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CyrA, a matricellular protein that modulates cell motility in *Dictyostelium discoideum*

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ABSTRACT

CyrA, an extracellular matrix (slime sheath), calmodulin (CaM)-binding protein in *Dictyostelium discoideum*, possesses four tandem EGF-like repeats in its C-terminus and is proteolytically cleaved during asexual development. A previous study reported the expression and localization of CyrA cleavage products CyrA-C45 and CyrA-C40. In this study, an N-terminal antibody was produced that detected the full-length 63 kDa protein (CyrA-C63). Western blot analyses showed that the intracellular expression of CyrA-C63 peaked between 12 and 16 h of development, consistent with the time that cells are developing into a motile, multicellular slug. CyrA immunolocalization and CyrA-GFP showed that the protein localized to the endoplasmic reticulum, particularly its perinuclear component. CyrA-C63 secretion began shortly after the onset of starvation peaking between 8 and 16 h of development. A pharmacological analysis showed that CyrA-C63 secretion was dependent on intracellular Ca^{2+} release and active CaM, PI3K, and PLA2. CyrA-C63 bound to CaM both intra- and extracellularly and both proteins were detected in the slime sheath deposited by migrating slugs. In keeping with its purported function, CyrA-GFP over-expression enhanced cAMP-mediated chemotaxis and CyrA-C45 was detected in vinculin B (VinB)-GFP immunoprecipitates, thus providing a link between the increase in chemotaxis and a specific cytoskeletal component. Finally, DdEGFL1-FITC was detected on the membranes of cells capped with concanavalin A suggesting that a receptor exists for this peptide sequence. Together with previous studies, the data presented here suggests that CyrA is a bona fide matricellular protein in *D. discoideum*.

1. Introduction

CyrA is the first extracellular matrix (ECM; slime sheath), calmodulin (CaM)-binding protein (CaMBP) identified in the model eukaryote *Dictyostelium discoideum* (Suarez et al., 2011). This 63 kDa cysteine-rich protein is proteolytically cleaved during asexual development to release two prominent epidermal growth factor-like (EGFL) repeat-containing cleavage products of molecular weights 45 and 40 kDa (named CyrA-C45 and CyrA-C40, respectively) (Suarez et al., 2011). EGFL repeat-containing proteins are often found to be membrane-bound or secreted components of the ECM (Campbell and Bork, 1993). The proteolytic cleavage of proteins to produce small signaling polypeptides and peptides has been observed in many systems. For example, the ECM glycoprotein laminin-5 possesses EGFL repeats in its ectodomain that when cleaved by matrix metalloproteinase 2 (MMP2), promote cell migration by binding to the EGF receptor (EGFR) and activating EGFR-dependent signaling (Giannelli et al., 1997; Schenk et al., 2003). Certain EGFL repeats of another ECM glycoprotein tenascin C (e.g. Ten14) have also been shown to enhance cell motility by binding to the EGFR (Swindle et al., 2001; Iyer et al., 2007, 2008). In *Dictyostelium*, this type of protein processing has been reported for the acyl-CoA binding protein (AcbA), which is secreted and cleaved by the membrane-bound serine protease TagC to generate the bioactive peptide spore differentiation factor-2 (SDF-2) (Anjard and Loomis, 2005). SDF-2 binds to a receptor on prespore cells in order to induce spore cell differentiation (Anjard and Loomis, 2005).

Dictyostelium is widely used as a model system for studying a number of cell and developmental processes (Williams, 2010). Upon starvation, single cells enter a developmental program that takes approximately 24 h to complete. Development begins with the aggregation of single cells to form multicellular aggregates or mounds. After approximately 16 h of development, aggregated cells form multicellular structures called pseudoplasmodia or slugs that can migrate on the substratum in response to light and temperature. When

Abbreviations: CaM, Calmodulin; CaMBP, CaM-binding protein; CaMBD, CaM-binding domain; CaMBOT, CaM-binding overlay technique; EGFL, Epidermal growth factor-like; EGFR, EGF receptor; ECM, Extracellular matrix; Ten14, 14th EGFL repeat of tenascin C; MMP2, Matrix metalloproteinase 2; PI3K, Phosphatidylinositol 3-kinase; PLA2, Phospholipase A2; CyrA-C63, Full-length 63 kDa CyrA; CyrA-C45, 45 kDa CyrA cleavage product; CyrA-C40, 40 kDa CyrA cleavage product; VinB, Vinculin B; AcbA, Acyl-CoA binding protein; SDF2, Spore differentiation factor 2; MHC, Myosin II heavy chain; GFP, Green fluorescent protein; ER, Endoplasmic reticulum; TSP, Thrombospondin; SPARC, Secreted protein, acidic and rich in cysteine; DB, Development buffer; kDa, kilodalton; WC, Whole cell; IP, Immunoprecipitate; PD, Protein-depleted; FITC, Fluorescein isothiocyanate.

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conditions are suitable, the slugs culminate into fruiting bodies composed of dead stalk cells supporting a mass of viable spores (Schaap, 2011). If development is allowed to proceed in the dark, slugs will not immediately culminate. Instead, they will migrate on the surface for an indefinite period of time leaving behind a trail of extracellular slime sheath (i.e. ECM) (Wilkins and Williams, 1995). CyrA is differentially secreted during development and localizes to the extracellular slime sheath of the migrating slug (Suarez et al., 2011). Antagonism of CaM with the CaM-specific inhibitor W7 enhances CyrA proteolysis suggesting that CaM protects CyrA from proteolytic cleavage (Suarez et al., 2011). A synthetic peptide (DdEGFL1) equivalent in sequence to the first 18 amino acids of CyrA EGFL repeat 1 (EGFL1) enhances both random cell motility and cAMP-mediated chemotaxis via a signaling pathway that does not require either of the two cAMP receptors that are active during early development (i.e. carA and carC) (Huber and O'Day, 2009, 2011a). These results suggest the existence of a novel pathway regulated by EGFL repeats/peptides in this model organism. The ability of DdEGFL1 to enhance cell movement is dependent on CaM function and intracellular Ca^{2+} release (Huber and O'Day, 2011a). In addition, DdEGFL1-enhanced cell movement requires active phosphatidylinositol 3-kinase (PI3K) and phospholipase A2 (PLA2), two proteins that mediate chemotaxis of *Dictyostelium* via two parallel compensatory pathways (Chen et al., 2007; Van Haastert et al., 2007; Huber and O'Day, 2009). Together these results indicate that CyrA may modulate *Dictyostelium* cell motility during early development and slug migration via one or more of its EGFL repeats.

A previous study using an antibody directed against the C-terminus of CyrA only weakly detected the full-length 63 kDa protein (CyrA-C63), leaving full-length CyrA largely uncharacterized (Suarez et al., 2011). To rectify this situation and to gain further insight into the function of this protein, we generated a rabbit polyclonal antibody against the N-terminus that detected the full-length protein. Western blot analyses showed that the protein was differentially expressed and secreted during asexual development and bound CaM both intra- and extracellularly. Pharmacological intervention showed that secretion of CyrA-C63 was dependent on intracellular Ca^{2+} release and active CaM, PI3K, and PLA2. Using a CyrA-GFP fusion protein and immunolocalization of endogenous CyrA, the protein was shown to localize to the endoplasmic reticulum (ER) prior to secretion. Full-length CyrA bound to CaM and both proteins were shown to localize to the slime sheath supporting an extracellular interaction. Support for a surface receptor for CyrA or its cleavage products was revealed when DdEGFL1-FITC was detected on the membranes of cells capped with concanavalin A and a link to the cytoskeleton was provided when vinculin B (VinB) was shown to co-immunoprecipitate with CyrA. Together, the results provide new insight into the function of CyrA during *Dictyostelium* asexual development and suggest that CyrA is functionally similar to matricellular proteins from mammals.

2. Results

2.1. Detection of full-length CyrA

A previous study on CyrA used an antibody (anti-C-CyrA) targeting the C-terminal end of the protein (amino acids 544–565; Fig. 1A) (Suarez et al., 2011). That antibody detected full-length CyrA (CyrA-C63) and specific cleavage products (CyrA-C45, CyrA-C40) during development (Suarez et al., 2011). However, anti-C-CyrA had a very low affinity for CyrA-C63 leaving full-length CyrA largely uncharacterized. For that reason, here we generated an antibody (anti-N-CyrA) targeting the N-terminus of the protein (amino acids 88–105; Fig. 1A). Anti-N-CyrA detected a 63 kDa protein in AX3 whole cell lysates, which is consistent with the molecular weight of full-length CyrA after the signal sequence (amino acids 1–20) is removed (Fig. 1A,B; http://www.dictybase.org). However, the antibody failed to detect CyrA-C45 and CyrA-C40. In order to confirm the specificity of anti-N-CyrA, a strain expressing CyrA-FLAG was generated (AX3/[act15]:cyrA:FLAG). CyrA-FLAG was immunoprecipitated with anti-FLAG. Immunoprecipitates were separated by SDS-PAGE and analyzed by western blotting with anti-N-CyrA. Anti-N-CyrA detected a 64 kDa protein in immunoprecipitates which corresponded to full-length CyrA (63 kDa) and the 1 kDa FLAG tag, however anti-FLAG did not completely immuno-deplete CyrA-FLAG since a large amount of the protein was still detected in the protein-depleted (PD) sample (Fig. 1C).

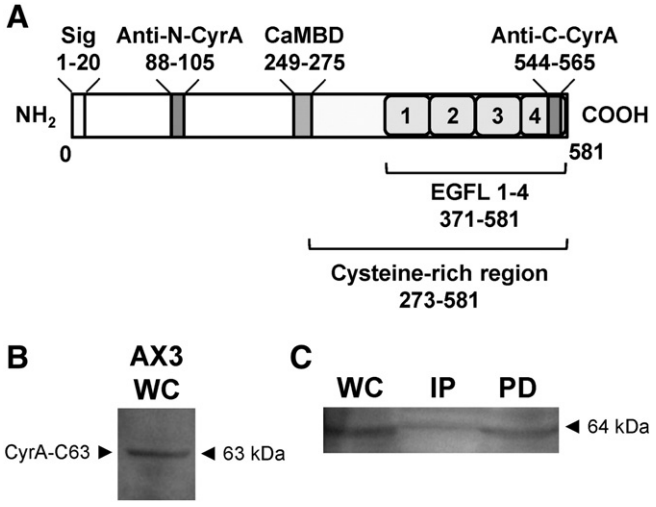

Fig. 1. Production of an antibody targeted against the N-terminus of CyrA. (A) Domain architecture of CyrA. Sig: signal sequence; CaMBD: CaM-binding domain; EGFL 1–4: EGF-like repeats 1–4. Amino acid sequences recognized by anti-N-CyrA and anti-C-CyrA are also shown. (B) Detection of full-length CyrA in an AX3 whole cell lysate. Western blot probed with anti-N-CyrA. WC, whole cell lysate (20 μg). (C) CyrA-FLAG was immunoprecipitated from an AX3/[act15]:cyrA:FLAG whole cell lysate with anti-FLAG. Immunoprecipitates were pulled down with ImmunoCruz™ E IP resin, separated by SDS-PAGE, and analyzed by western blotting with anti-N-CyrA. WC, whole cell lysate (20 μg); IP, immunoprecipitate (25 μl); PD, protein-depleted fraction (20 μg). Molecular weight markers (in kDa) are shown to the right of the blots in B and C.

2.2. Expression and secretion of full-length CyrA during asexual development

Western blots of whole cell lysates from AX3 cells developing on cellulose filters revealed that full-length CyrA was differentially expressed during development (Fig. 2A). The pattern of protein expression closely matched the RNA expression profile reported by dictyExpress, an online database with RNA expression data for *Dictyostelium* genes (http://dictyexpress.biolab.si) (Rot et al., 2009). Protein expression increased at a constant rate during the early stages of development reaching peak levels between 12 and 16 h, after which time the levels began to steadily decrease (Fig. 2A). An analysis of concentrated culture supernatant containing proteins secreted throughout development revealed that full-length CyrA, like its cleavage products, was detected extracellularly (Fig. 2B) (Suarez et al., 2011). Secretion increased during the early stages of development and peaked between 8 and 16 h, after which time the amount of secreted CyrA began to decrease. CyrA-C63 was secreted at its highest levels when cells were forming a motile aggregate (i.e. a slug; Fig. 2B). Blots probed with anti-tubulin controlled for the equal loading of whole cell samples and for the contamination of extracellular samples with proteins from lysed cells (Fig. 2A,B). Unless otherwise noted, from this point on, full-length CyrA will simply be referred to as CyrA.

2.3. Intracellular expression of CyrA during cell starvation

A previous study showed that DdEGFL1 treatment could feedback to regulate the conversion of CyrA-C45 to CyrA-C40, therefore suggesting