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The [U4/U6·U5] tri-snRNP-specific 27K protein is a novel SR protein that can be phosphorylated by the snRNP-associated protein kinase

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ABSTRACT

SR proteins play important roles in the recognition and selection of the 3′ and 5′ splice site of a given intron and contribute to the phosphorylation/dephosphorylation-mediated regulation of pre-mRNA splicing. Recent studies have demonstrated that the U1 snRNP is recruited to the 5′ splice site by protein/protein interactions involving the SR domains of the U1-70K protein and SF2/ASF. Recently, it was suggested that SR proteins might also contribute to the binding of the [U4/U6·U5] tri-snRNP to the pre-spliceosome (Roscigno RF, Garcia-Blanco MA, 1995, *RNA 1*:692–706), although it remains unclear whether these SR proteins interact with proteins of the tri-snRNP complex. As a first step toward the identification of proteins that could potentially mediate the integration of the [U4/U6·U5] tri-snRNP complex into the spliceosome, we investigated whether purified [U4/U6·U5] tri-snRNP complexes contain SR proteins. Three proteins in the tri-snRNP complex with approximate molecular weights of 27, 60, and 100 kDa were phosphorylated by purified snRNP-associated protein kinase, which has been shown previously to phosphorylate the serine/arginine-rich domains of U1-70K and SF2/ASF (Woppmann A et al., 1993, *Nucleic Acids Res 21*:2815–2822). These proteins are thus prime candidates for novel tri-snRNP SR proteins. Here, we describe the biochemical and molecular characterization of the 27K protein. Analysis of a cDNA encoding the 27K protein revealed an N-terminal SR domain strongly homologous (54% identity) to the SR domain of the U1 snRNP-specific 70K protein. In contrast to many other SR proteins, the 27K protein does not contain an RNA-binding domain. The 27K protein can be phosphorylated in vitro by the snRNP-associated protein kinase and exhibits several isoelectric variants upon 2D gel electrophoresis. Thus, the tri-snRNP-specific 27K protein could potentially be involved in SR protein-mediated protein/protein interactions and, additionally, its phosphorylation state could modulate pre-mRNA splicing.

Keywords: pre-mRNA splicing; protein kinase; snRNP; spliceosome assembly; SR protein

INTRODUCTION

Nuclear pre-mRNA splicing proceeds via two consecutive transesterification steps. In the first, the 5′ splice site is cleaved and a lariat/3′ exon intermediate is formed. The second step involves cleavage of the 3′ splice site, exon ligation, and release of the intron in the form of a lariat. The splicing reaction is catalyzed by a macromolecular complex termed the spliceosome. Nuclear pre-mRNA splicing is dependent upon the activity of a large number of *trans*-acting factors. These can be divided into two classes: the UsnRNPs, evolutionarily highly conserved ribonucleoprotein complexes, and the so-called non-snRNP protein splicing factors (for review, see Green, 1991; Guthrie, 1991; Moore et al., 1993).

The UsnRNPs consist of four distinct particles, U1, U2, U4/U6, and U5, that contain the snRNAs U1, U2, U4/U6, and U5, respectively. The protein composition of the snRNPs is well characterized in HeLa cells. Generally, the snRNP proteins fall into two classes. The first class comprises the so-called common proteins, denoted B′, B, D1, D2, D3, E, F, and G, which are shared by all snRNP particles. The second class consists of proteins that bind to the snRNPs in a particle-specific manner. For example, U1 snRNPs purified on Mono Q contain, in addition to the common proteins, three distinct U1-specific proteins termed A, C, and 70K (Hinterberger et al., 1983; Bach et al., 1990), whereas the U2 snRNP contains 11 specific proteins (Behrens et al., 1993; Krämer, 1996). The 20S U5 snRNP contains nine specific proteins with apparent molecular weights of 15, 40, 52, 100, 102, 110, 116, 200, and 220 kDa (Behrens & Lührmann, 1991), whereas two proteins with apparent molecular weights of 60 and 90 kDa are associated with the 12S U4/U6 snRNP (Gozani et al., 1994; J. Lauber, K. Gröning, S. Prehn, G. Plessel, & R. Lührmann, in prep.). The most complex composition is observed for the 25S [U4/U6·U5] tri-snRNP complex. The

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latter complex contains, in addition to the aforementioned 20S U5- and 12S U4/U6-specific proteins, five proteins with apparent molecular weights of 15.5, 20, 27, 61, and 63 kDa that are essential for its formation (Behrens & Lührmann, 1991).

Proper assembly of the spliceosome requires a group of non-snRNP splicing factors that contain domains rich in serine/arginine and are therefore denoted SR proteins. The majority of SR proteins possess a common epitope recognized by the monoclonal antibody (mAB104) (Roth et al., 1990). In addition to the non-snRNP SR proteins, an SR domain-containing protein, U1-70K, is also found associated with the U1 snRNP. SR proteins are highly conserved and share structural features including an RNA binding domain (the RNP motif) that is characteristic of a large family of RNA binding proteins (Birney et al., 1993).

In addition to RNA/RNA interactions, protein/protein interactions, particularly those between SR proteins, are essential for the formation of an active spliceosome. SR proteins play an important role at early stages of spliceosome assembly because they participate in the ordered integration of the U1 and U2 snRNP during commitment complex and pre-splicing complex formation. One of the earliest steps of spliceosome formation is the association of the U1 snRNP with the 5′ splice site. In addition to the base pairing interaction between the 5′ end of the U1 snRNA and the pre-mRNA, stable binding of U1 snRNP is mediated by protein/protein contacts involving the SR domains of SF2/ASF and U1-70K (Kohtz et al., 1994; Staknis & Reed, 1994). Commitment or E complex formation also appears to involve interactions between additional proteins containing an SR domain, such as U2AF and SC35 (Fu & Maniatis, 1992; Wu & Maniatis, 1993). Since U2AF binds the polypyrimidine tract (Ruskin et al., 1988; Zamore et al., 1992), SR protein interactions during E complex formation have been proposed to mediate communication between the 5′ and 3′ splice sites (Fu & Maniatis, 1992; Wu & Maniatis, 1993). That SR protein-mediated E complex formation may be a regulated event is suggested by the fact that serine residues present in the SR domains of both the U1-70K protein and SF2/ASF are phosphorylated in vivo (Woppmann et al., 1993; Colwill et al., 1996). Significantly, recent studies have suggested that the dephosphorylation of the U1-70K protein is a prerequisite for the first step of splicing, whereas the phosphorylation of the mAB104 SR proteins is required for spliceosome formation (Tazi et al., 1993; Mermoud et al., 1994).

Following E complex formation, U2 snRNP is recruited to the branch site, an event that requires the presence of U2AF and other splicing factors (e.g., SF1, Krämer & Utans, 1991). Finally, the 25S [U4/U6·U5] tri-snRNP complex associates with the pre-spliceosome to form the mature spliceosome that is catalytically active. Integration of the tri-snRNP complex into the spliceosome leads to the disruption and formation of a number of RNA/RNA interactions. For example, the base paired U4/U6 snRNAs within the U4/U6 snRNP dissociate and new U6/U2 snRNA intermolecular helices are formed (Hausner et al., 1990; Madhani et al., 1990; Datta & Weiner, 1991; Wu & Manley, 1991; Madhani & Guthrie, 1992; Sun & Manley, 1995). Moreover, the conserved loop I of the U5 snRNA is thought to interact with exonic sequences at both splice sites (Newman & Norman, 1991, 1992; Cortes et al., 1993; Sontheimer & Steitz, 1993) and the U6 snRNA recognizes intron sequences at the 5′ splice site (Kandels-Lewis & Séraphin, 1993; Lesser & Guthrie, 1993; Sontheimer & Steitz, 1993).

Due to the complex protein composition of the 25S [U4/U6·U5] tri-snRNP complex, protein/protein interactions most likely play an important role in the association of the tri-snRNP with the pre-spliceosome. Because SR proteins have been shown to function as molecular bridges that recruit U1 snRNP and U2 snRNP into the pre-spliceosome, it is tempting to suggest that functionally important protein interactions between SR and particle-specific proteins might also occur during the final step of spliceosome assembly. Recently, Roscigno and Garcia-Blanco (1995) have provided evidence that SR proteins also recruit the [U4/U6·U5] tri-snRNP to the spliceosome. In particular, gradient-purified pre-spliceosomes could be chased into spliceosomes when incubated with purified or recombinant, phosphorylated SR proteins, such as ASF/SF2 or SC35, and an snRNP-containing fraction. Furthermore, studies by Tarn and Steitz (1995) suggest that SR proteins might stabilize the interaction between the U2 and U6 snRNAs. However, it is presently not clear how SR proteins interact at the molecular level with the [U4/U6·U5] tri-snRNP complex.

In order to identify proteins that could potentially mediate the integration of the [U4/U6·U5] tri-snRNP complex into the spliceosome, we investigated whether purified [U4/U6·U5] tri-snRNP complexes contain SR proteins. Using purified snRNP-associated protein kinase, which phosphorylates the serine/arginine-rich domains of U1-70K and SF2/ASF (Woppmann et al., 1993), we identified the first SR protein that is associated with the 25S [U4/U6·U5] tri-snRNP complex, namely the tri-snRNP-specific 27K protein. Here, we report the sequence of the 27K cDNA and the biochemical characterization of the 27K protein.

RESULTS AND DISCUSSION

[U4/U6·U5] tri-snRNP-specific 27K protein is phosphorylated in vitro by the snRNP-associated protein kinase

In order to characterize the proteins associated with the 25S [U4/U6·U5] tri-snRNP complex in more detail, spliceosomal snRNPs were isolated from HeLa

nuclear extracts by immunoaffinity chromatography with anti-m_3G antibodies. The resulting snRNP mixture (U1, U2, U4/U6, U5 snRNPs) was subsequently separated by glycerol gradient centrifugation and the protein composition of gradient fractions was analyzed by SDS-PAGE. The typical pattern of proteins migrating at 25S in the gradient is shown in Figure 1A. In addition to the common (Sm) snRNP proteins (B′, B, D1, D2, D3, E, F, and G), the 25S [U4/U6·U5] tri-snRNP is comprised of the U5 snRNP- (15, 40, 52, 100, 102, 110, 116, 200, and 220 kDa) and U4/U6 snRNP-specific (60 and 90 kDa) proteins. Furthermore, the 25S [U4/U6·U5] tri-snRNP contains five tri-snRNP-specific proteins that exhibit molecular weights of 15.5, 20, 27, 61, and 63 kDa (Behrens & Lührmann, 1991; J. Lauber, pers. comm.). Because the SR domain of the U1-70K protein is phosphorylated by an snRNP-associated protein kinase activity (Woppmann et al., 1993), the latter activity was used to investigate whether integral proteins of the tri-snRNP complex also possess SR domains. Although the previously described snRNP-associated protein kinase activity is associated preferentially with U1 snRNPs, variable amounts of this kinase activity are observed in 25S [U4/U6·U5] tri-snRNPs, depending on the preparation. We have also observed previously that snRNP preparations may contain a kinase activity that is capable of phosphorylating a 52-kDa protein (Behrens & Lührmann, 1991). In order to analyze whether the tri-snRNP complex contains SR proteins that are phosphorylated by the snRNP-associated protein kinase, glycerol gradient-purified tri-snRNP particles, which were lacking kinase activity, were incubated with [γ-^{32}P]-ATP and purified snRNP-associated protein kinase (S. Fetzer, M. Krause, & R. Lührmann, in prep.). As shown in Figure 1B (lane 3), at least three proteins with apparent molecular masses of approximately 100, 60, and 27 kDa were phosphorylated significantly. No significant phosphorylation was observed when [γ-^{32}P]-ATP was incubated with the purified protein kinase or tri-snRNPs alone (data not shown). Based on the known molecular masses of proteins associated with the tri-snRNP

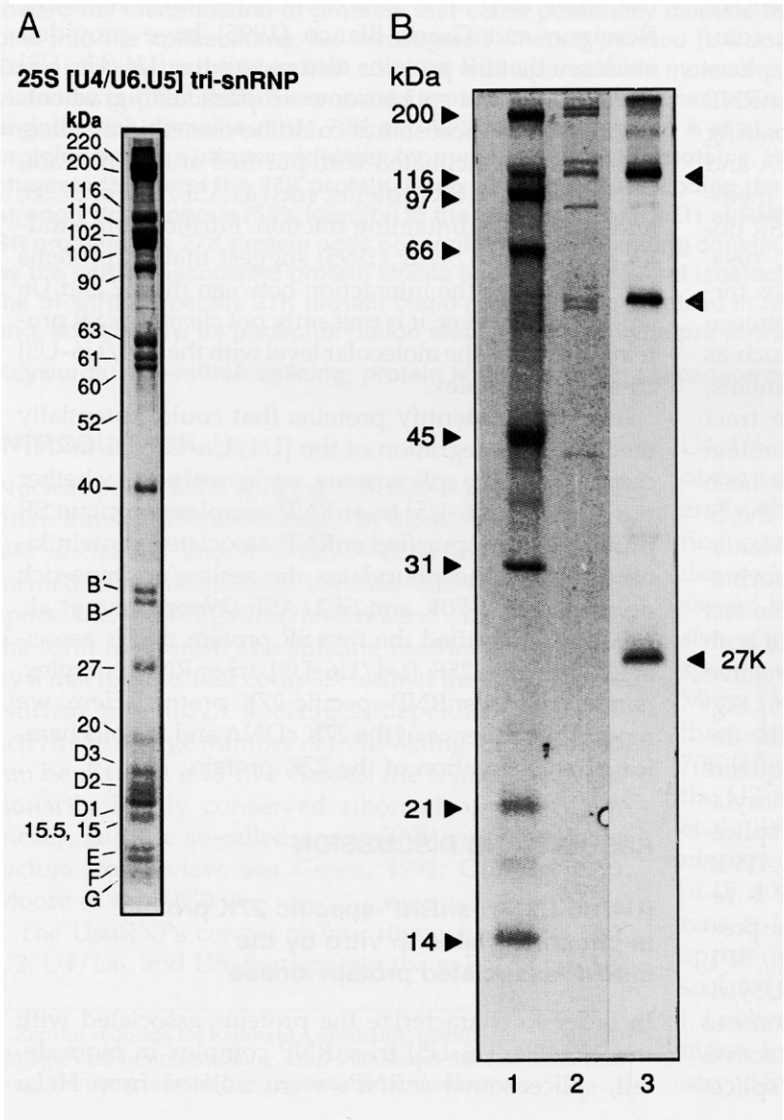

FIGURE 1. Protein composition and in vitro phosphorylation of glycerol-gradient purified 25S [U4/U6·U5] tri-snRNP. Total snRNPs (U1, U2, U4/U6, and U5) were isolated from HeLa nuclear extract by immunoaffinity chromatography using an anti-m_3G-antibody. 25S [U4/U6·U5] tri-snRNPs were isolated by gradient centrifugation on 10-30% glycerol gradients as described in Materials and Methods. **A:** Tri-snRNP proteins separated by SDS-PAGE on a 10% polyacrylamide gel and visualized by staining with Coomassie blue. **B:** Three micrograms of 25S [U4/U6·U5] tri-snRNP were phosphorylated with [γ-^{32}P]-ATP and purified snRNP-associated protein kinase as described in Materials and Methods. Proteins were then separated by SDS-PAGE on a 10% polyacrylamide gel and visualized by staining with Coomassie (lane 2) followed by autoradiography (lane 3). Molecular masses of protein standards (lane 1) are indicated at the left and the major phosphoproteins are indicated by an arrowhead on the right.

complex, the 27-kDa and 60-kDa phosphorylated bands could potentially represent the 27K and the 60-, 61-, or 63-kDa tri-snRNP proteins, respectively, whereas that with an apparent molecular mass of 100 kDa could represent one or more of the 100-, 102-, or 110-kDa U5 snRNP-specific proteins.

Analysis of the endogenous, in vitro-phosphorylated 27K protein by 2D gel electrophoresis

In light of our previous finding that the U1-70K protein, which is the first snRNP protein shown to harbor an SR domain, can be separated into 13 isoelectric variants (Woppmann et al., 1990), we analyzed tri-snRNP proteins by 2D gel electrophoresis. Because the amount of endogenous 27K protein in the tri-snRNP preparations used for 2D analysis was not sufficient to allow detection of different 27K variants by Coomassie staining, glycerol gradient-purified [U4/U6·U5] tri-snRNP proteins were phosphorylated in vitro in the presence of [γ-^{32}P]-ATP and purified snRNP-associated kinase. These in vitro-phosphorylated, radiolabeled tri-snRNP proteins were separated in the first dimension by nonequilibrium pH-gradient gel electrophoresis (NEPHGE), with a pH gradient ranging from 3 to 11, and subsequently fractionated in the second dimension by SDS-PAGE. As shown in Figure 2, at least five phosphorylated isoelectric variants could be resolved. In the absence of additional information regarding the ratio of phosphate incorporation/mole of isoelectric variant, it is presently unclear whether variants possessing lower pI values are phosphorylated more extensively. Because several isoelectric variants of the U1-70K protein appear to arise by differential phosphorylation (Woppmann et al., 1990), it seems likely that the existence of some of the 27K isoelectric variants is also a consequence of differential phosphorylation. In sum, these data suggest that the [U4/U6·U5] tri-snRNP-specific 27K protein, which is a novel SR protein, may be phosphorylated differentially by the snRNP-associated kinase activity and, thus, exhibits different isoelectric variants upon 2D gel electrophoresis.

Identification of a cDNA encoding the [U4/U6·U5] tri-snRNP-specific 27K protein and analysis of its primary structure

Because the phosphorylated protein migrating with an apparent molecular mass of 27 kDa most likely represents the 25S [U4/U6·U5] tri-snRNP-specific 27K protein, we were interested in characterizing this potential SR domain-containing protein at the molecular level and thus set out to isolate its cDNA. To this end, proteins isolated from glycerol gradient-purified 25S [U4/U6·U5] tri-snRNPs were separated by SDS-PAGE. After staining with Coomassie, the 27 kDa band was excised and electroeluted from the gel. Initial microsequencing by Edman degradation yielded two peptide sequences, 3 and 12 amino acids long (Fig. 3). These peptide sequences were used for a database search using the TBLASTN program (Altschul et al., 1990), which led to the identification of a putative 27K cDNA in the "Gene Bank" database (RY-1, accession number X76302). The putative cDNA sequence of the human 27K protein

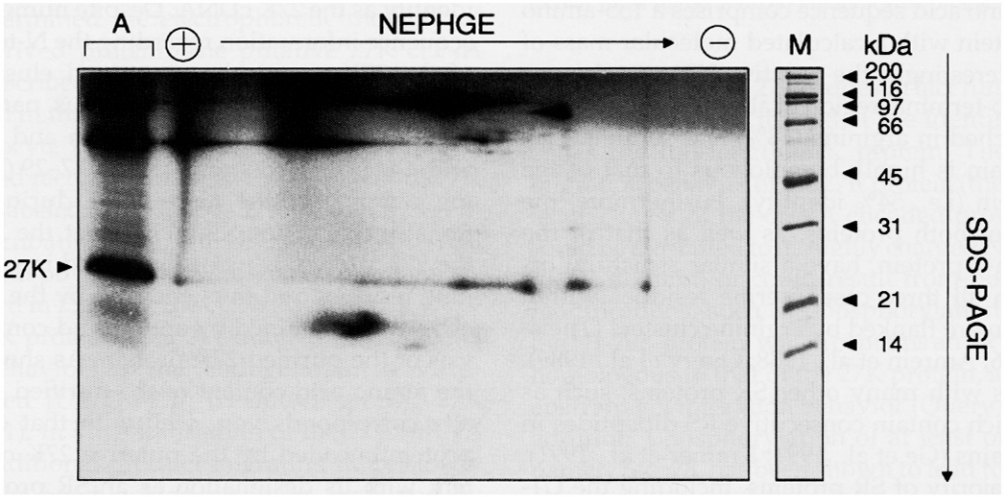

FIGURE 2. Characterization of the endogenous in vitro-phosphorylated 27K protein by 2D gel electrophoresis. Twenty microliters of in vitro-phosphorylated tri-snRNP protein (see Materials and Methods for in vitro phosphorylation conditions) were applied at the anode end in the first dimension and separated by migration toward the cathode. After NEPHGE with a pH gradient of 3–11, proteins were fractionated by SDS-PAGE in the second dimension. ^{32}P-labeled proteins were visualized by autoradiography. Ten percent of the in vitro-phosphorylated starting material, fractionated exclusively by SDS-PAGE, is shown in lane A. Protein molecular mass standards (lane M) were visualized by staining with Coomassie. Radiolabeled spots migrating below the 27K variants arise from nonspecific binding of [γ-^{32}P]-ATP to RNase A, which is used to ensure the isolation of highly purified protein from the in vitro phosphorylation preparations

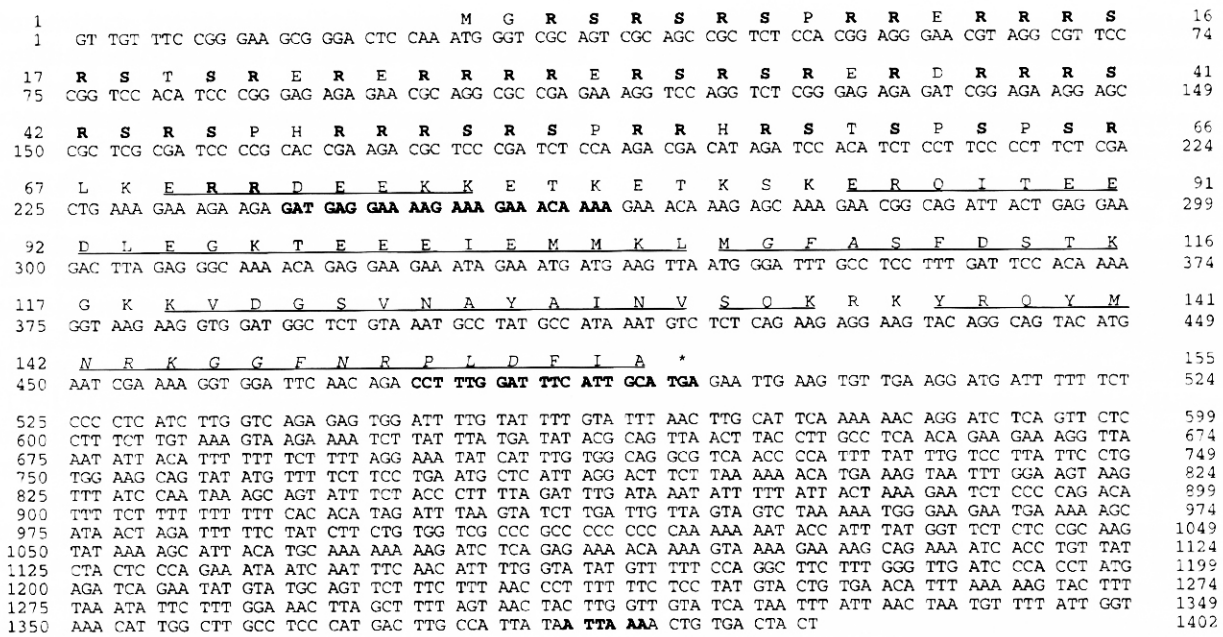

FIGURE 3. cDNA and predicted amino acid sequence of the [U4/U6·U5] tri-snRNP-specific 27K protein. The sequence of the 27K cDNA isolated from Jurkat T cells is shown. The peptide sequences obtained by initial microsequencing of the native protein are italicized. Additional sequences obtained by MALDI mass spectrometry of Lys-C digested protein are underlined. A potential polyadenylation signal in the 3′ untranslated region is marked by bold letters and the termination codon is marked with an asterisk. Oligonucleotides used for PCR cloning of a fragment of the HeLa 27K cDNA are marked by bold letters.

derived from Jurkat T cell lymphoblastic cells is shown in Figure 3 (Nakamura et al., 1994). It is 1,402 base pairs long, possesses a single open reading frame that begins near the 5′ end of the cDNA, and contains a potential AUG initiation codon at nt 27–29. The cDNA also contains a 910-bp 3′ untranslated region starting at position 492, which contains a probable polyadenylation signal (ATTAAA) at positions 1385–1390. The predicted amino acid sequence comprises a 155-amino acid long protein with a calculated molecular mass of 18,860 Da. Interestingly, the putative 27K protein contains an amino-terminal region of about 80 amino acids, which is enriched in arginine (48%) and serine (24%). This SR domain is highly homologous to that of the U1-70K protein (i.e., 54% identity). Furthermore, the SR domains of both proteins, as well as that of the *Drosophila* Tra-2 protein, have a similar structural organization. In all three cases, serine residues within the SR domain are flanked by arginine clusters (Theissen et al., 1986; Amrein et al., 1988; Query et al., 1989). This contrasts with many other SR proteins, such as SF2/ASF, which contain consecutive RS dipeptides in their SR domains (Ge et al., 1991; Krainer et al., 1991). Unlike the majority of SR proteins, including the U1-70K protein, the 27K protein does not contain an RNA-binding motif. In sum, consistent with our phosphorylation data (Fig. 1), the 27K protein appears to be a novel SR protein and, as such, is the first identified as an integral component of the 25S [U4/U6·U5] tri-snRNP.

To provide additional evidence that the cDNA shown in Figure 3 codes for the tri-snRNP-specific 27K protein, gel-purified 27K protein was analyzed additionally by MALDI mass spectrometry. Using the MALDI data, the identical cDNA (RY-1) was retrieved after a protein database search. Four peptide sequences were identified that encompass almost the entire C-terminal half of the cDNA (Fig. 3), consistent with its proposed identity as the 27K cDNA. Despite numerous attempts, sequence information regarding the N-terminal region of the 27K protein has remained elusive. However, two residues at the amino terminus, namely GR, were identified by Edman degradation and are consistent with the proposed start site at nt 27–29 (Fig. 3), assuming removal of the methionine during posttranslational modification. Evidence that the N-terminus of the 27K protein is indeed enriched in serine and arginine residues and thus encoded by the proposed 27K cDNA was obtained by amino acid composition analysis of the purified 27K protein. As shown in Table 1, the amino acid content of the purified protein generally corresponds very well with that deduced for a protein encoded by the putative 27K cDNA. Consistent with its designation as an SR protein, 27K possesses a high amount of arginine and serine. Although the number of serine residues determined experimentally differs considerably from the expected value (14.7 versus 22), this apparent discrepancy could arise from serine phosphorylation (consistent with the aforementioned phosphorylation studies), which would be ex-

TABLE 1. Amino acid composition of isolated 27K protein.

Amino acid	Number of residues	
	Expected[a]	Calculated
Asx[b]	10	10.0
Thr	7	5.4
Ser	22	14.7
Glx[c]	21	19.3
Ala	4	4.4
Val	2	3.2
Met	4	2.4
Ile	4	3.3
Leu	4	4.2
Tyr	3	2.4
Phe	3	2.7
Lys	14	6.7
Arg	36	33.5

[a]As deduced from the 27K cDNA sequence.
[b]Denotes both aspartate and asparagine.
[c]Includes both glutamate and glutamine.

pected to lead to an underestimation of serine content. Because the protein used for amino acid composition analysis was subjected initially to 10 cycles of automated N-terminal sequencing, the calculated amount of lysine is also less than the predicted amount (6.7 versus 14) due to modification of the ϵ-amino group of lysine by the coupling reagent PITC. Taken together, these data clearly indicate that the 27K protein is indeed encoded by the cDNA initially identified by the database search.

Gel migration behavior of the in vitro-translated 27K protein

Next, we determined the electrophoretic mobility of the cDNA-derived protein. The putative 27K cDNA was thus transcribed in vitro and the resultant mRNA was translated in the presence of [^{35}S]-methionine with rabbit reticulocyte lysate. In order to compare the in vitro-translated recombinant with the endogenous 27K protein, radiolabeled, endogenous 27K protein was generated by incubating [U4/U6·U5] tri-snRNPs with [γ-^{32}P]-ATP and purified snRNP-associated protein kinase. As shown in Figure 4, the cDNA-derived, in vitro-translated 27K protein (lane 2) exhibited the same gel migration behavior as the endogenous, in vitro-phosphorylated [U4/U6·U5] tri-snRNP-specific 27K protein (lane 1). In vitro translation of the 27K mRNA yielded an additional product migrating more slowly than the major band, which could be explained by differences in posttranslational modification of the same translation product. That the upper band is produced by use of an alternative, upstream initiation codon can be ruled out because the vector and the 5′ untranslated region of the cDNA do not contain such a signal. Furthermore, these data indicate that the putative start

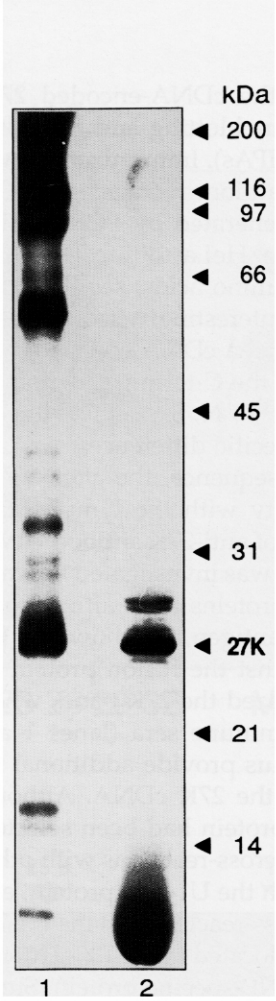

FIGURE 4. Gel migration behavior of the cDNA-derived in vitro-translated 27K protein and the [U4/U6·U5] tri-snRNP-associated, in vitro-phosphorylated 27K protein. Three micrograms of [U4/U6·U5] tri-snRNP proteins containing ^{32}P-labeled 27K protein (lane 1) or [^{35}S]-labeled, in vitro-translated 27K protein (lane 2) were fractionated by electrophoresis on a 12.5% SDS-polyacrylamide gel and visualized by autoradiography and fluorography, respectively. Molecular mass markers (kDa) are indicated on the right.

codon present at nt 27–29 does in fact function as an initiation codon and, thus, that the identified cDNA encodes the full-length 27K protein. The aberrant gel migration behavior of the 27K protein (the predicted molecular mass of the cDNA encoded protein is 19 kDa) could be attributed to its highly charged N-terminus (i.e., its SR domain) or could result from posttranslational modifications, such as phosphorylation. Interestingly, the U1-70K protein, which contains an SR domain highly homologous to that of the 27K protein, also exhibits an aberrant gel migration behavior (Query et al., 1989). In addition, phosphorylation of at least one SR protein, namely SRp75, has been shown to lead to a reduction in electrophoretic mobility (Zahler et al., 1993).

Immunoblot analysis of affinity-purified snRNPs with anti-27K antibodies

To confirm that the recombinant 27K protein is identical to the endogenous 27K protein, antibodies were

raised against the cDNA-encoded 27K protein and tested by immunoblotting and radio-immunoprecipitation assays (RIPAs). Immunizations were performed with a 27K-glutathione-S-transferase (GST) fusion protein that was generated by PCR cloning of a 255-bp fragment of the HeLa 27K cDNA (comprising the C-terminal 85 amino acids; see Fig. 3), using a HeLa cDNA library. Interestingly, sequence analysis of this portion of the HeLa cDNA revealed only three amino acid changes in the C-terminal region of the 27K protein ($T^{81} \rightarrow I$; $S^{93} \rightarrow N$; $S^{114} \rightarrow F$), suggesting that only minor tissue-specific differences exist. With regard to the nucleotide sequence, the cloned fragment exhibited 93% identity with the lymphoblast 27K cDNA. The interaction of anti-27K antibodies with the various snRNP proteins was investigated by immunoblot analysis using the proteins from affinity-purified snRNPs as a source of antigen. As shown in Figure 5, rabbit sera raised against the fusion protein (lanes 2 and 4) strongly recognized the 27K band, whereas the corresponding pre-immune sera (lanes 1 and 3) did not. These results thus provide additional support for the authenticity of the 27K cDNA. Although C-terminal regions of the protein had been selected for immunization to avoid cross-reactions with other SR proteins, particularly with the U1-70K protein, each of our anti-27K antisera cross-reacted with the U1-70K protein and another protein located in the 100–110 kDa region, most likely a U5 snRNP-specific protein. Similar cross-reactions were observed with antibodies raised against two synthetic peptides derived from the C-terminal domain of the 27K protein (data not shown). Due to lack of sequence information, is not clear whether the observed cross-reaction with the 100–110-kDa protein can be attributed to a homologous amino acid sequence in either the 100-, 102-, or 110-kDa U5-specific protein.

To provide further evidence that the anti-27K antisera derived from recombinant protein recognize an endogenous 25S [U4/U6·U5] tri-snRNP-specific protein specifically, anti-m_3G-immunoaffinity-purified snRNPs were fractionated on a 10–30% (w/w) glycerol gradient. The protein and RNA composition of such a gradient is shown in Figure 6A and B. The 12S region of the gradient (peak fraction 26) contains predominantly U1 and U2 snRNPs. The 20S region of the gradient (peak fraction 14) contains significant amounts of U5 snRNPs, whereas the majority of [U4/U6·U5] tri-snRNPs sediment at 25S (peak fraction 9). Equivalent protein amounts were separated by SDS-PAGE, and western blotting was performed with anti-27K/GST1 antiserum because it exhibits the least amount of cross-reactivity. Consistent with its designation as a tri-snRNP-specific protein, the vast majority of 27-kDa protein recognized by the anti-27K/GST1 antiserum co-migrated with 25S [U4/U6·U5] tri-snRNPs in gradient fractions 8–10 (Fig. 6C). In contrast, comparatively low reactivity was observed with proteins in the

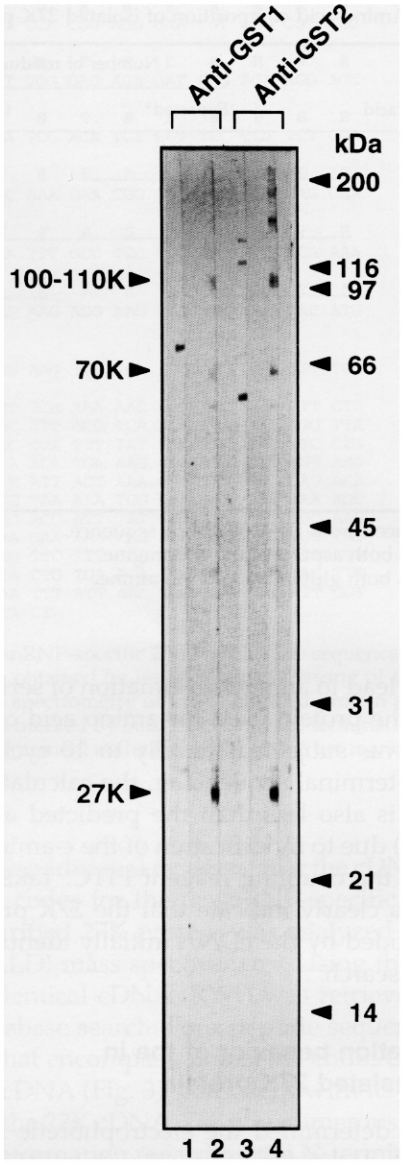

FIGURE 5. Specificity of anti-27K antibodies as determined by immunoblotting. Nitrocellulose strips containing 5 μg of snRNPs (U1–U6) were incubated with anti-27K/GST1 or anti-27K/GST2 preimmune (lanes 1 and 3, respectively) or immune serum (lanes 2 and 4, respectively). Immunostaining was performed as described in Materials and Methods. The position of molecular mass markers is indicated on the right and the major immunoreactive proteins are labeled on the left.

12S U1/U2 and the 20S U5 snRNP-containing fractions. The results presented in Figure 6 thus indicate that the 27K protein is a bona fide tri-snRNP-specific protein.

Immunoprecipitation of native [U4/U6·U5] tri-snRNPs with anti-27K antibodies

To investigate whether our anti-27K sera recognize the native, particle-associated 27K protein, immunoprecipitation studies were performed with native snRNP particles. Because the tri-snRNP-specific proteins dissociate

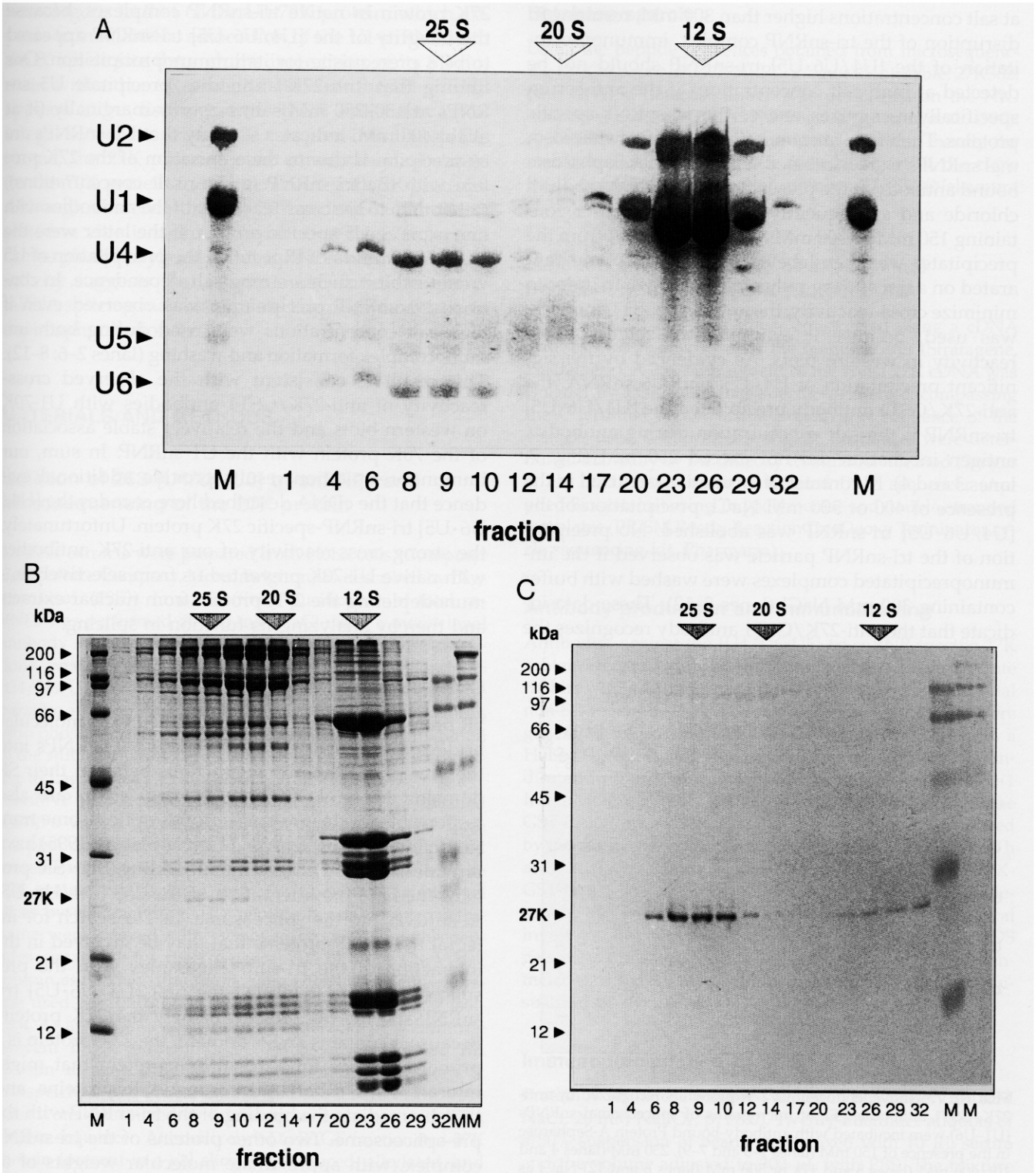

FIGURE 6. Antibodies raised against recombinant 27K protein specifically recognize glycerol gradient-purified tri-snRNP-specific 27K protein. Total snRNPs (U1, U2, U4/U6, and U5) were isolated from HeLa nuclear extract by immunoaffinity chromatography using an anti-m3G antibody. 12S U1 snRNPs, 20S U5 snRNPs, and 25S [U4/U6·U5] tri-snRNPs were fractionated by gradient centrifugation on 10–30% glycerol gradients as described in Materials and Methods. **A:** SnRNAs were separated by electrophoresis on a 10% denaturing polyacrylamide gel and visualized by silver staining. The position of the spliceosomal snRNAs is indicated on the left. Analyzed fractions and sedimentation values are indicated. **B:** Proteins of the corresponding fractions were separated by SDS-PAGE on a 10% polyacrylamide gel and visualized by staining with Coomassie blue. **C:** Proteins were separated by SDS-PAGE on a 10% polyacrylamide gel and subsequently transferred to nitrocellulose. The nitrocellulose was incubated with anti-27K/GST1 serum and immunostaining was performed as described in Materials and Methods. Lane M shows molecular mass marker proteins stained with Ponceau S. The position of molecular mass markers is indicated on the left.

at salt concentrations higher than 300 mM, resulting in disruption of the tri-snRNP complex, immunoprecipitation of the [U4/U6·U5] tri-snRNP should not be detected at high salt concentrations if the anti-serum specifically recognizes one of the tri-snRNP-specific proteins. Therefore, immunoaffinity-purified spliceosomal snRNPs were incubated with Protein A Sepharose-bound antibody in the presence of 150–500 mM sodium chloride and subsequently washed with buffer containing 150 mM or 300 mM NaCl. The snRNA from the precipitates was then labeled with [^{32}P]pCp and separated on a denaturing polyacrylamide gel. In order to minimize cross-reactivity, the anti-27K/GST1 antibody was used, because it exhibited the lowest cross-reactivity in western blots. As evidenced by the significant precipitation of U4, U5, and U6 snRNA, the anti-27K/GST1 antibody precipitated the [U4/U6·U5] tri-snRNP if the salt concentration during antibody/antigen incubation did not exceed 250 mM (Fig. 7, lanes 3 and 4). In contrast, if incubation occurred in the presence of 400 or 500 mM NaCl, precipitation of the [U4/U6·U5] tri-snRNP was abolished. No precipitation of the tri-snRNP particle was observed if the immunoprecipitated complexes were washed with buffer containing 300 mM NaCl (lanes 8–12). These data indicate that the anti-27K/GST1 antibody recognizes the 27K protein in native tri-snRNP complexes, because the integrity of the [U4/U6·U5] tri-snRNP appeared to be a prerequisite for its immunoprecipitation. Our finding that anti-27K antibodies precipitate U5 snRNPs at 150–250 mM salt, but only marginally (if at all) at 400 mM, indicates strongly that U5 snRNPs are co-precipitated due to the association of the 27K protein with the tri-snRNP (at low salt concentrations), rather than to the cross-reactivity of the antibodies with one or more U5-specific protein. If the latter were the case, one would not expect that the precipitation of U5 would exhibit such a strong salt dependence. In contrast, U1 snRNP precipitation was observed even if high salt concentrations were used during both immunocomplex formation and washing (lanes 2–6; 8–12). This result is consistent with the observed cross-reactivity of anti-27K/GST1 antibodies with U1-70K on western blots and the relatively stable association of the 70K protein with the U1 snRNP. In sum, our immunoprecipitation results provide additional evidence that the cDNA described here encodes the [U4/U6·U5] tri-snRNP-specific 27K protein. Unfortunately, the strong cross-reactivity of our anti-27K antibodies with native U1-70K prevented us from selectively immunodepleting the 27K protein from nuclear extracts and thereby analyzing its function in splicing.

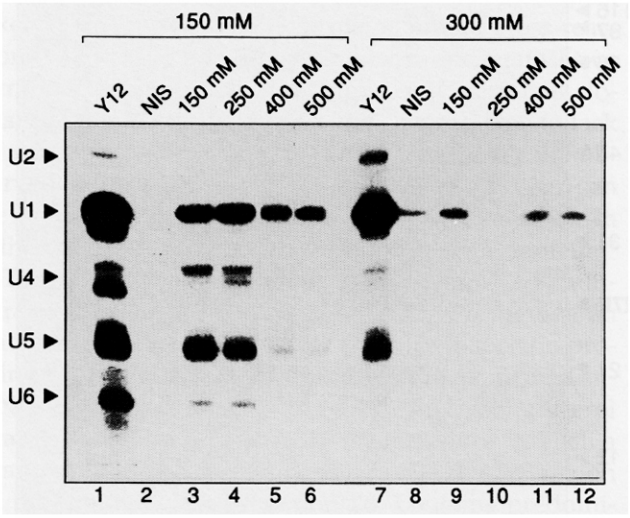

FIGURE 7. Native, endogenous 27K protein is recognized by anti-27K/GST1 antibodies. Eight micrograms of spliceosomal snRNPs (U1–U6) were incubated with antibody-bound Protein A-Sepharose in the presence of 150 mM (lanes 1–3 and 7–9), 250 mM (lanes 4 and 10), 400 mM (lanes 5 and 11), or 500 mM (lanes 6 and 12) NaCl as described in Materials and Methods. SnRNPs were immunoprecipitated and washed in the presence of either 150 mM (lanes 1–6) or 300 mM NaCl (lanes 7–12). SnRNA of the precipitates was extracted, radiolabeled with [^{32}P]pCp, and fractionated on a 10% polyacrylamide/7 M urea gel. RNA was detected subsequently by autoradiography. Lanes 1 and 7 shows immunoprecipitation using mAB Y12, which reacts with proteins common to all of the spliceosomal snRNPs, as a positive control. In lanes 2 and 8, immunoprecipitation was performed with pre-immune serum as a background control. The identity of the various snRNAs is indicated on the left. Similar results were obtained with the anti-27K/GST2 and anti-peptide antibodies.

CONCLUSIONS

SR proteins have been shown to function as molecular bridges that recruit U1 snRNPs and U2 snRNPs into the pre-spliceosome by interactions between their SR domains. Most likely, these splicing factors are also responsible for the pre-spliceosome/spliceosome transition. Indeed, Roscigno and Garcia-Blanco (1995) have provided evidence recently suggesting that SR proteins mediate the integration of the 25S [U4/U6·U5] tri-snRNP into the spliceosome. In our search for integral tri-snRNP proteins that may be involved in the interaction of the tri-snRNP complex with the pre-spliceosome, we identified the first [U4/U6·U5] tri-snRNP-specific SR protein, namely the 27K protein. Because it contains an SR domain, the 27K protein is a prime candidate for a tri-snRNP protein that might interact with other spliceosomal SR proteins and thereby mediate the binding of the tri-snRNP with the pre-spliceosome. Two other proteins of the tri-snRNP complex, with approximate molecular weights of 60 and 100 kDa, were also phosphorylated by the snRNP-associated protein kinase, suggesting that they too possess an SR domain and could also take part in SR protein interactions during spliceosome formation. Alternatively, the latter two putative SR proteins, as well as the 27K protein, could potentially play an important role in the interaction of the U5 and U4/U6 snRNPs during the formation of the tri-snRNP complex. Interestingly, the SR domain of the 27K protein is highly

homologous to that of the U1-70K protein, which appears to mediate the association of the U1 snRNP with the 5′ splice site through the interaction of its SR domain with other SR proteins. Because the U1 snRNP appears to be displaced from the 5′-splice site upon integration of the tri-snRNP complex into the spliceosome, the 27K protein could conceivably substitute for the 70K protein in its interaction with SR proteins. Although evidence that the 27K protein is phosphorylated in vivo is lacking, the fact that the 27K protein can be phosphorylated by an snRNP-associated kinase activity suggests that its presumed function in pre-mRNA splicing may be regulated by phosphorylation/dephosphorylation cycles.

MATERIALS AND METHODS

Isolation of 25S [U4/U6·U5] tri-snRNPs and microsequencing of the 27K protein

Nuclear extracts were prepared from HeLa cells (Computer Cell Culture Company, Mons, Belgium) as described by Dignam et al. (1983). Immunoaffinity purification of total snRNPs was performed at 0.25 mM NaCl using a monoclonal antibody raised against the m_3G cap. Under these conditions, predominantly the 12S U1, 12S U2, 20S U5, and 25S [U4/U6·U5] tri-snRNP are isolated (Will et al., 1994). Preparative amounts of 25S [U4/U6·U5] tri-snRNPs used for in vitro phosphorylation assays were isolated by centrifugation of the snRNP mixture on a 10–30% glycerol gradient according to Laggerbauer et al. (1996).

SnRNP proteins were extracted from 20 mg of purified snRNPs with 1 volume of phenol and precipitated from the phenolic phase with 5 volume of acetone. Precipitated proteins were separated by preparative SDS-PAGE and the 27K protein was isolated from the Coomassie-stained gel slices by electroelution. The peptide bonds next to methionine were cleaved with cyanogen bromide and the peptides separated by reverse-phase HPLC on a C8 Vydac column as described by Matsudaira (1989). The solvent system used was 0.1% (v/v) trifluoroacetic acid in water (solvent A) and 0.1% (v/v) trifluoroacetic acid in acetonitrile (solvent B). A gradient of 0–60% B was performed for 90 min using a flow rate of 1 mL/min (Eckerskorn & Lottspeich, 1989). Partial amino acid sequences of the peptides were determined by microsequencing on an ABI 477A protein sequencer. Alternatively, SDS-PAGE fractionated 27K protein (present in a gel slice) was reduced, alkylated, and subsequently digested with the endoproteinase Lys-C. The mass of the resulting peptides was then determined by MALDI mass spectrometry and a protein database search was performed using the MALDI data (Vorm et al., 1994). For amino-terminal sequencing, native 27K protein was blotted onto PVDP membrane and subjected to 10 steps of Edman degradation. The same protein preparation was used subsequently to determine the amino acid composition of the 27K protein by postcolumn derivatization with the reagent OPA on a Sykam analyzer.

Database search

Using the peptide sequences obtained from microsequencing (27K36.pep: NrKGGFNRPL, r = not identifiable; 27K362.pep: GFA), a database search was performed on the NIG mailserver using the TBLASTN program (Altschul et al., 1990). A cDNA with an open reading frame matching exactly the peptide sequences of the 27K protein is part of the clone RY-1, which has the accession number X76302 (Nakamura et al., 1994).

Isolation and sequencing of a C-terminal fragment of the HeLa 27K protein cDNA

A HeLa cell cDNA library was constructed using a cDNA synthesis kit as described by the manufacturer (Stratagene, Heidelberg). A 255-bp fragment of the HeLa 27K cDNA was isolated by PCR with gene-specific primers encompassing the C-terminal region of the protein (the sequence of the primers used is indicated in Fig. 3). Sequences were determined manually by the dideoxy termination method (Sanger et al., 1977) and with an automated DNA sequencer (Applied Biosystems) using Taq polymerase and double-stranded templates (PRISM Ready Reaction DyeDeoxy Terminator cycle sequencing kit, Pharmacia).

Antibody production and immunoblotting

Antibodies against the [U4/U6·U5] tri-snRNP-specific 27K protein were raised by immunization of New Zealand White rabbits with a GST fusion protein containing the C-terminal non-SR region of the 27K protein. Expression vectors were generated by isolating the 3′-half of the 27K cDNA from a HeLa cDNA library by PCR with gene-specific primers (indicated in Fig. 3) and cloning the resulting *Bam*H 1–*Xho* 1 fragment into pGEX-4T1 (Pharmacia). Overexpression of the GST-fusion protein in *Escherichia coli* XL1-Blue was induced by incubation with 1 mM isopropyl-D-thiogalactoside for 2 h at 30 °C. Cells were disrupted by sonication and the 27K-GST-fusion protein was isolated with GST-Sepharose as described by the manufacturer (Pharmacia). For immunological investigations, proteins fractionated on high-TEMED SDS gels were transferred to nitrocellulose as described by Lehmeier et al. (1990) and immunocomplex formation was visualized by the alkaline phosphatase reaction.

Immunoprecipitation

Protein A-Sepharose was pre-swollen in PBS buffer (130 mM NaCl, 20 mM $NaPO_4$, pH 8.0). Twenty-microliter aliquots of a 50% suspension of beads were mixed with 10 μL serum and the volume adjusted to 300 μL with PBS. The mixture was incubated overnight or for 2 h at 4 °C with continual end-over-end rotation of the vessel. After the beads had been washed four times with 1 mL of PBS, 8 μg snRNPs (U1, U2, U4/U6, U5) were added and the mixture was incubated for 3 h at 4 °C in PBS containing 150 mM, 250 mM, 400 mM, or 500 mM NaCl (as indicated in Fig. 7). The Protein A-Sepharose was then washed four times with IPP150 or IPP300 (10 mM Tris/HCl, pH 8.0, 150/300 mM NaCl, 0.1% NP-40). Bound RNA was extracted with phenol/chloroform, precipitated with ethanol, and 3′ end-labeled with [^{32}P]pCp (Amersham)

essentially as described by England and Uhlenbeck (1978). The RNA was fractionated on 10% polyacrylamide–7 M urea gels (Bringmann & Lührmann, 1986).

In vitro phosphorylation assays

Twenty micrograms (for 2D analysis) or 3 μg (for analytical phosphorylation) of 25S [U4/U6·U5] tri-snRNPs, prepared by glycerol gradient centrifugation as described above, were incubated for 30 min at 37 °C with 80 ng of purified snRNP-associated protein kinase (S. Fetzer, M. Krause, & R. Lührmann, in prep.), 66 nM [γ-^{32}P]-ATP (3,000 Ci/mmol) (Amersham) and 1 μM ATP in buffer P (20 mM Hepes/KOH, pH 7.9, 1.5 mM $MgCl_2$, 50 mM KCl, 5% (v/v) glycerol, 0.5 mM DTE). Proteins were separated by SDS-PAGE on a 12% high-TEMED gel (Lehmeier et al., 1990) and visualized by autoradiography.

In vitro translation

The plasmid containing the cDNA encoding the 27K protein was linearized with *Xho* 1 and transcribed with T3 RNA polymerase (Hermann et al., 1995). Two micrograms of the in vitro-transcribed mRNA was translated with rabbit reticulocyte lysate in the presence of [^{35}S]-methionine (Amersham) (end volume 75 μL) according to the manufacturer's instructions (Promega). Proteins were fractionated by SDS-PAGE on a 12% high-TEMED gel, which was treated subsequently with Amplify (Amersham). Proteins were visualized by fluorography, normally for 12 h.

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis of in vitro-phosphorylated 27K protein was performed by the NEPHGE method according to the manufacturer's instructions (Bio-Rad) with the following modifications. Twenty micrograms of gradient-purified 25S [U4/U6·U5] tri-snRNPs were applied at the anode end and separated on a 3–11 pH gradient using the Mini Protean II chamber (Bio-Rad, Munich). After NEPHGE, proteins were fractionated in the second dimension by SDS-PAGE on 12% high-TEMED gels and ^{32}P-labeled proteins were visualized by autoradiography.

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