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Carbohydrate cycling in signal transduction: Parafusin, a phosphoglycoprotein and possible Ca^{2+}-dependent transducer molecule in exocytosis in *Paramecium*

(secretion/membrane fusion/ciliates)

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ABSTRACT **Parafusin, a cytosolic phosphoglycoprotein of M_r 63,000, is dephosphorylated and rephosphorylated rapidly in a Ca^{2+}-dependent manner upon stimulation of exocytosis *in vivo* in wild-type (wt) *Paramecium*. In contrast, the temperature-sensitive exocytosis mutant nd9, grown at the nonpermissive temperature (27°C), does not exocytose or dephosphorylate parafusin upon stimulation in the presence of Ca^{2+}; grown at the permissive temperature (18°C), nd9 cells show a wt phenotype. Parafusin contains two types of phosphorylation sites: one where glucose 1-phosphate is added by an α-glucose-1-phosphate phosphotransferase and removed by a phosphodiesterase and one where phosphate from ATP is added directly to a serine residue by a protein kinase and removed by a phosphatase. We show here that, in cell fractions from wt *Paramecium*, both reactions can be carried out *in vitro* by using uridine(5′-[β-[^{35}S]thio])diphospho(1)-glucose (UDP[β^{35}S]-Glc) and [γ-^{32}P]ATP, respectively. The characteristics of these pathways are different. Specifically, in the presence of Ca^{2+}, the amount of UDP[β^{35}S]-Glc label in parafusin is reduced. In contrast, identical labeling experiments with [γ-^{32}P]ATP show that Ca^{2+} enhances labeling of parafusin. Mg^{2+} had no appreciable effect on either labeling. Removal of the UDP[β^{35}S]-Glc label on parafusin in the presence of Ca^{2+} correlates with the *in vivo* dephosphorylation seen upon exocytosis. Incubations with UDP[β^{35}S]-Glc were then performed with homogenates and nd9 cell fractions grown at 27°C under the ionic conditions used for wt cells. These labelings were not affected by Ca^{2+}, in contrast to results from wt cells but in accord with those obtained earlier with nd9-27 mutant cells *in vivo*. Factors responsible for both dephosphorylation and Ca^{2+} sensitivity were found in the high-speed pellet (P2) in wt cells, suggesting that the putative phosphodiesterase is in this fraction and that the defect in the mutant nd9-27 resides in the Ca^{2+} activation of the phosphodiesterase. We conclude that the *in vivo* dephosphorylation of parafusin that occurs upon exocytosis is a dephosphoglucosylation due to removal of the α-glucose 1-phosphate and more generally that carbohydrates on cytoplasmic glycoproteins may be cyclically added and/or removed in response to extracellular stimuli.**

This paper deals with an example of a posttranslational cyclic regulatory system: the glycosylation/deglycosylation of a cytosolic phosphoglycoprotein called parafusin that is potentially involved in membrane fusion in exocytosis. The possibility that carbohydrates on cytoplasmic glycoproteins may be cyclically added and/or removed rapidly in response to external stimuli seems worth exploring since it represents a regulatory mechanism that could be widely applicable.

The background data that tie parafusin to the process of membrane fusion and exocytosis come from studies using the ciliates *Paramecium tetraurelia* and *Tetrahymena thermophila*. *Paramecium tetraurelia* in axenic cultures take up $^{32}P_i$ and phosphorylate a number of polypeptides. The most heavily labeled polypeptide is parafusin, a minor component of M_r 63,000 that shows serine phosphorylation (1, 2). Gilligan and Satir (3, 4) showed that, when *in vivo*-prelabeled wild-type (wt) cells were stimulated to secrete the content of their docked dense-core secretory vesicles (trichocysts) with trinitrophenol, the labeled parafusin was dramatically dephosphorylated. Dephosphorylation was correlated with the massive exocytosis of secretory vesicles in two types of experiments: one testing ionic requirements for exocytosis (Ca^{2+} vs. Mg^{2+}) and the other using exocytosis mutants. Exocytosis and dephosphorylation required the presence of Ca^{2+} in the medium in that 5 mM $MgCl_2$, in the absence of added Ca^{2+}, inhibited both processes. Both membrane fusion and changes within the secretory vesicle content are Ca^{2+}-dependent processes, the former requiring an increase of cytosolic Ca^{2+}, probably via receptor-gated Ca^{2+} channels in the membrane (5). Further, the temperature-sensitive secretory mutant nd9 grown at the restrictive temperature (27°C) and then stimulated with trinitrophenol in the presence of 5 mM Ca^{2+} neither exocytosed nor dephosphorylated parafusin (3). Dephosphorylation and exocytosis, very rapid events, are normally followed by rephosphorylation within 10 sec (6).

Parafusin was isolated and purified to apparent homogeneity. An affinity-purified polyclonal antibody was made that recognizes both the phosphorylated and dephosphorylated forms. Two slightly acidic (pI = 5.8 and 6.2) phosphorylated forms of parafusin are found in a cytosolic fraction. The affinity-purified antibody recognizes a third isoelectric form at pI 6.3 that appears unlabeled and is probably membrane bound (1, 2). Parafusin shows immunological cross-reactivity with proteins from a variety of unicellular organisms and cells of metazoan groups of wide evolutionary divergence, as well as with rat tissues, including brain, heart, pituitary, and kidney. These results suggest that parafusin was present early in the history of eukaryotes and may be of functional importance in the general mechanism of exocytosis and membrane fusion (7, 8).

A M_r 62,000 phosphoglycoprotein in liver that possessed an α-glucose-1-phosphate (αGlc-1-*P*) moiety linked to a short chain of mannoses that was O-linked to a serine residue of the protein was independently discovered (9). The bond between glucose and mannose in this protein was a phosphodiester linkage formed by the action of a unique αGlc-1-*P* phosphotransferase (9). By incorporation of label from a thiophosphoro analogue of UDP-Glc {uridine(5′-[β-[^{35}S]thio])-diphospho(1)-glucose (UDP[β^{35}S]-Glc)} into parafusin, Satir

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Abbreviations: Glc-1-*P*, glucose 1-phosphate; wt, wild type; UDP[β^{35}S]-Glc, uridine (5′-[β-[^{35}S]thio])diphospho(1)-glucose.

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et al. (10) have determined that in *Paramecium* αGlc-1-*P* is similarly added to mannose residues in parafusin by an αGlc-1-*P* phosphotransferase and that parafusin from *Paramecium* and the liver phosphoglycoprotein have the same major characteristics.

Therefore, labeling of parafusin by P_i incorporation in living *Paramecium* could potentially occur by two pathways (Fig. 1): (*i*) by labeling the ATP pool and transferring phosphate directly onto a serine residue by using a protein kinase or (*ii*) by labeling the UDP-Glc pool and transferring αGlc-1-*P* onto a mannose residue by using an αGlc-1-*P* phosphotransferase. Similarly, the Ca^{2+}-dependent dephosphorylation of parafusin that accompanies exocytosis could be accomplished either by a protein phosphatase pathway or by removal of αGlc-1-*P* by an αGlc-1-*P* phosphodiesterase (11). In this paper, using an *in vitro* phosphorylation system, we demonstrate the existence of these two pathways. We show evidence that supports the hypothesis that the cycling of an oligosaccharide structure is involved in the regulation, by demonstrating the differential regulation by Ca^{2+} of the two pathways, and suggests that the latter pathway is involved in parafusin dephosphorylation during exocytosis.

METHODS

Homogenization and cell fractionation of *Paramecium tetraurelia*, wt and nd9 cells, were performed on axenically grown (27°C) cell cultures. Cultures were harvested at early stationary phase (≈20,000 cells per ml), washed twice in phosphate-free buffer (5 mM Tris·HCl, pH 7.0/10 mM $MgCl_2$/1 mM KCl), and resuspended in homogenization buffer [50 mM Tris·HCl, pH 7.2, containing the proteolytic inhibitors leupeptin (1 μg/ml), phenylmethylsulfonyl fluoride (0.5 mM), and aprotinin (0.012 trypsin inhibitor unit/ml)]. Cells were then homogenized on ice (or room temperature for the mix and match experiments) in a Dounce homogenizer (250 strokes). The homogenate was usually fractionated further by centrifugation for 10 min at 1500 × *g* into a low-speed pellet (P1) and supernatant (S1), and the S1 was centrifuged at 100,000 × *g* for 60 min to yield a high-speed pellet (P2) and supernatant (S2). The S2 was concentrated two to three times with a Centricon-30 (Amicon).

In general the labeling procedure of the cellular fractions, either individually or in combination, was performed as follows: identical amounts of protein were incubated for 30 min at room temperature with either 10 μCi (1 Ci = 37 GBq) of [γ-^{32}P]ATP or UDP[β^{35}S]-Glc in the presence of 100–200 μM unlabeled αGlc-1-*P*. Ca^{2+}, Mg^{2+}, or EGTA was added appropriately either at the beginning of labeling for 30 min or after 30 min of labeling. The appropriate fractions were subjected to immunoprecipitation (see Fig. 5) or SDS/PAGE followed by immunoblot analysis with the affinity-purified parafusin antibody. Details are given in Murtaugh *et al.* (1).

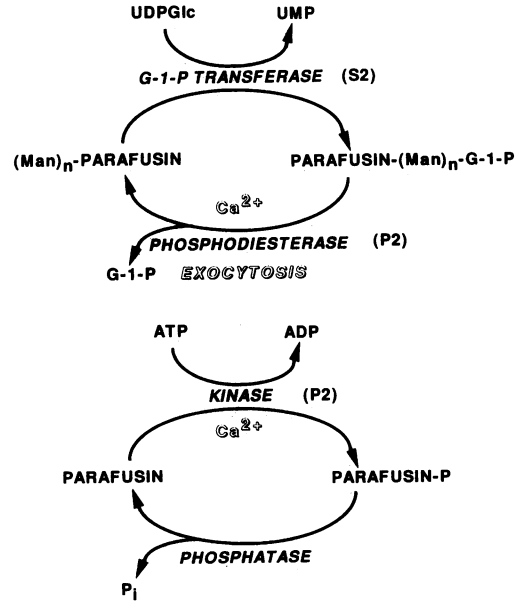

FIG. 1. Two cycles of covalent modification of parafusin indicating the localization and Ca^{2+} dependence of enzymes. (*Upper*) The phosphoglucosylation–dephosphoglucosylation cycle utilizing UDP-Glc. This cycle uses a cytosolic (S2) αGlc-1-*P* phosphotransferase and a Ca^{2+}-dependent phosphodiesterase that is associated with the membrane fraction (P2) and is presumably activated upon stimulation of exocytosis. (*Lower*) The kinase–phosphatase cycle utilizing ATP. The Ca^{2+}-dependent kinase is associated with the membrane fraction (P2).

RESULTS

Parafusin Can Be Labeled *in Vitro* Using UDP[β^{35}S]-Glc or [γ-^{32}P]ATP. Homogenates of axenically grown *Paramecium* incorporated label from UDP[β^{35}S]-Glc into a protein of M_r 63,000 (Fig. 2). A chase with 200 or 400 μM unlabeled Glc-1-*P* (lanes 2 and 3) or 200 or 400 μM unlabeled ATP (lanes 7 and 8) did not change the amount of label observed compared to controls (lane 1). In contrast, label was chased by 200 μM unlabeled Glc-1-*P* plus 200 μM UDP[βS]-Glc (lane 4) and by 200 and 400 μM UDP[βS]-Glc (lanes 5 and 6, respectively). Homogenates incubated with [γ-^{32}P]ATP phosphorylated a similar M_r 63,000 band, although other bands were also phosphorylated (Fig. 3*C*, lane 1). The kinetics of incorporation of either label into homogenates were similar to the kinetics of $^{32}P_i$ incorporation *in vivo* (data not shown). The identity of the M_r 63,000 protein labeled with either UDP[β^{35}S]-Glc or [γ-^{32}P]ATP was confirmed as parafusin by immunoprecipitation (see Fig. 5). Preimmune serum did not precipitate the labeled species.

Parafusin and Enzymes That Phosphorylate or Dephosphorylate Parafusin Can Be Localized to Specific Cell Fractions. Cell fractions, either individually or in combination, were incubated with either UDP[β^{35}S]-Glc or [γ-^{32}P]ATP. A representative autoradiogram of UDP[β^{35}S]-Glc incorporation is shown in Fig. 3*A*. The only major protein labeled was parafusin. The immunoblot (Fig. 3*B*) revealed that virtually all parafusin was found in the S2 (lane 2). By itself S2 incorporated label (Fig. 3*A*, lane 2), but incorporation of label was diminished when P2 was added to S2 (Fig. 3*A*, lane 3) compared to S2 alone (Fig. 3*A*, lane 2) even though the

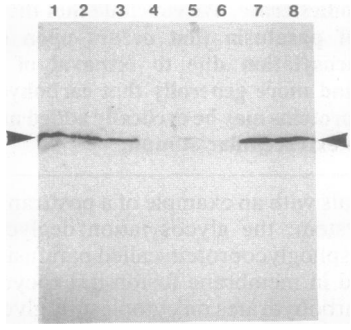

FIG. 2. Autoradiogram showing that ^{35}S label on parafusin from UDP[β^{35}S]-Glc is chased only by unlabeled UDP[βS]-Glc. *Paramecium* homogenates were labeled with UDP[β^{35}S]-Glc for 30 min. Equal aliquots were then incubated for 30 min with buffer alone (lane 1), 200 or 400 μM Glc-1-*P* (lanes 2 and 3), 200 μM Glc-1-*P* plus 200 μM UDP[βS]-Glc (lane 4), 200 or 400 μM UDP[βS]-Glc (lanes 5 and 6), and 200 or 400 μM ATP (lanes 7 and 8) and then subjected to SDS/PAGE and autoradiography. Arrowheads indicate M_r 63,000 in all figures.

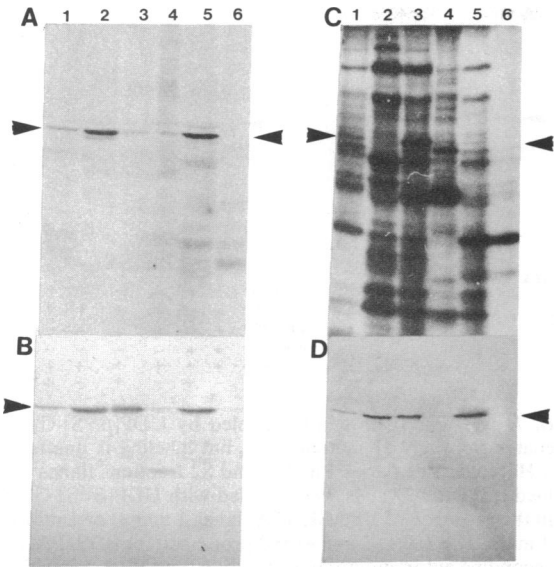

FIG. 3. Localization of parafusin and associated enzymes: autoradiograms (*A* and *C*) and immunoblots (*B* and *D*) of labeled *Paramecium* cell fractions. Equal protein concentrations of cell fractions were incubated with either UDP[β^{35}S]-Glc (*A* and *B*) or [γ-^{32}P]ATP (*C* and *D*) alone or combined with other fractions for 30 min at room temperature and then subjected to SDS/PAGE followed by autoradiography or immunoblot analysis with affinity-purified anti-parafusin antibody. Lanes: 1, homogenate; 2, S2; 3, S2 plus P2; 4, P2; 5, S2 plus P1; 6, P1.

amount of parafusin as judged from the immunoblot data was similar (Fig. 3*B*, lane 3 vs. lane 2). In contrast, if P1 was added, no change in labeling was observed (Fig. 3*A*, lane 5). These results suggest that the αGlc-1-*P* phosphotransferase is present predominantly in the S2 fraction. In addition, the homogenate contained a substance that segregated into the P2 fraction that either inhibited the action of the αGlc-1-*P* phosphotransferase or was itself a phosphodiesterase.

The corresponding results of [γ-^{32}P]ATP incorporation are shown in Fig. 3*C*. The ATP label was incorporated into many polypeptides including parafusin (see Fig. 5). Although the majority of parafusin was found in the S2 (Fig. 3*D*, lane 2), the S2 incorporated very little label from ATP (Fig. 3*C*, lane 2). The P2 fraction contained almost no parafusin (Fig. 3*D*, lane 4) and correspondingly incorporated little label (Fig. 3*C*, lane 4). However, the amount of incorporation increased synergistically when S2 was incubated with P2 (compare Fig. 3*C*, lanes 2 and 3). Addition of P1 to S2 had no effect. This result suggests that a kinase capable of phosphorylating parafusin is associated with P2.

Ca^{2+} Has a Differential Effect on Phosphorylation of Parafusin by UDP[β^{35}S]-Glc or by [γ-^{32}P]ATP. The ionic requirements affecting the labeling of parafusin were examined. UDP[β^{35}S]-Glc results are shown for homogenates in Fig. 4 *A* and *B*. Identical amounts of parafusin were present in each lane (Fig. 4*A*). Addition of 1 mM EGTA enhanced the ^{35}S labeling of parafusin significantly (Fig. 4*B*, lane 2) compared to controls (Fig. 4*B*, lane 1). This enhancement was prevented by addition of Ca^{2+}, whereas addition of Mg^{2+} had no effect (Fig. 4*B*, lanes 3 and 4).

[γ-^{32}P]ATP results are shown in Fig. 4 *C* and *D*. Identical amounts of parafusin were present in each lane (Fig. 4*C*). [γ-^{32}P]ATP labeling behaved quite differently from UDP[β^{35}S]-Glc labeling. Phosphorylation was virtually abolished in the presence of 1 mM EGTA (Fig. 4*D*, lane 2) compared to controls (Fig. 4*D*, lane 1). This was reversible if Ca^{2+} was added but not if Mg^{2+} was added (Fig. 4*D*, lanes 3 and 4).

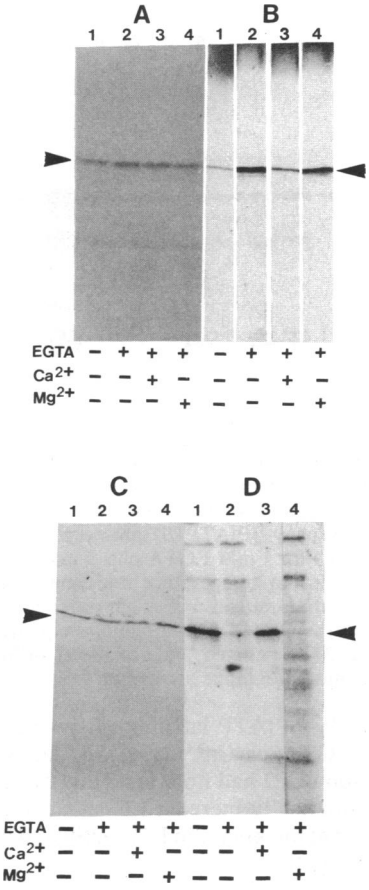

FIG. 4. Ca^{2+} differentially affects incorporation of label into parafusin: autoradiograms (*B* and *D*) and immunoblots (*A* and *C*) of labeled wt *Paramecium* homogenates in various ionic conditions. *Paramecium* wt cells were homogenized in Tris buffer and labeled with either UDP[β^{35}S]-Glc (*A* and *B*) or [γ-^{32}P]ATP (*C* and *D*) for 30 min in the presence of buffer alone (lanes 1), 1 mM EGTA (lanes 2), 1 mM EGTA plus 5 mM $CaCl_2$ (lanes 3), or 1 mM EGTA plus 5 mM $MgCl_2$ (lanes 4) and processed for SDS/PAGE followed by autoradiography or immunoblot analysis with affinity-purified anti-parafusin antibody. Immunoblots (*A* and *C*) show identical amounts of parafusin in all lanes. Ca^{2+} decreases the UDP[β^{35}S]-Glc labeling of parafusin enhanced by EGTA (*B*, lanes 2 and 3), whereas Ca^{2+} restores the [γ-^{32}P]ATP labeling of parafusin inhibited by EGTA (*D*, lanes 2 and 3).

A Ca^{2+}-Activatable Phosphodiesterase and a Ca^{2+}-Activated Kinase Are Present in the P2 Fraction. We examined which of the enzymes, localized in previous experiments, was responsible for the differential Ca^{2+} sensitivity observed in the homogenates. Individual preparations of S2 and S2 plus P2 were incubated with UDP[β^{35}S]-Glc or [γ-^{32}P]ATP in the presence of either EGTA alone or EGTA plus $CaCl_2$. Autoradiograms of immunoprecipitated parafusin are shown in Fig. 5. The lower molecular weight bands observed are breakdown products of parafusin. UDP[β^{35}S]-Glc labeled S2 parafusin in the presence of EGTA (Fig. 5*A*, lane 1). Addition of Ca^{2+} had little effect (Fig. 5*A*, lane 2). This suggests that the αGlc-1-*P* phosphotransferase in the S2 does not require Ca^{2+} as a cofactor and that this enzyme is active in low free-Ca^{2+} concentrations normally present in cytoplasm before stimulation. In EGTA, addition of P2 to the S2 had no effect (Fig. 5*A*, lane 3), but addition of P2 with Ca^{2+} reduced the amount of label dramatically (Fig. 5*A*, lane 4). This suggests either an inhibitor of αGlc-1-*P* phosphotransferase present in the P2 requires Ca^{2+} or activation of a phosphodiesterase in the P2 requires Ca^{2+}, either directly or indirectly.

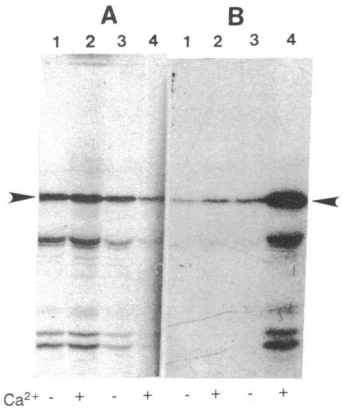

FIG. 5. Factors in the P2 fraction decrease labeling of parafusin by UDP[β^{35}S]-Glc but increase labeling by [γ-^{32}P]ATP in the presence of Ca^{2+}. Equal protein concentrations of S2 (lanes 1 and 2) and S2 plus P2 (lanes 3 and 4) fractions were incubated with either UDP[β^{35}S]-Glc (*A*) or [γ-^{32}P]ATP (*B*) in the presence of 1 mM EGTA alone (lanes 1 and 3) or 1 mM EGTA plus 5 mM $CaCl_2$ (lanes 2 and 4) for 30 min at room temperature and immunoprecipitated by incubating with anti-parafusin antiserum overnight at 4°C. Protein A-Sepharose was then added. The immunoprecipitated samples were shaken for 2 hr at 37°C, pelleted, and processed for SDS/PAGE. The corresponding autoradiographs are shown.

In contrast, [γ-^{32}P]ATP labeling of the S2 parafusin was negligible in EGTA or Ca^{2+} (Fig. 5*B*, lanes 1 and 2). In EGTA, addition of P2 had no effect (Fig. 5*B*, lane 3), but P2 plus Ca^{2+} dramatically increased labeling (Fig. 5*B*, lane 4). This suggests that the kinase in P2 is activated by Ca^{2+}, either directly or indirectly.

To determine whether the effect of Ca^{2+} was due to removal of αGlc-1-*P* from parafusin, homogenates were prelabeled. Then EGTA, $CaCl_2$, or $MgCl_2$ was added and incubations were continued for another 30 min. Fig. 6 shows autoradiograms of this experiment. Additions of EGTA or Mg^{2+} to UDP[β^{35}S]-Glc-prelabeled parafusin (Fig. 6*A*, lanes 1 and 3) had no effect, but labeling fell dramatically with Ca^{2+} (lane 2). Therefore, the Ca^{2+}-activated enzyme removes αGlc-1-*P* label from parafusin—i.e., it is a phosphodiesterase. Prelabeling of parafusin with ATP was enhanced after Ca^{2+} addition, consistent with Ca^{2+} activation of a kinase (Fig. 6*B*, lane 2).

Ca^{2+} Activation of the P2 Phosphodiesterase Is Defective in the nd9 Mutant. In homogenates from nd9-27 cells and in S2 prepared from these homogenates, UDP[β^{35}S]-Glc labeling was normal. However, in contrast to wt, addition of Ca^{2+} had no effect on this labeling (Fig. 7*A*, lane 2). The defect was found to reside in the P2 fraction. Addition of P2 from nd9-27 to S2 from nd9-27 had no effect on UDP[β^{35}S]-Glc labeling in the presence or absence of Ca^{2+} (Fig. 7*B*, lanes 1 and 2), but addition of wt P2 to nd9-27 S2 in the presence of Ca^{2+} lowered

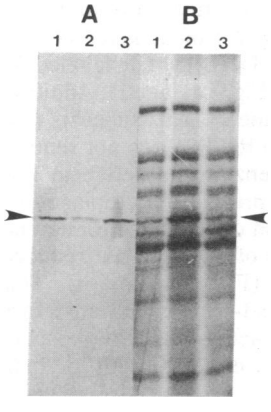

FIG. 6. Parafusin prelabeled with UDP[β^{35}S]-Glc or [γ-^{32}P]ATP shows that only the UDP[β^{35}S]-Glc label is dephosphorylated in the presence of Ca^{2+}. Cells were homogenized in Tris buffer and labeled with either UDP[β^{35}S]-Glc (*A*) or [γ-^{32}P]ATP (*B*) for 30 min in the presence of 1 mM EGTA after which equal aliquots were incubated with 1 mM EGTA buffer (lanes 1), 5 mM $CaCl_2$ (lanes 2), or 5 mM $MgCl_2$ (lanes 3) for an additional 30 min and then processed for SDS/PAGE. Corresponding autoradiographs are shown.

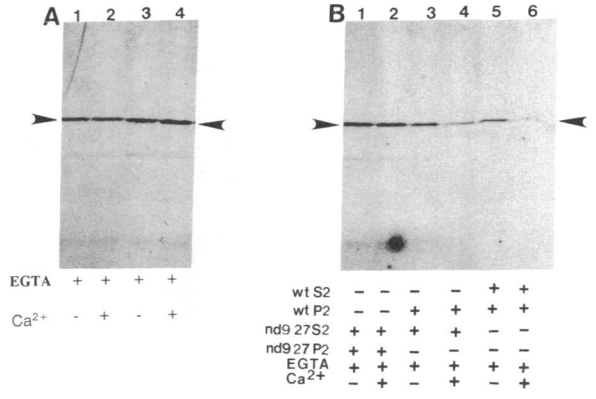

FIG. 7. (*A*) Parafusin can be labeled by UDP[β^{35}S]-Glc in homogenates from nd9-27 mutant cells, but labeling is unaffected by Ca^{2+}. Homogenates (lanes 1 and 2) and S2 fractions (lanes 3 and 4) obtained from nd9-27 cells were labeled with UDP[β^{35}S]-Glc for 30 min in the presence of 1 mM EGTA (lanes 1 and 3) or 1 mM EGTA plus 1 mM $CaCl_2$ (lanes 2 and 4) and processed for SDS/PAGE. The corresponding autoradiograph is shown. (*B*) wt P2 restores the Ca^{2+} sensitivity of UDP[β^{35}S]-Glc labeling of mutant S2. nd9-27 S2 was combined with equal amounts of protein from either mutant P2 (lanes 1 and 2) or wt P2 (lanes 3 and 4) in the presence of 1 mM EGTA alone (lanes 1 and 3) or 1 mM EGTA plus 1 mM $CaCl_2$ (lanes 2 and 4) and labeled with UDP[β^{35}S]-Glc. wt S2 and P2 were labeled with UDP[β^{35}S]-Glc in the presence of 1 mM EGTA alone (lane 5) or 1 mM EGTA plus 1 mM $CaCl_2$ (lane 6) as controls. Each sample was then subjected to SDS/PAGE. A corresponding autoradiograph is shown.

labeling (lane 4) though less efficiently than with wt S2 (lane 6). These results indicate that the αGlc-1-*P* phosphotransferase and the parafusin are unaffected but that the phosphodiesterase pathway is defective in the mutant. Addition of nd9-27 P2 in the presence of Ca^{2+} enhanced ATP labeling of parafusin, indicating that the kinase is unaffected (data not shown). When wt S2 was prelabeled with UDP[β^{35}S]-Glc and incubated with P2 from wt or nd9-27 cells (Fig. 8), labeling was reduced in the former (Fig. 8*B*) but not the latter case (Fig. 8*C*).

DISCUSSION

Phosphate can be incorporated into the cytosolic phosphoglycoprotein parafusin in homogenates and cell fractions of *Paramecium tetraurelia* by two distinct pathways: (*i*) from the γ-phosphate of ATP and (*ii*) from the αGlc-1-*P* of UDP-Glc. The conditions for incorporation are very different in the

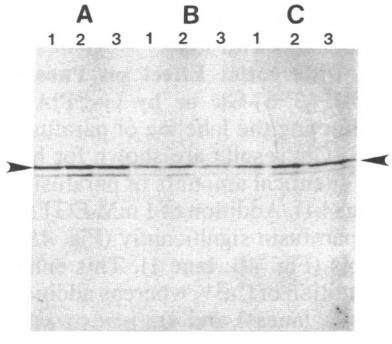

FIG. 8. nd9-27 P2 does not dephosphoglucosylate parafusin prelabeled with UDP[β^{35}S]-Glc. wt S2 fractions were labeled with UDP[β^{35}S]-Glc in the presence of 1 mM EGTA for 30 min at room temperature and then incubated with buffer (*A*), wt P2 (*B*), or mutant P2 (*C*), all in the presence of Ca^{2+}. Aliquots were taken for SDS/PAGE at 30 sec, 15 min, and 30 min (lanes 1, 2, and 3, respectively). After a 30-min incubation, label is reduced after addition of wt P2 (*B*, lane 3) but not mutant P2 (*C*, lane 3).

two pathways. The simplest explanation of our results is that the enzyme responsible for the phosphorylation by ATP is presumably a serine kinase found primarily in P2—the microsomal fraction—of the cell homogenate, and a Ca^{2+} concentration of $>10^{-7}$ M is required for its activation. In contrast, the enzyme responsible for the incorporation of αGlc-1-*P* onto the mannose chain(s) of parafusin, an αGlc-1-*P* phosphotransferase, is primarily cytosolic, found in the S2 fraction of the homogenate and is independent of Ca^{2+} (Fig. 1).

Both pathways are cyclic. The subcellular localization of the phosphatase is unknown. However, the Glc-1-*P* S-analog label is removable from parafusin by addition of wt P2 in the presence of Ca^{2+} ($>>10^{-7}$ M), suggesting that a Ca^{2+}-activatable αGlc-1-*P* phosphodiesterase (that can act on UDP[β^{35}S]-Glc) is present in P2.

Which cycle then corresponds to the *in vivo* situation? *In vivo*, in an unstimulated cell, cytoplasmic Ca^{2+} is low, but incorporation of P_i label into parafusin is extensive. Upon stimulation, intracellular Ca^{2+} increases locally to $>>10^{-7}$ M, presumably from Ca^{2+} entry through cell membrane channels, and parafusin loses its label. We conclude that the Ca^{2+}-activatable phosphodiesterase is responsible for the loss of label at exocytosis and that in unstimulated cells P_i is incorporated into parafusin largely as αGlc-1-*P* via the αGlc-1-*P* phosphotransferase pathway. Consistent with this hypothesis, Mg^{2+} cannot substitute for Ca^{2+} *in vivo* or *in vitro* in activation of the phosphodiesterase.

The increase in cytosolic Ca^{2+} could enhance phosphorylation of parafusin via the kinase pathway. This phosphorylation may be related to the observed rapid rephosphorylation of parafusin. If dephosphoglucosylation caused a redistribution of parafusin to membranes where the kinase was present, rephosphorylation might reverse this process causing dissociation of the molecule from plasma or vesicle membranes, as observed in other systems (12).

We have used the temperature-sensitive mutant nd9-27 to clarify these events. *In vivo*, this mutant incorporates P_i label into parafusin normally but, upon stimulation in the presence of Ca^{2+}, it is unable to exocytose or dephosphorylate. We have now shown that homogenates from nd9-27 add a UDP-Glc label to parafusin as does wt. When prelabeled nd9-27 parafusin is mixed with the phosphodiesterase-containing wt P2, label is removed in the usual Ca^{2+}-dependent fashion. However, when mixed with the mutant P2 in the presence of Ca^{2+}, label is not removed efficiently. This is consistent with the nd9 mutation being a defect in the Ca^{2+}-activatable phosphodiesterase pathway, either because the phosphodiesterase itself is defective or because Ca^{2+} activation is defective. nd9-27 parafusin and ATP labeling of parafusin are unaffected by the mutation.

Therefore, the *in vivo* results are completely consistent with the present *in vitro* results that indicate that an αGlc-1-*P* phosphotransferase–phosphodiesterase cycle of parafusin accompanies synchronous exocytosis in *Paramecium*. Removal of αGlc-1-*P* has an interesting consequence in that terminal mannose residues are exposed on the glycoprotein. The possibility that there is a cytosolic-facing mannose receptor that recognizes the exposed mannose residues of deglucosylated parafusin, thus targeting the molecule to a specific membrane site (for example, facilitating membrane fusion at exocytosis), is intriguing. Parafusin is an evolutionarily conserved molecule that is present in most eukaryotic cells examined. A glucosylation–deglucosylation cycle of parafusin may be physiologically significant in other cells as well as in the ciliates. It may be that parafusin is simply one of the cytosolic glycoproteins for which a rapid cycle of change in glucosylation can be demonstrated in response to an external stimulus. The addition and removal of terminal residues of such cytosolic glycoproteins may represent a previously unknown signal transduction mechanism with important implications for the targeting of these proteins in response to stimuli.

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