Parafusin, an Exocytic-sensitive Phosphoprotein, Is the Primary Acceptor for the Glucosylphosphotransferase in *Paramecium tetraurelia* and Rat Liver

Birgit H. Satir,* Chantragan Srisomsap,‡ Milaniya Reichman,* and Richard B. Marchase‡

*Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, New York 10461; and ‡Department of Cell Biology, University of Alabama at Birmingham, Birmingham, Alabama 35294

Abstract. Parafusin, the major protein in *Paramecium tetraurelia* to undergo dephosphorylation in response to secretory stimuli, appears to be the primary acceptor for the glucosylphosphotransferase in this species based on five independent criteria: identical molecular size of 63 kD; identical isoelectric points in the phosphorylated state of pH 5.8 and 6.2; identical behavior in reverse-phase chromatography; immunological cross-reactivity with an affinity-purified anti-parafusin antibody; the presence of a phosphorylated sugar after acid hydrolysis. It appears likely that the dephosphorylation observed with secretion reflects the removal of αGlc-1-P from parafusin's oligosaccharides and is consistent, therefore, with a regulatory role for this cytoplasmic glycosylation event. The glucosylphosphotransferase acceptor in rat liver is also immunoprecipitated by the anti-parafusin antibody and is very similar in physical characteristics to the paramecium protein. This conservation suggests a role for parafusin in mammalian exocytosis as well, at a step common to both the regulated and constitutive secretory pathways.

In cells exhibiting a regulated secretory pathway, exposure to anappropriate secretagogue leads rapidly to the fusion of secretory vesicles with the plasma membrane and consequently to the release of the contents of the vesicles. One approach toward identifying the proteins involved in this transduction sequence has been based on the assumption that at least some regulatory elements would undergo a change in their state of phosphorylation, in keeping with analogous changes seen with numerous other regulated processes (Cohen, 1989). Such changes in phosphorylation with exocytosis have been documented in intact mammalian cells (Amy and Kirschner, 1980; Burgoyne, 1984; Gutierrez et al., 1988), in synaptosomes (Wu et al., 1982; Dunkley et al., 1986; Greengard, 1987; Wang et al., 1988), and in *Paramecium tetraurelia* (Gilligan and Satir, 1982; Zieseniss and Plattner, 1985; Satir et al., 1988, 1989).

This ciliated protozoan is an attractive model system for the study of regulated secretion since massive and synchronous exocytosis can be initiated with an appropriate stimulus. In paramecium, parafusin, a cytoplasmic phosphoprotein of 63 kD, is the major protein to undergo dephosphorylation during exocytosis (Gilligan and Satir, 1982; Murtaugh et al., 1987; Satir et al., 1988). It appears to be rephosphorylated within seconds thereafter (Zieseniss and Plattner, 1985). Dephosphorylation of parafusin is inhibited in mutants defective in exocytosis and in wild-type cells under ionic conditions that inhibit exocytosis, such as in the presence of high extracellular Mg^{2+} (Gilligan and Satir, 1982; Zieseniss and Plattner, 1985). Functionally, parafusin may be involved in regulation of a membrane fusion step after the required influx of extracellular Ca^{2+} has taken place (Gilligan and Satir, 1983). Parafusin has been purified and a polyclonal antibody against it produced (Murtaugh et al., 1987). It has three isoforms, with isoelectric points of 5.8, 6.2, and 6.3, the former two being phosphorylated and cytosolic. Parafusin has been shown to be immunologically cross-reactive with proteins from a variety of sources including yeast, cockroaches, toad bladder, rat liver, and other tissues, and bovine and human brain (Satir et al., 1988, 1989).

Here, we report that paramecium parafusin is the principal acceptor for the UDP-glucose:glycoprotein glucose-1-phosphotransferase (Glc-phosphotransferase), an enzyme that catalyzes the transfer of αGlc-1-P from UDP-Glc to mannose residues on acceptor glycoproteins (Koro and Marchase, 1982; Hiller et al., 1987). In rat liver this membrane-associated enzyme has been shown to face the cytosol and to recognize a cytosolic glycoprotein of 62 kD (Srisomsap et al., 1988). The Glc-phosphotransferase and an acceptor of 62–64 kD have, like parafusin, been shown to be present in a wide variety of eukaryotes, including yeast, spinach, sea urchin, and a spectrum of mammalian tissues (unpublished results).

Our collaborative investigation exploring a possible relationship between parafusin and the Glc-phosphotransferase acceptor was begun because of similarities in their apparent size, in their subcellular distribution, and in their ubiquity

in eukaryotes. The evidence that in paramecium these phosphoproteins are one and the same includes identity of apparent molecular weights in SDS-PAGE and of pI's in analytical isoelectric focusing, and the finding that the acceptor labeled by the Glc-phosphotransferase can be immunoprecipitated with an affinity-purified anti-parafusin antibody. In addition, acid hydrolyses of paramecium parafusin labeled in vivo with $^{32}P_i$ yields a labeled sugar phosphate. The Glc-phosphotransferase acceptor in rat liver is similar in these physical characteristics to paramecium parafusin, and can also be immunoprecipitated by the paramecium anti-parafusin antibody.

These findings suggest that parafusin, which is highly conserved among eukaryotes, is a novel cytosolic phosphoglycoprotein and that reversible phosphoglycosylation of parafusin could play an important role in signal transduction during secretion in paramecium and perhaps in mammalian cells as well.

Materials and Methods

Culturing, Harvesting, and In Vivo $^{32}P_i$ Labeling of Paramecium

Wild-type *Paramecium tetraurelia* were grown to early stationary phase (~20,000 cells/ml) in axenic medium (Soldo et al., 1966) and then harvested by centrifugation in an IEC clinical centrifuge (500 *g*, 5 min), washed in phosphate-free buffer (5 mM 1,4-piperazinediethansulfonic acid, NaOH, 1 mM $CaCl_2$, 20 mM $MgCl_2$, 1 mM KCl, pH 7.0), and concentrated 100–200-fold. Cells were labeled in vivo with $^{32}P_i$ as described in Gilligan and Satir (1982).

Production of an Antibody to Parafusin

Preparation of paramecium parafusin and affinity-purified antibody to parafusin was performed as described in Murtaugh et al. (1987). Transfer to nitrocellulose and immunoblotting with this antibody were carried out as described in Satir et al. (1989).

Glc-Phosphotransferase Assays

Paramecium cultures were concentrated by centrifugation. The cells were resuspended in 100 mM sodium cacodylate, pH 6.8, and sonicated with a sonicator (three times for 2 s; power setting 6; Heat Systems-Ultrasonics, Inc., Farmingdale, NY). Glc-phosphotransferase assays, SDS-PAGE, and autoradiography were performed as described previously (Srisomsap et al., 1988). [β^{32}P]UDP-Glc (700–900 Ci/mmol) was synthesized and purified as described by Marchase et al. (1987). The ^{35}S-labeled phosphorothioate analogue of UDP-Glc [(β^{35}S)UDP-Glc] (1,100–1,300 Ci/mmol), which had been determined by Marchase et al. (1987) to be used with high efficiency by the Glc-phosphotransferase, was obtained from Dupont-NEN Products (Wilmington, DE) and used in some of the experiments reported here.

Acid Hydrolyses of ^{32}P-labeled Phosphoproteins

Phosphoproteins labeled via incubation with [β^{32}P]UDP-Glc were separated from precursor by chromatography in H_2O over Sephadex G-75, lyophilized, and hydrolyzed in 1 N HCl at 100°C for 1 h. The hydrolyzed products were diluted with H_2O, lyophilized, and subjected to chromatography in 0.05 N HCl over Dowex 50W (H^+ form) as described by Schaffer (1967). In vivo ^{32}P-labeled parafusin was immunoprecipitated as described above, and aliquots were subjected to either these hydrolytic conditions or to 2 N HCl, 100°C, 8 h. The samples were then diluted, lyophilized, and chromatographed as described above. [^{3}H]Man-6-P was prepared from [^{3}H]Man with hexokinase and ATP. This was used as a standard for calibrating the Dowex 50 W column, along with $^{32}P_i$, phosphoserine, phosphothreonine and phosphotyrosine. Phosphoamino acid standards were detected using a ninhydrin reagent (Marchase et al., 1990).

Aliquots of the first peak to elute from Dowex 50W were either subjected directly to high voltage paper electrophoresis (1% pyridine brought to pH 5.3 with glacial acetic acid for 90 min at 3,000 V) or first treated with phosphoglucose isomerase, phosphomannose isomerase, and glucose-6-phosphate dehydrogenase in the presence of 200 mM $NADP^+$ (Marchase et al., 1990).

Biochemical Comparisons

Analytical isoelectric focusing was carried out on a model 111 Mini IEF cell in pH 3–10 ampholytes as described by the manufacturer (Bio-Rad Laboratories, Inc., Richmond, CA). The dried gel was then subjected to autoradiography as described (Srisomsap et al., 1988). Reverse-phase high performance liquid chromatography was carried out on an Aquapore butyl column (Applied Biosystem Inc., Foster City, CA) in a linear 0–100% acetonitrile gradient in 0.1% trifluoroacetic acid.

Immunoprecipitation of Hepatocyte and Paramecium Parafusins

Paramecium cultures (10^7 cells) or primary cultures of rat hepatocytes (10^7 cells) were washed twice in 5 mM Tris-HCl, pH 7 containing 10 mM $MgCl_2$ and 1 mM KCl. Cells were homogenized on ice in 50 mM Tris, pH 7 containing 10 mM EDTA and proteolytic inhibitors (1 μg/ml leupeptin, 1 μg/ml antipain, 1.15 mM PMSF, and 0.03 TIU/ml aprotinin). The resulting homogenate (50 μl at 1 mg/ml) was incubated with 10 μCi [β^{35}S]UDP-Glc in the presence of 200 μM αGlc-1-P at room temperature for 1 h. After incubation the homogenate was spun for 10 min in a centrifuge (RC-5B; Dupont Sorvall, Newtown, CT) at 1,500 rpm and the supernatant collected. SDS was added to a final concentration of 2%, and the sample boiled for 4 min, cooled to room temperature, and diluted (1:4) in a buffer containing 190 mM NaCl, 50 mM Tris-HCl, pH 8.3, 6 mM EDTA, 2% Triton X-100, and all proteolytic inhibitors. The paramecium parafusin antiserum (final dilution 1:250) was added and the mixture rotated overnight at 4°C. The next day 25 μl of protein A–agarose was added, and the samples were shaken at 4°C for 4 h. The supernatant and pellet were collected by centrifugation in a microfuge in the cold for 2 min. The supernatant was saved and the immunoprecipitated pellet was washed three times in a buffer containing 150 mM NaCl, 0.1% Triton X-100, 10 mM Tris-HCl, pH 8.3, and 5 mM EDTA. 100 ml of SDS sample buffer was added to both pellet and supernatant and samples were boiled for 10 min. The beads were removed by centrifugation in a microfuge. The released protein from the pellets as well as the initial supernatants were subjected to 10% SDS-PAGE and then autoradiographed. Quantitation was obtained by excising the area around 63 kD and counting in a scintillation counter.

Results

The Glc-Phosphotransferase Adds αGlc-1-P to Mannose Residues on a 63-kD Acceptor in Paramecium

To determine if paramecium possessed the Glc-phosphotransferase and, if so, to determine the molecular masses of its acceptors, homogenates prepared from the cells were incubated with [β^{32}P]UDP-Glc, subjected to SDS-PAGE, and autoradiographed. An acceptor of 63 kD was found to be the primary band labeled in the autoradiograph (Fig. 1, lane *I*), nearly identical in molecular size to the primary acceptor for the Glc-phosphotransferase previously reported in rat liver (Srisomsap et al., 1988). These incubations were performed in the presence of 1 mM unlabeled ATP and P_i to dilute nonspecific kinase utilization of contaminants and breakdown products. In addition, 100 μM αGlc-1-P was included to dilute any possible labeling of phosphoglucomutase (Marchase et al., 1990), which also has a molecular mass of ~62 kD (Ray and Peck, 1972). The observed labeling appeared to be due to direct incorporation from UDP-Glc, since an additional 100 μM unlabeled UDP-Glc was found to effectively

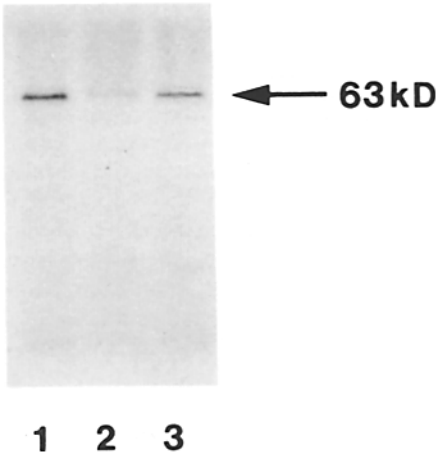

Figure 1. The Glc-phosphotransferase labels a 63-kD acceptor in paramecium homogenates. Lane *1*, autoradiograph after SDS-PAGE of proteins from paramecium homogenates after incubation for 30 min with 5 μCi [β^{32}P]UDP-Glc, 1 mM ATP, 1 mM P_i, and 100 μM αGlc-1-P. Lane *2*, autoradiograph following identical protocols except that an additional 100 μM unlabeled UDP-Glc was present during the incubation. Lane *3*, autoradiograph following protocol described for lane *1* except that αGlc-1-P concentration was 200 μM.

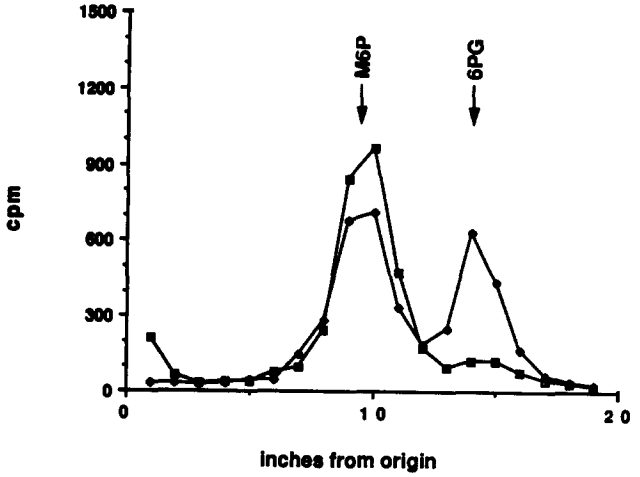

Figure 3. Enzymatic conversion of the ^{32}P-labeled product of the acid hydrolysis to 6-phosphogluconate suggests that it is [^{32}P]Man-6-P. The ^{32}P-labeled product of acid hydrolysis was found to co-migrate with [^{3}H]Man-6-P in paper electrophoresis carried out in H_2O/pyridine/acetic acid (□). After treatment with glucose-6-phosphate dehydrogenase, phosphomannose isomerase, and phosphoglucose isomerase (Gawehn, 1974), the unknown (●) was partially converted to 6-phosphogluconate (6PG), consistent with it initially being [^{32}P]Man-6-P.

dilute the observed labeling (Fig. 1, lane *2*), while increasing the αGlc-1-P concentration to 200 μM caused only limited loss of label (lane *3*) due to a slight inhibitory effect on the transferase (unpublished observation).

Further evidence that the observed label was due to the action of the Glc-phosphotransferase came from analyses of the labeled product of acid hydrolyses (1 N HCl, 100°C, 1 h) of the phosphoprotein, in which terminal Glc residues would be cleaved at the phosphodiester bond while the underlying

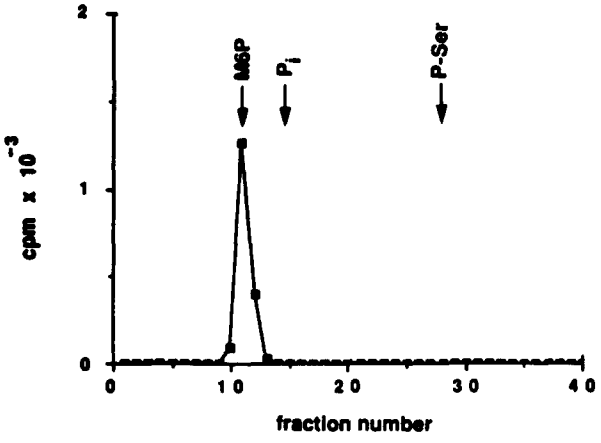

Figure 2. Acid hydrolysis of the paramecium protein labeled with [β^{32}P]UDP-Glc yields a phosphorylated sugar and no evidence of phosphorylated amino acids. Column chromatography over Dowex 50W (H^+ form) in 0.05 N HCl following acid hydrolysis of paramecium homogenates labeled with [β^{32}P]UDP-Glc yielded a ^{32}P-labeled moiety that co-chromatographed with [^{3}H]Man-6-P and not with phosphoserine, phosphothreonine, or phosphotyrosine. Arrows indicate elution peaks of standards. Phosphothreonine and phosphotyrosine eluted near phosphoserine.

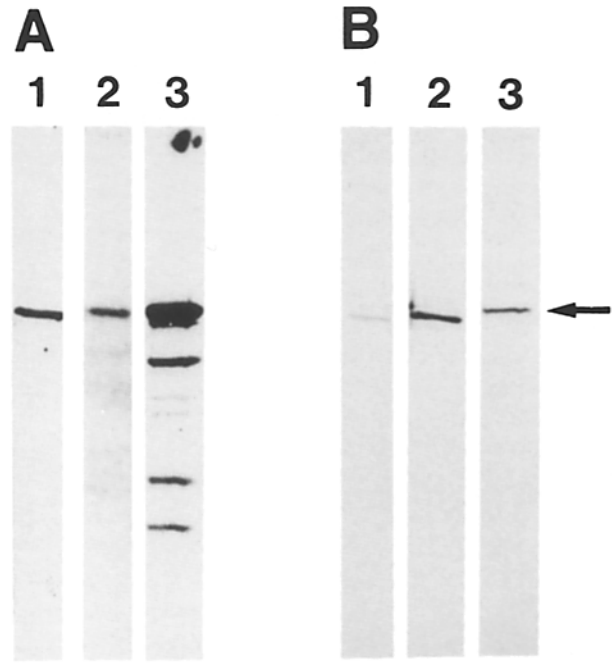

Figure 4. The Glc-phosphotransferase acceptor and parafusin are identical in apparent molecular mass in both paramecium and isolated rat hepatocytes. (*A*) Autoradiograph after SDS-PAGE and transfer to nitrocellulose. Lane *1*, homogenized paramecium labeled with [β^{35}S]UDP-Glc. Lane *2*, high speed supernatant (S2) after in vivo labeling of paramecium with $^{32}P_i$. Lane *3*, homogenized hepatocytes labeled with [β^{35}S]UDP-Glc. (*B*) Immunoblot using the paramecium anti-parafusin antibody of the same samples shown in *A*. Note in each case the samples show cross-reactivity at M_r 63,000 (*arrow*).

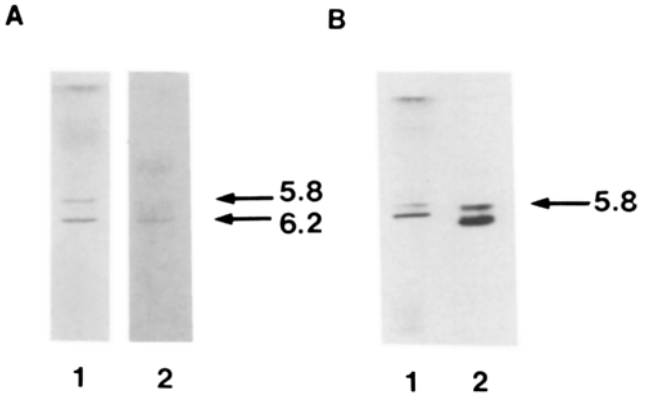

Figure 5. Similarities in analytical isoelectric focusing between paramecium parafusin and the Glc-phosphotransferase acceptor in paramecium and rat liver. Autoradiograph of two separate experiments in which labeled proteins were subjected to analytical isoelectric focusing. (*A*) The [β^{35}S]UDP-Glc-labeled product from paramecium homogenates (lane *1*) is compared with immunoprecipitated in vivo ^{32}P-labeled parafusin (lane *2*). Samples shown are from the same gel but exposed for 18 h (lane *1*) and 68 h (lane *2*). (*B*) The [β^{35}S]UDP-Glc-labeled products from paramecium (lane *1*) and rat liver (lane *2*) are compared. Labeled isoforms are found near pH 6.0.

phosphate would remain associated with the acceptor sugar or amino acid. The hydrolysis products were subjected to chromatography in 0.05 N HCl over Dowex 50W (H^+ form), a technique that clearly separates phosphosugars from phosphoamino acids. Nearly all of the counts were found to migrate with a [^{3}H]Man-6-P standard (Fig. 2). The ^{32}P-labeled material co-migrating with the [^{3}H]Man-6-P standard was pooled and incubated with glucose-6-phosphate dehydrogenase, phosphomannose isomerase, and phosphoglucose isomerase in the presence of $NADP^+$ (Gawehn, 1974). The enzyme-containing and control aliquots were then subjected to high voltage paper electrophoresis. When none of the enzymes was present, the ^{32}P unknown was found to co-migrate with the [^{3}H]Man-6-P standard. In the enzyme-treated sample, the ^{32}P-labeled unknown was partially converted to 6-phosphogluconate (Fig. 3). This result thus suggests that the underlying sugar on the acceptor for the Glc-phosphotransferase is identical to what was found in chick neural retina (Koro and Marchase, 1982) and rat liver (Marchase et al., 1990).

The Principal Acceptor for the Glc-Phosphotransferase in Paramecium Is Identical to Parafusin in Several of Its Physical Properties

To determine more precisely how the principal Glc-phosphotransferase acceptor in paramecium compared in molecular size to parafusin, homogenates prepared from the cells were incubated with the β^{35}S-labeled phosphorothioate analogue of UDP-Glc [(β^{35}S)UDP-Glc], subjected to SDS-PAGE, and blotted to nitrocellulose (Fig. 4 *A*, lane *1*). Parafusin, one of the most heavily phosphorylated proteins found after in vivo labeling of paramecium with $^{32}P_i$, was electrophoresed in an adjacent lane (Fig. 4 *A*, lane *2*). An apparent correspondence in molecular size was found, and more precisely confirmed by comparing the autoradiographs to Western blots (Fig. 4 *B*, lanes *1* and *2*) stained using an affinity purified antibody to parafusin (Murtaugh et al., 1987). The autoradiograph of the band labeled with (β^{35}S)-UDP-Glc corresponded precisely to the band visualized in the Western blot of the same lane.

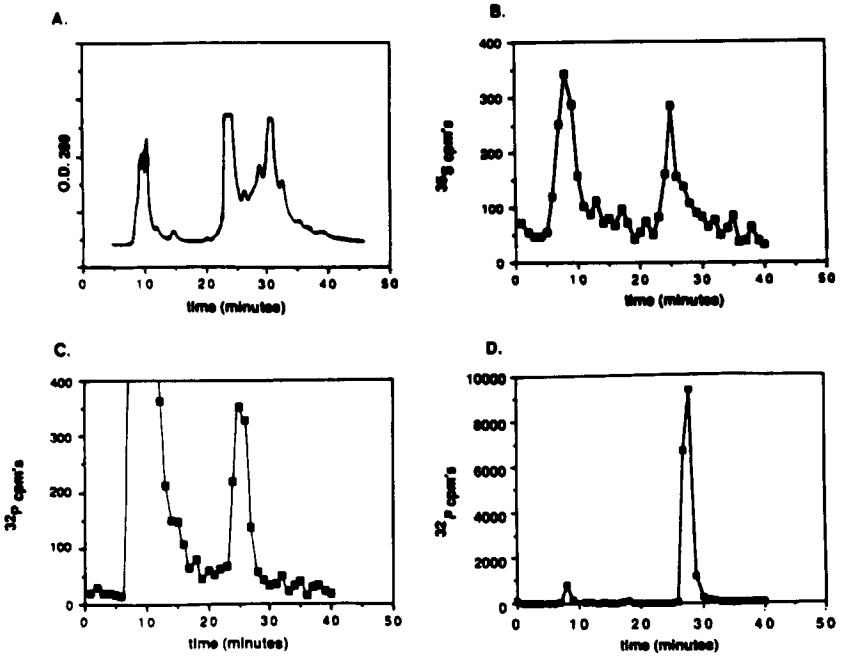

Figure 6. Similarities in elution profiles in C-4 reverse-phase chromatography between paramecium parafusin and the Glc-phosphotransferase acceptors of paramecium and rat liver. (*A*) Absorbance profile at 280 nm of paramecium homogenate after incubation with [β^{35}S]UDP-Glc during elution from a C-4 reverse-phase column with increasing acetonitrile concentrations. (*B*) Profile of radioactivity in the same experiment. The bulk of unincorporated [β^{35}S]-UDP-Glc was removed by molecular sieving, but some unincorporated radioactivity was eluted near 10 min. Only the fraction near 25 min displayed macromolecular incorporation after SDS-PAGE, with all of the radioactivity showing an apparent molecular mass of 63 kD (data not shown). (*C*) Profile of radioactivity from a paramecium high speed supernatant (S2) after in vivo labeling with $^{32}P_i$. Again the bulk of soluble radioactivity was removed by molecular sieving. SDS-PAGE showed that the radioactivity near 25 min was due to a 63-kD phosphoprotein, presumably parafusin. (*D*) Profile of radioactivity from rat liver homogenate incubated with [β^{35}S]UDP-Glc and then cleared of soluble label. SDS-PAGE confirmed that the radioactivity at 28 min was due to a 63-kD protein.

For comparison, homogenate prepared from rat hepatocytes was also incubated with [β^{35}S]UDP-Glc and electrophoresed (Fig. 4 *A*, lane *3*). A primary acceptor of nearly the same molecular size is seen (Srisomsap et al., 1988), as well as smaller peptides attributable to proteolysis (unpublished observations). Fig. 4 *B*, lane *3* demonstrates cross-reactivity of the anti-parafusin antibody with a liver protein of this apparent molecular mass (Satir et al., 1989).

Analytical isoelectric focusing of parafusin and the acceptor for the Glc-phosphotransferase in paramecium also suggested identity. Parafusin was labeled in vivo with $^{32}P_i$ and then purified by immunoprecipitation with the affinity-purified antibody. The ^{32}P-labeled sample was then compared by analytical isoelectric focusing and autoradiography with a paramecium homogenate labeled in vitro through incubation with [β^{35}S]UDP-Glc. As is shown in Fig. 5 *A*, lane *1*, the UDP-Glc-labeled sample produced predominantly two isoforms of pI 5.8 and 6.2, as did the immunoprecipitated in vivo $^{32}P_i$-labeled parafusin, although the more acidic form is only barely visible in the photograph (Fig. 5 *A*, lane *2*). This latter result confirms the finding reported by Murtaugh et al., (1987) of two phosphorylated forms of parafusin with precisely these pIs. In addition, the liver and paramecium proteins labeled in vitro with [β^{35}S]UDP-Glc are strikingly similar in isoforms (Fig. 5 *B*, lanes *1* and *2*).

The Glc-phosphotransferase acceptor labeled through incubation in paramecium homogenates with [β^{35}S]UDP-Glc was also compared with in vivo $^{32}P_i$-labeled parafusin on C-4 reverse-phase chromatography, in which separation is based predominantly on hydrophobicity. Their elution profiles (Fig. 6, *B* and *C*) both peaked at 25 min (corresponding to 58% acetonitrile), again suggesting identity. Similarity to the [β^{35}S]UDP-Glc protein labeled in rat liver is again seen (Fig. 6 *D*), although the liver protein displayed a slightly delayed retention time.

Anti-Parafusin Antibody Immunoprecipitates the Acceptor Labeled by the Glc-Phosphotransferase in Both Paramecium and Rat Liver

An additional test for the identity of parafusin and the acceptor for Glc-phosphotransferase in paramecium was performed using the criterion of immunoprecipitation with the affinity-purified anti-parafusin antibody (Murtaugh et al., 1987). Fig. 7 shows an autoradiograph of the results from an immunoprecipitation of a [β^{35}S]UDP-Glc-labeled paramecium homogenate. The first two lanes compare the supernatant (*S*) after precipitation with the anti-parafusin antibody (*Ab+*) to the supernatant from the control (*Ab−*). Radioactivity was completely removed from the supernatant exposed to anti-parafusin. The final two lanes compare the SDS-solubilized pellets (*P*) resulting from these precipitations. Radioactivity at 63 kD is greatly enhanced in the pellet exposed to antibody. Thus, both by the three independent physical characteristics described above and by immunocrossreactivity, the acceptor labeled by the Glc-phosphotransferase in paramecium and the phosphoprotein previously identified as parafusin are indistinguishable.

Fig. 8 is an autoradiograph of an SDS-PAGE showing an immunoprecipitation experiment after labeling of rat hepatocyte homogenate with [β^{35}S]UDP-Glc. The labeled hepatocyte homogenate (Fig. 8, lane *1*) was centrifuged to remove

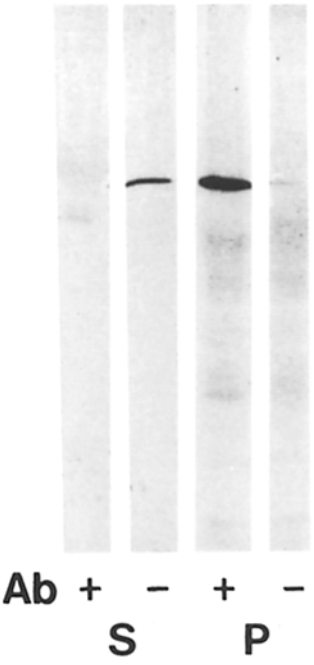

Figure 7. The Glc-phosphotransferase acceptor in paramecium is immunoprecipitated with affinity-purified anti-parafusin antibody. Paramecium homogenate was labeled with [β^{35}S]UDP-Glc and subjected to immunoprecipitation using the anti-parafusin antibody. A supernatant (*S*) and pellet (*P*) were obtained and analyzed by SDS-PAGE followed by autoradiography. A comparison between a preparation where anti-parafusin was added (*Ab+*) and a control (*Ab−*) is shown.

cell debris. As expected, the resulting supernatant (Fig. 8, lane *2*) was somewhat enriched in labeled protein, but some proteolysis was also evident. Following a mock immunoprecipitation (*Ab−*), all the 63-kD protein remained in the supernatant (Fig. 8, lane *3*). The corresponding pellet (Fig. 8, lane *8*) contained >4% of total counts, approximately background level. In contrast, after immunoprecipitation with the anti-paramecium parafusin antiserum (*Ab+*), ~40% of the labeled 63-kD protein from the hepatocytes was immunoprecipitated (Fig. 8, lanes *6* and *7*), the remaining label being found in the supernatants (lanes *4* and *5*).

Acid Hydrolyses of Parafusin Labeled In Vivo with $^{32}P_i$

Paramecium parafusin was labeled in vivo with $^{32}P_i$ and immunoprecipitated with the affinity-purified anti-parafusin antibody. This preparation was then split into halves and acid hydrolyzed under two conditions, one optimal for recovery of phosphoserine and the other for Man-6-P. They were then subjected to chromatography over Dowex 50W (H^+ form), as described above. After hydrolysis under the milder conditions (Fig. 9), 30% of the counts were found to migrate with an internal [^{3}H]Man-6-P standard, the remaining 70% being $^{32}P_i$. When the more stringent hydrolytic conditions were used, nearly all the radioactivity coeluted from the Dowex column with $^{32}P_i$ (data not shown). There was thus no evidence for the presence of a phosphorylated amino acid under either of these hydrolytic conditions.

Discussion

We have established that, like other eukaryotic cells, paramecium possesses a Glc-phosphotransferase that adds αGlc-1-P to mannose residues on a cytosolic 63-kD acceptor. Five independent criteria support the conclusion that parafusin, a protein that evidently is associated with membrane fusion during exocytosis in paramecium (Satir et al., 1989), is this

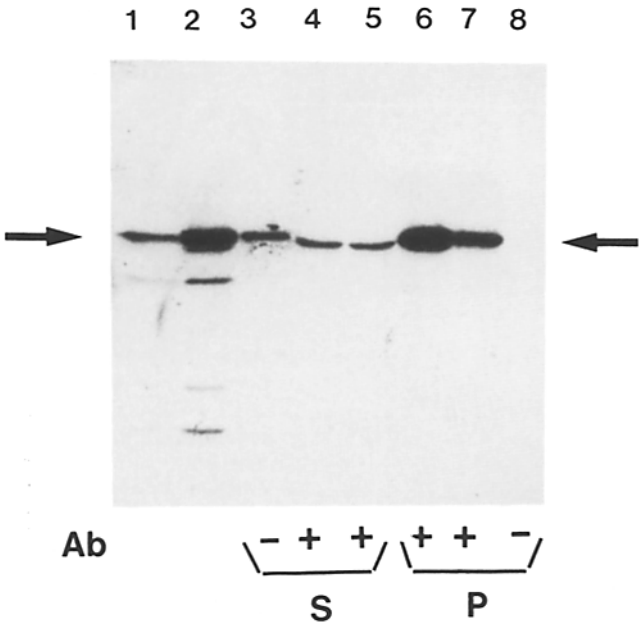

Figure 8. The Glc-phosphotransferase acceptor from rat hepatocytes is immunoprecipitated by anti-parafusin. Autoradiograph of SDS-PAGE supernatants (*S*) and pellets (*P*) obtained from rat hepatocyte homogenates incubated with [β^{35}S]UDP-Glc and subjected to immunoprecipitation with anti-parafusin antibody. Lane *1* shows initial homogenate. Lane *2* shows the low speed supernatant used in the immunoprecipitation. Some proteolysis of the 63-kD protein is evident. Lane *3* (*Ab*−) shows 1% of the supernatant after a mock (no primary antibody) immunoprecipitation, and lane *8* shows 10% of the corresponding pellet. Lanes *4* and *5* show 1% of the supernatant after immunoprecipitation with the anti-parafusin antibody (*Ab*+). Lanes *6* and *7* show 10% of the corresponding pellets. In lanes *4* and *6*, the sample was boiled in 2% SDS before exposure to the antibody; in lanes *5* and *7* the sample was not boiled.

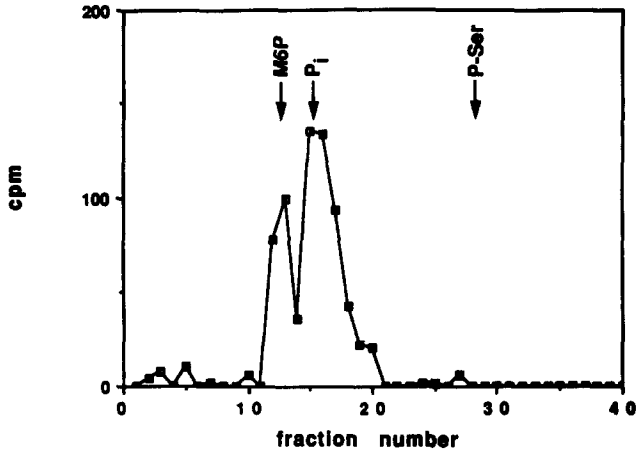

Figure 9. Acid hydrolysis of immunoprecipitated parafusin after in vivo labeling of paramecium with $^{32}P_i$ yields $^{32}P_i$ and [^{32}P]Man-6-P but no phosphorylated amino acids, as assessed by column chromatography over Dowex 50W (H^+ form) in 0.05 N HCl.

acceptor for the Glc-phosphotransferase. These criteria include: (*a*) identity in apparent molecular mass between parafusin and the Glc-phosphotransferase acceptor as determined by SDS-PAGE; (*b*) identity in hydrophobicity as assessed by their behavior in C4 reverse-phase chromatography; (*c*) identity in isoelectric points as determined by analytical isoelectric focusing; and more conclusively, (*d*) an affinity-purified antibody prepared against parafusin recognized the Glc-phosphotransferase acceptor in paramecium, and (*e*) in vivo ^{32}P-labeled immunoprecipitated parafusin was shown to contain labeled phosphosugar after acid hydrolysis. Until complete amino acid sequence analyses are obtained from both proteins, we tentatively conclude that the proteins are identical.

The acceptor for the Glc-phosphotransferase in rat liver and paramecium parafusin have also been found to be virtually identical with respect to molecular size, isoelectric profiles, and reverse-phase elution characteristics. Furthermore, the liver acceptor is immunoprecipitated by antibody prepared against parafusin isolated from paramecium. These results appear to reflect a remarkable conservation of parafusin (the Glc-phosphotransferase acceptor) in two evolutionarily widely divergent species. The ubiquity of a cytoplasmic phosphoglycoprotein of ~63 kD, as assessed by cross-reactivity with the antibody specific for paramecium parafusin (Satir et al., 1989) and by the presence of an acceptor of that size for the Glc-phosphotransferase across all eukaryotes examined including yeast, invertebrates, and a variety of mammalian tissues, suggests an even broader evolutionary and possibly functional conservation of the molecule.

The possible involvement of parafusin in exocytosis was first inferred in experiments with axenic cultures of paramecium, which take up $^{32}P_i$ and phosphorylate a small number of polypeptides. It was found that the most heavily labeled polypeptide was a minor component, parafusin. This polypeptide was linked to the process of membrane fusion and exocytosis in these cells in a series of in vivo studies (Gilligan and Satir, 1982, 1983) that showed that when prelabeled wild-type cells were stimulated to secrete the labeled parafusin was dramatically dephosphorylated.

In the present study in vivo $^{32}P_i$ incorporation into parafusin, after acid hydrolysis, yields a phosphorylated sugar. An earlier report suggested the presence of phosphorylated serine in paramecium parafusin (Satir and Murtaugh, 1988). The possibility exists that both phosphoserine and a phosphorylated oligosaccharide are present in parafusin and that conditions in its preparation are crucial for maintaining both. Phosphorylated serine may, for instance, be affected by phosphatases during preparation, as was found during isolation of parafusin (Murtaugh et al., 1987). Alternatively, phosphoglucomutase has a molecular size of 62 kD and contains phosphoserine (Ray et al., 1983). Since the initial hydrolyses were performed on labeled material enriched solely by SDS-PAGE, its presence could have influenced the previous result. In any case, since nearly a complete loss of phosphate from parafusin is seen after stimulation of exocytosis (Gilligan and Satir, 1982, 1983), removal of αGlc-1-P from parafusin seems likely. However, this point still remains to be clarified.

If, in fact, the dephosphorylation of parafusin that occurs as exocytosis is initiated is due to the removal of αGlc-1-P from its oligosaccharide, this would suggest that carbohydrates on cytoplasmic glycoproteins may be cyclically added and/or removed rapidly in response to external stimuli. In this case, such a removal could be catalyzed by an αGlc-1-P

phosphodiesterase similar to that described in rat liver (Srisomsap et al., 1989), which is apparently specific for the Glc-P-Man diester. This cycling in oligosaccharide structure thus could play a regulatory role in signal transduction during membrane fusion and exocytosis analogous to that played by phosphorylation in other physiological phenomena.

Satir et al. (1988) have reported that while the phosphate-containing form of parafusin is present primarily in the soluble pool of cytoplasmic proteins, the dephosphorylated form is present in membrane fractions. It is possible that the oligosaccharide remaining on parafusin after the removal of αGlc-1-P after exocytosis is triggered is important for its targeting to a membrane site relevant to the fusion event. Such a role would be topologically distinct, but analogous to the role played by Man-6-P residues on newly synthesized lysosomal hydrolases (Kornfeld, 1987).

Glycosylation is not normally thought of as a posttranslational modification that rapidly regulates a protein's behavior. The majority of glycosylation events occur as newly synthesized proteins mature while moving vectorially through the ER and Golgi apparatus. However, cytoplasmic glycosylation presents different possibilities. A cytoplasmic glycoprotein could be repeatedly exposed to glycosyltransferases and antagonistic glycosidases, free from compartmentalization constraints that discourage cyclical glycoprotein modifications within the ER and Golgi apparatus. The data presented here suggest that such a cycle could be involved in exocytosis. The presence of parafusin in liver may imply a role for the molecule at a step common to both constitutive and regulated secretion.

Cytoplasmic glycosylation may, therefore, function in a very different manner from glycosylation within the ER and Golgi apparatus. There are now several other examples of cytosolic glycosylation events (Hart et al., 1989), the best studied of which gives rise to O-linked GlcNAc's present, for instance, on nuclear pore (Davis and Blodel, 1987) and chromatin (Kelly and Hart, 1989) proteins. Any of these events could cyclically be exposed to antagonistic glycosidases and transferases within the cytosolic compartment. One rather special case is glycogenin, the core protein on which glycogen is synthesized and degraded in the cytosol in a regulated manner (Whelan, 1986). The data presented here may thus reflect but one example of a group of regulated posttranslational modifications in which sugars are used to modulate a cytosolic protein's behavior.

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