Signal Peptide for Protein Secretion Directing Glycophospholipid Membrane Anchor Attachment

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Decay accelerating factor (DAF) is anchored to the plasma membrane by a glycophospholipid (GPI) membrane anchor covalently attached to the COOH-terminus of the protein. A hydrophobic domain located at the COOH-terminus is required for anchor attachment; DAF molecules lacking this domain are secreted. Replacement of the COOH-terminal hydrophobic domain with a signal peptide that normally functions in membrane translocation, or with a random hydrophobic sequence, results in efficient and correct processing, producing GPI-anchored DAF on the cell surface. The structural requirements for GPI anchor attachment and for membrane translocation are therefore similar, presumably depending on overall hydrophobicity rather than specific sequences.

DECAY ACCELERATING FACTOR (DAF) is a complement-regulating protein that binds activated complement fragments C3b and C4b, thereby preventing amplification of the complement cascade on host cell membranes (*1*). DAF belongs to a small class of integral membrane proteins anchored to the lipid bilayer by a GPI membrane anchor containing phosphatidylinositol, carbohydrate, and ethanolamine, covalently linked to the COOH-terminus of the protein (*2*). Other proteins anchored in this way include Thy-1 (*3*), the variant surface glycoproteins of African trypanosomes (*4*), acetylcholinesterase (*5*), Qa-2 (*6*), and the Fcγ receptor (*7*). Attachment of the GPI anchor is thought to occur in the endoplasmic reticulum (*8*) after a proteolytic processing event that removes 17 to 31 residues, including a hydrophobic domain, from the COOH-terminus of the protein (*3, 9*). It has been established for DAF (*10*) and Qa-2 (*6*) that the COOH-terminus contains a signal for directing cleavage and attachment. These COOH-terminal signals display no obvious sequence homology, but do contain a short (15 to 20 residues) hydrophobic domain (*11, 12*) that is essential for attachment of a GPI anchor (*13, 14*). The process of GPI anchor attachment is similar to signal peptide–mediated protein secretion: both processes are involved in protein targeting and both require a hydrophobic domain of variable sequence that is cleaved during biosynthesis. Because of these similarities, we investigated whether a signal peptide, normally involved in protein secretion, could replace the COOH-terminal hydrophobic domain of DAF in directing GPI anchor attachment. We show that the signal peptide from human growth hormone (hGH) or a random hydrophobic peptide, when placed at the COOH-terminus of DAF, functions efficiently in directing attachment of a GPI membrane anchor.

The 17-residue COOH-terminal hydrophobic domain of DAF was removed by deletion mutagenesis (*15*) and replaced either with the hGH signal peptide or with a random hydrophobic sequence. The deletion mutant Δ1DAF (Fig. 1b), lacking the last 17 residues predicted by the DAF cDNA (the COOH-terminal hydrophobic domain) has been described (*13*). DAF-Sig1 (Fig. 1c) contains, in place of the COOH-terminal hydrophobic domain, a truncated signal sequence from the hGH gene (*16*) (residues −26 to −6) including the NH_2-terminal charged region and the hydrophobic core (approximately 13 residues), but lacking the signal peptidase cleavage site. DAF-Sig2 (Fig. 1d) is similar to DAF-Sig1 but contains the complete hGH signal sequence (residues −26 to −1). The hGH signal sequence thus was translocated, without altering its orientation, from the NH_2-terminus of hGH to the COOH-terminus of the secreted Δ1DAF mutant, the 13-residue hydrophobic core region forming the COOH-terminal domain of DAF-Sig1, whereas DAF-Sig2 contains an additional hydrophilic extension of five amino acids (the signal peptidase cleavage site). DAF-Rand17 (Fig. 1e) has at the COOH-terminus a random hydrophobic sequence derived by scrambling the order of the amino acids present in the COOH-terminal domain of wild-type DAF.

These modified forms of DAF were transiently expressed in COS cells under control of the cytomegalovirus promoter and localized by indirect immunofluorescence. As previously shown, wild-type DAF is expressed on the cell surface, whereas Δ1DAF can be detected only after permeabilization of the cells (*13, 17*). Surface labeling of intact (nonpermeabilized) cells indicated that DAF derived from DAF-Sig1, DAF-Sig2, or DAF-Rand17 cDNA is on the cell surface, as is wild-type DAF (Fig. 2). Cells expressing Δ1DAF are shown for reference and show no surface labeling. These observations suggest that whereas membrane DAF becomes targeted for secretion upon removal of the COOH-terminal hydrophobic domain (*13*), cell surface expression can be restored by fusing a signal peptide or a random hydrophobic peptide to the COOH-terminus of the truncated DAF protein.

To determine the nature of the attachment to the plasma membrane, we labeled

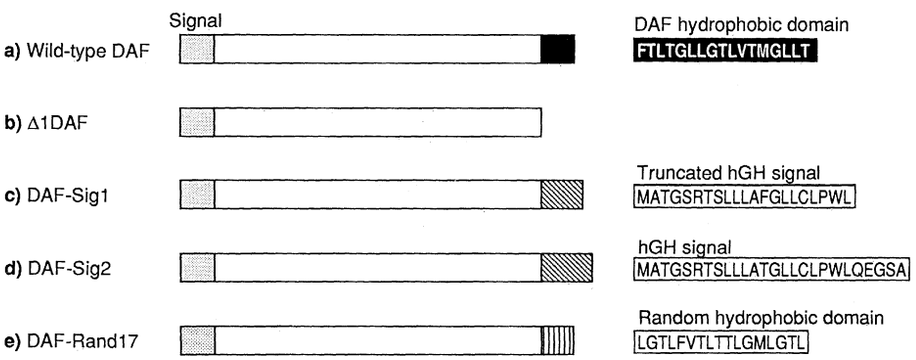

Fig. 1. A schematic diagram of the domain substitutions at the COOH-terminus of DAF. (**a**) Wild-type DAF, the COOH-terminal hydrophobic domain (residues 331 to 347) is depicted in black and the DAF signal sequence (residues −34 to −1) is shaded; (**b**) Δ1DAF deletion mutant (residues −34 to 330) without the hydrophobic domain; (**c**) and (**d**) DAF-Sig1 and DAF-Sig2, with the segment of DAF present in Δ1DAF fused in frame to the hGH signal peptide (hatched box, residues −26 to −6 or −26 to −1, respectively); (**e**) DAF-Rand17, which has the Δ1DAF segment fused in frame to a 17-residue random hydrophobic peptide (striped box) derived by scrambling the order of the amino acids present in the wild-type hydrophobic domain. The Δ1DAF mutant was constructed by deletion mutagenesis in M13 as described (*13*). DAF-Sig1, DAF-Sig2, and DAF-Rand17 were constructed by insertional mutagenesis (*15*), starting with Δ1DAF cloned into the M13 vector mp19 and with the use of synthetic oligonucleotides of 91, 106, and 79 base pairs, respectively, as insertion primers. Recombinant DNAs were verified by sequencing. The wild-type and modified DAF cDNAs were inserted into a mammalian expression vector between a cytomegalovirus enhancer-promoter and an SV40 polyadenylation sequence (*21*).

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transfected COS cells with [^{35}S]cysteine, washed, and resuspended in incubation medium, and then incubated with phosphatidylinositol-specific phospholipase C (PIPLC) from *Bacillus thuringiensis*. This enzyme specifically releases GPI-anchored but not conventionally anchored proteins from cell surfaces (*3, 10*). After a 1-hour incubation, cells and supernatants were separated by centrifugation and analyzed by immunoprecipitation. We also analyzed the culture medium collected after the 6-hour labeling period (Fig. 3). As previously noted (*13*), wild-type DAF is localized primarily in the cell lysate as an ~40-kD unglycosylated species and an ~70-kD mature form, both of which appear as doublets (possibly reflecting heterogeneity in the GPI anchor). In addition, a 68-kD soluble form accumulates in the culture medium collected after the labeling period. Pulse-chase experiments suggest that this soluble form is derived from membrane DAF by a cleavage within the GPI anchor (possibly by a phospholipase) that leaves [^{3}H]ethanolamine attached to the protein but removes [^{3}H]palmitate. In contrast, the mature form of Δ1DAF, which has no GPI anchor and is secreted, is present exclusively in the culture medium (*13*) (Fig. 3A, compare lanes 3 and 15). The expression patterns of DAF-Sig1, DAF-Sig2, and DAF-Rand17 were similar to wild-type DAF, showing a 70-kD mature form that was cell-associated (Fig. 3A, lanes 4 to 6) and a 68-kD form that accumulated in the culture medium (Fig. 3A, lanes 16 to 18). Incubation of the washed, labeled cells with PIPLC resulted in release of the ~70 kD DAF species from cells expressing DAF-Sig1, DAF-Sig2, or DAF-Rand17, as evidenced by a decrease in the amount of cell-associated mature DAF (Fig. 3A, lanes 10 to 12) and its recovery in the incubation supernatants (Fig. 3B, lanes 10 to 12). This result points to GPI anchorage as the mode of attachment of the modified DAF proteins. The levels of the ~40 kD unglycosylated species, presumably an intracellular protein, were not affected by PIPLC. PIPLC selectively released the larger of the two ~70 kD DAF species in the mutant as well as the wild-type DAF proteins, although both appear to be GPI-anchored as evidenced by [^{3}H]ethanolamine incorporation.

To confirm the presence of a GPI membrane anchor, we labeled transfected COS cells metabolically with [^{3}H]ethanolamine, a component of the GPI anchor. Analysis by immunoprecipitation revealed [^{3}H]ethanolamine-labeled bands corresponding to unglycosylated and mature DAF in the cell lysates and revealed that DAF had been released in the media from cells expressing DAF-Sig1, DAF-Sig2, or DAF-Rand17 (Fig. 4). The apparent molecular sizes and relative localization of the [^{3}H]ethanolamine-labeled, modified DAF proteins were essentially indistinguishable from that of wild-type DAF.

We conclude that replacement of the DAF COOH-terminal hydrophobic domain with a secretion signal peptide, or with a random hydrophobic sequence results in efficient and correct processing of the protein, producing GPI-anchored DAF on the cell surface. This result suggests that it may be the overall hydrophobicity of the COOH-terminus rather than the presence there of a specific sequence or structure that is critical for directing attachment of a GPI

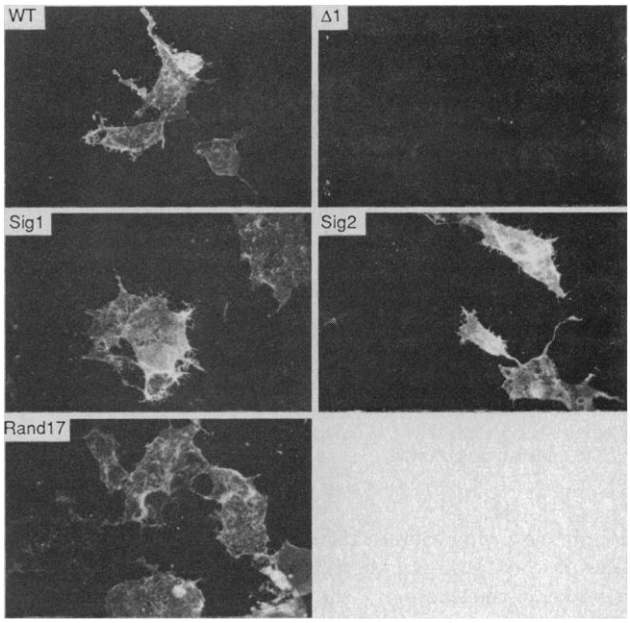

Fig. 2. Immunofluorescent labeling of DAF on the cell surface of transfected COS cells. Fixed, nonpermeabilized, transfected COS cells were incubated with monoclonal antibodies to human DAF, then with rhodamine-conjugated goat antibodies to mouse immunoglobulin G (IgG) as described (*17*). Cells expressing wild-type (WT) or Δ1DAF (Δ1) are shown for reference.

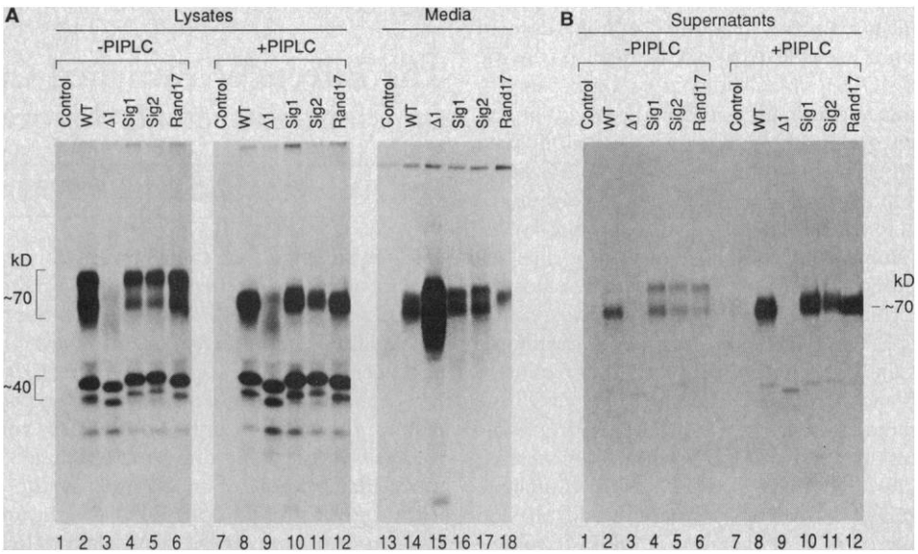

Fig. 3. Immunoprecipitation analysis of wild-type and mutant DAF proteins expressed in COS cells, showing both their relative distribution between cells and culture medium and release by PIPLC. Cells in 35-mm dishes were transfected with 3 μg of plasmid DNA as described (*22*) and labeled with [^{35}S]cysteine (200 μCi per 35-mm dish) for 6 hours. The culture medium was then collected and analyzed by immunoprecipitation as described (*23*). The cells were washed in phosphate-buffered saline (PBS) and resuspended in PBS containing 2% heat-inactivated fetal bovine serum with or without PIPLC (4 μg/ml). After incubation at 37°C for 60 min, the cells and incubation supernatants were separated by centrifugation and analyzed by immunoprecipitation. (**A**) Lanes 1 to 6, NP40 cell lysates showing cell-associated DAF; lanes 7 to 12, residual DAF in lysates after incubation of cells with PIPLC; and lanes 13 to 18, culture media collected after the labeling period. (**B**) Incubation supernatants from cells incubated without (lanes 1 to 6) or with (lanes 7 to 12) PIPLC. Control cells were mock transfected without DNA.

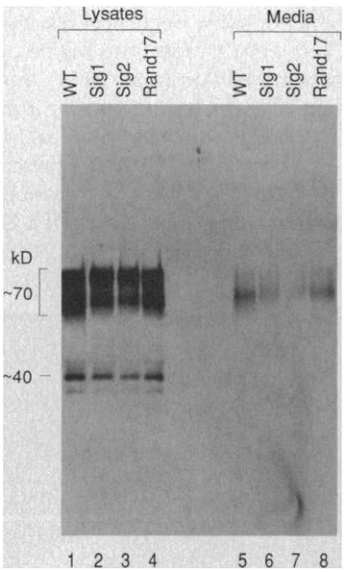

Fig. 4. Immunoprecipitation analysis of DAF proteins labeled metabolically with [^{3}H]ethanolamine. Transfected COS cells were incubated with [^{3}H]ethanolamine (200 μCi per 35-mm dish) for 16 hours. DAF was then immunoprecipitated from NP40 cell lysates (lanes 1 to 4) or culture media (lanes 5 to 8) that was collected at the end of the radiolabeling period.

membrane anchor. Similar conclusions have been reached regarding signal peptides for membrane translocation, whose function depends on their length and hydrophobicity rather than their specific sequence (*18*). It has been suggested that GPI anchor attachment requires a weakly hydrophobic domain since a single Asp → Val mutation in the COOH-terminal domain of Qa-2 converts this normally GPI-anchored protein into an integral membrane protein (*19*). Our data argue against this, since the hGH signal peptide contains a strongly hydrophobic core region. The length as well as the hydrophobicity of COOH-terminal domains appears to be important for GPI anchor attachment. Placental alkaline phosphatase (PLAP) synthesized with a hydrophobic COOH-terminal domain of 17 amino acids is anchored by a GPI anchor, whereas PLAP mutants that have 13 or fewer hydrophobic residues at the COOH-terminus are secreted (*14*). The hGH signal sequence contains a run of 13 hydrophobic amino acids that, in the context of the DAF COOH-terminus, appears to be sufficient to direct the attachment of a GPI anchor. Additional factors therefore may influence the precise minimal length requirement. DAF-Sig2 contains a COOH-terminal extension of five hydrophilic amino acids with an overall negative charge (Gln-Glu-Gly-Ser-Ala). This apparently does not affect processing and attachment of the GPI anchor.

Despite a wide degree of sequence diversity, signal peptides for membrane translocation are recognized by specific protein receptors (*20*). The finding that a secretion signal peptide can function in signaling GPI anchor attachment at the COOH-terminus of a protein suggests that the two processes may be related, mechanistically or evolutionarily. It is conceivable that the NH_2-terminal peptidase and the enzyme that cleaves the COOH-terminus of GPI-linked proteins have evolved from a common precursor. Whether the COOH-terminal signal for GPI attachment interacts with a protein receptor or membrane component, it appears that its overall conformation or character (hydrophobicity, length, secondary, or tertiary structure) rather than specific sequence is important for proper functioning.

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The Effects of Enriched Carbon Dioxide Atmospheres on Plant–Insect Herbivore Interactions

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Little is known about the effects of enriched CO_2 atmospheres, which may exist in the next century, on natural plant–insect herbivore interactions. Larvae of a specialist insect herbivore, *Junonia coenia* (Lepidoptera: Nymphalidae), were reared on one of its host plants, *Plantago lanceolata* (Plantaginaceae), grown in either current low (350 parts per million) or high (700 ppm) CO_2 environments. Those larvae raised on high-CO_2 foliage grew more slowly and experienced greater mortality, especially in early instars, than those raised on low-CO_2 foliage. Poor larval performance on high-CO_2 foliage was probably due to the reduced foliar water and nitrogen concentrations of those plants and not to changes in the concentration of the defensive compounds, iridoid glycosides. Adult pupal weight and female fecundity were not affected by the CO_2 environment of the host plant. These results indicate that interactions between plants and herbivorous insects will be modified under the predicted CO_2 conditions of the 21st century.

BECAUSE OF FOSSIL FUEL CONSUMPTION and tropical deforestation, global atmospheric CO_2 concentrations are rising. The current atmospheric CO_2 level is 350 ppm, and this is expected to reach 700 ppm by the mid- to late 21st century (*1*). In addition to potentially altering global climate (*2*), it is expected that enriched CO_2 atmospheres will influence

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