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Involvement of a 65 kDa phosphoprotein in the regulation of membrane fusion during exocytosis in *Paramecium* cells

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Antisera were raised against a phosphoprotein of 65 kDa (PP65) from *Paramecium* cells (shown before to be selectively dephosphorylated during synchronous exocytosis) and specified by immunoblotting. By immunofluorescence PP65 has been localized within the cortex, beneath the cell membrane. This corresponds to data obtained by cell fractionation, applying SDS-PAGE autoradiography to cortices prepared from ^{32}P-prelabeled cells. Antisera against PP65 inhibit exocytosis in vivo (microinjection). Applying anti-PP65 antisera in vitro to cortices we could demonstrate inhibition not only of exocytosis, but also of PP65 dephosphorylation. We conclude that PP65 is involved in the regulation of membrane fusion during exocytosis.

Exocytosis; Membrane fusion; Phosphoprotein; (*Paramecium*)

1. INTRODUCTION

The regulation of membrane fusion during exocytosis is poorly understood [1,2]. This also concerns the possible role of phosphoproteins [3–7].

Here we used *Paramecium tetraurelia* cells, since they allow us to analyze quite selectively exocytotic membrane fusion: their secretory organelles (trichocysts) are tightly docked at the cell membrane for immediate, synchronous (≤ 1 s) release by membrane fusion [8]. Furthermore, isolated cell cortices can be prepared to study exocytotic membrane fusion in vitro without the involvement of endocytosis coupling [9].

We have recently demonstrated that during exocytosis a phosphoprotein of 65 kDa (PP65) is selectively and reversibly dephosphorylated [10]. This process can be triggered by exogenous phosphatases [11,12]. Calcineurin might be the physiological equivalent that initiates membrane fusion by PP65 dephosphorylation [11].

We now present more direct evidence of the involvement of PP65 dephosphorylation in membrane fusion regulation. This is based on data obtained with antisera raised against PP65, which were used for functional tests in vivo and in vitro and for immunofluorescence localization.

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2. MATERIALS AND METHODS

2.1. *Cell cultures*

Sterile cultures of *P. tetraurelia* wild-type (*7S*) cells were grown to early stationary phase [10] and washed twice with Pipes buffer (5 mM; 1 mM $CaCl_2$, 1 mM KCl; pH 7.0) before further use.

2.2. *^{32}P labeling*

Two protocols, both using $^{32}PO_4^{3-}$ from NEN (Braunschweig) as in [10], were applied. Cells were transferred for 10 h to Pipes buffer (section 2.1) before ^{32}P labeling. (i) For thorough labeling of all

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endogenous protein substrates labeling was conducted over 4 h. (ii) Preferential labeling of PP65 was obtained by reducing the time period available for ^{32}P uptake to ≤ 120 min and subsequent synchronous (≤ 1 s) exocytosis triggering by aminoethyldextran (AED) as in [10]. Since rephosphorylation is rather selective for PP65 and since this takes only < 1 min [10], these cells were rapidly solubilized 1 min after AED triggering. For (i) and (ii) cells were inactivated and solubilized in boiling 'sample buffer' [125 mM Tris-HCl, 10% (w/v) SDS, 20% (v/v) glycerin, 10% (v/v) mercaptoethanol]. Protocol (i) has also been applied before isolating cortices (see below).

2.3. *Cortex preparations and exocytosis in vitro*

Cortices, i.e. cell surface complexes containing surface membranes and attached trichocysts, were prepared by gentle homogenization [9]; [9] also indicates the mode of triggering by decreasing the ratio of $[Mg^{2+}]$ to $[Ca^{2+}]$; final free $[Ca^{2+}]$ was 10^{-5} M.

The effect of anti-PP65 antisera or of pre-immune sera on membrane fusion was tested by counting the number of trichocysts released [9]. The distribution of PP65 between pellets and supernatants (precipitated with cold 15% trichloroacetic acid) obtained from cortex preparations, using different centrifugation steps ($180 \times g$ for 5 min, $5500 \times g$ for 15 min), as well as the effect of anti-PP65 antisera on PP65 dephosphorylation was quantified as indicated below.

2.4. *SDS-PAGE and electroelution*

Samples (cells or cortices) were dissolved in boiling sample buffer (see section 2.2) and developed on 2 mm thick polyacrylamide gels as in [10]. Coomassie blue-stained 65 kDa bands were cut out ($\pm$ 1 kDa, without any recognizable additional band), destained with isopropanol [13] and electroeluted with phosphate-buffered saline (PBS) applying 15 mA (48 h, 4°C). PP65 was washed and concentrated with a Centricon device (Amicon, Danvers, MA) using a limit pore size of 10 kDa. When this band was eventually subjected to re-electrophoresis, only one band could be recognized.

The ^{32}P labeling intensity was either directly visualized on autoradiograms or by liquid scintillation counting [10]. For this purpose the PP65 band (as well as other bands to ascertain the selectivity of PP65 dephosphorylation) has been cut out and solubilized as in [10].

2.5. *Protein staining and preparation of autoradiograms from SDS-PAGE preparations*

Coomassie blue staining and preparation of ARG also followed methods previously used [10].

2.6. *Antibody production*

Anti-PP65 antisera were produced in adult Balb/c mice in different ways. (i) Polyclonal sera were obtained by weekly injections of 50 μg electroeluted PP65 (see section 2.4) into the hind food pads. Primary and secondary injections were supplemented with complete Freund's adjuvant. (ii) Other sera were produced by weekly i.p. injections of homogenized gel bands (washed and destained) containing 50 μg PP65 (without adjuvant). Mice

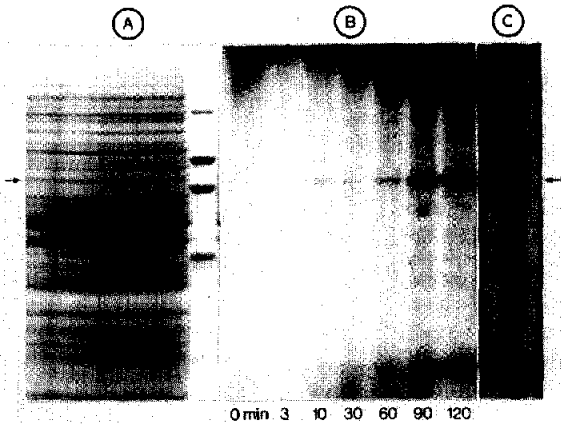

Fig.1. (A) SDS-PAGE from cell homogenates, as used for the preparation of PP65 (molecular mass standards: phosphorylase *a*, bovine serum albumin, catalase, aldolase and chymotrypsinogen A, 95, 67, 60, 40 and 25 kDa, respectively). The identity of PP65 (arrows) was ascertained in (B) by autoradiography after selective phosphorylation after AED triggering [see section 2.2(b)]. The time indicated below (B) refers to the duration of ^{32}P incubation of cells before a de-/rephosphorylation cycle. (C) Western blot; mouse anti-PP65-AS was applied to a cell homogenate blotted onto nitrocellulose and visualized as indicated in section 2.7 (left lane); in the right lane pre-immune serum has been used. Amounts of protein applied: 110 μg/cm lane width for cell lysate (A); 150 μg and 70 μg per lane in (B,C).

were bled ≥4 weeks after primary immunization (i,ii). (iii) The popliteal lymph nodes and spleens of mice immunized as in (i) were removed, and the cells were fused with the myeloma cell line P3-X63-Ag8.653 in a ratio of 2:1, according to [14]. Resulting hybridoma cells were cloned by limiting dilution. Cell lines were grown in mice to produce anti-PP65 antisera. Due to the restricted number of recloning cycles antibodies obtained cannot yet be considered as strictly monoclonal.

All sera were tested by methods outlined in sections 2.3 and 2.7–2.10. Given the restricted amount of sera and since no systematic differences could be ascertained as yet, sera were pooled from these different sources.

2.7. *Western blots*

Proteins were transferred onto nitrocellulose (60 V, 150–300 mA, 5 h) according to [15] and masked with 5% milk powder in Tris-buffered saline (TBS) with 0.05% Tween 20 added (pH 7.6, 20 min). This was followed by a wash step (2 × 5 min), 12 h incubation with anti-PP65 antisera (diluted 1/50) in TBS + 0.25% milk powder (omitted in controls), 3 × 10 min wash and an incubation with a rabbit anti-mouse IgG–peroxidase conjugate (7 μg/ml in the same solvent, 4 h) obtained from ICN Immuno-Biologicals (Eschwege, FRG). After 4 washes (5 min) peroxidase was visualized by a 4-chloro-1-naphthol (in methanol) reaction. TBS-Tween 20 was used for washes.

Western blots were prepared from cell homogenates (or, for controls, from re-electrophoresed PP65 bands, showing unaltered location). Protease inhibitors, as indicated in [9] and with additional leupeptin and pepstatin (20 or 1 μg/ml, respectively; from Sigma, St. Louis, MO), were added with the first homogenization stroke.

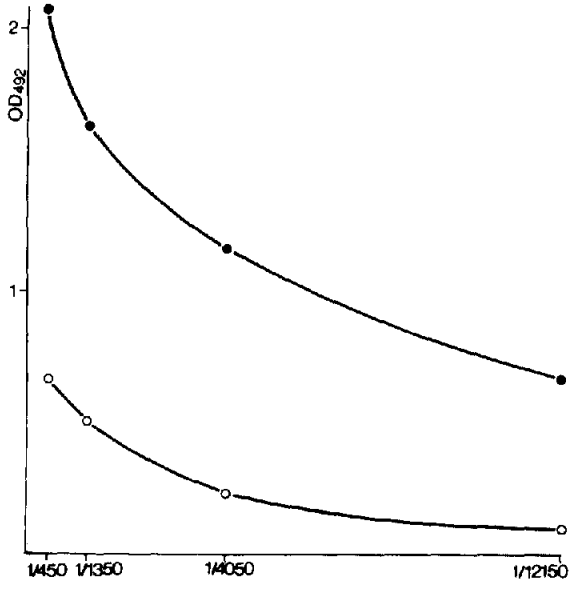

Fig.2. ELISA carried out by using electroeluted PP65 as an antigen and six pooled antibody preparations (●) or pre-immune sera (○), followed by rabbit anti-mouse IgG–peroxidase. Ordinate, absorbance of peroxidase reaction product; abscissa, dilution of sera.

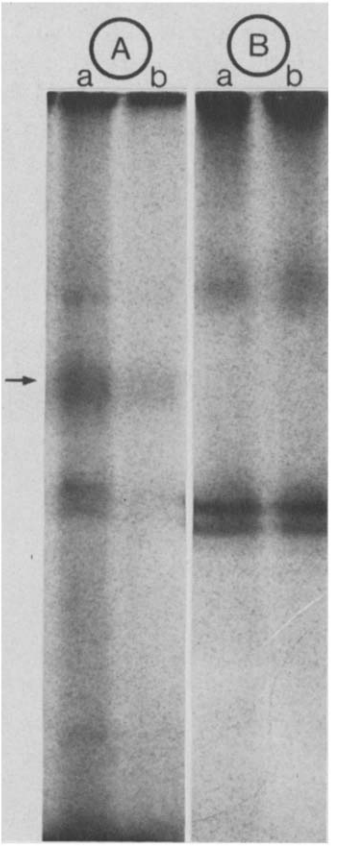

Fig.3. SDS-PAGE applied to cortices from ^{32}P-prelabeled cells. (A) shows the presence of PP65 (arrow) in isolated cortices after the first (a) and second (b) centrifugation steps (see section 2.3), respectively. No PP65 band is seen in the corresponding supernatants (B; a,b).

2.8. *ELISA*

This was performed according to [16], modified as before [17]. The antigen adsorbed to the test vials was electroeluted PP65 in PBS (see section 2.4)

2.9. *Microinjection*

The method applied was as in [18]. Volumes injected amounted to ~10% of the cell volume. 5–10 min after injection AED was added to the cell medium to trigger trichocyst exocytosis (see section 2.2) and the number of released or retained trichocysts was estimated in a microscope equipped with Nomarski optics.

2.10. *Immunofluorescence labeling*

Cells were fixed with 2.5% (w/v) formaldehyde and 0.25% (v/v) glutaraldehyde (0°C, 15 min) in 0.1 M cacodylate buffer (pH 7.0) containing 1% saponin for permeabilization. After two washes with Tris-maleate buffer (50 mM, pH 7.0), containing 50 mM NH_4Cl and glycine, cells were incubated with anti-PP65 antisera (1 h), washed and exposed to rabbit anti-mouse IgG–FITC (1 h) from Nordic (Tilburg, The Netherlands), all at 0°C, and washed before fluorescence microscopy as in [18].

The following controls were carried out: (i) omission of the primary antibodies, (ii) omission

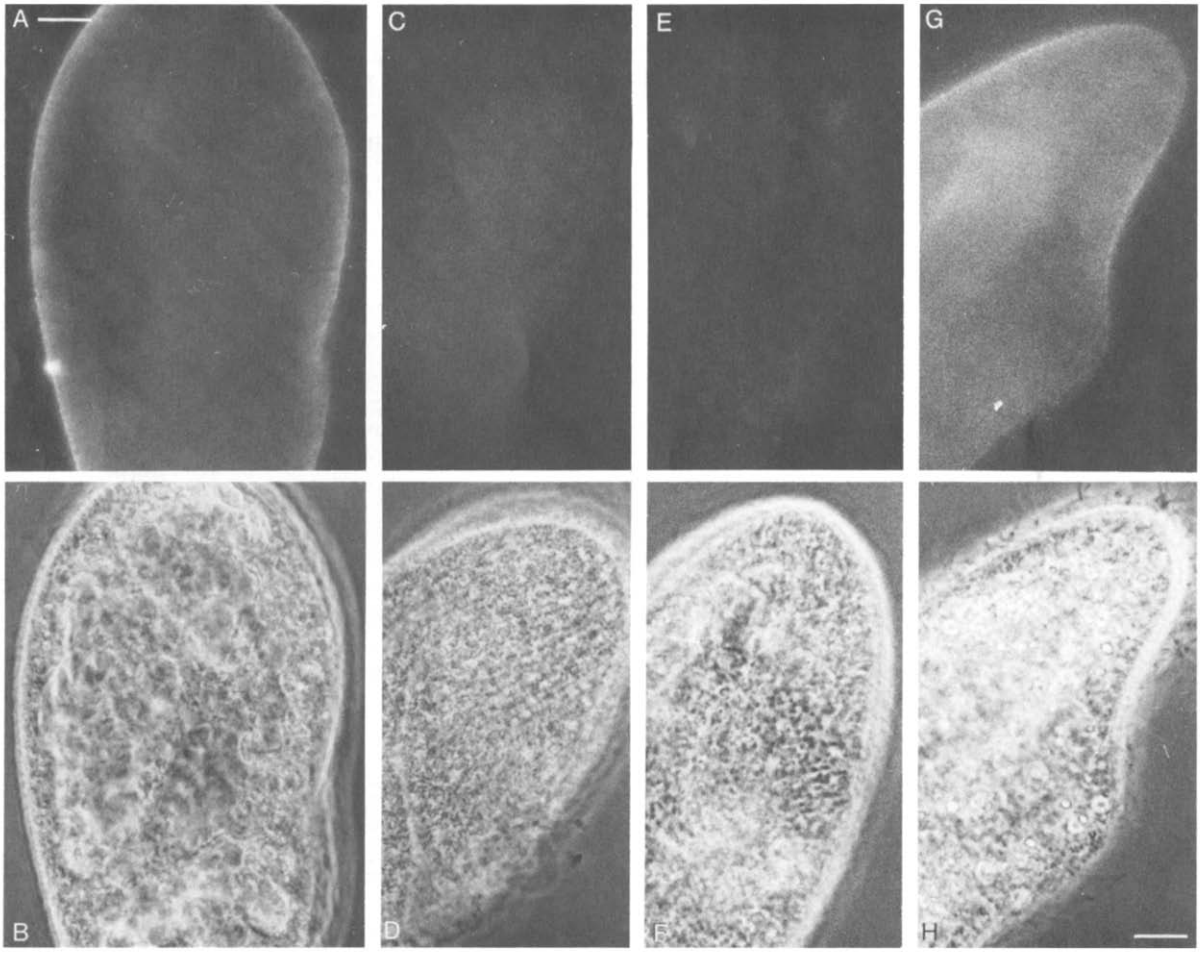

Fig.4. Subplasmalemmal localization of PP65 in slightly fixed and permeabilized cells, using indirect immunofluorescence (A) and phase contrast imaging (B). Controls involve omission of primary antibody (C,D) or of cell permeabilization (E,F) and the demonstration of calmodulin (G,H) in the cell cortex and throughout the cell body as in [17]. Bar, 10 μm (A–H).

of permeabilization or (iii) the use of anti-calmodulin antibodies (specified in [17]) as a primary antibody (to test the permeabilization protocol; see [17]).

3. RESULTS

Fig.1 shows that antisera raised against the 65 kDa phosphoprotein (anti-PP65-AS) band (panel A) selectively recognize the PP65 band in Western blots obtained from cell homogenates (panel C). This band coincides with the rather selective ^{32}P labeling of PP65 on autoradiograms obtained according to the following principle. Phosphorylation of other endogenous substrates (see [10]) was restricted by reducing $^{32}P_i$ uptake to ≤ 120 min, followed by synchronous exocytosis triggering by AED. This entails a dephosphorylation (≤ 1 s) $\longrightarrow$ rephosphorylation (≤ 1 min) cycle selective for PP65 [see section 2.2 (ii)]. This band (the autoradiogram of fig.1B) coincides with the immunoreactive band of PP65 on the Western blot (fig.1C).

Results from ELISA (fig.2) show concentration dependency of the binding of anti-PP65-AS to PP65.

The cortical localization of PP65 has been substantiated by two widely different methods. (i)

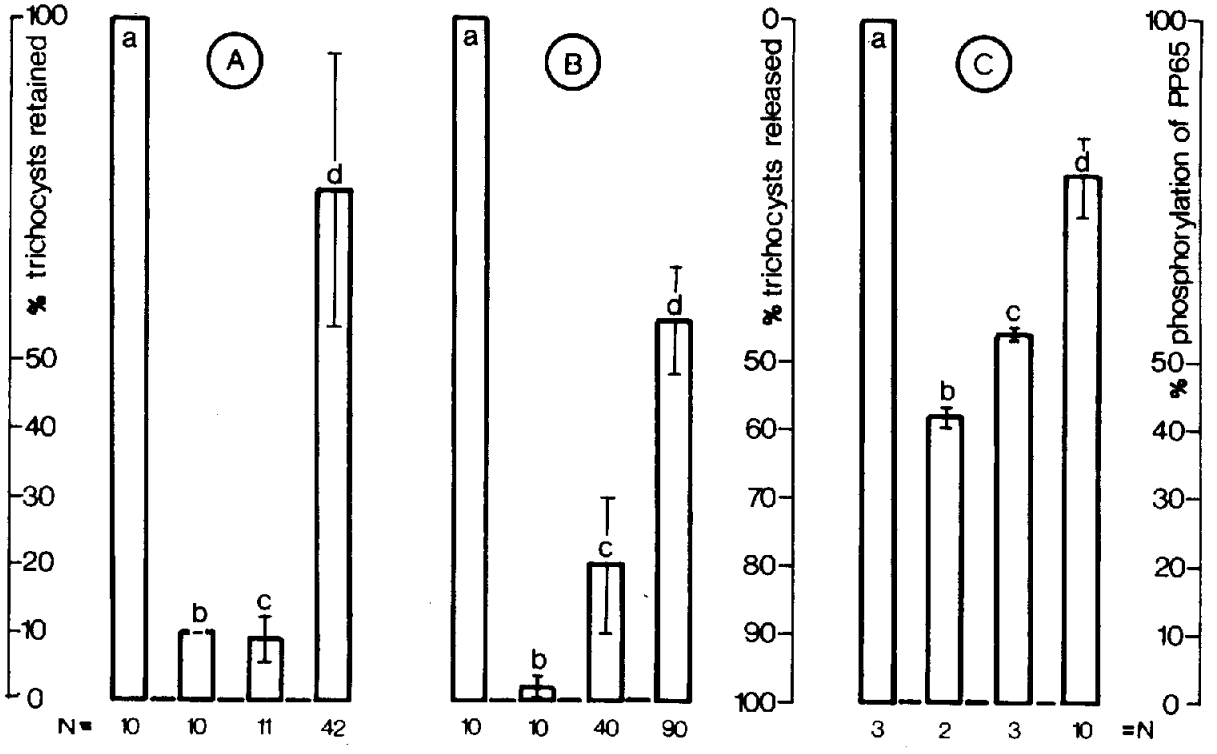

Fig.5. (A) Effect of anti-PP65-AS (after microinjection in a dilution 1:20) on exocytosis performance (or trichocyst retention, respectively) in vivo in response to exogenous AED (see section 2.2). (a) Non-injected, non-triggered control (reference value: 100% retention $\hat{=}$ 0% exocytosis). (b) Fully triggered non-injected control. (c) Control after pre-immune serum injection and AED triggering. (Similar negative results were obtained with non-specific antibodies or with 'injection buffer' alone; cf. [11].) (d) Exocytosis inhibition after anti-PP65-AS injection, followed by AED triggering. (B) Effect of anti-PP65-AS (dilution 1:50) on exocytosis in vitro; test object: isolated cortices; trigger procedure as outlined in section 2.3. Scales are as in (A). (a) Control without antibodies; no triggering (reference value). (b) Fully triggered control without antibodies. (c) Control with pre-immune serum and triggering. (d) Exocytosis inhibition by triggering in the presence of anti-PP65-AS. (C) Effect of anti-PP65-AS (dilution 1:50) on the dephosphorylation of PP65 in vitro. (a–d) As in (B). Scale: 100% = full phosphorylation of PP65. *N*, number of samples analyzed; bars, SD. Values for (a) are normalized to 100% in (A–C).

SDS-PAGE autoradiography, obtained from cortices prepared from ^{32}P-prelabeled cells, reveals the occurrence of PP65 in the cortex and its absence from the cytosol (fig.3). (ii) By indirect immunofluorescence we have demonstrated the subplasmalemmal localization of PP65 (fig.4A,B). (The methods currently applicable do not allow us yet to localize PP65 with a higher degree of precision, i.e. to determine whether PP65 would occur exclusively on the sites of trichocyst exocytosis.) The subplasmalemmal localization has been further verified by various controls contained in fig.4 (C–H): Omission of the anti-PP65-AS incubation step (fig.4C,D) or of cell permeabilization (fig.4E,F) prevents fluorescence labeling. Antibody penetration has been controlled by experiments using antibodies against calmodulin, since calmodulin had previously been shown to be localized also in deeper cell regions [17]; fig.4G,H assures that antibody penetration was not restricted by the fixation and permeabilization protocol used.

Anti-PP65-AS considerably inhibits exocytosis in vivo (fig.5A) and in vitro (fig.5B). Pre-immune sera or non-specific antibodies never exert a similar effect as do anti-PP65-AS. Experiments in vitro also allowed us to demonstrate that dephosphorylation of PP65, which in vivo parallels trichocyst release [10,19], is considerably inhibited by anti-PP65-AS, but not by pre-immune sera (fig.5C). Normally PP65 is selectively, though only partly, dephosphorylated also during exocytosis in vitro [11], as it also occurs in fig.5C(b). PP bands in the range of 26 and 39–50 kDa (cf. [10]), analyzed here for comparison, remained unaltered during exocytosis in vitro (not shown).

Although the anti-PP65-AS we used was obtained against denatured PP65, we can reasonably assume that it also reacts with the native antigen, since it simultaneously inhibits exocytosis and the dephosphorylation of native PP65 (fig.5B,C) in cortices, where it also is localized exclusively (fig.4).

4. DISCUSSION

4.1. *Aspects pertinent to the involvement of phosphoproteins in exocytosis regulation*

In different systems a variety of proteins become phosphorylated, whereas only some proteins are eventually dephosphorylated [6,20,21] in response to secretory stimuli. However, there is no conclusive evidence of a direct involvement of any of these phosphoproteins in membrane fusion regulation. Only with *Paramecium* cells has it been unequivocally established that a specific phosphoprotein of 65 kDa (PP65) is selectively dephosphorylated in the course of exocytosis performance [10,11,19]. The inhibitory effects of anti-PP65-AS on exocytosis and the cortical localization of PP65 reported here furnish the first direct evidence for a key role of PP65 in the regulation of membrane fusion during exocytosis.

In other (non-synchronous) exocytotic systems, there might occur considerable overlap of dephosphorylation/rephosphorylation processes (see [10,11] for a discussion) and a variety of quite different phenomena other than membrane fusion might be involved. This includes the phosphorylation of a variety of intracellular components as well as of proteins bound to secretory organelle membranes [22] and of cell membrane constituents, such as ion channels [23], ion pumps [24], receptors [25,26], etc. Proteins undergoing dephosphorylation have rarely been thoroughly analyzed. Moreover, as shown in fig.1B, dephosphorylation (if reversible) can result in net phosphorylation. Concomitantly, in mast cells protein phosphorylation seems to terminate rather than to initiate exocytosis in response to compound 48/80 [27] or to antibody triggering [28].

4.2. *Aspects pertinent to the present system*

The PP65 is clearly localized in the outermost zone of *Paramecium* cells and thus could be able to control exocytotic membrane fusion. The gross distribution pattern corresponds to that of calmodulin [17] and of calcineurin [11], the Ca^{2+}-calmodulin-stimulated phosphoprotein phosphatase EC 3.1.3.16 [29,30] which we had also identified with *Paramecium* cells [11]. Accordingly, we had shown before (by exocytosis inhibition using monospecific antibodies) that both of these antigens might be involved in PP65 dephosphorylation and, in parallel, in the regulation of membrane fusion in *Paramecium* cells [11]. Data obtained with different inhibitory drugs and compounds [12] support this hypothesis. It now appeared important to demonstrate that antibodies against the target molecule, PP65, are also able to

inhibit exocytosis performance and the dephosphorylation of PP65 (fig.5). In vitro, the effect of anti-PP65-AS on PP65 dephosphorylation was less pronounced than its effect on exocytosis. This is not surprising, since we also previously found only partial dephosphorylation with in vivo experiments [10].

4.3. *Aspects pertinent to a general mechanism of membrane fusion*

Membrane-integrated and -associated proteins are now generally assumed to occur at the sites of exocytotic membrane fusion, based on ultrastructural [31,32] and biochemical data [10,17]. While the identity and role of integral membrane proteins remain poorly understood, membrane-associated proteins could in part be identified (see section 4.2). According to studies with model systems synexin and related proteins were also considered as fusogenic proteins [22,33,34]. Yet, so far, no conclusive proof of the involvement of any of these proteins in membrane fusion regulation in vivo has been presented.

Dephosphorylated PP65 might induce membrane fusion by virtue of its temporarily increased hydrophobicity. This implication would correspond, in principle, to the fusion mechanism which is now generally accepted for myxoviruses ([35]; reviews [36,37]). This may or may not involve partial proteolysis (depending on the virus type; review [37]), a mechanism to be excluded for our system on the basis of an unaltered M_r of PP65 [10]. The triggering effect we observed with microinjected trypsin [12] might be due to its activation effect on calcineurin [30]. Since the structure and activation of fusogenic proteins are different even with ortho- and paramyxoviruses (see [37]), it would not appear surprising that this also holds for membrane fusion during trichocyst exocytosis. Interestingly, an increasing number of papers dealing with artificial systems also shows the induction of membrane fusion by more or less hydrophobic proteins [33,34,38,39]. Since immunoblots from adrenal medullae also reveal a 65 kDa band crossreacting with anti-PP65-AS (unpublished), this might indicate a widespread fusion mechanism. To our knowledge neither calcineurin nor PP65 has ever been envisaged before in the literature to regulate possibly exocytotic membrane fusion.

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