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The nucleolar RNA-binding protein B-36 is highly conserved among plants

Mark J. Guiltinan[a], Margaret E. Schelling[b], Nasreen Z. Ehtesham[a], John C. Thomas[a], Mark E. Christensen[1)][a]

[a] Department of Biology, Texas A & M University, College Station, TX/USA
[b] Department of Medical Physiology, Texas A & M University, College Station, TX/USA

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The nucleolar protein B-36 is an RNA-associated protein which has a number of properties in common with pre-mRNA-binding proteins (hnRNP proteins). Like the hnRNP proteins, B-36 appears to be evolutionarily conserved among various eukaryotes (protists and several animal species). The conservation of B-36 throughout the plant kingdom has been investigated using a panel of nine monoclonal antibodies previously shown to recognize a minimum of four different epitopes in Physarum B-36, the protein used to generate the monoclonal antibodies. Two of the epitopes (I and III) are widely conserved in 34 kDa proteins (presumably B-36 homologues) from the various species tested (Chlamydomonas, moss, fern, oat, onion, carrot, and bean). Using immunofluorescence localization in moss and carrot protoplasts, the cross-reacting proteins were shown to be restricted to the nucleolus, further confirming their probable homology to B-36. Epitopes I and III are also unique to the B-36 homologues as demonstrated by the failure of any other bands to cross-react. Another epitope (IV) was specifically recognized in the plant B-36 homologues but exhibited greatly reduced affinity for the monoclonal antibody relative to Physarum B-36. The remaining epitope (II), unlike the others, exhibited variable conservation in the plant B-36 homologues and, in addition, was present in several other seemingly unrelated proteins. Finally, several of the plant species exhibited two cross-reacting variants at roughly the 34 kDa position and in at least one of these cases a single monoclonal antibody was able to distinguish between the two variants, a result indicating that the variants do have bona fide structural differences. These results extend the demonstration of the high degree of conservation of B-36 to higher and lower plants and suggest that the protein is conserved throughout all eukaryotes; i.e., any organism which exhibits a nucleolar organization of the ribosomal gene transcription units.

Introduction

B-36 is a major nucleolar protein of 34-kDa molecular weight originally characterized from the slime mold Physarum polycephalum [3, 10, 19]. This protein is similar in many of its properties to structural proteins (A1, A2 and B1 core proteins) which are associated with mRNA precursors to form heterogeneous nuclear ribonucleoprotein (hnRNP) particles in higher eukaryotes [1, 3, 4, 9]. On this basis it is suggested that B-36 associates with newly synthesized pre-rRNA and performs an analogous structural role during the early stages of rRNA processing.

A possible rat homologue of B-36 protein (termed fibrillarin) has been found independently as a major antigen recognized by autoimmune sera from patients with scleroderma [11, 14]. In addition to several biochemical similarities [11], purified fibrillarin cross-reacts with several of the monoclonal antibodies raised against B-36 [5]. Fibrillarin was named for its localization in the "fibrillar" region of the nucleolus using the autoimmune antibodies [14], a localization also observed for B-36 by using a monoclonal antibody together with immunogold labeling (manuscript submitted). This pattern of localization is consistent with the protein's predicted association with newly transcribed rRNA molecules since the fibrillar region is enriched for such molecules.

A panel of monoclonal antibodies (Mabs), produced against purified B-36, has been found to have remarkable specificity for B-36 protein [5] and has been used to demonstrate that B-36 is a highly conserved protein among all the protist and animal species examined (rat, HeLa cells, Drosophila, dinoflagellates, [5] and unpublished observations). The only organism that has failed to exhibit a specific 34-kDa cross-reacting protein with the Mabs has been Escherichia coli. Although higher plants appear to possess a nucleolar protein with an epitope recognized by the anti-fibrillarin autoimmune antisera [15, 18], no characterization of the specific antigen(s) has been done. In order to evaluate the distribution and degree of conservation of B-36 among plants, the panel of nine Mabs previously generated against B-36 [5] has been tested against nuclear protein extracts of algal and several lower and higher plants. It is observed that B-36 is conserved in all the plant species tested and that three of the four epitopes studied are uniquely conserved in the 34-kDa homologues. This study also reveals the presence of B-36 microheterogeneity within several of the plant species investigated.

Materials and methods

Plant species and tissues

The following tissues were frozen in liquid nitrogen and stored at −80 °C prior to protein extraction: immature cotyledons of Pha-

[1)] Dr. Mark E. Christensen, Department of Biology, Texas A & M University, College Station, TX 77843/USA.

seolus vulgaris cv. Tendergreen (bean), cultured embryos of Daucus carota cv. Dv. 126 (carrot), roots of Avena sativa (oat) and Allium cepa (onion), immature leaves of Nephrolepis exalata (boston fern), thalli of local moss species Vetrichum heterostichum, and protoplasts of Chlamydomonas reinhardtii. For control purposes, a nuclear extract from microplasmodia of Physarum polycephalum (wild-type strain obtained from Carolina Biological Supply, Burlington, NC/USA) was also used.

Carrot protoplasts were prepared according to the procedure of Wallin and Eriksson [20]. Protoplasts from protonemata of the moss Polytrichum (Carolina Biological Supply) were prepared according to the procedure of Tanchak and Fowke [16].

Monoclonal antibodies

Nine Mabs representing at least four different epitopes (Epitope I - Mabs P1C5, P1D10, P1D11, P2B11, P2D7, and P2G1; Epitope II - Mab P1G12; Epitope III - Mab P2G3; and Epitope IV - Mab P3F11) in B-36 have been previously characterized [5]; all recognize purified B-36. Furthermore, Epitopes I, III and IV are unique to B-36 since they do not cross-react with any other Physarum proteins as tested in Western blots of total protein. Epitope II, in contrast, is found in several other nuclear proteins in Physarum. The six Mabs designated as recognizing Epitope I have been classified together due to their common pattern of reaction with the V8 protease digestion products of purified B-36 [5], and it therefore remains possible that one or more of these may be directed against different but adjacent epitopes.

Preparation of nuclear extracts

Tissues were extracted by a modification of the method of Dignam [6]. Briefly, tissues were ground with a mortar and pestle in the presence of liquid nitrogen and then homogenized using a Polytron tissue homogenizer (3 pulses for 30 s, setting of 8, with 30-s rests on ice between pulses) in 10 volumes (w/v) of buffer H (0.44 M sucrose, 2.5% Ficoll, 5% Dextran T-40, 25 mM Tris-HCl, *p*H 8.5, 10 mM $MgCl_2$, 10 mM beta-mercaptoethanol, 0.5% Triton X-100 (v/v), 2 mM spermine with the following protease inhibitors: 1 μg/ml alpha-2-macroglobulin, 10 μg/ml aprotinin, 10 μg/ml antipain, 10 μg/ml leupeptin and 0.5 mM phenylmethylsulfonyl fluoride; PMSF). The homogenate was filtered through three layers of 40-μm nitex screen, then centrifuged for 5 min at 3900*g* at 4 °C. The pellet was resuspended in 5 volumes of buffer H (no spermine), centrifuged again; the pellet resuspended in 1 volume of buffer C (20 mM Hepes, *p*H 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol (DTT)), homogenized ten strokes in a "Dounce" homogenizer using a "B" pestle and then rocked gently for 30 min at 4 °C. The solution was centrifuged for 30 min at 26900*g*, 4 °C, and the supernatants were dialyzed at 4 °C for 2 h against 50 volumes of buffer D (20 mM Hepes, *p*H 7.9, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF and 0.5 mM DTT). The resulting solution was frozen and stored at −80 °C. Protein concentrations were measured by the method of Bradford [2] and were between 0.1 to 1 μg/ml.

This method differs from the original Dignam procedure by the use of an initial homogenization buffer optimized for plant nuclear isolation (H buffer, [12]) but adjusted to *p*H 8.5 and containing protease inhibitors shown to be effective in the isolation of functional proteins from plants [13]. Fluorescence microscopy of ethidium bromide stained preparations was used to determine the presence and integrity of nuclei. Typically, many nuclei and/or nuclear fragments were observed.

Electrophoretic separation of proteins

Proteins present in the nuclear extracts were precipitated by the addition of 2.5 volumes of ethanol and storage at −20 °C overnight. The precipitates were collected by centrifugation at 16000*g* for 10 min. The pellets were washed once with ethanol, dried until only slightly damp, and dissolved in sodium dodecyl sulfate (SDS) sample buffer (0.1% SDS, 0.25 M sucrose, 10 mM sodium phosphate, *p*H 7.6, 0.1% beta-mercaptoethanol). Samples were heated in a boiling water bath for 3 min and electrophoresed in 0.75 mm, 12.5% acrylamide SDS gels essentially according to Laemmli [7], with minor modifications [8].

Electrophoretic transfer to nitrocellulose

Identical sets of electrophoretically separated plant proteins were transferred to nitrocellulose using the buffer system described by Towbin et al. [17] with the exception that 0.1% SDS was added. Transfer was done for 1 h using an American Bionetics Polyblot unit at a current density of 2.5 mA/cm^2.

Western immunoassay using B-36 Mabs

Nitrocellulose strips containing the transferred proteins were rinsed briefly in TBS (Tris-buffered saline: 20 mM Tris, *p*H 7.5, 0.5 M NaCl) and blocked for 30 min in 3% gelatin/TBS. The strips were then incubated overnight (approx. 15 h) with a specific Mab supernatant diluted 1:1 with 1% gelatin/TBS. Following Mab incubation, the strips were washed several times briefly with dH_2O and two times with TBS, 10 min each. The strips were subsequently incubated for 1 h with alkaline phosphatase-conjugated goat anti-mouse IgG (Promega Biotec, Madison, WI or Bio-Rad Labs, Richmond, CA) diluted 1:5000 in 1% gelatin/TBS, washed as above, and finally treated with the NBT and BCIP color development mixture (Promega Biotec) for 30 min. All steps in the assay were performed at room temperature and with constant gentle agitation using a reciprocal shaker. Following the 30-min incubation, the strips were rinsed thoroughly with dH_2O and dried under a heat lamp for 10 min in order to effectively stop the color development reaction and to prepare the strips for storage or photography.

Immunofluorescence localization of B-36

Protoplasts were placed on clean uncoated glass slides, covered with a coverslip, and gently squashed by application of pressure to the coverslip. The slides were immediately frozen in liquid nitrogen and, following quick removal of the coverslip while still frozen, the slides were placed in 95% ethanol containing 3.7% formaldehyde (in an ice bath). After a fixation of 5 min, the slides were transferred to acetone and treated for 5 min, also in an ice bath. The slides were carried through three, 3-min washes in PBS (phosphate-buffered saline: 0.14 M NaCl, 10 mM Na_2HPO_4, *p*H 7.2) and a single, 1-min wash in 3% Tween 20. These washes and all subsequent steps were carried out at room temperature. Following a rinse in PBS, the slides were incubated for 1 h in Mab P2G3 diluted 1:9 with PBS containing 0.5% bovine serum albumin (BSA). The slides were washed three times in PBS, incubated for 30 min with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Pel-Freez Biologicals, Rogers, AZ) diluted 1:50 in PBS containing 0.5% BSA, and washed again three times with PBS. Coverslips were mounted on the slides using 10 μl of 50% glycerol in PBS. The mounting medium also contained 1 μg/ml Hoechst stain no. 33342 (Sigma Chemical Co., St. Louis, MO) in order to detect DNA-containing structures (e.g., nuclei).

Results

Two epitopes in B-36 are highly conserved in plants

B-36 Mabs recognizing four different epitopes (I, II, III, and IV) were tested for cross-reactivity with proteins in

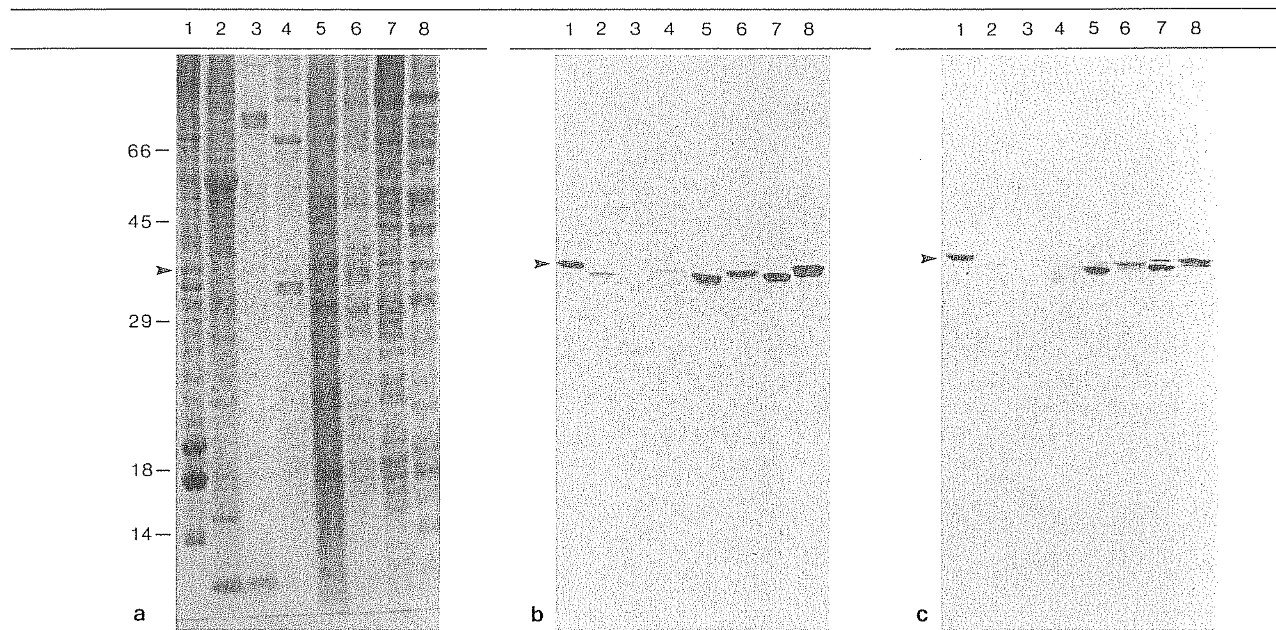

Fig. 1. Western immunoblot analysis of plant nuclear proteins using B-36 monoclonal antibodies specific for Epitope I. Proteins of nuclear extracts were run in 12.5% polyacrylamide gels in triplicate (*slot 1*, Physarum; *2*, Chlamydomonas; *3*, moss; *4*, fern; *5*, oat; *6*, onion; *7*, carrot; *8*, bean). One set was stained with Coomassie Blue (**a**), while the remaining two replicates were transferred to nitrocellulose paper and tested for cross-reactivity with two of the Epitope I Mabs: P1D10 (**b**) and P2B11 (**c**). The position of B-36 from Physarum is denoted in each case by the *arrowhead*. Positions of molecular weight standards (bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa; betalactoglobulin, 18 kDa; and lysozyme, 14 kDa) are given to the left.

plant nuclear extracts prepared from *(1)* Physarum plasmodia (the homologous source of the B-36 to which the Mabs are directed), *(2)* the green alga Chlamydomonas, *(3)* moss thalli, *(4)* fern immature leaves, *(5)* oat roots, *(6)* onion root-tips, *(7)* carrot embryos from tissue culture, and *(8)* bean cotyledons. The proteins were electrophoretically separated (Fig. 1a), transferred to nitrocellulose, and tested for cross-reaction with each Mab using an immunoblot assay.

Due to the diverse nature of the plant species and tissues used, it was not meaningful to standardize the protein loaded per gel slot on the basis of starting tissue weight, nor was it practical to determine the number of nuclei represented in each preparation. Furthermore, the transcriptional status of any given cell is known to influence the amount of B-36 present in its nucleus (unpublished observations). Therefore, since this investigation was not aimed at obtaining a quantitative comparison of B-36 among the different plants, the extracts were roughly equalized to the same amount of protein per slot. This was accomplished by running a preliminary gel of maximally concentrated protein samples and, on the basis of visual inspection of the resulting profiles, adjusting the samples accordingly. Due to very low yields for two of the plants (moss and fern) it was not possible to adjust the samples totally, and therefore the amount of protein loaded was less than for the other plant samples (Fig. 1a).

Two epitopes (I and III) are apparently completely conserved as shown by a specific and strong cross-reaction with polypeptides of nearly the same mobility as Physarum B-36 (Figs. 1b, c Epitope I Mabs P1D10 and P2B11, respectively; Fig. 2b Epitope III Mab P2G3). The four other Epitope I Mabs gave reaction patterns identical to that shown in Figure 1b. The moss sample failed to show a cross-reacting band (Figs. 1b, c, *slot 3*), but the lack of a reaction may be attributable to a low antigen level rather than to the absence of B-36-cross-reacting epitopes. To test for the presence of a B-36 homologue in moss, without having to rely on protein extraction, moss protoplasts were assayed directly by immunofluorescence. Using Mab P2G3 which recognizes the highly conserved Epitope III, a weak, but specific, nucleolar fluorescence was seen in intact protoplasts (Figs. 3e–g). The weakness of the reaction is probably due to poor accessibility to the antibody since, in cases where the protoplast was broken open and the nucleus clearly exposed (Figs. 3h–j), a much stronger signal was observed. Thus, moss does contain a B-36 homologue.

Epitope II exhibits a variable degree of conservation

The reactions obtained with the Epitope II Mab P1G12 show that this epitope is conserved in the 34-kDa band(s) of three of the higher plant species tested (onion, carrot and bean; Fig. 2a, *slots 6–8*, respectively) but is not conserved in the corresponding bands of oat (Fig. 2a, *slot 5*), fern (Fig. 2a, *slot 4*) or Chlamydomonas (Fig. 2a, *slot 2*) extracts. This epitope, unlike the others, is not entirely

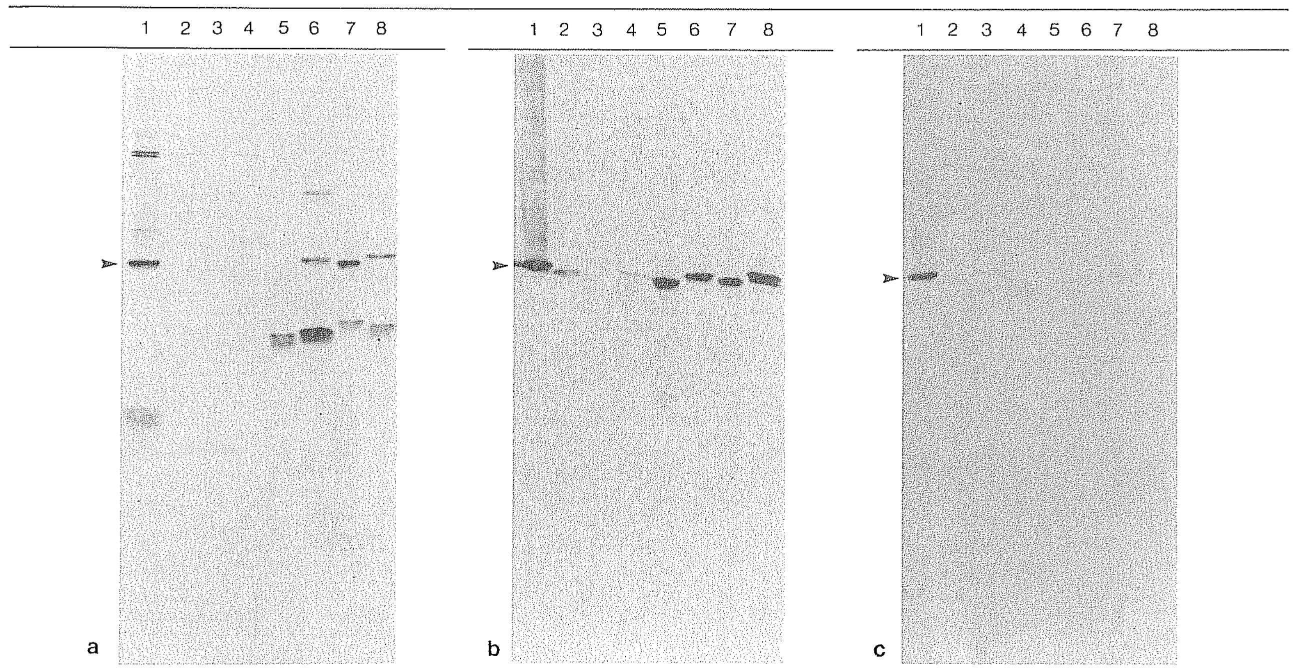

Fig. 2. Western immunoblot analysis of plant nuclear proteins using B-36 monoclonal antibodies recognizing Epitopes II, III and IV. Three nitrocellulose-bound replicas of the plant proteins were prepared as described under Figure 1 (*slot 1*, Physarum; *2*, Chlamydomonas; *3*, moss; *4*, fern; *5*, oat; *6*, onion; *7*, carrot; *8*, bean). These were treated with the Epitope II Mab P1G12 (**a**), the Epitope III Mab P2G3 (**b**), and the Epitope IV Mab P3F11 (**c**), respectively. The position of B-36 from Physarum is indicated by the *arrowheads*.

unique to B-36 among total Physarum proteins [5] but rather is found as well in several polypeptides of higher molecular weight (Fig. 2a, *slot 1*), i.e., a weakly reacting doublet at 38 kDa and a strongly reacting doublet at 55 kDa. It is interesting that, although Chlamydomonas and fern do not exhibit a 34 kDa cross-reacting band, both have a single cross-reacting band comigrating with the 55-kDa doublet seen in Physarum. In the seed plants, however, the 55-kDa band is not seen, and a new cross-reacting band of approximately 25 kDa appears. Due to the similarity in mobilities of the 25-kDa band in all of the seed plants and the lack of cross-reaction with any other bands, it is likely that the 25-kDa polypeptide is conserved in the seed plants. This band is not simply a degradation product of B-36 since the Mab P1G12, which reveals the 25-kDa band, does not react with the intact 34-kDa B-36 protein in oats but does react with the 25-kDa band in oats (Fig. 2a, *slot 5*).

Epitope IV Mab P3F11 exhibits specificity but reduced affinity

Mab P3F11 produced only a faint reaction with the 34-kDa bands of the various plant extracts (Fig. 2c), whereas its reaction with the homologous Physarum B-36 band (Fig. 2c, *slot 1*) was as intense as for any of the other Mabs. Since the faint cross-reaction at 34 kDa was seen in all the plant extracts whereas no other reactions were detected throughout the rest of the nitrocellulose strip, it is likely that this Mab is specifically recognizing a partially conserved epitope, i.e., one that has undergone an evolutionary change to a lower affinity than the one present in Physarum B-36. This characteristically weak reaction with Mab P3F11 was reproduced several times and was therefore not an artifact of a given Western assay.

Mab cross-reactions reveal microheterogeneity in the B-36 protein

In analyzing the cross-reacting 34-kDa "B-36" homologues in the various plant extracts, it was observed that the mobility of the cross-reacting band for a given extract was either very close to that of Physarum B-36 or slightly faster. Interestingly, in several cases there were cross-reacting bands at both of these mobilities within the same extract, a microheterogeneity most apparent in the bean cotyledon extract where a cross-reacting doublet was observed with both the Epitope I and III Mabs (e.g., Fig. 1b, *slot 8*, Fig. 2b, *slot 8*). Only the upper band of this doublet was able to cross-react with the Epitope II Mab P1G12 (Fig. 2a, *slot 8*), a result consistent with the less conserved nature of the epitope recognized in this case. This is of additional interest because the results show that this epitope is present in one isoform of B-36, but not the other, within the same species.

In carrot, the upper band (that with lower mobility) was only detected with Mab P2B11 specific for Epitope I (Fig. 1c, *slot 7*), while the other Mabs defined as specific for Epitope I failed to react with that band. This result suggests that Mab P2B11 may be directed against an epitope

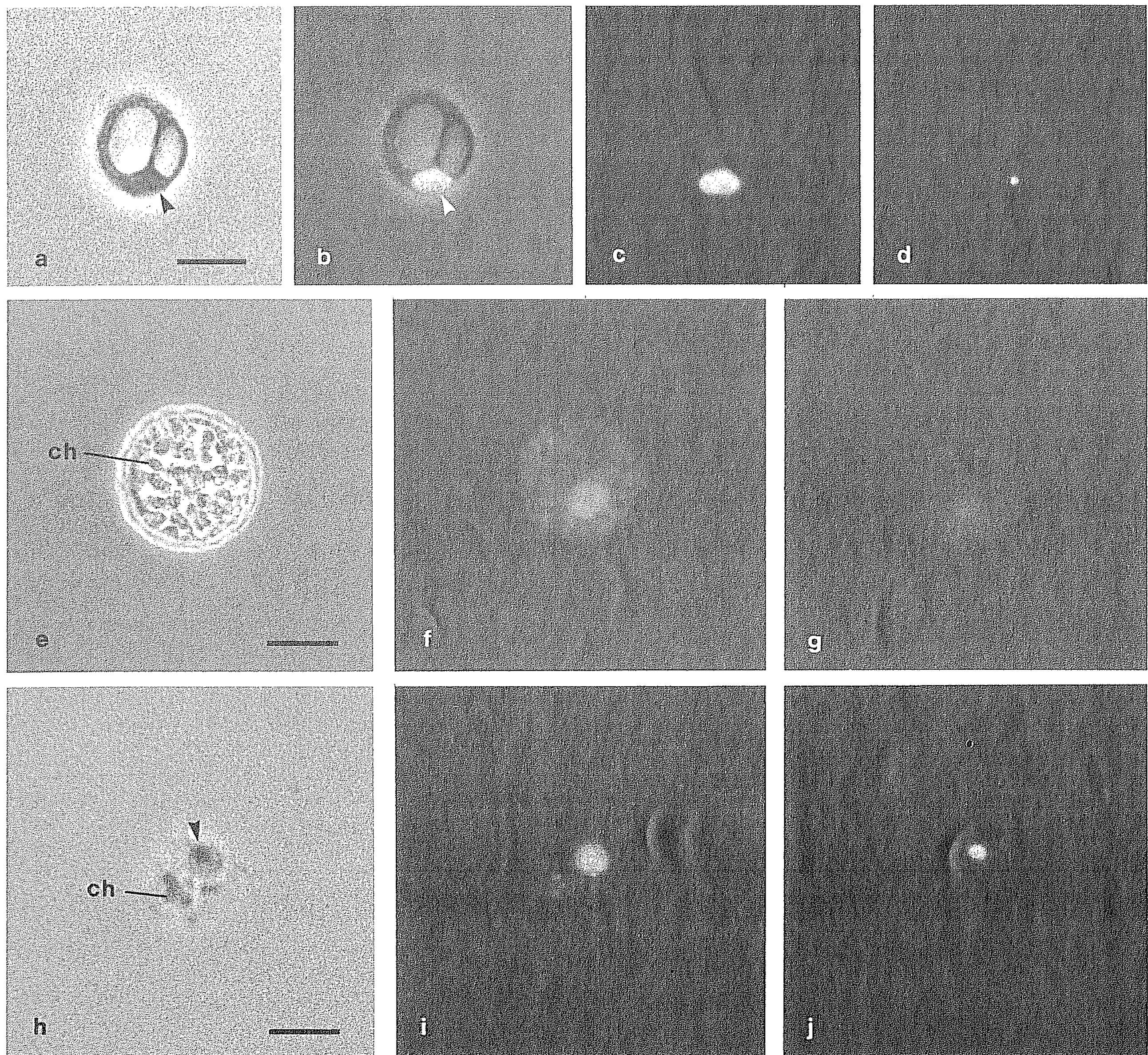

Fig. 3. Immunofluorescence localization in carrot and moss protoplasts. Carrot (**a–d**) or moss (**e–j**) protoplasts were fixed onto glass slides and treated with B-36 Mab P2G3. Bound antibodies were localized using FITC-conjugated goat anti-mouse antibodies. For the fields of protoplasts shown, **a**, **e**, **h** are phase-contrast images and **c**, **f**, **i** represent staining of nuclei and chloroplasts (ch) using the fluorescent DNA-binding dye Hoechst no. 33342. In (**b**) the phase contrast and Hoechst nuclear fluorescence images are superimposed. The right panels (**d**, **g**, **j**) represent the immunofluorescence localization of B-36 homologues using Mab P2G3 (specific for Epitope III). The nucleoli in several of the panels are denoted by *arrowheads*. — Bars 10 µm.

different from the one recognized by the other Epitope I Mabs. These Mabs were initially placed in a single epitope class on the basis of a similar reaction pattern with the V8 protease digestion products of B-36 [5]. However, two adjacent but distinct epitopes might be scored as a single epitope using this method of characterization. It follows therefore that Mab P2B11 may be directed against a fifth epitope, located near Epitope I in the protein.

B-36 is localized exclusively in the nucleoli of carrot and moss protoplasts

The fact that at least four different epitopes of B-36 are conserved specifically in 34-kDa polypeptides from a wide variety of plant species argues strongly that the cross-reacting protein in each case is the true homologue of B-36. However, to confirm this, the predicted nucleolar localiza-

tion was tested in a higher plant. B-36 was localized in carrot protoplasts using Epitope III Mab P2G3 together with fluorescent-conjugated second antibodies. Only a single Mab need be tested since the results of the Western assays clearly show that the same bands are reacting for each of the Mabs. P2G3 was chosen since it previously gave excellent immunofluorescence in Physarum and rat liver nucleoli [5]; several of the other Mabs gave poor signals in immunofluorescence in Physarum (unpublished observations) and therefore may not be good choices for studying localization in other species.

In Figure 3a a single carrot protoplast containing two large vacuoles is shown. The nucleus is located at the lower periphery of the protoplast as indicated by its staining with the DNA-specific Hoechst stain (Figs. 3b, c). The nucleolus is a dark circle within the nucleus when observed with phase contrast (Fig. 3a) and appears as a 'shadow' in fluorescence (Figs. 3b, c). The immunofluorescence localization of B-36 is clearly confined to the nucleolus (Fig. 3d). These findings confirm that the 34-kDa cross-reacting protein of the carrot extract is strictly nucleolar in localization, further substantiating it as the carrot B-36 homologue.

Discussion

B-36 protein was originally described in the slime mold Physarum polycephalum [3, 10]. However, with the production of anti-B-36 polyclonal sera this protein was shown to have probable homologues in several other eukaryotes including Drosophila [4]. The extensive degree to which B-36 is conserved among various lower and higher organisms (protists and animals) was more fully appreciated when a highly specific set of monoclonal antibody probes, representing several different epitopes, was produced and found to react uniquely with B-36 homologues in various species tested [5].

In this study, the strong conservation of B-36 is extended throughout the plant kingdom as well. Two epitopes (I and III) were found, using a Western blot assay, to be conserved in roughly 34-kDa proteins in all but one of the plant species tested. Moss failed to give a reaction in the Western blot assay with any of the Mabs, a result attributed to low protein level present during the assays. This interpretation was subsequently confirmed when a specific nucleolar immunofluorescence signal was obtained in moss protoplasts using the Epitope III Mab P2G3 and permits us to conclude that Epitope III is conserved in B-36 in all the plants tested, including moss. A third epitope (II) was conserved in the B-36 homologues in only three of the plants tested. The fourth epitope (IV), while exhibiting significantly reduced affinity, was still specifically recognized in all of the species except moss. These findings, together with previously published results using animal species, indicate that B-36 from widely varying eukaryotes is subject to a high level of evolutionary conservation. B-36 represents the most highly conserved nucleolar protein now known.

Two recently published reports showed that scleroderma-associated autoimmune sera which recognize the mammalian B-36 homologue, fibrillarin, reacted with nucleolar antigens in onion [15, 18] and soybean [18]. However, only immunofluorescence localization was done in both cases, and the identity of the cross-reacting antigen as the 34-kDa protein was never established. As a result, the possible cross-reaction of the autoimmune sera with other nucleolar proteins in the plant tissues could not be ruled out. Scleroderma autoimmune sera are often found to react with a variety of nucleolar proteins, in addition to the 34-kDa species.

The high degree of conservation of B-36 indicates a significant function in some nucleolar process. It is postulated that the protein has a role in the early posttranscriptional packaging of the pre-rRNA precursor since it shares several properties with a family of moderately conserved structural proteins which bind to pre-mRNA in the nucleoplasm, the hnRNP proteins [1, 3, 4, 9]. A number of independent approaches have shown B-36 to be associated with RNA in the nucleolus [5], and recent immunoprecipitation experiments using nuclear extracts prepared from Physarum have shown that B-36 is bound to pre-rRNA sequences (unpublished observations). If B-36 serves a packaging role it would presumably make numerous protein-protein and protein-nucleic acid interactions. Such interactions, assuming they are essential for the proper processing of rRNA or for some step in ribosome formation, could account for the high degree of conservation of the protein's structure. This is reminiscent of the highly conserved nature of the histones which make many complex interactions in organizing the nucleosome. Precisely how the conservation of B-36 might compare to other conserved families of proteins awaits compilation of sequence data from several divergent species. Such data are not yet available, but efforts to isolate cDNA clones for Physarum B-36 are underway.

Another important observation made in this study is that B-36 exhibits microheterogeneity in several of the species tested. This was particularly evident in the case of the bean. The microheterogeneity generally consisted of an additional band of slightly greater mobility than the main B-36 band (based on using the mobility of Physarum B-36 as a standard). This additional band is probably not an artifact of electrophoresis since in the case of the bean both bands reacted with Epitope I and III Mabs whereas only the upper band reacted with the Epitope II Mab. Furthermore, in the case of carrot the upper band was only detected by a single Mab (P2B11). Two forms of B-36 exhibiting similar mobility have previously been observed in Physarum, but only when longer gels were run in order to increase band separation. Multiple electrophoretic forms for this protein are likely due to differential posttranslational modification (this protein is subject to extensive arginine methylation) of a single gene product or to the presence of several related gene products.

The Epitope II Mab P1G12 gave results that were interesting for several reasons. First, the epitope recognized by this antibody has apparently been lost in the B-36 homologues from Chlamydomonas, fern and oat, but is conserved in the B-36 homologues from three of the higher plants tested (onion, carrot and bean). Secondly, unlike the

other epitopes (I, III and IV) which are apparently unique to the 34-kDa B-36 homologues, Epitope II is present in several other plant proteins of widely varying sizes. This has been previously observed in Physarum as well [5]. Two cross-reacting species, with molecular weights estimated to be 55 and 25 kDa, gave particularly interesting distributions. The 55-kDa band was observed in Physarum, Chlamydomonas and fern, but was absent from the four seed plants. In contrast, the seed plants all exhibited a 25-kDa band not seen in Physarum, Chlamydomonas or fern. What relationship these proteins have with each other or with B-36 is not known, other than the fact that they all share a common structural feature containing Epitope II. Current work to map and characterize Epitopes I to IV in Physarum B-36 protein should aid in determining functionally significant domains conserved in this and possibly related proteins.

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