The exosome is a complex of 3′→5′ exoribonucleases that functions in a variety of cellular processes, all concerning the processing or degradation of RNA. Paradoxically, the previously described cDNA for the human autoantigenic exosome subunit PM/Scl-75 (Alderuccio, F., Chan, E. K., and Tan, E. M. (1991) J. Exp. Med. 173, 941–952) encodes a polypeptide that failed to interact with the exosome complex. Here, we describe the cloning of a more complete cDNA for PM/Scl-75 encoding 84 additional amino acids at its N terminus. We show that only the longer polypeptide is able to associate with the exosome complex. This interaction is most likely mediated by protein-protein interactions with two other exosome subunits, hRrp46p and hRrp41p, one of which was confirmed in a mammalian two-hybrid system. In addition we show that the putative nuclear localization signal present in the C-terminal region of PM/Scl-75 is sufficient, although not essential for nuclear localization of the protein. Moreover, the deletion of this element abrogated the nucleolar accumulation of PM/Scl-75, although its association with the exosome was not disturbed. This suggests that this basic element of PM/Scl-75 plays a role in targeting the exosome to the nucleolus.
Characterization of the Complete PM/Scl-75 Protein

pEYFP vectors (Clontech) by PCR or by available restriction sites. The data base accession numbers of the cDNAs used are BC000474 (hRrp4p), AF281132 (hRrp4p), AF281133 (hRrp4p), D29958 (hRrp42p), AF281134 (hRrp46p), M58460 (PM/Scl-75a), L01457 (PM/Scl-100), AF151866 (hDia3p), RP7667 (hDia3p), AF025438 (OIP2), and NM_058219 (hMtrp). The sequences of the cDNAs encoding PM/Scl-75c-α and PM/Scl-75c-β were submitted under accession numbers AF150899 and AF157294, respectively. Deletion mutants of PM/Scl-75 were constructed using suitable internal restriction sites.

Bioinformatics—Predictions of functional domains and putative NLSs were done using the Pfam data base of Hidden Markov models and the SMART database (10). Predictions of functional domains and putative NLSs were done using the Pfam data base of Hidden Markov models and the SMART database (10). Predictions of functional domains and putative NLSs were done using the Pfam data base of Hidden Markov models and the SMART database (10). Predictions of functional domains and putative NLSs were done using the Pfam data base of Hidden Markov models and the SMART database (10). Predictions of functional domains and putative NLSs were done using the Pfam data base of Hidden Markov models and the SMART database (10). Predictions of functional domains and putative NLSs were done using the Pfam data base of Hidden Markov models and the SMART database (10).

Immunoblot Analysis—For immunoblot analysis, the proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. To visualize the proteins, the blots were incubated with autoimmune patient or rabbit sera diluted 5000- and 500-fold, respectively, in blocking buffer (4% skimmed milk, phosphate-buffered saline (PBS), 0.1% Nonidet P-40). As secondary antibody, horseradish peroxidase-conjugated rabbit anti-human IgG or swine anti-rabbit IgG (Dako Immunoglobulins) were used, 5000-fold diluted in blocking buffer. Visualization was performed by chemiluminescence.

In Vitro Translation—Radioactively labeled proteins were produced using a reticulocyte lysate system (Promega) according to the manufacturer’s instructions. A total volume of 25 μl containing ~1 μg of circular plasmid DNA (pCI-neo; Promega), containing the coding sequence of the protein, and in the presence of [35S]methionine.

Reverse Transcription-PCR—RNA was isolated from cells using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. Synthesis of cDNA from 1 μg of RNA was performed using a reverse transcription system (Promega) with 10 pmol of PM/Scl-75c-specific primer. The reaction mixture was incubated for 10 min at room temperature, followed by 90 min at 42 °C. For RT-PCR 1 μl of cDNA was added to 49 μl of the reaction mixture containing 75 mM Tris-HCl, pH 8.8, 20 mM (NH4)2SO4, 0.01% (v/v) Tween 20, 2 mM MgCl2, 0.2 mM dNTPs, 20 pmol of forward and reverse primer, and 8 units of Taq DNA polymerase. PCR was performed using a T3 thermocycler (Biometra): 2 C, 30 s at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C; and 5 min at 72 °C. 10 μl of PCR product was analyzed on a 1.5% agarose gel. The following primers were used: RT primer, 5′-CAGGT-GTAAAGAACCCTGTGAGGAGG-3′; PM/Scl-75 reverse primer, 5′-CACCAGGAGCATGGACTTCTGCTGAC-3′; PM/Scl-75α forward primer, 5′-AGATCTCGAGCCTTATGCGGCGGCGGATTGACGTC-3′; and PM/Scl-75c forward primer, 5′-AGATCTCGAGCCTTATGCGGCGGCGGATTGACGTC-3′.

Transient Transfection of HEP-2 Cells and Direct Immunofluorescence—For transfection, cDNAs were cloned into suitable pEYFP vectors (Clontech), allowing expression of the proteins fused to the N terminus of the EGFP protein. HEP-2 human epithelial carcinoma, ATCC number CCL-23) cells were grown to 80% confluent monolayers by standard tissue culture techniques in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum. For immunoprecipitation, ~10 × 105 cells were transfected with 20–30 μg of DNA in 1600 μl of Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum by electroporation, which was performed at 270 V and 950 microfarad using a Gene-Pulser II (Bio-Rad). After transfection, the cells were seeded in 75-cm² culture flasks and cultured overnight. After washing twice with PBS, the cells were resuspended in 500 μl of lysis buffer (25 mM Tris-HCl, pH 7.5, 100 mM KCl, 2 mM EDTA, 1 mM dithioerythritol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.05% Nonidet P-40) and homogenized. For fluorescent microscopy, ~2 × 106 cells were transfected with 10–20 μg of DNA in 800 μl of Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum by electroporation, as described above. After transfection, the cells were seeded onto cover slips and cultured overnight. The cells were washed twice with PBS, fixed with 4% paraformaldehyde in PBS for 20 min, washed in PBS twice, incubated in acetone, dried, and finally mounted with PBS/ glycero1. The expressed EGFP-tagged proteins were visualized by fluorescent microscopy.

Immunoprecipitation—Polyclonal antibodies from rabbits and patients were coupled to protein A-agarose beads (Biozym) in IPP500 (500 μl NaCl, 10 mM Tris-HCl, pH 8.0, 0.05% Nonidet P-40) by incubation of the beads with room temperature. The beads were washed twice with IPP500 and once with IPP150 (150 mM NaCl, 10 mM Tris-HCl, pH 8.0, 0.05% Nonidet P-40). For each immunoprecipitation, the cell extract was incubated with the antibody-coupled beads for 1 h at 4 °C. The beads were washed three times with IPP150, and the co-immunoprecipitated proteins were analyzed by immunoblotting.

Mammalian Two-hybrid Analysis—All of the interactions were analyzed using the CheckMate mammalian two-hybrid system (Promega), essentially according to the manufacturer’s protocol. Briefly, 3–4 × 104 COS-1 cells seeded in one well of a 6-well plate were transfected with three vectors (1 μg each), pACT and pBIND, either with or without insert, and the pCDNA3 reporter vector using 5 μl of FuGENE Transfection Reagent (Roche Applied Science), as described by the manufacturer. After 40–48 h of growth, the cells were harvested using 500 μl of Passive Lysis Buffer (Promega), and the activity of the two firefly luciferase and the control Renilla luciferase were determined using a dual luciferase reporter assay system (Promega) on a Berthold Lumat LB 9507 photometer.

RESULTS

Amino Acid Sequence of PM/Scl-75—Several amino acid sequences for the human PM/Scl-75 polypeptide have been reported previously. Besides the first sequence ever described (here referred to as PM/Scl-75a-α, accession number M58460) (1), a splicing variant containing the sequence encoded by an additional exon (PM/Scl-75a-β, accession number HSU09215) and a sequence containing 68 additional amino acid residues at the N terminus (PM/Scl-75b-α, accession number Q62656) have been reported. Moreover, the sequence reported for Mus musculus PM/Scl-75 (accession number Q8J1H7) contains, compared with the human PM/Scl-75a-α, 84 additional amino acid residues at the N terminus (23). The reported heterogeneity in sequences for the N-terminal region of the human PM/Scl-75 prompted us to screen the human EST data bases with the available human and mouse PM/Scl-75 cDNA sequences. These analyses demonstrated that in humans PM/Scl-75 sequences are expressed that fully correspond to the N-terminal region of the mouse protein. Thus, in comparison with PM/Scl-75a the human protein may also contain 84 additional amino acids. Based upon these data extended open reading frames for the human PM/Scl-75 were generated (PM/Scl-75c-α and PM/Scl-75c-β) and submitted to the EMBL data base under accession numbers AJ505899 and AJ517294. Fig. 1a shows the sequence of PM/Scl-75c, in which the differences with previously described PM/Scl-75 sequences are indicated. In Fig. 1b a schematic overview of the human polypeptides and their mRNAs is shown. It is important to note that PM/Scl-75a lacks a significant portion of the RNase PH domain as predicted by Pfam (21). The RNase PH domain is expected to be important for the interactions of PM/Scl-75 with other exosomal proteins in the core of the exosome (20).

When the cDNA sequences are compared with the human genome, it is clear that the difference between the sequences is due to the use of an apparent alternative promoter in the PM/Scl-75 gene or to alternative splicing of the PM/Scl-75 pre-mRNA, as is illustrated in Fig. 1c. The 5′ end of the cDNAs of PM/Scl-75 is encoded by an alternative first exon and lacks the first 234 nucleotides of the first exon of PM/Scl-75a. The protein sequence of PM/Scl-75b was derived from a cDNA very similar to that of PM/Scl-75a. The PM/Scl-75b cDNA contains a stretch of 8 instead of 7 thymidine residues at position 395–402 (which may be due to a sequencing error of the original clone), leading to a longer open reading frame (23). To investigate which of the corresponding mRNAs are actually expressed in human cell lines, RNA isolated from HEP-2, HeLa, 293, Jurkat, and MOLT-4 cells was analyzed by RT-PCR using primers specific for the PM/Scl-75a/b and PM/Scl-75c/mRNAs. Both mRNAs were found to be present in all cell lines (results not shown). To discriminate between PM/Scl-75a and PM/Scl-75b,
the RT-PCR products were cloned and sequenced. The results showed that the T-stretch consisted of 8 thymidine residues in all cases, indicating that the expressed mRNA corresponds to PM/Scl-75b. Strikingly, however, no evidence for the existence of mRNAs encoding either PM/Scl-75a or PM/Scl-75b was found in the EST data bases, strongly suggesting that PM/Scl-75c is the most abundantly expressed isoform of the protein. To generate cDNAs encoding PM/Scl-75c, we performed PCR on

Fig. 1. The different isoforms of PM/Scl-75. a, overview of the sequence of PM/Scl-75, in which the N termini of the different isoforms are indicated (arrows and sequence in italics), as well as the position of the sequence encoded by the extra exon 10* (sequence in italics indicated by PM/Scl-75-a/b). The RNase PH domain (underlined) and the putative NLS sequence (bold type) are also marked in the sequence. b, schematic representation of the domains present in the different isoforms of the PM/Scl-75 protein. The RNase PH domain (which is incomplete in PM/Scl-75a), the negatively charged region, the site at which the amino acids encoded by the extra exon 10* are inserted in PM/Scl-75-b, and the putative NLSs are indicated. Below the proteins schematic representations of the corresponding mRNAs are shown. The relative lengths of the coding sequence (CDS), the 5'- and 3'-untranslated regions (UTR), and the number of thymidine residues in the thymidine stretch are indicated. c, a schematic overview of the first four exons (boxes 1–4) of the PMSCL1 gene, its putative transcription initiation sites (marked >) and mode of splicing, leading to the various N-terminally different forms of PM/Scl-75. The first four amino acids of these open reading frames (a), (b), and (c) are indicated d, a schematic overview of the last four exons (boxes 10*, 11, and 12) of the PMSCL1 gene and its mode of splicing, leading to the α and β isoforms of PM/Scl-75. The exon present only in the β form of PM/Scl-75 is designated exon 10* (in the figure). The stop codon (TAA) is located in exon 12.
The migration of PM/Scl-75c was identical to that of HeLa cell PM/Scl-75. In vitro translated, 35S-labeled PM/Scl-75a- and PM/Scl-75c- cDNAs were produced by in vitro transcription/translation, and their migration in SDS-PAGE gels was compared with that of PM/Scl-75 from a cytoplasmic HeLa cell extract, which was detected by immunoblotting using anti-PM/Scl-75 rabbit antibodies (Fig. 2). The results showed that the migration of PM/Scl-75c was identical to that of HeLa cell PM/Scl-75. The migration of in vitro translated PM/Scl-75c was clearly different, in agreement with previous observations (1).

To investigate the occurrence of the extra exon (10*) that is present in the β variant of PM/Scl-75 (Fig. 1d, exon α), the GenBank™ EST data base was screened for sequences containing the exon-exon junctions 10*-11 and 10–11. In total, 52 ESTs encompassing this region were identified, 10 of which contained exon 10*, indicating that both splice variants of PM/Scl-75 are expressed but that the isoform lacking exon 10* may be more abundant.

Taken together, these results indicate that PM/Scl-75c-α is the predominant isoform of PM/Scl-75 but that at least four splice variants exist that differ by their N terminus and the presence or absence of 17 amino acids encoded by exon 10*.

The Subcellular Localization of PM/Scl-75—To investigate the subcellular localization of PM/Scl-75 polypeptides, constructs encoding PM/Scl-75a-α, PM/Scl-75c-α, and PM/Scl-75c-β tagged with EGFP were generated. The fusion proteins were expressed in transiently transfected HEp-2 cells and as a control the endogenous PM/Scl-75 protein in HEp-2 cells was visualized by immunofluorescence using rabbit anti-PM/Scl-75 antibodies. Whereas the highest concentration of the endogenous PM/Scl-75 protein was found in the nucleoli (Fig. 3A), EGFP-PM/Scl-75a-α failed to enter the nucleoli and accumulated in the nucleoplasm (Fig. 3B). In contrast, EGFP-tagged PM/Scl-75c-α and PM/Scl-75c-β efficiently entered the nucleolus of HEp-2 cells (Fig. 3, C and D). These data indicate that the N-terminal 84 amino acids of PM/Scl-75c are important for nucleolar accumulation and that the 17 amino acids encoded by exon 10* do not affect this process. To investigate whether the N-terminal region of PM/Scl-75c is sufficient for nucleolar targeting, the N-terminal 88 amino acids of PM/Scl-75c were fused to the N terminus of EGFP, and the subcellular localization of this fusion protein was analyzed in transfected HEp-2 cells. This fusion protein distributed throughout HEp-2 cells, similar to EGFP alone (data not shown). Previously, a sequence element (KKKKKKKKK) with similarity to NRLs has been reported to reside at the C-terminal end of PM/Scl-75 (1). To investigate the role of this element in nuclear and nucleolar entry, mutants of PM/Scl-75a-α and PM/Scl-75c-α lacking the C-terminal 39 amino acids were generated (ΔNLS). As can be seen in Fig. 3E, PM/Scl-75a-α ΔNLS failed to enter the nucleus. Remarkably, PM/Scl-75c-α ΔNLS still was transported to the nucleus but was unable to enter the nucleolus (Fig. 3F). Finally, an EGFP fusion protein was expressed containing only the C-terminal 39 amino acids of PM/Scl-75. Fig. 3G shows that this element is sufficient for transportation of EGFP to the nucleus. These data show that both N- and C-terminal elements of PM/Scl-75 are involved in its nuclear entry and that the C-terminal elements (also) play a role in nucleolar targeting.

The Association of PM/Scl-75 with the Exosome—In addition to elements directly involved in subcellular transport processes, the association with the exosome may also play a role in the subcellular localization. To investigate the effect of the mutations on the association with the exosome, immunoprecipitations with anti-EGFP antibodies were performed using lysates of HEp-2 cells transfected with constructs encoding the EGFP-tagged PM/Scl-75 mutants. The immunoprecipitated material was analyzed by Western blotting, using a human serum (Ven96) reactive with several exosome proteins (including PM/Scl-100, PM/Scl-75, hRrp4p, hRrp4p1p, and hRrp4p2p). The patient serum stained the different PM/Scl-75 fusion proteins very efficiently, which shows that all variants/mutants were expressed at similar levels. The results showed that many endogenous exosome proteins (although PM/Scl-100 was not present in the patient serum) stained the different PM/Scl-75 fusion proteins very efficiently, which shows that all variants/mutants were expressed at similar levels. The results showed that many endogenous exosome proteins.
detected in the immunoprecipitates) co-precipitated with both PM/Scl-75c-H9251 and PM/Scl-75c-H9251/H9004 NLS but not with PM/Scl-75a-H9251 (Fig. 4a). In a similar type of experiment, we demonstrated that both the H9251 and H9252 form of PM/Scl-75c can be incorporated in the exosome complex (Fig. 4b).

Previously, we demonstrated that PM/Scl-75a-H9251 was not able to interact directly with any other exosome component in a mammalian two-hybrid system, although its yeast counterpart (Rrp45p) is known to interact with yeast Rrp41p (20). The full-length cDNAs encoding PM/Scl-75a-H9251, PM/Scl-75c-H9251, and 10 (putative) human exosome components (hRrp4p, hRrp40p, hRrp41p, hRrp42p, hRrp46p, PM/Scl-100, hCsl4p, hRrp44p/hDis3p, OIP2, and hMtr3p) were cloned in both the pACT (in-frame with the sequence encoding the VP16 transcription activation domain) and pBIND (in-frame with the sequence encoding the GAL4 DNA-binding domain) vectors of the Checkmate mammalian two-hybrid system. COS-1 cells were co-transfected with each pair of these constructs, and after 40–48 h the luciferase activity of extracts prepared from these cells was determined. This revealed that, in contrast to PM/Scl-75a-α, PM/Scl-75c-α fused to the GAL4 DNA-binding domain was able to interact with hRrp41p fused to the VP16 activation domain (Fig. 5). This interaction was confirmed by a complementary experiment in which the fusion domains were exchanged (data not shown). The interaction of PM/Scl-75c with hRrp41 adds further evidence to the model for the human exosome, in which PM/Scl-75 is flanked by hRrp41p and hRrp46p (20).

**DISCUSSION**

In this study we have shown that the previously reported cDNA and amino acid sequences for the human polymyositis/scleroderma autoantigen PM/Scl-75 are probably incomplete. We cloned a cDNA with an extended N terminus, and only the longer form of PM/Scl-75 was able to associate with the exosome complex, to interact with at least one other exosome subunit, and to enter the nucleolus, three functional activities that are crucial for its role in RNA metabolism.
that are shared with other exosome subunits. The presence of exon 10* in the coding sequence of PM/Scl-75 (observed in a previously reported splice variant) did not affect the behavior of PM/Scl-75 with regard to its subcellular localization and exosome association. In addition, PM/Scl-75 was shown to contain both N- and C-terminal elements involved in its nuclear localization, the latter of which also appeared to be responsible for nucleolar accumulation of this protein.

The Amino Acid Sequence of PM/Scl-75—The cDNA of PM/Scl-75a-α (1) lacks the first exon compared with the cDNA of PM/Scl-75c-α, and as a consequence the open reading frame starts at another methionine. Note that the 5′ end of the PM/Scl-75b-α cDNA is identical to that of PM/Scl-75a-α, with the exception of one additional nucleotide in the former, leading to a longer open reading frame in the 5′ region (23). The expression of mRNAs corresponding to these two types of cDNAs is most likely due to the existence of alternative promoters and transcription initiation sites in the gene of this protein (Fig. 1c). In contrast to PM/Scl-75c-α the expression of PM/Scl-75a-α and PM/Scl-75b-α mRNAs is not supported by entries in the EST data bases. A TBLASTN search of the human EST data base with the N-terminal 80 amino acids of the mouse sequence resulted in 49 of 50 hits that fully matched human EST data base with the N-terminal 80 amino acids of entries in the EST data bases. A TBLASTN search of the nucleotide sequences of several cell lines by RT-PCR, followed by sequencing, revealed the sequence corresponding to the N terminus resulted in 49 of 50 hits that fully matched human EST data base with the N-terminal 80 amino acids of entries in the EST data bases. A TBLASTN search of the nucleotide sequences of several cell lines by RT-PCR, followed by sequencing, revealed the sequence corresponding to the N terminus of the major PM/Scl-75 isoform corresponds to the sequence encoded by PM/Scl-75c-α and that as a result of an alternative promoter PM/Scl-75b-α is produced. The expression of mRNAs either containing or lacking exon 10* (Fig. 1d) is supported by a series of data base entries and thus is consistent with the expression of two splicing variants (designated α and β). Western blot analyses of extracts of several primate cell lines indeed show a doublet for PM/Scl-75, in agreement with the simultaneous expression of these splice variants in a variety of cells. Interestingly, the results of HeLa cell fractionation experiments suggested that the β isoform might accumulate somewhat more efficiently in the nucleus than the α isoform.

Transport of PM/Scl-75 to Nucleus and Nucleolus—Previously, the C-terminal element of PM/Scl-75, rich in basic amino acids, was proposed to be a nuclear localization sequence. Here, we have shown that this element indeed can direct a reporter protein to the nucleus. However, this element did not appear to be essential for nuclear import of PM/Scl-75, as long as PM/Scl-75 was able to interact with the rest of the exosome complex. On the other hand, the basic sequence element of PM/Scl-75 is important for nucleolar accumulation of this protein. Based upon the presence of putative NLS sequences in at least two other exosome proteins, hRrp41p (amino acids 85–90, ERKRRP) and PM/Scl-100 (amino acids 752–758, AKKRERA), two mechanisms for nuclear entry of the exosome can be envisaged. First, the nuclear import of the completely assembled exosome may be mediated by the concerted action of several signals. Second, partially assembled parts of the exosome may enter the nucleus and assemble into a complete exosome in the nucleoplasm or nucleolus. All of these basic elements may also be involved in nucleolar targeting of the exosome, because elements rich in basic residues have been demonstrated to play an essential role in nucleolar accumulation (24).

Association of PM/Scl-75 with the Exosome—The direct interaction between PM/Scl-75c and hRrp41p supports the model for the human exosome that we recently proposed, as illustrated in Fig. 7 (20). The minor isoform of the protein, PM/Scl-75b, most likely is also capable of interacting with hRrp41p and the exosome, because it contains the complete RNase PH domain (Fig. 1b). Another interaction predicted by that model, between PM/Scl-75 and hRrp46p (Fig. 7), could not be detected in the mammalian two-hybrid system. PM/Scl-75 represents together with hRrp41p the equivalent of one PNPase subunit in the structurally related PNPase trimer, and two RNase PH domains of PNPase interact with
each other via their most C-terminal sequences. The failure of PM/Scl-75 to interact with its putative neighbor hRrp46p, which is predicted to be mediated by the N-terminal regions of their RNase PH domains, might be due to the fact that the hRrp46p cDNA that was used is also incomplete. Very recently an alternative sequence for hRrp46p was published (25) that contains an N terminus with 33 additional amino acids. Although the complete RNase PH domain is present in the original sequence of hRrp46p, it is possible that some flanking amino acids might be required for the interaction with PM/Scl-75, either by making direct contacts or by stabilizing the proper conformation of the RNase PH domain. Another explanation might be the possible interference of the fusion parts of the mammalian two-hybrid constructs, which are both attached to the N terminus of the proteins to be analyzed. Whether the AU binding properties of PM/Scl-75 (19) are also influenced by the extra N-terminal sequence remains to be investigated, although this activity is most likely mediated by the C-terminal part of the protein, because the RNase PH domain is responsible for the interaction of the protein with the exosome complex. Because no functional differences were found between the splice variants PM/Scl-75-α and PM/Scl-75-β, and both PM/Scl-75b and PM/Scl-75c contain the complete RNase PH domain, the function of this multiplicity of PM/Scl-75 splice variants remains to be identified, although the function of the β form might be specific for the nucleus given its increased nuclear accumulation.

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The Association of the Human PM/Scl-75 Autoantigen with the Exosome Is Dependent on a Newly Identified N Terminus

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