Journal of Cellular Biochemistry 93:497–502 (2004)

Simultaneous Detection of DsRed2-Tagged and EGFP-Tagged Human β-Interferons in the Same Single Cells

Masato Maruyama,[1] Teruko Nishio,[1] Toyokazu Yoshida,[1] Chisaki Ishida,[1] Kayo Ishida,[1] Yoshihiko Watanabe,[2] Makiya Nishikawa,[1] and Yoshinobu Takakura[1]*

[1]Department of Biopharmaceutics and Drug Metabolism, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan
[2]Department of Molecular Microbiology, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan

Abstract The red fluorescent protein DsRed2 is a useful fusion tag for various proteins, together with the enhanced green fluorescent protein (EGFP). These chromoproteins have spectral properties that allow simultaneous distinctive detection of tagged proteins in the same single cells by dual color imaging. We used them for tagging a secretory protein, human interferon-β (IFN-β). Expression plasmids for human IFN-β tagged with DsRed2 or with EGFP at the carboxyl terminal were constructed and their coexpression was examined in Mardin–Darby canine kidney epithelial cells. Although maturation of DsRed2 for coloration was slow and the color intensity was weak compared with EGFP, low temperature treatment (20°C) allowed DsRed2-tagged human IFN-β to be detected in the cells using color imaging. Consequently, the two chimeric proteins were shown to be colocalized in the same single cells by dual color confocal microscopy. This approach will be useful for investigating subcellular localization of not only cell resident proteins but also secretory proteins. J. Cell. Biochem. 93: 497–502, 2004.

Key words: epithelial cell; green fluorescent protein; interferon-β; red fluorescent protein; secretory protein

The enhanced green fluorescent protein (EGFP) is a variant of the green fluorescent protein (GFP) of the luminescent jellyfish *Aequorea victoria* [Tsien, 1998]. It contains two amino acid substitutions, F64L and S65T [Cormack et al., 1996], and is an excellent marker of gene expression and protein localization in various biological systems including mammalian cells using color imaging [Sugimoto et al., 2002; Lippincott-Schwartz and Patterson, 2003]. This provides new insights into protein functions and its cellular processes in the complex environment of mammalian cells [Lippincott-Schwartz et al., 2000]. The red fluorescent protein (DsRed or drFP583) cloned from *Discosoma* coral [Matz et al., 1999] is another promising color protein which shows red fluorescence with a maximum emission at 583 nm, although it displays slow maturation for luminescence and obligate harmful aggregation [Baird et al., 2000] compared with monomeric EGFP which matures rapidly [Cormack et al., 1996]. These properties of DsRed have been improved to some degree by introducing several mutations. DsRed2 [Terskikh et al., 2002; Yanushevich et al., 2002] contains six amino acid substitutions (A105V, I161T, S197A, R2A, K5E, and K9T) and its expression results in more rapid appearance of red fluorescence without cytotoxicity compared with DsRed, although it still forms a tetramer that is similar to

Abbreviations used: DsRed, red fluorescent protein from *Discosoma* coral; EGFP, enhanced green fluorescent protein; IFN, interferon; MDCK, Madin–Darby canine kidney.

Grant sponsor: Ministry of Education, Cultures, Sports, Science, and Technology, Japan (Grant-in-Aid for Scientific Research); Grant sponsor: Naito Foundation; Grant sponsor: 21st Century COE Program "Knowledge Information Infrastructure for Genome Science".

*Correspondence to: Yoshinobu Takakura, Department of Biopharmaceutics and Drug Metabolism, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan.
E-mail: takakura@pharm.kyoto-u.ac.jp

Received 19 May 2004; Accepted 20 May 2004
DOI 10.1002/jcb.20203

the wild type [Yarbrough et al., 2001]. DsRed2 as well as DsRed have been utilized as a tag for investigating subcellular localization of cell resident proteins [Lynch et al., 2002; Traweger et al., 2003].

In the present study, we generated an expression vector for human interferon-β (IFN-β) fused with DsRed2 and investigated the location of the DsRed2-tagged secretory protein, together with EGFP-tagged IFN-β [Maruyama et al., 2004] in epithelial Mardin–Darby canine kidney (MDCK) cells. Although DsRed2-fused IFN-β displayed slow coloration and weak red fluorescence under regular culture conditions, the fluorescence signals were significantly increased by treatment at 20°C for 16 h presumably due to retention of the protein in the cells for longer and sufficient time for maturation. Furthermore, following transfection with a mixture of the two expression plasmids, both chimeric proteins were detected in an overlapping manner in the same single cells by two color confocal microscopy. Our approach will be useful for investigating the subcellular spatial relationships of not only cell resident proteins but also secretory proteins.

MATERIALS AND METHODS

Cell Lines

The MDCK cells (type I; ATCC-CCL-34) were grown in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO-Invitrogen Co., Carlsbad, CA). The human FL cells, maintained in Eagle's minimum essential medium (MEM) (Nissui Pharmaceutical Co.) supplemented with 6% FBS, were used for IFN bioassay as previously described [Okamoto et al., 1999].

Plasmids

The expression plasmids for human IFN-β and EGFP-tagged human IFN-β (HuIFNβ-EGFP), respectively designated pCMV-HuIFNβ and pCMV-HuIFNβ-EGFP, have been described previously [Nakanishi et al., 2002; Maruyama et al., 2004]. The vectors pEGFP-N1 and pDsRed2-N1 were purchased from CLONTECH Laboratories (Palo Alto, CA). DsRed2-tagged human IFN-β expression plasmid, pCMV-HuIFNβ-DsRed2, was constructed by inserting the human IFN-β gene fragment, which was excised from pCMV-HuIFNβ-EGFP with restriction enzymes Bsh T1 and Nhe I, into pDsRed2-N1 double-digested with Bsh T1 and Nhe I.

Plasmid Transfection

MDCK cells were seeded at 5×10^4 cells/cm^2 in 24-well plates, cultured for 24 h, and then transfected with expression plasmid DNA (3 μg/ml) complexed with 10 μg/ml cationic liposomes, Lipofectamine-2000 (GIBCO-Invitrogen Co.) for 4 h, as previously described [Maruyama et al., 2004]. Then, cells were further incubated for 20 h and the culture media were subjected to IFN bioassay (see below).

In cotransfection, pCMV-HuIFNβ-DsRed2 (2.5 μg/ml) and either pCMV-HuIFNβ-EGFP or pCMV-EGFP (0.5 μg/ml) were admixed with 10 μg/ml Lipofectamine-2000 (GIBCO-Invitrogen Co.).

IFN Bioassay

Human IFN-β activities in the supernatants of the cell cultures were measured on FL cells and expressed in antiviral units (U) as described previously [Watanabe and Kawade, 1987; Nakanishi et al., 2002].

Immunostaining and Confocal Laser Scanning Microscopy

Cells, cultured on coverslips, were transfected with pCMV-HuIFNβ, pDsRed2-N1, or pCMV-HuIFNβ-DsRed2, complexed with cationic liposomes and incubated for 4 h. Cells expressing DsRed2 were further incubated for 20 h at 37°C. Then, 8 h after transfection, cells expressing HuIFN-β and HuIFNβ-DsRed2 were subjected to 20°C for a further 2 and 16 h, respectively. Cells were then fixed with 4% paraform-aldehyde in phosphate buffered saline (PBS) at room temperature for 20 min. As necessary, fixed cells were permeabilized with 0.2% Triton-X 100-PBS for 20 min and immunostained by treatment with mouse monoclonal antibodies against human IFN-β (8C3) (YAMASA Shoyu Co., Choshi, Japan) (at 1:100) followed by staining with Alexa Fluor 488- or Alexa Fluor 594-conjugated goat anti-mouse IgG (Molecular Probes, Inc., Eugene, OR) (at 1:100), in 1% BSA-1% Tween 20-PBS at 37°C for 1 h. After fixation or immunostaining, cells were immersed in 50% glycerol-2.5% DABCO (1,4-diazabicyclo-[2,2,2]octane) (Sigma-Aldrich Co., St. Louis, MO)-PBS. Emissions from the

optical x-y sections were collected using a confocal microscope (MRC-1024; BioRad, Hercules, CA) equipped with a Nikon Optiphot 2 microscope (Nikon, Tokyo, Japan) using 40× or 60× oil immersion objectives.

RESULTS

Transfection with pCMV-HuIFNβ-DsRed2 or pCMV-HuIFNβ-EGFP caused production of a similar amount of IFN activity in MDCK cells, although the activity was only about one fifteenth of that in the cells transfected with pCMV-HuIFNβ (Fig. 1). Both naive and empty vector-transfected MDCK cells displayed no detectable production of IFN activity (data not shown).

Next, HuIFNβ-DsRed2 and DsRed2 each expressed in MDCK cells were observed under confocal microscopy. Cells were transfected with pCMV-HuIFNβ-DsRed2, pDsRed2-N1, or pCMV-HuIFNβ for 4 h and then incubated at 37°C for 4, 8, 14, and 20 h. Weak red fluorescence was seen in pCMV-HuIFNβ-DsRed2 transfected cells at 14 h post transfection (p.t.) but became invisible at 20 h p.t. (Fig. 2a–d). Meanwhile, the red fluorescence in pDsRed2-N1 transfected cells was seen at 4 h p.t. and then gradually increased to a maximum level at 14 h p.t. (Fig. 2e–h). The red fluorescence was homogeneously distributed throughout the cytoplasm. When cells were transfected with pCMV-HuIFNβ followed by immunostaining with anti-HuIFN-β, the red fluorescence spots derived from HuIFN-β were observed at 4 h p.t. (Fig. 2i–l). These results indicate that the maturation of the auto-fluorescent moiety of DsRed2 is slow as reported [Bevis and Glick, 2002], or it takes a longer time duration for the maturation compared with EGFP.

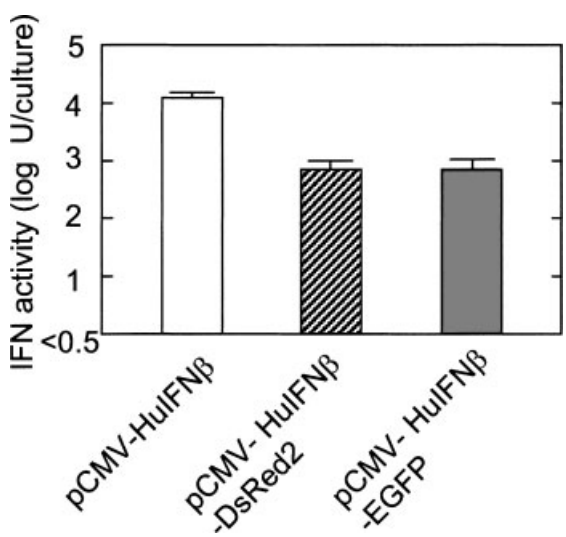

Fig. 1. Interferon (IFN) production in Madin–Darby canine kidney (MDCK) cells following transfection with expression plasmids. Cells grown in 24-well plates were transfected for 4 h with expression plasmid, pCMV-HuIFNβ, pCMV-HuIFNβ-DsRed2, or pCMV-HuIFNβ-EGFP. The IFN activity in the culture fluids was subjected to bioassay. Each value represents the mean ± SD, (n = 3).

These results indicate that HuIFNβ-DsRed2 needs to be retained in the cells before secretion to become clearly observable under confocal microscopy. To retard the secretion of the fusion protein after translation, the cells were treated at 20°C temperature, because low-temperature treatment leads to the accumulation of newly synthesized proteins in the trafficking systems, including the endoplasmic reticulum, Golgi and trans-Golgi network [Maruyama et al., 2004]. Eight hours after transfection with pCMV-HuIFNβ-DsRed2, the temperature of cell culture was reduced from 37 to 20°C for 2, 4, 8, or 16 h, and then the cells were fixed and subjected to confocal microscopy (Fig. 3). The red fluorescence became observable at 8 h after the temperature shift and the intensity was substantially augmented at 16 h.

To confirm whether HuIFNβ-DsRed2 expressed in MDCK is the fusion protein of intact IFN-β and DsRed2, the gene-transfected cells were stained with the specific monoclonal antibody for HuIFN-β. As shown in Figure 4a–c, the cellular red signals of DsRed2 were almost superimposed on the green signals of HuIFN-β, which were dispersed in the cytoplasm as aggregations of flecks. The cellular distribution pattern was similar to that of the signals for HuIFN-β in the pCMV-HuIFNβ-transfected cells (Fig. 4d–f), but different from the homogeneous distribution of red signals throughout the pDsRed2-N1 transfected cells (Fig. 4g–i). These results suggest that both HuIFN-β and DsRed2 moieties of the fusion protein retain their intact conformations in the cells.

Finally, we examined the simultaneous imaging of the two kinds of fluorescent proteins in the same single cells co-transfected with pCMV-HuIFNβ-DsRed2 and pCMV-HuIFNβ-EGFP or pEGFP-N1. Since the color intensity derived from DsRed2 is lower than that from EGFP, five-fold more DsRed2-related plasmid was used compared with the amount of EGFP-related plasmid. As shown in Figure 5, the cellular

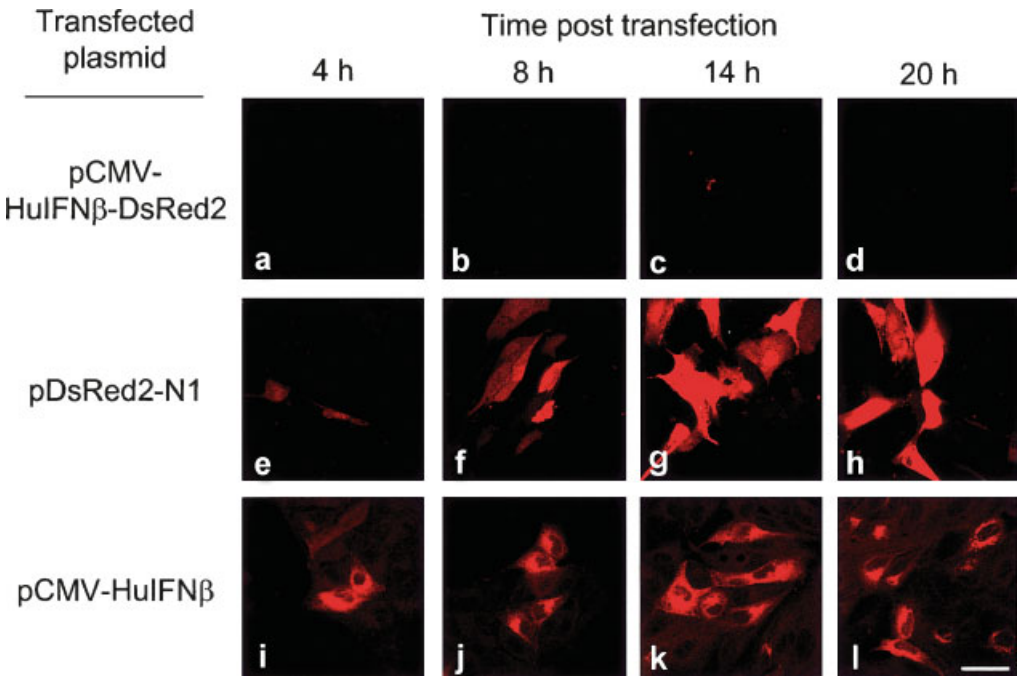

Fig. 2. Confocal microcsopy of HuIFNβ-DsRed2, DsRed2, and HuIFN-β expressed in MDCK cells. Cells cultured on coverslips were transfected with pCMV-HuIFNβ-DsRed2 (**a**–**d**), pDsRed2-N1 (**e**–**h**) and pCMV-HuIFNβ (**i**–**l**) for 4 h. Then, after incubation for indicated times, cells transfected with pCMV-HuIFNβ-DsRed2 or pDsRed2-N1 were fixed and subjected to confocal microscopy. Cells transfected with pCMV-HuIFNβ were fixed, permeabilized, and immunostained with the mouse anti-HuIFN-β (8C3) and the secondary Alexa Fluor 594-conjugated goat anti-mouse IgG, followed by confocal imaging. All images are of the same magnification; the white scale bar in panel l = 50 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

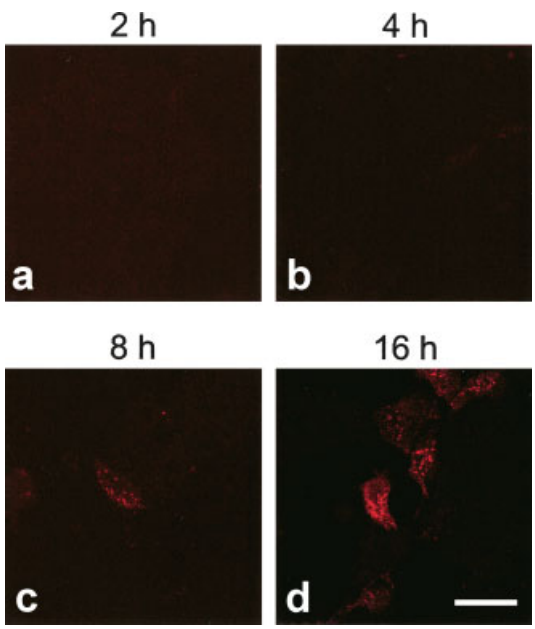

Fig. 3. Temperature control to visualize HuIFNβ-DsRed2 expressed in MDCK cells. Cells cultured on coverslips were transfected with pCMV-HuIFNβ-DsRed2 as in Figure 2. The temperature of the culture was reduced from 37 to 20°C at 4 h post transfection, and the cells were further incubated at 20°C for the indicated times, followed by fixation and confocal microscopy. All images are of the same magnification; the white bar in panel d = 50 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

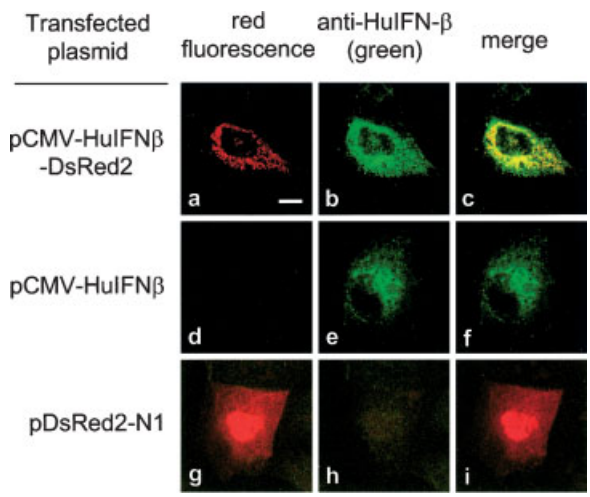

Fig. 4. Visualization of HuIFNβ-DsRed2, HuIFN-β and DsRed2 expressed in MDCK cells. Cells cultured on coverslips were transfected with plasmids, pCMV-HuIFNβ-DsRed2 (**a**–**c**), pCMV-HuIFNβ (**d**–**f**), or pDsRed2-N1 (**g**–**i**), and were fixed, permeabilized, and stained with the mouse antibody against human IFN-β (8C3) and the secondary Alexa Fluor 488-conjugated goat anti-mouse IgG, followed by confocal imaging of the red fluorescence (a, d, g) or green color (b, e, h). Those overlays (c, f, i) are shown. All images are of the same magnification; the white bar in panel a represents 10 μm.

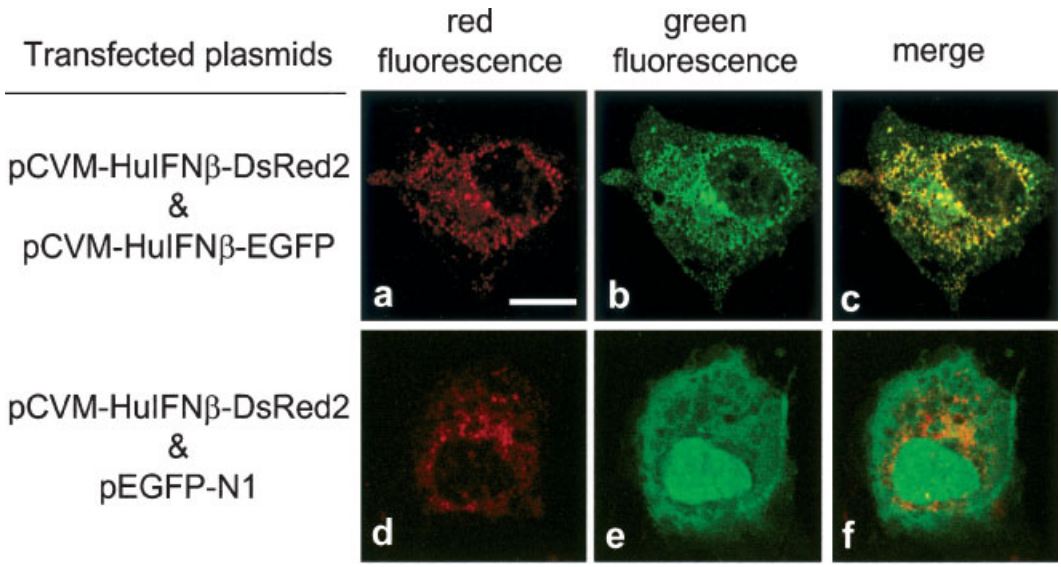

Fig. 5. Visualization of HuIFNβ-DsRed2 and HuIFNβ-EGFP in the same single cells. Cells cultured on coverslips were co-transfected with plasmids, pCMV-HuIFNβ-DsRed2 and pCMV-HuIFNβ-EGFP (**a**–**c**) or pEGFP-N1 (**d**–**f**), fixed and then analyzed by confocal microscopy. Red fluorescence (a, d), green fluorescence (b, e), and those over-lays (c, f) are shown. All images are of the same magnification; the white bar in panel a represents 10 μm.

signals of red and green fluorescence were mostly colocalized in the same single cells co-expressing HuIFNβ-DsRed2 and HuIFNβ-EGFP (a–c), while the two fluorescence signals did not overlap in the same single cells co-expressing HuIFNβ-DsRed2 and EGFP (d–f).

DISCUSSION

Previously, we constructed a plasmid encoding EGFP-fused HuIFN-β and used it for investigating cellular trafficking of HuIFN-β in MDCK cells [Maruyama et al., 2004]. In the present study, we engineered another expression plasmid for DsRed2-fused HuIFN-β and compared it with the former one. The transfection efficiency was similar in both plasmids as assessed by the production of antiviral activity, although the efficiency was very low compared with HuIFN-β expression plasmids. This apparent reduction in gene expression may be caused by the approximately two-fold longer coding sequence for the chimeric proteins, although fine gene construction (e.g., non-coding regions) may influence the expression efficiency. Also, the C-terminal chromoprotein portions may sterically affect the interaction between the IFN-β portion and its cellular receptor. Apart from this, it is likely that the chimeric proteins consist of the chromoprotein and the intact IFN-β which are linked by an 8-amino acid spacer [Maruyama et al., 2004], since a normal color image was obtained for the chromoprotein portion and staining occurred with specific monoclonal antibody against the HuIFN-β portion (Fig. 4).

While EGFP forms a chromophore rapidly after translation (half time = 25 min) [Cormack et al., 1996], DsRed2 takes longer to develop a coloration (half time = 6.5 h) [Bevis and Glick, 2002]. This is the reason why color signals in HuIFNβ-DsRed2-expressing cells are weak: the chimeric protein will be secreted before establishing a conformation for coloration. Thus, secretory proteins have not been examined so far by using the red color tag, and this is the first report that secretory proteins fused with DsRed2 can be visualized in mammary cells.

When the intracellular localization of HuIFNβ-DsRed2 was investigated, it was difficult to see the red fluorescence under normal conditions. This was considered to be due mainly to the slow maturation of the DsRed2 portion for coloration as mentioned above: the fusion protein should be secreted prior to maturation for fluorescence. To overcome this problem, the cells expressing HuIFNβ-DsRed2 were incubated at 20°C for a period. During the low temperature treatment, the cellular red fluorescence signals substantially increased to

become observable depending on the incubation time. These results suggest that DsRed2 is also useful for imaging secretory proteins in cells by modifying the temperature, although improved DsRed derivatives are needed which mature more rapidly to emit red fluorescence [Bevis and Glick, 2002; Campbell et al., 2002].

When cells were transfected with a mixture of plasmids for HuIFNβ-DsRed2 and HuIFNβ-EGFP followed by treament at 20°C, both fusion proteins were demonstrated to colocalize in the same single cells under dual color confocal microscopy (Fig. 5). Therefore, this approach will be useful for investigating the simultaneous subcellular localization of various proteins, including secretory proteins as well as membrane proteins, which are concomitantly expressed, e.g., by different gene-transfer modes.

REFERENCES

Baird GS, Zacharias DA, Tsien RY. 2000. Biochemistry, mutagenesis, and oligomerization of DsRed, a red fluorescent protein from coral. Proc Natl Acad Sci USA 97:11984–11989.

Bevis BJ, Glick BS. 2002. Rapidly maturing variants of the *Discosoma* red fluorescent protein (DsRed). Nat Biotechnol 20:83–87.

Campbell PR, Tour O, Palmer AE, Steinbach PA, Baired GS, Zacharias DA, Tsien RY. 2002. A monomeric red fluorescent protein. Proc Natl Acad Sci USA 99:7877–7882.

Cormack BP, Valdivia RH, Falkow S. 1996. FACS-optimized mutants of the green fluorescent protein (GFP). Gene 173:33–38.

Lippincott-Schwartz J, Patterson GH. 2003. Development and use of fluorescent protein markers in living cells. Science 300:87–91.

Lippincott-Schwartz J, Roberts TH, Hirschberg K. 2000. Secretory protein trafficking and organelle dynamics in living cells. Annu Rev Cell Dev Biol 16:557–589.

Lynch DT, Zimmerman JS, Rowe DT. 2002. Epstein-Barr virus latent membrane protein 2B (LMP2B) co-localizes with LMP2A in perinuclear regions in transiently transfected cells. J Gen Virol 83:1025–1035.

Maruyama M, Nishio T, Kato T, Yoshida T, Ishida C, Watanabe Y, Nishikawa M, Kaneda Y, Takakura Y. 2004. Subcellular trafficking of exogenously expressed interferon-β in Madin-Darby canine kidney cells. J Cell Physiol (in press).

Matz MV, Fradkov AF, Labas YA, Savitsky AP, Zaraisky AG, Markelov ML, Lukyanov SA. 1999. Fluorescent proteins from nonbioluminescent Anthozoa species. Nat Biotechnol 17:969–973.

Nakanishi K, Watanabe Y, Maruyama M, Yamashita F, Takakura Y, Hashida M. 2002. Secretion polarity of interferon-β in epithelial cell lines. Arch Biochem Biophys 402:201–207.

Okamoto S, Nakanishi K, Watanabe Y, Yamashita F, Takakura Y, Hashida M. 1999. Stimulation side-dependent asymmetrical secretion of poly I:poly C-induced interferon-β from polarized epithelial cell lines. Biochem Biophys Res Commun 254:5–9.

Sugimoto K, Urano T, Zushi H, Inoue K, Tasaka H, Tachibana M, Dotsu M. 2002. Molecular dynamics of Aurora-A kinase in living mitotic cells simultaneously visualized with histone H3 and nuclear membrane protein importinalpha. Cell Struct Funct 27:457–467.

Terskikh AV, Fradkov AF, Zaraisky AG, Kajava AV, Angres B. 2002. Analysis of DsRed mutants. Space around the fluorophore accelerates fluorescence development. J Biol Chem 277:7633–7636.

Traweger A, Fuchs R, Krizbai IA, Weiger TM, Bauer HC, Bauer H. 2003. The tight junction protein ZO-2 localizes to the nucleus and interacts with the heterogeneous nuclear ribonucleoprotein scaffold attachment factor B. J Biol Chem 278:2692–2700.

Tsien RY. 1998. The green fluorescent protein. Annu Rev Biochem 67:509–544.

Watanabe Y, Kawade Y. 1987. Induction, production and purification of natural mouse IFN-α and -β. In: Clemens MJ, Morris AG, Gearing AJH, editors. Lymphokines and interferons: A practical approach. Oxford: IRL Press. pp 1–14.

Yanushevich YG, Staroverov DB, Savitsky AP, Fradkov AF, Gurskaya NG, Bulina ME, Lukyanov KA, Lukyanov SA. 2002. A strategy for the generation of non-aggregating mutants of Anthozoa fluorescent proteins. FEBS Lett 511:11–14.

Yarbrough D, Wachter RM, Kallio K, Matz MV, Remington SJ. 2001. Refined crystal structure of DsRed, a red fluorescent protein from coral, at 2.0-A resolution. Proc Natl Acad Sci USA 98:462–467.