High levels of actin tyrosine phosphorylation: correlation with the dormant state of Dictyostelium spores

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SUMMARY

Upon removal of nutrients, the amoebae of the cellular slime mold Dictyostelium discoideum differentiate into dormant spores which survive starvation stress. In this study, we demonstrate that half of the actin molecules in the spores are tyrosine-phosphorylated. The phosphorylated actin is distributed around immobile crenate mitochondria and vesicles, as well as in the cytoplasm of the spores. The actin isolated from spore lysates contains phosphorylated and unphosphorylated forms at the same molar ratio as that of the original whole spore lysate. Under actin polymerizing conditions they form actin filaments and then they are completely depolymerized under actin depolymerizing conditions, indicating that tyrosine phosphorylation of actin may not prohibit actin polymerization nor stimulate depolymerization. The phosphorylation levels increase at the end of the culmination stage when spores have matured morphologically and physiologically, and reach maximum levels after an additional 12 hours of development. The levels are stable for 20 days following spore maturation, and decline to undetectable levels within the next 10 days. Spores having high levels of phosphorylation show high viability, and vice versa. Following activation of spores with nutrient medium containing spore germination promoters, the phosphorylation levels quickly decrease with a half-life of about 5 minutes. After 20 minutes spores begin to swell. At this later time, most of the phosphorylated actin already has been dephosphorylated. Also, in heat-activated spores actin dephosphorylation occurs prior to spore swelling. However, addition of phosphatase inhibitors following heat-activation, prevented spore swelling and dephosphorylation of actin. Our data indicate that the high levels of actin tyrosine phosphorylation, specific to the spore stage, may be required for maintaining dormancy to withstand starvation stress. The rapid dephosphorylation of actin leads to a reactivated dynamic actin system which participates in spore swelling, vesicle movement, and mitochondrial shape changes during the spore germination process.

Key words: Tyrosine phosphorylation, Actin, Dictyostelium, Spore, Dormancy, Mitochondria

INTRODUCTION

Dynamic functions of actin cytoskeletons are controlled by a great variety of actin binding proteins. In some of them, the regulatory activity depends on phosphorylation and dephosphorylation of the molecules (Bretscher, 1989; Moriyama et al., 1996; Kuwayama et al., 1996; Huang et al., 1997; De Corte et al., 1997). In recent years, there have been several reports indicating that the phosphorylation of actin regulates its functions. Stimulation of fibroblasts with epidermal growth factors induces phosphorylation of actin on serine residues as well as membrane ruffles in vivo (van Delft et al., 1995). In Physarum polycephalum, phosphorylation at Thr 203 and Thr 202 of actin in complex with fragmin may be involved in actin nucleation and F-actin capping activity. Actin-fragmin kinase has been found to specifically phosphorylate actin threonine residues in the actin-fragmin heterodimer (De Corte et al., 1996; Eichinger et al., 1996). It also has been reported that dry-stress induces actin phosphorylation at Thr-203 in Physarum, and the phosphorylated actin has no polymerizing activity (Furuhashi et al., 1998).

Details of conditions for actin phosphorylation have been examined in amoebae of the cellular slime mold Dictyostelium discoideum. Oxygen depletion, inhibition of ATP synthesis by 2,4-dinitrophenol (DNP) or azide, heat shock, and Cd^{2+} lead to tyrosine phosphorylation of actin, and upon removal of these stresses the actin is dephosphorylated (Jungbluth et al., 1994, 1995). The phosphorylation site found after treatment with azide is at Tyr-53 residue (Jungbluth et al., 1995). When starved cells are returned to nutrient medium (nutrient shift), 10 to 15% of the total actin is tyrosine phosphorylated. It has also been shown
that protein tyrosine phosphatase PTP1 is partially involved in the dephosphorylation of the actin (Schweiger et al., 1992; Howard et al., 1992, 1993, 1994; Gamper et al., 1996). Tyrosine and serine phosphorylation of actin occurs when phenylarsine oxide is used as an inhibitor of protein tyrosine phosphatases (Schweiger et al., 1992). Prior to the phosphorylation in the cells exposed to these stress conditions, cells quickly change shape, usually round up, and become immobile, indicating tyrosine phosphorylation of actin is correlated with the final step of actin cytoskeleton rearrangement. Currently, no data exist regarding the protein tyrosine kinases that are responsible for the phosphorylation of actin in Dictyostelium cells.

Upon exhaustion of food sources, vegetative cells of *D. discoideum* differentiate into dormant spores and stalk cells. The spores survive harsh environmental conditions such as dehydration, heat, osmotic pressure, ammonia as well as non-nutrient conditions (Raper, 1935). Morphologically and physiologically spores are static. In dormant spores spherical vesicles and mitochondria, which are transformed to crenate shapes, are both immobile, and the cytoplasm is condensed due to dehydration (Cotter et al., 1969; Gregg and Badman, 1970; Sameshima et al., 1994). In addition, oxygen consumption of spores is 10-fold less than that of amoebae, and the rate of oxidative phosphorylation as well as glycolysis and many synthetic reactions are restricted to low levels (Gregg, 1950; Liddel and Wright, 1961; Cotter, 1973).

Spores in the sori are prevented from precocious germination by an inhibitor which is an adenine derivative called discadenine that exists in the interspore matrix (Abe et al., 1976). Spores immediately begin to germinate in the presence of food bacteria. One such bacterium, *Klebsiella aerogenes*, secretes spore germination promoters that have been suggested to be polysaccharides originated from the bacterial cell wall (Ihara et al., 1990). Wild-type spores aged more than 10 days secrete a phosphorylated adenine derivative termed the autoactivator (Dahlberg and Cotter, 1978). When discadenine is washed from the surface of spores, the aged spores are spontaneously activated by the autoactivator. Protein denaturing conditions such as heat, urea, or dimethyl sulfoxide also induce spore germination (Cotter et al., 1992). After activation by any one of these factors, spores begin to swell concomitantly with vesicular movement and mitochondrial shape changes, and finally at the end of swelling, a nascent myxamoeba escapes from the spore case to begin a new life cycle. The entire spore germination process requires a high level of oxygen up-take, and has been shown to be inhibited at any point in the process by oxygen deprivation or respiratory poisons (Cotter and Raper, 1966, 1968; Cotter et al., 1976). In addition, Ca\(^{2+}\) is quickly released from an internal calcium pool at the earliest stage of the germination process, resulting in activation of the Ca\(^{2+}\)-dependent regulatory protein, calmodulin; the latter protein is required throughout the swelling phase (Lydan and Cotter, 1994, 1995).

The regulatory system of actin in dormant spores has not been extensively investigated. However, a 43 kDa protein in the spores is tyrosine-phosphorylated and subsequently dephosphorylated during germination (Gauthier et al., 1997). In this study, we demonstrate that the 43 kDa protein is actin and that 50% of the actin is tyrosine-phosphorylated in the dormant spores. The actin fraction isolated from a spore lysate contains phosphorylated actin and can form actin filaments in vitro. The distribution of the actin as well as changes in phosphorylation levels during development and germination are further analyzed. Our data indicate that tyrosine phosphorylation of actin is required for the maintenance of a dormant state and the viability of *Dictyostelium* spores.

**MATERIALS AND METHODS**

**Anti sera**

Anti-actin monoclonal antibody clone C4 (Boehringer Mannheim GmbH, Mannheim, Germany; Lessard, 1988), anti-phosphotyrosine monoclonal antibody PY54 (Transduction Laboratories, Lexington, KY) and PY20 (Takara Biomedicals, Kusatsu, Japan; Glenney et al., 1988), and anti-phosphotyrosine polyclonal antibody (Transduction Laboratories) were used. Alkaline phosphatase-conjugated goat anti-mouse IgG and alkaline phosphatase-conjugated goat anti-rabbit IgG were obtained from Cappel Research Products, Durham, NC. Horseradish peroxidase-conjugated sheep anti-mouse Ig was purchased from Amersham, Buckinghamshire, UK. Goat anti-mouse IgG conjugated to 10 nm or 15 nm gold particles and goat anti-rabbit IgG conjugated to 10 nm gold particles were obtained from Amersham or British Biocell, Cardiff, UK.

**Cell culture and development**

Spores of *Dictyostelium discoideum*, the wild-type strain NC-4 (Raper, 1935), were spread on 2% agar plates made with nutrient medium containing 5 g proteose peptone, 0.5 g yeast extract, 5 g glucose, 2.25 g KH\(_2\)PO\(_4\), and 0.5 g MgSO\(_4\)·7H\(_2\)O per liter in association with *Klebsiella aerogenes* as described elsewhere (Sameshima et al., 1978). Fruiting bodies formed after 3 days. For synchronous development, growing cells were freed from bacteria with low speed centrifugation, suspended in 65 mM phosphate buffer, pH 6.4 (PB), and shaken for 4 hours at 22°C. Starved cells were spun down, suspended in Bonner’s salt solution containing 11 mM KCl, 11 mM NaCl, and 3 mM CaCl\(_2\), and then spread on non-nutrient agar plates at a density of 2×10\(^6\) cells/cm\(^2\). After 24 hours fruiting bodies formed and spores at this time were designated as 0 day old spores. Sori were collected by using inoculating loops and washed with PB for small volumes of spores. To obtain a large volume of spores, agar plates were hit upside down against a table, and the spores transferred to the dish-lids were collected and washed with PB (Kessin and Newell, 1974). Spore suspensions were passed through nylon mesh to remove contaminating stalks. There were less than 0.1% amoebae in the spore preparations as observed by phase contrast microscopy. To examine spore viability, 100 spores were spread on a nutrient agar plate with bacteria. After 2 days the percentage of viable spores was determined by counting plaques formed on bacterial lawns. The average percentages of plaques of two or three independent experiments are presented in the figures.

**Spore germination**

The nutrient medium used for the bacterial culture contained germination promoter(s) (Hashimoto et al., 1976; Ihara et al., 1990). The medium was centrifuged to remove bacteria and condensed to a 1/20 volume by evaporation. The condensed medium was a kind gift from Dr Y. Hashimoto. Fresh nutrient medium supplemented with 10% of the condensed medium was designated as germination medium. For synchronous germination, spores were washed with PB to clear away the germination inhibitors (Abe et al., 1976), suspended in the germination medium at density of 1×10\(^7\) spores/ml and placed on a reciprocal shaker at 150 strokes/minute at 22°C. For heat activation (Cotter and Raper, 1968) spores were incubated with PB at 45°C for 30 minutes and then transferred to a shaker at 22°C. The time when spore suspensions were transferred to 22°C was defined as 0 hour. The percentage of spore swelling and amoeba emergence was quantified by counting swollen spores and emerged amoebae on a
phase contrast microscope (Olympus, Tokyo, Japan). Roundish and phase dark spores, and amoebae completely emerged from spore cases were regarded as swollen spores and emerged amoebae, respectively. Intracellular particle movements were observed by a differential interference microscope (Nikon, Tokyo, Japan). The average percentage of swollen spores and emerged amoebae for three independent experiments are indicated in the figures. For phosphatase inhibition, heat-activated spores were treated with 7 μM phenylarsine oxide (PAO) (Sigma-Aldrich, Mississauga, ON, Canada) or 200 μM potassium bisperoxo (1,10-phenanthroline) oxovanadate (BPV) (Calbiochem, La Jolla, CA.) for 5 hours and total proteins of the spores were immunoblotted with anti-phosphotyrosine antibody.

SDS-PAGE and immunoblotting
As soon as spores were pelleted, spores were frozen by dipping the centrifugation tubes into liquid nitrogen and stored at −80°C until use. To analyze total spore proteins, spores were thawed and resuspended in 20 times volume of SDS-sample buffer (60 mM Tris-HCl, pH 6.8, 1% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.5 mM diithiothreitol (DTT), 0.05% Bromophenol Blue, 20 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml Bowman-Birk Inhibitor, 10 μg/ml N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 10 μM sodium vanadate) and boiled for 3 minutes. Cooled samples were centrifuged to remove spore coats and the resultant supernatants were loaded on a 10 to 20% polyacrylamide gradient gel (Daiichi Pure Chemicals Co., Tokyo, Japan) transmission electron microscope (TEM).

Immunoblotting of 2D-PAGE was done as described above using the ECL system. Rinsed membranes were confirmed by exposing the membrane for 15 minutes using the ECL system. Rinsed membranes were blocked with 5% BSA in TBS and then stained with anti-phosphotyrosine PY20 antibody.

SDS-PAGE
Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and reprobing
Spores were suspended in SDS-sample buffer modified so that the concentration of SDS and glycerol was 0.3 and 0%, respectively, boiled for 3 minutes and centrifuged. The supernatant was diluted with 6% Amphioline (mixture of pH 5.7 and pH 3.5-10.0 at the ratio of 4:1) containing 6% Nonidet P40 and 15% β-mercaptoethanol, while urea powder was added and dissolved to give a final concentration of 8.5 M. The final concentration of Nonidet P40, Amphioline and β-mercaptoethanol is 2, 2 and 5%, respectively. Samples loaded on a 4% polyacrylamide gel containing 8.5 M urea, 2% Amphioline and 2% Nonidet P40 were run for 7 hours at 200 to 200 volts (O’Farrell, 1975). For the second dimension using PAGE, the focused gel was loaded on a 10 to 20% polyacrylamide gradient gel, and the proteins were detected by silver staining methods (2D-Silver Stain II, Daiichi Pure Chemicals Co.). Isoelectric points were measured by comparison with marker proteins (2D-Premarker, Daiichi Pure Chemicals Co.). Immunoblotting of 2D-PAGE was done as described above using monoclonal anti-actin antibody clone C4. The PVDF membrane stained by the ECL system was washed with 0.2% Tween in TBS and shaken in erasing buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM β-mercaptoethanol) for 30 minutes at 65°C (Tesfaigzi et al., 1994). After a rinse in a 0.2% Tween solution and then in TBS, erasure of the first antibody was confirmed by exposing the membrane for 15 minutes using the ECL system. Rinsed membranes were blocked with 5% BSA in TBS and then stained with anti-phosphotyrosine PY20 antibody.

Preparation of actin
Spores 2 days of age were suspended in 10 mM N2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (Hepes, pH 8.0) buffer (0.2 mM Na2-ATP, 0.1 mM CaCl2, 0.2 mM DTT, 0.2 mM PMSF, 10 μg/ml TPCK, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, 10 μg/ml chymostatin, and 100 μM sodium vanadate) and boiled for 3 minutes. Cooled samples were centrifuged to remove spores coats and the resultant supernatants were loaded on a 10 to 20% polyacrylamide gradient gel (Daiichi Pure Chemicals Co., Tokyo, Japan; Laemmli, 1970). After the proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (immobilon-P Millipore Co., Bedford, MA) at 2 mA/cm² for 90 minutes using a submerged transfer system, Mini-Tank Electrobobler (Owl Scientific, Woburn, MA; Towbin et al., 1979), the membrane was incubated with Tris-buffered saline (TBS; 10 mM Tris-HCl, pH 7.4, 150 mM NaCl) containing 5% bovine serum albumin (BSA) at 25°C for 60 minutes, and stained with anti-phosphotyrosine antibody or anti-actin antibody diluted to 1:1,000 with the same solution. The membrane was then incubated with alkaline phosphatase-conjugated goat anti-mouse or -rabbit IgG, or horseradish peroxidase-conjugated sheep anti-mouse Ig diluted to 1:2,000 with TBS containing 3% skim milk (Difco, Detroit, MI). Signals to the second antibody were detected with a colorimetric detection system (KPL, Gaithersburg, MD). In some experiments, the blots were developed using an enhanced chemiluminescent detection system (ECL, Amersham) and exposed on an X-ray film (RX-U, FUJI FILM, Tokyo, Japan) for 2 minutes. Phosphorylation levels of actin band were measured by autoradiometry Quantity One (PDI, Huntington Station, NY), and the value at day 1 in Fig. 7 or at time 0 in Fig. 9 was normalized to 100%. Averages calculated from the relative values of two or three independent experiments are indicated in the figures.

Actin polymerization and depolymerization in vitro
The fraction including actin was separated by using the ECL system. SDS-PAGE and the amount of actin in each fraction was quantified by using autodensitometry. 2D-PAGE was done as described above. To depolymerize precipitated actin, the precipitated fraction was resuspended in Hepes buffer as described above and dialyzed against this buffer for 48 hours at 4°C to clear the remaining actin polymers and aggregates.

Negative staining
Precipitated actin molecules were fixed with 1% paraformaldehyde in

Immunelectron microscopy
Spores were fixed with a mixture of 2% paraformaldehyde and 0.1% glutaraldehyde in PB (pH 6.8) for 1 hour at 25°C. Fixed cells embedded in phosphate-buffered saline (PBS) containing 10% gelatin were cut into blocks and kept in 1 M sucrose until the blocks were submersed. Blocks were transferred and immersed in PBS containing 1.84 M sucrose and 20% polyvinylpyrrolidone overnight at 4°C, and then frozen by dipping into liquid nitrogen. Ultrathin cryo-sections were cut on an ULTRACUL (Leica, Wien, Austria). Formvar-carbon coated nickel grids bearing ultrathin sections were rinsed for 15 minutes at 25°C with 5% BSA in PBS, and floated overnight at 4°C on drops of 1:20 diluted monoclonal anti-phosphotyrosine antibody clone PY20, clone PY54, or 1% BSA for controls. After washing with PBS, specimens were stained with goat anti-mouse IgG conjugated to 10 nm gold particles diluted 30-fold in PBS containing 3% skim milk. Washed grids were stained with uranyl acetate (Tokuyasu, 1989), and then observed with a JEM 1200EX (JEOL, Tokyo, Japan) transmission electron microscope (TEM).
Pipes buffer, dropped on Formvar-coated copper grids, stained with 2% uranyl acetate and observed with TEM. For immunonegative staining, the precipitated actin molecules fixed with 1.5% paraformaldehyde in Pipes buffer were dropped on Formvar-carbon coated nickel grids. The grids blocked with 1% BSA in TBS for 15 minutes at 25°C were washed with TBS and floated on drops of a mixture of monoclonal anti-actin antibody diluted 20-fold in TBS and polyclonal anti-phosphotyrosine antibody diluted 100-fold for 40 minutes. After washing with TBS, samples were stained with a mixture of goat anti-mouse IgG conjugated to 15 nm gold particles diluted 20-fold and goat anti-rabbit IgG conjugated to 10 nm gold particles diluted 100-fold for 40 minutes. Washed grids were stained with 2% uranyl acetate and observed with TEM.

RESULTS

Tyrosine phosphorylation of actin in Dictyostelium spores

As shown by Gauthier et al. (1997) in addition to the 43 kDa component expected to be actin, many other unknown bands were recognized by immunoblotting of total D. discoideum spore proteins using anti-phosphotyrosine RC20 antibody which is a recombinant form of the anti-phosphotyrosine PY20 antibody. In contrast, only the 43 kDa band, corresponding to that stained with anti-actin C4, was detected with PY20 (Fig. 1, lane 3 and 4), when the second antibody was diluted with TBS containing 3% skim milk instead of 5% BSA used in the earlier work. Other anti-phosphotyrosine antibodies also recognized only the 43 kDa component under the same conditions (Fig. 1, lane 4 and 5). Very faint staining was observed at the low molecular weight position when the membranes were exposed for 10 minutes which is 5 times longer exposure time than that used in Figs 7 and 8 for the detection of 43 kDa band by the ECL detection system (data not shown). Therefore, the 43 kDa component is the major molecule that is tyrosine-phosphorylated in spores.

To confirm that the 43 kDa protein is actin, total spore proteins were subjected to 2D-PAGE and were immunoblotted with anti-actin C4 antibody and then reprobed with anti-phosphotyrosine PY20 antibody. Actin molecules were composed of two major isoforms (Fig. 2A). The pI value of the acidic isoform is 5.6, and the other is 5.8. A minor isoform was observed with a pI value of 6.0 as shown previously (MacLeod et al., 1980). After reprobing with the anti-phosphotyrosine PY20 antibody, only the acidic isoform was detected (Fig. 2B). The ratio of the acidic isoform to total actin was estimated at about 50% by autodensitometry. Thus, half of the actin molecules were tyrosine-phosphorylated in dormant D. discoideum spores.

Distribution of tyrosine-phosphorylated actin in spores

When the spore homogenate was separated into supernatant and precipitate fractions by ultracentrifugation, both fractions contained the same amount of actin (Fig. 3A). Since half of the actin was tyrosine-phosphorylated in either fraction (Fig. 3B), tyrosine-phosphorylated actin seems to exist in both soluble and precipitable forms in dormant spores. The distribution of phosphorylated actin in spores was further analyzed by cryosection-immuno electron microscopy using anti-actin C4 and anti-phosphotyrosine PY20 antibodies. Immuno-gold signals to C4 were found in the cytoplasm, and around vesicles as well as mitochondria that were transformed to round and crenate shapes (Fig. 3Ca; Sameshima et al., 1994). Signals to PY20 were also located around vesicles and mitochondria as well as in the cytoplasm (Fig. 3Cb). A portion of the phosphorylated actin present in the precipitable fraction likely originates from these particulate structures.
Actin tyrosine phosphorylation in Dictyostelium spores

To examine the effect of the phosphorylation on actin polymerization, actin molecules were isolated from the supernatant of spore homogenates by an anion-exchange MonoQ column (Fig. 4A). The fraction number 39 solely contained actin, and the molar ratio of the phosphorylated actin to unphosphorylated form was very similar to that of the original spore lysate as well as the supernatant of the lysate (Fig. 4B,C). This fraction was incubated under actin polymerizing conditions and fractionated into supernatants and precipitates by ultracentrifugation. About 90% of the actin molecules were recovered as precipitates (Fig. 5A). The molar ratio of the phosphorylated actin to unphosphorylated form in the precipitate fraction was similar to that of the original actin fraction (Fig. 5B). The negative staining images of the precipitated fraction showed that the fraction contained the same fibers as usual actin filaments 5-7 nm in diameter (Fig. 5C; Pollard and Cooper, 1986) but not any agglomerates. The double-immunonegative staining of the microfilaments with anti-actin and anti-phosphotyrosine showed that phosphorylated actin was integrated randomly in the fibers (insert in Fig. 5C). After dialysis of the precipitated fraction under actin depolymerizing conditions, 100% of the actin was collected again into the supernatant by ultracentrifugation (Fig. 5A). Thus, phosphorylated and unphosphorylated actin molecules were co-polymerized without any selection. It seems that tyrosine phosphorylation of actin does not affect its polymerizability in vitro.

Polymerization and depolymerization of phosphorylated actin

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Increase in tyrosine phosphorylation levels of actin during the very late stages in development

Since tyrosine phosphorylation of actin had not been detected in cells growing and developing up to the late culmination stage (22 hours in development; Schweiger et al., 1990), phosphorylation levels of actin should be increased after the late stage of the culmination and dephosphorylated during germination (Gauthier et al., 1997).

Tyrosine phosphorylation in the actin band was first detected at the end of the culmination stage (23 to 24 hours in development) by immunoblotting with anti-phosphotyrosine antibody PY20. The phosphorylation levels reached a
Actin tyrosine phosphorylation in Dictyostelium spores

maximum in the next 12 hours (Fig. 6A). Corresponding to an increase of staining on actin bands, the acidic isoform of actin at pI 5.6 appeared on 2D-PAGE at 24 hours of development, and increased to 50% of the total actin during the next 12 hours (Fig. 6B). This increase is due to tyrosine phosphorylation of basic isoforms, because the amount of total actin in spores is relatively constant (Fig. 6A, column CBB).

In aged spores stored at 22°C or 4°C, the phosphorylation levels of actin tyrosine were well correlated with the spore viability. The high levels of actin phosphorylation were kept for 20 days and declined to undetectable levels in 30 day old spores stored at 22°C (Fig. 7, filled square symbols). It is worthwhile to note that the viability of aged spores at 22°C was retained at more than 75% for 20 days, and then decreased in the same manner as the actin tyrosine phosphorylation levels (Fig. 7, open square symbols). On the other hand, both the phosphorylation and spore viability remained at high levels when spores were maintained at 4°C for 30 days (Fig. 7, circle symbols).

**Actin tyrosine dephosphorylation and spore swelling during germination**

In contrast to actin tyrosine phosphorylation, the rate of the dephosphorylation is very rapid. Just after activating spores with germination medium, phosphorylation levels rapidly decreased with a half-life of about 5 minutes, and almost all actin was dephosphorylated after 60 minutes of activation (Fig. 8A). Corresponding to this, the acidic isoform of actin was found to return quickly to the basic isoform on 2D-PAGE (Fig. 8B).

Kinetics of spore swelling, which is one of the earliest morphological changes observed in the germination process, was compared with that of the actin dephosphorylation. When spores began to swell at 20 minutes after the activation treatment, more than 90% of phosphorylated actin already had been dephosphorylated (Figs 8A, 9A). In swollen spores, movement of intracellular spherical particles, 1-2 μm in diameter, was observed by differential interference microscopy (Kishi et al., 1994). These structures appear to be spore vesicles, other spherical vesicles, and crenate mitochondria (Cotter et al., 1969; Wessels et al., 1989; Sameshima et al., 1994). At 90 minutes after activation, amoebae began to emerge from spore cases, and most of the spores completely germinated within the next 1 to 2 hours (Kishi et al., 1994).

Actin tyrosine dephosphorylation also began before spore swelling, when spores are stimulated with heat activation (Figs 8A, 9B), although the rate of dephosphorylation and swelling was much slower than that found in spores activated with germination medium. By 2.5 hours after activation, phosphorylation levels already were decreased to one third of the original level, while only 10% of spores swelled. Nutrient free phosphate buffer did not induce either actin dephosphorylation or spore germination for at least 5 hours when the culture was kept at 22°C (Figs 8A, 9B). To make the

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**Fig. 7.** Correlation of actin tyrosine phosphorylation levels with viability in aged spores of *D. discoideum*. After sorocarp formation, the dishes were kept either at 22°C (square) or 4°C (circle). The relative values of actin tyrosine phosphorylation levels (filled symbols) and percentages of viable spores (opened symbols) are indicated.

**Fig. 8.** Actin tyrosine dephosphorylation during the germination of *D. discoideum*. Spores 2 days of age were activated by suspending in germination medium (GM) or phosphate buffer (PB) as a control and shaken at 22°C. A portion of the spore suspension in PB was incubated at 45°C for 30 minutes and then transferred to a shaker at 22°C (Heat). Time 0 hour was defined as the time when the spore suspension was moved to 22°C. (A) Total proteins of the activated spores were immunoblotted with anti-phosphotyrosine clone PY20. Incubation time (minutes) with GM is indicated at the top, and the time (hours) with PB is indicated at the bottom. The arrowheads on the right indicate actin bands. (B) Silver staining images after 2D-PAGE of total proteins from the spores activated by GM. Minutes at the left indicate time of incubation with GM. pH values determined by pI markers are shown at the top. The arrowhead at the top shows the point at pI 5.6.
correlation between actin dephosphorylation and spore swelling clearer, spores were treated with the phosphatase inhibitors, phenylarsine oxide and potassium bisperoxo (1,10-phenanthroline) oxovanadate after heat acitvation. As a result, the spores maintained high levels of actin tyrosine phosphorylation for at least 5 hours (Fig. 9C). In this case, spore swelling was completely inhibited and spores retained their viability (data not shown).

**DISCUSSION**

In this study, it has been found that half of the actin molecules are tyrosine-phosphorylated in D. discoideum spores, which have developed upon starvation stress. Such high levels of actin phosphorylation would indicate that most of the actin system was affected in the dormant spores. However, the tyrosine phosphorylation of actin may not prohibit actin polymerization nor stimulate the depolymerization in spores, becausethe isolated actin fraction, containing phosphorylated and unphosphorylated isoforms at the same molar ratio as that of whole spores, can form actin filaments and can usually be depolymerized in vitro (Fig. 5), and tyrosine-phosphorylated actin exists in both a soluble and a precipitable fraction containing particulate structures in the same ratio in vivo (Fig. 3).

It is clear that high levels of actin tyrosine phosphorylation are specific to the dormant spore stage in the life cycle of D. discoideum. At the end of the culmination stage, spores have matured by morphological and physiological criteria. The formation of a spore wall and spore vesicles, transformation of mitochondria to round and creenate shapes, and condensation of cytoplasm have been completed, and these vesicles become completely immovable (Cotter et al., 1969; Maeda and Takeuchi, 1969; Gregg and Badman, 1970; Sameshima, 1993; Sameshima et al., 1994; Kishi et al., 1994). In addition, levels of oxygen uptake as well as energy metabolism are restricted (Liddel and Wright, 1961). Actin tyrosine phosphorylation begins to be detected at the time when spores have matured. Therefore, the actin tyrosine phosphorylation is possibly one of the final events during sporulation. The increasing levels of actin phosphorylation may correlate with the processes leading up to the spore maturation and the maintenance of the resting state of spores. In addition, the actin phosphorylation seems to be involved in keeping spores viable, since aged spores having high levels of the phosphorylation show high viability, and vice versa (Fig. 7).

Amoebae of another lower eukaryote, Physarum polycephalum, are known to be converted from a plasmodium into a dormant macrocyt, known as the sclerotium, upon exposure to desiccating conditions. In this case more than half of the actin molecules also exist in a phosphorylated state. However, the phosphorylated site on actin is not at a tyrosine residue but rather at Thr-203, and the phosphorylated actin has completely lost its polymerizing activity. It is suggested that the phosphorylation is involved in the reorganization of the actin network for transforming plasmodia into sclerotia (Furuhashi et al., 1998).

In contrast to actin tyrosine phosphorylation in spores, the dephosphorylation is one of the earliest events in the germination process. In all cases of spores stimulated with germination medium (Figs 8, 9), heat activation or autoactivators (Gauthier et al., 1997), the dephosphorylation of actin occurs before the beginning of the earliest morphological
changes; i.e. spore swelling, vesicle movement, and shape changes of mitochondria (Cotter et al., 1969; Maeda and Takeuchi, 1969; Kishi et al., 1994). In addition to this, spore swelling is completely inhibited when the dephosphorylation of actin is blocked by phosphatase inhibitors after heat activation (Fig. 9C), indicating that the dephosphorylation of actin may be necessary for spore swelling. In the early stages of germination, internal Ca^{2+} levels transiently increase prior to spore swelling, and is followed by activation of calmodulin which is necessary for swelling (Lydan and Cotter, 1994, 1995). Therefore, the rapid dephosphorylation of actin, together with increased internal Ca^{2+} levels, may lead to reactivation of the actin-based morphological changes and motility of intracellular organelles during the germination process.

The possibility that actin tyrosine phosphorylation is required for maintaining cells and their intracellular particles in an immobile state is supported by experiments with *Dictyostelium* amoebae treated with oxygen depletion or inhibition of oxidative phosphorylation by DNP and azide (Gerisch, 1962; Jungbluth et al., 1994, 1995). Within a few minutes after the treatment, cells round up becoming immovable, and vesicles as well as mitochondria agglomerate also becoming immobile. Following this, tyrosine phosphorylation levels of actin are increased. The phosphorylation and the resting state are retained throughout the treatment. Combined with our data, restriction of mitochondrial function seems to be one of the signals for the onset of actin tyrosine phosphorylation.

It may appear contradictory that after actin tyrosine dephosphorylation activated spores begin to swell, whereas before the dephosphorylation in cells freed from DNP or azide, the cells reform normal cell-shape and regain motility of vesicles and mitochondria (Jungbluth et al., 1994). This discrepancy is possibly explained by the different phosphorylation levels between vegetative cells exposed to inhibitors of respiration and dormant spores. The level in the former is roughly 10% (Fig. 3 in Jungbluth et al., 1995), while in inhibitors of respiration and dormant spores. The level in the former is roughly 10% (Fig. 3 in Jungbluth et al., 1995); while inhibitors of respiration and dormant spores. The level in the former is roughly 10% (Fig. 3 in Jungbluth et al., 1995), while in inhibitors of respiration and dormant spores. The level in the former is roughly 10% (Fig. 3 in Jungbluth et al., 1995). Therefore, the rapid dephosphorylation of actin, which is necessary for swelling (Lydan and Cotter, 1994, 1995). Within a few minutes after the treatment, cells round up becoming immobile. Following this, tyrosine phosphorylation in *Dictyostelium* spores.

In *Dictyostelium* spores and stressed amoebae, the mechanism for actin phosphorylation is unknown. However, the *Dictyostelium* dual-specificity kinase, splA, may be a candidate kinase that phosphorylates actin tyrosine during the very late stages in development. In spores of the splA gene null mutant, phosphorylation of actin is not detected by immunoblots with anti-phosphotyrosine antibody (data not shown), and the spores spontaneously lyse before completing their differentiation, while they normally grow and develop to form fruiting bodies consisting of sori and stalks (Nuckolls et al., 1996).

In this study, we have demonstrated that high levels of actin tyrosine phosphorylation are related to the maintenance of the dormant state in *D. discoideum* spores. Very recently, we have found that actin in spores of other *Dictyostelium* species, *D. mucoroides* and *D. purpureum*, are also heavily tyrosine-phosphorylated (data not shown). In dormant and stressed cells, high levels of actin tyrosine phosphorylation may play a key role in surviving undesirable conditions.

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