Phosphorylation of algal centrin is rapidly responsive to changes in the external milieu

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Summary

Centrin, a calcium-sensitive contractile phosphoprotein, is the major component of the striated flagellar roots of the flagellate green alga, Tetraselmis striata. Flagellar roots contract in response to elevated calcium levels. Data presented here indicate that the level of centrin phosphorylation is rapidly responsive to changes in the cell's external environment. Centrin is dephosphorylated in response to elevated calcium or to heat shock. An increase in centrin phosphorylation accompanies pH shock or ethanol treatment. These changes are compared with flagellar excision, flagellar root contraction, and protein synthesis under the same treatments. We conclude that under certain conditions phosphorylation/dephosphorylation of centrin and extension/contraction of the flagellar root are uncoupled. Possible role(s) of centrin phosphorylation independent of force generation in the flagellar root are discussed.

Key words: centrin, flagellar root, phosphorylation, Tetraselmis.

Introduction

Protein phosphorylation is well established as a means by which cells can regulate both their internally driven processes (e.g. metabolism, cell division) and their responses to the external environment (e.g. hormone-induced responses, responses to changes in culture conditions). It is a specific, reversible modification of protein, which can affect assembly of structural proteins (e.g. see Inagaki et al. 1987), activity of enzymes (e.g. see Stefano-vic et al. 1986), specificity of molecular interactions (e.g. see Sorger et al. 1987), and response to regulatory agents (e.g. see Lai et al. 1987). It can be regulated by kinase regulation, phosphatase regulation, or both.

Centrin is a calcium-binding contractile protein that contains an antigenic epitope(s) ubiquitous in the centrosomes of eucaryotes (Salisbury et al. 1986; Koutoulis et al. 1988; Hohfeld et al. 1988; Wright et al. 1985; Schulze et al. 1987; Baron and Salisbury, 1988; Wick and Cho, 1988; Hiraoka et al. 1989). Hohfeld and coworkers (1988) suggest that centrin-mediated cell motility represents a phobic shock–motility system, on the basis of the involvement of centrin in a number of motility systems that are rapidly responsive to changes in the cell's environment (Salisbury et al. 1984; Wright et al. 1985; McFadden et al. 1986; Schulze et al. 1987; Salisbury et al. 1987; Hohfeld et al. 1988; Koutoulis et al. 1988). In Tetraselmis striata, centrin is the principal protein component of contractile flagellar roots and occurs as two acidic isoforms, α and β; β is phosphorylated (Salisbury et al. 1984). The degree of centrin phosphorylation is affected by calcium, a treatment that results in flagellar root contraction (Salisbury et al. 1984). Furthermore, the flagellar roots of T. striata cells undergo cycles of contraction and extension in the presence of Ca2+ and ATP, as determined by observation of inpocketing of the plasma membrane at the points of flagellar root attachment (Salisbury and Floyd, 1978). These findings taken together have led to the hypothesis that centrin phosphorylation is required either for contraction or for extension of the flagellar root (Salisbury et al. 1984, 1988).

Detergent-extracted cell models of Chlamydomonas reinhardtii clearly demonstrate a requirement for ATP to potentiate the effect of Ca2+ on centrin contraction (Salisbury et al. 1987). This requirement may be satisfied either by ATP added with calcium, or by ATP added in the presence of EGTA, followed, after washing, by addition of calcium to induce contraction. The observation that ATP potentiation is effective prior to the initiation of contraction suggests that the high energy of the phosphate bond has been preserved for later use by protein phosphorylation. The protein target of phosphorylation in detergent-extracted Chlamydomonas cell models may be centrin itself, although this point has not yet been experimentally confirmed (Salisbury et al. 1987).

T. striata has large striated flagellar roots that undergo dramatic rapid contraction from the resting extended state. Typically, approximately 50% of the centrin present in these cells is found to be in the unphosphorylated state, even in fully 'potentiated' extended flagellar roots (Salisbury et al. 1984). Conversion of flagellar roots from the extended to contracted configuration results in a shift of approximately 20% of the centrin from the phosphorylated to the unphosphorylated state; data presented earlier
(Salisbury et al. 1984) demonstrate a change in the ratio of $\alpha$ to $\beta$ from 1:1 for extended flagellar roots to 2:1 for contracted flagellar roots.

Evidence is presented here to show that although centrin contraction and centrin dephosphorylation are directly correlated in calcium-induced responses, they can be uncoupled under certain conditions. The state of centrin phosphorylation is responsive to a number of different treatments that ‘shock’ T. striata cells. A rapid increase in calcium concentration in the external medium, or addition of calcium ionophore A23187, were used to investigate the response of these cells to calcium. In addition, heat shock, pH shock and ethanol treatment were investigated. For each treatment, flagellar excision, flagellar root contraction/extension and the state of centrin phosphorylation were determined. The possible functional significance of centrin phosphorylation independent of contraction is discussed.

Materials and methods

Cell cultures

Tetraselmis striata cells were cultured in suspension in an artificial sea-water medium (33.4 g l$^{-1}$ Forty Fathoms Marine Mix, Marine Enterprises, Baltimore MD) supplemented with 10% soil extract at 18°C in a 16 h light, 8 h dark cycle with continuous bubbling of air. Cells were harvested from log phase cultures at densities of $10^5$ to $10^6$ cells ml$^{-1}$ by centrifugation either for 5 min in a 4-place DynaK centrifuge at high speed, or for 15 min in a Sorvall RC-2 centrifuge equipped with a GSA rotor at 2000 revs min$^{-1}$.

$^{32}$P-labeling of cells

Cells were incubated in phosphate-free artificial sea-water medium (ASW-P, 15 g NaCl, 0.7 g KCl, 0.3 g NaNO$_3$, 1.0 g Tris-base, 0.1 m glycerol, 8 g MgSO$_4$·7H$_2$O, 1 m molybdic acid stock and 5 ml PII metal stock as described by Bold and Wynne (1978, p. 577), brought to 11, pH 7.8) for 1.5 h. $[32P]$orthophosphate (carrier-free, from ICN) was then added to the medium to a final concentration of 0.1 mCi ml$^{-1}$. Cell density during labeling was $5 \times 10^7$ cells ml$^{-1}$. Except where indicated otherwise, labeling was carried out for 70-90 min. Samples were prepared either for immunoprecipitation or for two-dimensional gel electrophoresis.

$^{35}$S-labeling of cells

Cells were incubated in sulfate-free artificial sea-water medium (ASW-S, 15 g NaCl, 0.7 g KCl, 0.3 g NaNO$_3$, 1.0 g Tris-base, 0.1 g 2-glycerophosphate, 6.5 g MgCl$_2$, 1 ml molybdic acid stock and 5 ml PII metal stock as described by Bold and Wynne (1978, p. 577), brought to 11, pH 7.8) for 1.5 h. $[^{35}S]$orthophosphate (carrier-free, from Amersham or ICN) was then added to a final concentration of 0.1 mCi ml$^{-1}$. Cell density was $5 \times 10^7$ cells ml$^{-1}$. Labeling was allowed to proceed for 30 min, at the end of which cells were washed and then lysed in SDS reducing lysis buffer (0.125 M Tris–HCl, 4% SDS, 20% glycerol, 10% dithiothreitol, 2 mM EDTA, 0.04% Bromophenol Blue, 0.02% pyronin Y) and electrophoresed on a 15% polyacrylamide gel.

Two-dimensional electrophoresis

Two-dimensional (2-D) gel electrophoresis was performed essentially according to O'Farrell (1975) with the modifications described here. 0.5 ml samples of cells were pelleted for 20 s in a microfuge, and lysed in 0.1 ml 2-D lysis buffer (0.2% SDS, 50% 2-mercaptoethanol, 10 μg ml$^{-1}$ butylated hydroxytoluene) for 2 h at 20°C, followed by 4 min at 98°C. Samples were either frozen at this point or immediately precipitated by addition of 1 ml acetone at −20°C for 2 h. Acetone precipitate was fixed (5 min), aliquoted to add to the surface, then dissolved in 20 μl 2-D buffer A (2% CHAPS, 30% glycerol, 5% 2-mercaptoethanol, 10 μg ml$^{-1}$ butylated hydroxytoluene). After 2 h at 20°C 2-D buffer B (2% CHAPS, 10% glycerol, 5% 2-mercaptoethanol, 8% BioRad BioLytes 4/6, 2% BioRad BioLytes 3/10, 9.0 μl urea, 10 μg ml$^{-1}$ butylated hydroxytoluene) was added, and samples of 15 μl were run on first-dimension gels on a BioRad Mini-Protean II 2-D cell for 5.5 h, followed by SDS–polyacrylamide gel electrophoresis run according to Laemmli (1970).

Transfer to nitrocellulose, 0.45 μm pore size (Schleicher and Schuell, Keene, NH), was performed essentially according to Towbin and coworkers (1979), with transfer time of 1 h, and with the addition of 0.66 mM CaCl$_2$ in the transfer buffer.

Towbin & coworkers (1979).

Auroradiography

Gels were either stained with Coomasie Brilliant Blue and dried under a vacuum with heat, or transferred to nitrocellulose, which was then stained with Amido Black blue and air dried. $^{32}$P-labeled gels and blots were covered with a layer of plastic wrap. Dried gels or blots were laid flat on Kodak X-OMAT AR photographic film, exposed either at 20°C or at −70°C, and developed in an automated processor. Within a given experiment, all samples were treated in parallel.

Video densitometry

 Autoradiographic exposures were quantitated by video densitometry. A Techman Video VanGogh digitizing board in an IBM PC equipped with a color graphics board and a Mitsubishi color monitor was connected to a Hitachi video camera and black and white monitor. Images transmitted by the camera were digitized and stored on disk, where they could be manipulated mathematically. Autoradiographs were placed on a light box, with the area of interest framed with black paper to provide an automatic black level, and to defeat the gain circuit in the camera. The gain control was turned to minimum as well. For each spot digitized, a suitable background area was chosen and digitized. The optical density for each pixel, $i$, was calculated according to:

$$\text{Density} = \log_{10}(\text{background luminance})/(\text{sample luminance})$$

and summed over all pixels to give a single number for overall density. The method is critically dependent on choice of background. For this reason, autoradiographs with mottled backgrounds, or with poor separation of radioactive spots, could not be used. The final number is in arbitrary units, which may be converted to optical density units by use of a standard curve. Linearity of the method was checked using a Kodak calibrated optical density step wedge. All measurements used were made on the linear portion of the curve.

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**Electron microscopy**

Cells were treated exactly as for phosphate labeling, including preadaptation to phosphate-free medium and high-density resuspension, but without added phosphate. They were then subjected to the various treatments in the same manner, except that, in place of lysozyme buffer, cells were resuspended in labeling medium containing 2% glutaraldehyde and allowed to fix for 1 h. Cells were then washed with buffer, and mixed into warm 4% agarose.

After solidification, the agarose block was postfixed in 1% OsO4, 0.8% K3Fe(CN)6, in 4 mM phosphate buffer, pH 7.2, for 1 h on ice. The block was then rinsed in distilled water, treated with 0.15% tannic acid at room temperature for 1 min, washed again, and en bloc stained with ethanolic 2% uranyl acetate for 1 h in darkness. After another wash, the block was dehydrated through an ethanol series, cleared with propylene oxide, and embedded in PolyBed 812 (Polysciences). Polymerization proceeded at 60°C for 48 h. Silver sections were collected on Formvar-coated copper grids and examined by transmission electron microscopy.

**Results**

**Change in levels of phosphocentrin in response to acute cell shock**

In order to determine the effect of each perturbation on the state of centrin phosphorylation, *Tetraselmis striata* cells were labeled *in vivo* with 32P as described in Materials and methods. After 60–90 min of labeling time, samples were subjected to each of the perturbations described. Samples were prepared for two-dimensional gel electrophoresis, transfer to nitrocellulose, and autoradiography. Autoradiographs were exposed in parallel, for equal amounts of time. Density of exposure of the film by phosphocentrin was measured as described. Density determinations were normalized to the average of control values within each experiment. An example is shown in Fig. 1. A is an autoradiograph of control whole cell lysate (only the relevant portion of the gel is shown); B is an autoradiograph of whole cell lysate from calcium-treated cells. The difference in centrin phosphorylation is exaggerated by the photographic reproduction of the figure. Results are compiled for all treatments in Fig. 2.

As predicted by a previous observation (Salisbury et al., 1984), calcium shock and calcium ionophore treatment produced a sharp decrease in phosphocentrin, to less than 5% of control values. Heat shock had an effect similar to calcium shock. Both ethanol and acid treatments, however, produced dramatic and rapid increase in phosphocentrin, an average of 2.5- and 2.8-fold, respectively. The reason for the large spread of the data points for ethanol and pH treatments is unknown (see Discussion).

**Fig. 1. Autoradiographs of two-dimensional gels showing 32P-labeled centrin (only the appropriate region of the gel is shown).** A. Control cell lysate. The frames indicate areas used as background (upper left) and signal (center). B. Lysate from calcium-treated cells. Frames are as in A. Ovals indicate the positions of (nonradioactive) internal standards, /α-lactoglobulin A (left), with pl 5.13 and molecular weight 18.4K, and trypsin inhibitor (right), with pl 4.55 and molecular weight 20.1K. Centrin is reliably located between and slightly above these standards.

**Examination of living shocked cells**

In order to determine the effects of each treatment on cell motility and to rule out gross morphological changes, *T. striata* cells were subjected to the treatments described above, and then observed by phase-contrast microscopy. Table 1 summarizes these observations. Control cells were at least 40% flagellate and motile.

Addition of calcium to 50 mM in the external medium had no obvious effect on these cells. Addition of calcium ionophore caused cells to clump together, but they maintained motile flagella for as long as 30 min in the presence of ionophore and 15 mM Ca2+. Flagellar movement did slow, however, and eventually stop. Thus the effects of calcium shock and ionophore treatment on centrin phosphorylation are not due to catastrophic effects on cells.

**Table 1. Effects of different shock treatments on cell motility**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Observation at light microscope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium shock*</td>
<td>No effect on motility</td>
</tr>
<tr>
<td>Calcium ionophore</td>
<td>Cells clump together, maintain flagella, flagellar movement is slowed</td>
</tr>
<tr>
<td>pH&lt;4.0f</td>
<td>Immediate loss of flagella</td>
</tr>
<tr>
<td>pH&lt;4.0f</td>
<td>Loss of flagella within several seconds</td>
</tr>
<tr>
<td>Heat shock†</td>
<td>No effect on motility below 37°C, above 37°C</td>
</tr>
<tr>
<td>Heat shock†</td>
<td>Cells begin to deflagellate</td>
</tr>
<tr>
<td>Heat shock†</td>
<td>Loss of flagella within several seconds</td>
</tr>
</tbody>
</table>

* Cultures treated with calcium continue to grow normally.
† Heat-shocked cultures show no change after as long as 45 min at 32°C.
‡ Cultures exposed briefly to acid shock or ethanol shock recover overnight in fresh medium.

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in loss of flagella. If the active agent was washed out, and cells returned to their normal growth medium, they were capable of recovering to control levels of flagellation and motility. Loss of flagella was immediate in acid-shocked cells, but in ethanol shock a few seconds were required for all cells to be deflagellated. Cells cannot survive prolonged exposure to low pH or ethanol.

**Electron microscopic observation of shocked cells**

To test directly the hypothesis that centrin dephosphorylation is concomitant with root contraction, a survey of cells subjected to each treatment was performed at the level of the electron microscope. Representative images of flagellar roots from each treatment are shown in Fig. 3. Table 2 compares the number of extended, contracted and intermediate stage flagellar roots in surveys of cells that have undergone each treatment. (Flagellar roots were scored as intermediate if they displayed a banding pattern in which both extended, fibrous interzones and contracted, amorphous interzones were present.)

Control cells (Fig. 3A) displayed fully extended flagellar roots in all but one case, in which the cell appeared vacuolated (n=13). Several sections contained segments of flagella, indicating that these cells were not deflagellated by the fixation process. Cells shocked by sudden elevation of calcium to 50 mM (Fig. 3B) showed contracted flagellar roots in six out of seven cells examined. The remaining cell contained flagellar roots in an intermediate state of contraction. No sections of flagella were found. Although
Table 2. Comparison of different stages of flagellar root contraction in shocked cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Extended</th>
<th>% Intermediate</th>
<th>% Contracted</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>92</td>
<td>0</td>
<td>8</td>
<td>13</td>
</tr>
<tr>
<td>Calcium shock</td>
<td>0</td>
<td>14</td>
<td>96</td>
<td>7</td>
</tr>
<tr>
<td>Ionophore A23187</td>
<td>0</td>
<td>38</td>
<td>62</td>
<td>8</td>
</tr>
<tr>
<td>Heat shock</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>pH shock</td>
<td>89</td>
<td>11</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>Ethanol shock</td>
<td>0</td>
<td>14</td>
<td>86</td>
<td>7</td>
</tr>
</tbody>
</table>

calcium did not deflagellate cells in vivo, it has been noted previously that calcium is capable of deflagellating cells (Salisbury et al. 1984). It may be that calcium treatment made the cells more fragile, so that pelleting or pipetting caused the loss of flagella. Fixation itself has also been shown to deflagellate *T. striata* (Lewin and Lee, 1985). Of eight ionophore-treated cells surveyed, five contained contracted flagellar roots, and intermediate levels of contraction (Fig. 3C) were observed in the remaining three. Some ionophore-treated cells still bore flagella (see Discussion). Heat-shocked cells (Fig. 3D) contained fully extended flagellar roots (n=5) and sections of flagella were discernible. Cells shocked by acid treatment (Fig. 3E) displayed extended flagellar roots in eight out of nine cases, the remaining one being intermediate. No sections of flagella were found. Cells shocked with ethanol (Fig. 3F) displayed contracted flagellar roots in six out of seven cases, the remaining one being intermediate. No sections of flagella were found, indicating that deflagellation had been caused by these treatments. The survey did not reveal any gross changes in cells, such as breaches in cell walls or aggregation of cytoplasm, which might have been effects of the treatments used. The changes in flagellar root morphology are summarized in Table 2.

**Altered protein synthesis in response to treatments**

A role has been proposed for the nucleus–basal body connector in *C. reinhardtii* in the pathway initiating changes in protein synthesis following flagellar excision (Salisbury et al. 1987). Here, we investigate two experimental paradigms for inducing altered protein synthesis, heat shock and deflagellation, for effects on *T. striata* centrin phosphorylation.

In order to evaluate the degree of the heat shock response at 32°C, *T. striata* cells were prepared for 35S labeling. Samples were rapidly heated to each of the following temperatures for 15 min: 18, 23, 28, 32 and 37°C. [35S]Sulfate was then added to the medium and the elevated temperatures maintained for an additional 30 min.

At the end of this time, cells were pelleted and prepared for SDS–polyacrylamide gel electrophoresis. The dried gel was autoradiographed to reveal the pattern of protein synthesis following flagellar excision (Fig. 4). *Tetraselmis striata* normally grow at 18°C. Therefore the 18°C pattern of synthesis is that of normal interphase cells. A weak heat shock response is detectable at 28°C, but normal protein synthesis continues. At 32°C, normal protein synthesis has been shut down, and heat shock proteins are expressed preferentially. Approximate molecular weights of the induced proteins are 22, 33, 50, 69 and 76×10^3 M_r. At 37°C, cells were deflagellated before the end of the labeling period. The small amount of incorporation of 35S into proteins suggests that sulfate uptake and/or metabolism was shut down completely, and cells may have been dying.

Protein production in response to a brief deflagellating shock was determined in a similar manner (Fig. 5). Cells were subjected to 8% ethanol shock after presadaptation to sulfate-free medium. After 1 min, the shocked cells were pelleted and resuspended in fresh medium containing [35S]Sulfate for 30 min. Acid shock was also followed by...
centrifugation and resuspension in fresh medium containing $^{32}$P for 30 min. Controls at 18 and 32°C were prepared as for heat shock determination. Deflagellating shocks resulted in the induction of synthesis of proteins with the same molecular weights as some of those observed in the heat shock response. Additional protein synthesis beyond that of normal interphase cells, and not corresponding to heat shock proteins, was also revealed, possibly including flagellar precursor proteins.

**Discussion**

The present study investigates the response of flagellar root contraction and of centrin phosphorylation to a variety of agents. The state of centrin phosphorylation changes rapidly and dramatically in response to perturbations of the cell. Table 3 is a summary of the results of this study.

**Calcium and ionophore**

The coincidence of centrin dephosphorylation with flagellar root contraction, when root contraction is induced by calcium influx, supports the hypothesis that phosphorylation of centrin itself is required for cycles of contraction/extension. When cells are flooded with calcium in the external medium, flagellar roots contract, and centrin phosphorylation drops to 10% of the basal level. Similarly, cells treated with calcium ionophore A23187 possess flagellar roots that are in various stages of contraction, and a degree of centrin phosphorylation less than 5% of the basal level. These observations suggest the action of a calcium-activated phosphatase on centrin.

It is not surprising that calcium shock did not cause flagellar excision in this study, since cells were preadapted to 1 mM rather than zero free calcium in order to optimize labeling conditions. This was found to be necessary for efficient uptake of $^{32}$P. In this regard, it has also been noted by Segal and Luck (1985) in *C. reinhardtii* that uptake of phosphate into protein is greatly increased by the presence of calcium in the labeling medium. *T. striata* normally grows in seawater in calcium concentrations of the order of 10 mM.

**Heat shock**

Since contraction of the centrin-based cytoskeleton has been shown to be coordinated with the induction of flagellar precursor genes in *C. reinhardtii* (Salisbury et al. 1987) and since genes induced in response to deflagellation in this and other organisms include some of the heat shock genes (May and Rosenbaum, 1980, abstract; May and Rosenbaum, personal communication; Guttman et al. 1980), we investigated a possible correspondence between the state of centrin phosphorylation and alterations in protein synthesis. We chose heat shock and flagellar excision as well-documented models for comparison. Centrin phosphorylation was found to be correlated with shocks expected to induce flagellar precursor genes (see ‘Deflagellating Shocks’, below), but not with heat shock response.

Heat shock proteins in *T. striata* are mildly induced at 28°C, which is 10 deg. C above optimal growth temperature. At 32°C, a complete shift to heat shock protein synthesis occurs, in which normal protein synthesis is reduced to low levels. Cells remain flagellate and motile. At 37°C cells deflagellate, and at 42°C the cells are killed during the $^{35}$S-labeling interval (data not shown). No attempt was made to identify individual heat shock proteins, but the 69K (K=103 Mr) protein is probably the *T. striata* homolog of HSP 70, which has an *M*, of 69000 in *C. reinhardtii* (May and Rosenbaum, personal communication) and the 22K protein is probably a chloroplast membrane protein (Kloppstech and Ohad, 1986; Kloppstech et al. 1985).

Heat shock is detectable in many organisms at the RNA level within minutes after the inducing temperature change (Lindquist, 1986). In order to determine whether this environmental change provokes a change in centrin-based structures, the temperature of the medium was raised to 32°C for 4 min, and flagellar root structure and centrin phosphorylation were investigated. Phosphorylation drops precipitously, as in response to calcium. Preliminary evidence indicates that this drop is dependent on the presence of calcium in the medium (V. E. Martindale, personal observation). However, flagellar roots remain extended, suggesting that any calcium influx is too low to induce contraction.

Temperature alone has a considerable effect on enzyme activities, in some cases causing deactivation, and in others increasing enzyme efficiency. 32°C may be considered a physiologically relevant temperature, since cells produce a robust heat shock response, and are capable of surviving for at least 45 min under these conditions. More study on the role of centrin-based systems in the heat shock response is needed before the relevance of the state of centrin phosphorylation and flagellar root contraction in this situation can be evaluated.

**Deflagellating shocks**

A rapid drop in the pH of the medium causes a rapid increase in phosphorylation of centrin, about 2.8-fold on average. It is important to note that phosphate is exchanged rapidly on centrin, so that by 60 min, equilibration between hot and cold phosphate pools on centrin is about 70% complete (Martindale and Salisbury, unpublished data). By 90 min equilibration is about 77% complete. Thus a large change in centrin phosphorylation in response to acute shock is not attributable to continued basal phosphate exchange, particularly in the short time frame of sample preparation in these experiments.

The centrin-based nucleus–basal body connector of *C. reinhardtii* contracts in response to either calcium or acid shock (Salisbury et al. 1987; Sanders and Salisbury, 1989). In this study, the centrin-based *T. striata* flagellar roots remained extended even after acid shock, but contracted, as previously observed, after calcium shock. Acid shock took place in 1 mM Ca$^{2+}$, which is sufficient to cause contraction in isolated centrin-based organelles (Salisbury et al. 1984; Wright et al. 1985; McFadden et al. 1987). It is

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Calcium</th>
<th>Ionophore</th>
<th>32°C</th>
<th>pH shock</th>
<th>Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Centrin phosphorylation</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Flagellar excision</td>
<td></td>
<td></td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Heat shock proteins</td>
<td>ND</td>
<td></td>
<td>++</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Flagellar root contraction</td>
<td>+</td>
<td>+/−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
</tbody>
</table>

ND, not determined; ↓, decrease; ↑, increase.

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possible that calcium is not entering the cell in sufficient quantity to cause flagellar root contraction in the case of acid shock. Deflagellation is too rapid to be observed as a time course at the microscope, and the cell wall protects plasma membrane, except that of the flagella, from rapid environmental changes. Thus the major absorbing surfaces of the cell are lost immediately upon exposure to adverse conditions.

Following ethanol treatment of *T. striata*, flagellar roots contract. The flagella are lost over the course of a few seconds, which may be enough time to permit intracellular calcium concentrations to rise above pCa7, the level at which centrin contraction takes place (McFadden et al. 1987; Salisbury et al. 1987). Ethanol treatment induces an increase in centrin phosphorylation of 2.5-fold on average.

Hyperphosphorylation of centrin in response to acid and ethanol treatments may occur in either of two ways. There may be an increase in the number of centrin molecules bearing a phosphate on the same site that are partially phosphorylated in unperturbed cells. Alternatively, a new (inappropriate) site of phosphorylation may be activated under these particular conditions; in which case, an increase in overall phosphorylation may mask dephosphorylation occurring at the normal phosphorylation site. It is not clear what property these two treatments might share, which would bring into play a new site of phosphorylation. Although a hyperphosphorylated form of centrin, γ centrin, has been identified (Martindale and Salisbury, unpublished data), its intensity covaries with that of β centrin, regardless of treatment.

As ethanol treatment results in the induction of most of the heat shock proteins in a wide variety of organisms (for review see Lindquist, 1986), it is not surprising that a mild heat shock response was engendered in *T. striata* by 8% ethanol. Acid shock, as a deflagellating agent, was also expected to produce altered protein synthesis. In both cases, proteins with molecular weights identical to those of some of the heat shock-induced proteins were induced. Additional proteins, which correspond neither to constitutive protein synthesis nor to heat shock protein synthesis, were induced; these probably represent flagellar precursor proteins.

Heat shock protein induction, flagellar root contraction and deflagellation show no interdependence, each occurring in the absence of the others. It is possible that flagellar precursor induction, as distinct from heat shock induction, is tied to centrin phosphorylation: deflagellating shocks correlate with hyperphosphorylation of centrin.

The spread of data in the two cases of acid- and ethanol-induced increase in phosphorylation is quite large, ranging essentially from control to as much as five times control. Student’s t-test shows the difference of the means to be significant at the 75% confidence level. The reason for this spread is unknown. Possibly it is due to a time course for increase of phosphorylation which is of the same order as that of sample preparation, i.e. about 90–120 s from addition of acid or ethanol to cell lysis. This is supported by some data that suggest that phosphorylation continues to increase for at least the first 5 min after ethanol shock (unpublished observations).

There is a correspondence between phosphorylation and deflagellation. Deflagellation has been shown in *C. reinhardtii* to be associated with centrin contraction, specifically contraction of a centrin-based structure in the flagellar transition zone (Sanders and Salisbury, 1989). Because the vast majority of centrin in these cells is associated with the flagellar roots (Salisbury et al. 1984), the centrin of the transition zone is expected to make a negligible contribution to the overall state of phosphorylation in the total centrin pool as determined from whole-cell lysates. Therefore the correlation that appears to exist between deflagellation and the state of centrin phosphorylation cannot be due to changes in transition zone centrin (see Sanders and Salisbury, 1989). Roles for centrin phosphorylation

To summarize, centrin phosphorylation is rapidly and dramatically responsive to changes in the environment of the cell. Calcium influx, caused either by flooding the medium with calcium or by addition of calcium ionophore, induces a reduction in centrin phosphorylation accompanied by contraction of the centrin-based flagellar roots. Acid shock causes an increase in centrin phosphorylation, and flagellar roots are extended, either remaining so or rapidly (<2 min) re-extending under these conditions. These results support the hypothesis that centrin dephosphorylation accompanies centrin contraction. However, heat shock causes a reduction in centrin phosphorylation in the absence of flagellar root contraction, while ethanol treatment results in an increase in centrin phosphorylation and contracted flagellar roots. Therefore, following heat shock and ethanol treatment, centrin phosphorylation and flagellar root contraction have been uncoupled. The possibility of uncoupling extension/contraction from phosphorylation/dephosphorylation raises some interesting questions about the role(s) of centrin phosphorylation.

If centrin phosphorylation is not always directly related to its contraction/extension, what is its purpose? It is especially difficult to speculate on function in this case, since the function of the flagellar root itself is unknown. Suggested roles include initiator and/or coordinator of flagellar beat, shock absorber and force dissipater, signal transducer for flagellar excision, signal transducer for gene induction, a mechanism for correct segregation of basal bodies, and a storage form for elements necessary in spindle formation and mitotic machinery (Salisbury and Floyd, 1978; Salisbury et al. 1981; Sleigh, 1979; Schulze et al. 1987; Salisbury, 1988; Salisbury et al. 1987; Wright et al. 1988; McFadden et al. 1987; Stewart et al. 1974). This presents a multitude of possible roles for regulation of function by centrin phosphorylation. We will emphasize only a few of these.

The binding of calcium to centrin has a *Kₐ* between 10⁻⁶ and 10⁻⁷ M (Salisbury et al. 1987; Sanders and Salisbury, 1989), as determined by *in vitro* functional assays in centrin-based systems. Phosphorylation of centrin, a small, highly charged protein whose structure (Huang et al. 1988) is largely devoted to the calcium-binding domains known as EF hands, could easily effect a change in the binding constant for calcium. Such a modification would then provide a fine tuning of centrin function, playing an indirect role in force generation by preferentially stabilizing one conformation over the other.

The structures composed in part of centrin display a variety of morphologies and locations. In order to form such elaborate structures as the striated flagellar root, centrin must be capable of associating with a number of different connecting elements in specialized ways. Centrin in specialized locations might require specific modification in order to maintain its structural associations. In addition, centrin polymerization itself may require a regulation by phosphorylation. Phosphorylation has been shown to play a role in regulating the arrangement and

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polymerization of intermediate filaments (Inagaki et al. 1987).

Finally, the flagellar roots of Tetraselmis subcordiformis undergo a dramatic change in mitotic cells, losing their regular structure and becoming an amorphous, granular mass (Stewart et al. 1974), which forms the broad flat spindle poles in this organism. Transformation of flagellar roots, which in interphase are resistant to 0.5% Triton X-100, 100 mM salt solutions, Dounce homogenization and sonication (Salisbury et al. 1984), must require modification of its constituent elements. As protein phosphorylation is known to play key roles in the transition from interphase to mitosis in eukaryotic cells, it is possible that the dramatic alterations in flagellar root morphology during the mitotic phase of the cell cycle may be accompanied by changes in centrin phosphorylation.

Phosphorylation of centrin is rapidly responsive to changes in the external milieu. Treatments described here that perturb the state of centrin phosphorylation in T. striata include calcium influx, heat shock, pH shock and ethanol treatment. Changes in centrin phosphorylation do not correlate with heat shock protein production, but do correlate with flagellar excision, for the treatments investigated. Centrin phosphorylation is increased under conditions expected to induce flagellar precursors. Flagellar root contraction correlates with changes in phosphorylation in calcium-induced responses, but not in the cases of heat shock and ethanol treatment.

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