European Journal of Cell Biology 90 (2011) 115–127

Contents lists available at ScienceDirect

European Journal of Cell Biology

journal homepage: www.elsevier.de/ejcb

Novel invadopodia components revealed by differential proteomic analysis

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ARTICLE INFO

Article history:
Received 19 February 2010
Received in revised form 26 April 2010
Accepted 10 May 2010

Keywords:
DIfference Gel Electrophoresis (DIGE)
MALDI-MS
Peptide Mass Fingerprinting (PMF)
Invadopodia
Extracellular matrix degradation

ABSTRACT

When highly invasive cancer cells are cultured on an extracellular matrix substrate, they extend proteolytically active membrane protrusions, termed invadopodia, from their ventral surface into the underlying matrix. Our understanding of the molecular composition of invadopodia has rapidly advanced in the last few years, but is far from complete.

To accelerate component discovery, we resorted to a proteomics approach by applying DIfference Gel Electrophoresis (DIGE) to compare invadopodia-enriched sub-cellular fractions with cytosol and cell body membrane fractions and the whole cell lysate. The fractionation procedure was validated through step-by-step monitoring of the enrichment in typical actin-related invadopodia-associated proteins. After statistical analysis, 129 protein spots were selected for peptide mass fingerprinting analysis; of these 76 were successfully identified and found to correspond to 58 proteins belonging to different functional classes including aerobic glycolysis and other metabolic pathways, protein synthesis, degradation and folding, cytoskeletal components and membrane-associated proteins.

Finally, validation of a number of identified proteins was carried out by a combination of immunoblotting on cell fractions and immunofluorescence localization at invadopodia. These results reveal newly identified components of invadopodia and open further avenues to the molecular study of invasive growth behavior of cancer cells.

Introduction

The ability of cells to invade the extracellular matrix (ECM) is essential in the response to injury, pathogen infection, embryogenesis, differentiation, neo-angiogenesis and also during tumor cell invasion and metastasis (Basbaum and Werb, 1996). In particular, migration-associated proteolytic degradation of the ECM is a common feature of cancer cells (Wolf and Friedl, 2008). Invadopodia are stable actin-rich protrusions formed from the ventral surface of invasive tumor or transformed cells when cultured on appropriate ECM substrates (Kelly et al., 1994) and displaying focalized proteolytic activity towards the substrate (Chen, 1989; Mueller and Chen, 1991). A number of molecular components of invadopodia have been identified in the nineties including integrins, proteins of signaling machineries, soluble and membrane-bound proteases (including matrix metalloproteases), and actin and actin-associated proteins such as cortactin and others (Bowden et al., 1999; Chen, 1996; Monsky et al., 1994; Mueller et al., 1992; Nakahara et al., 1997). In recent years, many more molecular players have been defined through a combination of RNA interference and dominant-negative mutant expression. Among these, the ubiquitous Wiskott–Aldrich Syndrome Protein family member N-WASP (Mizutani et al., 2002), a major activator for the Arp2/3 complex-mediated actin nucleation machinery, the Arp2/3 complex itself (Yamaguchi et al., 2005a) and some of their upstream regulators such as the Rho-family member GTPase cdc42 (Artym et al., 2006; Ayala et al., 2008; Yamaguchi et al., 2005b), the Src family tyrosine kinases (Bowden et al., 2006; Hauck et al., 2002) and serine/threonine kinases such as PKD (Bowden et al., 1999) and ERK1/2 (Ayala et al., 2008; Tague et al., 2004) have been most intensively studied (see recent reviews on these and other findings (Buccione et al., 2009; Stylli et al., 2008)).

In summary, focal degradation of the ECM at invadopodia may thus very well recapitulate the initial steps of tumor cell invasion and involves the tight integration of the membrane remodeling, actin dynamics, trafficking and signaling machineries. The field of invadopodia biology is relatively new, hence there are still many open questions. A major obstacle is that no truly specific invadopodia markers are known and protein components of invadopodia are also shared with other cell structures such as podosomes, focal adhesions, pseudopodia, lamellipodia and filopodia, i.e. those

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doi:10.1016/j.ejcb.2010.05.004

structures whose function is dependent on actin cytoskeleton reorganization.

The aim of this study was to accelerate the discovery of novel proteins present in invadopodia that have functional activity and consequently may be related to cancer invasion. Our approach also provides proof of concept that it is possible to extract novel compositional information from invadopodia-enriched sub-cellular membrane fractions.

We decided to employ a proteomic-based approach by quantitative analysis of protein levels. This was achieved by refining a previously introduced (Mueller et al., 1992) sub-cellular fractionation protocol aimed at obtaining invadopodia-enriched fractions from cultured cells. Our model of choice was the A375MM invasive human melanoma line because these cells form stable, long-lasting invadopodia, have modest motility and form few focal adhesions.

This approach was integrated with the application of differential proteomic analysis, by DIfference Gel Electrophoresis (DIGE), which allows protein quantification based on gel image analysis with a high degree of confidence, and is compatible with mass spectrometric identification of proteins by Peptide Mass Fingerprinting (PMF).

We report on the identification of novel invadopodia-enriched proteins belonging to diverse functional classes including cell metabolism, protein synthesis, degradation and folding, cytoskeletal components and membrane-associated proteins.

Materials and methods

Reagents and antibodies

All chemical reagents, unless otherwise stated, were purchased from Sigma (St. Louis, MO). Alexa Fluor 633-, 546- and 488-conjugated secondary antibodies and phalloidin, as well as Alexa Fluor 555-conjugated cholera toxin subunit B, were from Invitrogen (Carlsbad, USA). Peroxidase-conjugated secondary antibodies were from Calbiochem (San Diego, CA).

Monoclonal mouse anti-cortactin antibody was from Upstate Biotechnology (Lake Placid, NY), while monoclonal anti-tubulin, anti-actin and anti-vimentin antibodies were from Sigma, monoclonal anti-paxillin was from Oncogene (Boston, MA), monoclonal anti-prohibitin from Neomarkers (Fremont, CA), monoclonal anti-dynamin 2, anti-gelsolin, anti-FAK, anti-14-3-3ε protein and anti-HSP70 kDa/BiP antibodies were from BD Transduction Laboratories (Lexington, KY). Polyclonal antibodies against glyceraldehyde-3-phosphate dehydrogenase were purchased from Biogenesis (Kingston, NH) and from Cell Signaling Technology (Beverly, MA), Caveolin 1, lamin B and G protein β1 subunit from Santa Cruz (Santa Cruz, CA), lactate dehydrogenase and muscle pyruvate kinase from Rockland (Gilbertsville, PA) and glucose-6P dehydrogenase from Bethyl (Montgomery, TX).

Cell culture

Wild-type human melanoma A375MM cells and A375MM stably transfected with actin-GFP were cultured in DMEM/F-12 (1:1) (Invitrogen) containing 10% FCS. Cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2 as previously described (Baldassarre et al., 2003).

Invadopodia fractionation

Purification of an invadopodia-enriched sub-cellular fraction was performed using previously published protocols with some modifications (Bowden et al., 1999; Mueller et al., 1992).

A375MM cells were cultured at 3.5 million cells/25 cm Petri dishes on cross-linked gelatin prepared as previously described (Baldassarre et al., 2003; Bowden et al., 2001). After 24 h, when cells were about 70/80% confluent, each plate was first washed in PBS containing 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, then in five times diluted PBS containing 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, and incubated for 15 min in the presence of 3 ml of the diluted PBS containing a protease inhibitor mixture to induce cell swelling. Cell bodies were then sheared away using an L shaped Pasteur pipette with sealed end, to leave invadopodia remnants embedded in the gelatin. The embedded invadopodia remnants were washed in PBS containing 0.5 mM $MgCl_2$, 1 mM $CaCl_2$ before being scraped away with the cross-linked gelatin into RIPA buffer (150 mM NaCl, 1% NP40, 0,5% sodium deoxycholate, 0,1% sodium dodecyl sulphate, 50 mM Tris base buffer pH 8, proteases inhibitor) and clarified by centrifugation (15 min, 13,000 rpm at 4 °C).

The cell body fraction was further separated into the cell body membranes and cytosol fractions by centrifugation at 9000 × *g* for 20 min at 4 °C. The supernatant (cytosolic fraction) was used directly whereas the cell body membrane pellet obtained after centrifugation was solubilized in RIPA buffer and clarified by centrifugation (15 min, 13,000 rpm at 4 °C). The whole lysate was obtained from cells grown in 10 cm Petri dishes on cross-linked gelatin. Cells were plated at 2 million cells/10 cm dish, washed after 24 h with PBS buffer containing 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, and finally solubilized in RIPA buffer and clarified by centrifugation (15 min, 13,000 rpm at 4 °C).

Hence, four different samples were obtained for differential proteomic analysis: invadopodia membrane fraction (I), cytosolic fraction (C), cell body membrane fraction (M) and the whole lysate (W).

Immuno-blotting analysis

Proteins (30 μg) from cell lysates and invadopodia fractionation were precipitated in 3 volumes of cold acetone overnight at −20 °C, centrifuged, denatured, separated by 10% acrylamide SDS-PAGE and finally electro-blotted onto nitrocellulose. Membranes were blocked with 5% milk, incubated with the primary antibodies of interest for 1 h and then with horseradish peroxidase-conjugated secondary antibodies for 45 min and finally proteins of interest were detected by enhanced chemiluminescence (ECL; GE Healthcare, Uppsala, Sweden).

Densitometric analysis of protein bands was performed with the Image J1.32 public domain software from NIH. Quantitative results, corresponding to three different biological replicate samples, are expressed in % and standard deviation from the mean. To circumvent problems with comparison of samples with heterogeneous protein composition we used Ponceau staining of total loaded protein as control for loading.

Immunofluorescence microscopy

The immunofluorescence analysis of invadopodia was performed according to published procedures (Baldassarre et al., 2003; Bowden et al., 2001). Briefly, A375MM cells were plated on coverslips coated with cross-linked fluorescent gelatin in the presence of 5 μM BB94 (British Biotech, UK) and incubated overnight to facilitate cell attachment. BB94 was then washed out to allow invadopodia formation, cells were then fixed after 1 h, 3 h and 6 h, in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA), for 15 min, permeabilized in PBS containing 0.02% saponin, 0.2% BSA and 50 mM NH_4Cl, incubated with the primary antibodies of interest for 1 h and then incubated with fluorophore-conjugated secondary antibodies for 45 min. Finally, coverslips were mounted in Mowiol (Calbiochem, La Jolla, CA). Invadopodia were identi-