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Localization of the 65-kDa Phosphoprotein in the Ciliary Axoneme of *Paramecium caudatum*

Tokushige OGAWA and Munenori NOGUCHI*

Department of Environmental Biology and Chemistry, Faculty of Science, Toyama University, Toyama 930-8555, Japan.

SUMMARY

The cAMP-dependent phosphorylation of the 29-kDa and 65-kDa axonemal proteins is closely correlated with the control mechanism of ciliary beat direction. Localization of the 65-kDa protein is still unknown whereas the 29-kDa protein has been identified as a light chain of the outer-arm 22S dynein. The localization of the 65-kDa phosphoprotein was determined by two-dimensional electrophoresis using a native gel in the first run. In the native gel, the 65-kDa phosphoprotein co-migrated with dynein heavy chains. This suggests that the 65-kDa phosphoprotein plays an important role in cAMP-dependent ciliary control as an intermediate chain of a dynein complex.

INTRODUCTION

The locomotor behavior of *Paramecium* depends on the ciliary beat direction and beat frequency. The beat direction is controlled by intracellular Ca^{2+} concentration (Naitoh and Kaneko, 1972, 1973; Naitoh and Sugino, 1984) and presumably by cyclic nucleotide concentration (Nakaoka and Ooi, 1985; Majima et al., 1986; Bonini and Nelson, 1988). Hamasaki et al. (1991) reported that cAMP-stimulated phosphorylation of the 29-kDa axonemal protein regulated the velocity of microtubule translocation and the swimming speed in *Paramecium*. Barkalow et al. (1994) reported that the 29-kDa protein functioned as a 22S dynein regulatory light chain. They showed a 22S dynein that was reconstituted with the phosphorylated 29-kDa protein translocated microtubules significantly faster than controls. We have reported that the cAMP-dependent phosphorylation of the 29-kDa and 65-kDa axonemal proteins was closely correlated with the control mechanism of ciliary beat direction (Noguchi et al., 2000).

Cilia on the cortical sheets from Triton-glycerol-extracted *Paramecium* lose their ability to change their orientation in response to cAMP and to removing Ca^{2+} by tryptic digestion within 1 min (Noguchi et al., 2000). The brief digestion removes axonemal cAMP-dependent protein kinase activity within 10 sec. Following the digestion,

*Corresponding author
Tel: +81-076-445-6675
Fax: +81-76-445-6549
E-mail: noguchi@sci.toyama-u.ac.jp

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not only the phosphorylated 29-kDa but also the 65-kDa axonemal proteins disappeared in 1 min (Noguchi et al., 2000). The disappearance of the kinase activity and phosphorylated proteins accompanied the disability of ciliary responses. The 29-kDa phosphoprotein has been thought to be involved in the ciliary response through controlling the sliding velocity of outer doublet microtubules. The 29-kDa phosphoprotein has been shown to co-purified with 22S dynein in a sucrose density gradient centrifugation and to be a light chain of the outer-arm 22S dynein (Bonini and Nelson, 1990; Hamasaki et al., 1991). On the other hand, the 65-kDa phosphoprotein is a strong candidate that sets the direction of ciliary beat antagonizing the effect of Ca^{2+} (Noguchi et al., 2000; 2003). However, the 65-kDa phosphoprotein has not been shown to co-purified with dynein heavy chains in a sucrose density gradient centrifugation and the localization of the 65-kDa phosphoprotein is still unknown (Bonini and Nelson, 1990; Hamasaki et al., 1991).

In this study, we tried to clarify the localization of the 65-kDa phosphoprotein in the ciliary axonemes from *Paramecium caudatum*. Two-dimensional electrophoresis using a native gel electrophoresis as a first run revealed that the 65-kDa phosphoprotein was presumably associated with a dynein complex.

MATERIALS AND METHODS

Isolation of axonemes

Paramecium caudatum (stock G3) was cultured in a hay infusion. Cells were grown to the late-logarithmic phase at 25ºC. Collected cells were washed three times with a washing solution (2 mM KCl, 2 mM $CaCl_2$, 0.3 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM Tris-maleate, pH 7.0). Cells were deciliated by the dibucaine treatment following the method of Mogami and Takahashi (1983) with slight modifications. Cilia were isolated from cell bodies by centrifugation repeated twice at 600 g for 5 min. The pellets were discarded. The supernatant was centrifuged at 7,700 g for 10 min to pellet the cilia. The pellet was resuspended in TMKE solution (10 mM Tris-maleate, pH 7.0, 5 mM $MgCl_2$, 20 mM potassium acetate, 1 mM EGTA) containing 0.3 mM PMSF and centrifuged. The pellet was rewashed with the TMKE solution. The isolation of cilia was monitored by dark-field microscopy. These cilia were then treated with a demembranation solution containing 0.1% Triton X-100 in TMKE solution for 10 min at 0ºC. The Triton was removed by washing twice with HKMED solution (30 mM HEPES, pH 7.3, 20 mM potassium acetate, 5 mM $MgSO_4$, 1 mM EGTA, 1 mM dithiothreitol (DTT), 0.3 mM PMSF, 10 μg/ml leupeptin).

Phosphorylation of the axonemes

In vitro phosphorylation of the axonemes was performed following the procedure described in our previous paper (Noguchi et al., 2000). The reaction mixture contained 0.15 mg axonemes in 80 μl of TMKE solution as well as 10 μM cAMP. Phosphorylation by endogenous protein kinases was started by addition of 20 μl of γ-[^{32}P]ATP to achieve a final concentration of 1 μM ATP. The ATP concentration of the γ-[^{32}P]ATP was 5 μM, and radioactivity was adjusted to 10 or 20 μCi with adenosine 5'-γ-[^{32}P]triphosphate (specific activity 6000 Ci/mmol from Du Pont-New England Nuclear). Immediately after 10 min incubation at 0ºC, the reaction mixture was centrifuged at 10,000g for 10 min. The pellet of the axonemes was used for the extraction of crude dynein.

Extraction of crude dynein

Extraction of dynein was carried out by the method of Larsen et al. (1991) with slight modifications. The pellet of axonemes was resuspended in HKMED solution containing 0.6 M KCl. The suspension was kept on ice for 30 min to extract

the dyneins. Axonemes were separated from extracted dynein by centrifugation. The high-salt extract (crude dynein) was divided into two parts. One part was immediately diluted with a buffer solution (HEPES, pH 7.3) to achieve a KCl concentration of 20 mM and concentrated using Centricon-10 (low-salt sample). Another part was concentrated without diluting the KCl concentration using Centricon-10 (high-salt sample).

Two-dimensional electrophoresis using native gel and SDS-PAGE

Two-dimensional electrophoresis was performed using agarose polyacrylamide slab gel electrophoresis (AG-PAGE) as a first run (Takaya et al., 1995). The concentrated crude dynein was mixed with 1/2 volume of 20% glycerol containing 1% bromophenol blue. The sample was then applied on the gel. The first run of a native gel electrophoresis was performed using the AG-PAGE (gel size, 16 × 16 × 0.1 cm). Electrophoresis was run at 5 mA and terminated when bromophenol blue ran to the end of the gel. A gel stripe was cut out of the gel, incubated in an SDS sample buffer (2% SDS, 5% 2-mercaptoethanol, 12% glycerol, 62.5 mM Tris-HCl, pH 6.8) for 7 min x 2, and then placed on a stacking gel of SDS-PAGE. The second run using SDS-PAGE was carried out in 3-15% linear gradient acrylamide gel containing a 0-19% glycerol gradient following a modification of the procedure of Laemmli (1970). Molecular-weight standards were obtained from Boehringer-Mannheim and Bio-Rad. The gels were stained with silver (Blum et al., 1987). To produce the autoradiograms, Fuji medical X-ray film RX (Fuji, Kanagawa) with intensifying screen (Kasei Optonix, Tokyo) was placed over the dried gels for 1 to a few days and developed.

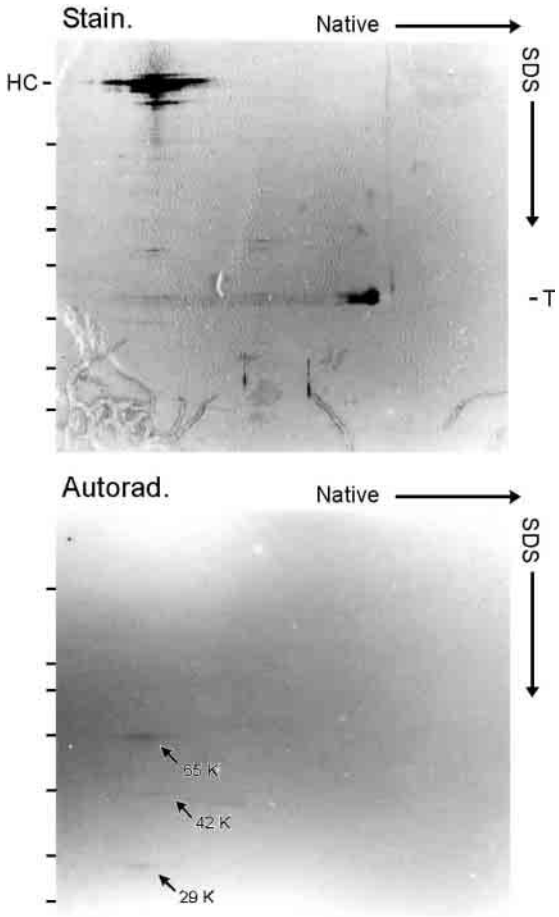

Fig. 1. Two-dimensional electrophoresis of phosphorylated crude dynein (low-salt sample). Extracted crude dynein from phosphorylated axonemes was diluted with a buffer solution (HEPES, pH 7.3) to achieve a KCl concentration of 20 mM and concentrated using Centricon-10 (low-salt sample). Stain.: Silver stained gel. Autorad.: Corresponding autoradiogram. HC and T indicate the position of heavy chains and tubulin in the second run. Markers at left indicate (from top) 200, 116, 97, 66, 45, 31, and 21.5 kDa. Both 29- and 65-kDa protein were electrophoresed in conjunction with heavy chains in the first run.

RESULTS AND DISCUSSION

The localization of the 65-kDa phosphoprotein has not been clarified. The 65-kDa protein is extracted together with the 29-kDa protein (Hamasaki et al., 1989), dyneins, and A-kinases. However, the 65-kDa protein is not co-purified with dynein heavy chains (HCs) in sucrose density gradient centrifugation.

We tried to clarify the localization of 65-kDa

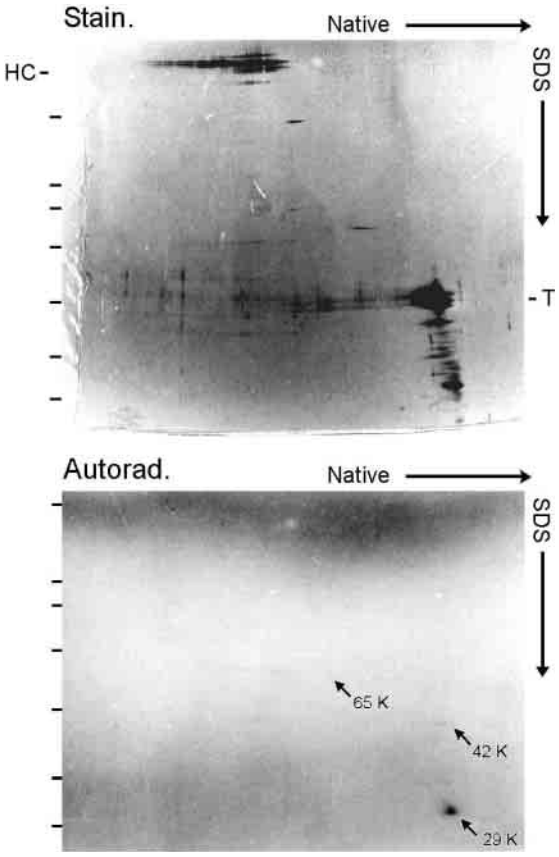

Fig. 2. Two-dimensional electrophoresis of phosphorylated crude dynein (high-salt sample). In this case, the high-salt extract was concentrated without diluting the KCl concentration. Stain.: Silver stained gel. Autorad.: Corresponding autoradiogram. HC and T indicate the position of heavy chains and tubulin in the second run. Markers at left indicate (from top) 200, 116, 97, 66, 45, 31, and 21.5 kDa. Both 29- and 65-kDa protein were not electrophoresed in conjunction with heavy chains in the first run in the high-salt condition.

phosphoprotein by another method. We analyzed using two-dimensional electrophoresis. In this study, the first run of the two-dimensional electrophoresis was performed with native AG-PAGE gel electrophoresis (Takaya et al.,1995). Using a native gel in the first run, the conformation of dynein complexes were expected to keep their conformation intact. The native gel electrophoreses were performed using both high-salt and low-salt samples. The high-salt extract from axonemes that had previously been phosphorylated with γ-[^{32}P]ATP in the presence of cAMP was immediately diluted to achieve a KCl concentration of 20 mM and concentrated (low-salt sample). In another case, the high-salt extract was concentrated without diluting the KCl concentration (high-salt sample).

Dynein HCs migrated as relatively compact bands when the low-salt sample was electrophoresed in a native gel (Fig. 1, STAIN). On the other hand, the dynein HCs of the high-salt sample migrated as wider bands (Fig. 2, STAIN). In the autoradiograms from each two-dimensional electrophoresis, the mobility of the labeled phosphoproteins in the first run was different.

Identified phosphorylated bands corresponded to 29-kDa, 65-kDa, and about 42-kDa proteins in the SDS-PAGE (indicated by arrows in Fig. 1 and 2, AUTORAD). These phosphorylated bands migrated with dynein HCs in the native gel of the low-salt sample (Fig. 1). This indicates that 65-kDa phosphoprotein co-migrates with dynein heavy chains as 29-kDa phosphoprotein in the low-salt condition (Fig. 1). On the other hand, the phosphorylated bands migrated far from dynein HCs in the case of the high-salt sample (Fig. 2). These results suggest that the 65-kDa phosphoprotein was released from the dynein complex with a long-time exposure to 0.6 M KCl (at least 2 h during the concentrating procedure). The 65-kDa phosphoprotein did not co-sedimented with dynein HCs in sucrose density gradient centrifugation (Bonini and Nelson, 1990; Hamasaki et al., 1991). This also may be due to a long time exposure to 0.6 M KCl before loaded on a sucrose density gradient and following overnight centrifugation.

The 65-kDa phosphoprotein presumably attach to some dynein complex in vivo and may play a significant role in cAMP-dependent regulation cooperating with 29-kDa light chain. In addition, 42-kDa phosphoprotein comigrated with dynein

heavy chains. The 42-kDa protein might be involved in the regulation of ciliary movement.

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