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Conversion of a PI-Anchored Protein to an Integral Membrane Protein by a Single Amino Acid Mutation

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Qa-2, a cell-surface glycoprotein anchored by phosphatidylinositol (PI), is structurally related to the class I transplantation antigens H-2 K, D, and L, which are integral membrane glycoproteins. The predicted transmembrane segment of Qa-2 differs from those of H-2 K, D, and L by the presence of an aspartate in place of a valine at position 295. A single base change that replaced this aspartate with valine resulted in cell-surface Qa-2 molecules that were insensitive to hydrolysis by a PI-specific phospholipase C and more resistant to papain cleavage, properties shared by H-2D. Cells expressing Asp → Val mutant Qa-2 proteins were still able to attach a PI anchor to endogenous proteins such as Thy-1 and J11D. It therefore appears that this single amino acid change converts Qa-2 from a PI-linked form into an integral membrane protein.

UNTIL RECENTLY IT WAS BELIEVED that all membrane-bound proteins are integrated into the lipid bilayer by a membrane-spanning segment of hydrophobic amino acids interacting noncovalently with the nonpolar tails of membrane lipids. In the past few years, however, it has become clear that a number of proteins are anchored instead to one leaflet of the lipid bilayer by covalent linkage to phosphatidylinositol (PI) (*1*). Although the signals mediating this novel posttranslational modification are not well understood, alignment of carboxyl-terminal regions of the proteins, predicted from their respective cDNA or genomic DNA sequences, has revealed common features among polypeptides with PI anchors. The common features are a truncated or weakly hydrophobic membrane-spanning segment and little or no cytoplasmic tail (*1*).

Qa-2, a cell-surface glycoprotein expressed on subpopulations of hematopoietic cells (*2*), was shown to be anchored by PI (*3–5*), since it could be released from the cell membrane by treatment with a PI-specific phospholipase C (PI-PLC). Qa-2 is otherwise structurally similar to the H-2 class I transplantation antigens K, D, and L, which are integral membrane glycoproteins with strongly hydrophobic transmembrane segments and cytoplasmic tails of about 30 amino acids (*6, 7*). Exon exchange between transplantation antigens and the *Q7* gene, which encodes Qa-2 (*4, 8, 9*), showed that the signals for PI modification of Qa-2 are located in the carboxyl-terminal region of the molecule (*4, 5*). By exchanging segments of the *Q7* and *H-2* genes of the C57BL/10 mouse (H-2^b haplotype), we mapped the signals for PI modification of Qa-2 to a region of 80 amino acids that differs from H-$2D^b$ most significantly by the presence of a charged aspartate residue (Asp^{295}) in place of valine in the transmembrane segment and by a cytoplasmic tail of only three basic amino acids (*5*). The transmembrane segment of all transplantation antigens that have been sequenced has a hydrophobic or neutral amino acid at this position (*7*), and the unusual features of *Q7* led Steinmetz *et al.* (*10*) to propose that *Q7* might be a pseudogene. To distinguish which of these features is responsible for the modification of Qa-2 by PI, we replaced the Asp^{295} with Val and treated transfected thymoma cells with PI-PLC to test whether the mutant Qa-2 is linked to PI.

The transmembrane Asp^{295} codon of a $Q7^b$ cDNA was converted to Val by site-directed mutagenesis (Fig. 1). A segment of DNA containing the mutated codon was inserted in place of a corresponding segment from the wild-type $Q7^b$ cDNA expression vector to ensure that no other mutations were introduced aberrantly. R1.1 thymoma cells were transfected with the mutant $Q7^b$ cDNA vector as described (*8*), and G418-resistant clones were screened for expression of cell-surface Qa-2. Two strongly positive clones (Q7V3R and Q7V6R) were independently isolated and used for further study.

```
H-2D^b   284 ...MVIVAVLGVLGAMAIIGAVVAFVM... 307
                |  ||  ||| ||||||||||
Q7^b     287 ...MATIAVVVDLGAVAIIGAVVAFVM... 310
                      ↑
```

Fig. 1. Alignment of the transmembrane peptide segments of H-$2D^b$ and $Q7^b$. The arrow refers to the $Q7^b$ Asp^{295} that was mutated to Val by antisense oligonucleotide 5′-TCCAAGGA CAACCAC-3′, which differs from the wild-type sequence (*8*) by a single base (underlined). A 672-bp Kpn I–Dra III fragment (positions 415 to 1087) (*8*) was removed from the $Q7^b$ cDNA expression vector and site-directed mutagenesis was performed by using a procedure for gapped-plasmid heteroduplexes (*17*). Colonies were screened with the ^{32}P-labeled oligonucleotide, and mutant DNAs were purified after retransformation of plasmids from positive clones. The Kpn I–Dra III segment containing the mutated codon was sequenced by a modified chain-termination method used for supercoiled plasmids (*18*). This segment was excised from the mutated plasmid and inserted in place of a corresponding segment from the wild-type $Q7^b$ cDNA expression vector. Mutant DNAs were electroporated into R1.1 cells, and stable transfectants were selected in growth medium containing G418 (1 mg/ml) (*8*).

The PI-PLC sensitivity of Qa-2 molecules on the surface of clones Q7V3R and Q7V6R was compared to that of clone Q7c26R, an R1.1 cell line transfected with a wild-type $Q7^b$ cDNA construct (*8*), which expresses the PI-anchored form of Qa-2 (*5*). As expected, the Q7c26R cells showed a marked reduction in staining (about tenfold) after PI-PLC treatment (Fig. 2A). In contrast, Asp^{295} → Val mutant Qa-2 molecules on Q7V3R and Q7V6R were completely resistant to PI-PLC hydrolysis in that virtually identical profiles were obtained from mock-treated and PI-PLC–treated groups (Fig. 2A). It therefore appears that the Asp^{295} → Val mutant Qa-2 is no longer modified by PI but, like H-$2D^b$, integrates into the lipid bilayer.

To be certain that the Q7V3R and Q7V6R cells retained the ability to covalently modify proteins with PI, two other proteins endogenous to R1.1 cells and known to be PI-anchored, Thy-1 (*11*) and J11D (*12*), were analyzed for their sensitivity to PI-PLC. There is little difference in the PI-PLC sensitivity of Thy-1 and J11D among the Q7c26R, Q7V3R, and Q7V6R cell lines (Fig. 2, B and C). Not all of these molecules are removed by PI-PLC treatment, although this unexplained phenome-

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non has been reported by others (*1*). The PI-PLC resistance of the $Asp^{295} \rightarrow$ Val Qa-2 mutants (Fig. 2A) is therefore a property of the molecule itself and not the result of a PI-modification defect in either Q7V3R or Q7V6R cells.

All mouse and human class I transplantation antigens appear to contain a papain cleavage site in the proline-rich peptide that connects the globular external domains to the transmembrane segment (*6*). In contrast, the globular domains of PI-anchored proteins appear to be connected to the phospholipid anchor by glycosidic linkage (*1*). The presence of glycans in place of amino acids in this region might cause the PI-anchored form to be more resistant to protease. Alternatively, the carboxyl-terminal region of the protein might be more accessible to papain.

We therefore measured the papain sensitivity of class I antigens expressed on R1.1 cells transfected with native or hybrid gene constructs. In hybrid $Q7^b/D^b$ molecules the extracellular domains of $Q7^b$ are anchored by the transmembrane segment of H-$2D^b$, and in hybrid $D^b/Q7^b$ molecules the extracellular domains of H-$2D^b$ are anchored by the PI-modified carboxyl-terminus of $Q7^b$ (*5, 8*). We found that PI-anchored molecules are reproducibly more sensitive than integral membrane forms by several orders of magnitude (Fig. 3). Analysis of the hybrid molecules indicates that the papain cleavage site maps to the region connecting the extracellular domains to the membrane anchor (Fig. 3). Like H-$2D^b$ and the $Q7^b/D^b$ hybrid, the $Asp^{295} \rightarrow$ Val mutant Qa-2 molecule is resistant to papain cleavage (Fig. 3). This implies that the $Asp^{295} \rightarrow$ Val mutant Qa-2 molecule is anchored in the plasma membrane by a carboxyl-terminal segment similar to that of H-$2D^b$. In addition, it appears that $Asp^{295} \rightarrow$ Val mutant Qa-2 molecules may be slightly more sensitive to higher amounts of papain than $Q7^b/D^b$ molecules. This may be a result of the three extra amino acids at positions 273 through 275 in the $Q7^b$ connecting peptide (*5*), which might make this region more accessible to papain.

One could argue that the PI-PLC resistance of the $Asp^{295} \rightarrow$ Val Qa-2 mutant does not necessarily indicate that it is an integral membrane protein. For example, it appears that human erythrocyte acetylcholinesterase is modified by PI in such a way that it is resistant to the bacterial enzyme used here, whereas bovine erythrocyte acetylcholinesterase is almost completely sensitive (*13*). In our system, the possibility of a PI anchor that is resistant to PI-PLC seems unlikely. We have shown elsewhere (*14*) that the sensitivity of PI-anchored proteins to PI-PLC from *Staphylococcus aureus* appears to be cell-specific, in that the same protein (for example, Thy-1) may be sensitive on one cell but resistant on another. Conversely, different PI-anchored proteins (for example, Thy-1, J-11D, and Qa-2) on the same cell show the same pattern of sensitivity or resistance to the PI-PLC from *S. aureus*. However, when PI-PLC from *Bacillus thuringiensis* was used, PI-anchored proteins on all cells examined were sensitive to hydrolysis. In this study, we have used PI-PLC from *B. thuringiensis* to treat transfectants derived from the same cell line, R1.1. The only molecule resistant to hydrolysis was the $Asp^{295} \rightarrow$ Val mutant Qa-2, even though Thy-1 and J11D on the same cells were sensitive. Hence it seems unlikely that the $Asp^{295} \rightarrow$ Val Qa-2 mutant is somehow modified by PI in such a way as to be resistant to *B. thuringiensis* PI-PLC. In addition, $Asp^{295} \rightarrow$ Val mutant Qa-2 molecules are expressed on the surface of transfected mouse fibroblasts (L cells) at levels similar to the $Q7^b/D^b$ hybrid (*15*) whereas wild-type (PI-anchored) Qa-2 molecules are secreted by L cells and are not expressed at high levels on the L cell surface (*4, 8, 16*).

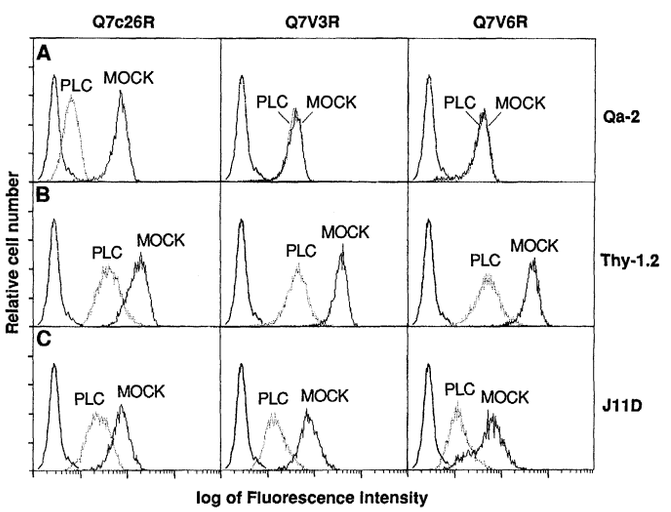

Fig. 2. PI-PLC sensitivity of molecules expressed on clones Q7c26R, Q7V3R, and Q7V6R. Cells (5×10^5) were incubated at 37°C for 60 min in 0.5 ml of RPMI 1640 containing 10% fetal bovine serum with or without 0.2 U of PI-PLC purified from *B. thuringiensis* (*5*). After treatment, cells were washed and then fixed in 0.04% formaldehyde. The fixed cells were reacted with monoclonal antibodies against (**A**) Qa-2, (**B**) Thy-1.2, and (**C**) J11D and then stained with fluorescein isothiocyanate–conjugated goat antiserum to mouse immunoglobulins (*19*). Samples were analyzed by flow cytometry on a FACStar fluorescence-activated cell sorter (Becton Dickinson). The profile seen on the far left side of each panel represents background fluorescence from the staining of R1.1 thymoma cells with antibody to Qa-2 and the fluorescein conjugate.

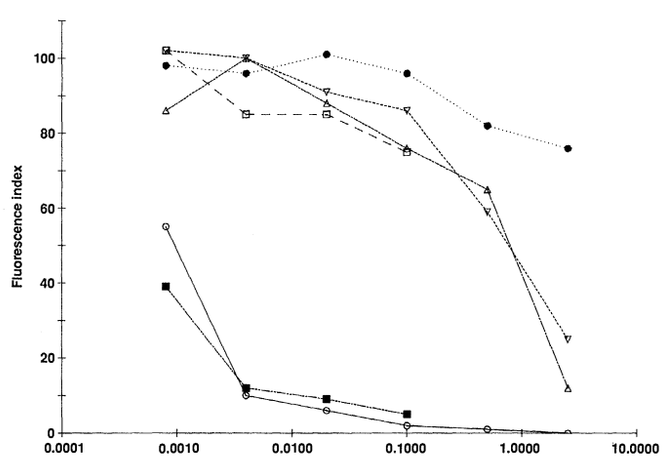

Fig. 3. Papain sensitivity of class I molecules on R1.1 transfectants. Cell lines used for analysis were as follows: Q7c26R (○), which expresses the PI-anchored form of Qa-2; Q7V3R (△) and Q7V6R (▽), which express $Asp^{295} \rightarrow$ Val mutant Qa-2 molecules; DbMoR (□), which expresses H-$2D^b$; B3R (●), which expresses $Q7^b/D^b$ hybrid molecules anchored by the H-$2D^b$ transmembrane segment; and C18R (■), which expresses $D^b/Q7^b$ hybrid molecules anchored by PI (*5*). Live cells (2×10^6) were resuspended in 0.2 ml of papain (*20*), diluted serially (fivefold) in RPMI 1640 containing 0.01*M* disodium EDTA and 0.01*M* 2-mercaptoethanol, and then incubated for 60 min at 37°C. Reactions were stopped by addition of 5 ml of ice-cold Hanks balanced salt solution containing 5% fetal bovine serum. Cells were washed, fixed, and stained with monoclonal antibodies to Qa-2 or H-$2D^b$ (*19*), and analyzed by means of flow cytometry (as described in the legend to Fig. 2). The fluorescence index for each sample was calculated from its mean fluorescence intensity (MFI) with the following formula:

$$\frac{(\text{MFI of papain-treated sample}) - (\text{MFI of negative control})}{(\text{MFI of mock-treated sample}) - (\text{MFI of negative control})} \times 100$$

We have shown, by two independent criteria (protease and phospholipase sensitivity), that the elimination of the negatively charged transmembrane aspartate residue abolishes the signal for PI modification of Qa-2. These results indicate that at least one requirement for PI linkage of proteins is the presence of a weakly hydrophobic transmembrane segment.

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20. Papain was from Cooper Biomedical. This lot (77621M) had a specific activity of 21 U/mg and a concentration of 31.6 mg/ml.

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Alpha-2-Antiplasmin: A Serpin with Two Separate but Overlapping Reactive Sites

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Although the proteinase inhibitor alpha-2-antiplasmin (α_2AP) is known to control the activity of plasmin through rapid formation of stable complexes, it also efficiently inactivates chymotrypsin. These interactions are shown to occur at adjacent, overlapping sites so that plasmin attacks the inhibitor at an Arg^{364}-Met^{365} peptide bond, while chymotrypsin interacts at a Met^{365}-Ser^{366} sequence one residue downstream. Thus, a naturally occurring plasma serine proteinase inhibitor can have multiple specificities through interactions at adjacent sites. It also illustrates the potential flexibility of the reactive site loop in this class of inhibitors.

HUMAN α_2AP IS ONE OF SEVERAL homologous proteins that comprise the serpin (SERine Proteinase INhibitor) superfamily (*1, 2*). Kinetic studies on the interaction of this inhibitor with a series of proteinases have shown that its major target enzymes are plasmin and trypsin ($K_a = 1.0 - 4.0 \times 10^7 M^{-1}$ sec^{-1}) (*3–5*). Complex formation occurs through enzyme-inhibitor interactions at the Arg^{364}-Met^{365} peptide bond (*3, 6*), and this is in agreement with the specificities of both plasmin and trypsin. However, α_2AP can also form stable complexes with bovine α-chymotrypsin [$K_a = 6.7 \times 10^5 M^{-1}$ sec^{-1} (*3*)] which dissociate only very slowly [$K_d = 5.6 \times 10^{-5}$ sec^{-1} (*7*)]. Thus, such interactions are not simply a mechanism for chymotrypsin inactivation of α_2AP by limited proteolysis. Because the structure of the inhibitor loop of α_2AP includes the sequence beginning -Ala-Met-Ser-Arg-Met-Ser-Leu-Ser- (residues 361–368), with the Arg-Met peptide bond representing the inhibitory site for plasmin and trypsin inhibition (*3, 6*), many other adjacent peptide bonds in this region would appear to be more favorable for chymotrypsin binding and inhibition. We have, therefore, determined the site of attack of chymotrypsin during complex formation with α_2AP.

Complexes of α_2AP with bovine α-chymotrypsin were formed by incubation of equimolar quantities of inhibitor and enzyme at *p*H 7.4 for 1 minute at 25°C. Human α-2-macroglobulin (α_2M) (5*M* excess) was added to trap any chymotrypsin released from complexes, and the reaction mixture was incubated for 15 hours. Under these conditions more than 90% of the chymotrypsin activity was released. The large α_2M-chymotrypsin complexes were separated from mixtures of native and modified α_2AP by gel filtration chromatography on Sephadex G-100. The two forms of inhibitor were then purified by fast protein liquid chromatography (FPLC) on a mono-Q column (Pharmacia). Modified α_2AP, which represented more than 75% of the starting material, migrated as two components of 60 kD and 14 kD, on SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1). Preparative gel electrophoresis was used to isolate each, and both were then analyzed for NH_2-terminal sequences. The first ten residues are given below:

60 kD:
LysSerProProGlyValCysSerArgAsp **1**
14 kD:
SerLeuSerSerPheSerValAsnArgPro **2**

The sequence of peptide **1** indicates that chymotrypsin attacked α_2AP between residues 24 and 25 while analysis of peptide **2** shows cleavage between residues 365 and 366. The former reaction is presumed to be noninhibitory, representing only enzymatic hydrolysis of peptide bonds in the NH_2-terminal region and has been reported for other serpins (*8, 9*). However, the latter cleavage indicates that the interaction between chymotrypsin and α_2AP has occurred between Met^{365} and Ser^{366} in the reactive site loop and that the 14-kD fragment represents the cleavage peptide released during slow dissociation of this complex. Thus, α_2AP has two inhibitory sites that overlap, Met^{367} being in the P_1'-position for plasmin/trypsin inhibition and in the P_1-position for chymotrypsin inhibition (Fig. 2). Upstream, human neutrophil elastase inactivates α_2AP by cleavage between Ala^{361} and Met^{362} (*3, 10*). This reaction occurs even though there are two nearby Met-Ser se-

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