Experimental Cell Research 355 (2017) 26–39

Contents lists available at ScienceDirect

Experimental Cell Research

journal homepage: www.elsevier.com/locate/yexcr

Insulin promotes cell migration by regulating PSA-NCAM

Hector J. Monzo[a,b], Natacha Coppieters[a,b], Thomas I.H. Park[a,c], Birger V. Dieriks[a,b], Richard L.M. Faull[a,b], Mike Dragunow[a,c], Maurice A. Curtis[a,b,*]

[a] Centre for Brain Research, Faculty of Medical and Health Sciences, The University of Auckland, Private Bag, 92019, Auckland, New Zealand
[b] Department of Anatomy and Medical Imaging, Faculty of Medical and Health Sciences, The University of Auckland, Private Bag, 92019, Auckland, New Zealand
[c] Department of Pharmacology, Faculty of Medical and Health Sciences, The University of Auckland, Private Bag, 92019, Auckland, New Zealand

ARTICLE INFO

Keywords:
PSA-NCAM
Insulin
Integrin
P-FAK
ECM
Migration

ABSTRACT

Cellular interactions with the extracellular environment are modulated by cell surface polysialic acid (PSA) carried by the neural cell adhesion molecule (NCAM). PSA-NCAM is involved in cellular processes such as differentiation, plasticity, and migration, and is elevated in Alzheimer's disease as well as in metastatic tumour cells. Our previous work demonstrated that insulin enhances the abundance of cell surface PSA by inhibiting PSA-NCAM endocytosis. In the present study we have identified a mechanism for insulin-dependent inhibition of PSA-NCAM turnover affecting cell migration. Insulin enhanced the phosphorylation of the focal adhesion kinase leading to dissociation of αv-integrin/PSA-NCAM clusters, and promoted cell migration. Our results show that αv-integrin plays a key role in the PSA-NCAM turnover process. αv-integrin knockdown stopped PSA-NCAM from being endocytosed, and αv-integrin/PSA-NCAM clusters co-labelled intracellularly with Rab5, altogether indicating a role for αv-integrin as a carrier for PSA-NCAM during internalisation. Furthermore, inhibition of p-FAK caused dissociation of αv-integrin/PSA-NCAM clusters and counteracted the insulin-induced accumulation of PSA at the cell surface and cell migration was impaired. Our data reveal a functional association between the insulin/p-FAK-dependent regulation of PSA-NCAM turnover and cell migration through the extracellular matrix. Most importantly, they identify a novel mechanism for insulin-stimulated cell migration.

1. Introduction

Neural cell adhesion molecules (NCAM), belonging to the immunoglobulin superfamily of cell adhesion molecules, are almost the exclusive carrier of an unusual posttranslational modification (polysialylation) resulting from the addition of polysialic acid (PSA; a neuraminic acid carbohydrate; PSA-NCAM). PSA-NCAM is abundant in neural tissues but can also be found in tumour cells with metastatic behaviour and in immune cells [1,10,33,4]. The dynamic amount of PSA at the cell surface is regulated by PSA-NCAM turnover, a mechanism comprising mainly two independent processes: 1, polysialylation of NCAM (PSA-NCAM synthesis) by ST8SiaIV (PST) and ST8SiaII (STX), two Ca^{2+}-dependent and Golgi-complex-associated polysialyltransferases [7]; and 2, endocytosis of PSA-NCAM into the intracellular compartment, a process regulated by components of the membrane-like extracellular matrix (ECM) [29] or ectodomain shedding of NCAM that could work in harmony with endocytosis to influence cell biology (Rutishauser, U. 2008). *In vivo*, cells procure the right amount of cell surface PSA at each specific developmental stage by tightly regulating PSA-NCAM synthesis through expression/activity of PST and STX [2,7]. Turnover of PSA-NCAM encompasses continuous recycling of the molecule at the cell surface. Newly synthesized NCAMs are polysialylated inside the Golgi complex before PSA-NCAM is transported to the cell surface. Subsequently, in an ECM-dependent manner, PSA-NCAMs are endocytosed, partially degraded into non-polysialylated NCAMs, sorted for re-polysialylation and, eventually, transported back to the cell surface where the process begins again. In this cycle, inhibition of PSA synthesis results in actively-endocytosed PSA-NCAM molecules being progressively substituted by non-sialylated NCAMs at the cell surface, thereby reducing the amount of cell-surface PSA. Conversely, inhibition of PSA-NCAM endocytosis prevents its progressive removal from the cell surface and

Abbreviations: PSA, polysialic acid; NCAM, neural cell adhesion molecule; ECM, membrane-like extracellular matrix; INS, insulin; WT, wortmannin; PF, PF-562271; p-Akt, phosphorylated Akt; p-FAK, phosphorylated focal adhesion kinase; RTKs, receptor tyrosine kinases; IR-β, insulin receptor-β INSR); CI, calcium ionophore; PI3-K, phosphoinositide 3-kinase

* Corresponding author at: Centre for Brain Research, Faculty of Medical and Health Sciences, The University of Auckland, Private Bag, 92019, Auckland, New Zealand.
E-mail address: m.curtis@auckland.ac.nz (M.A. Curtis).

http://dx.doi.org/10.1016/j.yexcr.2017.03.029
Received 14 November 2016; Received in revised form 2 March 2017; Accepted 14 March 2017
Available online 21 March 2017
0014-4827/

consequently promotes the presence of large amounts of PSA at the cell surface, even in conditions where the NCAM polysialylation machinery is inactive [14,29,5].

Dysregulated cell surface PSA abundance has been associated with tumour metastasis, ectopic cellular migration and altered neural development [1,14,15,33,4,5]. Mechanisms altering the proper regulation of PSA-NCAM turnover might therefore result in the expression of anomalous/malignant cellular phenotypes [44,7].

Because of its fundamental properties, such as a large volume and negative charge, PSA molecules at the cell surface produce steric impediment between receptors and extracellular ligands, thus modulating their interaction. The establishment of cellular interactions with the extracellular matrix is crucial for a number of cellular processes including cell migration. Cell-extracellular matrix interactions connect the extracellular milieu with the intracellular cytoskeleton and serve as physical adhesion points for traction generation during migration as well as antennae for sensory-input signalling [20]. Mechanisms modulating cell adhesion with the extracellular milieu, such as that involving PSA-NCAM, affect the establishment of cellular interactions and interfere with the cell migration process by elevating or decreasing the net cellular adhesive-load [1,22,33,37].

Insulin is actively involved in the coordination of migration and tumour progression [30,31,39]. In a previous study we reported a role for insulin as an inhibitor of PSA-NCAM removal from the cell surface [29]. In the present study, the human rhabdomyosarcoma TE671 cell line previously used to model PSA-NCAM turnover [29], NCAM lateral diffusion [11] and lung metastases in nude mice [13] has been used to study the involvement of the insulin-signalling pathway in the regulation of the PSA-NCAM turnover process and its influence on cell migration. Our results showed that insulin promoted an upregulation of p-FAK, which, in turn, caused the inhibition of cell surface PSA-NCAM endocytosis. From a functional perspective, regulation of PSA-NCAM turnover by insulin affected cell migration.

2. Material and methods

2.1. Cell culture

Human rhabdomyosarcoma TE671 cells (CRL-10636; ATCC-Cryosite) were cultured in complete media (DMEM/F12 supplemented with 10% foetal bovine serum (FBS), 1% penicillin streptomycin glutamine (PSG) and 1 mM sodium pyruvate) as previously described [29], and used at low passage. Briefly, cells were cultured in 1 ml of complete media overnight (o/n), washed twice with DMEM/F12 and overlaid with 100 μl/cm^2 of ECM diluted 1:4 in normal media (DMEM/F12 containing 1 mM sodium pyruvate, 1 X glutamax and no FBS or PSG) and were supplemented with different treatments as specified. All cell culture media and reagents were from LifeTechnologies. Cells were then assayed for 15 h (unless otherwise stated). For the experiments involving endoneuraminidase (EndoN; EurobioAbCys) cells were treated for 5 h with 2.5 units of the enzyme in 1 ml complete media, washed twice with DMEM/F12, overlaid with 200 μl ECM (1:4) containing 2.5 units of EndoN and supplemented as specified and assayed as described above. For PSA-NCAM recovery experiments, cells were pre-treated with EndoN (as described above); subsequently, cells were thoroughly washed to remove the enzyme, overlaid with ECM-dilutions supplemented as specified (without EndoN), and assayed as described above.

2.2. Reagents and antibodies

Nunc cell-culture treated plates (Thermo Scientific); Membrane-like extracellular Matrix (ECM; Matrigel Basement membrane matrix, growth factor reduced; main components according to manufacturer: laminin, collagen IV and entactin; BD Biosciences). Calcium ionophore (CI; 1 μM; Sigma A23187), Phenylarsine Oxide (PAO; 50 μM; Sigma P3075), Insulin from bovine pancreas (INS; 200 nM; Sigma I0516), wortmannin (WT; 1 μM; Sigma) and A66 (1 μM; Sigma-Aldrich SML1213). PF-562271 (PF; 15 nM; Symansis SY-PF56227). AKT inhibitor-IV (AKT-IV; 1 μM; Calbiochem; Millipore 124011). Mono/polyclonal primary antibodies (mAb, pAb): mouse mAb anti-PSA-NCAM (clone 2-2B; Merck Millipore); rabbit pAb anti-phospho-AKT (Ser473; p-Akt; Cell Signalling) and rabbit mAb anti-insulin receptor β (IR-β; INSR; clone 4b8; Cell Signalling); mouse mAb anti-GAPDH (Abcam), rabbit pAb anti-phospho-Fak (Tyr397; p-FAK; Abcam), mouse mAb anti-integrin alpha V (272-17E6; αv-integrin; LifeTechnologies), rabbit pAb anti-integrin alpha V (EPR16800; Abcam) and rabbit pAb anti-Rab5 (Abcam); rabbit pAb anti-NCAM (clone H-300; SantaCruzBiotechnology). Secondary antibodies: goat pAb anti-mouse IgM: HRP (Bio-Rad AbD Serotec;); sheep pAb anti-mouse IgG: HRP and donkey pAb anti-rabbit IgG: HRP (GE Healthcare). Goat anti-mouse-IgM: Alexa 488 (cross-adsorbed against IgG), goat anti-mouse IgG: Alexa 594 and goat anti-rabbit IgG: Alexa 633 (LifeTechnologies). ActinGreen 488 (for F-actin stain) and NucBlue (for Hoechst 33342 nuclear counterstain) ready probes (LifeTechnologies).

2.3. Small interfering RNA (siRNA)-mediated gene expression silencing

SiRNA experiments were performed using validated-specific, and universal-scrambled (control), Trilencer-27 Human siRNA duplexes (OriGene), and Lipofectamine RNAiMax transfection reagent (Life Technologies) following the manufacturer's instructions. Briefly, 1.0×10^5 cells were cultured in 24-well plates, washed once with serum free media and transfected for 6 h in 600 μl Opti-MEM medium (LifeTechnologies) containing 5 nM siRNA (for multiple gene silencing specified siRNA duplexes were mixed at final 5 nM each) and Lipofectamine RNAiMAX. Subsequently, siRNA solution was removed, and cells were cultured in complete media for 24 h. Then, cells were washed twice with DMEM/F12 (without serum), overlaid with ECM-dilutions supplemented as specified and assayed as described above. Human siRNAs used: ST8SIA4 (PST; locus ID 7903; Cat. Num.: SR305305); ST8SIA2 (STX; locus ID 8128; Cat. Num.: SR305373); ITGAV (αv-integrin; locus ID 3685; Cat. Num.: SR302468); INSR (IR-β; locus ID 3643; Cat. Num: SR302436); IGF1R (locus ID 3480; Cat. Num.: SR302344). Three different siRNA duplexes targeting the same gene, named numerically and alphabetically, were tested. Effective and specific gene silencing was tested by qPCR and protein immunoblot analysis as specified. The most effective siRNA duplex combinations used for subsequent experimentation were as follows: PST/STX 1/B; ITGAV 1, INSR 1 and IGF1R 1. Scrambled/nonsense siRNAs were used as negative controls and prepared to corresponding combinations at final 5 nM each (scrambled-siRNA duplex combinations are specified in text and figures as scrambled). Data is presented as mean percent values of n =3 independent experiments relative to control (set as value =100) ± s.d.

2.4. Western blot

Protein quantification assays (western blot; WB) were performed as described previously [29]. Briefly assayed cultures were collected and cells were pelleted by centrifugation. The ECM was removed by gentle aspiration, and cell pellets were lysed with a Bullet blender (NextAdvance) in lysis buffer (50 mM Tris-HCl +150 mM NaCl +5 mM EDTA +1% Triton X-100 and protease inhibitor). Proteins were transferred to a PVDF membrane and blotted with antibodies specific for PSA-NCAM (1:3000), GAPDH (1:1500), p-AKT (1:1000), p-FAK (1:1000), αv-integrin (1:1000), IR-β (1:1000), IGF1R (1:1000) followed by anti-mouse: IgM:HRP, anti-mouse: HRP or anti-rabbit: HRP (1:2000) antibodies. Target-protein integrated densities were normalised to corresponding GAPDH signals. Data is presented as