Two lipid-anchored cAMP-binding proteins in the yeast *Saccharomyces cerevisiae* are unrelated to the R subunit of cytoplasmic protein kinase A

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We show that the yeast, *Saccharomyces cerevisiae*, contains two cAMP-binding proteins in addition to the well-characterized regulatory (R) subunit of cytoplasmic cAMP-dependent protein kinase (PKA). We provide evidence that they comprise a new type of cAMP receptor, membrane-anchored by covalently attached lipid structures. They are genetically not related to the cytoplasmic R subunit. The respective proteins can be detected in *sra1* mutants, in which the gene for the R subunit of PKA has been disrupted and a monoclonal antibody raised against the cytoplasmic R subunit does not cross-react with the two membrane-bound cAMP-binding proteins. In addition, they differ from the cytoplasmic species also with respect to their location and the peptide maps of the photoaffinity-labeled proteins. Although they differ from one another in molecular mass and subcellular location, peptide maps of the cAMP-binding domains resemble each other and both proteins are membrane-anchored by lipid structures, one to the outer surface of the plasma membrane, the other to the outer surface of the inner mitochondrial membrane. Both anchors can be metabolically labeled by Etn, *myo*-Ins and fatty acids. In addition, the anchor structure of the cAMP receptor from plasma membranes can be radiolabeled by GlcN and Man. After cleavage of the anchor with glycosylphosphatidylinositol-specific phospholipase C from trypanosomes, the solubilized cAMP-binding protein from plasma membranes reacts with antibodies which specifically recognize the cross-reacting determinant from soluble trypanosomal coat protein, suggesting similarity of the anchors. Degradation studies also point to the glycosylphosphatidylinositol nature of the anchor from the plasma membrane, whereas the mitochondrial counterpart is less complex in that it lacks carbohydrates. The plasma membrane cAMP receptor is, in addition, modified by an *N*-glycosidically linked carbohydrate side chain, responsible mainly for its higher molecular mass.

In eukaryotes, cAMP controls the activities of a number of key regulatory proteins by phosphorylation and dephosphorylation through cAMP-dependent protein kinases (for recent reviews see [1, 2]). The substrates of these enzymes are distributed all over the cell, e. g. the cytoplasm, endoplasmic reticulum, Golgi apparatus, secretory granules, cytoskeleton and nucleus [3, 4]. Accordingly, cAMP-dependent protein kinases (PKA) occur both in solution and associated with a number of cellular membranes, thus facilitating access to their targets. cAMP-dependent phosphorylation of these substrates regulates certain metabolic pathways [5] as well as progression through the cell cycle [6−8]. In their inactive state, PKA are heterotetrameric, consisting of two regulatory (R) and two catalytic (C) subunits. Binding of cAMP to the R subunits results in the dissociation of the complex, yielding

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Abbreviations N₃-cAMP, 8-azidoadenosine 3′,5′-(cyclic)phosphate; PKA, cAMP-dependent protein kinase holoenzyme; TPA, 12-myristoyl-phorbol 13-acetate; R, regulatory subunit; C, catalytic subunit; *N*-glycanase, peptide *N*-glycosidase; MeMan, methyl α-mannopyranoside; GPI, glycosylphosphatidylinositol; VSG, variant surface glycoprotein from *Trypanosoma brucei*; CRD, cross-reacting determinant; GPI-PLC (D) glycosylphosphatidylinositol-specific phospholipase from trypanosomes (human serum).

two active C subunits [9] (reviewed recently in [1]). In mammals, at least two types of R subunits are known, R1 and R2 [2]. Among these, a number of isoforms exist which differ with respect to tissue distribution, primary structure, their ability to be autophosphorylated and the strength of interaction with their C subunits. According to this, the specific pattern of cAMP-dependent protein phosphorylation in a particular cell type or tissue, depends on the subcellular distribution of the PKA. Little is known about the interactions that govern the association of the PKA holoenzyme with a number of cellular membranes. The derived primary sequences of both R and C subunits do not contain obvious transmembrane or anchor sequences. Thus, PKA may be bound by high-affinity protein/ protein interactions to integral-membrane anchor proteins or by long-chain fatty acylation at the N-terminus of the C subunit. Although myristoylation of some C subunits has been observed, the role of this co-translational modification in the amphitropic behaviour of some PKA is unknown [10]. In any case, it has been observed that cAMP causes the release of the soluble active C subunit [11].

The yeast *Saccharomyces cerevisiae* also contains a PKA of the R2C2 type, with one unique R subunit [12−14] and three distinct C subunit isozymes [15] which structurally and functionally resemble their mammalian analogues. A cytoplasmic location has been reported for at least the major

portion of PKA in yeast [12], although a loose association of this enzyme with the inner face of the plasma membrane has also been observed [16]. Only a single structural gene for the R subunit (*SRA1*, *BCY1*) has been detected in this organism and sequenced [14, 17, 18].

We have been interested in whether, in yeast cells, regulation of cellular functions by cAMP exists which is not mediated by the R subunit of cytoplasmic PKA. In fact, we identified membrane-associated cAMP-binding proteins in mitochondria [19] and in the plasma membrane (our unpublished results). The vast majority of the mitochondrial protein was located at the outer surface of the inner mitochondrial membrane and constituted the R subunit of a new type of PKA. It failed to dissociate an active C subunit, even in the presence of cAMP [20]. Activation could only be achieved after lipolytic cleavage of the PtdIns-containing membrane anchor, suggesting that membrane association of this R subunit provides an additional control of the kinase activity which is epistatic over the regulation by cAMP [21–23]. Thus, the yeast mitochondrial protein kinase is amphitropic and inactive in its membrane-bound state. In the soluble form, the mitochondrial kinase phosphorylates acidic exogenous substrates as well as an endogenous inner membrane protein [20].

The recently identified third cAMP-binding protein species is located at the periplasmic side of the plasma membrane [23a]. So far no function has been attributed to this ectoprotein. Having established the existence of two cAMP-binding proteins in locations and with properties which differed from those of the R subunit of the cytoplasmic PKA, we studied the intriguing question of their genetic relationship. We report here that the two new species are genetically and structurally unrelated to the R subunit of the known PKA.

MATERIALS AND METHODS

Materials

[1-^{14}C]Stearic acid (50–60 Ci/mol), [1-^{14}C]myristic acid (50–60 Ci/mol), *myo*-[2-^{3}H]inositol (15 Ci/mM), D-[U-^{14}C]mannose (200 Ci/mol), D-[1-^{3}H]GlcN/HCl (2–5 Ci/mmol), [N-^{3}H]Etn/HCl (5–30 Ci/mmol) and [N-^{3}H]acetyl-concanavalin A (45 Ci/mmol) were from Amersham-Buchler, Braunschweig; 8-azidoadenosine 3′,5′-(cyclic)[^{32}P]phosphate (N$_3$-[^{32}P]cAMP, 20 Ci/mol) was purchased from ICN, Eschwege; N^2-(2-aminoethyl)-adenosine 3′,5′-(cyclic)phosphate–Sepharose and Percoll were obtained from Pharmacia, Freiburg; glycosylphosphatidylinositol phospholipase C (GPI-PLC, from *T. brucei*), polyclonal anti-(rabbit VSG) (VSG = variable surface glycoprotein) 117 and 221, anti-CRD serum (CRD = cross-reacting determinant) and the soluble antigens 117 and 221, were gifts from P. Overath, Tübingen; a polyclonal anti-(VSG-CRD) serum was also kindly donated by A. Mehlert and M. Ferguson, Dundee; GPI-PLD from rabbit serum was a gift from W. Gutensohn, München; 12-myristoyl-phorbol 13-acetate (TPA) was bought from Serva, Heidelberg; silica gel G and soft plus Si-60 thin-layer plates were from Merck, Darmstadt; all other chemicals were obtained as described [21].

Yeast strains, growth conditions and preparation of mitochondria and plasma membranes

Cells of the haploid protease-deficient strain ABYS1 *MATα pra1-1 prb1-1 prc1-1 cps1-3 ade*, lacking the vacuolar proteinases A and B, carboxypeptidases Y and S [24] and of the *SRA1*-disruption mutant EG273-5A *MATα leu2 ura3 his4 ade2-1 bcy1 :: HIS3* [17] were grown at 30°C to log phase in lactate medium and harvested at a titer of 10^7 cells/ml. Mitochondria were prepared from spheroplasts by osmotic lysis and differential centrifugation and purified by Percoll-density-gradient centrifugation [21]. Plasma membranes were isolated from whole-cell homogenates by two successive density-gradient centrifugations on Percoll (28%) and sucrose (15–28%), taking the binding of radiolabeled concanavalin A as a criterion for recovery. Finally, the crude plasma membranes were spun through a cushion of 0.5 M sucrose. The pellet was resuspended in 0.25 M sucrose, 1 mM EDTA and 20 mM Mops/KOH, pH 7.4, at 2.5 mg/ml. Mitochondrial inner membranes were prepared according to [19].

Metabolic labeling and TLC-analysis

ABYS1 cells were grown overnight in semisynthetic medium containing 1.8 g/l yeast nitrogen base (Difco), 5.2 g/l (NH$_4$)$_2$SO$_4$, 2% lactate and 0.1% glucose. After growth of the cells to an absorbance at 600 nm of 10–15, in the presence of one of either 1.0 mCi [^{14}C]stearic acid and 0.2 mCi [^{3}H]-stearic acid, 0.5 mCi [^{14}C]*myo*-Ins and 0.25 mCi [^{3}H]*myo*-Ins, 200 μCi [^{14}C]GlcN and 25 μCi [^{3}H]GlcN, 275 μCi [^{14}C]mannose and 125 μCi [^{3}H]mannose or 350 μCi [^{14}C]Etn and [^{3}H]Etn for 2 h at 30°C, a 1000-fold excess of the respective unlabeled compound was added as described previously [22] and mitochondria and plasma membranes were prepared. The cAMP-binding proteins were partially purified by affinity chromatography on cAMP-Sepharose, subjected to chemical and enzymic degradations and the cleavage products analysed by TLC (after extraction of lipids).

Partial proteolysis

Photoaffinity-labeled membranes or partially purified proteins were precipitated with 13% poly(ethylene glycol) 6000, washed with 0.5 mg/ml 8% poly(ethylene glycol), dissolved in either 20 mM (NH$_4$)$_2$CO$_3$, pH 7.8, containing 0.5% Mega 10 (for V8 protease) or 50 mM (NH$_4$)$_2$CO$_3$, pH 8.2, plus 0.2% Chaps (for trypsin) and incubated with either 15 μg/ml V8 protease from *Staphylococcus aureus* (25°C, 60 min) or 30 μg/ml trypsin (treated with *N*-tosyl-L-phenylalanine chloromethane 0°C, 90 min), in a total volume of 250 μl. After the addition of a twofold molar excess of α$_2$-macroglobulin, a fourfold molar excess of soya-bean trypsin inhibitor and 200 μM phenylmethylsulfonyl fluoride (0°C, 5 min), the samples were precipitated with 5% trichloroacetic acid, washed twice with acetone and resuspended in sample buffer for SDS/PAGE.

Western-blot analysis

The unstained gel was soaked in 100 ml 25 mM Tris/HCl, pH 8.3, 200 mM glycine, 20% methanol plus 0.02% (mass/vol.) SDS, for 15 min at 4°C, placed into a vertical electroblot apparatus and the proteins transferred to nitrocellulose. After electrophoresis (40 V, 14 h, 4°C) the nitrocellulose sheet was incubated in 100 ml 20 mM Tris/HCl pH 7.4 and 150 mM NaCl (buffer A) plus 5% (mass/vol.) fat-free milk powder for 1 h at 22°C. The sheet was then incubated with the antibody (1000-fold dilution in the above buffer) overnight at room temperature. Unbound antibody was removed by washing three times with 100 ml buffer A for 30 min. ^{125}I-labeled rabbit anti-(mouse F$_{ab}$) serum [0.75 μCi for anti-(R/C) serum]

or mouse anti-(rabbit IgG) serum [0.15 μCi for anti-(CRD) serum] was added to 15 ml 5% milk powder in buffer A and incubated with the filter for 1 h at 22 °C. Excess reagent was washed away with buffer A. The filter was exposed to Kodak X-Omat AR-5 film at −80 °C for 7 d using eight-times intensifier screen.

Immunoprecipitation

0.25 mg photoaffinity-labeled membrane protein (7500 dpm) from plasma membranes or mitochondria was solubilised in 0.5% Mega 10 and centrifuged at 150000 g for 1 h. Subsequent precipitation of the supernatant was with 13% poly(ethylene glycol) 6000 and, after dissolving in 10 mM Tris/HCl, pH 8.0, 150 mM NaCl and 0.05% Triton X-100, the solubilised material was dissolved in 1:100-diluted rabbit anti-(VSG-CRD) serum, directed against VSG-117 of trypanosomes. Purification was by affinity chromatography on VSG-121 (anti-CRD serum). Incubations were performed in the presence or absence of purified soluble VSG for 2 h at 4 °C. After the reaction with 50 μl mouse anti-(rabbit IgG) − agarose for 30 min at 4 °C, the agarose beads were washed three times with immunoprecipitation buffer, twice with buffer lacking Triton X-100, dissociated in 60 mM Tris/HCl, pH 6.8, 2% SDS, 5% mercaptoethanol, 10% glycine and analysed by SDS/PAGE and autoradiography.

Miscellaneous procedures

Preparation of shocked mitochondria [21], photoaffinity labeling with N_3-[^{32}P]cAMP [25], affinity purification on cAMP-Sepharose [26], hydrophilic/hydrophobic partitioning using Triton X-114 [22], protein determination [27], SDS/PAGE [28], native gel electrophoresis [29] and fluorography using ENHANCE [30] were performed according to published procedures.

RESULTS

Physical analysis of two membrane-bound cAMP-binding proteins

Yeast is generally agreed to have a single cAMP-binding protein [12, 14], which is the R subunit of the cytoplasmic type-II protein kinase and regulates three different C subunits [15]. cAMP has been observed to bind to receptor proteins occurring in three different compartments of the yeast cell: the cytoplasm [12], mitochondria [19] and plasma membranes [23a, 31]. Their structural and functional relationships are unknown. Previous [21−23] and the present studies (Fig. 1) show that, in contrast to the cytoplasmic R subunit of PKA, the latter two cAMP-receptor proteins are membrane-bound and exhibit properties which point to the nonidentity of the three. Therefore, we undertook a comparative study to elucidate whether they are derived from a single gene or constitute separate, structurally and genetically unrelated cAMP-binding proteins. For the cytoplasmic R subunit, a molecular mass of 52 kDa was reported [12]. Unfortunately, no such protein can be labeled with N_3-[^{32}P]cAMP to a significant extent in dialysed cytoplasmic fractions. (However, the presence of this species can be proven immunologically as shown in Fig. 3, or after affinity purification as shown in Fig. 4.) Purified plasma membranes, photoaffinity-labeled with N_3-[^{32}P]cAMP, display one major ^{32}P-labeled protein species which has an electrophoretic mobility corresponding to a species of 54 kDa
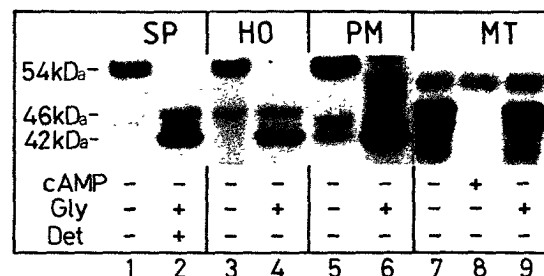

Fig. 1. *Photoaffinity labeling of cAMP-binding proteins from mitochondria and plasma membranes.* 100 μg intact or detergent-solubilized (0.5% Mega 10; Det) spheroplasts (SP), 100 μg osmotically lysed spheroplasts (HO), 25 μg plasma-membrane vesicles (PM) and 25 μg mitochondria (MT) were photoaffinity labeled with N_3-[^{32}P]cAMP in the presence or absence of unlabeled cAMP and N-glycanase (Gly). After adjusting to 0.5% Mega 10, the supernatant of a centrifugation 150000 × g, 30 min) was chromatographed on cAMP-Sepharose, the high-salt eluate precipitated with 8% poly(ethylene glycol) 6000 and the pellet analysed by SDS/PAGE. Molecular mass markers were run in parallel on the same gel

(Fig. 1, lane 5). Treatment with *N*-glycanase reduces the apparent molecular mass to about 42 − 44 kDa, indicative of the removal of (an) *N*-glycosidically linked carbohydrate side chain(s) (Fig. 1, lane 6). The molecular mass of the mitochondrial version was found to be 46 kDa [19]. SDS/PAGE of photoaffinity-labeled proteins from purified mitochondria is consistent with the published data (Fig. 1, lane 7). N-glycanase has no effect on the electrophoretic mobility of the mitochondrial species (Fig. 1, lane 9) in agreement with the presumed import pathway of this protein. The minor band seen in Fig. 1, lanes 7 − 9, is unlikely to represent a specific cAMP-binding species, because excess unlabeled cAMP does not compete (Fig. 1, lane 8). Fig. 1 also shows that mutual contamination of plasma membranes and mitochondria is low (compare Fig. 1, lanes 5 and 7). In cell homogenates, however, both species can be affinity-labeled simultaneously (Fig. 1, lane 3) whereas in spheroplasts only the cAMP receptor from plasma membranes can be labeled (lane 1). Since the mitochondrial 46-kDa species is readily affinity labeled in detergent-treated (Fig. 1, lane 2) or osmotically lysed spherosplasts (lane 3 and 4), this failure is likely to be due to an accessibility barrier for N_3-cAMP provided by the plasma membrane and suggests an outward orientation of the plasma-membrane protein. Thus, the data compiled in Fig. 1 demonstrates that the two membrane-bound proteins, which can be affinity-labeled with N_3-[^{32}P]cAMP are separate entities having different molecular masses and, residing in two different cellular compartments.

Immunological analysis of the cAMP-binding proteins

As the next step, we assayed the immunological relationship of the soluble R subunit of PKA to the two-membrane-bound cAMP receptor proteins. A monoclonal antibody against the cytoplasmic R subunit was used in order to test the possibility that yeast PKA is amphipathic and that the two membrane-embedded forms constitute structural and topological variants of the latter. In addition, an antibody against the cytoplasmic C1 subunit was used to test the possible identity with this protein of the putative C subunits associated with the two membrane-bound receptors. Fig. 2 shows that both antibodies recognize only the authentic cytoplasmic proteins against which they had been raised (lanes 9, 11 and 12). The reported molecular masses of both the
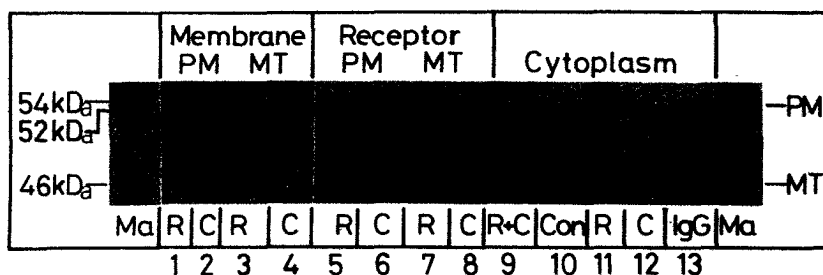
Fig. 2. *Immunological relationship between three cAMP-binding proteins from yeast.* 80 µg membranes from mitochondria (MT) or plasma membranes (PM), in 0.5% Mega 10 or 0.75% octyl glucoside, respectively, 25 µg affinity-purified cAMP-binding protein (Receptor) from mitochondria and plasma membranes and 150 µg cytoplasmic proteins (Cytoplasm) were separated by SDS/PAGE (14.5% gel), the proteins transferred to nitrocellulose and reacted with mouse antibodies directed against either the cytoplasmic isozyme C_1, anti-C mAbZ48, (C), or the R subunit of the cytoplasmic PKA, anti-R mAb427 (R). The blot was incubated in ^{125}I-labeled rabbit anti-(mouse Fab fragment) serum. In control incubations, 5 µg affinity-purified C or R subunits of PKA were present as competitors (Con). Another control was carried out using preimmune serum (IgG), photoaffinity-labeled mitochondrial (46 kDa; MT) and plasma membrane (54 kDa; PM). cAMP-binding proteins served as markers (Ma). Molecular masses of cytoplasmic R and C subunits were 52 kDa

cytoplasmic R and C1 subunits are 52 kDa [12, 15, 32]. No cross-reactivity was observed with the mitochondrial 46-kDa protein (Fig. 2, lanes 3 and 4) and the plasma membrane 54-kDa cAMP receptor proteins (lanes 1 and 2). Also, cellular subfractionation and purification by affinity chromatography (Fig. 2, lanes 5−8) did not change this results. This finding indicates that the known cytoplasmic R subunit is immunologically not related to either of the two membrane-bound cAMP-binding proteins and, in particular, makes it rather unlikely that the cytoplasmic R subunit is simultaneously the precursor of the other two. In addition, it is evident from these results that the C subunit, usually found tightly associated with the non-activated mitochondrial-membrane-embedded cAMP receptor [33], is not C1.

Genetic approach

To support this important finding, we also took a genetic approach. We used a strain in which the structural gene for the cytoplasmic R subunit of PKA, *SRA1*, had been partly deleted and replaced by the *HIS3* selective marker gene [17]. We prepared and labeled spheroplasts, plasma membranes and mitochondria with N_3-[32P]cAMP and, by SDS/PAGE, analysed the fractions for the presence of the membrane-bound cAMP-binding proteins (Fig. 3). We made three observations. (a) Specific signals were found in all three fractions obtained from the mutant (Fig. 3, lanes 3, 5 and 10). The 32P was competed for by excess unlabeled cAMP (Fig. 3, lanes 4, 6 and 11). The electrophoretic mobility of the protein from plasma membranes could be decreased by N-glycanase treatment as for the wild-type protein. These properties suggest that the affinity-labeled membrane proteins are related to those labeled by 32P in the wild type (Fig. 3, lanes 1 and 8) and indicate that the R subunit of PKA from plasma membranes and the cAMP-binding proteins from mitochondria are encoded by different genes. (b) It is evident from Fig. 3 that the apparent molecular mass of the deglycosylated cAMP-binding protein, which can still be 32P labeled in plasma membranes from *sra1* mutant cells (Fig. 3, lanes 3 and 5), is higher (50 kDa) than in the wild-type (47 kDa) (Fig. 3, lane 1, see also lane 7). Since this difference is maintained after the removal of N-glycosidic carbohydrate chains, this observation could mean that, in the *sra1* mutant, a (presumably C-terminal) processing event did not occur. A more trivial explanation that the different electrophoretic mobilities of the 32P-labeled plasma-membrane proteins from the two stains reflects strain-
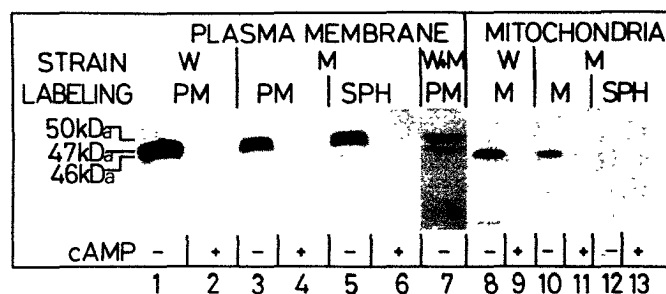

Fig. 3. *cAMP-binding proteins in plasma membranes and mitochondria of a yeast strain deficient in the R subunit of PKA.* Plasma membranes (PM), spheroplasts (SPH) and mitochondria (M) from the wild-type strain, ABYS1 (STRAIN W) and the SRA1/BCY1 disruption mutant, EG273-5A (STRAIN M), were photoaffinity-labeled with N_3-[32P]cAMP (LABELING) in the presence or absence of an excess of unlabeled cAMP and digested with N-glycanase. Subsequently, plasma membranes (lanes 5 and 6) or mitochondria (lanes 12 and 13) were prepared from the spheroplasts. The plasma membrane and mitochondrial fractions were analysed by SDS/PAGE and autoradiography. For comparison of molecular masses, aliquots of plasma membranes from wild-type and mutant cells (W + M, lane 7), containing equal amounts of radioactive material, were mixed together and analysed simultaneously. Molecular masses were derived from marker proteins run in parallel on the same gel. Identical amounts of plasma membranes (75 µg), mitochondria (125 µg) and spheroplasts (675 µg) from wild-type and mutant cells were used for the photoaffinity labeling

specific peculiarities in *O*-glycosylation cannot, however, be ruled out. (c) In comparison to the wild-type strain, little photoaffinity-labeled material is found in the plasma-membrane fraction and in spheroplast membranes from the mutant strain (Fig. 3, lanes 3 and 5). Moreover, this portion is significantly lower than that observed after photoaffinity-labeling of cell homogenates (data not shown). This finding argues that the receptor, or a precursor form thereof, is retained in an intracellular compartment, most likely the endoplasmic reticulum or the Golgi, due to the unregulated activity of the protein kinase in this mutant.

In contrast, the molecular mass of the mitochondrial cAMP-receptor protein is unchanged in the mutant compared to the wild type (Fig. 3, lane 8 versus lane 10), suggesting a different regulation of the biosynthetic processing pathway for the mitochondrial and the plasma-membrane protein. However, in contrast to the plasma-membrane isoprotein, th:
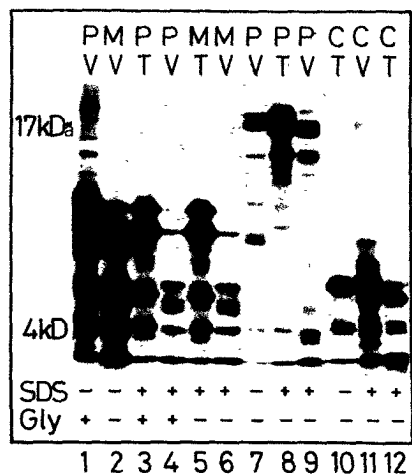
Fig. 4. *Comparative peptide mapping of three cAMP-binding proteins from yeast*. Solubilized photoaffinity-labeled cAMP-binding protein from cytoplasm (200 µg/lane; C), plasma membrane (125 µg/lane; P) and mitochondria (75 µg/lane; M), were digested with V8 protease (V) or trypsin (T) in the presence or absence of SDS and 6 M urea (SDS). Subsequently, aliquots were digested with N-glycanase (Gly) and all samples analysed by SDS/PAGE [58] (gel system of Schägger and von Jagow) and fluorography. The molecular masses indicated at the left margin were derived from marker proteins run in parallel on the same gel

total concentration of the mitochondrial cAMP receptor is diminished in a *sra1* mutant simultaneously in mitochondria and in cell homogenates (data not shown). Taken together, the results prove that the two membrane-bound cAMP-receptor proteins are encoded by gene(s) different from these coding for *SRA1*.

Peptide mapping

To prove the difference between the cytosolic R subunit and the two membrane-bound cAMP-binding proteins at the level of the protein structure, and to examine a possible structural relationship between these two proteins, a peptide-mapping study of all three photoaffinity-labeled proteins was undertaken (Fig. 4).

The native and the denatured forms were digested with two different proteases, either V8 protease or trypsin, respectively. All three species were partly purified by cAMP affinity chromatography. The peptide maps of the native (Fig. 4, lane 10) and denatured cytoplasmic species (lanes 11 and 12), can clearly be discriminated from the respective patterns of the isoproteins from mitochondrial membranes and plasma membranes. This result confirms that the cytoplasmic isoproteins and the two membrane-bound isoproteins differ.

However, the V8 digests of the native, affinity-purified and subsequently photoaffinity-labeled cAMP receptors from mitochondria and plasma membranes differ from one another (Fig. 4, lanes 2 and 7). This difference is not exclusively due to the fact that the plasma-membrane protein is *N*-glycosylated, whereas the mitochondrial species is not (evident from a comparison of Fig. 4, lanes 8 and 9 with lanes 5 and 6, respectively). The V8 peptide maps of the native cAMP-receptor proteins, affinity-purified from the two membrane fractions, also differed when plasma membranes were previously treated with N-glycanase (compare Fig. 4, lane 1 with lane 2). But, as soon as the N-glycanase-treated protein from plasma membranes and the mitochondrial species are compared unfolded, in the

presence of SDS, they yield degradation patterns with two different proteases which are indistinguishable by SDS/PAGE (compare Fig. 4, lanes 3 and 5 with lanes 4 and 6, respectively). This finding argues that the two membrane-bound isoproteins have identical or very closely related primary structures and that the differences observed between the native conformations of the two must be due to different folding structures. It has been observed previously that the presence or absence of a PtdIns-anchor significantly influences the tertiary structure of a cAMP-receptor protein [22]. Although the ^{32}P may be on only one cAMP-binding site of the receptor, [34] and thus the present results do not provide direct proof, the similarities in electrophoretic mobilities and in the peptide-degradation patterns of the deglycosylated, denatured proteins may be interpreted as a first indication of a close structural relationship between the two topologically different, membrane-bound isoproteins. Their genetic relationship, however, still has to be elucidated.

Characterization of the lipid membrane anchors by phospholipases

Previous work on the mitochondrial cAMP-receptor protein [20, 22] has demonstrated that it is anchored to the outer surface of the inner membrane by a PtdIns-containing lipid moiety. Similar lipid structures, i.e. GPI anchors, have so far only been observed with ectoproteins from a variety of organisms, including yeast [35—40]. We compared the susceptibilities of the two anchors to GPI-specific phospholipases *in vitro*, tested the metabolic labeling, identified the anchor constituents by TLC and analysed the immunological properties of the two structures.

An increasing number of GPI-anchored ectoproteins have been found at the extracellular face of a variety of cell types from many different organisms. They can be released from their resident cells by GPI-specific phospholipases and thereby rendered hydrophilic. This release by PtdIns or GPI-specific phospholipases serves as a diagnostic tool in proving anchorage by a GPI structure [35, 41, 42]. To analyse the nature of the anchoring structures of the two membrane-bound cAMP receptor proteins, we treated them with GPI-PLC from trypanosomes and phospholipase D from rabbit serum (Fig. 5). Both the release from the membrane fraction into the soluble supernatant after centrifugation (Fig. 5A) and the concomitant increase in hydrophilicity upon removal of the lipid part of the anchor (Fig. 5B), were measured. In addition, it was observed previously [23] that, with the mitochondrial cAMP-binding protein, a natural lipolytic liberation mechanism could be activated by lipids (particularly efficiently by TPA), in the presence of Ca^{2+} and ATP. Therefore, it was determined whether such a mode of release from the membrane also existed for the counterpart from plasma membranes. Hydrophilicity was assayed by Triton X-114/aqueous-phase partitioning. Salt (or MeMan, not shown) was used to assist membrane release of the plasma membrane protein.

Fig. 5 shows that, in contrast to the mitochondrial cAMP receptor [23], the isoprotein from plasma membranes fails to be liberated by a TPA-activated endogenous phospholipase. This observation reveals an additional difference between the two membrane-bound cAMP receptor proteins. Both anchors can be cleaved by GPI-PLD from rabbit serum, proving their phospholipid nature. Surprisingly, the releasing efficiency is poor with phospholipase A_2 and hydroxylamine which both cleave oxy esters. It is also unexpected that PtdIns-PLC from *B. cereus* and *Bacillus thuringiensis* fail completely to cleave
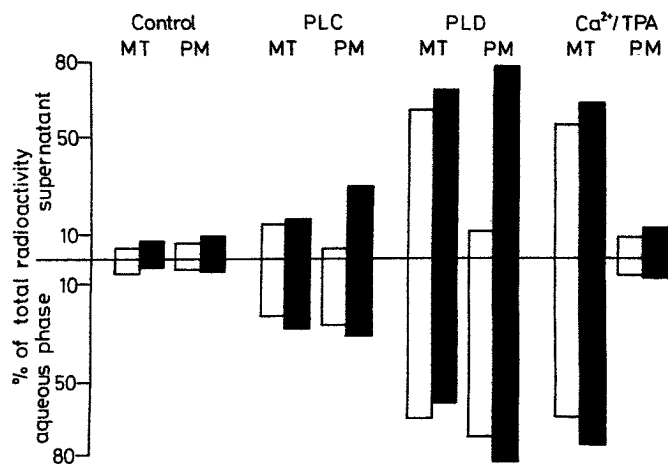
Fig. 5. *Membrane release of cAMP-binding proteins*. Photoaffinity-labeled shocked mitochondria (MT) were prepared by sixfold dilution of the ^{32}P-labeled mitochondria with 10 mM Hepes/KOH, pH 7.2, 0.5 mM EDTA, 25 mM KCl and gentle stirring on ice for 30 min, and plasma membranes (PM, 250 µg protein) were incubated with buffer (Control), 50 µM Ca^{2+} plus 0.5 µg/ml TPA (Ca^{2+}/TPA), phospholipase C from *T. brucei* (PLC) or phospholipase D from human serum (PLD) as described previously [23, 23a]. Half of the incubation mixture then received 1 M NaCl (filled bars) whereas to the other aliquot the same volume of water was added (open bars). Subsequently, the membranes were either centrifuged through 0.5 ml cushions of 0.5 M sucrose (150000 × *g*, 30 min) or subjected to Triton X-114 partitioning. The radioactivity of the supernatant and pellet fractions of the centrifugation and the aqueous and detergent phases of the partitioning was determined. The sum of both supernatant and pellet fractions, or of aqueous and detergent phases, were set at 100% of total radioactivity

Fig. 6. *Western-blot analysis of the cAMP-binding proteins with anti-VSG serum*. Plasma membranes (PM; 20 or 50 µg), intact or detergent-solubilized (Det) mitochondria (MT; 90 µg) and inner mitochondrial membranes (IM; 70 µg) were incubated with 10 U GPI-PLC from *T. brucei* (PLC). Where indicated, mitochondria were treated with 50 µM Ca^{2+} and 10 µg/ml TPA (Ca^{2+}/TPA) as described, then adjusted to 0.5% Mega 10. Samples were partially purified by cAMP-Sepharose affinity chromatography, precipitated with poly(ethylene glycol) 6000 (8%) and analysed by SDS/PAGE (14.5% gel). In parallel, on the same gel, purified soluble VSG-117 protein (VSG) was electrophoresed (lanes 13 and 14). After transfer to nitrocellulose and saturation of free binding sites with 50 mg/ml bovine serum albumin, the lanes were incubated separately with two different polyclonal rabbit anti-VSG sera (serum A, anti-VSG-117 serum; serum B, anti-VSG-221 serum; both from Overath, each diluted 1 : 1000). Molecular-mass standards were run in parallel on the same gel

the mitochondrial anchor and leave a significant portion of the anchor of the plasma-membrane cAMP receptor undigested (not shown). Nevertheless, the anchor from both the mitochondrial and plasma-membrane cAMP-binding protein can be cleaved by GPI-PLC from *T. brucei*, albeit with modest efficiency. Although it has been observed [43, 44] that in some instances GIP-anchored VSG are not efficiently cleaved by trypanosomal phospholipase C, the exact reason for this behaviour in the case of the yeast protein is not yet known. Taken together, cleavability of the two membrane anchors by GPI-PLC and GPI-PLD provide strong evidence for their anchorage by a lipid structure.

Fig. 5 also reveals differences between the two cAMP receptors with respect to interaction with the membrane (or an integral-membrane anchor protein) of the (glyco)protein moieties. After lipolytic cleavage of the membrane anchors by either phospholipase C or D, efficient release from the membranes of the plasma-membrane protein requires the presence of either high salt (Fig. 5) or MeMan or deglycosylation (not shown), whereas solubilization of the mitochondrial counterpart does not require further treatment. This indicates that the cAMP receptor from the plasma membrane interacts with the membrane additionally through the carbohydrate side chain.

Immunological study of anchor epitopes

The GPI anchor of the VSG from trypanosomes was observed to harbour a number of immunogenic determinants which were primarily responsible for the cross-reactivity be-
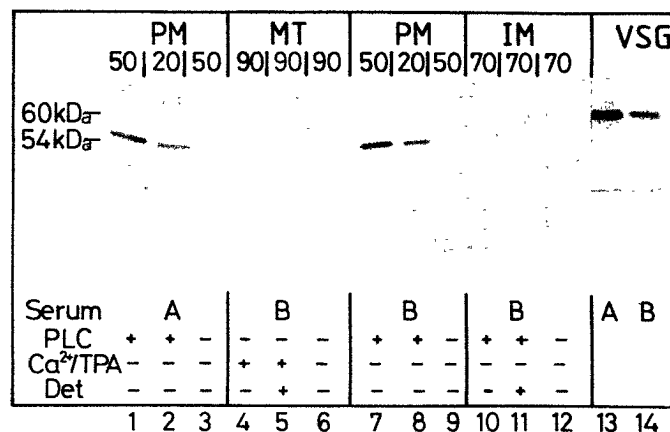
tween different VSG [42, 45, 46]. One epitope is constituted by unmodified GlcN, another one by the Ins-1,2-(cyclic)-phosphoester generated upon cleavage by GPI-PLC [46]. In order to verify the (glyco)lipid nature of the two yeast membrane anchors, we used polyclonal antibodies raised against two different soluble VSG and assayed for cross-reactivity with the yeast membrane anchor structures.

Fig. 6 shows a Western-blot experiment where the yeast proteins have been reacted with two polyclonal anti-CRD sera (A and B) raised against a GPI-PLC-treated soluble VSG and affinity-purified by using a different VSG. The affinity-purified cAMP-binding proteins from yeast were treated with GPI-PLC or, in the case of the mitochondrial PtdIns lipid-anchored protein, with TPA in the presence of Ca^{2+} and ATP, then electrophoresed and blotted. The plasma-membrane protein yields a single although weak signal after immunoprecipitation at a position which agrees with its apparent molecular mass of 54 kDa (Fig. 6, lanes 1, 2, 7 and 8). An immunoprecipitation signal is lacking where the treatment with PLC has been omitted (compare, e. g. lanes 1 and 3 in Fig. 6). This indicates that cleavage by GPI-PLC is required to generate at least one of the epitopes. The membrane anchor from the mitochondrial isoprotein does not immunoprecipitate (Fig. 6, lanes 4—6 and 10—12) indicative of the absence of at least one of the epitopes, most likely the non-acetylated GlcN [21] generally found in GPI (see also Figs 8 and 9B). In the control, authentic soluble (i.e. PLC-cleaved) VSG yields a strong immunoprecipitation signal at the expected position in the blot with the respective homologous antiserum (Fig. 6, lane 13) but a weak cross-reaction with the heterologous serum (lane 14). This reflects the fact that the sera recognize both protein and glycolipid determinants [45] and proves that the intensity with soluble heterologous VSG and with the affinity-
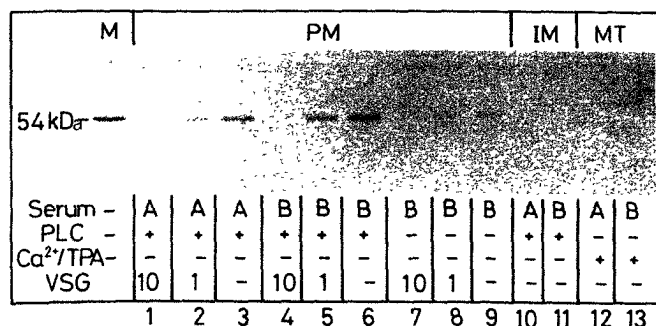

Fig. 7. *Immunoprecipitation of the cAMP-binding proteins with anti-VSG serum.* 500 µg plasma membranes (PM), mitochondria (MT) and inner mitochondrial membranes (IM) were photoaffinity-labeled, treated with PLC from *T. brucei* (PLC) or Ca^{2+}/TPA as above, solubilized by detergent, precipitated with poly(ethylene glycol) 6000, dissolved in 10 mM Tris/HCl, pH 8.0, 150 mM NaCl and 0.5% Triton X-100 and incubated with a 1:100 dilution of either the polyclonal rabbit anti-VSG-117 serum (serum A) or anti-VSG-221 serum (serum B) in the presence (1 or 10 µl) or absence of the homologous purified soluble VSG 117 antigen (in the case of serum A) or VSG-221 antigen (in the case of serum B) for 2 h at 4°C. After the reaction with 50 µl anti-(rabbit IgG)—agarose from mouse for 30 min at 4°C, the agarose beads were washed three times with buffer (as above), twice with buffer lacking Triton X-100, dissociated in sample buffer and analysed by SDS/PAGE and autoradiography. Authentic photoaffinity-labeled cAMP-binding protein from plasma membranes was run as a marker (M) on the same gel

purified yeast protein is due to the cross-reactivity with the anchor structure.

Fig. 7 confirms these results. It shows an autoradiogram of material from yeast, immunoprecipitated by polyclonal anti-VSG and anti-CRD sera (A and B). The signal is weak although significant in those cases where treatment with GPI-PLC has been omitted (Fig. 7, lanes 7—9). This faint signal may be ascribed to the reaction with the epitope constituted by non-acetylated GlcN (see below). Treatment of plasma membranes with trypanosomal PLC greatly enhances the immunoreactivity of the 54-kDa cAMP-binding protein, probably due to the generation of an additional cross-reacting determinant in the form of the *myo*-inositol-1,2-cyclic phosphoester (lanes 1—3 and 4—6 in Fig. 7). Since homologous soluble VSG competes (lanes 1, 2, 4 and 5 in Fig. 7), the immune reaction is considered specific. Again, no precipitate is formed with the protein from mitochondria (Fig. 7, lanes 12, 13) and mitochondrial inner-membrane vesicles (lanes 10, 11).

Comparative analysis of anchor constituents

The data shown in Figs 6 and 7 suggest a close structural similarity between the GPI anchor of VSG (the classical example of a GPI) and the structure linking the cAMP-binding protein to the plasma membrane of yeast. The anchor structure of the mitochondrial isoprotein is not recognized by these antibodies. To elucidate the structural basis for the different immune reactivity of the two anchors from yeast, we studied the constituent building bricks. We metabolically labeled yeast cells with compounds found in protozoan and vertebrate GPI and observed (Fig. 8) that [^{14}C]myristate pulse-labels the cAMP-receptor protein from plasma membranes (Fig. 8, lanes 4 and 5), whereas, in confirmation of previous observations [22], the mitochondrial isoprotein is preferentially labeled by [^{14}C]stearate (lane 1). Both ^{14}C atoms can be removed from

the core proteins by treatment with either GPI-PLD and, less efficiently, with trypanosomal PLC (not shown), indicating that the ^{14}C label resides in the lipid anchor and not in amino acids of the protein moiety. Results (G. Müller, unpublished results) show that after complete hydrolysis of the metabolically labeled anchor structure and subsequent TLC, most of the ^{14}C can be reisolated with the respective component used for labeling. Both anchor structures contain *myo*-Ins and Etn (Fig. 8, lanes 6, 7 and 8—10, respectively). Only the cAMP-binding protein from plasma membranes is labeled by [^{3}H]GlcN and [^{14}C]Man (Fig. 8, lanes 12 and 16, respectively). These labels are not removed by treatment with N-glycanase (Fig. 8, lanes 14 and 17), indicating that they are not exclusively contained in *N*-glycosidic side chains. Recent observations (our unpublished results) show that the residual carbohydrate label, in fact, is associated with the anchor and not with *O*-glycosidic side chains. Thus, the anchor structure of the cAMP receptor from plasma membranes can be labeled by components which are typical of GPI anchors, whereas the lipid anchor of the mitochondrial cAMP-binding protein lacks carbohydrates (Fig. 8, lanes 11 and 15). Since, during translocation through the secretory pathway, GPI are transferred to the protein (in a pseudo-transpeptidation reaction by means of the amino group of Etn) on the endoplasmic reticulum [37, 47, 48], the results from the metabolic-labeling experiments are in agreement with the presumed biosynthetic and transport pathways of the two proteins.

In order to identify the constituents of the two anchors directly and to study their order of linkage, we degraded metabolically labeled and partially affinity-purified cAMP-binding proteins by a number of anchor-specific enzymic and chemical-cleavage reactions and analysed the products by TLC. Cells were labeled with [^{3}H]Etn, the purified cAMP receptors digested to completion with pronase, then chromatographed by TLC in two adjacent lanes (Fig. 9A). The two lipid structures retain the ^{3}H and chromatograph to non-identical positions on the plate. This reflects the fact that they differ structurally from one another. None of the spots migrates as far as any of the marker substances used, suggesting that they are more complex and less hydrophobic than the marker substances. Particularly the anchor fragment from the plasma-membrane protein appears to be more hydrophilic than that from mitochondria, probably due to a higher carbohydrate content which is not altered by treatment with N-glycanase. This behaviour proves that the large carbohydrate side chain does not branch from the lipid anchor but is likely to be attached *N*-glycosidically to internal sequences of the protein moiety.

Nitrous acid cleaves nonacetylated GlcN residues [46]. Fig. 9B shows that, after *in vivo* labeling with [^{3}H]*myo*-Ins, only the anchor from the plasma-membrane protein is affected by nitrous acid, in agreement with the finding (Fig. 8, lanes 11—14) that only the anchor of this protein contains GlcN. Cleavage by nitrous acid simultaneously increases the hydrophobic character of the ^{3}H-labeled residual anchor fragment, consistent with the assumption that (some) carbohydrate residues have been removed by this treatment. The ^{3}H-labeled cleavage product co-migrates with PtdIns, in agreement with results obtained with GPI anchors from *T. brucei* and some vertebrate ectoproteins [37, 38, 46]. This also argues that the inositol ring is not substituted by an additional fatty acid. The anchor of the mitochondrial isoprotein is not visibly affected by this treatment and most likely remains attached to the core protein, since it does not migrate from the origin of the TLC plate (in contrast to the pronase-treated protein in Fig. 9A).
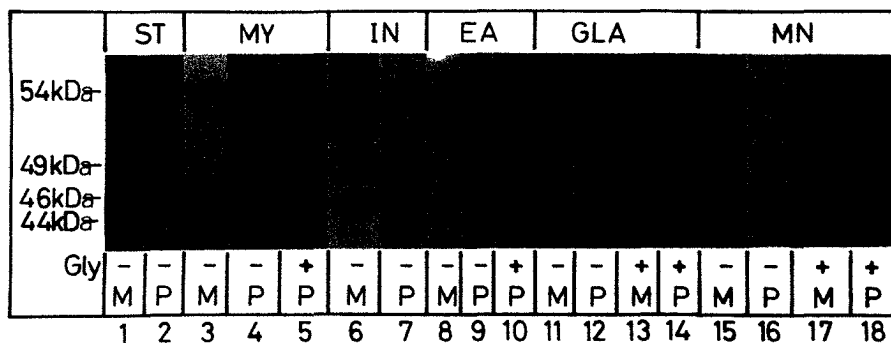
Fig. 8. *Metabolic labeling of the mitochondrial and plasma membrane cAMP-binding proteins.* Yeast cells were metabolically labeled with [14C]stearic acid (ST), [14C]myristic acid (MY), [14C]myo-inositol (IN), [14C]ethanolamine (EA), [14C]glucosamine (GLA) or [14C]mannose (MAN) and subsequently fractionated into mitochondria (M) and plasma membranes (P). The cAMP-binding proteins were enriched from detergent extracts of the mitochondria (0.5% Mega 10) and plasma membranes (0.75% octyl glucoside) by affinity purification using cAMP-Sepharose. Where indicated, half of the plasma membrane or mitochondrial fractions, eluted from the affinity column by 0.1 M cAMP. was digested with N-glycanase (Gly). All samples were subsequently dissolved in sample buffer, boiled for 5 min at 95°C and centrifuged. The supernatants were analysed by SDS/PAGE and fluorography

Degradation by GPI-PLD removes the inositol ring, releasing the fatty acid associated with the respective phosphatidic acids (Fig. 9C). (For these and the following experiments, Fig. 9D and E, cells have been labeled separately with either of the two 3H-labeled fatty acids.) The identification of phosphatidic acids as the residual fragments in both instances leads to the conclusion that, in the case of the mitochondrial protein, GPI-PLD must have removed a large constituent, most likely the core protein.

Acetolysis exchanges the phosphoric acid of the lipid for an acetyl residue, yielding 1,2-dimyristoyl-3-acetylglycerol and 1,2-distearyl-3-acetylglycerol (Fig. 9D), in agreement with the metabolic-labeling data (Fig. 8, lanes 1 and 4) and as a confirmation of the presumed structure.

Base hydrolysis cleaves fatty-ester bonds. The 3H left over from the mitochondrial protein anchor structure co-migrates with stearic acid and that from plasma membranes co-migrates with myristic acid, again in agreement with the in vivo 3H-labeling data (Fig. 8, lanes 1 and 4). This demonstrates the ester nature of the linkage to glycerol of at least one fatty acid. However, alkaline hydrolysis fails to remove the fatty-acid label completely from both anchors (data not shown). This behaviour may be in line with the observation that PLA₂ was also ineffective in the complete removal of the fatty-acid label, and leaves the possibility open that, in addition to one ester bond, an ether bond is involved (most likely at the sn-2-position), guaranteeing membrane attachment of the protein after PLA₂ treatment.

Taken together, the results obtained from this series of experiments demonstrate that the lipid-anchor structure of the cAMP-binding protein from plasma membranes contains constituents which are likely to be linked in a similar fashion as found in trypanosomal VSG and a number of GPI-anchored ectoproteins from vertebrates. The mitochondrial membrane anchor appears to constitute a new, less complex type of lipid anchor. Although it contains PtdIns and Etn, it lacks carbohydrate residues.

DISCUSSION

A number of indications for the existence of additional cAMP-binding proteins in the cytoplasm [50, 51], plasma membranes [31] and mitochondria of yeast [19, 33] have sporadically appeared in the literature. These cAMP-binding species were, however, mainly regarded as artifacts caused by contaminating soluble R subunits of cytoplasmic PKA or by degradation products thereof [12]. This interpretation was encouraged by a number of extraordinary properties of the well-known cytoplasmic cAMP-binding proteins. In higher organisms, this protein was found to be amphipathic. The R2 isoform, in particular, occurs as a soluble and a membrane-associated form, depending on the cell type and the organism [52 – 57]. A similar amphipathic nature was recently also reported for PKA from yeast, which was observed to occur associated with the inner surface of the plasma membrane [16]. Furthermore, smaller cAMP-binding entities, which subsequently turned out to be degradation products of the R subunit of PKA, were still capable of binding cAMP [12, 50, 51]. The omnipresence of proteolytic artifacts made it extremely difficult to prove the authenticity of cAMP-binding proteins with molecular masses below 52 kDa. The original publication on cAMP-binding proteins present in yeast plasma membranes [31] had described four photoaffinity-labeled species with, in part, very low molecular masses. Lastly, later intense research in this laboratory and others for the structural gene of an R-subunit isoprotein in yeast had remained unsuccessful. Since heterologous hybridizations with DNA fragments and degenerate oligonucleotides synthesized according to the conserved consensus of cAMP-binding domains failed, it had even prematurely been concluded that yeast contains only a single cAMP-binding protein, namely the R subunit of PKA [14, 15]. This conclusion was particularly facilitated by the fact that genetic approaches had also failed to disclose the gene(s) of possible isoproteins. Strains mutated in the gene for the R subunit of PKA (e. g. sra1) show an altered phenotype in which storage-carbohydrate metabolism, entry into mitosis and, in diploids, meiosis are severely affected [13]. This phenotype could not be healed by overexpression of any other yeast gene. Using both a genetic and a biochemical approach, we show in the present paper that yeast harbours at least two genetically distinct proteins capable of binding cAMP. The strongest argument in support of this conclusion is dereived from a sra1 mutant in which the structural gene encoding the R subunit of PKA has been destroyed and which nevertheless displays two membrane-bound protein species which can be labeled with a photoreactive cAMP derivative. One of these is even larger than the R subunit of PKA and is located at the outer surface
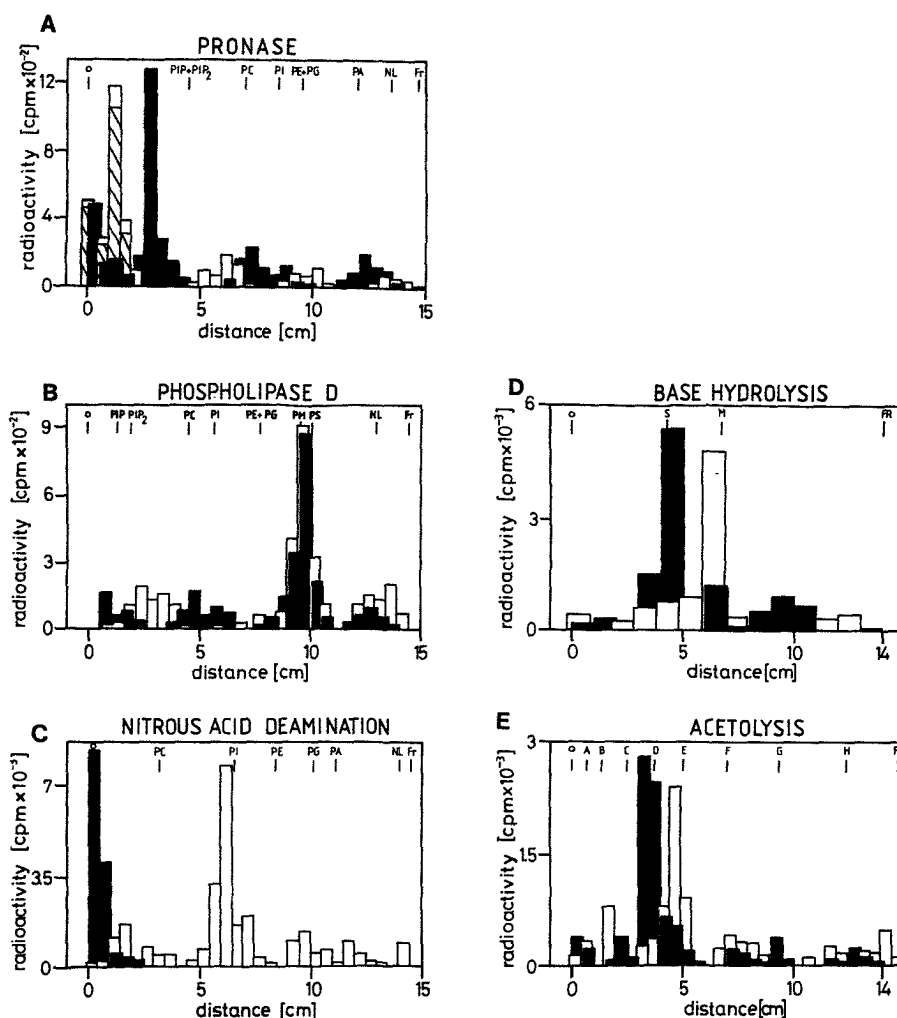
Fig. 9. *Chemical and enzymic analysis of the membrane anchors of the cAMP-binding proteins*. Yeast cells were metabolically labeled with [³H]ethanolamine (A), [³H]myo-inositol (B), [³H]myristic acid for isolation of plasma membranes (C, D and E; open bars) or [³H]stearic acid for the isolation of mitochondria (C, D and E; filled bars) and plasma membranes and mitochondria were prepared. Solubilized and affinity-purified cAMP-binding proteins were then subjected to digestion by pronase E from *S. aureus* (A); cross-hatched bars, additional digestion with N-glycanase, nitrous acid deamination (B), phospholipase D (from human serum) treatment (C), acetolysis (D) and base hydrolysis (E), respectively. After lipid extraction the organic phases were analysed by TLC. Radioactivity was determined using a linear TLC analyser. Each bar represents the mean of four independent experiments. Authentic fatty acids, lipids and lipid derivatives were separated in parallel lanes on the same plates and their positions indicated on top of each diagram. o, Origin; Fr, front; S, stearic acid; M, myristic acid; A, monomyristoylglycerol; B, 1,2-dimyristoylglycerol; C, 1,3-dimyristoylglycerol; D, 1,2-distearoyl-3-acetylglycerol; E, 1,2-dimyristoyl-3-acetylglycerol; F, 1,3-dimyristoyl-2-acetylglycerol; G, trimyristoylglycerol; H, methyl myristate; PIP, phosphatidylinositol phosphate; PIP_2, phosphatidylinositol bisphosphate; PC, phosphatidylcholine; PI, phosphatidylinositol; PE, dimyristoylglycerophosphoethanolamine; PG, dimyristoylglycerophosphoglycerol; PM, dimyristoylglycerol phosphate; PS, distearoylglycerol phosphate; NL, neutral lipids; PA, 1-myristoyl-2-acetylglycerol phosphate

[23 a] of the plasma membrane, whereas the other occurs in mitochondria. The location in compartments different from PKA may also explain the failure to isolate *sra1*-complementing genes. In addition to topology, we assayed a number of biochemical and immunological parameters in order to examine the structural relationship between these two cAMP-binding proteins and the R subunit of PKA. Support in favour of non-relatedness comes from experiments where all three cAMP-receptor proteins have been treated with proteases. Complete digestion of the affinity-purified, photolabeled, then denatured proteins yields cAMP-binding-peptide domains which differ strongly in size, dependent on whether they are derived from the cytoplasmic or from the two membrane-bound proteins. This reveals an obvious difference in primary structure which can also be the reason why heterologous hy-

bridization has failed to unmask the genes for further cAMP-binding proteins. The immunological data are not in conflict with this conclusion.

Comparison of the two-membrane-bound cAMP-receptor proteins reveals both differences and similarities. The two are located in different compartments [19] and our unpublished results and the mature proteins differ in molecular masses. They resemble one another, however, in that both are embedded in a membrane by a post-translationally attached lipid moiety. Interestingly, these two proteins represent the first examples of lipid-anchored cAMP-binding proteins.

Although the nature and exact arrangement of components appears to differ partly in the two anchors, they both contain PtdIns and Etn. In the cAMP receptor from plasma membranes, these two constituents are apparently linked by

a bridge containing non-acetylated GlcN and Man (absent from the mitochondrial protein). Thus, the anchor of the cAMP receptor from plasma membranes closely resembles the GPI found in the VSG from trypanosomes and in a rapidly increasing number of eukaryotic ectoproteins.

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