexin A1 antibody (Hirata et al., in press).

Isolation of DNA synthesomes. DNA synthesomes were isolated from L5178Y *tk*(+/−) lymphoma cells according to the method of Lin et al. (1997). Briefly, nuclei were isolated by differential centrifugation of homogenates of lymphoma cell. Nuclear extracts were obtained by incubating nuclei in 50 mM Tris–HCl, pH 7.5, 150 mM KCl, 1 mM dithiothreitol (DTT), 5 mM EDTA and EGTA, and 1 mM PMSF for 2 h at 4 °C, and were centrifuged at 100,000*g* for 1 h at 4 °C. The resulting supernatants were layered onto a 1 ml sucrose cushion containing 50 mM Tris–HCl, pH 7.5, 1 mM DTT, 5 mM EDTA and 2 M sucrose, and were centrifuged at 100,000*g* for 17 h at 4 °C. The lower fraction in the sucrose cushion (approximately 20% of the total volume) was collected, dialyzed against 50 mM Tris–HCl, pH 7.5 and 1 mM DTT, 1 mM EDTA, 10% glycerol, 50 mM KCl, and was used as DNA synthesomes.

ATPase assays. ATPase activity of nuclear annexin A1 helicase was measured by hydrolysis of [γ - ^{32}P]ATP according to the method described previously (Hirata and Hirata, 2002).

DNA substrates for helicase activity. For the assays of annealing and unwinding of DNA, the 80mer oligonucleotide, $^{5'}$GTCCACTATTAAAGA-ACGTGGACTCCAACG-TCAAAGGGCGAAAAACCGTCTATCAGG-GCGATGGCCCACTACGTGA-ACCA$^{3'}$, were synthesized. This oligonucleotide (P0G) was complementary to the replication origin, 5'-^{5790}GTCCACTATTAAAGAACGTGGACTCCAACGTCAAAGGGCGAA-AAACCGTCTATCAGGGCGATGGCCCACTACGTGAACCA^{5711}-3' of M13mp18. Thus, this oligonucleotide (80 n.b.) was able to be annealed to M13mp18 to form a duplex.

Helicase assays. For the DNA annealing assay, the 5'- end of P0G was labeled with [γ - ^{32}P] ATP by using T4 polynucleotide kinase as described previously and annealing of [^{32}P]-labeled P0G to M13mp18 was measured (Hirata and Hirata, 2002).

For the unwinding assay, [^{32}P]-labeled P0G was annealed to equimolar M13mp18 DNA prior to assay, and dissociation of [^{32}P]-labeled P0G from M13mp18 was measured (Hirata and Hirata, 2002).

After incubation at 32 °C for 20 min, the reaction products were separated on agarose gel with TAE buffer (40 mM Tris–acetate buffer, pH 8.5, and 1 mM EDTA). Gels were dried on DEAE-cellulose. Radioactivity was detected with Fuji Film BAS-1800 II (Fujifilm Life Science; Stamford, CT). Unwinding and annealing of the radioactive oligonucleotide were quantitated by utilizing Fuji Film Science Laboratory, Image Gauge (Fujifilm Life Science, Stamford, CT).

Electrophoretic mobility shift assays. DNA binding activity of annexin A1 was measured using the electrophoretic mobility shift assays (EMSA) as described previously (Hirata and Hirata, 2002). Autoradiography and quantitative analysis were carried out as described above.

Results and data analyses. Results were reported as mean ± standard error of the mean (number of observations in parentheses). Difference between values were assessed using Analysis of Variance (SigmaStat) with $p<0.05$ considered to be statistically significant.

Results

Properties of purified nuclear annexin A1

When lipocortin/annexin A1 was purified from rat liver as measured by poly$(dC)_{20}$ binding as well as by phospholipases A_2 inhibition (see Methods and experimental procedures), it exhibited a single band of an apparent molecular weight of 92,000 ± 2,000 Daltons on SDS-PAGE as detected by staining with Coomasie blue (Fig. 1). This protein was stained with anti-annexin A1 antibody, but not with anti-annexin A2 antibody. Since annexin A1, a 37,000 Dalton protein, is known to complex with S100 proteins such as S100A10 with molecular weight of approximately 10,000 Daltons, we initially thought that annexin A1 formed a heterotetrameric complex of $(\text{annexin A1})_2$ $(\text{S100})_2$ (Hirata and Hirata, 2002). However, our recent experiments demonstrated that annexin A1 is modified by ubiquitin(s) as demonstrated by immunoblot analysis with anti-ubiquitin and anti-annexin A1 antibodies (Fig. 1). SDS-PAGE demonstrated that this protein has a molecular weight of 45,000 Daltons under reducing conditions. Accordingly, we have concluded that purified nuclear annexin A1 is a homodimer of mono-ubiquitinated annexin A1.