A Rapid and Simple Method of Evaluating the Dimeric Tendency of Fluorescent Proteins in Living Cells Using a Truncated Protein of Importin α as Fusion Tag

Chika Nakagawa,1 Shigenori Nishimura, Kaori Senda-Murata, and Kenji Sugimoto

Laboratory of Molecular Biology and Cell Informatics, Division of Bioscience and Informatics, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1-1 Gakuen-cho, Naka-ku, Sakai, Osaka 599-8531, Japan

Received September 8, 2011; Accepted November 4, 2011; Online Publication, February 7, 2012 [doi:10.1271/bbb.110677]

Enhanced green fluorescent protein (EGFP) and its yellow variant (Venus) are weakly dimeric under physiological conditions. We designed a simple method to evaluate the dimeric tendency of fluorescent proteins in living mammalian cells. A novel single mutation, A206L, interfering with the hydrophobic interactions of the dimer interface in Venus, contributed to its monomerization, and was as effective as the A206K mutation in this assay.

Key words: fluorescent protein (FP); Venus; weak dimer; importin alpha (importin α); nuclear-pore complex (NPC)

Green fluorescent protein (GFP),1 derived from the Aequorea Victoria jellyfish, is widely used in the imaging of biological processes in living cells. Expressed as a fusion-tag, it is possible to visualize the molecular behavior of many proteins of interest.2,3) The molecular structures of Aequorea GFP and the red fluorescent protein from the coral Discosoma sp. (DsRed)4 have been extensively characterized. Since the tetrameric tendency of DsRed often hinders its use as a fusion tag, a monomeric mutant of DsRed, mRFP1, was generated,5) but some dimeric activity remained in mRFP1, and a second generation of true monomers, including mCherry and mOrange, was constructed by Shaner et al.6) In contrast, enhanced green and yellow fluorescent proteins (EGFP and EYFP)7) and the derivative Venus8) are known to be weakly dimeric.9) Here we report a rapid and simple method to evaluate the dimeric tendency of Venus in living cells.

Importin α is known to translocate into the nucleus through nuclear-pore complex (NPC), forming a ternary complex with importin β and the cargo protein containing the nuclear localization signal. Without the N-terminal importin β-binding domain, however, the truncated importin α protein itself migrates into the nucleus in an importin β-independent manner.10) Hubner et al. have reported that plant importin α has the ability to localize at the nuclear envelope (NE)/NPC in the absence of importin β.11) We also have found that human importin α protein localized to NE in living MDA435 cells.3,12) First we examined the subcellular localizations of DsRed, mKO,13) EGFP, Venus, mCherry, and mOrange that had been fused to the truncated protein of human importin α (residues 251–529) in mouse C3H10T1/2 cells. As shown in the left column of Fig. 1A, all the fluorescent protein (FP)-importin α fusions showed NE localization. Terameric DsRed-importin α and mKO-importin α were exclusively localized to NE, consistently with previous results,3,12) but we also observed nuclear localization patterns with weak dimeric Venus- and EGFP-importin α, and monomeric mCherry- and mOrange-importin α (Fig. 1A, right column). Figure 1B shows the relative NE localizations of the various FP-importin αs, calculated from the number of cells showing NE localization among total FP-importin α-expressing cells. The relative NE localization of Venus-importin α and EGFP-importin α was about 60–75%, and that of mCherry-importin α and mOrange-importin α was about 30–40%, which correlates well with dimeric or monomeric tendency of each of the various FPs. Since FP alone did not show any NE localization pattern (data not shown), these FPs-importin α fusions had some difficulty in passing through NPC, probably depending on the molecular conformation of a given FP. Rehm et al. found that YFP fused with second mitochondria-derived activator of caspase (YFP-Smac) was released from the mitochondria, whereas terameric DsRed-Smac remained there during apoptosis.14)

Next we examined to determine whether NE localization of the truncated importin α protein could be used to evaluate the dimeric tendency of Venus and its mutants. Figure 2A shows the amino acid residues predicted to be involved in the hydrophobic interactions of Venus, based on the reported three-dimensional structure.8) As enlarged in Fig. 2B, A206 (green sticks), L221 (magenta sticks), and F223 (cyan sticks) were close to each other in the dimer interface, and are likely to be important in intermolecular hydrophobic interactions (red dotted line). We examined all possible C–C contacts among the hydrophobic amino acids predicted to be involved in the dimeric interaction of Venus. Figure 2C shows pairs of amino acid residues with a

---

Note

Aequorea GFP and the fluorescent protein; DsRed, Discosoma sp. red fluorescent protein; mRFP1, monomeric red fluorescent protein; mKO, monomeric Kusabira Orange; NPC, nuclear-pore complex; NE, nuclear envelope; IBB, importin β-binding; Kd, dissociation constant
side-chain distance of <5 Å. Only three pairs, L221–L221, F223–A206, and F223–L221, were within this range. We expected that the dimerization tendency of Venus would be reduced if we weakened these hydrophobic interactions by site-directed mutagenesis. Zacharias et al. have reported that point mutation of A206K, L221K, and F223R reduced the dimeric tendency of YFP.15) They found that the dissociation constant \( K_d \) of the wild-type was 0.11 mM, while the \( K_d \)s values for A206K, L221K, and F223R mutants were 74 mM, 9.7 mM, and 4.8 mM respectively. They concluded that the A206K mutant was the most monomeric, according to the \( K_d \) value of each mutant in vitro. Hence we constructed these monomeric mutants of Venus by site-directed mutagenesis16) and evaluated the extent of their monomerization again in mouse C3H10T1/2 cells. The relative NE localization of wild-type Venus-importin \( \alpha \) was 71%, and those of Venus A206K-importin \( \alpha \), Venus L221K-importin \( \alpha \), and Venus F223R-importin \( \alpha \) were 53%, 58%, and 56% respectively (Fig. 3). As expected, the relative NE localization of all the mutants decreased significantly, indicating a correlation between \( K_d \) values in vitro and the NE distribution index in vivo.

Next, we replaced A206, L221, and F223 of Venus-importin \( \alpha \) with other hydrophobic amino acids, L, A, and A respectively. The relative nuclear localizations of Venus A206L-importin \( \alpha \), Venus L221A-importin \( \alpha \), and Venus F223A-importin \( \alpha \) were 54%, 58%, and 55% respectively (Fig. 3). Elongation and reduction of the side-chain length of the hydrophobic amino acids (A206L, L221A, F223A) had the same effect as replacement of them with basic amino acids (A206K, L221K, L223R) in reducing the dimeric tendency of Venus. No effect was observed with point mutations at L207 (L207V, L207A), next to A206. This is partly

---

**Fig. 1.** Nuclear Envelope (NE) Localization of Fluorescent Proteins (FPs) Fused with Importin \( \alpha \).

A. Cellular localization of various FPs-importin \( \alpha \). Plasmids, pmKO-imp \( \alpha \) and pEGFP-imp \( \alpha \), were constructed by inserting a 1-kb fragment of the EcoRI-XhoI fragment of DsRed-imp \( \alpha \), encoding a truncated protein of human importin \( \alpha \) (residues 251–529), into the EcoRI-SalI sites of pEGFP-AC2, a frame-shifted derivative of pEGFP-C1 (Clontech, Palo Alto, CA) and the EcoRI-XhoI sites of pmKO-MC1 (MBL, Nagoya) respectively. Other plasmids, pmCherry-imp \( \alpha \) and pmOrange-imp \( \alpha \), were constructed by replacing the AgeI-BglII fragment encoding DsRed of pDsRed-imp \( \alpha \) with amplified DNA fragments encoding mCherry or mOrange (pRSET-B-mCherry or pRSET-B-mOrange (provided by Professor Roger Y. Tsien, UCSD) respectively. Similarly, pVenus-imp \( \alpha \) was constructed with an amplified DNA fragment encoding Venus of pCS2-Venus (provided by Dr. Atsushi Miyawaki, RIKEN). Mouse C3H10T1/2 cells were transfected with plasmids encoding FPs-importin \( \alpha \) and observed under a fluorescence microscope, as described previously.17) FPs-importin \( \alpha \) was localized to the nuclear envelope (left column) or the nucleus (right column). B, Relative NE localization of FPs-importin \( \alpha \) was calculated from the number of cells indicating NE localization among total fluorescent cells. Standard deviations were calculated from images captured in three independent experiments.

---

**Fig. 2.** Dimeric Form of Venus.

A, Graphical view of the Venus dimer. This figure is based on the crystal structure of Venus (Protein Data Bank code 1MYW), and was created using the PyMOL molecular graphics system (http://www.pymol.org). Single mutations were introduced into A206 (green spheres), L221 (magenta spheres), and F223 (cyan spheres) at the dimer interface, and into V150, L201, and L207 (blue spheres) at the BiFC interphase.17) B, Zoom-in of the dimer interface created by PyMOL. Dotted lines (red) are the predicted hydrophobic interactions among A206 (green sticks), L221 (magenta sticks), and F223 (cyan sticks). C, C–C contacts between Venus. Pairs of hydrophobic amino acid residues with a side-chain C–C distance of <5 Å are listed.
because the side chain of L207 is directed internally to the \( \beta \)-barrel and hence the effect of its length is negligible in the dimerization of Venus (see Fig. 2B). No effect was observed either with the single mutations (V150A, L201V, L201A), located far from the dimer interphase, although they efficiently reduced the non-specific fluorescent background in the bimolecular fluorescence complementation (BiFC) assay in a previous study.\(^ {17} \)

In conclusion, A206, L221, and F223 were found to be important for the dimeric interactions of Venus in living cells. The novel point mutations of A206L, L221A, and F223A reduced the weak dimerization of Venus. Among these, the A206L mutation was most effective, and was as efficient as the A206K mutation\(^ {15,18} \) on this assay. Our rapid and simple method using a truncated protein of importin \( \alpha \) should be useful in evaluating the dimeric tendency of other FPs in living cells.

Acknowledgments

We thank Dr. Atsushi Miyawaki (RIKEN) for providing pCS2-Venus, and Professor Roger Y. Tsien (University of California at San Diego) for providing pRSET-B-mCherry and pRSET-B-mOrange. We also thank Dr. Takashi Fukada (Osaka Prefecture University) for helpful discussion.

References