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Nanobodies targeting cortactin proline rich, helical and actin binding regions downregulate invadopodium formation and matrix degradation in SCC-61 cancer cells

Laurence Bertier, Tim Hebbrecht, Elien Mettepenningen, Natasja De Wit, Olivier Zwaenepoel, Adriaan Verhelle, Jan Gettemans*

Department of Biochemistry, Faculty of Medicine and Health Sciences, Ghent University, Campus Rommelaere, Albert Baertsoenkaai 3, B-9000, Ghent, Belgium

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ABSTRACT

Cortactin is a multidomain actin binding protein that activates Arp2/3 mediated branched actin polymerization. This is essential for the formation of protrusive structures during cancer cell invasion. Invadopodia are cancer cell-specific membrane protrusions, specialized at extracellular matrix degradation and essential for invasion and tumor metastasis. Given the unequivocal role of cortactin at every stage of invadopodium formation, it is considered an invadopodium marker and potential drug target. We used cortactin nanobodies to examine the role of cortactin domain-specific function at endogenous protein level. Two cortactin nanobodies target the central region of cortactin with high specificity. One nanobody interacts with the actin binding repeats whereas the other targets the proline rich region and was found to reduce EGF-induced cortactin phosphorylation. After intracellular expression as an intrabody, they are both capable of tracing their target in the complex environment of the cytoplasm, and disturb cortactin functions during invadopodia formation and extracellular matrix degradation. These data illustrate the use of nanobodies as a research tool to dissect the role of cortactin in cancer cell motility. This information can contribute to the development of novel therapeutics for tumor cell migration and metastasis.

1. Introduction

During tumor dissemination or microbial infection, physiological processes are dysregulated or hijacked to enable disease progression [1,2]. Cancer cell invasion consists of different stages: disintegration of the surrounding basement membrane, invasion of the extracellular matrix, intravasation of the blood stream, and extravasation and colonization at distant sites [3]. Depending on the extracellular environment, cancer cells must adapt their shape and migration mode [4]. The actin cytoskeleton has a prominent role in the maintenance and regulation of cell shape, and the formation of cellular protrusions during cell migration and metastasis [5]. Different types of actin-mediated cellular protrusions are known to assist in cancer cell motility including lamellipodia, filopodia and invadopodia [6–8]. Lamellipodia and filopodia are found at the leading edge of migrating cells. The former are thin, sheet-like, cytoplasmic projections and mainly consist of branched actin networks [9]; the latter are finger-like protrusions of the plasma membrane that are built up by parallel, bundled actin filaments [10]. Cancer cell migration, intravasation and extravasation can be facilitated by actin rich protrusions that enable focussed secretion of proteases, known as invadopodia [8]. The formation of these structures requires the integration of different signals from the surrounding microenvironment to mediate changes in cytoskeletal structure. The cytoskeletal protein cortactin participates in many cellular events such as cell migration, endocytosis, cell adhesion and intracellular vesicle trafficking [11]. As a multidomain adaptor and actin binding protein,

Abbreviations: Abl, ableson kinase; ABR, actin binding repeat; Arg, ableson related gene kinase; Arp2/3, actin related protein 2/3; ATAT1, alpha-tubulin N-acetyltransferase 1; DMEM, Dulbecco's modified Eagle's medium; DOX, doxycycline; Erk, extracellular signal–regulated kinase; FHP, F-actin-helical-proline rich fragment; HDAC6, histone deacetylase 6; HNSCC, head and neck squamous cell carcinoma; HS1, hematopoietic lineage cell-specific protein; ITC, isothermal titration calorimetry; K_D, dissociation constant; MT1-MMP, membrane type-1 matrix metalloprotease; Nb, nanobody; NTA, N-terminal acidic; N-WASP, neural Wiskott-Aldrich syndrome protein; PAK, p21-activated kinase; PKC, protein kinase C; PKD, protein kinase D; PMSF, phenylmethylsulfonyl fluoride; SH2, src homology 2 domain; SH3, src homology 3 domain; Tip60, 60kDa Tat-interactive protein; Tks5, tyrosine kinase substrate 5; WIP, WASP interacting protein

* Corresponding author at: Nanobody Lab, Department of Biochemistry, Faculty of Medicine and Health Sciences, Ghent University, Campus Rommelaere, Albert Baertsoenkaai 3, B-9000 Ghent, Belgium.

E-mail addresses: laurence.bertier@ugent.be (L. Bertier), tim.hebbrecht@ugent.be (T. Hebbrecht), elien.mettepenningen@ugent.be (E. Mettepenningen), natasja.dewit@ugent.be (N. De Wit), olivier.zwaenepoel@ugent.be (O. Zwaenepoel), adriaan.verhelle@ugent.be (A. Verhelle), jan.gettemans@ugent.be (J. Gettemans).

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cortactin provides an excellent link between signaling/membrane trafficking and the actin cytoskeleton [12–14]. The N-terminal acidic (NTA) domain interacts with the Arp2/3 complex to initiate branched actin network formation, and the F-actin binding repeats (ABRs) bind to and stabilize these branched actin networks [15]. Next, there is a helical domain [16] and a proline rich domain that contains several residues susceptible to phosphorylation by serine/threonine and tyrosine kinases, including Src, Erk, Abl/Arg, PKD and atypical PKC [17–22]. Cortactin function is also regulated by posttranslational modifications at the ABRs, including phosphorylation and acetylation by PAK1, PAK3, ATAT1 and HDAC6, respectively [23–26]. The C-terminal SH3 domain acts as a scaffold for various interaction partners, for example with dynamin2 during vesicle trafficking and during actin reorganization [27,28], with ZO-1 at cell-cell junctions [29], and with N-WASP and WIP for synergistic activation of the Arp2/3 complex and actin assembly [30]. The cortactin gene (CTTN, 11q13) is frequently overexpressed in different cancer types [31–36], and is associated with increased cancer cell motility, invasion and metastasis [37–39]. Moreover, cortactin is involved in invadopodium formation, stability, maturation and turnover, and hence considered as a bona fide invadopodium marker [14,40]. Although the role of cortactin in invadopodium formation and cancer cell motility has been thoroughly studied, there are conflicting results on the role of different cortactin domains in cancer cell motility [12,41,42]. Moreover, new challenges that emerge are the contribution of the tumor microenvironment and tumor type on invadopodium dynamics and function such as exosome secretion, and the molecular pathways contributing to invadopodium formation *in vivo* during different phases of cancer cell dissemination [8,43–46].

Nanobodies are single-domain antibodies derived from heavy chain-only antibodies present in various species of the *Camelidae* family [47]. They have been shown to contribute to our understanding of domain specific functions of endogenous proteins in living cells, and can help pinpoint targets that are relevant for therapeutic intervention [48–55]. Additionally, they can be used as a tracer of endogenous targets [56] or for super-resolution microscopy [57,58].

Here, we describe two novel cortactin nanobodies against the central domains of cortactin (actin binding repeats and proline rich region). When expressed as an EGFP-tagged intrabody in SCC-61 head and neck squamous cell carcinoma cells, they colocalize with and bind their target. Morever, they affect cortactin function and decrease invadopodia formation and matrix degradation. These findings increase our understanding of the relative contribution of cortactin domains with respect to invadopodium formation. Together with earlier studies we conclude that virtually every functional domain in cortactin contributes to invadopodium formation or stability, pointing to the prominent role that this protein plays in generating these structures.

2. Material and methods

2.1. Antibodies and reagents

Mouse monoclonal anti-cortactin clone 4F11 (05–180) was purchased from Millipore (Watford, UK). Rabbit polyclonal anti-cortactin (H-222, #3503), rabbit monoclonal anti-hematopoietic lineage cell-specific protein 1 (HS1, 3890S) and rabbit polyclonal anti-EGFP (2555S) were from Cell Signaling (Danvers, MA, USA). Rabbit polyclonal cortactin phosphorylation-specific antibodies against Y421 (PA5-38129) and Y470 (PA5-38130) were from Thermo Fisher Scientific (Waltham, MA, USA), the antibody against Y486 (AB3853) was from Millipore. Rabbit polyclonal anti-Tks5/fish (M-300, sc-30122) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal anti-ARP2/3 subunit 1B (ab99314) was from Abcam (Cambridge, United Kingdom). Mouse monoclonal anti-actin clone C4 (0869100) was from MP Biomedicals (Santa Ana, CA, USA). Mouse monoclonal anti-V5 (R960-25) was purchased from Thermo Fisher Scientific. Rabbit polyclonal anti-EGFP antibody was obtained as previously described [59]. Secondary HRP-linked anti-mouse (NA931) and anti-rabbit IgG (NA9340V) were obtained from GE healthcare (Buckinghamshire, UK). Alexa Fluor 488/594-labeled secondary goat anti-rabbit (A11034/A11037) and anti-mouse (A11001/A11032) IgG antibodies were from Thermo Fisher Scientific. Acti-stain 670 phalloidin (PHDN1-A) was purchased from Cytoskeleton (Denver, CO, USA). DAPI (D8417) and anti-V5 agarose clone V5-10 (A7345) were from Sigma (St. Louis, MO, USA). Rabbit skeletal muscle actin (AKL95) was from Cytoskeleton. Protein G sepharose (17-0618-01) was obtained from GE healthcare (Buckinghamshire, UK). Bovine skin gelatin (G1393) and glutaraldehyde (G6257) were purchased from Sigma. QCM™ Gelatin Invadopodia Assay (ECM671) was from Millipore.

2.2. Generation of cortactin Nbs

Cortactin Nbs were produced in collaboration with the Nanobody Service Facility of the VIB. The SH3 Nb and GFP Nb were generated as described previously [48,60]. An alpaca was injected with 120 µg of human cortactin FHP domain (central regions of cortactin, see below) on days 0, 7, 14, 21, 28 and 35. On day 39, anticoagulated blood was collected for lymphocyte preparation. Next, total RNA from peripheral blood lymphocytes was isolated and used as template for first strand cDNA synthesis with oligo(dT) primer. The VHH encoding sequences were amplified by PCR and cloned into the phagemid vector pHEN4. A VHH library of independent transformants was obtained and subjected to four consecutive rounds of panning. Nbs were further analyzed by ELISA and sequence analysis.

2.3. Cloning and recombinant protein production

Cortactin cDNA was purchased from Origene (Rockville, MD, USA). Full length cortactin, and the fragment coding for the cortactin FHP domain, were cloned in the pTYB12 vector (New England Biolabs, Herts, UK). For full length cortactin forward primer, 5′-TGT-ACA-GAA-TGC-TGG-TCA-TAT-GTG-GAA-AGC-TTC-AGC-AGG-C-3′; reverse primer, 5′- TCA-CCC-GGG-CTC-GAG-GAA-TTC-TTA-CTG-CCG-CAG-CTC-CAC-ATA-3′ were used, the FHP domain was cloned with forward primer, 5′-TGT-ACA-GAA-TGC-TGG-TCA-TAT-GGG-CTA-TGG-AGG-GAA-ATT-TGG-TGT-GGA-AC-3′; reverse primer, 5′-TCA-CCC-GGG-CTC-GAG-GAA-TTC-TTA-GAT-CCC-CAG-ATC-GTT-CTC-GTA-CTC-ATC-GTA-G-3′. For protein production, BL21 *E. coli* cells were transformed with pTYB12 constructs, grown at 37 °C, induced with 0.5 mM IPTG and incubated overnight at 20 °C. The intein fusion protein was purified using the Intein Mediated Purification with an Affinity Chitin-binding Tag (IMPACT) system (New England Biolabs). Cortactin cDNA sequence for the N-term, C-term, helical- and the proline rich domain were cloned in the pTrcHis-TOPO vector (Thermo Fisher Scientific). The following primers were used: cortactin N-term, forward, 5′-AGC-ATG-TGG-AAA-GCT-TCA-GCA-GGC-CAG-G-3′; reverse, 5′-TGC-GGC-CGC-TTA-TTC-GAC-AGG-TAC-TGT-CTT-CTG-G-3′; cortactin C-term, forward, 5′-AGC-GCT-GTG-ACC-AGC-AAA-ACA-AGT-AAC-ATC-AGA-GC-3′; reverse 5′- TGC-GGC-CGC-TTA-CTG-CCG-CAG-CTC-CAC-ATA-GTT-GGC-TGG-GA-3′; helical domain, forward 5′-CGA-TGA-CGA-TAA-GGG-ATC-CGC-TGT-GAC-CAG-CAA-AAC-A-3′; reverse 5′-GCC-AGC-CAA-GCT-TCG-AAT-TGA-ATT-CTT-ATT-GCG-TTT-TGG-CTC-TGG-C-3′; proline rich domain, forward 5′-CGA-TGA-CGA-TAA-GGG-ATC-CAC-GCC-CCC-TGT-GTC-G-3′; reverse 5′-CCA-AGC-TTC-GAA-TTG-AAT-TCT-TAC-CCC-AGA-TCG-TTC-TCG-TAC-TC-3′. The vector encoding cortactin N-term, C-term, helical or proline rich domain were transformed in BL21 *E. coli* cells, grown at 37 °C and induced with 1 mM IPTG for 3 h at 37 °C. His_6-tagged N-term domain was purified with Immobilized Metal ion Affinity Chromatography (IMAC) and eluted via competitive elution with 250–500 mM imidazole.

GST- HS1 was produced and purified as described before [48].

Nb coding sequences were cloned into the pHEN6-V5-His_6 vector for recombinant protein production, and in the lentiviral expression pLVX-TP vector for the generation of stable, doxycycline (DOX)-inducible cell