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A cortical phosphoprotein ('PP63') sensitive to exocytosis triggering in *Paramecium* cells

Immunolocalization and quenched-flow correlation of time course of dephosphorylation with membrane fusion

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We had previously shown that a phosphoprotein of 63 kDa ('PP63') is rapidly and selectively dephosphorylated during synchronous ($\leqslant$ 1 s) trichocyst exocytosis in *Paramecium* cells and then rephosphorylated within $\leqslant$ 1 min [Zieseniss & Plattner (1985) J. Cell Biol. **101**, 2028–2035]. Using a new quenched-flow device, we now find a strict correlation between PP63 dephosphorylation and the process of membrane fusion, both occurring within 80 ms. Uptake of ^{32}P over 90 min, followed by exocytosis and rephosphorylation for 1 min, results in a rather selective phosphorylation of the dephosphorylated form, P63, to PP63. Solubilization by repeated freezing and thawing allows isolations of P63 and PP63. On isoelectric focusing autoradiograms they have pI values of 6.05, 5.95 (major spots), 5.85 and 5.75. All spots are sensitive to alkaline, but not to acidic, hydrolysis (except for the pI-6.05 spot). On two-dimensional-gel autoradiograms the most prominent spot, of pI 5.95, is most extensively de- and re-phosphorylated. This spot, from de- and re-phosphorylated samples, was used to produce monospecific antibodies. A cortical localization of PP63 was revealed by producing Western blots from isolated cell-surface fragments ('cortices') and by immunofluorescence labelling. We assume that both P63 and PP63 are attached to cortical structures, e.g. around trichocysts, though they are partly soluble. This localization and the strict correlation of PP63 dephosphorylation with exocytotic membrane fusion suggests a role in fusion regulation.

INTRODUCTION

Though a change in the degree of phosphorylation of different proteins is a key event accompanying triggered secretory activity in many cells, no phosphoprotein (PP) has so far been shown to be directly involved in the regulation of vesicle–cell-membrane interaction for fusion (for reviews, see Hemmings *et al.*, 1989; Plattner, 1989; Burgoyne, 1991). Some of the uncertainties come from superposition of different steps, such as organelle docking, membrane fusion, resealing and internalization. A synchronous system allowing for the selective analysis of specific steps would be of evident advantage.

This is the case with *Paramecium* cells, whose trichocysts can be released synchronously upon triggering with certain polyaminated secretagogues (Plattner *et al.*, 1985). The recent development of a quenched-flow procedure applicable to such fragile cells (Knoll *et al.*, 1991) now allows for simultaneous analysis by ultrastructural and biochemical methods, specifically of the membrane fusion process. The present investigation was focused on the dephosphorylation of a PP of 63 kDa (PP63, formerly 65 kDa), a salient feature of trichocyst exocytosis (Gilligan & Satir, 1982; Zieseniss & Plattner, 1985). We analysed the precise time course of PP63 dephosphorylation, the occurrence of PP63 isoforms of different pI values and their sensitivity to exocytosis triggering, as well as the subcellular localization of PP63 and its dephosphorylated form (P63). We prepared monospecific antibodies for Western blots and for immunocytochemical localization.

The time course of dephosphorylation of PP63 and its cortical localization strongly suggest a regulatory role in membrane fusion regulation.

MATERIALS AND METHODS

Cell cultures

Paramecium tetraurelia wild-type cells (strain *7S*) were grown in axenic medium (Kaneshiro *et al.*, 1979) to early stationary phase and transferred overnight to 5 mM-Pipes buffer, adjusted with HCl to pH 7.0 and supplemented with 1 mM-$CaCl_2$ and 1 mM-KCl.

Exocytosis stimulation

Synchronous exocytosis was induced (in the presence of 0.1 mM-Ca^{2+}) by 0.005% (w/v) aminoethyl-dextran (AED; 40 kDa, one amino group per kDa of dextran) as previously described (Plattner *et al.*, 1985), either manually or in the quenched-flow mode (see below).

^{32}P labelling of proteins

(A) General phosphoprotein labelling. Cells (6×10^4/ml; 10 ml) were incubated with 37×10^6 Bq (1 mCi) of carrier-free [^{32}P]P_i (Amersham–Buchler, Braunschweig, Germany) for 3 h at room temperature. Under these conditions, endogenous PPs are intensely labelled.

(B) Labelling of PP63 during de-/re-phosphorylation. Cells were ^{32}P labelled for only 90 min. Samples were taken without AED (0 s) or 1 s and 1 min after AED triggering, since this allows selective analysis of the de- and re-phosphorylated states, P63 and PP63 respectively (Stecher *et al.*, 1987). In some cases 7.5 mM-EGTA was added to cells 1 min before AED triggering. Inactivation was by manual injection into an excess of boiling 'sample buffer' (see below).

Abbreviations used: AED, aminoethyl-dextran; IEF, isoelectric focusing; PP, phosphoprotein; P63, dephosphorylated form of 63 kDa PP; PP63, phosphorylated form of 63 kDa PP.

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Sample processing by quenched-flow

Cells subjected to general ^{32}P labelling (see above) were diluted to 3×10^4/ml and processed in the quenched-flow apparatus designed by Knoll *et al.* (1991). This included mixing with an equal volume of 0.01 % AED or, for controls, with Pipes buffer, and rapid freezing at precisely defined time points after triggering (0 = untriggered controls, or 80 and 200 ms after mixing with AED). Propane (used as a cryogen) was evaporated at 231 K and ambient pressure, then the ice powder was immersed in methanol at 193 K for freeze-substitution. After a slow (approx. 5 K/h) rise in temperature to 278 K, the samples were centrifuged, and supernatant was decanted. The pellet was dried, suspended in boiling sample buffer (see below) and used for PAGE as indicated below. For autoradiography, the background was decreased by washing gels with HCl (see below).

The time course of exocytosis was determined in samples by electron-microscopic methods after quenched-flow freezing as described by Knoll *et al.* (1991).

Isolation of cell cortex fragments

Cortices were prepared as described by Vilmart-Seuwen *et al.* (1986). They were either used immediately by dissolving in boiling sample buffer or after further fractionation by freezing and thawing (up to five times), followed each time by centrifugation at 100000 ***g*** for 1 h (277K). Supernatants were additionally centrifuged as above, concentrated with a Centricon apparatus (Amicon, Beverly, MA, U.S.A.) with a cut-off of 10 kDa, precipitated with 10 % trichloracetic acid, re-centrifuged (21000 ***g*** for 30 min, 277K) and resuspended in 0.1 M-HCl in acetone, and then in methanol, each time again followed by the same centrifugation step. The resulting pellets and supernatants were dissolved in boiling sample buffer.

Solubilization of (P)P63

***(a)* Mechanical break-up.** After ^{32}P labelling (method B), without AED or after AED triggering, cells were homogenized in 50 mM-Tris/HCl, pH 7.4, with 10 mM-EDTA, 30 mM-NaF, 5 mM-phenylmethanesulphonyl fluoride and 1 mM-vanadate (both freshly dissolved) added. We used a glass homogenizer (type S135, from Braun, Melsungen, Germany) with a loosely fitting Teflon pestle, approx. 70 strokes at 10^3 rev./min. Centrifugations, precipitation with trichloracetic acid and dissolving were as for cortices (see above).

***(b)* Repeated freezing and thawing.** ^{32}P-labelled cells (method B) were repeatedly frozen (in liquid propane/nitrogen) and thawed, then centrifuged etc. as in the preceding protocol. For isoelectric focusing and two-dimensional gel electrophoresis, supernatants were concentrated with a Centricon and used in native form.

Protein determination

The standard method of Bradford (1976) was used.

Dissolving samples for application to gels

Samples were injected into boiling 'sample buffer', i.e. 125 mM-Tris/HCl buffer, pH 6.8, with 10 % (w/v) SDS, 20 % (v/v) glycerol and 10 % (v/v) β-mercaptoethanol added (inactivated by adding 50 mM-iodoacetamide for 1 h before application to gels).

Gel electrophoresis

SDS/PAGE. For this, 10 %-acrylamide gels were prepared as described by Laemmli (1970), routinely stained with Coomassie Blue R250 or processed for autoradiography or Western blots. Molecular-mass markers were cytochrome *c* (12.4 kDa), chymotrypsinogen A (25 kDa), aldolase (40 kDa), catalase (60 kDa), BSA (67 kDa) and phosphorylase *a* (95 kDa).

Some gels were processed as follows. To some samples we added 1 mM-$CaCl_2$ or 5 mM-EGTA before subjecting them to SDS/PAGE and Coomassie Blue staining or autoradiography. Occasionally 'Stains all' was applied, as described by Campbell *et al.* (1983).

Western blots. These were done as described by Towbin & Gordon (1984). For the purpose of purification, antibodies obtained from rabbits as specified below were applied in 1:50 dilution, followed by goat anti-rabbit IgG–peroxidase from Dianova (Hamburg, Germany). For immunolabelling of molecular-mass markers, an antiserum against thyroglobulin (330 kDa), ferritin (220 kDa subunit), BSA (67 kDa), catalase (60 kDa), lactate dehydrogenase (36 kDa) and ferritin (18.5 kDa) was produced in rabbits as described by Bjerrum & Hinnerfeldt (1987). Antibody–peroxidase labelling was detected by the 'enhanced chemiluminescence' (ECL) method (Pollard-Knight *et al.*, 1990) with an ECL kit from Amersham-Buchler.

Isoelectric focusing (IEF)

Proteins obtained by freeze–thaw solubilization after ^{32}P labelling (method B) were applied to Ampholine PAG plates pH 4.0–6.5 from LKB (Freiburg/Br., Germany). Calibration was with a coloured protein set (phycocyanin, pI 4.65; β-lactoglobulin B, pI 5.1; bovine or human carbonic anhydrase, pI 6.0 or 6.5 respectively) from Bio-Rad (Munich, Germany). Samples were placed in the middle or on the cathodic side of the gel, but results obtained by autoradiography were the same.

Alkaline or acid hydrolysis

Some SDS/PAGE gels or pI gels were subjected to alkaline or acid hydrolysis as described by Bourassa *et al.* (1988) and Huttner (1984) respectively.

Two-dimensional electrophoresis

After IEF, gel strips with samples or markers were put on to 4 % collecting polyacrylamide gels and transferred to 10 % gels, with SDS. Two-dimensional gels were stained with Coomassie Blue or with silver.

Autoradiography of gels

An Amersham Hyperfilm-MP was put on one- or two-dimensional M_r gels or on pI gels for exposure at 193 K in Kodak X-Omatic cassettes (Kodak, Stuttgart, Germany) with an intensifier screen. Relevant bands were scanned.

Antibody production

Cells were exposed to AED for 1 s or 1 min (allowing for de- and re-phosphorylation as outlined above) and solubilized by freezing/thawing for two-dimensional gel electrophoresis. After blotting on to nitrocellulose, the pI-5.95 spots of P63 and PP63 were localized by Ponceau S staining. Excised and destained spots were impregnated with complete Freund's adjuvant and implanted under sterile conditions close to inguinal lymph nodes in adult male CHbb:CH rabbits. Sera were prepared from blood samples collected after 30 days.

Monospecific antibodies were obtained by repeated absorption on blots and release by exposure to glycine/HCl buffer, pH 2.8 (Fisher & Smith, 1988; Roth *et al.*, 1985). Antibody solutions were neutralized by 3 M-Tris and dissolved in 100 mM-Tris/HCl buffer, pH 7.4, with 100 mM-$MgCl_2$, 0.005 % (v/v) Tween 20, 1 % (w/v) BSA and 2 % (v/v) fetal-calf serum added.

Immunofluorescence localization

Cells stimulated by adding AED (1 s or 1 min) were injected

into fixative at 273 K [2.5% (w/v) freshly depolymerized paraformaldehyde, with 0.25% (w/v) glutaraldehyde and 1% (w/v) saponin added, in 100 mM-cacodylate/HCl buffer, pH 7.0]. After 1 min the fixative was diluted 1:10 for 14 min. Samples were washed with NH_4Cl and glycine (50 mM each) and with 0.2% (w/v) fish gelatin from Sigma (Deisenhofen, Germany) before and after application of the first antibody (273 K, 1 h). Detection was with fluorescein isothiocyanate-labelled goat anti-rabbit IgG (1:300) from Dianova.

Negative controls were obtained (*a*) by omission of the first antibody or (*b*) by application of pre-immune sera from the same rabbits.

RESULTS

Rapid dephosphorylation of PP63

To analyse the correlation of PP63 dephosphorylation with membrane fusion [occurring as a distinct event within 100 ms after stimulation; Knoll *et al.* (1991)], ^{32}P-labelled samples were triggered and rapidly frozen in a new quenched-flow device. Since the final cell density was 15×10^3/ml, these dilute frozen cell suspensions required concentration by freeze-substitution in methanol for subsequent SDS/PAGE and autoradiographic analysis. Another critical methodological aspect is the following: our first autoradiograms of gels, although showing well-resolved protein bands after Coomassie Blue staining, had high background, obscuring individual bands. This background (presumably caused by some ^{32}P precipitation) was greatly decreased by acid treatment of gels. This treatment did not affect the labelling density of PP63, as we found (see below).

A typical autoradiogram of SDS/PAGE gels obtained from cells stopped at different time points after stimulation is shown in Fig. 1(*a*). For quantification of label we made densitograms. Thus we could correlate the degree of PP63 dephosphorylation with the degree of trichocyst exocytosis (membrane fusion, as analysed by structural methods; see Knoll *et al.*, 1991), using the same cell population under identical experimental conditions (but without ^{32}P label). Both parameters change strictly in parallel with time, both being practically completed within 80 ms after stimulation (Fig. 1*b*). During this time membrane resealing does not take place. It should be emphasized that, until now, it was not possible to analyse these aspects separately.

De- and re-phosphorylation are independent of extracellular Ca^{2+}

Unless indicated otherwise, trigger experiments were conducted in the presence of extracellular Ca^{2+} (0.1 mM). Quantitative evaluation of scans from one-dimensional Ca^{2+} (0.1 mM). Quantitative evaluations of scans from one-dimensional gel autoradiograms obtained from homogenates after application of standard trigger (+0.1 mM extracellular Ca^{2+}) and preparation (no addition of Ca^{2+} or EGTA etc.) conditions by manual pipetting gave the following results (Fig. 2). When ^{32}P labelling is standardized to 100% for untriggered controls, the degree of dephosphorylation achieved (1 s after AED) is 64% (i.e. with a residual level of 36 ± 5%), whereas rephosphorylation amounts to 161 ± 5% (1 min after AED). ^{32}P labelling after rephosphorylation exceeds that in the untriggered state, for reasons discussed by Stecher *et al.* (1987). When cells and secretagogue were mixed and samples taken by quenched-flow (Fig. 1), this clearly preserves a higher degree of PP63 dephosphorylation than does less synchronous manual operation.

EGTA (7.5 mM) was added to cells for 1 min. Then untriggered (0 s) and AED-triggered (1 s, 1 min) samples were subjected to SDS/PAGE and autoradiography (Fig. 2*a*). Quantitative evaluation of the 63 kDa band by density scans showed partial dephosphorylation (1 s) and rephosphorylation (1 min). Again, dephosphorylation is less pronounced than after quenched-flow (Fig. 1), but data are rather similar to those described above for

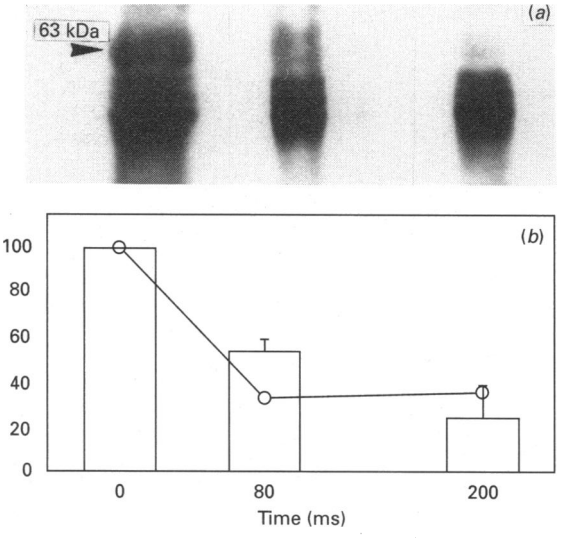

Fig. 1. Time course of PP63 dephosphorylation parallels AED-triggered exocytotic membrane fusion: quenched-flow analysis

(*a*) Autoradiograms of SDS/PAGE from cell homogenates after ^{32}P labelling according to method (A), without triggering (0 ms) and 80 or 200 ms after stimulation. (*b*) PP63 dephosphorylation (100% = degree of phosphorylation before stimulation as a reference value) relative to membrane fusion (100% trichocysts retained = all membranes unfused). Symbols: □, PP63 phosphorylation; ○, trichocyst contents. Bars indicate S.E.M. Protein applied: 30 μg/lane.

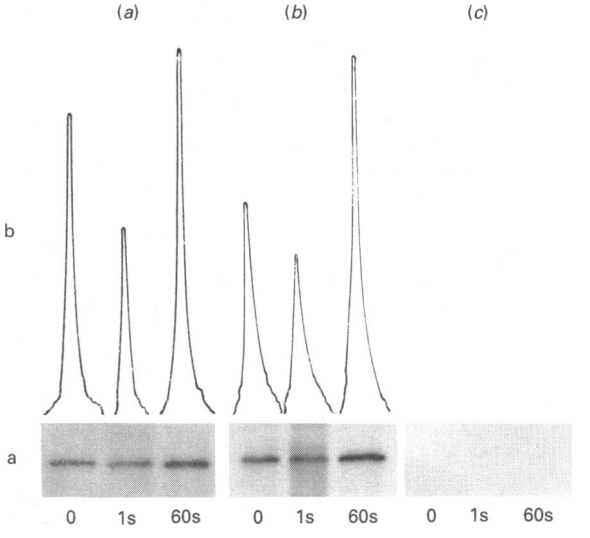

Fig. 2. (*a*) Absence of Ca^{2+} in the medium has no influence on PP63 dephosphorylation: AED-triggered exocytosis in presence of 7.5 mM-EGTA (with 1 min pre-incubation); (*b*) insensitivity of PP63 to acid hydrolysis (*a*) and (*c*) sensitivity to alkaline hydrolysis

(a) SDS/PAGE autoradiogram from homogenates from ^{32}P-labelled (method B) cells without trigger or 1 s (dephosphorylation) or 60 s (rephosphorylation) after adding AED. (b) Corresponding scans show that dephosphorylation is not complete and that rephosphorylation exceeds the original degree of phosphorylation [just as in samples plus extracellular Ca^{2+}; see (*b*) and the text]. In (*b*) and (*c*), preparations are as in (*a*), but with Ca^{2+} in the medium. (a) SDS/PAGE autoradiograms of controls and 1 or 60 s after AED stimulation. (b) Corresponding scans. Protein applied: 35 μg/lane.

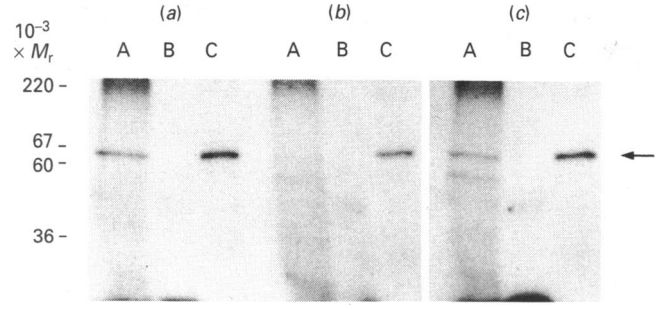

Fig. 3. Isolation of PP63 in supernatants obtained by repeated freezing/thawing from ^{32}P-labelled (method B) cells, either non-stimulated (*a*), or (*b*) 1 s or (*c*) 1 min after AED stimulation

SDS/PAGE autoradiograms: A, cell homogenates; B, 100000 *g* pellet; C, supernatant. Note selective detectability of partially de- and re-phosphorylated PP63 in the soluble fraction. Protein applied: 20 μg/lane.

manual pipetting and are equivalent to data obtained previously, also in the presence of extracellular Ca^{2+} (Zieseniss & Plattner, 1985).

P63 and PP63 are not Ca^{2+}-binding proteins

The 63 kDa band was not stained by 'Stains all', an indicator of Ca^{2+}-binding proteins (Campbell *et al.*, 1983). The same molecular-mass values were obtained on SDS/PAGE with or without added Ca^{2+} (results not shown).

PP63 is sensitive to alkaline, but not to acid, hydrolysis

SDS/PAGE gels were fixed and subjected to alkaline or acid hydrolysis, as indicated in the Materials and methods section. Autoradiograms then showed selective sensitivity of P63 and PP63 bands to alkaline, but not to acid, treatment (Figs. 2*b* and 2*c*). For two-dimensional gels, see below.

Solubilization of P63/PP63

Cells collected 90 min after ^{32}P incorporation and processed with or without previous stimulation by AED (0 s, 1 s, 1 min) were repeatedly frozen and thawed. Autoradiograms obtained by SDS/PAGE from solubilized samples again showed partial dephosphorylation and complete rephosphorylation (Fig. 3). The absence of PP63 from pellets is in contrast with results of immunoblotting experiments, as explained below.

Mechanical break-up with a Potter homogenizer in dilute salt solution, pH 7.0 (see the Materials and methods section), also solubilizes P63/PP63 (Murtaugh *et al.*, 1987), as we can confirm (results not shown). However, this does not allow isolation of distinct phosphorylation stages, i.e. during de- and re-phosphorylation. Therefore, further experiments were conducted with repeated freezing/thawing cycles.

IEF and two-dimensional-gel autoradiography analysis

After this solubilization protocol, the different phosphorylation stages were analysed on two-dimensional gels (Fig. 4). The only protein labelled on SDS/PAGE is of 63 kDa. Its labelling density visibly changes during de- and re-phosphorylation. All stages display spots of pI 5.95 (main spot), 5.85 and 5.75. The pI-

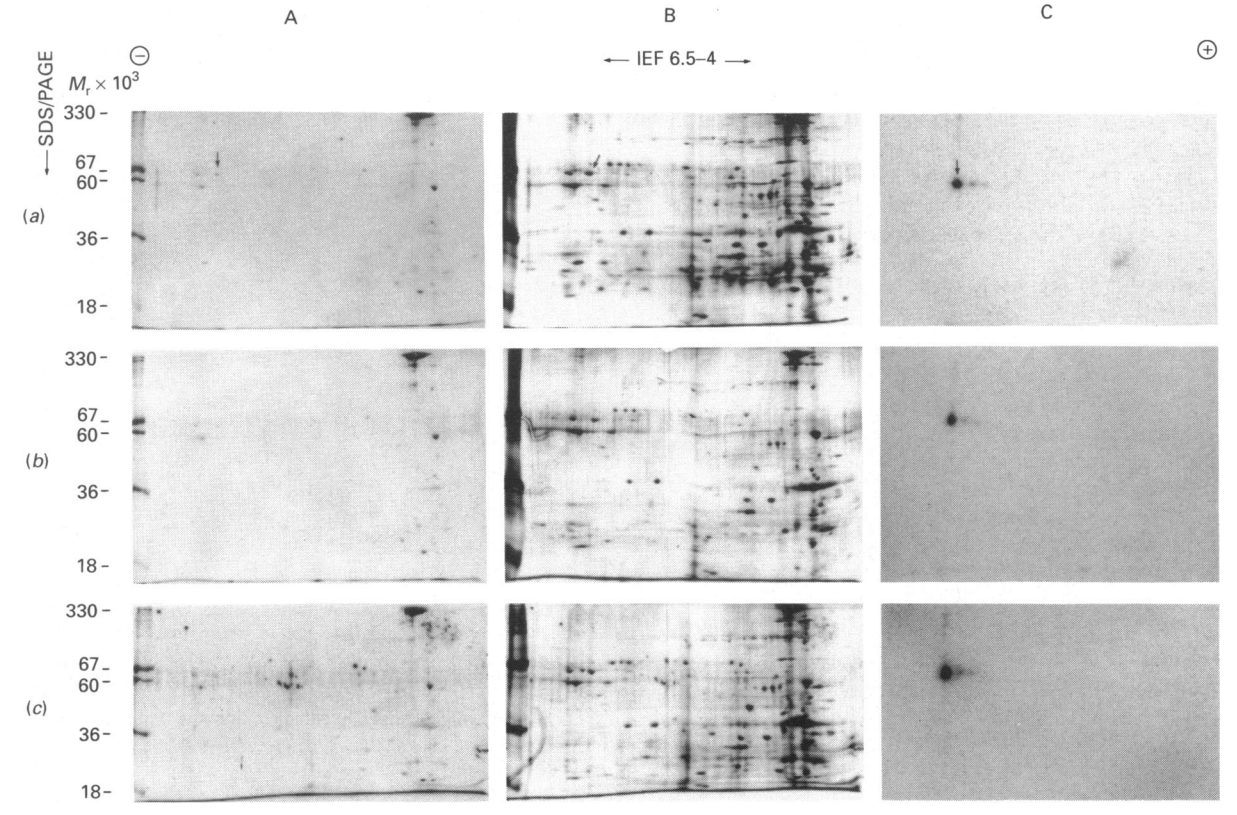

Fig. 4. Two-dimensional gel electrophoresis of supernatants prepared as in Fig. 3, i.e. without AED stimulation (*a*) or 1 s (*b*) or 1 min (*c*) after AED stimulation

(A) Coomassie Blue, (B) silver staining and (C) autoradiograms (with exclusive labelling of 63 kDa protein). Arrow points to prominent spot of pI 5.95 showing increased phosphorylation (*c*). Protein applied: 30 μg/lane.

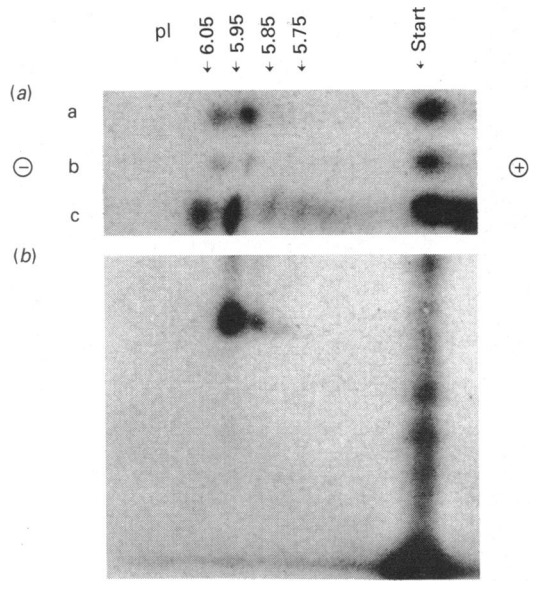

Fig. 5. (*a*) IEF autoradiogram of supernatants (as in Fig. 3) from unstimulated cells (a) and 1 s (b) or 1 min (c) after adding AED; (*b*) subsequent PAGE

In (*a*), note detectability of up to four or five spots after rephosphorylation, with the most intense spots of pI 6.05 and 5.95. (*b*) During subsequent SDS/PAGE the pI-6.05 spot is lost [rephosphorylated sample as in (c)].

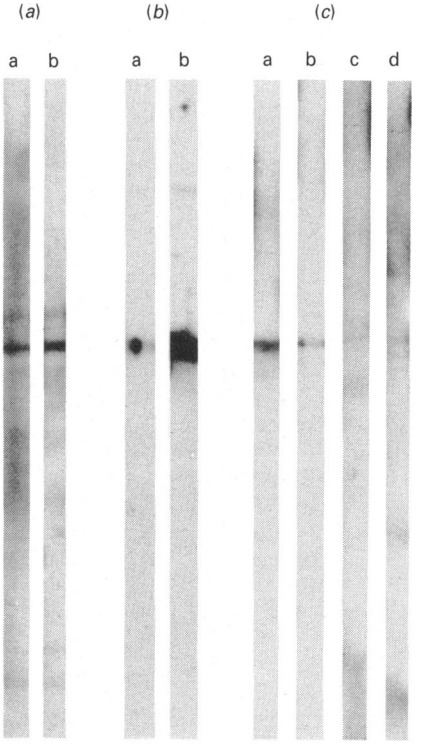

Fig. 6. (*a*) Western blots of cell homogenates [(a) anti-P63 and (b) anti-PP63 antibodies, the latter being more strongly reactive]; (*b*) immunodetection of PP63 on Western blots from cortices isolated from unstimulated cells using antibodies prepared (a) against P63 or (b) against PP63; (*c*) PP63 immunodetection after freezing–thawing

In (*c*), when subjected to rapid freezing and thawing, cortices still react strongly with antibodies (obtained against PP63) (a), whereas only part of PP63 goes into the 100000 ***g*** supernatant (b); (c) and (d) are pellets and supernatant from a second centrifugation, showing no additional sedimentation of PP63, but some additional solubilization. This indicates that PP63 is primarily a cortex-bound but soluble antigen.

5.95 spot is most intensely labelled 1 min after rephosphorylation (see the scan). This is impressive, considering the large number of spots detected in two-dimensional gels by protein stains (Fig. 4).

Surprisingly, IEF alone revealed an additional spot of pI 6.05, which also undergoes de- and re-phosphorylation during AED triggering (Fig. 5*a*). In two-dimensional gels, this spot consistently disappears from autoradiograms in the second dimension (Fig. 5*b*) and, on IEF gels, it disappears after alkaline as well as after acid hydrolysis (results not shown).

Western-blot analysis

Antisera were prepared against the most prominent spot, of pI 5.95, of the ^{32}P-labelled 63 kDa protein by using the phosphorylated and dephosphorylated forms (PP63 and P63) as antigens. Monospecific antibodies were isolated as indicated in the Materials and methods section.

Antibodies prepared against PP63 or P63 both recognize PP63 in homogenates prepared from untriggered cells (Fig. 6*a*). The same holds for cortex preparations derived from such cells (Figs. 6*b* and *c*). Antibodies prepared against PP63 appear more reactive, so that any difference in labelling intensity may not absolutely indicate phosphorylation-dependent antigen redistribution. (We note that these antibodies cannot be assumed to be specific for the phosphorylated or dephosphorylated form respectively.)

Western-blot analysis of P63/PP63 in particulate and soluble components (obtained from cortices by repeated freezing and thawing) consistently showed association of either phosphorylation form predominantly with pellets (Figs. 6*b* and 6*c*). This is in contrast with SDS/PAGE autoradiography (see above). For explanation, see the Discussion section.

Immunofluorescence localization

We also used antibodies to localize the 63 kDa protein by immunofluorescence in untriggered, slightly fixed and permeabilized cells. With either type of antibody, fluorescence was concentrated in cortical regions. As shown in Fig. 7 for the antibody prepared against P63, the size, form and pattern observed were consistent with labelling of components attached to trichocyst tips. Identical results were obtained with antibodies prepared against PP63. Controls with preimmune sera were negative.

DISCUSSION

Time course of PP63 dephosphorylation

Dephosphorylation of the phosphoprotein, PP63, is largely executed within 80 ms after stimulation of cells with AED. This is the fastest protein-dephosphorylation process reported in any system, on the basis of SDS/PAGE and autoradiography. Our analyses were facilitated by the synchrony of trichocyst exocytosis (Plattner, 1987) and by the new quenched-flow device (Knoll *et al.*, 1991). Otherwise de- and re-phosphorylation easily overlap, and may yield conflicting results (Plattner, 1989). The time course that we observed strictly parallels exocytotic membrane fusion, as found by electron-microscopic analysis (Knoll *et al.*, 1991). Since massive membrane resealing occurs only 200 ms after stimulation, the close temporal correlation of PP63 dephosphorylation strongly supports its possible role in membrane fusion (see also at the end of this Discussion section).

Manual pipetting allows recognition of PP63 dephosphorylation only in the range of 1 s and to a smaller extent (Zieseniss & Plattner, 1985). Use of a fixative for triggering trichocyst release

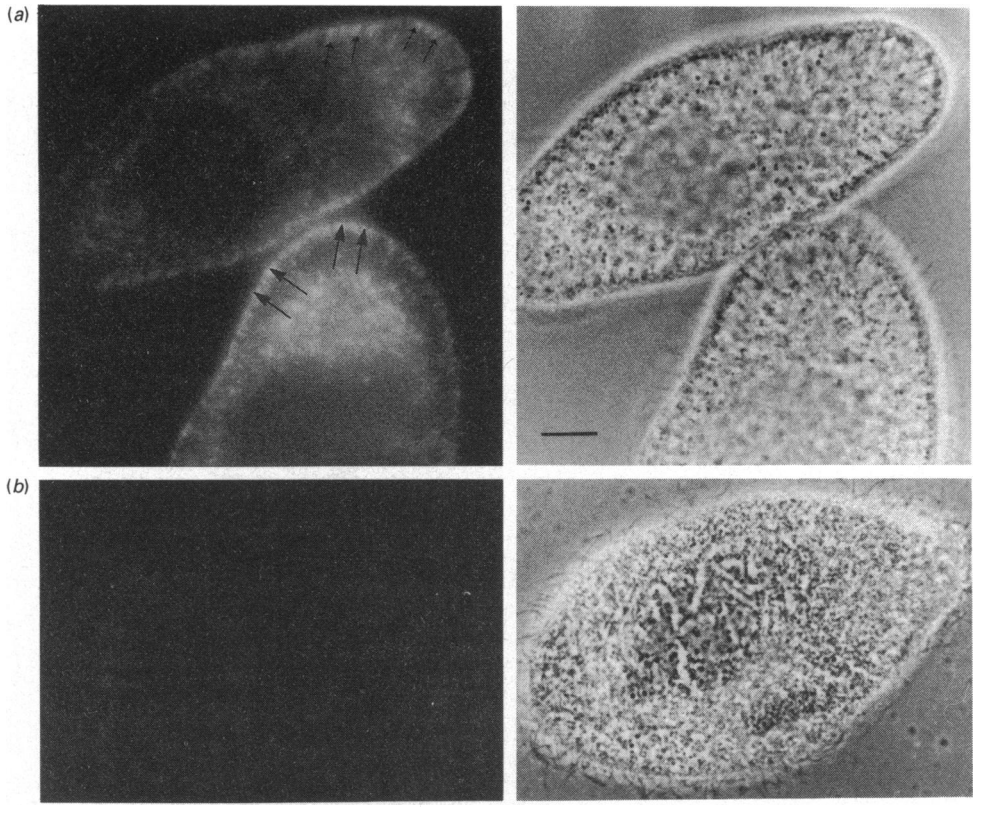

Fig. 7. Immunofluorescence localization in permeabilized cells using rabbit antibodies against P63 and anti-(rabbit IgG)—FITC (*a*); (*b*) control with first antibody omitted

The surface of trichocyst tips is labelled (arrows). Some labelling also occurs remote from the cortex. Bar = 10 μm. Left, fluorescence; right, phase contrast.

and conservation of the dephosphorylated state was another approach (Gilligan & Satir, 1982). Any other analyses reported in the literature were conducted on a broader time scale than that analysed here.

Localization of P63/PP63

On the basis of fractionation studies and SDS/PAGE autoradiography, the 63 kDa PP was originally considered to be a soluble cytosolic protein (Murtaugh *et al.*, 1987). In a recent review, immunofluorescence micrographs display a cortical localization (Satir, 1989), as we have also found. How can this discrepancy be reconciled?

Solubilization by repeated freezing and thawing allowed us to isolate both phosphorylation states. Both are soluble, according to SDS/PAGE and autoradiography. Yet by Western-blot analysis with antibodies against P63 or PP63 (showing cross-reactivity), we clearly found that the 63 kDa PP occurs partly in a structure-bound and partly in a soluble form. Since a phosphatase system is located in the cell cortex (Momayezi *et al.*, 1987), this might account for the loss of ^{32}P label from cortices and for the selective detectability of PP63 in supernatants by gel autoradiography, whereas detection by antibodies is less ambiguous. Satir *et al.* (1989) suggested the occurrence of a Ca^{2+}-dependent sedimentable pool which had previously remained undetected. Yet we found no indications of Ca^{2+} binding by P63 or PP63 (see the Results section). Therefore we assume that any Ca^{2+}-dependent functions during trichocyst exocytosis would have to be mediated in another way (for review, see Plattner *et al.*, 1991). Based on our data, we consider P63 and PP63 to be a soluble protein, which *in vivo* is structure-bound in a Ca^{2+}-independent way. Our immunofluorescence micrographs reveal association of P63 and PP63 with the outlines of trichocyst tips.

Isoforms of PP63

On precisely calibrated IEF gels the pI values we found were 6.05, 5.95 (main spots in autoradiograms) and 5.85 (with spurious spots at 5.75 and 5.65). Satir *et al.* (1989) have reported pI values of 6.2 and 5.8. During two-dimensional gel electrophoresis and autoradiography we consistently lost the first spot during SDS/PAGE. Interestingly, the ^{32}P label also was completely lost, probably for the same reason, with the isolation protocol used by Satir *et al.* (1989), although the antibodies that they prepared against this form recognize the phosphorylated 63 kDa band on blots from SDS/PAGE. Altogether, their data may be difficult to compare with ours, since the antibodies that we prepared were against the well-defined spot of pI 5.95 of 63 kDa. Previous indications on seryl- (or threonyl-) type phosphorylation sites (Murtaugh *et al.*, 1987) may be valid, as we also found them to be sensitive to alkaline, but insensitive to acid, hydrolysis. [It was not analysed in any detail why (P)P63 forms of different pI values occur, and whether one form is glycosylated, as suggested by Satir *et al.* (1990).]

For all these reasons we used the pI-5.95 form of P63 and of PP63 for immunolocalization studies.

Possible functional implications of PP63 dephosphorylation

The time course and localization of PP63 suggest a regulatory function during membrane fusion. A similar conclusion has been derived from the fusogenic effect of micro-injected phosphatases (Momayezi *et al.*, 1987) and from the inhibitory effect of ATP (Vilmart-Seuwen *et al.*, 1986; Lumpert *et al.*, 1990). We had inferred that a protein-phosphorylation step would prime fusogenic sites and that actual membrane fusion would require dephosphorylation (Momayezi *et al.*, 1987; Plattner, 1989;

Plattner *et al.*, 1991). This is not questioned by our new finding that EGTA in the culture medium during AED triggering does not inhibit PP63 de- and re-phosphorylation, since EGTA does not inhibit membrane fusion, either (Knoll *et al.*, 1991).

A potential role of a dephosphorylation step during exocytotic membrane fusion is corroborated by data recently obtained with other systems. (*a*) In non-equilibrium kinetic studies, mast cells display, depending on ATP concentration, a delay at the onset of exocytosis, but then an increase in the rate of exocytosis (Howell *et al.*, 1987; Churcher *et al.*, 1990). (*b*) Exocytosis is inhibited by phosphatase inhibitors in sea-urchin eggs (Whalley *et al.*, 1991). (*c*) In this system permanent thiophosphorylation also abolishes exocytotic membrane fusion (Whalley *et al.*, 1991), as it does in chromaffin cells (Brooks *et al.*, 1984).

Since (P)P63 is a soluble, though structure-bound, protein, we envisage the following possibilities of a regulatory function. (*a*) Perturbation of lipid bilayers after dephosphorylation has been considered (Plattner, 1989). (*b*) Dephosphorylation could also induce disassembly of the subunits of 'fusion rosettes', i.e. aggregates of freeze-fracture particles occurring in the cell membrane precisely at trichocyst exocytosis sites (Plattner *et al.*, 1973). These membrane-integrated proteins (Vilmart & Plattner, 1983), whose occurrence is linked to fusion capacity (Beisson *et al.*, 1976; Pouphile *et al.*, 1986; Pape *et al.*, 1988), disappear during trichocyst exocytosis, on account of a 6-fold larger population of smaller subunits (Knoll *et al.*, 1991). This aspect would be compatible with a fusion hypothesis recently proposed by Almers (1990), on the basis of surface conductance flickering phenomena. Along these lines, Kéryer *et al.* (1987) have demonstrated that in *Paramecium* transient phosphorylation of some cortical proteins (though their identity was not analysed in detail) is required for the assembly of cortical constituents. They showed that monoclonal antibodies directed against phosphorylation residues recognize different cortical PPs, including one of approx. 63 kDa, as we can confirm with the same antibodies (results not shown). Since PP63 occurs in many, including mammalian, cells (Satir *et al.*, 1989), it might exert a regulatory role in membrane fusion during exocytosis in many systems.

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