MEMBRANE-BOUND PROTEINS may be bound to cellular membranes in a number of different ways. Most are “integral”; that is, bound to the lipid portion of the bilayer by stable interactions that are not easily disrupted by changes in pH, ionic strength, or other environmental changes short of disruption of the membrane or the protein structure. These interactions generally involve hydrophobic interactions with the lipid moieties of the bilayer.

This is most commonly accomplished by a sequence of hydrophobic amino acids, commonly 23 to 24 residues, usually preceded and followed by a charged residue. This unit is long enough to span the lipid portion of the bilayer and anchor the entire protein into it. This sequence is part of the primary structure of the protein and is coded by the gene coding for the protein; in some instances, as in the case of the membrane-bound immunoglobulins, this anchor sequence arises by alternative splicing of the primary transcript, converting a protein that is normally secreted to one that remains on the membrane. Such proteins are well-anchored into the membrane by the thermodynamic stability of the hydrophobic interactions.

BINDING OF MEMBRANE PROTEINS BY A GLYCOLIPID ANCHOR

Recently, an alternative method for anchoring proteins to the lipid bilayer has been described for a large number of proteins1 (see Table 1), including the surface glycoproteins of trypanosomes2 and leishmanias,3 a number of immune regulatory proteins such as Thy-1,4 adhesive proteins such as neural cellular adhesion molecule (N-CAM),5 complement regulatory proteins such as decay accelerating factor,6 and membrane enzymes such as acetylcholinesterase7 and alkaline phosphatase.8

In these membrane-bound proteins, the protein itself is not anchored to the membrane by a transmembrane segment of amino acids reacting with the lipid of the bilayer; rather, it is anchored by a glycolipid moiety composed of several parts (Fig 1). The attachment to the lipid bilayer is through a phosphatidylinositol moiety that is inserted into the lipids of the membrane by the fatty acids of a diacyl glycerol.2 In trypanosomes, the fatty acids of this portion of the molecule are exclusively myristate, which provides a handy means of tracing the moiety, as these fatty acids are rarely incorporated into other membrane phospholipids; in mammals, this unusual biochemical finding does not obtain. In humans, one of the fatty acid groups may be an alkyl rather than an acyl moiety.

A variable number of sugars (usually four), the first of which is always non-acetylated glucosamine,9 are attached to this phosphatidylinositol moiety. At least three other hexoses are present; in trypanosomal proteins, these are usually mannoses. The final one is affixed to an ethanolamine moiety through a phosphate group. The carboxyl end of the attached protein is fixed to the amino group of this ethanolamine through an amide bond.10,11

Although there is sufficient similarity between glycan of the human and the trypanosomal form of this anchor, so that antibodies produced against one react with the other,12 there appear to be differences. In the trypanosome, a complex containing galactose residues is attached to the second mannose. In the human anchor for erythrocyte proteins, there may be an additional acyl (palmitoyl) group that is attached to the inositol residue;13 this additional acyl group, present in human erythrocyte acetylcholinesterase, is missing in the bovine anchor of this molecule.14

THE CONSTRUCTION OF PI-LINKED PROTEINS

The assembly of glycolipid-anchor proteins has been studied in the trypanosome; the variable glycoprotein that composes over 90% of the proteins of the external membrane are of this sort. In the endoplasmic reticulum (ER), the glycolipid anchor is synthesized separtaely.15 The first step of this synthesis appears to be the enzymatic addition of an N-acetylated glucosamine molecule to phosphatidylinositol; this is then deacetylated, leaving only the unusual sugar, N-aminoglucose.16 This is then followed by steps that add subsequent sugars (mannoses in the trypanosome) and ethanolamine.15 After the attachment of the protein (see below), a branched galactose-containing moiety may be added to the first mannose (Fig 1A).

The precursor of the protein to be anchored in this way is generated by ribosomes lined up on the external surface of...
that signals an enzyme that almost immediately attaches the ER. The protoprotein contains a hydrophobic segment that signals the enzyme to the anchor. The hydrophobic section that serves to signal the enzyme is of variable length (at least 8-12 amino acids) and is removed. The carboxyl residue of an asparagine molecule that results from the hydrolysis of the peptide bond is immediately attached to the ethanolamine of the preformed bridge; it appears likely that the cleavage and amide bond formation are catalyzed by a single enzyme, but this is not certain. The glycolipid-anchored protein is then processed normally through the Golgi apparatus and eventually appears on the cell surface.

Similar proteins are also described in mammalian systems, where a very similar anchor is made (Fig 1B); the fine structure is known for very few molecules, and there appears to be some variability depending upon the molecule and the cell. In some erythrocyte-derived proteins, an acyl (usually palmitoyl) group is affixed to the inositol moiety; this is inserted into the bilayer and makes difficult the removal of the molecule from the membrane by phosphatidyl inositol-specific phospholipase C. In addition, there may be another ethanolamine molecule whose role is not known.

The synthesis of these proteins in mammals has not been defined in detail but presumably has the same general format as in the trypanosome. The signal sequence for the enzyme appears to be a stretch of hydrophobic amino acids of variable length for different proteins; this segment, when fused along with the adjacent 20 residues to a protein that is usually secreted, results in the fixation of the protein to the membrane by a glycolipid bridge. The precise composition of this segment does not appear to be important since the substitution of one hydrophobic residue for another does not alter the reaction.

In some of these proteins, a single gene appears to result in proteins that are attached by the glycolipid anchor, as well as soluble molecules and molecules attached by the usual transmembrane anchor consisting of hydrophobic amino acids. These alternative forms may arise by differential splicing of mRNA transcripts so that the recognition site for the enzyme is spliced out and a hydrophilic tail (for water-soluble forms) or the membrane-attachment sequence (for membrane-bound forms) is spliced in; the data for these hypotheses is incomplete.

The Function and Metabolism of Glycolipid-Anchored Proteins

Proteins anchored by such a glycolipid bridge have at least two advantages over proteins anchored by a hydrophobic amino acid sequence. They are able to move more readily laterally in the plane of the membrane, in part because they have no intracytoplasmic portion to bind to internal proteins of the cytoskeleton. In fact, they are probably almost as mobile as phospholipids not attached to proteins. This lateral mobility is a great advantage in some cases since small numbers of protein molecules can “patrol” the membrane, as for instance, in the case of decay accelerating factor, where as few as 2,000 molecules are able to downregulate the activation of the convertase enzymes of complement very effectively.

Secondly, such proteins may be easily removed from the membrane by the enzymatic hydrolysis of the anchor. This is catalyzed by enzymes present in all life forms: phosphatidyl inositol-specific phospholipases, often phospholipase C (PIPLC), which removes the inositol from the diacylglycerol moiety. This enzyme has been characterized in the trypanosome and is found to have several hydrophobic sequences, suggesting that it is intimately associated with the membrane.

The factors controlling its activity are not known.

In many cases, the PI-linked proteins of human cells cannot be readily removed by PIPLC from bacterial or protozoan sources. This has been ascribed to differences in the chemical structure of the bridge, particularly an acyl (usually palmitoyl) group attached directly to the inositol. Although PIPLC enzymes have been identified on the cytoplasmic side of the membrane, where they play an important role in a second messenger system, specific

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**Table 1. A Partial List of Proteins Known to Be Linked to the Membrane Through a Glycosyl-Phosphatidylinositol Anchor**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypanosoma variable surface glycoproteins</td>
<td>2</td>
</tr>
<tr>
<td>Leishmania variable surface glycoproteins</td>
<td>3, 70, 71</td>
</tr>
<tr>
<td>Trypanosoma gondii surface antigen P30</td>
<td>72</td>
</tr>
<tr>
<td>Schistosoma mansoni surface antigens</td>
<td>74</td>
</tr>
<tr>
<td>Parmarumum temperature-specific antigens</td>
<td>75</td>
</tr>
<tr>
<td>Plasmodium 195 Kd antigen</td>
<td>1</td>
</tr>
<tr>
<td>N-CAM</td>
<td>5</td>
</tr>
<tr>
<td>Proteosepepin sulfate</td>
<td>76</td>
</tr>
<tr>
<td>Thy-1 (mouse)</td>
<td>4, 77</td>
</tr>
<tr>
<td>LFA-3</td>
<td>48</td>
</tr>
<tr>
<td>CD4 (human)</td>
<td>52</td>
</tr>
<tr>
<td>CD14 (human)</td>
<td>55</td>
</tr>
<tr>
<td>Thocyte surface proteins (2)</td>
<td>79</td>
</tr>
<tr>
<td>RT-6.2 (thymocyte differentiation antigen)</td>
<td>80</td>
</tr>
<tr>
<td>ThB and certain Qa antigens</td>
<td>81</td>
</tr>
<tr>
<td>Complement regulatory proteins</td>
<td>87</td>
</tr>
<tr>
<td>DAF (human)</td>
<td>6</td>
</tr>
<tr>
<td>MIRL (CD55)</td>
<td>40</td>
</tr>
<tr>
<td>C6 binding protein</td>
<td>44</td>
</tr>
<tr>
<td>Alkaline phosphatase (human)</td>
<td>8</td>
</tr>
<tr>
<td>Acetylcholinesterase (human)</td>
<td>12, 13</td>
</tr>
<tr>
<td>Acetylcholinesterase (Drosophila)</td>
<td>82</td>
</tr>
<tr>
<td>Alkaline phosphodiesterase I</td>
<td>83</td>
</tr>
<tr>
<td>Trehalase</td>
<td>84</td>
</tr>
<tr>
<td>Lymphocyte 5′-ectonucleotidase</td>
<td>85</td>
</tr>
<tr>
<td>Kidney microvillus ectoenzymes</td>
<td>85</td>
</tr>
<tr>
<td>Folate binding protein</td>
<td>86</td>
</tr>
<tr>
<td>Neural cell recognition protein</td>
<td>87</td>
</tr>
<tr>
<td>Carcinoembryonic antigen (CEA)</td>
<td>87</td>
</tr>
<tr>
<td>J11d (murine hematopoietic cell marker)</td>
<td>89</td>
</tr>
<tr>
<td>Scrapie prion protein</td>
<td>90</td>
</tr>
<tr>
<td>Placental p34 growth factor</td>
<td>91</td>
</tr>
</tbody>
</table>
mammalian cellular phospholipases active on the PI-linked proteins of the external surface have not been identified. A phospholipase D able to cleave some of these proteins has been found in human serum, but its role in cleaving cell-bound molecules is not known. Some proteins involved in intercellular binding have been found to have glycolipid anchors. Presumably, such binding could be regulated by cleavage of the phosphatidylinositol linkage.

When these proteins are purified with the glycolipid anchor intact (the so-called "membrane" form), they are readily re-incorporated in vitro into the membrane of intact cells by incubation with a very small amount of detergent. Proteins cleaved by PIPLC, and thus lacking the diacylglycerol (the so-called "soluble" form), do not readily incorporate into cells in this way. Presumably, these lipid moieties of the membrane form are able to insert into the lipid bilayer.

THE GLYCOLIPID ANCHORED PROTEINS IN PAROXYSMAL NOCTURNAL HEMOGLOBINURIA

An abnormality in the glycolipid-anchored proteins of the membranes of blood cells has recently been implicated in the pathogenesis of paroxysmal nocturnal hemoglobinuria (PNH). To date, at least nine such proteins have been demonstrated to be missing from the membrane of the abnormal blood cells in this disorder; the lack of these proteins appears to be the cause of the clinical syndrome.

Complement Control Proteins

At least three proteins involved in the regulation of the activation of complement have been found to be lacking on the abnormal cells in PNH: decay accelerating factor (DAF), membrane inhibitor of reactive lysis (MIRL or CD59), and C8 binding protein. All are attached to the membrane by the glycolipid anchor.

Decay accelerating factor. DAF is a glycoprotein of about 70 Kd that belongs to the complement regulator family of proteins. It is composed of four tandem repeats (sequences of about 60 amino acids with a characteristic folded structure) characteristic of the proteins of this family and a segment rich in serine and threonine, and is presumably heavily glycosylated and bears the phospholipid anchor. There may be a soluble non-membrane form that does not bear the anchor and that is generated by differential splicing of the mRNA, bears a hydrophilic sequence on the carboxy terminus.

The function of DAF is the disruption of the convertase complexes of complement (the C4b2a complex of the classical pathway and the C3bBb complex of the alternative pathway). Each of these is an enzymatic complex composed of two molecules noncovalently bound together, and both are responsible for much of the amplification of the activation of the complement cascade. DAF disrupts the bimolecular
complex and, thus, inactivates its amplificatory enzymatic action.

DAF is completely lacking on the most sensitive PNH III cells and is lacking or nearly so on the somewhat less sensitive PNH II cells, there is some evidence that the level may be somewhat low on the normal-like PNH I cells. This results in an increased stability of the convertase complexes, and increased fixation of C5b to the membrane, and increased lysis of the cell in vitro. The life span of these cells in vivo is only slightly shortened, and it is clear that the lack of DAF is not the major cause for the increased susceptibility to hemolysis of the cells in PNH.

Membrane inhibitor of reactive lysis (CD59). Several investigators, particularly Holguin et al., have described a protein of 18 Kd that normally controls the formation of the membrane attack complex, C5b-9. It is not entirely certain at what stage this inhibition occurs, but the result is a diminished initiation of these complexes when complement is activated by either the classical or the alternative pathway. The full structure of this protein is known, and it appears to be related to the Ly6 protein of mouse T cells, a protein that is used in intercellular communication. This protein is PI-linked and is missing on the abnormal PNH cells. Its functional importance for erythrocytes can be assessed from the fact that inhibition of its activity on normal red blood cells by polyclonal antibody results in cells that are nearly as sensitive to lysis by complement as PNH II cells, the most sensitive. The full structure of this protein is known, and it appears to be related to the Ly6 protein of mouse T cells, a protein that is used in intercellular communication. This protein is PI-linked and is missing on the abnormal PNH cells. Its functional importance for erythrocytes can be assessed from the fact that inhibition of its activity on normal red blood cells by polyclonal antibody results in cells that are nearly as sensitive to lysis by complement as PNH II cells, the most sensitive.

PNH II red blood cells (the moderately sensitive variant) appear to have a small amount of CD59 (and DAF) on the external membrane; this small amount appears to regulate C5b-9 formation sufficiently to render these cells less susceptible to complement lysis than PNH III cells. Conversely, the addition of the purified protein to PNH III cells renders them nearly normal in their sensitivity to complement.

PNH III red blood cells (the most sensitive variant) appear to have a small amount of CD59 and DAF on the external membrane; this small amount appears to regulate C5b-9 formation sufficiently to render these cells less susceptible to complement lysis than PNH III cells.

The abnormal PNH platelets and granulocytes appear to lack the 18 Kd MIRL or CD59, and yet the formation of the membrane attack complex is markedly inhibited, with the consequence of this lack are not clear but may be manifest in the control of hematopoiesis that is usually deficient in PNH.

The major Fc receptor (CD16) on neutrophils, called Fc,RII, is PI-linked and is missing in the abnormal cells of PNH. This protein bears the best-known alloantigens of neutrophils, the NA1 and NA2 antigens. The functional consequences of the lack of this protein have not been fully investigated.

CD14 is a protein antigen present on monocytes whose true function is not known; it is PI-linked and is missing in the abnormal PNH monocytes.

Membrane Enzymes

Three enzymes linked by a phosphatidylinositol anchor are missing from the abnormal PNH cells: acetylcholinesterase from erythrocytes, alkaline phosphatase from neutrophils, and 5'-ectonucleotidase from lymphocytes. Although the lack of these proteins is useful in defining the abnormality or origin of the cells, the functional importance of their absence is not clear.

THE DEFECT IN PAROXYSMAL NOCTURNAL HEMOGLOBINURIA

The abnormal cells in paroxysmal nocturnal hemoglobinuria lack the PI-linked proteins, but how this abnormality arises is not clear. It is probably a "somatic mutation"; an abnormality that has occurred in a somatic cell (a stem cell) and is passed to its progeny, as the abnormal cells are clonal in origin. The defect is clearly post-translational since the variety of proteins involved precludes a defective transcription or translation of the missing proteins. Further, the mRNA of DAF from PNH cells is fully expressed. The abnormality may be either a synthetic defect (the inability to preform the anchor, the inability to bind the preformed proteins to the anchor, etc) a catabolic defect (uncontrolled activity of a PIPLC, etc). Recent evidence would suggest that the defect is synthetic. Granulocytes and mononuclear leukocytes from PNH patients made a species of DAF that was 3 Kd smaller than that made by normal cells, that appeared to lack the PI anchor, and that was not inserted into the membrane.

Whatever the underlying defect, the manifestations of PNH are in all probability related directly or indirectly to the lack of these proteins. The lack of the complement control proteins results in the intravascular lysis of the abnormal cells. The unusual susceptibility of the platelets to aggregation in response to thrombin is probably due to the lack of these or other proteins. Finally, the lack of other proteins, possibly those of immune control, probably accounts for the increased incidence of infections and for the deficient hematopoiesis characteristic of the syndrome.

REFERENCES

results in resistance to phosphatidylinositol-specific phospholipase C. J Biol Chem 263:18766, 1988
39. Rosse WF: The life-span of complement-sensitive and
43. Rosse WF, Hoffman S, Campbell M, Borowitz MJ: Complement regulatory proteins on variant erythrocytes in paroxysmal nocturnal hemoglobinuria. (manuscript in preparation)
62. Devine DV, Rosse WF: Deficiency of 5'-ectonucleotidase on the lymphocytes in paroxysmal nocturnal hemoglobinuria. (manuscript in preparation)
74. Pearce EJ, Sher A: Three major surface antigens of Schistosoma mansoni are linked to the membrane by glycosyl-phosphatidylinositol. J Immunol 142:979, 1989
77. Conzelman A, Spiazzi A, Hyman R, Bruch C: Anchoring of
membrane proteins via phosphatidylinositol is deficient in two classes of Thy-1 negative mutant lymphoma cells. EMBO J 5:3291, 1986


86. Lacey SW, Sanders JM, Rothberg KG, Anderson RG, Kamen BA: Complementary DNA for the folate binding protein correctly predicts anchoring to the membrane by glycosylphosphatidylinositol. J Clin Invest 84:715, 1989


Phosphatidylinositol-linked proteins and paroxysmal nocturnal hemoglobinuria

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