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Real-Time Detection of Cellular Death Receptor-4 Activation by Fluorescence Resonance Energy Transfer

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Abstract

Targeted therapy involving the activation of death receptors DR4 and/or DR5 by its ligand, TRAIL, can selectively induce apoptosis in certain tumor cells. In order to profile the dynamic activation or trimerization of TRAIL-DR4 in live cells in real time, the development of an apoptosis reporter cell line is essential. Fluorescence resonance energy transfer (FRET) technology via a FRET pair, cyan fluorescence protein (CFP) and yellow fluorescence protein (YFP), was used in this study. DR4-CFP and DR4-YFP were stably expressed in human lung cancer PC9 cells. Flow cytometer sorting and limited dilution coupled with fluorescence microscopy were used to select a monoclonal reporter cell line with high and compatible expression levels of DR4-CFP and DR4-YFP. FRET experiments were conducted and FRET efficiencies were monitored according to the Siegel's YFP photobleaching FRET protocol. Upon TRAIL induction a significant increase in FRET efficiencies from 5 to 9% demonstrated the ability of the DR4-CFP/YFP reporter cell line in monitoring the dynamic activation of TRAIL pathways. 3D reconstructed confocal images of DR4-CFP/YFP reporter cells exhibited a colocalized expression of DR4-CFP and DR4-YFP mainly on cell membranes. FRET results obtained during this study complements the use of epi-fluorescence microscopy for FRET analysis. The real-time FRET analysis allows the dynamic profiling of the activation of TRAIL pathways by using the time-lapse fluorescence microscopy. Therefore, DR4-CFP/YFP PC9 reporter cells along with FRET technology can be used as a tool for anti-cancer drug screening to identify compounds that are capable of activating TRAIL pathways.

Keywords

apoptosis; TRAIL receptors; DR4; reporter cell line; FRET

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Introduction

Targeting tumor cells with limited detrimental consequences to normal cells is the main goal of cancer therapy (Kasibhatla and Tseng 2003). A variety of approaches aim to trigger cancer-selective cell death for the treatment of cancer. Apoptosis is a highly complicated process that includes two main initiating pathways, the extrinsic or the death receptor pathway and the intrinsic or the mitochondrial pathway. These two pathways initiate apoptosis both independently and interdependently (Igney and Krammer 2002). In extrinsic pathways, transmembrane receptors, known as death receptors, belong to the tumor necrosis factor (TNF) receptor superfamily (Locksley et al. 2001). The binding between death receptors and their ligands causes the recruitment of adaptor molecules such as FADD or TRADD, then the activation of initiator caspases 8 and 10, which in turn cleave and activate executioner caspases 3 and 7 (Krammer 2000), eventually leading to apoptosis. The important death ligand-receptor pairs that initiate this extrinsic pathway include FasL/FasR (Martinvalet et al. 2005), TNF-Related Apoptosis-Inducing Ligand (TRAIL)/DR4 (Suliman et al. 2001), TRAIL/DR5 and TNF- α /TNFR1 (Ashkenazi and Dixit 1988).

TRAIL is a homotrimeric type-II transmembrane protein that contains 281 amino acids with molecular weight of 24KDa (Pitti et al. 1996). TRAIL-mRNA is constitutively expressed in most of human tissues. However, the functional TRAIL that can induce apoptosis is mainly produced by immune effector cells such as T cells, natural killer cells, monocytes and dendritic cells. TRAIL has been shown to kill a wide variety of tumor cells with minimal effects on normal cells (Ashkenazi et al. 1999). This is because DR4 and DR5 are mainly expressed in transformed cells while its decoy receptors (i.e. DcR1, DcR2 and OPG) are expressed in normal cells (Clancy et al. 2005). Currently, TRAIL based therapies are under development using recombinant TRAIL or agonistic antibodies (e.g. Mapatumumab (Pukac et al. 2005)) for DR4 and DR5 to either enhance TRAIL toxicity (Sloot et al. 2006) or combine with chemotherapy and radiotherapy to sensitize tumor cells (Lashinger et al. 2005; Martin et al. 2005; Parrot 2005).

Despite TRAIL's ability against tumor cells, studies to date have failed to elucidate the exact physiological role of TRAIL in the human body. Some studies in mice models have implicated the possible role of TRAIL in tumor surveillance (Schmaltz et al. 2002), immune cell mediated targeted cell killing (Fangera et al. 1999; Kayagaki et al. 1999) and innate immune system regulation. In order to screen potential TRAIL related reagents either from natural extracts or synthetic chemistry and study the dynamic interaction between the ligands and DR4/DR5, a fluorescence reporter system which can detect the activation of TRAIL-receptors in live cells in real time using a widely accessible epi-fluorescence microscope is desirable.

Fluorescence resonance energy transfer (FRET) technology has been used to monitor dynamic protein-protein interactions in live cells using flow cytometry since 1980s (Szollosi et al. 1987; Szollosi et al. 1984; Trón et al. 1984). FRET is a physical phenomenon by which energy from a fluorophore (donor) at excited state is non-radioactively transferred to a neighboring fluorophore (acceptor) through dipole-dipole interactions (Förster 1984). The strong dependence of FRET on the distance enables the use of FRET to image molecular

interactions at 1–10nm distance. Since the TRAIL, a trimer, activates the homotrimerization or heterotrimerization of DR4/DR5, the real time activation of DR4 or DR5 in live cells can be monitored once a molecule of DR4 or DR5 is conjugated with a donor fluorophore and another DR4 or DR5 with an acceptor fluorophore. The development of green fluorescent proteins (GFP) and variants such as yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP) enables to use CFP/YFP as a FRET pair (Patterson et al. 2000). TNFR1 activation detected by flow cytometry and fluorescence microscopy FRET signals was reported in human embryonic kidney cells (293T) transiently expressing TNFR1-CFP/TNFR1-YFP and stimulated with TNF- α (Chan et al. 2001).

In this study, we developed a monoclonal DR4 FRET reporter human lung cancer cell line to monitor the dynamic activation/homotrimerization of DR4 receptors in real time via epi-fluorescence microscopy. Monoclonal DR4-CFP/YFP cell lines with similar levels of CFP and YFP expression were selected. Characterization of DR4-CFP/YFP colocalization on cell membranes and FRET efficiency under the TRAIL stimulation indicates that this monoclonal DR4-CFP/YFP reporter cell line could be used for screening potential anti-cancer drugs activating TRAIL pathways and for physiological studies of TRAIL signaling.

Materials and Methods

The DR4-CFP/YFP FRET reporter cell line was constructed by co-transfection with two plasmids encoding fusion protein genes of DR4-CFP and DR4-YFP in lung cancer cells (PC9). The characterization of the DR4 reporter cell line, such as the status of fusion proteins and their localization, was accomplished. The dynamic activation of TRAIL-DR4 complexes under the stimulation of TRAIL was monitored via CFP/YFP FRET signals in the reporter cells using an epi-fluorescence microscope with CFP and YFP filters. All reagents and chemicals without manufacture labels were purchased from Sigma-Aldrich (St Louis, MO).

Plasmid Isolation

Bacterial strain [DH5 α] were transformed with 2 recombinant plasmids containing genes encoding for human TRAIL receptor DR4 fused with genes encoding either cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP). The plasmids were kindly provided by Dr. Francis K. Chan's lab from University of Massachusetts Medical School, Worcester, MA. The plasmids are pcDNA3-HA-DR4 CD-CFP (pDR4-CFP) and pcDNA3-HA-DR4 CD-YFP (pDR4-YFP) (Clancy et al. 2005). A maxi prep kit (Qiagen, Valencia, CA) was used to purify plasmids using the manufacture's protocol.

Cell Culture

Non-small cell lung cancer cells (PC-9) were cultured using RPMI 1640 media with 10% heat-inactivated fetal bovine serum, and 1% penicillin-streptomycin (Invitrogen, Grand Island NY).

Construction of a DR4-CFP/YFP FRET reporter cell line

In order to obtain a DR4-CFP/YFP FRET reporter cell line, pDR4-CFP and pDR4-YFP were co-transfected in PC9 cells via electroporation using Gene Pulser Xcell (Bio-Rad, Hercules, CA) at capacitance of 960 μ F and voltage of 250V. Geneticin G418 (0.6mg/ml) was used to select stably transfected cells. However, the majority of the stable transfected PC9 cells expressed either DR4-CFP or DR4-YFP but not co-localized expression of both plasmids. Therefore, Fluorescence Activated Cell Sorting (FACS) (Becton Dickinson, Franklin Lakes, NJ) was used to sort cells with high DR4-CFP expression, followed by counter transfection of pDR4-YFP via electroporation. The second FACS sorting was performed to select PC9 population with both DR4-CFP and DR4-YFP expression. Limited dilution coupled with a Zeiss fluorescence microscope (Axio Observer) with a CFP filter (Excitation/Emission- 436/480nm) and an YFP filter (500/535nm) was conducted to obtain a monoclonal DR4-CFP/YFP reporter cell line with high and similar expression levels of both CFP and YFP.

Confirming the fusion protein expression of DR4-CFP or DR4-YFP

DR4-CFP/YFP reporter PC-9 cells were lysed with 1% SDS, 50 mM Tris-HCl, 5 mM EDTA, and protease inhibitors. The cell lysate was centrifuged at 21,000g for 10 min at 4°C and the supernatant was collected for Western Blot. Total protein concentrations were determined by the Bradford method with the protein assay kit (Bio-Rad Laboratories, Hercules, CA). 25–40 μ g of total proteins were loaded in 10% Tris-HCl Ready Gel (Bio-Rad, Hercules, CA) for electrophoresis, and polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Hercules, CA) were used for blot transfer. The membranes were incubated with a primary antibody, mouse anti-DR4 monoclonal antibody (Pierce Biotechnology, Rockford IL), at 1:1000 dilution. The secondary antibody was horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (Assay Designs) at 1:5000 dilution. Tetramethylbenzidine (TMB) substrate kit (Vector Laboratories, Burlingame, CA) was used to visualize the protein bands. Membranes were dried and scanned into digital images.

Verification of DR4-CFP and DR4-YFP colocalization by Confocal Microscopy

Co-localized expression of DR4-CFP and DR4-YFP in DR4 reporter cells was confirmed by observing cells under a confocal microscope (Leica TCS SP2). The 434 nm laser was used to excite the CFP and the 514 nm laser was used to excite the YFP. Sequential Z-stack images covering the whole volume of the cells were acquired. The acquired images were then processed using the Leica software (Leica TCS SP2). A 3D image demonstrating the colocalized expression of both fluorescent proteins, DR4-CFP and DR-YFP, was reconstructed using Mimics (Materialise HQ, Belgium).

Monitoring DR4 activation via FRET using photobleaching methods

DR4-CFP/YFP PC9 reporter cells were cultured in a 4-well slide chamber (Thermo Fisher Scientific) and induced with rhTRAIL (R&D Systems, USA) at 400ng/ml. Immediately after TRAIL induction, cells were exposed to dynamic or batch YFP photobleaching in separate experiments. The same Zeiss fluorescence microscope (Axio Observer) equipped with CFP and YFP filters as well as the AxioVision software with a Physiology Module was used to

monitoring the dynamic TRAIL-DR4 complex activation via FRET through Siegel acceptor photobleaching method (Chan et al. 2001).

For dynamic YFP photobleaching, a time lapse series of images of DR4-CFP/YFP cells using the CFP filter were taken using the Axiovision software. The Physiology Module of AxioVision was set-up in a way such that the acceptor (YFP) was bleached for 30 seconds by blasting the cells with the YFP filter itself. After 30 seconds of photobleaching, an image was acquired using the CFP filter. This cycle of YFP photobleaching for 30 seconds followed by a CFP image acquisition was repeated for 18 minutes to capture the FRET signal during the activation of the TRAIL- DR4 complex. For batch YFP photobleaching, the DR4 reporter PC9 cells were also subjected to batch YFP bleaching. The protocol was set up exactly in the same way as that of dynamic bleaching apart from the fact that in batch bleaching only 2 CFP images are acquired, one before the YFP photobleaching and one 15 minutes after the continuous YFP photobleaching.

Once the images from either dynamic or batch YFP photobleaching were acquired, the FRET measurements are performed using the following Siegel acceptor photobleaching formula.

$$F_{\text{eff}}(t) = \left[1 - \frac{FI_{\text{CFP}}(0)}{FI_{\text{CFP}}(t)} \right] * 100$$

Where F_{eff} is FRET efficiency, $FI_{\text{CFP}}(0)$ CFP fluorescence intensity before YFP bleaching, and $FI_{\text{CFP}}(t)$ CFP fluorescence intensity after time t of YFP bleaching. The FRET efficiency values were calculated in Physiology Module from different regions manually drawn around the cells. For the dynamic photobleaching, areas of interest (AOI) were drawn such that maximum cells were included in them; while for the batching photobleaching, these regions were drawn in such a way that each region contains one cell. However in some cases where cellular density was too high to separate individual cells, then these regions were drawn to include multiple cells. Both dynamic and batch YFP photobleaching experiments were repeated twice with or without TRAIL stimulation. In addition, FRET efficiency was calculated using CFP intensities only from cell membranes with Siegel's photobleaching formula to show the robustness of the method developed in this study. The analysis was done over 20 individual cells treated with TRAIL and 15 minute photobleaching, from which 10 cells had CFP expression all around their membranes while another 10 cells had CFP expression on their membranes partially.

Caspase 3 Activity Assay

PC9 and PC9 DR4-CFP cells were subjected to TRAIL (100ng/ml) stimulation for 4.5 hours. They were lysed in a cell lysis buffer (20 mM HEPES of pH 7.5, 20mM β -mercaptoethanol, 1 mM EDTA, 150 mM KCL). Cell lysate was centrifuged for 15 min at 4 °C, and the supernatant was collected for caspase 3 analysis. The total protein concentration was determined by the Bradford method with the Bio-Rad protein assay kit. 120 μ g total proteins of PC9 and PC9 DR4-CFP were transferred into a chilled 96-well plate. The caspase 3 activity was measured by the reaction with 1.5mM DEVD RH110 substrate (Anaspec, Fremont, CA), which generates fluorescent green molecules upon

caspace 3 cleavage. For the kinetic measurements, the plate was read at 485 nm/535 nm at 30°C every two minutes for 2 hours using an M2e Microplate Reader (Molecular Devices, Silicon Valley, CA). The average green fluorescence intensity was calculated from 3 biological samples for each time point in Excel, and the experiments were repeated twice.

Statistical Analysis

All values were expressed as mean $\pm$ standard deviation (SD) if not specifically labeled, and analyzed statistically using a two-tailed Student's t-test. The level of significance was set at $p < 0.05$.

Results

A well-characterized monoclonal DR4-CFP/YFP reporter cell line using non-small cell lung cancer cells was described below for future anti-cancer drug screening targeting the TRAIL pathways.

DR4-CFP/YFP FRET reporter cell line

Expression levels of DR4-CFP and DR4-YFP as well as their co-localization viewed by epifluorescence microscopy in monoclonal DR4-CFP/YFP PC9 reporter cells are shown in Figure 1. Figure 1A is a 20 $\times$ phase contrast image of the DR4-CFP/YFP PC9 reporter cells, while 1B and 1C are fluorescence images acquired via CFP and YFP channels respectively. The green fluorescent cells in the overlay image (Fig. 1D) clearly indicate the co-localized expression of both DR4-CFP and DR4-YFP in majority of cells. Though the fluorescence intensity may be different among individual cells, the expression levels between CFP and YFP in the same cell are similar except a small number of cells that have higher YFP expression than CFP. A rough estimation by yellow area vs. green area in Figure 1D reveals that more than 90% of the DR4-CFP/YFP reporter cells have co-localized CFP and YFP expressions at similar levels.

The fusion status of DR4 and CFP/YFP

Figure 2 shows that an anti-human DR4 monoclonal antibody recognized two prominent protein bands in DR4-CFP/YFP cell lysate, one near 89 KDa and another 50–60 KDa. The presence of two specific bands confirmed that the DR4-CFP/YFP PC9 reporter cells contain both endogenous DR4 (56 KDa) and the recombinant DR4-CFP and DR4-YFP, and the fusion of the fluorescence protein CFP/YFP to the DR4 had no hindrance on the DR4 antibody recognition site on the DR4 receptor.

Dynamics of DR4 activation detected by FRET signals in DR4-CFP/YFP reporter cells

The energy transferred between the DR4-CFP (donor) and DR4-YFP (acceptor) during the activation/trimerization of DR4 receptors due to the binding of TRAIL and DR4 molecules was monitored with the dynamic acceptor photobleaching method. Theoretically, if FRET is taking place, then upon photobleaching the YFP molecules the CFP's emission intensity should increase with time since there are no YFP molecules to quench the CFP's emission during bleaching. CFP image series in Figure 3A~F, which represent one area of interest (AOI3), show that there was an increase in CFP intensity over 18 min dynamic bleaching

upon TRAIL induction. The quantification of CFP image series using the FRET Siegel formula show 6–12% increases of FRET efficiency at the end of 18 minutes of acceptor photobleaching. The representative FRET efficiency curves of four AOIs from dynamic photobleaching experiments are demonstrated in Figure 3G.

In order to confirm the FRET efficiency of DR4-CFP and DR4-YFP under the TRAIL stimulation, 15 min batch photobleaching of YFP was performed right after adding TRAIL to the cell culture. Results from quantitative analysis of CFP images before and after photobleaching using the same Siegel formula are shown in Figure 4. When the analysis is over whole single cells or small cell patches, the average FRET efficiency for DR4-CFP/YFP reporter cells treated with TRAIL (n=149) is 9.29 ± 2.58 %, which is significantly greater than that of untreated DR4-CFP/YFP reporter cells (5.49 ± 1.65 %, n=142). The median values of FRET efficiency for both conditions are similar to their mean values shown in Figure 4A. The results obtained from batch and dynamic photobleaching experiments demonstrate that DR4 homotrimerization upon TRAIL treatment can be monitored in real time using the DR4-CFP/YFP reporter cell line via FRET measurements. When FRET efficiency is calculated only from cell membranes, it increases to 27.84 ± 7.76 % (n=20).

Detailed colocalization of DR4-CFP and DR4-YFP in a 3D view

Cross-section images of a reconstructed 3D confocal image in Figure 5 unveil exact locations of DR4-CFP and DR4-YFP in a DR4-CFP/YFP reporter cell attached to the glass bottom of a culture chamber. The approximate dimensions of the cell are $50 \mu\text{m} \times 30 \mu\text{m}$ round and $17 \mu\text{m}$ in height. The majority of DR4-CFP and DR4-YFP is expressed on the cell membrane. However, some of them do reside in cytoplasm. Figure 5C also shows that there is a great degree of DR4-CFP and DR4-YFP co-localization illustrated by green regions, but it is not 100%.

Effects of fused CFPs on the activation of downstream apoptosis pathway

CFP or YFP molecules in the DR4-CFP/YFP reporter cell line are fused at the C terminal of DR4 at the inner side of cell membranes. The possibility of their adverse effects on recruiting procaspase 8/10 molecules after DR4 activation was investigated by analyzing caspase 3 activities that are downstream indicators of an apoptosis pathway. Shown in Figure 6, PC9 and PC9 DR4-CFP cells have the same basal level of caspase 3 activities without TRAIL stimulation. However, there is about 4 times increase in caspase 3 activities in TRAIL treated PC9 cells compared to control cells while the increase is only about 1.3 folds for TRAIL treated DR4-CFP reporter cells. The reduced caspase 3 activities in the DR4-CFP cells indicate an adverse effect of CFP proteins fused at the C terminal of DR4 on the activation of apoptosis downstream signals after DR4 trimerization. As a reporter cell line, activation of DR4 without downstream caspase activation is desirable, because the apoptotic morphology associated with caspase activation, such as cell shrinkage and membrane blebbing, will interfere with the microscopic observation and FRET measurement.

Discussion and Conclusion

In this study, we have developed a FRET based early apoptosis reporting system via the detection of TRAIL-DR4 activation for the screening of potential anti-cancer compounds. The monoclonal DR4 reporter cell line consists of DR4-CFP and DR4-YFP fusion proteins (Fig. 2) colocalized on cell membranes of non-small cell lung cancer cells (Fig. 1&5). The FRET efficiency observed using YFP photobleaching methods with epi-fluorescence microscopy was $9.29 \pm 2.58\%$ when cells are treated with 400ng/ml TRAIL, which was significantly higher than the background FRET signal of $5.49 \pm 1.65\%$ without TRAIL (Fig. 3&4). The CFP/YFP fused at the C terminal of DR4 molecules inhibited the apoptotic signal transduction from the cell membrane to the downstream caspase 3 level (Fig. 6). Thus, the DR4-CFP/YFP FRET reporter cell line can be a valuable tool for anti-cancer drug screening targeting TRAIL pathways.

FRET determined through the acceptor photobleaching method is regarded as accurate and true FRET signal (Chan et al. 2001). However, the FRET signal from CFP/YFP pair can be also measured directly through FRET filters (436/535nm) on a fluorescence microscope. In our preliminary study, increased FRET signals were observed directly using a FRET filter set on Zeiss Axio Observer in HepG2 (human liver cancer) cells transiently co-transfected with pDR4-CFP and pDR5-YFP upon TRAIL stimulation (data not shown). In order to obtain true FRET signals using this direct FRET measurement, donor (CFP) cross-talk and acceptor (YFP) leakage effects have to be taken into account for the final FRET value calculation. The following Youvan's formula was used to calculate the true FRET signal intensity at time t (Youvan et al. 1997).

$$\text{FRET}(t) = FI_{\text{fret}}(t) - cf_{\text{cfp}} * FI_{\text{cfp}}(t) - cf_{\text{yfp}} * FI_{\text{yfp}}(t)$$

Where FI is fluorescence intensity and cf is a correction factor. The cf was chosen in such a way that there was minimum donor crosstalk and acceptor leakage in the FRET image. Two stable transfected PC9 cell lines with DR4-CFP only and DR4-YFP only were generated to obtain cf_{cfp} and cf_{yfp} , which were 40–50% and 10–20% in our systems, respectively. Appropriate settings of this correction factor value is very critical in the FRET Youvan method as over-compensation or under-compensation would result in a FRET signal that is not because of the energy transfer between CFP and YFP but due to fluorophore's crosstalk artifacts. Images from FRET, CFP and YFP channels have to be taken at each time to obtain a corrected FRET signal. Due to its simplicity and accuracy YFP photobleaching method was chosen for the study of monoclonal DR4-CFP/YFP reporter cells, which will ultimately be used in high throughput anti-cancer drug screening.

Though dynamic photobleaching produces the similar FRET efficiency as batch photobleaching does in DR4-CFP/YFP reporter cells upon a TRAIL treatment, these two methods have their own pros and cons. Data collected from dynamic photobleaching method unveils not only the maximum FRET signal but also the binding kinetics of ligands and receptors. However, special software and hardware are required to setup a dynamic photobleaching experiment. On the other hand, settings of batch photobleaching are simple

and standard. Limitations of batch photobleaching include that it cannot provide any ligand-receptor kinetics and the optimized photobleaching time has to be determined in advance.

The FRET efficiency of pure CFP and YFP proteins tagged on small beads in *in vitro* experiments is in the range of 40–50% (Vogel et al. 2006). In our monoclonal DR4-CFP/YFP reporter cells, the FRET efficiency observed was about 9% upon TRAIL treatment. One explanation for lower FRET efficiencies in cellular conditions is the presence of relatively higher amount of endogenous DR4 in the DR4-CFP/YFP reporter cells (see Fig. 2). Thus formation of hybrid states with endogenous and fluorescently tagged DR4 molecules may decrease the overall FRET efficiency. A high FRET efficiency is possible only if a trimeric DR4 contains only fluorescently tagged proteins. The FRET efficiency calculation method implemented in AxioVision software may also lead to an underestimated measurement. The FRET efficiencies are calculated based on the mean fluorescence intensity per pixel over the entire area of interest (AOI). Each AOI contains a single cell and most of the FRET signals are localized at cell membranes. When an AOI was selected only around cell membranes and CFP intensities were measured, the FRET efficiency was increased to about 27% calculated using the same Siegel's formula. However, specifically and manually selecting cell membrane regions for FRET analysis are time-consuming and demand a lot of manpower. It will be unlikely incorporated into an automated FRET image analysis in the future.

In our DR4-CFP/YFP reporter lung cancer cells, there was a background FRET signal at about 5% without TRAIL stimulation. The level of background FRET from endogenous TRAIL is cell-type dependent because different kinds of cells produce different amount of endogenous TRAIL. A background FRET efficiency of 9.78% was reported in human embryonic kidney (293T) cells that were transiently co-transfected with DR4-CFP and DR4-YFP (Chan et al. 2001). The background FRET signal was 7.46% in 293T cells transiently co-transfected with TNFR1-CFP and TNFR1-YFP, and the FRET signal was increased after TNF- α stimulation (Chan et al. 2001).

During the dynamic photobleaching experiment, the CFP intensity not only increased on cell membranes but also translocated into cytoplasm (Fig. 3). This is possibly due to the natural recycling of the receptor proteins between its original membrane location and protein degradation machinery in the cell. Membrane receptors undergo recycling process in which they are cleaved from the membrane and are then transported to endosomes and lysosomes for degradation. The images acquired during dynamic photobleaching suggest that the TRAIL-DR4 complex after activation also undergoes such recycling process. The existence of cytoplasmic DR4-CFP/YFP even without TRAIL stimulation was confirmed by confocal images of monoclonal DR4-CFP/YFP reporter cells (Fig. 5). One such possible experiment to further confirm our hypothesis could be modifying the TRAIL ligand with a red fluorescent dye such as Alexa 580 and tracking its movement after adding to the DR4 reporter cells. Such an experiment would demonstrate the dynamics behind this TRAIL-DR4 complex formation and also would explain the ultimate fate of this DR4 receptor after binding with TRAIL.

In summary, it is demonstrated in this study that the monoclonal DR4-CFP/YFP reporter cell line can be utilized to monitor the dynamic activation of the TRAIL pathway at the ligand-receptor level via FRET technique in real time. The DR4 molecules undergo a recycling process and translocate into cytoplasm after TRAIL treatment. The future study will be focused on using this reporter cell line to screen clinical and potential anti-cancer drugs including natural compounds in order to discover their action mechanisms related to the specific TRAIL pathways.

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Figure 1. Monoclonal DR4-CFP/YFP reporter PC9 cells. (A) 20 $\times$ phase-contrast image, (B) and (C) fluorescence images of CFP filter set and YFP filter set, (D) an overlay of fluorescence images of CFP and YFP channels. The overlay image illustrates co-localized expression of DR4-CFP and DR4-YFP in most of the cells. Scale bar is 50 μ m.

Figure 2.
Western Blot membrane of four samples of monoclonal DR4-CFP/YFP cell lysates with anti-human DR4 monoclonal antibody

Figure 3.

Time lapse CFP images of AOI3 (area of interest #3) acquired during dynamic YFP photobleaching at (A) 0, (B) 3.5, (C) 7, (D) 10.5, (E) 14 and (F) 17 minutes, (G) FRET efficiencies of 4 AOIs calculated from dynamic photobleaching CFP images. Scale bar is 20 μ m.

Figure 4.

The FRET efficiencies observed in DR4-CFP/YFP reporter cells from the batch YFP photobleaching method. (A) Histogram of FRET efficiencies calculated from areas of interest where areas were drawn to include single whole cells and occasionally patches of cells, (B) Bar graph of the same data as (A) and FRET efficiency along cell membranes using mean values and standard deviation. * means significant difference between the FRET efficiency of untreated samples and TRAIL treated cells analyzed.

Figure 5.

Panel (A), (B) and (C) are reconstructed confocal images from CFP channel, YFP channel and overlay of CFP and YFP channels, respectively. Image (i) in the panels is the Coronal (front) view, (ii) the Sagittal (side) view and (iii) the axial (top) view of one particular z-plane. Blue color represents DR4-CFP, Yellow color represents DR4-YFP and Green color represents the area with colocalized DR4-CFP and DR4-YFP

Figure 6.
Caspase 3 activities in non-transfected PC9 cells and stable transfected DR4-CFP cells.