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Pseudopodial and β-arrestin-interacting proteomes from migrating breast cancer cells upon PAR2 activation

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ABSTRACT

Metastatic cancer cells form pseudopodia (PD) to facilitate their migration. The proteinase-activated receptor-2 (PAR-2) transduces migratory signals from proteases, and it forms protein complexes with β-arrestin and other signalling molecules that are enriched in pseudopodia. More generally, however, pseudopodial regulation is poorly understood. Here, we purified the pseudopodial proteomes of breast cancer cells after activation of the endogenous PAR-2 and we combined gel-based approaches with label-free high-resolution mass spectrometry to identify proteins that accumulate at the pseudopodia upon PAR-2-mediated migration. We identified >410 proteins in the cell body and >380 in the pseudopodia upon PAR2 activation, of which 93 were enriched in the pseudopodia. One of the pathways strongly enriched in the PD was the clathrin-mediated endocytosis signalling pathway, highlighting the importance of the scaffolding function of β-arrestin in PAR-2 signalling via its endocytosis. We therefore immunoprecipitated β-arrestins, and with mass spectrometry we identified 418 novel putative interactors. These data revealed novel β-arrestin functions that specifically control PAR-2-regulated signalling in migrating breast cancer cells but also showed that some β-arrestin functions are universal between GPCRs and cell types. In conclusion, this study reveals novel proteins and signalling pathways potentially important for migration of breast cancer cells.

1. Introduction

Cell migration is important in embryonic development, tissue homeostasis and in the operation of the immune system. When it malfunctions, it can contribute to several pathological conditions, including cancer metastasis [1]. Several chemotactic signals are transduced into directional cell migration (chemotaxis) via numerous membrane receptors and intracellular signalling pathways. The main characteristic of a chemotactic cell is the formation of a leading edge, termed pseudopodium (PD), as a result of spatiotemporally regulated accumulation of proteins towards the gradient [2]. The identification of proteins important for gradient sensing, pseudopodia extension, attachment and retraction is of vital importance for the understanding of chemotaxis.

Numerous proteins are involved in pseudopodia formation, such as plasma membrane receptors, integrins, kinases and phosphatases, GTPases, scaffolding proteins and others, important for actin dynamics, binding of the cytoskeleton to the extra-cellular matrix (ECM) as well as the control of further signalling events [3–5]. However, the PD-enriched proteins, which are dependent on the type of the chemoattractant and the targeted receptor, as well as the cell type, are largely unknown.

In addition to their role in degrading the extracellular matrix, trypsin-like proteases secreted from cancer cells have

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been implicated in the promotion of migration via the proteolytic activation of the protease-activated receptor 2 (PAR-2) [6]. PAR-2 is a ubiquitously but unevenly expressed GPCR receptor, which is activated by trypsin and trypsin-like proteases, including tryptase, clotting factors VIIa and Xa, and has important functions in immune system responses [7,8]. PAR-2 has also been found overexpressed in cancer cell lines, human tumours and in cells of the tumour microenvironment *in situ* [9]. Ge et al. reported the secretion of biologically active trypsin-like proteases by the MDA-MB-231 breast cancer cell line that can promote cell migration [10]. PAR-2-mediated migratory signals have also been reported in pancreatic cells [11], human melanoma and prostate carcinoma cells [12] and breast cancer cells [13,14]. Similarly, increase on the migration and ERK signalling of BT549 cells showed the activation of PAR-2 by the clotting factors VIIa and Xa [15], suggesting that chronic inflammation contributes to tumour metastasis. However, little is known about the downstream signalling pathways regulated by this receptor. Following receptor activation, β-arrestins antagonize with G-proteins, bind to the receptor, uncouple it from G-proteins [16] and transfer it via clathrin-coated pits to early endosomes [17]. Interestingly, during this β-arrestin-dependent endocytosis of PAR-2, a multiprotein signalling complex is formed containing the receptor, β-arrestin, raf-1 and ERK [18]. In this complex, β-arrestin works as a scaffold and retains ERK1/2 in the cytoplasm, thus ERK activity is predominantly cytosolic. Notably, this protein complex is enriched in the pseudopodia, where it promotes actin assembly along with other unidentified proteins [10,19]. However, the proteins that interact with β-arrestin upon PAR-2 activation remain unknown.

In this study, we used a breast cancer cell line endogenously expressing the PAR-2 receptor and we applied gel-based, label-free proteomics approaches [20,21] in order to identify proteins and signalling pathways enriched in the pseudopodia. In addition, we immunoprecipitated β-arrestin and identified PAR-2-regulated β-arrestin partners in breast cancer cells (Fig. 1).

2. Materials and methods

2.1. Materials

MCF-7 and MDA-MB-231 were a kind gift from Dr. Elena Klenova and HEK293S were from Dr. Phillip Reeves. Vectors encoding FLAG-tagged β-arrestin-1 or FLAG-tagged β-arrestin-2 (βarr+FLAG) were kindly provided by Dr. Robert Lefkowitz and were described elsewhere [22,23]. PAR-2-activating synthetic peptide (P_2AP) SLIGRL-NH2, which is a more potent activator of human PAR-2 compared to SLIGKV-NH2 [24,25], was from Sigma. Anti-FLAG antibody was from Sigma and anti-phospho p44/p42 MAPK from Cell Signaling. All other antibodies were from Santa Cruz Biotechnology and were used in 1/1000 dilution.

2.2. Cell culture and plasmid preparations

All cells, except HEK293, were cultured in Roswell Park Memorial Institute (RPMI; Lonza) medium supplemented with Ultraglutamine 1, 10% foetal calf serum (FCS; Biosera) and 1% antibiotic mixture (penicillin and streptomycin; Cambrex) at 37 °C in a humidified environment (5% CO_2). Serum deprivation was performed in the same medium but without FCS and for 12–16 h. HEK293 were cultured in Dulbeco's Modified Eagle's Medium (DMEM; Cambrex) with L-glutamine, supplemented with 10% FCS and 1% antibiotic mixture in 37 °C and 5% CO_2.

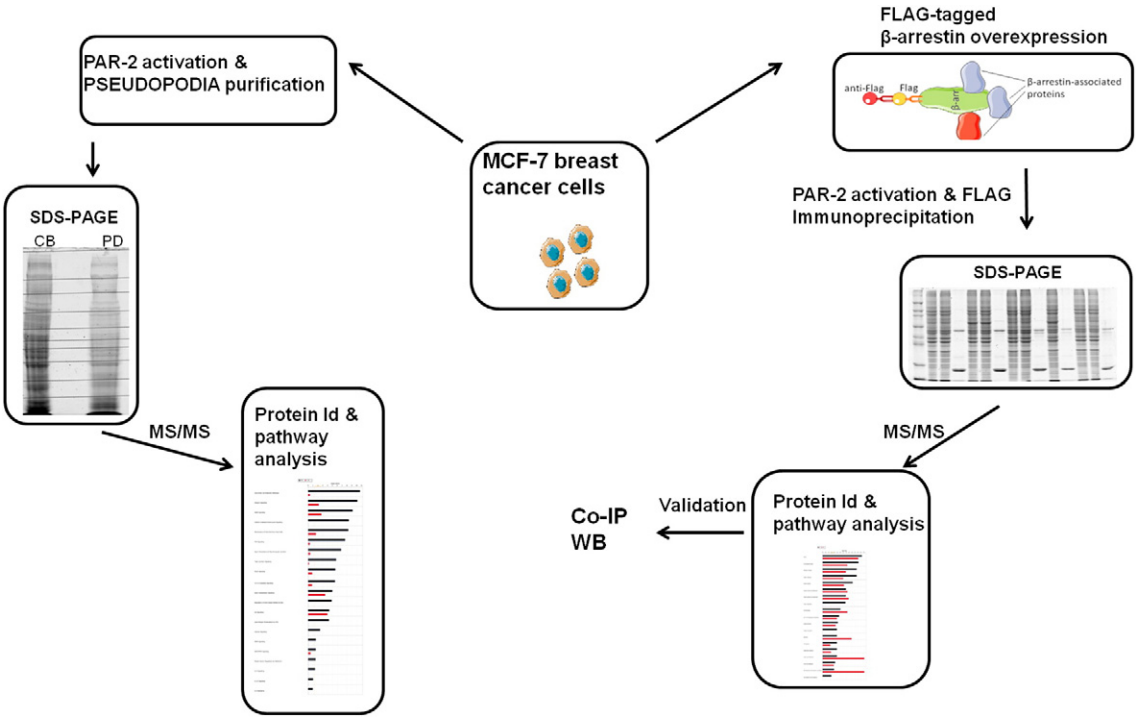

Fig. 1 – Schematic representation of the experimental strategies performed in this study.