The Signal Peptide of the Mouse Mammary Tumor Virus Rem Protein Is Released from the Endoplasmic Reticulum Membrane and Accumulates in Nucleoli*

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N-terminal signal sequences mediate endoplasmic reticulum (ER) targeting and insertion of nascent secretory and membrane proteins and are, in most cases, cleaved off by signal peptidase. The mouse mammary tumor virus envelope protein and its alternative splice variant Rem have an unusually long signal sequence, which contains a nuclear localization signal. Although the envelope protein is targeted to the ER, inserted, and glycosylated, Rem has been described as a nuclear protein. Rem as well as a truncated version identical to the cleaved signal sequence have been shown to function as nuclear export factors for intron-containing transcripts. Using transiently transfected cells, we found that Rem is targeted to the ER, where the C-terminal portion is translocated and glycosylated. The signal sequence is cleaved off and accumulates in nucleoli. In a cell-free in vitro system, the generation of the Rem signal peptide depends on the presence of microsomal membranes. In vitro and in cells, the signal peptide initially accumulates in the membrane and is subsequently released into the cytosol. This release does not depend on processing by signal peptide peptidase, an intramembrane cleaving protease that can mediate the liberation of signal peptide fragments from the ER membrane. Our study suggests a novel pathway by which a signal peptide can be released from the ER membrane to fulfill a post-targeting function in a different compartment.

Signal sequences are N-terminal extensions on nascent secretory and membrane proteins and mediate translocation across or insertion into the membrane of the endoplasmic reticulum. They typically include 15–25 amino acid residues and have a tripartite structure with a hydrophobic core region flanked by a positively charged n-region and a c-region. The latter includes the signal peptidase cleavage site. Cleaved signal sequences, named signal peptides, are thought to be degraded, but some accumulate or are further processed by an intramembrane cleaving protease named signal peptide peptidase (SPP)5. Signal peptides or signal peptide fragments can have a function beyond targeting. For example, the signal peptides of several arenaviral (Lassa, Junin, and lymphocytic choriomeningitis virus) glycoproteins remain membrane-inserted. They are necessary for glycoprotein processing, part of the mature glycoprotein complexes, and important for viral infection (3–9). The signal peptide of prolactin (10, 11), the HLA-A*0301 molecule (12), and the internal signal sequences of the hepatitis C virus polyprotein (13) are processed by SPP, which results in the liberation of signal peptide fragments into the cytosol. The HLA-A*0301-derived signal peptide fragments are presented at the cell surface and monitor the expression of their corresponding protein for immunosurveillance (12). For the hepatitis C virus polyprotein, SPP processing results in the release of the core protein into the cytosol (13) and affects the formation of virus-like particles (14–16).

The mouse mammary tumor virus (MMTV), a type B retrovirus, is a causative agent of murine mammary carcinomas and is also associated with T-cell lymphomas (17, 18). Its envelope protein (Env) is synthesized at the ER as a 73-kDa precursor, the signal sequence is cleaved, and the protein is further processed into the two glycoproteins, gp52 and gp36 (19, 20). The Env signal sequence is predicted to comprise 98 amino acid residues and includes a consensus sequence for a nuclear localization signal (NLS, Fig. 1A) in its extended n-region (21). Recently, an alternative splice variant of the env mRNA was identified. The translation product, named regulator of export/expression of MMTV mRNA (Rem), shares identity with Env in the predicted signal sequence, as well as the N-terminal 162 and the C-terminal 41 amino acid residues of Env (Fig. 1A) (22, 23). Rem was detected as a 39-kDa protein in mouse mammary tumor cells (GR) and CrFK cells stably transfected with a complete MMTV provirus (22), whereas Mertz et al. (23) describe a

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5 The abbreviations used are: SPP, signal peptidase peptidase; MMTV, mouse mammary tumor virus; Env, envelope protein; gp, glycoprotein; NLS, nuclear localization signal; Rem, regulator of export/expression of MMTV; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RM, rough microsomes; Prl, prolactin; HERV-K, human endogenous retrovirus K; ER, endoplasmic reticulum; EGFP, enhanced green fluorescent protein; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PBS, phosphate-buffered saline.
33-kDa protein in transiently transfected cells. In both studies, EGFP-tagged Rem localized to nucleoli (22, 23), which depended on the presence of the NLS (23). Rem and an experimentally truncated version of Rem identical to the signal sequence were shown to function as nuclear export factors for intron-containing transcripts. This activity requires the NLS within the nuclear export factor and the presence of the 3’ long terminal repeat as cis-regulatory sequence in the transcript, suggesting that the export of env transcripts is Rem-dependent (23).

Beyond Env and Rem, a 14-kDa nucleolar protein named p14 was detected in MMTV bearing S49 and EL-4 T-cell lymphomas using a monoclonal antibody that recognizes an epitope within the predicted signal sequence. Protein purification, mass spectrometry, and microsequencing revealed a mass of 11 kDa and sequence identity to, at least, the N-terminal 81 residues of the Rem/Env signal sequence, including the NLS. The mass of 11 kDa is fully consistent with the calculated molecular weight of the 98 amino acids, which are predicted for the entire signal sequence (21, 24). More recently, p14 was also detected in mouse mammary carcinoma-derived cell lines, and a polyclonal serum identified its immunogenic determinant in some paraffin-embedded human breast cancer sections (25).

Thus, Rem as well as p14 were detected in the nucleus. However, Rem contains a signal sequence, which suggests ER targeting and translocation of this molecule. Beyond that, the biogenesis of p14 is not known. Therefore, we wanted to investigate how Rem and p14 are generated, if they are processed, and how their localization within the cell is achieved. We have analyzed HeLa cells transiently expressing Rem and found the C-terminal portion of Rem as a glycosylated protein (gp32Rem) (26), whereas the cleaved signal peptide of Rem, here named SpRem, accumulated in nucleoli. Furthermore, we show in a cell-free in vitro system that SpRem is generated at and released from microsomal membranes independent of a processing by the intramembrane protease SPP.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—With the exception of pEGFP-N1 (Clontech), the plasmids used in this study contain the pRK5sr backbone, which has a cytomegalovirus and an SP6 promoter (26). An Env encoding plasmid was obtained by re-cloning a PCR amplification product from a full-size MMTV clone (GR strain, kindly provided by E. Buetti, University of Lausanne). pRK5sr-Env encodes MMTV Env amino acids 1–688 without the 5’- and 3’-untranslated regions. Rem cDNA was obtained by reverse transcription of total RNA prepared with the RNeasy kit (Qiagen) from HeLa cells transiently transfected with pRK5rs-Env. The cDNA was amplified using the primers 5’ GTT CAG TGT AGG ACA CTC TCG, including the Myc tag, and a fragment of about 500 bp cloned and sequenced. Rem cDNA was obtained by reverse transcription of total RNA prepared with the RNeasy kit (Qiagen) from HeLa cells transiently transfected with pRK5rs-Env. The cDNA was amplified using the primers 5’ GTT CAG TGT AGG ACA CTC TCG, including the Myc tag, and a fragment of about 500 bp cloned and sequenced. Rem cDNA was obtained by reverse transcription of total RNA prepared with the RNeasy kit (Qiagen) from HeLa cells transiently transfected with pRK5rs-Env. The cDNA was amplified using the primers 5’ GTT CAG TGT AGG ACA CTC TCG, including the Myc tag, and a fragment of about 500 bp cloned and sequenced.

Rem-dependent (23).

**Antibodies**—Monoclonal antibody M66 recognizes an epitope between amino acids 25 and 56 in the signal sequence of MMTV Env (see Ref. 24 and references therein). B23 antibody (sc-6013), GFP antibody (sc-8334), and lamin A/C antibody (sc-7292) were obtained from Santa Cruz Biotechnology. Myc antibodies were prepared from 9E10 hybridoma cell supernatant using standard procedures. The Sec61β serum is directed against the 9 N-terminal residues of the human protein and was prepared as described previously (27). GAPDH antibody (C10) was obtained from Cell Signaling. Sheep α-mouse antibodies conjugated with peroxidase and donkey α-mouse antibodies conjugated with Texas Red were from Dianova. Alexa Fluor® 488 goat α-mouse-, goat α-rabbit-, or donkey α-goat antibodies, Alexa Fluor® 568 goat α-mouse antibodies, and Alexa Fluor® 546 goat α-rabbit antibodies were from Molecular Probes.

**Cells and Transient Transfection**—HeLa cells (ATCC, CCL-2) were grown in Dulbecco’s modified Eagle’s medium containing 4.5 g/liter glucose, 10% fetal calf serum, and 2 mM l-glutamine. Cells were controlled for the absence of mycoplasma using standard PCR. 1 × 10⁴ cells were seeded in 12-well slots on glass coverslips for immunofluorescence, and 2 × 10⁵ cells were seeded in 6-well slots for Western blot analyses or metabolic labeling. 18 h after calcium phosphate transfection (28), cells were supplied with fresh medium.

**Immunofluorescence**—48 h post-transfection, HeLa cells were fixed for 5 min with 2% formaldehyde in PBS supplemented with 125 mM sucrose and subsequently for 20 min in ice-cold methanol at −20 °C. Unreacted groups were protected by treatment with 0.1 M glycine in PBS. Blocking and incubations with antibodies were done for 1 h at room temperature with 10% fetal calf serum in PBS and with 5% fetal calf serum, respectively. Coverslips were mounted with Mowiol containing 0.1 µg/ml 4’,6-diamidino-2-phenylindole. Confocal microscopy was done with a Leica TCS SP2, using a 63× HCX PL APO oil immersion objective (numerical aperture 1.4). Excitation laser lines were 488 nm (argon laser) and 561 nm (diode pumped solid state laser). All images are single plane images produced with the Leica confocal software.
Cell Lysis and Western Blot Analyses—42 h post-transfection cells were lysed in SDS sample buffer (50 mM Tris/HCl, pH 7.5; 500 mM NaCl; 1% (w/v) sodium deoxycholate; 1% (v/v) Nonidet P-40; 0.1% (w/v) SDS; 2 mM EDTA; 1 mM phenylmethylsulfonyl fluoride; 10 µg/ml leupeptin; 10 µg/ml chymostatin; 10 µg/ml pepstatin). The lysate was spun through a QIAshredder column (Qiagen, Hilden, Germany) according to the protocol of the manufacturer and, additionally, cleared by centrifugation at 16,000 × g for 20 min at 4 °C. For immunoprecipitation, the lysates were diluted with IP buffer (20 mM Hepes, pH 7.5; 500 mM NaCl; 10% (v/v) glycerol; 0.1% (v/v) Triton X-100), protein A-Sepharose beads (Amersham Biosciences) and antibodies were added. Samples were rotated for 3 h, and the beads were washed four times with IP buffer. Endoglycosidase H treatment was done as suggested by the manufacturer (New England Biolabs).

For fractionation, cells were first permeabilized for 5 min with 0.02% digitonin (50 mM Hepes, pH 7.5; 150 mM NaCl; 1.5 mM MgCl₂; 10% (v/v) glycerol; 0.02% (w/v) digitonin; 1 mM EGTA; 1 mM phenylmethylsulfonyl fluoride; 10 µg/ml leupeptin; 10 µg/ml chymostatin; 10 µg/ml pepstatin) to release cytosolic proteins. The permeabilized cells were pelleted (5 min, 16,000 × g, 4 °C) and lysed with 1% Triton X-100 (50 mM Hepes, pH 7.5; 150 mM NaCl; 1.5 mM MgCl₂; 10% (v/v) glycerol; 1% (v/v) Triton X-100; 1 mM EGTA; 1 mM phenylmethylsulfonyl fluoride; 10 µg/ml leupeptin; 10 µg/ml chymostatin; 10 µg/ml pepstatin) to solubilize membrane proteins. Nonsoluble material was pelleted as detailed above, and the pellet was resuspended in SDS-containing lysis buffer. This lysate was cleared by centrifugation (20 min, 16,000 × g, 4 °C). Following immunoprecipitation, proteins were separated on a Tris/Tricine SDS-PAGE as described above. Dried gels were analyzed by PhosphorImaging (Bio-Rad PMI system for Fig. 2B, Fuji BAS 1500 for every other autoradiogram). To control the fractionation, nontransfected cells or cells expressing EGFP were lysed as described, and lysates were analyzed by Western blotting.

Densitometric and Statistical Analyses—Densitometric analysis was done with ImageJ. Each signal, represented by a defined area, was corrected for background by subtracting a signal from an identical area within the same lane. For statistical analyses, relative amounts were calculated, i.e. signals from each time point were adjusted to 100%. Relative amounts are given in percent with standard deviation. Statistical analysis was done with JMP (SAS Institute). Data were analyzed by one-way analysis of variance and Student’s t tests for unpaired groups. p values are given in the legend.

In Vitro Translation/Translocation Assay—Rem encoding plasmid DNA was linearized, purified, and used for in vitro transcription with SP6 polymerase as described before (11). Transcripts were treated with DNase (Promega) and purified using G-25 columns (GE Healthcare). In vitro translation was done for 30 min at 30 °C in 10-µl reactions using rabbit reticulocyte lysate (Promega), Redivue Pro-mix L-35S cell labeling mix (GE Healthcare), and 1–1.5 eq canine pancreas rough microsomes produced according to the protocol from Walter and Blobel (30). Signal peptide peptidase inhibitor (Z-LL)₂-ketone (Calbiochem) was dissolved in Me₂SO and added as indicated. In vitro reactions were precipitated with ammonium sulfate by adding 2 volumes of saturated ammonium sulfate solution and precipitation for 20 min on ice. The precipitate was pelleted by centrifugation at 16,000 × g for 5 min and resuspended in distilled H₂O. Proteins were again precipitated with 2 volumes of ice-cold absolute ethanol, pelleted, and resuspended in SDS sample buffer (50 mM Tris/HCl, pH 6.8; 10 mM EDTA; 5% glycerol; 2% SDS; 0.01% bromphenol blue).

To separate the microsomes from the supernatant, in vitro reaction samples were layered on top of a 50-µl cushion (50 mM Hepes-KOH, pH 7.6; 750 mM KOAc; 10 mM Mg(OAc)₂; 1 mM dithiothreitol; 500 mM sucrose) and membranes were pelleted by 5 min of centrifugation at 100,000 × g and at 4 °C (Beckman TL100 rotor or Sorvall S100-AT3 rotor). For SDS-gel electrophoresis, the supernatant was precipitated with ammonium sulfate as described above, and the pellet was directly resuspended in SDS sample buffer. The samples were separated in 10–17% gradient gels (T, 30%; C, 2.6%) (31). For immunoprecipitation, the supernatant was diluted with lysis buffer containing 1% Triton X-100, whereas the pellet was directly resuspended in this lysis buffer. Immunoprecipitation was done as described above.

For the characterization of the release, in vitro translation was carried out as described above but stopped after 15 min by the addition of 1.25 mM cycloheximide (Sigma). Signal peptide release was analyzed for various periods of time as well as under different temperature conditions (either on ice or at 30 °C). The samples were analyzed by immunoprecipitation, SDS-PAGE, and autoradiography.

RESULTS

Cellular Localization of the Rem N- and C-terminal Portions—In previous studies, a Rem–EGFP fusion protein was used for localization, and a nucleolar EGFP staining was detected (22, 23). We wanted to determine the localization of both the N- and C-terminal portions of Rem by immunofluorescence microscopy. To this end, we transiently expressed C-terminally Myc-tagged Rem (Rem-myc) in HeLa cells and stained with the monoclonal antibody M66, which recognizes an epitope in the predicted signal sequence (21, 24), that is the N-terminal portion, as well as an antibody recognizing the C-terminal Myc tag.
ER Membrane Release of the MMTV Rem Signal Peptide

The M66 antibody detected its epitope mainly in the nucleus, where a major fraction co-localized with the nucleolar marker B23 (Fig. 1B, upper panel). The C-terminal Myc tag of Rem-myc was detected in a reticular structure, which was also stained by an antibody against Sec61β, an ER marker. Additionally, nucleolar staining was obtained with the anti-Myc antibody (Fig. 1B, bottom panel). Thus, the localization of the N- and C-terminal portions of Rem-myc in nuclei indicates that full-length Rem-myc is imported into the nucleus. However, Rem-myc is not exclusively a nuclear protein, because the C-terminal portion can also be found at the ER.

Next, we analyzed whether the predicted signal sequence of Rem/Env is able to direct an unrelated, otherwise cytosolic protein to the ER. To this end, we fused the N-terminal 101 amino acids of Rem/Env (including the 98-amino acid-long predicted signal sequence) to EGFP with a C-terminal KDEL motif. The KDEL motif retains translocated EGFP in the ER, where it can be detected. HeLa cells were transiently transfected with this construct named SpRem-EGFP-KDEL. By immunofluorescence microscopy, EGFP staining was observed primarily in a reticular structure typical for the ER, but it was also found in nucleoli (Fig. 1C). The M66 epitope was again detected in the nucleus, mainly in nucleoli. Thus, the Rem/Env-derived signal sequence can target EGFP-KDEL to the ER. As a consequence of targeting, the signal sequence may be cleaved, and nucleolar M66 staining may in part be due to the presence of cleaved signal sequences. The EGFP staining found in nucleoli along with the nucleolar M66 staining again suggests that a certain amount of the precursor molecules was transported to the nucleus.

Biochemical Identification of Rem and Its Derivatives—Next, we wanted to biochemically identify the antigens detected by the M66, anti-Myc, and anti-GFP antibody. We transfected HeLa cells with Rem-myc or SpRem-EGFP-KDEL and analyzed cell lysates by immunoblotting. In both cases, the M66 antibody detected a 14-kDa protein, termed p14 (Fig. 2A, p14, lanes 2 and 4). The signal sequence is the only common sequence of the proteins expressed from these two constructs, which strongly suggests that p14 is the cleaved signal sequence of Rem. Besides p14, the M66 antibody detected a 42-kDa protein in cells expressing SpRem-EGFP-KDEL (Fig. 2A, lane 4) and a low amount of a 38-kDa protein in cells expressing Rem-myc, respectively (lane 2). To identify the C-terminal portion, Rem-myc expressing cells were analyzed with the anti-Myc antibody. We detected a 38- and a 33-kDa protein (Rem-myc and p33Rem-myc, Fig. 2A, lane 7). Thus, the 38-kDa protein detected by both antibodies represents the precursor Rem-myc. Because the 33-kDa protein was not detected by the M66 antibody, it does not contain the signal sequence and represents the signal sequence-cleaved protein p33Rem-myc. Similarly, in cells expressing SpRem-EGFP-KDEL, the anti-GFP antibody detected a signal sequence-cleaved EGFP-KDEL protein (28 kDa) as well as the precursor protein (SpRem-EGFP-KDEL, 42 kDa), which was also detected with the M66 antibody (lane 9). In summary, precursor molecules as well as mature proteins along with cleaved signal sequences can be detected in cells overexpressing Rem-myc or SpRem-EGFP-KDEL.

Consistent with ER targeting and signal sequence cleavage, the C-terminal portion of Rem-myc (p33Rem-myc) is most likely translocated across the ER membrane. Because Rem contains two putative N-glycosylation sites (compare Fig. 1A), we investigated whether p33Rem-myc is a glycoprotein. To this end, we expressed Rem-myc in HeLa cells, metabolically labeled the cells, and analyzed lysates by immunoprecipitation and treatment with endoglycosidase H, which removes N-linked sugar moieties. A 38-kDa protein representing Rem-myc was detected independent of endoglycosidase H treatment. The 33-kDa protein detected in Rem-myc expressing cells was reduced to 27 kDa (p27Rem-myc) by treatment with endoglycosidase H (Fig. 2B). This indicates two glycosylation events, which alter the molecular mass by about 2–3 kDa each. In an independent experiment, we found a similar reduction in molecular mass by 5–6 kDa when analyzing cells treated with tunicamycin, an inhibitor of N-glycosylation (data not shown). Thus, p33Rem-myc (in Fig. 2A) is a glycoprotein, which confirms its translocation into the ER lumen. We named this protein gp33Rem-myc in contrast to the precursor that was previously named Rem or, in our case, Rem-myc.
Next, we wanted to determine whether the cleaved signal sequence can be detected in the nucleus and thus could give rise to the nucleolar staining observed by immunolocalization. We analyzed the subcellular localization of p14 in cells expressing Rem-myc by consecutively applying different detergents. We first released cytosolic proteins by permeabilizing the plasma membrane with 0.02% digitonin (32) and pelleted the cellular remnants by centrifugation. The supernatant was recovered and represents the cytosol. The pellet was treated with a lysis buffer containing 1% Triton X-100, which leads to the solubilization and release of membrane proteins, e.g., from the ER or nuclear membrane, as well as of nuclear proteins unless these are part of stable, higher order complexes. The solubilized proteins were separated by centrifugation to obtain a fraction containing membrane proteins. The remaining pellet was lysed in SDS-containing lysis buffer (1% sodium deoxycholate, 1% Nonidet P-40, and 0.1% SDS as well as an increased salt concentration) to give a nuclear fraction. To characterize the three fractions, the presence of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and lamin A as cytosolic and nuclear markers, respectively, was analyzed by Western blot. We found most GAPDH molecules in the cytosolic fraction and to a small amount in the membrane fraction but not in the nuclear fraction. Lamin A was detected in the nuclear fraction (Fig. 2C, lower panels). We also analyzed cells overexpressing EGFP by fractionation and Western blot, and we detected EGFP mainly in the cytosolic fraction and to a lower amount in the membrane fraction (Fig. 2C, lower panels). This suggests that the nuclear fraction is not contaminated with cytosolic GAPDH or EGFP. We applied the fractionation protocol on metabolically labeled cells expressing Rem-myc and analyzed proteins from the three fractions by immunoprecipitation with the M66 antibody. A prominent protein of about 14 kDa was detected in all fractions (Fig. 2C), suggesting that p14 can occur in the nucleus but also in the cytosol. Furthermore, we detected Rem-myc mainly in the cytosolic fraction and only in very low amounts in the membrane and nuclear fractions.

**SPRem Is Generated in the Presence of ER-derived Membranes and Released into the Cytosol**—Because of their hydrophobic nature, signal peptides are assumed to remain membrane-inserted. The observation that the Rem-derived signal peptide is detected in the cytosolic and nuclear compartment prompted...
us to confirm and further characterize SP\textsuperscript{Rem} generation and its putative release from the ER membrane. To this end, we took advantage of a cell-free translation/translocation assay to monitor ER targeting, signal sequence cleavage, and protein translocation. In this assay, Rem transcripts were translated in the absence of RMs in a rabbit reticulocyte lysate in the absence or presence of ER-derived RMs. When Rem transcripts were translated in the absence of RMs, a prominent 34-kDa Rem protein was synthesized and immunoprecipitated with the M66 antibody (Fig. 3A, lanes 2 and 4). In the presence of RMs, an additional protein of 32 kDa (gp32\textsuperscript{Rem}) appeared, whereas the 34-kDa protein was slightly reduced in amount (Fig. 3A, lane 3). After immunoprecipitation with the M66 antibody, a 34- and a 14-kDa protein were detected, representing the precursor and the cleaved signal peptide named SP\textsuperscript{Rem} (Fig. 3A, lane 5). SP\textsuperscript{Rem} is hardly visible without immunoprecipitation, because the high amount of globin of the reticulocyte lysate distorts the gel region of 12–14 kDa (Fig. 3A, lanes 2 and 3). The apparent molecular weights of Rem and the Rem-derived glycoprotein are slightly lower than the ones found in cell lysates. This difference is partly because of the absence of the Myc tag, which accounts for about 1 kDa, and partly because of the use of a different gel system (compare “Experimental Procedures”). The molecular weights described here correlate with the theoretical masses of 34 and 32 kDa for Rem and its glycoprotein, respectively. In summary, our findings using the in vitro assay indicate that the generation of SP\textsuperscript{Rem} (Fig. 3B, compare lanes 4 and 5) and gp32\textsuperscript{Rem} requires the presence of ER-derived membranes.

To characterize the size of SP\textsuperscript{Rem}, we synthesized in vitro a marker peptide identical to the signal sequence of Rem/Env. The marker peptide was co-separated by SDS-gel electrophoresis with the in vitro translation/translocation reactions and co-migrated with the SP\textsuperscript{Rem} derived from Rem (Fig. 3B, lanes 2, 4, and 5).

Next, we wanted to analyze whether SP\textsuperscript{Rem} is released from the microsomal membranes. To this end, we synthesized Rem in the presence of RMs and separated RM membranes from the cytosol by centrifugation through a sucrose cushion. As shown in Fig. 3B, lanes 4 and 5, the 34-kDa-sized Rem is hardly visible without immunoprecipitation, because the high amount of globin of the reticulocyte lysate distorts the gel region of 12–14 kDa (Fig. 3A, lanes 2 and 3). In the presence of RM membranes, the SP\textsuperscript{Rem} is generated and found in the supernatant as well as in the pellet (Fig. 3B, lanes 4 and 5). Unprocessed Rem is mainly found in the supernatant consistent with this protein not being inserted into the membranes...

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**FIGURE 3. In vitro characterization of SP\textsuperscript{Rem} release from microsomal membranes.** A, in vitro translation/translocation reaction of Rem. Rem mRNA was translated in the absence (lanes 2 and 4) or presence of RMs (lanes 3 and 5). For immunoprecipitation (IP) (lanes 2 and 5), the M66 antibody was used. The samples were analyzed by standard SDS-PAGE and autoradiography. B, in vitro translation/translocation reaction of SP\textsuperscript{Rem}, Rem, and Rem\textsuperscript{mut}. For SP\textsuperscript{Rem}, a plasmid was constructed encoding the 98-amino acid residues of the Rem/Env signal sequence terminated by a stop codon, and transcripts were derived (lane 2). SP\textsuperscript{Rem}, Rem, or Rem\textsuperscript{mut} were translated in the absence or presence of RMs as indicated. In the presence of membranes, the separation of membranes from cytosol was achieved by centrifugation through a high salt sucrose cushion resulting in a cytosolic supernatant (s) and a membrane-containing fraction (pellet, p). Both fractions were lysed in the presence of 1% Triton X-100. In vitro translation reactions as well as fractionated reactions were used for immunoprecipitation with the M66 antibody and analyzed as described above. To characterize both fractions, samples were separated by SDS-PAGE, blotted, and analyzed with anti-Sec61\beta antibody (bottom panel). WB, Western blot.
However, by metabolic labeling after a rather short time of transient transfection, we could detect a 38- and a 42-kDa protein but never a 14-kDa protein (data not shown). In summary, we conclude that cleavage by signal peptidase is required to generate SPRem.

Although to a much lower extent than full-length Rem, two proteins of about 30 and 28 kDa were detected independent of the presence of membranes and immunoprecipitation (Fig. 3B, also compare Fig. 4). These additional products are most likely the result of alternative translation initiation from either of the two further methionine residues within the 98-amino acid-long signal sequence.

The Release of SPRem from Microsomal Membranes Is Independent of SPP Activity but a Time- and Temperature-dependent Process—

Signal peptide fragments resulting from processing of signal peptides by SPP are known to be released into the cytosol. To analyze whether SPRem is processed by SPP, we synthesized Rem in vitro in the presence of RMs and an SPP inhibitor (Z-LL)2-ketone (10) or the solvent Me2SO (Fig. 4A). We found no effect of the inhibitor on the size of SPRem or on its distribution between the membrane and the cytosolic fractions (Fig. 4A, lanes 6–9 in comparison to lanes 1–5). As a control for the activity of the inhibitor, we translated the known SPP substrate prolactin (Prl) in the presence of RMs and the SPP inhibitor. In the presence of the inhibitor, SPPrl accumulated in the membrane fraction, whereas no SPPrl was detected in the absence of the inhibitor (Fig. 4A, lanes 10–15). We also analyzed the effect of SPP on SPRem generation in transiently transfected cells by co-transfection with SPP or an enzymatically inactive SPP mutant, SPP-D265A (34). Again, we did not observe any effect of SPP or its inactive mutant on SPRem generation or size.6 In summary, we conclude that the generation and release of SPRem from the membrane is independent of processing by SPP.

To further characterize the release of SPRem from microsomal membranes, we allowed only a rather short time (15 min) for in vitro translation of Rem in the presence of RMs. We arrested further synthesis by the addition of cycloheximide and incubated the translation/translocation reactions for 0, 15, 30, or 60 min on ice (0 °C) or at 30 °C (Fig. 4B). At each time point, an aliquot was fractionated by centrifugation, and antigens were immunoprecipitated with the M66 antibody.

6 B. Schrul, K. Kapp, and B. Dobberstein, unpublished data.
that after 60 min of incubation at 0 °C, SPRem was evenly distributed between the RM pellet and supernatant fraction (Fig. 4B, lanes 14 and 15). However, the incubation at 30 °C for increasing periods of time resulted in a gradual increase of SPRem in the supernatant, and after 60 min essentially all SPRem was released from the RMs and found in the supernatant fraction (Fig. 4B, lanes 16 and 17). This suggests that SPRem initially accumulates in the RM membranes and is then released in a time- and temperature-dependent process.

Subcellular Localization of SPRem in a Time Course Analysis—Next, we wanted to analyze SPRem generation and its localization to the cytosolic and nuclear compartment in cells transiently expressing Rem-myc. We pulse-labeled the cells and at different times thereafter applied the fractionation protocol as detailed above (compare Fig. 2C) to detect SPRem in the cytosolic, membranous, and nuclear fractions, respectively. Fig. 5A shows an autoradiogram of a pulse-chase experiment and the densitometric analysis. After a 5-min pulse labeling and immediate lysis, Rem-myc was predominantly detected in the cytosolic fraction, and only small amounts occurred in the membrane and nuclear fractions. After increasing chase periods, the amount of Rem-myc decreased in the cytosolic fraction, was rather constant in the membrane fraction, and slightly increased in the nuclear fraction. In parallel, a high amount of SPRem was present in the membrane fraction after 5 min of labeling and immediate lysis. A slightly reduced amount was detected in the cytosolic fraction, whereas the nuclear fraction only contained small amounts of SPRem. When cells were pulse-labeled and chased for 5 min, the amount of SPRem within the membrane fraction decreased but increased in the cytosolic and nuclear fraction. After further chase times, the amount of SPRem in the cytosolic and membrane fractions decreased but stayed rather constant in the nuclear fraction for at least 90 min.

To further investigate the initial events, i.e., in the first 15 min after labeling, we quantified the results from three replicate experiments. We found that the initial increase of SPRem in the cytosolic fraction and the decrease in the membrane fraction within the first 5 min after the labeling period is significant (Fig. 5B). The increase in the nuclear fraction is significant, when comparing the nonchased samples with samples from the 15-min chase period. In conclusion, the initially high amount of SPRem in the membrane fraction suggests that SPRem is generated at the ER and is subsequently relocated to the cytosol, whereas Rem-myc is predominantly generated in the cytosol. Both molecules,
ER Membrane Release of the MMTV Rem Signal Peptide

**DISCUSSION**

We show here that Rem is the precursor of a secretory type glycoprotein (gp32Rem). It is targeted to the ER membrane by a signal sequence and inserted. The signal sequence is cleaved off, and the C-terminal portion is translocated and glycosylated. We refer to the glycoprotein as gp32Rem, because the name Rem was previously assigned to the signal sequence containing precursor molecule (22, 23). The cleaved signal peptide is released from the ER membrane and transported into the nucleus. Besides ER targeting, we also find a fraction of Rem to accumulate in the nucleus as precursor molecules.

ER targeting of Rem has not been described previously. Instead, Mertz et al. (23) and Indik et al. (22) identified Rem as a nuclear/nucleolar protein when expressing Rem/EGFP fusion constructs with the EGFP tag at either terminus (22, 23). When EGFP is fused to Rem at the C terminus, ER targeting and signal sequence cleavage can result in ER lumenal gp32Rem-EGFP. This protein is possibly secreted so that it can no longer be detected in cells. However, nuclear localization would be obtained with Rem-EGFP precursor proteins that are directly imported into the nucleus. Alternatively, when Rem is tagged at its N terminus, nuclear staining can result from the cleaved EGFP-tagged signal peptide as a consequence of ER targeting and nuclear import. Again, nuclear staining would also result from direct nuclear import of the precursor protein. Thus, our findings that a fraction of Rem molecules is targeted to the nucleus and another fraction is inserted into the ER and gives rise to gp32Rem are consistent with the previous findings.

In our study, Rem and gp32Rem have molecular masses of about 34 and 32 kDa, respectively, when analyzed by standard SDS-PAGE. These protein sizes correlate with the description of Rem as a 33-kDa protein by Mertz et al. (23). The protein they identify may either represent Rem or gp32Rem, or even both. Indik et al. (22) detect Rem as a 39-kDa protein in MMTV bearing cells with an antibody against the signal sequence. The authors suggest that this protein may be glycosylated; however, for a further conclusion, an experimental verification is necessary.

The 14-kDa protein found in Rem-myc expressing cells and by in vitro analyses is the Rem-derived signal peptide, SPRem. This can be deduced from several lines of evidence as follows. 1) A 14-kDa protein was detected in cells expressing either Rem-myc or a fusion of the predicted signal sequence to EGFP. 2) Exclusively Rem is synthesized in the in vitro assay containing Rem transcripts. The generation of the 14-kDa SPRem requires the presence of ER-derived membranes. 3) Signal sequence cleavage by signal peptidase is a prerequisite for the generation of SPRem. Furthermore, alternative mechanisms for the generation of the 14-kDa protein like alternative splicing, truncated transcripts, or other cell-based mechanisms can be largely ruled out because we confirmed SPRem generation by the cell-free in vitro assay. Thus, we suggest that p14 as identified previously in T-cell lymphomas bearing MMTV (21, 24) and in murine, MMTV-bearing mammary gland-derived cells (25) is also the signal peptide derived from signal sequence cleavage of ER-inserted proteins.

SPRem is released in vitro from microsomal membranes and in cells from the ER membrane. One explanation for SPRem release would have been SPP-dependent processing within the hydrophobic region as shown for other signal peptides (35). However, we found that the release of SPRem from the membrane is independent of SPP activity.

Alternatively, SPRem might be processed by a different enzyme. However, we found no indication for a processing event when applying a broad set of protease inhibitors. Furthermore, SPRem co-migrates with a marker peptide representing the full-length signal peptide in a high resolution gel. In addition, p14/SPRem isolated from nuclear lysates of MMTV-bearing lymphomas was previously shown by mass spectrometry to have a mass of 11 kDa, arguing that this is the full-length signal sequence of 98 amino acid residues (21). Therefore, we suggest that SPRem is the unprocessed, entire signal peptide.

An open question is how SPRem is released from the membrane. One possible mechanism could be retrotranslocation, a process that mediates the transport of ER luminal and transmembrane proteins back into the cytosol. However, retrotranslocation has been described as part of the ER-associated degradation, where it is directly coupled with protein degradation by the proteasome (36). The release of SPRem by such a mechanism would therefore require an additional mechanism to circumvent degradation. Alternatively, the release could be mediated by a cytosolic factor, for example nuclear import factors or B23. Because nuclear import receptors are abundant in reticulocyte lysate (37), a release may also occur in the in vitro translation/translocation assay. B23 has previously been identified as an interaction partner of SPRem (21). It is an abundant nucleolar protein but is also found in the cytosol. Furthermore, B23 is known to bind NLS-containing peptides (38), stimulates the nuclear import of human immunodeficiency virus Rev (39), and has molecular chaperone activities (40), rendering it a possible factor for SPRem release from the membrane.

With a biochemical approach, we detected SPRem in the cytosolic and nuclear compartment. In the nucleus, SPRem accumulates over time, which correlates with the nuclear localization as identified by immunofluorescence microscopy. The cytosolic pool detected biochemically is presumably at the detection limit for immunofluorescence microscopy, especially in comparison to the more concentrated localization in nucleoli. Mertz et al. (23) have shown that Rem functions as nuclear export factor for intron-containing transcripts and that an experimentally truncated form, representing SPRem, is even more potent. Our finding that SPRem is generated in cells suggests that, besides Rem, SPRem functions as a nuclear export factor. Because such factors shuttle between the cytosolic and nuclear compartment, a nuclear export sequence is required, characterized by hydrophobic amino acid residues. In SPRem, the hydrophobic region may be primarily important for ER targeting, and also may function as a nuclear export signal. Furthermore, the cytosolic and nuclear occurrence of SPRem is consistent with a nuclear export factor that shuttles between the two compartments.
Our findings suggest the following model (Fig. 6): 1) Rem can be targeted to the ER and is translocated and glycosylated giving rise to gp32Rem; 2) the signal sequence is cleaved and accumulates as a 14-kDa protein termed SPRem; 3) SPRem is released into the cytosol; and 4) finally it accumulates in nucleoli where it could function as nuclear export factor. Because the signal sequences of Env and Rem are identical, the expression of Env is also expected to result in an accumulating signal peptide. Thus, a sequence of events within a viral infection can be envisaged, where first Rem and SPRem are responsible for the nuclear export of intron-containing transcripts, e.g. the env transcript. SPase, signal peptidase.

In transiently transfected cells, some Rem molecules are not targeted to the ER but accumulate in nucleoli as precursor proteins, suggesting a dual targeting. It is yet unclear if dual targeting also occurs under physiological conditions. However, it is conceivable because the N terminus of Rem is a signal sequence for ER targeting and also contains a nuclear localization signal (compare Fig. 1A). Thus, binding of the signal recognition particle for ER targeting and binding of nuclear transport receptors for nuclear import can compete as soon as the signal sequence emerges from the ribosome. Moreover, nuclear localization of the precursor protein could also result from overloading of the ER targeting pathway with large amounts of proteins during overexpression driven by a strong promoter or upon viral infection. Until now, p14/SPRem was found in MMTV-bearing cells (21, 24, 25), and in another study Rem but not SPRem was found in extracts from MMTV-bearing cells (22). Thus, both pathways, ER targeting and nuclear import, seem to take place in virus-infected cells, and it will be interesting to determine to what extent they compete.

Beyond that, gp32Rem may have a function. Because gp32Rem is located in the ER lumen, we expected it to be secreted. However, we were not able to detect secreted protein in the cell culture medium under various conditions (data not shown). Thus, the fate and function of gp32Rem remain to be determined.

A further, unusually long signal sequence, also including an NLS, is found in human endogenous retrovirus K (HERV-K) Env. Rec, regulator of expression encoded by corf, is a splice variant of Env (41), and similar to Rem it functions as a nuclear export factor. However, for HERV-K only a large portion of the signal sequence rather than the entire signal sequence is also part of Rec. Nevertheless, it will be interesting to test whether the HERV-K Env signal peptide can also be released from the ER membrane and function as a nuclear export factor.

In conclusion, the MMTV-derived SPRem is a further example of a signal peptide that has a post-targeting function and the first signal peptide found to be transported into the nucleus. In contrast to previously described signal peptides that either accumulate in membranes or are released from the membrane after processing by SPP (1, 2), SPRem appears to be released from the membrane without processing by SPP. Future experiments will show if this novel pathway is also used by other signal peptides, independent of whether they do or do not have a post-targeting function.

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REFERENCES
ER Membrane Release of the MMTV Rem Signal Peptide
