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The cAMP-binding Ectoprotein from *Saccharomyces cerevisiae* Is Membrane-anchored by Glycosyl-Phosphatidylinositol*

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Günter Müller‡§, Karin Schubert¶, Franz Fiedler¶, and Wolfhard Bandlow¶

From the ‡Hoechst Aktiengesellschaft/Frankfurt am Main, Post Office Box 80 03 20, D-6230 Frankfurt am Main 80, Federal Republic of Germany, and the ¶Institut für Genetik und Mikrobiologie der Universität München, Maria-Ward-Strasse 1a, D-8000 München 19, Federal Republic of Germany

Saccharomyces cerevisiae **contains an amphiphilic cAMP-binding glycoprotein at the outer face of the plasma membrane (M_r = 54,000). It is converted to a hydrophilic form by treatment with glycosyl-phosphatidylinositol-specific phospholipases C and D (GPI-PLC/D), suggesting membrane anchorage by a covalently bound glycolipid. Determination of the constituents of the purified anchor by gas-liquid chromatography and amino acid analysis reveals the presence of glycerol, *myo*-inositol, glucosamine, galactose, mannose, ethanolamine, and asparagine (as the carboxyl-terminal amino acid of the Pronase-digested protein to which the anchor is attached). Complementary results are obtained by metabolic labeling, indicating that fatty acids and phosphorus are additional anchor constituents. The phosphorus is resistant to alkaline phosphatase, whereas approximately half is lost from the protein after treatment with GPI-PLD or nitrous acid, and all is removed by aqueous HF indicating the presence of two phosphodiester bonds. Inhibition of *N*-glycosylation by tunicamycin or removal of protein-bound glycan chains by *N*-glycanase or Pronase does not abolish radiolabeling of the anchor structure by any of the above compounds. Analysis of the products obtained after sequential enzymic and chemical degradation of the anchor agrees with the arrangement of constitutents in GPIs from higher eucaryotes. Evidence for anchorage of the yeast cAMP-binding protein by a GPI anchor is strengthened additionally by the reactivity of the GPI-PLC-cleaved anchor with antibodies directed against the cross-reacting determinant of trypanosomal variant surface glycoproteins.**

An increasing number of eucaryotic cell surface proteins has been found embedded in the plasma membrane by carboxyl-terminally attached glycolipidic membrane anchors. Among them are coat and structural proteins, receptors, cell adhesion and regulatory molecules, and ectoenzymes (*e.g.* hydrolases) (for reviews see Refs. 1–4). These proteins face the extracellular space and behave as integral membrane proteins. In most cases the glycolipidic anchor structure is constituted by phosphatidylinositol linked to the carboxyl terminus of the polypeptide via nonacetylated glucosamine, a glycan portion composed of 3 mannosyl residues (to which, in some proteins, further carbohydrate moieties like galactose may be attached) and phosphate ester-bound ethanolamine (5, 6, 8, 9).

Recently, covalent modification of proteins by lipids has also been described in lower eucaryotes. The contact site A cell adhesion protein of *Dictyostelium discoideum* (10), possibly identical with antigen 117 (11), is attached to the outer leaflet of the plasma membrane by an inositol-phosphoceramide-based lipid glycan instead of by phosphatidylinositol. In *Saccharomyces cerevisiae* several phosphatidylinositol-anchored membrane proteins have been identified, *e.g.* a cell cycle-modulated 115-kDa glycoprotein gp115 (12), an abundant 125-kDa glycoprotein (13–16), and a cAMP-binding protein (17). These amphiphilic plasma membrane proteins, the physiological functions of which are as yet unknown, can be metabolically labeled by *myo*-inositol and fatty acids and released from the membrane in hydrophilic form by enzymic and chemical cleavages of GPI[1] membrane anchors. Although the yeast proteins exhibit characteristics of GPI-anchored proteins, in no case has the structure of their anchors been studied in greater detail. In the present study we prove the GPI nature of the cAMP-binding plasma membrane protein from yeast.

EXPERIMENTAL PROCEDURES

Materials

[1-^{14}C]Stearic acid (50–60 mCi/mmol), [9,10-^{3}H]palmitic acid (30–60 Ci/mmol), D-*myo*-[2-^{3}H]inositol (15 Ci/mmol), D-[1-^{3}H]glucosamine hydrochloride (2–5 Ci/mmol), [1-^{3}H]ethan-1-ol-2-amine hydrochloride (5–30 Ci/mmol), and *N*-[^{3}H]acetyl-concanavalin A (45 Ci/mmol) were provided by Amersham-Buchler, Braunschweig. 8-N_3-[^{32}P]cAMP (20 Ci/mmol) was purchased from ICN, Eschwege. *myo*-[^{14}C(U)]Inositol (200–250 mCi/mmol), D-[^{14}C(U)]galactose (250–360 mCi/mmol), D-[^{14}C(U)]mannose (200 mCi/mmol), D-[^{14}C(U)]glucosamine hydrochloride (250–350 mCi/mmol), [^{14}C(U)]glycerol (100–150 mCi/mmol), $^{32}P_i$ (8,500–9,120 Ci/mmol), mouse ^{125}I-anti-rabbit IgG (2–10 μCi/μg), Aquasol II, and autoradiography enhancer EN^{3}HANCE (liquid and spray) were bought from Du Pont-New England Nuclear, Dreieich. N^6-(2-Aminoethyl)-cAMP-Sepharose, Percoll, and phenyl-Sepharose were obtained from Pharmacia, Freiburg. Bacitracin was from Calbiochem. PI-PLC (*Bacillus cereus*) alkaline phosphatase (calf intestinal mucosa) was from Boehringer,

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§ To whom correspondence should be addressed: Hoechst AG Frankfurt am Main, Pharmaceutical Research Division, SBU Metabolism H 825, P. O. Box 80 03 20, D-6230 Frankfurt am Main 80, Federal Republic of Germany. Tel.: 069-305-4271; Fax: 069-311454.

[1] The abbreviations used are: GPI, glycosyl-phosphatidylinositol; 8-N_3-[^{32}P]cAMP, 8-azidoadenosine 3′:5′-mono[^{32}P]phosphate; PI, phosphatidylinositol; PL, phospholipase; GPI-PLC(D), glycosyl-phosphatidylinositol-specific phospholipase C(D); VSG, variant surface glycoprotein; CRD, cross-reacting determinant; PEG, polyethylene glycol; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; MOPS, 3-(*N*-morpholino)propanesulfonic acid; (P)P_i, inorganic (pyro)phosphate; Triton X-100, octylphenol(ethylene glycolether)$_n$; Triton X-114, poly(ethylene glycol)$_8$mono(octylphenylether); *N*-glycanase, peptide *N*-glycohydrolase.

Mannheim. Highly purified GPI-PLC (*Trypanosoma brucei*), polyclonal anti-VSG rabbit antiserum 117, and the soluble VSG antigen 117, as well as anti-CRD antibody were gifts from P. Overath, Tübingen. GPI-PLD from rabbit serum and PI-PLC from *Bacillus thuringiensis* were donated by W. Gutensohn, München. 12-*O*-Tetradecanoylphorbol-13-acetate was bought from Serva, Heidelberg. Mixed ester cellulosic filters (HAWP, 24-mm diameter and Millex HV, 0.45 μm) were purchased from Millipore, Eschborn. cAMP, PEG 4000, tunicamycin, PLD (cabbage), and rhodamine 6G were bought from Sigma, Deisenhofen. Silica Gel G and "soft-plus" Si-60 plates were from Merck, Darmstadt. HF and trifluoroacetic acid were from Riedel de Haen, Seelze. All other chemicals were obtained as described previously (18–22).

Yeast Strains, Growth Conditions, Preparation of Plasma Membranes

Plasma membranes were isolated from total homogenates of lactate-grown cells (strain ABYS1; Ref. 23) in the presence of protease inhibitors (20) by two successive density gradient centrifugations on Percoll (28%) and sucrose (15–28%), using the binding of radiolabeled concanavalin A as a criterion for recovery (17). Finally, the plasma membranes were spun through a cushion of 0.5 M sucrose and resuspended in SEM buffer (0.25 M sucrose, 1 mM EDTA, 20 mM MOPS/KOH, pH 7.4) at 2.5 mg/ml. Aliquots were frozen in liquid N_2 and stored at −70 °C.

Metabolic Labeling

ABYS1 cells were grown overnight (labeling to equilibrium) or for 2 h (short term) at 30 °C in semisynthetic medium as described (17) or in phosphate-free lactate (3%) medium (for labeling with P_i) to an A_{600} of 0.4, harvested and labeled with 0.75 (0.15) mCi of [^{14}C]([^{3}H])stearic acid or 0.5 (0.2) mCi of *myo*[^{14}C]([^{3}H])inositol or 0.4 (0.1) mCi of [^{14}C]([^{3}H])glucosamine or 0.35 mCi of [^{14}C]ethanolamine or 0.25 (0.1) mCi of [^{14}C]([^{3}H])galactose or 0.1 (0.04) mCi of [^{14}C]([^{3}H])mannose or 0.08 mCi of $^{32}P_i$ or with 0.5 mCi of [^{3}H]palmitic acid plus 0.1 mCi of *myo*-[^{14}C]inositol (double labeling) for 1 h (short term labeling: A_{600} = 0.10–0.15). For P_i labeling, NaF (25 mM, final concentration), glycerol 3-phosphate (50 mM, final concentration), ocadaic acid (0.5 μM, final concentration), and KPP_i (100 mM, final concentration) were added 2 min before termination of the labeling. Tunicamycin (final concentration of 30 μg/ml) was added 30 min before the labeled compound and maintained throughout the labeling period.

Affinity Purification

Up to 500 μg of plasma membrane protein was solubilized in 25 mM MOPS/KOH, pH 7.0, 150 mM NaCl, 4 mM $MgCl_2$, 0.4 mM EGTA, 0.5 mM DTT, 0.5% decanoyl-*N*-methylglucamide, 0.1 mM 3-isobutyl-1-methylxanthine, 0.1 mM phenylmethanesulfonyl fluoride, 50 μM leupeptin, 0.1 mM aprotinin at 2 mg/ml, centrifuged (150,000 × *g*, 30 min) and the supernatant applied to a 2-ml column of N^6-(2-aminoethyl)-cAMP-Sepharose as described (21) with the following modifications. The column was washed five times with 2 ml of 25 mM MOPS/KOH, pH 7.2, 100 mM sodium citrate, 5 mM DTT, 5 mM $MgCl_2$, 150 mM NaCl, 250 mM sucrose, 7.5% ethylene glycol, 10% glycerol, 1 mg/ml bovine serum albumin, 1 mM 3-isobutyl-1-methylxanthine and eluted with 2 ml of the same buffer supplemented additionally with 100 mM cAMP at 4 °C. The initial 250 μl of the eluate was desalted by centrifugation through a 1-ml column of Sephadex G-25 equilibrated with 25 mM MOPS/KOH, pH 7.0, 50 mM KCl, 5 mM $MgCl_2$, 10 mM DTT, 50 μM EDTA, 50 μM phenylmethanesulfonyl fluoride, 0.1% deoxycholate, 5% glycerol and precipitated with an equal volume of 8% PEG 4000 in 10 mM MOPS/KOH, pH 7.2, 1 mM EDTA (30 min, 4 °C). After centrifugation (15 min, 4 °C, microcentrifuge) the pellet was dissolved in 20 mM MOPS/KOH, pH 7.2, 1 mM EDTA, 100 μM phenylmethanesulfonyl fluoride, 0.5% deoxycholate at 2 mg/ml.

Digestion with Phospholipases

5 units of partially purified PI-PLC (*B. cereus*) (the activity corresponds to 5 μmol of bovine acetylcholinesterase cleaved per min) in 300 μl of 20 mM Tris/HCl, pH 7.5, 120 mM NaCl, 2 mM EDTA, 1 mM DTT, 0.05% (v/v) Triton X-100, 0.5 mg/ml phosphatidylcholine at 37 °C for 2 h (24); 0.2 units of purified PI-PLC (*B. thuringiensis*) (1 unit defined as the activity cleaving 1 μmol of PI/min in Triton X-100) in 200 μl 5 mM potassium phosphate, 10 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM DTT, 0.1% Triton X-100 at 37 °C for 2 h (25); 5 μl of partially purified GPI-PLC (from *T. brucei* ILTat 1.25, obtained from P. Overath, Tübingen (26)) in 100 μl of 25 mM Tris/HCl, pH 8.0, 3 mM EDTA, 0.1% Triton X-100 at 37 °C for 1 h (27); 15 μl of crude GPI-PLD (heparinized human serum) in 250 μl of 100 mM Tris maleate, pH 7.4, 30 mM NaCl, 2 mM $CaCl_2$, 0.1% Nonidet P-40 at 37 °C for 60 min (28); 50 units (1 unit defined as the activity which cleaves 1 μmol of phosphatidylcholine/min) PLD (cabbage) in 500 μl of 20 mM potassium phosphate, pH 5.6, 125 mM $CaCl_2$, 0.05% Triton X-100 at 30 °C for 90 min (29) were alternatively used to cleave the anchor or fragments thereof.

Pronase Treatment

Biosynthetically labeled and affinity-purified cAMP-binding protein (from 250 μg of plasma membranes), in 70 μl of 25 mM MOPS/KOH, pH 7.4, 0.5 mM EDTA, 0.5% deoxycholate, 5 mM DTT, was precipitated with 8% of PEG 6000, washed with acetone, dried under N_2 gas, and dispersed in 150 μl of 100 mM $(NH_4)_2CO_3$, pH 8.2, 1 mM $CaCl_2$, 0.05% octyl glucoside. Pronase was added initially and after 12 h (10 mg each) and the mixture incubated at 37 °C for 24 h with constant shaking. The digest was lyophilized and extracted three times with methanol/pyridine/water (2:1:1). The pooled extracts were adjusted to pH 5 with acetic acid and loaded onto a column (0.5 × 18 cm) of octyl-Sepharose equilibrated with 0.1 M sodium acetate buffer, pH 5, containing 5% 1-propanol. The column was washed with 10 ml of the same buffer and eluted with a 5–55% 1-propanol gradient at a flow rate of 1 ml/h. The GPI-peptide was repurified on octyl-Sepharose using batch elution with 32% 1-propanol in water. The GPI peptide-containing fraction was filtered through a Millex HV 0.45-μm membrane, dried, redissolved in 500 μl of the sodium acetate buffer and refiltered.

Glycanase Treatment

Denatured Membrane Proteins (According to Ref. 30 with the Following Modifications—^{3}H- or ^{14}C-labeled, affinity-purified and precipitated (8% PEG 6000) cAMP-binding protein (approximately 20 μg of protein) was dissolved in 0.1 M phosphate buffer, pH 8.6, 50 mM KCl, 25 mM DTT, 0.5% deoxycholate, 0.1% SDS and 0.75 M NaSCN and boiled for 5 min. The sample was diluted 10-fold with the same buffer lacking SDS and NaSCN and incubated with *N*-glycanase (5 units/ml) in a total volume of 500 μl for 18 h, terminated by the addition of acetone, 0.07 M HCl together with 50 μg of hemoglobin as a carrier, followed by centrifugation at 12,000 × *g* for 10 min. Acetone, 0.07 M HCl, and ether-washed dry precipitates were analyzed by SDS-PAGE or subjected to further enzymic or chemical analyses as indicated.

Native, Metabolically Labeled Membrane Proteins—Intact yeast cells (75,000 dpm) or spheroplasts (60,000 dpm) or isolated plasma membrane vesicles (30,000 dpm) were incubated with 15 units/ml *N*-glycanase in 0.01 M sodium acetate, pH 5.1, 0.1 M β-mercaptoethanol, 0.15 M NaSCN (total volume, 400 μl) at 30 °C. After 90 min another 15 units/ml *N*-glycanase was added and the incubation continued for 3 h. Subsequently the cells/spheroplasts/vesicles were washed two times (centrifugation through a cushion of 0.25 M sucrose). Subsequent cellular subfractionation was performed in the presence of a 4-fold molar excess of bovine trypsin inhibitor and 100 μM phenylmethanesulfonyl fluoride.

Binding to Phenyl-Sepharose (According to Ref. 31 with the Following Modifications

100 μl of sample in 900 μl of phosphate-buffered saline was incubated (25 °C, 1 h) with 75 μl of phenyl-Sepharose beads. After washing (two times) in phosphate-buffered saline containing 0.01% Nonidet P-40 unbound material was recovered from the supernatant (10,000 rpm, 1 min, microcentrifuge). Material bound to the beads was extracted from the pellet by two washes (15 min, 37 °C) with 450 μl of 2% Nonidet P-40 each.

TLC Analysis

Purification of the Anchor Structure—Pronase-digested anchor was chromatographed by TLC in chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, v/v). It was scraped off its position near the origin of the plate, extracted, and rechromatographed in chloroform/methanol/ammonia/water (45:45:3.5:10, v/v) on oxalate-impregnated Silica Si-60 plates. Radiolabeled standard lipids were localized by autoradiography of TLC plates sprayed with EN^{3}HANCE. Radioac-

tivity was quantitated by linear TLC analyzer (Berthold). Alternatively, the radioactive material was scraped off the plate and evaluated by liquid scintillation counting. Nonradioactive phospholipid standards were localized by spraying the plate with rhodamine 6G or exposing to iodine vapor.

TLC Analysis of Chemical and Enzymic Cleavage Products of the Anchor Structure—TLC was performed on Silica G plates activated at 125 °C for 2 h. The plates were developed with solvent systems I–V as described: I, petroleum ether/diethyl ether/acetic acid (70:30:2, v/v); II, acetone/petroleum ether (1:3, v/v) for the first development and chloroform/methanol/acetic acid/water (25:15:4:2, v/v) for the second development in the same direction); III, chloroform/methanol/acetone/acetic acid/water (30:10:40:10:5, v/v); IV, petroleum ether/diethyl ether/acetic acid (80:20:1, v/v); V, petroleum ether/diethyl ether/acetic acid (80:20:1, v/v) for the first development and toluene/methanol/acetic acid (80:20:1, v/v) for the second development in the same direction. Radioactivity was determined by scraping 0.5- × 0.5-cm wide areas from the sample lanes and liquid scintillation counting in 10 ml of Aquasol 2. Alternatively, the ^{3}H- or ^{14}C-labeled products were detected by a TLC scanner. The positions of authentic unlabeled lipid standards, which cochromatographed as a mixture in a parallel lane on the same plate, were identified by spraying the plates with 80% (w/v) H_2SO_4 and heating at 180 °C for 30 min. Hydroxamates were identified by spraying the chromatograms with $FeCl_2$ reagent.

Extraction from Silica

Radiolabeled material was extracted from silica gel (TLC plates or powder) by three washes with 2 ml of methanol followed by one wash with 2 ml of chloroform/methanol/25% ammonia (2:1:0.075, v/v). After evaporation of combined extracts the lipids were mixed with 50 μl of H_2O, 150 μl of HCl and extracted with 300 μl of chloroform/methanol (1:1, v/v). The organic phase was washed twice with 150 μl of methanol, 1 N HCl (1:1, v/v), evaporated, and the residue dissolved in the desired buffer.

Extraction of the cAMP-binding Protein from Polyacrylamide

Metabolically labeled and affinity-purified cAMP-binding protein (localized after SDS-PAGE by fluorography) was eluted from the respective gel pieces by electrophoresis and purified by gel filtration (Sephadex G-50). Residual protein was eluted from the minced gel slices by two extractions with five gel volumes of 0.1 M Hepes/KOH, pH 8.0, 0.1% SDS, 6 M urea and mixing overnight at 56 °C. Combined eluates were dialyzed against water, concentrated (SpeedVac), and suspended in 25 mM MOPS/KOH, pH 7.4, 0.5 mM EDTA, 0.5% deoxycholate, 5 mM DTT.

Purification of Polar Anchor Fragments

The Pronase-digested cAMP-binding protein (GPI-peptide) was dephosphorylated with 60% (w/v) aqueous HF. Anions were removed by a passage through 0.6 ml of AG3X4(OH^-) on top of 0.2 ml of QAE-Sephadex A-25. The flow-through was adjusted to pH 5 with 0.1 M sodium acetate, pH 5, filtered (0.2 μm membrane), dried, dissolved in the same buffer, and purified by a passage through a small column (1.5 × 20 cm) of Bio-Gel P-4 (400 mesh). The polar GPI core eluted from Bio-Gel P-4 at a position equivalent to 6.8 glucose units. Carbohydrate-containing fractions were detected by spotting 2-μl aliquots onto silica TLC plates and staining with α-naphthol/H_2SO_4. The anchor core was dried.

Gas-Liquid Chromatographic Analyses

The GPI-glycopeptides prepared from tunicamycin-treated cells were purified by octyl-Sepharose chromatography. Alternatively, the GPI-glycopeptide was dephosphorylated and the polar glycan fragment separated from the apolar lipid fragment (see above). Dried samples (5–10 nmol of anchor) were treated with 60% (w/v) aqueous HF in polyethylene tubes at 0 °C for 16 h (32). HF was volatilized *in vacuo* over NaOH pellets in a glass-free system. Oligosaccharides were hydrolyzed (2 M trifluoroacetic acid, 4 h, 100 °C), sugars reduced (1% $NaBH_4$ in 0.1 M NH_4OH, 1 h, 37 °C) and acetylated (pyridine:acetic acid anhydride (1:1), 1 h, 60 °C). Each sample received 25 nmol of xylose and 10 nmol of *scyllo*-inositol as internal yield standards before the HF treatment. A separate standard mixture containing 10 nmol each of glycerol, xylose, mannose, galactose, glucose, *myo*-inositol, *scyllo*-inositol, glucosamine, and galactosamine was treated identically and run in parallel with each analysis. A blank sample was analyzed in addition. For the identification of glycerol, neutral sugars, and amino sugars the corresponding per-acetylated derivatives were separated using 3% SP 2340 on 100/120 Supelcoport (Supelco Inc., Bellefonte, PA) and a Packard gas chromatograph, model 428, as described (33, 34).

Amino Acid Analysis

Anchor fragments (2 nmol) were sequentially hydrolyzed with 60% HF (see above) and 4 M HCl (16 h, 100 °C). Dried and resuspended samples were applied to the amino acid analyzer (Biotronic LC 6001) together with 2 nmol of *m*-diaminopimelic acid as an internal standard. A separate standard mixture contained 2 nmol each of *m*-diaminopimelic acid, ethanolamine, glucosamine, and asparagine. Evaluations were made by a Shimadzu integrator, model C-R1A.

Miscellaneous Methods

Preparation of mitochondria, cytoplasm, separation of inside-out and outside-out plasma membrane vesicles (18, 20), the radioassay for acetylcholine esterase activity (35), photoaffinity labeling with 8-N_3-[^{32}P]cAMP or 8-N_3-[^{3}H]cAMP (17), hydrophobic/hydrophilic partitioning between a Triton X-114 and an aqueous phase (36), GPI analytical procedures such as base hydrolysis (37), acetolysis (38), nitrous acid deamination (9), periodate oxidation (8), dephosphorylation by aqueous HF (40), and mild acid treatment for cleavage of inositol-1,2-cyclic phosphate esters (41), sodium carbohydrate extraction of membrane proteins (42), Western blotting, immunoprecipitation (43), PAGE (5% (w/v) stacking gel and 14% (w/v) running gel in the presence of SDS and urea), and fluorography (44) and protein determination (45) were carried out according to published procedures.

RESULTS

The cAMP-binding Protein from Plasma Membranes Is Converted to a Soluble, Hydrophilic Version by Lipolytic Cleavage—The cAMP-binding protein from yeast plasma membranes (54 kDa) can be specifically photoaffinity labeled with membrane-impermeable 8-N_3-[^{32}P]cAMP in spheroplasts (Fig. 1, *lane 1*) and in isolated plasma membrane vesicles (*lanes 5* and *6*). It is accessible to digestion of *N*-glycosidic carbohydrate side chains by *N*-glycanase in intact or sonicated spheroplasts (*lanes 2* and *4*) and outside-out plasma membrane vesicles (*lane 7*). This is evident from the reduction of its molecular mass to 41–44 kDa. Carbohydrate chains are inaccessible in inside-out plasma membrane vesicles where inversion of the membrane topology prevents access of the enzyme (*lane 5*). These results confirm the previously described glycoprotein nature and location of the cAMP receptor protein at the outer face of the plasma membrane (17). Interestingly, the *N*-glycosidic carbohydrate side chains are not

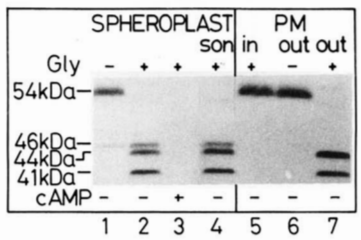

FIG. 1. **Photoaffinity labeling of the plasma membrane cAMP-binding protein.** Yeast spheroplasts (*lanes 1–4*) or plasma membrane vesicles (*PM*, *lanes 5–7*) with outside-out (*out*) or inside-out (*in*) orientation were incubated in the presence or absence of *N*-glycanase (*Gly*) under native conditions. A portion of the spheroplasts was sonified during the digestion (ultrasonic bath, maximal intensity, three times for 10 s; *son*, *lane 4*). The spheroplasts (50 μg of protein) and plasma membranes (5 μg of protein) were solubilized with 0.5% deoxycholate, photoaffinity labeled with 8-N_3-[^{32}P]cAMP in the absence or presence of excess of unlabeled cAMP as indicated, and subjected to SDS-PAGE and fluorography. Molecular masses, indicated on the *left margin*, were derived from marker proteins run in parallel on the same gel.

required for specific binding of cAMP since the efficiencies of photoaffinity labeling do not differ significantly between the intact (54 kDa, *lanes 1* and *6*) and deglycosylated (44- and 41-kDa fragments in *lanes 2* and *7*) cAMP-binding protein. The occurrence of a similar cAMP-binding isoprotein (46 kDa, 18–22) in spheroplasts (*lanes 1, 2,* and *4*) provides a versatile marker (in addition to adenylate cyclase) to assess mutual contamination of the two membrane fractions (see *e.g.* the absence of the mitochondrial receptor from plasma membranes (*lanes 5–7*). Photoaffinity labeling of intact spheroplasts provides a convenient tool for the selective labeling of the plasma membrane cAMP receptor. The mitochondrial isoprotein is poorly labeled under these conditions.

Previous studies (17) have shown that the cAMP-binding plasma membrane protein is tightly membrane-associated since it can be extracted neither with alkaline carbonate nor with high salt. In addition, some evidence has been obtained that membrane anchorage of this protein is mediated by a lipid structure. In Triton X-114 partitioning, it behaved as an amphiphilic protein and is recovered from the detergent-enriched phase. It is converted into a hydrophilic form by cleavage with PLC. To analyze phospholipase specificity in greater detail we treated solubilized plasma membranes (Fig. 2, *filled bars*) with phospholipases of different origin and assayed cleavage of the anchor in comparison with a typical GPI-modified protein, bovine erythrocyte acetylcholine esterase (*hatched bars*). Amphiphilicity of the cleavage products was monitored in two aliquots. In one the capacity to bind to phenyl-Sepharose was measured (*panel A*) and with the other the behavior in Triton X-114/aqueous phase partitioning (*panel B*). With phenyl-Sepharose only the bound fraction, with partitioning only the fraction recovered from the detergent-depleted (aqueous) phase, is shown. It can be seen that the bovine and the yeast proteins behave similarly. They are converted to hydrophilic forms which partition into the aqueous phase and are unable to bind to phenyl-Sepharose when treated with PI-PLC from *B. cereus* or *B. thuringiensis* or, most efficiently, with GPI-PLC from *T. brucei*. GPI-PLDs from both human and rabbit serum also generate the hydrophilic version of the two proteins, albeit to a slightly lower extent. PLD from cabbage, which is not GPI-specific, has almost no effect. The generation of hydrophilic forms of both the yeast cAMP-binding protein and bovine acetylcholine esterase by the lipolytic treatment strongly suggests anchorage by a GPI structure also of the cAMP-binding protein. The lower efficiency of cleavage of the yeast anchor as compared with the bovine anchor, particularly evident with PLC from *B. cereus*, may reflect some structural heterogeneity (14).

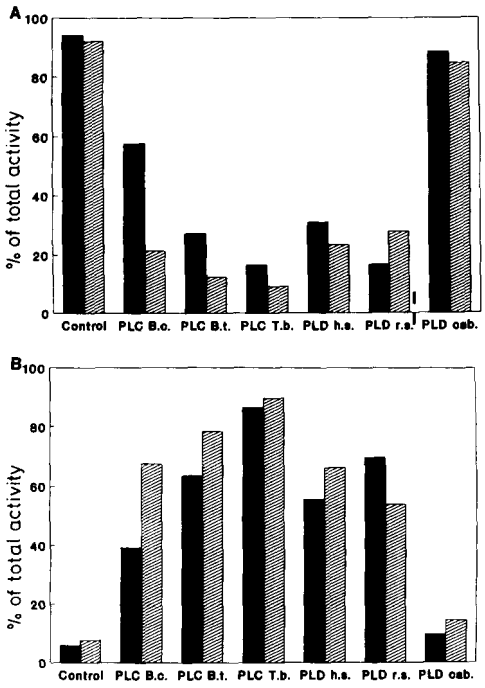

FIG. 2. **Lipolytic cleavage of the plasma membrane cAMP-binding protein.** Solubilized, photoaffinity-labeled plasma membranes from yeast (50 μg of protein, 7,000 dpm; *filled bars*) and bovine erythrocyte membranes (10 μg of protein; *hatched bars*) were mock treated (*Control*) or incubated with PI-PLC from *B. cereus* (*PLC B.c.*) or PI-PLC from *B. thuringiensis* (*PLC B.t.*) or GPI-PLC from *T. brucei* (*PLC T.b.*) or GPI-PLD from human (*PLD h.s.*) or rabbit serum (*PLD r.s.*) or PLD (*cabbage*). To one half of the reaction mixture phenyl-Sepharose was added, and the material bound or not bound was analyzed (*panel A*). The other half was analyzed by Triton X-114 partitioning (*panel B*). The radioactivity of the cAMP-binding protein and the enzymic activity of acetylcholine esterase were measured in the aqueous and detergent phases as well as in the fractions bound and not bound to phenyl-Sepharose, respectively. The sum was set at 100% in both instances. Only the fractions of cAMP-binding protein (*filled bars*) and acetylcholine esterase (*hatched bars*) bound to phenyl-Sepharose and partitioned into the aqueous phase are shown. Results were derived from four independent experiments.

Metabolic Labeling of the Anchor Structure—To identify compounds of the lipidic anchor we used metabolic labeling of cells with typical anchor constituents, D-*myo*-inositol, glucosamine, mannose, ethanolamine (Fig. 3*A*) and phosphate (Fig. 3*B*). A minor component (54 kDa) of the total plasma membrane fraction (*panel A, lane 6*) is labeled with all the sugar compounds and ethanolamine. This protein is highly enriched by affinity purification on cAMP-Sepharose. It is photoaffinity labeled with 8-N_3-[^{32}P]cAMP (*lane 17*) and represents the major metabolically labeled plasma membrane protein eluted from the cAMP affinity column (*lanes 5, 11, 16,* and *22*). A separate series of experiments (not shown) reveals some metabolic interconversion of the carbohydrates so that the radioactivity used for labeling is recovered from constituents of the Pronase-treated, purified anchor other than the one used for labeling. However, in any case we identified the compound used for labeling as the major radioactive constituent (> 70%) of the cleaved (Pronase), reisolated (two consecutive TLC runs using different solvent systems), and completely hydrolyzed anchor.[2] The comigration with the authentic, photoaffinity-labeled protein in SDS-PAGE, cellular location, binding to cAMP-Sepharose, and simultaneous labeling by components characteristic of GPI-moieties strongly argue in favor of the identity of the labeled protein species displayed in Fig. 3*A* with the cAMP-binding protein.

Radiolabeled putative GPI anchor compounds can be discriminated from label residing in *N*-glycosidic side chains of the cAMP receptor by metabolic labeling of yeast cells in the presence of tunicamycin which prevents attachment of *N*-glycosidic carbohydrate side chains to the protein or by their subsequent digestion with *N*-glycanase. Fig. 3*A* shows the fluorogram of an SDS-PAGE of the affinity-purified cAMP-binding proteins. It can be seen that both, labeling in the presence of tunicamycin and treatment with *N*-glycanase, reduce the size of the labeled cAMP affinity-purified protein as expected for a glycoprotein (*lanes 2–4, 8–10, 13–15, 19–21*). However, in all cases the core protein (41–44 kDa) has retained at least some label as compared with the intact protein (*lanes 5, 11, 16,* and *22*). To test whether the label is associated with *O*-glycosidic side chains or with a glycolipidic anchor, the protein from tunicamycin-treated cells (44 kDa) was additionally exposed to aqueous HF, which cleaves phos-

[2] G. Müller and W. Bandlow, unpublished data.

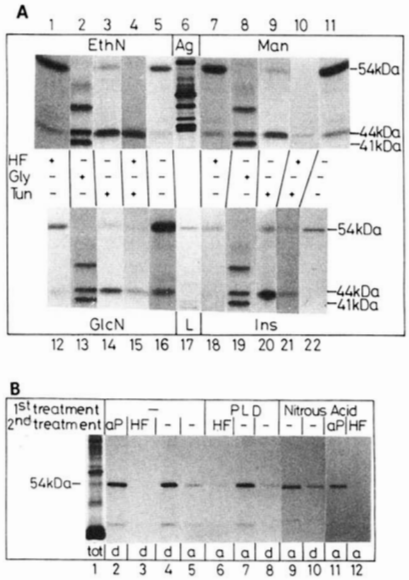

FIG. 3. **Metabolic labeling of the plasma membrane cAMP-binding protein.** *Panel A*, yeast cells were metabolically labeled (short term) with [^{14}C]ethanolamine (*EthN*), [^{14}C]mannose (*Man*), [^{14}C]glucosamine (*GlcN*), or *myo*-[^{14}C]inositol (*Ins*) in the absence or presence of tunicamycin (*Tun*). Aliquots of the affinity-purified cAMP-binding protein (10 μg) were left (*lanes 3, 5, 9, 11, 14, 16, 20,* and *22*) or treated with aqueous HF (*lanes 1, 4, 7, 10, 12, 15, 18,* and *21*) or digested with *N*-glycanase (*lanes 2, 8, 13,* and *19*) as indicated. All samples were analyzed by SDS-PAGE and fluorography. Total plasma membranes photoaffinity labeled with 8-N_3-[^{32}P]cAMP (*L*) and silver-stained (*Ag*) were shown as controls. Molecular masses shown at the *left margin* are derived from marker proteins run in parallel on the the same gel. *Panel B*, yeast cells were metabolically labeled (to equilibrium) with [^{32}P]P_i. Aliquots of affinity-purified cAMP-binding protein (5 μg of protein) were digested with GPI-PLD from rabbit serum (*PLD*) or subjected to nitrous acid deamination or left untreated (first treatment). For the second treatment, the samples were incubated with 2.5 units/ml alkaline phosphatase (*aP*) for 60 min at 37 °C or with aqueous HF or left untreated. Each sample was partitioned into a detergent (*d*) or aqueous phase (*a*). Relevant phases were analyzed by SDS-PAGE and autoradiography. As a control, total ^{32}P-labeled plasma membrane proteins (*tot*) are shown (*lane 1*). The molecular mass, indicated on the *left margin*, was derived from a parallel run of the authentic photoaffinity-labeled cAMP-binding protein.

phodiesters and removes the complete anchor with the exception of ethanolamine. This dual treatment removes most of the residual carbohydrate label including inositol (*lanes 10, 15,* and *21*) whereas ethanolamine remains unaffected (*lane 4*). In agreement, treatment of the intact cAMP-binding (54-kDa) protein with aqueous HF alone releases most of the inositol (*lane 18*), significantly reduces but does not abolish the glucosamine and mannose labeling (*lanes 7* and *12*), and leaves the ethanolamine labeling unaffected (*lane 1*).

To examine incorporation of phosphate into the anchor structure we metabolically labeled yeast cells with [^{32}P]P_i overnight and purified the cAMP-binding protein by cAMP affinity chromatography. To discriminate between phosphate bound to the protein and to the lipid moiety, the cAMP-binding protein was treated first with nitrous acid or GPI-PLD (Fig. 3*B*, first treatment) and subsequently with alkaline phosphatase or aqueous HF (second treatment). The autoradiogram of the SDS-PAGE of the resulting amphiphilic (*d*) and hydrophilic (*a*) forms of the cAMP receptor separated by Triton X-114 partitioning demonstrates that the amphiphilic version of the cAMP receptor can be labeled with [^{32}P]P_i (*lane 4*). It represents only a minor fraction of the total phosphate-labeled proteins from the plasma membrane (*lane 1*) but constitutes the major ^{32}P-labeled plasma membrane protein eluted from the cAMP-Sepharose column. Comigration of the photoaffinity-labeled and affinity-purified ^{32}P-labeled protein strongly suggests identity (data not shown). The ^{32}P label is largely resistant to treatment with alkaline phosphatase (*lane 2*) (under conditions which completely reverse autophosphorylation of the mitochondrial cAMP-binding protein.[2] This indicates that the majority of the [^{32}P]P_i is not bound to amino acids (serine or threonine). On the other hand, treatment with aqueous HF leads to complete loss of the phosphorus label (*lane 3*). Treatment of the ^{32}P-labeled protein with GPI-PLD from rabbit serum or with nitrous acid converts it to the hydrophilic form (*lanes 7* and *9*) and simultaneously removes about 50% of the total phosphorus label (detergent plus aqueous phases) (compare *lanes 4* and *5* (8,730 dpm) with *lanes 7* and *8* (4,250 dpm) and *lanes 9* and *10* (3,120 dpm)). This suggests the presence of two phosphorus atoms in the anchor.

Analysis of the Isolated Anchor Structure—To confirm and extend the results obtained in the previous section and to identify constituents of the lipidic anchor directly, we subjected the purified and hydrolyzed structure to gas-liquid chromatography and amino acid analysis. For this purpose plasma membranes were isolated from yeast cells grown in the absence or presence of tunicamycin. The latter condition was chosen to avoid analytical interference with constituents of *N*-glycosidic side chains and to test possible effects of the antibiotic on the biosynthesis of the anchor structure. After solubilization of plasma membranes, the cAMP-binding protein was purified by several steps including cAMP affinity and phenyl-Sepharose chromatographies and digested to completion with Pronase. The TLC-purified lipidic anchor fragment from the preparation derived from tunicamycin-treated cells was reisolated, hydrolyzed, and directly analyzed by gas-liquid chromatography (Fig. 4*A* and Table I). The anchor prepared from untreated cells was first cleaved with HF, the polar fragment reisolated from an additional TLC, and then analyzed as above (Fig. 4*B* and Table I). Ninhydrin-positive compounds were identified by an amino acid analysis (Fig. 5, *A* and *B*, and Table I).

Although the two preparations shown in Fig. 4, *A* and *B*, were derived from two different yeast cultures and represent differently treated anchor preparations, the results are qualitatively (Figs. 4 and 5) and quantitatively (Table I) comparable. Fig. 4 shows that the complete anchor contains glycerol, *myo*-inositol, glucosamine, galactose, and mannose. Fig. 5 reveals the presence of glucosamine and (in the complete anchor (Fig. 5*A*) but not in the polar, HF-cleaved fragment (Fig. 5*B*) of ethanolamine, aspartate, and ammonia. The latter two compounds are derived from the hydrolysis of asparagine. Ethanolamine is coupled by an amide bond to the carboxyl-terminal amino acid of the protein which in several cases, including a yeast plasma membrane protein of unknown function, has been identified as asparagine (15). Figs. 4*A* and 5*A* show the occurrence of constituents also found in GPIs from vertebrates and some forms of trypanosomal VSGs including galactose, which branches off from mannose proximal to the glucosamine residue (5–9). The quantitative recoveries of the constituents of the anchor are displayed in Table I. Based on 1 mol of *myo*-inositol (which is a very stable component in the analytical procedure), the complete anchor contains 1.8 mol of glycerol, 2.5 mol of mannose, 1.8 mol of galactose, 0.7 mol of glucosamine, 0.9 mol of ethanolamine, and 0.4 mol of aspartate. The amino acid appears to have been removed, at least in part, from the unpolar anchor during the rigorous treatment with Pronase. Taking into consideration that triacetyl glycerol is volatile and difficult to quantitate and that

glucosamine may have been decomposed more in the anchor sample than in the standard mixture, the molar ratio may be approximated to 1 glycerol:1 *myo*-inositol:1 glucosamine:3 mannose: 2 galactose. This ratio of carbohydrates is additionally confirmed by the analysis of the polar fragment which lacks glycerol, ethanolamine, and aspartate. The approximate stoichiometry agrees fairly well with a molecular mass corresponding to 6.8 glucose units determined from elution of the metabolically labeled (*myo*-[^{14}C]inositol) polar glycan fragment (generated by aqueous HF treatment) from a Bio-Gel P-4 column (data not shown).

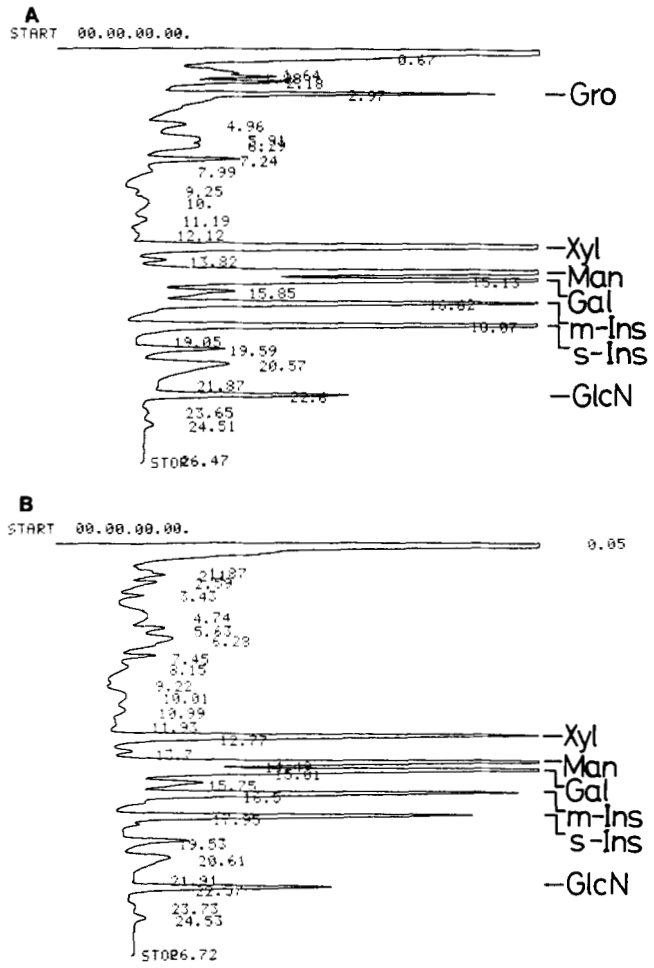

FIG. 4. **Gas-liquid chromatography of components of the anchor.** The cAMP receptor protein was isolated from plasma membranes of cells grown in the absence or presence of tunicamycin (80 μg/ml, added 6 h before harvesting of the cells), purified, and digested with Pronase. The anchor structure was purified by TLC, hydrolyzed, and, after reduction and acetylation, the per-acetylated polyols analyzed by gas-liquid chromatography together with xylose and *scyllo*-inositol as internal standards. *Gro*, triacetyl glycerol; *Man*, hexaacetyl mannitol; *Gal*, hexaacetyl galactitol; *m-Ins*, hexaacetyl *myo*-inositol; *GlcN*, heptaacetyl glucosaminitol; *Xyl*, pentaacetyl xylitol; *s-Ins*, hexaacetyl *scyllo*-inositol. *Panel A*, analysis of the complete anchor from tunicamycin-treated cells. *Panel B*, analysis of the polar anchor fragment from untreated cells obtained after HF cleavage and reisolation from TLC. Further hydrolysis as above.

Analytical Approach to the Structural Arrangement of the Anchor—To study the structural arrangement of the constituents, we cleaved the anchor sequentially by five sequential chemical and enzymic reactions and examined the cleavage products. In this experiment (Fig. 6) yeast cells were metabolically double-labeled with [^{3}H]stearic acid (*open bars*) and *myo*-[^{14}C]inositol (*filled bars*).

In TLC analysis (Fig. 6) of the purified anchor (after affinity purification and Pronase digestion of the protein), the [^{3}H]stearic acid and the *myo*-[^{14}C]inositol labels comigrate in one spot close to the origin on TLC (*panel A*, spot I; recovered almost quantitatively). After nitrous acid deamination of this material, the cleaved double-labeled anchor structure has a less polar character and migrates to a position coincident with the phosphatidylinositol standard (*panel B*, spot III; spot II contains uncleaved starting material). This suggests direct linkage of phosphatidylinositol to nonacetylated glucosamine. The material reisolated from spot III was then cleaved by PLD from cabbage (*panel C*) or, in a different aliquot, with PI-PLC from *B. cereus* (*panel D*). The almost complete loss of the *myo*-inositol label is observed in both cases. The ^{14}C-labeled polar material recovered at the origin of the TLC plate in both instances most likely is comprised of inositol (*panel C*) and 1,2-cyclic inositol phosphate (*panel D*), respectively. After the alternative cleavage with PLD, the [^{3}H]stearic acid label is retained in a compound which exhibits the migration behavior of phosphatidic acid consistent with the enzyme's cleavage specificity (*panel C*, spot V). Treatment of the other aliquot with PI-PLC shifts the ^{3}H-labeled spot to the position of the diacylglycerol standard as expected (*panel D*, spot VII; the efficiency of both lipolytic cleavages is about 50% as can be seen from the amount of uncleaved double-labeled PI, spots IV and VI, respectively). Subsequent acetolysis converts the major portion of the stearic acid-labeled PLD reaction product (eluted from spot V) to a less polar structure, comigrating with the diglyceride acetate standard in agreement with the exchange of phosphate in phosphatidic acid for acetate (*panel E*, spot VIII; again a significant portion of the starting material, spot IX, remains unaltered). The final base hydrolysis of the residual [^{3}H] stearic acid-labeled anchor structures (spots VIII and VII combined) generates a single ^{3}H radioactive spot (about 5% recovery of counts relative to the starting material) comigrating with the stearic acid standard (*panel F*, spot X). This is consistent with the almost complete hydrolytic release of [^{3}H]

TABLE I

Quantitative evaluation of anchor analyses

The total anchor of the cAMP-binding protein isolated from cells grown in the presence of tunicamycin (see legend to Fig. 4) and the polar HF fragment isolated from cells grown in the absence of tunicamycin were purified as described under "Experimental Procedures" and analyzed by gas liquid chromatography and amino acid analysis as described in Figs. 4 and 5. Gror, triacetyl glycerol; Man, hexaacetyl mennitol; Gal, hexaacetyl galactitol; m-Ins, hexaacetyl *myo*-inositol; GlcN, heptaacetyl glucosaminitol; EthN, ethanolamine.

	Gas-liquid chromatography					Amino acid analyzer			
	Gro	Man	Gal	m-Ins	GlcN	GlcN	Asp	NH_3	EthN
	nmol/200 μl					*nmol/200 μl*			
Total anchor (tunicamycin)	54.4	74.5	53.3	30.6	18.8	27.9	12.3	32.8	27.9
Polar fragment	0	69.3	68.9	39.6	26.4	48.1	0	0	0

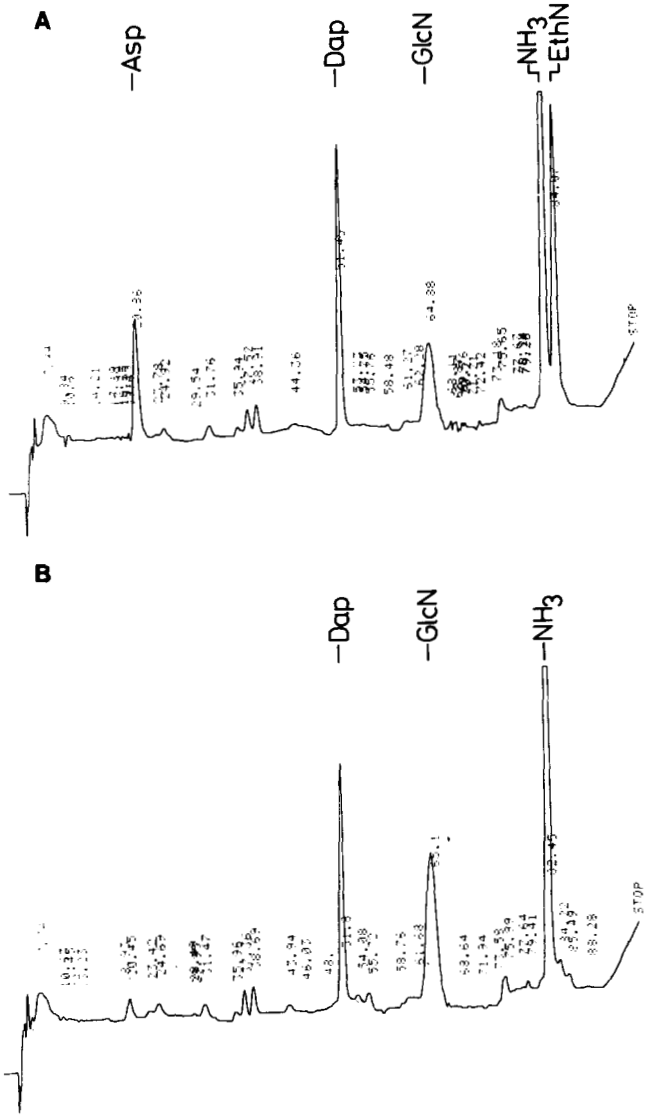

FIG. 5. **Analysis of ninhydrin-positive components.** The anchor was purified as described under Fig. 4 and hydrolyzed. *Panel A*, analysis of the complete anchor from tunicamycin-treated cells. *Panel B*, analysis of the polar anchor fragment from untreated cells obtained after HF cleavage, reisolation from TLC, and subsequent hydrolysis. *Asp*, aspartic acid; *Dap*, diaminopimelic acid; *GlcN*, glucosamine; *EthN*, ethanolamine.

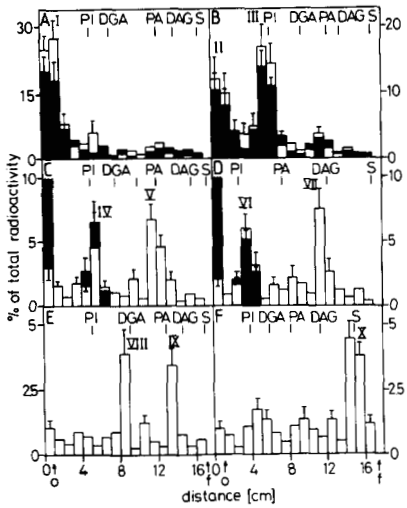

FIG. 6. **TLC analysis of the constituents of the membrane anchor after sequential degradation.** Affinity-purified cAMP-binding protein (from 500 μg of plasma membranes), double-labeled (short term) with [^{3}H]stearic acid (*open bars*) and [^{14}C]inositol (*filled bars*), was digested with Pronase. The anchor-containing structure was analyzed by TLC (*panel A*, solvent system I). The anchor-derived, radiolabeled material was eluted and subjected to the next degradative step as detailed under "Experimental Procedures." Material recovered from spot I was sequentially subjected to nitrous acid deamination (*panel B*, solvent system II), digestion with PLD (cabbage) (*panel C*, solvent system III) or, alternatively, with PI-PLC (*B. cereus*) (*panel D*, solvent system III). The material eluted from spot V was subjected to acetolysis (*panel E*, solvent system IV) and finally to base hydrolysis (*panel F*, solvent system V). Radioactivity values after treatment with PLD and PLC were corrected for the splitting of the samples. Unlabeled phosphatidylinositol (*PI*), 1,2-distearyl-3-acetylglycerol (*DGA*), distearyl phosphatidate (*PA*), distearyl glycerol (*DAG*), and stearic acid (*S*) standards were localized on the same plate with rhodamine 6G. The ^{3}H and ^{14}C radioactivities of the affinity-purified cAMP-binding protein (before Pronase treatment) was taken as 100% (9,500 and 37,400 dpm, respectively). *o*, origin; *f*, front.

stearic acid from stearic acid-labeled diacylglycerol (spot VII) and diglyceride acetate (spot VIII). Taken together, the amenability of the lipidic anchor structure to digestion with PLD and PI-PLC, acetolysis, and base hydrolysis demonstrates that the plasma membrane cAMP-binding protein is covalently modified by phosphatidylinositol. The lipid is linked glycosidically to nonacetylated glucosamine as revealed by cleavage at the glucosaminyl residue after nitrous acid deamination as well as by carbohydrate analysis of the anchor. The arrangement is consistent with the structure of a GPI.

To elucidate also the number and arrangement of phosphorus atoms present in the glycan portion of the lipid anchor in addition to the one in phosphatidylinositol, affinity-purified cAMP-binding protein from ^{32}P-labeled cells was again digested with Pronase, the anchor purified by two successive TLC runs with different solvent systems and then chromatographed a third time by TLC using the basic solvent system (Fig. 7*A*, spot I). The reisolated anchor was then digested with GPI-PLD from rabbit serum (*panel B*) or GPI-PLC from *T. brucei* (*panel C*), respectively, followed again by TLC analysis of the cleavage products.

After treatment with GPI-PLD spot I is converted to two new, about equally strongly radiolabeled spots (Fig. 7*B*). The one (spot IV) comigrates with phosphatidic acid, whereas the other (spot II) hardly leaves the origin. We conclude that this latter highly polar material presumably consists of the oligosaccharide head group labeled with ^{32}P at the nonreducing end of its terminal sugar residue. The sum of radioactivity recovered in ^{32}P-labeled phosphatidic acid plus head group is approximately 45% (about 25% of the anchor structure is not attacked by GPI-PLD, spot III). This finding suggests that the oligosaccharide head group harbors one phosphorus atom/mol, presumably as terminal phosphorylethanolamine, whereas the other is present in the PI moiety. After digestion with GPI-PLC from *T. brucei* (Fig. 7*C*) the major portion of ^{32}P label of the anchor structure is recovered from a single polar cleavage product (spot V). It is likely to harbor both phosphorus atoms, since it contains about the sum of ^{32}P radioactivity of spots II plus IV. The only other ^{32}P-labeled compound (spot VI) comigrates with the uncleaved anchor structure. This suggests that the second cleavage product is unlabeled, presumably consisting of diacylglycerol. The occurrence and structural arrangement of two phosphorus atoms in the lipidic anchor agree with a GPI structure.

Cross-reacting Immunological Determinants in the GPI Anchors of the cAMP-binding Protein from Yeast Plasma Membranes and VSG from T. brucei—The above analyses suggest that the anchor of the cAMP-binding protein contains all

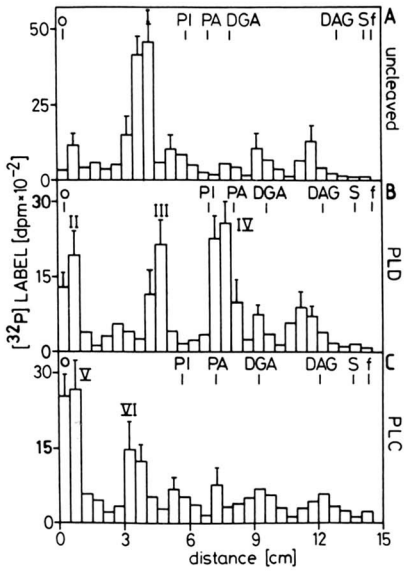

FIG. 7. **Incorporation of [$^{32}P_i$] into the anchor structure of the cAMP-binding protein.** Yeast cells were metabolically labeled (to equilibrium) with [^{32}P]P_i. The affinity-purified cAMP-binding protein was digested with Pronase, the anchor purified by two sequential TLCs, and then chromatographed (solvent system II) for a third time (*panel A*, uncleaved). Reeluted anchor material was digested with GPI-PLD from rabbit serum (*panel B*, *PLD*) or GPI-PLC from *T. brucei* (*panel C*, *PLC*). Radioactivity (triplicates) was evaluated by a linear TLC analyzer. Standard deviations are indicated. The positions of the standards (see legend to Fig. 6), indicated at the *top* of each run, were determined separately.

components typically present in GPI anchors. To confirm further the similarity to GPIs from higher organisms in terms of structural arrangement and to prove identity of epitopes, we examined immunological cross-reactivity to trypanosomal CRD. Cross-reacting determinants were found to reside in the GPI anchors of the VSGs from different lines of trypanosomes expressing different VSGs (47, 48). One of the determinants is constituted by the unmodified glucosamine of the anchor. Another one is formed by *myo*-inositol 1,2-cyclic phosphate generated upon cleavage by GPI-PLC (48). Since both of these determinants are presumably also contained in the yeast anchor, we reacted a protein blot of the affinity-purified cAMP receptor, pretreated with GPI-PLC or GPI-PLD, with an anti-CRD antiserum (Fig. 8*A*).

In the control, the authentic, soluble VSG generates a strong signal at the expected mobility corresponding to 60 kDa (*lane 1*). Indeed, the affinity-purified, otherwise untreated cAMP receptor yields a weak signal corresponding to a 54-kDa molecular mass (*lane 3*) which comigrates with the authentic photoaffinity-labeled cAMP-binding protein (*lane 2*). The signal is enhanced severalfold upon cleavage of the anchor with GPI-PLC dependent on the concentration of the lipase (*lanes 4* and *5*). This indicates that removal of diacylglycerol generates or exposes an additional strong epitope, most likely the 1,2-cyclic phosphate diester at the *myo*-inositol which is formed by cleavage with GPI-PLC. Accordingly, no signal is obtained after cleavage with GPI-PLD (*lane 11*). The PLC-treated cAMP receptor was subjected to various treatments known to affect the 1,2-cyclic phosphodiester bonds at the *myo*-inositol. As expected, oxidation of the carbohydrate moiety with periodate (*lanes 6* and *10*), phosphodiester cleavage with aqueous HF (*lane 7*), and removal of phosphatidylinositol by nitrous acid (*lane 9*) eliminate recognition by the antibody. This reflects the fact that the anti-CRD antiserum recognizes cross-reacting determinants

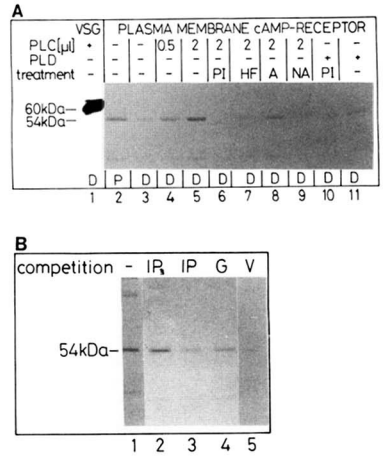

FIG. 8. **Immunological characterization of the anchor structure.** *Panel A*, affinity-purified cAMP-binding protein (from 100 µg of plasma membranes each) was incubated in the presence or absence of GPI-PLC (*T. brucei*; *PLC*) or GPI-PLD (rabbit serum; *PLD*) and subsequently subjected (*treatment*) to periodate oxidation (*PI*), aqueous HF dephosphorylation (*HF*), mild acid hydrolysis (*A*), nitrous acid deamination (*NA*), or left untreated (−). Samples were separated by SDS-PAGE, transferred to nitrocellulose, incubated with polyclonal rabbit anti-117 VSG antiserum (dilution 1:500), and decorated with ^{125}I-labeled mouse anti-rabbit IgG (*D*). As controls, 0.5 µg of soluble (GPI-PLC treated) VSG protein (*lane 1*) and 8-N_3-[^{32}P]cAMP photoaffinity-labeled plasma membranes (5,000 dpm, *lane 2*) were run in parallel on the same gel. Following transfer, *lane 2* was cut off and directly autoradiographed without immunodecoration (*P*). Molecular masses were derived from marker proteins run in parallel on the same gel. *Panel B*, solubilized plasma membranes photoaffinity labeled with 8-N_3-[^{32}P]cAMP (5,500 dpm) were incubated with GPI-PLC from *T. brucei*, concentrated, incubated with a 1:100 dilution of anti-117 VSG antiserum in the presence or absence of a 5 mM concentration of either *myo*-inositol 1,4,5-trisphosphate (IP_3), *myo*-inositol 1,2-cyclic phosphate (*IP*), glucosamine HCl (*G*), or 10 µg of soluble VSG (*V*) (*competition*), precipitated with mouse anti-rabbit IgG-agarose and analyzed by SDS-PAGE and autoradiography. The molecular mass indicated is derived from total photoaffinity-labeled cAMP-binding protein run in parallel on the same gel.

within the glycolipid and not within the protein moiety. Interestingly, also decyclization of the 1,2-cyclic phosphate ring by mild acid treatment reduces the reaction with anti-CRD antiserum to about 20% (*lane 8*), comparable with the antibody reactivity of the uncleaved structure (*lane 3*). These results indicate that approximately 80% of the anti-CRD antibodies recognize inositol 1,2-cyclic phosphate in agreement with the data obtained with authentic soluble VSG from trypanosomes (48) and suggest that the anti-CRD epitope is located within the glucosaminyl-inositol phosphate portion of the anchor structure of the cAMP-binding protein.

Since it is important to characterize this epitope from the yeast anchor in greater detail to be able to demonstrate similarity to the respective structure from higher eucaryotes we measured the competition of antibody binding by constituents present in GPI. Binding was assayed by indirect immunoprecipitation using anti-rabbit IgG-agarose. Fig. 8*B* shows an autoradiogram of the electrophoretically separated immunoprecipitates.

The anti-CRD antiserum immunoprecipitates the GPI-PLC-cleaved, photoaffinity-labeled cAMP-binding protein (*lane 1*). The specificity of the immunoprecipitation and the immunological relationship between the GPI anchors from VSG and from the cAMP-binding protein are best demonstrated by the efficient competition by excess soluble VSG (*lane 5*). The weakening of the immunoprecipitation signals

observed in the presence of *myo*-inositol-1,2-cyclic phosphate (*lane 3*) and glucosamine (*lane 4*) but not in the presence of inositol trisphosphate (*lane 2*) proves that these constituents comprise highly antigenic determinants of the anchors of both soluble VSG (48) and the cAMP-binding protein from yeast. This confirms that the two structures are either identical or very closely related.

DISCUSSION

This study provides the first detailed structural analysis of the lipidic membrane anchor of a plasma membrane protein from a lower eucaryote, showing its close relatedness to GPIs from higher eucaryotes. *S. cerevisiae* harbors a 54-kDa glycoprotein bound to the outer face of the plasma membrane which binds cAMP with high affinity (17). It is genetically unrelated to the regulatory subunit of cytoplasmic protein kinase A (43), which has been reported to occur both in a soluble and membrane-associated state. The following observations provide strong evidence that anchorage of the cAMP receptor protein is achieved through a GPI moiety. (i) The cAMP-binding protein is amphiphilic like typical GPI-modified proteins. It is converted hydrophilic by bacterial PI-specific and trypanosomal GPI-specific phospholipases. (ii) The protein can be metabolically labeled with all GPI-specific constituents. The label is preserved in the presence of tunicamycin and resists deglycosylation by *N*-glycanase. All label, except ethanolamine, is lost from the deglycosylated protein after dephosphorylation with aqueous HF. (iii) The metabolic labels can be recovered, at least in part, together with the anchor after digestion of the protein with Pronase. Analysis of the anchor reveals the presence of all components occurring in GPIs from higher eucaryotes. The almost quantitative release of the radiolabeled fatty acids from the diglyceride (acetate) moiety by alkaline hydrolysis suggests that both fatty acids are linked to glycerol via ester bonds. Since nitrous acid deamination generates a ^{3}H-fatty acid-labeled fragment which comigrates on TLC with PI, the inositol ring seems to be unmodified. (iv) The hydrophilic form of the cAMP-binding protein obtained after GPI-PLC digestion is recognized by antibodies raised against the (CRD of) soluble VSG from *T. brucei* both in immunodecoration and immunoprecipitation. Cross-reacting determinants reside in the anchor, not in the protein moiety.

These results strongly support the conclusion that the cAMP receptor protein is attached to the plasma membrane by a typical GPI anchor. The presence of glycerol and stearic acid excludes that the anchor contains sphingolipid. The occurrence of lipidic membrane anchors other than GPI (*e.g.* by fatty acids, ceramide-based anchors) has been reported (10, 14). In fact, it was observed that the GPI-peptide prepared by Pronase digestion of the cAMP affinity-purified protein, which yielded a single protein band in SDS-PAGE, was eluted in two peaks from a octyl-Sepharose column using a 1-propanol gradient. Only the major more hydrophobic second fraction was analyzed in this paper. Gas chromatographic analysis showed that the first did not contain glycerol but a less volatile, unidentified component instead (data not shown). This could be interpreted to mean that the same protein carries to alternative anchors, one of which is a GPI, whereas the other could be a ceramide-based structure, as recently described by Conzelmann *et al.* (14).

The variation in the labeling patterns after labeling with anchor-specific compounds and the difference to the Coomassie Blue staining pattern, in addition, provide solid evidence against the argument that the label may have been converted to other compounds like amino acids. The proteins are not uniformly labeled. This is particularly evident from the observation that three essentially unlabeled proteins (molecular masses of 68, 64, and 30 kDa) can be eluted from the cAMP affinity column together with the cAMP receptor after labeling cells with inositol, glucosamine or ethanolamine.[2] The nature of these polypeptides, which were coeluted with the 54-kDa protein from the cAMP affinity column although they do not bind cAMP, is unknown. Perhaps they represent components of a multisubunit cAMP receptor complex not dissociated during affinity purification.

The GPI-specific arrangement of the anchor of the cAMP-binding protein is particularly underlined by the cross-reactivity with a serum raised against soluble VSG from trypanosomes. Anti-CRD antibodies immunoprecipitate GPI-modified proteins only if a galactose branch is present in the carbohydrate moiety of the PI-PLC-cleaved anchor (46, 47) which, in fact, has been found by gas-liquid chromatography of the anchor constituents and by results obtained from metabolic labeling of the anchor with [^{14}C]galactose (data not shown). Specific chemical destruction of individual epitopes of the soluble cAMP receptor and competitive immunoprecipitation with single constituents of GPI structures indicate the presence of at least three overlapping epitopes which are responsible for the cross-reactivity with the anti-VSG antibodies. They are comprised by *myo*-inositol 1,2-cyclic phosphate, the non-*N*-acetylated-glucosamine residue, and the galactose branch, consistent with the analysis of the VSG anchor (48).

Thus, GPI anchors are similar or even identical in lower and higher eucaryotes. The identification of GPI anchorage of an increasing number of ectoproteins from higher eucaryotes and the conservation of such structures in evolution imply some important biological function about which little is known (for a review see Ref. 49). Yeast provides a versatile and genetically manipulable model organism for the study of the biosynthesis and physiological role of GPI anchor structures. An example in which lipidic anchorage has regulatory implications on the protein's activity has been described recently in yeast mitochondria (21, 22). The protein binds cAMP and is linked to the outer face of the inner mitochondrial membrane by a covalently attached PI moiety lacking carbohydrates. It appears likely that it comprises the regulatory subunit of a cAMP-dependent protein kinase but fails to release an active catalytic subunit in the membrane-bound state despite the presence of cAMP. Incubation of isolated mitochondria with phospholipids or phorbolester in the presence of Ca^{2+} and ATP causes lipolytic cleavage of the anchor and release of the cAMP receptor into the soluble intermembrane space and simultaneously leads to the activation of a soluble protein kinase in the intermembrane space which phosphorylates a 40-kDa inner membrane protein in a cAMP-dependent manner. This implies that in this case membrane anchorage controls the activity of a cAMP-dependent protein kinase in a fashion in which lipolytic membrane release is epistatic to the dissociation of the complex by cAMP. Unfortunately, such a regulatory role could not yet be established for the membrane anchorage of the cAMP receptor from plasma membranes of yeast.

It has been observed, however, that lipolytic cleavage of the GPI anchor of the yeast plasma membrane cAMP receptor by PI-PLC (*B. cereus*) causes a significant change in the pattern of amino acids, which can be photoaffinity labeled with 8-N_3-[^{32}P]cAMP.[3] This may indicate a conformational alteration also in the plasma membrane protein moiety, presumably accompanied by a functional change, occurring upon

[3] G. Müller and W. Bandlow, manuscript submitted for publication.

cleavage of the anchor. Similarly, in mammalian cells, the hormone-controlled release of GPI-anchored proteins from the membrane surface has been described for alkaline phosphatase, heparansulfate proteoglycan, lipoprotein lipase, and 5′-nucleotidase (7, 39, 50, 51). Currently we are searching for physiological conditions causing lipolytic cleavage of the GPI anchor of the cAMP-binding protein from yeast plasma membranes.

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