PROTOCOL 9

Monitoring Protein-Protein Interactions with GFP Chimeras

THESE PROTOCOLS WERE CONTRIBUTED BY Sang-Hyun Park (Department of Cellular and Molecular Pharmacology, University of California, San Francisco, California) and Ronald T. Raines (Department of Biochemistry, University of Wisconsin, Madison, Wisconsin).

Green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has exceptional physical and chemical properties besides its spontaneous fluorescence. These properties, which include high thermal stability and resistance to detergents, organic solvents, and proteases, endow GFP with enormous potential for biotechnical applications. Since the cDNA of GFP was cloned (Prasher et al. 1992), a variety of GFP variants have been generated that broaden the spectrum of its application (Cubitt et al. 1995; Delagrave et al. 1995; Ehrig et al. 1995; Heim et al. 1995; Crameri et al. 1996; Ward et al. 2000). Among those variants, S65T GFP is unique in having increased fluorescence intensity and faster fluorophore formation than that of the wild-type protein, as well as altered excitation and emission spectra (Heim et al. 1995; Ormö et al. 1996). Moreover, the wavelengths of the excitation and emission maxima of S65T GFP (490 and 510 nm, respectively) are close to those of fluorescein. The fluorescein-like spectral characteristics of S65T GFP enable its use with instrumentation that had been designed specifically for use with fluorescein.

We describe here the use of S65T GFP to probe protein-protein interactions in vitro (Park and Raines 1997, 2000). This method requires fusing S65T GFP to a target protein (X) to create a GFP chimera (X~GFP[S65T]). The target protein is fused to the amino terminus of GFP, rather than to the carboxyl terminus; otherwise, heterogeneity can occur (Park and Raines 1997, 2000). The interaction of the fusion protein with another protein (Y) can be analyzed with common instrumentation by two distinct methods.

The first method is a fluorescence gel-retardation assay. The gel-retardation assay has been used widely to study protein-DNA interactions (Carey 1991). This assay is based on the electrophoretic mobility of a protein-DNA complex being less than that of either molecule alone. In a fluorescence gel-retardation assay, electrophoretic mobility is detected by the fluorescence of S65T GFP. This assay is a rapid method to demonstrate the existence of a protein-protein interaction and to estimate the equilibrium dissociation constant (K_d) of the resulting complex.

The second method is a fluorescence polarization assay, which provides an accurate means to evaluate K_d in a specified homogeneous solution. Fluoresence polarization assays usually rely on fluorescein as an exogenous fluorophore. S65T GFP can likewise serve in this role. Furthermore, the fluorescence polarization assay can be adapted for the high-throughput screening of protein or peptide libraries.

METHOD 1: Fluorescence Gel-retardation Assay

MATERIALS

CAUTION: See Appendix 3 for appropriate handling of materials marked with <!>.

Reagents

Native polyacrylamide gel (6% w/v) <!>
Tris-HCl buffer (10 mM, pH 7.5) containing glycerol (5% v/v)

Equipment

Software for quantifying fluorescence (ImageQuant, Amersham Biosciences)
Fluorescence imaging system (e.g., FluorImager System, Amersham Biosciences)
UV/visible spectrophotometer
Vertical gel electrophoresis system

Biological Molecules

Purified protein of interest (Y)
Purified S65T GFP chimera (X~GFP[S65T]) (CLONTECH)
For details on the construction and purification of the chimera, see Park and Raines (1997, 2000).

METHOD

1. Estimate the concentration of purified X~GFP(S65T) using the extinction coefficient (ε = 39.2 $mM^{-1}cm^{-1}$ at 490 nm) of S65T GFP (Heim et al. 1995).
2. To begin the gel-retardation assay, mix 1.0 µM of X~GFP(S65T) with varying amounts of Y in 10 µl of 10 mM Tris-HCl buffer (pH 7.5) containing 5% glycerol.
3. Incubate the mixtures for 20 minutes at 20°C. Load the mixtures onto a 6% native polyacrylamide gel, and subject the loaded gel to electrophoresis at 10 V/cm for 30 minutes at 4°C.
4. Immediately after electrophoresis, scan the gel with a fluorescence imaging system at 700 V using a built-in filter set (490 nm for excitation and ≥515 nm for emission).
5. Quantify the fluorescence intensities of bound and free X~GFP(S65T) using appropriate software, such as ImageQuant.
6. Determine the value of **R** (= fluorescence intensity of bound X~GFP[S65T]/total fluorescence intensity) for each gel lane from the measured fluorescence intensities.
7. Calculate the value of K_d for each lane with Equation 1, and average those values.

$$K_d = \frac{1-\mathbf{R}}{\mathbf{R}} ([Y]_{total} - \mathbf{R}[X\sim GFP]_{total}) \quad (1)$$

CASE STUDY: S65T GFP CHIMERAS

To demonstrate the potential of S65T GFP chimeras in the exploration of protein-protein interactions, we use as a model system the well-characterized interaction of the S peptide and S-protein fragments of bovine pancreatic ribonuclease (RNase A; EC 3.1.27.5) (Raines 1998; Raines et al. 2000). (GFP chimeras have also been used to explore the interaction of [1] the BALF-1 protein and Bax/Bak [Marshall et al. 1999]; [2] CREB and importins [Forwood et al. 2001]; and [3] a cyclophilin and the capsid protein p24 of HIV-1 [Kiessig et al. 2001].) Subtilisin treatment of RNase A yields two tightly associated polypeptide chains: S peptide (residues 1–20) and S protein (residues 21–124) (Richards 1955). Residues 1–15 of S peptide (S15) are necessary and sufficient to form a complex with S protein (Potts et al. 1963).

The fluorescence gel-retardation assay is used to quantify the interaction between S protein and S15. A GFP chimera, S15~GFP(S65T)~His_6, is produced by standard recombinant DNA techniques and purified by Ni^{++}-affinity chromatography (Park and Raines 1997, 2000). A fixed quantity of S15~GFP(S65T)~His_6 is incubated with a varying quantity of S protein prior to electrophoresis in a native polyacrylamide gel. After electrophoresis, the gel is scanned with a fluorescence imaging system, and the fluorescence intensities of bound and free S15~GFP(S65T)~His_6 are quantified (Figure 10.31, top). The value of K_d for the complex formed in the presence of different S-protein concentrations is calculated from the values of **R** and the total concentrations of S protein and S15~GFP(S65T)~His_6 (Equation 1). The average (±SE) value of K_d is $(6 \pm 3) \times 10^{-8}$ M.

A competition assay is used to probe the specificity of the interaction between S15~GFP(S65T)~His_6 and S protein. S15~GFP(S65T)~His_6 and S protein are incubated to allow for complex formation. Varying amounts of S peptide are added, and the resulting mixture is incubated further (20 minutes), and then subjected to native gel electrophoresis. The addition of S peptide converts bound S15~GFP(S65T)~His_6 to the free state (Figure 10.31, bottom). Thus, S15~GFP(S65T)~His_6 and S peptide bind to the same region of S protein.

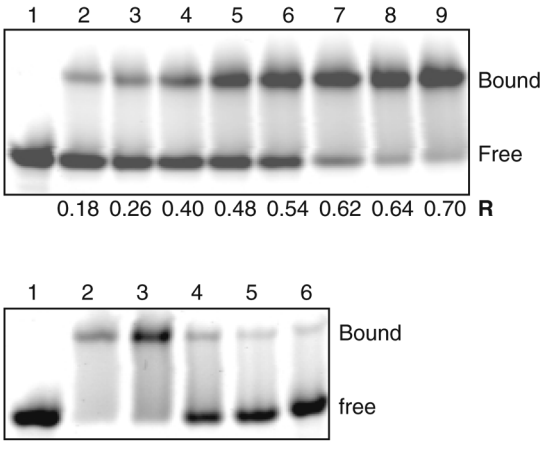

FIGURE 10.31. Gel-retardation assay of a protein-protein interaction. (*Top*, Lanes *1–9*) 1 μM S15~GFP(S65T)~His_6 and 0, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, and 0.9 μM S protein, respectively. The relative mobilities of free and bound S15~GFP(S65T)~His_6 are 0.72 and 0.47, respectively. The value of **R** is obtained for each lane, and values of K_d are calculated by using Equation 1, with the average being $K_d = (6 \pm 3) \times 10^{-8}$ M. (*Bottom*) Gel-retardation assay demonstrating that S peptide competes with S15~GFP(S65T)~His_6 for interaction with S protein. (Lane *1*) 1 μM S15~GFP(S65T)~His_6 and no S protein or S peptide; (lanes *2–6*) 1 μM S15~GFP(S65T)~His_6, 1 μM S protein, and 0, 0.3, 1.0, 3.0, and 10 μM S peptide, respectively.

METHOD 2: Fluorescence Polarization Assay

The fluorescence gel-retardation assay described above is a convenient method to visualize a protein-protein interaction as well as to estimate the value of K_d for the resulting complex. Still, gel-retardation assays have an intrinsic limitation in evaluating equilibrium dissociation constants. In a gel-retardation assay, it is assumed that a receptor-ligand interaction remains at equilibrium during sample loading and electrophoresis. Yet, as samples are loaded and migrate through a gel, complex dissociation is unavoidable and results in an underestimation of the value of K_d. Moreover, if the conditions (e.g., pH, salt type, and concentration) encountered during electrophoresis differ from those in the incubation, then the measured value of K_d may not correspond to the true value.

In a fluorescence polarization assay, the formation of a complex is deduced from an increase in fluorescence polarization, and the equilibrium dissociation constant is determined in a homogeneous aqueous environment. Many fluorescence polarization assays have used fluorescein as the fluorophore (LeTilly and Royer 1993; Heyduk et al. 1996; Malpeli et al. 1996; Fisher et al. 1998). Like a free fluorescein-labeled ligand, a free S65T GFP chimera is likely to rotate more rapidly and therefore to have a lower rotational correlation time than does a bound chimera. An increase in rotational correlation time upon binding results in an increase in fluorescence polarization, which can be used to assess complex formation (Jameson and Sawyer 1995). In contrast to a gel-retardation assay, the fluorescence polarization assay is performed in a homogeneous solution in which the conditions can be dictated precisely.

MATERIALS

Reagents

Tris-HCl buffer (20 mM, pH 8.0) containing NaCl (at various concentrations)

Equipment

Fluorescence spectrometer capable of measuring polarization (e.g., the Beacon fluorescence polarization system, PanVera, Madison, Wisconsin)

Software capable of nonlinear regression analysis (e.g., DeltaGraph, DeltaPoint, Monterey, California)

Biological Molecules

Purified protein of interest (Y)

Purified S65T GFP chimera (X~GFP[S65T]) (CLONTECH)

For details on the construction and purification of the chimera, see Park and Raines (1997, 2000).

METHOD

1. Mix 0.50–1.0 nM X~GFP(S65T) with various concentrations of Y in 1.0 ml of 20 mM Tris-HCl buffer (pH 8.0), with or without NaCl at 20°C.

   Conditions such as buffer, pH, temperature, and salt can be varied to test their effect on the binding interaction.

2. Immediately after mixing, make five to seven polarization measurements at each concentration of Y using a fluorescence spectrometer capable of measuring polarization. For a blank measurement, use a mixture that contains the same components but lacks X~GFP(S65T). Fluorescence polarization (P) is defined as:

$$P = \frac{I_{||} - I_{\perp}}{I_{||} - I_{\perp}} \tag{2}$$

   where $I_{||}$ is the intensity of the emission light parallel to the excitation light plane and $I_{\perp}$ is the intensity of the emission light perpendicular to the excitation light plane. P, being a ratio of light intensities, is a dimensionless number and has a maximum value of 0.5.

3. Determine values of K_d by using software, such as DeltaGraph, to fit the data to the equation:

$$P = \frac{\Delta P \cdot F}{K_d + F} + P_{min} \tag{3}$$

   where P is the measured polarization, ΔP ($= P_{max} - P_{min}$) is the total change in polarization, and F is the concentration of free Y. The fraction of bound protein (f_B) is obtained by using the equation:

$$f_B = \frac{P - P_{min}}{\Delta P} = \frac{F}{K_d + F} \tag{4}$$

   The familiar binding isotherms are obtained by plotting f_B versus F.

CASE STUDY: FLUORESCENCE POLARIZATION

Fluorescence polarization is used to determine the effect of salt concentration on the formation of a complex between S protein and S15~GFP(S65T)~His_6. The value of K_d increases by fourfold when NaCl is added to a final concentration of 0.10 M (see Figure 10.32). A similar salt dependence for the dissociation of RNase S had been observed previously (Schreier and Baldwin 1977). The added salt is likely to disturb the water molecules hydrating the hydrophobic patch in the complex between S peptide and S protein, resulting in a decrease in the binding affinity (Baldwin 1996). Finally, the value of $K_d = 4.2 \times 10^{-8}$ M observed in 20 mM Tris-HCl buffer (pH 8.0) containing NaCl (0.10 M) is similar (i.e., threefold lower) to that obtained by titration calorimetry in 50 mM sodium acetate buffer (pH 6.0) containing NaCl (0.10 M) (Connelly et al. 1990).

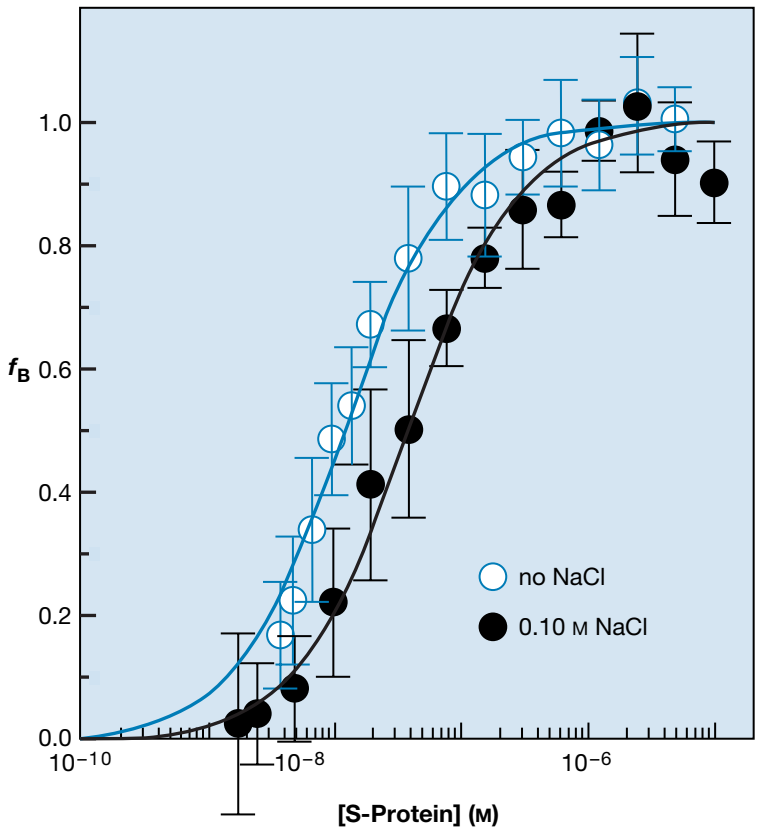

FIGURE 10.32. Fluorescence polarization assay of a protein-protein interaction. S protein (20 μM to 1.0 nM) is added to 20 mM Tris-HCl buffer (pH 8.0) containing S15~GFP(S65T)~His_6 (0.50 nM) at 20°C in a volume of 1.0 ml. Each data point is an average of 5–7 measurements. Binding isotherms are obtained by fitting the data to Equations 3 and 4. The values of K_d in the presence of 0 and 0.10 M NaCl are 1.1×10^{-8} M and 4.2×10^{-8} M, respectively. (Courtesy of Sang-Hyun Park, University of California, San Francisco, and Ronald T. Raines, University of Wisconsin, Madison.)