IDENTIFICATION OF N^G, N^G-DIMETHYLARGININE IN A NUCLEAR PROTEIN FROM THE LOWER EUKARYOTE PHYSARUM POLYCEPHALUM HOMOLOGOUS TO THE MAJOR PROTEINS OF MAMMALIAN 40S RIBONUCLEOPROTEIN PARTICLES

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SUMMARY: A nuclear protein apparently homologous to the two major proteins of 40S heterogeneous nuclear ribonucleoprotein particles from mammalian cells has been isolated from the lower eukaryote Physarum polycephalum, purified, and found to contain a substantial amount of the unusual amino acid N^G, N^G-dimethylarginine. The apparent homology is based on similar molecular weights, basic isoelectric points and amino acid compositions including the dimethylarginine and a high content of glycine. The implications of the presence of this protein in Physarum polycephalum and the possible significance of the N^G, N^G-dimethylarginine are discussed.

Recently evidence has accumulated which indicates that heterogeneous nuclear RNA (precursor to cytoplasmic messenger RNA) in eukaryotic cells is bound with proteins in a stoichiometric fashion (1-9). The HnRNP[1] is extracted in the form of 40S, 200 Å spherical particles (1,5,8), the protein component of which appears to be simple and moderately conserved (1,2,4-9); although some reports suggest a more heterogeneous protein composition (3,10). In HeLa cells the two major HnRNP proteins migrate as a doublet in SDS polyacrylamide gels with molecular weights of 32,000 and 34,000 (8). These two proteins are always observed in a 1:1 stoichiometry within the HnRNP particles and they possess similar amino acid compositions including two defining characteristics: a high content of glycine and the presence of an unusual basic residue(s) (8). These characteristics have also been observed in the amino acid compositions of the total HnRNP protein complement from rat liver (2,9,11), duck liver (5), and mouse ascites hepatoma (5). The two major HnRNP proteins from HeLa cells both have basic isoelectric points (8). On the basis of the preceding properties it is believed that the interaction of the HnRNP proteins with HnRNA is analogous to the interaction of the histones with DNA in nucleosomes; the two major HnRNP proteins being structural in nature.

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[1]Abbreviations used: HnRNP, heterogeneous nuclear ribonucleoprotein; SDS, sodium dodecyl sulfate; HnRNA, heterogeneous nuclear RNA.

The data presented in this paper concern a major nuclear protein isolated from the lower eukaryote Physarum polycephalum which, on the basis of several criteria, appears to be homologous to the HnRNA-associated proteins described above. The protein has a similar molecular weight, isoelectric point and amino acid composition including the unusual basic residue which is identified in this paper as N^G, N^G-dimethylarginine.[2]

MATERIALS AND METHODS:

A single isolate of the Myxomycete Physarum polycephalum M_3C VII (12) was used in these studies. Plasmodia were cultured as previously described (12, 13). Nuclei were isolated by the method of Mohberg and Rusch (14) with several modifications. Following filtration of the homogenate the nuclei were pelleted by centrifugation at 2000 RPM for 15 min. and washed with the nuclear homogenizing solution. The suspension was then centrifuged at 300 RPM for 5 min. afterwhich the slime pellet was discarded. The supernatant was centrifuged at 1500 RPM for 15 min. and the pelleted nuclei were washed with distilled H_2O and repelleted as above.

Procedures for purifying the homologous HnRNP protein consisted of extracting isolated nuclei with five volumes of 1 M KCl, 20 mM Tris-HCl (pH 7.2), 10 mM 2-mercaptoethanol for 3 min. followed by overnight dialysis of the extract against 0.025 M KCl, 20 mM Tris-HCl (pH 7.2), 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol. The precipitate formed was pelleted, solubilized in 8 M urea, 20 mM Tris-HCl (pH 7.2), 10 mM EDTA, 10 mM 2-mercaptoethanol and loaded onto a 1 cm X 8 cm phosphocellulose column. Following an initial wash the protein of interest was eluted with a single step consisting of the same solution as above with the addition of 0.12 M KCl. The eluate was dialized extensively against distilled H_2O, lyophilized, and solubilized in 0.25 M sucrose, 10 mM sodium phosphate buffer (pH 7.2), 0.1% SDS, 0.14 M 2-mercaptoethanol. This solution of proteins was separated by preparative SDS polyacrylamide gel electrophoresis using a unit designed by Wallace M. LeStourgeon [described in detail elsewhere (15)] which utilizes a 2 cm X 10 cm gel prepared according to the procedures of Laemmli (16). Fractions were collected subsequent to the migration of the marker dye front off the bottom of the gel and assayed by analytical SDS gel electrophoresis also according to the procedures of Laemmli as described elsewhere (17).

Purified protein was hydrolyzed in 6 N HCl for 21 hours at 105°C in a sealed tube. Amino acid compositions were obtained using a Beckman 120C automatic amino acid analyzer according to the method of Spackman, Stein, and Moore (18).

The unusual basic residue was purified by BioRad Aminex A 5 chromatography of the basic amino acids present in the hydrolyzate of the Physarum phenol-soluble residual nuclear protein fraction prepared as previously described (19). The fractions containing the residue, clearly resolved from both the ammonia and arginine peaks, were pooled and loaded onto a column of BioRad Ag 50W X 8 cation exchange resin for final purification (removal of contaminating buffers). Following an extensive wash with 0.2 N HCl the basic residue was eluted with 2.0 N HCl. The HCl was removed by evaporation.

Standards of N^G-monomethylarginine, N^G, N^G-dimethylarginine, and N^G, N'^G-dimethylarginine were obtained from Calbiochem. Cochromatography comparing the

[2] L. C. Boffa and G. Vidali have identified this residue in the major HnRNP proteins of rat liver (personal communication).

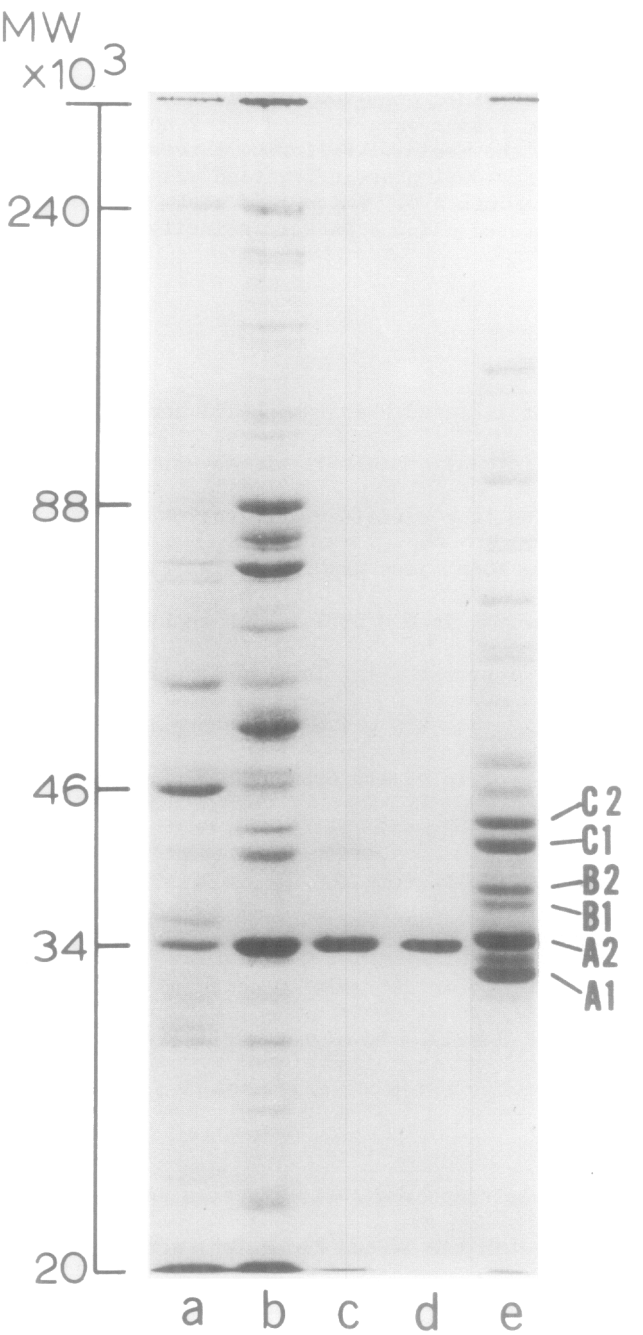


Fig. 1. Electrophoretic profiles of various protein fractions: Gels (a) - (d) represent stages in the purification of the Physarum homologous HnRNP protein. (a) 1 M KCl extract, (b) low salt precipitated proteins, (c) phosphocellulose fraction, (d) purified protein via preparative SDS gel electrophoresis. Gel (e) shows the 40S HnRNP particle proteins from Hela cells with corresponding designations (8).

purified basic residue with these standards was done with the automatic amino acid analyzer as described above.

(^{14}C)-L-arginine was obtained from New England Nuclear. Physarum microplasmodia growing in shaker flasks were labeled for 2 hours by the addition of 50 microcuries of label to the medium. Following harvesting and nuclear isolation the phenol-soluble residual protein fraction was obtained, hydrolyzed and chromatographed using Aminex A 5. Amino acid peaks were assayed as above and radioactivity was monitored using a Packard scintillation counter.

RESULTS:

Purification of the Protein

The 1 M KCl extract of isolated Physarum nuclei contains a heterogeneous mixture of polypeptides (Fig. 1a). Dialysis of the extract into low salt results in the selective and reproducible precipitation of certain proteins among which is the putative homologous HnRNP protein (Fig. 1b). Following phosphocellulose chromatography in 8 M urea, which results in a many-fold enrichment in the protein of interest (Fig. 1c), an electrophoretically-pure protein is obtained with preparative SDS gel electrophoresis (Fig. 1d). For purposes of comparison a gel profile of the 40S HnRNP proteins from HeLa cells is given in Fig. 1e.

Partial Characterization of the Protein

The Physarum protein has a molecular weight of approximately 34,000 as estimated by its relative migration in SDS gels as compared to that for a series of molecular weight standards (19,21). It migrates closely with the upper band (A2) of the HeLa HnRNP major doublet proteins which also has an estimated molecular weight of around 34,000 (Fig. 1e).

Two dimensional gel electrophoresis (isoelectric focusing in the first dimension and SDS gel electrophoresis in the second dimension) according to the procedure of O'Farrell (22) has demonstrated that the Physarum protein is basic with an isoelectric point of approximately 8.3 - 8.4 (data not shown). This same technique has shown that the HeLa HnRNP doublet proteins (A1 and A2) are also basic with approximate isoelectri points of 9.2 and 8.4, respectively (8).

The amino acid composition of the purified Physarum protein is given in the first column of Table 1. Outstanding features of the composition are the high

TABLE 1

AMINO ACID COMPOSITIONS OF THE *PHYSARUM* HOMOLOGOUS HnRNP PROTEIN, HeLa HnRNP PROTEINS A1 AND A2, AND TOTAL HnRNP PROTEIN COMPLEMENT FROM SEVERAL SOURCES

(in mole %)

Amino acid	*Physarum* protein	HeLa A1 protein (8)	HeLa A2 protein (8)	Mouse Ascites HnRNP Proteins(5)	Duck Liver HnRNP Proteins(5)	Rat liver HnRNP Proteins a.(11)	Rat liver HnRNP Proteins b.(2)
Lys	6.6	7.7	6.0	6.3	5.4	6.5	7.4
His	1.0	1.5	1.6	2.0	2.0	2.6	2.4
Arg	3.7	3.3	5.3	6.0	5.8	7.6	6.2
Asx	7.8	7.6	10.3	10.8	9.5	10.6	10.4
Thr	3.0	3.5	2.7	3.3	3.7	3.9	4.6
Ser	10.0	13.0	10.4	7.6	9.0	7.6	6.1
Glx	10.6	12.6	10.7	10.5	13.1	10.6	11.9
Pro	5.3	3.5	4.0	3.7	3.9	5.7	4.6
Gly	29.0	23.2	24.9	22.4	17.9	17.1	14.0
Ala	7.8	8.1	5.8	4.0	7.2	5.5	5.5
Cys/2	-	Trace	Trace	Trace	Trace	0.9	0.9
Val	4.0	3.2	2.5	4.8	4.4	4.1	5.7
Met	-	0.7	1.5	1.8	1.6	-	2.0
Ile	2.6	3.3	2.7	2.5	2.6	2.4	3.3
Leu	3.3	4.8	4.3	3.3	4.2	4.8	5.2
Tyr	Trace	1.3	2.6	5.6	3.7	4.5	4.0
Phe	2.3	2.6	4.3	5.4	4.1	4.2	5.1
**	3.1	0.1	0.4	-	2.0	1.4	x

** Unusual basic residue identified for the *Physarum* protein as N^G, N^G-dimethylarginine.
x Unspecified amount present.

content of glycine (29%) and the substantial amount (3%) of an unusual basic residue which elutes between ammonia and arginine when chromatographed on sulfonated polystyrene cation exchange resins (Fig. 2).[3] Table 1 also lists the amino acid compositions of the HeLa HnRNP proteins, A1 and A2, and the compositions of the total HnRNP protein complement from other eukaryotic sources. The differences that exist between the composition of the *Physarum* protein and the compositions of the higher eukaryotic HnRNP proteins are no greater than the differences among the various HnRNP protein compositions themselves.

Identification of N^G, N^G-dimethylarginine

Identification of the unusual basic residue present in the *Physarum* homologous HnRNP protein as N^G, N^G-dimethylarginine is based on two lines of evi-

[3] On the basis of identical positions of elution it is believed that the unusual basic residue in the HeLa HnRNP proteins A1 and A2 is the same as that found in the *Physarum* protein.

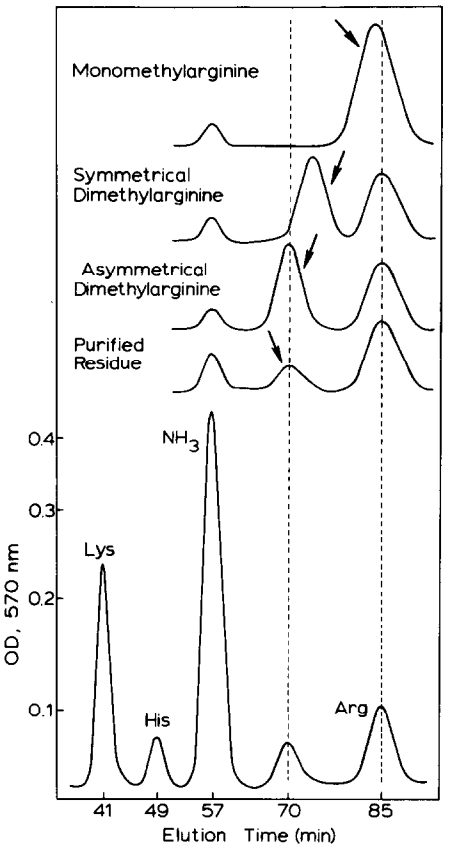


Fig. 2. Comparative chromatography of the purified residue and the three derivatives of arginine known to occur naturally in proteins. In each of the upper four curves the ammonia peak and the arginine peak (arg. standard added) act as internal markers enabling precise comparison of the elution positions (indicated by arrows) of the compounds listed in the figure. Monomethylarginine elutes closely enough to arginine that the two appear as a single broad peak. The symmetrical and the asymmetrical dimethylarginines are clearly resolved and the latter coelutes exactly with the purified residue. The curve at the bottom of the figure is the basic amino acid profile of the purified Physarum protein.

dence. The first consists of comparative amino acid chromatography using a purified sample of the basic residue and known standards of the three derivatives of arginine known to naturally occur in proteins: N^G-monomethylarginine, N^G, N^G-dimethylarginine (asymmetrical), and N^G, N'^G-dimethylarginine (symmetrical). The results shown in Fig. 2 indicate that all three methylated arginine derivatives are resolved and that the position of elution of the purified basic

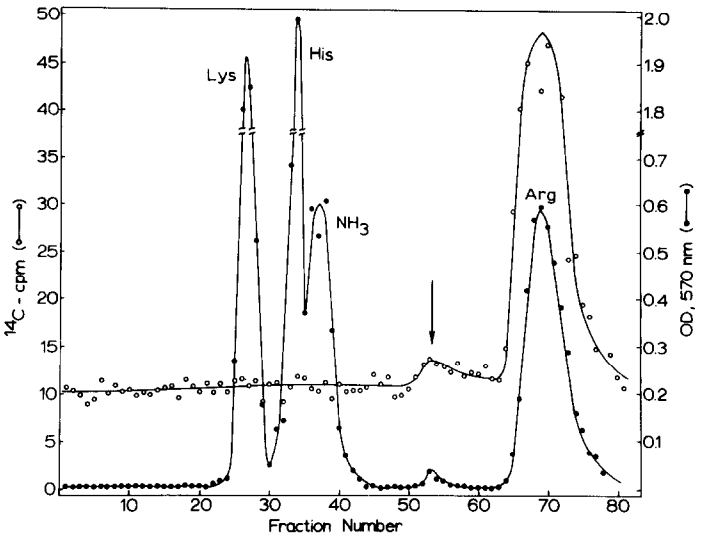
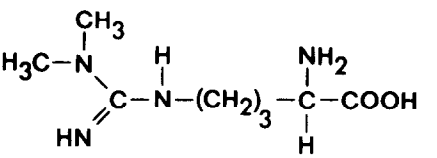


Fig. 3. Chromatographic profile of the basic amino acids in the hydrolyzate of the phenol-soluble residual nuclear protein fraction containing the homologous HnRNP protein and labeled with (^{14}C)-L-arginine. Essentially all of the unusual residue present in this phenol fraction can be accounted for by that present in the HnRNP protein. The profile is overlaid with the graph of the radioactivity present in the various amino acid peaks. Arrow indicates the unusual residue peak and corresponding radioactivity. The only other peak containing radioactivity is that of arginine; as would be expected with the label used.

N^G, N^G - Dimethylarginine



Structure of "asymmetrical" or N^G, N^G-dimethylarginine. In this form both of the additional methyl groups are located on the same guanidino nitrogen.

residue is identical to that for asymmetrical or N^G, N^G-dimethylarginine. The second is the demonstration that when Physarum plasmodia are labeled with (^{14}C)-L-arginine, radioactivity is detected only in the arginine and the basic residue peaks following chromatographic separation of a hydrolyzed sample containing the homologous HnRNP protein (Fig. 3). Based on the relatively short labeling time and the lack of

radioactivity in the lysine, histidine and ammonia peaks it can be reasonably concluded that the presence of radioactivity in the basic residue peak is due to the translational uptake of (^{14}C)-arginine at those positions in the protein molecule where subsequent methylation yields the unusual basic residues.

DISCUSSION:

The proteins associated with HnRNA have attracted considerable attention recently due to the increasing significance of nuclear mRNA processing as a possible control mechanism in gene expression. Little is known concerning the specific mechanisms whereby the nuclear HnRNA primary transcripts are structurally edited and physically transported to their cytoplasmic site of translation. It is becoming increasingly apparent that the HnRNA-associated proteins probably play a major role in these processes. Therefore it is important that these proteins and their interaction with HnRNA be fully characterized.

40S HnRNP particles similar to those isolated from higher eukaryotes have not been extractable from Physarum using currently available procedures. This is consistent with published data suggesting that slime molds may not possess high molecular weight HnRNA molecules analogous to those apparently present in mammalian cells (23). The presence of a homologous HnRNP protein in Physarum, however, suggests that this lower eukaryote may possess at least some "HnRNA" packaging, processing or transport events similar to those occurring in higher eukaryotes.

It has recently been suggested that the guanidino group of arginine may be important in the specific recognition of nucleic acids by proteins (24,25), and methylation of this group could have a stabilizing or destabilizing effect upon this interaction. Chang et al. (26) have recently shown that the major modification in HeLa ribosomal proteins is also N^G, N^G-dimethylarginine and, since ribosomes contain several species of RNA, the possibility arises that the methylated arginine residues are involved in a generalized RNA-protein interaction. Whatever the case may be, this modification appears to be well-conserved in higher and lower eukaryotes and is undoubtedly of fundamental importance to the function of the HnRNP proteins.

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