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STAGE-SPECIFIC CHANGES IN PROTEIN PHOSPHORYLATION DURING SPORE GERMINATION IN DICTYOSTELIUM: ROLE OF CALMODULIN

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Summary: Extensive protein phosphorylation occurs during all phases of spore germination in Dictyostelium discoideum. The developmental changes were prevented when germination was inhibited by inhibitors of calmodulin function. In addition, differences in patterns of phosphorylation were detected based upon the method of spore activation. Several phosphoproteins were lost in heat activated as compared to autoactivated spores. In spite of the fact that several aspects (i.e. autoactivation, emergence) are calmodulin-dependent, there was no evidence that calcium- or calmodulin-dependent protein kinase activity is present during any phase of spore germination. This suggests that other CaM-dependent processes mediate the diverse aspects of spore germination in D. discoideum.

In Dictyostelium discoideum, both exogenous physical factors and endogenous molecules play roles in maintaining dormancy and promoting germination. High osmotic stress and the action of an autoinhibitor, discadenine enforce dormancy of spores within the sorus (1,2). Once spores are placed under suitable osmotic conditions and the autoinhibitor is removed, they can be activated by various physical means including treatment with dimethyl sulfoxide and elevated temperature (2-5). However, fully developed spores can also autoactivate, via the action of a diffusible, endogenous autoactivator (2,6-9). Recent evidence suggests that spore activation, especially autoactivation, in D. discoideum occurs via a calcium-dependent signal transduction process similar to that seen during cell to cell signalling in other eukaryotes (10-13). One of the essential intracellular targets for calcium during spore germination in D. discoideum is the ubiquitous eukaryotic regulatory protein CaM (12,13). Spore swelling and amoebal emergence in are CaM-

Abbreviations used: calmodulin, CaM; trifluoperazine, TFP; ethyleneglycol-bis-(ß-aminoethylether) N,N,N',N'-tetraacetic acid, EGTA; ethylenediaminetetraacetic acid, EGTA; phenylmethylsulfonylfluoride, PMSF; phosphoprotein, PP.

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dependent regardless of the mode of spore activation (12,13). In contrast, autoactivation is CaM-dependent while activation by heat is not (12,13). While it is clearly beneficial for spores to have alternative routes to germinating, the biochemical and molecular mechanisms that underlie them are only recently becoming clear. A CaM-binding protein of 64,000 M_r (CaMBP64) is developmentally linked to autoactivation (13). No other biochemical comparisons between activation mechanisms have been carried out.

One major class of targets for CaM activity following cell stimulation are CaM-dependent kinases (14,15). In D. discoideum, a CaM-dependent kinase activity, which may be CaM kinase III, is present during fertilization (16). Recently, a CaM-dependent kinase of about 55,000 M_r has been partially purified from asexually developing D. discoideum cells (17). To date, no studies of protein kinase activity have been carried out during spore germination. In this study, an attempt was made to elucidate possible biochemical events that underlie the different forms of activation by comparing the developmental changes in calcium-and CaM-dependent protein kinase activities after autoactivation and heat activation. Differences in patterns of protein phosphorylation were observed between spores activated by the different methods. This may reflect differences in the underlying mechanisms of germination. Furthermore, the developmental changes in kinase activity were prevented when germination was inhibited by the CaM inhibitors TFP and calmidazolium. However, CaM-dependent protein kinase activity was not evident, suggesting that other CaM-dependent processes mediate the diverse aspects of spore germination in D. discoideum.

MATERIALS AND METHODS

Strains and Culturing

Spores of D. discoideum strain SG2 (ATCC 44841) were maintained on glucose-salts agar with E. coli B/r as a food source. SG2 is derived from V12 (ATCC 44842) and autoactivates within 1 day of spore formation rather than after 10-16 days as seen in V12 (7,8). SG2 is identical to the parental strain with regards to its response to heat activation (7,8). Spores were harvested at one to three days post culmination in ddH_2O, washed twice with 10 mM phosphate buffer pH 6.5 (germination buffer), to remove the autoinhibitor, and resuspended in this buffer at 5 x 10^7 spores/mL (9). Some spores were allowed to autoactivate in germination buffer. Here, time 0 is defined as the time immediately following the first wash of the harvested spores. Spores which were heat activated were heated to 45°C for 30 minutes with time 0 immediately following the heat-activation period. 20 mL of each spore suspension was shaken at 175 rpm and 20 µL aliquots were examined every 30 minutes after the first 60 minutes. The proportion of swollen spores and nascent amoebae was determined following survey of at least 200 spores/amoebae per aliquot via phase-contrast microscopy. Each trial was repeated at least three times and the mean value and standard error of that mean was presented for each trial.

Inhibitor Studies

For studies examining the effect of anti-CaM drugs on spore germination, TFP (Sigma Chemical Co., St. Louis, Mo.) and calmidazolium (Sigma Chemical Co., St. Louis, Mo.) were prepared as 5 mM stock solutions in germination buffer and absolute ethanol, respectively. Both drugs were diluted to working concentrations with germination buffer and added prior to heat-activation or, in the case of autoactivation, both drugs were added at time 0. Ethanol at 0.5% was used as a control for the calmidazolium trials.

Phosphoprotein Identification

Germination, harvesting and processing of SG2 spores was performed as per Lydan et al and North and Cotter (13,18). The endogenous phosphorylation activity of the spore extracts was determined as per Lydan and O'Day (1993b). 40 µg of total spore protein was phosphorylated in reaction mixtures containing 50 mM Tris, pH 6.8, 15 mM $MgCl_2$, 0.8 mM EDTA, 0.8 mM PMSF, 0.8 µg/mL leupeptin, 0.1% BSA, 5 µM ^{32}P-γ-ATP (5Ci/mmol) with one of: a) 1 mM $CaCl_2$/1 µg/mL VU-1-CaM (19), b) 1 mM EGTA, c) 10 µM TFP or d) 20 µM calmidazolium. In addition, proteins from spores treated with either TFP or calmidazolium were phosphorylated in reaction mixtures containing 1 mM $CaCl_2$/1 µg/mL VU-1-CaM.

RESULTS AND DISCUSSION

Both CaM antagonists TFP and calmidazolium completely inhibited spore swelling and subsequent amoebal emergence (Figure 1). The concentrations of each agent used here were the minimum required to completely inhibit germination of both autoactivated and heat activated spores at a spore concentration of 5 x 10^7/mL (data not shown). In vitro phosphorylation reactions were carried out to determine whether 1) CaM-dependent kinase activity plays a role during spore activation in D. discoideum and 2) if an inhibition of CaM activity interferes with the change in pattern of the endogenous proteins phosphorylated by kinases found within the spore.

However, CaM-dependent kinase activity was not visualized in either autoactivated or heat shocked spores since there was no effect on the PP pattern caused by the addition of either calcium, CaM or CaM inhibitors to the phosphorylation reactions (Figures 2A,B,C,D). However, there were some interesting changes in the detection of certain PPs during germination. A 41,000 M_r PP showed a germination-dependent loss in autoactivated, but not heat activated spores (Figures 2A,B,C,D; closed arrow, left panel Figure 2A). Several PPs of about 49,000, 45,000 and 42,000 M_r appeared during amoebal emergence in both autoactivated and heat activated spores (Figures 2A,B,C,D; open arrows, right panel Figure 2A). Finally, two PPs of about 33,000 and 31,000 M_r appeared during amoebal emergence in heat activated spores but were present throughout germination in autoactivated spores (Figures 2A,B,C,D; closed arrows, right panel Figure 2B). None of these changes occurred in any spore which were inhibited from germinating by TFP and calmidazolium (Figure 2). The roles for any of these PPs cannot be

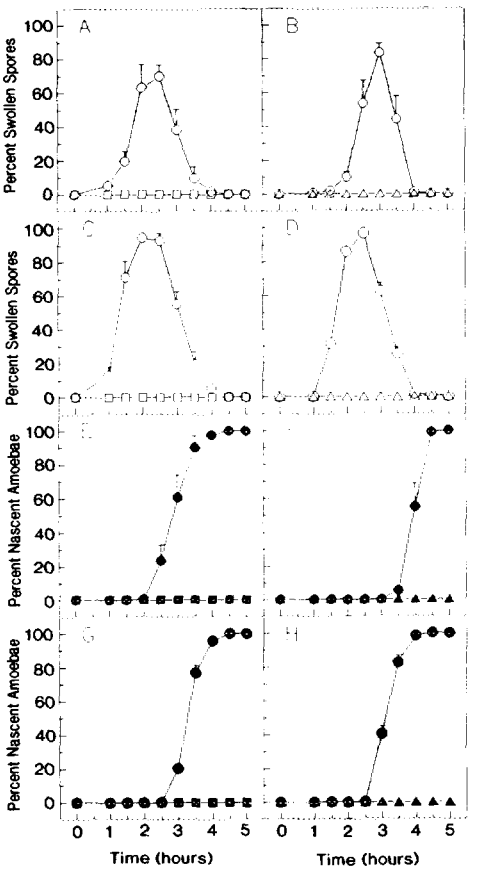

Figure 1. Effects of TFP and calmidazolium on the germination of autoactivated and heat activated spores of Dictyostelium discoideum strain SG2. Spores of D. discoideum strain SG2 were either autoactivated or heat activated in the presence of either TFP (Figures 1A,C,E,G) at 10 (□, ▩) μM or calmidazolium (Figures 1B,D,F,H) at 20 (△, ▲) μM. The open symbols represent swollen spores (Figures 1A,B,C,D) and the closed symbols represent nascent amoebae (Figures 1E,F,G,H). Aliquots were removed and the percentages of swollen spores and nascent amoebae in both treated cultures and untreated controls (○, ●) were determined as per the Materials and Methods. The error bars represent the s.e.m.

determined from this data, however, PP41 was temporally associated with activation and swelling of autoactivated spores and PPs 31,33,42,45,49 were associated with amoebal emergence.

The data here show that CaM-dependent kinase activity does not play a role during spore gemination. In addition, these data support a previous study which showed, via a pharmacological approach, that protein kinase C does not mediate spore germination (13). Since there was no change in the PP pattern between extracts phosphorylated in the presence of calcium or EGTA, no calcium-dependent kinase activity was seen. While PKC from many organisms is both calcium- and phospholipid-dependent, the D. discoideum form is stimulated in vitro by calcium

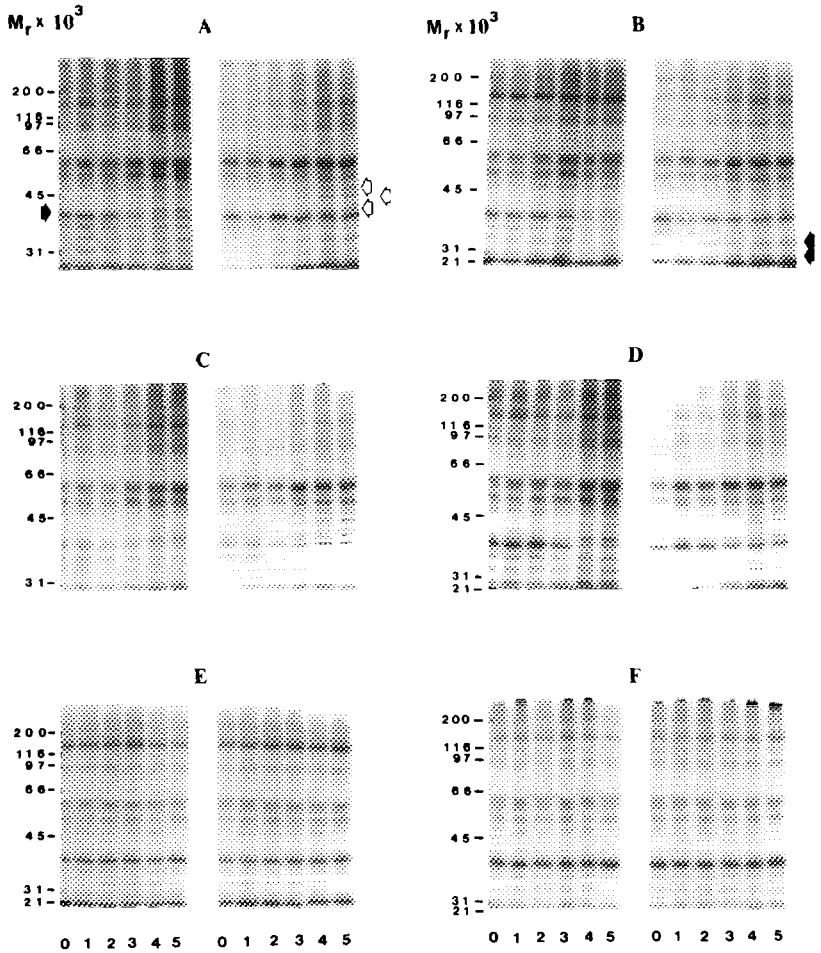

Figure 2. Endogenous kinase activities and substrates in germinating Dictyostelium discoideum spores. Spores of D. discoideum strain SG2 were either allowed to autoactivate (left panels) or heat activated (right panels). The soluble proteins were extracted as per the Materials and Methods and phosphorylation reactions were carried out in the presence of 1 mM $CaCl_2$/1 µg/mL VU-1-CaM (Figure 2A), 1 mM EGTA (Figure 2B), 10 µM TFP (Figures 2C) or 20 µM calmidazolium (Figure 2D). In addition, autoactivating spores were treated, at time 0, with either 10 µM TFP (Figure 2E) or 20 µM calmidazolium (Figure 2F) and phosphorylation reactions were carried out in the presence of 1 mM $CaCl_2$.

alone (20). By default, the kinase activity seen in these spore protein preparations may be interpreted to be primarily protein kinase A-dependent since cAMP plays an important role during spore germination (Virdy, K.J., Sands T.W., Kopko, S.H. and Cotter, D.A., unpublished observations).

While CaM-dependent kinase activity does not regulate spore germination in D. discoideum, CaM function is essential. First, specific inhibitors of CaM activity inhibit germination (Figure 1; 11,13). Second, an inhibition of CaM activity inhibits

the normal change in the pattern of the endogenous PPs seen during spore germination (Figure 2). The role that CaM plays here appears to be as a mediator of spore activation. Since the spore cannot be activated, due to the effect of the CaM inhibitors, the changes in PPs observed in untreated spores do not occur. However, a direct role for other CaM-dependent proteins is not clear yet since the pattern of CaM-binding protein does not markedly change during spore germination in D. discoideum (13). Elucidation of the specific CaM-dependent mechanisms which either initiate or directly mediate the changes in PPs seen here will require disruption of the genes for the various CaMBPs and an examination of each for its effect on both germination and the pattern of endogenous PPs.

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