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EGF-like peptide-enhanced cell movement in *Dictyostelium* is mediated by protein kinases and the activity of several cytoskeletal proteins

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ABSTRACT

DdEGFL1, a synthetic epidermal growth factor-like (EGFL) peptide based on the first EGFL repeat of the extracellular matrix, cysteine-rich, calmodulin-binding protein CyrA, has previously been shown to sustain the threonine phosphorylation of a 210 kDa protein during the starvation of *Dictyostelium* cells. Immunoprecipitation coupled with a LC/MS/MS analysis identified the 210 kDa protein as vinculin B (VinB). VinB shares sequence similarity with mammalian vinculin, a protein that links the actin cytoskeleton to the plasma membrane. Both threonine phosphorylated VinB (P-VinB) and VinB-GFP localized to the cytoplasm and cytoskeleton of *Dictyostelium* amoebae. VinB-GFP was also shown to be threonine phosphorylated and co-immunoprecipitated with established vinculin-binding cytoskeletal proteins (e.g. myosin II heavy chain, actin, alpha-actinin, talin). P-VinB and VinB-GFP were detected in DdEGFL1 pull-down assays, which also identified a 135 kDa phosphothreonine protein and two phosphotyrosine proteins (35 and 32 kDa) as potential components of the DdEGFL1 signaling pathway. DdEGFL1-enhanced cell movement required the cytoskeletal proteins talin B and paxillin B and tyrosine kinase activity mediated by PKA signaling, however VinB threonine phosphorylation was shown to be independent of PI3K/PLA2 signaling and PI3K and PKA kinase activity. Finally, VinB-GFP over-expression suppressed DdEGFL1-enhanced random cell movement, but not folic acid-mediated chemotaxis. Together, this study provides the first evidence for VinB function plus new insight into the signaling pathway(s) mediating EGFL repeat/peptide-enhanced cell movement in *Dictyostelium*. This information is integrated into an emerging model that summarizes existing knowledge.

1. Introduction

Dictyostelium is a social amoebozoan that is used as a model system for studying a number of cell and developmental processes, especially cell motility and chemotaxis [1]. It has been suggested that the *Dictyostelium* genome encodes a higher percentage of epidermal growth factor-like (EGFL) domains than any other sequenced eukaryote [2]. EGF is a polypeptide that regulates a variety of processes in mammalian cells, including cell movement and chemotaxis [3–5]. Ten14, the 14th EGFL repeat from the extracellular matrix (ECM) glycoprotein tenascin C, increases mammalian cell movement by binding to the EGF receptor (EGFR) and activating EGFR-dependent signaling [6–8].

CyrA, an ECM, cysteine-rich, calmodulin (CaM)-binding protein (CaMBP) in *Dictyostelium*, possesses four tandem EGFL repeats in its C-terminus [9,10]. The protein is secreted during development, localizes to the slime sheath of the migrating slug, and is proteolytically cleaved to release EGFL repeat-containing cleavage products [9,10]. A synthetic EGFL peptide (DdEGFL1), whose sequence is identical to the first 18 amino acids of the first EGFL repeat (EGFL1) of CyrA, functions extracellularly to increase both random cell motility and cAMP-mediated chemotaxis in *Dictyostelium* [11]. FITC-conjugated DdEGFL1 has been detected on the surface of cells treated with concanavalin A suggesting that a receptor exists for this peptide sequence [10]. DdEGFL1 increases *Dictyostelium* cell movement via a signaling pathway that does not require the cAMP receptor or the heterotrimeric G-protein used in cAMP signaling [12]. The signaling pathway does however involve RasG, CaM, and intracellular Ca^{2+} release and DdEGFL1 stimulation increases the amount of polymeric actin and myosin II heavy chain (MHC) in the cytoskeleton [12].

Movement of both mammalian and *Dictyostelium* cells involves dynamic changes in the cytoskeleton. In mammalian cells, vinculin

Abbreviations: EGF, epidermal growth factor; EGFL, EGF-like; EGFL1, 1st EGFL repeat of CyrA; EGFR, EGF receptor; EGFRL, EGFR-like protein; Ten14, 14th EGFL repeat of tenascin C; ECM, extracellular matrix; CaM, calmodulin; CaMBP, CaM binding protein; MHC, myosin II heavy chain; PKC, protein kinase C; VinA, vinculin A; VinB, vinculin B; GFP, green fluorescent protein; kDa, kilodalton; P-Thr, phosphorylated threonine; P-Ser, phosphorylated serine; P-Tyr, phosphorylated tyrosine; PI3K, phosphatidylinositol-3-kinase; PLA2, phospholipase A2; P-VinB, threonine phosphorylated VinB; TalA, talin A; TalB, talin B; PaxB, paxillin B; AbpA, actin-binding protein A; AbpC, actin-binding protein C; PKA, protein kinase A.

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associates with actin, alpha-actinin, talin, and paxillin to regulate cytoskeletal dynamics during cell adhesion and migration and to anchor the actin cytoskeleton to the cell membrane [13]. Vinculin has also been reported to interact with myosin, but not with tubulin [14,15]. The *Dictyostelium* genome encodes one alpha-actinin homologue (AbpA), two talin homologues (TalA and TalB), and one true homologue of mammalian paxillin (PaxB). *Dictyostelium* alpha-actinin cross-links actin filaments and increases the actin-stimulated ATPase activity of MHC [16]. *abpA* − cells appear phenotypically normal, however cell–substrate adhesion, growth, pinocytosis, and development are slightly impaired [17–19]. *talA* − cells adhere weakly to the substratum and display slightly impaired cytokinesis in the vegetative state, however development proceeds normally [20]. In contrast, *talB* − cells display reduced motility during development which arrests at the tight mound stage [21]. PaxB has been shown to be important for cell–substrate adhesion and multicellular development by regulating the actin cytoskeleton and actin-dependent processes [22]. *Dictyostelium* also possesses a homologue of mammalian filamin (encoded by the *abpC* gene). This 120 kDa F-actin binding protein cross-links the actin cytoskeleton in *Dictyostelium*, but is not absolutely necessary for growth, shape, development, motility, or chemotaxis towards cAMP [23].

The structure of mammalian vinculin comprised four head domains (D1–D4) and a tail domain (D5) [24,13]. A proline-rich region (amino acids 838–878) lies between D4 and D5 and links the globular heads of the protein to the rod-like tail [25,13]. The binding of F-actin to vinculin occurs in the D5 domain, specifically at a domain confined by amino acids 893–1016 [26,27,13]. Binding of talin and alpha-actinin to vinculin occurs within the D1 domain (amino acids 1–258) [28,29,24,13]. In mammals, vinculin has been shown to be primarily phosphorylated on serine and threonine residues within the C-terminal tail however tyrosine phosphorylation has also been reported [30–34]. Vinculin phosphorylation has also been shown to be enhanced by growth factor stimulation [35].

DdEGFL1 is the first identified peptide regulator of cell motility and chemotaxis in *Dictyostelium*. DdEGFL1 has previously been shown to sustain the threonine phosphorylation of a 210 kDa protein during starvation [11]. In this study, immunoprecipitation coupled with a LC/MS/MS analysis identified the unknown 210 kDa protein as vinculin B (VinB), a protein that shares sequence similarity with mammalian vinculin. The subcellular localization of the protein was analyzed using cell fractionations and a VinB-GFP fusion protein. VinB-GFP co-immunoprecipitated with established cytoskeletal proteins in *Dictyostelium* and DdEGFL1 pull-down assays supported the interaction between VinB and DdEGFL1. The signaling pathway mediating DdEGFL1-enhanced cell movement was also shown to involve TalB, PaxB, protein kinase A (PKA), and tyrosine kinase activity. Together, these results extend our understanding of EGFL repeat/peptide signal transduction in *Dictyostelium*.

2. Materials and methods

2.1. Cells, experimental peptides, chemicals

All *Dictyostelium discoideum* strains were grown either in the presence of *Escherichia coli* on SM agar pH 6.5 at 22 °C in the dark for 24–30 h as previously described or axenically in HL-5 medium [12]. All parental and mutant strains were obtained from the Dicty Stock Center (http://dictybase.org/StockCenter/StockCenter.html). The following mutants were used in this study: *abpA* −, DBS0235459 [36]; *abpC* −, DBS0236167 [23]; *abpA* −/*abpC* −, DBS0235456 [36]; *talA* −, DBS0236180 [20]; *talB* −, DBS0302472 [37]; and *paxB* −, DBS0236728 [22]. DdEGFL1 was provided as a gift from Dr. Yali Wang (Advanced Syntech Corporation, Mississauga, ON, Canada) [11]. LY294002 and quinacrine were purchased from EMD Biosciences Incorporated (La Jolla, CA, USA). H-89 and KT5720 were purchased from Sigma-Aldrich Canada Limited (Oakville, ON, Canada). Myristoylated PKI (14–22) amide was purchased from Enzo Life Sciences Incorporated (Farmingdale, NY, USA).

2.2. Subcellular localization and fractionation

Cells were fixed and imaged as previously described [38]. The following primary and secondary antibodies were used for immunolocalizations: mouse monoclonal anti-GFP (1:50; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and goat anti-mouse Alexa-488 (1:100; Invitrogen Canada Inc., Burlington, ON, Canada). Whole cell lysates were fractionated into cytoplasmic and cytoskeletal samples as previously described except for a few minor changes [12]. Vegetative amoebae were harvested from SM agar plates and fractionated. The cytoskeleton was isolated from 1 mg of total cell protein. The final pellet containing the cytoskeleton was resuspended in 30 μl of wash buffer and 10 μl sample loading buffer and stored at −80 °C for future use.

2.3. Immunoprecipitation

Immunoprecipitations were performed as previously described [38]. The following IP antibodies were used: mouse monoclonal anti-P-Thr (5 μg; Cell Signalling Technology Inc., Danvers, MA, USA) or mouse monoclonal anti-GFP (5 μg; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Immunoprecipitates were pulled-down with Protein G-PLUS agarose or Immunocruz™ B, C, or E IP resins (100 μl slurry; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Samples were stored at −80 °C for future use. For protein identification, anti-P-Thr immunoprecipitates (40 μl) were separated by SDS-PAGE. Gels were stained with Coomassie blue overnight at room temperature with gentle agitation and then destained with destaining solution (40% acetic acid, 10% methanol, 50% ddH_20). Protein bands were excised with a razor blade and stored at 4 °C in 1% acetic acid. Western blots were performed in parallel to confirm the pull-down of the desired 210 kDa protein. Protein bands were submitted for analysis to the Mass Spectrometry Facility at the Advanced Protein Technology Centre of the Hospital for Sick Children (Toronto, ON, Canada). Samples were subjected to an in-gel trypsin digestion followed by a LC/MS/MS analysis. All MS/MS samples were analyzed using Mascot set up to search the NCBInr database for *D. discoideum* (Matrix Science Inc., Boston, MA, USA).

2.4. Phosphoprotein analysis

Cells were harvested from SM agar plates and starved at 22 °C and 160 rpm in KK2 phosphate buffer (9×10^6 cells/ml) ± DdEGFL1 (450 μM) and the appropriate chemical as detailed in the Results and elsewhere [11,12,9]. In separate experiments, cells were grown in HL-5 medium ± H-89 (10 or 50 μM) for 5 and 24 h. Cells were harvested and lysed at the indicated times with a buffer containing 50 mM Tris–HCl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 5 mM EDTA, 10 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium fluoride, and a protease inhibitor cocktail (Hoffmann-La Roche Limited, Mississauga, ON, Canada). Samples were stored at −80 °C for future use.

2.5. DdEGFL1 pull-down assay

All steps were carried out according to the manufacturer's instructions unless otherwise stated. DdEGFL1 (2–4 mg) was dissolved in 20 mM EDTA pH 8.0 and reduced with TCEP disulphide reducing gel (Fisher Scientific, Ottawa, ON, Canada). Reduced DdEGFL1 was conjugated to SulfoLink® coupling resin (Fisher Scientific, Ottawa, ON, Canada). Non-specific binding sites on the resin were blocked with 50 mM L-Cysteine·HCl. The resin was washed several times with 1 M NaCl and stored in PBS containing 0.05% sodium azide.