Cytokine 64 (2013) 272–285

Contents lists available at SciVerse ScienceDirect

Cytokine

journal homepage: www.journals.elsevier.com/cytokine

Improving the spectral analysis of fluorescence resonance energy transfer in live cells: Application to interferon receptors and Janus kinases

Christopher D. Krause [a,*], Gina Digioia [a], Lara S. Izotova [a,b], Sidney Pestka [a,b]

[a] Department of Biochemistry and Molecular Biology, Robert Wood Johnson Medical School, The University of Medicine and Dentistry of New Jersey, 675 Hoes Lane West, Piscataway, NJ 08855, USA
[b] Pestka Biomedical Laboratories, 131 Ethel Road West, Suite 6, Piscataway, NJ 08854, USA

ARTICLE INFO

Article history:
Available online 21 June 2013

Keywords:
Interferon
FRET
Receptor
Equilibrium
Janus kinase

ABSTRACT

The observed Fluorescence Resonance Energy Transfer (FRET) between fluorescently labeled proteins varies in cells. To understand how this variation affects our interpretation of how proteins interact in cells, we developed a protocol that mathematically separates donor-independent and donor-dependent excitations of acceptor, determines the electromagnetic interaction of donors and acceptors, and quantifies the efficiency of the interaction of donors and acceptors. By analyzing large populations of cells, we found that misbalanced or insufficient expression of acceptor or donor as well as their inefficient or reversible interaction influenced FRET efficiency *in vivo*. Use of red-shifted donors and acceptors gave spectra with less endogenous fluorescence but produced lower FRET efficiency, possibly caused by reduced quenching of red-shifted fluorophores in cells. Additionally, cryptic interactions between jellyfish FPs artefactually increased the apparent FRET efficiency. Our protocol can distinguish specific and nonspecific protein interactions even within highly constrained environments as plasma membranes. Overall, accurate FRET estimations in cells or within complex environments can be obtained by a combination of proper data analysis, study of sufficient numbers of cells, and use of properly empirically developed fluorescent proteins.

1. Introduction

1.1. Analyzing protein:protein interactions in cells

Studying protein:protein interactions is crucial to understand how signals are propagated and integrated into signaling networks in cells. The vast majority of technologies that are used to analyze protein:protein interactions require the separation of these proteins from the complex biological environments in which they reside. Although a detailed understanding of these interactions in chemically defined conditions is important, these interactions must also be observed in their natural environments to correlate *in vitro* data with *in vivo* data and to realize any modifications of this interaction by components in intact cells. The implementation of variants of the green fluorescent protein (GFP) greatly facilitated the comparison of protein interactions *in vivo* and *in vitro*.

1.2. The interferon-gamma receptor complex

Some protein complexes cannot be purified intact from their biological environment; this prevents a detailed analysis of their structure using current technologies, and requires noninvasive technologies to determine their structure. Among these protein complexes are receptors for transmembrane cellular receptors such as growth factors, interleukins, and interferons (IFNs). The IFN-γ receptor complex is composed of two genetically distinct transmembrane polypeptide chains [1–4]. IFN-γR1 binds the IFN-γ ligand with its extracellular domain; its intracellular domain binds the tyrosine kinase Jak1 to empower it with enzymatic activity. IFN-γR2 binds IFN-γ by its extracellular domain only after IFN-γ binds to IFN-γR1; the intracellular domain of IFN-γR2 binds the tyrosine kinase Jak2 to similarly gain enzymatic activity. Unlike observations performed *in vitro* implying that neither IFN-γR1 and IFN-γR2 nor IL-10R1 and IL-10R2 interact, our group used

Abbreviations: CFS, confocal fluorescence spectroscopy; ChFP, mCherry/cherry fluorescent protein; CiFP, mCitrine/monomeric citrine fluorescent protein; EBFP, enhanced blue fluorescent protein; ECFP, enhanced cyan fluorescent protein; EGFP, enhanced green fluorescent protein; ESaFP, enhanced Sapphire fluorescent protein; EYFP, enhanced yellow fluorescent protein; FL, FLAG epitope (DYKDDDD); FP, fluorescent protein; FRET, fluorescence resonance energy transfer; IFN, interferon; IFN-α, interferon-α; IFN-γ, interferon-γ; OFP, mOrange/orange fluorescent protein; ORF, open reading frame; PEI, polyethyleneimine; StFP, mStrawberry/strawberry fluorescent protein; TFP, mTeal/teal fluorescent protein.

* Corresponding author. Tel.: +1 732 278 7289, fax: +1 732 235 5223.
E-mail addresses: christopher.d.krause@gmail.com (C.D. Krause), ginadigioia9879@gmail.com (G. Digioia), izotovls@umdnj.edu (L.S. Izotova), sp@pblbio.com (S. Pestka).

1043-4666/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.cyto.2013.05.026

noninvasive fluorescent technologies to determine that the both the interferon-gamma (IFN-γ) receptors and the interleukin-10 (IL-10) receptors interact *in vivo* [5–7].

Because the IFN-γ receptor complex is structurally different *in vivo* vs. *in vitro*, and the pharmacological target of the receptor is *in vivo*, we felt that further analysis of receptor interactions must be done *in vivo*. We used a mutagenic approach to find that significant FRET between IFN-γR1 and IFN-γR2 requires (1) that the extracellular domains of IFN-γR1 and IFN-γR2 be species-matched, and (2) that the Jak1 association site of IFN-γR1 be intact [6].

1.3. Variation of protein levels in cells

Inspection of flow cytometric data from a wide variety of systems reveals that the levels of a given protein can vary considerably from cell to cell within a population. Similarly, we found early on that the fluorescence emission of cells expressing a given pair of fluorescent receptors varies from point to point within a cell as well as among cells within a transfected population (unpublished). Furthermore, the observed FRET between a given pair of receptor chains [7] and between cytoplasmic components of an RNA stability complex [8,9] varies within each population of cells. Although variations of protein levels and of FRET are unavoidable consequences of analyzing protein:protein interactions in cells that must be addressed, we hypothesized that quantifying this variation by deconvolution of populations of spectra will assist our understanding of how a given interaction may vary throughout a population of cells and that this analysis will reveal the optimal conditions that produce a desired interaction.

2. Materials and methods

2.1. Reagents

Restriction endonucleases, shrimp alkaline phosphatase and T4 DNA Ligase was purchased from New England BioLabs. Recombinant Taq DNA polymerase was purified as described previously [10]. Turbo Pfu DNA polymerase was purchased from Stratagene. Polyethyleneimine (PEI), in the linear 25 KDa form was purchased from Fluka as a powder. Solutions of PEI were prepared by dissolution in water, for 24 h, titration with hydrochloric acid until turbidity has disappeared for 1 h, followed by neutralization with NaOH and filtration; aliquots were stored at −70 °C until needed [11]. Diethyldioctadecylammonium bromide (DDAB) and dioleoylphosphatidylethanolamine (DOPE) were purchased from Sigma–Aldrich as powders, dissolved in absolute ethanol, and stored at −20 °C until used. Fluorescent proteins (with amino-terminal hexahistidine tags) were purified as described previously [12] with further purification by reverse-phase chromatography.

2.2. DNA constructions

Ligations were performed with T4 DNA Ligase. All PCR products were sequenced after they were subcloned into their host vectors and suitable isolates identified. Plasmids are purified using the alkaline lysis method, except that 7.5 M ammonium acetate instead of 5 M potassium phosphate is used. The synthesis of pEF3-IFN-γR1/γR1/STOP (hereafter referred to as pEF3-IFN-γR1), pcDNA3-FLAG-IFN-γR2/γR2 (hereafter referred to as pc3-FL-IFN-γR2), pcDNA3-FL-IL-10R2/IL-10R2/STOP (hereafter referred to as pc3-FL-IL-10R2), pEF3-IFN-γR1/ECFP, pEF3-IFN-γR1/EGFP, pEF3-IFN-γR1/EYFP, pc3-FL-IFN-γR2/ECFP, pc3-FL-IFN-γR2/EGFP, and pc3-FL-IFN-γR2/EYFP is described elsewhere [5,6]. The details of constructions synthesized with these starting plasmids are described in Supplemental Text 1.

2.3. Cell lines and transfections

Human kidney epithelial 293T and 293TT cells and green monkey kidney epithelial COS-7 cells were grown in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% (v:v) fetal bovine serum. All cell lines were grown in HEPA-filtered tissue culture incubators under an environment of 37 °C, 5% CO_2, and 95% relative humidity.

Multiple transfection protocols were used according to the cell line used as well as the favored and optimized method at the time. Unless explicitly stated, all transfections were done with 2 μg each co-transfected plasmid or 4 μg of tandem vector per well of cells (about 1 million) plated at approximately 70% confluence in six-well tissue culture dishes. The transfection conditions are described in Supplementary Text 2.

2.4. Fluorescence confocal spectroscopy, spectral deconvolution and data analysis

Details of the confocal microscope and spectrophotometer are described in Supplementary Text 3 but were published previously [6,8,9]. Spectroscopic values for the various fluorophores were obtained from published literature [12–16]. Excitation and emission spectra were generously provided either by the webpage of the laboratory of Tsien, by members of the laboratories of Tsien, Shaner and Campbell, or were digitized from published figures.

The process of adding scalable reference spectra to optimize a fit to an obtained cellular emission spectrum, after correcting for spectrometer noise and laser emission, is described elsewhere [7,8].

In Supplementary Text 4, we describe the protocol we now follow to estimate the relative levels of donor, acceptor, donor:acceptor pairs, the FRET efficiency, the fractions of donor or of acceptor in paired complexes, and the efficiencies of coupling of donor and acceptor.

2.5. Fluorescence Lifetime imaging microscopy

A 405 nm picosecond diode pulse laser with a 1 kHz repetition rate, (Courtesy of Prof. Sergei Vinogradov, University of Pennsylvania Department of Chemistry) was used to excite the enhanced sapphire fluorescent protein. A Chameleon Ultra II Ti:Sapphire oscillator (Coherent, Inc., Santa Clara, CA., USA), with a pulse width of ~100 fs and a 80 MHz repetition rate was tuned to 880 nm to excite the teal fluorescent protein.

Samples were placed on 1 mm coverslips and fluorescence emission was obtained on a scanning confocal microscope with the sample and stage mounted on an inverted, epi-illumination microscope (Nikon Diaphot 300). A Nikon FLUOR 40, NA = 1.3 objective focused the excitation light and directed the fluorescence emission to either a monochromator (Acton Research) equipped with a back-illumination liquid nitrogen cooled CCD camera (Princeton Instruments, Trenton, NJ) for spectral measurement to confirm the identity of the excited fluorescent species or to a single photon counting board, SPC 730 (Becker & Hickl, Inc.) to record the fluorescence lifetime decay curve. Both the CCD camera and the time-resolved photon counting board were coupled to Windows-based personal computers to process data.

2.6. Statistical analysis

The arithmetic means and standard deviations of either acceptor levels, donor levels, acceptor:donor molar ratio, acceptor:donor pairs, FRET efficiency or reaction quotients were calculated using SigmaPlot 10.0 (Systat Software, Inc). These values are shown graphically as two-dimensional error bars that bisect at the