The MADS-box transcription factor SrfA is required for actin cytoskeleton organization and spore coat stability during

ARTICLE *in* MECHANISMS OF DEVELOPMENT · FEBRUARY 2004

Impact Factor: 2.44 · DOI: 10.1016/j.mod.2003.11.001 · Source: PubMed

Mechanisms of Development 121 (2004) 51–56

www.elsevier.com/locate/modo

The MADS-box transcription factor SrfA is required for actin cytoskeleton organization and spore coat stability during *Dictyostelium* sporulation

Ricardo Escalante[a], Yohko Yamada[b], David Cotter[c], Leandro Sastre[a,*], Masazumi Sameshima[b]

[a]*Instituto de Investigaciones Biomédicas CSIC/UAM, Gene Expression, Arturo Duperier, 4, 28029-Madrid, Spain*
[b]*Department of Biofunctional Science, Faculty of Agriculture and Life Science, Hirosaki University, 3 Bunkyo-cho, Hirosaki, Aomori 036-8561, Japan*
[c]*Department of Biological Sciences, University of Windsor, Windsor, Ontario N9B 3P4, Canada*

Received 9 May 2003; received in revised form 29 August 2003; accepted 21 October 2003

Abstract

The MADS-box transcription factor SrfA is involved in spore differentiation in *Dictyostelium* [Development 125 (1998) 3801]. Mutant spores show an altered morphology and loss of viability. A detailed structural analysis of mutant spores has been performed to gain insight into the specific aspects of spore differentiation in which SrfA is involved. Two main structural defects have been observed. One is the formation of high order actin structures, the so-called actin rods. SrfA mutant spores showed the initial stages of rod formation but no mature rods were found in older spores either in the nucleus or the cytoplasm. Moreover, phosphorylation of actin, that is believed to stabilize the actin rods, is strongly reduced in the mutant. The other defect observed was the formation of the spore coat. Young *srfA*$^-$ spores show basically normal trilaminar coat structures suggesting that release of prespore vesicles and basic assembly of the coat takes place in the absence of SrfA. However, the outer layer gets wavier as the spore ages and suffers a progressive degradation suggesting a late defect in the stability of the spore coat. Taken together, these results suggest that SrfA is involved in late events of spore maturation necessary for spore stability.

Keywords: Actin; Cytoskeleton; *Dictyostelium*; Differentiation; Spore; Spore coat; Serum response factor

1. Introduction

Sporulation in *Dictyostelium* involves a tightly regulated process of morphogenesis that generates a fruiting body composed essentially of two specialized cell types: stalk cells that support the structure and spores resting on top of the stalk. Spores are highly specialized cells adapted to survive under adverse environmental conditions. The modifications include a marked decrease in metabolic activity, dehydration of the cells, compaction of intracellular structures and organelles and the synthesis of a semi-impermeable spore coat. Spore differentiation initially involves the release of prespore vesicles containing components of the spore coat. These components are organized into a tri-lamellar case composed of two proteinaceous layers and a middle layer of cellulose (West, 2003; Srinivasan et al., 2000; Cotter et al., 2000). This process, often referred to as encapsulation, is very rapid and is concomitant with the onset of expression of SpiA, a culmination protein with four strong transmembrane domains that has been described to localize into inner coat layers (Richardson et al., 1991, 1994; Richardson and Loomis, 1992). Although morphogenesis of the fruiting body and spore encapsulation is completed in 24 h, spores continue to mature for at least 12 h more. Spore maturation is necessary to make a fully resistant spore. Spore maturation is not well characterized but involves accumulation of certain components and changes in the cytoskeleton that are necessary for the spore to survive under extreme environmental conditions (Cotter et al., 2000).

The organization of actin molecules changes dramatically in the course of spore differentiation. Rods of actin tubules are formed both in the nucleus and cytoplasm of immature spores starting at mid-culmination. These rods are formed of actin tubules hexagonally cross-linked. As the spores mature modules of rods are associated into thicker rods (Sameshima et al., 1994, 2000, 2001). In addition, a large fraction of actin

* Corresponding author. Tel.: +34-91-585-4437; fax: +34-91-585-4401.
E-mail address: lsastre@iib.uam.es (L. Sastre).

0925-4773/$ - see front matter © 2003 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.mod.2003.11.001

molecules (50%) are phosphorylated on tyrosine during spore maturation. Actin tyrosine phosphorylation is coincident with rod maturation and might contribute to the formation and stability of elongated actin rods in the spores (Kishi et al., 1998). During germination the level of tyrosine phosphorylation rapidly declines and, shortly after, the actin rods disaggregate (Sameshima et al., 2001).

As occurs during metazoan embryogenesis, *Dictyostelium* cell type differentiation and morphogenesis are coordinated to ensure correct temporal and spatial differentiation of stalk and spore cell types (Escalante and Vicente, 2000). SrfA, a *Dictyostelium* protein highly homologous to the MADS-box family of transcription factors, plays an essential role in this coordination (Escalante and Sastre, 1998, 2002; Escalante et al., 2001). The expression of this gene is activated by the cAMP-dependent protein kinase (PKA) during mid-culmination as the prespore cells are being lifted halfway up the stalk (Escalante and Sastre, 2002). PKA activity depends on the level of intracellular cAMP that is regulated by a balance between synthesis and degradation (Loomis, 1998; Shaulsky et al., 1998; Soderbom et al., 1999; Wang et al., 1999). At this stage prespore cells begin to differentiate into spores. SrfA-dependent gene expression is essential for spore differentiation since strains lacking *srfA* display abnormal spore morphology and loss of viability (Escalante and Sastre, 1998). The mRNA level of expression of the major spore coat proteins is normal in the mutant during morphogenesis. However, the expression of *spiA* is strongly reduced, suggesting that *srfA* might be involved only in late events of spore differentiation (Escalante and Sastre, 1998).

Multiple studies have shown that serum response factor (SRF) is involved in the regulation of actin cytoskeleton in several animal systems. SRF-binding sites have been found in several actin genes (Chai and Tarnawski, 2002). The expression of several actin-binding proteins including β1-integrin, Talin and Zyxin were found to be dependent on SRF. Embryonic stem cells where the SRF-coding gene has been deleted are defective in actin cytoskeleton organization (Schratt et al., 2002). Moreover, the activity of SRF itself is regulated by actin dynamics (Sotiropoulos et al., 1999; Posern et al., 2002). Since a similar interplay could exist in *Dictyostelium discoideum*, a ultrastructural study of *srfA*$^-$ spores was undertaken. Our results suggest that *srfA* is involved in the formation of the actin rods during late spore maturation and that this transcription factor is also necessary for the stability of the spore coat.

2. Results

2.1. Initial stages of actin rod formation are not dependent on srfA but rod elongation and maturation are compromised in the mutant

Spores were taken at different times of development and visualized by EM. Mutant spores taken at 26.5 h of development were a mixture of prespore cells not yet encapsulated and encapsulated spores. Parallel arrangements of actin tubules, each composed with three actin filaments, were observed in the nucleus of both in WT and *srfA*$^-$ spores. These structures, designated as modules, are composed of 20–30 parallel actin tubules arranged hexagonally in transverse sections (Sameshima et al., 2001). Actin modules appeared in prespore cells that were not encapsulated (Fig. 1A,E), as well as in newly encapsulated spores (Fig. 1B,F). In 7% of the sections observed from Wild Type spores, several modules were associated symmetrically (Fig. 1C) to form elongated actin rods that stretched across the nucleus over the next hours after encapsulation (Fig. 1D). Cytoplasmic rods were also visible in 5% of the sections from WT spores (data not shown). This process of rod formation was not observed in mutant *srfA*$^-$ spores. Instead, 7% of the sections from mutant spores taken at 35 h (a time in which elongated rods should be visible) still showed modules not arranged into rods (Fig. 1G,H). Moreover, the observation of more than 1000 sections of *srfA*$^-$ spores taken at 45 h of development did not allow the detection of any actin rod module, compared to 10% of WT spores that showed them (data not shown).

2.2. Actin phosphorylation is reduced in srfA spores

Actin phosphorylation is coincident with the process of rod maturation, from association of single modules to elongation and tight packing of rods. Since actin phosphorylation is believed to contribute to the process of rod maturation, we checked the level of actin phosphorylation in spore extracts at different times of development. As expected, tyrosine phosphorylation was drastically reduced in *srfA* mutant spores at any of the developmental times tested (Fig. 2).

2.3. Spore coat assembly and stability of srfA spores

The majority (70%) of *srfA*$^-$ spores collected at 26.5 h of development displayed the characteristic trilaminar coat composed of two electron-dense layers (the outer and inner layers) and a middle cellulose layer (Hohl and Hamamoto, 1969), although it was not obvious in spores frozen quickly and freeze-substituted (Fig. 3A). However, a proportion of mutant spores taken at 26.5 h (30%) showed anomalies in the outer spore coat layer. While WT spores show a smooth, continuous outer layer (Fig. 3E), mutant spores displayed wavy and sometimes broken layers (Fig. 3B,C). Mutant spores taken at 45 h of development were clearly abnormal. Some of them showed rounded morphology instead of the Wild Type ellipsoid shape. Spore coats were partially or almost totally lost in 60% of the spores (Fig. 3D, arrowheads) and some of them (30%) showed extensive vacuolization (Fig. 3D, double arrow). Spore coat disruption might affect spore resistance to detergent treatment. Accordingly, *srfA*$^-$ spores taken at 30 h and plated without further treatment show up to 30% viability versus less than

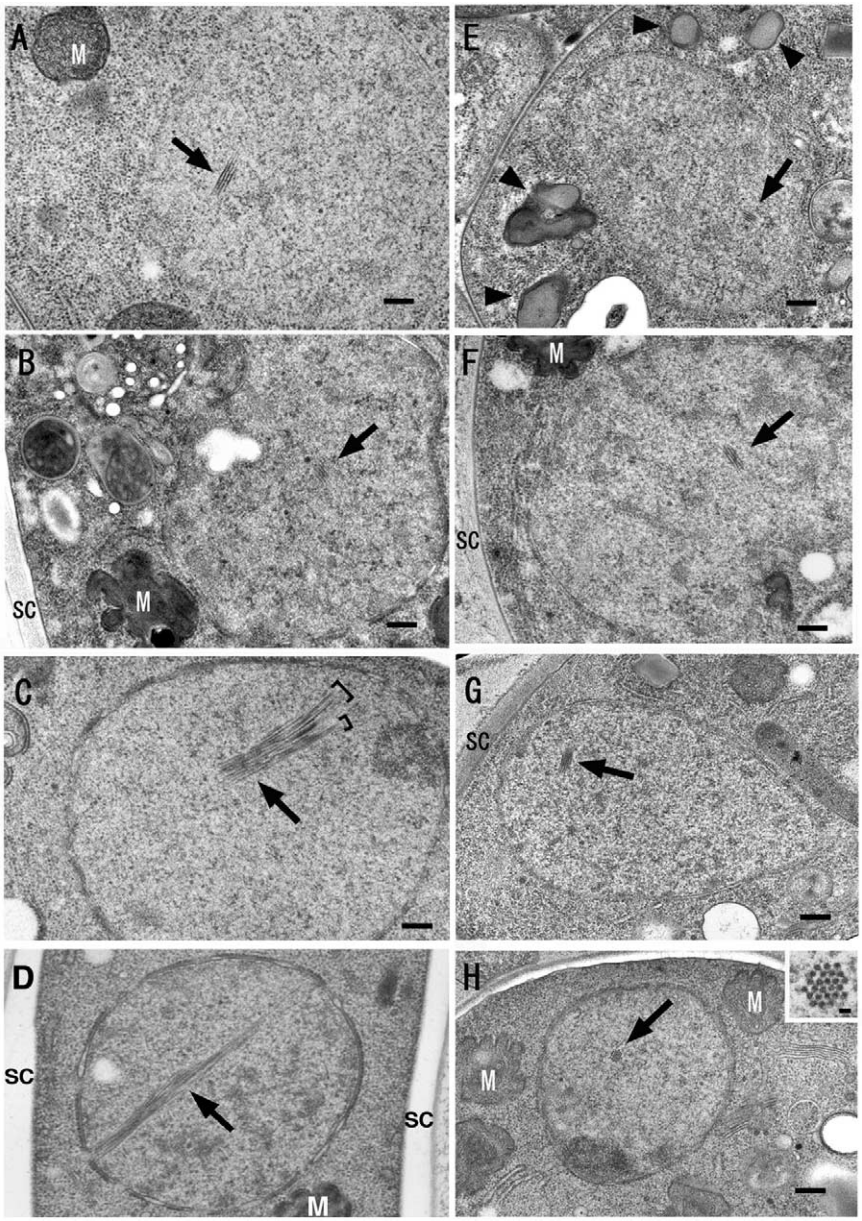

Fig. 1. Presence of actin rods in spores from Wild Type and *srfA*$^-$ strains. Spores were frozen and freeze-substituted after 26.5 (A, B, E, F), or 35 (G, H) or 45 (C, D) h of development from Wild Type (A–D) or *srfA*$^-$ (E–H) *Dictyostelium* strains. Longitudinal sections of spore actin rods were examined on a transmission electron microscope. Inset in panel H shows a higher magnification cross-section that shows the hexagonal arrangement of actin tubules. Spore sections in (C, D) show a gap between the spore coat and the plasma membrane probably due to artifacts generated during sectioning of the samples. Arrows, actin rods in the nucleus; arrow heads in panel E, prespore vesicles; brackets in panel C, modules of actin rods; SC in panel B, D, F and G, spore coat; white M, mitochondria; bars, 200 nm (A–H) and 20 nm (inset of H).

0.5% viability if the spores were previously treated with detergent. Wild Type spores showed 100% of viability after detergent treatment.

3. Discussion

Spores are highly specialized cells resistant to extreme environmental conditions. The capacity of spores to resist heat for example is not fully developed until several hours after the fruiting body has differentiated. This process of maturation is poorly understood. SrfA seems to play an important role at this late stage of differentiation since two of the best characterized structures formed in terminally differentiated spores, actin rods and the spore coat, are altered in *srfA*$^-$ spores. The phenotype of *srfA*$^-$ spores can be explained, at least partially, by the deficiencies observed in these structures. Sameshima et al. (2000) showed that spores lacking actin rods had a round shape and very low viability, similar to *srfA*$^-$ spores. The progressive degradation of the spore coat must affect permeability

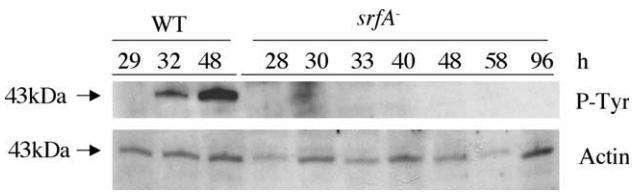

Fig. 2. Analyses of actin tyrosine phosphorylation in Wild Type and *srfA*$^{-}$ strains. Wild type and *srfA*$^{-}$ spores were collected at the times indicated over each lane, lysed and analyzed by Western blot using either anti-phosphotyrosine antibody PY20 (upper blot) or anti-actin antibody C4 (lower blot). Migration of the 43 kDa molecular weight marker is indicated to the left.

and might also explain the low viability of *srfA*$^{-}$ spores and their decreased resistance to stress conditions.

The alterations observed could be the consequence of spore degeneration after differentiation or premature germination. However, our results strongly suggest that the primary defect is that *srfA*$^{-}$ spores do not complete the differentiation process and never acquire the characteristics of mature spores. Spore markers, including SpiA and some new markers recently characterized in our laboratory, are not expressed in *srfA*$^{-}$ spores or are expressed at very low levels (Escalante and Sastre, 1998; Escalante et al., 2003). The cellulose layer is also not completely formed, as shown by calcofluor staining (Escalante and Sastre, 1998). The absence of a completely differentiated spore coat is also detected by the sensitivity to detergent treatment of *srfA*$^{-}$ spores. Moreover, mutant spores with relatively normal internal ultrastructure (no abnormal vacuolization and normal organization of organelles) showed no mature rods at any time tested, which suggests that SrfA is involved in rod maturation.

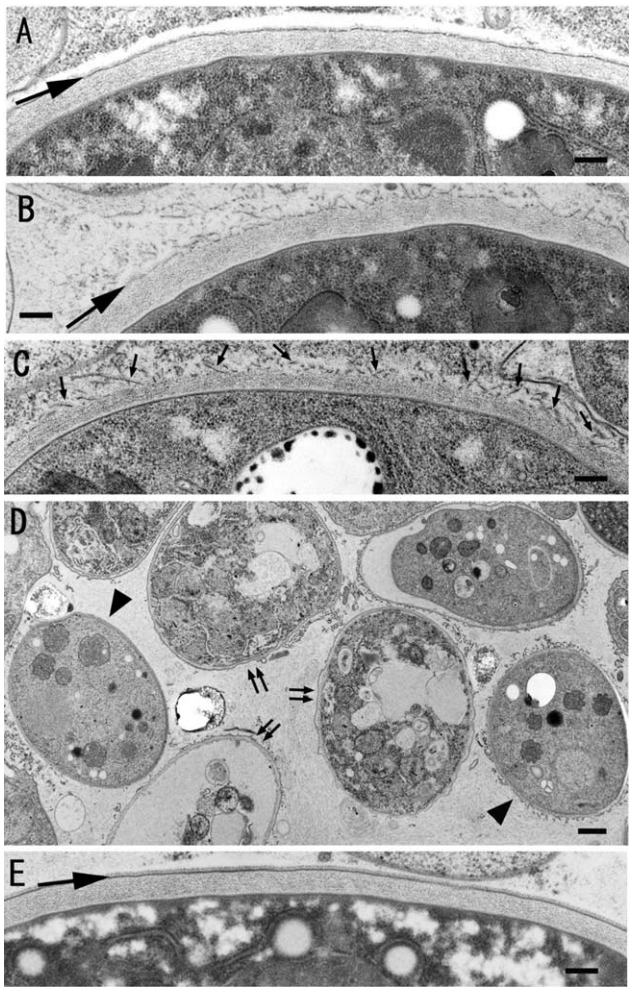

Fig. 3. Ultrastructural analyses of the coat from Wild Type and *srfA*$^{-}$ spores. Spores of *srfA*$^{-}$ after 26.5 (panels A–C) or 45 (D) h and Wild Type spores (panel E) after 26.5 h of development were frozen and freeze-substituted. High magnification of longitudinal sections of the spores, showing the spore coats, is presented in panel A–C and E. Panel D showed disruption of the outer layer of the spore coat (arrow heads) as well as other alterations of spore structure, such as vacuolation (double arrow). Arrows, the outer layer of spore coat; small arrows in panel C, fragmented outer layer of spore coat; bar, 200 nm (A–C and E) and 1 μm (D).

The mechanisms that result in the impaired spore differentiation in *srfA*$^{-}$ strains could be the consequence of altered expression of different genes. As mentioned in Section 1, it has been recently reported that mammalian SRF is involved in actin cytoskeletal organization. Our results in *Dictyostelium* may suggest a possible ancient origin for the interplay between actin and SRF. Since SrfA is a transcription factor, the expression of critical components necessary for rod maturation and/or actin phosphorylation might be dependent on the function of SrfA. Consistent with the last hypothesis we have recently found that expression of cofilin-1, an actin-binding protein that was previously found to be a component of the cytoplasmic rods in *Dictyostelium* (Sameshima et al., 2000), was greatly reduced in the *srfA* mutant strain during late culmination (data not shown).

A possible function for actin phosphorylation in the maturation or stability of actin rods has been proposed. In agreement with this idea we have found both aspects to be affected in the mutant *srfA*$^{-}$. SrfA might regulate both aspects independently or perhaps one aspect might be depend on the other one in a functional interdependent relationship.

The defects observed in spore coat structure also seem to be due to alterations in late steps of coat formation. The expression of the major spore coat proteins (SP60, SP70, SP96 and psB) are normally induced in *srfA*$^{-}$ prespore cells (Escalante and Sastre, 1998 and unpublished results). Moreover, prespore vesicles can be visualized in *srfA*$^{-}$ prespore cells (Fig. 1E). Release of these prespore vesicles and spore coat assembly is essentially normal since the trilaminar structure of the coat can be seen during late culmination. However, the stability of the outer proteinaceous layer seems to be compromised as disruption of this layer becomes apparent soon after coat assembly. In older spores the subjacent cellulose layer is probably very scarce since staining with calcofluor showed reduced fluorescent intensity (Escalante and Sastre, 1998).

It is possible that the lack of expression of other components that are deposited in the coat at later stages

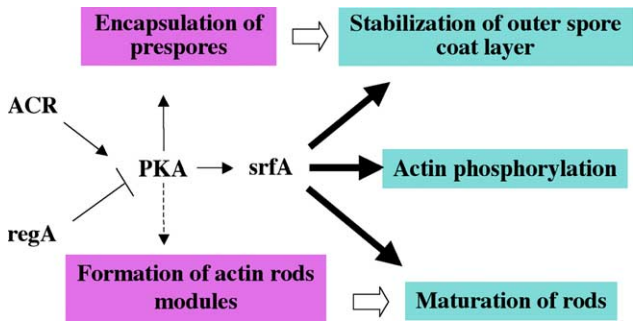

Fig. 4. Schematic model of the proposed pathway through which the transcription factor SrfA participates in the regulation of terminal spore differentiation. Encapsulation of prespores and the formation of actin rods modules are early events of spore maturation that are not dependent on SrfA, whereas late events such as tyrosine phosphorylation of actin, stabilization of the outer spore coat layer and maturation of actin rods are regulated by SrfA. The presence of pathways indicated by thick arrows was suggested in this work. The pathway shown by a dashed line is not yet verified.

might be responsible for the phenotype observed. A subtle defect in prespore vesicle-dependent coat assembly cannot be ruled out either. A protein known to be important for spore viability and whose expression is dependent on *srfA* is SpiA. Reduced expression of *spiA* in *srfA*$^-$ mutant cannot account by itself for the defect in spore coat. It has been described that *spiA* mutants show normal spore coat structure and the reduced viability can only be detected after long time exposure to submerged conditions (Richardson and Loomis, 1992). At least two spore coat genes are expressed too late in development to be included in the classical prespore vesicles and must reach the coat by an independent unknown mechanism (unpublished results). The expression, secretion and/or spore coat association of some of these proteins could be dependent on SrfA.

In summary, the results obtained in this analyses, together with previous results on *srfA* regulation, lead us to suggest a scenario as shown in Fig. 4. During mid-culmination, the activity of the adenylyl cyclase ACR and the inhibition of the phosphodiesterase RegA lead to the activation of PKA. PKA in turn leads to the onset of SrfA expression in the prespore cells at this stage of development. Events that occur during mid and late culmination such as release of prespore vesicles, assembly of the coat and initial stages of actin rod formation can occur in the absence of SrfA. However, other aspects that occur later, such as actin rod maturation, actin phosphorylation and spore coat stability, seem to be compromised. Taken together our results suggest that SrfA plays a major role in different aspects of late spore maturation.

4. Experimental procedures

4.1. Cell culture and development

D. discoideum cells, strain Ax4 and a *srfA*$^-$ mutant (Escalante and Sastre, 1998) were grown axenically in HL5 medium (Cocucci and Sussman, 1970) without or with 5 μg/ml Blasticidin S hydrochloride. For development cells were washed twice by brief centrifugations with 20 mM phosphate-buffered solution containing 1 mM $MgSO_4$ and plated at 5×10^5 cells/cm^2 on a Millipore HA filters underlaid with Whatmann 3MM filters that absorbed 40 mM phosphate-buffered solution containing 20 mM KCl, 2.5 mM $MgCl_2$ and 20 μg/ml of streptomysin sulfate. Spore resistance to detergent treatment was tested by incubating the spores in 0.3% cemusol (NP12) for 30 min. The viability of the spores was determined by counting the plaques formed after plating them on *Klebsiella aerogenes* in SM plates.

4.2. Electron microscopy

Sori of fruiting bodies were sandwiched between copper grids and quickly frozen by submersion in liquid propane using Leica WM CPC (Wien, Austria). Specimens were freeze-substituted with acetone containing 2% OsO_4 at −83 °C for 4 days, warmed to −25 °C, and transferred on ice (Sameshima et al., 2001). Washed specimens with cold absolute acetone were kept at room temperature for 30 min and then embedded in epoxy resin (Poly/Bed 812, Polyscience Inc., Warrington, PA, USA). Ultrathin sections doubly stained with uranyl acetate and lead citrate were examined with a JEOL JEM-1010 transmission electron microscope (JEOL, Tokyo, Japan). The diameter of microtubules was normalized to 25 nm. At least 1000 spore sections were analyzed for each sample.

4.3. Detection of phosphorylated actin

Sorocarps from structures at different times of development were collected and transferred to SDS-sample buffer (60 mM Tris–HCl pH 6.8, 1% SDS, 10% glycerol, 5% β-mercaptoetanol, 0.5 mM Dithiothreitol (DTT), 0.05% Bromophenol blue, 20 μg/ml Leupeptin, 2 μg/ml Aproteinin, 0.1 mM AEBSF). Samples were boiled for 5 min and loaded on 10% polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with TBST (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) containing 0.5% skim milk and then incubated with anti-phosphotyrosine (PY20, ICN) or anti-actin (C4, Santa Cruz Biotechnology) antibodies, diluted 1:1000 in TBST containing 5% Bovine Serum Albumin, overnight at 4 °C. After three 10-min washes with TBST, membranes were incubated for 1 h at room temperature with the peroxidase-conjugated secondary antibody, diluted 1:2000 with TBST containing 3% skim milk. After three washes with TBST, blots were developed using the enhanced chemiluminescent detection system (ECL, Amersham).

Acknowledgements

Research by R.E. and L.S. was financed by Grants from the Dirección General de Investigación (Grants PB98-0517 and BMC2002-01501).

References

Chai, J., Tarnawski, A.S., 2002. Serum response factor: discovery, biochemistry, biological roles and implications for tissue injury healing. J. Physiol. Pharmacol. 53, 147–157.

Cocucci, S.M., Sussman, M., 1970. RNA in cytoplasmic and nuclear fraction of cellular slime mold amoebas. J. Cell Biol. 45, 399–407.

Cotter, D.A., Mahadeo, D.C., Cervi, D.N., Kishi, Y., Gale, K., Sands, T., Sameshima, M., 2000. Environmental regulation of pathways controlling sporulation, dormancy and germination utilizes bacterial-like signaling complexes in *Dictyostelium discoideum*. Protist 151, 111–126.

Escalante, R., Sastre, L., 1998. A serum response factor homolog is required for spore differentiation in *Dictyostelium*. Development 125, 3801–3808.

Escalante, R., Sastre, L., 2002. Regulated expression of the MADS-box transcription factor SrfA mediates activation of gene expression by protein kinase A during *Dictyostelium* sporulation. Mech. Develop. 117, 201–208.

Escalante, R., Vicente, J.J., 2000. *Dictyostelium discoideum*: a model system for differentiation and patterning. Int. J. Dev. Biol. 44, 819–835.

Escalante, R., Vicente, J.J., Moreno, N., Sastre, L., 2001. The MADS-box gene *srfA* is expressed in a complex pattern under the control of alternative promoters and is essential for different aspects of *Dictyostelium* development. Develop. Biol. 235, 314–329.

Escalante, R., Moreno, N., Sastre, L., 2003. *Dictyostelium discoideum* developmentally regulated genes whose expression is dependent on the MADS-box transcription factor SrfA. Eukaryotic Cell in press.

Hohl, H.R., Hamamoto, S.T., 1969. Ultrastructure of spore differentiation in *Dictyostelium*: the prespore vacuole. J. Ultrastruct. Res. 26, 442–453.

Kishi, Y., Clements, C., Mahadeo, D.C., Cotter, D.A., Sameshima, M., 1998. High levels of actin tyrosine phosphorylation: correlation with the dormant state of *Dictyostelium* spores. J. Cell Sci. 111, 2923–2932.

Loomis, W.F., 1998. Role of PKA in the timing of developmental events in *Dictyostelium* cells. Microbiol. Mol. Biol. Rev. 62, 684–694.

Posern, G., Sotiropoulos, A., Treisman, R., 2002. Mutant actins demonstrate a role for unpolymerized actin in control of transcription by serum response factor. Mol. Biol. Cell 13, 4167–4178.

Richardson, D.L., Loomis, W.F., 1992. Disruption of the sporulation-specific gene *spiA* in *Dictyostelium discoideum* leads to spore instability. Genes Develop. 6, 1058–1070.

Richardson, D.L., Hong, C.B., Loomis, W.F., 1991. A prespore gene, Dd31, expressed during culmination of *Dictyostelium discoideum*. Develop. Biol. 144, 269–280.

Richardson, D.L., Loomis, W.F., Kimmel, A.R., 1994. Progression of an inductive signal activates sporulation in *Dictyostelium discoideum*. Development 120, 2891–2900.

Sameshima, M., Chijiiwa, Y., Kishi, Y., Hashimoto, Y., 1994. Novel actin rods appeared in spores of *Dictyostelium discoideum*. Cell Struct. Funct. 19, 189–194.

Sameshima, M., Kishi, Y., Osumi, M., Mahadeo, D., Cotter, D.A., 2000. Novel actin cytoskeleton: actin tubules. Cell Struct. Funct. 25, 291–295.

Sameshima, M., Kishi, Y., Osumi, M., Minamikawa-Tachino, R., Mahadeo, D., Cotter, D.A., 2001. The formation of actin rods composed of actin tubules in *Dictyostelium discoideum* spores. J. Struct. Biol. 136, 7–19.

Schratt, G., Philippar, U., Berger, J., Schwarz, H., Heidenreich, O., Nordheim, A., 2002. Serum response factor is crucial for actin cytoskeletal organization and focal adhesion assembly in embryonic stem cells. J. Cell Biol. 156, 737–750.

Shaulsky, G., Fuller, D., Loomis, W.F., 1998. A cAMP-phosphodiesterase controls PKA-dependent differentiation. Development 125, 691–699.

Soderbom, F., Anjard, C., Iranfar, N., Fuller, D., Loomis, W.F., 1999. An adenylyl cyclase that functions during late development of *Dictyostelium*. Development 126, 5463–5471.

Sotiropoulos, A., Gineitis, D., Copeland, J., Treisman, R., 1999. Signal-regulated activation of serum response factor is mediated by changes in actin dynamics. Cell 98, 159–169.

Srinivasan, S., Alexander, H., Alexander, S., 2000. Crossing the finish line of development: regulated secretion of *Dictyostelium* proteins. Trends Cell Biol. 10, 215–219.

Wang, N., Soderbom, F., Anjard, C., Shaulsky, G., Loomis, W.F., 1999. SDF-2 induction of terminal differentiation in *Dictyostelium discoideum* is mediated by the membrane-spanning sensor kinase DhkA. Mol. Cell Biol. 19, 4750–4756.

West, C.M., 2003. Comparative analysis of spore coat formation, structure, and function in *Dictyostelium*. Int. Rev. Cytol. 222, 237–293.