may be the case for human erythrocyte glycosphingolipids transferred into murine cells expressing human CD4.[6]

Acknowledgments

This work was supported by the Fondation pour la Recherche Médicale (SIDACTION grant).

[42] Analysis of Ceramides Present in Glycosylphosphatidylinositol Anchored Proteins of *Saccharomyces cerevisiae*

By ISABELLE GUILLAS, MARTINE PFEFFERLI, and ANDREAS CONZELMANN

Principle

Glycosylphosphatidylinositol (GPI) anchored proteins are found on the cell surfaces of virtually all eukaryotic organisms.[1] The genome of *Saccharomyces cerevisiae* contains about 70 open reading frames encoding proteins that would be predicted to be anchored by a GPI anchor.[2,3] The presence of a GPI anchor has been confirmed in several yeast proteins, and the detailed structures of the GPI anchor of a pool of GPI proteins as well as of individual proteins such as Gas1p or cAMP binding protein have been reported.[4,5] The carbohydrate core structure linking the C-terminal end of yeast GPI proteins to a lipid moiety is the same as in all other eukaryotic organisms, namely, protein-CO-NH$(CH_2)_2$-PO_4-6Manα1-2Manα1-6Manα1-4GlcNα1-6*myo*-inositol-PO_4-lipid (Fig. 1). The lipid moiety consists of either a ceramide or a mono- or diacylglycerol.

Ceramides are found on the majority of yeast anchors; they mainly consist of C18:0- or C20:0-phytosphingosine (PHS) and a C26:0 fatty acid. In contrast, the lipid moiety of Gas1p, an abundant GPI protein, is sensitive to PI-PLC and to mild alkaline hydrolysis, and contains C26:0 fatty acids.[5] Thus, yeast GPI proteins are made with two different kinds

[1] M. J. McConville and M. A. Ferguson, *Biochem. J.* **294,** 305 (1993).
[2] K. Hamada, S. Fukuchi, M. Arisawa, M. Baba, and K. Kitada, *Mol. Gen. Genet.* **258,** 53 (1998).
[3] L. H. Caro, H. Tettelin, J. H. Vossen, A. F. Ram, H. van den Ende, and F. M. Klis, *Yeast* **13,** 1477 (1997).
[4] G. Muller, K. Schubert, F. Fiedler, and W. Bandlow, *J. Biol. Chem.* **267,** 25337 (1992).
[5] C. Fankhauser, S. W. Homans, J. E. Thomas Oates, M. J. McConville, C. Desponds, A. Conzelmann, and M. A. Ferguson, *J. Biol. Chem.* **268,** 26365 (1993).

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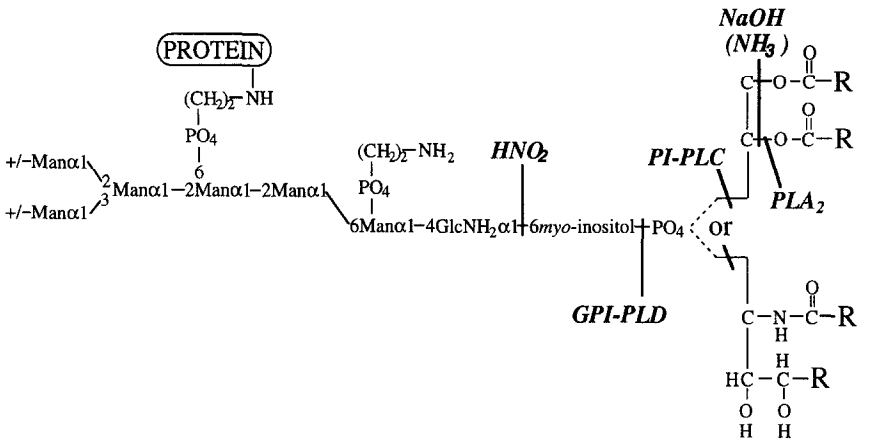

FIG. 1. Structural variants of the yeast GPI anchor. Relevant cleavage procedures are indicated. Anchors can contain an optional fifth mannose residue linked either to α1,2 or α1,3. This residue is added to GPI proteins in the Golgi [C. Fankhauser, S. W. Homans, J. E. Thomas Oates, M. J. McConville, C. Desponds, A. Conzelmann, and M. A. Ferguson, *J. Biol. Chem.* **268,** 26365 (1993); G. Sipos, A. Puoti, and A. Conzelmann, *J. Biol. Chem.* **270,** 19709 (1995)]. They also can bear a second ethanolaminephosphate on the α1,4-linked mannose (unpublished observation). GPI-PLD, GPI-specific phospholipase D; PI-PLC, phosphatidylinositol-specific phospholipase C.

of lipids, both of which contain very long fatty acids. In both types of lipids, the C26:0 fatty acid may be hydroxylated on C_2. The complete GPI lipids that are transferred onto newly synthesized proteins shortly after their translocation into the endoplasmic reticulum (ER), however, contain only diacylglycerol as a lipid moiety.[6,7] This suggests that lipids of GPI anchors are exchanged by a process called *anchor remodeling.* Data so far indicate that three types of enzymes are intimately involved in the elaboration of the mature lipid moiety of GPI proteins: (1) enzymes exchanging diacylglycerol for ceramide, (2) enzymes exchanging C_{16} or C_{18} for C26:0 fatty acids, and (3) enzymes responsible for the presence of monohydroxylated C_{26} fatty acids, the latter being localized in the Golgi apparatus.[7,8]

The functional significance of lipid remodeling is not clear, in part because there are no mutants that are deficient specifically in these processes and the enzymes. Because the majority of GPI anchored proteins of *S. cerevisiae* eventually lose part of the anchor, including the lipid moiety,

[6] A. Conzelmann, A. Puoti, R. L. Lester, and C. Desponds, *EMBO J.* **11,** 457 (1992).
[7] G. Sipos, F. Reggiori, C. Vionnet, and A. Conzelmann, *EMBO J.* **16,** 3494 (1997).
[8] F. Reggiori, E. Canivenc-Gansel, and A. Conzelmann, *EMBO J.* **16,** 3506 (1997).

and become covalently attached to the β1,6-glucans of the cell wall,[9–11] it may be that the lipid remodeling of GPI proteins contributes to the correct transport and sorting of GPI proteins rather than altering their function per se. The study of remodeling events requires methods that can analyze the lipid moieties of individual proteins at different stages of maturation. The methods described here rely on procedures that allow for rapid, specific, and efficient metabolic radiolabeling of the lipid moiety of GPI proteins.

The analytical methods that provide structural information on the labeled anchor lipids have been developed in other labs. The procedure described here is useful for biosynthetic studies using secretory mutants or for kinetic studies as recently described,[6–8,12] but results obtained with these methods will eventually have to be corroborated by mass spectrometry.

Overall Approach

A typical experiment will follow the various steps denoted in Fig. 2. Metabolic labeling with *myo*-[2-^{3}H]inositol ([^{3}H]Ins) specifically labels GPI proteins but not other proteins.[6] Metabolic labeling with [4,5-^{3}H]dihydrosphingosine ([^{3}H]DHS) is also specific for GPI proteins and labels the ceramide-containing GPI proteins but not the ones that contain a diacylglycerol, although part of the [^{3}H]DHS is degraded to [^{3}H]palmitaldehyde and is subsequently used for the biosynthesis of phosphatidic acid and phospholipids.[13,14] Metabolic labeling with [^{3}H]palmitate or [^{3}H]myristate leads to extensive and almost specific labeling of GPI proteins, but curiously, after [^{3}H]palmitate labeling, the protein-associated label has been found almost exclusively in the C_{18}-phytosphingosine component of GPI anchors containing ceramide.[6] Note that the labeled anchor lipids isolated after labeling cells with [^{3}H]DHS or ^{3}H-labeled fatty acids may not be representative of the lipids that are normally added to GPI proteins. Thus, after labeling with [^{3}H]DHS, it has been noted that about 15% of anchor ceramides contained [^{3}H]DHS; the others contained [^{3}H]phytosphingosine. When analyzed by mass spectrometry, the proportion of DHS-containing

[9] C.-F. Lu, R. C. Montijn, J. L. Brown, F. Klis, J. Kurjan, H. Bussey, and P. N. Lipke, *J. Cell Biol.* **128,** 333 (1995).
[10] H. de Nobel and P. N. Lipke, *Trends Cell Biol.* **4,** 42 (1994).
[11] R. Kollar, B. B. Reinhold, E. Petrakova, H. J. Yeh, G. Ashwell, J. Drgonova, J. C. Kapteyn, F. M. Klis, and E. Cabib, *J. Biol. Chem.* **272,** 17762 (1997).
[12] F. Reggiori and A. Conzelmann, *J. Biol. Chem.* **273,** 30550 (1998).
[13] C. Mao, M. Wadleigh, G. M. Jenkins, Y. A. Hannun, and L. M. Obeid, *J. Biol. Chem.* **272,** 28690 (1997).
[14] J. D. Saba, F. Nara, A. Bielawska, S. Garrett, and Y. A. Hannun, *J. Biol. Chem.* **272,** 26087 (1997).

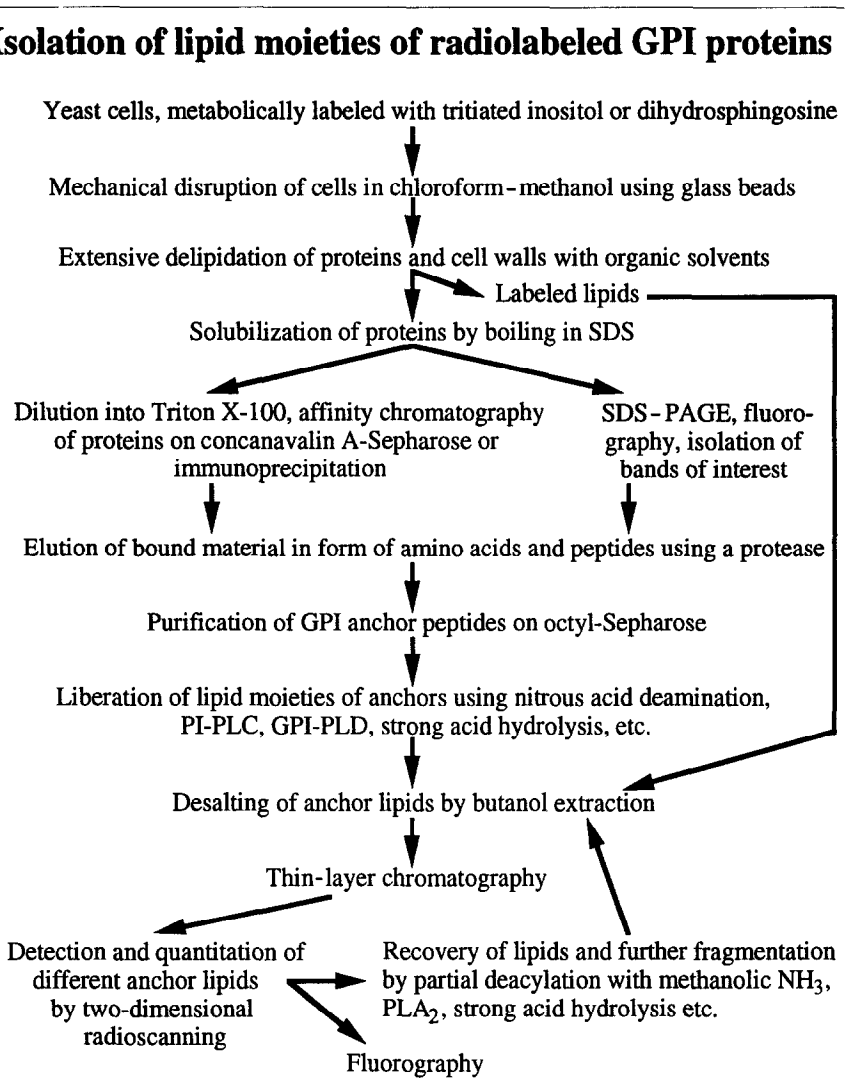

FIG. 2. Outline of a typical experiment investigating the lipids of GPI anchors. The recovery of total anchor lipids typically amounts to 0.2–0.5% of the [^{3}H]Ins initially added to cells.

anchor ceramides amounted to only 5%.[5,8] No bias is expected if anchors are labeled with [^{3}H]Ins.

Extensive extraction with organic solvents will not achieve a complete delipidation of proteins; further delipidation steps (e.g., affinity chromatography on concanavalin A-Sepharose) must also be used. All of the [^{3}H]Ins-labeled proteins of *S. cerevisiae* are retained on concanavalin A-Sepharose,

which indicates that all of the major GPI proteins are glycoproteins. Alternatively, proteins may be further delipidated by immunoprecipitation or by gel electrophoresis. In all cases, GPI peptides can conveniently be recovered using protease digestion as long as the protease is not contaminated with enzymes that would hydrolyze the anchor components under study. To analyze anchor lipids, we use pronase, because it does not contain phospholipase activity, which would remove the labeled inositol from the lipid. The subsequent chromatography on octyl-Sepharose removes amino acids, hydrophilic glycopeptides, and detergent.

Experimental Procedures

Radiolabeling of Cells with Myo-[2-^{3}H]Inositol or [4,5-^{3}H]Dihydrosphingosine and Preparation of Delipidated Proteins

[^{3}H]Ins and [^{3}H]DHS are available at specific activities of 15–20 Ci/mmol and 30–60 Ci/mmol, respectively, commercially (e.g., Anawa Trading SA, Wangen, Switzerland). Catalytic reduction of C_{18}-sphingosine with tritium gas can also be performed (in our case, by NEN-Du Pont, Les Ulis, France) to yield C_{18}-[4, 5-^{3}H]DHS that is purified by preparative thin-layer chromatography (TLC) as recently described.[15] Cells are grown in synthetic minimal medium containing 2% glucose,[16] but inositol is omitted from the vitamin supplement, and the medium is supplemented with 1% casein hydrolyzate (casamino acids, GibcoBRL, Paisley, Scotland). Inositol must be omitted to induce the inositol transporter Itr1p.[17–19] Casamino acids contain small amounts of inositol that do not interfere with the induction of the transporter, but better incorporation of [^{3}H]Ins is achieved if cells are labeled in glucose containing minimal medium supplemented with a mixture of pure amino acids (i.e., SC medium).[16]

Labeling is performed by resuspending exponentially growing cells in fresh medium at about $1–2 \times 10^8$ cells/ml and adding 40–150 μCi of [^{3}H]Ins or 100 μCi of [^{3}H]DHS/ml of medium from concentrated stocks kept in water or methanol, respectively. With both radiotracers, the bulk of radioactivity is incorporated into cellular lipids within 10–30 min and fresh medium can be added if labeling is to be continued. Note that under the indicated conditions, the incorporation of these radiotracers into GPI

[15] F. Reggiori, E. Canivenc-Gensel, and A. Conzelmann, *Methods Mol. Biol.* **116,** 91 (1998).
[16] F. Sherman, *Methods Enzymol.* **194,** 3 (1991).
[17] K. Lai and P. McGraw, *J. Biol. Chem.* **269,** 2245 (1994).
[18] J. Nikawa, K. Hosaka, and S. Yamashita, *Mol. Microbiol.* **10,** 955 (1993).
[19] K. Lai, C. P. Bolognese, S. Swift, and P. McGraw, *J. Biol. Chem.* **270,** 2525 (1995).

proteins continues even while the labeled lipids are being chased by lipids made by cells from unlabeled, endogenously synthesized Ins and DHS. To stop the incorporation of radiolabel into proteins, protein synthesis inhibitors such as cycloheximide must be used. Note, however, that [^{3}H]DHS continues to be incorporated into mature GPI proteins for as long as 60 min after protein synthesis has been arrested, apparently because the ceramides of mature proteins continue to be exchanged.[8]

In contrast, [^{3}H]Ins incorporation into proteins is rapidly arrested by cycloheximide.[20] Typically, we label 0.25–1 ml of cell suspension for 120 min in a shaking water bath. At the end, cells can be washed in water but we prefer to process them immediately after centrifugation in a screw-topped Eppendorf tube. The cell pellet is resuspended in 500 μl of chloroform–methanol (1:1, v/v) by sonication and vortexing, and 200 μl of acid washed glass beads is added. Cells are broken by repeated vortexing (5 times for 1 min each). After sedimentation of glass beads the supernatant is transferred to a fresh tube, the glass beads are rinsed twice with 400 μl of chloroform–methanol–water (10:5:3, v/v), and the supernatants are pooled with the first supernatant. Precipitated proteins and cell wall fragments are then sedimented at 15,000*g* for 5 min at 4°. The supernatant contains lipids that can serve as a reference for the cell's free (i.e., not protein bound) lipids and is stored at −20°.

The protein pellet is further delipidated by resuspension in 0.5 ml chloroform–methanol–water (10:10:3), sonicated in a bath-type sonicator, centrifuged, and supernatant removed. This is done four times. Proteins are then resuspended in 0.5 ml ethanol–water–diethyl ether–pyridine–ammonia (15:15:5:1:0.018 v/v) and incubated at 37° for 15 min[21] and the solvent is removed in a Speed-Vac evaporator. This treatment may solubilize some labeled lipids since it seems to render the subsequent delipidation steps more efficient. The protein pellet is then delipidated once more with chloroform–methanol–water (10:10:3). The dried pellet is boiled for 5 min in solubilization buffer (80 m*M* Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol). When starting with 1–2 × 10^8 cells, about 200 μl of this buffer must be used.

Affinity Chromatography of Proteins on Concanavalin A-Sepharose and Generation of Anchor Peptides

All of the following procedures will be described for a sample starting with 1–2 × 10^8 cells. To remove insoluble material, add 1 ml of concanavalin

[20] A. Conzelmann, C. Fankhauser, and C. Desponds, *EMBO J.* **9,** 653 (1990).
[21] B. A. Hanson and R. L. Lester, *J. Lipid. Res.* **21,** 309 (1980).

A (ConA)-Sepharose buffer (50 m*M* Tris-HCl, 1% Triton X-100, 150 m*M* NaCl, 1 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, pH 7.4), mix, and centrifuge at 15,000*g* for 5 min. Transfer the supernatant to a 15-ml plastic tube. To avoid denaturation of ConA by SDS, a 20-fold excess of Triton X-100 should be added. Thus, 8 ml of ConA-Sepharose buffer is added to neutralize the SDS of 200 μl of solubilization buffer. ConA-Sepharose (Pharmacia, Uppsala, Sweden) is sedimented two times in ConA-Sepharose buffer by centrifugation at 200*g* for 5 min to remove free ConA before use. The washed ConA-Sepharose is added to the sample (100 μl of packed beads for material from 1–2 $\times$ 10^8 cells) and the sample is incubated at room temperature for 2 hr on a rotating wheel.

The Sepharose is then sedimented and washed by repeated resuspension in large volumes of ConA-Sepharose buffer. Beads are left standing a few minutes to allow for lipid exchange, and sedimentation is terminated by centrifugation for 3 min at 12*g*. Washing the Sepharose in this manner should continue until the radioactivity in the supernatant becomes constant (at approximately 300–500 cpm/ml). Sepharose may be washed once more with ConA-Sepharose buffer containing Triton X-100 at 0.02% to reduce the amount of detergent carried over into subsequent steps. At this stage proteins may be eluted intact by incubation with 1 *M* α-methylmannoside or by boiling in SDS sample buffer.[22]

Several of the procedures for liberating the GPI anchor lipid moieties can be done on the intact proteins.[8] However, results seem to be more quantitative when GPI anchor peptides are prepared using protease first. For this, ConA-Sepharose is resuspended in pronase buffer (100 m*M* Tris-HCl, 1 m*M* $CaCl_2$, 0.02% Triton X-100, 10 m*M* NaN_3, 20 μg/ml gentamycin, pH 8.0; 0.5 ml/0.1 ml of Sepharose) and treated for 16 hr with 0.5 mg of pronase at 37°. Pronase should be preincubated for 30 min to digest contaminating hydrolases. Samples are boiled for 5 min, centrifuged, and the supernatant is transferred to a fresh tube. Concanavalin A-Sepharose beads are rinsed 2 times with 0.4 ml of 0.1 *M* ammonium acetate, 5% 1-propanol and these washes are added to the pronase eluate.

Purification of GPI Anchor Peptides on Octyl-Sepharose

This method is commonly used to purify GPI anchor peptides and has been described elsewhere in this series.[23] Our procedure is similar, however, we use 1 ml of octyl-Sepharose per column, adjust the pH of the sample first to 4.5 by adding 50-μl aliquots of 10% acetic acid, reapply the sample twice to ensure complete adsorption, and elute columns either with a gradi-

[22] U. K. Laemmli, *Nature* **227,** 680 (1970).
[23] P. Schneider and M. A. J. Ferguson, *Methods Enzymol.* **250,** 614 (1995).

ent of propanol or with 5% 1-propanol, then 3 ml of 25% 1-propanol, then 4 ml of 50% 1-propanol. When using a gradient, we generally obtain three peaks of radioactivity: the first, small peak of uncharacterized material contains the bulk of detergents (SDS and Triton X-100); the two other peaks eluting between 30 and 40% propanol are not well separated and correspond to GPI peptides, which contain the same lipid moieties and the same number of mannose residues on their GPI anchor but which may differ by the number of residual amino acids. When using stepwise elution with 25 and 50% 1-propanol, the anchor peptides are found in the first 1.5 ml of the 50% 1-propanol eluate.

Liberation of Lipid Moieties

In all procedures it is important to perform an incubation control that documents that the lipids derive from the cleavage of GPI anchor peptides and are not simply due to incomplete delipidation of the anchor peptide preparation under study (e.g., see Fig. 3, lane 3). Nitrous acid (HNO_2) deamination liberates phosphatidylinositol (PI) or inositolphosphorylceramide (IPC) and is the method of choice for [^{3}H]Ins-labeled samples (Fig. 1). Nitrous acid treatment is carried out exactly as previously de-

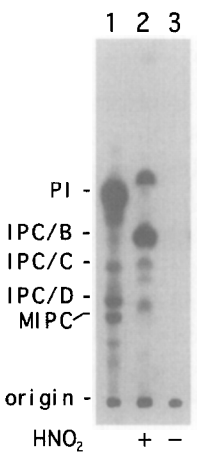

FIG. 3. [^{3}H]Ins-labeled GPI anchor lipids of W303 wild-type cells. 10^8 cells were labeled for 2 hr with 40 μCi [^{3}H]Ins. Lipids were extracted and desalted by butanol–water partitioning. Proteins were completely delipidated by affinity chromatography over ConA-Sepharose and their lipid moieties were liberated using HNO_2. Free lipids (lane 1) and liberated anchor lipids (lane 2) were separated by TLC using solvent (55:45:5). One-half of the material was control incubated without HNO_2 (lane 3). The fluorogram was exposed during 4 weeks. IPC/B, IPC/C, and IPC/D denote subclasses of IPC. MIPC is mannosylated IPC.

scribed.[24] If cells have been labeled with [^{3}H]DHS, enzymes such as GPI-PLD and PI-PLC will also liberate radiolabeled lipid moieties. For GPI-PLD treatment, the anchor peptides are resuspended in 100 μl of GPI-PLD buffer (50 m*M* Tris-HCl, 10 m*M* NaCl, 2.6 m*M* $CaCl_2$, 20% 1-propanol, pH 4.5), split into two equal aliquots, and incubated with or without 0.5 U of GPI-PLD[25] at 37° overnight. PI-PLC treatment is carried out in the same way but PI-PLC buffer (20 m*M* Tris-HCl, 0.2 m*M* EDTA, 20% 1-propanol) and 0.05 U of PI-PLC (ICN, Costa Mesa, CA) are used. For treatment with PLA_2 from bee venom (Sigma, St. Louis, MO), anchor peptides or anchor lipids are resuspended in 30 μl PLA_2 buffer (25 m*M* Tris-HCl, pH 7.2, 2 m*M* $CaCl_2$, 0.1% sodium deoxycholate) and incubated with or without 2 U of PLA_2 for 5 hr at 37°.

After HNO_2 or enzyme treatments, the liberated lipids are desalted by partitioning between butanol and water. For this, 400 μl of water saturated 1-butanol is added at the end of the incubation. After vortexing and centrifugation at 15,000*g* for 1 min, the upper, butanol phase is removed. After a second, identical butanol extraction, the pooled butanol phases are back extracted 2 or 3 times with 500 μl of water.

To free the long-chain bases one can use strong acid hydrolysis. For this, peptides are resuspended in 90% methanol, 1*M* HCl and are incubated at 80° for 16 hr. They are then dried in the Speed-Vac and residual HCl is flash evaporated twice with 200 μl dry methanol.

Thin-Layer Chromatography

Dried lipids are taken up in chloroform-methanol (1 : 1) and spotted on 0.2-mm-thick silica gel plates (20 × 20 cm, Merck, Darmstadt, Germany, silica gel 60). Ceramides, diacylglycerol, and long-chain bases are well separated using the solvent chloroform–methanol–2*M* ammonium hydroxide (40 : 10 : 1); PIs and IPCs are well separated by using the solvent chloroform–methanol–0.25% KCl (55 : 45 : 5). Radioactive bands can be localized and quantitated by one- and two-dimensional radioscanning (LB2842, Berthold AG, Regensdorf, Switzerland). At this stage, interesting bands can be scraped and eluted from the silica for further enzymatic and chemical treatments as depicted in Fig. 2. Alternatively, plates are sprayed with EN^{3}HANCE (NEN, Cambridge, MA) and exposed to film (X-OMAT; Eastman Kodak Co., Rochester, NY) at −80°. A typical example of the latter is shown in Fig. 3. Note that the main IPC of GPI anchors is IPC/B, a species that represents only a minor fraction of the cell's free IPC (Fig. 3, lane 2). Note also that delipidation of anchor peptides ap-

[24] M. L. Güther, W. J. Masterson, and M. A. Ferguson, *J. Biol. Chem.* **269,** 18694 (1994).
[25] M. A. Davitz, J. Hom, and S. Schenkman, *J. Biol. Chem.* **264,** 13760 (1989).

pears to have been complete since no lipids are visible if HNO_2 is omitted (Fig. 3, lane 3).

Acknowledgments

I.G. is supported by grant 3100-049664.96/1 from the Swiss National Foundation for Scientific Research. M.P. works for the EUROFAN2 project and is supported by OFES contract 97.0537-2.

[43] Preparation of Functionalized Lipid Tubules for Electron Crystallography of Macromolecules

By ELIZABETH M. WILSON-KUBALEK

Introduction

Sample preparation still remains the critical step for obtaining high-resolution structural information about biological macromolecules. The methodology for preparing two-dimensional (2D) crystals is maturing rapidly both for membrane proteins and for soluble proteins that have been ordered on lipid layers (reviewed in Refs. 1 and 2). The latter method, introduced by Uzgiris and Kornberg,[3] is particularly versatile due to the possibility of modifying the lipid substrate to facilitate protein binding. Binding and crystallization of the protein at the lipid surface can be achieved by the use of specific ligand-derivatized headgroups[3] or by nonspecific, electrostatic interactions with charged lipids.[4,5] The method has been further generalized through the introduction of Ni^{2+}-chelating moieties to the headgroups of lipids, expanding the application of the lipid layer crystallization method to a large variety of histidine-tagged proteins.[6–9] Streptavidin crys-

[1] B. K. Jap, M. Zulauf, T. Scheybani, A. Hefti, W. Baumeister, U. Aebi, and A. Engel, *Ultramicroscopy* **46,** 45 (1992).
[2] W. Chiu, A. J. Avila-Sakar, and M. F. Schmid, *Adv. Biophys.* **34,** 161 (1997).
[3] E. E. Uzgiris and R. D. Kornberg, *Nature* **301,** 125 (1983).
[4] S. A. Darst, H. O., Ribi, D. W. Pierce, and R. D. Kornberg, *J. Mol. Biol.* **203,** 269 (1998).
[5] S. A. Darst, E. W. Kubalek, A. M. Edwards, and R. D. Kornberg, *J. Mol. Biol.* **221,** 347 (1991).
[6] E. W. Kubalek, S. F. J. Le Grice, and P. O. Brown, *J. Struct. Biol.* **113,** 117 (1994).
[7] E. Barklis, J. McDermott, S. Wilkens, E. Schabtach, M. F. Schmid, S. Fuller, S. Karanjia, Z. Love, R. Jones, Y. Rui, X. Zhao, and D. Thompson, *EMBO J.* **16,** 1199 (1997).
[8] C. Venien-Bryan, F. Balavoine, B. Toussaint, C. Mioskowski, E. A. Hewat, B. Helme, and P. M. Vignais, *J. Mol. Biol.* **274,** 687 (1997).
[9] N. Bischler, F. Balavoine, P. Milkereit, H. Tschochner, C. Mioskowski, and P. Schultz, *Biophys. J.* **74,** 1522 (1998).

0076-6879/00 $30.00