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Overexpression and purification of human myosins from transiently and stably transfected suspension adapted HEK293SF-3F6 cells

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ABSTRACT

The myosin family of motor proteins is an attractive target of therapeutic small-molecule protein inhibitors and modulators. Milligrams of protein quantities are required to conduct proper biophysical and biochemical studies to understand myosin functions. Myosin protein expression and purification represent a critical starting point towards this goal. Established utilization of *Dictyostelium discoideum*, *Drosophila melanogaster*, insect and mouse cells for myosin expression and purification is limited, cost, labor and time inefficient particularly for (full-length) human myosins. Here we are presenting detailed protocols for production of several difficult-to-purify recombinant human myosins in efficient quantities up to 1 mg of protein per liter of cell culture. This is the first time that myosins have been purified in large scales from suspension adapted transiently and stably expressing human cells. The method is also useful for expressing other human proteins in quantities sufficient to perform extensive biochemical and biophysical characterization.

Introduction

Myosins are molecular motors that utilize ATP to perform mechanical work [1]. This is a large and diversified gene family with at least 31 different known classes across all eukaryotes [2,3], which have been shown to be essential for many cellular functions [4]. Human genome contains approximately 40 myosin genes representing 12 classes [5]. Their functional significance is accented by the discovery that mutations in myosin genes cause severe phenotypes such as deafness, blindness, cancer, cardiomyopathy, sterility and many neurological disorders [6]. Thus, the myosin family of motor proteins is an attractive target of therapeutic small-molecule protein inhibitors and modulators [7–9]. However, we can improve treatment of the diseases occurring due to myosin dysfunction with much better knowledge of myosin structure-function and reaction mechanism. For this, milligrams of protein are required [10] and thus myosin protein expression and purification represent a critical starting point towards profound understanding of myosin function in health and disease.

A viscous protein was extracted from muscle with concentrated salt solution by Kühne already in 1864, who coined it “myosin” and considered it to be responsible for the rigor state of muscle [11,12]. At first, myosins were purified directly from primary tissues such as rabbit skeletal muscle [13], brain [14] and tuna [15]. Although the extraction from tissues can provide significant amount of protein it is often hard to obtain homogenous single myosin isoform and furthermore, it limits the possibilities to consequently study specific mutations [16]. Despite attempts to express myosins in *E.coli* [17] it is generally accepted that functional vertebrate myosins cannot be expressed and purified from bacterial expression systems.

Expression and purification system based on *Dictyostelium discoideum* represents an important development towards recombinant myosin [18,19]. The *Dictyostelium* expression system is able to produce stable clones expressing myosin and its mutants [20,21] even in inducible/regulated manner [22] and in milligram quantities. However, despite the authors' intention to use the system to express also mammalian myosins [20] the expression ability seems to be limited to *Dictyostelium* myosins.

Alternative expression platform has emerged based on insect cells and baculovirus as a vector [23,24]. This system proved to be the optimal for expression of sufficient amounts of active proteins of many myosin classes including mammalian unconventional myosin class V [25], class VI [26], VII [27] and others. With the recent optimization of the production process the purification yields of milligrams of pure protein per liter of cell culture are sufficient for detailed biochemical

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and biophysical studies [28]. Despite that many myosin classes were successfully expressed and purified, this system did not perform well for every myosin isoform. The lack of certain chaperones was identified as a source of unsuccessful purification of mouse myosin 15 [29] and *Toxoplasma gondii* class XIV myosin [30]. Co-expression of chaperones was required to obtain sufficient amount of both proteins in soluble form. Beside that the expression and purification is mostly limited to myosin constructs consisting of head and neck domains in large quantities. The purification of full-length myosins, thus, remains a considerable challenge despite the fact that insect cells can incorporate most of the posttranslational modifications known to date. However, the modification patterns are different than in mammalian cells [31]. A major difference is with regard to N-linked glycosylation. Insect cells mainly synthesize oligomannosidic and paucimannosidic glycans with low levels of galactose and sialic acid [32,33]. In contrast, mammalian cells synthesize complex glycans containing mannose, N-acetylglucosamine, galactose, and sialic acid [34,35]. There are examples where non-homogenous glycosylation in insect cells resulted in immature protein [36]. Additionally, the recombinant protein production in insect cells is a time-consuming procedure. It can take up to a month to produce adequate recombinant baculovirus for large-scale expression. The baculovirus infection of insect cells is cytolytic, limiting the production phase to a relatively short period (2–5 days) and unfit for the construction of producing stable cell lines. In addition, the growth medium is complex and expensive and the growth conditions are highly delicate and very sensitive to temperature changes [31].

It is therefore expected that mammalian expression system could overcome the above-mentioned limitation. In 1995 Collins and Matsudaria have already expressed and purified chicken brush border Myosin I in monkey COS-7 cells [37]. Transiently transfected cells expressed 1 μg of protein per each 35 mm dish and cells from approximately *sixty* of 100 mm dishes were harvested per single prep. Despite the fact that the recombinant myosin catalyzed calcium-sensitive, actin-activated MgATPase activity similar to that of the native enzyme, [37] the very limited amount of the protein obtained prevented any detailed biochemical and biophysical studies. In 1996 Kinose with coworkers from the Winkelmann group developed a unique expression system for skeletal muscle myosin [38]. This expression system is based on the mouse muscle myoblast C2C12 that differentiates into myotubes. The stable cell line with integrated full-length chicken embryonic fast skeletal muscle myosin was generated and the expression was induced by placing the cells into differentiation media. About 20–40 μg of recombinant myosin was purified from 0.25 to 0.5 g of cell pellet prepared from *several* 100 mm dishes. Despite limited amount obtained the actin-activated ATPase activity of the wild-type recombinant myosin was very similar to that of myosin isolated from chicken breast muscle. The system was later modified utilizing transient adenovirus expression to analyze equivalent point mutations associated with familial hypertrophic cardiomyopathies by (low protein concentration demanding) *in vitro* motility assay [39]. Using the same expression system platform Resnicow with coworkers further optimized it and purified all six recombinant His-tagged Subfragment 1 (S1) of human type II skeletal muscle myosin isoforms [40]. Evidently the purification yield of C2C12 expression system in its final form (1 mg of homogenous protein at up to 10 μM final concentration from 1500 to 3000 cm^2 cultured cells) allows for the first time transient kinetic analysis of recombinant human skeletal and cardiac muscle isoforms with their mutants [41–43]. The considerably higher ATPase activities were achieved for both WT and mutant β-cardiac myosin than in previous report [44] where human β-cardiac myosin mutants were purified from insect cells. This and other discrepancies between studies are probably due to the difficulties in obtaining active recombinant human β-cardiac myosin in heterologous expression systems (200 μg per 10 g of insect cell pellet), most likely due to the lack of mammalian muscle-cell–specific chaperones [45]. Presently, the C2C12 expression system is the only one able to produce properly folded and functional S1 fragments of human muscle myosins in efficient quantities. Recently, a conventional human β-cardiac heavy meromyosin (HMM, a soluble fragment of myosin consisting of two S1 heads attached to coiled-coil subfragment 2, S2) albeit with shortened S2 tail was successfully purified [46].

The currently available system for expression of mammalian muscle myosins is the onlyone of its kind, but it is much less efficient in terms of time, workload and cost. Partially this is due to the infection method using transient expression of proteins based on adenoviruses as well as adherent mode of growing of C2C12 cells. These requirements make the system much less accessible for routine use [47] as well as the lack of effective transfection methods for C2C12 system. These are further characterized by low transfection efficiency (e.g. PEI) or are too expensive (e.g. Fugene) to be used in large scale experiments [48]. For large scale protein purification, it is crucial that transfection method is cost effective and highly efficient. In order to overcome C2C12 based expression system disadvantages Caldwell with coworkers has described the improvement of another expression system based on *Drosophila melanogaster* [47] from where histidine-tagged recombinant version of a *Drosophila* embryonic body wall (EMB) myosin isoform was purified from whole extracts of transgenic flies. Ability to use extract from whole flies significantly improve the standard method where purification was done on dissected muscles. Despite relatively high yield (10 mg of full length EMB myosin per run) the time course (from injection of DNA into embryos to the receipt of transgenic lines ~ 6 weeks; from crosses to generate homozygous lines in Mhc10 background, assessment of expression levels to culturing of gram quantities of flies ~ 4 months) can be challenging.

Mammalian cells are able to carry out proper protein folding for mammalian gene products with their specific chaperons and the physiologically relevant posttranslational modifications. However, posttranslational modifications differ greatly between different mammalian cells. In a key study, specific differences in glycosylation were observed when a mAb was expressed in cells from 13 different species, including humans [49]. On the other hand, Xigris, an activated protein C, could be manufactured exclusively using HEK293 cells since two essential PMT modifications, were not adequate in CHO cells [50,51]. Furthermore, mammalian cells can support expression of large proteins complexes composed of multiple subunits, cofactors and disulphide bonds. Lipid content of mammalian membranes provides native environment for membrane proteins [31]. This aspect is important also in the context of myosins since some of the family members are also associated with cell or organelle membranes [52,53]. Traditionally, however, mammalian expression and purification platforms are associated with critical drawbacks of lower purification yield, high cost (i.e. complicated and expensive culture media formulations) and they are labor and time consuming (i.e. cells growing in adherent mode, like C2C12 cells described above) comparing to other systems. Recent improvements in technology of cell line development through effective selection methods, media optimization and process control [54] have increased significantly the productivity of protein expression in mammalian cells [55,56].

Results

Development of the protocol

Choice of suitable host cell line

Table 1 shows a few mammalian cell lines derived from different species and different tissues that have been successfully used to express recombinant proteins [51,57]. Moreover, there are severalderived or engineered variations of HEK293 and CHO cells to increase volumetric productivity and to improve posttranslational modifications [58]. Generally, mammalian cell lines produce proteins with large deviation in their quantity and quality. Our choice of the cell line after considering the above mentioned factors is the specific derivative of HEK293, termed HEK293SF-3F6 [59,60]. The HEK293SF-3F6 cells are