DMD Fast Forward. Published on June 24, 2005 as doi:10.1124/dmd.105.005538

DMD #5538

Bimolecular Fluorescence Complementation Analysis of Cytochrome P450 2C2, 2E1 and NADPH-Cytochrome P450 Reductase Molecular Interactions in Living Cells

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Number of text pages: 31

Number of tables: 0

Number of figures: 5

Number of references: 40

Number of Words in Abstract: 236

Number of Words in Introduction: 748

Number of Words in Discussion: 1498

Abbreviations: P450, Cytochrome P450; BiFC, bimolecular fluorescence complementation; ER, endoplasmic reticulum; FRET, fluorescence resonance energy transfer; YFP, yellow fluorescent protein; CFP, cyan fluorescent protein; PCR, polymerase chain reaction; GFP, green fluorescent protein

Abstract

Interactions between cytochromes P450 (P450s) and P450 reductase are required for enzymatic activity, and homo- or hetero-oligomerization of P450s may also be functionally important. Bimolecular fluorescence complementation (BiFC) was used to examine P450 interactions in a natural membrane context within living cells. BiFC detects protein interactions in living cells by reconstitution of a fluorescent protein from two fragments that are fused to the two interacting proteins. Non-specific protein-protein interactions were detected if proteins were expressed at high levels. At low protein expression levels, homooligomerization of P450 2C2, but not P450 2E1, and interactions of these P450s with P450 reductase were detected by BiFC consistent with interactions detected previously by fluorescence resonance emission transfer. Weak interaction of P450 2C2 with P450 2E1 and homooligomerization of P450 reductase were also detected by BiFC. Homooligomerization of the N-terminal P450 2C1 signal anchor sequence and interactions between the signal anchor and full length P450 2C2 were detected suggesting that homooligomerization of P450 2C2 is mediated by the signal anchor. However, interactions between the signal anchor and either P450 2E1 or P450 reductase were not detected by BiFC. Although high concentrations of the substrate lauric acid increased BiFC for both P450 2E1 and P450 2C2 with P450 reductase, the concentration-dependence did not correlate with reported Km's. These results demonstrate that BiFC is an effective method to study the complex protein interactions that occur within the microsomal P450 system in living cells.

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The hepatic microsomal cytochrome P450 (P450) monooxygenase system, which consists of P450s, NADPH-dependent P450 reductase, and cytochrome b_5, catalyzes the oxidation of a large number of endogenous and xenobiotic compounds (Gonzalez, 1990). There are 50-100 P450s in mammals of which about 90% are present in hepatic microsomal membranes, with the remainder in mitochondria (Nelson et al., 2004). In contrast, there is only one microsomal P450 reductase, which forms a functional 1:1 complex with P450 (Miwa and Lu, 1984). The concentration of P450 reductase in the membrane has been estimated to be 1/10 to 1/100 that of total P450s (Estabrook et al., 1971; Watanabe et al., 1993). Since there are multiple P450s in excess of one P450 reductase in the same membrane, the reductase cannot interact with all P450s at the same time, so that either the P450s need to be organized in complexes to interact with P450 reductase or monomeric P450s and P450 reductase must interact stochastically. Further, in addition to interaction with P450 reductase, a P450 may interact with another P450 which potentially could affect the activity of the P450s or facilitate sequential metabolic steps of a single substrate by different P450s. Knowledge of the arrangement of the P450s and P450 reductase in the membrane is, therefore, important in understanding the mechanisms of catalysis by these enzymes.

The observations that P450 reductase is limiting relative to P450, that P450s aggregrate *in vitro,* and that there is a rapid and slow phase of P450 reduction in reconstituted systems led to the suggestion that multimeric complexes of P450s bound to the reductase account for the fast reaction and P450s not complexed require diffusion to the P450 reductase for the slow reaction (Estabrook et al., 1971; Peterson et al., 1976). Purified P450s have been shown to aggregate *in vitro* (Myasoedova and Berndt, 1990; Schwarz et al., 1990; Kanaeva et al., 1992a) and rotational studies of P450s in microsomal membranes indicate that P450s form oligomeric complexes in

the membranes (Kawato et al., 1982; Schwarz et al., 1982). Oligomeric P450s have been reported to have increased affinity for substrates and increased functional interaction with P450 reductase i*n vitro* (Dean and Gray, 1982) although conflicting results have been reported for the latter observation (Kanaeva et al., 1992a; Kanaeva et al., 1992b). An alternative hypothesis is that monomeric P450s randomly interact with P450 reductase which was supported by studies in which increased levels of P450 reductase caused disaggregation of P450s (Gut et al., 1982; Kawato et al., 1982; Gut et al., 1983; Wu and Yang, 1984). These in vitro studies, thus, have not resolved the nature of in vivo interactions of P450. Further the in vitro conditions may influence the interactions, for example, phospholipid composition and concentration affect the interaction between P450 and P450 reductase (Causey et al., 1990).

Co-immunoprecipitation of cross-linked P450s in microsomal membranes has shown that P450s form heterologous oligomers and that there is specificity to the interactions (Alston et al., 1991). Studies with reconstituted systems or membranes containing exogenously expressed P450s and P450 reductase have shown that these heterooligomeric interactions can affect the activity of the P450s (reviewed in (Backes and Kelley, 2003)). Addition of P450 1A2 to P450 2B4 in reconstituted systems was strongly inhibitory for dealkylation of 7-pentoxyresorufin by P450 2B4 at limiting P450 reductase concentrations, but not for metabolism of other substrates (Cawley et al., 2001; Backes and Kelley, 2003). Similar results were observed for microsomal membranes from rats treated with inducers for these P450s suggesting that the effects were not artifacts of the reconstituted reaction conditions (Cawley et al., 2001). Kinetic models suggested that the inhibition was the result of the formation of a heterooligomeric complex between the P450 1A2 and P450 2B4 that increased affinity for P450 reductase at the P450 1A2 reductase site, rather than simple competition between the two monomeric P450s for the reductase.

Further, either inhibition or synergism was observed for other combinations of P450s (Backes and Kelley, 2003).

Complex interactions may, therefore, occur in the natural endoplasmic reticulum (ER) membrane *in vivo* which contains multiple P450s at subsaturating P450 reductase concentrations. We have shown that fluorescence resonance energy transfer (FRET) can be used to examine interactions of P450s and P450 reductase in living cells (Szczesna-Skorupa et al., 2003). FRET is a powerful method, but is also technically difficult and prone to artifacts. In the present study, P450 interactions in living cells were successfully detected by a technically simpler method, bimolecular fluorescence complementation (BiFC) (Hu et al., 2002).

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Methods

Construction of chimeras for BiFC analysis— The plasmids pBiFC-YN154 and pBiFC-YC155 (Hu et al., 2002) were provided by Dr. T. Kerppola (University of Michigan, Ann Arbor, MI, U.S.A.) and encode amino acid residues 1-154 (YN) and 155-238 (YC) of EYFP, respectively. The sequences encoding YN and YC are preceded by sequences encoding the linkers RPACKIPNDLKQKVMNH and RSIAT, respectively, and an epitope tag. To construct chimeric proteins fused at the C terminus with either the YN or YC fragments of YFP, the cyan fluorescent protein (CFP) encoding sequences in chimeras of the signal-anchor sequence (1-29)P450 2C1, full-length 2C2, 2E1 or P450 reductase fused to CFP (Szczesna-Skorupa et al., 2003) were replaced with either YN or YC including the preceding linker sequence. The sequence encoding YN and YC plus the linkers were amplified by polymerase chain reaction (PCR) from pBiFC-YN154 and pBiFC-YC155. For full length P450 2C2, the 5' PCR primers, 5'-CCGCGGGATCCAGATCCATCGCCACCATGGTGAGCAAGGG-3' for YN and 5'-CCGCGGGATCCACGTCCGGCGTGCAAAATCCCG-3' for YC, introduced a BamHI site and the 3' primers, 5'-AGAGTCGCGGCCGCCTAGGCCATGATATAGACGTGGCTGTTG-3' for YN and 5'-AGAGTCGGGCCGCTTACTTGTACAGCTCGTCCATGCCGAGAGTG-3' for YC, introduced a NotI site. The YN and YC PCR products were digested with BamHI and NotI and inserted into the 2C2/CFP vector, digested with the same enzymes to excise CFP, to produce 2C2YN and 2C2YC. The same procedure was used to construct the YN and YC chimera fused to the P450 2C1 signal anchor (amino acids 1-29), P450 reductase, and full length P450 2E1 except that 5' PCR primer sequences contained HindIII sites for the signal anchor and P450 reductase and an ApaI site for P450 2E1. The CFP plasmids were digested with the

corresponding restriction enzymes for each and the PCR fragments were inserted to produce (1-29)2C1YN, (1-29)2C1YC, reductaseYN, reductaseYC, 2E1YN, and 2E1YC.

Cell culture and transfections – Cos-1 and HEK293 cells were grown and co-transfected at 60% confluence with DNA of expression vectors for YN and YC chimera by Lipofectamine 2000 (Invitrogen Inc.) on a 6 well plate as described (Szczesna-Skorupa and Kemper, 2001). After transfection, the cells were incubated at 37^{o} C for 24 hr followed by incubation for 4-7 hr at 30^{o}C (Hu et al., 2002). Cells from the same transfection were analyzed by flow cytometry, confocal microscopy and western blotting.

Western blotting – Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a 10% or 15 % (w/v) gel and electrophoretically blotted to nitrocellulose. Western blot analysis was performed using a mixture of two polyclonal rabbit green fluorescent protein (GFP) antibodies (AntiGFP(FL) and AntiGFP(T-19), Santa Cruz Biotechnology, Inc.). The secondary antibody was goat horseradish peroxidase conjugated anti-rabbit IgG (Santa Cruz Biotech.) which was detected using a chemiluminescence kit as described by the manufacturer (Pierce Biotechnology, Inc.).

BiFC analysis by flow cytometry - For flow cytometry, the transfected Cos-1 cells were collected from the plates by trypsinization and centrifugation. The cells were washed in 1 mL of Dulbecco's phosphate-buffered saline and about 10^5 cells were resuspended in 0.5 mL of the same solution at room temperature. The cells were analyzed in a Coulter EpicsXL-MCL flow cytometer, equipped with an argon laser emitting at 488 nm, using Epics XL-MCL System II Version 3.0 Software (Beckman Coulter, Fullerton, CA). Analysis was restricted to live cells by gating cells that exhibited forward and side scatter features typical of live cells. Results from 50,000 cells were analyzed using Summit V3.1 software (Cytomation inc.) and the results were

plotted as a distribution of cells versus YFP fluorescence in arbitrary units. The percentage and average fluorescent intensity of cells above the indicated threshold were indicated in the figures.

BiFC analysis by confocal microscopy - For analysis of individual cells by confocal microscopy, HEK293 cells transfected with the YN and YC expression vectors were grown on coverslips in the 6-well plates. As an internal standard the expression vector for a coral-derived red fluorescent protein tHcRed (pHcRED-Tandem-N1, Evrogen Joint Stock Company, Moscow, Russia) was cotransfected. The coverslips were placed directly on a drop of culture media on a glass slide and examined with a Zeiss Confocal Microscope LSM510. YFP was excited at 514 nm and detected at 535-545 nm and HcRED was excited at 563 nm and detected at 590 nm. Images were analyzed with Image J software (Wayne Rasband, National Institute of Mental Health, Bethesda, MD).

Results

Nonspecific BiFC as a function of time and level of expression – In the BiFC assay, protein interactions are detected by the recombination of two non-fluorescent fragments of YFP, each of which is fused to one of the two interacting proteins (Hu et al., 2002). This assay has been used primarily for soluble proteins with only two reported studies of membrane-bound proteins (Hynes et al., 2004; Remy and Michnick, 2004). To optimize conditions for the assay, we first examined the expression of just the two fragments of YFP, YN and YC which are soluble proteins and should not interact in the cells. BiFC was first measured by analysis of single cell images in a confocal microscope and later by analysis of a population of cells in a fluorescence flow cytometer. In the confocal studies, HcRED was cotransfected with the YFP chimera as an internal standard for transfection efficiency and there was no cross-bleed of red fluorescence into the yellow channel or of yellow fluorescence from a YFP chimera, 2C2YFP into the red channel (Fig. 1B). Transfection of HEK293 cells with 100 or 250 ng of DNA for each expression vector for YN or YC resulted in very low fluorescence as expected (Fig. 1A). However, strong fluorescence was observed when 500 ng or 1000 ng of vector DNA was transfected. The fluorescence that was observed was distributed throughout the cell as expected for a soluble small protein (Fig. 1B). Strong fluorescence was also observed 48 hr after transfection when 100 ng of DNA for each expression vector was transfected (data not shown). For analysis by flow cytometry, Cos-1 cells were used because of higher transfection efficiencies. Consistent with the confocal microscopy results, transfection of 100 ng of DNA for each YN and YC expression vector resulted in very little fluorescence with 7% of cells above the threshold fluorescence compared to 5% for cells expressing only YN (Fig. 2A). In contrast, 16% and 30% of the cells were above the threshold when 500 ng or 1000 ng of vector DNA were transfected,

respectively. Likewise, if 100 ng of vector DNA was transfected and cells were analyzed 48 hr rather than 24 hr after transfection, 27% of cells were above the threshold fluorescence (Fig. 2B). Western analysis of the expressed proteins demonstrated a good correlation between the amount of vector DNA cotransfected or the time of expression and the amount of protein expressed in the cells (Fig. 2E). These results indicate that nonspecific interactions occur between the YFP fragments which lead to a concentration and time-dependent accumulation of fluorescence for these soluble proteins. Appropriate expression levels of chimeric proteins, thus, are critical to avoid false positives for the interaction of two proteins.

We also examined whether similar nonspecific interactions with soluble partners occur for the membrane-bound proteins P450 and P450 reductase. Full length 2C2YN or P450 reductaseYN with YC or P450 reductaseYC with YN were co-expressed. In HEK293 cells, little fluorescence was observed by confocal microscopy for 2C2YN and the YC fragments when less than 500 ng of DNA for each expression vector was transfected, but strong fluorescence was observed with 1000 ng of transfected vector DNA (Fig. 1A). Similar results were observed for P450 reductase with substantial fluorescence observed only with transfection of greater than 250 ng of DNA for each vector (Fig. 1A, B). The reductaseYC and reductaseYN chimera and the respective soluble YC and YN partners were also analyzed by flow cytometry in Cos-1 cells. In both cases little fluorescence was observed when 100 ng of vector DNA for each construction was transfected, but strong fluorescence was observed if 100 ng of P450 reductase vector DNA and 1000 ng of the soluble YC or YN vector DNA was transfected (Fig. 2C, D). Based on western analysis of the P450 reductaseYC and YN pair, the amount of protein detected was proportional to the amount of vector DNA transfected (Fig. 2F). These results indicate that nonspecific interactions of soluble proteins with membrane proteins also occur at high

concentrations of protein demonstrating that the level of protein expression in BiFC assays has to be carefully controlled to avoid false positive interactions.

As a result of these initial studies, the flow cytometry method was chosen to analyze protein interactions, since this method measures the fluorescence in thousands of cells at one time while the confocal method requires the analysis of the cells one at a time and the level of expression varies in individual cells. The ability to analyze large numbers of cells averages the variability and provides more consistent reproducible results. In addition, because the amount of expressed proteins is critical to the interpretation of results, the expression levels of the different chimeras were analyzed by western blotting using GFP antisera that recognize the YFP fragments and the amount of vector DNA transfected was adjusted so that the protein expression levels were similar to that of YN or YC at 24 hr after transfection with 100 ng of vector DNA. At this concentration and time, there was no interaction detected between the YN and YC fragments. Amounts of transfected expression vector DNA that resulted in the desired levels of protein expression were 1500 ng, 500 ng, and 100 ng for P450 2C2, P450 2E1, and P450 reductase or (1-29)P450 2C1, respectively (Fig. 3G). In each experiment, cells expressing only one of the two YFP fragment chimera were analyzed as a control for intrinsic fluorescence, and a threshold for fluorescence was set such that 5% of these control cells were above the threshold. The percentage of experimental cells above the threshold and the mean fluorescence intensity of these cells provide a semi-quantitative measure of the strength of the interaction between the proteins.

Interactions among P450 2C2, P450 2E1, and P450 reductase detected by BiFC –P450 2C2 with GFP fused to its C terminus was shown to retain its normal subcellular localization in the ER membrane and full catalytic activity (Szczesna-Skorupa et al., 1998; Szczesna-Skorupa et

al., 2000). Thus, fusion of YN and YC fragments to the C-terminus of P450s would not be expected to alter their function. For the proteins pairs of P450 and P450 reductase that resulted in BiFC, the distribution of fluorescence was consistent with localization of the proteins in the ER (Fig. 3H). In contrast, the soluble YN and YC complementation fluorescence was distributed evenly throughout the cell.

The interactions among the P450s and reductase at optimized protein expression levels as analyzed by flow cytometry are shown in Fig. 3A-F. Strong fluorescence is observed in cells expressing P450 2C2YN and P450 2C2YC such that 36% of cells are above the threshold (Fig. 3D). In contrast, only 7% compared to 5% for the control of cells are above the threshold in cells expressing P450 2E1YN and P450 2E1YC (Fig. 2E). Similarly, both 2C2YN and 2E1YN interact with P450 reductaseYC (Fig. 2B,C). These results are consistent with FRET studies which detected homooligomerization of P450 2C2, but not P450 2E1, and interactions of both P450s with P450 reductase (Szczesna-Skorupa et al., 2003). In addition, an interaction between P450 2C2 and P450 2E1 was also detected with 28% of the cells above the threshold but with a lower mean intensity than that for the homologous interaction of P450 2C2 suggesting that the heterologous interaction is weaker (Fig. 3F). This interaction was not detected by FRET analysis (Szczesna-Skorupa et al., 2003). Self oligomerization of P450 reductase, which was not examined in the FRET studies, was also detected with BiFC. About 20% of the cells expressing P450 reductase fused to YN and to YC were above the threshold with an average intensity comparable to that detected for P450 reductase interaction with either 2E1 or 2C2 (Fig. 3A). While the distribution of the fluorescence for the reductase homooligomers was partially reticular, consistent with ER localization, large punctuate fluorescence was also observed (Fig. 3H). The cellular basis for the punctuate fluorescence is not known.

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The role of the P450 signal anchor sequence in interactions of P450s and P450 reductase – FRET analysis suggested that formation of homooligomers was mediated by the P450 signal anchor sequence while interactions between the reductase and P450s were mediated by the large cytoplasmic domain of P450 (Szczesna-Skorupa et al., 2003). As was observed in the FRET studies, the (1-29)P450 2C1 fragments also strongly interacted in the BiFC assay with a mean fluorescence intensity which was greater than that observed for interactions of full length P450 2C2 (Fig. 4A). The increased fluorescence intensity probably results from a decreased distance between the two YFP fragments fused to the truncated 29-amino acid fragment compared to full length P450 2C2. An interaction between (1-29)P450 2C1 and full length P450 2C2 was also observed by BiFC as would be expected if homooligomerization of P450 2C2 was mediated by the signal anchor sequence (Fig. 4B). The distributions of fluorescence for the (1-29)P450 2C1 interactions were consistent with ER localization (Fig. 4F, G). In contrast, little or no increased fluorescence was observed for (1-29)P450 2C1 with either full length P450 2E1 or P450 reductase. The latter observation is consistent with the previous conclusion that the interactions of P450 2C2 with P450 reductase are mediated by the cytoplasmic domain of P450 (Szczesna-Skorupa et al., 2003). The lack of interaction of (1-29)P450 2C1 with P450 2E1 suggests that the cytoplasmic domain is most important in the interaction of these two P450s. However, the inability of the YFP fragments to interact because of the different sizes of the fused proteins cannot be ruled out.

Effect of substrate on the interaction between P450s and P450 reductase – Since P450 concentrations are in excess of that of P450 reductase, the control of P450 reductase interactions with individual P450s is an important question. Productive interactions require that a substrate be bound to the P450 so it is reasonable to speculate that binding of the substrate to the P450

may increase its affinity for the reductase. We examined the effects of the addition of lauric acid, a substrate for P450 2C2 and P450 2E1, and ethanol and chlorzoxazone, substrates for P450 2E1. No effect of ethanol and chlorzoxazone on the interaction between P450 2E1 and P450 reductase could be detected by BiFC (data not shown). No increase in the interaction of P450 2C2 or P450 2E1 with P450 reductase was detected by BiFC with the addition of up to 15 μM lauric acid (Fig. 5A,B). At 50 μM lauric acid, an increase in fluorescence with both P450 2C2 and P450 2E1 paired with P450 reductase was observed (Fig. 5C,D). The Km's of P450 2C2 and P450 2E1 have been reported to be 7-9 μM (Imai, 1988) and 84 μM (Amet et al., 1995), respectively. While the increased BiFC with 50 μM lauric acid would be consistent with substrate mediation of P450 interaction with P450 reductase, the lack of effect for P450 2C2 at lauric acid concentrations up to 15 μM, well above the Km, and the similar concentration dependence for P450 2E1 and P450 2C2, for which Km's differ about 10-fold, suggest that the changes at 50 μM are not specific.

Discussion

Many studies of interactions of P450s with P450 reductase have been done with purified proteins or in reconstituted systems of purified proteins, but rarely in native membranes in which characteristics of the proteins may be substantially different. We have previously studied the interactions of P450s and P450 reductase in living cells using FRET (Szczesna-Skorupa et al., 2003) and in this report we have used BiFC to study the interactions among P450 2C2, P450 2E1 and P450 reductase in living cells. The results demonstrate that this method is an effective alternative to FRET studies for the analysis of these membrane-bound proteins. Homooligomeric interactions of either full length P450 2C2 or the signal anchor sequence of P450 2C1 and heteroligomeric interactions of P450 2E1 and P450 2C2 with P450 reductase were detected by BiFC, consistent with earlier FRET studies (Szczesna-Skorupa et al., 2003). Likewise, homooligomeric interactions of P450 2E1 were not detected by either method. In contrast, interaction between P450 2E1 and P450 2C2 was observed by BiFC, but not by FRET. The discrepancy probably is explained by the fact that BiFC measures a summation of protein interactions whereas FRET measures the interactions at steady state and thus is more sensitive to the binding affinity of the two proteins. Although the YFP fragments do not contribute to protein interaction, once the two YFP fragments are brought together and recombine, the protein complex is irreversibly stabilized (Hu et al., 2002). Relatively weak transient interactions would likely produce little or no FRET signal but could result in an accumulation of recombined YFP fragments.

Most demonstrations of protein-protein interactions by BiFC have been for soluble proteins, for example, transcription factors Jun and Fos in nuclei (Hu et al., 2002), bromodomain protein Brd2 , and histone H4 (Kanno et al., 2004), Agrobacterium VirD4 and VirE2 proteins

(Atmakuri et al., 2003) although membrane proteins have also been studied (Hynes et al., 2004; Remy and Michnick, 2004). Surprisingly, expression of high levels of either the soluble YN and YC fragments or membrane-bound P450 reductase or P450 2C2 with the soluble YN or YC fragments, which should not interact specifically, resulted in substantial fluorescence. The fluorescence under these conditions presumably results from random nonspecific interactions. To minimize this possibility, however, the amount of expression vector was adjusted so that expression levels were similar for each protein and to the level of YN and YC expression that did not result in nonspecific interactions. The possibility remains that the interactions observed for the P450s and P450 reductase are non-specific as a result of concentration of these proteins in the ER membrane compartment. However, the ER is a large cellular compartment with a volume of 15% to 30% that of the cytoplasm and diffusion constants of membrane proteins in the ER are 100-fold, or more, lower than for soluble proteins (Levin et al., 2001) which should compensate for any increased concentration. In addition, BiFC was not observed for P450 2E1 or for the (1-29)2C1/P450 reductase pair at comparable expression levels. Although the possibility cannot be completely eliminated, it seems unlikely that nonspecific interactions as a result of concentration in the ER compartment underlie the BiFC observed.

Homooligomerization of P450 2E1 was not observed, but the possibility must be considered that, due to steric considerations, interactions of the YFP fragments or P450 2E1 are hindered. However, P450 2E1 is closely related to P450 2C2, the basic fold is very similar for all P450 structures, and the C-terminus of P450s is at the opposite end of the molecule from the putative membrane-interaction domain so that the YFP fragments attached by linkers should not be constrained in their interactions. Further, P450 2E1 interaction with P450 reductase was detected, indicating that the orientation of P450 2E1 in the membrane is similar to that of P450

2C2. It seems likely, therefore, that the lack of BiFC detected for P450 2E1 correctly reflects the lack of homooligmerization.

In the previous FRET studies, both the full-length P450 2C2 and the cytoplasmic domain of P450 2C2, without the signal anchor, interacted with P450 reductase (Szczesna-Skorupa et al., 2003) which was consistent with *in vitro* studies that the cytoplasmic domain mediated interaction with P450 reductase (Voznesensky and Schenkman, 1994; Bridges et al., 1998; Omata et al., 2000). A role of the signal anchor sequence in the interaction could not be directly tested by FRET because the size difference between the P450 signal anchor and P450 reductase would likely not bring the two fluorescent proteins together close enough for FRET to occur. For example, the expected FRET between (1-29)2C1 and P450 2C2 was not observed (unpublished). For efficient FRET to occur, the two chromophores must be less than 10 nm, usually about 6 nm apart (Eidne et al., 2002), but BiFC can occur if the two YFP fragments are within 10 nm with appropriate linkers (Hu et al., 2002). Although interaction of the signal anchor of P450 2C1 with full length P450 2C2 could be detected, its interaction with P450 2E1 or P450 reductase could not be detected by BiFC which suggests that only the P450 2C2 cytoplasmic domain interacts with the reductase or with P450 2E1 to form heterooligomers. It remains possible, however, that BiFC was not observed because of the size difference of the proteins or the orientation of YFP fragments relative to each other. Nevertheless, the lack of interaction of the reductase with (1-29)P450 2C1 complements the FRET studies and strengthens the conclusion that the cytoplasmic domain of the P450 2C2 predominantly mediates the interaction with P450 reductase.

Self oligomerization of P450 reductase was also detected by BiFC. The strength of the interaction based on the intensity of fluorescence was similar to that between P450 reductase and

the P450s. Since P450 reductase is limiting in the membrane, it is generally considered to be in a monomeric form during interactions with P450s although the reductase does form aggregates *in vitro* (French et al., 1980). The oligomerization of P450 reductase in the membrane adds complexity to the kinetics of the interaction with the P450s. If the P450 reductase must be monomeric to interact with P450, then the binding of a P450 to P450 reductase would require not only dissociation of the P450 reductase from other P450s, but also dissociation of the P450 reductase oligomers. On the other hand, it is possible that the oligomeric reductase is functional and can bind to multiple P450s in a large complex that facilitates metabolism.

An increased affinity of P450 reductase for P450s as a result of substrate binding could not be clearly demonstrated by BiFC. Changes in the structure of P450 2B4 bound to an inhibitor included the F and G helices and the C helix, which are thought to form part of the P450 reductase interaction surface (Scott et al., 2004). These changes have been interpreted as a structural basis for increased affinity of the P450 for P450 reductase upon substrate binding. Similar, but more subtle, changes have been observed in other mammalian and bacterial P450s (see (Scott et al., 2004) for references). In a reconstituted system, modest increases in affinity of P450 2B4 for reductase were observed by addition of substrate (French et al., 1980), but no effect of substrate was observed on binding of P450 reductase to 4 purified mammalian P450s attached to a polystyrene plate (Shimada et al., 2005). In the latter study, it was proposed that the observed substrate-induced conformational changes increased the efficiency of electron transfer rather than affinity for P450 reductase. In the present studies, while increased BiFC was observed at the highest concentrations of lauric acid examined, changes of BiFC could not be detected at concentrations of lauric acid above the Km of P450 2C2 and a similar concentration dependence was observed for both P450 2E1 and P450 2C2, even though the reported Km's of

these P450s differ by 10-fold (Imai, 1988; Amet et al., 1995). This suggests that the apparent increased interaction was not specific for substrate binding. More detailed studies of additional substrates and inhibitors will be required to establish a role for BiFC in analysis of substrate-P450 interactions.

These results demonstrate that both BiFC and FRET are useful and complementary measures of P450 protein-protein interactions. Advantages of BiFC are its relative simplicity and the less stringent geometrical and distance constraints for interaction of the fluorescent proteins. A major difference between the methods is that BiFC essentially measures the extent of protein interactions, because the formation of the recombined YFP irreversibly stabilizes the complex. The complementation complex for GFP has been shown to be irreversible with a half-life of 9.8 years (Magliery et al., 2005). FRET, on the other hand, is dependent on the dynamic equilibrium of protein interactions. Thus, weak and transient interactions, undetectable by FRET, may be detected by BiFC but the possibility of detecting nonspecific weak transient interactions is also greater with BiFC. The ability to detect interactions among P450s and between P450s and P450 reductase in living cells should allow a better understanding of the functional implications of these interactions.

Acknowledgements

We thank T.K. Kerppola for supplying the YFP fragment expression plasmids. Flow cytometry analysis was done in the Flow Cytometry Facility in the Biotechnology Center of the University of Illinois at Urbana-Champaign.

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Footnotes

This work was supported by a grant from the National Institutes of Health, GM35897.

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Figure Legends

Figure 1. BiFC as a function of protein expression levels detected by confocal microscopy. Cells were transfected with varying amounts expression plasmid DNA for the YN or YC fragments, P450 2C2YN, P450 reductaseYN or P450 reductaseYC and with the expression vector for tHcRed. The yellow and red fluorescence were determined for individual cells and yellow fluorescence was normalized to the red fluorescence as described in Methods. YN, N-terminal YFP fragment, YC, C-terminal YFP fragment, 2C2YN, full length P450 2C2 fused to YN, RdYN, P450 reductase fused to YN, RdYC, P450 reductase fused to YC. A. The averages of normalized yellow fluorescence for 75 cells are plotted in the bar graphs and the standard deviation is indicated. The ng of DNA transfected for each vector are indicated in the insert box. B. Examples of fluorescent cells are shown at the right. Control cells with only 2C2YFP or tHcRed transfected are shown to demonstrate the lack of bleed-through by red fluorescence in the yellow channel and vice versa. The amount of DNA for each expression vector is indicated for RdYN, YN, and YC.

Figure 2. BiFC detected by flow cytometry as a function of the amount of expression plasmid DNA transfected and time of expression. Cells were transfected as described in the legend to Fig. 1 and yellow complementation fluorescence was detected by flow cytometry as described in Methods. The distribution of cells as a function of the fluorescence intensity is shown (A-D). In each case, expression of only one of the YFP fragments or chimera was expressed as a control for intrinsic fluorescence and a threshold for cells exhibiting BiFC was chosen so that only 5% of the control cells were included. The percentage of cells above the threshold and the average fluorescent intensity of those cells are shown next to the names of the expression vectors transfected. Increasing amounts of expression vector DNA for YN and YC were transfected (A)

or the time of expression after transfection for the YN/YC pair was increased from 24 hr to 48 hr (B). The effect of increasing the YN (C) or YC (D) expression plasmid DNA from 100 ng to 1000 ng with a constant 100 ng of expression vector DNA for P450 reductase was examined. E, F. The protein expression levels in cells transfected in parallel with those analyzed by BiFC were determined by western blotting using a combination of two GFP antisera that detect both the YN and YC fragments. Positions of molecular weight markers are indicated at the left. The proteins expressed are indicated above the images and the amounts of vector DNA transfected below.

Figure 3. BiFC analysis of interactions among P450 2C2, 2E1 and P450 reductase. Cells were transfected and analyzed by flow cytometry as described in the legend to Figure 2. Cells were transfected with 100 ng, 500 ng, or 1500 ng of expression plasmid DNA for P450 reductase, P450 2E1, or P450 2C2 chimera, respectively. A-F. The distribution of cells as a function of the fluorescence is shown for the pairs P450 reductaseYN/P450 reductaseYC (A), P450 reductaseYN/P450 2C2YC (B), P450 reductaseYN/P450 2E1YC (C), P450 2C2YN/P450 2C2YC (D), P450 2E1YN/P450 2E1YC (E), and P450 2C2YN/P450 2E1YC (F). G. The amount of each of the proteins expressed was determined by western blotting as described in the legend to Figure 2. H. Distribution of fluorescence in representative cells imaged by confocal microscopy is shown for the indicated chimeric pairs. An image of a representative cell expressing high amounts of YN/YC is shown for comparison.

Figure 4. Role of the signal anchor sequence in the interactions among P450s and reductase. Cells were transfected and analyzed by flow cytometry as described in the legend to Figure 2. A-D. The distribution of cells as a function of the fluorescence is shown for the pairs (1-29)2C1YN/(1-29)2C1YC (A), P450 2C2YN/(1-29)2C1YC (B), P450 2E1YN/(1-29)2C1YC (C), and P450 reductaseYN/(1-29)2C1YC (D). E. The amount of each of the proteins expressed was

determined by western blotting as described in the legend to Figure 2. F-G. Distribution of fluorescence in representative cells imaged by confocal microscopy is shown for the indicated chimeric pairs.

Figure 5. Effect of the substrate lauric acid on the interaction of P450 2C2 with P450 reductase. Cells were transfected with expression vector DNA for P450 reductase, P450 2C2 and P450 2E1 as indicated and fluorescence was analyzed by flow cytometry as described in the legend to Figure 2. Lauric acid was added to the medium 6 hr after transfection and cells were cultured for an additional 16 hr at 37^{o}C and 5 hr at room temperature. Lauric acid concentrations from 1 to 15 μM (A, B) or 10 and 50 μM (C, D) or ethanol vehicle (0 μM) were added as indicated. The distribution of cells as a function of the fluorescence is shown for the pairs P450 reductaseYN/P450 2C2YC (A, C) or P450 reductaseYN/P450 2E1YC (B, D).

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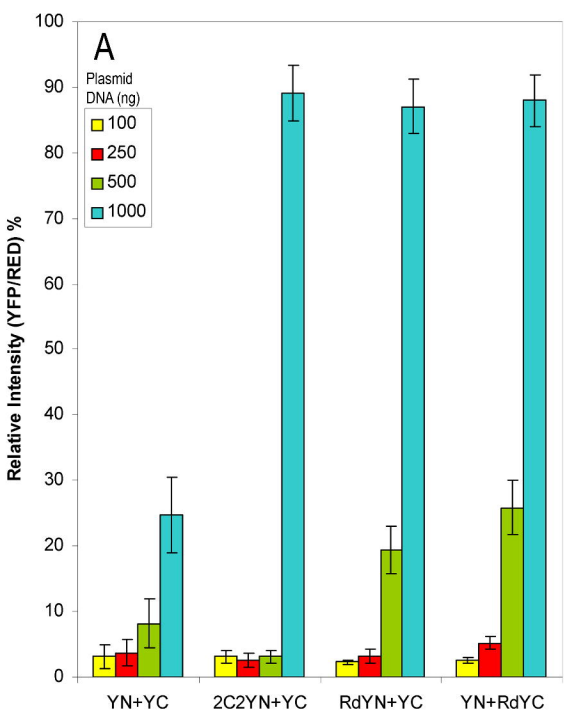
Figure 1



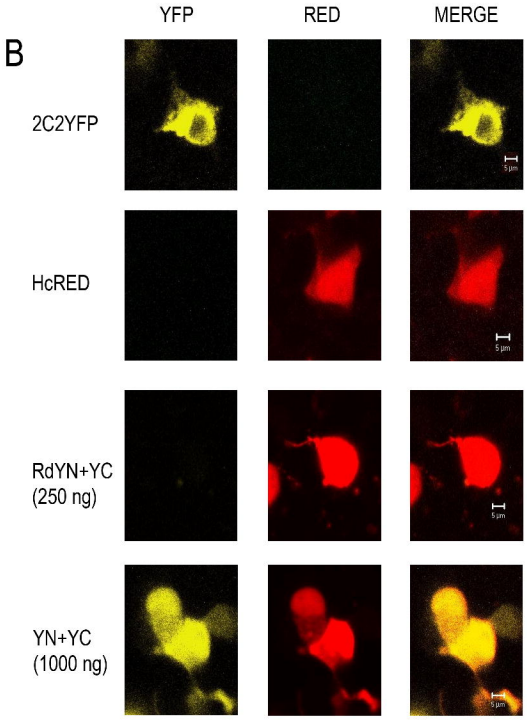


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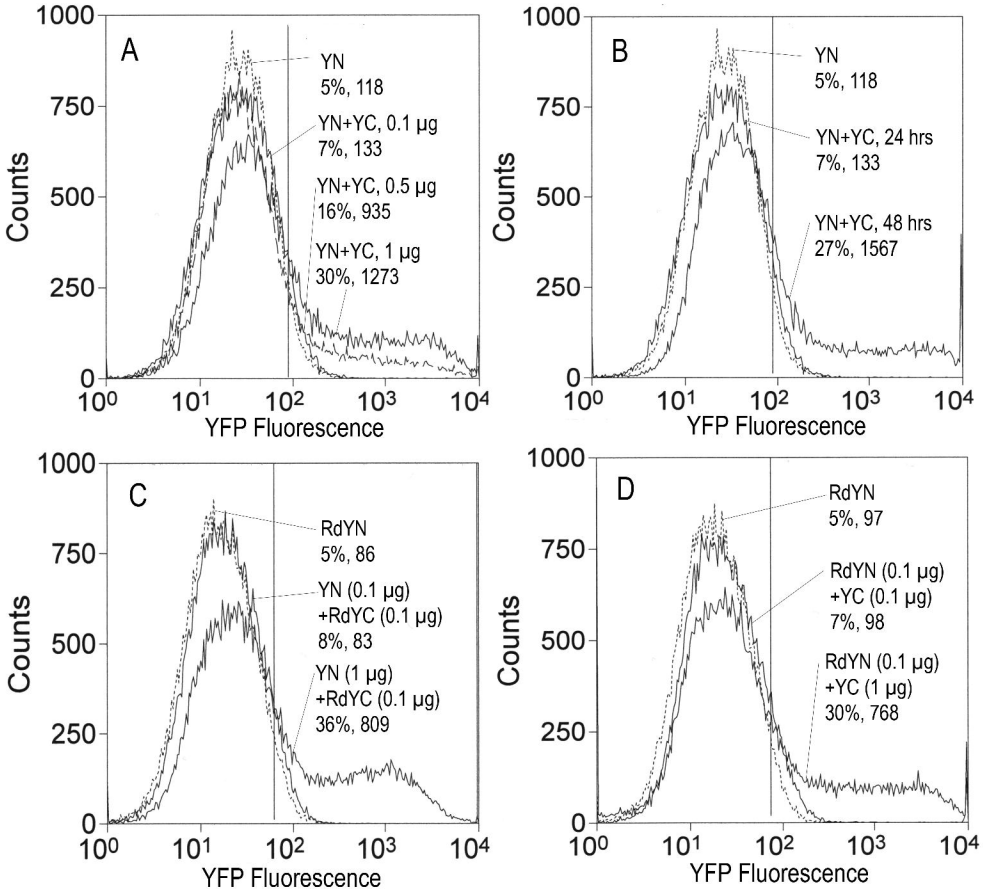
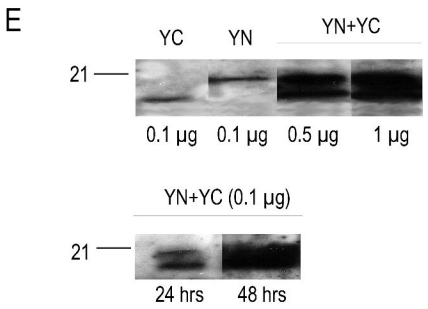
Figure 2





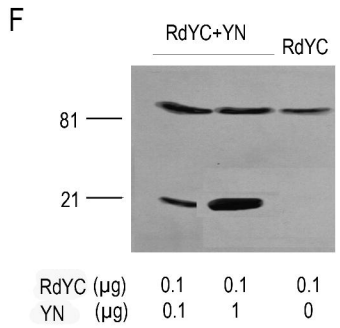


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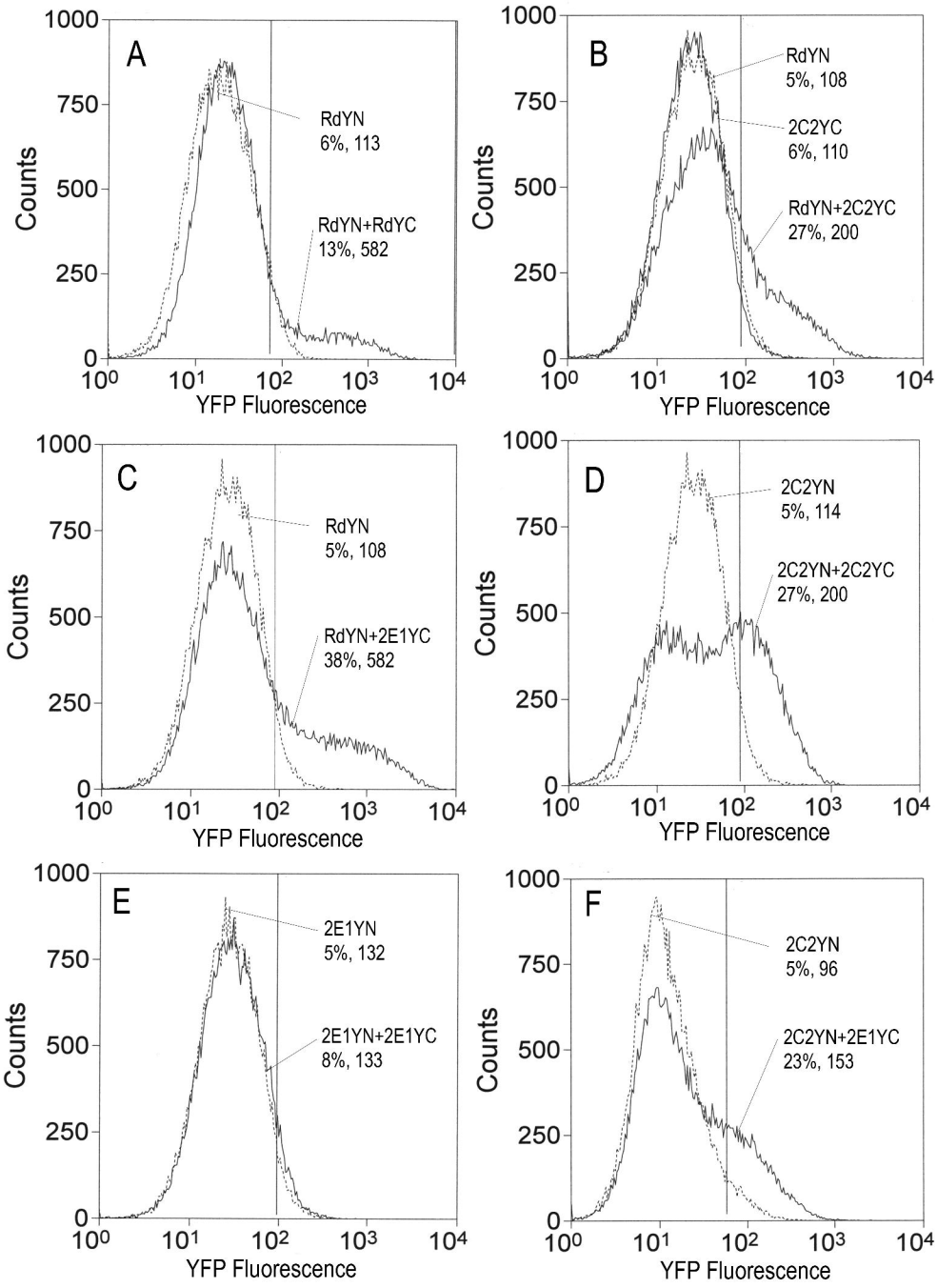
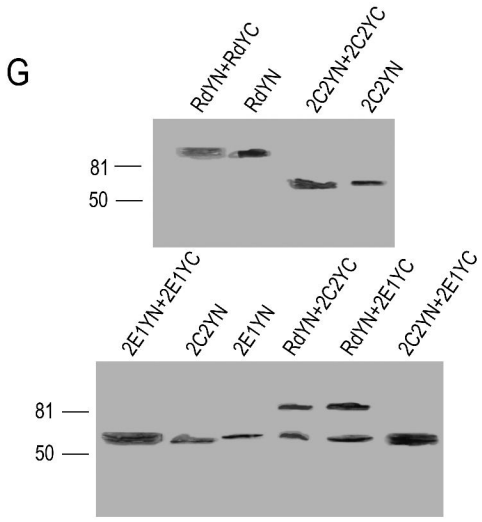
Figure 3





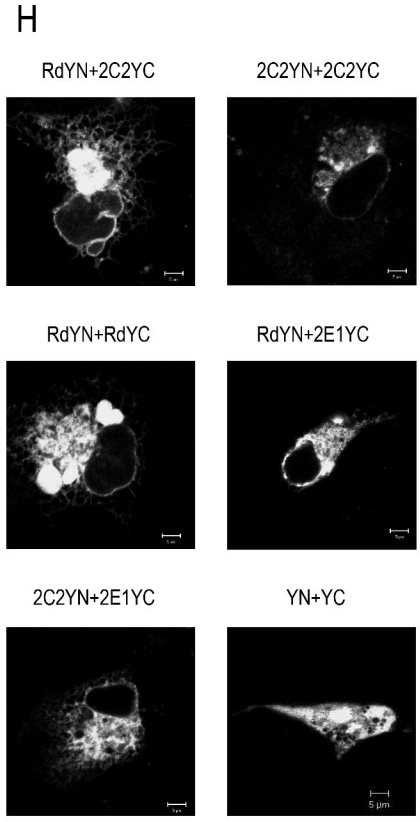


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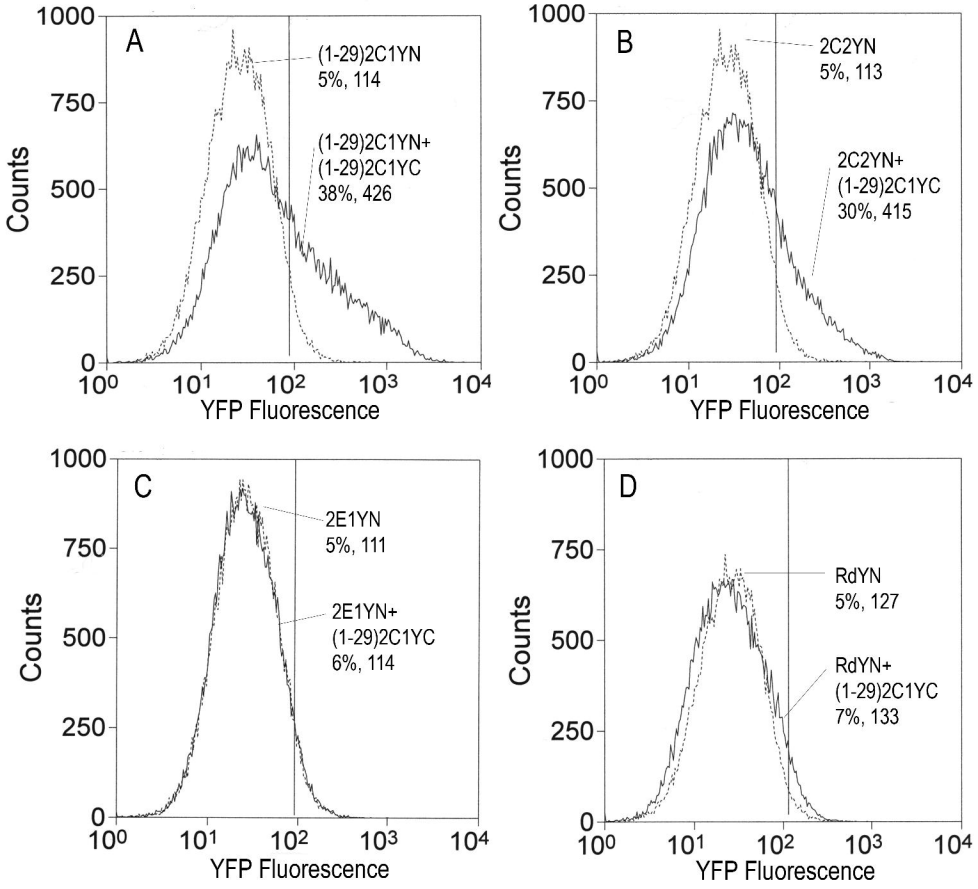
Figure 4



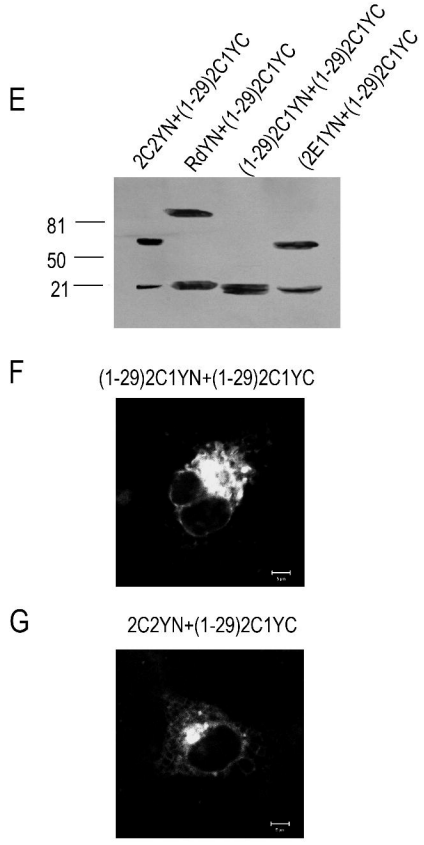


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Figure 5