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Overexpression of myosin motor domains in *Dictyostelium:* screening of transformants and purification of the affinity tagged protein

DIETMAR J. MANSTEIN* and DEBORAH M. HUNT

National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK

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Summary

The eukaryotic organism *Dictyostelium discoideum* has become one of the organisms of choice for the overexpression of recombinant myosins and myosin fragments. Here, we describe a protocol that facilitates the screening of cells that have been transformed with myosin expression constructs and allows the rapid purification of recombinant myosins.

Depletion of cellular ATP is used to recruit most of the endogenous and recombinant myosin into a rigor-like complex with actin. Following cell lysis the insoluble actomyosin complex is precipitated by centrifugation, washed, and Mg^{2+}-ATP is added to extract the recombinant protein from the pellet. More than 90% of the protein in the resulting supernatant corresponds to actin, myosin, and the recombinant myosin fragments. Therefore, it is easy to detect any differences in expression level between individual myosin constructs on SDS-polyacrylamide gels. Additionally, the dependence of expression on external factors, such as cell density, can be readily determined. Furthermore, the presence of a band corresponding to the recombinant protein indicates that the overexpressed protein has at least some of the functional properties that are characteristic for a myosin motor.

This rapid and selective extraction protocol can also be utilized to facilitate the purification of recombinant myosin motors on a preparative scale and has proved particularly useful in the purification of myosin head fragments, that are tagged with histidine residues, by Ni^{2+}-chelate affinity chromatography.

Introduction

We are using the cellular slime mould *Dictyostelium discoideum* as expression system for recombinant myosins. *Dictyostelium* has a number of features which makes it an attractive expression system. The motile amoeba lack a cell wall and resemble higher eukaryotic cells in behaviour and appearance. Furthermore, *Dictyostelium* cells carry out mammalian-like post-translational modifications and can be readily cultured in petri dishes, shaken flasks, and fermentors. Functional myosin head fragments can be expressed and purified in milligram quantities using this organism (Manstein *et al.*, 1989), and detailed kinetic studies (Ritchie *et al.*, 1993), as well as complete structural studies (Schröder *et al.*, 1993), have been carried out using myosin fragments expressed in *Dictyostelium*.

However, there are also problems associated with the use of *Dictyostelium* as an expression host. The abundance of proteolytic enzymes in *Dictyostelium* poses a significant obstacle to the quantitative analysis of levels at which recombinant proteins are expressed in transformed cells and can generate artefacts concerning their activity, structure, and intracellular location. Conditions that are commonly used for protein analysis, like the boiling of the samples in SDS, further increase the problem as they result in enhanced protease activity. Therefore, minimizing the exposure of the overexpressed protein of the action of endogenous *Dictyostelium* proteases is a prerequisite for any useful screening protocol. This can in part be achieved by milder lysis conditions that do not result in the disruption of lysosomes and other vacuolar organelles, as the lysosomal proteases constitute the major source of problems. In the special case of functional myosin motor-fragments, additional protection of the overexpressed protein from post-lysis proteolytic degradation can be achieved by the formation of a tight, insoluble complex with actin. As actin is one of the most abundant proteins in *Dictyostelium*, formation of such a complex can be accomplished simply by depletion of cellular ATP.

The formation of a rigor-like actomyosin complex facilitates also the rapid enrichment and purification of recombinant myosins, both on the analytical and

*To whom correspondence should be addressed.

preparative scale. The complex can be precipitated and washed to remove the bulk of the cell lysate. Subsequently, functional myosin motors can be selectively released into the supernatant by extraction with Mg^{2+}-ATP.

The screening of cells transformed with a variety of different myosin expression constructs and the purification of a myosin head fragment that is affinity tagged with a histidine octamer are described.

Materials and methods

Strains and growth conditions

Dictyostelium transformants were grown at 21° C in DD-Broth 20 containing (per litre): 20 g protease peptone (Oxoid), 7 g yeast extract (Oxoid), 8 g glucose, 0.33 g $NaH_2PO_4 \cdot 7H_2O$, and 0.35 g KH_2PO_4. Cells were either grown on 9 cm plastic petri dishes or in 100 ml conical flasks on a gyratory shaker at 190 rpm. Transformants were obtained either by the calcium-phosphate transformation procedure (Nellen *et al.*, 1984) or by electroporation (Egelhoff *et al.*, 1991) and continuously grown in the presence of 10 μg ml^{-1} of the aminoglycoside G418. Plasmids used for transformation were either derivatives of the integrating vector pB10TP2 (Early & Williams, 1987) or the extrachromosomal vector pDXA-3H. The vector pDXA-3H carries the origin of replication of the *Dictyostelium* high copy number plasmid Ddp2 (Chang *et al.*, 1990; Leiting *et al.*, 1990), an expression cassette consisting of the strong, constitutive *actin*15 promoter, a translational start codon upstream from a multiple cloning site (MCS), and sequences for the addition of a histidine octamer at the carboxy terminus of any protein. Plasmids derived from pDXA-3H were transformed into orf$^+$-cells (P. Morandini, manuscript in preparation). These cells carry several integrated copies of the *rep* gene which is essential in *trans* for the replication of plasmids that carry the Ddp2 origin (Leiting *et al.*, 1990; Slade *et al.*, 1990). The myosin-α-actinin fusion constructs were created by linking codon 754 of the *Dictyostelium mhc*A gene to codon 264 of the *Dictyostelium* α-actinin gene. Constructs fused to one central α-actinin repeat extended to codon 387, those tagged with two repeats to codon 505, and constructs tagged with three repeats to codon 618 of the *α-actinin* gene. Details of plasmid construction will be described elsewhere (Manstein *et al.*, 1995).

Screening of transformants

Mg^{2+}-ATP extractable cytoskeletal proteins were isolated starting from 2 to 5 × 10^7 cells. Cells were washed in 15 ml MMC-buffer (20 mM MES, 2 mM $MgCl_2$, 0.2 mM $CaCl_2$, pH 6.8), resuspended in 1 ml MMC-buffer, transferred in an Eppendorf microfuge tube, and pelleted by centrifugation at 5000 rpm for 2 min in an Eppendorf Model 5415C microcentrifuge or Heraeus Biofuge 13. The pellet was resuspended and washed once with Lysis Buffer (50 mM Tris HCl, 2.5 mM EDTA, 1 mM DTT, 5 mM benzamidine, pH 8.1). Lysis was performed by resuspending the cells in 0.6 ml Lysis Buffer containing 5 units ml^{-1} alkaline phosphatase (Boehringer Mannheim), 40 μg ml^{-1} N-tosyl-L-lysine chloromethyl ketone (TLCK), and 10 μg ml^{-1} leupeptin, followed by the addition of 0.6 ml of the same buffer containing 1% (w/v) Triton-X100. Lysis was followed by incubation for 1 h on ice or 10 min at 21° C. The insoluble fraction was collected by centrifugation for 30 min at 14000 rpm and washed with 0.5 ml Lysis Buffer. Myosin and recombinant myosin fragments were extracted by homogenizing the pellet with the aid of an Eppendorf micropistille in 50–70 μl Lysis Buffer containing 10 mM Mg^{2+}-ATP. The suspension was centrifuged for 20 min at 14000 rpm. Forty μl of the supernatant were carefully removed and mixed with 40 μl SDS-gel loading buffer. Samples were heat-denatured and 10–40 μl were loaded per lane on an SDS-polyacrylamide gel. *Dictyostelium* whole cell lysates were generated as described by De Lozanne and Spudich (1987). All samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gels. Gels were stained with a solution that was made up from 2 g Coomassie R250, 0.5 g Coomassie G250, 50 ml methanol, 450 ml ethanol, 100 ml glacial acetic acid, and 400 ml water. All centrifugations were carried out at 4° C.

Purification of affinity tagged myosin head fragments

Cells expressing a histidine octamer tagged fusion protein, consisting of the catalytic domain of *Dictyostelium* myosin and the first two central repeats of *Dictyostelium* α-actinin (DCD-2R) were grown in 5 l flasks containing 2.5 l DD-Broth 20. The flasks were incubated on a gyratory shaker at 200 rpm and 21° C. Cells were harvested at a density of 6 × 10^6 ml^{-1} by centrifugation for 7 min at 2700 rpm in a Beckman J-6 centrifuge and washed once in phosphate buffered saline. The wet weight of the resulting cell pellet was determined. Typically, 35 g were obtained from a 15 l shaking culture. The cells were resuspended in 140 ml of Lysis Buffer (50 mM Tris HCl, pH 8.0, 2 mM EDTA, 0.2 mM EGTA, 1 mM dithiothreitol (DTT), 5 mM benzamidine, 40 μg ml^{-1} TLCK, 20 μg ml^{-1} N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 200 μM phenylmethylsulfonyl fluoride (PMSF), 200 μM 1, 10-phenanthroline, 0.04% NaN_3. Cell lysis was induced by the addition of 70 ml of Lysis Buffer containing 1% Triton-X100, 15 mg ml^{-1} RNaseA (Sigma) and 100 units of alkaline phosphatase (Boehringer Mannheim). The lysate was incubated on ice for 1 h. Upon centrifugation (230000 g, 1 h), the recombinant protein remained in the pellet. The pellet was washed in 100 ml of HKM buffer (50 mM HEPES, pH 7.3, 30 mM KAc, 10 mM $MgSO_4$, 7 mM β-mercaptoethenol, 5 mM benzamidine, 40 μg ml^{-1} PMSF) and centrifuged for 45 min at 230000 g. The recombinant protein was released into the supernatant by extraction of the resulting pellet with 60 ml HKM buffer containing 10 mM ATP. After centrifugation (500000 g, 45 min), the supernatant was loaded using a peristaltic pump onto a Ni^{2+}-nitrilotriacetic acid (Ni^{2+}-NTA) affinity column (1.5 × 10 cm) (Qiagen). The flow-rate was adjusted to approximately 3 ml min^{-1}. After loading was completed the column was connected to a Waters 650M chromatography system. The column was washed briefly in Low Salt buffer (50 mM HEPES, pH 7.3, 30 mM KAc, 3 mM benzamidine), High Salt buffer (as Low Salt Buffer, but with 300 mM KAc), and Low Salt Buffer containing 50 mM imidazole. The recombinant myosin was eluted using a linear gradient of Low Salt Buffer and

Imidazole Buffer (0.5 M imidazole, pH 7.3, 3 mM benzamidine), starting with 10% Imidazole Buffer and reaching 100% after 15 min. The flow rate was 3 ml min^{-1} and 3 ml fractions were collected. Absorbance at 280 nm was monitored. SDS gels were run to check the purity of the eluted protein (Fig. 4). The pooled fractions were dialysed immediately against Storage Buffer (20 mM HEPES, 0.5 mM EDTA, 1 mM DTT, pH 7.0) containing 3% sucrose and the purified protein could be stored at −80° C for several months without apparent loss of enzymatic activity. Actin activated ATPase activity was measured as described by White (1982).

Results

Screening of transformants

Figure 1 shows the results obtained by SDS-PAGE with a transformant cell line that expresses an 88 kDa catalytic domain fragment of the myosin head at a level of 0.7 mg per g cells. The recombinant protein was either enriched by recruitment into a rigor-like complex with actin and selective solubilization upon addition of Mg^{2+}-ATP (lane B, C) or whole cell lysate was directly loaded onto the gel (lane D). The enriched fractions not only gave much clearer information about the expression of a recombinant product by the transformant, but the presence of an 88 kDa band also indicates that the recombinant protein behaves like a functional myosin. The effect of centrifugal force on the purity of the recombinant myosin fragment can be judged from the differences observed between lane B, where all sedimentation steps following lysis were performed at 125 000 g in a Beckman Optima TLX benchtop ultracentrifuge equipped with a TLA-45 rotor, and lane C, where post-lysis sedimentation steps were carried out at 13 800 g in a Heraeus Biofuge 13. Comparison of lanes B and C shows that higher sedimentation speeds result in a greater enrichment of the recombinant product. However, in regard to the information that can be gained about the expression level displayed by a given cell line, both high and low speed centrifugations yield comparable results.

In regard to the effectiveness of different protocols in recruiting myosin head fragments into the insoluble actomyosin fraction, we found that addition of alkaline phosphatase to the Lysis Buffer improved the reproducibility of the protocol, especially when screening for the expression of constructs that are produced at lower levels. However, the protocol can accommodate a number of changes and good results were also obtained when a variety of other agents are used to deplete cellular ATP (Fig. 2). In fact, a 10 min incubation of the cell lysate at room temperature without any external ATP-depleting factors produced almost the same result as the standard protocol for constructs that show high levels of expression (data not shown).

The result of a typical expression screen performed on 20 transformants expressing nine different con-

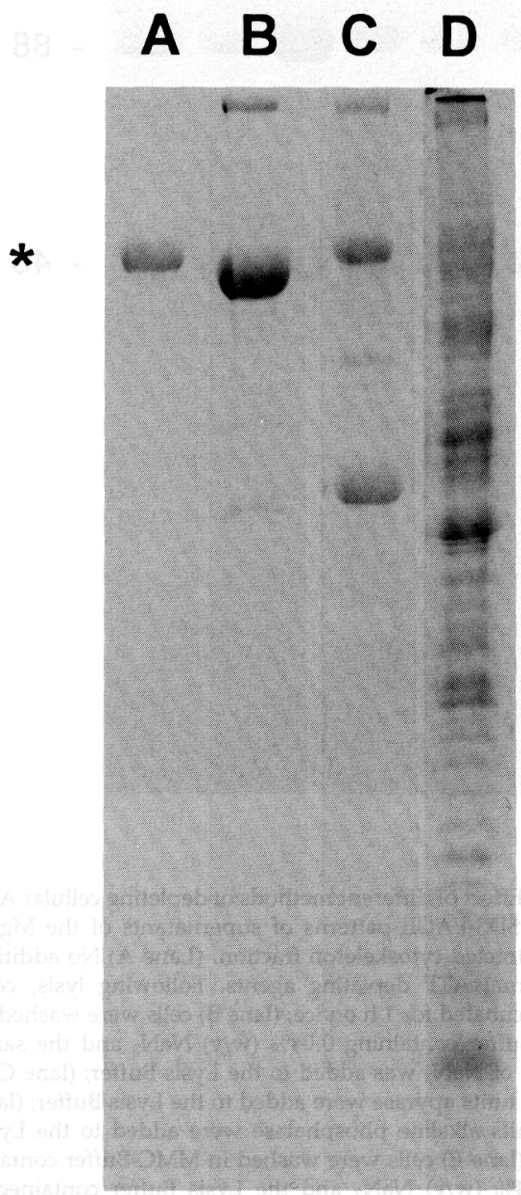

Fig. 1. SDS-PAGE patterns of the whole cell lysate and the supernatant of the Mg^{2+}-ATP extracted cytoskeleton fraction from a *Dictyostelium* transformant overexpressing the catalytic domain of myosin. (Lane A) Purified catalytic domain of *Dictyostelium* myosin; (lane B) supernatant of Mg^{2+}-ATP extracted cytoskeleton fraction obtained following the protocol described in Materials and Methods, but with all post-lysis centrifugations performed at 125 000 g; (lane C) same as lane B but centrifugations carried out at 13 800 g; (lane D) whole cell lysate. Twelve per cent acrylamide gels were used and Coomassie Blue was used as protein stain. The asterisk marks the electrophoretic mobility of the recombinant myosin fragment on the gels.

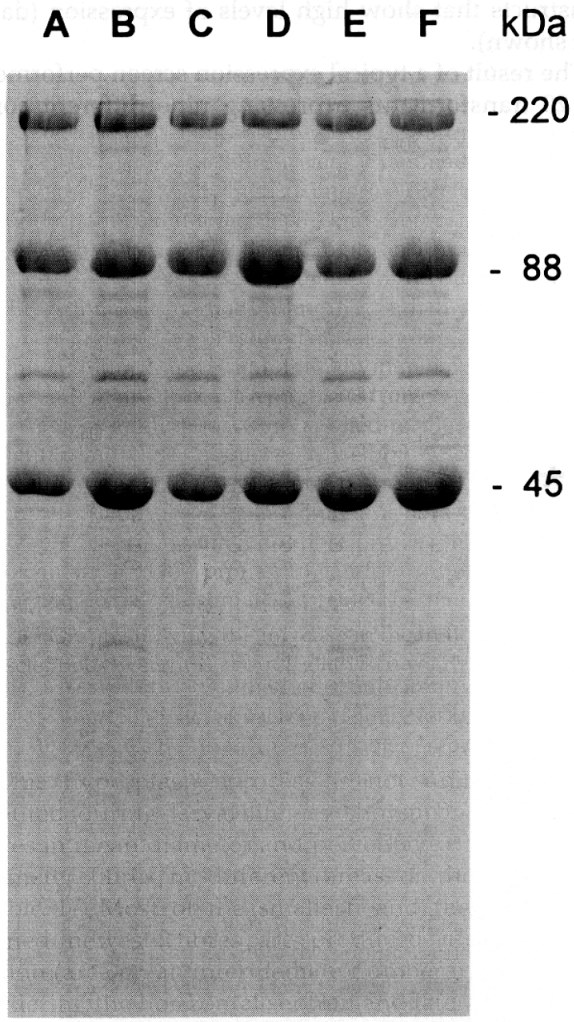

Fig. 2. Effect of different methods of depleting cellular ATP on the SDS-PAGE patterns of supernatants of the Mg^{2+}-ATP extracted cytoskeleton fraction. (Lane A) No addition of external ATP depleting agents. Following lysis, cells were incubated for 1 h on ice; (lane B) cells were washed in MMC-Buffer containing 0.04% (w/v) NaN_3 and the same amount of NaN_3 was added to the Lysis Buffer; (lane C) 5 ATPase units apyrase were added to the Lysis Buffer; (lane D) 5 units alkaline phosphatase were added to the Lysis Buffer; (lane E) cells were washed in MMC-Buffer containing 0.04% (w/v) NaN_3 and the Lysis Buffer contained 5 units apyrase; (lane F) same as E but 5 units alkaline phosphatase were added instead of apyrase. A 12% acrylamide gel was used and Coomassie Blue was used as protein stain.

structs is shown in Fig. 3. The transformants can be grouped into two classes based on the type of construct. CD19, CD29.1, CD29.2, 1R-a2, 1R-a3, 1R-a4, 1R-b1, 2R-b2, 2R-b3, 2R-b4, 2R-c1, 2R-c2, 2R-c3, and 2R-c4 express a catalytic domain fragment which in the case of the 1R and 2R transformants is fused to one or two central α-actinin repeats, respectively. All other transformants express myosin head fragments that lack the first 80 amino acids of the myosin heavy chain, a part of the molecule that is assumed to be non-essential for motility as it is missing in several single headed myosins (Pollard *et al.*, 1991). In the case of chicken skeletal muscle myosin this N-terminal part of the molecule is known to form a fairly independent domain that shares topological similarities with the *Src*-homology 3 (SH3) domain of spectrin (Rayment *et al.*, 1993). Transformants DH-1R-1, DH-1R-2, DH-2R-2, DH-3R-3 express catalytic domain-like fragments that are fused to one, two, or three central α-actinin repeats and MHF-67 and MHF-68 produce S1-like fragments. The recombinant myosin head fragment produced by MHF-67 lacks the binding site for the essential light chain.

Figure 3 shows that all nine constructs can be expressed in *Dictyostelium* in functional form, as each of the recombinant proteins binds to actin in the absence of ATP and is released from actin by the addition of Mg^{2+}-ATP. The amount of recombinant myosin produced varied from approximately 1 mg per g of cells for cell lines CD19, CD29.1 and CD29.2 to levels ≤ 10 μg per g of cells for cell lines DH1R-1, DH1R-2 and DH3R-3. However, the level of expression varied only slightly between cells that were transformed with the same extrachromosomal plasmid. This is the case for all cell lines shown in Fig. 3 with the exception of MHF-67 and MHF-68 which were transformed with plasmids derived from the integrating vector pB10TP2. We found that extrachromosomal vectors are in most instances preferable to integrating vectors. They give rise to 10–100 times high transformation efficiencies and transformants obtained with a particular extrachromosomal construct produce the same level of recombinant protein (see Fig. 3, lanes 2R-b2 to 2R-c4). However, this is not always the case. Extrachromosomal vectors harbouring N-terminally truncated myosins generally fail to give high expression levels (Fig. 3, lanes DH-1R-1–DH-3R-3) while a small subpopulation of the transformants that were obtained using integrating vectors (MHF-67, MHF-68) express the protein at high level. It should be noted that both the extrachromosomal and integrating constructs carry the same translational fusion of the eighth codon of the *Dictyostelium actin*15-promoter and the 81st codon of the *mhc*A gene. Furthermore, extrachromosomal vectors for the production of myosin-α-actinin fusion proteins with intact N-terminus, but otherwise identical to those used in the transformation of cell lines DH-1R-1 and DH-2R-2, gave high levels of expression (see Fig. 3, lanes 1R-a2–2R-c4).

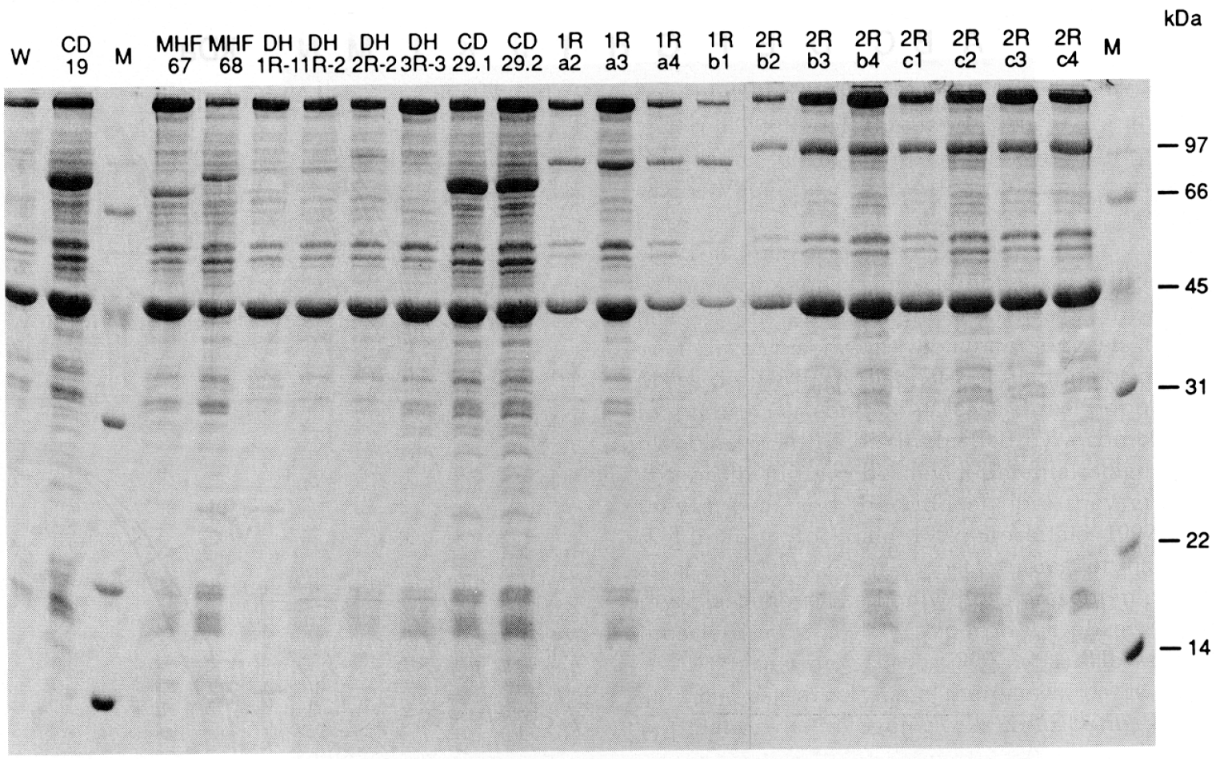

Fig. 3. Screening of *Dictyostelium* transformants expressing a variety of different myosin motor fragments. The 12% SDS-polyacrylamide gel shows the range of expression levels obtained with different constructs. CD19, CD29.1, CD29.2, 1R-a2, 1R-a3, 1R-a4, 1R-b1, 2R-b2, 2R-b3, 2R-b4, 2R-c1, 2R-c2, 2R-c3, and 2R-c4 express a catalytic domain fragment which in the case of the 1R and 2R transformants is fused to one or two central α-actinin repeats, respectively. Transformants DH-1R-1, DH-1R-2, DH-2R-2, DH-3R-3 express catalytic domain-like fragments that lack the first 80 amino acids of the myosin heavy chain and are fused to one, two, or three central α-actinin repeats, receptively. Transformants MHF-67 and MHF-68 produce S1-like fragments that lack the first 80 amino acids of the myosin heavy chain. The fragment produced by MHF-67 lacks the binding site for the essential light chain. The recombinant myosin fragments were enriched as described in Materials and Methods. Coomassie Blue was used as protein stain. (M) molecular mass markers; (W) wild-type *Dictyostelium* cells; all other lane headings refer to the name of the individual transformed cell line.

Expression of affinity tagged myosin motor domains

The purification of a fusion protein (DCD-2R) composed of a 88 kDa myosin catalytic domain fragment, a 27 kDa α-actinin fragment corresponding to two central repeats of the protein, and a C-terminal histidine octamer is shown in Fig. 4. Transformant cell line 2R-b1 was used for the production of the protein (see Fig. 3). The enrichment of the recombinant protein by recruitment into a rigor-like complex with actin and selective solubilization upon addition of Mg^{2+}-ATP was approximately the same as that achieved on the analytical scale (Fig. 4, lane D). The recovery of DCD-2R was almost complete at this stage, as the protein could not be detected by immunoblot analysis in either the supernatant of the first centrifugation step after cell lysis or the pellet after ATP-extraction (data not shown). The supernatant after ATP extraction (Fig. 4, lane D), which contained DCD-2R at ~ 50% of the soluble protein, was loaded onto a Ni^{2+}-NTA column and the column was extensively washed with Low and High Salt Buffer. An additional wash with Low Salt Buffer containing 50 mM imidazole was essential for the removal of most of the non-specifically bound protein (Fig. 4, lanes F & G). DCD-2R was eluted using a gradient from 50–500 mM imidazole. The protein was > 95% pure as estimated from the Coomassie Blue stained protein gel (Fig. 4, lanes H–N) and fully functional based on actin-activated ATPase activity. The basal ATPase rate for DCD-2R was 0.02 s^{-1} and at 25 μM actin the ATPase rate was 1.8 s^{-1}, a 90-fold increase. In the absence of actin and at high ionic strength, DCD-2R displayed a high ATPase activity in the presence of Ca^{2+} (1.1 s^{-1}) similar to that of the native *Dictyostelium* myosin. The molecular weight of DCD-2R was calculated as 114665 and a molar extinction coefficient $\varepsilon^{1cm}_{280nm}$ of 92500 ± 4000 was determined. An average of 0.25% of the total cell protein was recovered as purified DCD-2R, which corresponds to a yield of 1.2 mg from 10 g of cells.

Discussion

The lack of reliable and efficient methods for the screening of transformants is one of the major limitations of *Dictyostelium* as a biotechnological

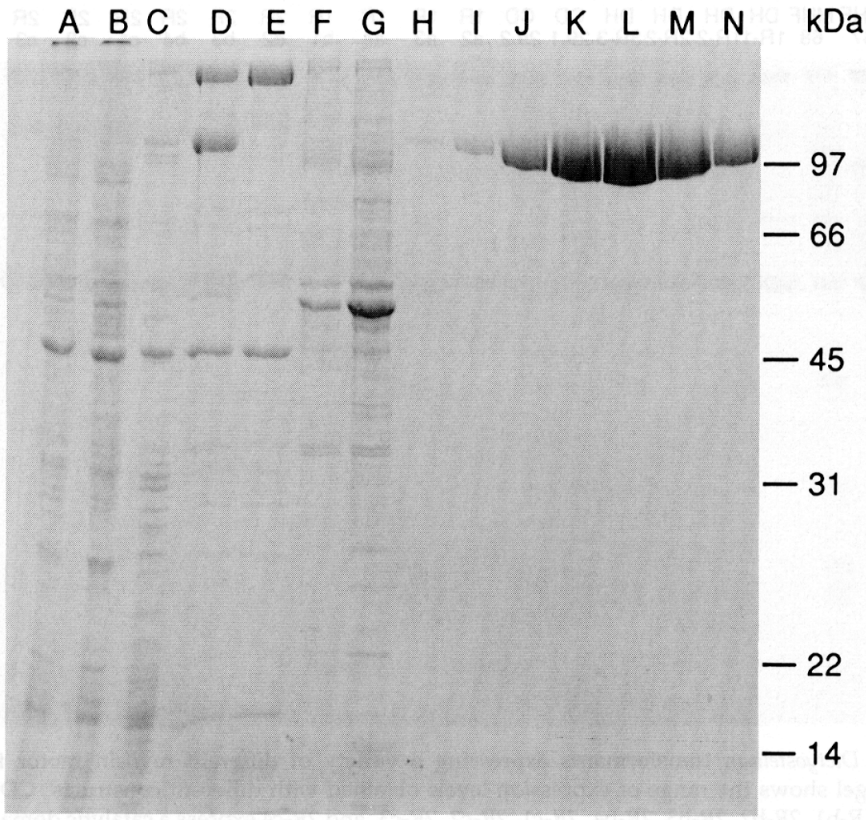

Fig. 4. Purification of a histidine-octamer tagged myosin motor. The purification of a myosin catalytic domain fragment fused to two central α-actinin repeats and tagged with a C-terminal histidine octamer is shown. (Lane A) whole cell lysate; (lane B) supernatant of whole cell lysate; (lane C) supernatant of wash with extraction buffer; (lane D) supernatant after extraction with 10 mM Mg^{2+}-ATP; (lane E) flow through of Ni^{2+}-chelate affinity column; (lanes F, G) wash with 50 mM imidazole; (lanes H–M) elution of the recombinant protein using a gradient from 50 to 500 mM imidazole. Ten μl aliquots were mixed with 10 μl SDS-gel loading buffer and 10 μl of this mixture where loaded per lane, with the exception of lanes A and B where 3 and 5 μl were loaded, respectively. A 12% polyacrylamide gel was used and Coomassie Blue was used as protein stain.

system. This is indicated by the fact that although very reliable transformation systems exist for *Dictyostelium* (Nellen *et al.*, 1984; Howard *et al.*, 1988; Egelhoff *et al.*, 1989) only a small number of homologous and heterologous genes have been overexpressed. Of the heterologous genes whose overexpression has been reported, most served as selective markers or phenotypic reporters (Dingermann *et al.*, 1991). Amongst these genes are the bacterial Tn5 and Tn903 aminoglycoside phosphotransferases (Nellen & Firtel, 1985; Knecht *et al.*, 1986), *Escherichia coli* hygromycin phosphotransferase (Egelhoff *et al.*, 1989). *E. coli* β-galactosidase (Dingermann *et al.*, 1989), *E. coli* β-glucuronidase (Dittrich *et al.*, 1994), firefly luciferase (Howard *et al.*, 1988), and mouse thymidylate synthase (Chang *et al.*, 1989; Dynes & Firtel, 1989). The bias towards proteins that are very easy to detect in the transformed cell may be caused, at least in part, by the phagocytic nature of *Dictyostelium*. An abundance of lysosomal proteases can make the detection of recombinant proteins in whole cell lysates very difficult. Even if the expression of a protein can be established unambiguously, is it often impossible to quantitate expression levels which makes it difficult to identify transformants producing the highest levels of the recombinant protein and to optimise the conditions affecting production.

Screening of transformants

The results presented here demonstrate that, in the special case of functional myosin motor domains, these limitations of the *Dictyostelium* system can be readily overcome. Sequestration of the recombinant protein into a complex with endogenous actin almost completely prevents the proteolytic degradation that would otherwise inevitably occur during cell lysis and subsequent treatment. On the analytical scale a single confluent 9 cm petri dish yields enough pro-

tein for 4–6 lanes on a SDS-polyacrylamide gel and the screening of large numbers of transformants is further facilitated by the fact that all sedimentation steps can be carried out at 13 000 g with equipment that is readily available in most molecular biology and biochemistry laboratories.

Variations in expression levels are not only encountered with different constructs but can depend on parameters such as cell density, growth conditions, and the presence of external factors (Maniak & Nellen, 1990; Blusch *et al.*, 1992; Wetterauer *et al.*, 1993a, b; Blusch & Nellen, 1994). The development and application of the screening protocol described here, therefore, not only enhanced our ability to identify the best expression constructs but also helped to optimise conditions for the expression of new and existing constructs. Currently, we are extending this work to the expression of human β-cardiac myosin and rabbit skeletal muscle myosin. These investigations should provide us with useful information about the general potential of expressing complex proteins of mammalian origin in *Dictyostelium*.

Expression of affinity tagged myosin motor domains

The rapid and selective enrichment of functional myosin motors by binding to actin and release by addition of Mg^{2+}-ATP did considerably help in the purification of histidine-tagged myosins. The high selectivity of Ni^{2+}-chelate affinity chromatography for proteins carrying a tag, consisting of six or more consecutive histidine residues, is used in a variety of prokaryotic and eukaryotic expression systems for the rapid purification of proteins under native conditions (Janknecht *et al.*, 1991). For cells that are devoid of histidine-rich proteins, purification of the tagged protein can be accomplished by a one-step procedure. However, in the case of a host like *Dictyostelium* that contains many histidine-rich proteins, the usefulness of this method is greatly reduced by the requirement to separate the recombinant protein from the bulk of the endogenous histidine-rich proteins prior to the affinity chromatography step. The selective enrichment of functional myosins by recruitment into a complex with actin and solubilization by addition of Mg^{2+}-ATP provides a very effective means to overcome this problem and preliminary studies with a variety of myosin head constructs have shown that the incorporation of a histidine-tag at the C-terminus does not interfere with enzymatic activity. Consequently, this two-step protocol consisting of a myosin specific enrichment step followed by Ni^{2+}-NTA chromatography provides a convenient and rapid method for the purification of large quantities of recombinant myosins in a native and functional state and may greatly facilitate the structural and functional dissection of myosin motor activity. The protcol may also prove to be useful for the production of a wider range of proteins in *Dictyostelium*, particularly proteins that require mammalian-like post-translational modifications to make them functionally and immunogenically similar to the native protein. We have generated vectors for the expression of double-tagged fusion proteins that carry a myosin catalytic domain fragment at their N-terminus and a histidine-octamer at their C-terminus. As shown above for the expression of a 27 kDa α-actinin fragment as part of DCD-2R (Fig. 4), these fusion proteins can be purified by the same rapid procedure involving a single chromatographic step.

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References

BLUSCH, J., MORANDINI, P. & NELLEN, W. (1992) Transcriptional regulation by folate-inducible gene-expression in *Dictyostelium* transformants during growth and early development. *Nucleic Acids Res.* **20**, 6235–8.

BLUSCH, J. H. & NELLEN, W. (1994) Folate responsiveness during growth and development of *Dictyostelium*: separate but related pathways control chemotaxis and gene regulation. *Mol. Microbiol.* **11**, 331–5.

CHANG, A. C. M., WILLIAMS, K. L. WILLIAMS, J. G. & CECCARELLI, A. (1989) Complementation of a *Dictyostelium discoideum* thymidylate synthase mutation with the mouse gene provides a new selectable marker for transformation. *Nuclei Acids Res.* **17**, 3655–61.

CHANG, A. C. M., SLADE, M. B. & WILLIAMS, K. L. (1990) Identification of the origin of replication of the eukaryote *Dictyostelium discoideum* nuclear plasmid Ddp2. *Plasmid* **24**, 208–17.

DE LOZANNE, A. & SPUDICH, J. A. (1987) Disruption of the *Dictyostelium* myosin heavy-chain gene by homologous recombination. *Science* **236**, 1086–91.

DINGERMANN, T., REINDL, N., WERNER, H., HILDEBRANDT, M., NELLEN, W., HARWOOD, A., WILLIAMS, J. & NERKE, K. (1989) Optimization and *in situ* detection of *Escherichia coli* β-galactosidase gene-expression in *Dictyostelium discoideum*. *Gene* **85**, 353–62.

DINGERMANN, T., TROIDL, E. M., BROKER, M. & NERKE, K. (1991) Expression of human antithrombin-III in the cellular slime-mold *Dictyostelium discoideum*. *Appl. Microbiol. Biotech.* **35**, 496–503.

DITTRICH, W., WILLIAMS, K. L. & SLADE, M. B. (1994) Production and secretion of recombinant proteins in *Dictyostelium discoideum*. *Biotech.* **12**, 614–18.

DYNES, J. L. & FIRTEL, R. A. (1989) Molecular complementation of a genetic-marker in *Dictyostelium* using a genomic DNA library. *Proc. Natl Acad. Sci. USA* **86**,

7966–70.

EARLY, A. E. & WILLIAMS, J. G. (1987) Two vectors which facilitate gene manipulation and a simplified transformation procedure for *Dictyostelium discoideum*. *Gene* **59**, 99–106.

EGELHOFF, T. T., BROWN, S. S., MANSTEIN, D. J. & SPUDICH, J. A. (1989) Hygromycin resistance as a selectable marker in *Dictyostelium discoideum*. *Mol. Cell. Biol.* **9**, 1965–8.

EGELHOFF, T. T., TITUS, M. A., MANSTEIN, D. J., RUPPEL, K. M. & SPUDICH, J. A. (1991) Molecular genetic tools for study of the cytoskeleton in *Dictyostelium*. *Meth. Enzymol.* **196**, 319–34.

HOWARD, P. K., AHERN, K. G. & FIRTEL, R. A. (1988) Establishment of a transient expression system for *Dictyostelium discoideum*. *Nucleic Acids Res.* **16**, 2613–23.

JANKNECHT, R. DE MARTYNOFF, G., LOU, J., HIPSKIND, R. A., NORDHEIM, A. & STUNNENBERG, H. G. (1991) Rapid and efficient purification of native histidine-tagged protein expressed by recombinant vaccinia virus. *Proc. Natl Acad. Sci. USA*. **88**, 8972–6.

KNECHT, D., COHEN, S., LOOMIS, W. & LODISH, H. (1986) Developmental regulation of *Dictyostelium discoideum* actin gene fusions carried on low-copy and high-copy transformation vectors. *Mol. Cell. Biol.* **6**, 3973–83.

LEITING, B., LINDNER, I. J. & NOEGEL, A. A. (1990) The extrachromosomal replication of *Dictyostelium* plasmid Ddp2 requires a cis-acting element and a plasmid-encoded trans-acting factor. *Mol. Cell. Biol.* **10**, 3727–36.

MANIAK, M. & NELLEN, W. (1990) Evidence for a feedback regulated backup promoter which controls permanent expression of a *Dictyostelium* gene. *Nuclec Acids Res.* **18**, 5375–80.

MANSTEIN, D. J., RUPPEL, K. M. & SPUDICH, J. A. (1989) Expression and characterization of a functional myosin head fragment in *Dictyostelium discoideum*. *Science* **246**, 656–8.

MANSTEIN, D. J., SCHUSTER, H.-P., MORANDINI, P. & HUNT, D. M. (1995) Cloning vectors for the production of proteins in *Dictyostelium discoideum*. *Gene*, in press.

NELLEN, W. & FIRTEL, R. A. (1985) High-copy-number transformants and co-transformation in *Dictyostelium*. *Gene* **39**, 155–63.

NELLEN, W., SILAN, C. & FIRTEL, R. A. (1984) DNA-mediated transformation in *Dictyostelium discoideum* – regulated expression of an actin gene fusion. *Mol. Cell. Biol.* **4**, 2890–8.

POLLARD, T. D., DOBERSTEIN, S. K. & ZOT, H. G. (1991) Myosin-I. *Ann. Rev. Physiol.* **53**, 653–81.

RAYMENT, I., RYPNIEWSKI, W. R., SCHMIDT-BÄSE, K. SMITH, R. TOMCHICK, D. R., BENNING, M. M., WINKELMANN, D. A., WESENBERG, G. & HOLDEN, H. M. (1993) 3-dimensional structure of myosin subfragment-1: a molecular motor. *Science* **261**, 50–8.

RITCHIE, M. D., GEEVES, M. A., WOODWARD, S. K. A. & MANSTEIN, D. J. (1993) Kinetic characterization of a cytoplasmic myosin motor domain expressed in *Dictyostelium discoideum*. *Proc. Natl Acad. Sci. USA*. **90**, 8619–23.

SCHRÖDER, R. R., MANSTEIN, D. J., JAHN, W., HOLDEN, H. M., RAYMENT, I., HOLMES, K. C. & SPUDICH, J. A. (1993) Three-dimensional atomic model of F-actin decorated with *Dictyostelium* myosin S1. *Nature* **364**, 171–4.

SLADE, M. B., CHANG, A. C. M. & WILLIAMS, K. L. (1990) The sequence and organization of Ddp2, a high-copy-number nuclear plasmid of *Dictyostelium discoideum*. *Plasmid* **24**, 195–207.

WETTERAUER, B., JACOBSEN, G., MORANDINI, P. & MACWILLIAMS, H. (1993a) Mutants of *Dictyostelium discoideum* with defects in the regulation of discoidin-I expression. *Dev. Biol.* **159**, 184–95.

WETTERAUER, B. W., SALGER, K. & MACWILLIAMS, H. K. (1993b) Use of a transactive regulatory mutant of *Dictyostelium discoideum* in a eukaryotic expression system. *Nucleic Acids Res.* **21**, 1397–401.

WHITE, H. D. (1982) Special instrumentation and techniques for kinetic studies of contractile systems. *Meth. Enzymol.* **85**, 698–708.