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Direct Observation of Ligand Colocalization on Individual Receptor Molecules

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ABSTRACT We have exploited the novel methodology of far-field fluorescence microscopy at the single molecule level to study colocalization of two different ligand molecules on an individual receptor. The use of dual-wavelength single molecule imaging allows discrimination between isolated and colocalized ligands with an accuracy of 40 nm. In the case of very close proximity of the two ligands, below 7 nm, single pair Förster energy-transfer was observed. The latter finding unequivocally demonstrates colocalization of two ligands on an individual receptor.

INTRODUCTION

Molecular signaling is mediated by a myriad of interactions between proteins and ligands. Typically, the specific binding of one or more ligand molecules induces a conformational change of a protein. A detailed description of this process requires time-resolved information on receptor-ligand and ligand-ligand interactions. Conventional biochemical and immunochemical assays using optical microscopy have been developed in a large variety in order to study such association phenomena. However, the inherent structural and temporal inhomogeneities of biological samples largely limit those methods to the determination of ensemble averages, thereby masking the broad distribution of molecular parameter. For a much more detailed picture of dynamic processes in biosystems, optical assays have been developed recently, which are based on the observation of individual molecules (Soper et al., 1991; Betzig and Chichester, 1993; Xie and Dunn, 1994; Eigen and Rigler, 1994; Nie et al., 1994). Properties of motor molecules (Funatsu et al., 1995), the dynamics of lipids in phospholipid bilayers (Schmidt et al., 1996), the dynamics of a molecular rotor (Noji et al., 1997), and the conformational change in oligonucleotides (Ha et al., 1996a; Wennmalm et al., 1997) are examples of the power of single molecule techniques. In the current report we have determined (dynamic) colocalizations of two types of ligands on a single receptor molecule, the latter being anchored to a phospholipid membrane. Highly sensitive far-field video-rate fluorescence microscopy was used for the detection of ligand co-binding. Our far-field approach complements a similar approach by near-field optical microscopy and spectroscopy for the detection of oligonucleotide hybridization (Ha et al., 1996b) and colocalization of two membrane proteins (Enderle et al., 1997). The lateral accuracy of ~40 nm, which can be obtained solely by single molecule techniques, provides sufficient reliability for the detection of colocalization at low concentrations. The unequivocal test for very close proximity of a few nanometer was finally achieved by detection of single-pair resonant energy transfer.

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MATERIALS AND METHODS

Materials

The protein streptavidin (Sigma) was immobilized on supported phospholipid membranes of 1,2-dimyristoyl-sn-glycero-3-phosphocholin (Sigma) at a surface density $<0.1\ \mu m^{-2}$ using standard Langmuir-Blodgett techniques. Membranes were incubated with 50 nM of a mixture of two biotin-fluorophore conjugates (64% biotin-poly(ethylene glycol)(800)-tetramethylrhodamine (TMR), 36% biotin-poly(ethylene glycol)(1900)-Cy5) for about 10 s. The fluorescent conjugates have been synthesized in our group (Gruber et al., 1997). No binding of the biotin-poly(ethylene glycol)-fluorophores to the phospholipid membranes was observed when the preincubation step with streptavidin was omitted.

Microscopy

Samples were observed while illuminated alternately for 5 ms by 12 ± 3 kW/cm^2 with 514 nm of light from an Ar$^+$-laser (C306, Coherent Radiation, Mountain View, CA) and with 630 nm of light from a dye-laser (CR599, DCM dye, Coherent Radiation) using a ×100 objective (Plan-Neofluar, NA = 1.3, Zeiss, Oberhausen, Germany) in an epifluorescence microscope (Axiovert 135TV, Zeiss). The fluorescence was effectively separated from scattered light by appropriate filter combinations (custom made dichroic and emission filters, Omega, Brattleborough, OR). Cross talk between both wavelength channels was smaller than 5%. The fluorescence was collected on a liquid-nitrogen cooled slow-scan CCD-camera system (AT200, Photometrics, Tuscon, AZ) equipped with a TK512CB-chip, (Tektronix) and stored on a personal computer (Schmidt et al., 1995). Seven frames were averaged for a total illumination time of 35 ms per observation, yielding a signal of 2000 ± 500 counts and 1600 ± 400 counts for the TMR- and Cy5-labeled biotin molecules, respectively. At a background noise of 56 and 40 counts RMS for green and red illumination, respectively, these signals permitted to detect individual molecules at a signal-to-background noise ratio of S/B ~40. Subsequent data analysis determined the lateral position of each signal to within 29 nm by fitting the fluorescence intensity profile to a two-dimensional gaussian surface (Schmidt et al., 1996). The distance between two objects can thus be determined to a $\sqrt{2} \times 29$ nm = 41 nm accuracy. Systematical errors caused by mechanical instabilities of the set up and chromatic aberration were less than 20 nm over the whole field of view of $\sim 10 \times 10\ \mu m^2$ as

determined by imaging heavily stained and immobilized dual-color fluorescent latex beads.

RESULTS AND DISCUSSION

We investigated the highly specific ligand-receptor recognition between biotin and streptavidin (Green, 1990). Streptavidin is a protein with four binding sites for biotin, two of which were used to immobilize the protein on a supported phospholipid membrane. The streptavidin concentration on the membrane surface was adjusted to values smaller than one molecule per 10 μm^2. The membranes were observed in a wide-field fluorescence microscope capable of single molecule detection (Schmidt et al., 1996). Two different biotin-fluorophore conjugates were used as ligands, one with a rhodamine label (excited in the green at 514 nm) and the second with a cyanine label (excited in the red at 630 nm). After incubation with a mixture of both biotin-conjugates, the membranes were illuminated alternately by green (514 nm, Fig. 1 *A*) and red light (630 nm, Fig. 1 *B*). Three signal-types are distinguished in Fig. 1: one appears at both excitation wavelengths (signals on the right in Fig. 1, *A* and *B*) and the two others appear only in the green (left Fig. 1 *A*) and red (left Fig. 1 *B*), respectively. The first signal is of particular interest because it constitutes a candidate for colocalization of two different biotin ligands on a single streptavidin molecule as receptor. For a quantitative assignment of colocalization the lateral positions of the biotin-conjugates were determined (Schmidt et al., 1995). The distance between the green and red signal was 27 nm, which is below the lateral accuracy of the experiment of 41 nm. The latter value was determined by Monte Carlo simulations (2σ-criterion) of the imaging process for the signal-to-background ratio obtained in our experiments. The close proximity between the red- and the green-labeled ligand is a strong indication for colocalization of both ligand molecules on an individual receptor, taking into account that the probability for random colocalization of two receptors is negligible (the probability is calculated from the surface density of the receptor, n, and the actual positional accuracy of our experiment, $\Delta r = 41$ nm (Chandrasekar, 1943): $p = 1 - \exp(-\pi n \Delta r^2) = 5 \times 10^{-4}$).

In total 241 receptor-ligand complexes were observed in 60 experiments. On $p_{rg} = 20\%$ of the observed (fluorescence labeled) complexes colocalization of two ligands was found, $p_g = 54\%$ ($p_r = 26\%$) of the complexes carried green (red)-labeled ligands only. These values are in excellent agreement with the predictions based on the model of two independent binding sites on each streptavidin (Green, 1990)

$$p_g = \frac{r_g(2 - 2p_1 + p_1 r_g)}{2 - p_1},$$

$$p_r = \frac{(1 - r_g)(2 - p_1 - p_1 r_g)}{2 - p_1},$$

$$p_{rg} = \frac{2\, p_1 r_g (1 - r_g)}{2 - p_1}.$$

The factor $1/(2 - p_1)$ accounts for the occurrence of two unoccupied binding sites that are not observable. Considering the ratio between green-, $r_g = 0.64$, and red-labeled biotin, $r_r = 1 - r_g = 0.36$, determined spectrometrically, and a binding probability of $p_1 = 0.61$, the model yields $p_{rg} = 20\%$, $p_g = 54\%$, and $p_r = 26\%$. The binding probability, p_1, was estimated from the concentrations used (50 nM), the binding constants of the receptor-ligand pair ($K_D = 1$ nM, $k_{off} = 10^{-3}$ s^{-1} (Gruber et al., 1997)), and the incubation time (10 s).

Optical microscopy on individual molecules is the technique of choice for determination of in vivo molecular structures. Its exceedingly high positional accuracy is far

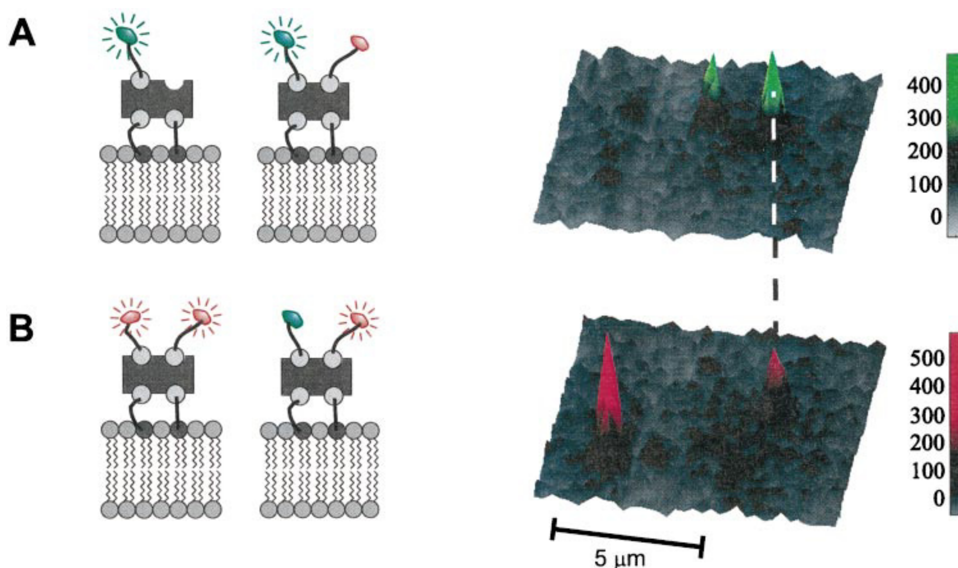

FIGURE 1 Dual-wavelength single molecule microscopy. (*A*), A 9.5×9.5-μm^2 area of a phospholipid membrane illuminated in the *green* (514 nm) for 35 ms. Two signals were observed, corresponding to rhodamine-labeled biotin molecules bound to individual streptavidin receptors. (*B*) The same area of the membrane was illuminated in the *red* (630 nm). Two signals were observed corresponding to cyanine-labeled biotins bound to streptavidin receptors. The signals on the *right* of *A* and *B* were colocalized ($\Delta r = 27$ nm).

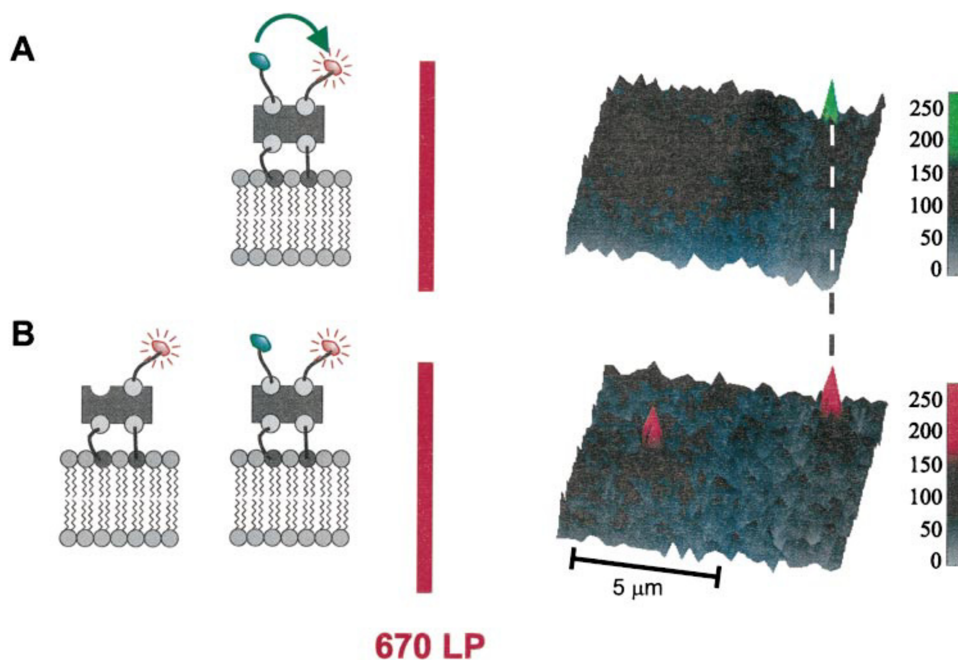

FIGURE 2 Ligand colocalization on an individual receptor molecule monitored by single-pair Förster energy transfer. (*A*) A 9.5 × 9.5-μm^2 area of a phospholipid membrane illuminated in the *green* (514 nm) for 35 ms. The signal observed corresponds to an acceptor ligand (cyanine) excited by its donor (rhodamine) via Förster energy transfer. Long-pass filtering (670LP) assured selective detection of the emission from the acceptor molecules only. (*B*) Image of the same area illuminated in the *red* (630 nm). In addition to the acceptor molecule excited directly, a second molecule on the *left* was observed without counterpart at *green* illumination.

below the Abbé-limit of typically 270 nm obtained in light microscopy and is only limited by the signal-to-noise ratio (Bobroff, 1986). The dual-wavelength approach presented here is a logical extension (Betzig, 1995) of one-wavelength experiments performed earlier (Betzig and Chichester, 1993; Xie and Dunn, 1994; Eigen and Rigler, 1994; Nie et al., 1994; Funatsu et al., 1995; Schmidt et al., 1995; Dickson et al., 1996; Noji et al., 1997). It allows determination of the spatial organization of multicomponent systems on length scales of ~40 nm. For determination of colocalization below 40 nm on molecular length scales, we have further designed a methodology based on single-pair Förster energy transfer. Its strong dependence on intermolecular distance (Förster, 1948; Stryer and Haugland, 1967) makes Förster energy transfer a very sensitive tool for the determination of colocalizations on the order of a few nanometers (Stryer and Haugland, 1967). The latter length scale ideally matches the size of many proteins.

Selective observation of the emission from an acceptor molecule (cyanine) during illumination of a donor (rhodamine) allows to test for energy transfer. We selected the acceptor emission by insertion of a 670-nm long-pass filter into the detection pathway, which effectively blocked (<5%) the donor fluorescence (maximum at 570 nm). Fig. 2 shows images of the same sample as used in Fig. 1. Again, the illumination was altered between green (Fig. 2 *A*, 514 nm) and red (Fig. 2 *B*, 630 nm) light. The observation of a signal when illuminated in the green (Fig. 2 *A*) provides unambiguous evidence for energy transfer between a biotin-rhodamine donor and a biotin-cyanine acceptor. Taking into account the Förster radius of 7.6 nm for the fluorophores used, this finding definitively demonstrates the colocalization of two differently labeled biotin ligands on an individual streptavidin receptor molecule of ~4 nm in size (Green, 1990) (the probability for random colocalization of two receptors on the length scale of the Förster radius is 2×10^{-5}). For control, the sample was illuminated in the red, which directly and selectively excites the acceptor molecules (cyanine). Two signals were observed on red excitation (Fig. 2 *B*). One (right) is colocalized with the signal in Fig. 2 *A* in terms of positional analysis as expected for a donor-acceptor pair. The second signal (left), without counterpart at green illumination, reflects a streptavidin receptor to which only one type of ligand (the biotin-cyanine conjugate) has bound. In total, we have observed 27 receptor-ligand complexes in 80 experiments. The statistical results corroborate the findings of the dual wavelength studies discussed earlier. $p_{rg}/(p_r + p_{rg}) = 48\%$ of all observed biotin-cyanine ligands were colocalized with a biotin-rhodamine ligand on their respective receptor molecule.

The inherent structural and temporal heterogeneity of biological systems requires methodologies capable for studies at the level of the individual molecule. In the current report we have used high resolution optical microscopy to directly observe ligand colocalization on an individual receptor molecule. This development opens up a new perspective for the study of molecular structures in biological cells with the potential to directly observe conformational changes and dynamics of ligand binding in vivo.

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