1P-191 フォルミルペプチド受容体 (Gタンパク質共役型受容体) は、生理学的な条件下で2量体を形成する: 一分子追跡法による研究
Dimers of formyl peptide receptor, a GPCR, exist under physiological conditions; a study using single-molecule tracking

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Dimerization and oligomerization of receptor molecules in the plasma membrane are often the key step for triggering intracellular signaling responses. In the present research, we developed a single-molecule tracking method for counting the number density of monomers and dimers (after subtracting the number density of coincidentally overlapping spots). Using this method, we found that 57% of CD28 conjugated with GFP, a dimer-control molecule, are in dimers or 72% of the GFP is fluorescent if all of the CD28-GFP were in dimers. We then quantitated the dimer fraction of formyl peptide receptor (FPR), a G-protein coupled receptor (GPCR) responsible for chemotaxis of neutrophils and other immune cells, in the plasma membrane of the living cell that expresses FPR at physiological levels (~6000 copies per cell). The presence of GPCR dimers has been a subject of controversy in recent years, mostly due to the lack of quantitative analysis and the use of overexpression conditions. Non-internalization mutant of FPR, D71A-FPR, was bound by the ligand labeled with Alexa594 dye precisely at the 1:1 mole ratio, exhibiting the dissociation constant of 1.8 nM, consistent with the published values. Approximately 30% of D71A-FPR molecules were found to be in dimers. Using the wild-type FPR conjugated with GFP, and assuming the actually fluorescent fraction of GFP of 72% based on the CD28 data, we found that approximately 28% of wild-type FPR molecules are in dimers, in good agreement with the D71A-FPR data.

1P-192 細胞内フラジェリンの生化学的特性
Biochemical characteristics of intracellular flagellin

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Terminal regions of flagellin (FliC), which constitutes the flagellar filament, can have two thermostable, reversible conformations (active- and inactive-form) albeit these regions are unfolded in the monomeric form. The polymerization ability of inactive-form produced by interaction with HAP3 is much higher than that of active-form. Results of conformational analysis and the polymerization ability of FliC isolated from various transport stages suggest that the switching from active- to inactive-form is caused by the interaction with HAP1/3 of the hook-filament junction. These results suggest that the local conformational states of FliC modify the polymerization efficiency and that the central channel is not a mere tunnel but has a gate function. Flagellar protein export apparatus is another gate for FliC transport. The local conformational change of FliC may also occur during the passage through this gate. We therefore analyzed CD spectra and polymerization ability of FliC before passing through the export gate, i.e., intracellular FliC. Interestingly, we found that the conformation of FliC overproduced from plasmids differs from batch to batch of purified specimen. The reason for this unstable conformation is not clear, although it could be due to misfolding by overproduction. Or, intracellular FliC may be intrinsically sensitive to slight changes in the intracellular environment. We will present the data on overproduced FliC and genome-produced FliC from a fliS mutant, and discuss biochemical characteristics of intracellular FliC.

1P-193 細胞膜において上皮成長因子受容体が形成する階層的クラスタリングの高分解能解析
A High-resolution Analysis of the Hierarchical Clustering of Epidermal Growth Factor Receptor in the Cell Membrane

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Epidermal growth factor receptor (EGFR), which is responsible for cell proliferation, transduces signals forming homo-dimers in the cell membrane. Several studies have suggested the presence of EGFR clusters larger than dimers even before EGF binding. However, the role of the EGFR clustering in the signal transduction has been still unclear. We are exploring the EGFR clustering by imaging single-molecules of fluorescent protein (FP) fused with EGFR in the cell membrane. The fluorescence intensities of EGFR-FP clusters were measured for quantification of receptors in the clusters. But the intensities of monomer and oligomers widely distributed and could not be distinguished clearly by effects of overlapping of fluorescence spots and large deviation of single-fluorophore intensities. To overcome the former problem, we used total internal reflection microscopy (TIRF) with 2D deconvolution processing to improve resolution up to 170nm, 75% of diffraction-limited resolution. For further improvement, we are applying fluorescence photoactivation localization microscopy (FPALM) on novel photo-convertible FP, mKikGR, fused to EGFR. As reported in other studies, FPALM can increase the resolution dramatically to several-tenth of nanometers. Moreover, we can digitally count the receptors in a cluster by activating molecules one-by-one to avoid the problem of fluorescence deviation.

Properties and mechanism of EGFR clustering, such as dependency on EGFR density in the cell membrane and the effects of ligand binding, will be discussed.

1P-194 二重蛍光蛋白質標識による MAP4 アイソフォームの細胞内局在観察

Subcellular localizations of MAP4 isoforms analyzed by double fluorescent labeling

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Coordinated movements of microtubules and filamentous actin (F-actin) are thought to be required in the protruding and the retracting of neuronal processes. The interaction between microtubules and F-actin has been postulated to be mediated by molecules binding the two cytoskeletons. Recently, we identified a neuron-specific isoform, named SP, of microtubule-associated protein 4 (MAP4), and reported that SP as well as a ubiquitously expressed MAP4 isoform, LP, can bind to F-actin *in vitro* (45th annual meeting), raising the possibility that MAP4 variants can regulate the interaction between microtubules and F-actin in neuron. Here, we have engineered SP and LP constructs fused with fluorescent proteins, and analyzed with their localization in the growth cone of a single NG108-15 cell. We found that both SP and LP were concentrated in the central region with a dense microtubule network; SP was relatively more abundant. Since we have reported that SP is less potent in stabilizing microtubules, the concentration of SP in this region may contribute to the dynamic microtubule reorganization. Actin-microtubule double staining was also performed using SP or LP constructs fused with green fluorescent protein and an actin construct fused with DsRed-Monomer. The LP-associated microtubules and F-actin were found almost exclusively in the growth cone, while a few microtubules which associated with SP sometimes invaded actin-rich regions. MAP4-SP may mediate the interaction between microtubules and F-actin in this region.

1P-195 AFM Study of Mycoplasma mobile's Gliding Motility.
AFM Study of Mycoplasma mobile's Gliding Motility.

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Mycoplasma mobile (M. mobile) are parasitical bacteria that lack peptidoglycan layer but still present recognizable flask-shape cell morphology. One of their most striking features is the ability to glide along cell or glass surfaces at an average speed of 2.0 to 4.5 $\mu\text{m/s}$ towards the tapered end of the cell called the head. However, the genome of M. mobile showed no evidence of known motility systems such as flagella or pili, nor expression of any classic eukaryotic motor protein. Recent studies have identified four proteins Gli23, Gli349 and Gli521 that are involved in this novel gliding system, and a model for gliding motility has been presented, but more experimental data are needed to confirm the arrangement and precise role of each protein in this system. In this study, we propose a novel approach to investigate the gliding motility system of M. mobile using an AFM (Atomic Force Microscope) both as an imaging and a force measurement device. AFM Images of biotinylated M. mobile cells were obtained through immobilization on a streptavidin modified mica surface. Pictures showing the morphology of individual Gli349 and Gli521 molecules in dried and liquid conditions were also obtained and were consistent with previous electron micrographs of the proteins. Investigation of the interaction between Gli molecules and sialic acid on various sample surfaces was also conducted using an AFM tip modified with Gli349 or Gli521 molecules and the results showed specific interaction for Gli349, whereas Gli521 did not show any interaction.

1P-196 細胞接着パターンニングによる細胞移動挙動の変化

Spatial control of cell adhesion by micro-patterning regulates cell locomotion behavior

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Cell movement requires the spatio-temporal reorganization of cytoskeletal actin structure such that the formation of new adhesions and protrusion occur at the front of the cell while detachment and retraction occur at the rear. In addition, cell movement is affected by assembly and disassembly of focal adhesions which mediate between the substrate and the cytoskeleton. Therefore, controlling the distribution of cell adhesions is expected to lead to the regulation of cell locomotion. In this study, we utilized the technique of micro-patterning to investigate the effect of controlling cell adhesion on the locomotion of fish epidermal keratocytes. We designed a PDMS stamp, and used it to transfer cell-scale square arrays of fibronectin to glass substrates to spatially separate protrusion and retraction during cell locomotion. It was observed that the geometrical constraints imposed on the cell by the patterns induced periodic changes in cell morphology, such that cells exhibited crescent and elongated shapes alternately, whose frequency correlated with that of periodic increase in intercellular fluorescent intensity of calcium indicator. This illustrates that retraction, as defined by morphological changes, was triggered by the increase in calcium concentration during cell translocation from one square pattern to the next. This shows that spatial control of cell adhesion by the micro-patterning can regulate the calcium influx, thereby regulating cell locomotion through the modulation of cytoskeletal actin structure remodeling.