ISOLATION OF ACIDIC LIPOPROTEINS FROM BRAIN CHROMATIN THEIR RELATION TO THE ACIDIC NONHISTONE PROTEINS

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1. Introduction

Isolated nuclei from diverse tissues contain a moderate amount of phospholipid, a substantial portion of which occurs in the membranous envelope [1–4]. Phospholipids are also thought by some to be associated with the nuclear chromatin [5] and nucleolus [6]. However, the structural localization, molecular organization and functional significance of the intranuclear lipids are obscure. Active, diffuse chromatin fractions are greatly enriched in phospholipid compared with inactive, dense chromatin fractions [7, 8]. Phospholipids, presumably in the form of lipoproteins (LPs), have been implicated in the regulation of RNA transcription [7] and DNA replication [9].

Acidic LPs have been previously prepared from nuclear residual proteins, i.e. cell nuclei that have been freed of deoxyribonucleoprotein by extraction of the nuclei with 1–2 M NaCl or KCl [10, 11]. However, LPs have not been hitherto obtained from isolated chromatin. We now describe the isolation and partial characterization of soluble acidic LPs in a chromatin fraction derived from nuclei of cat brain cells. The results show that these acidic LPs are an integral part of the chromatin and constitute a major portion of the acidic chromatin nonhistone proteins. A preliminary account of this work has appeared [12].

2. Materials and methods

Cell nuclei were isolated from cat cerebrum according to Lǿvtrup-Rein and McEwen [13]. Protein and associated lipids and nucleic acids were precipitated by adding trichloroacetic acid to a final concentration of 10% (v/v). Protein was determined by the method of Lowry et al. [14]. Phosphorus was determined in lipid extracts [15] by the method of Bartlett [16] and multiplied by 25 to give phospholipid values. Cholesterol was determined by gas–liquid chromatography. RNA was measured according to Hutchinson and Munro [17], and DNA according to Giles and Meyers [18]. Fractions were also fluorochromed with acridine orange for fluorescence microscopy and processed for electron microscopy as described earlier [19].

To extract the soluble proteins the nuclear pellet was washed three times in 0.075 M NaCl, 0.024 M EDTA, pH 8, and twice in 0.005 M Tris, pH 8.0, 0.005 M sodium bisulfite. The nuclear residue was centrifuged at 7 700 *g* for 15 min after each wash and a final pellet at 150 000 *g* for 1 hr. The chromatin was solubilized by a modification of the method of Franke et al. [2] for isolating nuclear membranes. The washed nuclear residue was suspended in 5 ml of 0.3 M sucrose, 0.135 M KCl, 0.01 M citrate-phosphate buffer, pH 7.4,

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and sonicated for 20 sec in a Branson Sonifier Model W185D (4°C, microtip, 35 W, 2 sec periods alternating with 15 sec rest periods to avoid overheating). 20 vol of 1.5 M KCl, 0.01 M Tris, pH 7.2, 0.3 M sucrose was added to the nuclear sonicate and the nuclear suspension stirred for 18 hr at 4°C. The insoluble residue was removed by centrifugation at 110 000 *g* for 1 hr. The soluble chromatin was dialyzed overnight against 0.01 M Tris, pH 7.2, 0.024 M EDTA at 4°C and lyophilized.

The chromatin LPs were solubilized by extracting the dialyzed, lyophilized chromatin in 0.2% Triton X-100, 0.01 M Tris buffer, pH 7.5, 0.001 M EDTA for 15 min at 4°C and clarified by centrifuging at 133 000 *g* for 1 hr. The Triton extract of chromatin, designated the whole LP (WLP) fraction, was fractionated by ultracentrifugal flotation in aqueous KBr by a modification of the procedure described earlier [19]. The WLP fraction was adjusted to a density of 1.35 g/ml by adding KBr and centrifuged at 122 000 *g* for 18 hr at 4°C in the No. 50 Rotor in the Spinco L2 ultracentrifuge. The floating layer (1 ml) containing the HDLP fraction ($d < 1.35$ g/ml) was separated from the infranatant (7 ml) containing the VHDLP ($d > 1.35$ g/ml). These LP fractions were dialyzed against 0.001 M EDTA, 0.002 M Tris, pH 7.2, for 24 hr at 4°C and lyophilized. In some experiments an acidic protein (AP) fraction was prepared by dissociating the Triton X-100-insoluble chromatin (or whole chromatin) in 1.5 M KCl. The salt concentration was reduced to 0.15 M by overnight dialysis at 4°C against 10 vol of water, and the reconstituted nucleohistones removed by centrifugation at 30 000 *g* for 15 min. The extraction was repeated once and the pooled supernatants dialyzed at 4°C against 0.002 M Tris–HCl buffer, pH 7.2, 0.001 M EDTA to give the AP fraction. For amino acid composition, LP fractions were precipitated with 10% trichloroacetic acid, the lipids extracted with chloroform–methanol (2:1, v/v), and the residue hydrolyzed in 6 N HCl at 110°C for 72 hr. Protein hydrolysates were analyzed in a Beckman automatic amino acid analyzer.

LP fractions were analyzed by electrophoresis in 7% polyacrylamide gels containing 5 M urea and 0.25% Triton X-100 at pH 2.7 according to Lim and Taddyon [20]. Proteins were demonstrated in gels by staining with Amido Black (1% in 7% acetic acid). Gels were fixed overnight in 10% cold trichloroacetic acid and stained for lipid with Sudan black B (0.5% in propylene glycol with 2% HCHO) and acidic groups with acridine orange (0.003% in 0.01 M glycine buffer, pH 8, with 2% HCHO). Gels were destained by soaking in large volumes of 7% acetic acid (3.5% acetic acid and 50% ethanol for Sudan black B). Stained gels were densitometered with a Gilford 2000 absorbance recorder and a Gilford 2410 linear transport.

3. Results and discussion

The nuclear fraction consisted solely of intact, naked nuclei when examined by phase microscopy, and in the fluorescence microscope after staining with the cationic fluorochrome, acridine orange. On electron microscopic examination, the nuclear pellet consisted exclusively of nuclei, mostly still intact and surrounded by the outer nuclear membrane with its attached ribosomes. Contamination by cytoplasm, myelin and other extranuclear elements was negligible. Purified nuclear fractions harvested from 50 g of brain (3 animals) contained approx. 30 mg of protein and 6 mg of DNA. About 70% of the total DNA was recovered in the nuclear fraction. The ratio of protein to DNA in the nuclear fraction was 5.0, and of DNA to RNA was 4.0 (table 1). Cell nuclei from rat cerebral cortex exhibit similar ratios [21]. The phospholipid content of the nuclear fraction was 0.079 per mg of protein. This value is within the range reported for isolated liver nuclei, 0.04–0.10 mg phospholipid/mg protein [1–4].

Chromatin fractions prepared from whole tissue or isolated nuclei by the commonly used procedure of mechanical shearing and density gradient centrifugation of the nucleohistone complex evidently contain membranes of cytoplasm and/or nuclear origin [22, 23]. To avoid such contamination we chose to prepare an unsheared soluble chromatin from isolated brain nuclei through the use of 1.5 M KCl which should not dissociate the nucleoli and nuclear membranes. The 1.5 M KCl-insoluble residue from brain nuclei was centrifuged at 110 000 *g* for 1 hr to ensure complete removal of membrane fragments from the supernatant chromatin, and found to contain 21% of the protein, 43% of the phospholipid, 3.5% of the DNA, and 44% of the RNA in the nuclear fraction. On light and electron microscopic examination, this residue consisted mainly of nuclear membrane fragments and nucleoli.

Table 1
Composition of nuclei, chromatin, and chromatin fractions isolated from cat brain.

Fractions	Protein	Phospholipid		RNA		DNA	
	% Of total	% Of total	Concn.*	% Of total	Concn.*	% Of total	Concn.*
Nuclei	100	100	0.079 (±0.002)	100	0.049 (±0.002)	100	0.209 (±0.004)
Chromatin	69.8 (± 3.4)	49.5 (± 2.8)	0.062 (±0.002)	47.1 (± 2.5)	0.037 (±0.003)	96.0 (± 0.4)	0.332 (±0.015)
WLP+	34.5 (± 1.8)	84.7 (± 1.8)	±0.152 (±0.005)	32.6 (± 1.3)	0.034 (±0.001)	0.2 (± 0.13)	0.006 (±0.005)
Ap+	10.0 (± 0.6)	3.0 (± 1.0)	0.021 (±0.005)	8.8 (± 1.7)	0.036 (±0.002)	2.0 (± 0.9)	0.074 (±0.034)

* Concentration in mg/mg protein.
+ % Of total in chromatin
Mean ± S.E. of 4 experiments (2 for cholesterol).

This finding confirms earlier observations of others that high salt media do not dissociate nucleoli [25] and nuclear membrane [2, 10] of liver cells and are useful for freeing nuclear membranes of chromatin [2, 4]. The 1.5 M KCl-soluble chromatin exhibited the spectral properties characteristic of chromatin with the absorption maximum at 260 nm and a trough at 240 nm (fig. 1). The A_{260}/A_{240} was 1.54; the A_{260}/A_{280} was 1.40. This chromatin solution contained 96% of the DNA, 70% of the protein, 50% of the phospholipid and 47% of the RNA in the nuclear fraction and showed extremely low scattering (the A_{310}/A_{260} was 0.046), indicating the essential absence of membrane fragments and other insoluble particles. Moreover, studies to be reported separately (Lu and Koenig, in preparation) indicate that the LP constituents present in the 1.5 M KCl-insoluble residue from brain nuclei differ biochemically and electrophoretically from the chromatin LPs that are described in this report. We conclude, therefore, that the chromatin used in these studies was indeed free of contamination by envelope membranes and nucleoli.

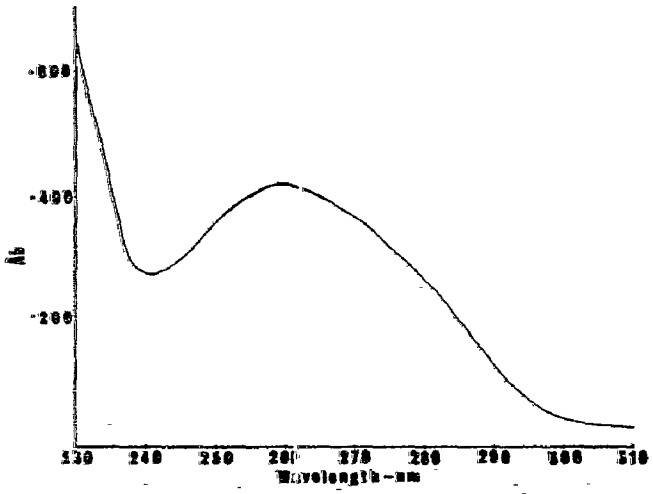

Fig. 1. Absorption spectrum of soluble chromatin from cat brain nuclei.

Table 2
Composition of brain chromatin lipoprotein fractions prepared by ultracentrifugal flotation.

Fraction	Protein	Phospholipid		Cholesterol		RNA	
	% Of total	% Of total	Concn.*	% Of total	Concn.*	% Of total	Concn.*
WLP	100	100	0.152 (±0.005)	100	0.038 (±0.003)	100	0.031 (±0.003)
HDLP	59.9 (± 1.9)	91.2 (± 0.5)	0.241 (±0.009)	94.4 (± 0.6)	0.060 (±0.003)	31.7 (± 1.1)	0.017 (±0.002)
VHDLP	40.1 (± 1.9)	8.1 (± 0.5)	0.035 (±0.003)	5.6 (± 0.6)	0.004 (±0.003)	68.3 (± 3.3)	0.049 (±0.009)
% Recovery	88.6 (± 1.5)	87.7 (± 3.5)		86.0 (± 4.1)		85.0 (± 4.4)	

* Concentration in mg/mg protein.
Mean ± S.E. of 4 experiments (2 for cholesterol).

The whole chromatin LP (WLP) fraction extractable in 0.2% Triton X-100 contained about 85% of the phospholipid, 35% of the protein and 32% of the RNA in the chromatin, and was enriched 2.5-fold in phospholipid over the latter (table 1). Ultracentrifugal flotation of the WLP yielded two LP fractions: a high density LP (HDLP, d < 1.35 g/ml), 60%, and a very high density LP (VHDLP, d > 1.35 g/ml) fraction, 40% of the total protein (table 2). The HDLP fraction was recovered as a yellowish floating layer in about 1 ml volume, while the VHDLP fraction was present in a colorless infranatant of about 7 ml volume. The protein and phospholipid concentrations in the HDLP fraction were increased 10- and 66-fold, respectively, over their concentrations in the VHDLP fraction. The HDLP fraction contained 0.24 mg phospholipid and 0.06 mg cholesterol, and the VHDLP fraction, 0.035 mg phospholipid and 0.004 mg cholesterol/mg protein. These findings strongly suggest that the lipid constituents in Triton X-100 extracts of chromatin are associated with the proteins as LP complexes.

Table 3
Amino acid composition of lipoprotein and acidic protein fractions from brain chromatin.

Amino acid	WLP	HDLP	VHDLP	AP
Aspartic acid	10.6	10.2	11.4	9.9
Threonine	3.5	4.4	4.4	7.3
Serine	2.9	5.2	3.9	10.7
Proline	6.8	6.3	5.0	trace
Glutamic acid	16.6	13.6	15.7	15.7
Glycine	10.0	10.2	10.5	12.0
Alanine	7.4	7.7	5.3	8.5
Valine	5.7	6.7	8.1	9.7
Methionine	trace	1.4	1.1	0.4
Isoleucine	4.2	4.4	5.2	4.2
Leucine	7.9	8.8	7.9	8.1
Tyrosine	2.2	1.6	1.3	1.1
Phenylalanine	3.6	3.6	5.0	1.9
1/2 Cystine	trace	trace	trace	trace
Lysine	11.6	8.7	9.9	9.4
Histidine	0.5	1.9	trace	trace
Arginine	4.9	5.5	4.1	1.1
Acidic A.A./basic A.A.	1.59	1.48	1.93	2.44
% Polar A.A.	50.6%	50.1%	50.8%	54.1%

Results as moles %. Polar amino acids (A.A.) = aspartic acid, threonine, serine, glutamic acid, lysine, histidine, arginine.

The amino acid composition of the apoLPs shown in table 3 indicates that they are acidic proteins. The molar ratio of acidic to basic amino acid residues was 1.48 in the HDLP and 1.92 in the VHDLP. These apoLPs also are rich in polar amino acids, the latter comprising somewhat more than 50% of the total amino acids. Such high polarities are characteristic of soluble, nonmembrane proteins, e.g. the various apoproteins from human serum LPs have polarities ranging between 46 and 52% [25].

On polyacrylamide gel electrophoresis at least 20 protein bands were discernible in gel electropherograms of the HDLP and VHDLP fractions (fig. 2). A similar pattern of staining was observed in gels stained for lipids with Sudan black B and iodine (not shown)

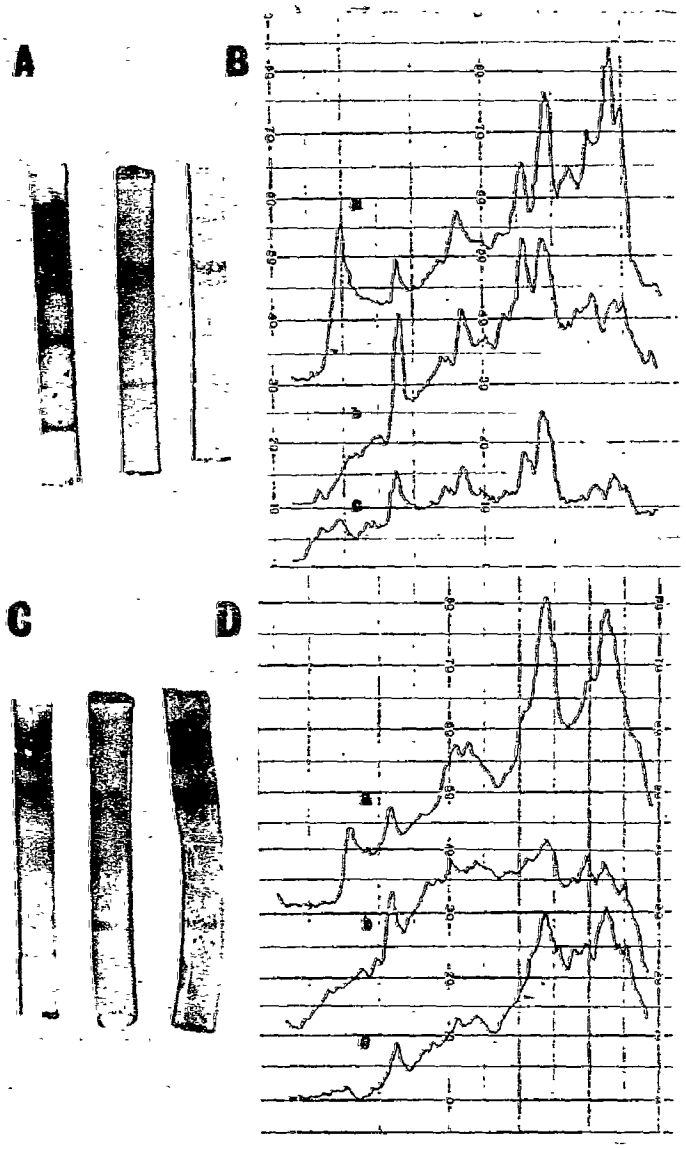

Fig. 2. A and C. Gel electropherograms of the HDLP (A) and VHDLP (B) fraction from cat brain chromatin. From left to right, the gels are stained with Amido black (protein) Sudan black B (lipid) and acridine orange (acidic groups). B and D. Densitometric tracings of the electropherograms shown in A and C, respectively. Gel stains are: a: Amido black; b: Sudan black B; c: acridine orange.

and for acidic groups with acridine orange. Comparison of densitometric scans of gel electropherograms stained by these various dyes indicated that essentially all of the protein components exept the leading band in the HDLP and VHDLP fractions contained lipid and anionic groups (fig. 2). LP constituents with corresponding mobilities were present in both the HDLP and VHDLP fractions. However, they differed in their relative concentration in these fractions due to differences in lipid content. In accord with the greater lipid content of the HDLP fraction, its components stained much more intensely for lipid than the corresponding components in the VHDLP. The lipid moieties of these LPs apparently exert little influence on their electrophoretic mobilities.

The chromatin LPs closely resemble the acidic chromatin nonhistone proteins of liver [26] and brain cell nuclei [27] in their solubility in 1–2 M NaCl or KCl, amino acid composition, and electrophoretic mobility in polyacrylamide gels. However, an association of phospholipids and acidic chromatin proteins has not, to the best of our knowledge, been previously reported. We thus measured the phospholipid and protein content in an acidic protein (AP) fraction isolated from brain chromatin by dissociation in high salt solution [26]. The AP fraction from whole chromatin contained 62% of the protein and 68% of the phospholipid (table 4). The phospholipid concentration was 0.07 mg/mg protein. Ultracentrifugal flotation of this AP fraction in aqueous KBr of density 1.35 g/ml yielded a high density AP (HDAP, $d < 1.35$ g/ml) fraction containing 26% and 49% of the protein and phospholipid, and a very high density AP (VHDAP, $d > 1.35$ g/ml) fraction containing 74% and 51% of the protein and phospholipid in the AP fraction (table 4). The protein and phospholipid concentrations in the HDAP fraction were increased 2.5-fold and 6.6-fold over their concentrations in the VHDAP fraction. The HDAP and VHDAP fractions contained 0.14 mg and 0.05 mg of phospholipid/mg protein. These findings show that the phospholipid in the AP fraction is firmly complexed with protein. When the chromatin was extracted with 0.2% Triton X-100 prior to isolation of the AP fraction, the protein and phospholipid content of the AP fraction declined to 10% and 3% of the total in the chromatin, and the phospholipid concentration diminished to 0.021 mg/mg protein (table 1). The high phospholipid content of the AP fraction and the drastic reduction in protein and phospholipid retrieved in this fraction when isolated from the Triton-extracted chromatin unequivocally demonstrate that the Triton X-100-soluble LPs occur mainly in this AP fraction. More-

Table 4
Composition of acid protein fraction and its subfractions from whole brain chromatin.

Fraction	Protein	Phospholipid		RNA		DNA	
	% Of total	% Of total	Concn.*	% Of total	Concn.*	% Of total	Concn.*
Chromatin	100	100	0.060 (±0.006)	100	0.043 (±0.006)	100	0.349 (±0.018)
Acidic Protein	62.4 (± 4.2)	67.9 (± 7.0)	0.070 (±0.006)	36.4 (± 1.5)	0.034 (±0.009)	1.70 (± 0.2)	0.022 (±0.008)
HDAP+ (d < 1.35 g/ml)	26.4 (± 3.9)	48.7 (± 3.7)	0.140 (±0.007)	N.D.		N.D.	
VHDAP+ (d > 1.35 g/ml)	73.6 (± 3.9)	51.3 (± 3.7)	0.050 (±0.005)	N.D.		N.D.	

* Concentration in mg/mg protein.
N.D., not determined.
+ % Of total in acidic protein fraction.
Mean ± S.E. of 3 experiments (2 for HDAP and VHDAP).

over, experiments to be reported separately indicate that the acidic nonhistone proteins which remain in the Triton X-100-extracted residue of brain chromatin are mostly acidic LPs with approx. 2% phospholipid.

On the basis of these findings we suggest that the great majority of acidic nonhistone proteins of chromatin can be regarded as members of a large and heterogeneous family of acidic LPs which vary widely in buoyant density, lipid composition and electrophoretic mobility. The LP nature of the acidic chromatin nonhistone proteins, which are now generally considered to play an important role in the control of genetic function, accounts for some of the distinctive physicochemical properties of these macromolecules, notably, their tendency to aggregate with histones, DNA and one another, that have previously hampered their isolation and fractionation [28]. The amphophilic nature of these LPs and their electronegativity would favor coulombic, hydrophobic and other types of interactions of the kind that have been postulated for the acidic chromatin proteins with histones, DNA and various gene-modifying ligands [28].

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