JOURNAL OF BACTERIOLOGY, May 1990, p. 2808–2811
0021-9193/90/052808-04$02.00/0

Vol. 172, No. 5

A 57-Kilodalton Protein Associated with *Spiroplasma melliferum* Fibrils Undergoes Reversible Phosphorylation

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Received 11 October 1989/Accepted 7 February 1990

Phosphorylation of a major 57-kilodalton protein substrate was observed in cell lysates of *Spiroplasma melliferum* BC3 incubated with [γ-^{32}P]ATP. Only serine phosphates have been isolated from the acid hydrolysate of the phosphorylated protein. The 57-kilodalton protein substrate was found, to a large extent, in the cytosolic fraction and, to a lesser extent, associated with cell membranes and was detected in the Triton X-100-insoluble fraction that contained fibrils.

The involvement of protein phosphorylation cascades in intracellular signaling is a well-known phenomenon in eucaryotes (4, 9, 13). Despite intensive studies, the biochemical nature of signaling in bacteria remains poorly understood. It was recently suggested that such signaling may involve protein phosphorylation cascades similar to those implicated in many eucaryotic systems (5, 6, 16, 21, 22, 25, 29). Although the mollicutes lack a rigid cell wall and locomotive organelles, some *Mycoplasma* species show a peculiar flask-shaped appearance (12, 15) and are capable of gliding on solid surfaces (2). Recently, the possibility that protein phosphorylation in the mollicutes is associated with the unusual morphology and motility was raised (7, 18). Among the mollicutes, the *Spiroplasma* species are unique in having helical morphology and rotary motility (31). Questions concerning the structural basis of these properties have been raised previously (18–20). Several spiroplasmas were found to release, upon cell lysis, fibrils 3 to 4 nm in diameter (3, 30). In *Spiroplasma melliferum* BC3, these fibrils were found to be composed of a single protein (27, 28). Nevertheless, the regulatory mechanisms that govern assembly and disassembly of spiroplasmal fibrils are obscure. In view of the recent findings that reversible phosphorylation controls the assembly of intermediate filaments in eucaryotes (10, 11), we undertook the present study to investigate the interrelations of protein phosphorylation and cell fibrils in spiroplasmas.

S. melliferum BC3 cells were grown for 24 to 72 h at 32°C in a modified Saglio medium (24). Cells were harvested at the mid-exponential phase of growth (A_{640}, ~0.2) by centrifugation at 12,000 × *g* for 20 min, washed once, and suspended in 0.25 M NaCl in 20 mM Tris hydrochloride buffer (pH 7.4). Cells were osmotically lysed following preloading with glycerol as previously described (20), and membranes were separated from the soluble fraction by centrifugation at 37,000 × *g* for 45 min. Protein in the membrane and soluble fractions was determined by the method of Bradford (1) and ATPase activity as previously described (20).

In vitro phosphorylation was assayed as previously described (18) in a reaction mixture (final volume of 50 μl) containing 20 mM Tris-maleate buffer (pH 6.0), 1 mM dithiothreitol, 50 mM NaF, 2 mM $CaCl_2$, 10 to 40 μg of spiroplasma cell protein, and 1 mM [γ-^{32}P]ATP (5 to 20 Ci/mol; The Radiochemical Center, Amersham Corp.). In some experiments, the reaction mixture was supplemented with the soluble fraction derived from *Acholeplasma axanthum* cells (25 μg of protein). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (14). The gels were stained with Coomassie blue R-250 in methanol-acetic acid-water (12.5:2.5:10 by volume) at 50°C, destained for 5 h with frequent changes of the same solvent, dried, and exposed to X-Omat (Eastman Kodak Co.) at −70°C for autoradiography. Treatment of the gels with NaOH, HCl, trichloroacetic acid, ribonuclease, or pronase was performed as described before (18). Phosphoamino acids were identified as described by Wang and Koshland (29).

Incubation of cell lysates of *S. melliferum* BC3 with [γ-^{32}P]ATP revealed a major 57-kilodalton (kDa) phosphorylated protein band. This protein was not phosphorylated by exogenous kinases from eucaryotic origins (M. Platt, unpublished data) but was efficiently phosphorylated by a protein kinase present in the soluble fraction of *A. axanthum* (17). No phosphorylation was obtained when washed, isolated membranes were utilized as the sole cellular protein in the reaction mixture (Fig. 1, lane A2). Nevertheless, when the soluble fraction of *A. axanthum* was added to a reaction mixture containing either native or heat-denatured washed *S. melliferum* membranes, a 57-kDa phosphorylated protein band was apparent (Fig. 1, lane A4). These observations suggest that the 57-kDa protein, at least in its nonphosphorylated form, is attached to the cell membrane. Comparison of the labeling intensity of the phosphorylated band obtained from the heat-denatured membrane preparation (Fig. 1, lane A4) with that obtained from the heat-denatured soluble preparation (Fig. 1, lane B4) revealed that about 25% of the 57-kDa protein substrate was associated with the membrane fraction. About 80% of the radioactivity was released from the phosphorylated native membranes upon a single washing in 25 mM Tris-HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (pH 7.5), whereas only 7% of the membrane-bound ATPase activity was released by the same treatment, suggesting that the phosphorylated and unphosphorylated 57-kDa proteins differ in their affinities to their membrane receptors. Such differences may be due to a conformational change that affects binding to membrane fragments. Differences in the affinities of phosphorylated and

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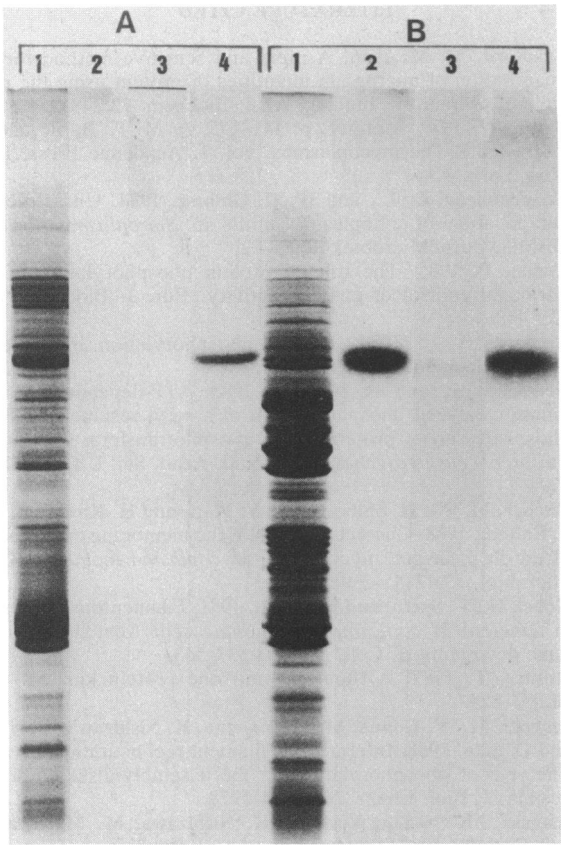

FIG. 1. Phosphorylation of the 57-kDa protein in the soluble and membrane fractions of *S. melliferum*. Phosphorylation was carried out with isolated membranes (A) or the soluble fraction (B). Lanes: 1, Coomassie blue-stained gel prepared from the complete reaction mixture; 2, autoradiogram of lane 1 after 24 h of exposure; 3, autoradiogram of a gel prepared from a reaction mixture containing heat-denatured membrane or soluble protein (80°C for 10 min); 4, autoradiogram of a gel prepared from a reaction mixture described for lane 3 supplemented with 25 μg of native soluble fraction of *A. axanthum*.

dephosphorylated heavy meromysin to F-actin were recently demonstrated (26).

The phosphorylation of the 57-kDa protein of *S. melliferum* was linear for about 2 min. Longer incubation periods resulted in a decrease in the phosphorylation level depending on the concentration of NaF in the reaction mixture. Decreasing the NaF from 50 to 10 mM resulted in a 50% decrease in the level of phosphorylation within 8 min, and in a reaction mixture without NaF, phosphorylation could not be detected. As the phosphorylation of the 57-kDa protein was detected only in the presence of NaF, a nonspecific phosphatase inhibitor, it seems that phosphorylation is reversible through the activity of a phosphoprotein phosphatase.

The phosphorylation of the 57-kDa protein was pH dependent, with the highest levels of phosphorylation obtained in the pH range of 5.5 to 6.0. Little or no phosphorylation was observed in the more alkaline pH range. ^{32}P incorporation into the 57-kDa *S. melliferum* protein was found with [γ-^{32}P]ATP but not with [α-^{32}P]ATP. During the course of preparing the soluble fraction, it became apparent that the kinase activity was considerably more stable in extracts

TABLE 1. Binding of soluble proteins to *S. melliferum* membranes[a]

Soluble protein (μg/mg of membrane protein)	Binding	
	cpm/mg of membrane protein ± SD	% ± SD
^{125}I labeled		
900	9,790 ± 1,200	6.5 ± 0.8
450	3,220 ± 450	4.2 ± 0.6
^{32}P labeled		
900	630 ± 250	1.3 ± 0.5
450	390 ± 120	1.6 ± 0.4

[a] Isolated membranes (1 mg/ml) were incubated for 15 min at 37°C with equal volumes of the soluble protein fraction (2 μg/ml) labeled by either the ^{125}I-chloramine-T procedure (20) or in vitro phosphorylation as described in Materials and Methods. The resulting membrane-soluble protein complex was collected by centrifugation (37,000 × *g* for 45 min) and washed once, and the amount of soluble protein bound to the membrane was estimated by measuring the respective isotope. Values are means from three independent measurements.

where dithiothreitol was present; therefore, dithiothreitol was included in all buffers utilized.

The phosphoryl moiety of the 57-kDa *S. melliferum* protein was completely released by pronase treatment (50 μg/ml for 1 h at 37°C) or by incubating the phosphorylated gels in 1 N NaOH at 60°C for 15 min. The phosphoryl moiety was unaffected by RNase treatment (25 μg/ml for up to 2 h at 37°C), was stable during staining and destaining of the gels at 50°C, and was only slightly affected by boiling in 1 N HCl for 15 min, suggesting that like in *Mycoplasma gallisepticum* (18), the phosphorylated protein contains on acid-stable phosphoaminoacyl residue. To identify the phosphoamino acid residue, total hydrolysis of the ^{32}P-labeled protein was performed and the hydrolysate was subjected to electrophoresis. Autoradiography of the electrophoretogram revealed that the phosphorylated 57-kDa protein contained almost exclusively (>95%) phosphoserine.

When the total soluble fraction of *S. melliferum* was ^{125}I labeled with chloramine-T and incubated with the isolated membrane fraction, about 5% of the ^{125}I-labeled protein was bound to the membrane fragments (Table 1). Because the addition of 0.5 M NaCl to the binding medium strongly inhibited the binding and because washing the membrane-soluble protein complex with 1 M NaCl released over 85% of the bound soluble proteins, it appears that the soluble proteins bind to the membrane fragments by electrostatic bonds. When the ^{32}P-labeled soluble fraction was incubated with the isolated membrane fraction, the binding of the ^{32}P-labeled protein to the membrane fragments was found to be only about 20% of that of the ^{125}I-labeled protein. As with the ^{125}I-labeled proteins, the ^{32}P-labeled proteins were readily released by washing with 1 M NaCl.

A fibril preparation, prepared by extracting intact *S. melliferum* cells with 1% Triton X-100 (28), contained a major 57-kDa protein band (Fig. 2, lane B). When the fibrils were the sole protein in the phosphorylation reaction mixture, no phosphorylation was detected (data not shown). However, when the fibrils were added to the reaction mixture together with *A. axanthum* soluble fractions, a 57-kDa protein band was intensively labeled (Fig. 2, lane E). When whole cell lysates were phosphorylated prior to the Triton X-100 extraction, relatively little labeling was obtained in the Triton X-100-insoluble fraction (Fig. 2, lane D). Intracellular fibrils were demonstrated in various *Spiroplasma* species (3, 27, 28, 30) as well as in flask-shaped

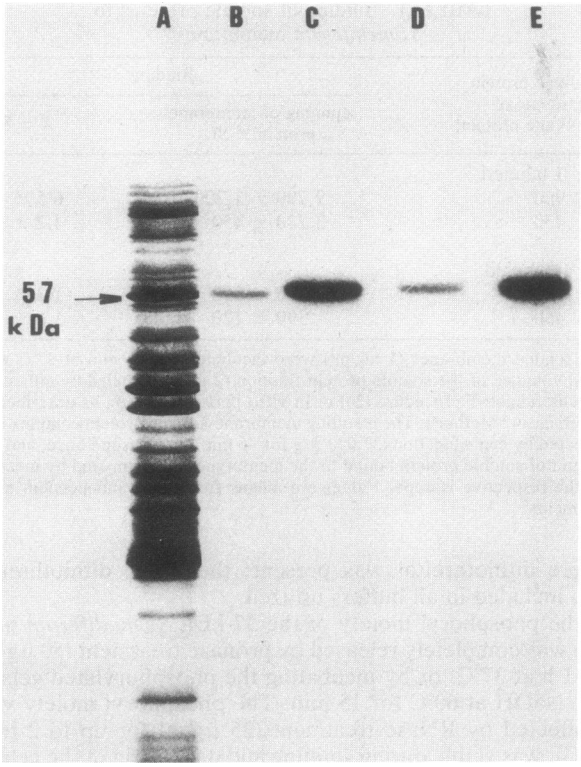

FIG. 2. Phosphorylation of a 57-kDa protein associated with *S. melliferum* fibrils. Phosphorylation was carried out as described in Materials and Methods. Lanes: A, Coomassie blue-stained gel of total cellular protein fraction; B, Coomassie blue-stained gel of the Triton X-100-insoluble fraction; C, autoradiogram of a gel obtained after sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the complete reaction mixture containing the total cellular protein fraction; D, autoradiogram of a gel after sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the Triton X-100-insoluble fraction obtained by extracting the reaction mixture described for lane C; E, autoradiogram of a gel after sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the Triton X-100-insoluble fraction extracted and then phosphorylated in a reaction mixture containing 25 μg of *A. axanthum* soluble fraction.

Mycoplasma species (8, 23). It was suggested that if anchored to the cell membrane, these fibrils play a major role in motility and shape maintenance (3, 31). Nevertheless, the mechanism(s) controlling anchoring to the cell membrane as well as assembly and diassembly of spiroplasmal fibrils is yet unknown. Our findings that a 57-kDa protein associated with spiroplasmal fibrils underwent a reversed phosphorylation, the fibril fraction contained the 57-kDa protein mainly in its nonphosphorylated form, the nonphosphorylated form was found to be associated with the cell membranes, and the phosphorylated 57-kDa protein was poorly bound to isolated membranes are in support of the notion that a reversible phosphorylation of a 57-kDa protein is involved in such a mechanism(s). This protein is either identical to or associated with the major fibrilar protein described before (27, 28).

This work was supported by the U.S.-Israel Binational Agricultural Research and Development Fund and by the U.S.-Israel Binational Science Foundation.

We thank A. Katzenell for technical assistance and B. Chronis-Anner for helpful discussions.

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