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BIOCHEMISTRY

Biochemistry General

Real-time monitoring of polyacrylamide gel electrophoresis by the shadowgraph technique

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Polyacrylamide gel electrophoresis (PAGE) with sodium dodecyl sulphate (SDS) and Coomassie brilliant blue (CBB) staining is widely used in protein research and requires time for electrophoresis, staining and destaining. Because the protein bands electrophoresed in the gel are invisible in most cases, the results cannot be observed until the whole process is complete. In this study, shadowgraph was applied to detect biomolecules such as proteins during electrophoresis. A simple optical system andcamera-enabledreal-time monitoring of Graphical Abstract migration and separation of individual protein bands in polyacrylamide gels without staining. The visibility was high enough that it was possible to visualize substances other than proteins, such as DNA. This method provides protein profiles instantly in the early stage of electrophoresis. The elimination of the staining and destaining steps will help save researchers' time. The method is also environmentally friendly and will help reduce the generation of waste solutions containing synthetic dyes.

Keywords: shadowgraph, SDS-PAGE, protein analysis, polyacrylamide gel electrophoresis, direct observation

Phosphorylation of PBX2, a novel downstream target of mTORC1, is determined by GSK3 and PP1

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Weinvestigated the molecular details of the role of protein tyrosine phosphatase (PTP)-PEST in cell migration. PTP-PEST knockout mouse embryonic fibroblasts (KO MEFs) and MEF cells expressing a dominant negative mutant of PTP-PEST showed significant suppression of cell migration compared to MEF cells expressing wild-type PTP-PEST (WT MEFs). Moreover, MEF cells harbouring a constitutively active mutant of PTP-PEST (S39A MEFs) showed a marked decrease in cell migration. In addition, MEF cells with no PTP-PEST or little PTP activity rapidly adhered to fibronectin and made many focal adhesions compared to WTMEF cells. In contrast, S39A MEF cells showed weak adhesion to fibronectin and formed a few focal adhesions. Furthermore, investigating the subcellular localization showed that Ser39-phosphorylated PTPPEST was favourably situated in the adherent area of the pseudopodia. Therefore, we propose that suppression of PTP-PEST enzyme activity due to Ser39phosphorylationinpseudopodiaandattheleadingedge of migrating cells induces rapid and good adherence to the extracellular matrix. Thus, suppression of PTP activity by Ser39-phosphorylation is critical for cell migration. Three amino acid substitutions in human PTP-PEST have been previously reported to alter PTP Graphical Abstract activity. These amino acid substitutions in mouse PTPPEST altered the migration of MEF cells in a positive correlation.

Keywords: Ser39 phosphorylation, PTP-PEST, mutational analysis, focal adhesion, cell migration

Protein Structure

Epitope mapping of a blood–brain barrier crossing antibody targeting the cysteine-rich region of IGF1R using hydrogen-exchange mass spectrometry enabled by electrochemical reduction

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Pathologies of the central nervous system impact a significant portion of our population, and the delivery of therapeutics for effective treatment is challenging. The insulin-like growth factor-1 receptor (IGF1R) has emerged as atarget for receptor-mediated transcytosis, a process by which antibodies are shuttled across the blood–brain barrier (BBB). Here, we describe the biophysical characterization of VHH-IR4, a BBB crossing single-domain antibody (sdAb). Binding was confirmed by isothermal titration

calorimetry and an epitope was highlighted by surface plasmon resonance that does not overlap with the IGF-1 binding site or other known BBB-crossing sdAbs. The epitope was mapped with a combination of linear peptide scanning and hydrogen–deuterium exchange mass spectrometry (HDX-MS). IGF1R is large and heavily disulphide bonded, and comprehensive HDX analysis was achieved only through the use of online electrochemical reduction coupled with amultiprotease approach, which identified an epitope for VHHIR4 within the cysteine-rich region (CRR) of IGF1R spanning residues W244-G265. This is the first report of an sdAb binding the CRR. We show that VHHIR4 inhibits ligand induced auto-phosphorylation of IGF1R and that this effect is mediated by downstream conformational effects. Our results will guide the selection of antibodies with improved trafficking and optimized IGF1R binding characteristics. Graphical Abstract

Keywords: blood–brain barrier; epitope mapping, hydrogen–deuterium exchange mass spectrometry, IGF1R, single-domain antibody

Structural insights into the rational design of a nanobody that binds with high affinity to the SARS-CoV-2 spike variant

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The continuous emergence of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) variants associated with the adaptive evolution of the virus is prolonging the global coronavirus disease 2019 Graphical Abstract (COVID-19) pandemic. The modification of neutralizing antibodies based on structural information is expected to be a useful approach to rapidly combat emerging variants. A dimerized variable domain of heavy chain of heavy chain antibody (VHH) P17 that has highly potent neutralizing activity against SARSCoV-2 has been reported but the mode of interaction with the epitope remains unclear. Here, we report the X-ray crystal structure of the complex of monomerized P17 bound to the SARS-CoV-2 receptor binding domain (RBD) and investigated the binding activity of P17 toward various variants of concern (VOCs) using kinetics measurements. The structure revealed details of the binding interface and showed that P17 had an appropriate linker length to have an avidity effect and recognize a wide range of RBD orientations. Furthermore, we identified mutations in known VOCs that decrease the binding affinity of P17 and proposed methods for the acquisition of affinity toward the Omicron RBD because Omicron is currently the most predominant VOC. This study provides information for the rational design of effective VHHs for emerging VOCs.

Keywords: VHH, SARS-CoV-2, recognition mechanism, Crystal structure

Protein Interaction and Recognition

Differential interactions of *α*-synuclein conformers affect refolding and activity of proteins

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The accumulation of protein aggregates as intracellular inclusions interferes with cellular protein homeostasis leading to protein aggregation diseases. Protein aggregation results in the formation of several protein conformers including oligomers and fibrils, where each conformer has its own structural characteristic and proteotoxic potential. The present study explores the effect of alpha-synuclein (*α*-syn) conformers on the Graphical Abstract activity and spontaneous refolding of firefly luciferase. Of the different conformers, *α*-syn monomers delayed the inactivation of luciferase under thermal stress conditions and enhanced the spontaneous refolding of luciferase. In contrast, the *α*-syn oligomers and fibrils adversely affected luciferase activity and refolding, where the oligomers inhibited spontaneous refolding, whereas a pronounced effect on the inactivation of native luciferase was observed in the case of fibrils. These results indicate that the oligomers and fibrils of *α*-syn interfere with the refolding of luciferase and promote its misfolding and aggregation. The study reveals the differential propensities of various conformers of a pathologically relevant protein in causing inactivation, structural modifications and misfolding of other proteins, consequently resulting in altered protein homeostasis.

Keywords: α-synuclein, toxicity, protein folding, neurodegenerative disorder, chaperone

Glycobiology and Carbohydrate Biochemistry

β-1, 4-Galactan suppresses lipid synthesis in sebaceous gland cells via TLR4

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Sebum is a lipid mixture secreted from sebaceous glands of the skin. The excessive secretion of sebum causes acne vulgaris and seborrheic dermatitis, while its deficiency causes xerosis. Therefore, the appropriate control of sebum secretion is crucially important to keep the skin healthy. In the present study, we evaluated the effects of naturally occurring polysaccharides on lipid biosynthesis in hamster sebaceous gland cells. Among the tested polysaccharides, β-1,4-galactan, the main chain of type I arabinogalactan, most potently suppressed lipid synthesis in the sebaceous gland cells as analysed by oil red O staining. Tolllike receptor (TLR)4 inhibitors counteracted this suppressive effect and lipopolysaccharide, a TLR4 ligand, mimicked this effect, suggesting the involvement of the TLR4 signalling pathway. In the cells β1,4-galactan significantly decreased mRNA levels of lipogenesis-related transcription factors (peroxisome Graphical Abstract proliferator-activated receptor γ and sterol regulatory element-binding protein 1) and enzymes (acetyl-CoA carboxylase and fatty acid synthase) as well as the glucose transporter GLUT4. Furthermore, β-1,4galactan increased the production of lactic acid serving as a natural moisturizing factor and enhanced the proliferation of sebaceous gland cells. These results suggest potential of β-1,4-galactan as a material with therapeutic and cosmetic values for the skin.

Keywords: arabinogalactan, lactic acid, lipopolysaccharide, sebum, skin

CELL

Cytoskeleton/Cell Motility and Cell Shape

Phosphorylation/dephosphorylation of PTP-PEST at Serine 39 is crucial for cell migration

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Mechanistic target of rapamycincomplex1(mTORC1) is a serine–threonine kinase that is activated by extracellular signals, such as nutrients and growth factors. It plays a key role in the control of various biological processes, such as protein synthesis and energy metabolism by mediating or regulating the phosphorylation of multiple target molecules, some of which remain to be identified. We have here reanalysed a large-scale phosphoproteomics data set for mTORC1 target molecules and identified pre-B cell leukemia transcription factor 2 (PBX2) as such a novel target that is dephosphorylated downstream of mTORC1. We confirmed that PBX2, but not other members of the PBX family, is dephosphorylated in an mTORC1 activity–dependent manner. Furthermore, pharmacological and gene knockdown experiments revealed that glycogen synthase kinase 3 (GSK3) and protein phosphatase 1 (PP1) are responsible for the phosphorylation and dephosphorylation of PBX2, respectively.

Our results thus suggest that the balance between the antagonistic actions of GSK3 and PP1 determines the phosphorylation status of PBX2 and its regulation by mTORC1.

Keywords: protein phosphatase 1 (PP1), pre-B cell leukemia transcription factor 2 (PBX2), phosphorylation, mechanistic target of rapamycin complex 1 (mTORC1), glycogen synthase kinase 3 (GSK3)

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JB SPECIAL ISSUE-STROMAL CELL BIOLOGY

***GUEST EDITORS: MASANORI AIKAWA, YUMIKO OISHI AND ICHIRO MANABE JB SPECIAL ISSUE*—REVIEW**

Recent topics regarding macrophage in the central nervous system

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The central nervous system (CNS) is a highly complex collection of neurons with a variety of stromal cells, such as glia cells, immune cells, vascular cells and fibroblasts. Microglia are a resident macrophage and a type of glial cells located in the parenchyma of the CNS and play a pivotal role in the maintenance of tissue homeostasis. They are early responders to the abnormality of the CNS, leading to the adaptation of their phenotypes by virtue of their plasticity, after which they give an impact on neuronal functions. Besides microglia, there are anatomically

and phenotypically distinct macrophage populations at the border of the CNS, such as meninge, perivascular space and choroid plexus, where they show distinct morphology and gene expression profiles when compared with microglia. This review will summarize the recent advance in our knowledge regarding their heterogeneity, plasticity, ontogenetic relationship of these CNS-resident macrophage populations.
Keywords: CNS, stroma, microglia, macrophage, heterogeneity

Immunity in the brain and surrounding tissues
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Immune reactions in the brain, the most complex organ that directly or indirectly regulates almost every part of the body and its actions, need to be tightly regulated. Recent findings in the field of neuroimmunology have enhanced our understanding of immune cells not only inside the brain but also in adjacent tissues. Multiple types of immune cells exist and are active in neighboring border tissues, even in the steady state. In addition, advances in technology have allowed researchers to characterize a broad range of cell types, including stromal cells that support immune reactions. This review presents a short overview of the roles of the immune system in the brain during health and disease, with focus on adaptive immunity and anatomical sites of action. We also discuss potential roles of stromal cells.
Key words: brain, meninges, cerebrospinal fluid

Microglia regulate neuronal and behavioural functions under physiological and pathological conditions
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Microglia are immune cells in the central nervous system that engulf unnecessary synapses during development. In vivo imaging has substantially improved in recent years, besides the development of tools for manipulating microglia and neurons. These techniques reveal the novel functions of microglia. Microglia regulate neuronal activity to prevent synchronization. This neuron–microglia interaction is mediated by adenosine triphosphate—P2Y12 and adenosine—adenosine A1 receptor signalling in the striatum. Moreover, microglia release inflammation-related molecules that suppress neuronal activity, thus leading to lipopolysaccharide-induced aversion. Prostaglandin E2 (PGE2)—PGE receptor 1 signalling in the striatum underlies this behavioural alteration. Chronic stress activates microglia through toll-like receptor (TLR) 2 and TLR4 to release pro-inflammatory cytokines in the medial prefrontal cortex, thereby causing social avoidance. Microglia play multiple functions under physiological conditions, as well as pathological and psychological stress.
Key words: stress, neuron, microglia, inflammation

Hyperosmotic stress response regulates interstitial homeostasis and pathogenic inflammation
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Hyperosmotic stress triggers an evolutionally preserved, fundamental cellular response. A growing body of evidence has highlighted the role of extra-renal, interstitial hyperosmolality in maintaining local tissue immune homeostasis and potentially driving tissue inflammation in human diseases. The hyperosmotic stress response initiates cellular shrinkage, oxidative stress, metabolic remodeling and cell cycle arrest, all of which are adjusted by a counteractive adaptative response that includes osmolyte synthesis, upregulation of ion transporters and induction of heat shock proteins. Recent studies have revealed that high osmolality can impact immune cell differentiation and activation pathways in a cell type specific manner. The fine-tuning of the immune response depends on the tissue microenvironment. Accordingly, novel therapeutic approaches that target hyperosmolality-mediated inflammation may be identified by furthering our understanding of hyperosmotic response in the context of disease. In this review, we discuss the cellular and molecular mechanisms by which hyperosmotic stress response regulates interstitial homeostasis and pathogenic inflammation.
Key words: immune response, hyperosmotic stress, tissue inflammation

REGULAR PAPER
BIOCHEMISTRY
Biochemistry in Diseases and Aging

Post-translational modifi cation of lysine residues in erythrocyte *α*-synuclein
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α-Synuclein is a protein linked to various synuclein-associated diseases (‘synucleinopathies’), including Parkinson’s disease, dementia with Lewy Bodies and multiple system atrophy, and

is highly expressed in the central nervous system and in erythrocytes. Moreover, α-synuclein-containing erythrocyte-derived extracellular vesicles may be involved in the pathogenesis of synucleinopathies and their progression across the blood–brain barrier. Several post-translational modifications of α-synuclein have been reported in brain inclusions, including S129 phosphorylation, but fewer have been found in erythrocytes. In this study, we analysed the post-translational modifications of erythrocyte α-synuclein using liquid chromatography–mass spectrometry. We found that all lysine residues in the α-synuclein protein could be modified by acetylation, glycation, ubiquitination or SUMOylation but that phosphorylation, nitration and acylation were uncommon minor post-translational modifications in erythrocytes. Since the post-translational modification of lysine residues has been implicated in both membrane association and protein clearance, our findings provide new insight into how synucleinopathies may progress and suggest possible therapeutic strategies designed to target α-synuclein.

Key words: erythrocyte, Parkinson's disease, post-translational modification, synucleinopathy, α-synuclein.Abbreviations: aa, amino acids; AGE, advanced glycation end product; BBB, blood–brain barrier; CML, Nε-(1-carboxymethyl)-L-lysine; CNS, central nervous system; EVs, extracellular vesicles; IP, immunoprecipitation; LC–MS/MS, liquid chromatography–mass spectrometry; PBS, phosphate buffered saline; PD, Parkinson's disease; PTM, post-translational modification; SUMO, small ubiquitin-related modifier

Immuno-chemistry

Sensitive immunoassay of Legionella using multivalent conjugates of engineered VHHs

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VHH antibodies or nanobodies, which are antigen binding domains of heavy chain antibodies from camelid species, have several advantageous characteristics, including compact molecular size, high productibility in bacteria and easy engineering for functional improvement. Focusing on these advantages of VHHs, we attempted to establish an immunoassay system for detection of Legionella, the causative pathogen of Legionnaires' disease. A VHH phage display library was constructed using cDNA from B cells of alpacas immunized with Legionella pneumophila serogroup1 (LpSG1). Through biopanning, two specific VHH clones were isolated and used to construct a Legionella detection system based on the latex agglutination assay. After engineering the VHHs and improving the assay system, the sensitive detection system was successfully established for the LpSG1 antigen. The immunoassay developed in this study should be useful in easy and sensitive detection of Legionella, the causative agent of Legionnaires' disease, which is a potentially fatal pneumonia.

Keywords: alpaca, antibody, engineering, immunoassay, VHH

Biochemical Pharmacology

Availability of aldo-keto reductase 1C3 and ATP-binding cassette B1 as therapeutic targets for alleviating paclitaxel resistance in breast cancer MCF7 cells

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Paclitaxel (PTX) is frequently utilized for the chemotherapy of breast cancer, but its continuous treatment provokes hyposensitivity. Here, we established a PTX-resistant variant of human breast cancer MCF7 cells and found that acquiring the chemoresistance elicits a remarkable up-regulation of aldo-keto reductase (AKR) 1C3. MCF7 cell sensitivity to PTX toxicity was increased by pretreatment with AKR1C3 inhibitor and knockdown of this enzyme, and decreased by its overexpression, inferring a crucial role of AKR1C3 in the development of PTX resistance. The PTX-resistant cells were much less sensitive to 4-hydroxy-2-nonenal and acrolein, cytotoxic reactive aldehydes derived from ROS-mediated lipid peroxidation, compared with the parental cells. Additionally, the resistant cells lowered levels of 4-hydroxy-2-nonenal formed during PTX treatment, which was mitigated by pretreating with AKR1C3 inhibitor, suggesting that AKR1C3 procures the chemoresistance through facilitating the metabolism of the cytotoxic aldehyde. The gain of PTX resistance additively promoted the aberrant expression of an ATP-binding cassette (ABC) transporter ABCB1 among the ABC transporter isoforms. The combined treatment with AKR1C3 and ABCB1 inhibitors overcame the PTX resistance and cross-resistance to another taxane-based drug docetaxel. Collectively, combined treatment with AKR1C3 and ABCB1 inhibitors may exert an overcoming effect of PTX resistance in breast cancer.

Key words: paclitaxel, docetaxel, chemoresistance, breast cancer, ATP-binding cassette B1, Aldo-keto reductase 1C3

BIOTECHNOLOGY

Biomaterials

A novel peptide isolated from Catla skin collagen acts as a self-assembling scaff old promoting nucleation of calcium-defi cient hydroxyapatite nanocrystals

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Catla collagen hydrolysate (CH) was fractionated by chromatography and each fraction was subjected to HAnucleation, with the resultant HA-fraction composites being scored based on the structural and functional group of the HA formed. The process was repeated till a single peptide with augmented HA nucleation capacity was obtained. Thepeptide(4.6 kDa),exhibited high solubility, existed in polyproline-II conformation and displayed a dynamic yet stable hierarchical selfassembling property. The 3D modelling of the peptide revealed multiple calcium and phosphate binding sites Graphical Abstract andahighpropensitytoself-assemble. Structural analysis of the peptide-HA crystals revealed characteristic diffraction planes of HA with mineralization following the (002) plane, retention of the self-assembled hierarchy of the peptide and intense ionic interactions between carboxyl groups and calcium. The peptideHA composite crystals were mostly of 25–40 nm dimensions and displayed 79% mineralization, 92% crystallinity, 39.25% porosity, 12 GPa Young's modulus andenhancedstability in physiological pH. Cells grown on peptide-HA depicted faster proliferation rates and higher levels of osteogenic markers. It was concluded that the prerequisite for HA nucleation by a peptide included: a conserved sequence with a unique charge topology allowing calcium chelation and its ability to form a dynamic self-assembled hierarchy for crystal propagation.

Keywords: structure, self-assembly, nano-hydroxyapatite, collagen peptide, biomimetic synthesis