Novel Fluorescence Labeling and High-Throughput Assay Technologies for In Vitro Analysis of Protein Interactions

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We developed and tested a simple method for fluorescence labeling and interaction analysis of proteins based on a highly efficient in vitro translation system combined with high-throughput technologies such as microarrays and fluorescence cross-correlation spectroscopy (FCCS). By use of puromycin analogs linked to various fluorophores through a deoxycytidylic acid linker, a single fluorophore can be efficiently incorporated into a protein at the carboxyl terminus during in vitro translation. We confirmed that the resulting fluorescently labeled proteins are useful for probing protein–protein and protein–DNA interactions by means of pulldown assay, DNA microarrays, and FCCS in model experiments. These fluorescence assay systems can be easily extended to highly parallel analysis of protein interactions in studies of functional genomics.

[Online supplementary material available at http://www.genome.org.]
dues, because the protein terminal regions have large flexibility in general and tend to be located far from the active site (Thornton and Sibanda 1983).

Here we describe the establishment of a fluorescence assay system for in vitro analysis of protein interactions by combining a simple protein labeling method, an efficient cell-free protein synthesis system, and miniaturized assay systems such as microarray and FCCS technologies. We have used classical oncogenes, Fos and Jun, as model proteins, because they are known to form a heterodimer that specifically binds to a DNA sequence (Angel and Karin 1991) and thus they can be used to examine both protein–protein and protein–DNA interactions.

RESULTS AND DISCUSSION

Fluorescence Labeling with Puromycin Analogs

Puromycin is an antibiotic that mimics the aminoacyl end of tRNA and acts as a translation inhibitor by being linked to the nascent peptide by the peptidyl transferase activity of the ribosome. Recently, it has been shown that puromycin and its derivatives at low concentrations can bind to the carboxyl terminus of full-length proteins (Miyamoto-Sato et al. 2000) and that a fluorescein–puromycin conjugate was successfully used for the fluorescein labeling of proteins (Nemoto et al. 1999). To confirm the general utility of this labeling method, we chemically synthesized various puromycin analogs containing a different fluorophore such as fluorescein, rhodamine green (RhG), tetramethyl-rhodamine (TAMRA), Cy3, or Cy5. Further, one or two (deoxy-)cytidylic acid(s) (i.e., dC, dCdC, rC, or rCrC) was inserted between fluorophore and puromycin as a linker (Fig. 1A) to enhance the incorporation of puromycin analogs into proteins by mimicking the CCA sequence at the 3' end of tRNA. These puromycin analogs were added to the wheat germ in vitro translation system (Madin et al. 2000) supplemented with a template RNA transcribed from a part of the *c-fos* (118–211 amino acids) or *c-jun* (216–318 amino acids) gene encoding DNA-binding and leucine-zipper regions (Fig. 1B).

The Fos and Jun proteins were labeled with all the fluorophores. A typical result for RhG-dC-puromycin is shown in Figure 1C. The intensities of the fluorescent bands depended on the concentration of fluorescent puromycin and the labeling efficiencies at the peak concentration ranged from 10% to 30%. With increasing dye concentration, extra bands of lower molecular weight than that of the target band appeared. These bands might originate from the incorporation of puromycin analogs into the nascent polypeptides as they could be removed by affinity purification of translated products with carboxy-terminal His6-tag sequences (Fig. 1C, Pure), but not with the amino-terminal His6-tag (data not shown).

Figure 2 shows the relative yields of Jun proteins labeled with fluorescein, RhG, and Cy5. These values were highly sensitive to the linker structure. For example, the maximum yields of proteins labeled with fluorescein-, RhG-, and Cy5-dC-puromycin were 140–2– and 25-fold higher than those of proteins labeled with fluorescein-, RhG-, and Cy5-puromycin, respectively. For all the fluorophores, use of the dC linker resulted in the highest yields of labeled Jun proteins, and thus fluorophore-dC-puromycin conjugates were used in further experiments. By use of these fluorescent reagents, >10 proteins derived from human, mouse, and other organisms could also be labeled with various fluorophores (N. Doi, H. Takashima, R. Oyama, E. Miyamoto-Sato, and H. Yanagawa, unpubl.).

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**Figure 1** Fluorescence labeling of proteins. (A) The structure of fluorescent puromycin. A fluorophore (fluorescein, RhG, TAMRA, Cy3, or Cy5) was chemically joined to puromycin through a linker (dC, dCdC, rC, rCrC, or none). (B) Template DNA containing SP6 promoter (Morigita et al. 1999), AMV (alfalfa mosaic virus) leader sequence (Jobling and Gehlke 1987), Kozak sequence (Kozak 1986), and *c-fos* (116–211 amino acids) or *c-jun* (216–318 amino acids) gene with T7-tag (11 amino acids, MASMTGGQQMG) and His6-tag sequences. (C) RhG-labeled Fos and Jun proteins. In vitro translation reaction was carried out in the presence of the template RNA and 0–48 µM of RhG–dC–puromycin. The products were analyzed by 16.5% Tricine-SDS-PAGE with an imaging analyzer. The labeled proteins with His6-tag were purified by affinity chromatography (Pure).
Detecting Protein–Protein Interactions by Pulldown Assay

As the bait protein, Fos was fused with the IgG-binding domain of protein A (ZZ domain) and calmodulin-binding peptide (CBP) for a tandem affinity purification method (Rigaut et al. 1999; Fig. 3A). The prey Jun and Fos (as a negative control) were carboxy-terminally labeled with Cy5–dC–puromycin as described above. Further, as a control with another labeling method, Jun and Fos proteins labeled with Bodipy-FL at their lysine residues were also prepared by means of an in vitro translation reaction containing fluorescently labeled lysine tRNA. As shown in Figure 3B, the carboxy-terminally labeled Jun (Cy5) bound to ZZ–CBP–Fos and was purified by tandem affinity beads (lanes 3 and 5, top), whereas the internal lysine-labeled Jun (Bodipy-FL) did not bind to the bait Fos (lanes 3 and 5, bottom). This is a typical example of the advantage of the carboxy-terminal labeling of proteins.

Detecting Protein–DNA Interactions with DNA Microarrays

DNA microarrays have been used widely for monitoring gene expression patterns (Schena et al. 1995) but also can potentially be used for studying sequence-specific protein–DNA interactions (Bulyk et al. 1999; Iyer et al. 2001). We have tested this application by using as probes Fos/Jun proteins carboxy-terminally labeled with TAMRA-conjugated puromycin. The Fos–Jun heterodimer is known to bind to DNA containing a TGA(G/C)TCA consensus sequence (Angel and Karin 1991). We arrayed Cy5-labeled DNA containing or not containing the target sequence on poly-L-lysine-coated slides using commercially available arrayers (Fig. 4A). The slides were then probed with the TAMRA-labeled proteins, washed, and scanned with a fluorescence scanner. As anticipated, the heterodimer of TAMRA-labeled Jun with nonlabeled Fos bound to the DNA in the sequence-specific manner (Fig. 4B, C), whereas no binding was observed in control experiments (Fig. 4D–F). In a complementary experiment, the sequence-specific binding of the complex of TAMRA-labeled Fos with unlabeled Jun to the DNA was also observed (data not shown).

Kinetic Analysis of Protein Interactions with FCCS

Fluorescence correlation spectroscopy (FCS) is a new, highly sensitive method that can, in principle, detect the motion or fluctuation of a single fluorescent molecule in a solution volume of <1 fl (Eigen and Rigler 1994; Rigler 1995; Maiti et al. 1997). By autocorrelation function analysis of the fluorescence fluctuations, the diffusion time and the molecular weight of the fluorescent molecule can be determined. Although FCS was invented >25 years ago, it has been applied only recently in the field of biology; for example, in vitro studies of DNA hybridization (Schwille et al. 1997), DNA amplification (Kinjo 1998), protein–protein interaction (Pack et al. 2000), protein aggregation (Pitschke et al. 1998; Bieschke et al. 2000), and protein dynamics (Haupts et al. 1998), and in vivo studies of protein transport (Terada et al. 2000) and flagellum rotation (Cluzel et al. 2000). The detection of protein interactions with single-color FCS requires drastic changes of the size or shape of the protein complex (Pack et al. 2000). In contrast, the FCCS (dual-color FCS) analysis is independent of the size and shape of the fluorescent molecule, but rather is based on the iden-
tification of fluctuations that occur simultaneously in two detected channels (Schwille et al. 1997). Hence, this method makes it possible to monitor whether two molecules labeled with different fluorophores bind or not, with higher sensitivity, specificity, and speed than those of single-color FCS.

Here we have shown the quantitative analysis of protein–DNA interactions with FCCS (Fig. 5). The Fos and Jun proteins were labeled with RhG–dC–puromycin at the carboxyl terminus, and the target DNA was labeled with Cy5 at the 5’-ends. As shown in Figure 5C, only the Fos–Jun–DNA complex molecule carrying both RhG and Cy5 was observed in the cross-correlation and quantified, but not the Fos–Fos and Jun–Jun pairs. The apparent dissociation constant $K_d$ for the binding of the Fos–Jun heterodimer to DNA can be obtained from a single FCCS measurement (Földes-Papp and Kinjo 2001; Jankowski and Janka 2001): The $K_d$ value was found to be 30 nM (see supplemental data on the web site), which is consistent with a value of 50 nM independently derived from the results of gel shift assay (John et al. 1996).

In summary, we have established a simple method for fluorescence labeling of proteins and confirmed that the resulting fluorescently labeled proteins are useful for probing protein–protein and protein–DNA interactions by means of pulldown assay, DNA microarrays, and FCCS measurements. To our knowledge, this work is the first to describe the successful detection of protein–DNA interactions with FCCS and DNA microarray by using fluorescently labeled proteins. These assay systems should also be applicable to the detection of protein–protein interactions. Our labeling method is so simple that it can easily be extended to the large-scale analysis of proteins, and, hence, we believe that these technologies will contribute greatly to studies of genomic function.

**Figure 4** Detecting protein–DNA interactions. (A) Two types of DNA labeled with Cy5 (650/670 nm) were spotted on a glass slide (represented as false-colored red), one containing a Fos–Jun bound sequence (+) and the other not (−). (B) The sequence-specific binding of the complex of the Jun labeled with TAMRA (542/568 nm) and unlabeled Fos to the DNA (+) was detected as false-colored green spots. (C) The same result as in B is represented as a superposition of red and green color. (D–F) As negative controls, the solution of TAMRA-labeled Jun (D) without Fos, (E) with unlabeled Fos, and an excess of competitive DNA, and (F) with unlabeled Jun were probed, and no binding was observed (shown by the superposition of red and green color).

**Figure 5** FCCS setup and measured cross-correlation curves. (A) The beampath of the ConfoCor 2 FCCS setup. A sample droplet is excited with two-color laser beams through the objective lens. The emission light is divided and detected by two avalanche photodiodes (APD1 and APD2), and the signals are analyzed by a computer with a correlator. DM, dichroic mirror 488/633 nm; BP, bandpass filter 530–600 nm; LP, longpass filter 650 nm. (B) Schematic diagram of FCCS detection apparatus. (Left) Laser beams are focused in the sample droplet on the coverglass. (Right) In the superimposed confocal volume, RhG-labeled proteins and Cy5-labeled DNA are simultaneously excited. (C) Typical cross-correlation curves obtained from samples of DNA–protein complexes. Fos–Jun heterodimers were bound to Cy5-labeled DNA (top curves), whereas no or little formation of the Fos–Fos–DNA or Jun–Jun–DNA complexes was observed (bottom curves).
New Technologies for Analysis of Protein Interactions

METHODS

Synthesis of Fluorescent Puromycin Derivatives

Protected nucleoside phosphoramidites, fluoroephosphoramidites, 5'-amino-modifier C6 phosphoramidite, and Poly-pak II cartridges were purchased from Glen Research. Rhodamine green succinimidyl ester (RhG-OSu) was purchased from Molecular Probes. N-Fluorenylmethoxycarbonyl-puromycin and N-t-butoxycarbonylpuromycin attached to controlled pore glass supports [Purumycin(Fmoc)-CPG and Purumycin(Boc)-CPG] were synthesized according to the published procedure (Ikeda et al. 1998). Fluorescein-[X]-puromycin, Cy5-[X]-puromycin and TAMRA-dC-puromycin were synthesized from Purumycin(Fmoc)-CPG according to the standard solid-phase phosphoramidite method (Ikeda et al. 1998). After deprotection according to the recommended protocol (Glen Research) for nucleosides and fluorophores, fluorescent puromycin analogs were purified by reverse-phase HPLC on a YMC-Pack ODS-A column (2 cm x 30 cm, YMC, Kyoto, Japan) with 0.1 M triethylammonium acetate (pH 7.0) as solvent A and acetonitrile as solvent B at a flow rate of 10 mL/min. A linear gradient of 10%-60% solvent B over 30 min was used for elution. The 5'-amino-modifier C6-[X]-puromycin(Boc) was synthesized from Purumycin(Boc)-CPG according to the standard solid-phase phosphoramidite method. After deprotection according to the recommended protocol for each nucleoside, the puromycin analogs were purified by reverse-phase HPLC under the elution condition described above. The 5'-amino-modifier C6-[X]-puromycin(Boc) was then reacted with RhG-OSu (5 equivalent) in 0.1 M NaHCO3 (pH 8.3)/dimethylformamide (1:1, vol/vol) at 25°C for 2 h. Desalting by the Poly-pak II cartridge, acid treatment (60% CF3COOH-H2O, 25°C, 30 min), and following HPLC purification under the elution condition described above gave RhG-[X]-puromycin. The structures of fluorescent puromycin analogs were confirmed by MALDI-TOF mass spectrometry (Lasermat 2000, Thermo Finnigan). A list of structures (Supplementary Fig. 1), and UV and Mass spectral data (Supplementary Table 1) of fluorescent puromycin analogs were provided as supplemental data on the web site.

Fluorescence Labeling of Proteins

The template DNAs encoding c-Fos (118-211 amino acids) and c-Jun (216-318 amino acids) genes (Fig. 1B) were prepared by PCR with Ex Taq DNA polymerase (Takara Shuzo) and purified with a QIAquick PCR purification kit (Qiagen). Detailed methods and a list of the primer sequences (Supplementary Table 2) are provided as supplemental data on the web site. RNAs were transcribed from the DNAs by use of a RibomAX large-scale RNA production system (Promega) and purified by using an RNeasy mini kit (Qiagen). In vitro translation reaction solution containing TAMRA-labeled Fos or Jun (10 nM) and unlabeled Fos or Jun (1 μM) was taken up in a buffer [50 mM Tris-HCl at pH 7.5, 2 mM MgCl2, 1 mM EDTA, 1 mM DTT, 20% glycerol, 0.01% NP-40, 50 mM NaCl, and 10 μg/mL poly(dIdC)] and applied to the DNA-arrayed slides (Ready Type II slides, Clontech) with a MicroGrid arrayer (BioRobotics). The slides were subjected to heating, UV cross-linking, and blocking according to the recommended protocols (Clontech) and then treated with a PBS solution containing 3% skim milk and 0.02% sodium azide at 4°C overnight and washed twice with PBS. As the fluorescent probe, in vitro translation reaction solution containing TAMRA-labeled Fos or Jun (10 nM) and unlabeled Fos or Jun (1 μM) was taken up in a buffer [50 mM Tris-HCl at pH 7.5, 2 mM MgCl2, 1 mM EDTA, 1 mM DTT, 20% glycerol, 0.01% NP-40, 50 mM NaCl, and 10 μg/mL poly(dIdC)] and applied to the DNA-arrayed slides. After a 10-min incubation at room temperature, the slides were washed twice with the previous buffer, twice with PBS, and then centrifuged at 200g for 1 min to remove excess buffer. The slides were scanned with a laser fluorescence scanner GenePix 4000A (Axon Instruments) to detect the interaction signals from the TAMRA-labeled Jun proteins (excited at 532 nm) and the Cy5-labeled DNA (excited at 635 nm).

Fluorescence Cross-Correlation Spectroscopy

The RhG-labeled Fos and Jun with carboxy-terminal His6-tag were purified on a Ni-NTA affinity column (Qiagen), followed by size exclusion chromatography on a PD-10 column (Amersham Pharmacia Biotech) twice. The Cy5-labeled dsDNA was prepared by the hybridization of two complementary 20-nucleotide ssDNAs (CTTGCATGACTCATCGA and AATGGATGACTCATCAGA) and applied to the DNA-arrayed slides (in italics), which were synthesized with Cy5-label at their 5'-ends and HPLC-purified by Sigma Genosys Japan. Unlabeled Fos and Jun proteins were synthesized by use of the wheat cell-free dialysis system (Madin et al. 2000) and incubated with the fluorescently labeled Jun or Fos (negative control) in a buffer (10 mM Tris-HCl at pH 8.0, 150 mM NaCl, and 0.1% NP-40) at 37°C for 30 min. According to the protocol described previously (Rigaut et al. 1999), the ZZ-CBP–Jun complex was captured on IgG Sepharose (Amersham Pharmacia Biotech), digested with TEV protease (Invitrogen), and applied on a calmodulin beads (Stratagene). Each of the flowthrough and eluate fractions was analyzed by Tricine-SDS-PAGE with a Molecular Imager FX.

DNA Microarrays

The 1.9-kb fragments of double-stranded DNA (dsDNA) were prepared by PCR with Cy5-labeled primers from pET20b (Novagen) or from its plasmid derivative, pET20-f, containing a Fox/Jun binding sequence, in vitro translation reaction solution containing TAMRA-labeled Fos or Jun (10 nM) and unlabeled Fos or Jun (1 μM) was taken up in a buffer [50 mM Tris-HCl at pH 7.5, 2 mM MgCl2, 1 mM EDTA, 1 mM DTT, 20% glycerol, 0.01% NP-40, 50 mM NaCl, and 10 μg/mL poly(dIdC)] and applied to the DNA-arrayed slides. After a 10-min incubation at room temperature, the slides were washed twice with the previous buffer, twice with PBS, and then centrifuged at 200g for 1 min to remove excess buffer. The slides were scanned with a laser fluorescence scanner GenePix 4000A (Axon Instruments) to detect the interaction signals from the TAMRA-labeled Jun proteins (excited at 532 nm) and the Cy5-labeled DNA (excited at 635 nm).

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