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Lipocortin (Annexin) I Heterotetramer Binds to Purine RNA and Pyrimidine DNA

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Lipocortin I-like protein with a molecular weight of 94,000 Da as judged by Western analysis was found to bind to ssDNA rather than to dsDNA in a Ca^{2+}-dependent manner. This protein was also bound to [^{32}P]poly(rA) and [^{32}P]poly(rG) as measured by EMSA. Poly(rG), poly(rA), poly(dC), and poly(dT) were competitive against binding of either [^{32}P]poly(rA) or [^{32}P]poly(rG), while poly(rC), poly(rU), and poly(dA) were less effective binding competitors. The binding of this protein to poly(rA) or poly(rG) was inhibited by immunoprecipitable anti-lipocortin I (calpactin II) and anti-S100 protein antibodies, but not by an anti-Ig antibody. Phospholipids such as phosphatidylserine and phosphatidylinositol enhanced the binding of lipocortin I to poly(rA). Taken together, our present observations suggest that the lipocortin I-S100 protein heterotetramer binds to either purine RNAs or pyrimidine ssDNAs in a Ca^{2+}- and phospholipid-dependent manner.

Lipocortin I is a member of the lipocortin (also termed annexin) family of proteins that bind to phospholipids in a Ca^{2+}-dependent manner (1). Our laboratory first reported that lipocortin I in murine thymocytes is phosphorylated at a tyrosine residue after stimulation with mitogens such as Con A (2). Since tyrosine phosphorylation parallels mitogenesis of murine lymphocytes as measured by [^{3}H]thymidine uptake, it was proposed that lipocortin I is involved in signals of mitogenesis (2). This proposal was recently substantiated by the findings that the treatment of A386 cells with an antisense oligonucleotide reduces the synthesis and subsequent phosphorylation of lipocortin I, thereby inhibiting cell proliferation after stimulation with HGF, a growth factor (3). Several members of the lipocortin family including I and II are shown to exist in the nuclei, especially within the nuclear matrix (4). Translocation of lipocortins from the cytosols to the nuclei apparently requires their phosphorylation at a tyrosine residue(s) (5). Although the exact role of lipocortins in nuclear function remains poorly understood, anti-lipocortin II antibody blocks cell free DNA replication in *Xenopus* oocytes, and purified lipocortin II stimulates the synthesis of DNA lagging strand in HeLa cell nuclear extracts (4, 6). Essentially similar results were obtained with lipocortin I in isolated rat hepatocyte nuclei (7). Therefore, lipocortins are proposed to function as a primer recognition protein that stimulates DNA polymerase α (6). While replication protein A (RP-A), a single strand DNA binding protein (SSB) that stimulates DNA polymerase α, is the essential component of the replication fork in the SV40 replication system, other SSBs such as glyceraldehyde dehydrogenase are also reported to stimulate DNA polymerase α by substituting RP-A (8). These findings suggest that lipocortins are capable to bind to ssDNA. Although lipocortins have been reported to bind to DNAs such as Z-DNA and Alu-DNA (9, 10), the nature of the interaction between lipocortins and DNA has not been established. To extend our efforts in signal transduction of growth factors for cell proliferation and ultimately DNA replication, we investigated whether lipocortin I can bind to ssDNA and/or RNA. In this communication, we report that the lipocortin I-S100 protein heterotetramer binds to purine clusters of RNAs and pyrimidine clusters of ssDNA in a Ca^{2+} and phospholipid dependent manner.

MATERIALS AND METHODS

Materials. Polynucleotides were purchased from Sigma Chemicals (St. Louis, MO). Poly(dC) of 5, 10, 15, 20, 25, 30, 50, and 75 nt were synthesized by Beckman Oligo1000 according to the manufacturer's instruction. Anti-calpactin II and I (lipocortins I and II, respectively), anti-S100 protein and anti-Igs antibodies were products of Oncogene (Rockville, MD), Sigma Chemicals and Miles (Tarrytown, NY), respectively. Anti-annexin I and II antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). ssDNA (bovine

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thymus)– and dsDNA (bovine thymus)–cellulose were products of Pharmacia (Piscataway, NJ).

Purification of lipocortin I heterotetramer. Lipocortin I heterotetramer was purified from rat (6 week old, male, 150 g body weight) livers according to a modification of the method previously described (11). Briefly, rat livers were homogenized in four-time volumes of 50 mM Tris–HCl, pH 7.4, containing 1 mM $CaCl_2$, and membrane fractions were obtained by centrifugation at 27,000*g* for 60 min at 4°C. Crude lipocortins were obtained by extraction of the membrane fractions with the same volume of 50 mM Tris–HCl, pH 7.4, containing 10 mM EDTA, and were applied on DEAE-Sepharose column for chromatography. After washing the column with 50 mM Tris–HCl, pH 7.4, containing 1 mM EDTA, lipocortin I was eluted with 50 mM Tris–HCl, pH 7.4, 1 mM EDTA, and 250 mM NaCl. Following concentration by an Amicon Bioseparator (Millipore) with a YM10 membrane, eluates from DEAE-Sepharose column were applied to Sephacryl 200-column equilibrated with 50 mM Tris–HCl, pH 7.4, 1 mM $CaCl_2$ and 1 mM β-mercaptoethanol, and fractions corresponding to molecular weights in the vicinity of 94,000 Da were collected. After concentration by an Amicon Bioseparator with a YM10 membrane, samples were chromatographed on Q-Sepharose column with a gradient ranging from 0 to 500 mM NaCl in 50 mM Tris–HCl, pH 7.4. Lipocortins were assayed by both *in vitro* inhibition of phospholipase A_2 (11) and binding to poly(rA).

Electrophoretic mobility shift assays (EMSA). Poly(rA) (3.5 ng) (Sigma Chemical) was dephosphorylated by alkaline phosphatase, and was then labeled with 8 pmol (50 μCi) [γ-^{32}P]ATP (NEN Life Science Products, Boston, MA), in 50 μl of a reaction mixture containing 50 μg/ml acetylated bovine serum albumin (BSA) (Promega, Madison, WI), 10 units of T4 polynucleotide kinase (New England Bio Lab., Beverly, MA), 70 mM Tris–HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT) at 37°C for 2 hr. Following extraction with phenol, [^{32}P]poly(A) was purified on Sephadex G50 spun column. Lipocortin I (25–250 ng) was incubated with 0.2 ng [^{32}P]-labeled poly(rA) (7.5×10^5 cpm) in 20 μl of a reaction buffer containing 20 mM Hepes, pH 7.4, 100 mM KCl, 1 mM DTT, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 20% glycerol at 30°C for 25 min. The reaction mixtures were applied on 4% polyacrylamide gel containing 2.5% (v/v) glycerol, and were electrophoresed with Tris-glycine/EDTA buffer (25 mM Tris-base, 190 mM glycine, 1 mM EDTA) at 4°C. Gels were dried, and autoradiography was performed with Kodak BioMax MR films (Eastman Kodak Co., Rochester, NY).

Determination of base length of poly(rA). Lipocortin I was incubated with poly(rA) as described above, and then poly(rA) unbound to lipocortin in the reaction mixture was digested at 37°C, 30 min by S1 nuclease (40 units), followed by digestion at a room temperature overnight with 1% SDS, 1 mM EDTA and 1 mg proteinase K. Poly(rA) remaining was labeled with [^{32}P]. After ethanol precipitation following phenol extraction, [^{32}P]poly(rA) was run on 8 M urea–24% polyacrylamide gels. Gels were fixed with methanol-acetic acid and autoradiography was performed as described above. Alternatively, lipocortin was incubated with [^{32}P] labeled poly(dC) of various lengths, and EMSAs were carried out.

RESULTS

Ca^{2+}- and Mg^{2+}-dependent binding of lipocortin I heterotetramer to ssDNA–cellulose. When purified lipocortin I was added to isolated rat hepatocyte nuclei or their extracts, it stimulated the synthesis of DNA as measured by incorporation of [α-^{35}S]CTP into a TCA precipitable fraction (7). Subsequent identification of the product(s) suggested that Okazaki's fragments are accumulated under these conditions (7). These observations prompted us to determine whether lipocortin I stimulates the synthesis of DNA as a single strand DNA binding protein (SSB) by replacing RP-A, thus stimulating DNA primase-polymerase α. Thymus dsDNA– and ssDNA–cellulose were employed to test whether lipocortin I binds to dsDNA or ssDNA. Purified lipocortin I (approximately 10 μg), in 1 ml of 50 mM Tris–HCl buffer, pH 7.4, and 100 mM KCl with and without 5 mM $MgCl_2$, and/or 0.1 mM $CaCl_2$, was applied to 1 ml dsDNA- and ssDNA- cellulose columns. Lipocortin I attached to the columns was eluted in 1-ml fractions with 50 mM Tris–HCl, pH 7.4, 100 mM KCl and 5 mM EDTA. Absorbance at 280 nm was monitored to detect lipocortin. Lipocortin I apparently bound to ssDNA–cellulose in a Ca^{2+}- and Mg^{2+}-dependent manner. To confirm it, the eluates were immunoprecipitated with anti-calpactin II (lipocortin I) antiserum, followed by adding protein A-agarose. After thoroughly washing the immunoprecipitates with a phosphate buffered saline solution (PBS), the immunoprecipitates were applied on SDS polyacrylamide gel electrophoresis (Fig. 1). Western analysis demonstrated that lipocortin attached to DNA–cellulose has an apparent molecular weight of 94,000 Da.

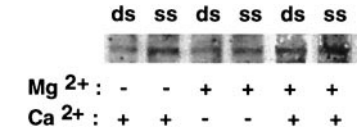

FIG. 1. Western analysis of EDTA eluates from ssDNA– and dsDNA–cellulose columns. Lipocortin I attached in the absence and presence of Ca^{2+} and/or Mg^{2+} was eluted from ssDNA– and dsDNA–cellulose columns with 5 mM EDTA, and was immunoprecipitated with anti-calpactin II (lipocortin I) antibody as described in the text. The immunoprecipitates were run on SDS–PAGE and Western analysis was performed.

Specific binding of poly(rA) to lipocortin I. When purified lipocortin I heterotetramer was incubated with various polyribonucleotides, it bound to poly(rA) and poly(rG) as detected by EMSA. Mobility shift of [^{32}P] labeled poly(rA) was not observed, when lipocortin I was omitted from the reaction mixture. To determine whether this mobility shift is attributable to the specific binding of lipocortin I to poly(rA), a monospecific antibody against lipocortin I was included in the reaction mixture (Fig. 2). As a control, anti-Ig antibody was added. Anti-calpactin II (lipocortin I) antiserum and anti-S100 protein antiserum, which immunoprecipitate lipocortin I and S100 protein, respectively, blocked the mobility shift. Anti-annexin I and II (lipocortin I and II heavy chain) antisera (for immunohistochemical detection) were less effective to block the binding. Essentially similar results were obtained with [^{32}P] labeled poly(rG). Therefore, we concluded that the lipocortin I-S100 protein complex (heterotetramer) specifically binds to poly(rA) or poly (rG).

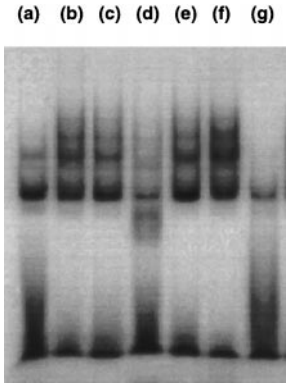

FIG. 2. Effects of various antibodies on binding of lipocortin I to poly(rA). The binding reactions were run in the (a) absence and presence of (b) anti-Igs, (c) anti-annexin I (lipocortin I), (d) anti-calpactin II (lipocortin I), (e) anti-annexin II (lipocortin II), (f) anti-calpactin I (lipocortin II), and (g) anti-S100 protein antibodies. The antibodies were included in the reaction mixtures before [^{32}P]poly(rA) was added to initiate the binding reactions. EMSA was carried out as described in the text.

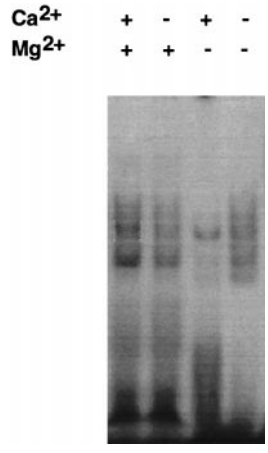

FIG. 4. Effects of Ca^{2+} and Mg^{2+} on binding of lipocortin I to poly(rA). $CaCl_2$ and/or $MgCl_2$ were added to the reaction mixture at the final concentrations of 0.1 and 5 mM, respectively. EMSA was performed as described in the text.

Specificity of lipocortin I for polynucleotides. To determine the specificity of the lipocortin I heterotetramer for polynucleotides, competitive assays against poly(rA) or poly(rG) binding were employed. Poly(rA) and poly(rG) reduced the binding of [^{32}P] labeled poly(rA) (Fig. 3). Essentially similar results were obtained with [^{32}P] labeled poly (rG). Poly(rU) and poly (rC) possibly hybridized to poly(rA) and poly(rG), respectively, thus impairing the binding of lipocortin to poly(rA) and poly(rG), or altering the mobility of the lipocortin I-poly(rA) complex. While the binding of poly(rG) to lipocortin I was relatively weaker than that of poly(rA), it was blocked by poly(rA) and poly(rG) but much less by poly(rU). These results suggest that lipocortin I is more specific for purine-polyribonucleotides rather than for pyrimidine-polyribonucleotides. To investigate whether polydeoxynucleotides can bind to lipocortin I, poly(dA), ploy(dT), poly(dG)·poly(dC) and poly(dC) were used for the competitive assays with [^{32}P]poly(rA) (Fig. 3). Poly(dG) was not used because of solubility problems. Poly(dC) was effective to compete against poly(rA). Poly(dA) provided a weak but significant competition against binding of lipocortin I to poly(rA) or poly(rG). Poly(dT) was found to compete against binding of [^{32}P]poly(rG) to lipocortin I. These observations suggest that lipocortin I can particularly bind to pyrimidine clusters of DNA. It should be noted that the affinity (1 nM) of lipocortin I for poly(dC) in the absence of phospholipids is one order of magnitude higher than that for poly(rA) (10 nM).

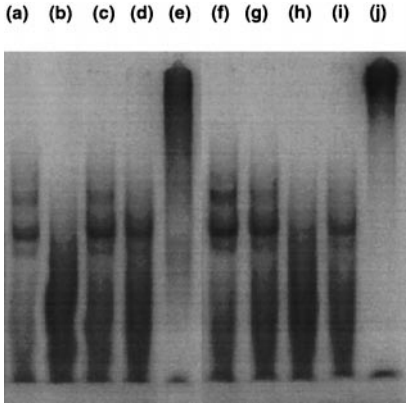

FIG. 3. Effects of various polynucleotides on binding of lipocortin I to poly(rA) and poly(rG). EMSA was carried out with [^{32}P] labeled poly(rA) (a, f) without or with (b) poly(rA), (c) poly(rC), (d) poly(rG), (e) poly(rU), (g) poly(dA), (h) poly(dC), (i) poly(dG).poly(dC), and (j) poly(dT).

Effects of Ca^{2+} and phospholipids on the binding of poly(rA) to lipocortin I. To establish the requirement of Mg^{2+} and Ca^{2+} for the binding of lipocortin I to polynucleotides, Ca^{2+} (0.1 mM) and/or Mg^{2+} (5 mM) were added to the reaction mixture (Fig. 4). The presence of both Ca^{2+} and Mg^{2+} maximized the binding of lipocortin I to poly(rA). The K_a values of Ca^{2+} and Mg^{2+} for the binding of poly(rA) to lipocortin I were approximately 10 μM and 0.2 mM, respectively, in the absence of phospholipids. To determine whether the binding of poly(rA) to lipocortin I is attributable to ionic interaction, thereby competing against phospholipids, we added various phospholipids to the reaction mixture for EMSA. Phosphatidylserine (PtdSer) markedly enhanced the binding of poly(rA) to lipocortin I, while phosphatidylinositol (PtdIns) was less effective (Fig. 5). Phosphatidylethanolamine (PtdEtn) and phosphatidylcholine (PtdCho) exhibited small but significant effects on the binding of poly(rA) to lipocortin I. These results suggest that the binding site(s) of lipocortin I for phospholipids is distinct from that for polynucleotides. Since PtdSer is reported to increase the affinity of

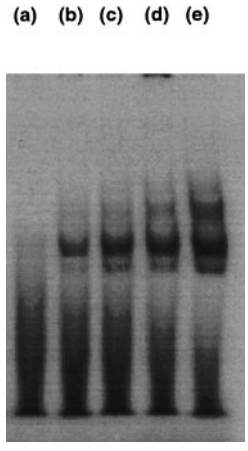

FIG. 5. Effects of phospholipids on binding of lipocortin I to poly (rA). Chloroform–methanol solutions of various phospholipids were dried in a N_2 gas, and were sonicated. One microgram of each phospholipids was added to the reaction mixture. EMSA was performed in the (a) absence and presence of (b) PtdCho, (c) PtdEtn, (d) PtdIns, or (e) PtdSer.

lipocortin I for Ca^{2+} (1), we examined whether this phospholipid alters the K_a for Ca^{2+} and the K_m for polynucleotides. In the presence of PtdSer, the K_a for Ca^{2+} and the K_m for poly(dC) were 1 μM and 0.01 nM, respectively. These results suggest that PtdSer may act as an allosteric effector, thus enhancing the binding of lipocortin to Ca^{2+} and polynucleotides.

Length of polynucleotides bound to lipocortin I. Upon urea–gel electrophoresis, poly(rA) employed in these experiments were found to be a mixture of various nucleotide lengths ranging 1 to 50 nucleotides (nt). To determine the base length of poly(rA) bound to lipocortin I, free poly(rA) in the reaction mixture was digested by S1 nuclease. Nondigested poly(rA) was isolated following the SDS-proteinase K treatment, and was subsequently labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase. Upon urea–gel electrophoresis, poly(rA)s of 15 to 30 nt were found to specifically bind to lipocortin I (data not shown). Since this method caused relatively high backgrounds, we used chemically-synthesized poly(dC) of various lengths, 5, 10, 15, 20, 25, 30, 50, and 75 nt. After labeling with [γ-^{32}P]ATP, EMSA was carried out to determine the length of poly(dC) bound to lipocortin I (Fig. 6). Results obtained in these experiments substantiated our conclusion that the lipocortin I heterotetramer binds to polynucleotides of 15–30 nt with the maximal affinity for 20- to 25-nt polynucleotides.

DISCUSSION

Results of the present communication have demonstrated that the lipocortin I-S100 protein heterotetramer binds to pyrimidine polydeoxynucleotides of 15 to 30 nt. Furthermore, this protein can bind to purine clusters of RNA. This binding is stimulated by Ca^{2+} and phospholipids. Since these characteristics of polynucleotide binding share some common features with those of replication protein A (RP-A), the lipocortin I heterotetramer may exert similar functions to those of RP-A on DNA metabolism, replication, repair and recombination (12).

Lipocortins have a common internal structure comprising four repetitions of a conserved 70 amino acid domains, and differ significantly in the length and composition of the amino-terminal domains (1). At this amino-terminal domain, lipocortins can make the complex with S100 proteins such as calcyclin (13). A defining feature of lipocortins is their ability to bind, in a Ca^{2+}-dependent manner, to negatively charged phospholipids such as PtdSer. This function is attributed to the conserved sequence of the repeats (1). The nature of the interaction between lipocortins and phospholipids is proposed to be ionic. Therefore, it has been conceived that through such ionic interactions, these proteins are able to bind not only to glycosaminoglycans such as heparin and chondroitin sulfate but also to DNAs such as Z-DNA and alu-DNA (9, 10).

Lipocortin II, another member of the family proteins, is proposed to function as a primer recognition protein, thereby increasing the synthesis of DNA lagging strand by stimulation of DNA polymerase α (6). To confirm the stimulation of synthesis of DNA lagging strand by lipocortin I, we investigated DNA synthesis in isolated rat liver nuclei (7). Phosphorylated lipocortin I stimulated DNA synthesis as measured by incorporation of [α-^{35}S]dCTP into acid insoluble fractions, and caused the accumulation of short-length DNAs with 120–170 nt. Since these short-length DNAs were sensitive to DNase and RNase treatments, we con-

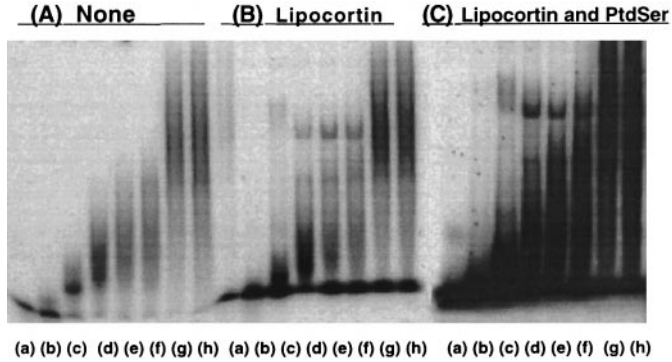

FIG. 6. Length of nucleotides bound to lipocortin I. Poly(dC) of various lengths, (a) 5, (b) 10, (c) 15, (d) 20, (e) 25, (f) 30, (g) 50 and (h) 75 nt were labeled with [γ-^{32}P]ATP by polynucleotide kinase as described in the text. Twenty femtomoles of each poly(dC) was included in the binding reaction mixture in the absence (a) and presence of lipocortin without (b), and with (c) 1 μg PtdSer. EMSA was performed as described in the text.

cluded that lipocortin I stimulates the synthesis of Okazaki fragments (7).

The first step of Okazaki fragments is catalyzed by DNA primase, an enzyme that initiates the synthesis of RNA primers for DNA polymerase α. Primase has preferential initiation sites located at or near the 3′-end of the pyrimidine clusters where pyrimidine-rich stretches are generally 6–14 bases long. Eukaryotic primase has affinity for the 3′-CC(C/A)-5′ motif, in which -CC- can be replaced by -TT- (14). Therefore, the lipocortin I heteroterotetramer apparently binds to the initiation sites of DNA templates for DNA primase as well as to RNA primers. Furthermore, lipocortins are able to associate with various acidic protein including DNA methyltransferase and helicase (15). RP-A, an essential component of the replication fork for the SV40 replication system, is known to bind to pyrimidine clusters of DNA. This protein also interacts with acidic domains of various nuclear proteins, thereby playing an important role in replication, repair and recombination of DNA (12). Accordingly, it is likely that lipocortins are capable of replacing RP-A.

Although nuclear functions and nuclear subcompartments are closely associated, no molecular and biochemical links have been presented. Membrane structures of the nuclear envelope and nuclear matrix are crucial for the assembly of the replication apparatus and chromatins (16). DnaA protein, the initiator of chromosomal DNA replication, is known to interact with acidic phospholipids, and its activity is regulated by membrane binding (17). Nuclear lipocortins are attached to nuclear membranes in a Ca^{2+}-dependent manner (4, 6). Since nuclear Ca^{2+} is an important factor for various nuclear functions including replication and transcription of DNA (18), it is likely that lipocortins may be involved in the initiation signal of DNA replication in the nuclei. Furthermore, lipocortin may present a biochemical link between the nuclear matrix compartmentation and DNA replication in mammalian cells, responding to nuclear phospholipid and Ca^{2+} signals for DNA synthesis.

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