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A Retrotranslocation Assay that Predicts Defective VCP/p97-Mediated Trafficking of a

Retroviral Signal Peptide

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ABSTRACT

Studies of viral replication have provided critical insights into host processes, including protein trafficking and turnover. Mouse mammary tumor virus (MMTV) is a betaretrovirus that encodes a functional 98-amino acid signal peptide (SP). MMTV SP is generated from both Rem and envelope precursor proteins by signal peptidase cleavage in the endoplasmic reticulum (ER) membrane. We previously showed that SP functions as an HIV-1 Rev-like protein that is dependent on the AAA ATPase VCP/p97 to subvert ER-associated degradation (ERAD). SP contains a nuclear/nucleolar localization sequence (NLS/NoLS) within the N-terminal 45 amino acids. To directly determine the SP regions needed for membrane extraction and trafficking, we developed a quantitative retrotranslocation assay with biotin acceptor peptide (BAP)-tagged SP proteins. Use of alanine substitution mutants of BAP-tagged MMTV SP in retrotranslocation assays revealed that mutation of amino acids 57 and 58 (M57-58) interfered with ER membrane extraction, whereas adjacent mutations did not. The M57-58 mutant also showed reduced interaction with VCP/p97 in co-immunoprecipitation experiments. Using transfection and reporter assays to measure activity of BAP-tagged proteins, both M57-58 and an adjacent mutant (M59-61) were functionally defective compared to wild-type SP. Confocal microscopy revealed defects in SP nuclear trafficking and abnormal localization of both M57-58 and M59-61. Furthermore, purified GST-tagged M57-58 and M59-61 demonstrated reduced ability to oligomerize compared to tagged wild-type SP. These experiments suggest that SP amino acids 57-58 are critical for VCP/p97 interaction and retrotranslocation, whereas residues 57-61 are critical for oligomerization and nuclear trafficking independent of the NLS/NoLS. Our results emphasize the complex host interactions with long signal peptides.

IMPORTANCE

Endoplasmic reticulum-associated degradation (ERAD) is a form of cellular protein quality control that is manipulated by viruses, including the betaretrovirus, mouse mammary tumor virus (MMTV). MMTV-encoded signal peptide (SP) has been shown to interact with an essential ERAD factor, VCP/p97 ATPase, to mediate its extraction from the ER membrane, also known as retrotranslocation, for RNA-binding and nuclear function. In this manuscript, we developed a quantitative retrotranslocation assay that identified an SP substitution mutant, which is defective for VCP interaction as well as nuclear trafficking, oligomer formation, and function. An adjacent SP mutant was competent for retrotranslocation and VCP interaction, but shared the other defects. Our results revealed the requirement for VCP during SP trafficking and the complex cellular pathways used by long signal peptides.

INTRODUCTION

Mouse mammary tumor virus (MMTV) is a complex murine betaretrovirus that encodes both regulatory and accessory proteins for efficient replication and transmission in mice (1–5). Rem is a precursor protein that is translated from a doubly spliced version of envelope (*env*) mRNA on the ER membrane (1, 2). Rem then is cleaved by signal peptidase into an N-terminal signal peptide (SP) (6, 7) and a C-terminal product (Rem-CT) (4, 5). Since Rem and MMTV envelope protein are made from the same open reading frame, SP is synthesized from both singly spliced *env* mRNA and doubly spliced *rem* mRNA (2). The cleaved Env protein serves as the viral anti-receptor for mouse transferrin receptor 1 (8), whereas failure to express Rem C-terminal sequences leads to hypermutation of the MMTV genome by the Apobec family member, activation-induced cytidine deaminase (AID) after infection of BALB/c mice (4). Rem-

CT is primarily located in the ER, but traffics to early and late endosomes for an unknown

function (5).   Our results suggest that Rem has a human immunodeficiency virus type 1 (HIV-1)

Vif-like accessory function (9–11). Rem-CT also has an accessory function that is not required

for MMTV replication (4, 5). MMTV SP expression is necessary for viral replication in tissue

culture (1, 2, 6), whereas Rem C-terminal sequences are needed for efficient viral transmission

in vivo (4, 12).

Conventional signal peptides are short, 20-30 amino-acid sequences that are bound by

the cytosolic signal recognition particle to allow docking and co-translational transfer of ER

membrane-spanning or secreted polypeptides (13, 14). Typically signal peptides are cleaved by

ER-luminal signal peptidase (15) and then degraded by intramembrane signal peptide

peptidases (16). MMTV-encoded SP is 98 amino acids in length and has an arginine-rich motif

(ARM), typical of RNA-binding proteins, as well as a nuclear/nucleolar localization sequence

(NLS/NoLS) and a leucine-rich nuclear export signal (NES) (Fig. 1A) (1, 17). These features

are typical of viral nuclear export proteins, such as HIV-1 Rev (18–20). Our previous

experiments have shown that SP levels are highest in the nucleolus (1). Analysis of mutants

combined with transfection and reporter assays revealed that SP trafficking and function require

an intact NLS and cleavage by signal peptidase (1, 2). We also have demonstrated that

uncleaved Rem accumulates in the presence of proteasome inhibitors and that a dominant-

negative form of the AAA ATPase family member, VCP/p97, inhibits SP activity (2, 21). These

characteristics suggested that Rem is a target of the cellular protein quality control system, ER-

associated degradation (ERAD) (22, 23). However, after cleavage from the precursor in the ER

membrane, SP is stable and avoids proteasomal degradation (2, 21, 23). Knockdown and

knockout methods in tissue culture indicate that SP activity does not need Derlin proteins (21)

as observed for most ERAD substrates (24). Our experiments indicate that only Rem, not SP,

is ubiquitylated, but both require the p97/VCP ATPase for activity (2, 21).

VCP/p97/Cdc48 ATPase is well conserved in eukaryotes from yeast to humans (25, 26)

and is used for multiple cellular functions, including ERAD (22, 22). VCP mutations are

associated with human diseases, such as multisystem disease inclusion body myopathy,

Paget's disease of bone, and frontotemporal dementia (IBMPFD), amyotropic lateral sclerosis,

and Parkinson's disease (27–29). The p97 ATPase provides the energy required for the

energetically unfavorable retrotranslocation step of the ERAD pathway (25). Ubiquitylated

substrates are marked for degradation by specific E3 ligases within the ER membrane and then

are bound to p97 through a variety of mediators, including the heterodimer UFD1–NPL4 and

UBX2 (30, 31). Retrotranslocation of ubiquitylated substrates by p97/VCP to the cytosol leads

to their interaction with the proteasome and degradation in the cytosol. Nevertheless,

retrotranslocated SP survives p97-assisted ER membrane extraction relative to ERAD

substrates (2, 3). Further, our results from a UBAIT interaction screen in human cells

suggested that MMTV SP binds p97/VCP, but other ERAD-associated proteins were not

identified (21).

In this report, we further explored our hypothesis that the ATPase p97 interacts with

MMTV-encoded SP to mediate its retrotranslocation into the cytosol. To detect and quantify the

fraction of retrotranslocated SP, we developed an assay based on specific biotin labeling of SP

bearing a biotin acceptor peptide (BAP) tag on the C-terminus (SP-BAP) by the cytosolic

enzyme BirA (32). Using the retrotranslocation assay and cellular extracts expressing SP-BAP

mutants, we identified a 2-amino-acid region of SP that is critical for retrotranslocation and

function in reporter assays. We also showed that wild-type SP interacts with p97/VCP using co-

immunoprecipitation experiments and that the mutant with compromised retrotranslocation has

defective interactions with p97. Reporter assays, confocal microscopy, and oligomerization

assays suggested amino acids 57-61 are important for nuclear trafficking. These experiments

support the idea that MMTV-encoded SP avoids proteasomal degradation by direct or indirect

binding to the VCP/p97 ATPase for retrotranslocation and function of a long signal peptide.

RESULTS

MMTV SP retrotranslocation is detectable using a biotinylation assay. Our

published experiments indicate that SP is synthesized as a type II transmembrane protein on

ER membranes as part of the Env or Rem precursors and retrotranslocated to the cytosol using

the p97 ATPase (2, 21, 23). These conclusions were based on the observed glycosylation of

the Rem C-terminus, the ability of mutations in the predicted signal peptidase cleavage site

between SP and Rem-CT to abolish SP activity, and decreased SP-reporter activity in the

presence of dominant-negative VCP/p97 expression (2, 21). The SP reporter assay depends

on transfection of the pHM*Rluc* plasmid, which has the *Renilla* luciferase gene inserted between

splice acceptor and donor sites within the 3' end of the MMTV genome containing the Rem-

responsive element (RmRE) (1, 6). Co-expression of MMTV SP in the presence of pHM*Rluc*

leads to luciferase expression if the unspliced *Renilla*-containing mRNA is exported from the

nucleus for translation (1). Although highly sensitive, quantitative, and specific, this method

provides an indirect measure of SP retrotranslocation.

To develop a quantitative SP retrotranslocation assay, we used an approach similar to

that adopted by Petris et al. for four different cellular proteins (33, 34). A BAP tag (35) was

inserted after amino acid 96 of the N-terminally GFP-tagged SP sequence (GFP-SP-BAP) using

site-directed mutagenesis (Fig.1A). The placement of the tag was designed to delete the Rem

signal peptidase cleavage site that would remove the BAP tag during cotranslational transfer

across the ER membrane (2). We predicted that co-expression of GFP-SP-BAP with *E. coli*

biotin ligase (BirA) (32) in the presence of biotin would allow BirA-mediated biotinylation of the

single acceptor lysine within the C-terminal BAP tag after SP retrotranslocation to the cytosol.

Biotinylation increases the mass of proteins after incubation of cellular lysates with streptavidin,

which has a high affinity and specificity for biotin (36). Since the BirA ligase and the BAP tag are

initially in different cellular compartments (cytosol and ER lumen, respectively), we expected

that biotinylation of GFP-SP-BAP would confirm that SP is retrotranslocated (Fig.1B, left).

Our previously published experiments indicated that both N-terminally GFP-tagged Rem

or SP alone are functional in the pHM*Rluc* reporter assay (2, 21). To determine whether

addition of the C-terminal BAP tag interferes with SP activity, we co-transfected human

embryonic kidney (HEK) 293 cells in triplicate with GFP-SP or GFP-SP-BAP expression

plasmids in the presence of the pHM*Rluc* reporter expressing *Renilla* luciferase. Transfections

also contained a firefly luciferase reporter vector lacking an RmRE to control for differences in

DNA uptake or expression (6). Cell lysates then were assayed for *Renilla* and firefly luciferase

activities relative to lysates derived from transfections lacking SP expression vectors (pcDNA3).

The results indicated that the BAP-tagged GFP-SP construct was functional, but had slightly

lower activity compared to GFP-SP (Fig. 1C).

To directly test the SP retrotranslocation assay, we co-transfected a negative control

vector (pcDNA3) or expression vectors for GFP-SP lacking a BAP tag or GFP-SP-BAP together

with the BirA expression plasmid. Although a low level of biotin is present in normal cells (37), it

is usually insufficient to allow efficient biotinylation by BirA.   Therefore, after 24 h, we added

biotin to the media for 12 h, and cell lysates were prepared. Streptavidin was added to one

portion of each extract, and treated and untreated extracts were analyzed by gel electrophoresis

and Western blotting using GFP-specific antibody (Fig. 1D). As expected, lysates transfected

with pcDNA3 alone lacked GFP-SP expression (lanes 1 and 2). Samples derived from

transfection of the GFP-SP expression vector lacking a BAP tag showed a band consistent with

GFP-SP, a product of signal peptidase cleavage, both in the presence or absence of

streptavidin (compare lanes 3 and 4). As expected, lysates expressing GFP-SP-BAP revealed

a band with a slightly higher mass than untagged GFP-SP. In the presence of streptavidin, we

observed that a portion of GFP-SP-BAP was retarded within the gel, consistent with the tight

binding of streptavidin to the biotinylated form of the protein (Fig. 1D, compare lanes 5 and 6).

Only a portion of GFP-SP-BAP was biotinylated due to the addition of biotin after SP

retrotranslocation to the cytoplasm and prior to its import into the nucleus for MMTV RNA

binding (1). As expected, SP tagged at the N-terminus (see Fig. 1B), was biotinylated by the

cytosolic BirA as detected by streptavidin binding, (Fig. 1D, compare lanes 7 and 8). These

data strongly suggest that the C-terminal BAP tag of GFP-SP-BAP is biotinylated by BirA after

retrotranslocation into the cytosol.

To verify that SP biotinylation occurred in transfected cells and not after lysis, we

transfected HEK 293 cells with the GFP-SP-BAP expression plasmid together with the BirA

expression vector or cells were transfected separately with each vector. After addition of biotin,

lysates from cells individually transfected with the expression vector for GFP-SP-BAP or BirA

were combined and compared to lysates from co-transfected cells. Streptavidin was added to

an equal portion of each lysate prior to gel electrophoresis and Western blotting with antibodies

specific for GFP and GAPDH (Fig. 1E). Lysates obtained from cells co-transfected with

expression vectors for GFP-SP-BAP and BirA revealed a biotinylated band in the presence of

streptavidin, whereas the combined lysate from separately transfected cells did not (Fig. 1E,

compare lanes 2 and 4, respectively). These results indicated that biotinylation of GFP-SP-

BAP occurs inside transfected cells, and not after cell lysis.

To confirm the SP membrane orientation and specificity of the retrotranslocation assay,

we used a BirA expression construct engineered with an N-terminal signal peptide and a C-

terminal KDEL sequence to allow expression and retention in the ER lumen (ER-BirA) (38).

Cells were transfected with N-terminally or C-terminally BAP-tagged SP expression plasmids

(GFP-BAP-SP or GFP-SP-BAP, respectively) and vectors expressing either BirA or ER-BirA.

After addition of biotin, lysates were prepared and equal amounts were incubated with or

without streptavidin prior to Western blotting. As anticipated for N-terminally BAP-tagged SP,

biotinylated protein was detected in the presence of BirA and streptavidin (Fig. 1F, lane 2), but

not when ER-BirA was expressed (lane 4). In contrast, biotinylated SP was detected when

GFP-SP-BAP was expressed in the presence of ER-BirA (Fig. 1F, compare lanes 4 and 6).

Interestingly, C-terminally BAP-tagged SP appeared to be more stable relative to the N-

terminally BAP-tagged protein (Fig. 1F, compare lanes 5 and 6 and lanes 1-4). This result is

consistent with the lower functional activity of GFP-BAP-SP in reporter assays (Fig. 1C).

Together, our experiments support the type II transmembrane orientation of SP and verify the

specificity of SP retrotranslocation assays.

SP retrotranslocation requires the VCP/p97 ATPase. We previously have shown that

SP function in a transient assay requires both the nuclear localization signal on the SP

expression plasmid and the presence of the RmRE on the pHM*Rluc* reporter vector (1). Using

this assay, we demonstrated that a dominant-negative form of p97/VCP inhibited SP activity, but

not Rem cleavage by signal peptidase (2). To confirm that our retrotranslocation assay was

dependent on p97, we used a chemical inhibitor (CB-5083) that binds selectively to the D2

domain of the ATPase (39). HEK 293T/17 cells were transfected with vectors expressing GFP-

SP-BAP and BirA for 24 h.   Media containing 1 μM of inhibitor was added for 8 h prior to

addition of biotin. We then treated an equal amount of each lysate with streptavidin before

analysis by denaturing gel electrophoresis and Western blotting with GFP-specific antibody (Fig.

2A). Use of GAPDH-specific antibody confirmed equal loading of the lanes. Gels then were

scanned and retrotranslocation was quantified.   The results revealed that inhibition of p97

ATPase activity interfered with SP retrotranslocation (Fig. 2A, compare lanes 2 and 4).

Retrotranslocation assays with the VCP/p97 inhibitor also were performed in HC11

mouse mammary cells, a cell type that is a physiological target of MMTV infection (40). HC11

cells were transfected with an expression vector for GFP-SP-BAP together with the BirA

expression plasmid and treated with either of two different amounts of CB-5083 for 9 h. Lysates

were analyzed with and without streptavidin (Fig. 2B). As expected, quantitation of the results

revealed that CB-5083 inhibited SP retrotranslocation in a dose-dependent manner (Fig. 2B,

compare lane 2 to lanes 4 and 6). Since CB-5083 blocks VCP enzyme function, these results

confirmed that p97 ATPase activity was required for SP retrotranslocation in both human and

mouse cells.

Biotinylated SP is sensitive to trypsin during retrotranslocation. To confirm that SP

biotinylation is specific to proteins that have been extracted from the ER membrane, we used a

trypsin-sensitivity assay with cell lysates containing intact microsomes. In this assay, we

anticipate that SP extracted from the membrane and biotinylated in the cytosol will be

susceptible to digestion with trypsin. In contrast, proteins that reside inside membranes are

expected to resist trypsin digestion. HEK 293T/17 cells co-transfected with plasmids expressing

C-terminally BAP tagged SP (GFP-SP-BAP) and BirA were harvested and subjected to freeze-

thaw in a hypotonic buffer to isolate intact ER-derived microsomes. Lysates then were treated

with increasing concentrations of trypsin. Streptavidin was added to one of the duplicate

samples prior to gel electrophoresis and Western blotting. As a positive control for isolation of

intact ER-derived vesicles, we used antibody against BiP, a cellular ER-resident protein (41).

Samples treated with different concentrations of trypsin or with streptavidin showed little

difference in the levels of BiP (Fig. 3A). These data confirmed that proteins within the ER lumen

were protected from trypsin digestion under these conditions.

To determine whether biotinylated GFP-SP was susceptible to trypsin digestion, we

incubated the blot with GFP-specific antibody. A significant portion of the signal in the absence

of trypsin was shifted in the presence of streptavidin (Fig. 3B, compare lanes 1 and 2).

However, the biotinylated fraction of GFP-SP-BAP showed a gradual decline with increasing

trypsin concentration, indicating that the biotinylated GFP-SP is exposed to the cytosol.

Quantitation of the trypsin sensitivity of the biotinylated protein confirmed this result (Fig. 3C).

However, the non-biotinylated GFP-SP was trypsin resistant, suggesting that GFP-SP

synthesized prior to the addition of biotin is protected from enzymatic digestion by the presence

of a membrane. Since the steady state levels of SP are highest within nucleoli (1), these results

suggest that non-biotinylated GFP-SP is protected from trypsin by the nuclear membrane.

SP co-immunoprecipitation with VCP/p97 is ATP-dependent. Our previously

published work indicates that VCP is required for efficient SP function (2, 21). We also have

shown that VCP/p97 was identified in a covalent interaction screen using an SP-ubiquitin-

activated interaction trap (SP-UBAIT) (21), and that a p97 inhibitor interfered with SP

biotinylation in a retrotranslocation assay (see Fig. 2). Therefore, our expectation was that VCP

interacts with SP during retrotranslocation and that p97-specific antibody would

immunoprecipitate SP.

HEK 293 cells were co-transfected with expression vectors for GFP-SP and, in some

cases, VCP/p97. After 48 h, cells were fixed in formaldehyde to preserve protein interactions,

and a portion of the cell lysate was used for co-immunoprecipitation with p97-specific antibody.

Immunoprecipitates and cell lysates were analyzed by Western blotting with p97 and GFP-

specific antibodies. Little difference was observed with or without exogenous p97 expression

(Fig. 4, right panel). GFP-SP was precipitated both in the presence and absence of exogenous

p97 (Fig. 4, left panel). However, SP co-immunoprecipitation was increased when ATP and

Mg^{2+} were added to the lysates (Fig. 4, compare lanes 2 and 3 to lanes 4 and 5). This result

was not unexpected since the N-terminal domain of p97 is known to change its conformational

state dependent on ATP binding (42). These experiments confirm that SP interacts with

endogenous p97. In addition, our data suggest that SP binds to the ATP-bound form of

VCP/p97.

SP amino acids 57-58 are required for retrotranslocation and efficient interaction

with p97. To identify SP regions essential for the retrotranslocation process, we prepared a

series of N-terminal deletion mutants (Fig. 5A), including the nuclear/nucleolar sequence

(NLS/NoLS) (1, 7). Our previous experiments have shown that the NLS/NoLS is required for SP

function in reporter assays (1).   Plasmids expressing GFP-SP-BAP or SP deletion mutants and

BirA were co-transfected into HEK 293 cells.   Transfected cells were treated with biotin prior to

extract preparation. Extracts were subjected to Western blotting in the presence or absence of

streptavidin. Deletion of the N-terminal 50 amino acids of SP within the GFP-SP-BAP construct

retained SP retrotranslocation activity (Fig. 5B). Deletion of the N-terminal 60 amino acids of SP

produced an unstable protein despite the presence of the GFP tag (Fig. 5B, lanes 13 and 14).

Biotinylation assays using these mutants suggested that the SP region C-terminal to amino acid

50 was important for retrotranslocation (Fig. 5B, even lanes 4-12 compared to 14). We also

assayed the functional activity of the deletion mutants. Cells were transfected in triplicate with

expression vectors for wild-type and SP mutants in addition to the pHM*Rluc* reporter plasmid. In

contrast to retrotranslocation assays, N-terminal deletions of 30 amino acids or more eliminated

detectable SP function (Fig. 5C), consistent with removal of a portion of the NLS/ARM (Fig. 5A).

285   To avoid potentially destabilizing effects of large deletions, we performed alanine-

scanning mutagenesis to delineate further SP sequences needed for ER membrane extraction.

We prepared two-to-three amino-acid substitutions in the region between 50 to 61 amino acids,

which is localized between the NLS/NoLS/ARM and the NES (Fig. 6A). Co-transfections of

plasmids expressing wild-type SP or mutants and BirA were used for biotinylation in HEK 293

cells. Extracts incubated with streptavidin showed that mutants M50-52, M53-55, and M59-61

had retrotranslocation efficiencies similar to wild-type GFP-SP-BAP (Fig. 6B). Since wild-type

SP has an alanine at position 56, only two amino acids were mutated for M57-58 (WQ to AA).

Alteration of these two amino acids decreased retrotranslocation activity, which varied

depending on the efficiency of transfection and the amount of expression plasmid used (Fig.

6C). In multiple experiments (n = 9) subjected to quantitation relative to the total GFP signal

detected, M57-58 showed significantly reduced retrotranslocation compared to wild-type SP

(Fig. 6D).

The M57-58 defect in retrotranslocation predicted that this mutant would be defective in

the SP reporter assay. Therefore, we tested substitution mutants for SP activity. Cells were co-

transfected in triplicate with expression vectors for GFP-SP-BAP (wild-type) or mutants together

with the pHM*Rluc* plasmid. As anticipated, no activity was detected for M57-58 using the same

amount of the expression vector that gave a 6-fold increase in luciferase activity for the wild-

type plasmid (Fig. 7A). No statistical difference was observed between the wild-type SP and the

mutants, M50-52 and M53-55. Surprisingly, the M59-61 mutant, which showed no defect in the

retrotranslocation assay, had greatly reduced SP reporter activity compared to the wild-type

expression vector. We repeated the activity assays for both M57-58 and M59-61 with higher

concentrations of the expression plasmids (Fig. 7B). The M57-58 mutant showed minimal

activity even at 5-fold higher amounts of DNA in the reporter assay, but remained significantly

lower than wild-type SP. The M59-61 mutant had detectable activity using DNA amounts that

were significantly more compared to the wild-type expression vector (Fig. 7B). These data

suggest that M59-61 has a defect in a function subsequent to SP retrotranslocation to the

cytosol.

Since SP extraction from the ER membrane is likely to be dependent on p97 based on

the involvement of the ATPase in ERAD (43), we performed co-immunoprecipitation

experiments with lysates obtained from cells transfected with plasmids expressing wild-type

GFP-SP or the GFP-tagged versions of the mutants M57-58 and M59-61 (Fig. 7C). Precipitates

and cell lysates were subjected to Western blotting with antibodies to p97 and GFP. Antibody

specific for VCP/p97 precipitated both the wild-type SP as well as M59-61. However, the

mutant M57-58 interacted very weakly with p97 in cellular lysates containing ATP and Mg^{2+}

under the same conditions (compare lanes 2 and 3). Co-immunoprecipitations with lysates from

cells transfected with EGFP expression vector served as a negative control (Fig. 7C, lane 5).

Analysis of cell lysates revealed similar expression of the wild-type and mutant SP proteins (Fig.

7C, lanes 7-9). Furthermore, GFP alone did not immunoprecipitate with p97 (lane 5). Our

experiments suggest that SP amino acids 57 and/or 58 (WQ) are essential for interaction

directly or indirectly with the cellular ATPase p97.

SP mutants 57-58 and 59-61 show aberrant nuclear localization. SP mutants M57-

58 and M59-61 had decreased activity in our reporter assay and amino acids 57-58 appear to

be critical for SP retrotranslocation. Therefore, we anticipated increased co-localization of M57-

58 with a fluorescent ER marker, ER-mCherry, compared to wild-type SP. ER-mCherry has an

N-terminal signal peptide linked to monomeric Cherry and a C-terminal KDEL to facilitate

retention in the ER lumen (44). HC11 mouse mammary cells were co-transfected with plasmids

expressing ER-mCherry and either wild-type GFP-SP or SP mutants prior to fixation, nuclear

staining with 4′,6-diamidino-2-phenylindole (DAPI), and confocal microscopy (Fig. 8A). Our

published observations have shown that wild-type SP is localized primarily in nucleoli similar to

other HIV-1 Rev-like proteins (19, 20), in agreement with the results observed here (Fig. 8A; see

top right, WT Merge). In contrast, M57-58 appeared in perinuclear regions that overlapped with

the ER-mCherry signal as well as the nucleus. M59-61 was co-localized with the mCherry

signal, but also outside the ER in both nuclear and extranuclear locations. Compared to the

wild-type GFP-SP, neither mutant appears to localize to the nucleolus (Fig. 8A, middle and

lower panels). Quantitation of the fluorescent signals in multiple cells from two independent

experiments revealed some wild-type SP co-localized with the ER marker (Fig. 8B), which is

consistent with SP synthesis in the ER prior to retrotranslocation and nuclear trafficking.

However, the mutant M57-58 showed low, but significantly higher, co-localization with the ER

marker relative to wild-type SP (0.03 and 0.00, respectively) (Fig. 8B). The mutant M59-61,

which had normal activity in the retrotranslocation assay, showed a similar localization pattern

as M57-58 (Fig. 8A and 8B), indicating that M59-61 also had defective nucleolar trafficking.

These results explain the low activity of M57-58 and M59-61 in reporter assays, which depend

on SP binding to the RmRE, presumably in nucleoli.

Our previous retrotranslocation assays were performed with lysates obtained from

transfected cells treated with biotin for 4 to 12 h to maximize detection of SP extraction from the

ER membrane. To determine the timing of the retrotrotanslocation process, we performed

shorter pulses of biotin after co-transfection of HEK 293T cells with plasmids expressing the

wild-type SP (GFP-SP-BAP) or the mutants with a BirA expression vector. At 24 h post-

transfection, cells were treated with 100 µM biotin for either 1 h or 3 h. Lysates were incubated

with streptavidin and used for Western blotting with GFP-specific antibody prior to quantitation

(Fig. 8C). Retrotranslocated SP constituted approximately 40 to 50% of the total wild-type SP

signal and was similar for lysates obtained at 1 h and 3 h after biotin addition. As expected, the

M57-58 mutant showed <20% retrotranslocation during the 1 h-biotin treatment, and the longer

incubations did not significantly change this result. In contrast, the M59-61 mutant showed an

initial defect in retrotranslocation that was detectable after 1 h of biotin treatment, whereas

retrotranslocation of this mutant recovered to wild-type SP levels after 3 h. Together with

previous results, our data suggest that both M57-58 and M59-61 have defects in

retrotranslocation to different extents (Fig. 7C). Nevertheless, M57-58 has a weaker interaction

with VCP/p97, which explains its greater defect in ER membrane extraction.

Conformational modeling predicts defective multimerization of SP mutants. SP is

a Rev-like protein that likely shares a similar secondary structural conformation. To test this

idea, we performed molecular modeling of SP using AlphaFold2, a highly sophisticated machine

learning-based modeling tool (45, 46). SP is predicted to contain a long disordered N-terminus

and three α-helices designated as α1, α2 and a transmembrane helix (as detected by MEMSAT-

SVM (47) (Fig. 9A). Since SP is a type II transmembrane protein, the disordered N-terminus

faces the cytosol, and the very short, disordered C-terminus localizes within the ER. The α1

helix contains the NLS/ARM and is connected to the α2 helix by a loop. This structure is

reminiscent of HIV-1 Rev, which is known to form a helix-loop-helix (helical hairpin)

conformation. The α-helices of HIV-1 Rev are known to form a coiled-coil structure that acts as

a nucleation site for oligomerization (48).

The coiled-coil motifs in SP were modeled using the CCHMM_PROF coiled-coil server

(49). Like Rev, SP was predicted to have two coiled-coil motifs localized in the α1 and α2

helical regions. Since M57-58 and M59-61 differ from WT SP and each other, similar modeling

predicted changes within the α-helices. The α1-helix of M57-58 was extended with no major

conformational change in the α2-helix (Fig. 9B, left). However, mutation in the 59-61 region

destabilized the α2-helix (Fig. 9B, right). Neither mutation affected the conformation of the

transmembrane helix (Fig. 9B and 9C). Conformational changes in either α-helix could affect

SP stability and function. Likewise, both predicted coiled-coils were extended in M57-58,

whereas the mutation in M59-61 shortened the coiled-coil of the α2-helix (Fig. 9C). If SP forms

the helical hairpin for RNA binding, changes in the α-helices and/or length of coiled-coil region

might create stearic hindrance to interfere with the formation of oligomers/multimers.

To determine whether amino acid changes in M57-58 and M59-61 affect the ability of SP

to form multimers, we expressed the wild-type and mutant SP proteins fused to glutathione-S-

transferase (GST) in *E. coli*. Cell lysates were used for purification and analysis on SDS-

containing gels prior to staining. The results revealed similar yield and purity of the proteins

(Fig. 9D). To assess the ability of purified SP proteins as well as GST to oligomerize, we

resolved the purified wild-type or mutant proteins using polyacrylamide gradient gel

electrophoresis in the absence of denaturing agents. After Coomassie blue staining, the wild-

type SP protein showed a band expected of the GST-SP monomer (Fig. 9E, lane 1), although

most of the protein was located near the gel origin, indicative of higher oligomeric conformation.

As expected, the purified GST control did not oligomerize (lane 4). In contrast, the majority of

M57-58 appeared to be monomeric, whereas M59-61 migrated between the monomers and

oligomeric wild-type SP. These experiments suggested that both M57-58 and M59-61 are

defective in multimerization.

DISCUSSION

402   Unlike other signal peptides, MMTV-encoded SP is unusually long (98 amino acids) and

has an arginine-rich motif typical of RNA-binding proteins, an NLS/NoLS, and a leucine-rich

NES typical of proteins that use the Crm1 export pathway (50–52). We previously showed that

the NLS is required for SP localization to the nucleolus (1). SP must be extracted from the ER

membrane by retrotranslocation prior to its entry into the nucleus for binding to the RmRE on

MMTV RNA (6, 53). Retrotranslocation is a critical component of ERAD, which provides cellular

quality control of protein function (24, 54, 55).

409   To directly measure SP retrotranslocation to the cytosol, we developed a quantitative

assay. This assay is based on the biotinylation of a lysine residue in a C-terminally BAP-tagged

SP protein during expression of the *E.coli* ligase BirA in the cytosol of mammalian cells. Similar

assays have been used to measure the retrotranslocation of both membrane-bound and

secretory proteins to the cytosol for ERAD (33, 34, 56). Our previous experiments indicate that

SP is a type II transmembrane protein with the C-terminus in the ER lumen based on

glycosylation of the Rem C-terminus (2). C-terminal BAP tagging of SP does not greatly

compromise its function based on reporter assays (Fig. 1C). Biotinylation of the C-terminal BAP

tag by the cytosolic BirA provides direct evidence that MMTV-SP is retrotranslocated from the

ER to the cytosol. Moreover, this assay represents a simple, reliable, and quantitative measure

of SP extraction from the ER membrane (Fig. 1D). The assay was validated by comparisons to

functional reporter assays (Fig. 1C), the requirement for VCP/p97 ATPase activity (Fig. 2), the

use of biotin ligases that were specific for either the cytosol or the ER lumen (Fig. 1F), and

trypsin sensitivity of the biotinylated products not protected by the ER membrane (Fig. 3).

Multiple experiments support the idea that VCP/p97 is essential for efficient SP

retrotranslocation. First, using our retrotranslocation assay, we confirmed that inhibition of p97

activity reduced SP extraction from membranes in two different cell lines (Fig. 2). Second, we

showed that p97 interacted with MMTV-encoded SP by co-immunoprecipitation, and

interactions were dependent on the presence of ATP and Mg2+ (Fig. 4). Our published results

using a UBAIT screen support an SP-p97 interaction (21). Third, we identified a two amino-acid

sequence (WQ) in mutant M57-58 that greatly decreased association with p97 (Fig. 6). Fourth,

the same mutation that inhibited SP interaction with p97 showed a defect in retrotranslocation

as well as SP function in our reporter assay (Fig. 7). Together with our previously published

results that SP function is inhibited by expression of a dominant-negative p97 (2, 21), we

conclude that VCP/p97 provides the energy required for extraction of SP from the ER

membrane.

Although p97 is required for SP retrotranslocation, we cannot rule out the need for an

adapter protein to mediate their association. Our previous high-throughput screen failed to

identify any known p97 adapters (21), such as UFD1/NPL4 or UBX2, which often mediate

interactions between ubiquitylated ERAD substrates and VCP/p97 (30, 57). Therefore, we

speculated that SP interacts with p97 directly (21). However, our attempts to use binding

assays with purified GST-tagged SP and His-tagged p97 purified from *E. coli* have not been

successful.   In support of our hypothesis, published data suggest that p97 associates with both

ubiquitylated and non-ubiquitylated polypeptides (25, 58). In addition, the yeast homologue of

p97, Cdc48 (30, 57, 59), has been shown to recognize monoubiquitylated substrates that are

not degraded (60, 61). MMTV-encoded SP may mimic the p97 adapters, NPL4 and/or UFD1

(62, 63), to compete directly for p97 binding in the absence of ubiquitylation or an adapter may

yet be identified.

The mutant M59-61, which showed a limited defect in retrotranslocation (Fig. 8C), had

reduced activity in an assay for SP function (Figs. 7A and 7B). This mutant also showed normal

interactions with p97 under the co-immunoprecipitation conditions used (Fig. 7C). Both M57-58

and M59-61 likely have defects in retrotranslocation as determined from limiting the biotinylation

period to 1 h (Fig. 8C), but M57-58 is missing critical interactions with VCP/p97 that more

dramatically hamper ER extraction (Fig. 7C). Confocal microscopy of both M57-58 and M59-61

showed slightly, but significantly, increased ER localization compared to wild-type SP and

abnormal distribution at steady state (Fig. 8). Molecular modeling predicted that wild-type SP

has three α-helices (Fig. 9A). Two of those helices likely form a coiled-coil structure.

Furthermore, independent modeling of the functionally inactive mutants M57-58 and M59-61

revealed that each mutant changed the length of the α-helical and coiled-coil regions, predicting

altered interactions (Figs. 9B and C). Direct testing of purified mutant proteins on non-

denaturing gels confirmed this prediction, although M57-58 appeared to form less multimers

than did M59-61 compared to wild-type SP (Fig. 9E). This result may reflect additional defects

of M57-58 in membrane extraction that limit multimerization. Therefore, mutants in the SP

region from 57-61 showed a slight increase in ER association and abnormal nuclear localization

due to defects in interaction with p97 and adapters, and/or multimer formation.

In summary, MMTV-encoded SP interacts with a critical cellular factor, VCP/p97. Point

mutations in VCP induce multi-system proteinopathy type 1 (MSP1), which is known as

inclusion body myopathy associated with Paget's disease and frontotemporal

dementia/amyotrophic lateral sclerosis (IBMPFD/ALS) (27, 28, 64, 65). Disease mutations

increase p97 ATPase activity and change interactions with p97 adapter proteins (62). Since

MMTV SP is highly related to the human endogenous retrovirus type K (HERV-K) Rec protein

(66–68), it is possible that Rec interacts with VCP/p97 to affect ERAD and normal protein quality

control. Our results highlight the unique characteristics of long signal peptides specified by host

and viral genes (13, 69).   Many long signal peptides, including MMTV SP, are known to be

signal recognition particle-dependent and are co-translationally transferred across the ER

membrane (69). Nevertheless, these unique signal peptides are more than zip codes,

suggesting that additional characterization will provide translational benefits for development of

biotherapeutics (13).

MATERIALS AND METHODS

Constructs. The coding sequence of the BAP tag (GLNDIFEAQKIEWHE) (70) was

inserted after amino acid 96 of N-terminally GFP-tagged SP followed by a stop codon using site-

directed mutagenesis with the CloneAmp HiFi PCR Premix (Takara Bio). The BirA and ER-BirA

constructs were obtained from Addgene (38, 71).   The N-terminal deletion mutants of GFP-SP-

BAP (d10-d60) were generated by PCR, and the products were cloned between the *Xho*I and

*Bam*HI sites of GFP-SP. Alanine scanning mutagenesis was used to prepare specific GFP-SP-

BAP mutants between SP amino acids 50 to 61. In this approach, two to three amino acids in

GFP-SP-BAP were replaced by alanine for each construct using the CloneAmp HiFi PCR

Premix (Takara Bio) according to the manufacturer.

Cell lines and transfections. HEK 293, 293T, or 293T/clone 17 (293T/17) cells

(purchased from ATCC) were cultured at 37°C and 7.5% CO_2 in Dulbecco's modified Eagle's

medium (DMEM; Life Technologies, Inc.) supplemented with 10% fetal bovine serum (FBS),

100 U/ml penicillin, 100 U/ml streptomycin, 50 µg/ml gentamicin, and 2 mM L-glutamine. Cells

were seeded in 6-well plates at 1×10^6 cells/well for 24 h prior to transfection.

Polyethylenimine (PEI) transfection reagent (72) was added with the indicated amounts of DNA

and adjusted to a total of 3 µg of DNA with the plasmid vector pcDNA3. Most biotinylation

experiments used 300 ng each of GFP-SP-BAP and BirA constructs. After 24 h, the media was

replaced with 2 ml of serum-free media supplemented with 0.1 mM biotin and further incubated

for 1 to 12 h as noted. In transfections using luciferase reporter vectors, 250 ng each of the

pHM*Rluc* (1) and pGL3-control plasmids (Promega) were added in addition to the indicated

amounts of SP expression constructs.

Cell lysate preparation, biotinylation assay and Western blotting.   Transfected

HEK 293, 293T, or clone T17 cells were washed with phosphate-buffered saline (137 mM NaCl,

2.7 mM KCl, 10 mM NaH_2PO4, and 1.8 mM KH_2PO4) to remove free biotin. Cell pellets were

lysed in SDS-sample buffer (25 mM Tris-HCl, pH 6.8, 1% SDS, 10% glycerol, and 5% 2-

mercaptoethanol). For gel retardation assays, samples were boiled for 10 min, cooled to room

temperature, and incubated with 1 µg of streptavidin (Sigma) for 30 min before separation on

SDS-containing polyacrylamide gels. Proteins were then transferred to nitrocellulose

membranes. Membranes were pre-incubated with Tris-buffered saline [20 mM Tris-HCl, pH 7.4,

137 mM NaCl (TBS)] plus 0.1% Tween 20 (TBS-T) buffer containing 5% nonfat dry milk (TBS-

TM) for 1 h. Membranes were incubated with primary antibody in TBS-TM for 2 h at room

temperature or overnight at 4°C, depending on the antibody. Following three washes, the

membranes were treated with horseradish peroxidase-conjugated secondary antibody in TBS-

TM for 1 h, and then washed again thrice with TBS-T. Secondary antibody binding was

detected using the ECL Prime Western Blotting Detection Reagent (Amersham) as described by

the manufacturer. For LI-COR detection, membranes were incubated with Odyssey Blocking

Buffer (OBB) and TBS (1:2 ratio by volume) followed by incubation with primary antibody diluted

in OBB–TBS-T (1:2 ratio by volume). The membranes were then washed thrice with TBS-T and

incubated with fluorescently labeled IRDye secondary antibodies in OBB–TBS-T (1:2 ratio by

volume) for 1 h. Following the incubation, membranes were washed again thrice in TBS-T and

once in TBS. Signals were then detected using the Odyssey Imaging System according to the

manufacturer's instructions.

The sources of the antibodies were: GFP (Clontech); GAPDH, BiP, goat anti-mouse IgG,

goat anti-rabbit IgG (all from Cell Signaling); VCP (Novus Biologicals or Invitrogen); IRDye 800CW

goat anti-mouse IgG and IRDye 680RD goat anti-rabbit IgG (LI-COR). Band intensities were

quantitated either using ImageJ or Image Studio software.

Trypsin sensitivity assays. To maintain intact microsomes for trypsin sensitivity assays,

cells were resuspended in a buffer containing 50 mM Tris-HCl, pH 8.0, 250 mM sucrose, and 10

mM N-ethyl-maleimide (to block BirA post-lysis activity). Cell suspensions were subjected to one

freeze-thaw cycle and centrifugation at 5,000 Xg for 5 min at 4°C. Supernatants (microsome-

containing lysates) containing ~100 µg of total protein were incubated with 0.01 µg or 0.025 µg

trypsin in reaction volumes of 100 µl for 1 h at 37°C. Microsome preparations then were used for

retardation assays.

Co-immunoprecipitation experiments. For co-immunoprecipitation studies, different

GFP-tagged SP constructs (either wild-type or mutants) (1 µg) were used for transfection of HEK

293T/17 cells. After 48 h, cells were harvested and fixed in 4% formaldehyde solution. Cells were

lysed in NP-40 lysis buffer (0.1 M Tris-HCl, pH 7.5, 0.1 M NaCl, 1% NP-40, and 1 mM dithiothreitol

[DTT]) containing 1 mM ATP, 5 mM MgCl$_2$, and complete protease inhibitor (Thermo Fisher

Scientific) on ice for 1 h with intermittent vortexing. The lysates were then cleared by

centrifugation at 13,000 Xg at 4°C, and the protein yield was determined. Lysates containing

~200 to 500 µg of total protein were incubated with rabbit polyclonal p97-specific antibody (Novus

Biologicals) overnight at 4°C. Antibody-containing mixtures were incubated with magnetic Protein

G beads for 3 h at 4°C and washed thrice in a detergent-containing buffer (1 M Tris-HCl, pH 7.5,

5 M NaCl, 0.5 M EDTA, 0.1 M EGTA, and 1% Triton X-100). Immunoprecipitates were then boiled

in SDS-containing loading buffer for 10 min prior to analysis by SDS-containing polyacrylamide

gel electrophoresis and Western blotting. Antibodies used for Western blotting were p97

(Invitrogen) and GFP (Clontech).

Confocal microscopy and image analysis. One day prior to transfection, HC11 cells

were seeded at 1×10^6 cells in 6-well plates. Equal amounts of plasmids expressing GFP-SP

WT, M57-58, or M59-61 were co-transfected with ER-mCherry using Lipofectamine 3000

(Thermo Fisher Scientific). At 24 h post-transfection, 6×10^5 cells were seeded onto 22 X 22

1mm coverslips and, after another 24 h, fixed with 4% paraformaldehyde followed by

permeabilization with 0.1% Triton-X 100. Cells were then washed thrice with PBS containing

0.1% Tween 20 (PBS-T). Blocking was performed with the same buffer containing 2% FBS.

Fixed cells were incubated with 150 nM DAPI for 10 min followed by three washes with PBS-T.

Finally, the coverslips were mounted on slides with VECTASHIELD mounting medium (Vector

Laboratories) and sealed for imaging. The Zeiss LSM 710 Confocal and Elyra S.1 Structured

Illumination Super Resolution Microscopes were used to capture Z-stack images of the

samples. All slices in Z-stacks across samples were kept at 0.33 µm per slice interval. Lasers

405 nm, 488 nm, and 561 nm were used with the same power and gain across samples to

capture images. Quantitation and image processing were performed using Zeiss software ZEN,

Graphpad (Prism), and ImageJ. Briefly, Manders Colocalization Coefficient (MCC) (**73**) was

used to quantify the percentage of GFP-SP pixels colocalized with ER-mCherry pixels in single

slices. Mann-Whitney's t tests were used to compare different samples in Graphpad software.

Images were processed using ImageJ.

Cloning and purification of GST-tagged SP proteins. GST-tagged wild-type or mutant

SP expression constructs were generated by cloning the coding sequence into the *XhoI* and *NotI*

restriction enzyme sites within the PGEX-6-GST vector. The constructs were transformed into

BL21 cells, and a single colony was inoculated in 5 ml 2X YT media (16 g/L tryptone, 10 g/L yeast

extract, and 5 g/L sodium chloride) containing ampicillin (100 µg/ml). After overnight incubation

at 37°C with constant shaking at 200 rpm, the initial culture was inoculated into 500 ml of 2X YT

media containing 100 µg/ml ampicillin and again grown at 37°C with constant shaking at 200 rpm

to an optical density of ~0.6. Induction of GST-SP proteins was performed by addition of 100 µM

IPTG and overnight incubation at 16°C with constant shaking. The culture was then subjected to

centrifugation at 7,000 rpm for 10 min at 4°C, and the pellet was lysed in PBS buffer containing 5

mM EDTA, 1X protease inhibitor cocktail (Thermo Fisher Scientific), 1 mM PMSF, and 1 mg/ml

lysozyme. The lysate was centrifuged at 13,000 Xg for 30 min at 4°C, the cleared supernatant

was collected and incubated with 100 µl of GST Sepharose beads (GE Healthcare) for 1-2 h at

4°C. The GST beads were washed four times with PBS. The bound protein was then eluted from

the beads using PBS containing 10 mM reduced glutathione. The integrity and purity of the

proteins were verified by SDS-polyacrylamide gel electrophoresis.

Native gel electrophoresis. Self-association of GST-SP proteins was determined by

native gel electrophoresis using Mini-PROTEAN TGX 4–20% precast polyacrylamide protein

gels (Bio-Rad). Purified wild-type or mutant GST-SP (5 µg) were mixed with 2X native sample

buffer (Laemmli buffer without SDS) (74) and subjected to electrophoresis using Tris-glycine

running buffer at constant voltage (100V). Proteins were stained using Coomassie Blue. The

results were repeated at least three times.

Molecular modeling. Structural modeling of wild-type SP and mutants M57-58 and

M59-61 was performed using the open-source ColabFold pipeline, which combines the multiple

sequence alignment of MMseq2 with structure predictions by AlphaFold2 and RoseTTAFold

(45, 46). Individual models were selected based on the average predicted local distance

difference test (LDDT). The predicted structures were further visualized and analyzed using

ChimeraX (47). The transmembrane helix was predicted using MEMSAT-SVM (48) and the

coiled-coil was predicted using the CCHMM_PROF server using default settings (49).

Statistical analyses and reproducibility. Either Mann-Whitney or Student's t tests

were performed as indicated. All experiments were performed at least twice with similar

results.

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FIGURE LEGENDS

FIG 1. Biotinylation assay to detect MMTV SP biotinylation inside cells. (A) Diagram of GFP-

tagged Rem and SP proteins. The GFP-tagged Rem precursor is shown with an arrow indicating

the position of host signal peptidase cleavage between SP and the Rem C-terminus (CT). The

relative positions of the nucleolar/nuclear localization sequences (NoLS/NLS), arginine-rich motif

(ARM), and nuclear export sequence (NES) within SP are indicated. A BAP tag was inserted

after amino acid 96 at the C-terminus of GFP-tagged SP for expression of GFP-SP-BAP.

Alternatively, BAP was inserted at the N-terminus of SP to allow expression of GFP-BAP-SP. (B)

Rationale for SP retrotranslocation assays. Expression vectors for GFP-SP-BAP and a cytosolic

biotin-ligase (BirA) (orange Pac-Man) were co-transfected into HEK 293 cells. Following

retrotranslocation, BirA covalently adds biotin (red circles) to the single acceptor lysine within the

BAP tag of GFP-SP-BAP. The purple transmembrane shapes represent retrotranslocons. Co-

transfection of expression vectors for GFP-BAP-SP and BirA do not require retrotranslocation for

detection of biotinylated SP. Biotinylation is detected by an increased mass after incubation with

streptavidin. (C) Luciferase assays confirm the activity of BAP-tagged SP proteins. Each bar

represents the mean of triplicate transfections ± standard deviations in HEK 293 cells. The *Renilla*

luciferase values are normalized to values for co-transfected firefly luciferase vector lacking a

Rem-responsive element and in the absence of SP expression (assigned a relative value of 1).

The amounts of expression plasmids used for transfection are shown in nanograms (ng). NS =

not significant. * = p-value <0.05. (D) Western blots confirm SP retrotranslocation. Addition of

streptavidin to extracts increases the molecular mass of GFP-SP-BAP by ~50 kDa. Extracts from

HEK 293 cells transfected with pcDNA3 (empty vector) (lanes 1 and 2) or a GFP-SP expression

plasmid lacking a BAP tag (lanes 3 and 4) and BirA expression vector were used as negative

controls. The upper and lower blots were incubated with antibodies specific for GFP and actin,

respectively. The biotinylated and non-biotinylated forms of N-terminally and C-terminally BAP-

tagged proteins are indicated by arrows. Non-biotinylated GFP-SP is detectable since biotin was

added for the last 12 h of a 48 h transfection period. (E) Biotinylation of GFP-SP-BAP occurs

within co-transfected cells and not after cell lysis. Lysates from co-transfections of expression

plasmids for GFP-SP-BAP and BirA (lanes 1 and 2) or separate transfections (lanes 3 and 4)

were prepared. Lysates from separate transfections were combined post-lysis prior to

streptavidin addition as indicated. Western blots were incubated with antibodies for GFP or

GAPDH. (F) ER-localized BirA biotinylates C-terminally, but not N-terminally BAP-tagged SP.

HEK 293T cells were co-transfected with expression vectors for GFP-BAP-SP or GFP-SP-BAP

and either BirA (cytosolic) or ER-BirA (ER localized) as indicated. Streptavidin was added to

lysates in even lanes, and samples were analyzed by Western blotting using GFP- and GAPDH-

specific antibodies and LI-COR detection.

FIG 2. VCP/p97 is essential for SP retrotranslocation. (A) The VCP/p97 inhibitor CB-5083

reduces SP retrotranslocation in HEK 293T/17 cells. Cells were co-transfected with plasmids

expressing GFP-SP-BAP and BirA. After 24 h, cells were treated with 1 µM CB-5083 for 8 h prior

to addition of 100 µM biotin for 1 h. Cell lysates were incubated in the presence and absence of

streptavidin prior to Western blotting and incubation with GFP or GAPDH-specific antibodies. The

amount of biotinylated GFP-SP-BAP was quantitated relative to the total GFP-SP-BAP signal and

given as % retardation. (B) CB-5083 blocks SP retrotranslocation in HC11 mouse mammary

cells. The inhibitor was added at 24 h post-transfection for 9 h at the indicated concentrations.

Biotin was added in the presence of CB-5083 for 1 h prior to removal of cells for lysate preparation.

Streptavidin was added to one portion of each lysate and subjected to Western blotting. Blots

were incubated with antibodies specific for GFP or GAPDH as indicated.   The amount of

biotinylated GFP-SP was quantitated relative to the total SP signal (% retardation).

FIG 3. Trypsin sensitivity assay confirms that retrotranslocated SP is susceptible to

enzymatic digestion. (A) BiP levels provide a measure of intact microsomes. Microsomes

isolated from HEK 293T cells co-transfected with vectors for GFP-SP-BAP and BirA were treated

with increasing trypsin concentrations. Western blots of microsome preparations were analyzed

with BiP-specific antibody. (B) Biotinylated SP is trypsin sensitive. Microsomes were treated with

the indicated concentrations of trypsin prior to addition of streptavidin and Western blotting with

GFP-specific antibody. The majority of SP is localized in the nucleus (1) and is protected from

trypsin digestion.   (C) Quantitation of retrotranslocated GFP-SP-BAP in the presence of

increasing trypsin concentrations. The level of biotinylated GFP-SP was quantitated relative to

the total GFP signal and shown as percentage of retardation in the presence of streptavidin.

FIG 4. SP interaction with VCP/p97 is ATP-dependent. Cells (HEK 293) were transfected with

pcDNA3 and an expression plasmid for GFP-SP in the presence or absence of an exogenous

VCP/p97 expression vector. Co-mmunoprecipitation of cell lysates with p97-specific antibodies

was performed with or without exogenous ATP and its cofactor Mg^{2+} as indicated. Western

blotting with GFP-specific antibody is shown for co-immunoprecipitates (lanes 1-6) or cell lysates

(lanes 7-12). M = molecular mass markers. The positions of immunoglobulin heavy chain (Ig HC),

p97, and GFP-SP are indicated with arrows.

FIG 5. The N-terminal 50 amino acids of SP, including the NLS, are not required for

retrotranslocation. (A) Diagram of N-terminal SP deletions. Each of the mutants is tagged at

the N-terminus and C-terminus with GFP and BAP, respectively. Sequential increments of 10

amino acids were deleted from the N-terminus of SP (shown as d10-d60). The positions of the

NLSs, ARM, and NES are indicated. Two amino acids of the SP C-terminus were deleted to

prevent cleavage of the BAP tag by signal peptidase. (B) Retrotranslocation assays with SP

deletion mutants. Cells (HEK 293) were co-transfected with expression vectors for BirA and GFP-

SP-BAP or deletion mutants. After addition of biotin, lysates were used for streptavidin addition

and Western blotting with GFP and GAPDH-specific antibodies. The position of biotinylated and

non-biotinylated GFP-SP proteins are shown with arrows. (C) Reporter assays for GFP-SP-BAP

deletion mutants. Cells were co-transfected in triplicate with the reporter vector pHM*Rluc* and

expression vectors for wild-type GFP-SP-BAP or deletion mutants. Results were reported as

described in Fig. 1C. NS = not significant. * = p<0.05.

FIG 6. Amino acids 57-58 of SP are critical for retrotranslocation. (A) Diagram and

nomenclature for sequential alanine substitution mutations in GFP-SP-BAP starting from SP

amino acid 50. (B) Retrotranslocation assays with alanine substitution mutants. HEK 293T cells

were co-transfected with expression vectors for BirA and wild-type or mutant GFP-SP-BAP. Biotin

was added for 12 h prior to extract preparation and addition of streptavidin in alternate lanes.

Western blots were incubated with GFP and GAPDH-specific antibodies. Arrows indicate the

positions of biotinylated and non-biotinylated GFP-SP. (C) Retrotranslocation assay with

increasing levels of mutant M57-58 compared to wild-type SP. Transfections were performed as

in panel B. The amounts of the wild-type and mutant expression vector used for transfection are

provided in micrograms. The % retardation is calculated by dividing the signal of biotinylated

GFP-SP compared to the total GFP signal. (D) Quantitation of multiple retrotranslocation assays

comparing wild-type GFP-SP-BAP and M57-58. Transfections of HEK 293T cells were performed

with 300 and 600 ng of wild-type and mutant SP expression vectors, respectively, to ensure equal

SP levels together with the BirA expression plasmid. Biotin was added from 2 to 12 h prior to

lysate preparation in different experiments. The means and standard deviations of nine

quantitated assays showed that M57-58 has a significantly decreased retrotranslocation

efficiency compared to the wild-type. * = p<0.05.

FIG 7. Amino acids needed for p97 interaction are a subset of sequences required for

optimal SP function. (A) Mutants M57-58 and M59-61 are defective for SP function. HEK 293T

cells were co-transfected in triplicate with expression vectors for GFP-SP-BAP (WT) alanine

scanning mutants (2.5 ng each), the SP reporter vector pHM*Rluc* (250 ng), and pGL3-control (250

ng). Luciferase values were normalized for pGL3-control expression in each transfection and

expressed relative to transfections with pcDNA3 lacking SP expression (assigned a relative value

of 1). Two-tailed Student's t tests were performed (* p<0.05). NS = not significant. (B) Titration

of M57-58 and M59-61 functional activity compared to WT GFP-SP-BAP. Expression vectors

(DNA levels given in nanograms) were co-transfected with pHM*Rluc* reporter and pGL3-control

as described in panel A. (C) Co-immunoprecipitation of GFP-SP and mutants with VCP/p97.

HEK 293T cells were transfected with expression vectors for GFP, GFP-SP (WT), or mutants.

Extracts were immunoprecipitated with antibody specific for p97. Immunoprecipitates (left panel)

or cell lysates (right panel) were analyzed by SDS-polyacrylamide gel electrophoresis before

Western blotting with antibodies specific for p97 and GFP. The positions of p97, GFP-SP, GFP,

and immunoglobulin heavy and light chains are indicated by arrows.

FIG 8. SP mutants 57-58 and 59-61 show increased ER co-localization and reduced

retrotranslocation relative to wild-type SP. (A) Colocalization of wild-type or mutant SP with

an ER marker in individual cells. HC11 mammary cells were co-transfected with expression

vectors for GFP-SP (WT) or mutants (green) together with ER-mCherry (red). Cells were fixed,

and the nuclei were stained with DAPI (blue). A merged image is provided on the right. Yellow

areas in the merged images indicate red and green overlapping pixels determined by the

colocalization analysis using ZEN software. Cells expressing GFP-SP (WT) lack colocalization

between the red and green signals, so no yellow areas were observed. (B) Quantitation of the

GFP-SP signal colocalization with ER-mCherry. Each dot represents the Mander's colocalization

coefficient (MCC) of GFP-SP with the ER signal for an individual cell as suggested by Manders

under conditions in which signal intensities are unequal (73). The median signal was calculated

based on the numbers of cells analyzed (n). Mann-Whitney t tests were used to compare wild-

type and mutant MCCs. ** = $p < 0.01$; NS = non-significant. (C) Retrotranslocation assays with

pulses of biotin indicate defects in the ER membrane extraction of both M57-58 and M59-61. HEK

293T cells were co-transfected with 300 ng of expression vectors for wild-type GFP-SP-BAP or

M59-61 or 600 ng of the M57-58 expression vector together with a BirA expression plasmid. Cells

were pulsed for either 1 h or 3 h prior to lysate preparation and Western blotting.

Retrotranslocation was quantified as described in the Fig. 2 legend. The results of triplicate

assays were expressed as the means and standard deviations for the 1 h and 3 h biotin pulses.

Student's t tests were performed between the wild-type and mutants. The asterisk shows a p-

value of <0.05, whereas NS indicates a non-significant value.

FIG 9. Structural models of SP mutants predict conformational changes related to wild-

type SP and reduced multimerization in native gels. A) Molecular modeling of SP using

AlphaFold2. The two α-helices (α1 and α2) and the transmembrane helix are indicated; α1 and

α2 are separated by a loop. The coiled-coil motifs in SP as predicted by the CCHMM_PROF

coiled-coil server are shown in aqua. The p97-interacting residues W57 and Q58 are depicted in

red. The model was analyzed and represented using ChimeraX. (B) Predicted α-helices in wild-

type relative to mutant SP. Wild-type (WT), M57-58 and M59-61 have been represented by gray,

blue, and red, respectively. M57-58 has an extended α1-helix (left image), whereas M59-61 has

a shortened α2-helix (right image). (C) Amino acid sequence of WT and mutants comparing the

α- helical regions and coiled-coil motifs. The sequence of α1 and α2 helices are highlighted in

yellow, whereas those forming the coiled-coil motifs are depicted in aqua. M57-58 is predicted to

have an increased length of both coiled-coils. The second coiled-coil formed by the α2-helix is

predicted to be shorter for M59-61. (D) Integrity of purified GST-tagged versions of wild-type and

SP mutants. The purity and integrity of purified proteins were confirmed by analysis on a

denaturing SDS-containing polyacrylamide gel. Cell lysates have been shown on the left and

compared to purified proteins on the right. The position of GST-SP is shown with an arrow. (E)

Gradient gel electrophoresis of purified WT GST-SP or M57-58 and M59-61 mutants under native

conditions. Purified proteins (5 µg) were analyzed on a 4-20% Tris-buffered gradient gel lacking

SDS. The relative positions of multimers and monomers as well as the unfused GST protein are

shown with arrows.

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Das et al. Fig. 1

Das et al. Fig. 2

A ←BiP

B

kDa
95 -
72 -   Biotinyl.
            GFP-SP-BAP
55 -

43 -   ←GFP-SP-BAP

	1	2	3	4	5	6
Trypsin (µg/µl)	-	-	0.01	0.01	0.025	0.025
Streptavidin	-	+	-	+	-	+

C

% Retardation

Trypsin (µg/µl)   -   0.01   0.025

Das et al. Fig. 3

Das et al. Fig. 4

Das et al. Fig. 5

A

```
                 50              61
GFP-SP-WT   P I K E K T A W Q H L Q ─[BAP]
M50-52      A A A . . . . . . . . . ─[BAP]
M53-55      . . . A A A . . . . . . ─[BAP]
M57-58      . . . . . . . A A . . . ─[BAP]
M59-61      . . . . . . . . . A A A ─[BAP]
```

B

C

Das et al. Fig. 6

Das et al. Fig. 7

Das et al. Fig. 8

A

α2-helix

transmembrane-helix

α1-helix

N

loop

C

B

N

N

WT

C

C

M57-58

N

N

M59-61

C

C

WT

C

WT
MPNHQSGSPTGSSDLLLSGKKQRPHLALRRKRRREMRKINRKVRRMNLA
PIKEKTAWQHLQALIFEAEEVPKTSQTPQTSLTLFLALLSVLGPPPVTG

M57-58
MPNHQSGSPTGSSDLLLSGKKQRPHLALRRKRRREMRKINRKVRRMNLA
PIKEKTAAAHLQALIFEAEEVPKTSQTPQTSLTLFLALLSVLGPPPVTG

M59-61
MPNHQSGSPTGSSDLLLSGKKQRPHLALRRKRRREMRKINRKVRRMNLA
PIKEKTAWQAAAALIFEAEEVPKTSQTPQTSLTLFLALLSVLGPPPVTG

D

Cell lysate   Purified protein

kDa
-55
-43
GST-SP→   -37
-26
-17

1 2 3   4 5 6

WT M57-58 M59-61 WT M57-58 M59-61

E

GST-SP/mutant
multimers

GST-SP/mutant
monomer

GST

1 2 3 4

WT M57-58 M59-61 GST

Das et al. Fig. 9