

# Glucose Induces Lipolytic Cleavage of a Glycolipidic Plasma Membrane Anchor in Yeast

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**Abstract.** In the yeast *Saccharomyces cerevisiae* an amphiphilic cAMP-binding protein has been found recently to be anchored to plasma membranes by virtue of a glycolipid structure (Müller and Bandlow, 1991a, 1992). The cAMP-binding parameters of this protein are affected by the lipolytic removal of the glycosyl-phosphatidylinositol (GPI) membrane anchor by exogenous (G)PI-specific phospholipases C or D (PLC or PLD) (Müller and Bandlow, 1993) suggesting a regulatory role of glycolipidic membrane anchorage. Here we report that transfer of yeast cells from lactate to glucose medium results in the conversion of the amphiphilic form of the cAMP receptor protein into a hydrophilic version accompanied by the rapid loss of fatty acids from the GPI anchor of the [<sup>14</sup>C]palmitic acid-labeled protein. Analysis of the cleavage site identifies [<sup>14</sup>C]inositol phosphate as the major product after treatment of the soluble, [<sup>14</sup>C]inositol-labeled protein with nitrous acid which destroys the glucos-

amine constituent of the anchor. Together with the observed cross-reactivity of the hydrophilic fragment with antibodies directed against the cross-reacting determinant of soluble trypanosomal variable surface glycoproteins (i.e., *myo*-inositol-1,2-cyclic phosphate) this demonstrates that, in membrane release, the initial cleavage event is catalyzed by an intrinsic GPI-PLC activated upon transfer of cells to glucose medium. Release from the plasma membrane in soluble form requires, in addition, the presence of high salt or α-methyl mannoside, or the removal of the carbohydrate moieties, because otherwise the protein remains associated with the membrane presumably at least in part via its N-glycosidic carbohydrate side chains. The data point to the possibility that cleavage of the anchor could play a role in the transfer of the signal for the nutritional situation to the interior of the cell.

VARIOUS proteins have been found to be anchored to the outer face of the plasma membrane by virtue of a glycolipid (GPI)<sup>1</sup> structure. The anchor, typically containing phosphatidylinositol and a characteristic core glycan consisting of non-acetylated glucosamine and mannose, is linked via a phosphate ester bridge and ethanolamine to the COOH terminus of the respective polypeptides. During the biogenesis of this type of cell surface proteins, the preformed anchor is transferred as a whole to the protein thereby replacing a short COOH-terminal transmembrane domain (Caras, 1991; Kodukula et al., 1992; for review see Doering et al., 1990). This type of anchor is conserved in

evolution from yeast to man. In yeast, several proteins have been characterized which behave as integral plasma membrane proteins, but can be released from their resident membrane by exogenous (G)PI-specific phospholipases. They can be metabolically labeled with certain lipid constituents and carbohydrates and have been identified as glycosyl-phosphatidylinositol-anchored (Conzelmann et al., 1988, 1990; Vai et al., 1990; Müller and Bandlow, 1991a,b). In one case the anchor of a yeast protein has been analyzed in some detail and shown to be structurally and immunologically closely related or even identical to typical GPI anchors from protozoa and higher eucaryotes (Müller et al., 1992).

The group of GPI-anchored proteins is functionally extremely heterogeneous. The most extensively studied example is the variable surface glycoprotein from the parasitic protozoon, *Trypanosoma brucei* (Bülow and Overath, 1986; Zamze et al., 1988; Mayor et al., 1990). In vertebrates the group of GPI-anchored proteins comprises several surface receptors, cell adhesion and extracellular matrix molecules, the prion protein, and a number of hydrolytically active ectoenzymes. Some of these proteins exist in soluble extracellular and in plasma membrane-associated forms. The two ver-

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1. Abbreviations used in this paper: CRD, cross-reacting determinant; GPI, glycosyl-phosphatidylinositol; GPI-PLC(D), glycosyl-phosphatidylinositol-specific phospholipase C(D) from *Trypanosoma brucei* (rabbit serum); α-MMP, α-methyl mannoside; VSG, variable surface glycoprotein from *Trypanosoma brucei*.

sions may be related by alternative splicing or may even be encoded by separate, although very similar genes, and may be expressed or processed differently in different cell types (for review see Low, 1989). In most cases the enzymic activities of the alternative forms of the proteins are very similar, and no specific difference has been found to correlate with the presence or absence of the GPI anchor. These observations suggest that it is not a general requirement for a change of the properties of the protein that dictates the necessity of anchorage by GPI. Why some of these proteins use GPI structures for membrane anchorage in one type of cells but not in another, i.e., the question for the physiological significance of GPI membrane anchorage, is a pendent problem. The observation that in some cell lines surface expression of subsets of GPI-anchored proteins can be modulated dramatically by starvation for serum factors or by treatment with insulin, whereupon some of them are released from the plasma membrane in soluble form (Romero et al., 1988; Chan et al., 1988; Lisanti et al., 1989) together with the evolutionary conservation of the glycosyl-phosphatidylinositol anchor implies an important role for this structure.

To study glycolipidic membrane anchorage and to assign it a physiological role, the detection of GPI anchors in yeast as a microorganism amenable to genetic manipulation may be helpful. In this organism three glycoproteins of 125 kD (Conzelmann et al., 1988), 115 kD (Vai et al., 1990), and 54 kD (Müller and Bandlow, 1991a; Miller et al., 1992), respectively, have been shown to be bound to the outer face of the plasma membrane by virtue of glycolipidic membrane anchors. No specific biological function could so far be attributed to any of these proteins. The latter has been characterized by its property to bind cAMP specifically and with high affinity (Müller and Bandlow, 1991a). Interestingly, the cleavage of the GPI anchor of this protein results in a change of ligand binding kinetics and affinity as well as in the alteration of the amino acid residues amenable to photoaffinity labeling (Müller and Bandlow, 1993). Therefore, we asked whether the GPI anchor of this protein is cleaved in vivo under certain conditions so that anchor cleavage may have a functional meaning.

In this paper, we report that the cAMP receptor protein is lipolytically released from the plasma membrane of oxidatively growing cells upon addition of glucose to the medium. We show that the primary cleavage is effected by a PLC activity and that thereby the originally amphiphilic protein is turned hydrophilic, but remains peripherally associated with the plasma membrane from its periplasmic side. We discuss that lipolytic cleavage could play a role in the generation or transfer of the signal for nutritional upshift across the plasma membrane of yeast cells.

## Materials and Methods

### Materials

*L*-[U-<sup>14</sup>C]myo-inositol-1-phosphate (50 mCi/mmol), *L*-3-[U-<sup>14</sup>C]phosphatidylinositol (220 mCi/mmol), myo-[2-<sup>3</sup>H]inositol (15 Ci/mmol), goat anti-rabbit IgG coupled to horseradish peroxidase (affinity-purified), and scintillation cocktail ACS II were bought from Amersham-Buchler, Braunschweig; 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP (20 Ci/mmol) was from ICN, Radioisotope Division, Eschwege; myo-[<sup>14</sup>C(U)]inositol (200–250 mCi/mmol), [<sup>2</sup>-<sup>14</sup>C]-ethan-1-ol-2-amine hydrochloride (54 mCi/mmol), and EN<sup>3</sup>HANCE™ (liquid and spray) were purchased from New England Nuclear, Dreieich;

N<sup>6</sup>-(2-amino-ethyl)-cAMP-Sepharose, phenyl Sepharose, Ficoll, and concanavalin A Sepharose were obtained from Pharmacia, Freiburg; PI-PLC (*Bacillus cereus*), N-glycanase was from Boehringer, Mannheim; TX-114 was bought from Fluka, Neu-Ulm; polyclonal anti-CRD anti-bodies raised against purified soluble variant surface glycoprotein (sVSG) from MIT at 1.2 in New Zealand White rabbits were gifts from P. Overath, Tübingen; crude glycosyl-phosphatidylinositol-specific phospholipase (GPI-PLD) from heparinized rabbit serum was donated by W. Guttensohn, München; silica gel Si-60 plates were from Merck, Darmstadt; α-methyl manno-pyranoside (α-MMP) was provided by Sigma, Deisenhofen; mixed-ester cellulose filters Millipore HV (0.45 μm) were purchased from Millipore, Eschborn; and all other reagents were obtained as described (Müller and Bandlow, 1991a,b).

### Yeast Strain and Culture

Strain ABYS1 defective in proteases A and B and carboxypeptidases Y and S (Achstetter et al., 1984) was grown overnight in semisynthetic lactate medium at 30°C to log phase and harvested at a titer of 10<sup>7</sup> cells/ml. Spheroplasts were prepared by digestion with Zymolase 20,000 (Müller and Bandlow, 1989).

### Metabolic Labeling

Cells were harvested, converted to spheroplasts (Müller and Bandlow, 1989), and suspended in twice the volume of synthetic lactate medium lacking yeast extract. 10-ml portions of spheroplasts (corresponding to an *A*<sub>600</sub> of 5–7) were incubated in the presence of either 0.5 mCi [<sup>14</sup>C]palmitic acid, 0.1 mCi myo-[<sup>14</sup>C]inositol, or 1 mCi [<sup>14</sup>C]ethanolamine for 1 h at 30°C. For double-labeling, 0.3 mCi myo-[<sup>3</sup>H]inositol was added in addition. The labeling was terminated by a 1,000-fold excess of the respective unlabeled compound (chase). Finally, the spheroplasts were spun through a 5-ml cushion of 0.8 M sucrose, 1.5% Ficoll, 25 mM Tris/HCl (pH 7.4), 1 mM EDTA (1,000 g, 5 min), suspended in semisynthetic medium containing yeast extract, and either 2% lactate but no glucose or various concentrations of glucose at a titer of 5 × 10<sup>7</sup> cells/ml and incubated at 30°C for the times indicated in the figure legends.

**Photoaffinity Labeling.** Photo affinity labeling of spheroplasts was carried out as described previously (Müller and Bandlow, 1991a) with the following modifications: 1.5-ml portions of spheroplasts (7.5 × 10<sup>7</sup> cells) were incubated with 10 μCi of 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP in the wells of a microtiter plate (24 wells) at 4°C for 5 min, and then irradiated at 312 nm with a UV hand lamp for 2 min at a distance of 2 cm. After addition of 100 μM cAMP, spheroplasts were centrifuged through a sucrose/Ficoll cushion and suspended as described for the metabolic labeling.

**Preparation of Plasma Membranes.** Preparation of plasma membranes was carried out as described previously but omitting the sucrose density gradient centrifugation (Müller and Bandlow, 1991a). Briefly, the spheroplasts were homogenized in the presence of protease inhibitors. The cell lysate was centrifuged (1,000 g, 3 min) and the supernatant was subjected to density gradient centrifugation on 28% Percoll, 0.25 M sucrose, 5 mM MgCl<sub>2</sub>, 1 mM DTT, 25 mM Tris/HCl (pH 7.4) (0.5 ml per 10<sup>6</sup> spheroplasts). The plasma membrane band (0.5 ml) was diluted with 4 vol of 0.25 M sucrose, 25 mM Tris/HCl, 1 mM EDTA, 1 mM DTT (STE-buffer), and supplemented with Con A Sepharose beads (50 μg per 0.5 ml). After incubation (5 min, 4°C), the beads were collected by centrifugation (10,000 g, 2 min) and suspended in 1.5 ml STE-buffer supplemented with 0.5 M mannose, 100 mM KCl. After incubation (5 min, 4°C) and centrifugation of the beads (10,000 g, 2 min), the released plasma membrane vesicles were collected by centrifugation (100,000 g, 15 min), washed once, and suspended in STE-buffer containing 200 μM PMSF at 2 mg protein/ml.

### TX-114 Partitioning

Plasma membrane proteins were separated into amphiphilic and hydrophilic ones by partitioning between a detergent (TX-114)-enriched and depleted (aqueous) phase according to Bordier (1981) with the following modifications: 25 μl of plasma membranes (50 μg of protein in STE-buffer) were solubilized by incubation (30 min, 4°C) in 1 ml ice cold TX-114 (2%) in 25 mM Tris/HCl (pH 7.4), 144 mM NaCl. The mixture was layered onto a cushion of 0.4 ml of 0.25 M sucrose, 25 mM Tris/HCl (pH 7.4) on ice. Phase separation was induced by warming up to 37°C and centrifugation (microfuge, 2 min). The detergent-enriched phase was reextracted two times. Aqueous phases were pooled.

## Retention on Phenyl Sepharose

The amphiphilic nature of the cAMP-binding protein was studied by binding to phenyl Sepharose beads. 100  $\mu$ l of solubilized plasma membranes (0.5% octyl glucoside; 150,000 g, 15 min) were supplemented with 900  $\mu$ l of PBS and 75  $\mu$ l phenyl Sepharose beads, and further processed as described previously (Müller and Bandlow, 1991a).

## Release of the cAMP-binding Protein from Spheroplasts

1 ml of spheroplasts ( $5 \times 10^7$  cells) were layered onto a 1-ml cushion of 0.8 M sucrose, 1.5% Ficoll, 20 mM Hepes/KOH (pH 7.2), 1 mM EDTA, 200  $\mu$ M PMSF (2.5 ml Eppendorf cup). After centrifugation (1,000 g, 2 min, swing out rotor), the layer on top of the sucrose cushion (soluble fraction) was carefully removed and proteins precipitated with 5% TCA (30 min on ice; 10,000 g, 5 min; three acetone washes).

## Affinity Purification

The cAMP-binding protein was purified either from total solubilized plasma membranes or from the detergent-enriched and hydrophilic phases after TX-114 partitioning by binding to N<sup>6</sup>-(2-aminoethyl)-cAMP Sepharose as described (Müller and Bandlow, 1991a) but using a batch procedure: 100–500  $\mu$ g of solubilized plasma membrane proteins in up to 500  $\mu$ l were incubated with 1 ml of cAMP-Sepharose beads. The beads were washed five times with 1.5 ml washing buffer, finally extracted with 0.5 ml of the same buffer containing 10 mM cAMP for 5 min at 4°C and centrifuged (10,000 g, 2 min). The soluble cAMP-binding protein was precipitated with an equal volume of 8% PEG 4000 in 10 mM MOPS/KOH (pH 7.2), 1 mM EDTA (30 min, 4°C; 10,000 g, 5 min; two washes with 0.8% PEG 4000). For SDS-PAGE, the pellet was directly solubilized in sample buffer (5 min, 95°C).

## Thin Layer Chromatography

Neutralized samples were concentrated by lyophilization, suspended in 50  $\mu$ l tetrahydrofuran and 5–10  $\mu$ l applied to a silica gel Si 60 TLC plate which was developed twice in pyridine/ethyl acetate/acetic acid/H<sub>2</sub>O (5:5:1:3.5, by vol). Radioactive spots were visualized by fluorography using ENHANCE™ spray.

## Miscellaneous Procedures

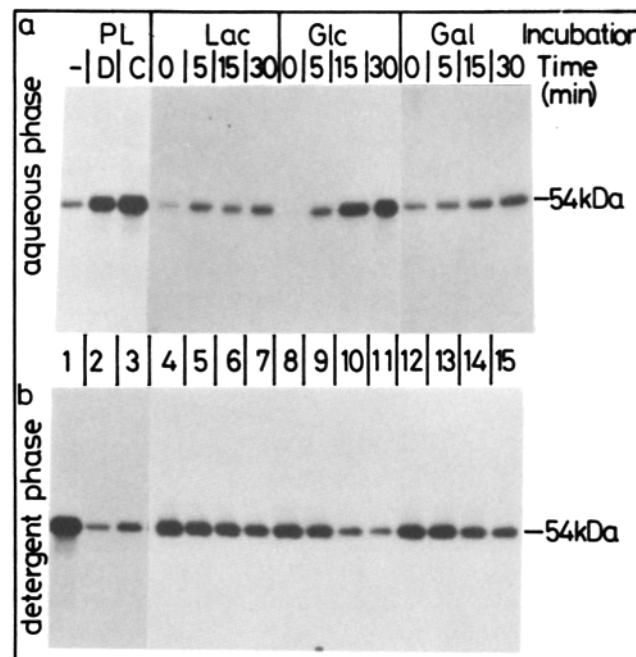
Published procedures were used for Western blotting with rabbit anti-CRD antibodies (1:1,000 dilution) using 100  $\mu$ g mouse anti-(rabbit IgG) serum coupled to horseradish peroxidase in 25 ml of 20 mM Tris/HCl (pH 7.4), 150 mM NaCl, 5% fat-free milk powder for 1 h at 22°C (Müller and Bandlow, 1991b) followed by a chemiluminescent detection method; N-glycanase treatment of spheroplasts ( $5 \times 10^7$  cells in 0.5 ml) with 5 U N-glycanase under native conditions (Müller et al., 1992) followed by centrifugation of the spheroplasts through a 0.5-ml cushion of sucrose/Ficoll (see above); acid hydrolysis of dried samples with 4 M HCl at 110°C for 6 h, followed by neutralization, lyophilization, suspension in 50  $\mu$ l of tetrahydrofuran, centrifugation (10,000 g, 2 min), and analysis of 5  $\mu$ l portions by TLC; nitrous acid deamination of the detergent-solubilized (0.5% octyl glucoside) or soluble cAMP-binding protein (after TX-114 partitioning) (Müller and Bandlow, 1991a) followed by passing of the deaminated samples through a Millex HV (0.45  $\mu$ M) for removal of the protein moiety of the cAMP-binding protein; SDS-PAGE (5% [wt/vol] stacking gel and 14% [vol/wt] running gel in the presence of urea) (Müller and Zimmermann, 1987), fluorography using ENHANCE™; densitometry with an LKB densitometer; liquid scintillation counting using scintillation cocktail ASC II; protein determination using the amidoblack staining method (Popov et al., 1975); and digestion with GPI-PLD (rabbit serum) and PI-PLC (*Bacillus cereus*) (Müller et al., 1992).

## Results

### Glucose Effects Amphiphilic to Hydrophilic Conversion of the cAMP-binding Protein

Previously, a GPI-anchored glycoprotein of 54 kD molecular mass from yeast plasma membranes, genetically unrelated to

the well known cytoplasmic R subunit of protein kinase A, has been shown to be amenable to photoaffinity labeling by 8-N<sub>3</sub>-cAMP (Müller and Bandlow, 1991a, 1993). Since cAMP plays an important role in the regulation of carbohydrate metabolism in both lower and higher eucaryotes, we examined if the cAMP-binding protein is involved in nutritional signaling. We tested if a change of the nutritional situation of yeast cells results in the cleavage of a GPI anchor in a similar fashion as the deprivation of vertebrate cells of serum factors (Lisanti et al., 1989). We found that, indeed, a change of media from non-fermentable carbon sources to glucose causes a rapid partial conversion of the photoaffinity-labeled amphiphilic cAMP-binding protein into its hydrophilic form (Fig. 1). The opposite shift of media or a change from nitrogen-rich to poor media or vice versa has no effect (not shown). Three essential points can be derived from the figure (Fig. 1, a and b): (1) A small but significant portion of the GPI-anchored amphiphilic cAMP-binding protein is continuously converted into a hydrophilic form and can be recovered from the aqueous phase after TX-114 partitioning, irrespective of the carbon source used for growing the cells (lanes 1, 4, 8, and 12 in Fig. 1 a). (2) A transfer of the cells from lactate to glucose greatly enhances the velocity and extent of the conversion and of the appearance of the protein



**Figure 1.** Generation of a hydrophilic version of the cAMP-binding protein. Spheroplasts from lactate-grown yeast cells were photoaffinity-labeled with 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP under conditions saturating for the label and subsequently transferred to either 2.5% lactate (Lac, lanes 4–7), 2% glucose (Glc, lanes 8–11), or 2% galactose (Gal, lanes 12–15) media and incubated for the periods indicated. Then, plasma membranes were prepared from equal numbers of spheroplasts (2 mg of protein). As control (PL), plasma membranes were isolated from spheroplasts incubated in lactate medium (lane 1), detergent-solubilized and digested with GPI-PLD from rabbit serum (lane 2) or PI-PLC (*Bacillus cereus*) (lane 3). All samples were subjected to TX-114 partitioning. Equivalent volumes of the aqueous (a) and detergent phases (b) were analyzed by SDS-PAGE and fluorography.

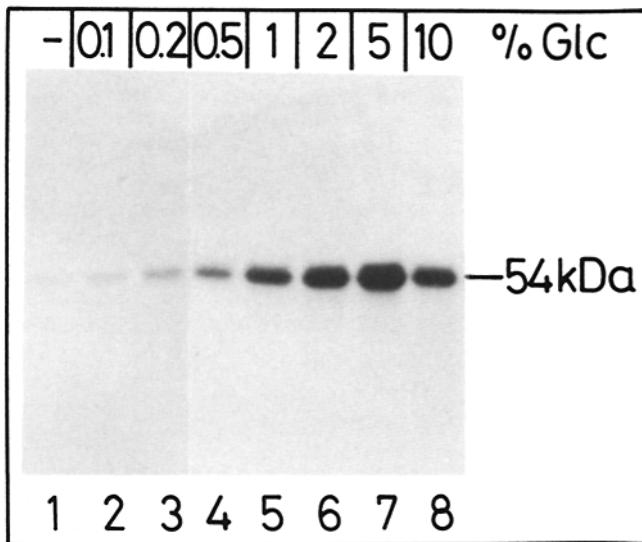
in the aqueous phase (lanes 8–11), whereas the effect of galactose is intermediate (lanes 12–15). (3) The molecular mass of the protein is not noticeably altered, making proteolytic removal of large parts from the protein's COOH terminus a less likely mechanism underlying this amphitropic change. Rather, the hydrophilic version exerts identical electrophoretic mobility and partitioning behavior as the protein liberated by either bacterial PI-PLC or GPI-PLD from serum (lanes 2 and 3 in Fig. 1, *a* and *b*). As a control and for reasons of quantitation, the TX-114 detergent phase is also shown (Fig. 1 *b*). These data suggest that glucose accelerates the amphiphilic/hydrophilic interconversion of the GPI-anchored protein.

If conversion of the membrane-anchored form of the cAMP-binding protein to a soluble version relies on a glucose-inducible hydrolytic enzyme, we suspected that it is activated proportionally to the extrinsic glucose concentration over a certain range. To examine this possibility, we transferred spheroplasts from lactate-grown cells to media containing increasing concentrations of glucose and measured the partitioning of the photoaffinity-labeled cAMP receptor protein between TX-114 and an aqueous phase. It can be seen that the glucose effect, indeed, is concentration-dependent (Fig. 2). A plateau value is reached at ~5% glucose. Again, galactose is much less efficient, whereas other sugars like mannose up to 5%, are ineffective (not shown).

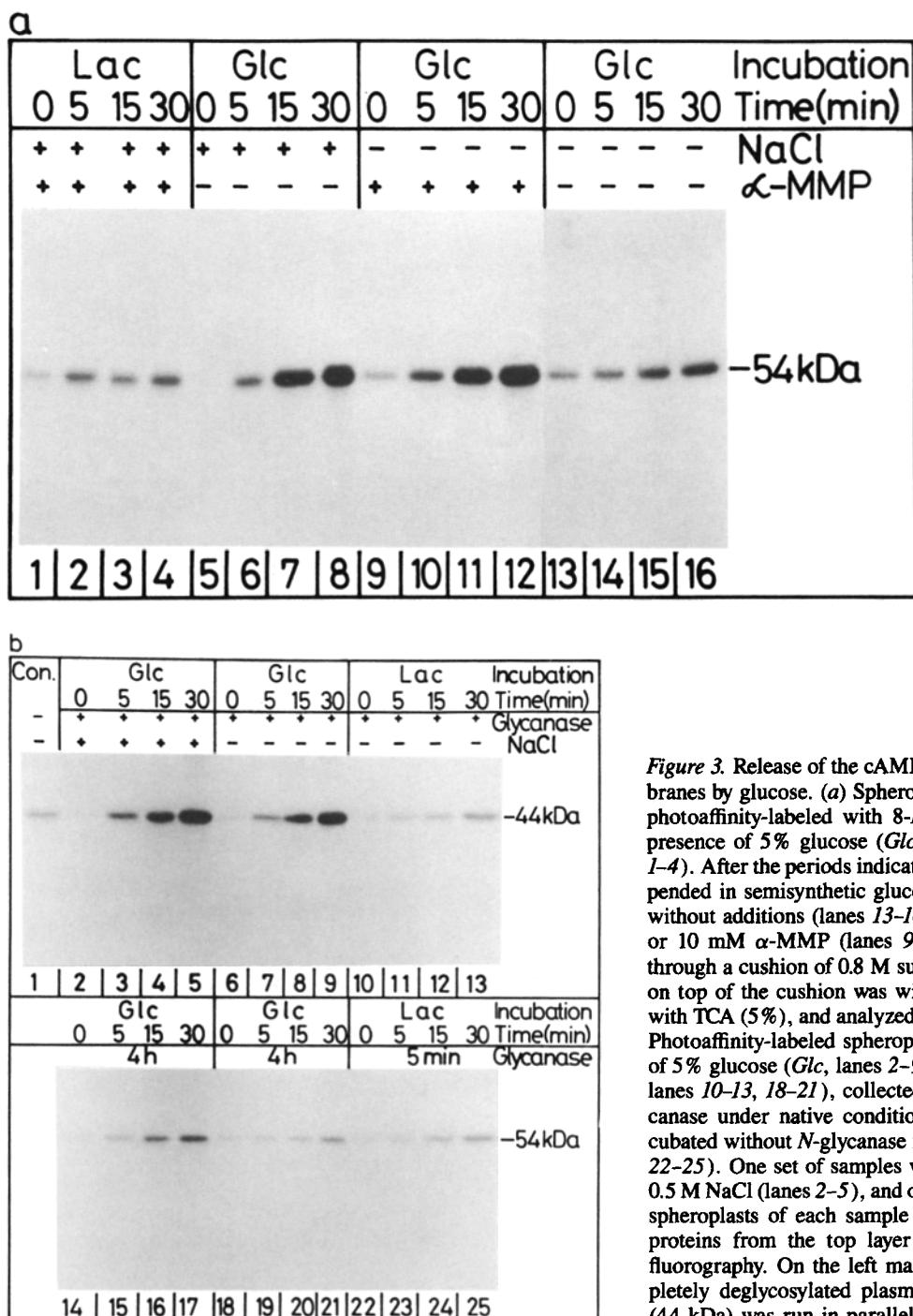
#### **The Hydrophilic Form of the Protein Remains Attached to the Membrane**

The GPI-anchored cAMP-binding protein has been shown to reside at the outer face of yeast plasma membranes (Müller and Bandlow, 1991a). In intact cells, amphiphilic to hydrophilic conversion by hydrolytic removal of the GPI mem-

brane anchor would release the protein to the periplasmic space. Therefore, we assayed if the protein is freely diffusible when liberated from plasma membrane vesicles (absence of detergents, in contrast to Figs. 1 and 2). For this purpose lactate-grown cells were protoplasted, photoaffinity-labeled with N<sub>3</sub>-cAMP, and subsequently incubated in either lactate (Lac, control) or glucose (Glc)-containing medium as detailed in the legend to Fig. 3. Membrane release was then assayed by determining the soluble cAMP receptor in the supernatant fraction after centrifugation of isolated plasma membranes through a cushion of sucrose. Fig. 3 *a* shows that the amount of photoaffinity-labeled protein released and the kinetics of its liberation upon transfer to glucose (lanes 13–16) are not dramatically enhanced over the controls (e.g., lanes 1–4). When, however, after incubation in glucose, the spheroplasts were supplied with NaCl (at concentrations high enough to perturb polar interactions) or α-MMP (as a competitor of interactions involving carbohydrate), and then centrifuged, a significantly enhanced release of the protein from the membrane in hydrophilic form was observed (compare Fig. 3 *a*, lanes 5–8 and 9–12 with both, lanes 13–16 and 1–4). This indicated that, in the presence of physiological salt concentrations, the major portion of the cAMP-binding protein remained associated with the membrane despite cleavage of the anchor and despite the hydrophilic nature of the anchor-free protein. It can be released by either high salt or α-MMP. The suspicion that this peripheral membrane attachment involves the carbohydrate moieties of this glycoprotein is underlined by the fact that, after their removal by N-glycanase (yielding a core protein of 44 kD molecular mass), glucose-induced liberation from the membrane is significantly more pronounced (Fig. 3 *b*, compare lanes 6–9 with lanes 10–13 and lanes 22–25). Inclusion of NaCl in the wash buffer supports membrane release severalfold with the holoprotein (Fig. 3 *a*), but has only a small, however significant, additional effect on the membrane release of the core protein (Fig. 3 *b*, compare lanes 6–9 with lanes 2–5). This may reflect the possibility that, in addition to the N-glycosidic side chains, other bipolar interactions, possibly comprising the anchor core structure, are involved. Mock incubation for up to 4 h without N-glycanase fails to solubilize the protein to a significant extent (Fig. 3 *b*, lanes 14–21) indicating that denaturation or degradation of a presumptive receptor protein is not responsible for the release during the treatment with the N-glycanase (compare lanes 22–25 with lanes 14–17 in Fig. 3 *b*). Fig. 4 compiles the quantitative evaluations of these results. It demonstrates that, without further treatment, the percentages of membrane-associated (*filled columns*) as well as of the soluble (*open columns*) cAMP-binding protein are about comparable after incubating spheroplasts in either lactate or glucose (columns 1 and 2 in Fig. 4, *a* and *b*). When spheroplasts were incubated only in lactate medium (Fig. 4 *b*), the ratio of membrane associated over soluble cAMP-binding protein is not significantly affected by the presence of NaCl (columns 3 and 4) or α-MMP (columns 5 and 6), or by treatment with N-glycanase (columns 7 and 8). Most of the photoaffinity-labeled material behaves as an amphiphilic protein in both TX-114 partitioning (columns 9 and 10) and phenyl Sepharose chromatography (columns 11 and 12). Only incubation with exogenous PI-PLC renders a significant portion of the material soluble and hydrophilic in spheroplasts kept in lactate (not



**Figure 2.** Conversion efficiency depends on the glucose concentration. Spheroplasts from lactate-grown cells were photoaffinity-labeled with 8-N<sub>3</sub>-[<sup>32</sup>P]cAMP and incubated with the glucose concentrations indicated. After 15 min, plasma membranes were prepared and subjected to TX-114 partitioning. Aqueous phases were analyzed by SDS-PAGE and fluorography.

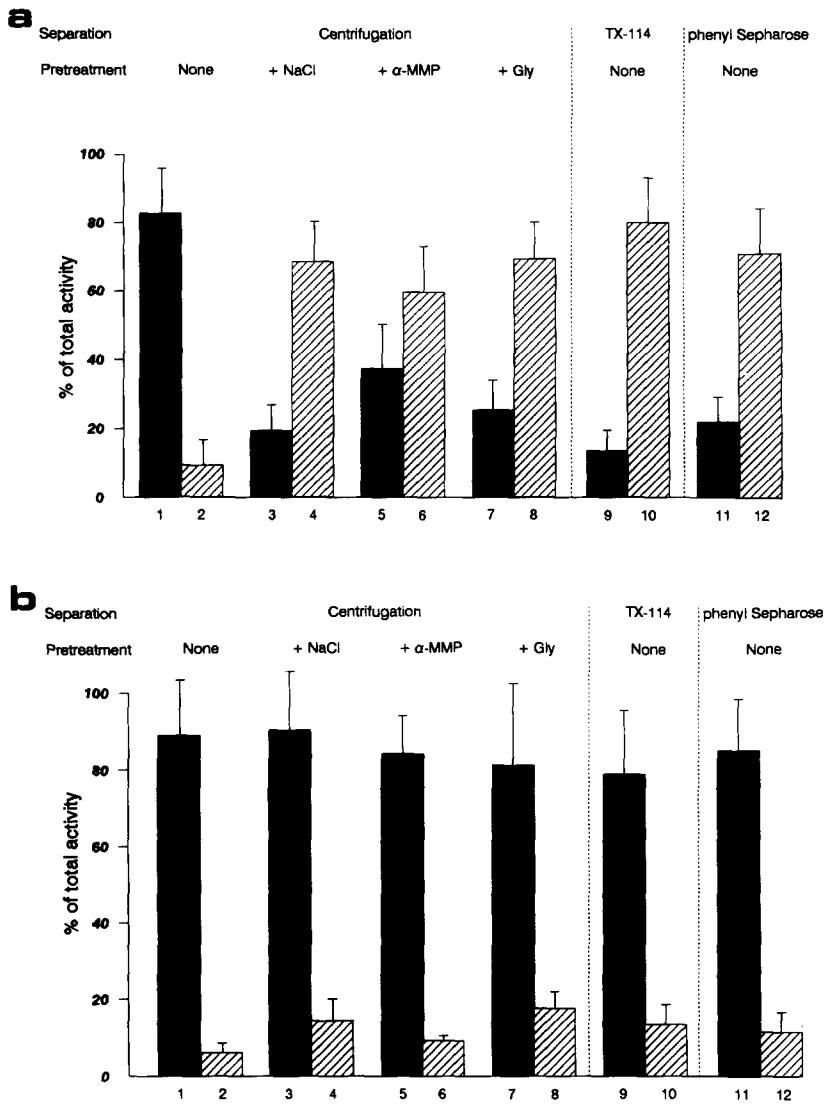


**Figure 3.** Release of the cAMP-binding protein from plasma membranes by glucose. (a) Spheroplasts from lactate-grown cells were photoaffinity-labeled with  $8-N_3-[^{32}P]cAMP$  and incubated in the presence of 5% glucose (Glc, lanes 5–16) or lactate (Lac, lanes 1–4). After the periods indicated, spheroplasts were collected, suspended in semisynthetic glucose or lactate medium, respectively, without additions (lanes 13–16) or with 0.5 M NaCl (lanes 5–8), or 10 mM  $\alpha$ -MMP (lanes 9–12) or both (lanes 1–4), and spun through a cushion of 0.8 M sucrose and 1.5% Ficoll. The medium on top of the cushion was withdrawn, proteins were precipitated with TCA (5%), and analyzed by SDS-PAGE and fluorography. (b) Photoaffinity-labeled spheroplasts were incubated in the presence of 5% glucose (Glc, lanes 2–9, 14–17, and 22–25) or lactate (Lac, lanes 10–13, 18–21), collected, and incubated for 4 h with  $N$ -glycanase under native conditions (lanes 2–13). Controls were incubated without  $N$ -glycanase for 4 h (lanes 14–21) or 5 min (lanes 22–25). One set of samples was subsequently supplemented with 0.5 M NaCl (lanes 2–5), and omitted in the other (lanes 6–25). The spheroplasts of each sample were centrifuged as above. Soluble proteins from the top layer were analyzed by SDS-PAGE and fluorography. On the left margin, photoaffinity-labeled and completely deglycosylated plasma membrane cAMP-binding protein (44 kDa) was run in parallel as a control (ma, lane 1).

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shown). By contrast to the situation with lactate, in those spheroplasts shifted to glucose medium NaCl,  $\alpha$ -MMP and treatment with  $N$ -glycanase support membrane release of a hydrophilic form of the protein significantly (Fig. 4 a, columns 3–8). These results suggest that *in vivo* transfer of the cells to glucose induces the cleavage of the anchor but leaves the protein moiety attached to the outer face of the plasma membrane presumably via carbohydrate interactions involving, at least in part, the  $N$ -glycosidic side chains. Interestingly,  $\alpha$ -MMP is much more efficient in supporting glucose-induced membrane release as compared to man-

nose: 0.5 M of the sugar are less efficient in this respect (~30%) than 10 mM of the mannose. Addition of Con A Sepharose beads, as used for the preparation of plasma membrane vesicles, further reduces liberation of the protein (to 3–5% of what can be released with  $\alpha$ -MMP, data not shown). The molecular basis of this behavior remains unknown, but these observations explain why the hydrophilic version of the cAMP receptor is almost quantitatively recovered with the plasma membrane fraction during adsorption to Con A Sepharose and elution with 0.5 M mannose (see Materials and Methods).



**Figure 4.** Efficiencies of membrane release and amphiphilic/hydrophilic conversion of the cAMP-binding protein. Photoaffinity-labeled spheroplasts from lactate-grown cells were incubated for 15 min in the presence of 2% glucose (a) or 2.5% lactate (b), and crude plasma membranes were prepared. Six aliquots were centrifuged (150,000 g, 60 min) in the absence (columns 1 and 2) or presence of 500 mM NaCl (columns 3 and 4) or 10 mM  $\alpha$ -MMP (columns 5 and 6), or after digestion with N-glycanase (Gly, columns 7 and 8). The other four aliquots were subjected to TX-114 partitioning (columns 9 and 10) or adsorbed to phenyl Sepharose (columns 11 and 12). The membrane-associated, amphiphilic (filled columns) and soluble hydrophilic cAMP-binding protein (hatched columns) is given as percentage of the total photoaffinity-labeled cAMP-binding protein before the incubation ( $\sim$ 5,500–6,000 dpm in each case).

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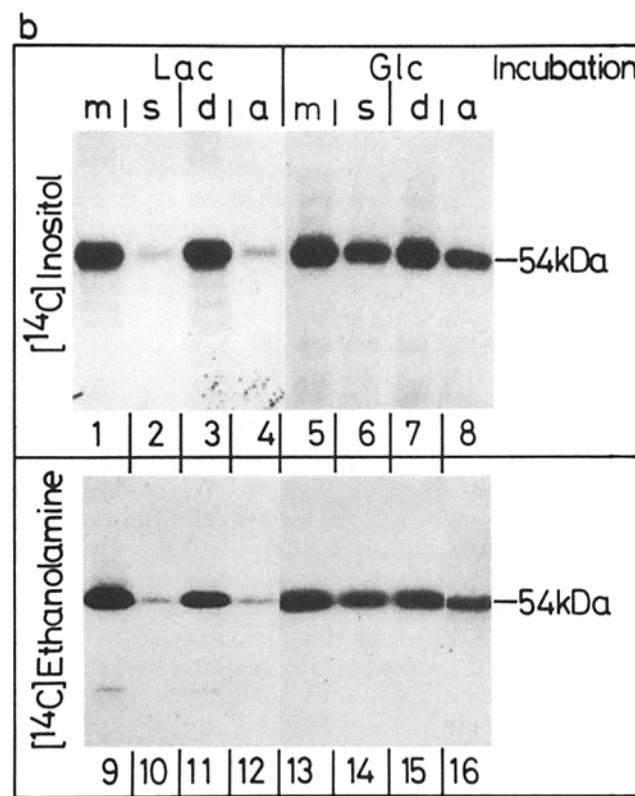
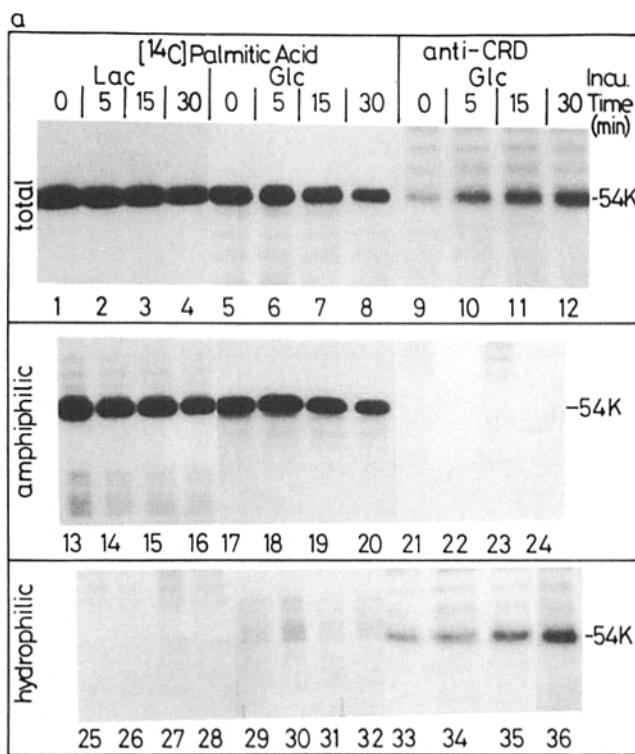
### Anchor Cleavage Is Effected by a Phospholipase C

The results described above point to the involvement of a glucose-inducible enzyme in anchor cleavage. To test this idea and to localize the cleavage site within the anchor, we metabolically labeled the protein with radiolabeled constituents of the GPI anchor: palmitic acid, *myo*-inositol, and ethanolamine. Subsequently, spheroplasts were exposed to glucose in the presence of excess unlabeled constituent, and the portions of the protein, recovered from both the detergent and the aqueous phase after TX-114 partitioning of total plasma membrane proteins, purified by cAMP affinity chromatography. Both fractions were assayed for retention or release of label by SDS-PAGE and fluorography.

To detect not only the palmitic acid-labeled complete anchor, but also the hydrophilic, protein-containing cleavage product from which the metabolic label should have been removed, this experiment (Fig. 5 a) was divided into two parts. One aliquot was used for autoradiographic detection of the metabolically labeled, anchor-containing protein (left side of Fig. 5 a). From this figure it can be seen that, after incubation in either glucose or lactate, palmitic acid exclusively

labels the amphiphilic form isolated from plasma membranes after solubilization by detergent (lanes 13–20). The label is lost from the hydrophilic version of the protein isolated from the aqueous phase after TX-114 partitioning of plasma membranes (lanes 25–32) (or from the supernatant fraction after plasma membrane centrifugation in the presence of  $\alpha$ -MMP, not shown), again widely independent of the conditions of incubation of the spheroplasts, lactate (lanes 25–28), or glucose (lanes 29–32).

To monitor also the unlabeled, soluble cAMP-binding protein and to test whether the hydrophilic conversion involves a lipolytic cleavage event, the second aliquot was reacted with an anti-trypanosomal VSG anti-cross-reacting determinant (CRD) antibody in a Western blot (right side of Fig. 5 a). The cross-reactivity of this polyclonal antiserum with soluble VSGs from different trypanosome variants (Zamze et al., 1988) as well as with the cAMP-binding protein from yeast (Müller et al., 1992) depends on the fact that the antibodies recognize distinct epitopes in the glycan portion of the GPIs from the respective proteins which are generated by the cleavage with (G)PI-PLC. Fig. 5 a shows that only in



**Figure 5.** (a) Loss of palmitic acid from the metabolically labeled cAMP-binding protein during incubation with glucose. Spheroplasts from lactate-grown cells were metabolically labeled with [<sup>14</sup>C]palmitic acid and subsequently incubated in lactate (lanes 1–4) or 5% glucose medium (lanes 5–12) supplemented with an excess unlabeled palmitic acid for the periods indicated. One half of the plasma membranes prepared was directly used for affinity purification of the cAMP-binding protein (*total*). The other half was first separated into amphiphilic and hydrophilic proteins by TX-114 partitioning, and the cAMP-binding protein was affinity-purified from the detergent and aqueous phases, respectively. Half of the glucose-treated samples was analyzed by Western blotting, immunodecoration with anti-CRD antiserum and chemiluminescent detection (lanes 9–12, 21–24, and 33–36). The other half was analyzed directly for the retention of [<sup>14</sup>C]palmitic acid by SDS-PAGE and autoradiography (lanes 1–8, 13–20, and 25–32). The molecular mass indicated on the right margin is derived from photoaffinity-labeled cAMP-binding protein run in parallel. (b) Retention of *myo*-inositol and ethanolamine in the hydrophilic version of the metabolically labeled cAMP-binding protein. Spheroplasts from lactate-grown cells were metabolically labeled with [<sup>14</sup>C]*myo*-inositol (lanes 1–8) or [<sup>14</sup>C]ethanolamine (lanes 9–16), and subsequently incubated under chase conditions in the presence of lactate (*Lac*, lanes 1–4, 9–12) or 5% glucose (*Glc*, lanes 5–8, 13–16) for 10 min. One half of the plasma membranes received 0.5 M NaCl and was centrifuged (150,000 g, 60 min) (lanes 1, 2, 5, 6, 9, 10, 13, and 14). The other half was subjected to TX-114 partitioning (lanes 3, 4, 7, 8, 11, 12, 15, and 16). The cAMP-binding protein was affinity-purified from the membrane (*m*) and soluble fractions (*s*) as well as from the detergent (*d*) and aqueous (*a*) phases, and analyzed by SDS-PAGE and fluorography. The molecular mass (54 kD) indicated on the right margin is derived from photoaffinity-labeled cAMP-binding protein run in parallel.

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the soluble version of the cAMP-binding protein, recovered either from the aqueous phase after TX-114 partitioning (lanes 33–36) or from the supernatant fraction after centrifugation of the plasma membranes in the presence of  $\alpha$ -MMP (not shown), immunoreactivity with anti-CRD antibodies can be demonstrated. This cross-reactivity has been shown to depend on the presence of *myo*-inositol-1,2-cyclic phosphate ester as the major epitope generated by (G)PI-PLC cleavage (Zamze et al., 1988). Immunodetection of the soluble version of the cAMP-binding protein is, thus, in accordance with the cleavage of the GPI by an intrinsic PLC activity. Consistently, the major epitope is lost upon mild acid hydrolysis which destroys the 1,2-cyclic phosphate ester (not shown here, but see, e.g., Zamze et al., 1988; Müller et al., 1992). Conversely, the amphiphilic material does not cross-react (lanes 21–24) because, without cleavage by PLC,

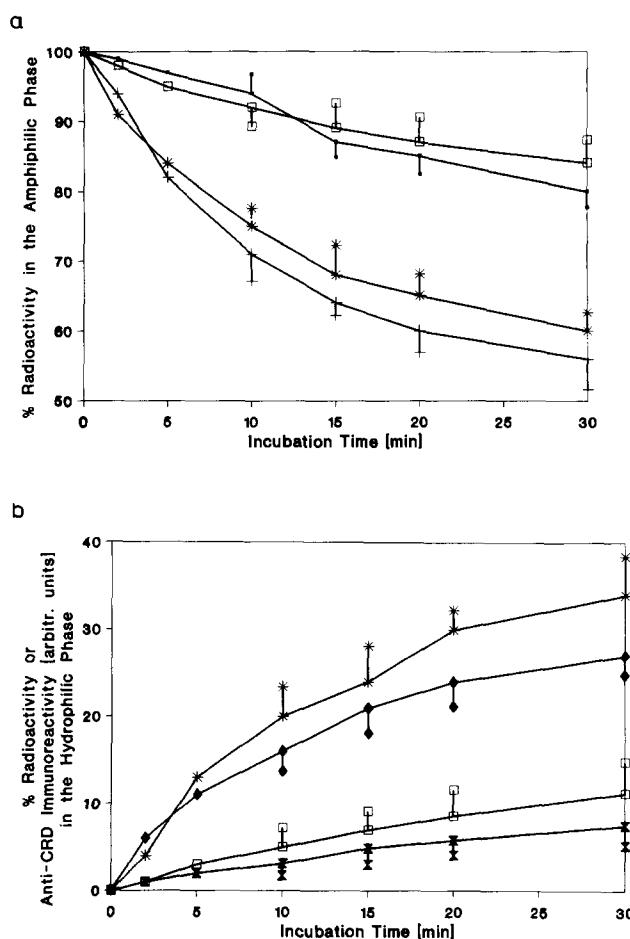
the major epitope is lacking. In addition, this result demonstrates that the antiserum does not recognize any epitopes residing in the protein moiety. As a control for quantitative recovery of the palmitic acid label and of the cross-reactive material, respectively, from TX-114 partitioning, the same amount of total plasma membranes was analyzed directly without partitioning (lanes 1–12). The sum of <sup>14</sup>C-labeled or anti-CRD-cross-reactive hydrophilic and amphiphilic cAMP-binding protein correlates nicely with the total amount used for each time point and growth condition (compare “*total*” with “*hydrophilic*” plus “*amphiphilic*” in Fig. 5 a). In immunoprecipitation of the metabolically labeled material neither the soluble nor the amphiphilic material displayed higher than background radioactivity after labeling with [<sup>14</sup>C]palmitate (not shown).

By contrast to the above results demonstrating the loss of

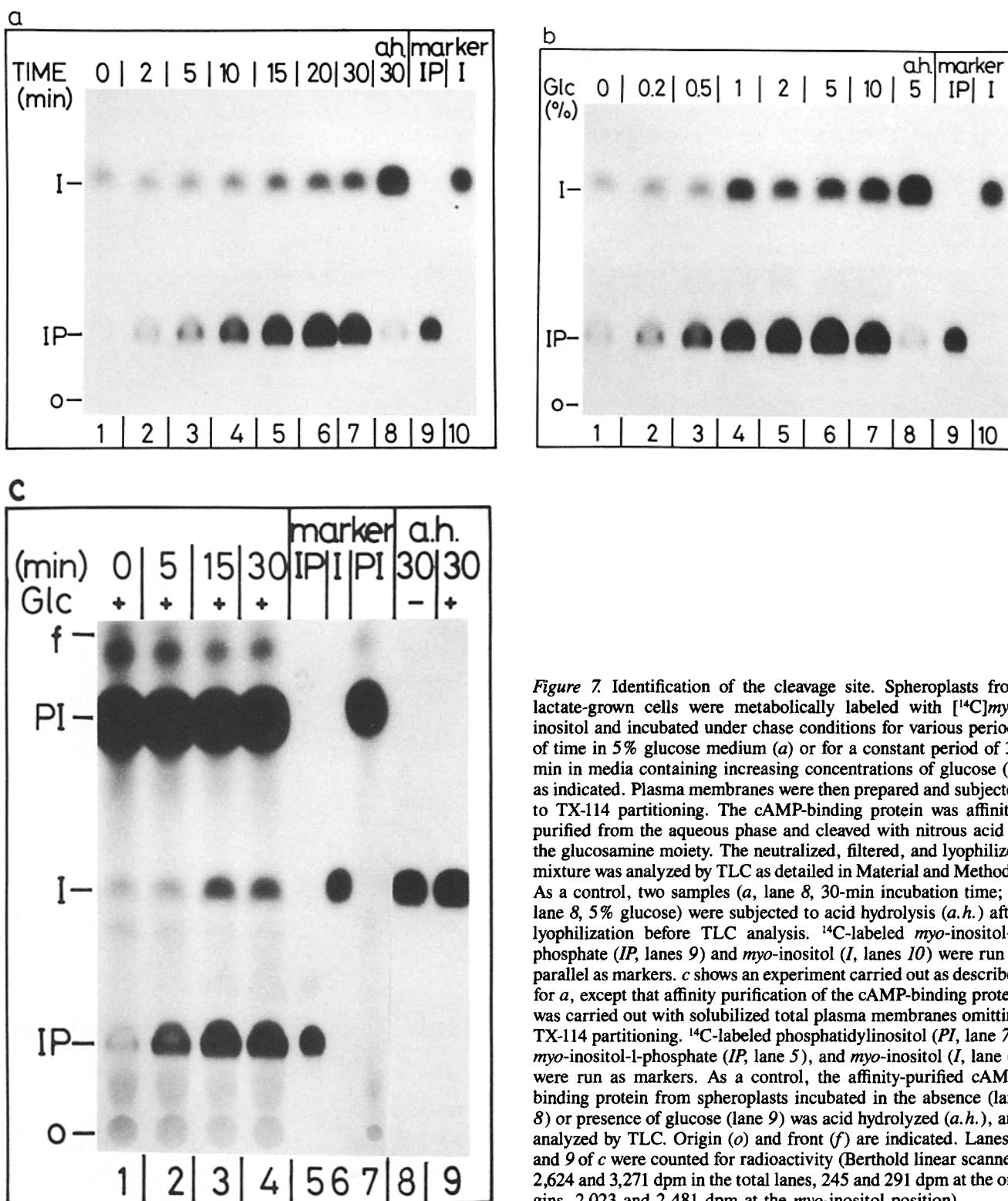
the [<sup>14</sup>C]palmitic acid label after the transfer of cells to glucose medium, *myo*-[<sup>14</sup>C]inositol (Fig. 5 b, upper panel) and [<sup>14</sup>C]ethanolamine (Fig. 5 b, lower panel) are both retained simultaneously in the hydrophilic (lanes 8 and 16) and in the amphiphilic version (lanes 7 and 15) as well as in the soluble form of the cAMP-binding protein isolated from the supernatant after centrifugation of plasma membranes in the presence of high salt (lanes 6 and 14), and in the membrane-attached form in the pellet fraction (lanes 5 and 13). This indicates that glucose induces a phospholipase which cleaves the anchor within the phosphatidylinositol moiety. The cross-reactivity with an anti-trypanosomal anti-CRD antibody (see above) suggests phospholipase C specificity of the cleaving enzyme.

To demonstrate the time course of the cleavage and to quantitate the loss of the amphiphilic character of the protein and the simultaneous emergence of the CRD in the hydrophilic version, we double-labeled the protein *in vivo* with [<sup>14</sup>C]palmitic acid and [<sup>3</sup>H]*myo*-inositol. After transfer of spheroplasts to glucose medium, the disappearance of both labels from the TX-114 phase in partitioning (Fig. 6 a) and the simultaneous appearance of the inositol label and the CRD, but not of the palmitic acid label (not shown), in the aqueous phase (Fig. 6 b) were monitored. Anti-CRD cross-reactivity was at the limit of detection and about constant with time in the amphiphilic phase (not shown). The results corroborate that the cleavage is effected by a phospholipase presumably of type C. It can be seen that glucose stimulates the lipolytic cleavage about threefold and that within 30 min a total of ~30% of the double-labeled material is converted from the amphiphilic to a hydrophilic version.

Next, we confirmed the lipolytic nature of the anchor degradation and identified the cleavage site in order to determine directly the specificity of the phospholipase involved. For this purpose we undertook a kinetic study of the cleavage and, in parallel, monitored the dependence of the hydrophilic conversion on the glucose concentration. After induction of the cleavage by glucose, the hydrophilic version of the cAMP-binding protein, metabolically labeled with *myo*-[<sup>14</sup>C]inositol and affinity-purified on cAMP-Sepharose as above, was chemically cleaved with nitrous acid at the glucosamine and, after removal of the protein by ultrafiltration, the filtrate analyzed for the labeled polar end group by TLC. The respective standards were treated with nitrous acid in the same manner to exclude differences in migration behavior that might have been caused by the way of sample preparation. Fig. 7, a and b reveal that the <sup>14</sup>C-label is associated with a component comigrating with *myo*-inositol 1-monophosphate when isolated and analyzed directly from the filtrate. The concentration of inositol monophosphate increases in a time- and glucose-dependent fashion as expected for a glucose-induced enzymatic process (Fig. 7, a and b, lanes 1–7). In addition, some non-derivatized *myo*-inositol emerges, which may have formed by degradation of the product by a phosphatase or from unspecific hydrolysis of the lipolytically cleaved anchor during the isolation of the hydrophilic protein by affinity-chromatography and subsequent preparation of the anchor fragment. As a control, the substance comigrating with *myo*-inositol phosphate can be converted to inositol by complete hydrolysis (lanes 8 in Fig. 7, a and b) proving the identity of the original material with inositol monophosphate. Since *myo*-inositol monophosphate is



**Figure 6.** Time course of the glucose-induced loss of palmitic acid and appearance of anti-CRD cross-reactivity in the released cAMP-binding protein. Spheroplasts from lactate-grown cells were metabolically labeled simultaneously with [<sup>14</sup>C]palmitic acid (+, ■) and [<sup>3</sup>H]*myo*-inositol (\*, □), and subsequently incubated in lactate or 5% glucose medium under chase conditions. At the periods of time indicated, plasma membranes were prepared and subjected to TX-114 partitioning. The cAMP-binding protein was affinity-purified from the TX-114 phase (a) and from the aqueous phase (b). a shows the analysis of the TX-114 phase: the affinity-purified cAMP-binding protein from spheroplasts incubated in lactate (□, ■) or glucose (+, \*) was analyzed for the retention of [<sup>14</sup>C]palmitic acid (+, ■) and [<sup>3</sup>H]*myo*-inositol (□, \*). b shows the analysis of the aqueous phase: half of the affinity-purified cAMP-binding protein from spheroplasts incubated in lactate (□, \*) or glucose (♦, \*) was analyzed for retention of [<sup>3</sup>H]*myo*-inositol (□, \*). The other half was analyzed by Western blotting, immunodecoration with anti-CRD antiserum, chemiluminescent detection and densitometry of the exposed film (LKB densitometer) for the appearance of anti-CRD immunoreactivity (♦, \*). The amount of <sup>14</sup>C- and <sup>3</sup>H-radioactivity in the amphiphilic phase (a) at time point 0 was set at 100%. The amount of <sup>3</sup>H-radioactivity in the hydrophilic phase (b) at time point 0 was set at 0% (\*, □) and anti-CRD immunoreactivity was set at 0 arbitrary units (♦, \*). The <sup>14</sup>C-radioactivity is shown for the amphiphilic phase, and the anti-CRD immunoreactivity for the hydrophilic phase, only (the respective other phases contained <sup>14</sup>C-radioactivity and anti-CRD immunoreactivity at the limit of detection which did not change significantly over the entire period of time). The curves represent the results of three averaged independent experiments ( $\pm$  SEM, only the + or - bar is shown for each point).



**Figure 7.** Identification of the cleavage site. Spheroplasts from lactate-grown cells were metabolically labeled with [<sup>14</sup>C]myo-inositol and incubated under chase conditions for various periods of time in 5% glucose medium (*a*) or for a constant period of 30 min in media containing increasing concentrations of glucose (*b*) as indicated. Plasma membranes were then prepared and subjected to TX-114 partitioning. The cAMP-binding protein was affinity-purified from the aqueous phase and cleaved with nitrous acid at the glucosamine moiety. The neutralized, filtered, and lyophilized mixture was analyzed by TLC as detailed in Material and Methods. As a control, two samples (*a*, lane 8, 30-min incubation time; *b*, lane 8, 5% glucose) were subjected to acid hydrolysis (*a.h.*) after lyophilization before TLC analysis. <sup>14</sup>C-labeled myo-inositol-1-phosphate (*IP*, lanes 9) and myo-inositol (*I*, lanes 10) were run in parallel as markers. *c* shows an experiment carried out as described for *a*, except that affinity purification of the cAMP-binding protein was carried out with solubilized total plasma membranes omitting TX-114 partitioning. <sup>14</sup>C-labeled phosphatidylinositol (*PI*, lane 7), myo-inositol-1-phosphate (*IP*, lane 5), and myo-inositol (*I*, lane 6) were run as markers. As a control, the affinity-purified cAMP-binding protein from spheroplasts incubated in the absence (lane 8) or presence of glucose (lane 9) was acid hydrolyzed (*a.h.*), and analyzed by TLC. Origin (*o*) and front (*f*) are indicated. Lanes 8 and 9 of *c* were counted for radioactivity (Berthold linear scanner; 2,624 and 3,271 dpm in the total lanes, 245 and 291 dpm at the origins, 2,023 and 2,481 dpm at the myo-inositol position).

clearly present in all samples as soon as the spheroplasts have been exposed to glucose, these findings confirm the results from the immunodecoration and argue that glucose leads to the activation of an intrinsic phospholipase C.

Two additional controls were performed. First, lanes 1–4 of Fig. 7*c* show that most of the label (>90%) is incorpo-

rated into phosphatidylinositol, when total affinity-purified cAMP-binding protein is directly treated with nitrous acid and the ultrafiltrate analyzed by TLC as above. PI may, in part, be derived from cAMP receptor molecules not lipolytically degraded upon transfer of spheroplasts to glucose. More likely, most of it may stem from free PI. Since in this

experiment TX-114 partitioning has been omitted so that lipid-lipid interactions have not been perturbed, it appears feasible that also some membrane-derived PI copurifies with the protein. After treatment with nitrous acid, again a small, presumably GPI-derived portion is converted to inositol monophosphate (and inositol) in a time-dependent fashion. Only insignificant amounts of radiolabel are found associated with other components. Secondly, lanes 8 and 9 of Fig. 7 c reveal that, if total inositol-labeled cAMP-binding protein is hydrolyzed to completion by acid and analyzed by TLC without previous deproteination, the label is recovered almost exclusively with inositol, independent of the presence or absence of glucose. Since in this experiment most, if not all compounds amenable to inositol labeling have been analyzed, this result reflects the metabolic stability of the applied inositol label and proves that it is not metabolically converted to other compounds to a significant extent such as, e.g., amino acids.

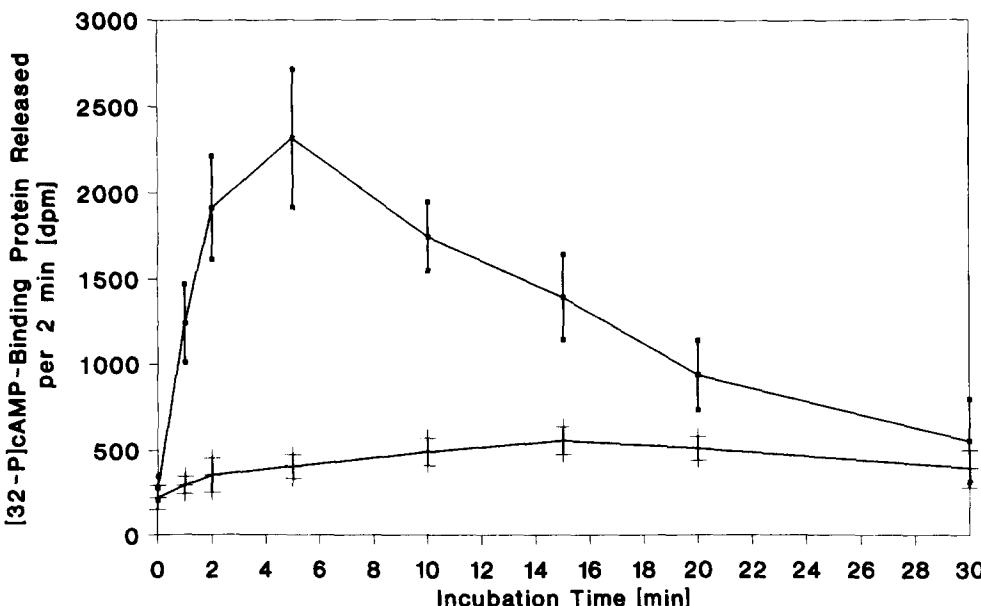
#### *Transient Activation of the Phospholipase by Glucose*

The above data point to the possibility that glucose induces the activation of a phospholipase C which then cleaves the GPI anchor of at least one plasma membrane protein in yeast, a cAMP-binding ecto-protein. To study whether the transfer to glucose results in the permanent activation of the lipase or rather in a transient increase in the rate of anchor cleavage which could generate a signal linked to nutritional upshift, we monitored the rate of the amphiphilic to hydrophilic conversion after exposure of cells to glucose medium. For this kinetic study,  $8-N_3-[^{32}P]cAMP$ -labeled spheroplasts from lactate-grown cells were transferred to glucose for various periods of time as indicated in Fig. 8. Then they were washed in the presence of NaCl at a concentration high

enough to free them of any adherent cAMP-binding protein which might have been cleaved off during this incubation. Two equal aliquots of these glucose-preincubated and washed spheroplasts from each time point were resuspended in either glucose or lactate medium for a constant period of 2 min and liberation rates measured by following the release of the labeled protein during this interval. Fig. 8 shows that the transfer of these spheroplasts to glucose (*upper curve*) results in a rapid increase in the rate of anchor cleavage relative to the rate observed with the other aliquot of spheroplasts transferred to lactate medium (*lower curve*). The rate of anchor cleavage reaches its maximum 5 min after the transfer to glucose and declines to the initial rate after 30 min. These data, together with the rapid decrease of the cleavage rate observed after quenching the spheroplasts by an immediate shift from glucose to lactate for the 2 min assay period, indicates a sharp increase in lipolytic activity tightly coupled to the nutritional upshift, but also a slow decline to the original activity upon adaptation to glucose.

#### *Discussion*

The functional relevance of GPI membrane anchorage is not known. Evolutionary conservation of this particular mode of anchoring implies that it confers specific, physiologically useful properties on the respective protein which are not achieved by other anchoring mechanisms. However, despite this expectation, apart from a few examples (Braun-Bretton et al., 1988; Espinoza et al., 1988) which rather appear to be exceptions to the rule, no difference in activity or function of the respective protein appears to be attributable to the lipolytic degradation of GPI anchors. For example, membrane-embedded and soluble versions of mammalian eryth-



**Figure 8.** Transient activation of the releasing enzyme. Spheroplasts from lactate-grown cells were photoaffinity-labeled and incubated in glucose medium (2%) under chase conditions. After various periods of time, NaCl (0.5 M final conc.) was added, and the spheroplasts were spun through a cushion of sucrose/Ficoll, resuspended in medium containing 2% glucose (■) or lactate (+), and incubated for a constant period of 2 min. After supplementation with 0.5 M NaCl, the spheroplasts were spun again through a cushion of sucrose/Ficoll. The medium above the cushion was concentrated by TCA precipitation and analyzed by SDS-PAGE and fluorography. A densitometric quantitation of the released cAMP-binding protein (54-kD protein band) is shown. The values represent means of four different experiments ( $\pm$  SEM).

rocyte acetylcholine esterase and bovine cerebral cortex 5'-nucleotidase have identical activities (Barton et al., 1985; Vogel et al., 1992), and transmembrane and GPI-anchored forms of N-CAM mediate cell adhesion of transfected mouse fibroblasts in a similar fashion (Pizsey et al., 1989). It appears that GPI anchorage does, in most cases, not significantly modulate the properties of the anchored protein. In support of such an interpretation, it has been shown that in mice the physiological function of neutrophils and NK cells is not impaired although both cell types, in contrast to the respective human cells, express only the transmembrane form of Fc $\gamma$ RIII (Perussia et al., 1989). On the other hand, treatment of chicken myoblasts with PLC impairs cell adhesion and myotube formation (Knudson et al., 1989), and crosslinking of Thy-1, Qa-2, or Ly-6 with an antibody activates T-cells only from transgenic mice expressing the GPI-anchored forms of these T-lymphocyte-specific receptors (Robinson et al., 1989; Su et al., 1991; Zhang et al., 1991). These observations demonstrate the indispensability of the anchor structure for the specific function of these proteins in differentiation and transmembrane signaling in the respective cell types. However, the direct function in signal transduction of GPI-anchored proteins could in no case be demonstrated. The interpretation of these seemingly controversial observations could be that for certain transmembrane signaling events, presumably those yielding a mitotic vs. differentiation signal, GPI-anchorage is indispensable, whereas it is not essential, where primarily the receptor function of the respective protein is required. This would additionally imply that the release of certain proteins from the plasma membrane leads to the generation of a signal which subsequently is transmitted to the interior of the cell and induces the responses observed.

The present study was undertaken in order to develop a model system for the analysis of the significance of membrane anchorage by a glycolipid. Because no other common property of GPI-anchored proteins is apparent apart from the lipolytic cleavability of the anchor structure, we suspected that it could be just the cleavage event which leads to the generation of the signal. Such a model would pass on the role of the key player in signaling through the involvement of GPI anchorage to GPI-specific phospholipases the activity of which might be modulated in response to environmental signals. We have chosen baker's yeast as a system to analyze this problem because it has been shown by several groups (Conzelmann et al., 1988; Vai et al., 1990; Müller and Bandlow, 1991a) to contain GPI-anchored plasma membrane surface proteins. In addition, this organism provides two decisive advantages over other systems in order to study the physiological significance of anchor cleavage: (1) it is simple to grow at a large scale and to analyze the cleavage event(s) within the anchor structure and (2) it is easily amenable to manipulation by genetic and recombinant DNA technologies.

In this paper, we have been able to provide evidence that, in this organism, membrane anchorage is modulated in response to an extracellular signal generated upon glucose upshift. A portion of at least one GPI-anchored protein, the ecto-receptor protein for cAMP, is released from the membrane through the activation of an endogenous PLC. This anchor cleavage following a nutritional upshift qualitatively resembles the lipolytic membrane release of some GPI-anchored proteins (e.g., 5'-nucleotidase, alkaline phospha-

tase, lipoprotein lipase, and heparan sulfate proteoglycan) observed with cultured cells and tissues from vertebrates in response to glucose and insulin (Ishihara et al., 1987; Romero et al., 1988; Chan et al., 1988; Klip et al., 1988; Lisanti et al., 1989). In both cases, yeast as shown here and—as far as is known—vertebrate cells, a phospholipase is activated in response to extrinsic signals.

The observations made in the present study imply that GPI cleavage is involved in glucose-mediated signaling in yeast. Experiments are underway to elucidate whether glucose-induced cleavage of GPI membrane anchors occurs in higher eucaryotic cells as well and applies to other GPI-anchored proteins and whether and how it could be related to the generation of a transducible signal. Although some evidence has been found in favor of the idea, originally put forward by Larner and coworkers (Larner, 1988; Romero et al., 1988), that nutritional and other transmembrane signaling events could involve a second messenger(s) derived from the polar head group of the cleaved anchor, a direct proof of this attractive hypothesis is still lacking.

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