Carboxy-Terminal Processing of the Urokinase Receptor: Implications for Substrate Recognition and Glycosylphosphatidylinositol Anchor Addition†

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ABSTRACT: Proteins linked to cell membranes by a glycosylphosphatidylinositol (GPI) anchor must first undergo cleavage by a putative transamidase between the ω and $\omega + 1$ positions within a proposed small amino acid (SAD) domain in the carboxy terminus of the nascent polypeptide. The requirements for such processing, defined in an engineered placental alkaline phosphatase construct (miniPLAP), suggest the SAD domain functions as an autonomous unit within the context of an otherwise permissive carboxy-terminal sequence with only certain amino acids tolerated at the ω, $\omega + 1$, and $\omega + 2$ positions. To test whether this hypothesis could be generalized, we engineered a chimeric molecule containing the extracellular domain of miniPLAP and the carboxy-terminal portion of the urokinase receptor (MP/uPAR) into which various amino acid substitutions were introduced. The variant proteins were translated and metabolically labeled in vitro using a cell-free translation system that contains the enzymatic machinery required for carboxy-terminal processing and GPI anchor addition. The results of this study indicate that the SAD domain functions as an independent, but not an autonomous, unit. The requirements for processing in miniPLAP and MP/uPAR differed markedly in some respects, in part due to the influence of the amino acid at the $\omega + 4$ position which both modified cleavage between the ω and $\omega + 1$ positions and permitted a second cleavage site to be generated in some cases. In addition, substitution of bulky hydrophobic amino acids in series at the $\omega + 2$ and $\omega + 3$ positions inhibited carboxy-terminal processing in a dose-dependent manner, suggesting the presence of a critical docking site adjacent to the cleavage site. These results suggest the carboxy-terminal transamidase recognizes a more extended structure similar to the mechanism proposed for serine proteases. Further, the data provide a potential means for isolating the transamidase.

Over the past two decades, a class of proteins has been identified that are linked to the plasma membranes of eukaryotic cells through a glycosylphosphatidylinositol (GPI)[1] moiety attached to the C-terminal amino acid rather than through a transmembrane anchor (see refs *1* and *2* for review). More than 100 such proteins have been identified to date. Diverse functions have been ascribed to the GPI anchor, including protein retention and degradation intracellularly (*3*, *4*), vectorial transport of proteins to the apical membrane of polarized cells (*5*, *6*), increased mobility of the proteins on the cell surface (*7*, *8*), endocytosis in caveolae (*9*), exchange of proteins between plasma and cells (*10*, *11*), changes in affinity for their cognate ligands (*12*, *13*), and initiation of intracellular signaling (*14*−*18*), among others. The inability to express GPI-anchored proteins on hematopoetic cells results in the condition paroxysmal nocturnal hemoglobinuria (PNH) which is manifest by complement-mediated hemolytic anemia, marrow aplasia, and thrombosis (*19*). Patients with PNH are unable to complete the synthesis of the GPI anchor as a result of a defect in the enzyme responsible for the synthesis of *N*-acetylglucosaminylphosphatidylinositol (*20*−*22*). The synthesis and composition of the GPI anchor have been the subject of extensive study and several excellent reviews (*23*−*25*).

Considerable uncertainty remains with respect to the mechanism by which the GPI anchor is added to specific proteins. An early step in the process involves the removal of 17−31 C-terminal amino acids from the designated proteins within the lumen of the rough endoplasmic reticulum (*26*−*28*). All of the determinants required for this cleavage reside within the C terminus of the nascent proteins (*29*); substitution of these sequences for those encoding the transmembrane portion of other proteins results in their being expressed on cell surfaces with a GPI anchor (*30*, *31*). Despite pronounced differences in their linear amino acid sequences (*1*, *32*), several important clues concerning the requirement for cleavage or attachment have been identified. All proteins which become GPI-anchored share certain

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[1] Abbreviations: GPI, glycosylphosphatidylinositol; SAD, small amino acid domain; miniPLAP, recombinant truncated construct of human placental alkaline phosphatase; uPAR, human urokinase receptor; MP/uPAR, recombinant construct containing the amino terminus of miniPLAP and the carboxy terminus of uPAR; PNH, paroxysmal nocturnal hemoglobinuria; RM, rough microsomal membranes; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; RIPA, radioimmunoprecipitation assay buffer.

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features, including a sequence of three to five small amino acids, the so-called small amino acid domain (SAD) (*29*, *33*−*35*) within which the cleavage occurs. The SAD domain invariably precedes a stretch of 8−12 hydrophilic residues which are often rich in charged amino acids and proline followed by a C-terminal tail comprised of 8−20 hydrophobic amino acids (*31*, *33*, *34*). Deletion of this tail prevents GPI anchoring, presumably because anchor addition can only occur when the protein has been inserted correctly into the lumenal membrane of the rough endoplasmic reticulum. However, the extent of flexibility tolerated within this region and the mechanism by which it contributes to processing have been the subject of conflicting reports (*31*, *33*, *36*).

Recent studies suggest that each of the first three amino acids in the SAD domain makes a specific contribution that is required for cleavage to proceed efficiently (*29*, *32*, *34*, *35*). It has been proposed that the enzyme responsible for C-terminal processing is a transamidase (*37*, *38*) that mediates a nucleophilic attack on any of six potential small amino acids that are found in what has been designated the omega (ω) position of susceptible proteins. This step involves the donation of a proton from the enzyme, leading to the formation of a reactive intermediate that contains a highly active carbonyl group (*39*). This activated intermediate serves to accept the nucleophilic amino group of the ethanolamine residue of the fully formed GPI (*40*, *41*). The first step in this process cannot occur if a proline is present in the adjacent $\omega + 1$ position, presumably because the cleavage site is distorted (*32*, *34*, *42*). Strict limitations in the amino acids tolerated at the $\omega + 2$ position have been identified as well, although their exact contribution to the biochemical process has not been as well defined (*32*, *35*, *42*).

Most of these conclusions have been derived from the analysis of a single protein, placental alkaline phosphatase (PLAP), and there are reasons to suspect that additional factors are involved in substrate recognition. First, the proposed rules do not identify a single site of cleavage in some GPI-anchored proteins (*43*). Second, there are notable differences in the amino acid sequence within and C-terminal to the SAD domains among GPI-anchored proteins (*32*). Third, there is heterogeneity in the level of expression of various GPI-anchored proteins on individual cells from patients with PNH and on cell lines with similar defects in GPI synthesis, suggesting a hierarchy of substrates exists that becomes evident when the enzyme level is limiting (*44*−*46*). Fourth, mutations in single amino acids at sites in some cases quite distant from the SAD domain can have profound and thus far unexplained effects on C-terminal processing and GPI anchor addition (*35*, *36*, *47*, *48*).

For these reasons, we analyzed the amino acid composition of the C-terminal portion of PLAP and two other GPI-anchored proteins, decay accelerating factor and the urokinase receptor (uPAR), which differ considerably in primary amino acid sequence, for potential similarities in secondary structure using homology searches in a crystallographic database (*49*). As a result of this analysis, we proposed a model in which the conformation of the SAD domain, and its susceptibility to cleavage, is dependent, in part, on interactions with the spacer sequence and specific amino acids in the hydrophobic tail. In this study, we begin to test this model by asking whether the three amino acids that comprise the classic SAD domain function as a totally autonomous entity or whether the cleavage site is influenced by regional geometry.

EXPERIMENTAL PROCEDURES

Construction of MiniPLAP/uPAR. The cDNA encoding miniPLAP in pGEM-4Z (Promega Biotec, Madison, WI) (*50*) was provided by S. Udenfriend (Nutley, NJ). The cDNA encoding the full-length urokinase receptor (uPAR), amino acids 1−313, was generously provided by F. Blasi (Milan, Italy). uPAR cDNA was ligated into the multicloning site of pGEM-3Z using *Hin*dIII at the 5′ end and *Eco*RI at the 3′ end of the insert. The plasmid, designated uPAR, lacks the 5′ untranslated region and begins one nucleotide N-terminal to the initiator methionine. The 3′ end extends 30 nucleotides C-terminal to the termination codon. uPAR and miniPLAP were then used as templates for the first step in a two-step PCR. Both clones were in similar orientations relative to their SP6 and T7 promoters. In step 1, SP6 and the internal primer CAGCCCCACTGGTGGTGCCGG were used with miniPLAP cDNA as the template for the first reaction. Simultaneously, T7 and the internal primer CGGCACCACCAGTGGGGCTGC were used with uPAR cDNA as the template. These and all oligonucleotides used were synthesized on an ABI 394 four-column synthesizer (University of Pennsylvania DNA sequencing facility).

In step 2, the purified products from each reaction were used as overlapping templates together with primers SP6 and T7 to generate a 763 bp fragment designated miniPLAP/uPAR (MP/uPAR). MP/uPAR contains the first 637 bp of miniPLAP (encoding residues −17 to +178 of the mature protein) followed by the last 96 bp of uPAR cDNA (encoding amino acids 282−313 of the mature protein) and 29 bp from its 3′ untranslated region and was oriented in the SP6 direction to generate sense mRNA. The fragment was digested with *Eco*RI, purified, and ligated into pGEM 4z. All PCRs were performed using the GeneAMP PCR 2400 system (Perkin-Elmer). The standard manufacture's parameters for all cycling reactions included initial denaturing at 94 °C for 10 min (one time), cycling at 94, 60, and 72 °C for 30 s at each temperature (25 times), and final extension at 72 °C for 7 min (one time). All PCR products were column purified using the QIAquick PCR Purification Kit (Qiagen, Chatsworth, CA). The complete nucleotide sequence and orientation within the vector of MP/uPAR and the variants listed below were confirmed using automated fluorescence-based dideoxy sequence analysis (Perkin-Elmer).

Construction of Variant Mini-PLAP/uPAR and Variant mini-PLAP. All changes within the C-terminal region of MP/uPAR were introduced by cassette mutagenesis. Silent restriction sites *Spe*I and *Afl*II were created at positions 638 and 670, respectively, using a two-step PCR. The internal primers GGCACCACTAGTGGGGCT and AGCCCCACTAGTGGTGCC were used to introduce the *Spe*I position, and GAGGCTTAAGTGGGCAGGGCCAGG and CCTGGCCCTGCCCACTTAAGCCTC were used to introduce the *Afl*II position. Both sites were introduced with external primers SP6 and T7. The final construct is diagrammed in Figure 1A.

To introduce changes in the peptide sequence of MP/uPAR, the chimera was digested with *Spe*I and *Afl*II, dephosphorylated, and isolated using low-melting point

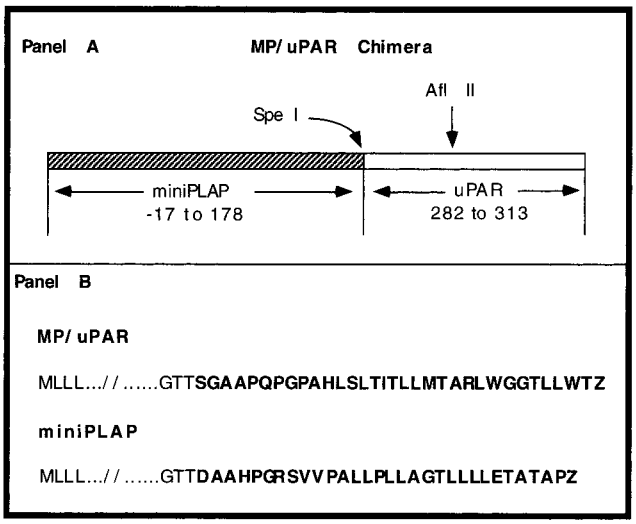

FIGURE 1: Amino acid sequence of MP/uPAR and miniPLAP. (A) MP/uPAR contains the first 195 amino acids of miniPLAP (−17 to 178) and amino acids 282−313 of uPAR. The silent restriction enzyme sites *Spe*I and *Afl*II were introduced on either side of the flanking region. (B) The peptide sequence of the carboxy terminus of both MP/uPAR and miniPLAP is shown. The amino acid sequences of the two constructs are identical from amino acid −17 to 178, after which the wild type uPAR sequence has been substituted for the wild type sequence in miniPLAP (179 to the end).

agarose electrophoresis. Oligonucleotides corresponding to the desired peptide sequence were synthesized with cohesive *Spe*I and *Afl*II ends. Ligation into the cut plasmid was carried out according to standard procedures (Current Protocols in Molecular Biology, Secaucus, NJ). Introduction of the mutant sequence was confirmed by cleavage of a unique *Sma*I site located within the oligonucleotide and dideoxy sequence analysis. A similar cassette strategy was followed to introduce mutations into miniPLAP at unique sites such as *Nae*I at position 573 and *Rsr*II at position 603.

Preparation of Rough Microsomal Membranes from CHO Cells. Rough microsomal membranes (RM) were prepared as described previously (*50*). Briefly, Chinese hamster ovary (CHO) cells or LMTK$^-$ fibroblasts (American Type Culture Collection, Rockville, MD) were grown in Iscove's modified Dulbecco's medium (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Labs, Logan, UT), penicillin/streptomycin, L-glutamine, gentamicin (each from Life Technologies), and hypoxanthine/thymidine supplement. When the cells reached 80% confluency, they were harvested, washed with ice-cold phosphate-buffered saline (PBS), and scraped into PBS with a rubber policeman. Harvested cells were pelleted at 2000*g* for 3 min at 4 °C and washed twice with PBS to remove any remaining culture medium. The cells were resuspended to ∼7.5 times the total wet volume of the cells in 10 mM triethanolamine (Sigma, St. Louis, MO) (pH 7.5) and incubated on ice for 15 min. An equal volume of 600 mM sucrose/6 mM dithiothreitol was added to the suspension, mixed, and homogenized at 4 °C using 10 strokes in a Dounce homogenizer. The homogenates were transferred to ultracentrifuge tubes for sequential differential centrifugation at 4 °C at 7700*g* for 10 min, then at 7700*g* for 20 min, and finally at 17300*g* for 10 min. After each centrifugation, the pellet was discarded and the supernatant transferred. RM were obtained from the final supernatant by centrifugation at 100000*g* for 90 min. The supernatant was removed, and the membrane pellet was resuspended in buffer B [250 mM sucrose, 60 mM triethanolamine, and 2.5 mM dithiothreitol (pH 7.5)]. Membranes were quantified on the basis of their optical density (A_{280}) and were stored at 200 OD/mL at −80 °C until they were used.

Transcription and Translational Processing. Plasmids were linearized using *Hin*dIII (New England Biolabs, Beverly, MA) and column purified (QIAquick Purification Kit). mRNA was reverse transcribed using SP6 RNA polymerase to generate sense mRNA using the Riboprobe-II Kit (Promega) as described previously (*50*). mRNA was purified for in vitro translation using the RNaid Kit (Bio101, La Jolla, CA). The purified mRNA was quantified (A_{260}) and its integrity confirmed on a 1.5% agarose gel.

Cell-free translations from miniPLAP/uPAR mRNA were performed exactly as described previously (*50*−*52*). Briefly, a 25 μL translation mixture was prepared which contained 50% nuclease-treated rabbit reticulocyte lysate (Promega Biotec), 76.4 μM potassium acetate, 4.0 mM magnesium acetate, 1.8 mM dithiothreitol, 40.0 mM sucrose, 8.0 mM triethanolamine (pH 7.5), a 0.03 mM amino acid mixture without cysteine, 20 units of RNAse inhibitor (40 units/μL), 1.75 μL of [^{35}S]cysteine (15 mCi/mL, 1100 Ci/mmol; Amersham, Arlington Heights, IL), 200 ng of mRNA, and a cocktail of six protease inhibitors, each at 2.0 μg/mL (aprotinin, antipain, leupeptin, chymostatin, bestatin, and pepstatin). C-terminal processing was initiated by adding 8.0 *A* units (A_{260}) of membranes from a stock preparation suspended in buffer B. All reactions were performed at 30 °C for 90 min unless otherwise stated, by which time translation and C-terminal processing had reached completion for all wild type and variant cDNAs tested (see below). Control reaction mixtures devoid of RM received an equal volume of buffer B alone. Reactions were terminated by adding 25 μL of 8% SDS/10% β-mercaptoethanol, after which the samples were boiled for 5 min. Translations from miniPLAP were performed in an identical manner using an amino acid mixture lacking methionine supplemented with [^{35}S]methionine.

Immunoprecipitation. Immunoprecipitation of the metabolically labeled proteins was then performed using site specific antibodies to miniPLAP and miniPLAP/uPAR. The antibodies used to recognized nascent and C-terminally processed miniPLAP (provided by S. Udenfriend) have been described previously (*50*). Affinity-purified IgG anti-PLAP (Accurate Chemical and Scientific Corp., Westbury, NY) recognizes nascent miniPLAP as well as miniPLAP that has undergone both N-terminal and C-terminal processing. A second anti-PLAP antibody, generated against a peptide encompassing amino acids 200−208 which lies within the carboxy terminus of miniPLAP, recognizes nascent and N-terminally processed miniPLAP, but the amino acid sequence recognized by this antibody is removed during C-terminal processing. A rabbit anti-uPAR antibody (designated Ab 9029) was generated to a peptide which corresponds to residues 301−313 of the mature protein. This antibody recognizes nascent (pre-pro) and N-terminally processed uPAR but does not recognize either C-terminally processed uPAR from which this amino acid sequence has been deleted or full-length soluble uPAR (amino acids −12

to 281). A second rabbit antibody (designated 9677) was raised against a peptide which corresponds to amino acids 25−44 in the ectodomain of uPAR which immunoprecipitates uPAR but not MP/uPAR (see below).

To perform the immunoprecipitation, each reaction mix containing denatured, labeled protein was diluted 1:1 with *d*-H_2O. Ten microliters of the diluted reaction mix and 1 μL of antibody were added to 500 μL of Radio-Immunoprecipitation Assay Buffer (RIPA), containing 50.0 mM Tris-HCl (pH 7.5), 1% Triton X-100, 0.5% sodium deoxycholate, 0.2% SDS, 100 mM sodium chloride, and 1.0 mM EDTA. Preliminary titration experiments were performed to determine the amount of antibody required to completely precipitate MP or uPAR from the supernatant (i.e., no bands were evident upon repeat precipitation) without precipitating detectable amounts of the other (i.e., nonspecific) protein. The mixture was incubated overnight at 4 °C. Thirty microliters of Protein A-Sepharose CL-4B (Pharmacia Biotech, Uppsala, Sweden) prewashed in RIPA was added to each sample and the solution mixed and incubated at room temperature for 60 min with constant, gentle rotation. The sample was centrifuged at 14000*g* for 5 min using a CS-15R Beckman Centrifuge. The pellet was washed with 750 μL of RIPA (five times), extracted with 20 μL of 2× SDS−PAGE sample buffer, and boiled for 5 min. The pellet was centrifuged, and supernatants containing the ^{35}S translation products were analyzed using SDS−PAGE (*53*). A 15% separating gel was used to obtain satisfactory resolution among proteins with M_rs of 6−30 kDa. Separating gels were 0.75 mm thick and 16.0 cm long with a 2.0 cm stacking gel. Electrophoresis was completed at 4 °C under a constant power of 15 W for 3−4 h. Gels were fixed and visualized by autoradiography. The immunoprecipitates were quantified by densitinometric scanning of the autoradiographs. Each band was scanned using a Hoefer GS 300 Scanning Densitometer (Hoefer Scientific Instruments, San Francisco, CA), and the results are expressed in arbitrary units as a percentage of the total specific translation product (pre-pro plus N-terminally processed plus N- and C-terminally processed) as well as the total amount of labeled protein in the precipitate. Because the translation efficiency and N-terminal processing efficiency of all constructs were essentially identical, the same inferences could be drawn from data expressed in either manner (see Table 1).

Table 1: Efficiency of C-Terminal Processing of MiniPLAP and MP/uPAR[a]

SAD domain	miniPLAP	MP/uPAR
DAA[b]	32 (15)	37 (21)
SGA[c]	36 (18)	40 (24)
DPA	0 (0)	0 (0)
SDAA	—[d]	40 (26)
SDPA	−	0 (0)
SPAA	22 (13)	43 (34)
SPAH	16 (8)	27 (19)
PGAA	−	9 (5)
SGPA	−	22 (19)
SGAP	−	38 (18)
PPAA	−	0 (0)
SPAP	−	43 (24)
SPVA	−	47 (26)
SGVA	−	30 (21)
SVVA	−	0 (0)
SLLA	−	0 (0)
SVAA	−	38 (23)
SIIA	−	0 (0)

[a] Metabolically labeled miniPLAP and MP/uPAR wild type and variant cDNAs were reverse transcribed and translated in vitro as described. The amount of each preprocessed precursor protein, amino-terminally processed protein, and protein that had undergone both amino- and carboxy-terminal processing was determined after immunoprecipitation using laser scanning densitometry. The data are expressed as the amount of fully processed protein expressed relative to the total amount of miniPLAP or MP/uPAR in the lysate. The data within parentheses represent the amount of fully processed miniPLAP or MP/uPAR relative to the total amount of translation product to ensure equal translational efficiency. The mean of three experiments is shown. [b] Wild type miniPLAP sequence. [c] Wild type MP/uPAR sequence. [d] Variant not studied.

RESULTS

The primary goal of this study was to compare the requirements for C-terminal processing of uPAR with those previously described for miniPLAP. To accomplish this, we took advantage of data which indicate that the information required for C-terminal processing is primarily contained within the deleted segment plus the single amino acid immediately N-terminal to the cleavage site (*29*, *30*), but that amino acids at the $\omega + 4$ and $\omega + 5$ positions may influence the efficiency of GPI attachment (*35*). Thus, to establish whether the determinants for the processing of miniPLAP and uPAR are identical, we first prepared a chimera designated MP/uPAR which contains the entire N terminus of miniPLAP (amino acids −17 to 178 of the mature protein) followed by the complete C terminus of uPAR (amino acids 282−313) (Figure 1). Study of this construct eliminated the possibility that any differences in the requirements for C-terminal processing of uPAR and miniPLAP could be the result of differences in the external domains of the two proteins.

MP/uPAR Undergoes C-Terminal Processing and GPI Anchor Addition. Experiments were first performed to determine whether MP/uPAR undergoes C-terminal processing and condensation with the GPI anchor in the manner described previously for miniPLAP. To address this question, miniPLAP/uPAR and miniPLAP cDNA were each transcribed in a cell-free system and the proteins were synthesized and metabolically labeled in the presence of rough endoplasmic membranes (RM) as the source of both the enzymes that mediate N- and C-terminal processing and the GPI moiety, using methods described previously (*50*−*52*). The labeled proteins were immunoprecipitated with antibodies that recognize distinct sequences within the two proteins, and the precipitates were analyzed using SDS−PAGE.

MP/uPAR mRNA was translated in the presence of RM, immunoprecipitated with a polyclonal antibody to the ectodoamins of PLAP separated by SDS−PAGE, and analyzed by autoradiography. Three bands were identified that migrated with M_rs of ∼29.8, 28.4, and 25.8 kDa which correspond to nascent, N-terminally processed, and fully processed forms of MP/uPAR, respectively (deduced molecular masses of ∼24.2, 22.4, and 20.5 kDa) (Figure 2). In the absence of RM, only the highest-molecular mass band was present. Three bands with virtually identical molecular masses were generated from miniPLAP in the presence of RM as expected (M_r = ∼29.8, 27.8, and 24.6 kDa) which correspond to nascent, N-terminally processed, and fully processed MP, respectively (deduced molecular masses of

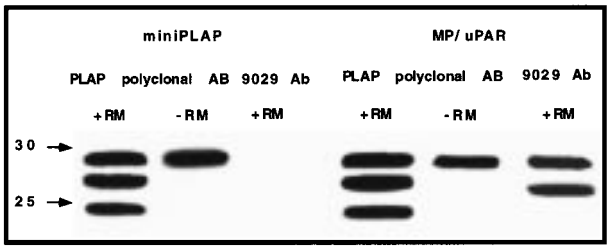

FIGURE 2: Immunoprecipitation of metabolically labeled miniPLAP and MP/uPAR. cDNA encoding miniPLAP and MP/uPAR was reverse transcribed, translated in the presence or absence of rough microsomal membranes (RM) from CHO cells, and metabolically labeled ^{35}[S]cysteine. The labeled proteins were immunoprecipitated using either a polyclonal antibody to miniPLAP or a polyclonal antibody (9029) which recognizes amino acids 301−313 of uPAR and analyzed by autoradiography, and the bands were quantified by densitometry. The same protocol was used in all subsequent experiments. In the presence of RM, the polyclonal antibody immunoprecipitates protein bands corresponding to the pre-pro (translated, unprocessed), pro- (N-terminally processed), and mature (N- and C-terminally processed) miniPLAP (top to bottom). In the absence of RM, only the pre-pro form is recognized. A polyclonal antibody specific to the carboxy-terminal region of uPAR (Ab 9029) recognizes pre-pro and pro MP/uPAR but does not recognize mature (carboxy-terminal processed) MP/uPAR or miniPLAP. The migration of molecular mass markers is shown on the left.

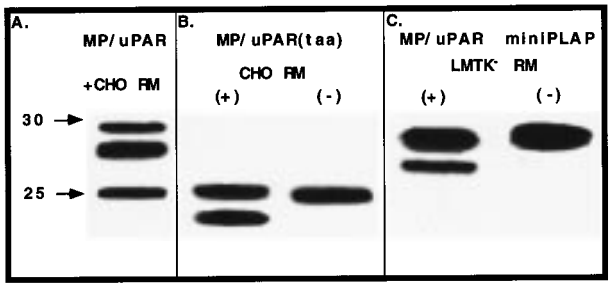

FIGURE 3: MP/uPAR undergoes carboxy-terminal cleavage and condensation with the GPI anchor. (A) C-Terminal processing of wild type MP/uPAR in the presence of CHO-RM. (B) A mutant MP/uPAR having a stop codon introduced at the cleavage site Ser$_{179}$ [MP/uPAR(taa)] was transcribed and translated in the presence and absence of CHO-RM. This mutant is translated and undergoes N-terminal processing normally but cannot undergo C-terminal cleavage or GPI anchor addition because the remainder of the SAD domain has been deleted. Note that the N-terminally processed MP/uPAR(taa) migrates more rapidly that the fastest migrating band of MP/uPAR. This difference is due to deletion of the 32 C-terminal amino acids without the addition of the GPI anchor which is added to MP/uPAR but not to the truncated MP/uPAR(taa). (C) Processing of MP/uPAR using RM from LMTK$^-$ cells. Only the upper two bands are seen because these cells are unable to mediate carboxy-terminal processing or GPI anchor addition. Molecular mass standards are shown on the left.

∼23.9, 22.0, and 20.6 kDa, respectively). This divergence between gel migration and predicted M_rs corresponds exactly to that reported previously by Udenfriend et al. (*50*) for MP. Again, only the highest-molecular mass band was seen in the absence of RM (Figure 2). These three bands from miniPLAP have been identified previously as representing nascent (or pre-pro using the nomenclature of Udenfriend et al.) miniPLAP, N-terminally processed miniPLAP (Pro-miniPLAP), and miniPLAP that has undergone N- and C-terminally processing and addition of the 1.7 kb GPI anchor (mature miniPLAP), respectively (*32*). Thus, the three bands generated from MP/uPAR showed essentially a migration pattern identical to those derived from miniPLAP as would be expected if the pattern of processing of the two proteins was identical. Further, an antibody specific for the carboxy-terminal region of uPAR (amino acids 301−313) immunoprecipitated the two highest-molecular mass bands, but not the band predicted to have undergone C-terminal processing with loss of this peptide sequence, whereas a polyclonal anti-uPAR antibody to the ectodomain of uPAR (amino acids 25−44) did not immunoprecipitate miniPLAP or MP/uPAR at all, as expected (not shown).

The fastest migrating (M_r ∼ 26 kDa) band in MP/uPAR was characterized further by comparing its migration to that of a soluble form of the chimera that contains a stop codon immediately 3′ to the nucleotide sequence corresponding to serine$_{179}$ which is the putative C-terminal cleavage site in MP/uPAR. Thus, the protein derived from this construct (MP/uPAR-Ser$_{179}$) cannot undergo C-terminal processing or anchor addition, and thereby identifies the migration of nascent and N-terminally processed MP/uPAR. Translation and immunoprecipitation of MP/uPAR-Ser$_{179}$ yielded two bands which migrated at ∼25 and ∼23 kDa (Figure 3), neither of which was recognized by the antibody to the uPAR sequence of amino acids 301−313. Thus, the fastest migrating band derived from MP/uPAR, which has been N- and C-terminally processed, migrates more slowly than does the fastest migrating band derived from MP/uPAR-Ser$_{179}$, as would be expected if the 32 C-terminal amino acids of uPAR were removed and the 1.7 kb GPI anchor was added (*52*).

A third, independent means was used to determine whether the fastest migrating band derived from MP/uPAR represented the C-terminally processed and GPI-anchored protein. We previously reported that this band was absent from the translation products of miniPLAP when RM from LMTK$^-$ fibroblasts, which lack the ability to synthesize a complete GPI moiety (*54*, *55*), were used (*52*); this outcome enabled us to conclude that GPI is an obligatory cosubstrate for C-terminal processing (*52*). MP/uPAR processed in the presence of LMTK$^-$ RM generated only the upper two bands, i.e., the pre-pro and N-terminally processed pro form (Figure 3). Taken together, these studies show by migration on SDS−PAGE, by immunoprecipitation using site specific antibodies, and by the pattern seen with mutant RM that MP/uPAR undergoes in vitro C-terminal processing and GPI anchor addition in a manner qualitatively similar to that reported previously for miniPLAP itself.

We then studied the time course of the appearance of the three bands derived from miniPLAP and MP/uPAR using CHO-RM as the source of enzyme and anchor. The nascent and N-terminally processed bands became evident by 15 min at 30 °C, reaching a peak within 30 min, whereas the C-terminally processed protein was first detected at 30 min (Figure 4, top). The amounts of C-terminally processed protein formed from miniPLAP and MP/uPAR were then measured at each time point relative to the total amount of protein synthesized (Figure 4, bottom). By 30 min, detectable amounts of fully processed miniPLAP and MP/uPAR were evident. The total amount of C-terminally processed MP and MP/uPAR reached a plateau by 90 min. However, by 60 min it was apparent that the chimeric MP/uPAR was more efficiently processed than miniPLAP ($n = 3$, $P < 0.05$). Additional studies were then performed to help understand this difference.

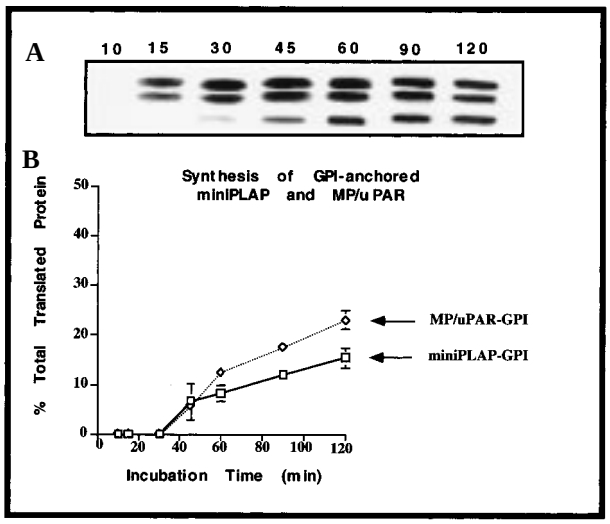

FIGURE 4: Time course of carboxy-terminal processing and GPI anchor addition to MP/uPAR. (A) The typical time course for the appearance of the three forms of MP/uPAR is shown. (B) Comparison of the appearance of the GPI-anchored form of miniPLAP and MP/uPAR. The data shown represent the mean ± SEM of three such experiments and represent the amount of GPI-anchored protein expressed as a percentage of the total amount of immunoprecipitated protein. The error bars at the 90 min point were too small to be seen graphically.

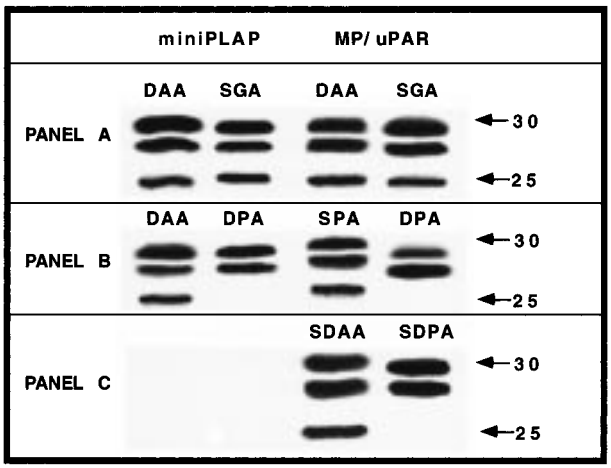

FIGURE 5: Effect of exchange of SAD domains of miniPLAP and MP/uPAR. (A) The SAD domains of uPAR (SGA) and miniPLAP (DAA) were exchanged, and the processing of both wild type and variant proteins was compared. In these and all subsequent legends, the letters denote the amino acids in the SAD domain (amino acids 179 and 180 or 181 of the construct). (B) The effect of introducing a proline into the $\omega + 2$ position of miniPLAP and MP/uPAR. Also shown is the effect of introducing the resultant DPA sequence from miniPLAP into MP/uPAR. (C) The effect of introducing the DAA sequence from MP into the $\omega + 1$ position in uPAR and the effect of introducing the proline into the $\omega + 3$ position.

Exchange of SAD Domains. Studies in which the amino acids in the SAD domain of miniPLAP were mutated individually to those found in other GPI-anchored proteins led to the prediction that C-terminal processing and anchor addition would be unaltered were the entire SAD domain of two GPI-anchored proteins to be exchanged (*56*), although this hypothesis was not tested directly. To do so, we next studied the C-terminal processing of two chimeric proteins, one in which the SAD domain of uPAR, SGA, was introduced into miniPLAP at positions 179−181 and one in which the SAD domain of miniPLAP, DAA, was introduced into MP/uPAR at the same positions; the remainder of the extracellular domains and the remainder of the C terminus of the proteins were left undisturbed. Both variant proteins in which the SAD domains had been exchanged underwent C-terminal processing (Figure 5A). This experiment confirms the hypothesis of Udenfriend et al. (*56*) whereby the SAD domain functions as an independent unit in the context of a permissive C-terminal sequence.

Comparison of the SAD Domains of MiniPLAP and MP/uPAR. The SAD domain of miniPLAP contains the sequence DAAH, whereas the corresponding sequence in MP/uPAR is SGAA. Theoretically, C-terminal processing in MP/uPAR could occur between Ser_{179} and Gly_{180}, between Gly_{180} and Ala_{181}, or at both sites with the same or with different efficiencies. It is also possible that the second site is used only in situations where the primary cleavage site is made unavailable. The observation that MP/uPAR underwent C-terminal processing more efficiently than miniPLAP (Figure 4, bottom) could result from either intrinsic differences in the recognition of Asp versus Ser which are in the sole ω position of the two proteins, the presence of an additional cleavage site in MP/uPAR which is not present in miniPLAP, or differences in the suitability of the cleavage site in MP/uPAR as a substrate that result from the influence of Ala_{182} or more distant portions of the C terminus of uPAR. A series of experiments were therefore performed to explore these various non-mutually exclusive possibilities.

It is known that introduction of a proline into the $\omega + 1$ position of miniPLAP (which generates the sequence DPA) prevents C-terminal processing totally (*32*). Therefore, we asked whether proline would exert a similar effect in MP/uPAR (which generates the sequence SPA). Although miniPLAP-Pro_{180} did not undergo C-terminal processing (Figure 5B), consistent with previous studies (*32*), MP/uPAR-Pro_{180} underwent C-terminal processing to the same extent as did wild type MP/uPAR (compare Figure 5B with Figures 3 and 4).

There are at least two possible explanations for the unexpected finding that MP/uPAR-Pro_{180} underwent C-terminal processing normally. First, the difference in the capacity of the transamidase to cleave Ser-Pro, but not Asp-Pro, may be intrinsic to structure assumed by these two amino acids in the context of the remainder of either C terminus. This interpretation implies that Ser-Pro is seen as well as the naturally occurring Ser-Gly in this position, whereas Asp-Pro assumes a radically different conformation. Alternatively, Ser-Pro may assume a permissive structure in the context of the C terminus of uPAR specifically.

Two variants were generated to distinguish between these possibilities further. First, the sequence DPA was introduced into MP/uPAR at positions 179−181. MP/uPAR-$D_{179}P_{180}A_{181}$ did not undergo C-terminal processing (Figure 5B). This result suggests that the transamidase distinguishes between Ser-Pro and Asp-Pro in this position. In accord with this notion, MP/uPAR-Ser_{179}-Asp_{180}-Pro_{181}-Ala_{182} did not undergo C-terminal processing (Figure 5C), consistent with the reported effect of introducing a proline into the $\omega + 2$ position (*32*, *56*). However, this explanation is too facile because when the sequence SPA was introduced into miniPLAP (miniPLAP-Ser_{179}-Pro_{180}-Ala_{181}), little (<10%) mature (C-terminally processed) protein was generated

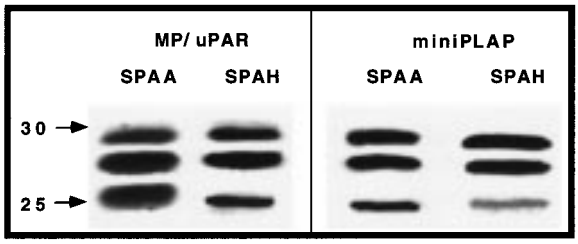

FIGURE 6: Effect of histidines substituted into the $\omega + 3$ position in miniPLAP and MP/uPAR.

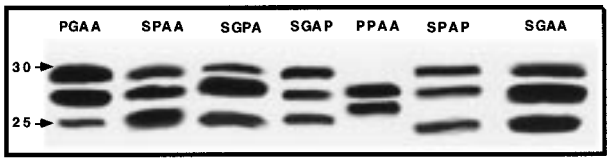

FIGURE 7: Effect of single and double proline substitutions in MP/uPAR. The sequences PGAA, SPAA, SGPA, and SGAP correspond to substitution of proline at the ω, $\omega + 1$, $\omega + 2$, and $\omega + 3$ positions, respectively. Double proline substitutions are introduced at ω and $\omega + 1$ (PPAA) and $\omega + 1$ and $\omega + 3$ (SPAP). Note the anomalous migration of PPAA.

relative to wild type miniPLAP (Figure 6 and Table 1), whereas the identical SAD domain was processed efficiently in the context of uPAR. This result shows unequivocally that the cleavage site within the SAD domain is modified by its interaction with other portions of the C terminus of the molecule.

The SAD domain in uPAR contains an alanine at the $\omega + 3$ position, a site where a histidine is present in miniPLAP. The additional small amino acid might contribute to the observed difference in the recognition of the SPA sequence in uPAR and PLAP. However, the construct MP/uPAR-Ser_{179}-Pro_{180}-Ala_{181}-His_{182} underwent processing almost to the same extent as did MP/uPAR-Ser_{179}-Pro_{180}-Ala_{181}-Ala_{182} (Figure 6 and Table 1). To the extent that SPAH assumes the same secondary structure as SPAA, this outcome implies that the difference seen in the processing of SPA in the content of uPAR versus in miniPLAP is due to the impact of the remainder of the carboxy terminus on the structure of the SAD domain, as proposed previously (*49*), or that the enzyme recognizes a broader structure in which these additional carboxy-terminal epitopes participate.

Role of the Fourth Small Amino Acid in MP/uPAR. We next asked whether the protein containing the sequence MP/uPAR-Ser_{179}-Pro_{180}-Ala_{181} underwent C-terminal cleavage, not because the Ser−Pro bond was recognized, but because cleavage occurred within a SAD domain comprised of Pro_{180}-Ala_{181}-Ala_{182} with the proline unexpectedly functioning as an acceptor (ω) site for the transamidase. To examine this possibility, prolines were individually substituted for each of the naturally occurring four small amino acids in MP/uPAR which may comprise a potentially "expanded" SAD domain (residues 179−182), and prolines were introduced at two sites simultaneously to eliminate cleavage at either potential acceptor site (Figure 7 and Table 1).

Introduction of a proline into the ω position is said to preclude processing because the R group of this amino acid is not a nucleophile acceptor (*39*, *40*). Yet introduction of a proline into the ω position of miniPLAP/uPAR (Pro_{179}-Gly_{180}-Ala_{181}-Ala_{182}) markedly reduced the level of, but did not totally inhibit, C-terminal processing (Figure 7 and Table 1). If indeed proline cannot function as a proton acceptor, this finding suggests that although the primary cleavage site in uPAR lies between Ser_{179} and Pro_{180}, a potential secondary cleavage site must exist in this variant as well. Consistent with this notion, substitution of proline for the small amino acid alanine at position 182, in the form of either SGAP or SPAP, both of which eliminate the potential SAD sequence GAA, had no appreciable effect on processing (Figure 7 and Table 1). Further, the mutant SGPA was processed normally (Figure 7 and Table 1). Had the cleavage between Gly and Pro contributed significantly to process, then the expected result would have been the markedly reduced level of processing observed with PGAA.

However, the observation that miniPLAP/uPAR-Pro_{179}-Gly_{181}-Ala_{180}-Ala_{182} underwent C-terminal processing at all suggests that cleavage can occur between Gly_{180} and Ala_{181} at least when the primary cleavage site is blocked by proline. This hypothesis was confirmed by the finding that residual C-terminal processing of this variant was eliminated by the addition of a second proline at the $\omega + 1$ position (Pro_{179}-Pro_{180}-Ala_{181}-Ala_{182}) which blocks both potential acceptor sites (Figure 7 and Table 1). Thus, these experiments taken together indicate that Ala_{182} participates in the formation of the SAD domain.

Next, the potential implications of having a small amino acid in the fourth position were studied in the context of miniPLAP itself. As noted above, the construct miniPLAP−SPAH was processed little whereas SPAH in the context of uPAR was processed relatively efficiently. In both cases, processing could only occur between Ser and Pro. We therefore asked whether the substitution of Ala for His as occurs in the $\omega + 3$ position in uPAR would modulate the cleavage of the SPA sequence in PLAP. The construct miniPLAP−SPAA was processed as efficiently as did wild type miniPLAP and at a level comparable to that of MP/uPAR−SPAA (Figure 7 and Table 1). Thus, the conformation of the Ser-Pro sequence is highly susceptible to modulation by the amino acid in the $\omega + 3$ position.

Substitution of Neutral and Hydrophobic Amino Acids in the SAD Domain. Moller et al. (*47*) reported that substitutions of valines at positions $\omega + 1$ or $\omega + 2$ (SVAA and SGVA) did not prevent the expression of uPAR on the surface of transfected cells as a GPI-anchored protein, whereas similar substitutions were not tolerated in the analogous positions in miniPLAP (*32*, *42*). Further, substitution of valines at both positions (SVVA) prevented surface expression of uPAR (*47*). Why cleavage occurred between Ser and Val when Ala was present at the $\omega + 2$ position but not when Val was present at this position was not explained.

Therefore, we investigated the effect of the single and double valine substitutions in uPAR in greater detail. Consistent with the observations of Moller et al., the constructs miniPLAP-uPAR−SVAA, −SGVA, and −SPVA each underwent C-terminal processing with the same efficiency as did both wild type and the SPAA variant of MP/uPAR, whereas SVVA did not (Figure 8 and Table 1).

One potential explanation for the effect of the double valine substitution lies in the linear, rigid conformation that neutral hydrophobic amino acids may assume when they occur in series, a structure that may affect recognition by proteolytic enzymes (*57*). To test this hypothesis, we studied

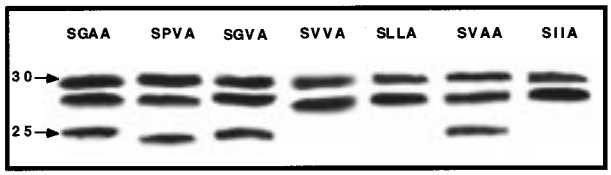

FIGURE 8: Effect of introducing neutral, hydrophobic amino acids into MP/uPAR. Single valine substitutions were introduced in wild type SGAA at the $\omega + 1$ (SVAA) and $\omega + 2$ (SGVA) positions; a single valine was substituted at the $\omega + 2$ position in the SPAA mutant (SPVA), and double substitutions with branched, neutral, and hydrophobic side chains were introduced at the $\omega + 1$ and $\omega + 2$ positions (SVVA, SLLA, and SIIA).

the effect of introducing two other neutral hydrophobic amino acids of similar size into the same positions in MP/uPAR, i.e., Leu and Ile. Neither MP/uPAR−SLLA nor MP/uPAR−SIIA underwent C-terminal processing (Figure 8 and Table 1).

We then asked whether these variants were not recognized by the transamidase or were recognized but were not cleaved. To test these possibilities, we compared the effect of the enzyme (RM) on wild type MP/uPAR in the presence of increasing amounts of MP/uPAR−SVVA. To do this, we added various amounts of MP/uPAR−SVVA mRNA (or wild type MP/uPAR or $uPAR_{1-281}$ as controls) to a fixed, optimal amount of wild type MP/uPAR mRNA determined in pilot experiments, and in vitro translation and processing were examined as before. We found that the addition of MP/uPAR−SVVA mRNA to wild type MP/uPAR mRNA inhibited the C-terminal processing of the resultant, labeled wild type protein in a dose-dependent manner, whereas processing was unaffected by the addition of the same amount of either control mRNA (Figure 9).

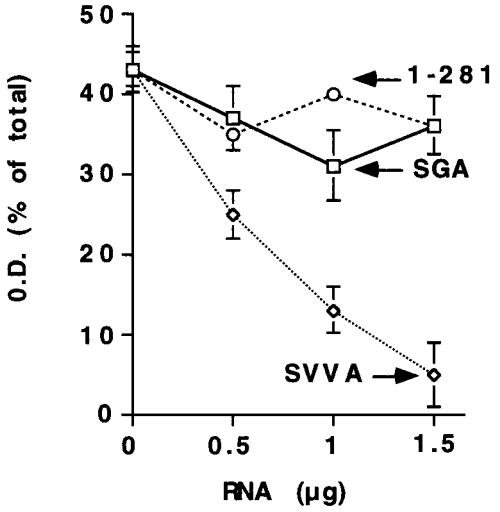

FIGURE 9: SVVA mutant of MP/uPAR competes for the processing of wild type MP/uPAR. Increasing amounts of MP/uPAR−SVVA (●), MP/uPAR−SGA (wild type) (■), or MP/uPAR (Taa) (■) mRNAs were added as indicated to 0.4 μg of MP/uPAR−SGA mRNA, and the relative amount of the GPI-anchored product was determined as described in Figure 4B. The data are expressed as the amount of C-terminally processed protein in OD units relative to the total amount of uPAR expressed. The means ± SEM of three experiments are shown.

DISCUSSION

We tested the hypothesis that the SAD domain of proteins that undergo C-terminal processing and condensation with a GPI anchor functions as a fully autonomous unit. To do this, we compared the effect of mutations introduced into the C terminus of two GPI-anchored proteins, i.e., the urokinase receptor and placental alkaline phosphatase which itself has been the subject of extensive study (*43*). All constructs shared the identical signal and N-terminal sequences to ensure that any differences in processing observed were due principally to the structure of the C-terminal portions of these molecules themselves, although a more detailed comparison of MP/uPAR and wild type uPAR would be required to formally exclude any contribution of the ectodomains of the native proteins. Further, the efficiency with which the variant proteins undergo C-terminal processing which we describe in this paper rests on the assumptions that the reactions have gone to completion and the immunoprecipitation is complete, assumptions met for each variant studied (*50*−*52*).

Our findings confirm those of others which indicate that C-terminal cleavage occurs within a short stretch (three to five) of small amino acids that precedes a spacer region of fixed length and a hydrophobic tail (*35*, *43*). We also confirmed the prediction based on substitutions of individual amino acids in miniPLAP that the entire SAD domain of one GPI-anchored protein could be exchanged with that of another without a loss of processing (*42*).

However, we found that the SAD domain does not function as an entirely autonomous unit. Rather, the efficiency of processing and GPI anchor addition depends on structural information present in other portions of the C terminus of these molecules. This is best exemplified by the observation that the introduction of a proline into the $\omega + 1$ position of miniPLAP totally precluded C-terminal processing but had no discernible effect on the processing of uPAR. In part, this outcome may be due to differences in the recognition of the resultant Asp-Pro sequence in miniPLAP compared with Ser-Pro in uPAR. Such an explanation is consistent with the finding that a variant uPAR containing DPA in the $\omega + 2$ position did not undergo processing.

However, this cannot be the entire explanation because little C-terminal processing occurred when the sequence SPA was introduced into the same positions in miniPLAP. This result shows unequivocally that the cleavage site within the SAD domain is influenced by other portions of the C terminus of the molecule. One such modifier is the presence of a histidine in the ω to $\omega + 3$ positions in miniPLAP since substitution of Ala at this position, as is found normally in uPAR, restores processing of the SPA variant.

There are two mechanisms by which the fourth small amino acid may have such a profound effect on processing. On one hand, it may alter the conformation of the $\omega - \omega + 1$ cleavage site. Alternatively, the presence of an additional small amino acid may permit cleavage to occur between positions $\omega + 1$ and $\omega + 2$ (*36*). Indeed, there is indirect evidence that cleavage at this second site does occur. Specifically, the variant MP/uPAR−PGAA undergoes limited, but discernible, C-terminal processing having an amino acid that cannot function as a nucleophilic acceptor (*32*) at the ω position, whereas no processing of the variant MP/uPAR−PPAA is seen in which both the ω−$\omega + 1$ and the potential $\omega + 1$−$\omega + 2$ sites are blocked. This finding

suggests that alanine at the $\omega + 3$ position of uPAR facilitates C-terminal processing primarily through an effect on the conformation of the $\omega-\omega + 1$ site in SPAA (*35*, *49*) and secondarily by permitting cleavage to occur between the $\omega + 1-\omega + 2$ amino acids. However, the observation that the sequence SPAH is cleaved efficiently in the context of MP/uPAR, but only to a limited extent in miniPLAP, suggests that other portions of the C-terminal sequence that lie outside the extended SAD domain may affect the activity of the transamidase as well. This concept helps to explain why substitution of a single amino acid in the hydrophobic tail of uPAR (*47*) or other proteins (*31*, *35*, *36*, *48*) prevents addition of the GPI anchor, suggesting the amino acid sequence in this region is not random as has been hypothesized. However, additional experimentation will be required before concluding with certainty that the remaining portions of the spacer region and hydrophobic tail indeed contribute to the efficiency of C-terminal processing.

The enzymatic activity of the transamidase may depend, in part, upon its ability to dock onto its substrate at a location(s) outside of the cleavage site as has been described for other enzymes such as serine proteases (*58*). In accord with this possibility, not only did the construct MP/uPAR−SVVA fail to undergo processing confirming previous findings that relied upon on cell surface expression of the protein (*47*), but also actually inhibited the processing of wild type MP/uPAR in a dose-dependent manner. One explanation for this latter finding is that the transamidase and the GPI moiety bind to MP/uPAR−SVVA but are unable to cleave it.

The fact that the uPAR variants SVAA and SGVA are processed normally makes it less likely that binding without cleavage occurs exclusively at the $\omega-\omega + 1$ position. Rather, the transamidase is more likely to bind over a broader interface with the substrate but is unable to cleave the $\omega-\omega + 1$ bond if valines or similar hydrophobic amino acids are present in series at the $\omega + 1$ and $\omega + 2$ positions. This is reminiscent of the situation with serine proteases and other enzymes that donate a proton to the carbonyl group of serine or other acceptors. The addition of the proton leads to the formation of a reactive intermediate which acts as a nucleophilic acceptor forming an oxyanion bond. In the case of C-terminal processing, the amino group of the terminal ethanolamine in the fully formed GPI anchor is the nucleophile (*39*, *40*). The enzyme must bind to adjacent sites on the substrate to form an oxyanion pocket that permits the nucleophilic attack to proceed efficiently. Substitution of neutral hydrophobic amino acids in series may generate a rigid, planar structure which precludes formation of the oxyanion pocket and thereby limits completion of the cleavage step (*57*). Confirmation of this hypothesis would require isolation and knowledge of the crystal structure of the enzyme−substrate complex. Proteins such as miniPLAP/uPAR−SVAA which are recognized but do not undergo cleavage may facilitate isolation of the C-terminal transamidase.

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