

GENERATION AND APPLICATION OF IMMUNOLOGICAL PROBES FOR THE STUDY OF MEMBRANE-BOUND PROTEINS AND ENZYMES — A REVIEW

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ABSTRACT

Immunological techniques are widely used for the detection of enzymes in food systems and for elucidating protein structure and function. The generation of antibodies recognizing membrane-bound proteins is difficult due to their hydrophobic nature, and reduced antigenicity. This review summarizes strategies for the production and purification of polyclonal antibodies recognizing membrane proteins. Applications such as enzyme immunoprecipitation, use of site-specific anti-peptide antibodies for determination of the topology of membrane-embedded proteins, immunocytochemistry, and expression vector cloning are described.

INTRODUCTION

Immunological research methods are widely used to address problems in microbial, plant and muscle systems that would be difficult or impossible to approach by traditional techniques. Antibodies have become essential tools in all phases of protein biochemistry. In 1970 the percentage of articles published in *The Journal of Cell Biology* in which antibodies were used was 1%, while in 1990 that percentage rose to 70% (Drenckhahn *et al.* 1993).

The original methods available for the generation of antibodies were created with hydrophilic, antigenic proteins in mind. Membrane-bound proteins, however,

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are hydrophobic and therefore require different approaches for antibody generation. The biological membrane is an invaluable structure in the cell serving numerous functions, including protection, containment, transport, recognition, and biosynthesis (Gennis 1989). Due to the hydrophobic nature of proteins embedded within the membrane, purification, and subsequently antibody production, have been difficult to achieve. This is especially true with plant-derived membrane proteins, where many important enzymes are limited in abundance (Sussman 1994).

Immunological procedures such as immunocytochemistry and immunoprecipitation are absolutely necessary in order to identify protein complexes associated with the membrane, therefore the generation of polyclonal or monoclonal antibodies against membrane-bound proteins lies at the heart of elucidating membrane structure and function. The objective of this review is to summarize the strategies used to generate polyclonal antibodies to membrane-bound proteins and to illustrate some of the available applications once these antibodies have been produced. While most of the selected examples cited in this review are based upon living systems, the approaches described should serve as important tools for investigating mechanisms of senescence and quality maintenance in postharvest and postmortem food systems (Haard 1995). Some examples of important membrane-bound proteins which occur in edible tissue are summarized in Table 1. Diagnostic membrane-associated marker enzymes used in subcellular fractionation studies to identify plant organelles are listed in Table 2.

Classes of Membrane-Bound Proteins

Proteins and lipids comprise the major components of the biological membranes of living systems. Proteins occur in varying amounts, ranging from about 20% to 80% dry weight (Gennis 1989). Membrane-localized proteins serve as enzymes, transporters, receptors, and pores. Individual membrane proteins are distinguished by their primary structures, as well as by post-translational modifications such as glycosylation (Faye *et al.* 1993; Herscovics and Orlean 1993), prenylation (Maltese 1990) and glycoposphatidylinositol anchoring (Cross 1990; Takeda and Kinoshita 1995).

Membrane-bound proteins are classified as either integral or peripheral. Integral membrane proteins are largely embedded in the membrane and are therefore strongly hydrophobic. Detergents must be used to solubilize integral membrane proteins from the membrane (Hjelmeland and Chrumbach 1984). Integral proteins virtually always contain multiple transmembrane segments, connected by hydrophilic sequences which loop out of the membrane bilayer (Gennis 1989). The number of transmembrane helices and surface loops is determined by hydrophathy analysis, which requires a full-length cDNA clone (Kyte and Doolit-

TABLE 1.
MEMBRANE-BOUND ENZYMES WHICH MAY INFLUENCE FOOD QUALITY

Enzymes Important for Preservation of Cellular and Membrane Integrity

| | |
|--------------------------------|---|
| Aquaporins/MIPs | (Water channels; major intrinsic proteins) |
| ATPases | (V- & P-type H ⁺ -ATPases; metal-activated ATPases) |
| Electron transport proteins | (Cytochromes) |
| Hormone binding receptors | (Ethylene and auxin binding proteins) |
| Ion channel proteins | (Voltage-regulated channels) |
| Lipases and phospholipases | |
| Metal-binding proteins | |
| Phospholipid synthetic enzymes | |
| Protein kinases | (Signal transduction pathway) |
| Sucrose transport proteins | |

Cell Wall Polymer Synthetic Enzymes

| | |
|---------------------------------|--|
| Chitin synthase | Assembly of microbial cell walls |
| (1,3)- β -Glucan synthase | Cell wall assembly in yeast Wound-induced callose formation in plants |

Miscellaneous

| | |
|-------------------------------|---|
| Chloroplast membrane proteins | Plant growth and development |
| Cytochrome P-450 | Detoxification system |
| Ethylene receptor proteins | Signal transduction during fruit ripening |
| Lipoxygenases | Off-flavor formation |

TABLE 2.
DIAGNOSTIC MARKER ENZYMES USED IN SUBCELLULAR FRACTIONATION STUDIES TO IDENTIFY PLANT ORGANELLES

| <u>Organelle</u> | <u>Marker Enzyme</u> |
|---------------------------|--|
| Endoplasmic reticulum | NADH cytochrome c reductase |
| Golgi endomembrane system | Inosine diphosphatase |
| Mitochondria | Cytochrome c oxidase |
| Plasma membrane | (1,3)- β -Glucan synthase Vanadate-sensitive ATPase |
| Tonoplast | Nitrate-sensitive ATPase H ⁺ -Pyrophosphatase |

tle 1982). Peripherally-associated membrane proteins can be hydrophobic or hydrophilic but are not embedded within the membrane. They associate with the membrane by either electrostatic or hydrophobic interactions, or via a hydrophobic "tail" anchored in the membrane, and can be dissociated from the membrane by washing with salts or chaotropic ions (Gennis 1989).

Some membrane-bound proteins, such as plasma membrane H^+ -ATPases (Serrano 1989; Sussman 1994), members of the major intrinsic protein (MIP) family (Chrispeels and Maurel 1994; Agre *et al.* 1995; King and Agre 1996; Verkman *et al.* 1996) and the vacuolar H^+ -pyrophosphatase of higher plants (Rea *et al.* 1992; Kim *et al.* 1994) exist as individual subunits or multisubunit complexes embedded largely within the membrane. On the other hand, large multisubunit complexes such as the chloroplast ATPase (Feng and McCarty 1990a; Feng and McCarty 1990b; Avital and Gromet-Elhanan 1991), chloroplast photosystems (Cohen *et al.* 1995), coated vesicle ATPase (Arai *et al.* 1988; Adachi *et al.* 1990a; Adachi *et al.* 1990b) and tonoplast ATPase of plants (Rea *et al.* 1987; Lai *et al.* 1988; Parry *et al.* 1989; Sze *et al.* 1992) and fungi (Bowman *et al.* 1989; Moriyama and Nelson 1989) contain a combination of integral and peripheral subunits, and can be visualized by "ball and stalk-like" protrusions from the membrane (Dschida and Bowman 1992; Getz and Klein 1995). Examples of well-characterized peripherally-bound proteins would include the plasma membrane GTP-binding subunits (Rho 1p) of yeast (1,3)- β -glucan synthases (Drzonova *et al.* 1996; Qadota *et al.* 1996), and a 62-kDa sucrose binding protein associated with the external surface the plasma membrane of soybean (Overvoorde and Grimes 1994; Overvoorde *et al.* 1996).

Since membrane-bound proteins are often large multisubunit enzyme complexes, it is often necessary to dissociate the complex and generate antibodies against individual subunits before immunological studies can be conducted. The presence of detergent to prevent aggregation and to maintain the protein in a solubilized state is necessary at all times when preparing antigen, as well as the appropriate reducing agent to maintain dissociated subunits. Oftentimes, however, antibodies produced against unfolded or partially unfolded membrane proteins do not recognize target proteins in their native-folded conformations. In these cases, uses of the antibodies is generally limited to diagnostic analysis using immunoblots.

Sources of Antigens

Native Purified Protein. Proteins used for immunization must be of high purity to avoid generation of antibodies against contaminating antigens. An adjuvant is added to the protein, which acts to stimulate the immune response nonspecifically. The antigen, coupled to a carrier protein, in the adjuvant mixture is injected into an animal, most often a rabbit. To obtain the antibody, the animal is bled at various intervals and serum is then purified (Harlow and Lane 1988; Dunbar and Schwoebel 1990).

This procedure has proven successful for an increasing number of membrane-bound proteins found in the tonoplast, or vacuolar membrane. For example, antibody was raised against a purified preparation of a 23-kDa major integral protein of the vacuolar membrane (VM 23) of radish (Maeshima 1992). This same group previously produced an antibody raised in rabbit against total peak chromatographic fractions of a 73-kDa H^+ -translocating inorganic pyrophosphatase found in the vacuolar membrane of mung bean hypocotyl tissue (Maeshima and Yoshida 1989). Working with maize tonoplast H^+ -ATPase, Mandala and Taiz (1986) prepared antisera using partially purified ATPase from sucrose gradient centrifugation peak fractions.

In the case of membrane-bound proteins the transmembrane domain contains hydrophobic residues which reduce antigenicity, deeming successful production of antibody against an intact hydrophobic protein unlikely. Separate studies conducted by Dr. Emil R. Unanue and Dr. Howard M. Grey have led to the discovery of antigen processing, whereby extracellular proteins must first be ingested and fragmented into peptides by an antigen-presenting cell before an immune response can occur (Engelhard 1994). Consequently, it should be possible to carry out peptide digestion with an insoluble enzyme such as trypsin attached to polyacrylamide beads in order to improve antigenicity. Short peptides containing the hydrophilic loop portions of the protein may be produced during trypsinization. Since the trypsin is insoluble, it can be centrifuged, pelleted, and removed, leaving only the fragmented protein. Trypsinized peptides can then be used as an antigen for immunization. This approach was used to generate antibodies recognizing plasma membrane intrinsic proteins from *Beta vulgaris* L. (Wasserman *et al.* 1996).

SDS-PAGE Gel Slices. If an antigen is not available in pure form, it can be purified further by separation on denaturing polyacrylamide gels, excised and electroeluted. Antigens purified in this way often induce good immunological responses. The resulting antibody is useful for techniques that need or benefit from denaturation-specific antibodies (Harlow and Lane 1988).

Several different approaches exist for injection of protein from gel slices. After an SDS gel is run, the band representing the protein of interest is cut out. The gel is then fragmented by passing it repeatedly through a syringe, emulsified with adjuvant, and injected into the animal as with the native protein (Harlow and Lane 1988). This procedure works only with larger animals, such as rabbits. In order to prepare antibodies to maize tonoplast ATPase, Coomassie-stained gel slices of both 62 kDa and 72 kDa proteins were excised, crushed with buffer in a glass homogenizer, and then mixed with Freund's adjuvant and injected into New Zealand White rabbits (Mandala and Taiz 1986).

In another procedure gel slices are dried, ground into a powder, and then resuspended for injection (Harlow and Lane 1988), as was done with a 62-kDa

band corresponding to a membrane protein associated with sucrose transport in soybean (Ripp *et al.* 1988).

Proteins can also be electroeluted from the gel in the presence of reducing agent as a further purification step and used to immunize the host animal (Harlow and Lane 1988). This method has been utilized for the generation of antibodies to several membrane-bound proteins, such as TP 25, an abundant vacuolar integral membrane protein from soybean (Johnson *et al.* 1989), peroxisome membrane proteins in oilseed glyoxysomes (Corpas *et al.* 1994), and a 43 kDa polypeptide located in heart gap junctions (Yancey *et al.* 1989).

Site-Specific Anti-Peptide Antibodies. As more DNA sequences and their corresponding protein sequences have become available due to technological advances such as peptide sequencing and PCR, synthetic or microbially-expressed peptides are now increasingly used. These antibodies can be targeted against specific domains of a protein. Thus, many previously uncharacterized proteins are now amenable to topological analysis. These peptides are synthesized by Merrifield solid-phase techniques, or produced genetically. Once obtained, the purified antigens can be coupled to carrier proteins, and injected into an animal. An antibody produced in this manner is termed an anti-peptide antibody (Harlow and Lane 1988).

To carry out this procedure, a synthetic peptide of at least ten residues must be constructed. The longer the peptide, the greater the chance of obtaining an antibody which reacts with the native protein. A peptide synthesized towards the N- or C-terminal end of a protein sequence is particularly immunogenic and will usually produce antibodies recognizing the native protein (Drenckhahn *et al.* 1993). Several systems utilizing peptides with polyhistidine tails to facilitate purification by Ni^{2+} -affinity chromatography prior to immunization are available commercially.

Several site-specific antibodies to membrane-bound proteins have been raised against either the N- or C-terminus of membrane proteins, including glycerol intrinsic protein (GLIP) (Ma *et al.* 1994), aquaporin 3 (AQP-3) from kidney collecting duct cells (Ishibashi *et al.* 1994), nodulin 26 (NOD26) from nitrogen-fixing soybean nodules (Weaver *et al.* 1991; Weaver *et al.* 1994) and channel-forming integral membrane protein of 28 kDa (CHIP28) (AQP-1) from erythrocytes (Denker *et al.* 1988; Smith and Agre 1991).

Two important advantages of site-specific anti-peptide antibodies are that they can be prepared immediately after determining the amino acid sequence of a protein, and that particular regions of a protein can be targeted specifically for antibody production. A number of disadvantages pertaining to use of site-specific anti-peptide antibodies should be noted. First, they may not recognize the native protein if certain domains are deeply embedded in the membrane and inaccessi-

ble to the antibody. This means that they may be unable to immunoprecipitate enzyme activity and can therefore be used only for immunoblotting. In addition, due to their small size, the peptides may not possess sufficient immunogenicity to induce a satisfactory immune response. In this case, they must be coupled to carrier proteins (Harlow and Lane 1988).

An example of a synthetic peptide coupled to a carrier protein before immunization is P40, a synthetic peptide corresponding to amino acid residues 40–50 of the beef heart ADP/ATP carrier from mitochondria (Marty *et al.* 1992). This peptide was synthesized by the Merrifield solid-state technique and coupled to an ovalbumin carrier protein prior to injection. Similarly, a peptide corresponding to nine amino acid residues of a K^+ -conducting protein in chloroplasts (Mi *et al.* 1994) was synthetically conjugated to maleimide-activated keyhole limpet hemacyanin (Tsao *et al.* 1991).

Bacterial Overexpression Vectors. When antibodies are needed against a protein for which a cloned gene is available, bacterial overexpression systems represent an excellent antigen source. Coding regions may be expressed in bacteria either alone or as fusion proteins. Vectors producing glutathione *S*-transferase, β -galactosidase or tryptophan fusion proteins are the most commonly used. Several vectors are commercially available for this purpose.

An antibody was raised against tonoplast intrinsic protein (TIP) from *Arabidopsis thaliana* using a glutathione *S*-transferase- γ -TIP fusion construct introduced into *Escherichia coli* (Hofte *et al.* 1992). This culture was then grown, and the fusion protein isolated and purified by centrifugation and gel electrophoresis. Electroeluted protein from SDS polyacrylamide gels served as antigen, and antiserum was prepared in rabbit. Antibodies against water channel proteins in the *A. thaliana* plasma membrane (PIP1 and PIP2) were generated from a fragment of PIP1a encoding the 42 N-terminal amino acids cloned into a plasmid (Kammerloher *et al.* 1994). This fusion protein was then used to affinity-purify specific anti-PIP1 antibodies from the anti-plasma membrane serum, and to characterize the subcellular location of the PIP proteins.

Affinity Purification. Crude immune serum often contains contaminating antibodies which may cross-react with proteins other than the targeted antigen. Ammonium sulfate precipitation is one of the most common methods for partial purification of antibodies (Harlow and Lane 1988; Dunbar and Schwoebel 1990). Saturated ammonium sulfate is added to a batch volume of antiserum while stirring. The mixture is then centrifuged, and the pellet resuspended and dialyzed to remove the salt. This method does not yield an antibody of high purity, but is convenient for large volumes.

Affinity columns may be used to yield pure antibodies. The crude antiserum

is passed through a column to which is bound protein A-, antigen-, or anti-immunoglobulin-coupled beads. Elution occurs at low pH. The diluted purified serum is then concentrated and stored in sodium azide (Harlow and Lane 1988).

Blot-affinity purification is a simple method to rid serum of contaminating antibodies (Olmsted 1981; Smith and Fisher 1984; Mishkind *et al.* 1987; Tang 1993). This method utilizes antigen-containing nitrocellulose strips to capture and purify antibodies recognizing a specific polypeptide. In the first step purified antigen is run on SDS-PAGE. Following electrotransfer to nitrocellulose, horizontal strips corresponding to desired antigens are excised from the membrane. Antibodies are then captured, eluted and neutralized. A desirable feature of the blot-affinity purification procedure is that both crude serum and the nitrocellulose strips are reusable.

Applications

Once specific antibodies have been produced against a membrane-bound protein, they can be used: (1) To associate an integral membrane protein with a corresponding enzymatic activity by immunoprecipitation. (2) To probe membrane topology using vesicles of defined sidedness. (3) To determine the subcellular localization of a protein using immunocytochemistry. (4) To probe cDNA expression libraries to immunoselect clones encoding these proteins. Use of preimmune serum as a control to ensure against nonspecific binding and thus artifactual results is imperative to any of these applications.

Immunoprecipitation. Immunoprecipitation has various uses, including the determination of the molecular mass of an antigen, characterization of the specificity of an antibody, and identification of molecules associated with antigens (Otto and Lee 1993). In principle, an enzyme preparation is incubated with antiserum generated against the polypeptide of interest. If the antigen-antibody complex cannot be pulled down by centrifugation alone, a secondary agent which has affinity for IgG, such as protein A coupled to agarose beads, is added to effect precipitation. Supernatants are then assayed to detect any loss in enzyme activity, or the immunoprecipitates are then analyzed for a dose-dependent increase in the corresponding protein band.

The use of polyclonal antibodies is advantageous in that they bind to multiple sites on the antigen and provide multiple binding sites for protein A molecules, which effects precipitation of antigen-antibody complexes. These multiple interactions provide an extremely stable complex (Harlow and Lane 1988). However, in the case of membrane proteins, it must be ensured that the protein remains solubilized. If the protein were to aggregate in the absence of adequate detergent, it could be pelleted during the centrifugation step of the reaction and falsely show

a loss in activity. Effective solubilizing agents for each membrane-bound protein differ, necessitating a sound characterization of the enzyme system. Furthermore, membrane proteins tend to be very "sticky" and may bind nonspecifically to the protein A-agarose. Therefore, proper control samples must be prepared to ensure the protein is precipitated due to antigen-antibody interactions.

Once the immunoprecipitation reaction has occurred, pellet fractions can be analyzed by SDS-PAGE. Potato membrane protein pp34 was immunoprecipitated using protein A-Sepharose in Nonidet P-40 buffer and the immunoadsorbed proteins were analyzed using SDS-PAGE to visualize the presence of the 34-kDa band (Jacinto *et al.* 1993). *Neurospora crassa* plasma membrane H⁺-ATPase activity was neutralized using protein A-Sepharose, but in Triton X-100 buffer (Lin and Addison 1994). Fusion proteins corresponding to different transmembrane segments of the ATPase were incubated with polyclonal antibodies directed against either the N- or C-terminus. Immunoprecipitation and subsequent SDS-PAGE analysis enabled the authors to discern that the carboxyl terminus was extracytoplasmic and the amino terminus was on the cytoplasmic side of microsomal membranes.

The effects of immunoprecipitation can also be quantified by assaying an aliquot of the supernatant after centrifugation and observing a significant decrease in enzyme activity. Glucan synthase II probed with antibodies to 55 and 70 kDa polypeptides showed a decrease in activity at increasing concentrations of IgG (Dhugga and Ray 1994). Antibodies raised against partially purified (1,3)- β -D-glucan synthase from *Saccharomyces cerevisiae* were able to immunoprecipitate enzyme activity and react with a 200-kDa polypeptide on Western blots, implicating the 200-kDa polypeptide as a subunit of yeast glucan synthase (Inoue *et al.* 1995).

Simple addition of antisera to an assay mixture without performing the precipitation reaction is another way to observe the binding of antibodies to an enzyme. An aliquot of antibody to maize tonoplast ATPase was incubated with purified ATPase, then assayed for activity. A dose-dependent neutralization of activity was observed (Mandala and Taiz 1986).

Membrane Topology. Membrane vesicles of defined sidedness produced by freeze-thaw techniques can be useful for the determination of membrane topology. Antibodies generated against a specific region of the protein are incubated with either inside-side out (IOV) or right-side out vesicles (ROV) to localize a protein domain to a defined region on the membrane. This procedure has been used to identify the orientation of both the N- and C-termini (Botfield and Wilson 1989; Kerppola and Ames 1992) of membrane proteins. In addition, limited proteolysis of membrane vesicles followed by analysis of digestion products by immunodetection (Marty *et al.* 1992) can similarly identify membrane topology.

Figure 1 illustrates the use of limited proteolysis to probe the topology of plasma membrane intrinsic proteins (PMIPs) in isolated vesicles from red beet storage tissue. In an earlier study, plasma membrane proteins of 31- and 27-kDa were established as members of the major intrinsic protein (MIP) superfamily (Qi *et al.* 1995). To establish if these proteins were proteolyzed similarly or differentially, affinity purified antibodies to each species were produced. This study enabled the identification of specific fragmentation products derived from PMIPs 31 and 27 following hydrolysis by Pronase E (Fig. 1). To examine the effect of partially permeabilizing the vesicles, protease digestions were conducted in the absence and presence of digitonin.

Several key observations are noted. First, PMIP31 was resistant to proteolysis in the absence of detergent (Fig. 1A, lanes 2, 4 and 6). In contrast, PMIP27 formed a digitonin-independent proteolytic fragment of 25 kDa (Fig. 1B, lanes 2, 4 and 6), and additional fragments at 20 and 21 kDa. Secondly, addition of digitonin caused a dose-dependent decrease in PMIP31, with its eventual complete disap-

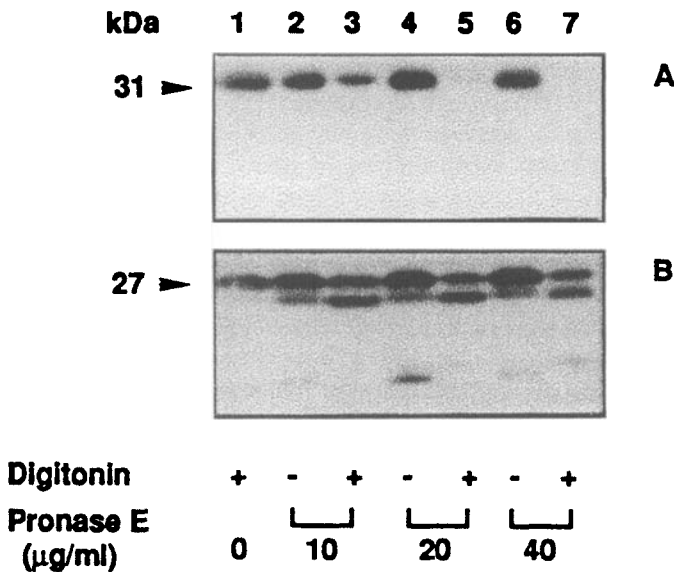


FIG. 1. LIMITED PROTEOLYSIS OF PLASMA MEMBRANE VESICLES FROM RED BEET STORAGE TISSUE

Plasma membrane vesicles (18 µg) prepared by aqueous two-phase partitioning were digested with Pronase E (0–40 µg mL⁻¹) in the absence or presence of 0.1% digitonin. After SDS-PAGE, gels were electrotransferred to nitrocellulose and probed with either affinity purified anti-PMIP31 (Panel A) or anti-PMIP27 (Panel B) at a dilution of 1:2000. Blots were developed by enhanced chemiluminescence (ECL, Amersham, Arlington Heights, IL).

pearance at $40 \mu\text{g mL}^{-1}$ of Pronase E (Fig. 1A, lanes 3, 5 and 7). These results demonstrated that PMIPs 31 and 27 each yield unique proteolytic fragmentation patterns and are either structurally distinct, or that they each reside in specialized domains of the plasma membrane.

The topology of fungal plasma membrane H^+ -ATPase was investigated using limited proteolysis of sided vesicles. The N- and C-termini of the *N. crassa* plasma membrane H^+ -ATPase were determined to be cytoplasmically located by trypsinizing reconstituted cytoplasmic-side out proteoliposomes (Hennessey and Scarborough 1990). In *S. cerevisiae*, the binding of C-terminal antibodies to H^+ -ATPase in 60% ROVs increased in the presence of Tween-20 at low levels (Monk *et al.* 1991). These results confirmed the cytoplasmic orientation of the C-terminus.

To investigate the topology of the melibiose carrier of *E. coli*, membrane vesicles were incubated with an antibody (anti-MBct 10) generated against a synthetic peptide corresponding to the C-terminus of the melibiose carrier (Botfield and Wilson 1989). Proposed models had suggested the presence of either ten or eleven transmembrane domains, causing uncertainty as to the orientation of the C-terminus. After incubation with anti-MBct 10, membrane suspensions were centrifuged and pellets were resuspended in TBS buffer containing [^{125}I]-protein A. Following a 2 h incubation, the mixtures were again centrifuged and pellets washed in buffer. Radioactivity was quantified, and it was found that only IOVs bound anti-MBct 10, suggesting a cytoplasmic orientation of the C-terminus and supporting a topological model for the melibiose carrier with an even number of transmembrane domains.

Antibodies raised against the C-termini of hydrophobic components (HisQ and HisM) of the histidine periplasmic permease were incubated with ROVs and IOVs and their binding measured radioactively, using [^{125}I] (Kerppola and Ames 1992). The antisera preferentially bound to IOVs, exhibiting binding to ROVs only 10–20% of the level of IOV binding. These results indicate that HisQ and HisM are located on the cytoplasmic side of the membrane.

The topology of the beef heart ADP/ATP carrier in the inner mitochondrial membrane was explored using proteolysis and immunodetection of inside-out submitochondrial particles and mitoplasts, whose inner membranes are in the right-side-out conformation (Marty *et al.* 1992). These mitochondrial particles were digested with a range of proteases. The cleavage products were separated by SDS-PAGE, transferred to nitrocellulose and probed with antibodies raised against a peptide corresponding to the N-terminal point of the ADP/ATP carrier chain. The membrane-bound carrier in mitochondria devoid of an outer membrane (mitoplasts) reacted with the antibodies, while inside out submitochondrial particles did not. This suggests that the N-terminus of the protein is exposed to the cytosol.

The membrane topology of aquaporin-1 (AQP-1) was assessed using limited proteolysis of *Xenopus laevis* oocyte membranes in an inside-out conformation (Preston *et al.* 1994). A proposed model had suggested the presence of six transmembrane domains with five connecting loops (Preston and Agre 1991). An epitope from the coronavirus E1 glycoprotein was inserted into AQP-1 at various locations on the loops. Its specific orientation was determined by proteolytic digestion of intact oocytes or inside-out membrane vesicles. Membranes from oocytes expressing wild-type AQP-1 or an E1 epitope-tagged AQP-1 were isolated and digested with α -chymotrypsin. Results were analyzed using SDS-PAGE and immunoblotting with an anti-N-terminal peptide. By observing which epitope-tagged regions were protease sensitive, it was found that both the N- and C-termini of AQP-1 were intracellular (Smith and Agre 1991; Nielsen *et al.* 1993), indicating that three connecting loops are extracellular and two are cytoplasmic.

Immunocytochemistry. Cells can be incubated with antibodies and visualized using electron or light microscopy in order to determine the presence and subcellular localization of membrane constituents. Briefly, the tissue to be stained is embedded into a solid support, cells are fixed by cross linking, and permeabilized to allow free access to labeled antibodies. After incubation with antibodies and removal of unbound antibody, labeled tissue is visualized (Harlow and Lane 1988).

Two methods of immunostaining are immunogold labeling and immunofluorescence. Gold staining allows for high resolution, is permanent and can be visualized with either a light or electron microscope. Photochemical silver amplification must be used for high sensitivity at the light microscopy level. Colloidal gold particles bind tightly to proteins at pH values close to the pI. Gold particles are conjugated to anti-immunoglobulin antibodies, protein A, or streptavidin (Harlow and Lane 1988).

Immunogold electron microscopy was used to determine the distribution of acyl carrier protein (ACP) in *E. coli* and plant tissues (Slabas and Smith 1988). Antibody-labeled tissue was detected using goat anti-rabbit/colloidal gold. ACP was localized to the cytoplasm of *E. coli* and not to membranes. However, in rape leaf 80% of ACP was localized to the chloroplast thylakoid membrane.

The subcellular localization of a tonoplast integral membrane protein of 25 kDa (TP 25) from soybean was investigated using immunogold labeling (Johnson *et al.* 1989). Tonoplast membranes, but not the vacuolar matrix, were heavily labeled with gold particles following incubation with TP 25 antiserum and subsequent treatment with a colloidal-gold-coupled secondary antibody.

Immunolocalization of the plasma membrane H^+ -ATPase in leaves of broad bean was determined using colloidal gold particles (Bouche-Pillon *et al.* 1994). The antibody used for incubation was generated against an isoform of the *A. thaliana* H^+ -ATPase expressed in *E. coli*. The density of gold particles was

greatest on the plasma membrane of transfer cells and least dense on the plasma membrane of sieve tube, phloem parenchyma cells, and bundle sheath cells, localizing the proton pump to transfer cell membranes.

Immunofluorescence has the advantage of high resolution and the possibility to stain live cells. However, a specially equipped microscope is required and the signal is lost over time (Harlow and Lane 1988). In addition, this technique is incompatible with most histochemical stains, which autofluoresce. Fluorochromes can be conjugated to the same reagents as colloidal gold particles. AQP-3, a water channel of kidney collecting ducts, has been found to transport urea and glycerol in addition to water (Ishibashi *et al.* 1994). An antibody raised against a synthetic peptide corresponding to the C-terminus of AQP-3 was incubated with sections of rat kidney fixed onto slides. After treatment with a fluorochrome conjugate and visualization, staining was detected only at the basolateral membranes of collecting duct cells and not in other nephron segments.

Immunofluorescence has been widely employed to investigate the subcellular localization of membrane-bound proteins. A glycerol intrinsic protein (GLIP) was localized to the basolateral membrane of kidney collecting duct cells and brain meningeal cells by immunofluorescence techniques (Ma *et al.* 1994). The polyclonal GLIP antibody was generated against a synthetic C-terminal peptide. The membrane topology of the (Na^+ , K^+)-ATPase was investigated by epitope-tagging the N- and C-termini of both the α - and β subunits (Yoon and Guidotti 1994). The orientation of the epitopes was determined by immunofluorescence with cells expressing the (Na^+ , K^+)-ATPase. The localization of the C-terminus of the yeast plasma membrane H^+ -ATPase to the cytoplasmic side of the membrane was determined by immunofluorescence of isolated plasma membrane vesicles (Monk *et al.* 1991). The antibody was prepared against the C-terminus of the yeast ATPase.

Immunoselection. Antibodies can be used as probes to screen cDNA expression libraries when conventional cloning methods are unsuccessful. Those antibodies that recognize a short linear sequence in both unfolded and folded structures are most useful in this technique (Obar and Holzbaaur 1993). Either bacterial (Zimniak *et al.* 1988; Sarafian *et al.* 1992) or mammalian (Kammerloher *et al.* 1994) expression systems can be employed. Following primary and secondary screening, positive clones are identified, isolated and characterized.

The carrot vacuolar H^+ -ATPase contains a 69 kDa subunit which is closely related to the β -chain of F_0F_1 -ATPases and is thought to contain the catalytic site (Zimniak *et al.* 1988). A carrot root λ gt11 cDNA library was screened with antibodies generated against the purified 72-kDa subunit of the tonoplast H^+ -ATPase of corn coleoptiles. Five clones were isolated and reacted positively with antibody to the native as well as to the denatured enzyme. These positive

clones were plaque affinity purified and the eluted antibodies used for immunodetection of corn tonoplast proteins. The antibodies for three different positive clones specifically bound to the 70-kDa band on Western blots. The clones were then sequenced and it was confirmed that the 70-kDa subunits of plant, fungal and vacuolar ATPases are highly conserved.

To clone cDNAs encoding the *A. thaliana* tonoplast H⁺-translocating inorganic pyrophosphatase (PPase), an *A. thaliana* cDNA expression library in λ ZAP was screened with affinity-purified antibodies generated against tonoplast H⁺-PPase purified from etiolated mung bean hypocotyls (Sarafian *et al.* 1992). A primary partial clone was obtained and then used to screen a cDNA library constructed in λ gt10 for the isolation of a near-full-length clone. Sequence information of the tonoplast H⁺-PPase revealed no homology with other characterized ion translocases, suggesting a novel category of H⁺-translocase.

Bacterial expression systems often do not provide correct folding and high expression levels of hydrophobic proteins. Therefore, mammalian COS cell expression systems have been developed when working with integral plasma membrane proteins. In order to identify genes encoding plant integral plasma membrane proteins, COS cells were transfected using a DEAE-dextran method with an *A. thaliana* root cDNA library (Kammerloher *et al.* 1994). Antibodies raised against purified deglycosylated integral plasma membrane proteins from *A. thaliana* roots were used to screen the mammalian library. Sequence analysis of positive clones revealed several genes encoding a family of five related proteins. Antibodies immunopurified against a 27-kDa plasma membrane antigen detected each of the five proteins when individually expressed in COS cells. These proteins were determined by sequence comparisons to belong to the MIP superfamily.

SUMMARY

Due to their hydrophobicity and complexity, membrane-bound proteins, require distinct methods of purification and characterization. The application of immunological techniques is the best way to unequivocally identify the subcellular localization, membrane topology and association with enzyme activities. In addition, antibodies may be used as probes to screen cDNA libraries when traditional methods are unsuccessful. The most important aspect of using an immunological approach to a problem is the generation of an antibody which is specific, free of contaminants and of high titer. Once pure antibodies have been obtained against either an integral membrane protein or specific regions of the protein, a myriad of studies including immunoaffinity purification, expression-vector cloning and immunolocalization, can be conducted.

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REFERENCES

- ADACHI, I., ARAI, H., PIMENTAL, R. and FORGAC, M. 1990a. Proteolysis and orientation on reconstitution of the coated vesicle proton pump. *J. Biol. Chem.* **265**, 960-966.
- ADACHI, I., PUOPOLO, K., MARQUEZ-STERLONG, N., ARAI, H. and FORGAC, M. 1990b. Dissociation, cross-linking, and glycosylation of the coated vesicle proton pump. *J. Biol. Chem.* **265**, 967-973.
- AGRE, P., BROWN, D. and NIELSEN, S. 1995. Aquaporin water channels: Unanswered questions and unresolved controversies. *Curr. Opinion Cell Biol.* **7**, 472-483.
- ARAI, H., TERRES, G., PINK, S. and FORGAC, M. 1988. Topography and subunit stoichiometry of the coated vesicle proton ATPase. *J. Biol. Chem.* **263**, 8796-8802.
- AVITAL, S. and GROMET-ELHANAN, Z. 1991. Extraction and purification of the β -subunit and an active $\alpha\beta$ -core complex from the spinach chloroplast CF_0F_1 -ATP synthase. *J. Biol. Chem.* **266**, 7067-7072.
- BOTFIELD, M.C. and WILSON, T.H. 1989. Peptide-specific antibody for the melibiose carrier of *Escherichia coli* localizes the carboxyl terminus to the cytoplasmic face of the membrane. *J. Biol. Chem.* **264**, 11649-11652.
- BOUCHE-PILLON, S., FLEURAT-LESSAR, P., FROMONT, J., SERRANO, R. and BONNEMAIN, J. 1994. Immunolocalization of *Vicia faba* in relation to phloem loading. *Plant Physiol.* **105**, 691-697.
- BOWMAN, B.J., DSCHIDA, W.J., HARRIS, T. and BOWMAN, E.J. 1989. The vacuolar ATPase of *Neurospora crassa* contains an F_1 -like structure. *J. Biol. Chem.* **264**, 15606-15612.
- CHRISPEELS, M.J. and MAUREL, C. 1994. Aquaporins: The molecular basis of facilitated water movement through living plant cells? *Plant Physiol.* **105**, 9-13.
- COHEN, Y., YALOVSKY, S. and NECHUSHTAI, R. 1995. Integration and assembly of photosynthetic protein complexes in chloroplast thylakoid membranes. *Biochim. Biophys. Acta* **1241**, 1-30.

- CORPAS, F.J., BUNKELMANN, J. and TRELEASE, R.N. 1994. Identification and immunochemical characterization of a family of peroxisome membrane proteins (PMPs) in oilseed glyoxysomes. *Eur. J. Cell Biol.* 65, 280-290.
- CROSS, G.A.M. 1990. Glycolipid anchoring of plasma membrane proteins. *Ann. Rev. Cell Biol.* 6, 1-39.
- DENKER, B.M., SMITH, B.L., KUHAJDA, F.P. and AGRE, P. 1988. Identification, purification and partial characterization of a novel M_r 28,000 integral membrane protein from erythrocytes and renal tubules. *J. Biol. Chem.* 263, 15634-15642.
- DHUGGA, K.S. and RAY, P.M. 1994. Purification of 1,3- β -D-glucan synthase activity from pea tissue: Two polypeptides of 55 kDa and 70 kDa copurify with enzyme activity. *Eur. J. Biochem.* 220, 943-953.
- DRENCKHAHN, D., JONS, T. and SCHMITZ, F. 1993. Production of polyclonal antibodies against proteins and peptides. Ch. 2. In *Antibodies in Cell Biology*. Vol. 37 (D.J. Asai, ed.) pp. 8-54, Academic Press, San Diego, CA.
- DRGONOVA, J., DRGON, T., TANAKA, K., KOLLAR, R., CHEN, G.C., FORD, R.A., CHAN, C.S.M., TAKAI, Y. and CABIB, E. 1996. Rho 1p, a yeast protein at the interface between cell polarization and morphogenesis. *Science* 272, 277-279.
- DSCHIDA, W.J. and BOWMAN, B.J. 1992. Structure of the vacuolar ATPase from *Neurospora crassa* as determined by electron microscopy. *J. Biol. Chem.* 267, 18783-18789.
- DUNBAR, B.S. and SCHWOEBEL, E.D. 1990. Preparation of polyclonal antibodies. *Meth. Enzymol.* 182, 663-670.
- ENGELHARD, V.H. 1994. How cells process antigens. *Sci. Am.* 271(2), 54-61.
- FAYE, L., FITCHETTE-LAINE, A.C., GOMORD, V., CHEKKAFI, A., DELAUNAY and DRIOUICH, A. 1993. Detection, biosynthesis and some functions of glycans, N-linked to plant secreted proteins. In *Post-Translational Modifications in Plants*, (N.H. Battey, H.G. Dickinson and A.M. Hetherington, eds.) pp. 213-242, Cambridge Univ. Press, Cambridge, UK.
- FENG, Y. and MCCARTY R.E. 1990a. Chromatographic purification of the chloroplast ATP Synthase (CF_0 - CF_1) and the role of the CF_0 subunit IV in proton conduction. *J. Biol. Chem.* 265, 12474-12480.
- FENG, Y. and MCCARTY, R.E. 1990b. Subunit interaction within the chloroplast ATP synthase (CF_0 - CF_1) as deduced by specific depletion of CF_0 polypeptides. *J. Biol. Chem.* 265, 12481-12485.
- GENNIS, R.B. 1989. *Biomembranes: Molecular Structure and Function*. pp. 1-35, Springer-Verlag, New York.
- GETZ, H.P. and KLEIN, M. 1995. The vacuolar ATPase of red beet storage tissue: Electron microscopic demonstration of the "head-and-stalk" structure. *Bot. Acta* 108, 14-23.

- HAARD, N.F. 1995. Foods as cellular systems: Impact on quality and preservation. A review. *J. Food Biochemistry* 19, 191-238.
- HARLOW, E. and LANE, D. 1988. *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- HENNESSEY, J.P. and SCARBOROUGH, G.A. 1990. Direct evidence for the cytoplasmic location of the NH₂- and COOH-terminal ends of the *Neurospora crassa* plasma membrane H⁺-ATPase. *J. Biol. Chem.* 265, 532-537.
- HERSCOVICS, A. and ORLEAN, P. 1993. Glycoprotein biosynthesis in yeast. *FASEB J.* 7, 540-550.
- HJELMELAND, L.M. and CHRAMBACH, A. 1984. Solubilization of functional membrane proteins. *Meth. Enzymol.* 104, 305-308.
- HOFTE, H., HUBBARD, L., REIZER, J., LUDEVID, D., HERMAN, E.M. and CHRISPEELS, M.J. 1992. Vegetative and seed-specific forms of tonoplast intrinsic protein in the vacuolar membrane of *Arabidopsis thaliana*. *Plant Physiol.* 99, 561-570.
- INOUE, S.B., TAKEWAKI, N., TAKASUKA, T., MIO, T., ADACHI, M., FUJII, Y., MIYAMOTO, C., ARISAWA, M., FURICHI, Y. and WATANABE, T. 1995. Characterization and gene cloning of 1,3- β -glucan synthase from *Saccharomyces cerevisiae*. *Eur. J. Biochem.* 231, 845-854.
- ISHIBASHI, K., SASAKI, S., FUSHIMI, K., UCHIDA, S., KUWAHARA, M., SAITO, H., FURUKAWA, T., NAKAJIMA, K., YAMAGUCHI, Y., GOJOBORI, T. and MARUMO, F. 1994. Molecular cloning and expression of a member of the aquaporin family with permeability to glycerol and urea in addition to water expressed at the basolateral membrane of kidney collecting duct cells. *Proc. Natl. Acad. Sci. USA* 91, 6269-6273.
- JACINTO, T., FARMER, E.E. and RYAN, C.A. 1993. Purification of potato leaf plasma membrane protein pp34, a protein phosphorylated in response to oligogalacturonide signals for defense and development. *Plant Physiol.* 103, 1393-1397.
- JOHNSON, K.D., HERMAN, E.M. and CHRISPEELS, M.J. 1989. An abundant, highly conserved tonoplast protein in seeds. *Plant Physiol.* 91, 1006-1013.
- KAMMERLOHER, W., FISCHER, U., PIECHOTTKA, G.P. and SCHAFFNER, A.R. 1994. Water channels in the plant plasma membrane cloned by immunoselection from a mammalian expression system. *Plant J.* 6, 187-189.
- KERPPOLA, R.E. and AMES, G.F. 1992. Topology of the hydrophobic membrane-bound components of the histidine periplasmic permease. *J. Biol. Chem.* 267, 2329-2336.
- KIM, Y., KIM, E.J. and REA, P.A. 1994. Isolation and characterization of cDNAs encoding the vacuolar H⁺-pyrophosphatase of *Beta vulgaris*. *Plant Physiol.* 106, 375-382.
- KING, L.S. and AGRE, P. 1996. Pathophysiology of the aquaporin water channels. *Annu. Rev. Physiol.* 58, 619-648.

- KYTE, J. and DOOLITTLE, R.F. 1982. A simple method for displaying the hydrophobic character of a protein. *J. Mol. Biol.* *157*, 105–132.
- LAI, S., RANDALL, S.K. and SZE, H. 1988. Peripheral and integral subunits of the tonoplast H⁺-ATPase from oat roots. *J. Biol. Chem.* *263*, 16731–16737.
- LIN, J. and ADDISON, R. 1994. Topology of the *Neurospora* plasma membrane H⁺-ATPase. *J. Biol. Chem.* *269*, 3887–3890.
- MA, T., FRIGERI, A., HASEGAWA, H. and VERKMAN, A.S. 1994. Cloning of a water channel homolog expressed in brain meningeal cells and kidney collecting duct that functions as a stilbene-sensitive glycerol transporter. *J. Biol. Chem.* *269*, 21845–21849.
- MAESHIMA, M. 1992. Characterization of the major integral protein of vacuolar membrane. *Plant Physiol.* *98*, 1248–1254.
- MAESHIMA, M. and YOSHIDA, S. 1989. Purification and properties of vacuolar membrane proton-translocating inorganic pyrophosphatase from mung bean. *J. Biol. Chem.* *264*, 20068–20073.
- MALTESE, W.A. 1990. Posttranslational modification of proteins by isoprenoids in mammalian cells. *FASEB J.* *4*, 3319–3328.
- MANDALA, S. and TAIZ, L. 1986. Characterization of the subunit structure of the maize tonoplast ATPase. *J. Biol. Chem.* *261*, 12850–12855.
- MARTY, I., BRANDOLIN, G., GAGNON, J., BRASSEUR, R. and VIGNAIS, P.V. 1992. Topography of the membrane-bound ADP/ATP carrier assessed by enzymatic proteolysis. *Biochemistry* *31*, 4058–4065.
- MI, F., PETERS, J.S. and BERKOWITZ, G.A. 1994. Characterization of a chloroplast inner envelope K⁺ channel. *Plant Physiol.* *105*, 955–964.
- MISHKIND, M.L., PLUMLEY, F.G. and RAIKHEI, N.V. 1987. Immunohistochemical analysis of plant tissue. In *Handbook of Plant Cytochemistry*. Vol. 2. (K.C. Vaughn, ed.) pp. 6–119, CRC Press, Boca Raton, FL.
- MONK, B.C., MONTESINOS, C., FERGUSON, C., LEONARD, K. and SERRANO, R. 1991. Immunological approaches to the transmembrane topology and conformational changes of the carboxyl-terminal regulatory domain of yeast plasma membrane H⁺-ATPase. *J. Biol. Chem.* *266*, 18097–18103.
- MORIYAMA, Y. and NELSON, N. 1989. Cold inactivation of vacuolar protein-ATPase. *J. Biol. Chem.* *264*, 3577–3582.
- NIELSEN, S., SMITH, B.L., CHRISTENSEN, E.I., KNEPPER, M.A. and AGRE, P. 1993. CHIP28 water channels are localized in constitutively water-permeable segments of the nephron. *J. Cell Biol.* *120*, 371–383.
- OBAR, R.A. and HOLZBAUR, E.L.F. 1993. Immunoselection and characterization of cDNA clones. Ch. 18. In *Antibodies in Cell Biology*. Vol. 37. (D.J. Asai, ed.) pp. 362–402, Academic Press, San Diego, CA.
- OLMSTED, J.B. 1981. Affinity purification of antibodies from diazotized paper blots of heterogeneous protein samples. *J. Biol. Chem.* *256*, 11955–11957.

- OTTO, J.J. and LEE, S. 1993. Immunoprecipitation methods. Ch. 7. In *Antibodies in Cell Biology*. Vol. 37. (D.J. Asai, ed.) pp. 119-126, Academic Press, San Diego, CA.
- OVERVOORDE, P.J. and GRIMES, H.D. 1994. Topographical analysis of the plasma membrane-associated sucrose binding protein from soybean. *J. Biol. Chem.* **269**, 15154-15161.
- OVERVOORDE, P.J., FROMMER, W.B. and GRIMES, H.D. 1996. A soybean sucrose binding protein independently mediates nonsaturable sucrose uptake in yeast. *Plant Cell* **8**, 271-280.
- PARRY, R.V., TURNER, J.C. and REA, P.A. 1989. High purity preparations of higher plant vacuolar H⁺-ATPase reveal additional subunits: Revised subunit composition. *J. Biol. Chem.* **264**, 20025-20032.
- PRESTON, G.M. and AGRE, P. 1991. Isolation of the cDNA for erythrocyte integral membrane protein of 28 kilodaltons: Member of an ancient channel family. *Proc. Natl. Acad. Sci. USA* **88**, 11110-11114.
- PRESTON, G.M., JUNG, J.S., GUGGINO, W.B. and AGRE, P. 1994. Membrane topology of aquaporin CHIP. *J. Biol. Chem.* **269**, 1668-1673.
- QADOTA, H., PYTHON, C.P., INOUE, S.B., ARISAWA, M., ANRAKU, Y., ZHENG, Y., WATANABE, T., LEVIN, D.E. and OHYA, Y. 1996. Identification of yeast rho 1p GTPase as a regulatory subunit of 1,3- β -glucan synthase. *Science* **272**, 279-281.
- QI, X., TAI, C.Y. and WASSERMAN, B.P. 1995. Plasma membrane intrinsic proteins of *Beta vulgaris* L. *Plant Physiol.* **108**, 387-392.
- REA, P.A., BRITTEN, C.J. and SARAFIAN, V. 1992. Common identity of substrate-binding subunit of vacuolar H⁺-translocating inorganic pyrophosphatase of higher plant cells. *Plant Physiol.* **100**, 723-732.
- REA, P.A., GRIFFITH, C.J., MANOLSON, M.F. and SANDERS, D. 1987. Irreversible inhibition of H⁺-ATPase of higher plant tonoplast by chaotropic anions: Evidence for peripheral location of nucleotide-binding subunits. *Biochim. Biophys. Acta* **904**, 1-12.
- RIPP, K.G., VIITANEN, P.V., HITZ, W.D. and FRANCHESCHI, V.R. 1988. Identification of a membrane protein associated with sucrose transport into cells of developing soybean cotyledons. *Plant Physiol.* **88**, 1435-1445.
- SARAFIAN, V., KIM, Y., POOLE, R.J. and REA, P.A. 1992. Molecular cloning and sequence of cDNA encoding the pyrophosphate-energized vacuolar membrane proton pump of *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA* **89**, 1775-1779.
- SERRANO, R. 1989. Structure and function of plasma membrane ATPase. *Ann. Rev. Plant Physiol.* **40**, 61-84.
- SLABAS, A.R. and SMITH, C.G. 1988. Immunogold localization of acyl carrier protein in plants and *Escherichia coli*: Evidence for membrane association in plants. *Planta* **175**, 145-152.

- SMITH, B.L. and AGRE, P. 1991. Erythrocyte M_r 28,000 transmembrane protein exists as a multisubunit oligomer similar to channel proteins. *J. Biol. Chem.* **266**, 6407–6415.
- SMITH, D.E. and FISHER, P.A. 1984. Identification, developmental regulation and response to heat shock of two antigenically related forms of a major nuclear envelope protein in *Drosophila* embryos: Application of an improved method for affinity purification of antibodies using polypeptides immobilized on nitrocellulose blots. *J. Cell Biol.* **99**, 20–28.
- SUSSMAN, M.R. 1994. Molecular analysis of proteins in the plant plasma membrane. *Ann. Rev. Plant Physiol. Mol. Biol.* **45**, 211–234.
- SZE, H., WARD, J.M. and LAI, S. 1992. Vacuolar H^+ -translocating ATPases from plants: Structure, function and isoforms. *J. Bioenerg. Biomemb.* **24**, 371–381.
- TAKEDA, J. and KINOSHITA, T. 1995. GPE-anchor biosynthesis. *Trends in Biochem. Sci.* **20**, 367–370.
- TANG, W.J.Y. 1993. Blot-affinity purification of antibodies. Ch. 5. In *Antibodies in Cell Biology*. Vol. 37. (D.J. Asai, ed.) pp. 95–104, Academic Press, San Diego, CA.
- TSAO, J., LIN, X., LACKLAND, H., TOUS, G., WU, Y. and STEIN, S. 1991. Internally standardized amino acid analysis for determining peptide/carrier protein coupling ratio. *Anal. Biochem.* **197**, 137–142.
- VERKMAN, A.S., VAN HOEK, A.N., MA, T., FRIGERI, A., SCACH, W.R., MITRA, A., TAMARAPPOO, B.K. and FARINAS, J. 1996. Water transport across mammalian cell membranes. *Am. J. Physiol.* **270**, C12–C30.
- WASSERMAN, B.P., QI, X., BARONE, L.M. and WU, A. 1996. Biosynthesis of cell wall polysaccharides: Probing the subunit composition and topology of plasma membrane-bound (1,3)- β -glucan (callose) synthases. In *Plant Cell Wall Analysis. Modern Methods of Plant Analysis*. Vol. 17. (H.F. Linskens and J.F. Jackson, eds.) pp. 181–197, Springer-Verlag, New York.
- WEAVER, C.D., CROMBIE, B., STACEY, G. and ROBERTS, D.M. 1991. Calcium-dependent phosphorylation of symbiosome membrane proteins from nitrogen-fixing soybean nodules. *Plant Physiol.* **95**, 222–227.
- WEAVER, C.D., SHOMER, N.H., LOUIS, C.F. and ROBERTS, D.M. 1994. Nodulin 26, a nodule-specific symbiosome membrane protein from soybean, is an ion channel. *J. Biol. Chem.* **269**, 17858–17862.
- YANCEY, S.B., JOHN, S.A., LAL, R., AUSTIN, B.J. and REVEL, J. 1989. The 43-kD polypeptide of heart gap junctions: Immunolocalization (I), topology (II), and functional domains (III). *J. Cell Biol.* **108**, 2241–2254.
- YOON, K.L. and GUIDOTTI, G. 1994. Studies on the membrane topology of the (Na, K)-ATPase. *J. Biol. Chem.* **269**, 28249–28258.
- ZIMNIAK, L., DITTRICH, P., GOGARTEN, J.P., KIBAK, H. and TAIZ, L. 1988. The cDNA sequence of the 68-kDa subunit of the carrot vacuolar H^+ -ATPase. *J. Biol. Chem.* **263**, 9102–9112.