Pattern of phosphoproteins during cell differentiation in *Streptomyces collinus*

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Received 4 November 1997; accepted 6 November 1997

Abstract

Transition from vegetative cells to aerial mycelium and spores of *Streptomyces collinus* is accompanied by changes in the pattern of proteins phosphorylated. Preparation from spores exhibits lower phosphorylation activity than those of vegetative cells and aerial mycelium. Phosphorylation of proteins from aerial mycelium was markedly stimulated by the presence of Mn²⁺. Our data indicate that phosphorylation of proteins on Ser/Thr residues is involved in transition of vegetative cells to aerial mycelium.

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Keywords: *Streptomyces collinus*; Protein phosphorylation; Cell differentiation

1. Introduction

The involvement of reversible covalent modification of proteins in cell growth and differentiation of streptomycetes are only beginning to be understood. Experiments with eukaryotic protein kinase inhibitors in *Streptomyces griseus* revealed presence of multiply phosphorylated proteins. These experiments suggest that *S. griseus* possesses several protein kinases of the eukaryotic type and that inhibitors of these enzymes block the morphogenesis and pigment production [1]. Recently, two protein kinases PkaA and PkaB were cloned and sequenced from *S. coelicolor*. The N-terminal part of both proteins showed significant similarity with the catalytic domain of eukaryotic Ser/Thr protein kinase. The *pkaB* gene was overexpressed in *Escherichia coli* and the gene product PkaB was phosphorylated on Thr. Phosphoamino acids of PkaA were analyzed in a similar way and Thr and Ser but not Tyr residues were found to be phosphorylated [2]. *Streptomyces collinus* BSM 40733 is a Gram-positive kirromycin-producing microorganism which undergoes complex morphological differentiation. In an attempt to study molecular events accompanying transitions from filamentous vegetative cells to aerial mycelium and spores, we began to investigate proteins which are phosphorylated in various cell types from [γ-³²P]-ATP. The overall IEF-PAGE gel electrophoretic patterns provide an analysis of in vivo distribution of phosphoproteins in vegetative cells and aerial spores. Monoclonal antibodies against phosphoserine and phosphothreonine were used for the detection of phosphoproteins.
2. Materials and methods

2.1. Microbial strain and cultivation

Aerial spores of S. collinus DSM 40733 were used to inoculate complex medium containing 0.4% yeast extract (Difco), 1% malt extract (Oxoid), 0.4% glucose, pH 7.2 (before sterilization). After 20 h cultivation at 28°C, cells were used for inoculation of fresh medium. Cells were harvested by centrifugation at 10,000 g for 10 min and washed with standard buffer (20 mM Tris-HCl pH 7.6, 10 mM MgCl₂, 0.5 mM phenylmethylsulfonyl fluoride) at 4°C. Harvested cells were frozen and stored at −70°C.

2.2. Aerial mycelium and spores

Sporulation plates were prepared with the above medium and contained 2.5% agar (Oxoid). Agar plates were covered with sterile cellophane discs and inoculated with suspension of submerged cells. White aerial mycelium was harvested after 48 h of cultivation at 28°C and homogenized in ice-cold water at 15,000 rpm for 30 s in the Ultra-Turrax turbomixer. The mixture was centrifuged at 7000 g for 10 min at 4°C. Substrate mycelium sediments at the bottom of the centrifugation tube and white hydrophobic aerial mycelium floats in the supernatant. Aerial mycelium was collected and washed three times with standard buffer. Spores were prepared as previously described [3].

2.3. Preparation of cell-free extracts from vegetative cells and spores

Cells were suspended in the standard buffer to a concentration of about 1 g of wet cells per 2 ml of buffer. Suspensions of vegetative cells and aerial mycelium were disintegrated by sonication and spores were disrupted with glass beads in a precooled mortar. Homogenates were centrifuged to remove unbroken cells and glass. Membranes were removed by centrifugation at 30,000×g for 30 min and ribosomes from the supernatant fraction (S30 fraction) after centrifugation at 150,000×g for 2 h and 4°C. Final supernatants (S150 fractions) were frozen in liquid nitrogen and stored at −70°C.

2.4. Protein phosphorylation in vitro

Fresh, unfrozen S30 fractions were used for the protein kinase assay. Reaction mixtures (20 µl) contain 50 mM Tris-HCl pH 7.4, 50 mM NaCl, 10 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 40 µg proteins, 10 mM [γ-³²P]ATP (2000 cpm/pmol) and where indicated 10 mM MnCl₂ and 10 mM NaF. Reaction mixtures were incubated 10 min at 28°C. Assays were terminated by the addition of 5 µl of 5× SDS sample buffer and analyzed by SDS-PAGE. After

Fig. 1. In vitro phosphorylation of proteins in cell-free extracts of S. collinus. The assays were performed as described in Section 2 and analyzed: A: in 10% SDS-PAGE; B: in 17% SDS-PAGE. All reactions contained 40 µg of S30 fractions from vegetative cells (slots 1–3), aerial mycelium (slots 4–6) and spores (slots 7–9). In control experiments Mn²⁺ was absent (slots 1, 4 and 7). Complete reaction mixtures with 10 mM MnCl₂ (slots 2, 3, 5, 6, 8, and 9). Experiments where complete reaction mixtures were supplemented with 10 mM NaF (slots 3, 6 and 9). Dry gels were exposed to X-ray film.
staining and drying the gel was exposed to X-ray film.

2.5. High resolution two-dimensional IEF-PAGE

Samples for electrophoresis (S150 fractions) were extracted with a solution containing 9.5 M urea, 2% CHAPS, 1.6% ampholyte 5–7, 0.4% ampholyte 3–10 and 2% 2-mercaptoethanol. IEF gel rods were run at 400 V for 18 h. The gels were equilibrated in SDS sample buffer and layered on top of 10% polyacrylamide gel slabs and run at 30 mA.

2.6. Detection of phosphoamino acids in S150 proteins

S150 supernatant proteins were transferred to nitrocellulose membranes and incubated with 5% serum albumin and 1% ovalbumin in TBST (20 mM Tris-HCl pH 7.6, 137 mM NaCl, 0.2% Tween 20) for 2 h at room temperature. Monoclonal anti-phosphoserine and anti-phosphothreonine, respectively (Sigma) were used for detection of phosphorylated proteins. The blot was incubated with the first antibody in the above buffer with 5% serum albumin and 1% ovalbumin for 1 h at room temperature. Secondary mouse Ig, horseradish peroxidase-linked whole antibody from sheep was incubated with blots in the buffer for 1 h at room temperature. ECL Western blotting reagents (Amersham) were used for detection of phosphoproteins. Blots were washed between steps five times with 40 ml of the TBST buffer.

3. Results

We have examined phosphorylation of proteins of S. collinus from [γ-32P]ATP in fresh, unfrozen S30 fractions prepared from vegetative cells, aerial myce-

Fig. 2. Immunodetection of phosphorylated proteins after high resolution 2-dimensional gel electrophoresis. Vegetative cells: Pattern of proteins (S150 fraction) from 8-h-old cells after silver staining (A). Western blots were probed with monoclonal antibodies (Sigma) against phosphothreonine (B), phosphoserine (C). Aerial mycelium: Protein pattern of supernatant proteins (S150 fraction) after silver staining (D). Western blots were probed with anti-phosphothreonine (E), anti-phosphoserine (F). The binding of the first antibody was monitored by the ECL system.
lum and spores. Protein kinase activity of S30 fractions was explored under optimum salt concentrations in the presence or absence of Mn$^{2+}$ and NaF. Reaction mixtures were analyzed using 10% and 17% SDS-PAGE and radioactivity of phosphorylated proteins was detected on dried gels by autoradiography. As shown in Fig. 1A and B, there are differences in the pattern of in vitro phosphorylated proteins of vegetative cells, aerial mycelium and spores. In the preparation from vegetative cells proteins of 16, 24, 27, 33, 36, 40, 45, 55, 66, 72, 78, 92, and 120 kDa were phosphorylated, the major labeled band corresponds to the 66-kDa protein. The data also show that phosphorylation activity of the preparation from vegetative cells was not dependent on the presence of Mn$^{2+}$ or NaF (slots 1–3). Transition from vegetative cells to aerial mycelium was accompanied by changes in pattern of proteins phosphorylated. Phosphoproteins from aerial mycelium were resolved in 10% polyacrylamide gel into bands of 21, 27, 29, 34, 36, 43, 45, 55, 66, 72 and 120 kDa (Fig. 1A slots 4–6). The pattern of phosphorylated proteins in 17% gel (Fig. 1B, slots 4–6) displays major $^{32}$P-labeled proteins of 66, 55, 29, 27, 21, and 12 kDa. The phosphorylation of proteins from aerial mycelium was stimulated by the presence of Mn$^{2+}$ (Fig. 1A and B, slots 5 and 6) and one of the heavily labeled proteins of vegetative cells (16 kDa) was substituted for a protein of 12 kDa. In spite of the fact that all experiments were performed with an equal amount of proteins (40 μg), S30 fraction from spores exhibited relatively low phosphorylation activity which was not enhanced by the presence of Mn$^{2+}$, and only faintly visible phosphoproteins of 120, 80, 66, and 55 kDa were detected (Fig. 1A and B, slots 7–9).

To obtain information on the role of threonine/serine protein kinases in protein phosphorylation during cell differentiation, we combined analysis of proteins by high resolution two-dimensional electrophoresis with immunodetection of proteins containing phosphorylated threonine and serine. Equal amounts of protein extracts from vegetative cells and aerial mycelium were analyzed and Western blots were incubated with monoclonal antibodies raised against phosphoserine and phosphothreonine. As shown in Fig. 2, transition from vegetative cells to aerial mycelium is accompanied by differences in protein patterns as well as by changes in phosphorylated proteins. Results of these experiments indicate that cell differentiation in S. collinus involves post-translational modification of proteins catalyzed by serine/threonine protein kinases.

4. Discussion

Introduction of a phosphate group into protein molecules can induce changes in enzymatic activity, binding properties to ligand molecules or localization of proteins. Relatively little is known about protein kinase activity in vegetative cells, aerial mycelium and spores of streptomycetes. This is the first report describing the protein kinase activity and pattern of phosphorylated proteins in vitro from this type of cells. Our data show that protein kinases of vegetative cells and aerial mycelium differ in sensitivity to the stimulatory effect of Mn$^{2+}$. Differences in the pattern of in vitro phosphorylated proteins were found in E. coli when ATP or GTP were used as phosphate donors [4].

Protein phosphorylation and dephosphorylation is one of the principal mechanisms by which cellular activities are regulated in response to external stimuli. The protein kinases are tightly regulated and maintained in a catalytic inactive form in the absence of specific activating signal. We used monoclonal antibodies to rapid detection of phosphothreonine and phosphoserine residues of proteins. To eliminate nonspecific interactions, all antibodies were incubated in the presence of 5% serum albumin and 1% ovalbumin. The control experiments with phosphoserine and phosphothreonine indicate that binding of monoclonal antibodies to phosphoproteins was specific. Phosphorylation of proteins on Ser/Thr residues acts through simple charge alterations and its main function is to modify the conformation or substrate binding sites, whose activities are consequently altered. Our data show that this type of modification is involved in transition of vegetative cells to aerial mycelium.

References


