Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 930–932, February 1989
Cell Biology

Species distribution of a phosphoprotein (parafusin) involved in exocytosis

(membrane/fusion/secretion/*Paramecium*)

B. H. SATIR*, T. HAMASAKI, M. REICHMAN, AND T. J. MURTAUGH

Department of Anatomy & Structural Biology, Albert Einstein College of Medicine, Bronx, NY 10461

Communicated by Berta Scharrer, November 16, 1988 (received for review September 26, 1988)

ABSTRACT **A cytosolic phosphoprotein that appears to function in membrane fusion during exocytosis of secretory products has previously been isolated from *Paramecium tetraurelia*. This phosphoprotein, parafusin, with M_r 63,000, is rapidly dephosphorylated via a Ca^{2+}-dependent process when secretagogues induce exocytosis in competent cells. Dephosphorylation does not occur in exocytosis-incompetent cells. Polyclonal antibodies against purified parafusin have now been used to show that this protein is present in unicellular organisms and cells of metazoan groups of wide evolutionary divergence, such as yeast, insects, and mammals, including humans. These results suggest that parafusin was present early in the history of eukaryotes and that it is of functional importance in the general mechanism of exocytosis and membrane fusion.**

Exocytosis is a common process used by eukaryotic cells for release of most protein-rich secretory products. During biogenesis, the products are packaged in membrane-bounded vesicles and provided with an "address" guiding them to their docking sites below the cell membrane. In regulative, stimulus-coupled exocytosis, the vesicle remains docked until reception of an appropriate stimulus, whereupon the membranes of the vesicle and cell fuse. Unlike constitutive exocytosis, regulative exocytosis takes place only when an appropriate external ligand binds to a cell membrane receptor that stimulates exocytosis. Specific signals and/or messengers generated by this process transduce the received signal into cellular responses. Some of the regulatory molecules or messengers are present in the cytoplasm between the two partner membranes destined to fuse. Changes in the concentrations, conformations, or other properties of these molecules are postulated to induce or facilitate fusion.

The search for specific cytosolic proteins that appear intimately associated with regulation of exocytosis in living cells has proven extremely difficult. However, by using the ciliated protozoan *Paramecium tetraurelia* as a model system, such a molecule has been isolated. This is a phosphoprotein (M_r 63,000), parafusin, which in living cells undergoes a covalent modification (dephosphorylation) upon stimulation of exocytosis (see Fig. 1 and refs. 1–3).

In the regulative exocytosis of trichocyst matrices in *Paramecium*, thousands of release events can be induced synchronously, resulting in a very high signal-to-noise ratio for biochemical, structural, or other studies. The structure of the membrane domain involved in *Paramecium* exocytosis is very well defined, and this enhances the possibility of characterizing the critical components of each compartment (3). In wild-type (WT) cells the exocytotic cell membrane domain is marked by a specific intramembrane particle array, the fusion rosette. Exocytosis and dephosphorylation require the presence of sufficient external Ca^{2+}; in WT cells, stimulated in the absence of Ca^{2+} and in the presence of Mg^{2+}, exocytosis is blocked, even though the membrane microdomain is fusion-competent.

Mutants in the signal-transduction mechanism of regulative exocytosis are available, making it possible to assign changes in structure and biochemistry to specific steps in the sequence of events leading to exocytosis (4, 5). For example, ultrastructural studies using a temperature-sensitive signal transduction mutant (nd9) have revealed that the exocytotic membrane domain is defective at the nonpermissive temperature. Where exocytosis is blocked, the fusion rosette remains unassembled. However, at the permissive temperature the rosettes assemble and exocytosis occurs normally (6). Gilligan and Satir (1) found that parafusin was dephosphorylated upon stimulation of exocytosis in WT and nd9 cells grown at the permissive temperature but that dephosphorylation was inhibited in WT cells stimulated in the presence of high external Mg^{2+} and in nd9 cells at the nonpermissive temperature. Similar results were obtained with a series of additional *Paramecium* mutants (7). Furthermore, Satir and Murtaugh (8) demonstrated the presence of a similar phosphoprotein in *Tetrahymena thermophila* that became dephosphorylated upon stimulation of exocytosis and membrane fusion.

Because of the potential importance of parafusin in secretion in *Paramecium*, we tested whether the molecule was evolutionarily conserved. In this paper we report the presence of immunologically similar proteins of molecular weights similar to parafusin in a wide variety of species and tissues. Therefore, this molecule may be of widespread importance in exocytosis and membrane fusion processes of eukaryotic cells in general.

METHODS

Parafusin was isolated from axenically (sterile medium) grown *P. tetraurelia* and a rabbit polyclonal antibody was raised against it (2). The affinity-purified antibody to parafusin was used as a probe for the presence and size of immunoreactive proteins in homogenates or high-speed supernatants of a wide variety of eukaryotic cells. Protein concentrations were measured by the method of Lowry *et al.* (9). In general, homogeneous populations of cells or tissues of higher organisms were obtained. These were homogenized in 50 mM Tris buffer (pH 7.4) containing 10 mM EDTA and protease inhibitors (aprotinin, leupeptin, and phenylmethylsulfonyl fluoride) in a Polytron (Brinkmann) for 15 sec, three times on ice. In many cases the homogenate was further fractionated by centrifugation for 5 min at 1500 × *g* into a low-speed pellet (P1) and supernatant (S1), and the S1 was spun at 100,000 × *g* for 60 min to yield a high-speed pellet (P2)

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: WT, wild type; S2, 100,000 × *g* supernatant; P2, 100,000 × *g* pellet.

*To whom reprint requests should be addressed.

and supernatant (S2). The appropriate fractions were subjected to SDS/PAGE followed by immunoblotting with the affinity-purified parafusin antibody. Details of these procedures are given in ref. 2.

A crossreactive species at $M_r \approx 63,000$ was considered preliminary evidence that a preparation contained a molecule similar to parafusin. To clarify this further, we performed a series of control experiments in which undiluted affinity-purified parafusin antibody was mixed 1:1 (vol/vol) with either S2 preparations of each tissue or S2 preparations from *Paramecium* and the mixtures were used as antibody for immunoblotting. Immunologically reactive molecules similar to parafusin will bind antibody during this preabsorption, and the reactive band should not be detected when the preabsorbed antibody is used to probe an immunoblot.

RESULTS AND DISCUSSION

Fig. 1 confirms previous observations (1–3). Axenically grown *P. tetraurelia* incorporate $^{32}P_i$ into parafusin. When exocytosis is stimulated by a specific secretagogue, such as trinitrophenol, in the presence of sufficient external Ca^{2+} (0.5 mM), parafusin is dephosphorylated (Fig. 1 *Left*). An affinity-purified rabbit polyclonal antibody prepared against isolated parafusin recognizes both the phosphorylated and dephosphorylated forms of parafusin (Fig. 1 *Right*). Phosphorylated parafusin has two forms that can be distinguished by isoelectric focusing, with pI values of 5.8 and 6.2. In addition, a third, unphosphorylated form was identified, with a pI of 6.3 (2).

From the very first experiments on the isolation of parafusin, it was clear that the phosphorylated protein was present in extremely small amounts in the cytoplasm. Subcellular fractionation of unstimulated WT and nd9 cells in the presence of EDTA, followed by SDS/PAGE and immunoblotting, showed that the phosphoprotein was cytosolic, found primarily in the S2 fraction (Fig. 1). The P2 fraction prepared in the presence of 5 mM Ca^{2+} contained the dephosphorylated parafusin (8). This might mean that dephosphorylated parafusin binds specifically to membranes in the fusion process.

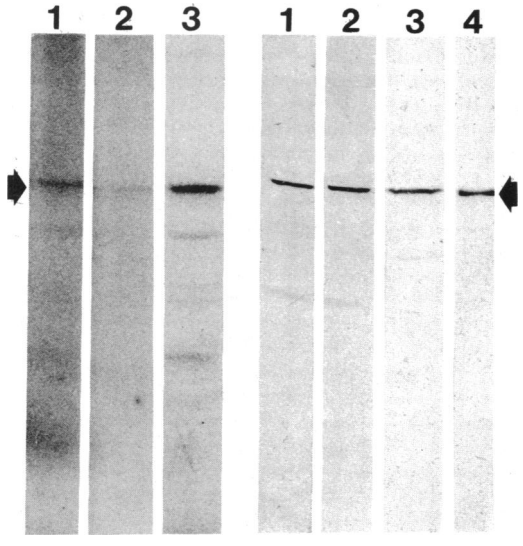

FIG. 1. (*Left*) ^{32}P autoradiogram after SDS/PAGE of three different *P. tetraurelia* samples (10 μg of protein per lane). Lane 1, homogenate of cells labeled metabolically with $^{32}P_i$; note the phosphorylated parafusin (arrow). Lane 2, homogenate of cells labeled with ^{32}P and then stimulated to secrete with trinitrophenol, resulting in dephosphorylation of most of the parafusin. Lane 3, S2 fraction prepared from unstimulated *in vivo* $^{32}P_i$-labeled cells; the major phosphorylated band represents parafusin. See refs. 2 and 8 for experimental details. (*Right*) Corresponding immunoblot probed with affinity-purified parafusin antibody. The antibody recognizes both the phosphorylated and the dephosphorylated form of the protein. Lanes 1–3, as described above. Lane 4, purified parafusin standard (*ca.* 5 μg). See ref. 2 for details of antibody production, affinity purification, and immunoblot procedures.

Fig. 2 shows a typical immunoblot from an experiment in which various rat tissues (lanes 4–11) were tested for the presence of proteins that were similar both in size and in immunological reactivity to parafusin. The reactions with purified parafusin (lanes 1 and 12), *Paramecium* S2 fraction (lane 2), and *T. thermophila* S2 fraction (lane 3) were used as controls. Although there was considerable variation in intensity, all lanes showed reactions at M_r 63,000. The differences in intensity probably reflect differences among the various tissues, although in part they may have resulted from differences in the amount of material applied to the gel, which varied from 0.5 to ≈10 μg. *Paramecium* (10 μg), liver (10 μg), and spleen (7 μg) samples represent the largest amounts of protein loaded. Heart (4.4 μg; lane 8) and liver (lane 10) showed more intense reactions, whereas spleen gave only a faint reaction (lane 11).

In each case the immunologically crossreactive species at M_r 63,000 was not detected when purified antibody was preabsorbed with *Paramecium* S2 or with the individual tissue (rat brain, liver, heart, and adrenal glands) S2 fractions. The resulting mixtures bound neither to *Paramecium* S2 parafusin nor to the corresponding proteins in tissue S2 fractions on immunoblots (data not shown). Thus, the M_r 63,000 polypeptide is able to compete specifically with authentic M_r 63,000 *Paramecium* parafusin for the purified antibody. Hence, rat tissues contain parafusin or a closely related molecule.

Because of the evolutionary divergence between the ciliates and mammals, we investigated whether immunologically crossreactive molecules of similar size to parafusin might be present in other eukaryotic organisms. Fig. 3 shows an immunoblot that addresses this question. Affinity-purified parafusin antibody reacted with polypeptides of $M_r \approx 63,000$ in homogenates of yeast (lane 2), cellular slime mold (lane 3),

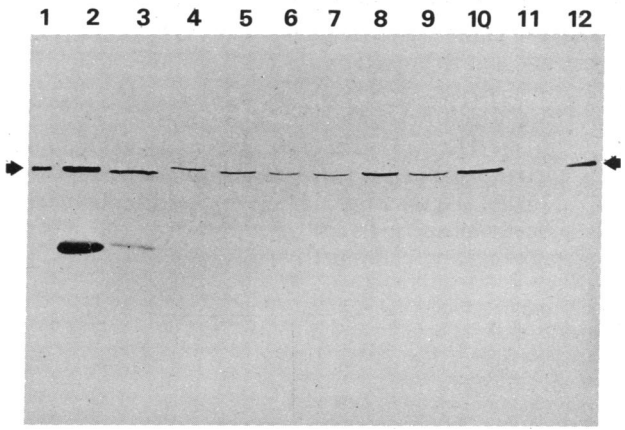

FIG. 2. Immunoblot analysis of various rat tissues probed with purified anti-parafusin. Lanes 1 and 12, purified parafusin (*ca.* 5 μg of protein). Lane 2, *Paramecium* S2 fraction (10 μg); lower molecular weight band (M_r 46,000) presumably represents a breakdown product of parafusin, as can often be detected in freshly prepared S2 fractions. Lane 3, *T. thermophila* S2 [protein not determined (ND)]. Lanes 4–11, S2 fractions of various rat tissues: 4, brain (1.4 μg); 5, pituitary (ND); 6, thyroid (ND); 7, adrenal gland (1.2 μg); 8, heart (4.4 μg); 9, kidney (4.6 μg); 10, liver (10 μg); 11, spleen (7.0 μg). The anti-parafusin detected the presence of a protein similar to parafusin in all the samples tested with the exception of the spleen, where the reaction was not totally negative but was too faint for reproduction in the figure.

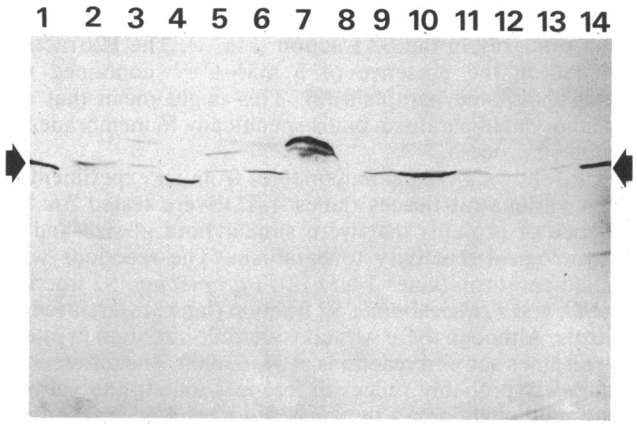

FIG. 3. Immunoblot probed with purified anti-parafusin showing the variety of organisms in which proteins immunologically similar to parafusin are present. Lane 1, parafusin standard (*ca.* 5 μg); lane 2, homogenate of yeast (*Saccharomyces cerevisiae*, kindly provided by S. Henry and J. Marmur) [protein not determined (ND)]; lane 3, homogenate of *Dictyostelium discoideum* (kindly provided by J. Condeelis) (0.5 μg); lane 4, homogenate of ootheca from cockroach (*Leucophaea maderae*, kindly provided by B. Scharrer) (≈ 4 μg); lane 5, homogenate of bladder from toad (*Bufo marinus*, kindly provided by R. Hays) (ND); lane 6, homogenate of isolated calf photoreceptor cells (kindly provided by D. Cohen and P. Satir) (ND); lane 7, homogenate of rat pancreas (5.9 μg); lane 8, S2 fraction of bull sperm (kindly provided by G. Orr) (ND); lane 9, S2 of rat brain (1.4 μg); lane 10, S2 of calf brain (5.6 μg); lane 11, S2 of human brain (1.8 μg) (brain samples kindly provided by S. Goldfischer); lane 12, S2 from rat testes (4.3 μg); lane 13, S2 from cultured neuroblastoma cells (kindly provided by R. Morrison) (ND); lane 14, *Paramecium* S2 (10 μg). All cells and tissues tested except bull sperm show crossreactive polypeptides of apparent molecular weight very similar to that of parafusin (M_r 63,000). In *Dictyostelium*, toad bladder, calf photoreceptor cells, and especially rat pancreas, crossreactive polypeptides at $M_r \approx 72{,}000$ are present. This band is not present in *Paramecium* S2. Bands at $M_r < 63{,}000$ seen with yeast and *Paramecium* S2 are probably breakdown products.

cockroach ootheca (lane 4), toad bladder (lane 5), and isolated calf photoreceptors (lane 6) and in S2 fractions of rat, bovine, and human brain (lanes 9–11). Reaction was also seen with rat pancreas homogenate (lane 7) and testis S2 fraction (lane 12) and with an S2 fraction of a human neuroblastoma cell line (lane 13). In several cases there was an additional crossreactive band at a somewhat higher molecular weight ($M_r \approx 72{,}000$). We were unable to detect significant reaction with isolated bull sperm (lane 8), but this may mean only that the amount of S2 preparation from this source was too small.

Therefore, in addition to the ciliates, a M_r 63,000 polypeptide that is immunologically similar to parafusin seems to be present in other unicellular organisms and in several metazoan groups of wide evolutionary divergence, including insects and mammals. These results suggest that such a protein was present early in the history of eukaryotes. A functional role for parafusin has been demonstrated for stimulus-coupled exocytosis in *Paramecium* and *Tetrahymena*, although elucidation of the effects of dephosphorylation and cellular redistribution of the molecule need further exploration. In this regard it is of interest that preliminary data show that parafusin is the major substrate for glucose-1-phosphotransferase in *Paramecium* (10). Further, a protein of similar molecular weight has been shown to be the predominant acceptor recognized by this enzyme in a variety of cells and tissues of other species including rat liver (11). One can speculate that perhaps in all eukaryotic cells, parafusin may be the substrate for this enzyme. It would be interesting to know how the glycosylation affects the distribution of parafusin in the cell and its potential interaction with membranes. Given the ubiquity of the species immunologically related to parafusin, it is probable that parafusin is of fundamental importance in the mechanism of membrane fusion and exocytosis or related phenomena in most eukaryotic cells.

This work was supported by U.S. Public Health Service Grant GM32762 to B.H.S.

1. Gilligan, D. M. & Satir, B. H. (1982) *J. Biol. Chem.* **257**, 13903–13906.
2. Murtaugh, T. J., Gilligan, D. M. & Satir, B. H. (1987) *J. Biol. Chem.* **262**, 15734–15735.
3. Satir, B. H., Busch, G., Vuoso, A. & Murtaugh, T. J. (1988) *J. Cell. Biochem.* **36**, 429–443.
4. Pollack, S. (1974) *J. Protozool.* **21**, 352–362.
5. Beisson, J. & Rossignol, M. (1975) in *Molecular Biology of Nucleocytoplasmic Relationships*, ed. Puiseaux-Dao, S. (Elsevier-North Holland, Amsterdam), pp. 291–294.
6. Beisson, J., Lefort-Tran, M., Pouphile, M., Rossignol, M. & Satir, B. H. (1976) *J. Cell Biol.* **69**, 126–143.
7. Zieseniss, E. & Plattner, H. J. (1985) *J. Cell Biol.* **101**, 2028–2035.
8. Satir, B. H. & Murtaugh, T. J. (1988) in *Molecular Mechanism of Membrane Fusion*, eds. Ohki, S., Doyle, D., Flanagan, T. D., Hūi, S. W. & Mayhew, E. (Plenum, New York), pp. 513–520.
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
10. Satir, B. H., Reichman, M., Srisomsap, C. & Marchase, R. B. (1989) *J. Cell Biol.* **107**, 404a (abstr.).
11. Srisomsap, C., Richardson, K. L., Jay, J. C. & Marchase, R. B. (1989) *J. Biol. Chem.* **263**, 17792–17797.