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RACK1 stabilises the activity of PP2A to regulate the transformed phenotype in mammary epithelial cells

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ABSTRACT

Conflicting reports implicate the scaffolding protein RACK1 in the progression of breast cancer. RACK1 has been identified as a key regulator downstream of growth factor and adhesion signalling and as a direct binding partner of PP2A. Our objective was to further characterise the interaction between PP2A and RACK1 and to advance our understanding of this complex in breast cancer cells. We examined how the PP2A holoenzyme is assembled on the RACK1 scaffold in MCF-7 cells. We used immobilized peptide arrays representing the entire PP2A-catalytic subunit to identify candidate amino acids on the C subunit of PP2A that might be involved in binding of RACK1. We identified the RACK1 interaction sites on PP2A. Stable cell lines expressing PP2A with FR69/70AA, R214A and Y218F substitutions were generated and it was confirmed that the RACK1/PP2A interaction is essential to stabilise PP2A activity. We used Real-Time Cell Analysis and a series of assays to demonstrate that disruption of the RACK1/PP2A complex also reduces the adhesion, proliferation, migration and invasion of breast cancer cells and plays a role in maintenance of the cancer phenotype. This work has significantly advanced our understanding of the RACK1/PP2A complex and suggests a pro-carcinogenic role for the RACK1/PP2A interaction. This work suggests that approaches to target the RACK1/PP2A complex are a viable option to regulate PP2A activity and identifies a novel potential therapeutic target in the treatment of breast cancer.

1. Introduction

RACK1 plays a critical role in many fundamental cellular processes including cell adhesion, proliferation, migration and protein synthesis through its ability to act as a scaffold within signalling pathways [1–4]. RACK1 is known to interact with a diverse array of proteins and functions to recruit and shuttle these proteins to their substrates or other binding partners [1,2]. Alterations in RACK1 expression and function are associated with a variety of disease states including Alzheimer's disease [5], bipolar disorder [6] and cancer including hepatocellular carcinoma, ovarian cancer and cancers of the prostate and breast [7–11]. The connection between RACK1 and cancer is complex, since RACK1 interacts with over 80 binding partners, either directly or indirectly in large complexes, thereby impacting on multiple signalling pathways. Many of the proteins in the RACK1 interactome are phosphatases and kinases whose activity is altered in cancer. For example, RACK1 plays a critical part in cell adhesion and migration, in particular through its role in regulating focal adhesion kinase (FAK) activity and focal adhesion assembly [12,13]. RACK1 is also a component of the signalling pathways downstream of FAK and phosphodiesterase 4D5 (PDE4D5) that control both cell spreading and the direction sensing mechanisms required to establish cell polarity, which is an important element in the process of cell migration [14,15]. RACK1 has also been shown to promote both cell migration and invasion in both oesophageal and lung cancers through a variety of different signalling mechanisms [16–18].

Conflicting reports suggest a role for RACK1 in breast cancer. High RACK1 expression has been reported in breast cancer patients, and this has been correlated with a poor clinical outcome [10]. RACK1 has also been found to promote breast cancer proliferation and invasion both *in vitro* and in mouse models through interaction with RhoA and activation of the RhoA/Rho kinase pathway [19]. This suggests that RACK1 has potential as a valuable prognostic indicator of advanced disease in breast cancer. However, other studies report findings that are in direct contrast to this. For example, decreased RACK1 is reported in a cohort of breast cancer patients and associated with a good clinical outcome in follow-up studies [11]. This apparent conflict in findings of RACK1 expression in cancer tissue could be due to the heterogeneous

Abbreviations: CI, cell index; EGF, epidermal growth factor; FAK, focal adhesion kinase; IGF-1, Insulin-like Growth Factor-1; LCMT-1, Leucine carboxyl methyltransferase 1; MAP kinase, Mitogen Activated Protein; P4DED5, Phosphodiesterase 4D5; PP2A, Protein Phosphatase 2A; RACK1, Receptor for Activated C Kinase; WT, wild type.

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nature of breast cancer and the inconsistencies may be explained by a lack of information on breast cancer subtypes within these studies [20]. Also, because RACK1 is involved in the scaffolding of such a large number of proteins within such diverse signalling pathways, it is acknowledged that any change in RACK1 expression, either up or down, has the potential to have serious consequences for the tight regulation of these pathways and as a result, on the processes regulating the establishment, development and progression of cancer [1].

Protein Phosphatase 2A (PP2A) is a major Ser/Thr phosphatase in cell signalling pathways. PP2A exists as a holoenzyme with three individual components. Once assembled, the structural subunit (A) and catalytic subunit (C) form the core dimer while the regulatory subunit (B) confers substrate specificity, full activity and subcellular location of the PP2A holoenyme when bound to the core dimer. There are two A isoforms (α and β) which share 87% sequence homology and two C subunits (α and β) which share 97% homology [21–23]. Currently, there are up to 26 known regulatory B subunits [24]. As a phosphatase, PP2A is essential to promote the post translational modifications that reverse kinase activity in many of the major cell signalling pathways, including those that regulate the cell cycle, metabolism, cell migration and survival [24–27]. It is also widely accepted that PP2A negatively regulates growth factor signalling and MAP kinase activation downstream of growth factor signalling [28,29].

PP2A directly interacts with RACK1 in an IGF-1 dependent manner where RACK1 serves to stabilise PP2A activity [30,31]. A reduction in RACK1 expression decreases the phosphatase activity of PP2A, which has been shown to promote cell migration in cancer cells [31]. This indicates that RACK1 has a role to play in keeping a specific pool of PP2A 'active' and thus facilitating the regulatory role of PP2A.

PP2A has a well-established role in cancer. It is largely recognised as a tumour suppressor and has been found to be mutated in many cancer types [27,32–34]. However, inhibition of PP2A has shown potential as an anti-cancer strategy in some cell models [23,35–38]. This apparent contradiction may arise, in part, because PP2A inhibition increases tumour chemo-sensitivity to many chemotherapeutic drugs (reviewed in [39]) but also because PP2A has been shown to play an anti-apoptotic role within signalling pathways (reviewed in [23]).

Here, we further characterise the RACK1/PP2A interaction in breast cancer cells by identifying and mapping the interaction site of RACK1 on the catalytic (C) subunit of PP2A (PP2A-C). Using mutations of PP2A that disrupt the binding with RACK1, we show that RACK1 stabilises PP2A activity and regulates the transformed phenotype in breast cancer.

2. Materials and methods

2.1. Cell culture

MCF-7 and HEK cells were maintained in DMEM supplemented with 5% L-glutamine, 5% penicillin/streptomycin and 10% FBS (Sigma-Aldrich Ltd).

2.2. Preparation of cellular protein extracts

Cellular protein extracts were prepared by placing cells on ice, removing media and washing three times in ice cold PBS. Cells were scraped into ice cold lysis buffer (20 mM Tris HCl pH 7.4, 50 mM NaCl, 50 mM NaF, 1% NP40) plus the tyrosine phosphatase inhibitor Na_3VO_4 (1 mM), protease inhibitors PMSF (1 mM), pepstatin (1 μM) and aprotinin (1.5 μg/ml). Lysates were incubated on ice for 20 min before centrifugation at 14,000 rpm for 15 min at 4 °C to remove nuclei and cellular debris. Lysates were analysed for protein concentration using the Bradford assay and boiled in sample buffer for SDS-PAGE or used in immunoprecipitation experiments.

2.3. Immunoprecipitation of proteins and western blotting

Protein extracts were precleared with 20 μl Protein G beads by incubation at 4 °C for 1 h rotating. The lysates were recovered from the beads by centrifugation at 3000 rpm for 3 min and transferred to a new tube containing primary antibody (2 μg), 40 μl Protein G beads, 500 μl lysis buffer and made up to 1 ml with dH_2O. Samples were incubated at 4 °C rotating overnight. Immune complexes were pelleted with the beads by centrifugation at 3000 rpm for 3 min at 4 °C. The beads were washed three times with ice cold lysis buffer and removed from beads by boiling for 5 min in 25 μl of 2× SDS PAGE sample buffer for electrophoresis and western blot analysis. Protein samples for western blot analysis were separated by 12% SDS-PAGE gels. Following separation on the gel, proteins were transferred using electrophoresis onto a nitrocellulose membrane and blocked for 1 h at room temperature shaking in 5% milk (*w*/*v*) in TBS containing 0.5% Tween-20 (TBS-T). Membranes were incubated overnight at 4 °C with the appropriate primary antibody; Anti-PP2A-Cα subunit (Cell Signalling), Anti-RACK1 (BD Biosciences). Appropriate secondary antibodies (IRDye® 680LT and 800CW-Infrared Dye coupled anti-rabbit or anti-mouse (LI-COR Biosciences)) were diluted 1:10,000 in TBS-T/5% milk for 1 h. Antibody reactive bands were detected with the Odyssey® infrared imaging system (LI-COR Biosciences).

2.4. Spot synthesis of peptides, overlay analysis and alanine substitution array analysis

Peptide arrays of PP2A-C on nitrocellulose were generated as previously described [40–42]. Scanning libraries of overlapping 23-mer peptides covering the entire sequence of a protein were produced by automatic SPOT synthesis and synthesized on Whatman 50 cellulose using Fmoc (9-fluorenylmethyloxycarbonyl) chemistry with the AutoSpot-Robot ASS 222 (Intavis Bioanalytical Instruments). The interaction of GST and GST-tagged proteins, *e.g.* GST-RACK1 with the protein array was investigated by overlaying the cellulose membranes with 10 μg/ml concentrations of each recombinant protein. Bound protein was detected with specific mouse antisera for each protein and a secondary anti-mouse antibody coupled with LICOR dye 680 and scanned on the Odyssey Infrared Scanner. Once candidate binding regions for RACK1 on the full-length PP2A-C subunit array had been determined, specific alanine scanning substitution arrays were generated for the relevant sequences using the same synthesis procedure. The progeny peptide arrays were synthesized in 18-mer format such that each of the 18 amino acids of the PP2A-C sequence were sequentially substituted with alanine (or aspartic acid where the wild-type sequence exhibited alanine). The array was probed with GST-RACK1 at a concentration of 10 μg/ml, which was detected by immunoblotting with anti-RACK1 antibody. Bound protein was detected with specific mouse antisera for the protein and a secondary anti-mouse antibody coupled with LICOR dye 680 and scanned on the Odyssey Infrared Scanner. A decrease in intensity in binding to the peptides after alanine substitution is indicative of decreased binding of the PP2A-C subunit to RACK1. The binding of RACK1 to each alanine-substituted PP2A-C subunit peptide was quantified by densitometry and presented as a percentage of the control "parent" sequence. A cut off of <50% binding was applied.

2.5. Generation of stable cell lines

To generate stable transfectants of PP2A mutants, MCF-7 cells were transfected with pcDNA3/HA-Empty Vector, pcDNA3/HA-PP2A (Wild Type), pcDNA3/HA-FR69/70AA, pcDNA3/HA-R214A and pcDNA3/HA-Y218F using Lipofectamine 2000 transfection reagent. Then, 24 h post transfection, the cells were split into DMEM medium containing 10% FBS, 10 mM l-glu and G418 (1 mg/ml) and maintained for 14 days, with regular replenishment of medium and drug. At this time the pool was expanded, and screened for expression of the HA tagged plasmids

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