MOLECULAR AND CELLULAR BIOLOGY, Jan. 1996, p. 442–456 Vol. 16, No. 1
0270-7306/96/$04.00+0

Glucose-Induced Sequential Processing of a Glycosyl-Phosphatidylinositol-Anchored Ectoprotein in *Saccharomyces cerevisiae*

GÜNTER MÜLLER,[1]* EVA GROß,[2] SUSANNE WIED,[1] AND WOLFHARD BANDLOW[2]

Hoechst Aktiengesellschaft Frankfurt am Main, D-65926 Frankfurt,[1] and Institut für Genetik und Mikrobiologie, D-80638 München,[2] Germany

Received 9 June 1995/Returned for modification 19 July 1995/Accepted 25 September 1995

Transfer of spheroplasts from the yeast *Saccharomyces cerevisiae* to glucose leads to the activation of an endogenous (glycosyl)-phosphatidylinositol-specific phospholipase C ([G]PI-PLC), which cleaves the anchor of at least one glycosyl-phosphatidylinositol (GPI)-anchored protein, the cyclic AMP (cAMP)-binding ectoprotein Gce1p (G. Müller and W. Bandlow, J. Cell Biol. 122:325–336, 1993). Analyses of the turnover of two constituents of the anchor, *myo*-inositol and ethanolamine, relative to the protein label as well as separation of the two differently processed versions of Gce1p by isoelectric focusing in spheroplasts demonstrate the glucose-induced conversion of amphiphilic Gce1p first into a lipolytically cleaved hydrophilic intermediate, which is then processed into another hydrophilic version lacking both *myo*-inositol and ethanolamine. When incubated with unlabeled spheroplasts, the lipolytically cleaved intermediate prepared in vitro is converted into the version lacking all anchor constituents, whereby the anchor glycan is apparently removed as a whole. The secondary cleavage ensues independently of the carbon source, attributing the key role in glucose-induced anchor processing to the endogenous (G)PI-PLC. The secondary processing of the lipolytically cleaved intermediate of Gce1p at the plasma membrane is correlated with the emergence of a covalently linked high-molecular-weight form of a cAMP-binding protein at the cell wall. This protein lacks anchor components, and its protein moiety appears to be identical with double-processed Gce1p detectable at the plasma membrane in spheroplasts. The data suggest that glucose-induced double processing of GPI anchors represents part of a mechanism of regulated cell wall expression of proteins in yeast cells.

An increasing number of surface proteins are found anchored at the plasma membrane of various tissues and cell types by virtue of a glycosyl-phosphatidylinositol (GPI) structure instead of a transmembrane protein domain. This kind of membrane anchor typically contains, in addition to the *myo*-inositol-harboring phospholipid, a carbohydrate core consisting of one nonacetylated glucosamine and three mannose residues in characteristic linkage. The latter may be branched and carry additional mannose, galactose, and/or phosphoethanolamine moieties. The preformed anchor structure is linked by a phosphodiester and an ethanolamine bridge to the carboxy terminus of the respective protein, thereby replacing a proteinaceous transmembrane domain present in the newly synthesized protein precursor (for reviews, see references 14, 17, 31, 52, and 56). The physiological significance of membrane anchorage by a glycolipidic structure has remained greatly obscure. Since GPI-anchored proteins have been detected recently also in lower eucaryotes, including the yeast *Saccharomyces cerevisiae* (9, 35, 43, 54), the evolutionary conservation from *S. cerevisiae* to humans of the GPI anchor argues that it confers properties on the respective proteins that are not achieved by a proteinaceous transmembrane domain.

It has been proposed that the spatial requirements, clustering behavior, and diffusion properties of GPI-anchored proteins in the outer leaflet of plasma membranes are the parameters critical for the use of either a transmembrane domain or a GPI moiety for membrane anchorage of a protein (7, 13, 61, 62). In addition, it has been proposed, on the basis that, in situ, GPI anchors may be cleaved upon activation of endogenous (glycosyl)-phosphatidylinositol-specific phospholipases ([G]PI-PLs), that GPI anchorage plays a role in regulating the concentration of certain proteins at the cell surface or in controlling protein localization and topology: in HeLa cells and in cultured bone marrow stromal cells, GPI-anchored decay-accelerating factor and heparan sulfate proteoglycan, respectively, are apparently removed constitutively from the plasma membrane and released into the culture medium by the action of an endogenous of type D (G)PI-PL ([G]PI-PLD) (32). In the yeast *S. cerevisiae*, expression of α-agglutinin at the cell wall depends on its synthesis as a GPI-anchored protein and occurs via an intermediate which lacks the *myo*-inositol moiety of the GPI anchor glycan (28, 59). However, other than (G)PI-PLs, which leave the *myo*-inositol residue attached to the glycan structure, processing enzymes of GPI anchors have not been identified. In addition to these cases of constitutive GPI anchor processing, in some vertebrate cells certain external signals, provided by serum factors like insulin and growth factors or by drugs, have been found to activate phospholipases which are capable of cleaving GPI anchors (20, 26, 40, 48). In the case of the insulin- or sulfonylurea-induced lipolytic cleavage of the GPI anchors of lipoprotein lipase, 5′-nucleotidase, and the cyclic AMP (cAMP)-binding ectoprotein Gce1p (54 kDa) in cultured or isolated adipocytes, we observed a continuous loss of the *myo*-inositol radiolabel from the hydrophilic versions of these GPI-anchored proteins (39, 40). Taken together, these observations suggest that in lower as well as in higher eucaryotes, complex processing events within or near the glycan structure of certain GPI anchors occur. The cleavage reactions involved as well as the resulting truncated forms of the affected GPI-anchored proteins, however, have in no case been ana-

* Corresponding author. Mailing address: Hoechst AG, Pharmaceutical Research, TD Metabolism H825, D-65926 Frankfurt, Germany; Phone: 069-305-4271. Fax: 069-311454.

lyzed in detail. As a consequence, the physiological importance of GPI anchor processing is poorly understood.

Recently we found that in the yeast *S. cerevisiae*, a GPI-anchored cAMP-binding ectoprotein, named Gce1p, is lipolytically cleaved by a (G)PI-PLC when spheroplasts are transferred from lactate to glucose medium (37). In this study, the metabolic stability of the lipolytically cleaved GPI anchor of Gce1p was studied. We present evidence that establishment of glucose repression in *S. cerevisiae* is accompanied by the en bloc removal of the complete glycan structure from the GPI anchor. The resulting double-processed Gce1p, detected in spheroplasts, behaves as a biosynthetic intermediate in the expression of a cAMP-binding protein at the cell wall, identified in intact cells.

MATERIALS AND METHODS

Materials. [^{3}H]leucine (25 Ci/mmol), [1-^{3}H]ethan-1-ol-2-amine hydrochloride (5 to 30 Ci/mmol), *myo*-[2-^{3}H]inositol (10 to 20 Ci/mmol), scintillation cocktail ACSII, and Amplify were bought from Amersham-Buchler, Braunschweig, Germany; 8-N_3-[^{32}P]cAMP (20 Ci/mmol) was obtained from ICN, Meckenheim, Germany; *myo*-[U-^{14}C]inositol (200 to 250 mCi/mmol), [2-^{14}C]ethan-1-ol-2-amine hydrochloride (54 mCi/mmol), and En^{3}Hance (spray) were purchased from DuPont-New England Nuclear, Dreieich, Germany; N^6-(2-aminoethyl)-cAMP–Sepharose, Ficoll, Ampholines, Pharmalyte, and protein A-Sepharose were obtained from Pharmacia-LKB, Freiburg, Germany; and Bio-Gel P4 was from Bio-Rad, Munich, Germany. Purified PI-PLC (*Bacillus cereus*), phosphodiesterase I from bovine intestinal mucosa, laminarinase (*Penicillium* sp.), cetyltriethylammonium bromide (CTAB), 3-isobutyl-1-methylxanthine, and glass beads were supplied by Sigma, Deisenhofen, Germany; proteinase K and protease inhibitors were supplied by Boehringer, Mannheim, Germany; deoxycholate, goat anti-rabbit immunoglobulin G antiserum, and pronase were provided by Calbiochem, Bad Soden, Germany; polyclonal anti-cross-reacting determinant (CRD) antibodies, raised in MITat 1.2 New Zealand White rabbits against purified soluble variant surface glycoprotein from *Trypanosoma brucei*, were the kind gift from P. Overath, Tübingen, Germany; silica gel Si-60 high-performance thin-layer chromatography (HPTLC) plates, polyethylene glycol (PEG) 6000, urea, and all buffer reagents (highest purity available) were from Merck, Darmstadt, Germany; 4′-aminobenzamidotaurocholic acid (BATC), ^{3}H-labeled glucose oligomers, and the ^{14}C-labeled oligosaccharide standards Mn_1A to Mn_3A (for structures, see the legend to Fig. 7) were synthesized by the Pharma Synthesis Department of Hoechst AG, Frankfurt, Germany. All other materials were obtained as described previously (37, 43).

Photolabeling of intact cells. Identical numbers of cells from *S. cerevisiae* ABYS-1 (1) grown in lactate or glucose medium were washed four times with 0.1 M morpholineethanesulfonic acid (MES; pH 6.2)–1.1 M sorbitol–140 mM KCl–100 μM phenylmethylsulfonyl fluoride (PMSF) and then suspended in 25 mM MES (pH 6.2)–50 mM KCl–10 $MgCl_2$–2 mM $MnCl_2$–1 mM 3-isobutyl-1-methylxanthine–0.5 mM dithiothreitol (DTT)–1 mM EDTA–200 μM 5′-AMP–100 μM PMSF at 5×10^5 cells per ml. Aliquots of 0.5 ml were transferred to cuvettes and incubated with 25 μCi of 8-N_3-[^{32}P]cAMP (0.5 to 1 μM, final concentration) in the dark (15 min, 20°C). Irradiation was performed in a Rayonet RPR-100 photochemical reactor (Southern Ultraviolet, Hamden, Conn.) equipped with 16 RPR-2530 A lamps at a distance of 10 cm from the lamps (according to data of the manufacturer, the RPR-2530 A lamps emit 84% of their radiation at 2,530 A with an intensity of 12,800 μW/cm^2 and 1.65×10^{16} photons per s per cm^3) for 5 min (20°C). The cross-linking was terminated by the addition of 100 μM cAMP. The cells were washed three times with 0.1 M Tris-SO_4 (pH 8.5)–1.1 M sorbitol–100 μM PMSF. The final pellet was used for preparation of cell wall fragments and laminarinase treatment.

Metabolic labeling of intact cells. Cells from *S. cerevisiae* ABYS-1 were grown in semisynthetic lactate medium as described previously (34) to 5×10^6 cells per ml at 30°C, washed two times in 0.1 M Tris-SO_4 (pH 8.5)–1.1 M sorbitol, then resuspended in amino acid-depleted or inositol-free semisynthetic lactate medium supplemented with 0.05% yeast extract at the same titer, and grown to 10^7 cells per ml. The cells were centrifuged, resuspended in the above-described medium lacking yeast extract at 7.5×10^7 cells per ml, and incubated (2 h, 30°C). After addition of [^{14}C]leucine (12.5 μCi/ml) or *myo*-[^{14}C]inositol (3.5 μCi/ml) or [^{14}C]ethanolamine (17.5 μCi/ml) to 5-ml aliquots, the suspensions were incubated for 120 min (leucine labeling), 60 min (inositol labeling), or 30 min (ethanolamine labeling) at 30°C. The labeling was terminated by addition of a 1,000-fold excess of the corresponding nonradioactive compound and further incubation (10 min, 30°C) followed by supplementation with NaN_3 (5 mM, final concentration) and PMSF (200 μM, final concentration). The cells were centrifuged and washed three times with 0.1 M Tris-SO_4 (pH 8.0)–1.1 M sorbitol–200 μM PMSF. The final pellet was used for preparation of cell wall fragments and laminarinase treatment.

Metabolic labeling of yeast spheroplasts. Aliquots (100 ml) of spheroplasts (9×10^8 cells) from strain ABYS-1 cells prepared as described previously (34) were incubated in semisynthetic lactate medium containing 0.05% yeast extract (60 min, 30°C), collected by centrifugation through a Ficoll cushion (37), and suspended in 10 ml of synthetic lactate medium containing 0.01% yeast extract. Labeling was initiated by the addition of 25 μCi of *myo*-[^{14}C]inositol (250 mCi/mmol; 90 min), 150 μCi of [^{14}C]ethanolamine hydrochloride (3.5 mCi/mmol; 45 min), or 75 μCi of [^{14}C]leucine (300 mCi/mmol; 2 h) at 30°C. For metabolic double labeling 5×10^8 spheroplasts were incubated in 10-ml cultures with 10 μCi of *myo*-[^{14}C]inositol plus 0.3 mCi of [^{3}H]ethanolamine (25 Ci/mmol) or 0.5 mCi of [^{3}H]inositol (100 Ci/mmol) plus 75 μCi of [^{14}C]ethanolamine for 60 min. Labeling was terminated by adding a 1,000-fold molar excess of the respective unlabeled compound(s) and further incubation for 10 min. Washing of the spheroplasts and subsequent glucose induction were performed as described elsewhere (37).

Proteinase K treatment of intact cells. Aliquots (1 ml) of 2×10^8 photoaffinity-labeled cells were washed two times in digestion buffer (25 mM Tris-HCl [pH 7.4], 1.1 M sorbitol, 0.5 mM EDTA), suspended in the same buffer, and incubated in the presence of DTT (200 mM, final concentration) and cycloheximide (25 μg/ml) for 30 min at 25°C. The cells were harvested, washed two times with digestion buffer lacking DTT, and suspended in the same buffer containing cycloheximide. After addition of various amounts of proteinase K, incubation was continued for 30 min. Digestion was terminated by adding PMSF (200 μM, final concentration) and washing the cells two times with cold 25 mM Tris-HCl (pH 7.4)–1.1 M sorbitol–200 μM PMSF. The final cell pellet was used for lysis and separation of cell wall fragments from cellular material.

Preparation of cell wall fragments. Cell wall fragments were prepared as described previously (28), with the following modifications. The cell pellets were suspended in extraction buffer containing 50 mM Tris-HCl (pH 7.4), 140 mM KCl, 2 mM EDTA, 0.5 mM DTT, 200 μM PMSF, 40 μg each of leupeptin, pepstatin, antipain, and E-64 per ml, and either 2% sodium dodecyl sulfate (SDS) (denaturing conditions) or 5% BATC (native conditions) at 2×10^6 cells per ml and incubated (15 min, 15°C) in order to increase the efficiency of extraction for material noncovalently associated with the cell wall. After addition of 2 volumes of cold glass beads (0.25-mm diameter), the cells were broken by vortexing six times for 30-s intervals interrupted by cooling on ice for 30 s. The cell lysate was then incubated for 10 min at 95°C (in the case of denaturing conditions) or at 15°C (in the case of native conditions). The total cellular extract was centrifuged ($15,000 \times g$, 10 min, 4°C) for collecting cell wall fragments. The pellet was reextracted two times with extraction buffer by extensive vortexing and resuspension in the same conditions (denaturing or native). The final pellet of insoluble cell wall fragments was washed three times under resuspension with 25 mM Tris-HCl (pH 7.4)–1 M NaCl–0.5 mM EDTA–100 μM PMSF–30 μg each of leupeptin, pepstatin, and antipain per ml and finally two times with 50 mM sodium acetate (pH 5.5)–1 mM EDTA–200 μM PMSF–protease inhibitors for treatment with laminarinase.

Laminarinase treatment of cell wall fragments. The procedure was as described previously (27, 28), with the following modifications. The cell wall pellet (from 9×10^9 cells) was suspended in 200 μl of digestion buffer–50 mM sodium acetate (pH 5.5)–1 mM EDTA–200 μM PMSF–15 μg each of leupeptin, pepstatin, antipain, and E-64 per ml and incubated with 1.2 U of laminarinase (30°C, 2 h). After addition of a second aliquot of enzyme, incubation was continued for 2 h. The extracted and insoluble materials were separated by centrifugation ($15,000 \times g$, 10 min, 4°C). The pellet fraction was suspended in an equal volume of digestion buffer and recentrifuged. The combined supernatants were precipitated (58). The washed pellets were either dissolved in sample buffer for SDS-polyacrylamide gel electrophoresis (PAGE) or used for affinity purification.

The efficiencies of cell wall isolation and laminarinase treatment were controlled by protein determination of the final laminarinase extract and did not differ significantly between lactate-grown and glucose-induced cells during the induction period (0 to 30 min) and remained about constant for 0 to 60 min of incubation.

Preparation of PI-PLC-cleaved Gce1p. Spheroplasts from lactate-grown cells were metabolically labeled with *myo*-[^{14}C]inositol. Isolated plasma membranes (75 to 150 μg of protein) were suspended in 150 μl of 20 mM Tris-HCl (pH 7.2)–1 mM EDTA–120 mM NaCl, incubated (2 h, 30°C) with 0.2 U of PI-PLC (*B. cereus*), and then centrifuged ($48,000 \times g$, 30 min). The released protein was precipitated from the supernatant with PEG 6000, and the pellet was subjected to Triton X-114 (TX-114) partitioning. The aqueous phase was used for affinity purification of Gce1p. The eluate was precipitated with PEG 6000 and finally dissolved in 20 mM Tris-HCl (pH 7.2)–10% glycerol–0.5 mM DTT–0.5 mM EDTA–100 μM PMSF at 50 to 100 dpm/μl.

Treatment with phosphodiesterase I. A trichloroacetic acid (TCA)-precipitated (5%, 30 min, 4°C, followed by a spin at $15,000 \times g$ for 10 min), washed (three times with acetone), and dried protein pellet of a Gce1p preparation was dissolved in 100 μl of 50 mM Tris-HCl (pH 9.0)–1 mM $MgCl_2$ and incubated (30 min, 37°C) with 0.5 U of phosphodiesterase I from bovine intestinal mucosa. After addition of a second aliquot of enzyme and further incubation (15 min, 37°C), the reaction was terminated by addition of 10 μl of 100 mM Tris-HCl (pH 8.0)–1 M NaCl–10 mM EDTA–5% (wt/vol) SDS and heating (15 min, 65°C). After centrifugation ($10,000 \times g$, 10 min) the supernatant was diluted 10-fold and precipitated (58). The pellet was washed extensively and dissolved in isoelectric

focusing (IEF) sample buffer containing detergent as specified in the figure legends.

PEG precipitation. The sample was adjusted to a protein concentration of 0.1 mg/ml with γ-globulin, supplemented with the same volume of ice-cold 12% PEG 6000 (in 10 mM Tris-HCl [pH 7.2]–0.5 mM EDTA), and kept on ice for 15 min. After centrifugation (12,000 × *g*, 10 min, 4°C), the pellet was washed three times with ice-cold 0.8% PEG.

IEF. IEF was performed with TCA-precipitated radiolabeled protein and 6% polyacrylamide gels, 15% *N*,*N*′-diallyltartardiamide cross-linker, and ampholines (pH range, 5.5 to 8.5) in the presence of 6 M urea with 0.5% BATC or with the detergent indicated as described previously (41). pH was monitored with a semiautomatic contact electrode (WTW, Weilheim, Germany). The precipitated samples were dissolved in IEF sample buffer containing 0.48 g of urea per ml, 5% β-mercaptoethanol, 1% ampholines (pH 3 to 10, 40%), 1% Pharmalyte (pH 3.5 to 10, 40%), and 0.5% BATC or, where indicated, 2% TX-100.

Charge shift electrophoresis. Precipitated plasma membrane proteins, prepared as described in the relevant figure legends (500 to 1,000 dpm/μl), were dissolved in 20 μl of twofold gel buffer containing the respective detergent (equilibration overnight at 4°C) and applied to slots (1 by 10 mm) in the centers of 1.2% agarose gels (19, 30), using a horizontal water-cooled (4°C) chamber (Pharmacia-LKB). Gel and running buffers contained 0.1 M NaCl, 50 mM succinic acid, and 50 mM MES-NaOH, (pH 6.8), supplemented with detergent as indicated. Prior to sample application, the gels were electrophoresed for 60 min at 8.5 V/cm. After electrophoresis at 8.5 V/cm for 4.5 to 5 h, gels were dried under vacuum at 50°C and fluorographed (Kodak X-Omat AR film).

High-resolution TLC. The radiolabeled pronase fragment of the (G)PI-PLC-cleaved Gce1p was deaminated with HNO_2 and reduced with $NaBH_4$ or dephosphorylated with aqueous HF (see below), dialyzed (Spectra/Por cellulose ester membrane; MWCO 500), and extracted with water-butanol. The aqueous-phase material was dried (SpeedVac vacuum concentrator), suspended in 20 μl of 0.05 M pyridine acetate (pH 5.0), and applied to Silica Gel 60 HPTLC plates (20 by 20 cm). The plates were developed two times with chloroform-methanol-H_2O (10:10:3, by volume) and two times with pyridine-ethyl acetate-acetic acid-H_2O (5:5:1:3.5, by volume) with air drying between two developments, sprayed with En^{3}Hance, and subjected to fluorography.

Bio-Gel P4 chromatography. The radiolabeled pronase fragment of the GPI-cleaved Gce1p was dialyzed and extracted with water-saturated butanol. The dried aqueous-phase material was resuspended in 200 μl of water, applied to a Bio-Gel P4 column (120 cm by 0.4 cm^2), and eluted with phosphate-citrate buffer (20 mM Na_2HPO_4, 100 mM citrate, 0.5 M KCl [pH 2.6]) at a flow rate of 4 ml/h. Fractions were collected every 3 min, and aliquots of each fraction were counted for radioactivity. The column was calibrated with a mixture of ^{3}H-labeled glucose oligomers of various chain lengths.

Pronase digestion. The radiolabeled hydrophilic and affinity-purified Gce1p (38) was precipitated with TCA, and the pellet was dissolved in 100 mM $(NH_4)_2CO_3$ (pH 9.5) and supplemented with 2% (wt/wt) pronase (10 mg/ml in 10 mM calcium acetate). After incubation (16 h, 37°C) under a drop of toluene, additional 0.2% pronase was added and incubation was continued (8 h). The reaction was terminated by addition of 0.1 volume of 2 M acetic acid and subsequent butanol extraction. The aqueous phase was dried in a SpeedVac vacuum concentrator.

Trifluoroacetic acid hydrolysis. The dry aqueous-phase material of the pronase digestion mixture was dissolved in 200 μl of 4 M trifluoroacetic acid and incubated (4 h, 100°C). The hydrolysate was neutralized, desalted, dried, and resuspended in 20 μl of H_2O as described previously (11).

Miscellaneous procedures. Published procedures were used for yeast culture and preparation of spheroplasts (1, 34), photoaffinity labeling with 8-N_3-[^{32}P]cAMP (35, 36), centrifugation of spheroplasts through a Ficoll cushion and preparation of plasma membranes (37), nitrous acid deamination followed by reduction with $NaBH_4$ and dephosphorylation with aqueous HF (16), TX-114 partitioning (37), affinity purification of Gce1p by using N^6-(2-aminoethyl)-cAMP–Sepharose (37, 43), immunoprecipitation with the anti-CRD antibody (36), HPTLC (40), SDS-PAGE (with 10% separating gels except as otherwise indicated in the figure legends) in the presence of urea and fluorography using Amplify and Kodak X-Omat-AR films (45), liquid scintillation counting of sliced gel bands (23), and protein determination (46).

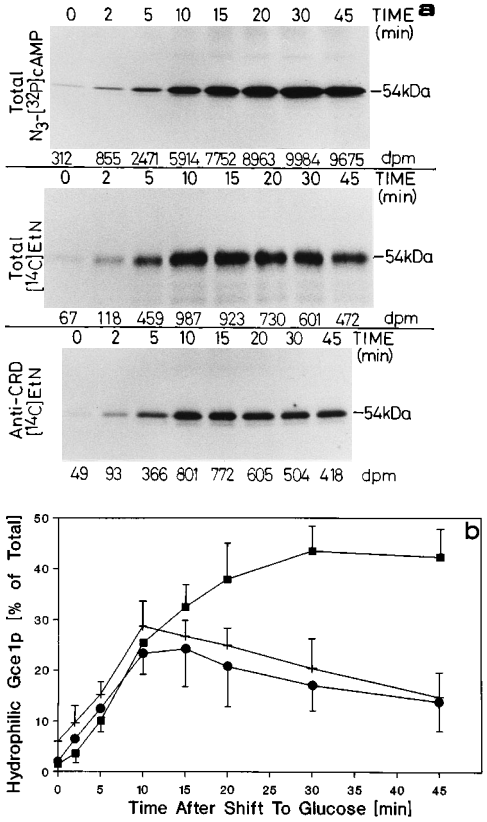

FIG. 1. Kinetics of the loss of constituents of the GPI anchor from soluble Gce1p. (a) Aliquots of spheroplasts from lactate-grown cells were either photoaffinity labeled with 8-N_3-[^{32}P]cAMP (upper panel) or metabolically labeled with [^{14}C]ethanolamine ([^{14}C]EtN; middle and lower panels). Subsequently the spheroplasts were incubated in 5% glucose under chase conditions for the periods indicated. Plasma membranes were isolated and subjected to TX-114 partitioning. Hydrophilic Gce1p (recovered from the aqueous phase) was visualized either directly (photoaffinity-labeled samples) or after affinity purification on cAMP-Sepharose (metabolically labeled samples) by SDS-PAGE and autoradiography-fluorography. Upper and middle panels, analysis of total samples (corresponding to material isolated from 6 × 10^7 spheroplasts per lane); lower panel, analysis of immunoprecipitates with an anti-CRD antiserum obtained from equivalent sample volumes. The numbers below each lane indicate the radioactivity in each Gce1p gel band evaluated by two-dimensional scanning of the lanes of the dried gel with a Berthold scanner (the background radioactivity above and below each band was subtracted as a medium value in each case; the linearity between the gel-associated radioactivity within the range of radiolabeled Gce1p and the response obtained by the scanning procedure was confirmed by control experiments). (b) The amount of radiolabeled hydrophilic Gce1p contained in each gel band was calculated as a percentage of the sum of the radiolabeled hydrophilic and amphiphilic material. ■, total 8-N_3-[^{32}P]cAMP-labeled Gce1p; +, total [^{14}C]ethanolamine-labeled Gce1p; ●, anti-CRD-immunoprecipitated [^{14}C]ethanolamine-labeled Gce1p. The values represent the means ± standard deviations of three independent experiments (only one-half of the standard deviation values are given for reasons of clarity).

RESULTS

Different turnover rates of the protein moiety and core glycan in the lipolytically cleaved Gce1 protein. Cleavage of Gce1p as a relatively easy to test model GPI-anchored protein by a glucose-activated endogenous (G)PI-PLC has been demonstrated (37). In addition, the previous data indicated metabolic instability of the anchor glycan still attached to the protein moiety via the phosphoethanolamine bridge. To characterize this finding in greater detail, we examined whether all or part of the anchor glycan is removed by a secondary cleavage event. As a first approach, the turnover rates of the anchor constituents relative to the protein moiety were measured after a nutritional upshift. For this purpose, the protein portion of Gce1p was photoaffinity labeled with 8-N_3-[^{32}P]cAMP in one aliquot of spheroplasts from lactate-grown cells, whereas [^{14}C]ethanolamine served as the anchor-specific metabolic label in another aliquot (Fig. 1). Metabolic labeling was terminated by a chase, and simultaneously both samples received 5% glucose. (Previous control experiments verified that glucose does not stimulate vesicular transport of Gce1p. Furthermore, it has been shown that the Zymolyase used for spheroplast formation does not cause any processing of Gce1p [3a].) After the incubation periods indicated, plasma membranes

were isolated ([G]PI-PLC-cleaved Gce1p remains associated with the membrane through specific, noncovalent interactions and is dissociable only in the presence of high salt [37, 44]) and subjected to TX-114 partitioning. The processed hydrophilic fraction of Gce1p was isolated from the aqueous phase and, in the case of metabolically labeled samples, partially purified by affinity chromatography on cAMP-Sepharose. All samples were analyzed by SDS-PAGE and autoradiography-fluorography (Fig. 1a). The radioactivity associated with each Gce1p in each band in the gel was measured, and the corresponding value is shown below each lane. In addition, the percentage of the radiolabeled hydrophilic fraction of the total radiolabeled amphiphilic plus hydrophilic Gce1p for each time point and for each type of radiolabel is given in Fig. 1b.

The amount of photoaffinity-labeled hydrophilic Gce1p increased continuously with time up to 30 min after induction by glucose (Fig. 1). On the other hand, hydrophilic Gce1p containing the ethanolamine radiolabel increased (with velocity similar to that of the protein label) only initially and reached a plateau after 10 min (Fig. 1). Analogous results were obtained when [^{14}C]leucine was used as the protein-specific label and *myo*-[^{14}C]inositol was used as the anchor-specific label, which proved that the kinetics of the processing was independent of the compound used for the labeling (data not shown).

These data were confirmed by a double-labeling experiment. For this, Gce1p was metabolically labeled in both the glycan and protein portions by either *myo*-[^{14}C]inositol together with [^{3}H]leucine or [^{14}C]ethanolamine together with [^{3}H]leucine or, as a control, only in the glycan portion by [^{3}H]ethanolamine together with [^{14}C]inositol. After activation of the endogenous (G)PI-PLC by addition of glucose to the spheroplasts, the hydrophilic forms of Gce1p were affinity purified from isolated plasma membranes, and the turnover rates of the radiolabeled anchor constituents relative to the protein moiety were determined. The quantitative evaluations of these experiments confirm that the labels residing in the polar head group were lost significantly faster than the protein label, yielding a sharp decline of the ratio of glycan label to protein label with both pairs of label. The loss of radioactivity from the polar head group became prominent after a lag of 10 to 15 min and persisted through at least the next 50 min of glucose induction. On the other hand, the losses of radiolabeled ethanolamine and inositol followed very similar kinetics so that the ratio in the respective double labeling experiment remained about constant. The observation that the *myo*-inositol- and ethanolamine-containing hydrophilic forms reached this steady state simultaneously after about 10 min of incubation in glucose medium, whereas the leucine-labeled Gce1p accumulated steadily in the hydrophilic fraction for up to 30 min, is compatible with the assumption of two cleavage events operating on Gce1p.

These data together with those shown in Fig. 1 exclude the possibility that photoaffinity labeling and metabolic labeling of Gce1p label different pools of molecules, accounting for the different labeling kinetics of the protein moiety by the photolabel and of the anchor moiety by the metabolic label. Moreover, it seems unlikely that the apparent different turnovers of the phosphoinositolglycan moiety of the anchor and of the residual protein are due to different chase efficiencies. Since the leucine label is chased into the protein directly, whereas inositol and ethanolamine run through different lipid pools before becoming attached to a GPI-anchored protein, one would expect labeling and chase with the anchor constituents to be delayed relative to the protein label. Thus, if pool size affects the turnover of the GPI-anchored protein in any way, then the anchor-specific label should persist longer than the protein label so that the turnover kinetics will be skewed in a direction opposite that observed. In addition, both types of chase are sensitive to brefeldin A (3a). This finding indicates that the mechanisms by which the protein and anchor labels are transported to the cell surface are identical or very similar. In conclusion, the results shown in Fig. 1 and the data from the double labeling provide a first hint that 20 min after the transfer of spheroplasts to glucose, processed Gce1p molecules which have lost both the ethanolamine and inositol radiolabels emerge.

The turnover of the anchor constituents was directly confirmed by the use of an anti-CRD antibody. This antiserum is directed against the CRD of the soluble variant surface glycoprotein from trypanosomes and exclusively recognizes epitopes in the glycan portion of GPI anchors provided that they have been cleaved by a (G)PI-PLC (60). Accordingly, it cross-reacts with the (G)PI-PLC-cleaved Gce1p from *S. cerevisiae* (35), but it does not recognize any epitopes in the protein moiety of Gce1p (43). This antiserum was used to immunoprecipitate the protein label or the label residing in the phosphoinositolglycan structure of the hydrophilic version of Gce1p generated as described above. The immunoprecipitates were analyzed by SDS-PAGE and evaluated by radioactivity scanning of the gels as described above (Fig. 1).

The fact that these antibodies precipitated labeled Gce1p confirms that the anchor had been cleaved by an endogenous PLC to generate the terminal inositol-1,2-cyclic-phosphate epitope. The kinetics of release of the total and of the immunoprecipitated ethanolamine-labeled hydrophilic material were very similar, but they differed from the kinetics of the amphiphilic-to-hydrophilic conversion of the protein label. The maximal amphiphilic-hydrophilic conversion of Gce1p labeled in the protein moiety did not exceed 45% of total amphiphilic molecules within the time range studied. Therefore, substrate limitation cannot explain why a steady-state level of the amount of Gce1p labeled in the anchor portion is reached after 10 to 15 min. These findings suggest that a hydrolytic cleavage reaction removes the anti-CRD epitope together with some anchor constituents from the lipolytically cleaved Gce1p molecules. Since the maximal fraction of hydrophilic Gce1p approached 45% of total Gce1p, glucose-induced processing of Gce1p may be fundamental for the function and biosynthesis of this GPI-anchored protein.

Anchor processing changes the isoelectric point of the protein. Irrespective of whether amphiphilic Gce1p is processed by two independent or coupled cleavage events, we expect to observe a total of at least three different forms of Gce1p: (i) the uncleaved amphiphilic u form, (ii) the lipolytically cleaved hydrophilic intermediate (l form), and (iii) a double-processed hydrophilic mature version (p form). At about neutral pH, the anchor of the u form will have two negative charges on the two phosphodiesters and one positive charge on the glucosamine. The expected hydrophilic l form presumably has a 1,2-cyclic phosphate on the inositol (since it is readily recognized by the anti-CRD antibody). Therefore, it should have about the same charge distribution as the u form. The p form has lost all three anchor-associated charges and gained a partial negative charge on the newly formed carboxyl terminus. Analysis of the three forms is therefore hampered by two major problems, the lack of major charge differences between u, l, and p forms and the lack of differences in electrophoretic mobility (Fig. 1a). One possible solution was based on the assumption that the uncleaved GPI anchor of the u form will bind ionic detergents, whereas both hydrophilic versions, the l and p forms, will not.

In a charge shift electrophoresis carried out in the presence or absence of various detergents (adapted from reference 19), uncleaved Gce1p (metabolically labeled with *myo*-[^{14}C]inosi-

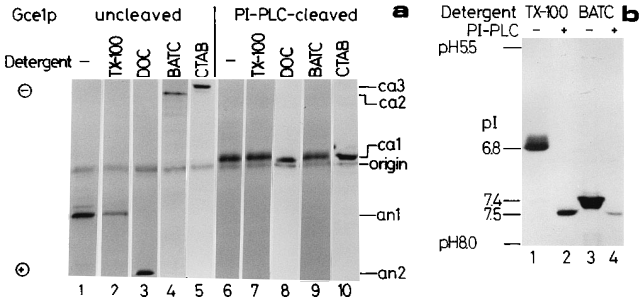

FIG. 2. Influence of detergent on the pI of Gce1p. (a) Charge shift electrophoresis. Spheroplasts from lactate-grown yeast cells were metabolically labeled with *myo*-[^{14}C]inositol. Plasma membranes were isolated, solubilized with 0.2% TX-100, and incubated with PI-PLC (*B. cereus*) or left untreated prior to TX-114 partitioning. From the detergent phase of the untreated sample (after *n*-butanol extraction) and from the aqueous phase of the PI-PLC-treated sample, Gce1p was affinity purified, precipitated with PEG 6000, dissolved in twofold gel buffer (see Materials and Methods) containing 0.01% TX-100, and then analyzed by charge shift electrophoresis either with no detergent present (lanes 1 and 6) or in the presence of 0.5% TX-100 (lanes 2 and 7), 0.2% deoxycholate (DOC) plus 0.5% TX-100 (lanes 3 and 8), 0.1% BATC plus 0.5% TX-100 (lanes 4 and 9), or 0.05% CTAB plus 0.5% TX-100 in the agarose gels and the running buffers. Gels were evaluated by fluorography. Positions of the various cationic (ca1 to ca3) and anionic (an1 and an2) forms of amphiphilic Gce1p and of PI-PLC-cleaved Gce1p are indicated. (b) IEF. Spheroplasts from lactate-grown cells were metabolically labeled with *myo*-[^{14}C]inositol. Isolated plasma membranes were solubilized with 0.2% TX-100 or 0.1% BATC. One half was left untreated, while the other was incubated with bacterial PI-PLC prior to TX-114 partitioning. From the detergent phase of the untreated sample (PI-PLC −) and from the aqueous phase of the PI-PLC-cleaved sample (PI-PLC +), Gce1p was affinity purified as described above, precipitated with PEG 6000, and dissolved in 0.05% TX-100 or BATC. The hydrophilic material (PI-PLC +) was dephosphorylated with phosphodiesterase I-alkaline phosphatase, and all samples were analyzed by IEF (in the presence of TX-100 or BATC as indicated) and fluorography.

tol) migrated at pH 6.8 to the anode after equilibration with TX-100 (Fig. 2a, lane 2, band an1). Its negative charge was significantly increased in the presence of deoxycholate (lane 3, band an2), whereas it moved to the cathode in the presence of BATC (which is zwitterionic and weakly positively charged at pH 6.8; lane 4, band ca2) and cationic CTAB (lane 5, band ca3). By contrast, the migration behavior of Gce1p cleaved by bacterial PI-PLC was the same in all detergents (lanes 6 to 10, band ca1). Since this form does not bind detergent and in consequence cannot obtain the partial positive charges of BATC and CTAB (compare lane 4 with lane 9 and lane 5 with lane 10) or the negative charge of deoxycholate (compare lane 3 with lane 8) introduced by detergent binding to the uncleaved anchor. Besides charge, other parameters affected by the bound detergent seem to determine the migration behavior of GPI-anchored proteins, since a considerable difference in migration between uncleaved and PI-PLC-cleaved Gce1p was observed in the presence of the nonionic detergent TX-100 (compare lanes 2 and 7). Regardless of the detergent present during the charge shift electrophoresis, a small amount of [^{14}C]inositol-labeled affinity-purified material did not leave the site of sample application (lanes 1 to 10, origin). It may represent aggregates of uncleaved Gce1p (present in the PI-PLC-cleaved samples as a contaminant) which resisted solubilization by detergent, a property characteristic of some GPI-anchored proteins (7).

Since detergent is required to solubilize and purify Gce1p from plasma membranes and since BATC has a high selectivity toward the solubilization of Gce1p (and other GPI-anchored proteins) (42), this detergent was chosen to discriminate between the u and l forms also in IEF. However, even in the presence of BATC, the pI values of the l and p forms were very similar; therefore, the terminal cyclic phosphate moiety was removed from the inositol residue of the glycan structure in all lipolytically cleaved samples. The analysis was additionally hampered by the observed spontaneous opening of the cyclic phosphodiester bond to a minor but variable degree during preparation of the samples containing the l form for IEF and by the fact that the l form with the opened or intact cyclic monophosphate residue remaining attached to *myo*-inositol is not separated from the u form by IEF, since the two forms do not differ sufficiently in charge (data not shown). Removal of the phosphate was achieved by incubation with phosphodiesterase I and alkaline phosphatase. The latter activity is a contaminant of the phosphodiesterase preparation from bovine intestinal mucosa used and is responsible for the release of the phosphate residue after opening of the inositol-1,2-cyclic phosphate ester. The internal phosphodiester bridge is, however, stable under these conditions. Thus, to separate the three forms, plasma membranes from yeast spheroplasts metabolically labeled with [^{14}C]inositol were prepared, solubilized with BATC or TX-100, and not treated or treated with bacterial PI-PLC. After TX-114 partitioning, Gce1p was affinity purified from the respective phases, incubated with phosphodiesterase, and analyzed by IEF (in the presence of BATC or TX-100) and fluorography (Fig. 2b).

The uncleaved u form of Gce1p focused at pH 6.8 in the presence of TX-100 (lane 1) and at pH 7.4 in the presence of BATC (lane 3), whereas the PI-PLC-cleaved version was found at pH 7.5 (after treatment with phosphodiesterase) in both cases, regardless of the detergent (lanes 2 and 4). The shift of the dephosphorylated l form to a more alkaline pH compared with the u form in the presence of BATC seems due to the loss of one phosphate residue and of the partial positive charge on the detergent. Thus, charge shift electrophoresis and IEF for the first time allowed the separation and identification of two distinct versions of Gce1p.

Next we tried to identify the hydrophilic version of Gce1p emerging from the additional hydrolytic cleavage event. This p form should focus at a more acidic pI compared with the dephosphorylated l form as a result of the additional loss of one phosphodiester and the glucosamine residue and the gain of a negative charge on the C-terminal carboxyl group. In comparison with the u form, the p form should have lost two phosphates, the glucosamine and the partial charge on the BATC, and gained a C-terminal carboxyl group so that the p form should be a little more acidic than the u form as a result of the net loss of the partially positively charged detergent. To examine these predictions, spheroplasts were labeled with either 8-N_3-[^{32}P]cAMP in the protein or *myo*-[^{14}C]inositol in the anchor (Fig. 3a) or with [^{14}C]leucine or [^{14}C]ethanolamine, respectively (Fig. 3b) and exposed to glucose for various periods of time. All of the metabolically labeled forms of Gce1p present at each given time point were affinity purified together before separation from one another by TX-114 partitioning, whereas incubation mixtures containing photoaffinity-labeled Gce1p were directly subjected to TX-114 partitioning. All samples were analyzed by IEF and autoradiography-fluorography.

Prior to the glucose pulse, the majority of Gce1p was amphiphilic, constituting the major form in the detergent phase after TX-114 partitioning. This form carried both anchor-specific labels and had a pI of 7.4 (Fig. 3a and b, at the zero time point, lanes 1 and 2 and lanes 8 and 9, respectively). After the transfer to glucose, labeled material dramatically decreased with time in the detergent phase (lanes 4, 6, 11, and 13) and emerged in the hydrophilic fraction (lanes 5, 7, 12, and 14). After 10 and 30 min, two hydrophilic forms emerged. At first a more alkaline form (pI 7.5) which still carried either label

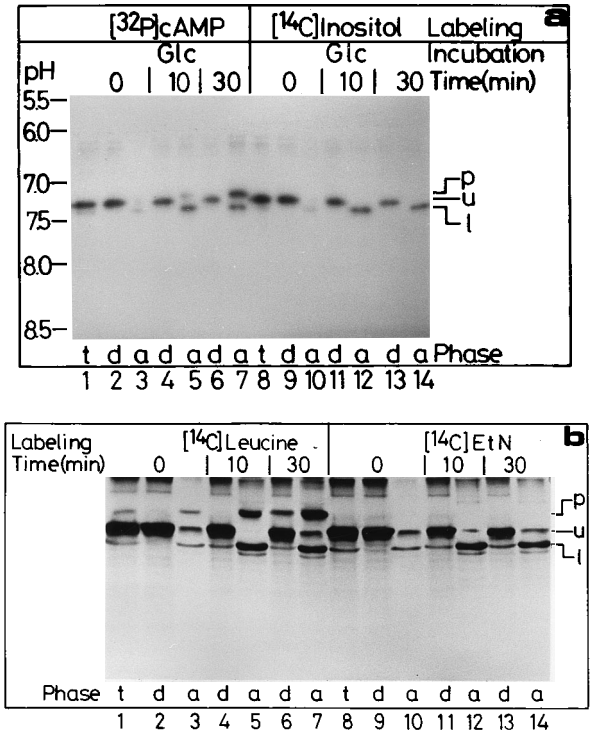

FIG. 3. IEF of soluble Gce1p. (a) Aliquots of spheroplasts from lactate-grown cells were either photoaffinity labeled with 8-N_3-[^{32}P]cAMP (lanes 1 to 7) or metabolically labeled with *myo*-[^{14}C]inositol (lanes 8 to 14) and subsequently incubated in 5% glucose medium (Glc) under chase conditions for different periods of time. Plasma membranes were isolated. For each radiolabel and for each time point, identical amounts of membranes (with respect to radioactivity) were divided into two halves. One half of each sample was partitioned between detergent (d) and aqueous (a) phases. The other half was left intact (total [t]; only the zero time point is shown). The [^{14}C]inositol-labeled samples were then subjected to affinity purification. All samples were PEG precipitated, treated with phosphodiesterase-alkaline phosphatase, again PEG precipitated, and analyzed by IEF (presence of BATC) and autoradiography-fluorography. pH values were determined with a semiautomatic pH measuring apparatus. (b) Aliquots of spheroplasts from lactate-grown cells were metabolically labeled with either [^{14}C]leucine (lanes 1 to 7) or [^{14}C]ethanolamine ([^{14}C]EtN; lanes 8 to 14) and then incubated for the periods indicated in 5% glucose medium under chase conditions. Samples were processed as described for panel a and analyzed by IEF (in the presence of BATC) and fluorography. The pI values determined were as follows: u form, 7.4; l form, 7.5; and p form, 7.3.

appeared (lanes 5 and 12). Because of the small alkaline shift of its pI compared with that of the u form, it should be identical with the l form identified in Fig. 2a. After 30 min in glucose, we observed a second hydrophilic form (pI 7.3) which carried only the protein-specific radiolabels (Fig. 3, lanes 7, cAMP label in panel a and leucine label in panel b) and evidently had lost all anchor constituents, as judged from the absence of the respective signals (lanes 14) after incubation with either *myo*-inositol (Fig. 3a) or ethanolamine (Fig. 3b). The kinetics of the amphiphilic-to-hydrophilic conversion and of the emergence of the pI 7.3 form lacking the anchor-specific radiolabel indicate that the latter form is derived from the hydrophilic intermediate (pI 7.5) and that the pI 7.3 species presumably is the double-processed product (p form).

Photoaffinity-labeled and metabolically labeled forms of Gce1p focus at about the same pH values. The pI values of the different forms of photoaffinity-labeled Gce1p are in each case only slightly more acidic than those of metabolically labeled Gce1p (u form, 7.3 versus 7.4; l form, 7.4 versus 7.5; p form, 7.2 versus 7.3). This is presumably due an almost complete compensation of the negative charge on the cyclic phosphodiester of 8-N_3-cAMP by the positive charge on the adenine ring. The relative migration and the resolution from one another of the u, l, and p forms from differently labeled Gce1p are not affected by this general shift of the pI. In addition to the three major bands attributable to the u, l, and p forms of Gce1p, particularly in Fig. 3b, some additional bands are present. The band at the u position in the aqueous phase may be due to some contaminating uncleaved material, the more acidic signal in the IEF of the aqueous phase (l position) may be caused by incomplete removal of the phosphate in this experiment, and the small band at high pI could be derived from labeled material not related to Gce1p contaminating this preparation. Since the additional bands are well separated from the dephosphorylated u, l, and p forms of Gce1p, they do not interfere with the interpretation of the results from the IEF.

Precursor-product relationship between l and p forms of Gce1p. To corroborate sequential processing, we tried to convert the hydrophilic l form into the hydrophilic p form. For this purpose, photoaffinity-labeled spheroplasts from lactate-grown cells were transferred to glucose medium and incubated under chase conditions for various periods of time. After TX-114 partitioning, only the hydrophilic forms of Gce1p were analyzed by IEF and autoradiography. The amount of l form increased only initially during the first 10 min after the transfer to glucose (Fig. 4a, lanes 8 to 11) and then declined (lanes 12 to 14). The amount of the p form increased steadily after administration of glucose (lanes 12 to 14), suggesting a chase of the l form into the p form. In contrast, incubation of the spheroplasts in lactate caused the generation of both the l and p forms with time at a basal level (lanes 1 to 7). The amount of p form at each time point and of l form up to 10 min was significantly higher in glucose-induced cells than in lactate-grown cells (at least 3.9- and 5.2-fold, respectively). These data demonstrate a significant (four- to fivefold) stimulation of the production of l and p forms of Gce1p in spheroplasts in the presence of glucose compared with lactate. Figure 4b quantitates the data and demonstrates the transient accumulation of the lipolytically cleaved intermediate. Figure 4c shows the close to inverse correlation between the decrease of the uncleaved amphiphilic starting material and the increase in the sum of the hydrophilic cleavage products (l plus p forms). The glucose-dependent decrease of the amount of u form approached up to 40% of the uncleaved starting material after 30 min. This processing efficacy of metabolically labeled Gce1p correlates well with the amphiphilic-to-hydrophilic conversion rate of photoaffinity-labeled Gce1p 30 min after transfer of spheroplasts from lactate to glucose medium (Fig. 1), demonstrating that the pool of unlabeled and newly synthesized labeled Gce1p molecules is homogeneous. The fact that the sum of the processed forms accounted for a maximum of only 28% (at 15 min) of the uncleaved starting material and then declined (30 min) may be due to gradual degradation of a total of about 15% (difference between u form converted and l plus p forms). Spheroplasts incubated in lactate (Fig. 4a, lanes 1 to 7; Fig. 4b, lower panel) continuously produced low amounts of both hydrophilic l and p forms. This observation suggests that the anchor processing is operating under both growth conditions, albeit with vastly different efficiencies.

The amount of affinity-purified material focusing at a lower pI than the p form of Gce1p increased with the chase time, but only in the presence of glucose, with a kinetics similar to that of the appearance of the p form (Fig. 4a, lanes 9 to 14). This material may contain Gce1p molecules derived from the glucose-induced l form, which is resistant to dephosphorylation at the inositol moiety. The failure of this Gce1p species to be

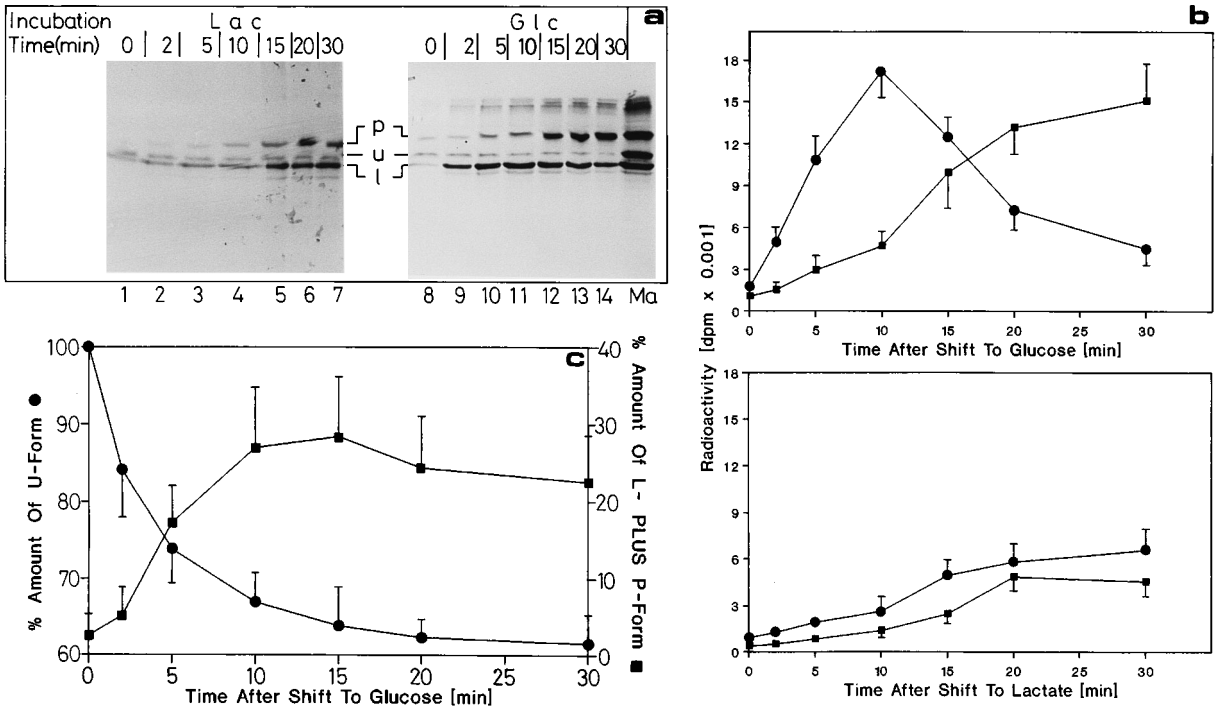

FIG. 4. Time course of glucose-induced primary and secondary processing of Gce1p. (a) Aliquots of spheroplasts from lactate-grown cells were photoaffinity labeled with 8-N_3-[^{32}P]cAMP and subsequently incubated in lactate (lanes 1 to 7) or 5% glucose medium (lanes 8 to 14) under chase conditions for the periods indicated. Aliquots of the total spheroplasts (containing identical amounts of radioactivity) were subjected to TX-114 partitioning. Aqueous phases were PEG precipitated, treated with phosphodiesterase-alkaline phosphatase, again PEG precipitated, and analyzed by IEF (in the presence of BATC) and autoradiography. Ma, affinity-purified amphiphilic Gce1p (marker). (b) Quantitative evaluation of the two processed forms from panel a by liquid scintillation counting of the radioactivity contained in the excised individual gel bands. ●, l form; ■, p form; top, lanes 8 to 14, transfer to glucose; bottom, lanes 1 to 7, lactate-grown cells. Each value represents the mean ± standard deviation of four independent experiments. (c) Precursor-product relationship between the various forms of Gce1p in glucose-induced cells. Data for the u form were taken from the analysis of the TX-114 phase from the experiment represented in panel a (not shown); data for the l and p forms were taken from panel a. The total amount (disintegrations per minute) of the u form at time zero was set at 100% and used for calculation of the percentage of u form left and of the sum of l and p forms generated at each time point.

accepted as a substrate for the second processing reaction would explain the observed accumulation throughout the chase. This possibility is compatible with the finding that this low-pI form of Gce1p is hydrophilic in nature and is metabolically labeled by both protein- and anchor-specific labels (Fig. 3b, lanes 3, 5, and 7 and lanes 10, 12, and 14, respectively) and that its emergence depends on the glucose-induced processing of Gce1p (Fig. 4a).

The two steps of GPI-processing can be uncoupled. As further evidence of the occurrence of the l form as an intermediate between amphiphilic u form and hydrophilic p form, we assayed whether the isolated and radiolabeled l form could be chased into the p form. By the same experimental approach of uncoupling of the two steps of the processing cascade, we tested whether the second processing occurs constitutively or is activated by glucose, i.e., whether glucose stimulates both processing steps directly or, alternatively, relieves only the rate-limiting step. For this, a constant (radioactive) amount of photoaffinity-labeled l form (prepared by digestion in vitro of radiolabeled Gce1p with bacterial PI-PLC and subsequent TX-114 partitioning) was added to unlabeled spheroplasts from lactate- or glucose-grown yeast cells.

Figure 5 illustrates that with incubation time, increasing amounts of the added radiolabeled l form were converted into the p form regardless of the carbon source present (lanes 3 to 12; compare L with G). No significant difference in the kinetics of the second processing between glucose and lactate medium was apparent. Uncleaved amphiphilic Gce1p, added to glucose-cultured spheroplasts, was not processed (lane 13). These data confirm the sequential order of the lipolytic and the additional hydrolytic processing events and suggest that the enzyme involved in the secondary step is not regulated by the nutritional situation. Apparently the second cleavage reaction ensues once Gce1p is processed by the endogenous (G)PI-PLC. Thus, lipolytic cleavage seems to be the glucose-controlled and rate-limiting step in the double processing of the GPI anchor of Gce1p in yeast cells.

Most interesting was the observation that obviously the cyclic phosphate ester bond on the *myo*-inositol moiety was required for the secondary cleavage to occur. Lane 14 of Fig. 5 demonstrates that after cleavage with GPI-PLD, which removed the terminal phosphate group, isolated photolabeled Gce1p failed to display the subsequent acidic shift, demonstrating that the processing of the l to the p form did not take place. (PLD- and PLC-cleaved materials focus at the same pH as a result of the subsequent enzymatic removal of the phosphate from the inositol prior to IEF; the cleavage of Gce1p by a PLD activity of crude rabbit serum was confirmed with [^{14}C]inositol-labeled amphiphilic Gce1p as a substrate. No significant loss of radiolabel was observed from the hydrophilic version of Gce1p generated under these conditions of incubation [data not shown].) Complementary to this observation, the anti-CRD antibody, preincubated with secondary anti-rabbit Fc-specific immunoglobulin G and then reacted with the PI-PLC-cleaved l form of Gce1p, likewise prevented the secondary cleavage event (lane 15). Omission of the secondary

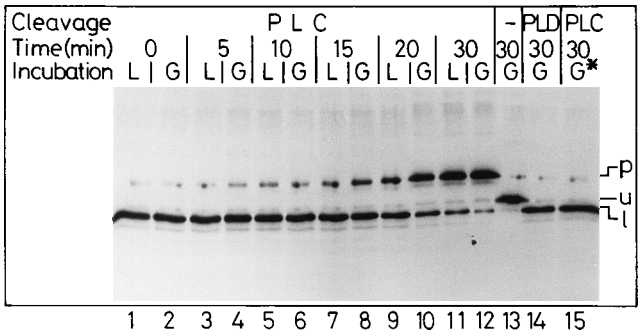

FIG. 5. Chase of PI-PLC-cleaved l form of Gce1p into its p form upon incubation with spheroplasts. Partially purified Gce1p (38) photoaffinity labeled with 8-N_3-[^{32}P]cAMP was either left untreated (control) or treated with PI-PLC (*B. cereus*), with crude PLD (rabbit serum), or with PI-PLC and then incubated (4 h, 4°C) with an anti-CRD antiserum (1:200) preincubated (16 h, 4°C) with secondary goat anti-rabbit immunoglobulin G (1:500). Separately, spheroplasts were prepared from unlabeled lactate-grown cells and incubated (10 min, 30°C) in lactate (L) or 5% glucose (G) medium. Then the photoaffinity-labeled Gce1p, pretreated as described above, was added (15 μg of protein, corresponding to 25,000 dpm per 5×10^5 spheroplasts), and samples were withdrawn at the time points indicated. All samples were PEG precipitated, treated with phosphodiesterase-alkaline phosphatase, again PEG precipitated, and analyzed by IEF (in the presence of BATC) and autoradiography. Lanes 1 to 12, PI-PLC-cleaved Gce1p; lane 13, uncleaved Gce1p; lane 14, PLD-cleaved Gce1p; lane 15, PI-PLC-cleaved Gce1p complexed to anti-CRD and secondary antibodies (G*).

antibody in an analogous experiment demonstrated the requirement for preformation of complexes between primary and secondary antibodies in order to efficiently inhibit secondary processing (data not shown). Since the *myo*-inositol–cyclic phosphate ester constitutes the major epitope recognized by these antibodies (60), the results shown in lanes 14 and 15 indicate that the terminal phosphate group is indispensable for the secondary processing to ensue.

The anchor glycan structure is likely removed en bloc. Since the secondary processing event removes both the inositol label and the ethanolamine label but does not affect the electrophoretic mobility of lipolytically cleaved Gce1p, it is likely to occur close to the carboxyl terminus of the protein. To discriminate between a possible very rapid, stepwise sequential degradation of the anchor glycan and a processing at one unique site, we studied whether, after activation of the endogenous (G)PI-PLC by glucose, an anchor fragment comprising the complete glycan structure together with the phosphate-linked ethanolamine (phosphoinositolglycan) potentially including one or a few carboxy-terminal amino acids (phosphoinositolglycan-peptide) can be prepared from the isolated l version of Gce1p by proteolytic removal of the protein moiety in vitro. For this purpose, spheroplasts were metabolically double labeled with [^{14}C]ethanolamine and *myo*-[^{3}H]inositol and exposed to glucose under chase conditions. Plasma membranes were isolated at the time points indicated. Only the hydrophilic versions of Gce1p were recovered from the aqueous phase after TX-114 partitioning, further purified by affinity chromatography on cAMP-Sepharose, and then subjected to exhaustive digestion by pronase. The sizes of the labeled products were evaluated by Bio-Gel P4 chromatography with a resolving capacity of 12 glucose units to 1 glucose unit. In the absence of multiple secondary cleavage events within the anchor glycan in vivo, i.e., in the case of a defined proteolytic cleavage, a single species of a pronase-resistant polar glycan structure will be generated. By contrast, in the case of stepwise degradation beginning from the inositol-phosphate moiety, a heterogeneous set of glycan-linked Gce1p molecules will arise following the addition of glucose. Upon pronase treatment, they will be converted into an array of glycan-peptides of variable size that have lost the inositol radiolabel but retained the ethanolamine label.

It can be seen from Fig. 6a that the double-labeled material was eluted from the column in a single peak at a volume equivalent to 8 to 8.5 glucose units. No smaller single- or double-labeled anchor-derived fragments emerged during 10 to 30 min after the induction of the primary cleavage by glucose, demonstrating that the glycan moiety is not degraded stepwise. In agreement with the proposed structure of this material, its elution maximum peaked between the two phosphoinositolglycan-peptides consisting of the complete consensus core glycan (glucosamine plus three mannose residues) with either one or two mannose carbohydrate side chains attached, which is coupled via a phosphodiester-linked ethanolamine bridge to a terminal asparagine residue (18a). These molecules (M_rs of 1,253 and 1,415) have been synthesized by chemical means according to the known structural features of the GPI anchors of Gce1p (43) and Gas1p (15). The amount of pronase fragment containing both the [^{14}C]inositol and [^{3}H] ethanolamine radiolabels which can be isolated from equal numbers of metabolically labeled spheroplasts (chase conditions) decreased with time from 10 to 30 min after the transfer to glucose medium. Thus, the amount of Gce1p molecules which had retained the ethanolamine and the complete glycan portion (as defined by the terminal inositol residue) declined during this time interval. Furthermore, the ethanolamine radiolabel displayed a turnover similar to that of the *myo*-inositol label (Fig. 6b), suggesting that both anchor components are removed by the same secondary cleavage event. These findings are consistent with the assumption that in response to glucose, the glycan structure is removed as a whole from the lipolytically cleaved GPI anchor of Gce1p.

The identity of the pronase fragment generated was confirmed by HPTLC analysis after dephosphorylation with aqueous HF, which should yield the neutral glycan fragment consisting of a core structure of three mannoses plus possibly a side chain with one or two mannose residues which is linked via glucosamine to the radiolabeled inositol (15, 16). High-resolution TLC of the HF cleavage products (Fig. 7) identified a [^{14}C]inositol-labeled pronase fragment which comigrated with the corresponding HF-cleaved authentic pronase fragment prepared from purified Gce1p by treatment with exogenous PI-PLC in vitro (ma1). It migrated markedly more slowly than the standard, Mn_3A (see the legend to Fig. 7), but faster than marker ma2, the *myo*-inositol-labeled product obtained after successive treatment of the pronase fragment with bacterial PLC and trifluoroacetic acid, which consists of glucosamine 1,6 linked to *myo*-inositol-phosphate (11). The amount of the HF cleavage product increased from 2 to 10 min after transfer to glucose (lanes 1 to 4), in agreement with the activation of an endogenous (G)PI-PLC by glucose, generating the intermediary Gce1p molecules used as material for cleavage with aqueous HF. The amount of HF-cleaved neutral glycan fragment declined subsequently with time, confirming the loss of the complete glycan structure from the lipolytically cleaved (intermediary) form of Gce1p (lanes 5 to 7). The kinetics resembled that observed with the Bio-Gel P4 chromatography (Fig. 6). Again, no other less polar fragments were observed after HF treatment of Gce1p material isolated 2 to 30 min after the transfer to glucose, strongly suggesting the en bloc removal of the complete glycan structure from the PLC-cleaved anchor of Gce1p (l form).

Processed versions of Gce1p in intact cells. Next we wanted to exclude the possibility that the glucose-induced processing of Gce1p depends on removal of the cell wall. For identifica-

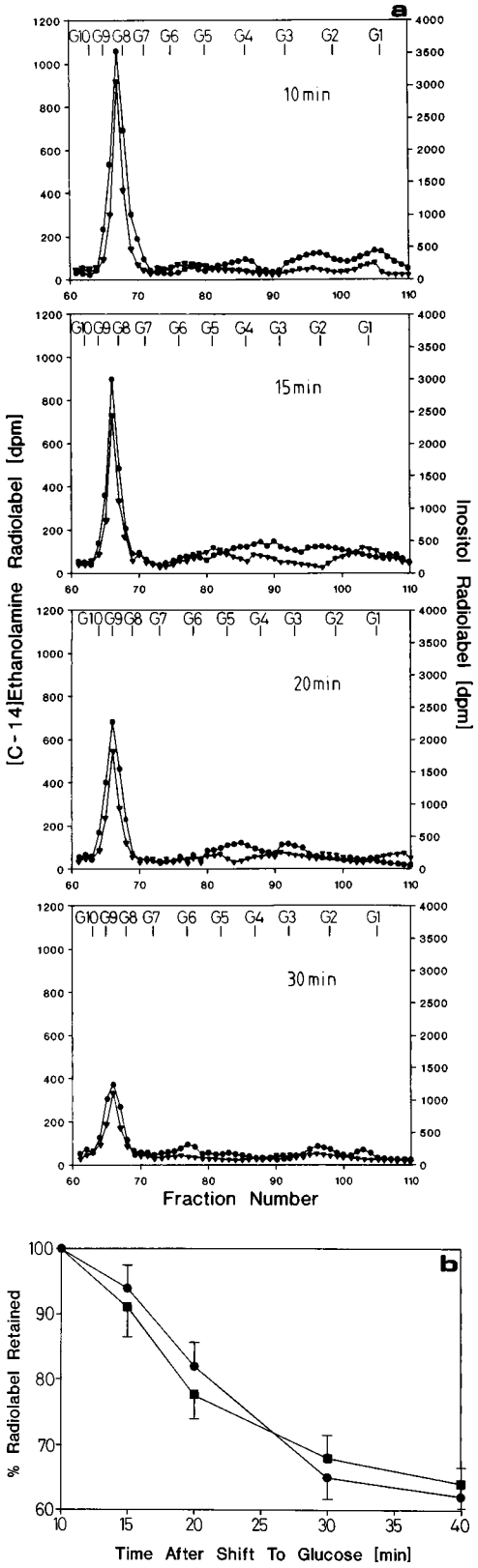

FIG. 6. Chromatographic analysis of the pronase fragment prepared from hydrophilic Gce1p. (a) Aliquots of spheroplasts from lactate-grown cells were metabolically double labeled with *myo*-[^{3}H]inositol (▼) and [^{14}C]ethanolamine (●) and subsequently incubated in 2% glucose medium under chase conditions for 10, 15, 20, and 30 min. Isolated plasma membranes were subjected to TX-114 partitioning. Hydrophilic Gce1p affinity purified from the aqueous phase at each time point was digested to completion with pronase and chromatographed on Bio-Gel P4 columns. Fractions were counted for ^{14}C and ^{3}H radioactivity. G1 to G10, standards of 1,4-linked [^{3}H]glucose oligomers with chain lengths of 1 to 10 glucose units. (b) Kinetic evaluation of the experiment from panel a. The radiolabels of *myo*-[^{3}H]inositol (■) and [^{14}C]ethanolamine (●), retained with Gce1p from peak fractions 63 to 69 (from panel a), were each arbitrarily set at 100% for the 10-min time point after the addition of glucose. Data for only the 40-min incubation are given in panel b. Results of one typical experiment is shown. (The chromatography was repeated three times with comparable results.)

tion of hydrophilic versions of Gce1p in intact yeast cells, the cells were grown in the presence or absence of [^{14}C]leucine for metabolic labeling of the protein moiety of Gce1p and of [^{14}C]ethanolamine or *myo*-[^{14}C]inositol for labeling of its GPI anchor. The cells were broken with glass beads in the presence of the detergent BATC, which preferentially solubilizes GPI-anchored proteins. The total extracted proteins were subjected to TX-114 partitioning. From the hydrophilic proteins recovered in the aqueous phase, Gce1p was affinity purified (in the case of metabolically labeled cells) or photoaffinity labeled (in the case of unlabeled cells). Half of the latter material was immunoprecipitated with the anti-CRD antiserum, and all samples were analyzed by SDS-PAGE and fluorography-autoradiography.

The amount of affinity-purified hydrophilic Gce1p metabolically labeled in the anchor moiety with [^{14}C]inositol (Fig. 8, lanes 16 to 20) or [^{14}C]ethanolamine (lanes 21 to 25) increased 5- to 6-fold with time above the basal level from 0 to 10 min after induction with glucose and thereafter decreased to 0.8- to 2.5-fold above the basal level at 60 min. The time courses of the emergence and disappearance of the lipolytically cleaved Gce1p closely resembled the kinetics of the glucose-induced generation of the l form of Gce1p at the plasma membrane of spheroplasts; thus, the hydrophilic product formed due to the retention of inositol and ethanolamine may be attributed to the intermediate l form. However, whereas in spheroplasts hydrophilic Gce1p labeled in the protein moiety was continuously generated with time from 0 to 60 min after glucose induction, in intact cells the amount of hydrophilic Gce1p metabolically labeled with [^{14}C]leucine (lanes 1 to 5) or photoaffinity labeled with 8-N_3-[^{32}P]cAMP (lanes 6 to 10) was comparable to that of Gce1p labeled in the GPI anchor for each time point after glucose induction (increase of three- to sixfold above the basal level from 0 to 10 min and decrease to near the basal level to twofold above the basal level at 60 min). Thus, in intact yeast cells, all of the hydrophilic Gce1p molecules which can be identified in a detergent-extractable form after glucose induction harbor the complete glycan structure of the GPI anchor, including *myo*-inositol and ethanolamine. They are presumably derived from amphiphilic Gce1p by a single lipolytic cleavage event mediated by the glucose-inducible (G)PI-PLC. The level of leucine- or photoaffinity-labeled Gce1p lacking the anchor glycan is undetectably low. This may be explained by a short half-life of the double-processed p form as a detergent-extractable species in intact yeast cells. To confirm the absence of the p form, photoaffinity-labeled hydrophilic Gce1p from glucose-induced cells was quantitatively immunoprecipitated with the anti-CRD antibody. A comparison of the fluorograms of the SDS-PAGE analysis of the total (lanes 6 to 10) and immunoprecipitated (lanes 11 to 15) samples demonstrated roughly identical time courses for appearance and disappearance of total and anti-CRD-cross-reactive hydrophilic photoaffinity-labeled Gce1p.

Identification of a cAMP-binding protein associated with the cell wall. The failure to detect the long-lived double-pro-

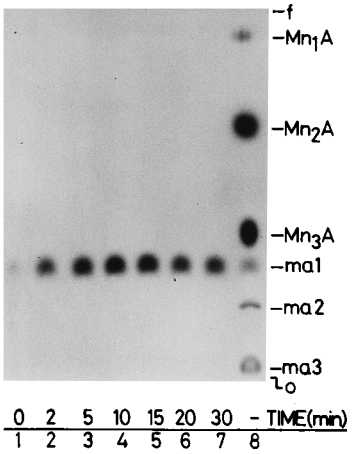

FIG. 7. HPTLC analysis of the polar glycan structure of lipolytically cleaved Gce1p. Spheroplasts from lactate-grown cells were metabolically labeled with *myo*-[^{14}C]inositol and incubated in 5% glucose under chase conditions for the periods indicated. Plasma membranes were prepared and subjected to TX-114 partitioning. Hydrophilic Gce1p was affinity purified from the aqueous phase, TCA precipitated, and treated with aqueous HF. The samples were desalted by dialysis (Spectra/Por cellulose ester membrane; MWCO 500), dried, and analyzed by HPTLC and fluorography. Chemically synthesized ^{14}C-labeled standards were as follows: Mn_3A (Manα1→2Manα1→6Manα1→4anhydromannitol); Man_2A (Manα 1→6Manα1→4anhydromannitol), Mn_1A (Manα1→4anhydromannitol); and ma1, ma2, and ma3 (HF-, trifluoroacetic acid-, and PLC-treated pronase fragments prepared from *myo*-[^{14}C]inositol-labeled affinity-purified amphiphilic Gce1p). f, front; o, origin.

cessed p form of Gce1p after glucose induction in total cell extracts (which consist of all the detergent-soluble proteins of spheroplasts along with the proteins of the periplasmic space) is in apparent conflict with its presence in glucose-induced spheroplasts. One explanation could be that double-processed Gce1p, after its release from the outer face of the plasma membrane into the periplasmic space, rapidly associates with the cell wall in a manner which resists extraction by detergent. To test this possibility, we looked for a cAMP-binding protein covalently bound to the cell wall of the yeast *S. cerevisiae*. Some cell wall proteins resist extraction by SDS and can be released only by digestion of the cell wall with β-glucanase (27, 50). This class of cell wall proteins has been shown to have glucose residues attached and to be covalently linked to β-glucan fibers of the cell wall (33, 55; for a review, see reference 22). For instance, the cell wall form of α-agglutinin extractable by β1,3-glucanase is covalently linked to β1,6-glucan (28). To study a possible covalent linkage of Gce1p to the cell wall glucan, intact yeast cells were incubated with glucose or lactate, then photoaffinity labeled with 8-N_3-[^{32}P]cAMP, mechanically broken, and subsequently extracted with hot SDS to solubilize contaminating membrane proteins and to remove noncovalently associated cell wall proteins. The insoluble material collected by centrifugation was digested with laminarinase, which contains mainly β1,3-glucanase activity. The material released by the laminarinase treatment was analyzed by SDS-PAGE and autoradiography (Fig. 9).

Only a single major photolabeled band was observed migrating as a broad area between 95 to 130 kDa. The specificity of the photoaffinity labeling of this protein was demonstrated by inclusion of a 1,000-fold molar excess of cAMP during the incubation with 8-N_3-[^{32}P]cAMP, which led to a complete inhibition of its labeling (data not shown). After glucose induction of intact cells, the amount of this material increased seven- to eightfold with time from 0 to 30 min (lanes 5 to 8), whereas it remained about constant in lactate-grown cells (lanes 1 to 4). A certain amount of photolabeled material did not migrate into the resolving gel (arrow). No prominent photoaffinity-labeled cAMP-binding protein was detectable comigrating with authentic detergent-solubilized plasma membrane Gce1p from yeast (54 kDa).

Similar observations were obtained with intact cells metabolically labeled with [^{14}C]leucine and subsequently incubated with lactate or glucose. A cAMP-binding protein was affinity purified from the laminarinase extract of cell wall fragments prepared under native conditions. It migrated as a broad smear with an electrophoretic mobility corresponding to 95 to 130 kDa in SDS-PAGE (data not shown). Again the time-dependent generation of this protein was significantly stimulated by glucose induction, up to 6.5-fold after 30 min. On the other hand, incubation with lactate did not cause an increase of the leucine-labeled high-molecular-weight cAMP-binding protein from 5 to 30 min. The high-molecular-weight cAMP-binding protein constituted only a minor species of the total leucine-labeled cell wall proteins recovered in the laminarinase extract, which predominantly are larger and hardly migrated into the separating gel. The amount of total leucine-labeled proteins which could be extracted by laminarinase digestion from the

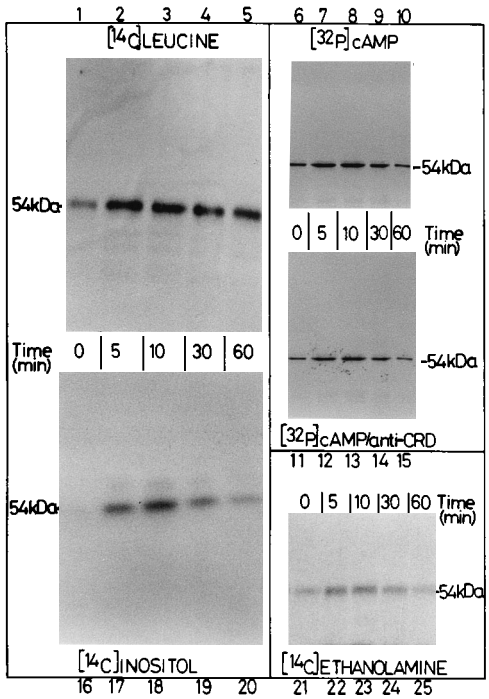

FIG. 8. Identification of processed Gce1p in intact cells. Lactate-grown yeast cells were metabolically labeled with *myo*-[^{14}C]inositol or [^{14}C]ethanolamine or incubated in the absence of radiolabeled compound for 60 min at 30°C. The cultures were supplemented with 4% glucose, and then aliquots of the suspension (5×10^8 cells) were incubated for the periods of time indicated. Subsequently washed cells were pelleted and used for extraction under native conditions (see Materials and Methods). The supernatants of the total cellular extract and of the reextraction were combined, diluted 10-fold with 50 mM Tris-HCl (pH 7.4)–150 mM NaCl–0.5 mM EDTA–200 μM PMSF, and precipitated (58). The pellet was washed once with the same buffer and subjected to TX-114 partitioning. The aqueous phases of the metabolically labeled cells were used for affinity purification of Gce1p, and those of the unlabeled samples were used for photoaffinity labeling of Gce1p. The photolabeled samples were divided into two halves. One half was subjected to quantitative immunoprecipitation with an anti-CRD antibody, and the other half was untreated. The metabolically labeled affinity-purified samples and the photoaffinity-labeled total or immunoprecipitated samples were PEG precipitated and analyzed by SDS-PAGE and autoradiography-fluorography. The molecular mass indicated was derived from photoaffinity-labeled amphiphilic Gce1p run in parallel.

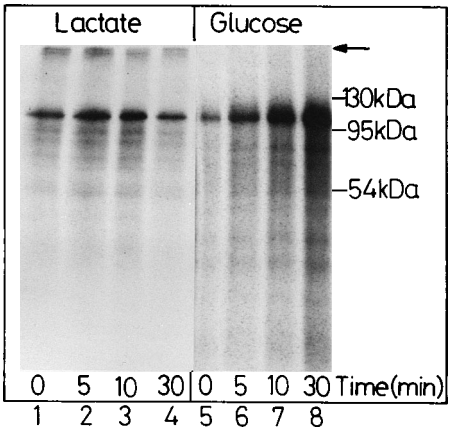

FIG. 9. Photoaffinity labeling of a cAMP-binding protein in the yeast cell wall. Yeast cells grown in lactate medium at 2×10^7 cells per ml were collected, suspended in lactate or glucose medium to 2×10^6 cells per ml, and then incubated for the periods indicated at 30°C. Identical numbers of cells were used for subsequent photoaffinity labeling with 8-N_3-[^{32}P]cAMP. Cell wall fragments were prepared from the washed cells under denaturing conditions (see Materials and Methods) and treated with laminarinase. The total laminarinase extracts were analyzed by SDS-PAGE (7.5% gel) and autoradiography. Molecular masses indicated on the right were derived from marker proteins and photoaffinity-labeled Gce1p run in parallel. The amount of protein applied onto each lane was 200 to 250 μg. The experiment was repeated three times with comparable results. ←, borderline between stacking and running gels.

cell wall fragments was 1.5- to 2.1-fold higher for cells incubated with glucose than for cells incubated with lactate. The general enhancement of the incorporation of proteins into the cell wall of yeast cells incubated in glucose medium may be mechanistically linked to increased synthesis of β-glucan structures for the cell wall under these metabolic conditions.

Topology of the cell wall cAMP-binding protein. The cellular location of the high-molecular-weight cAMP-binding protein was confirmed by assaying its accessibility to protease added to intact cells in concentrations which do not damage the plasma membrane. For this, lactate-grown yeast cells were transferred to glucose medium, incubated, then photoaffinity-labeled with 8-N_3-[^{32}P]cAMP, pretreated with DTT to enable penetration of exogenous protease into (and through) the cell wall, and finally incubated with various concentrations of proteinase K prior to preparation of total cellular extracts and cell wall fragments under denaturing conditions. After separation into amphiphilic and hydrophilic fractions by TX-114 partitioning, the laminarinase extract of the cell wall fragments and the BATC-extracted cellular proteins were analyzed by SDS-PAGE and autoradiography (Fig. 10). Control experiments demonstrated that high concentrations of DTT are required for complete proteinase K digestion of the cell wall protein α-agglutinin (data not shown).

With increasing concentrations of proteinase K, the photolabeled high-molecular-weight cAMP-binding protein (molecular mass of 95 to 130 kDa) progressively disappeared from the laminarinase extract of the cell wall fragments (lanes 1 to 6). At 20 μg of proteinase K per ml, about 95% of the cell wall-associated cAMP-binding protein was degraded (lane 3). Uncleaved amphiphilic Gce1p (lanes 19 to 24) and processed hydrophilic Gce1p (lanes 25 to 30) were also digested in a concentration-dependent manner but obviously exhibited a significantly lower sensitivity to limited proteolysis in intact yeast cells compared with the high-molecular-weight form. At 20 μg of proteinase K per ml, only 20% of SDS-extractable amphiphilic (lane 21) and less than 5% of hydrophilic Gce1p (lane 27) were degraded. This difference in protease sensitivity between the high-molecular-weight cAMP-binding protein and 54-kDa Gce1p is presumably due to partial protection of the proteins of the periplasmic space and the outer face of the plasma membrane by the cell wall in intact cells to attack by exogenous proteinase K, whereas in the presence of DTT, proteins of the cell wall are fully accessible. The modest difference in protease sensitivity between the u and l forms of Gce1p (compare lanes 19 to 24 with 25 to 30) may be caused by a conformational change in the protein moiety upon anchor cleavage. PI-PLC-induced refolding of Gce1p has been deduced previously from a study of the alteration of the patterns of photoaffinity-labeled amino acids in which the labeling was performed before and after lipolytic cleavage of Gce1p (38). However, it cannot be excluded that the relative resistance of the l form of Gce1p to proteolytic degradation is due to its uptake into the interior of the cell.

Analysis of the photoaffinity-labeled hydrophilic proteins from the total SDS extract by IEF and autoradiography revealed that the major portion of hydrophilic Gce1p represented the l form (lanes 13 to 18), whereas only minute amounts of the p form could be detected. This finding is in accordance with the difficulties in visualizing the p form in intact yeast cells in the previous experiments. The integrity of the plasma membrane under each condition was demonstrated by determination of the amount of full-size cytosolic hexokinase in the hydrophilic SDS-extracted proteins. Immunoblot analysis with polyclonal anti-yeast hexokinase antibodies did not reveal any significant loss of hexokinase (52 kDa) even with the highest proteinase K concentration applied (lanes 7 to 12). This finding indicates that the plasma membrane remained impermeable for the protease under the conditions used. The

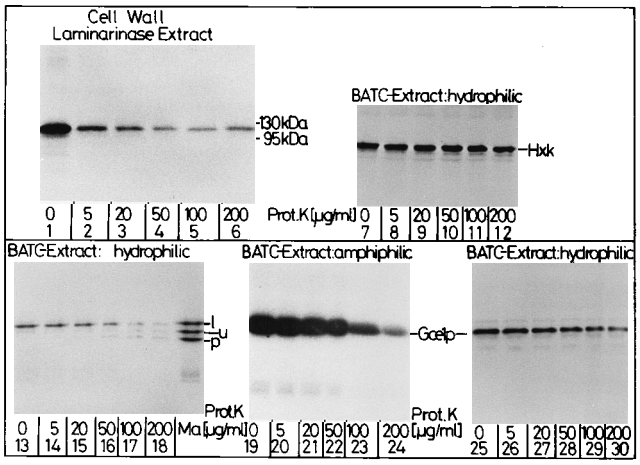

FIG. 10. Accessibility of cAMP-binding proteins to proteinase K in intact cells. Lactate-grown yeast cells were transferred to glucose medium, incubated (30 min, 30°C), and photoaffinity labeled with 8-N_3-[^{32}P]cAMP. Washed cells were treated with DTT and then incubated with the concentrations of proteinase (Prot.) K indicated (see Materials and Methods). After termination of the digestion, cells were lysed by using glass beads and extracted with SDS. The insoluble cell wall fragments were treated with laminarinase (lanes 1 to 6). The total SDS-extracted cellular proteins were subjected to TX-114 partitioning (lanes 7 to 30). The laminarinase-extracted and SDS-extracted amphiphilic and hydrophilic proteins were precipitated (58) and analyzed by SDS-PAGE (7.5% gel) and autoradiography (lanes 1 to 6, 19 to 24, and 25 to 30). In addition, samples of the hydrophilic proteins were analyzed by IEF and autoradiography (lanes 13 to 18) or by SDS-PAGE and immunoblotting with anti-yeast hexokinase (Hxk) antibodies, using a chemiluminescence detection system (ECL; Amersham-Buchler) (lanes 7 to 12). Molecular masses indicated were derived from marker proteins and photoaffinity-labeled Gce1p run in parallel. u, l, and p forms of photoaffinity-labeled Gce1p were run in parallel during IEF as markers (Ma).

pronounced protease sensitivity of the high-molecular-weight cAMP-binding protein, particularly in comparison with a cytosolic enzyme and with the u and l forms of Gce1p, is in agreement with a location external to the outer face of the plasma membrane, presumably at the cell wall. Vice versa, the relative protection against protease of the u and l forms compared with a cell wall protein points to their intracellular and/or plasma membrane location. Furthermore, the peptide patterns obtained by limited proteinase K digestion of the amphiphilic and hydrophilic forms of photoaffinity-labeled Gce1p under denaturing conditions are very similar to that of the high-molecular-weight cAMP-binding protein (data not shown), arguing that their protein moieties are identical and that the GPI-anchored 54-kDa plasma membrane Gce1p is the precursor of the 95- to 130-kDa cAMP-binding protein at the cell wall.

The following experiments were performed to control the specificity of the release of the cAMP-binding protein from the yeast cell wall by laminarinase. (i) Only minor amounts of the photoaffinity-labeled cAMP-binding protein migrating as a broad 95- to 130-kDa band in SDS-PAGE were liberated from cell wall fragments of mechanically lysed and BATC-solubilized cells by multiple extractions with either hot SDS (8 to 13% of the total), 100 mM DTT (3 to 7% of the total), or 10 mM EDTA (2 to 4% of the total) in the absence of laminarinase. Extraction of the major portion of the photolabeled cAMP-binding protein from the cell wall (up to 65 to 80% of the total) was strictly dependent on the amount of laminarinase added and on the digestion time (data not shown). (ii) The u, l, and p forms of plasma membrane Gce1p from yeast spheroplasts were incubated in vitro with laminarinase to test the possibility that despite the presence of a mix of protease inhibitors during the extractions, the high-molecular-weight cAMP-binding protein was released from the cell wall by cleavage within the carboxy-terminal portion. The criteria used were the conversion of photoaffinity-labeled detergent-solubilized Gce1p from the amphiphilic to the hydrophilic form as assayed by TX-114 partitioning, the release of [^{14}C]inositol from metabolically labeled affinity-purified and PI-PLC-cleaved Gce1p, and the release of [^{14}C]leucine from metabolically labeled affinity-purified p form of Gce1p from glucose-induced spheroplasts as assayed by both IEF and SDS-PAGE. The gels were scanned for radioactivity.

A 10-fold-higher concentration of laminarinase compared with that used for routine laminarinase extraction for similar amounts of radiolabeled substrate during incubation for 2 h at 37°C in the presence of protease inhibitors caused amphiphilic-hydrophilic conversion of up to 6 to 8% of the detergent-solubilized u form of Gce1p, released less than 2% of the total inositol radiolabel from the hydrophilic l form of Gce1p, and liberated 11 to 18% of the total leucine radiolabel from the hydrophilic p form of Gce1p. In no case was a reduction of the apparent molecular mass of Gce1p observed (data not shown). Thus, under our experimental conditions, hydrolytic activities possibly contaminating the laminarinase preparation do not seem to cleave within the glycan structure of the GPI anchor or near the carboxyl terminus of plasma membrane Gce1p regardless of whether the anchor is complete or partially degraded. These data argue that the release of the high-molecular-weight cAMP-binding protein was due to the β1,3-glucanase activity of laminarinase and, in analogy to the situation with α-agglutinin, suggest covalent coupling of the cAMP-binding protein to β1,6-glucan structures of the yeast cell wall. However, definite proof for this specific type of linkage will require the demonstration of β1,6-glucan moieties covalently bound to the cAMP-binding protein after digestion by laminarinase, e.g., by means of cross-reactivity with glucan-specific antibodies or by the use of yeast mutants defective in glucan synthesis as shown successfully for α-agglutinin (27, 28).

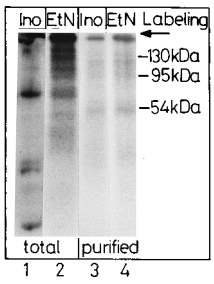

FIG. 11. Metabolic labeling of the cell wall cAMP-binding protein. Lactate-grown yeast cells were metabolically labeled with *myo*-[^{14}C]inositol (Ino) or [^{14}C]ethanolamine (EtN) for 60 or 30 min, respectively, at 30°C and then incubated with 4% glucose for 30 min. From equal numbers of washed cells cell wall fragments were prepared under native conditions (see Materials and Methods) and treated with laminarinase. From one half of the laminarinase extracts, the cAMP-binding protein was affinity purified (purified); the other half was left intact (total). The total extract and the material eluted from cAMP-Sepharose were analyzed by SDS-PAGE (7.5% gel) and fluorography, using Amplify and Kodak X-Omat AR film (exposure for 7 days at −80°C). Molecular masses indicated are derived from marker proteins and photoaffinity-labeled Gce1p run in parallel. A representative fluorogram of an experiment repeated two times with similar results is shown. ←, borderline between stacking and running gels.

The high-molecular-weight cAMP-binding protein may represent the cell wall-associated form of double-processed Gce1p. This would explain the apparent lack of the 54-kDa p form of Gce1p in intact cells despite the glucose-induced disappearance of the 54-kDa l form (Fig. 8 and 9). If this is true, then the cAMP-binding protein located in the cell wall should not contain constituents of the GPI anchor. To analyze this assumption, cells were metabolically labeled with [^{14}C]inositol or [^{14}C]ethanolamine. Laminarinase extracts were prepared and analyzed either in total or after affinity purification on cAMP-Sepharose by SDS-PAGE and fluorography (Fig. 11). Some inositol- and/or ethanolamine-labeled proteins were detected in total laminarinase extracts of glucose-induced cells (lanes 1 and 2) which, in part, may constitute GPI-anchored proteins associated with the β-glucan via the complete or a portion of the glycan structure of their lipolytically cleaved GPI anchors. However, significant incorporation of radiolabeled *myo*-inositol or ethanolamine into proteins of 95 to 130 kDa was observed neither with the total laminarinase extracts (lanes 1 and 2) nor after cAMP affinity purification of the proteins extracted by laminarinase (lanes 3 and 4). Obviously the cell wall-associated cAMP-binding protein does not contain components of a typical GPI anchor and is therefore not linked to the β-glucan via a residual anchor glycan. The 54-kDa l form containing the *myo*-inositol and ethanolamine moieties of the complete anchor glycan was not detected in the laminarinase extracts and is obviously not a component of the yeast cell wall. However, after long-term exposure of the fluorograms as shown in Fig. 11, some affinity-purified [^{14}C]inositol- and [^{14}C]ethanolamine-labeled high-molecular-weight material was detectable at the border between the stacking and running gels. It may be composed of Gce1p molecules which have not been digested by laminarinase to completion or which resist solubilization in the presence of 5% BATC (during extraction) and of even 2% SDS (during SDS-PAGE). Formation of clusters of mammalian GPI-anchored proteins within the plasma membrane which are stable in the presence of 1% TX-100 has been described previously (7).

DISCUSSION

The evolutionary conservation of GPI anchors indicates that they fulfill important biological functions which seem to be

based on two principal properties: (i) the ability of GPI-anchored proteins to associate in clusters together with glycosphingolipids and certain transmembrane and long-chain acylated proteins may be involved in the transmission of mitogenic signals across the membrane in vertebrate cells, most importantly in T-cell activation via GPI-anchored receptor proteins (24, 47, 53) as well as in the formation of caveolae thought to mediate specific endocytotic and transcytotic processes (for reviews, see reference 2 and 3); and (ii) the accessibility of GPI-anchored proteins for cleavage of their GPI anchors by specific phospholipases may provide the basis for controlled cell surface expression and/or degradation of their protein moieties. In some instances, GPI anchorage seems to control the topology of certain amphitropic proteins occurring either at the outer face of the plasma membrane or soluble in the extracellular space via release from the membrane after lipolytic processing of the GPI anchor (32, 44, 51). Eventually, the change in topology of a GPI-anchored protein with enzymatic function may be accompanied by alterations of its catalytic properties and by accessibility to novel substrates, as has been proven in a few cases (4, 5, 6, 34, 38, 57).

In addition, Larner and colleagues previously speculated that controlled GPI anchor cleavage plays a role in signal transduction (25, 48). In this context, it appears important that in yeast cells, the anchor of a GPI-anchored protein, Gce1p, is lipolytically cleaved in response to a nutritional signal, i.e., the transfer of the cells to glucose (37). Particularly compelling is the notion that anchor cleavage following nutritional upshift qualitatively resembles the lipolytic membrane release in response to insulin or to sulfonylurea drugs (in the presence of glucose) reported for some GPI-anchored proteins (e.g., 5′-nucleotidase, alkaline phosphatase, lipoprotein lipase, and heparan sulfate proteoglycan) in cultured BC_3H1 myocytes, 3T3-L1 adipocytes, rat hepatocytes, and the isolated rat diaphragm (8, 20, 21, 26, 37, 48). It has been proven recently that in yeast cells, Gce1p is processed by a GPI-PL of type C. In mammals, GPI-PLs of types C and D could be identified and partially purified or isolated (10, 18, 32).

The present study hints at a third property of GPI anchors which may have implications for the physiological function of GPI anchorage. Some of the GPI-anchored proteins seem to serve as substrates for enzymes other than phospholipases cleaving within the glycan structure of the anchor or at or near the carboxyl terminus of the protein moiety. In yeast cells, following the addition of glucose, two distinct cleavage events occur within the anchor and the immediate carboxy-terminal context of the Gce1 protein. Glucose-induced cleavage by a (G)PI-PLC constitutes the key event in this reaction cascade. The secondary cleavage ensues independently of the nutritional situation, presumably by proteolysis (for a possible candidate for the protease involved, see reference 29), resulting in the loss of the entire anchor glycan from the protein moiety. No evidence for unspecific anchor degradation could be obtained. Rather, the time courses of the two cleavages and the fact that the isolated lipolytically cleaved intermediate can be chased into the proteolytically processed version suggest that the two cleavage events are strictly consecutive. Such a mechanism would generate a fragment of the anchor comprising the complete polar glycan structure presumably linked to a small peptide (phosphoinositolglycan-peptide).

The secondary processing reaction may take place at the outer face of the plasma membrane, since Gce1p molecules located at the cell surface of yeast spheroplasts and labeled with 8-N_3 [^{32}P]cAMP function as a substrate for the endogenous (G)PI-PLC, even in the presence of brefeldin A, which in spheroplasts inhibits vesicular transport including endocytosis (3a). However, internalization of plasma membrane Gce1p into an intracellular processing compartment via a rapid brefeldin A-insensitive pathway (e.g., caveolae) cannot be excluded. It is unlikely that the secondary processing is caused by an enzymatic activity contaminating the Zymolyase used for preparation of the spheroplasts, since we did not observe any degradation of the glycan structure of the l form of [^{14}C]inositol-labeled Gce1p after detergent solubilization and incubation in vitro with high amounts of Zymolyase (data not shown). The observations that (i) PLD-cleaved Gce1p cannot be chased into the double-processed version and (ii) anti-CRD antibody complexes impair the secondary cleavage of Gce1p suggest that the corresponding processing enzyme mainly recognizes the PLC-cleaved GPI anchor, i.e., in particular the inositolphosphate epitope.

The physiological relevance of the double processing of GPI anchors as demonstrated here for Gce1p in the yeast *S. cerevisiae* remains to be elucidated. The following possibilities deserve further attention. (i) The phosphoinositolglycan-peptides generated in response to glucose may function as soluble messenger molecules mediating nutritional signaling during glucose repression in yeast cells. This situation would parallel the postulated signaling function of the insulin-induced GPI anchor processing in insulin-sensitive mammalian cells for certain metabolic and growth effects of insulin. At present, we are trying to identify phosphoinositolglycan-peptides produced in yeast cells after glucose induction and to study the effects of synthetic phosphoinositolglycan-peptides on glucose and cAMP metabolism in yeast cells. (ii) The glucose-induced removal of the complete GPI anchor glycan presumably including one or a few carboxy-terminal amino acids of Gce1p may alter its enzymatic properties. We have previously shown that the mere lipolytic cleavage dramatically alters the cAMP-binding characteristics of Gce1p independent of the concomitant membrane release (38). (iii) The double processing of GPI anchors may represent a biosynthetic step in the maturation of certain GPI-anchored proteins which ultimately will result in a change of their topology or location, e.g., in yeast cells in incorporation into the cell wall. Consequently, in yeast cells, double processing of GPI anchors will take place only with those GPI-anchored proteins with final destination at the cell wall. In agreement with this assumption are the findings that the resident plasma membrane protein Gas1p as well as the majority of [^{14}C]inositol-labeled amphiphilic GPI-anchored proteins in yeast cells do not undergo glucose-induced lipolytic cleavage by the endogenous (G)PI-PLC above a basal level.

In this respect, the observed extraction of a cAMP-binding protein from cell wall fragments by digestion with laminarinase (but not by SDS treatment) seems to be of relevance. Thus, *S. cerevisiae* harbors a cAMP-binding protein covalently attached to the cell wall which according to the following data may be derived from the lipolytically cleaved Gce1p of the plasma membrane via a double-processed intermediate lacking GPI anchor constituents. (i) The increase of the amount of cAMP-binding protein at the cell wall from 5 to 30 min after glucose induction is inversely correlated with the decrease of lipolytically processed Gce1p containing the terminal inositol(-phosphate) moiety. (ii) The p form of Gce1p and the high-molecular-weight form of the cAMP-binding protein lack *myo*-inositol and ethanolamine but exhibit similar peptide patterns. (iii) The similarity in kinetics of the glucose-induced accumulation of the 54-kDa p form of Gce1p in spheroplasts and of the high-molecular-weight form of a cAMP-binding protein in intact cells is compatible with a pathway whereby the double-processed p form represents the immediate (periplasmic) precursor for mature Gce1p at the cell wall. Functional cell wall

biosynthesis may prevent transient accumulation of the precursor, so that significant amounts of it can be detected only after partial degradation of the cell wall β-glucan.

According to the recently proposed model for the biosynthesis of α-agglutinin in the yeast *S. cerevisiae* (12, 27), we suggest that one physiological function of the glucose-regulated sequential double processing of Gce1p relies on the cell wall expression of a cAMP-binding protein in response to glucose: release of GPI-anchored Gce1p from the outer face of the plasma membrane via cleavage by the glucose-inducible (G)PI-PLC generates a lipolytic intermediate. This is apparently cross-linked in a subsequent reaction in the periplasmic space to certain building blocks of the cell wall, presumably to β1,6-glucan structures. These protein-containing structures are then incorporated into the cell wall, possibly via covalent coupling to β1,3-glucan fibers, leading to the final anchorage of Gce1p in the cell wall. During or immediately after secondary cleavage, which is obviously accompanied by the release of the complete anchor glycan containing one or a few amino acids (phosphoinositolglycan-peptide), the newly generated carboxy-terminal amino acid is linked to glucose units of the cell wall. Cleavage and linkage may be coupled intrinsically in whole cells, possibly during a transesterification reaction, whereas in spheroplasts, only the hydrolytic reaction can proceed. Remarkably, a direct covalent linkage between an amino acid and β-glucose has recently been described for mammalian laminin (49). The modified amino acid is asparagine and thus identical with the carboxy-terminal amino acid of Gce1p (43).

According to our present working hypothesis for Gce1p processing and biosynthesis, which has to be tested by further experimentation, the glucose-induced double processing of Gce1p in the yeast *S. cerevisiae* seems to represent a novel mode of the regulated cell wall expression of proteins, one which does not depend on the control of synthesis and vesicular transport of the protein (3a). As a final consequence, the glucose-induced double processing of Gce1p may coordinate transmembrane nutritional signaling during glucose repression with the glucose-induced cell wall expression of Gce1p via coupling the production of phosphoinositolglycan-derived messenger molecules to the generation of the precursor for cell wall-associated Gce1p.

REFERENCES

1. **Achstetter, T., O. Emter, C. Ehmann, and D. H. Wolf.** 1984. Proteolysis in eukaryotic cells. Identification of multiple proteolytic enzymes in yeast. J. Biol. Chem. **259:**13334–13343.
2. **Anderson, R. G. W.** 1993. Plasmalemmal caveolae and GPI-anchored membrane proteins. Curr. Opin. Cell Biol. **5:**647–652.
3. **Anderson, R. G. W., B. A. Kamen, K. G. Rothberg, and S. W. Lacey.** 1992. Potocytosis: sequestration and transport of small molecules by caveolae. Science **255:**410–411.

3a. **Bandlow, W., S Wied, and G. Müller.** Glucose induces amphiphilic to hydrophilic conversion of a subset of glycosyl-phosphatidylinositol-anchored ectoproteins in yeast. Arch. Biochem. Biophys., in press.

4. **Barton, P. L., A. H. Futerman, and I. Silman.** 1985. Arrhenius plots of acetylcholine esterase activity in mammalian erythrocytes and in Torpedo electric organ. Effect of solubilization by proteinases and by phosphatidylinositol-specific phospholipase C. Biochem. J. **231:**237–240.
5. **Braun-Breton, C., T. L. Rosenberry, and L. P. Da Silva.** 1988. Induction of the proteolytic activity of a membrane protein in *Plasmodium falciparum* by phosphatidylinositol-specific phospholipase C. Nature (London) **332:**457–459.
6. **Brewis, I. A., A. J. Turner, and N. M. Hooper.** 1994. Activation of the glycosyl-phosphatidylinositol-anchored membrane dipeptidase upon release from pig kidney membranes by phospholipase C. Biochem. J. **303:**633–638.
7. **Brown, D. A., and J. K. Rose.** 1992. Sorting of GPI-anchored proteins to glycolipid-enriched membrane subsomains during transport to the apical cell surface. Cell **68:**533–544.
8. **Chan, B. L., M. P. Lisanti, E. Rodriguez-Boulan, and A. R. Saltiel.** 1988. Insulin-stimulated release of lipoprotein lipase by metabolism of its phosphatidylinositol anchor. Science **241:**1670–1672.
9. **Conzelmann, A., H. Riezman, C. Desponds, and C. Bron.** 1988. A major 125-kd membrane glycoprotein of *Saccharomyces cerevisiae* is attached to the lipid bilayer through an inositol-containing phospholipid. EMBO J. **7:**2233–2240.
10. **Davitz, M. A., D. Hereld, S. Shak, J. Krakow, P. T. Englund, and V. Nussenzweig.** 1987. A glycan-phosphatidylinositol-specific phospholipase D in human serum. Science **238:**1–84.
11. **Deeg, M. A., D. R. Humphrey, S. H. Yang, T. R. Ferguson, V. N. Reinhold, and T. L. Rosenberry.** 1992. Glycan components in the glycoinositol phospholipid anchor of human erythrocyte acetylcholinesterase. J. Biol. Chem. **267:**18573–18580.
12. **De Nobel, J. G., and P. N. Lipke.** 1994. Is there a role for GPIs in assembly of yeast cell wall? Trends Cell Biol. **4:**42–45.
13. **Edidin, M., and I. Stroynowski.** 1991. Differences between lateral organization of conventional and inositol phospholipid-anchored membrane proteins. A further definition of micrometer scale membrane domains. J. Cell Biol. **112:**1143–1150.
14. **Englund, P. T.** 1993. The structure and biosynthesis of glycosyl-phosphatidylinositol protein anchors. Annu. Rev. Biochem. **62:**121–138.
15. **Fankhauser, C., S. W. Homans, J. E. Thomas-Oates, M. J. McConville, C. Desponds, A. Conzelmann, and M. A. J. Ferguson.** 1993. Structures of glycosylphosphatidylinositol membrane anchors from *Saccharomyces cerevisiae*. J. Biol. Chem. **268:**26365–26374.
16. **Ferguson, M. A. J., S. W. Homans, R. A. Dwek, and T. W. Rademacher.** 1988. Glycosyl-phosphatidylinositol moiety that anchors *Trypanosoma brucei* variant surface glycoprotein to the membrane. Science **239:**753–759.
17. **Field, M. C., and A. K. Menon.** 1993. Biosynthesis of glycosyl-phosphatidylinositol membrane protein anchors, p. 83–134. *In* M. J. Schlessinger (ed.), Lipid modification of proteins. CRC Press, Boca Raton, Fla.
18. **Fox, J. A., N. M. Soliz, and A. R. Saltiel.** 1987. Purification of a phosphatidylinositol-glycan specific phospholipase C from liver plasma membranes: a possible target of insulin action. Proc. Natl. Acad. Sci. USA **84:**2663–2667.

18a. **Frick, W., et al.** Unpublished data.

19. **Helenius, A., and K. Simons.** 1977. Charge shift electrophoresis: simple method for distinguishing between amphiphilic and hydrophilic proteins in detergent solution. Proc. Natl. Acad. Sci. USA **74:**529–532.
20. **Ishihara, M., N. S. Fedarko, and H. E. Conrad.** 1987. Involvement of phosphatidylinositol and insulin in the coordinate regulation of proteoheparan sulfate metabolism and hepatocyte growth. J. Biol. Chem. **262:**4708–4716.
21. **Klip, A., T. Ramlal, A. G. Douen, E. Burdett, D. Young, G. D. Cartree, and J. O. Holoszy.** 1988. Insulin-induced decrease in 5′-nucleotidase activity in skeletal muscle membranes. FEBS Lett. **238:**419–423.
22. **Klis, F. M.** 1994. Review: cell wall assembly in yeast. Yeast **10:**851–869.
23. **Kramer, W., G. Müller, F. Girbig, U. Gutjahr, S. Kowalewski, D. Hartz, and H.-D. Summ.** 1988. Differential interaction of glimepiride and glibenclamide with the β-cell sulfonylurea receptor. II. Photoaffinity labeling. Biochim. Biophys. Acta **1191:**278–290.
24. **Kroczek, R. A., K. C. Gunter, R. N. Germain, and E. M. Shevach.** 1986. Thy-1 functions as a signal transduction molecule in T lymphocytes and transfected B lymphocytes. Nature (London) **322:**181–184.
25. **Larner, J.** 1988. Insulin signaling mechanisms: lessons from the old testament of glycogen metabolism and the new testament of molecular biology. Diabetes **37:**262–275.
26. **Lisanti, M. P., J. C. Darnell, B. L. Chan, E. Rodriguez-Boulan, and A. R. Saltiel.** 1989. The distribution of glycosyl-phosphatidylinositol anchored proteins is differentially regulated by serum and insulin. Biochem. Biophys. Res. Commun. **164:**824–832.
27. **Lu, C.-F., J. Kurjan, and P. N. Lipke.** 1994. A pathway for cell wall anchorage of *Saccharomyces cerevisiae* α-agglutinin. Mol. Cell. Biol. **14:**4825–4833.
28. **Lu, C.-F., R. C. Montijn, J. L. Brown, F. Klis, J. Kurjan, H. Bussey, and P. N. Lipke.** 1995. Glycosyl phosphatidylinositol-dependent cross-linking of α-agglutinin and β1,6-glucan in the Saccharomyces cerevisiae cell wall. J. Cell Biol. **128:**333–340.
29. **Magdolen, U., G. Müller, V. Magdolen, and W. Bandlow.** 1993. A yeast gene (*BLH1*) encodes a polypeptide with homology to vertebrate bleomycin hydrolase, a family member of thiol proteinases. Biochim. Biophys. Acta **1171:** 299–303.
30. **Massoulie, J., J. P. Toutant, and S. Bon.** 1988. Characterization of amphiphilic forms of cholinesterases by their interaction with non-denaturing detergents in centrigugation and charge shift electrophoresis, p. 132–142. *In* U. Brodbeck and C. Bordier (ed.), Posttranslational modifications of proteins by lipids. Springer-Verlag, Heidelberg, Germany.
31. **McConville, M. J., and M. A. J. Ferguson.** 1993. The structure, biosynthesis and function of glycosylated phosphatidylinositols in parasitic protozoa and higher eukaryotes. Biochem. J. **294:**305–324.
32. **Metz, C. N., G. Brunner, N. H. Choi-Muira, H. Nguyen, J. Gabrilove, I. W. Caras, N. Altszuler, D. B. Rifkin, E. L. Wilson, and M. A. Davitz.** 1994. Release of GPI-anchored membrane proteins by a cell-associated GPI-specific phospholipase D. EMBO J. **13:**1741–1751.
33. **Montijin, R. C., J. Van Rinsum, F. A. Van Schagen, and F. M. Klis.** 1994. Glucomannoproteins in the cell wall of *Saccharomyces cerevisiae* contain a novel type of carbohydrate chain. J. Biol. Chem. **269:**19338–19342.
34. **Müller, G., and W. Bandlow.** 1989. An amphitropic cAMP-binding protein in

yeast mitochondria. I. Synergistic control of the intramitochondrial location by calcium and phospholipid. Biochemistry **28:**9957–9967.
35. **Müller, G., and W. Bandlow.** 1991. A cAMP-binding ectoprotein in the yeast *Saccharomyces cerevisiae*. Biochemistry **30:**10181–10190.
36. **Müller, G., and W. Bandlow.** 1991. Two lipid-anchored cAMP-binding proteins in the yeast *Saccharomyces cerevisiae* are unrelated to the R subunit of cytoplasmic protein kinase A. Eur. J. Biochem. **202:**299–308.
37. **Müller, G., and W. Bandlow.** 1993. Glucose induces lipolytic cleavage of a glycolipidic plasma membrane anchor in yeast. J. Cell Biol. **122:**325–336.
38. **Müller, G., and W. Bandlow.** 1994. Lipolytic membrane release of two phosphatidylinositol-anchored cAMP receptor proteins in yeast alters their ligand-binding parameters. Arch. Biochem. Biophys. **308:**504–514.
39. **Müller, G., E. A. Dearey, A. Korndörfer, and W. Bandlow.** 1994. Stimulation of a glycosyl-phosphatidylinositol-specific phospholipase by insulin and the sulfonylurea, glimepiride, in rat adipocytes depends on increased glucose transport. J. Cell Biol. **126:**1267–1276.
40. **Müller, G., E. A. Dearey, and J. Pünter.** 1993. The sulfonylurea drug, glimepiride, stimulates release of glycosylphosphatidylinositol-anchored plasma membrane proteins from 3T3 adipocytes. Biochem. J. **289:**509–521.
41. **Müller, G., A. Korndörfer, U. Kornak, and W. Malaisse.** 1994. Porin proteins in mitochondria from rat pancreatic islet cells and white adipocytes: identification and regulation of hexokinase-binding by the sulfonylurea, glimepiride. Arch. Biochem. Biophys. **308:**8–23.
42. **Müller, G., A. Korndörfer, K. Saar, B. Karbe-Thönges, H. Fasold, and S. Müllner.** 1994. 4′-Amino-bezamido-taurocholic acid selectively solubilizes glycosyl-phosphatidyl-inositol-anchored membrane proteins and improves lipolytic cleavage of their membrane anchors by specific phospholipases. Arch. Biochem. Biophys. **309:**329–340.
43. **Müller, G., K. Schubert, F. Fiedler, and W. Bandlow.** 1992. The cAMP-binding ectoprotein from *Saccharomyces cerevisiae* is membrane-anchored by glycosyl-phosphatidylinositol. J. Biol. Chem. **267:**25337–25346.
44. **Müller, G., E.-M. Wetekam, C. Jung, and W. Bandlow.** 1994. Membrane association of lipoprotein lipase and a cAMP-binding ectoprotein in rat adipocytes. Biochemistry **33:**12149–12159.
45. **Müller, G., and R. Zimmermann.** 1987. Import of honeybee prepromelittin into the endoplasmic reticulum: structural basis for independence of SRP and docking protein. EMBO J. **6:**2099–2107.
46. **Popov, N., M. Schmitt, S. Schulzeck, and H. Matthies.** 1975. Eine störungsfreie Mikromethode zur Bestimmung des Proteingehalts in Gewebehomogenaten. Acta Biol. Med. Ger. **34:**1441–1446.
47. **Robinson, P. J., M. Millrain, J. Antoniou, E. Simpson, and A. L. Mellor.** 1989. A glycophospholipid anchor is required for Qa-2-mediated T cell activation. Nature (London) **342:**85–87.
48. **Romero, G., L. Luttrell, A. Rogol, K. Zeller, E. Hewlett, and J. Larner.** 1988. Phosphatidylinositol-glycan anchors of membrane proteins: potential precursors of insulin mediators. Science **240:**509–512.
49. **Schreiner, R. E., E. Schnabel, and F. Wieland.** 1994. Novel N-glycosylation in eucaryotes: laminin contains the linkage unit β-glucosyl asparagine. J. Cell Biol. **124:**1071–1081.
50. **Schreuder, M. P., S. Brekelmans, H. Van Den Ende, and F. M. Klis.** 1993. Targeting of a heterologous protein to the cell wall of *Saccharomyces cerevisiae*. Yeast **9:**399–409.
51. **Spooner, P. M., S. S. Chernick, M. M. Garrison, and R. O. Scow.** 1979. Insulin regulation of lipoprotein lipase activity and release in 3T3-L1 adipocyces. J. Biol. Chem. **254:**10021–10029.
52. **Stevens, L. S.** 1995. Biosynthesis of glycosylphosphatidylinositol membrane anchors. Biochem. J. **310:**361–370.
53. **Su, B., G. L. Waneck, R. A. Flavell, and A. L. M. Bothwell.** 1991. The glycosylphosphatidylinositol anchor is critical for Ly-6A/E-mediated T cell activation. J. Cell. Biol. **112:**377–384.
54. **Vai, M., L. Popolo, R. Grandori, E. Lacana, and L. Alberghina.** 1990. The cell cycle modulated glycoprotein GP115 is one of the major proteins containing glycosylphosphatidylinositol. Biochim. Biophys. Acta **1038:**277–285.
55. **Van Rinsum, J., F. M. Klis, and H. Van Den Ende.** 1991. Cell wall glucomannoproteins of *Saccharomyces cerevisiae* mnn9. Yeast **7:**717–726.
56. **Vidugiriene, J., and A. K. Menon.** 1995. Biosynthesis of glycosylphosphatidylinositol anchors. Methods Enzymol. **250:**513–535.
57. **Vogel, M., H. Kowalewski, H. Zimmermann, N. M. Hooper, and A. J. Turner.** 1992. Soluble low-K_M 5′-nucleotidase from electric-ray (*Torpedo marmorata*) electric organ and bovine cerebral cortex is derived from the glycosylphosphatidylinositol-anchored ectoenzyme by phospholipase C cleavage. Biochem. J. **284:**621–624.
58. **Wessel, D., and U. I. Flügge.** 1984. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Anal. Biochem. **138:**141–143.
59. **Wojciechowicz, D., C.-F. Lu, J. Kurjan, and P. N. Lipke.** 1993. Cell surface anchorage and ligand-binding domains of the *Saccharomyces cerevisiae* cell adhesion protein, α-agglutinin, a member of the immunoglobulin superfamily. Mol. Cell. Biol. **13:**2554–2563.
60. **Zamze, S. E., M. A. J. Ferguson, R. Collins, R. A. Dwek, and T. W. Rademacher.** 1988. Characterization of the cross-reacting determinant (CRD) of the glycosyl-phosphatidylinositol membrane anchor of *Trypanosoma brucei* variant surface glycoprotein. Eur. J. Biochem. **176:**527–534.
61. **Zhang, F., B. Crise, B. Su, Y. Hou, J. K. Rose, A. Bothwell, and K. Jacobson.** 1991. Lateral diffusion of membrane-spanning and glycosylphosphatidylinositol-linked proteins: toward establishing rules governing the lateral mobility of membrane proteins. J. Cell Biol. **115:**75–84.
62. **Zurzolo, C., W. van't Hof, G. van Meer, and E. Rodriguez-Boulan.** 1994. VIP21/caveolin, glycosphingolipid clusters and the sorting of glycosyl-phosphatidylinositol-anchored proteins in epithelial cells. EMBO J. **13:**42–53.