Medical Proteomics Laboratory
疾患プロテオミクスラボラトリー

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The mission of our laboratory is to develop advanced technologies for integrative proteomic analyses from a physicochemical, structural and systems biology point of view. Currently, we mainly focus on functional protein-protein interaction networks related to a variety of diseases including cancer and infection. We are also engaged in collaborative researches regarding mass spectrometry and electron microscopy, which have made a substantial contribution to many scientific achievements.

<Group I>

1. Integrative analysis of cancer cell signaling networks by high-resolution proteomics and systems biology

Post-translational modifications (PTMs), such as phosphorylation, ubiquitination and acetylation, are known to be widely involved in the regulation of various biological processes through extensive diversification of each protein function at the cellular network level. Previous functional analyses of cancer cell signaling under a variety of experimental conditions revealed many of the key molecules and their associated protein modifications in relation to each type of cancer. In order to systematically discover critical modulators from diversified signaling molecules, we have developed a high-resolution mass spectrometry-based proteomics platform for integrative identification and quantification of multiple post-translational modifications from various types of cancer cells.

1-1. High-resolution proteomic analysis of EGF-regulated ubiquitination dynamics in human cancer cells

Hiroko Kozuka-Hata, Tomoko Hiroki, Aya Kitamura, Aiko Aizawa, Naoaki Miyamura, Kouhei Tsumoto, Jun-ichiro Inoue, and Masaaki Oyama.

Protein ubiquitination is one of the most prevalent post-translational modifications (PTMs) and plays critical roles in regulating protein degradation, signal transduction and DNA repair in cooperation with other PTMs such as phosphorylation and acetylation. Recent mass spectrometry-based proteomics coupled with efficient enrichment technologies for each type of the modified peptides has enabled us to identify precise modification sites and measure their quantitative changes on a global scale. Our previous lysine-modification proteomic analyses of thirteen representative human cancer cell lines led us to identify thousands of ubiquitination (Ub) and acetylation (Ac) sites in total and revealed that their system-wide modification status was mutually different at the cel-

lular network level. In this study, we further applied SILAC (Stable Isotope Labeling by Amino acids in Cell culture) for quantitative description of EGF-dependent lysine-modification site dynamics in HeLa cells in a time-resolved manner. Through integration of large-scale SILAC-encoded data on six time points upon EGF stimulation, we successfully quantified approximately 1,000 kinds of Ub-sites as well as 700 kinds of Ac-sites and found that one-third of these Ub-modified molecules, including several EGF signaling effectors, were subjected to downregulation by proteasomal inhibition.

1-2. Proteome-wide analysis of lysine acetylation and ubiquitination reveals critical signaling regulation in cancer cells

Hiroko Kozuka-Hata, Aya Kitamura, Tomoko Hiroki, Aiko Aizawa, Kouhei Tsumoto, Jun-ichiro Inoue, and Masaaki Oyama.

Post-translational modifications (PTMs), such as phosphorylation, ubiquitination and acetylation, are known to be widely involved in the regulation of various biological processes through extensive diversification of each protein function at the cellular network level. Previous functional analyses of cancer cell signaling under a variety of experimental conditions revealed many of the key molecules and their associated protein modifications in relation to each type of cancer. In order to systematically discover critical modulators from diversified signaling molecules, we have developed a high-resolution mass spectrometry-based proteomics platform for integrative identification and quantification of multiple post-translational modifications from various types of cancer cells. Our large-scale proteomic analysis enabled us to identify more than 5,000 kinds of ubiquitinated sites and 1,600 kinds of acetylated sites from representative human cancer cell lines, leading to identification of approximately 900 novel lysine modification sites in total. Very interestingly, 236 lysine residues derived from 141 proteins were found to be modified with both ubiquitination and acetylation. As a consequence of the subsequent motif extraction analyses, glutamic acid (E) was found to be highly enriched at the position (-1) for the lysine acetylation sites, whereas the same amino acid was relatively dispersed along the neighboring residues of the lysine ubiquitination sites.

1-3. System-wide perturbation of the proteome and phosphoproteome dynamics in glioblastoma stem cells through mTOR signaling inhibition

Hiroko Kozuka-Hata, Tomoko Hiroki, Ryo Koyama-Nasu, Kouhei Tsumoto, Jun-ichiro Inoue, Tetsu Akiyama, and Masaaki Oyama.

As glioblastoma is the most common and aggressive brain tumor with poor prognosis, systematic elucidation of signaling networks causally linked to the tumorigenesis is very crucial for developing more effective treatments for this intractable cancer. In our previous study, we applied a high-resolution mass spectrometry-based proteomics technology in combination with SILAC quantitative methods to understand EGF-dependent phosphoproteome dynamics in patient-derived glioblastoma stem cells. We demonstrated that the phosphorylation levels of the representative mTOR signaling molecules such as RPS6 and PRAS40 were dramatically up-regulated upon EGF stimulation. As EGFR signaling has been reported to play a pivotal role in regulating the maintenance of cancer stem cells, we next carried out mTOR inhibitor-dependent signaling perturbations to unravel stemness-related pathways at the network level.

In the present study, we identified a total of 3,726 proteins including 49 up-regulated and 436 down-regulated factors by Torin 1 treatment. Interestingly, we found that one of the well-known cancer stem cell markers was significantly down-regulated through mTOR signaling inhibition. Our in-depth phosphoproteome analysis also led to identification of 6,250 unique phosphopeptides derived from 2,221 proteins and unveiled a variety of dynamic changes regarding phosphorylation levels of cancer and neural stem cell markers in a comprehensive manner. The integrative view of the mTOR inhibitor-dependent proteome and phosphoproteome dynamics in glioblastoma stem cells presents us with further prospects towards understanding previously unrecognized regulations at the system level.

1-4. System-level analysis of CagA-dependent signaling network dynamics by Helicobacter pylori infection

Hiroko Kozuka-Hata, Masato Suzuki, Kotaro Kiga, Shinya Tasaki, Jun-ichiro Inoue, Tadashi Yamamoto, Chihiro Sasakawa, and Masaaki Oyama.

The signal transduction system within a cell regulates complex biological events in response to bacterial infection. The previous analyses of cell signaling in Helicobacter pylori-infected gastric epithelial cells have revealed that CagA, a major virulence factor of Helicobacter pylori, is delivered into cells via the type IV secretion system and perturbs signaling networks through the interaction with the key signaling molecules such as SHP-2, Grb2, Crk/Crk-L, Csk, Met, and ZO-1. Although the biological activity of tyrosine-phosphorylated CagA has intensively been studied, system-wide effects of the virulence factor on cellular signaling have yet to be analyzed. Here we performed time-resolved analyses of phosphoproteome and CagA-interactome in human gastric AGS

cells by CagA-positive/negative Helicobacter pylori infection. Our highly sensitive nanoLC-MS/MS analyses in combination with the Stable Isotope Labeling by Amino acids in Cell culture (SILAC) technology defined CagA-dependent perturbation of signaling dynamics along with a subset of CagA-associated possible modulators on a network-wide scale. Our result indicated that the activation level of the phosphotyrosine-related signaling molecules in AGS cells was suppressed overall in the presence of CagA during Helicobacter pylori infection. As Helicobacter pylori infection plays pivotal roles in the progression of gastric diseases including carcinogenesis, a comprehensive and fine description of the signaling dynamics would serve as a fundamental platform to theoretically explore for the potential drug targets through analyzing the regulatory mechanisms at the system-level.

2. Mass spectrometry-based annotation of the human short ORFeome

Masaaki Oyama, Hiroko Kozuka-Hata, Sumio Sugano, Tadashi Yamamoto, and Jun-ichiro Inoue.

In parallel with the human genome projects, human full-length cDNA data has also been intensively accumulated. Large-scale analysis of their 5'-UTRs revealed that about half of these had a short ORF upstream of the coding region. Experimental verification as to whether such upstream ORFs are translated is essential to reconsider the generality of the classical scanning mechanism for initiation of translation and define the real outline of the human proteome. Our previous proteomics analysis of small proteins expressed in human K562 cells provided the first direct evidence of translation of upstream ORFs in human full-length cDNAs (Oyama et al., **Genome Res**, 14: 2048-2052, 2004). In order to grasp an expanded landscape of the human short ORFeome, we have performed an in-depth proteomics analysis of human K562 and HEK293 cells using a two-dimensional nanoLC-MS/MS system. The results led to the identification of eight protein-coding regions besides 197 small proteins with a theoretical mass less than 20 kDa that were already annotated coding sequences in the curated mRNA database. In addition to the upstream ORFs in the presumed 5'-untranslated regions of mRNAs, bioinformatics analysis based on accumulated 5'-end cDNA sequence data provided evidence of novel short coding regions that were likely to be translated from the upstream non-AUG start site or from the new short transcript variants generated by utilization of downstream alternative promoters. Protein expression analysis of the *GRINL1A* gene revealed that translation from the most upstream start site occurred on the minor alternative splicing transcript, whereas this initiation site was not utilized on the major mRNA, resulting in translation of the downstream ORF from the second initiation codon. These findings reveal a novel post-transcriptional system that can augment the human proteome via the alternative use of diverse translation start sites coupled with transcriptional regulation through alternative promoters or splicing, leading to increased complexity of short protein-coding regions defined by the human transcriptome (Oyama et al., **Mol Cell Proteomics**, 6: 1000-1006, 2007).

3. Real-Time Search-Assisted Multiplexed Quantitative Proteomics Reveals System-Wide Translational Regulation of Non-Canonical Short Open Reading Frames

Hiroko Kozuka-Hata, Tomoko Hiroki, Naoaki Miyamura, Aya Kitamura, Kouhei Tsumoto, Jun-ichiro Inoue, and Masaaki Oyama.

Abnormal expression of histone deacetylases (HDACs) is reported to be associated with angiogenesis, metastasis and chemotherapy resistance regarding cancer in a wide range of previous studies. Suberoylanilide hydroxamic acid (SAHA) is well known to function as a pan-inhibitor for HDACs and recognized as one of the therapeutic drug candidates to epigenetically coordinate cancer cell fate regulation on a genomic scale. Here, we established a Real-Time Search-assisted mass spectrometric platform for system-wide quantification of translated products encoded by non-canonical short open reading frames (ORFs) as well as already annotated protein coding sequences (CDSs) on the human transcriptome and applied this methodology to quantitative proteomic analyses of SAHA-treated human HeLa cells to evaluate proteome-wide regulation in response to drug perturbation. Very intriguingly, our RTS-based in-depth proteomic analysis enabled us to identify approximately 5000 novel peptides from the ribosome profiling-based short ORFs encoded in the diversified regions on presumed 'non-coding' nucleotide sequences of mRNAs as well as lncRNAs and nonsense mediated decay (NMD) transcripts. Furthermore, TMT-based multiplex large-scale quantification of the whole proteome changes upon differential SAHA treatment unveiled dose-dependent selective translational regulation of a limited fraction of the non-canonical short ORFs in addition to key cell cycle/proliferation-related molecules such as UBE2C, CENPF and PRC1. Our study provided the first system-wide landscape of drug-perturbed translational modulation on both canonical and non-canonical proteome dynamics in human cancer cells.

4. Ultra-deep single-cell proteomic analysis of patient-derived glioblastoma cells

Hiroko Kozuka-Hata, Tomoko Hiroki, Aya Kitamura, Naoaki Miyamura, Tetsu Akiyama, Jun-ichiro Inoue, Kouhei Tsumoto, and Masaaki Oyama.

Single-cell analysis is an essential technique for understanding cellular diversity by analyzing mutually unique data from individual cells. The heterogeneity of cancer cells is known to be deeply involved in drug resistance and poor prognosis, thus there is a strong demand for the development of effective cancer therapies based on single-cell analysis. While single-cell transcriptome analysis has rapidly become widespread with the evolution of next-generation sequencers, single-cell proteome analysis is still an emerging approach due to technical limitations in detection sensitivity. However, since proteins more directly reflect cellular functions compared to RNA molecules, it is highly anticipated that in-depth single-cell proteome analysis, based on recent advancements in mass spectrometry technology, will provide more detailed molecular network information on individual cell regulation. Previously, we conducted a high-precision quantitative phosphoproteome analysis of cancer stem cells (GB2) derived from glioblastoma patients to elucidate the signaling mechanisms leading to very high malignant properties with a five-year survival rate of less than 10%. Our analysis revealed that stimulation by epidermal growth factor (EGF), which controls stemness maintenance of these cells, activated mTORC1 and highly phosphorylated the downstream ribosomal protein S6 (Kozuka-Hata et al., PLoS One, 2012). Since mTOR inhibitors exert antitumor effects by inhibiting the functions of various factors necessary for cancer cell proliferation and angiogenesis, we applied Torin 1, a representative mTOR inhibitor, for EGF signaling perturbation of GB2 cells and newly established an experimental system to evaluate EGF-dependent protein expression changes at the single-cell level. Our integrative single-cell proteomic measurement using advanced Orbitrap MS instruments unveiled Torin 1-dependent global dynamics of more than 6,000 proteins including novel peptides encoded by non-canonical translation at single-cell resolution.

5. PTM-directed high-resolution plasma proteomics uncovers system-wide alteration of protein acetylation associated with Alzheimer's Disease

Hiroko Kozuka-Hata, Aya Kitamura, Tomoko Hiroki, Naoaki Miyamura, Kouhei Tsumoto, and Masaaki Oyama.

Protein lysine acetylation is a well-known post-translational modification that broadly affects structural and functional properties and significantly contributes to the regulation of diverse biological processes. In this study, we aimed to gain insights into the pathophysiology of Alzheimer's disease (AD) by conducting a comprehensive analysis of the lysine acetylation proteome using plasma samples obtained from AD patients and healthy controls. High-resolution nano-flow liquid chromatography-tandem mass spectrometry (nanoLC-MS/MS) enabled the detection of over 7,600 peptide-spectrum matches (PSMs) corresponding to 350 lysine acetylation sites, not only from known plasma proteins but also from peptide sequences derived from novel open reading frames (ORFs) defined by ribosome profiling. Comparative analysis of AD patients and healthy controls revealed extensive alterations regarding acetylation modifications on Apolipoprotein B-100. Further structural predictions using AlphaFold3 suggested conformational changes at the interaction interface with Low-Density Lipoprotein Receptor (LDLR). These findings provide new perspectives on functional changes in plasma proteins mediated by post-translational modifications in the context of AD pathology and may serve as a valuable foundation for the discovery of novel disease biomarkers and therapeutic targets.

<Group II>

Biomolecular recognition arises from collective and specific non-covalent interactions between discrete biological molecules. Our laboratory investigates diverse protein systems, including antibody-antigen and protein-ligand complexes, to quantitatively understand how these coordinated non-covalent interactions govern their specific recognition in biological and artificial systems. We seek to elucidate the molecular mechanisms by which biological molecules obtain high-specificity and affinity from multiple perspectives by employing advanced experimental techniques. To produce functional molecules with higher performance and better properties, we aim to build a solid foundation from which to develop drugs that modulate specific interactions between biomolecules and ultimately to understand the principles of molecular interactions in our lives.

1. Generation of antagonistic biparatopic anti-CD30 antibody from an agonistic antibody by precise epitope determination and utilization of structural characteristics of CD30 molecule

Akiba H, Ise T, Satoh R, Abe Y, Tsumoto K, Ohno H, Kamada H, and Nagata S.

CD30 is a member of the tumor necrosis factor receptor superfamily. Recently, blocking CD30-dependent intracellular signaling has emerged as potential strategy for immunological regulation. Development of antibody-based CD30 antagonists is therefore of significant interest. However, a key challenge is that the bivalent form of natural antibody can crosslink CD30 molecules, leading to signal transduction even in the absence of specific ligand, CD153. Biparatopic antibodies (BpAbs) offer a solution, using

two different variable fragments (Fvs) to bind distinct epitopes on a single antigen molecule. BpAbs format is an attractive alternative of natural antibody by potentially avoiding unwanted crosslinking and signaling induction

2. Specific recognition mechanism of an antibody to sulfated tyrosine and its potential use in biological research

Ujiie K, Nakakido M, Kinoshita S, Caaveiro JMM, Entzminger K, Okumura CJ, Maruyama T, Miyauchi K, Matano T, and Tsumoto K.

Post-translational modification of proteins is a crucial biological reaction that regulates protein functions by altering molecular properties. The specific detection of such modifications in proteins has made significant contributions to molecular biology research and holds potential for future drug development applications. In HIV research, for example, tyrosine sulfation at the N-terminus of C-C chemokine receptor type 5 (CCR5) is considered to significantly enhance HIV infection efficiency. However, antibodies specific to sulfated CCR5 still need to be developed. In this study, we successfully generated an antibody that specifically recognized the sulfated N-terminal peptide of CCR5 through rabbit immunization and panning via phage display using a CCR5 N-terminal peptide containing sulfate modification. We used various physicochemical methods in combination with molecular dynamics simulation to screen for residues that could be involved in recognition of the sulfated peptide by this antibody. We also confirmed that this antibody recognized the sulfated full-length CCR5 on the cell surface, which suggested it should be useful as a research tool that could lead to the development of novel therapeutics. Although the antibody binding did not inhibit HIV infection, it could be also described as sulfation site-specific binding, beyond sulfation-specific binding.

3. The pericellular function of Fibulin-7 in the adhesion of oligodendrocyte lineage cells to neuronal axons during CNS myelination

Yamada M, Sasaki B,Yamada N, Hayashi C, Tsumoto K, de Vega S, and Suzuki N.

Myelin is an electrical insulator that enables saltatory nerve conduction and is essential for proper functioning of the central nervous system (CNS). It is formed by oligodendrocytes (OLs) in the CNS, and during OL development various molecules, including extracellular matrix (ECM) proteins, regulate OL differentiation and myelination; however, the role of ECM proteins in these processes is not well understood. Our present work is centered on the analyses of the expression and function of fibulin-7 (Fbln7), an ECM protein of the fibulin family, in OL differentiation. In the expression analysis of Fbln7 in the CNS, we found that it was expressed at early postnatal stage and localized in the processes of OL precursor cells (OPCs), in the inner region of myelin, and in axons. The functional analysis using recombinant Fbln7 protein (rFbln7) revealed that rFbln7 promoted OPC attachment activity via β1 integrin and heparan sulfate receptors. Further, rFbln7 induced the adhesion to neurites and the differentiation of OLs. Altogether, our results show that Fbln7 promotes the adhesion between OLs and axons and OL differentiation.

4. FASTIA: A rapid and accessible platform for protein variant interaction analysis demonstrated with a single-domain antibody.

Matsunaga R and Tsumoto K.

Antibodies are critical tools in medicine and research, and their affinity for their target antigens is a key determinant of their efficacy. Traditional antibody affinity maturation and interaction analyses are often hampered by time-consuming steps such as cloning, expression, purification, and interaction assays. To address this, we have developed FASTIA (Fast Affinity Screening Technology for Interaction Analysis), a novel platform that integrates rapid gene fragment preparation, cell-free protein synthesis, and bio-layer interferometry with non-regenerative analysis. Using this approach, we can analyze the intermolecular interactions of over 20 variants over 2 days, requiring only the parent protein expression plasmid and basic equipment. We have demonstrated the ability of FASTIA to discriminate between single-domain antibody variants with different binding affinities using the anti-HEL VHH antibody D2-L29, and mapped the results to the crystal structure to identify key interaction sites. FASTIA provides results comparable to those obtained using traditional methods. Our system bypasses the need for genetic engineering facilities and can be easily adopted by laboratories, accelerating the protein engineering and optimization processes. In addition, FASTIA is applicable to other protein-protein interactions, making it a versatile tool for studying molecular recognition. FASTIA facilitates efficient affinity maturation, protein engineering, and analysis of protein-protein interactions. This provides a rapid and accessible route for improving antibodies and a broader understanding of protein interactions.

5. Affinity-stability trade-off mechanism of residue 35 in framework region 2 of VHH antibodies with β-hairpin CDR3

Yamamoto K, Nagatoishi S, Matsunaga R, Nakakido M, Kuroda D, and Tsumoto K.

Single-domain V_HH antibodies are promising therapeutic and diagnostic tools. The third complementarity-determining region (CDR3) is usually the most critical region for antigen recognition by V_HH antibodies. When CDR3 adopts a short and extended β-hairpin conformation, framework region 2 (FR2) often interacts directly with the antigen. However, the importance of these interactions in antigen recognition remains unclear. In this research, we investigated the role of FR2 residues in V_HH antibodies with β-hairpin CDR3s. We found that several FR2 residues, particularly at positions 35 and 37, are critical for high-affinity antigen binding. Notably, a trade-off was observed: introducing a charged residue at position 35 enhanced binding affinity but reduced thermal stability. These findings provide insights into optimizing FR2 in single-domain antibodies to improve their functionality for diagnostic and therapeutic applications.

6. Mitochondrial complexity is regulated at ER-mitochondria contact sites via PDZD8-FKBP8 tethering

Nakamura K, Aoyama-Ishiwatari S, Nagao T, Paaran M, Obara CJ, Sakurai-Saito Y, Johnston J, Du Y, Suga S, Tsuboi M, Nakakido M, Tsumoto K, Kishi Y, Gotoh Y, Kwak C, Rhee HW, Seo JK, Kosako H, Potter C, Carragher B, Lippincott-Schwartz J, Polleux F, and Hirabayashi Y.

Mitochondria-ER membrane contact sites (MERCS) represent a fundamental ultrastructural feature underlying unique biochemistry and physiology in eukaryotic cells. The ER protein PDZD8 is required for the formation of MERCS in many cell types, however, its tethering partner on the outer mitochondrial membrane (OMM) is currently unknown. Here we identify the OMM protein FKBP8 as the tethering partner of PDZD8 using a combination of unbiased proximity proteomics, CRISPR-Cas9 endogenous protein tagging, Cryo-electron tomography, and correlative light-electron microscopy. Single molecule tracking reveals highly dynamic diffusion properties of PDZD8 along the ER membrane with significant pauses and captures at MERCS. Overexpression of FKBP8 is sufficient to narrow the ER-OMM distance, whereas independent versus combined deletions of these two proteins demonstrate their interdependence for MERCS formation. Furthermore, PDZD8 enhances mitochondrial complexity in a FKBP8-dependent manner. Our results identify a novel ER-mitochondria tethering complex that regulates mitochondrial morphology in mammalian cells.

7. Design of Aromatic Interaction Networks in a Protein Cage Modulated by Fluorescent Ligand Binding

Hishikawa Y, Suzuki T, Maity B, Noya H, Yoshizawa M, Asanuma A, Katagiri Y, Abe S, Nagatoishi S, Tsumoto K, and Ueno T.

Dynamic behavior of proteins, such as orientation changes of aromatic residues, plays an important role in controlling biomolecular functions. Protein design that can precisely control such dynamic behavior at the atomic level is a challenging issue. The study reports the development of a system capable of orientational changes of aromatic side chains upon ligand binding. Aromatic pockets are constructed on the inner surfaces of protein cages to bind polycyclic aromatic fluorescent molecules to the targeted position by π-π stacking interactions. X-ray crystal structural analysis indicated the cooperative orientation changes of the aromatic clusters around the pocket triggered by the ligand binding. A comparison of various ligands shows that the movement of aromatic clusters can be controlled depending on the ligand structures. Fluorescence quantum yield and fluorescence lifetime are enhanced due to isolation of the fluorescent molecules in an aromatic pocket. These findings provide an understanding of the unique molecular behavior and fluorescence properties of ligands due to the assembly of aromatic residues and a guideline for developing dynamically controlled supramolecular biomaterials.

8. Antiviral potential of proton-type zeolite

Kimura Y, Takemoto M, Nakamura N, Ichinohe T, Nakakido M, Iyoki K, Ohta S, Miyamae N, Egami Y, Sato K, Okubo T, Tsumoto K, and Wakihara T.

This study reports the potent antiviral ability of proton-type zeolites without the aid of any metal cations. Antiviral activities of zeolites with different topologies and chemical compositions are investigated using M13 phage and influenza virus. Proton-type zeolites exhibit excellent antiviral activity, equivalent to 99% inactivated. Antiviral tests using a centricon device suggest that direct contact of viruses on the external surface of zeolites is required to inactivate the viruses. This discovery is of importance in an interdisciplinary research field covering both zeolite science and virological science and shed light on the possibility of the development of low-cost and environmentally friendly antiviral zeolites.

9. Accelerating antibody discovery and optimization with high-throughput experimentation and machine learning

Matsunaga R and Tsumoto K.

The integration of high-throughput experimentation and machine learning is transforming data-driven antibody engineering, revolutionizing the discov-

ery and optimization of antibody therapeutics. These approaches employ extensive datasets comprising antibody sequences, structures, and functional properties to train predictive models that enable rational design. This review highlights the significant advancements in data acquisition and feature extraction, emphasizing the necessity of capturing both sequence and structural information. We illustrate how machine learning models, including protein language models, are used not only to enhance affinity but also to optimize other crucial therapeutic properties, such as specificity, stability, viscosity, and manufacturability. Furthermore, we provide practical examples and case studies to demonstrate how the synergy between experimental and computational approaches accelerates antibody engineering. Finally, this review discusses the remaining challenges in fully realizing the potential of artificial intelligence (AI)-powered antibody discovery pipelines to expedite therapeutic development.

10. The Structural Fingerprint of Therapeutic Monoclonal Antibodies Determined Using a Combination of Near-UV Circular Dichroism and Statistical Approach for Comparative Analysis

Kiyoshi M, Oyama T, Shibata H, Suzuki S, Higuchi Y, Tsumoto K, and Ishii-Watabe A.

The higher-order structure (HOS) of therapeutic monoclonal antibodies is one of the important quality characteristics in terms of efficacy and safety. However, the HOS of therapeutic monoclonal antibodies has been assessed using low- to medium-resolution spectroscopy. Furthermore, because the analysis of the obtained spectra has been performed by visual evaluation in many cases, their specificity, structural similarity, and statistical significance have not been thoroughly discussed. Therefore, there is an increasing need for advanced, statistically comparable, and highly specific analytical methods for the HOS of therapeutic monoclonal antibodies. Herein, we describe an analytical method for the HOS of therapeutic monoclonal antibodies using a powerful combination of near-UV circular dichroism (CD) and statistical analysis. The obtained near-UV CD spectra of 14 therapeutic monoclonal antibodies were employed for principal component analysis. Moreover, the spectral data were converted into Euclidean distances to perform equivalence tests and significance tests. The results clearly demonstrated that each antibody had a unique near-UV CD spectrum, like a structural fingerprint. All antibodies were judged to be not equivalent to other antibodies and were also judged to be significantly different from other antibodies. Moreover, the equivalence tests were performed on several lots of antibodies, and each lot of the antibodies were judged to be equivalent. We believe that our methods are useful for identity testing and also for comparative analysis of HOS of therapeutic monoclonal antibodies.

11. Development of a small compound that regulates the function of a maltodextrin-binding protein of Streptococcus pyogenes by multi-faceted screenings

Yamawaki T, Nakakido M, Nagatoishi S, Caaveiro JMM, Kuroda D, Aikawa C, Nakagawa I, and Tsumoto K.

Group A Streptococcus (GAS) are gram-positive bacteria that cause various symptoms. The treatment of GAS infections currently relies on antibiotics, but new treatment options are needed due to the spread of antibiotic resistance. To develop novel treatment methods that circumvent the generation of antibiotic resistance, we used virtual screening followed by several biophysical-based screening methods to identify antibacterial compounds that target SPs0871, which is a maltodextrin-binding protein that is involved in carbohydrate catabolism in GAS. We narrowed down the list of compounds in the library via multi-step screening and finally isolated a compound that bacteriostatically inhibited the growth of GAS. Together with our previous study showing that an anti-SPs0871 variable heavy domain of heavy chain antibody, which completely blocked ligand binding, did not suppress bacterial growth, our results provide guidelines for designing an antistreptococcal therapeutic.

12. Developing FGF2 Mutants with Selectively Reduced Heparan Sulfate Affinity to Explore their Impact on FGFR1 Signaling

Okada Y, Eguchi A, Kuroda D, Tsumoto K, Ueki R, and Sando S.

Fibroblast growth factor 2 (FGF2) regulates signal transduction by forming complexes with its receptors, FGF receptors (FGFRs), and heparan sulfate (HS), playing a crucial role in biological systems. Although HS has been suggested to modulate FGF/FGFR signaling as a coreceptor, multiple hypotheses exist regarding how HS affects FGF/FGFR signaling and the mechanism remains unclear. Herein, to highlight the role of FGF2/HS interaction in FGF2/FGFR1 signaling, FGF2 mutants with reduced HS-binding affinity are rationally designed through in silico analysis. These FGF2 mutants exhibit reduced HS affinity by more than two orders of magnitude while maintaining binding affinity to FGFR1. In addition, these mutants retain their thermal stability. Cellular assays using the FGF2 mutant suggest that, contrary to previous reports, the contribution of the FGF2/HS interaction in FGF2/FGFR1 signaling may be limited. The mutant FGFs that specifically alter the interaction

with HS, achieved in this study, would contribute to an understanding of the role of FGF/HS interaction in FGF/FGFR signaling.

13. PRELP functions via multiple interactions with intrinsically weak affinity relying on ECM anchoring and remodeling

Kosuge H, Nakakido M, de Vega S, Ohnuma SI, and Tsumoto K.

A small leucine-rich repeat proteoglycan PRELP is responsible for various biological functions. Here, to quantitatively assess the ligand binding of PRELP and its relevance to physiological activities, we validated the premise that PRELP multi-specifically binds to TGFβ1, IGFI-R, and p75NTR with relatively weak, micromolar range of affinities using surface plasmon resonance analysis. Results of a direct binding assay using N-terminal-truncated PRELP and chimeric PRELP and a dual injection assay to evaluate the binding regions and competitiveness suggested that PRELP interacts with the ligands via different but partially overlapping regions in the leucine-rich repeat domain. RNA-seq analysis revealed that PRELP greatly promotes gene expression of various extracellular matrix (ECM) components in A549 lung carcinoma cells, also at micromolar concentration. Since we reasoned that ECM anchoring contributes to an increase of apparent local concentrations of PRELP required for the weak affinity interactions, we validated the direct binding and co-localization of PRELP with ECM proteins using ELISA analysis and immunofluorescence staining. Results of this study suggest that PRELP modulates multiple interactions with intrinsically weak binding affinities through the anchoring to ECM proteins and also promotes the ECM protein expression to maintain the preferred environment to exert the molecular functions.

14. Improving the solubility of single domain antibodies using VH-like hallmark residues

Uto Y, Nakakido M, Yokoo T, Fernandez-Perez J, Entzminger K, Maruyama T, Okumura CJ, Kuroda D, Caaveiro JMM, and Tsumoto K.

Single domain antibodies (sdAbs) can be generated from variable regions of heavy-chain antibodies, which lack light chain and CH1 region. They have attracted attention due to their small size and molecular characteristics. Hydrophilic hallmark amino acids at framework region 2 (FR2) are key residues involved in the solubility of sdAbs. Nevertheless, previous studies reported that several sdAbs with human VH-like hydrophobic hallmark residues were soluble in a monomeric state and suggested that solubility also depends on the amino acid sequences in the complementarity-determining region. In this study, we obtained two sdAbs (sdAb A and B) with VH-like hallmark residues and low solubility from an alpaca immune library. We introduced VHH-like mutations (V37Y, G44E, L45R, W47L) into the hallmark residues in FR2 of both sdAb A and B. We were able to prepare sdAb A as a monomer without an additive in the buffer, but sdAb B was polydispersed when arginine was not added to the buffer. We also predicted the hydrophobicity of the sdAb B surface by spatial aggregation propensity calculations and identified W99 as the residue responsible for its low solubility. Subsequently, we obtained the sdAb B mutant as a monomer by introducing the W99A mutation. We characterized the engineered sdAbs using structural, physicochemical, and biophysical analyses and found that the solubility-improved sdAbs retained their functionality. Our findings can be applied to improving the solubility of sdAbs even in the absence of structural information.

15. The hook-like adaptor and cargo-binding (HAC) domain in the kinesin-2 tail enables adaptor assembly and cargo recognition

Jiang X, Danev R, Ichinose S, Niu B, Ohtsuki S, Yanagisawa H, Nagatoishi S, Tsumoto K, Hirokawa N, and Kikkawa M.

Intracellular transport relies on motor proteins such as kinesins to deliver cargo along microtubules, yet how they recognize cargo remains unclear. Here, we present high-resolution cryo-electron microscopy structures of the heterotrimeric kinesin-2 complex (KIF3A/KIF3B/KAP3) bound to the cargo protein APC. Our findings reveal a previously uncharacterized KIF3 tail hook-like motif, termed the "HAC" domain, which mediates binding to both KAP3 adaptor and APC cargo. Within this domain, the KIF3A helical regions ensure cargo specificity, while a β-hairpin and KIF3B provide structural support. Biochemical and neuronal experiments confirm its functional importance. Notably, the HAC/KAP3 structure resembles hook-like architectures seen in kinesin-1 and dynein, suggesting a shared cargo recognition framework. These findings also shed light on kinesin-2 cargo specificity and offer a structural framework for understanding related neuronal transport mechanisms.

16. Megalin-Mediated Direct and Indirect Pathways for the Development of Contrast-Induced Proximal Tubule Epithelial Cell Injury

Goto S, Hosojima M, Kabasawa H, Takemoto K, Aoki H, Komochi K, Kobayashi R, Sugita N, Endo T, Kuwahara S, Tanaka T, Kaseda R, Nagatoishi S, Yoshida Y, Narita I, Yamamoto S, Tsumoto K, Hirayama Y, and Saito A.

Megalin is a multiligand endocytic receptor in proximal tubule epithelial cells (PTECs) that facilitates the reabsorption of certain nephrotoxic drugs. Notably, cilastatin competes with these drugs for binding to megalin. Patients with chronic kidney disease are at increased risk of contrast-induced (CI) acute kidney injury, in which iodine-mediated PTEC toxicity is a crucial pathogenic factor. This study investigated the interplay between megalin and CI-PTEC injury, along with the protective effect of cilastatin, utilizing electron probe microanalysis, a mouse CI-PTEC injury model, quartz crystal microbalance analysis, and binding assays with magnetic beads immobilized with iomeprol, a water-soluble nonionic contrast medium (CM). Electron probe microanalysis revealed the reduced renal uptake of CMs in the kidney cortex of kidney-specific conditional megalin-knockout mice compared with controls. In the CI-PTEC injury model using kidney-specific conditional or mosaic megalin-knockout mice, a significant reduction of vacuolization was observed in PTECs in the cortex compared with controls in the former, while co-expression of megalin and kidney injury molecule-1 was observed in the latter. These findings indicate that megalin is involved in the development of CI-PTEC injury. Cilastatin inhibited the renal uptake of CMs and ameliorated CI-PTEC injury in wild-type mice. Of note, two distinct pathways of renal CM uptake were delineated, both of which cilastatin inhibited: the direct binding of CMs to megalin and an indirect route involving transcobalamin-2, an endocytic megalin ligand, functioning as a CM carrier. Collectively, megalin governs the direct and indirect pathways in the development of CI-PTEC injury, and cilastatin effectively impedes both.

17. Development of a VHH that inhibits the binding of neuronal pentraxin 2 to a postsynaptic glutamate receptor, AMPAR

Yokoo T, Nakakido M, Matsuda K, Caaveiro JMM, Fernandez-Perez J, Yuzaki M, and Tsumoto K.

Neurons connect to each other via synapses to form neural circuits. Recent research has shown that neuropsychiatric disorders and neurological disorders, such as autism spectrum disorders and Alzheimer's disease, are synaptic diseases caused by abnormalities of synapses. Synaptic organizers are molecules responsible for synapse formation. Neuronal pentraxin 2 (NP2) is a synaptic organizer and a secreted protein that is expressed mainly in the hippocampus and cerebellum, and it contributes to synaptic plasticity. NP2 forms clusters with its family proteins, NP1 and neuronal pentraxin receptor, and binds to postsynaptic amino-3-hydroxy-5-methyl -4-isoxazolepropionic acid-type receptors. In recent years, research has revealed the disease relevance of NP2. For example, it can be a biomarker of Alzheimer's disease, and its overexpression in the peripheral nervous system has been reported to cause chronic itch. However, the mechanism of NP2 function has not been well described at the molecular level. In this study, we developed a variable domain of a heavy-chain antibody (VHH) against NP2 to elucidate its molecular mechanism of action and to regulate its function of NP2. The obtained VHH N1 showed high specificity and affinity to NP2, and its binding mechanism was elucidated by X-ray crystallography. Furthermore, VHH N1 inhibited the binding of NP2 to amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-type receptors, and this inhibitory activity was confirmed in cells. These results provide useful insights into the molecular mechanism of NP2 function and highlight the potential application of VHH N1 as a detection agent for NP2 or as a therapeutic agent for chronic itch.

18. Solubilization and refolding of inclusion bodies by detergents

Arakawa T, Akuta T, Ejima D, and Tsumoto K.

Recombinant proteins play many important roles in development of biological reagents and biopharmaceuticals. Here, we will mainly review refolding of recombinant proteins when expressed in inclusion bodies, although strategies to enhance soluble expression are described as an alternative to refolding inclusion bodies. These strategies include, but not limited to, adding chemical chaperones in cell culture media, modifying cell lysis buffer and using solubility-enhacing fusion tags. Another solubility enhancement was to generate lipid complex for membrane proteins that form insoluble proteins without lipid. Among various solubilization and refolding technologies, those using denaturant, alkaline pH and pressure are also desribed, while we focus on solubilization and refolding using detergents, which are effective and cost-friendly. Sodium dodecylsulfate, lauroyl-glutamate, sarkosyl and cetyltrimethylammonium have been extensively used, as summarized in this review. Slow or step-wise removal of denaturants or ionic detergents used to solubilize appears to play a critical role in successful refolding by maintaining the solubility of proteins during refolding. In alkaline refolding, slow pH adjustment also helps maintain protein solubility. In pressure refolding, small amount of guanidine hydrochloride assisted refolding.

19. Structural basis for heme binding by the Shr protein from Streptococcus pyogenes

Seki K, Senoo A, Nagatoishi S, Yanaka S, Nakakido M, Tsumoto K, and Caaveiro JMM.

Streptococcus pyogenes causes a range of infectious diseases. In an era of increasing antibiotic resist-

ance, new antimicrobial strategies targeting virulence factors, rather than essential survival mechanisms, are being explored. A key virulence factor in S. pyogenes is the bacterial iron acquisition system, because iron is essential but limited in the host due to sequestration by proteins like hemoglobin. The bacteria S. pyogenes possesses the Shr protein that acquires heme from host hemoglobin and transfers it to Shp, a membrane proximity protein. Shr comprises multiple domains, including two NEAr-Transporter (NEAT) domains that directly bind to heme. While structural information of NEAT domains from other bacteria are available, the structure of NEAT domains from Shr remains unknown. In this study, crystal structures of Linker-NEAT1 and NEAT2 domains were determined to 2.35 Å resolution and 2.66 Å resolution, respectively. Structural and mutational analyses revealed that methionine residues play a key role in heme binding, which seems to be a characteristic of heme-binding proteins from S. pyogenes, but not of NEAT domains from other gram-positive species. These findings enhance our understanding of heme acquisition in S. pyogenes and may guide novel therapeutic approaches.

20. myCRP, Cysteine-Rich Protein in the Yesso Scallop Mizuhopecten yessoensis, Is a Novel High-Molecular Weight Cd-Binding Protein

Zheng Z, Namikawa Y, Awaji M, Kato Y, Iimura K, Nakakido M, Tsumoto K, Negishi L, Kurumizaka H, Homma-Takeda S, and Suzuki M.

Heavy metal bioaccumulation in marine organisms is a critical concern for the oceanic environment and food safety. Scallops, as filter-feeding bivalves, accumulate cadmium (Cd) in their midgut gland to toxic concentrations that may pose risks to human health. Despite extensive research on Cd bioaccumulation in the Yesso scallop Mizuhopecten yessoensis, a major Cd-binding protein has not yet been functionally characterized. In this study, we used high-performance liquid chromatography coupled with inductively coupled plasma mass spectrometry (HPLC-ICP-MS) to isolate Cd-binding proteins, and identified myCRP, a novel high-molecular-weight Cd-binding protein. myCRP contains a cysteine-rich sequence, resembling metallothionein Cd-binding motifs, and is expressed exclusively in the midgut gland. Cd-binding assays demonstrated that myCRP binds Cd in a dose-dependent manner. Immunostaining revealed the presence of a small cluster of myCRP-containing cells in the connective tissue surrounding digestive tubules in the midgut gland. Synchrotron radiation X-ray fluorescence (SR-XRF) analyses showed the distribution of Cd throughout the entire midgut gland tissue. We conclude that myCRP is partly responsible for Cd binding in the midgut gland. Our findings clarify a new mechanism of Cd-binding in marine organisms from both biological and molecular perspectives.

<Group III>

1. Development of new methods for analyzing neural circuits in the retina

Neural circuits in the central nervous system are the basis of various higher-order brain functions. It is also true in case of retina. In the retina, six main classes of neural cells connect systematically to make up complex neural circuits. Characteristics of the retinal neural cells have been examined mainly by the electrophysiological methods and models of cell connectivity have been proposed. Morphological studies of the actual neural connection, which constitute the physiological properties of retinal neurons, have been desired. Until recently the only method to reveal the three-dimensional (3D) connectivity of actual neural cells morphologically was to collect ultrathin serial sections and observe them in transmission electron microscope (TEM). But the technical difficulties discouraged us from such a troublesome procedure. Recent progress in scanning electron microscope (SEM) equipment allowed us to develop a new method to observe ultrathin TEM sections in SEM (thin section scanning electron microscopy: TSSEM) and construct serial section images into three dimensional structures (SEM array tomography). To collect huge number of serial sections stably and efficiently, we have been developing new equipment and techniques. By using these techniques, it became possible to collect more than 1000 serial sections of less than 30 nm thickness much easier. We have analyzed about 500 serial thin sections of zebrafish retinal outer plexiform layer by this method and succeeded in tracing thin processes of bipolar cells into the photoreceptor terminals. In fish retina, four kinds of color specific cone photoreceptors are arranged in a very regular pattern, forming cone mosaics. Therefore, the first step of color recognition circuit is expected to be morphologically analyzed by using SEM array tomography method.

The SEM array tomography method developed here was expanded to be used in analyzing mitochondrial 3D structure in neural stem cells as a collaborative work. Aside from getting 3D information, TSSEM method can provide us precise information of much wider areas of thin sections more effectively and more easily than transmission electron microscopy. Such studies are also in progress.

2. Collaborative and supportive works as electron microscope core-laboratory

This group is also engaged in collaborative works using electron microscope. We offer supports for the research projects those need electron microscopic

analysis. The services available in this group are the conventional thin section transmission electron microscopy, immuno-electron microscopy, negative staining techniques and scanning electron microscopy. By using individual technique or combination of some of these, we can offer direct visual evidence that cannot be acquired by other methods. This year, 14 projects in 10 laboratories were performed as core-laboratory works.

a. Thin section transmission electron microscopy

Thin section transmission electron microscopy is the most widely used technique to observe the inner structure of cells and tissues. In this method, samples are fixed and embedded in epoxy resin, thin sections of about 70 nm thickness are cut and observed in the transmission electron microscope. In case of immuno-electron microscopy, thin sections are obtained by similar procedure and the antigen epitopes exposed on the surface of the sections are marked by sequential reaction with appropriate primary antibodies and colloidal gold labeled secondary antibodies. This year, thin section electron microscopy and those combined with immuno-electron microscopy were used in many collaborative works.

a-1. Ultrastructural analysis of entry and assembly of Herpes Simplex Virus

We have been performing several studies with research groups in Dr. Kawaguchi[1]'s laboratory: [1]Division of Molecular Virology, Department of Microbiology and Immunology, regarding the infection/replication processes of herpes simplex virus (HSV). Thin section electron microscopy has been used to analyze the function of viral proteins in trans-nuclear membrane processes of the newly formed viruses. By analyzing the virus forming processes in some mutant host cells, we could analyze viral proteins as well as candidate host molecules those may be involved in the trans-nuclear process of the HSV. TSSEM method was also used to observe virus infected whole single cell and three-dimensional distribution of newly formed viruses are tried to be analyzed.

a-2. Other collaborative works using transmission electron microscope

Some other collaborative research works using thin section electron microscopy and/or immuno-electron microscopy were performed with Dr. Takekawa[2] in [2]Division of Cell Signaling and Molecular Medicine, about the uneven distribution of nuclear pore in cultured cells and changes of the pore size in relation to culture conditions, Dr. Iwama[3] in [3]Division of Stem Cell and Molecular Medicine, about the fine morphology of stem cells, Dr. Kono[4] in [4]Laboratory of Health Chemistry, Graduate School of Pharmaceutical Sciences, about the effects of aberration in lipid molecule synthesis on cellular structure, Dr. Nakahara[5] in [5]Department of Life Science Dentistry, The Nippon Dental University, about the structure of dental stem cells, Dr. Katayama[6], in [6]Laboratory of Viral Infection, Ohmura Satoshi Memorial Institute, Kitasato University, about the fine structure of SARS-CoV-2 infected culture cells and Dr. Watanabe[7] in [7]Course of Applied Marine Biosciences, Graduate School of Marine Science and Technology, Tokyo University of Marine Science and Technology, about the mitochondrial structure in freeze-thawed fish muscle cells.

b. Negative staining techniques

Negative staining techniques are simple and quick method to observe the morphology of macro-molecules such as viruses, exosomes and protein particles. This year, negative staining techniques were used to analyze newly developed adjuvant structure in collaboration with Dr. Hayashi[8] in [8]Division of Vaccine Science, Laboratory of Adjuvant Innovation.

c. Scanning electron microscopy

Conventional scanning electron microscopy is a technique used to examine surface structure of the cells, tissues or other non-biological materials. Thin section scanning electron microscopy (TSSEM) is a recently developed method to observe sectioned cells or tissues in scanning electron microscope. Serial thin section images collected with TSSEM method can construct into three-dimensional (3D) structures (SEM array tomography).

This year, conventional scanning electron microscopy was used to observe surface structure of inner retina as a collaborative work with Dr. Yamagami[9], [9]Division of Ophthalmology, Department of Visual Sciences, Nihon University School of Medicine.

SEM array tomography methods are used as a collaborative work with Dr. Kobayashi[10], [10]Division of Protein Metabolism, to analyze the 3D organization of mitochondria in neural stem cells in various conditions.

Publications

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