Members of a family of proteins (the RD family) detected by a U1 70K monoclonal antibody are present in spliceosomal complexes

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ABSTRACT

We have characterized a monoclonal antibody (mAb) to the U1 snRNP component U1 70K. We find that this antibody recognizes several proteins, in addition to U1 70K, in purified spliceosomal complexes and in total HeLa cell nuclear extract preparations. The novel mAb U1 70K antigens can also be specifically immunoprecipitated by the antibody. Similarly to U1 70K, many of the mAb U1 70K antigens can be phosphorylated by a co-purifying kinase activity. The epitope recognized by mAb U1 70K was previously shown to be a repeating arginine/aspartate (RD) dipeptide. Thus we have designated the novel mAb U1 70K antigens the RD family. Comparison of mAb U1 70K with a recently characterized antibody, mAb 16H3, whose epitope is a repeating R/D or R/E motif, showed that a large subset of the antigens are common. In contrast, most of the mAb U1 70K antigens are distinct from the proteins detected by mAb 104, an antibody to the SR family of splicing factors.

INTRODUCTION

In order to elucidate the mechanisms involved in pre-mRNA splicing it is necessary to identify and characterize the components of the splicing machinery. In the absence of the genetic approach being used in yeast, metazoan splicing factors are being identified using a variety of biochemical approaches. *In vitro* complementation assays have led to the purification of several essential splicing factors, including U2AF⁶⁵ (1), SF1 (2), SF3 (3), PSF (4) and SF2/ASF (5,6). In addition, analysis of the protein components of highly purified spliceosomal complexes has revealed >50 distinct proteins (7,8). Purification of the snRNPs required for splicing (U1, U2 and U4/U5/U6) has also identified a large number of proteins, many of which are a subset of the spliceosomal proteins (8–14). Another approach that has been valuable for identifying metazoan splicing factors is the production of monoclonal antibodies to spliceosomal components. Two antibodies, mAb 104 (15,16) and mAb SC35 (17), recognize members of the SR family of essential splicing factors. SR proteins contain a characteristic C-terminal domain of repeating arginine/serine (RS) dipeptides (16). This domain is thought to be

the epitope recognized by mAb 104, which detects at least six SR family members (SRp20, SRp30b/SC35, SRp30c/SF2/ASF, SRp40, Srp55 and SRp75) (16). MAb SC35 recognizes at least one of these (SRp30b/SC35). Other SR family members include 9G8 (18) and, possibly, two nuclear matrix-associated proteins (19).

Recently another monoclonal antibody, mAb 16H3, was isolated and shown to detect spliceosome components (20). This antibody recognizes U2AF⁶⁵, U2AF³⁵ and the U1 snRNP component U1 70K, a subset of SR proteins, as well as unknown proteins present in purified spliceosomal complexes (20). The epitope of mAb 16H3 is repeating R/D or R/E dipeptides (20). In this report we have characterized a monoclonal antibody, mAb U1 70K (21), previously shown to react specifically with the repeated RD-rich motif in U1 70K (22). Our analysis of mAb U1 70K shows that it reacts with a large family of proteins, many of which are also mAb 16H3 antigens.

MATERIALS AND METHODS

Antibodies

Tissue culture supernatants containing mAb U1 70K (21; a gift from S. Hoch), mAb 16H3 (20; a gift from M. Roth) or mAb 104 (15) were used undiluted, except for in Figure 4, where a dilution of 1:1000 mAb U1 70K was used. The secondary antibody for Western blots was a horseradish peroxidase-coupled goat anti-mouse antibody (1:10 000 dilution; BioSource International).

Western analysis

Affinity-purified spliceosomal complexes (7,23), nuclear extract or gel filtration fractions were treated with RNase A and then fractionated on 9% SDS gels or two-dimensional gels. Proteins were immobilized on PVDF membranes using standard methods (24). Blots were blocked for 1 h in phosphate-buffered saline, 0.1% Tween 20 (PBS-Tween) containing 5% non-fat dry milk. After a brief rinse with PBS-Tween blots were incubated for 2 h at room temperature or overnight at 4°C in tissue culture supernatants containing the antibodies. Blots were rinsed in PBS-Tween, incubated in the secondary antibody for 1 h at room temperature and then washed over a period of 30–60 min in PBS-Tween. Antibody-bound proteins were detected using the ECL system (Amersham).

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Immunoprecipitations

Total HeLa cell nuclear extract (720 μ l) was fractionated on a Sephacryl S-500 column (1.5 $\times$ 50 cm) under the same conditions used for isolation of spliceosomal complexes (7,23). Fractions enriched in U1 snRNP (see Fig. 7C, fractions 64–66) were pooled and then rotated overnight with mAb U1 70K coupled to protein A–Tris-acryl (with a rabbit anti-mouse secondary antibody). Bound material was washed five times in 150 mM NaCl, 20 mM Tris, pH 7.6. Proteins were eluted using high pH (50 mM glycine, pH 11), acetone-precipitated and then run on SDS or two-dimensional gels as indicated in the figure legends. Proteins in the mAb U1 70K immunoprecipitate were phosphorylated by incubation in 50 μ l reactions containing 50 mM NaCl, 20 mM Tris–HCl, pH 7.6, 2 μ l [γ - 32 P]ATP (1:32 dilution of 7000 Ci/mmol) and 3.2 mM MgCl₂ for 15 min at 30°C.

RESULTS

Spliceosomal complexes assemble on pre-mRNA in a stepwise pathway and the four functional complexes in this pathway assemble in the order E, A, B, C (25). The pre-mRNA also assembles into an hnRNP complex designated the H complex, which is not thought to be a functional spliceosomal complex (26). Unexpectedly, we detected several proteins, in addition to U1 70K, when blots of spliceosomal complexes E and C were probed with mAb U1 70K (Fig. 1, lanes 2 and 3). These proteins were not detected in the H complex (Fig. 1, lane 1) nor with secondary antibodies alone (Fig. 1, lanes 4–6). To characterize these additional proteins further we asked whether they are components of U1 snRNP. To do this we partially purified U1 snRNP by fractionating nuclear extract on a gel filtration column (see Materials and Methods). U1 snRNP was then immunopurified from the gel filtration fractions using mAb U1 70K. Consistent with previous work, U1 snRNA is the only RNA immunoprecipitated from the gel filtration fractions (Fig. 2A, lane 2). However, we found that a large set of proteins in addition to the expected U1 snRNP proteins (U1 A, U1 70K, B and B'), was immunoprecipitated by mAb U1 70K (Fig. 2B; note that the U1 70K protein stains negatively with silver). These proteins were not detected when the immunoprecipitation was carried out using the rabbit anti-mouse secondary antibody alone (data not shown). In addition, when the gel filtration-isolated U1 snRNP was treated with RNase A prior to immunoprecipitation with mAb U1 70K we detected the same set of proteins as observed in Figure 2B (data not shown).

This result suggested that these proteins either have an epitope recognized by mAb U1 70K or are associated with U1 snRNP via protein–protein interactions. To distinguish between these possibilities we probed a Western blot of the immunoprecipitated proteins with mAb U1 70K. As shown in Figure 3, a large number of proteins are specifically detected by mAb U1 70K (lane 1), but not by the secondary antibody alone (lane 2). Comparison of the Western blot pattern (Fig. 3, lane 1) to an ink stain of the blot (data not shown) revealed that most of the proteins immunoprecipitated by mAb U1 70K are also detected by this antibody. We conclude that mAb U1 70K specifically reacts with a large number of proteins in addition to U1 70K.

One of the proteins identified in the mAb U1 70K immunoprecipitate is U2AF⁶⁵ (Fig. 2B; data not shown). In contrast to most of the other proteins present in the immunoprecipitate (Fig.

Figure 1. MAb U1 70K recognizes novel proteins in spliceosomal complexes. Affinity-purified H, E and C complexes were fractionated on a 9% SDS gel, transferred to a PVDF membrane and probed with mAb U1 70K followed by a horseradish peroxidase-coupled goat anti-mouse secondary antibody (lanes 1–3) or with the secondary antibody alone (lanes 4–6). Proteins were detected with enhanced chemiluminescence. Protein size markers are indicated.

2B), U2AF⁶⁵ is recognized by antibodies to U2AF⁶⁵, but not by mAb U1 70K (data not shown). Thus U2AF⁶⁵ is probably associated with the immunoprecipitated proteins via protein–protein interactions.

Previous work showed that U1 snRNP co-purifies with a kinase activity that specifically phosphorylates U1 70K (27). Consistent with this, we detect a kinase activity in the mAb U1 70K immunoprecipitate (Fig. 2C). Incubation of the immunoprecipitate with [γ - 32 P]ATP and magnesium results in phosphorylation of U1 70K, as well as several of the other proteins (Fig. 2C). Thus, similarly to U1 70K, many of the mAb U1 70K antigens can be phosphorylated (Fig. 2C, indicated by *).

The observations that mAb U1 70K detects proteins other than U1 70K in spliceosomal complexes (Fig. 1) and specifically immunoprecipitates many proteins from gel filtration fractions (Fig. 2) prompted us to characterize mAb U1 70K further. Using a 1000-fold range of antibody to probe different amounts of nuclear extract we discovered that a distinct set of mAb U1 70K-reactive proteins is reproducibly detected (Fig. 4). Mostly background bands are detected when mAb U1 70K is diluted further (1:10 000; data not shown). With low amounts of nuclear extract and with a light exposure of the film it is possible to detect U1 70K as a major reactive protein (Fig. 4, lane 5). However, even under these conditions the other antigens are apparent. We have found that the same set of proteins are detected with an independent preparation of mAb U1 70K grown from a frozen stock and with mAb U1 70K obtained from another source. Together these data provide strong evidence that mAb U1 70K reacts with a large number of antigens. In a previous study in which mAb U1 70K was used to probe total nuclear extract U1 70K and one higher molecular weight protein were detected (28). Possible reasons that the other proteins were not detected include differences in the gels, blotting conditions and/or Western conditions. In another study a 44 kDa nuclear protein (29–31) that reacts with mAb U1 70K was reported (30). This protein contains the repeating RD motif that is the epitope of mAb U1 70K (22).

Figure 2. MAb U1 70K immunoprecipitates a set of novel proteins along with U1 snRNP. Gel filtration fractions enriched in U1 snRNP (see Materials and Methods) were incubated with immobilized mAb U1 70K, and bound RNA (A) or protein (B and C) was analyzed. (A) Total RNA was extracted from the bound material and fractionated on an 8% denaturing polyacrylamide gel (lane 2, IP). RNA was detected by ethidium bromide staining. Total RNA isolated from nuclear extract was run as a marker (lane 1). The positions of the snRNAs are shown. (B and C) Total protein was extracted from the bound material, incubated with [γ - 32 P]ATP and Mg and then run on a two-dimensional gel. The silver stained (B) and phosphorimager (C) patterns are shown. Bands designated lc and hc are the light chain and heavy chain of mAb U1 70K, respectively. The arrows indicate novel proteins immunoprecipitated by mAb U1 70K. The * indicates the subset that is phosphorylated (C). U1 70K, U1 A, B, B' and U2AF⁶⁵ are designated. Protein size markers are shown.

Figure 3. Many of the novel proteins immunoprecipitated by mAb U1 70K are detected by this antibody on a Western blot. Proteins prepared as in Figure 2B were fractionated on a 9% SDS gel, immobilized on PVDF and probed with mAb U1 70K followed by a horseradish peroxidase-coupled goat anti-mouse secondary antibody (lane 1) or with the secondary antibody alone (lane 2). Protein size markers and the heavy and light chains of the antibody are indicated.

Figure 4. Western analysis of nuclear extract using mAb U1 70K. The indicated amounts of nuclear extract were probed first with mAb U1 70K or a 1:1000 dilution of mAb U1 70K followed by a goat anti-mouse secondary antibody. The panel designated Control was probed with the secondary antibody alone. The U1 70K protein and protein size markers are indicated.

On the basis of these observations we have designated the novel U1 70K antigens detected in our study the RD family.

Two other monoclonal antibodies that react with protein families required for splicing have been characterized, mAb 16H3 (20) and mAb 104 (15,16). To determine the relationships between these families and the RD family we performed side-by-side Western blots of total nuclear extracts probed with the three antibodies (Fig. 5). These data revealed that many of the proteins detected by mAb U1 70K appear to be the same as those detected by mAb 16H3 (Fig. 5, lanes 1 and 2). In contrast, only

a few possible similarities are seen between mAbs 104 and U1 70K (or 16H3) (Fig. 5, lanes 1–3). We confirmed that mAbs 16H3 and U1 70K detect a similar set of proteins by carrying out Western analysis of nuclear extract on two-dimensional blots (Fig. 6A). Although some of the proteins are detected more strongly by one or the other antibody, the patterns are strikingly similar. The two main differences are an 89 kDa protein and U2AF⁶⁵ (identified by Western analysis; data not shown), which are detected by mAb 16H3 only.

Figure 5. Comparison of Western blots of nuclear extract probed with mAbs U1 70K, 16H3 or 104. Western blots of total nuclear extract (3 μ l) were probed with mAb U1 70K (lane 1), an antibody to nuclear antigens with a repeating R(D/E) epitope (lane 2), or an antibody to the SR protein family (lane 3). Protein size markers, U1 70K and the SR proteins are indicated.

A comparison of two-dimensional blots probed with mAb 104 versus U1 70K is shown in Figure 6B. These data indicate that the set of proteins recognized by mAb 104 is largely distinct from that recognized by mAb U1 70K. One protein, RD 53 (U1 70K, Fig. 6B), appears to be recognized by both antibodies. This protein migrates to the basic side of SRp55 (bracket, 104, Fig. 6B) and thus does not appear to correspond to any of the known SR protein family members. Consistent with previous studies (16,32), our two-dimensional blot of nuclear extract using mAb 104 indicates that there are many more mAb 104 antigens than the currently known SR family members. Whether all of these proteins play a role in splicing remains to be determined.

The observation that mAb U1 70K detects antigens in purified spliceosomal complexes indicates that at least some of the RD family members play a role in splicing. To determine whether the RD family members might associate with one another to form a particle or whether they might associate with SR family members we fractionated total nuclear extract by gel filtration and probed

the fractions with mAb U1 70K (Fig. 7). No evidence for a discrete particle containing the RD family members is seen (Fig. 7A). In contrast, when mAb 104 was used to probe an identical blot co-fractionation of the SR proteins in a broad peak was observed (Fig. 7B). These data suggest that, unlike the RD family members, the SR proteins may reside in a particle. This is consistent with previous work, which indicated that SR family members can interact directly with one another (33).

DISCUSSION

We have shown that a monoclonal antibody directed against the U1 snRNP component U1 70K (21) recognizes a large set of proteins, some of which are specifically associated with purified spliceosomal complexes. In previous work a cDNA encoding a 44 kDa protein, designated the RD protein, was isolated when mAb U1 70K was used to screen an expression library (30,31). This protein contains a repeating RD motif, which is the epitope recognized by mAb U1 70K (22). A comparison of the RD domain of U1 70K and the 44 kDa RD protein is shown in Figure 8. We have designated the antigens recognized by mAb U1 70K the RD family. We have no evidence that members of the RD family, other than those detected in the spliceosomal complexes, are involved in splicing. In addition, we were not able to detect a discrete particle containing RD family members or any direct associations between RD and SR family members. However, our data do indicate that the RD family is a major subset of the proteins detected by the recently described 16H3 monoclonal antibody (20). This is reasonable, as the epitope of 16H3 is repeating R(D/E) dipeptides (20). Two obvious differences between mAbs U1 70K and 16H3 are U2AF⁶⁵ and an 89 kDa protein, which are detected by mAb 16H3, but not mAb U1 70K.

We detect no overlap between RD and SR family members. In previous work mAb 16H3 was shown to detect a subset of the SR family members (SRp20, SRp40, SRp55 and SRp75; 20). We do not detect significant levels of these SR proteins when total nuclear extract is probed with either mAb 16H3 or U1 70K. However, large quantities of purified SR proteins were probed with mAb 16H3 in a previous study (20). Thus it is possible that mAb 16H3 has a low affinity for the SR family members and they are below the level of detection in total nuclear extract.

Figure 6. Comparison of two-dimensional Western blots of nuclear extracts probed with mAbs U1 70K, 104 or 16H3. Blots were probed with the indicated antibodies or with the secondary antibody alone (control). SR proteins, U1 70K, U2AF⁶⁵ and RD family members (designated by protein size) are indicated. Background bands are labeled with a lower case b. Proteins marked with an asterisk (*) are detected by mAb 16H3 only (A). The bracket indicates a protein that appears to correspond to RD53 (104) (B). Protein size markers are indicated on the left. An SDS gel lane containing nuclear extract was run on the second dimension of each of the two-dimensional gels (in B only). In the blot probed with mAb 104 (B) a lane containing baculovirus-expressed SC35 was run next to the total extract. SDS lanes and two-dimensional gels contain 3 and 10 μ l of nuclear extract, respectively.

Figure 7. Distribution of RD and SR family members in gel filtration-fractionated nuclear extract. Nuclear extract was fractionated on a gel filtration column and total RNA (C) or protein (A and B) was isolated from the indicated fractions across the column. Western blots of proteins were probed with mAb U1 70K (A) or mAb 104 (B). The SR proteins and U1 70K are indicated for the respective blots. RNA from the fractions was run on an 8% denaturing gel and visualized by ethidium bromide staining. snRNAs and tRNA are labeled. The bracket indicates the fractions used to immunoprecipitate U1 snRNP in Figure 2.

U1 70K 407 RDRDRDRERERRERSRERDKERERRRSRSRDRRRRSRSRDKEERRRSRERSKDKRDRKRSSRSR 472
524 RDRDRERRRRSHRSERERRRRDRDRDRDRDRE 553

44 kD RD 184 RSRSRDRSHERNRDRDRDRERDRDRDRDRDRDRERDRDRDRDRDRDRERDRDRERDRDRDRE 243

Figure 8. Comparison of the RD domain in U1 70K and the 44 kDa RD protein. The RD dipeptides are underlined. The numbers indicate the amino acids. U1 70K has two stretches of RD-rich regions.

Previous immunofluorescence studies using mAb U1 70K suggested that U1 snRNP is localized throughout the nucleus, as well as in the discrete speckle domains where all of the other splicing snRNPs are detected (34,35). However, our data raise the possibility that a portion, if not all, of the general nucleoplasmic staining is attributable to RD family members other than U1 70K. Consistent with this, in a study in which antisense probes were used for *in situ* hybridization, U1 snRNA and U2 snRNA were distributed essentially identically in speckle domains (36). Thus U1 snRNP, similarly to the other splicing snRNPs, may be localized almost exclusively in the speckle domains.

NOTE ADDED IN PROOF

K. Neugebauer and M. Roth have found (unpublished observation) that the U1 70K monoclonal antibody recognizes the polypeptide bound by mAb 16H3 (20), which consists primarily of alternating arginine and glutamate.

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