FEBS Letters 346 (1994) 225–228

FEBS 14093

The lamin B receptor-associated protein p34 shares sequence homology and antigenic determinants with the splicing factor 2-associated protein p32

George Simos, Spyros D. Georgatos*

Programme of Cell Biology, European Molecular Biology Laboratory, 1 Meyerhofstrasse, 69012 Heidelberg, Germany

Received 5 April 1994; revised version received 27 April 1994

Abstract

The lamin B receptor (p58) is an inner nuclear membrane protein that forms an in vivo complex with the nuclear lamins, a nuclear envelope kinase, and two other nuclear proteins with apparent M_r of 18,000 (p18) and 34,000 (p34). We now report the isolation of p34 by partial dissociation of the immunoaffinity-purified p58 protein complex. Determination of the N-terminal amino acid sequence of purified p34 shows that this polypeptide is homologous to p32, a splicing factor 2 (SF2)-associated protein. The relatedness between p34 and p32 can be further established by showing that antibodies raised against N- and C-terminal peptides of p32 cross-react with purified p34. As the amino acid sequence of p58 contains an arginine/serine (RS)-rich region similar to the RS-rich region found in SF 2, we speculate that these domains provide binding sites for p34 and that this protein may be a linker between the nuclear membrane and intranuclear spliceosomal substructures.

Key words: Turkey erythrocyte; Immunoaffinity chromatography; Lamin B receptor; p34; p32; Splicing factor SF2

1. Introduction

Higher eukaryotic cells possess a fibrous lamina which lines the inner nuclear membrane. The nuclear lamina is tethered to the inner nuclear membrane through several integral membrane proteins [1,2]. One such protein is the polypeptide p58, also known as the lamin B receptor [1]. p58 posseses a long N-terminal domain, which is nucleoplasmically disposed, and eight predicted membrane-spanning segments [3].

p58 binds in vitro to nuclear lamin B with an affinity of ~200 nM and in a phosphosphorylation-dependent manner [1,4]. Furthermore, recent work has shown that p58 forms during interphase a multimeric complex which includes the nuclear lamins A and B, a specific p58 kinase and two other polypeptides with molecular masses of 18 kDa and 34 kDa [5]. The identity of the non-lamin partners of p58 remains unknown; however, it is likely that these proteins regulate the p58–lamin interactions and affect the aggregation state of p58. Another potential function of the p58-associated proteins may be the regulation of interactions between p58 and DNA or other intranuclear structures. The N-terminal domain of p58 possesses three DNA-binding motifs and a report has appeared claiming specific binding to DNA in vitro [6]. Prompted by these findings, we decided to characterize the 34 kDa protein found in the p58 complex. Results presented below show that this protein (p34) is homologous to p32, a previously identified protein associated with splicing factor 2.

*Corresponding author. Fax: (49) (6221) 387 306.

2. Materials and methods

2.1. Peptides and antibodies

Peptides p34-N (CGGDFLSDEIKEERK) and p34-C (CGGTGESEWKDTNYTLNTDS), corresponding to N-terminal residues 11–22 and to the C-terminal residues 141–157 of HeLa p32 [7], were made in the Protein Sequencing and Peptide Synthesis Facility of EMBL (Heidelberg, Germany). The tripeptide CGG was added to the sequences for coupling purposes. For immunization, the synthetic peptides were coupled to keyhole limpet hemocyanin (Sigma) using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) as a cross-linker [8]. The immunization procedure was as previously described [5]. To purify the anti-peptide antibodies from whole sera, an affinity matrix was prepared by coupling the two peptides to Epoxy-activated Sepharose 6B (Sigma) [9]. The antisera were passed through the affinity column and the bound antibodies were eluted by washing the column with 0.2 M glycine-HCl, pH 2.3, and 0.5 M NaCl. The collected fractions were neutralized by the addition of 1 M Tris-HCl, pH 8, pooled and dialysed against PBS. The anti-p58 antibodies αR1 and αR6 were raised against peptides R1 (KQRKSQSSSSSPSRRSR SRS) and R6 (KPSENNTYNGEPDSTERND) corresponding to residues 61–80 and 119–139 of chicken liver p58 [3], respectively. The preparation and affinity purification of these antibodies has been described previously [5,10]. Both antibodies were previously shown to be specific for avian p58 [5,10]. For construction of the preparatory immunoaffinity column, 1 mg of affinity purified αR1 IgG was bound to 0.5 ml of protein A–Sepharose (PAS) and the antibodies were covalently coupled to protein A using dimethylpimelimidate (DMP) as a cross-linker [8].

2.2. Purification of p34 from turkey erythrocytes

Turkey erythrocyte ghosts were prepared from 80 ml of turkey blood by hypotonic lysis in 5 mM Na_3PO_4, 2 mM $MgCl_2$, 1 mM DTT and 1.3 mM PMSF, pH 7.4, and extracted with 300 ml 1% Triton buffer (1% Triton X-100, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl) containing protease inhibitors (1 μg/ml leupeptin, 1 μg/ml pepstatin, 5 μg/ml aprotinin and 1 mM PMSF) for 30 min on ice. After centrifugation at 12,000 × *g* for 15 min, the detergent-soluble extract was applied to the immunoaffinity column and recycled three times. The column was subsequently washed with 1% Triton buffer and then with 0.1% Triton buffer. Weakly bound proteins were eluted by applying a solution of 0.5 M NaCl in 0.1% Triton buffer. Finally, the column was treated either with a solution of the R1 peptide (2 mg/ml in 0.1% Triton buffer) (to elute the lamin B receptor and associated proteins including p34)

SSDI 0014-5793(94)00479-F

or with 1 M NaCl in the same buffer (to release and elute only p34). p34 was further purified by passing the 1 M NaCl eluate of the immunoaffinity column through a Sephacryl S-100 gel-filtration column equilibrated in 0.01% Triton X-100, 20 mM Tris-HCl, pH 7.6, 50 mM Nacl. Fractions containing p34 were pooled and concentrated by ultrafiltration.

2.3. Microsequencing

Purified p34 was concentrated by TCA precipitation, resolved in a SDS-PAGE gel, transferred to a ProBlott membrane (Applied Biosystems) and stained by Coomassie blue. The band at 34 kDa was excised from the blot and used for microsequencing. Protein sequence was dtermined by Edman degradation employing the Applied Biosystems, Model 477A, system.

2.4. Other methods

HeLa cells were fractionated into post-nuclear supernatant (PNS), nuclear extract (NE) and nuclear pellet (NP) essentially as described by Krainer et al. [11]. SDS-PAGE was performed according to Laemmli [12]. Immunoblotting was performed as previously described using [^{125}I]protein A for the detection of the primary antibodies [13]. The FASTA programme was used for data bank searches [14].

3. Results

To isolate p58 and associated proteins, a Triton X-100 extract of turkey erythrocyte ghosts was applied to an immunoaffinity column containing anti-peptide, anti-p58 antibodies (αR1; for details see section 2). Elution of the immunoaffinity column with 2 mg/ml of the R1 peptide yielded p58 and associated proteins, including p34 (Fig. 1, lane 1). To purify p34 away from other components, we took advantage of the fact that this protein remains associated with the p58 complex in 0.5 M salt, but dissociates from it in 1 M salt [5]. Therefore, we repeated the previous experiment and, before elution with R1, we applied to the column a buffer containing 1 M NaCl/0.1% Triton X-100. SDS-PAGE analysis and silver staining showed that the 1 M salt eluate was highly enriched in p34 (Fig. 1, lane 2). To examine whether any of the p34 protein remained bound to the column, the R1 peptide was applied after the salt elution and the peptide eluate was analyzed by SDS-PAGE. As shown in Fig. 1, lane 3, the R1 eluate contained all of the known components of the p58 complex, except p34. From these experiments we concluded that p34 could be quantitatively recovered by washing the immunoaffinity matrix with 1 M NaCl.

Fractions enriched in p34, after an additional purification step through a Sephacryl S100 column (not shown), were electrophoresed and blotted onto PVDF membranes. To obtain protein sequencing information, the band at 34 kDa was excised and subjected to Edman degradation. Fig. 2A shows the sequence of the first 20 N-terminal residues of turkey erythrocyte p34 (16 amino acids certain, 3 amino acids unidentified and 1 amino acid uncertain). A search in the GenEMBL databank revealed that the deduced N-terminal sequence is highly homologous to the N-terminal sequence of p32, a splicing factor 2 (SF2)-associated protein, originally characterized in HeLa cells [7]. The two proteins show 12 identities, 3 conservative substitutions and one probable match in a stretch of 20 residues (Fig. 2A). No other proteins were found with significant homology to the p34 N-terminus.

To confirm these results, we have taken an immunochemical approach. Two peptides corresponding to the N-terminal residues 11–22 and to the C-terminal residues 141–157 of HeLa p32 were synthesized, coupled to KLH, and used as immunogens to raise specific antibodies. The resultant antisera (anti-N and anti-C, respectively) were affinity-purified and tested by Western blotting on HeLa cell subcellular fractions. Consistent with previous results [7], the anti-N and anti-C antibodies recognized a 32 kDa protein present in HeLa nuclear extracts (Fig. 3, lanes 2). Some p32 was also present in the post-nuclear supernantant and the residue obtained after salt extraction of HeLa cell nuclei (Fig. 3, lanes 1 and 3). Testing of subcellular fractions of turkey erythrocytes by the same method did not yield a detectable signal, probably because p34 represented a low abudance protein. However, both anti-p32 antibodies readily recognized turkey erythrocyte p34 in the enriched high salt eluate fractions

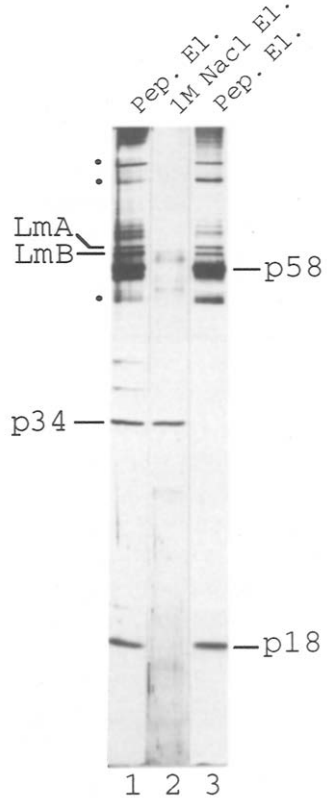

Fig. 1. Partial purification of p34 from turkey erythrocytes. SDS-PAGE analysis and silver staining of material eluted from the anti-R1 immunoaffinity column with the antigenic peptide (lane 1), material eluted with 1 M NaCl before applying the peptide (lane 2), and proteins eluted by the peptide after previous salt elution (lane 3). Pep. El., peptide eluate; 1 M NaCl El., 1 M NaCl eluate. The bands corresponding to lamins A and B, p58, p34 and p18 are indicated by bars (from top to bottom). Dots point to proteins that specifically co-isolate with p58.

A

```
                                   Q
   p34:  H2N-LXTEGXKAFAQFLTDEIXEE...
(turkey RBC)                       E
   p32:  H2N-LHTDGDKAFVDFLSDEIKEE...
  (HeLa)      1                   20
```

B

```
          71                                  101
p58:..SPSRRSRSRSRSRSPGRPAKGRRRSSSHSRE..

         202
SF2:..YGRSRSRSRSRSRSRSRSNSRSRSYSPRRSR
                        248
      GSPRYSPRHSRSRSRT..
```

Fig. 2. (A) Comparison between the N-terminal amino acid sequence of turkey erythrocyte p34, as deduced by protein sequencing, and the N-terminal amino acid sequence of HeLA p32 [7]. Solid lines indicate identities and dashed lines indicate conservative substitutions. (B) Part of the amino acid sequence of p58 [3] and an analogous sequence in SF2 [7], showing the existence of RS-rich regions in the two proteins.

(Fig. 3, lanes 4). This suggests that p34 and p32 are antigenically related and contain common epitopes present at both the N- and the C-terminal parts of the two molecules.

4. Discussion

The lamin B receptor (p58) has been localized in the inner nuclear membrane [15] and shown to associate with the nuclear lamins A and B, a protein kinase that modifies p58 and a set of other polypeptides [5]. One of the latter proteins, p34 can be released from the p58 complex by high salt and does not co-isolate with p58 when extraction and purification are performed under conditions that favor phosphorylation (in the presence of ATP and phosphatase inhibitors) [5]. In this report we have described the partial purification of turkey erythrocyte p34 and demonstrated that it is homologous to the HeLa protein p32 [7]. The possibility that p34 is co-isolated with p58 because it is directly recognized by the αR1 antibody can be safely ruled out since the same results have been obtained using another anti-p58 antibody, αR6 (data not shown). Unfortunately, our anti-p32 antibodies do not seem to recognize the native form of p32 as they give no signal in indirect immunofluoresence assays.

The human homologue of p34, i.e. the p32 protein, was initially identified as an element co-purifying with SF2, a well-characterized splicing factor [7]. It has been suggested that p32 is synthesized as a longer polypeptide containing 282 amino acids that is post-translationally processed by removal of the initial 73 residues to a mature protein of 209 amino acids [16]. The actual molecular weight of mature p32, as deduced from its cDNA, is 23.8 kDa and its calculated pI is ~4.0 [7]. Bacterially expressed p32 does not have a splicing activity itself and does not affect the splicing activity of SF2 [7]. However, the fact that p32 and SF2 co-purify through several chromatographic steps suggests a specific interaction between the two proteins [7].

Our anti-p58 antibodies do not react with the mammalian form of p58 and thus we were unable to examine whether p32 is also associated with p58 in HeLa cells.

At first glance, the presence of a p32 homologue in the lamin B receptor complex seems puzzling. However, closer inspection of the p58 and the SF2 sequences reveals certain common characteristics. First, as previously reported, SF2 and the N-terminal domain of p58 are highly charged and contain numerous basic amino acids [3,7]. Furthermore, we have noticed that p58 contains in its N-terminal domain an arginine/serine (RS)-rich region, a motif also found near the C-terminus of SF2 (Fig. 2B). RS domains have also been identified in other essential splicing factors such as the family of SR proteins (which comprises SF2, SC35, SRp20, SRp55 and SRp75) and U2AF, in *Drosophila* splicing regulators, in the integral U1 snPNP protein U1–70K [17,18], and in a 54 kDa nuclear protein of unknown function which, however, co-localizes with spliceosome compo-

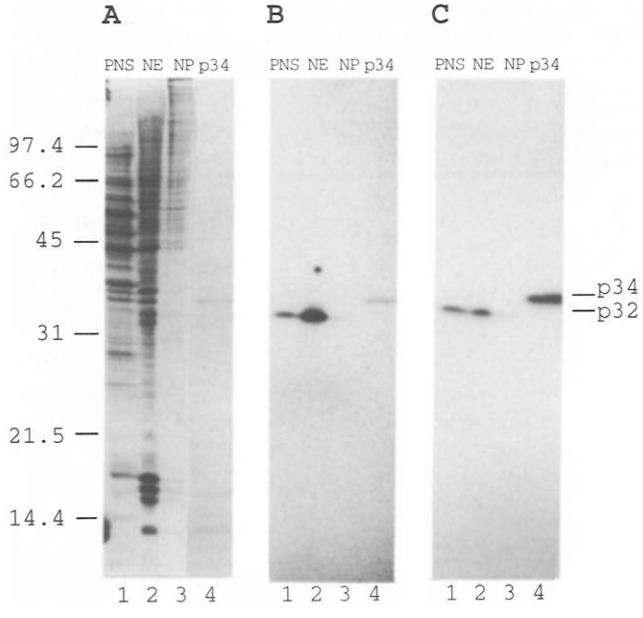

Fig. 3. HeLa cell subcellular fractions (lanes 1–3) and partially purified turkey erythrocyte p34 (1 M salt eluate of the αR1 immunoaffinity column; lanes 4) were analyzed by SDS-PAGE and either stained with Coomassie blue (A), or blotted and immunostained with affinity-purified antibodies raised against the N-terminal (B) or C-terminal (C) peptide of HeLa p32. Lanes 1, post-nuclear supernatant (PNS); lanes 2, nuclear extract (NE); lanes 3, nuclear pellet (NP). Bars on the left correspond to molecular mass (in kDa). The positions of p34 and p32 are indicated.

nents [19]. It has been suggested that the RS domains are responsible for targeting proteins to the speckled subnuclear compartments implicated in splicing [20]. More recently, it has been shown that the RS domains mediate protein–protein interactions between components of the splicing machinery [21,22], probably in a phosphorylation-dependent manner [23]. The presence of a RS domain in the lamin B receptor, a membrane protein of the nuclear envelope with no obvious involvement in RNA processing, suggests a wider role for this motif. Taking into account the acidic nature of p32/p34 and their association with the lamin B receptor and SF2, one could speculate that the p32/p34 function as RS binding proteins. An alternative explanation for the presence of a splicing factor-associated protein in the lamin B receptor complex is provided by previous immunoelectron microscopy data which demonstrated that the nuclear speckled substructures that contain spliceosomal components extend to, and come into contact with, the nuclear envelope [24]. Furthermore, upon in vitro-induced differentiation of murine erythroleukemia (MEL) cells and before loss of the nucleus, splicing snRNPs migrate to the nuclear periphery, associate with the nuclear envelope and form a membrane-enclosed domain (SCIM, SnRNP Clusters Inside a Membrane domain) [25]. p32/p34 may therefore function as a linking component between the nuclear membrane and intranuclear stuctures involved in RNA splicing.

Acknowledgements: This article is dedicated to Stavros and Adamantia Politis. We thank R. Kellner,T. Houthaeve and D. Nalis in the EMBL Protein Peptide Group for performing protein sequencing and peptide synthesis, C. Ouzounis for help with sequence searches, and J. Meier for useful discussions. G.S. is supported by a research bursary granted by the Commission of the European Communities in the framework of the BIOMED 1 programme.

References

[1] Worman, H.J., Yuan, J., Blobel, G. and Georgatos, S.D. (1988) Proc. Natl. Acad. Sci. USA 85, 8531–8534.
[2] Foisner, R. and Gerace, L. (1993) Cell 73, 1267–1279.
[3] Worman, H.J., Evans, C.D. and Blobel, G. (1990) J. Cell Biol. 111, 1535–1542.
[4] Appelbaum, J., Blobel, G. and Georgatos, S.D. (1990) J. Biol. Chem. 265, 4181–4184.
[5] Simos, G. and Georgatos, S.D. (1992) EMBO J. 11, 4027–4036.
[6] Ye., Q., Courvalin, J.-C. and Worman H.J. (1993) Mol. Biol. Cell 4, 308a.
[7] Krainer, A.R., Mayeda, A., Kozak, D. and Binns, G. (1991) Cell 66, 383–394.
[8] Harlow, E. and Lane, D. (1988) Antibodies: A Laboratory Manual, CSH, Cold Spring Harbor Laboratory, NY.
[9] Simons, P.C. and Vander Jagt, D.L. (1977) Anal. Biochem. 82, 334–341.
[10] Meier, J. and Georgatos, S.D. (1994) EMBO J. 13, 1888–1898.
[11] Krainer, A.R., Conway, G.C. and Kozak, D. (1990) Genes Dev. 4, 1158–1171.
[12] Laemmli, U.K. (1970) Nature 227, 680–685.
[13] Georgatos, S.D. and Blobel, G. (1987) J. Cell Biol. 105, 105–115.
[14] Pearson, W.R. and Lipman, D.J. (1988) Proc. Natl. Acad. Sci. USA 85, 2444–2448.
[15] Bailer, S.M., Eppenberger, H.M., Griffiths, G. and Nigg, E.A. (1991) J. Cell Biol. 114, 389–400.
[16] Honore, B., Madsen, P., Rasmussen, H.H., Vandekerckhove, J., Celis, J.E. and Leffers, H. (1993) Gene 134, 283–287.
[17] Lamm, G.M. and Lamond, A.I. (1993) Biochim. Biophys. Acta 1173, 247–265.
[18] Horowitz, D.S. and Krainer, A.R. (1994) Trends Genet. 10, 100–106.
[19] Chaudhary, N., McMahon, C. and Blobel, G. (1991) Proc. Natl. Acad. Sci. USA 88, 8189–8193.
[20] Li, H. and Bingham, P.M. (1991) Cell 67, 335–342.
[21] Wu, J.Y. and Maniatis, T. (1993) Cell 75, 1061–1070.
[22] Kohtz, J.D., Jamison, S.F., Will, C.L., Zuo, P., Luhrmann, R., Garcia-Blanco, M.A. and Manley, J.L. (1994) Nature 368, 119–124.
[23] Woppmann, A., Will, C.L., Kornstadt, U., Zuo, P., Manley, J.L. and Lurhmann, R. (1993) Nucleic Acids Res. 21, 2815–2822.
[24] Spector, D.L., Fu, X.-D. and Maniatis, T. (1991) EMBO J. 10, 3467–3481.
[25] Antoniou, M., Carmo-Fonseca, M., Ferreira, J. and Lamond, A.I. (1993) J. Cell Biol. 123, 1055–1068.