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FMDV–2A sequence and protein arrangement contribute to functionality of CYP2B1–reporter fusion protein

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Abstract

Two optimized forms of green fluorescence proteins (GFP), enhanced GFP (EGFP) and humanized *Renilla* GFP (hrGFP), were used to track expression of cytochrome P450 2B1 (CYP2B1), an endoplasmic reticulum membrane-bound protein. In transiently expressing HEK293 cells we show that CYP2B1–GFP fusion proteins are stable and functional, whereas the vice-versa-arranged GFP–CYP2B1 fusions are not. The CYP2B1–hrGFP fusion protein is characterized by reduction in mean fluorescence intensity (MFI) to less than 20% of that of the hrGFP protein alone, accompanied by a 50% loss of CYP2B1 activity. Exchanging the linker for an α-helical peptide structure between CYP2B1 and hrGFP does not improve fusion protein activity. Insertion of a short linker (five amino acids) increases reporter protein fluorescence intensity twofold without improving CYP2B1 activity. Introduction of the foot and mouth disease virus 2A sequence providing cotranslational cleavage led to an unstable hrGFP–2A protein, whereas the corresponding EGFP–2A protein was stable and yielded an MFI superior to those of all other fusion constructs tested. CYP2B1 activity of the EGFP–2A–CYP2B1 protein was in the range of that of the unmodified CYP2B1. These data indicate that the protein arrangement EGFP–2A–CYP2B1 is superior to others, since it is most active and visible, which is essential for an effective tracking of the CYP2B1 enzyme.

Keywords: CYP2B1; hrGFP; EGFP; FMDV–2A; Fusion protein; Reporter gene; Suicide gene

Marker genes are versatile tools for characterizing gene expression. In the past decade, green fluorescence proteins (GFP)[1] have replaced enzymatic reporter genes such as chloramphenicol acetyl transferase, β-glucuronidase, peroxidase, and β-galactosidase, largely because GFP is rapidly detectable after gene transfer without any staining procedures [1]. Two GFPs, from jellyfish *Aequorea victoria* and sea pansy *Renilla reniformis*, have been characterized [2]. Their optimized forms, enhanced GFP (EGFP) and humanized *Renilla* GFP (hrGFP), respectively, appear brighter in mammalian cells [3].

To date there are several approaches tracking transgene expression and localization using a marker gene. The classic method is the expression of the gene to be tracked and a marker gene, each under the control of its own promoter. However, a disadvantage of this strategy is that different quantities of proteins will be expressed, even when both genes are on the same construct and the ratio may also vary from cell type to cell type. Additionally for

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[1] *Abbreviations used:* GFP, green fluorescence protein; EGFP, enhanced GFP; hrGFP, humanized *Renilla* GFP; CYP2B1, cytochrome P450 2B1; ER, endoplasmic reticulum; FMDV, foot-and-mouth disease virus; FACS, fluorescence-activated cell sorting; MFI, mean fluorescence intensity; PBS, phosphate-buffered saline; MCS, multiple cloning site; SRP, signal recognition particle; CYP2C2, cytochrome P450 2C2.

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some applications, e.g., the use of viral vectors, there may not be sufficient space for two expression cassettes, since they are often quite large and their activity can vary dependent on the cell type used. Similar limitations have to be taken into account in polycistronic gene constructs using internal ribosome entry sites.

An attractive alternative is therefore the use of fusion proteins, which guarantee a monocistronic expression of both proteins in equal amounts and the efficient use of available space. EGFP and hrGFP marker genes have been used in various fusion proteins to trace the expression and subcellular localization of proteins of interest [2,4], but it has been observed that the arrangement of the fusion proteins can affect the function of the individual protein portions [5].

In recent studies, an approximately 20-amino-acid-long 2A sequence of either aphto- or cardiovirus, members of Picornaviridae, was used to enable the expression of two proteins from one cistron [6,7]. Fusion constructs harboring the 2A sequences allow the separation of the proteins by a ribosomal skipping mechanism, which leads to impairment of a peptide bond without stopping the translation [8].

The rat cytochrome P450 2B1 (CYP2B1) enzyme is bound to and acts at the endoplasmic reticulum (ER) [9]. CYP2B1 is predominately expressed in the liver and is able to metabolize various drugs [10]. In anticancer suicide therapy CYP2B1 metabolic activity is utilized by activating systemically administered prodrugs such as ifosfamide and cyclophosphamide, which are oxidized into their toxic 4-hydroxy forms causing cell damage and death [11]. CYP2B1 enzymatic activity can be determined either by measuring the conversion of an alternative substrate in the resorufin assay or by measuring the accumulation of the 4-hydroxy form [12].

In this study we analyzed the expression of CYP2B1 and different reporter proteins in various fusion arrangements to guarantee stoichiometric protein production without losing either enzymatic activity or fluorescence intensity of the respective proteins. Fusion constructs harboring the EGFP or hrGFP marker gene at either the 5′ or 3′ terminus of the CYP2B1 gene, connected via different linker sequences or the foot-and-mouth disease virus (FMDV) 2A sequence, were compared with respect to their expression level, their activity, and the subcellular localization of the respective gene products.

Materials and methods

Cloning of fusion constructs

The cDNA for the CYP2B1 enzyme, which is encoded on plasmid pSW1 [13], was cloned into the *Xho*I/*Xba*I restriction sites of plasmid pcDNA3 (Invitrogen, San Diego, CA) yielding plasmid pCMV–CYP2B1. In addition, the CYP2B1 cDNA was PCR amplified from plasmid pSW1 using primers 5′-ATC CCC GCG GAC CGG TCC ACC ATG GAG CCC AGT ATC TTG CTC-3′ and 5′-TCC GGA ATT CCC GAG CTG AGA AGC AGA TCT G-3′ (VBC-Genomics, Vienna, Austria) and inserted into the expression plasmid phrGFP–C (Stratagene, Amsterdam, The Netherlands) via *Sac*II/*Eco*RI restriction sites resulting in the plasmid pCYP2B1–hrGFP. To generate plasmid pCYP2B1–ll–hrGFP (long linker), complementary primers 5′-CAG GAA TTC GTC GAC CCC TTA TCG TCA ATC TTC TCA AGG ATT GGG GAC CCT CTC GAG ACC-3′ were annealed and introduced into the *Eco*RI/*Xho*I linearized plasmid pCYP2B1–hrGFP. Plasmid pCYP2B1–ll–hrGFP was modified to give the plasmid pCYP2B1–sl–hrGFP (short linker) by deleting a part of the linker sequence via *Sal*I/*Xho*I restriction digest and subsequent religation. Plasmid pCYP2B1–2A–hrGFP was obtained by annealing complementary oligonucleotides encoding the FMDV–2A sequence (5′-ATC ACG AAT TCC AGC TGT TGA ATT TTG ACC TTC TTA AGC TTG CGG GAG ACG TCG AGT CCA ACC CCG GGC CCG AAT TCG TCG AGA CC-3′) and introduced into the *Eco*RI site of plasmid pCYP2B1–hrGFP. To obtain plasmid phrGFP–CYP2B1 the hrGFP coding sequence was PCR amplified from plasmid phrGFP–C with the primers 5′-ACG TAA CCG GTC GAG ACC ATG GTG AGC AAG CAG-3′ and 5′-TAG AGT CGC GGC CGC TGA ATT CCA CCC ACT CGT GCA GGC TGC-3′, digested with *Age*I/*Not*I, and inserted into the plasmid pCMV–DsRed Express (Clontech, Heidelberg, Germany) to replace the DsRed coding sequence. Then the resulting plasmid was linearized with *Eco*RI and *Not*I and ligated to the CYP2B1 coding sequence, which had been PCR amplified from plasmid pCYP2B1–hrGFP using the oligonucleotides 5′-ACG TAG AAT TCG AGC CCA GTA TCT TGC-3′ and 5′-TAC GTG CGG CCG CTC ACC GAG CTG AGA AGC AGA TCT GG -3′ and then digested with *Eco*RI/*Not*I. The FMDV–2A sequence was inserted into plasmid phrGFP–CYP2B1 similarly to the cloning of plasmid pCYP2B1–2A–hrGFP, creating plasmid phrGFP–2A–CYP2B1.

The EGFP coding sequence was PCR amplified from plasmid pPCEmCMV [14] using specific oligonucleotides 5′-TTG TAC ACC CTA AGC CTC CG-3′ and 5′-AGT CAG AAT TCC TTG TAC AGC TCG TCC ATG CCG-3′. The PCR fragment was inserted into plasmid phrGFP–CYP2B1 after *Bam*HI/*Eco*RI digestion to replace the hrGFP sequence and to generate the plasmid pEGFP–CYP2B1. Subsequently, the FMDV–2A encoding sequence prepared as described above was inserted into the *Eco*RI-linearized plasmid pEGFP–CYP2B1, resulting in the plasmid pEGFP–2A–CYP2B1.

To replace the CYP2B1 3′ part and the entire hrGFP gene with the EGFP coding sequence, the EGFP coding