Monoclonal Antibody Characterization of the C Proteins of Heterogeneous Nuclear Ribonucleoprotein Complexes in Vertebrate Cells

YANG DO CHOI AND GIDEON DREYFUSS
Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, Illinois 60201

ABSTRACT The C proteins (C1 and C2) are major constituents of the 40S subparticle of heterogeneous nuclear ribonucleoprotein complexes (hnRNPs) (Beyer, A. L., M. E. Christensen, B. W. Walker, and W. M. LeStourgeon, 1977, Cell, 11:127-138) and are two of the most prominent proteins that become cross-linked by ultraviolet light to heterogeneous nuclear RNA (hnRNA) in vivo. Studies are described here on the characterization of the C proteins in vertebrate cells using monoclonal and polyclonal antibodies. Monoclonal antibodies to genuine RNP proteins, including the C proteins, were obtained by immunizing mice with purified complexes of poly(A)+hnRNA and poly(A)+mRNA with their contacting proteins in vivo obtained by ultraviolet cross-linking the complexes in intact cells (Dreyfuss, G., Y. D. Choi, and S. A. Adam, 1984, Mol. Cell. Biol., 4:1104-1114). One of the monoclonal antibodies identified the C proteins in widely divergent species ranging from human to lizard. In all species examined, there were two C proteins in the molecular weight range of from 39,000 to 42,000 for C1, and from 40,000 to 45,000 for C2. The two C proteins were found to be highly related to each other; they were recognized by the same monoclonal antibodies and antibodies raised against purified C1 reacted also with C2. In avian, rodent, and human cells the C proteins were phosphorylated and were in contact with hnRNA in vivo. Immunofluorescence microscopy demonstrated that the C proteins are segregated to the nucleus. Within the nucleus the C proteins were not found in nucleoli and were not associated with chromatin as seen in cells in prophase. These findings demonstrate that C proteins with similar characteristics to those in humans are ubiquitous components of hnRNPs in vertebrates.
These two proteins were shown (12) to correspond to the C proteins of the 40S hnRNP subparticle previously described by Beyer et al. (6).

The monoclonal antibody 2B12 recognized the C proteins (41,000 and 43,000 mol wt) only in higher mammals (12). In an attempt to define and study further the C proteins of other vertebrate species, we screened the anti-hnRNP monoclonal antibody-secreting hybridoma library and found monoclonal antibodies that recognize these proteins also in other species.

This report makes use of such an antibody to identify the C proteins across vertebrate species and describes immunological studies which further characterize the C proteins of hnRNPs.

MATERIALS AND METHODS

Cell Culture and Labeling: HeLa (human), CV-1 (monkey), and Madin-Darby bovine kidney (bovine) cells were grown in monolayer culture in Dulbecco's modified Eagle's medium (DME) containing 10% fetal calf serum (FCS) at 37°C in 5% CO2 atmosphere. Cultures were supplemented with penicillin and streptomycin and used at subconfluent densities. Chinese hamster ovary cells were grown similarly in DME supplemented with nonessential amino acids. Chicken (MSB) cells (1) were grown in RPMI 1640 containing 10% FCS and 1% chicken serum. Lizard (Anolis carolinensis) myogenic cells (3) were cultured in F10 medium containing 10% horse serum and 4% chick embryo extract at 30°C in 5% CO2. Rat kangaroo P1K cells were cultured in Eagle's minimum essential medium with nonessential amino acids and Earle's balanced saline solution with reduced bicarbonate (0.85 g/liter) supplemented with 10% FCS. Cells were labeled with [35S]methionine at 10 ~Ci/ml for 12 h in DME containing 1/10 the normal methionine level and 2% FCS. Labeling with [32P]orthophosphate (50 ~Ci/ml) was for 2 h in phosphate-free DME containing 2% FCS.

Preparation and Analysis of UV-cross-linked Poly(A)* hnRNP: Irradiation of cells on culture dishes was carried out in PBS for 3 min at room temperature as recently described (11, 12). After UV irradiation, the PBS was removed and the cells were allowed to swell for 5 min in ice-cold RSB (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 1.5 mM MgCl2) containing the protease inhibitors aprotinin (0.5%; Sigma Chemical Co., St. Louis, MO), pepstatin A (1 ~g/ml), leupeptin (1 ~g/ml), and RNase inhibitors vanadyl-adenosine (10 mM) (4, 9). Samples were made 0.5% (vol/vol) Triton X-100, 0.1% SDS, 0.1% 5S RNA, 0.4% dextran sulfate, and 1% NaCl, and 1% bovine serum albumin (BSA). The cells were homogenized by four passages through a 25-gauge needle. Nuclei and cytoplasmic fractions were separated by low-speed centrifugation. The cytoplasmic fraction was discarded and the nuclei were resuspended in RSB and digested with DNase I (50 ~g/ml for 15 min at 37°C in the presence of 10 mM vanadyl-adenosine (11, 12). The DNPase I (DPFF grade, Worthington Biochemical Corp., Lakewood, NJ) was treated with iodoacetamide (51) and further purified on an aminophenylphosphoryluridine-2'(3')-phosphate-agarose column to remove RNase contamination (34). The nuclear fraction was then adjusted to 10 mM EDTA, 1% mercaptoethanol, and 0.5% SDS. After heating to 65°C for 5 min, rapid chilling, and addition of LiCl to 0.5 M, the nuclear material was incubated for 15 min with oligo(dT)-cellulose (Type 3, Collaborative Research Inc., Lexington, MA) with constant agitation. The oligo(dT)-cellulose was then packed in a column and washed with >10 column volumes of binding buffer (10 mM Tris-HCl, pH 7.4, 500 mM LiCl, 1 mM EDTA, 0.5% SDS). The performance of the column was monitored by liquid scintillation counting. The eluted poly(A)* material (in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.05% SDS) was reheat to 65°C for 5 min and the oligo(dT)-cellulose chromatography was repeated. The poly(A)* material was precipitated overnight at -20°C with 3 vol of ethanol.

RNAse Digestion of Poly(A)* RNP: The poly(A)* material was pelleted by centrifugation at 12,500 g and resuspended in 75 ~l of 10 mM Tris-HCl, pH 7.4, containing 1 mM CaCl2, and digestion with RNase was carried out with 25 ~g/ml pancreatic RNase A (Worthington Biochemical Corp.), and 400 ~U/ml micrococcal nuclease (P-L Biochemicals, Inc., Milwaukee, WI) for 60 min at 37°C (11, 12). To inhibit possible traces of protease, the pancreatic RNase A treatment was preceded by aprotinin (0.5 ~g/ml), pepstatin A (1 ~g/ml), and leupeptin (1 ~g/ml) (Sigma Chemical Co.) were included in the digestion mixture. After the RNase digestion, the proteins were precipitated by addition of 3 vol of ethanol at -20°C for at least 2 h.

SDS PAGE: Protein samples were electrophoresed on an SDS-contain-
RESULTS

A library of monoclonal antibody-secreting hybridomas was generated by fusion of SP2/O myeloma cells with spleen lymphocytes of mice immunized with in vivo UV-cross-linked protein-RNA complexes of poly(A)\(^+\)hnRNA and poly(A)\(^+\)mRNA. Of these hybridomas, 80 recognized the 41,000- and 43,000-mol-wt C proteins of HeLa cells as assayed by immunoblotting (12). One of these, designated 2B12, was further characterized and it was found to recognize the C proteins in human and monkey cells but not in lower vertebrate species (12). In an attempt to obtain an anti-C proteins antibody that would identify these proteins in other species, the culture supernatants of the colonies that recognized HeLa C, and C2 by immunoblotting were tested by immunofluorescence on chick embryo fibroblasts. One colony, designated 4F4, which was positive and provided nuclear staining, was recloned and expanded. Immunoprecipitations of HeLa cell material with 4F4 and 2B12 are shown in Fig. 1 and demonstrate that the two monoclonal antibodies recognized two similar polypeptides.

Immunofluorescence microscopy on HeLa cells (Fig. 2, b and d) indicates that the cellular distribution of the proteins recognized by 2B12 and 4F4 was also similar. In addition 4F4, unlike 2B12, stained other vertebrate cells and extended the previous observation (12) that the C proteins (Fig. 2) in vertebrates are segregated to the nucleus but are not found in nucleoli. Nucleoli can be stained with monoclonal antibodies using the same fixation and staining procedures (data not shown) and, therefore, the absence of nucleolar stain indeed reflects the absence from nucleoli of the C proteins. Furthermore, since 4F4 is positive in human cells and in lizard cells whereas 2B12 is positive only in higher mammals (Fig. 2), the two monoclonal antibodies are apparently directed against two different epitopes. To determine whether the two antigenic sites reside on the same polypeptide chain of the 41,000- and the 43,000-mol-wt proteins (C1 and C2), or whether they are on different polypeptides of similar molecular weights, the antibodies were assayed by crossed immunoprecipitation-immunoblotting assay. HeLa cell material was immunoprecipitated with either 2B12 or 4F4, resolved by SDS PAGE, and electroblotted, and the blot was probed for each immunoprecipitation with the other antibody (Fig. 3). Since each antibody recognized the same two polypeptides that are immunoprecipitated by the other, the epitopes the two antibodies recognize must be on the same polypeptide chains.

That two different monoclonal antibodies recognize both C1 and C2 further suggests that the two polypeptides are highly related. To examine this relatedness, we purified the 41,000-mol-wt polypeptide (C1) from HeLa cells by immunoprecipitation with 2B12 and SDS PAGE. The 41,000-mol-wt band was excised from the gel and used to raise antibodies in mice. Immunoblotting analysis with the mouse polyclonal antibody (Fig. 4) shows that these polyclonal antibodies also recognized both 41,000- and 43,000-mol-wt components.

The immunofluorescence data (Fig. 2) indicated that 4F4 would be useful as a probe for the identification of the C proteins in different species. An immunoblot showing the proteins detected by 4F4 in different vertebrate species is shown in Fig. 5. It is clear that proteins corresponding to the C proteins which were initially defined in human HeLa cells (6) are found also in other vertebrates. Furthermore, in all species tested, at least two related proteins were detected. The size of the C proteins was not entirely conserved. The hamster C proteins (42,000 and 45,000 mol wt) were found to be slightly larger, and the chicken C proteins (39,000 and 40,000 mol wt) slightly smaller, than the C proteins of primates. The lizard C proteins were a closely spaced doublet at 41,000-mol wt. The mouse C proteins had a similar size to those found in human, monkey, and bovine cells, but 4F4 reacted with them at very low avidity (data not shown). Occasionally, 4F4 also reacted with lower molecular weight polypeptides. In hamster cells (from both baby hamster kidney and Chinese hamster ovary lines) a minor band at 41,500 mol wt was consistently seen in addition to the major ones at 42,000 and 45,000 mol wt. Since the lower molecular weight forms are somewhat variable, they probably represent proteolytic fragments of the C proteins. These forms are usually much more pronounced after immunoprecipitation experiments (cf. Fig. 8). No signal could be detected by either immunoblotting or immunofluorescence with 4F4 in Xenopus laevis cultured cells (data not shown).

The C proteins of HeLa cells have been shown by UV cross-linking to be in contact with hnRNA in vivo (12). To determine if this is also the case in other species, we carried out UV cross-linking in hamster and chicken cells. The cross-linked poly(A)\(^+\)hnRNP fraction from these cells was selected, digested with RNase, and probed by immunoblotting with 4F4 after SDS PAGE. The results, shown in Fig. 6, demonstrate that, as is the case in HeLa cells, the C proteins in other species are in contact with poly(A)\(^+\)hnRNA in vivo. The bands at higher molecular weight are presumably homotypic or heterotypic oligomeric forms of the C proteins (12).

The immunofluorescence microscopy (Fig. 2) and the UV cross-linking data indicate that the C proteins are concentrated in the nucleus where they interact with hnRNA and are excluded from nucleoli. Immunofluorescence microscopy of rat kangaroo cells Pk2 (Fig. 7) shows that chromosomes...
in prophase cells did not stain. It can therefore be concluded that although hnRNP proteins, possibly including the C proteins, probably associate with chromatin-bound nascent RNA polymerase II transcripts (13), they nevertheless are not bona fide chromatin proteins. The hnRNP C proteins in human HeLa cells have previously been shown to be phosphorylated in vitro (18, 28) and in vivo (12, 18). The autoradiogram shown in Fig. 8 demon-
FIGURE 3 Immunoprecipitation and cross-immunoblotting of 41,000- and 43,000-mol-wt proteins (41 and 43) with 2B12 and 4F4. Immunoprecipitations were from unlabeled HeLa cells. The immunoprecipitate was dissolved in sample buffer and subjected to SDS-PAGE and blotted. Immunoblot analysis was carried out as described in Materials and Methods and antibodies iodinated directly were used. (A), 41,000- and 43,000-mol-wt proteins were immunoprecipitated with 2B12 (1) or with SP2/O (2) and probed with 4F4. (B) The two proteins were immunoprecipitated with 4F4 (1) or with SP2/O and probed with 2B12.

FIGURE 4 Immunoblot analysis of total cellular material of HeLa (human) and monkey CV-1 (monkey) with anti-41,000-mol-wt protein polyclonal antibodies. These antibodies were prepared as ascitic fluid as described in Materials and Methods.

FIGURE 5 Identification of group C proteins of hnRNP in different vertebrate cells with monoclonal antibody 4F4. Cells grown in culture were washed twice with PBS and dissolved in SDS-PAGE sample buffer. An aliquot was resolved by SDS-PAGE and immunoblotting was carried out as described in Materials and Methods. The cell lines used were: HeLa (human), CV-1 (monkey), Madin-Darby bovine kidney (bovine), Chinese hamster ovary (hamster), MSB (chicken), and Anolis carolinensis myogenic cells (lizard). Values at left, molecular weight x 10^-3.

FIGURE 6 Immunoblot analysis of group C proteins cross-linked in vivo to poly(A)+hnRNA by UV irradiation of intact cells in different species. Cells grown in monolayer were irradiated for 3 min with UV and the nuclei were isolated as described in the text. The poly(A)+hnRNA was selected by oligo(dT)-cellulose chromatography and digested with RNAses, and the released proteins were resolved by SDS-PAGE, blotted onto nitrocellulose paper, and probed with monoclonal antibody 4F4.
FIGURE 7 Immunofluorescence microscopy of rat kangaroo cells PtK2 with 4F4. a, phase contrast; b, immunofluorescence. × 630.

...strates that, as in HeLa cells, the C proteins of hnRNP in other species are also phosphorylated in vivo. Human, hamster, and chicken cells were incubated with $[^{32}\text{P}]$orthophosphate and the C proteins were immunoprecipitated from the disrupted cells in the presence of phosphatase inhibitors. The labeling experiment shown in Fig. 8 was carried out in the presence of 5 μg/ml actinomycin D to inhibit transcription of RNA polymerase II. This was found to be helpful because otherwise the background was too high as a result of the RNA that was precipitated with the C proteins. However, there was no effect on the intensity of the label in the C proteins due to actinomycin D (Y. D. Choi and G. Dreyfuss, unpublished results).

DISCUSSION

The most prominent structural feature of hnRNP complexes described so far is the proteinaceous 40S subparticles. Of the three recognized subgroups of proteins that are major components of the 40S particles (A, B, and C), the C proteins are unique. They remain associated with the hnRNA in vitro under salt conditions where the other subgroup proteins dissociate (6), they are phosphorylated (12, 18, 28), and they are acidic whereas the others are basic (6, 12). The classification of the A, B, and C proteins was based on observations made in human HeLa cells and likely candidates were pointed out also in rodents (6). The data obtained with the monoclonal antibody 4F4 demonstrate that proteins similar to the human C proteins are found across vertebrate species and the antibody defines them from humans to reptiles. Although the C proteins in the different species must have some common and highly conserved features, such as the antigenic site recognized by 4F4 and an hnRNA binding site, they are not completely conserved across vertebrates. Their apparent molecular weight in different species can differ by a few thousand per polypeptide chain (approximately a 40 amino acid size difference between the chicken and hamster C2 proteins) and the site against which antibody 2B12 is directed is conserved only in higher mammals. This is somewhat surprising because other proteins that are part of a complex higher-order structure (e.g., histones, actins, and tubulins) are usually of almost identical size in different species. Other proteins of the 40S subparticle of hnRNP, which most likely correspond to the A and B proteins, were recently identified in different species using polyclonal and monoclonal antibodies (19, 27, 31), and these also differ in size between human, rodent, and avian cells (27).

The two C proteins, C1 and C2, are very similar to each other and share antigenic sites. In all cases, with every anti-
body so far encountered, the same antibody recognizes both proteins. In addition, their partial peptide maps are somewhat similar to poly(A)+ and poly(A)-hnRNA in vivo. The immunofluorescence microscopy data on the C proteins, the 120,000-Mr complexes of the C proteins are indicated by arrowheads. 1, immunoprecipitations with 4F4; 2, control immunoprecipitations with Sp 2/0.

As is the case in HeLa cells (12, 18, 28), the C proteins in other species are also phosphorylated and are associated with both poly(A)* and poly(A) hnRNA in vivo. The immunofluorescence microscopy data on the C proteins, the 120,000-Mr complexes of the C proteins are indicated by arrowheads. 1, immunoprecipitations with 4F4; 2, control immunoprecipitations with Sp 2/0.

Within the nucleus these hnRNPs proteins are excluded from nucleoli, which suggests that they are not involved in ribosomal RNA transcription or processing. The immunofluorescence microscopy of PtK2 cells in prophase indicate that the C proteins and possibly also the other major hnRNPs proteins are not chromatins proteins although they are likely to be associated with actively transcribing regions through association with nascent hnRNPs transcripts.

The data presented here demonstrate that C1 and C2 have conserved properties across vertebrates. Taken together with the recent findings of Lesser et al. (27) on the relative conservation of the A and B proteins, they suggest that higher-order structures of hnRNA with nuclear proteins are highly conserved in these species.

We are grateful to Ermone J. Hussain and Anjali Patel for excellent technical assistance. Empigen BB was a generous gift from Albright and Wilson, Ltd. (Marchon Division).

This work was supported by grants from the National Institutes of Health (GM3188), the National Science Foundation, the Leukemia Research Foundation, Inc., and the Searle Leadership Fund.

Received for publication 1 June 1984.

REFERENCES


