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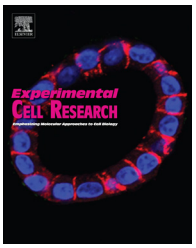

Research Article

Myosin 1e is a component of the invadosome core that contributes to regulation of invadosome dynamics

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ABSTRACT

Myosin 1e (myo1e) is an actin-based motor protein that has been implicated in cell adhesion and migration. We examined the role of myo1e in invadosomes, actin-rich adhesion structures that are important for degradation and invasion of the extracellular matrix. RSV-transformed BHK-21 cells, which readily form invadosomes and invadosome rosettes, were used as the experimental model. Myo1e localization to the actin-rich core of invadosomes required the proline-rich Tail Homology 2 (TH2) domain. During invadosome rosette expansion, we observed myo1e recruitment to newly forming invadosomes via Tail Homology 1 (TH1)-dependent interactions with the plasma membrane, where it preceded actin and paxillin. Dominant-negative inhibition of myo1e resulted in mislocalized invadosome formation, usually at the center of the rosette. We propose that TH2 domain of myo1e provides the key signal for localization to invadosomes, while TH1 domain interactions facilitate myo1e targeting to the plasma membrane-proximal locations within the rosettes. Myo1e may then act as a scaffold, linking the plasma membrane with the actin cytoskeleton and helping direct new invadosome formation to the periphery of the rosette.

Introduction

Myosin 1e (myo1e) is an actin-dependent molecular motor broadly expressed in vertebrate tissues. Myo1e belongs to myosin class I, which includes several single-headed myosin motors that interact with both actin filaments and membranes/vesicles [1]. Class I myosins consist of a single heavy chain and one or more calmodulin-like light chains. Myo1e heavy chain includes an N-terminal motor or head domain, responsible for actin binding, a neck domain that contains a single IQ motif for binding to light chains, and a C-terminal tail domain. Most class I myosins have tails that consist of a single domain known as TH1 (Tail Homology 1). TH1 domains are rich in positively charged amino acids and bind to acidic phospholipids, promoting myosin localization to the plasma membrane. Myo1e and a closely related myosin, myo1f, form a distinct subclass of class I myosins, known as long-tailed myosins I. These proteins contain two additional domains in their tails: a proline-rich TH2 domain and a C-terminal SH3 domain also referred to as TH3.

The functional roles previously identified for myo1e include involvement in clathrin-mediated endocytosis [2,3], maintenance of normal glomerular filtration in the kidney [4,5], and regulation of cell–cell adhesion [6]. Myo1f, the only other member of long-tailed myosin I subclass in vertebrates, has previously been shown

Abbreviations: BHK, Baby Hamster Kidney cells; FRAP, fluorescence recovery after photobleaching; Myo1e, m1e, myosin 1e; N-WASP, Neural Wiskott Aldrich Syndrome protein; RSV, Rous sarcoma virus; TH, Tail Homology domain; WIP, WASP interacting protein

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to play an important role in regulating cell-substrate adhesion and cell migration in neutrophils via modulation of integrin exocytosis [7]. These findings suggest that vertebrate long-tailed class I myosins contribute to a variety of actin-dependent processes. Intriguingly, myo1e has been detected as a component of integrin adhesion complexes in a large-scale proteomic study [8], suggesting that, similarly to myo1f, it may be involved in regulation of cell–substrate adhesion. Since myo1e role in cell–substrate adhesion has not been characterized, we set out to examine its localization and functions in cell-substrate contacts.

Initial observation of myo1e localization in several cell lines showed that it was not highly enriched in focal adhesions or focal complexes. However, we found that myo1e was highly concentrated in podosomes/invadopodia, specialized sites of cell-substrate attachment involved in matrix degradation and invasion [9–11]. Therefore, we focused on characterization of myo1e role in podosomes/invadopodia to determine how it contributes to cell adhesion, using Rous sarcoma virus (RSV)-transformed BHK-21 cells as a model system. BHK-21 is a fibroblast cell line derived from Syrian hamster kidneys and widely used to study virus replication. RSV-transformed BHK-21 cells form numerous invadopodia-like structures arranged in clusters or ring-shaped aggregates [12]. Tyrosine kinase Src, and, in particular, the active form of Src contributed by the RSV v-src gene, plays a key role in assembly of invadopodia. Although invadopodia-like structures formed by RSV-transformed BHK-21 cells are often referred to as podosomes in the literature, in this manuscript we will refer to them as "invadosomes", using a more general term to describe these invasive structures.

Invadosomes are actin-rich adhesion structures containing a dense meshwork of actin filaments at their center, or core, and a ring of adhesion adapter proteins, such as paxillin and vinculin, at their periphery [13,14]. Invadopodia play an important role in tumor cell invasion, representing the site of intense matrix degradation by tumor cells [15–17]. Invadosomes in RSV-transformed cells frequently form superstructures, or rosettes, consisting of multiple invadosomes. Treatment with the tyrosine phosphatase inhibitor sodium orthovanadate, or vanadate, has been shown to accelerate formation of new invadosomes and promote expansion of the rosettes towards the cell periphery [18], resulting in formation of large invadosome rings, similar to podosome belts in osteoclasts. The rosettes expand due to the assembly of new invadosomes at the periphery of the rosette and paxillin-regulated disassembly of invadosomes along the internal rim [18]. The dynamics of invadosome rosette expansion is reminiscent of the process that results in formation of podosome belts in osteoclasts, where new podosomes are assembled along the outer edge of a podosome cluster [19]. Observing rosette expansion in this model allows direct visualization of invadosome dynamics, allowing us to examine the sequence of protein recruitment to the sites of new invadosome assembly at the periphery.

In this study, we used RSV-transformed BHK-21 cells as a model for invadosome dynamics and assembly. We examined localization of myo1e in individual invadosomes and invadosome rosettes. Next, we mapped the domains of myo1e necessary for localization to invadosomes. Finally, we inspected myo1e localization within expanding invadosome rosettes relative to other invadosome markers and analyzed myo1e role in expansion of invadosome rosettes following vanadate treatment.

Materials and methods

Cells, plasmids, antibodies and reagents

RSV-transformed BHK-21 cells were a generous gift from the De Camilli lab, Yale University [20]. Cells were maintained in DMEM with 10% fetal bovine serum (FBS) in 5% CO_2 atmosphere at 37 °C.

All GFP-tagged human myo1e constructs were cloned into pEGFP-C1 vector (Clontech Laboratories, Inc., Mountain View, CA). Cloning of the myo1e full-length and tail constructs was previously described [2]. Domain deletion mutants of myo1e were made by inserting mutations using QuikChange Lightning site-directed mutagenesis kit (Agilent, Santa Clara, CA) or by In-fusion cloning (Clontech Laboratories, Inc., Mountain View, CA). Primers used to subclone TH1 domain surround AA717-921, TH2 domain – AA921-1056, TH3 domain – AA1051-1108. Murine myo1e-mApple [21] was provided by Dr. Christien Merrifield via Addgene (Cambridge, MA).

Monoclonal antibody against paxillin was purchased from BD Transduction Laboratories (Franklin Lakes, NJ). Anti-myo1e polyclonal antibody was previously described [22]. Gelatin was purchased from Sigma (St. Louis, MO) and prepared as previously described [23]. Sodium orthovanadate was purchased from Sigma (St. Louis, MO) and prepared as a 200 μM solution. The solution was activated as previously described [24] and stored at −20 °C. Prior to use, the 200 μM sodium orthovanadate was heated in a 55 °C water bath for 10 min and diluted in DMEM with 10% FBS to a final concentration of 5 μM.

Live cell imaging and immunostaining

RSV-transformed BHK-21 cells (60,000 cells/dish) were plated in 35 mm glass bottom dishes (MatTek Corportaion, Ashland, MA) 48 h prior to imaging. Cells were transfected with 2 μg of DNA plasmid per dish using Jet PEI reagent (Polyplus-Transfection Inc., Illkirch, France) 24 h before imaging. Cells were incubated at 37 °C in medium containing 5 μM activated vanadate for 10 min prior to imaging and during imaging. Cells were plated on 18 mm round glass coverslips 24 h before staining. Prior to staining, cells were treated for 25 min with 5 μM sodium orthovanadate at 37 °C. The staining protocol was similar to the previously described protocols [4,18]. In brief, cells were fixed in 4% paraformaldehyde for 10 min, permeabilized using 0.25% Triton X-100 and blocked in 3% Bovine Serum Albumin in PBS. This was followed by a one-hour incubation with primary antibody and a thirty-minute incubation with a secondary antibody.

To compare the probability of rosette formation in cells expressing either full-length myo1e or myo1e tail construct, cells transfected with the GFP-tagged myo1e constructs were treated with vanadate for 15 min, fixed, and stained with phalloidin. All cells expressing GFP-tagged proteins were counted and scored for the presence or absence of an invadosome rosette. A total of 1300 cells (from approximately 4 22×22 mm^2 coverslips) was scored for each construct. As a control, the number of rosette-forming cells among 1300 untransfected, vanadate-treated cells was also determined. Data are represented as percentages.

All imaging was performed using Perkin Elmer UltraView VoX Spinning Disk Confocal system mounted on a Nikon Eclipse Ti