CHAPTER 7

Recombinant p50/Dynamitin as a Tool to Examine the Role of Dynactin in Intracellular Processes

Torsten Wittmann and Tony Hyman
Cell Biology Program
EMBL
Heidelberg D-69117, Germany

I. Introduction

Dynein is a minus-end-directed motor responsible for many intracellular functions. Traditionally it has been difficult to interfere with dynein function and thus test its role in intracellular organization. It has become clear that most dynein functions in cytoplasmic organization are mediated through dynactin, a complex of proteins which appears to target dynein to different intracellular locations and regulate its function. Thus, a simple way of disrupting dynactin function would be to prevent dynein localization and therefore to disrupt its specific functions. Recently, such a tool has been described: the overexpression of one of the components of dynactin, p50/dynamitin. When overexpressed in cells by transfection, p50/dynamitin has been shown to disrupt the dynactin complex (Echeverri *et al.*, 1996) and therefore the function of dynein in spindle

METHODS IN CELL BIOLOGY, VOL. 61

0091-679X/99 $30.00

assembly and Golgi organization (Burkhardt *et al.*, 1997). While this is a valuable tool, in many systems it is not possible to transfect cells.

Here we describe a simple two-step method for the production of large amounts of active p50/dynamitin in bacteria using ammonium sulfate precipitation and subsequent anion-exchange chromatography. We show that the recombinant protein disrupts the dynactin complex in *Xenopus* egg extracts. Expressed p50/dynamitin has been used to block spindle pole assembly in *Xenopus* egg extracts (Wittmann *et al.*, 1998) and rearrangement of microtubules during neuronal differentiation (Ahmad *et al.*, 1998). Microinjection of p50/dynamitin also leads to the dispersion of the Golgi apparatus in mammalian as well as *Xenopus* tissue culture cells (Ahmad *et al.*, 1998; Nathalie Le Bot, personal communication).

II. Production of Recombinant p50/Dynamitin

A. Solutions

Phosphate-buffered saline (PBS): 137 m*M* NaCl, 2.7 m*M* KCl, 4.3 m*M* Na_2HPO_4, and 1.4 m*M* KH_2PO_4 (pH 7.4).

Lysis buffer: PBS containing 1 m*M* ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 1 m*M* ethylenediaminetetraacetic acid (EDTA), 0.1% 2-mercaptoethanol, 10 μg/ml leupeptin, aprotinin, pepstatin, and 1 m*M* phenylmethylsulfonyl fluoride (PMSF). PMSF should be added just before use from a stock solution in an anhydrous solvent (e.g., absolute ethanol) stored at −20°C.

Mono Q buffer: 40 m*M* bis-tris propane, pH 7.0, 10% glycerol, 1 m*M* EDTA, 1 m*M* dithiothreitol (DTT), and 0.01% Tween 20.

B. Procedure

1. The full-length p50/dynamitin cDNA was cloned into a T7 expression vector (Way *et al.*, 1990) by PCR. For protein expression this plasmid is transformed into BL21(DE3)pLysS *Escherichia coli* cells (Studier, 1991) and an overnight culture grown at 37°C in LB medium supplemented with 100 μg/ml ampicillin and 25 μg/ml chloramphenicol. The next morning the bacteria are diluted 1:5 into fresh LB medium containing antibiotics and grown to an OD_{600} of 0.4–0.5 at 20°C. Protein expression is induced by addition of 0.1 m*M* isopropylthio-β-galactoside (IPTG) and the bacteria are grown overnight at 20°C. We sometimes observed that expression of p50/dynamitin was induced before the addition of IPTG leading to a very slow growth of the bacteria. However, this did not seem to affect the high level of expression. We also keep a glycerol stock of the BL21 strain overexpressing p50/dynamitin without any reduction of the expression level.

2. The bacteria are harvested by centrifugation (5000*g*, 30 min, 4°C), resuspended in a small volume of PBS and centrifuged again, and the bacterial pellet is frozen in liquid nitrogen. The pellet can be stored at −70°C for several months.

3. All subsequent steps are carried out at 4°C. The bacterial pellet (from 1 liter of culture) is thawed and resuspended in 10 ml of lysis buffer. We lysed the bacteria either by sonication (three times 30 sec in the presence of 1 mg/ml lysozyme) or by using a French press with comparable results. If the extract appears to be very viscous due to DNA contamination, 10 μg/ml DNAse I can be added followed by an incubation on ice for a few minutes. The extract is then diluted to 20 ml with lysis buffer and precleared by centrifugation for 15 min at 30,000*g* at 4°C (Fig. 1, lane 1).

4. The precleared extract is filled in a beaker and placed in an ice bath on a magnetic stirring plate. While stirring the extract, finely ground ammonium sulfate powder is added slowly to a saturation of 20% (2.12 g ammonium sulfate for 20 ml of extract). The incubation on ice is continued with slight agitation for approximately 1 hr and the precipitate recovered by centrifugation for 10 min at 20,000*g* at 4°C. Under these conditions most of the p50/dynamitin is recovered in the precipitate, whereas most bacterial proteins remain soluble (Fig. 1, lane 2).

5. The ammonium sulfate pellet is then redissolved in 40 ml Mono Q buffer containing protease inhibitors for 30 min at 4°C with slight agitation. This volume of buffer sufficiently reduces the salt concentration to allow binding of p50/dynamitin to the Mono Q resin. Remaining particulate material is removed by centrifugation for 10 min at 30,000*g* at 4°C and subsequent filtration through a Millipore low-protein-binding filter unit.

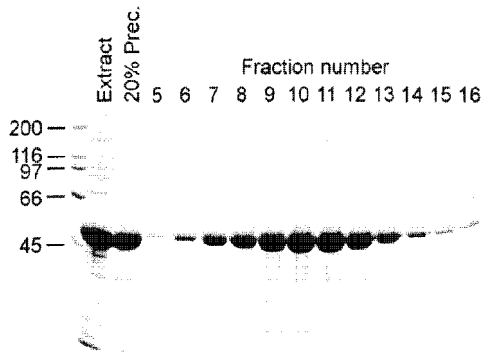

Fig. 1 Purification of bacterially expressed p50/dynamitin. Coomassie-stained 10% SDS–PAGE of precleared extract, 20% ammonium sulfate precipitate, and fractions of the Mono Q chromatography (0.5-μl aliquots of the fractions indicated were loaded on the gel). Fraction 10 contained about 12 mg/ml protein. The molecular mass of marker proteins is indicated on the left.

6. Typically, per run 20 ml of this protein solution is applied to a 1-ml Mono Q FPLC column (Pharmacia) equilibrated in Mono Q buffer. Use of more than this amount tends to overload the column and a substantial amount of protein is lost in the flowthrough. The column is washed extensively until the OD_{280} returns to zero and the column is eluted with a 20-ml linear gradient of 0–500 m*M* KCl in Mono Q buffer. One-milliliter fractions are collected. The peak of p50/dynamitin elutes at around 200 m*M* KCl (Fig. 1). The Mono Q peak fractions usually contain more than 10 mg/ml p50/dynamitin making further concentration unnecessary for most applications. If necessary, p50/dynamitin can be further purified on a Superose 12 gel filtration column.

III. Disruption of the Dynactin Complex by p50/Dynamitin in *Xenopus* Egg Extracts

When p50/dynamitin is overexpressed in tissue culture cells, this leads to the dissociation of the dynactin complex (Echeverri *et al.*, 1996). This has been demonstrated by analysis of the sedimentation behavior of different components of the dynactin complex in p50/dynamitin overexpressing cells. The most dramatic effect is observed for the $p150^{Glued}$ subunit of the dynactin complex, which normally sediments at around 18S together with other dynactin subunits but is shifted to 9S upon overexpression of p50/dynamitin. We used *Xenopus* egg extract to examine whether addition of the bacterially expressed p50/dynamitin would have the same effect and to test whether the recombinant protein is active

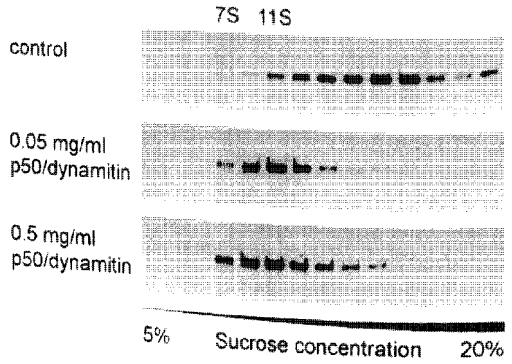

Fig. 2 Sedimentation analysis of the $p150^{Glued}$ dynactin subunit after addition of p50/dynamitin to *Xenopus* egg extract. The indicated amounts of Mono Q fraction 10 were added to 150,000*g* CSF-arrested *Xenopus* egg extract and subjected to 5–20% sucrose density gradient sedimentation. Two microliters of Mono Q buffer was added to the control reaction. The peak positions of two marker proteins are indicated on top (aldolase, 7.35 S; catalase, 11.3 S).

CSF-arrested *Xenopus* egg extract (Murray, 1991) was centrifuged for 60 min at 150,000*g* at 4°C. The high-speed cytosol (50 μl) was supplemented with p50/dynamitin and incubated for 30 min at 20°C. The reactions were then diluted with 200 μl CSF-XB (10 m*M* K-Hepes, pH 7.7, 50 m*M* sucrose, 100 m*M* KCl, 2 m*M* $MgCl_2$, 0.1 m*M* $CaCl_2$, and 5 m*M* EGTA) containing 1 m*M* DTT and protease inhibitors and layered on top of a 4.8-ml 5–20% sucrose gradient in CSF-XB containing 1 m*M* DTT and protease inhibitors. The gradients were centrifuged at 100,000*g* (28,000 rpm in a Beckman SW50.1 rotor) at 4°C for 18 hr. We collected and analyzed 350-μl fractions by Western blotting probed with an antibody against $p150^{Glued}$ (Fig. 2). We observed the same sedimentation

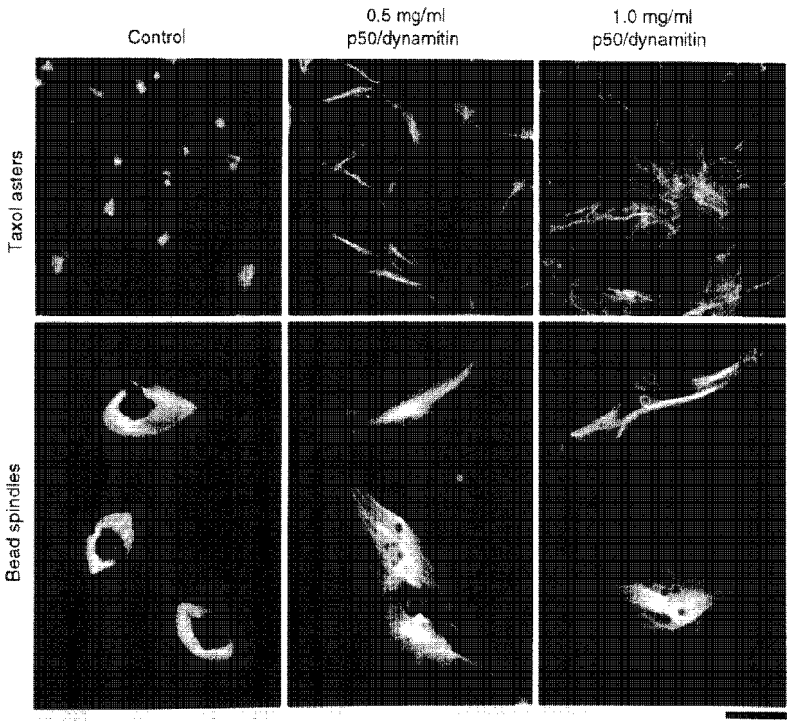

Fig. 3 Effect of p50/dynamitin on mitotic spindle poles. p50/dynamitin was added to 20 μl CSF-arrested *Xenopus* egg extract as indicated. An equivalent amount of Mono Q buffer was added to the control reaction. Microtubules were visualized by the addition of 0.2 mg/ml rhodaminated tubulin (Hyman *et al.*, 1991). Taxol asters were assembled by the addition of 1 μ*M* taxol (paclitaxel, Molecular Probes) and incubation for 30 min at 20°C. Bead spindles were assembled according to Heald *et al.*, (1996) for 60–90 min at 20°C. The reactions were fixed with 1 ml BRB80 (80 m*M* K-Pipes, pH 6.8, 1 m*M* EGTA, and 1 m*M* $MgCl_2$) containing 10% glycerol, 0.25% glutaraldehyde, 1 m*M* GTP, and 0.1% Triton X-100 and centrifuged on coverslips according to Sawin and Mitchison (1991). Scale bar = 20 μm.

pattern for p150^{Glued} as previously reported, even at rather low concentrations of exogenously added p50/dynamitin (0.05 mg/ml).

IV. Disruption of Spindle Poles Using p50/Dynamitin

In the absence of centrosomes, spindles can form in mitotic *Xenopus* egg extracts by self-organization of microtubules into poles. We have shown that this is due to cytoplasmic dynein using an antibody, m70.1, against the dynein intermediate chain (Heald *et al.*, 1996). Cytoplasmic dynein is also required for the formation of mitotic asters in the absence of centrosomes when microtubules are stabilized by taxol (Verde *et al.*, 1991).

To determine whether the dynactin complex was required for the formation of spindle poles, we added p50/dynamitin (0.5 and 1.0 mg/ml) to mitotic *Xenopus* egg extracts and then induced the assembly of taxol asters or bead spindles. We found that taxol-stabilized microtubules fail to organize into asters in the presence of p50/dynamitin. Bead spindles showed a phenotype similar to what has been observed upon addition of the m70.1 antibody (Fig. 3). Arrays of microtubules formed around the beads but they failed to form focused poles. This indicates that a dynein–dynactin interaction is required for spindle pole formation in *Xenopus* egg extracts. However, the concentration of p50/dynamitin required to observe a phenotypic effect in concentrated *Xenopus* egg extract appeared to be about 10 times higher compared to that of the biochemical dynactin disruption assay described previously.

Acknowledgments

We thank Christophe J. Echeverri for the original p50/dynamitin clone, Michael Way for help with subcloning, Trina Schroer for the p150^{Glued} antibody, and Rebecca Heald for her help with the bead spindles. T. W. is an EMBL predoctoral fellow.

References

Ahmad, F. J., Echeverri, C. J., Vallee, R. B., and Baas, P. W. (1998). Cytoplasmic dynein and dynactin are required for the transport of microtubules into the axon. *J. Cell Biol.* **140,** 391–401.

Burkhardt, J. K., Echeverri, C. J., Nilsson, T., and Vallee, R. B. (1997). Overexpression of the dynamitin (p50) subunit of the dynactin complex disrupts dynein-dependent maintenance of membrane organelle distribution. *J. Cell Biol.* **139,** 469–484.

Echeverri, C. J., Paschal, B. M., Vaughan, K. T., and Vallee, R. B. (1996). Molecular characterization of the 50-kD subunit of dynactin reveals function for the complex in chromosome alignment and spindle organization during mitosis. *J. Cell Biol.* **132,** 617–633.

Heald, R., Tournebize, R., Blank, T., Sandaltzopoulos, R., Becker, P., Hyman, A., and Karsenti, E. (1996). Self-organization of microtubules into bipolar spindles around artificial chromosomes in *Xenopus* egg extracts. *Nature* **382,** 420–425.

Hyman, A. A., Drechsel, D., Kellogg, D., Salser, S., Sawin, K., Steffen, P., Wordeman, L., and Mitchison, T. J. (1991). Preparation of modified tubulins. *Methods Enzymol.* **196,** 478–485.

Murray, A. W. (1991). Cell cycle extracts. *Methods in Cell Biol.* **36,** 581–605.

Sawin, K. E., and Mitchison, T. J. (1991). Mitotic spindle assembly by two different pathways in vitro. *J. Cell Biol.* **112,** 925–940.

Studier, F. W. (1991). Use of bacteriophage T7 lysozyme to improve an inducible T7 expression system. *J. Mol. Biol.* **219,** 37–44.

Verde, F., Berrez, J. M., Antony, C., and Karsenti, E. (1991). Taxol-induced microtubule asters in mitotic extracts of *Xenopus* eggs: Requirement for phosphorylated factors and cytoplasmic dynein. *J. Cell Biol.* **112,** 1177–1187.

Way, M., Pope, B., Gooch, J., Hawkins, M., and Weeds, A. G. (1990). Identification of a region in segment 1 of gelsolin critical for actin binding. *EMBO J.* **9,** 4103–4109.

Wittmann, T., Boleti, H., Antony, C., Karsenti, E., and Vernos, I. (1998). Localization of the kinesin-like protein Xklp2 to spindle poles requires a leucine zipper, a microtubule-associated protein, and dynein. *J. Cell Biol.*, in press.