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Semliki Forest virus vectors for overexpression of 101 G protein-coupled receptors in mammalian host cells

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Abstract

Semliki Forest virus vectors were applied for the evaluation of 101 G protein-coupled receptors in three mammalian cell lines. Western blotting demonstrated that 95 of the 101 tested GPCRs showed positive signals. A large number of the GPCRs were expressed at high levels suggesting receptor yields in the range of 1 mg/L or higher, suitable for structural biology applications. Specific binding assays on a selected number of GPCRs were carried out to compare the correlation between total and functional protein expression. Ligands and additives supplemented to the cell culture medium were evaluated for expression enhancement. Selected GPCRs were also expressed from mutant SFV vectors providing enhanced protein expression and reduced host cell toxicity in attempts to further improve receptor yields. © 2005 Elsevier Inc. All rights reserved.

Keywords: G protein-coupled receptors; Semliki Forest virus; Structural genomics; Structural biology; Recombinant expression; Large-scale production; Mammalian cell lines; Immunodetection; Binding assays

G protein-coupled receptors (GPCRs)¹ are targets for 60–70% of drugs developed today [1]. Traditionally, the drug discovery process has relied on screening chemical libraries in search of compounds interacting favorably with the GPCR of interest [2]. Although this procedure has been relatively successful, there is still a huge demand on drugs with improved affinity and selectivity. One solution has been to apply structure-based drug design and there are several examples of novel drugs developed based on the information of the three-dimensional (3D) structure of their target protein [3]. However,

the paradox with GPCRs is that of the therapeutically interesting targets, not a single high-resolution structure is available today. The only 3D structure on a GPCR has been solved for bovine rhodopsin [4] in which case the high receptor abundance in the cow retina made isolation from native tissue possible. In this context, one of the major bottlenecks for obtaining high-resolution structures for recombinant GPCRs has been the difficulties in producing quantitatively and qualitatively satisfactory material. Moreover, the need of detergents for receptor purification has an additional negative impact on the stability of GPCRs. The crystallization in the presence of detergents as well as the receptor flexibility and small hydrophilic loops, which reduce the potential crystal contacts, has further hampered the progress in structure determination of membrane proteins.

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¹ Abbreviations used: GPCRs, G protein-coupled receptors; 3D, three dimensional; MBP, maltose binding protein; SFV, Semliki Forest virus; MePNet, Membrane protein network.

The recombinant expression of GPCRs and other membrane proteins has been evaluated in all possible expression systems including bacterial, yeast insect, and mammalian vectors, and was recently reviewed by Saramegna and co-workers [5]. The results have been quite variable with generally relatively moderate yields in bacterial membranes. However, modification of the coding sequence and fusion to appropriate partner proteins such as maltose binding protein (MBP) has resulted in significantly improved expression yields of, for example, the rat neurotensin receptor [6]. Alternatively, GPCRs can be directed to inclusion of bodies in bacteria, which results in high yields, but require a refolding process to restore functional activity of the receptor. Although refolding has generally proven to be inefficient, success was achieved for the leukotriene BLT1 receptor [7]. Yeast-based GPCR expression has been relatively successful especially when applying *Pichia pastoris* vectors, which has resulted in yields of 40 pmol/mg protein [8]. One advantageous feature of yeast expression is that the extremely high biomasses are achieved in large-scale production although breaking the thick cell wall is a challenge. Moreover, insect cells have been frequently applied for GPCR expression using baculovirus vectors. For instance, the human neurokinin-1 receptor has been expressed in milligram quantities at 60 pmol/mg protein [9]. Very recently, a study on 16 GPCRs expressed from baculovirus in three insect cell lines exhibited binding activities from 1 to 250 pmol/mg suggesting excellent applicability of insect cell-based expression to structural studies on GPCRs [10]. Expression of GPCRs in mammalian cells has been hampered by time-consuming and labor-intensive procedures with disappointingly low yields. However, rhodopsin expression from a tetracycline-inducible stable HEK293 cell line resulted in yields of 10 mg/L opsin [11]. One system that has frequently been applied for GPCR expression in mammalian cells is based on Semliki Forest virus (SFV) vectors [12]. The attractive features of SFV, a member of the alphavirus family, are the rapid high-titer virus generation, the broad range of mammalian host cells and the high yields of recombinant GPCRs production. Several GPCRs have been expressed with a specific binding activity of 50–200 pmol/mg protein and the yields in large-scale production in mammalian cell suspension cultures have been up to 10 mg/L [13].

In this study, we have applied the SFV system for the overexpression of 101 GPCRs as part of the Membrane Protein Network (MePNet) program. In this consortium, this large number of GPCRs has been expressed in parallel in three expression systems based on *Escherichia coli*, *P. pastoris*, and SFV vectors [14]. Here, we concentrate on reporting on the results of the SFV expression by evaluating the expression levels for all targets by immunodetection. Selected targets were subjected to radioligand binding assays to determine the functional-

ity of the expressed GPCRs. Furthermore, some GPCRs were subjected to expression optimization by addition of ligands, previously shown to enhance the binding activity [15]. Finally, a comparison was made on the expression of a few GPCRs from the conventional SFV vector and a mutant SFV vector [16] with enhanced expression levels and prolonged host cell survival.

Experimental procedures

Materials

Restriction enzymes, SP6 RNA polymerase, RNase inhibitor, and the CAP analogue m⁷G(5')ppp(5')G were purchased from New England Biolabs. Chymotrypsin and aprotinin were from Sigma.

Mammalian cell cultures

BHK-21 (baby hamster kidney), CHO-K1 (Chinese hamster ovary), and HEK293 (human embryonic kidney) cells were cultured in a 1:1 mixture of Dulbecco's modified F-12 medium and Iscove's modified Dulbecco's medium supplemented with 4 mM glutamate and 10% FCS using standard cell culture techniques. DU-145 cells (human prostate tumor cell line) were grown in Dulbecco's modified F-12 medium, 4 mM glutamate and 10% FCS and C8166 cells (human T lymphocyte cell line) in RPI medium, 4 mM glutamate and 10% FCS. All cell culture reagents were from Gibco BRL.

Engineering of SFV vectors, subcloning of GPCR cDNAs, and generation of recombinant SFV

The pSFV2genB and pSFV2genC vectors (Fig. 1) were engineered based on the previously described pSFV2gen vector [17]. The pSFV-PD vector has been previously described [16]. All 101 GPCRs were originally cloned into the pCR4Blunt-TOPO vector (Invitrogen) and their sequences verified to those sequences available in publications and public databases before subcloning into the *Bam*HI–*Spe*I region of the SFV expression vectors. Each clone was verified by restriction endonuclease digestions to confirm the accuracy of obtained clones. Recombinant SFV particles were generated as described previously [17]. Briefly, SFV vector constructs were linearized by *Nru*I or *Sap*I and confirmed by restriction digestion analysis. In vitro transcribed RNA molecules from SFV expression and pSFV-helper2 vectors [18] were co-electroporated into BHK-21 cells and recombinant SFV particles harvested 24 h later. The conditionally infectious SFV particles were activated by α -chymotrypsin treatment and the reaction terminated by addition of aprotinin (trypsin inhibitor). Titers were

Fig. 1. Schematic presentation of various SFV vector constructs. nsP1-4, SFV non-structural genes; K, Kozak sequence; ss, signal sequence from influenza hemagglutinin (HA) gene; FLAG, FLAG-epitope tag; Tev, Tev protease cleavage site; His, 10 histidine-tag; Biotin, biotin tag from *Propriobacterium shermanii*; **, point mutations in nsP2 (S256P and R650D).

determined by verification of morphology changes on infected cells. Generally, titers in the range of 5×10^8 to 1×10^9 infectious particles per milliliter were obtained. Due to temperature sensitivity, virus stocks prepared from the pSFV-PD vector were cultured at 31 °C instead of the conventional temperature of 37 °C.

SFV-based GPCR expression in mammalian cell lines

Preliminary expression evaluation was done in adherently cultured BHK-21, CHO-K1, and HEK293 cells on 12- and 24-well plates. Cells at 60–70% confluency were infected with various concentrations of recombinant SFV and cultured for 24, 36, 48, and 72 h before harvesting. Selected well-expressed targets were also tested for expression in DU-145 cells and in suspension cultures of BHK-21, CHO-K1, and C8166 cells. The human prostate tumor cell line DU-145 was chosen because of the high SFV-infection rate and high reporter gene expression previously observed [19]. As the human T lymphocyte cell line C8166 natively represents suspension growth, the expression of a few GPCRs was compared to those expressed in BHK and CHO cells adapted to suspension culture. Expression scale-up was performed in spinner or roller flask cultures.

Immunodetection by Western blotting

Western blotting was performed as follows. Cell samples from various virus concentrations and harvest times were lysed as previously described and subjected to 10% SDS-PAGE [16]. A 30 min electrotransfer to Hyband ECL nitrocellulose membrane was followed by treatment with 5% milk in TBST (TBS with 0.1% Tween 20) at +4 °C, the primary antibodies (anti-FLAG and/or anti-His) (Sigma) and the secondary anti-mouse antibody (Sigma). The visualization of specific bands was carried out using the ECL chemiluminescence kit from Amersham.

Radioligand binding assays

The membrane fraction was isolated from SFV-infected mammalian cells as previously described [20]. Briefly, cell pellets were resuspended in 10 mM HEPES, 1 mM EDTA (pH 7.4) and homogenized with an Ultra-Turrax homogenizer for 20 s. Membranes were centrifuged at 55,000g and resuspended in 50 mM Tris, 150 mM NaCl, and 20% glycerol. Radioligand binding assays were performed as previously described [15,21–40]. The binding experiments were carried out as single point measurements in triplicates at radioligand concentrations close to the 10-fold theoretical K_d value.

Expression optimization by ligand addition

The ligand Atropin (Sigma) was added at 100, 300, and 500 μ M concentrations to cell culture medium at the time of infection with recombinant SFV particles expressing the human muscarinic M1 (ACM1_HUMAN) and the pig muscarinic M2 (ACM2_PIG) receptors, respectively. Radioligand binding was performed as described above to determine the effect of ligand addition on the specific binding activity. Expression optimization was also evaluated by comparison of the expression of the mouse muscarinic M1 receptor from the pSFV2genC vector to the less cytopathogenic pSFV-PD vector [16].

Results

Novel SFV expression vectors

Although previously developed SFV vectors had proven successful for the expression of a large number of GPCRs [12], and additional vectors had been engineered to contain tags for identification and purification [13] two novel SFV vectors were generated within the MePNet

program (Fig. 1). These vectors contain at the N-terminus an optimized Kozak translation sequence [41], a signal sequence from the influenza hemagglutinin (HA) gene to potentially enhance the receptor transport to the plasma membrane and a FLAG-epitope for immunodetection. Downstream of the gene of interest (GPCR), a deca-histidine (His-10) tag was engineered for immunodetection and metal affinity chromatography purification. The pSFV2genC vector differs from pSFV2genB by an additional biotinylation sequence from *Propriobacterium shermanii* downstream of the His-10 tag. The biotin tag was introduced into the vector because it would allow streptavidin affinity purification and the presence of a biotin tag has been demonstrated to provide improved stability of GPCRs expressed in yeast cells [8]. Previously, the expression of the human neurokinin-1 receptor with a C-terminal biotin tag from SFV vectors in CHO cells showed that the expression yields were as high as for the wild-type receptor [42]. Moreover, the receptor binding characteristics and the coupling to G proteins were unaffected. Both pSFV2genB and pSFV2genC contain Tev protease cleavage sites flanking the GPCR insert, which makes it possible to cleave off all tags of the purified receptor prior to crystallization.

Expression evaluation by immunodetection

Initial expression evaluation was performed by immunodetection in western blots (Fig. 2). Of the 101 GPCR targets, 47 were evaluated for the pSFV2genB vector and 99 for the pSFV2genC vector (Table 1). The expression levels were divided into three categories: low (+), medium (++), and high (+++). Combining the results from both pSFV2genB and pSFV2genC vectors showed that in only six cases no significant signal of the expected molecular weight was detected for the specific GPCR (–). Even so, despite being negative in western blots, one of these GPCRs, the rat serotonin 5-HT₇ receptor (5H7_RAT) showed specific binding activity (see below). As the expression levels were similar for the

two SFV vectors, in interest of the progress of the MeP-Net program, a large number of GPCRs were only tested in one of the vectors, mainly pSFV2genC. For this reason, of those six GPCRs with no signal from pSFV2genC only one was tested and also found negative in pSFV2genB. Among the positive signals obtained, 14 showed low (+), 10 medium (++), and 11 high (+++) from the pSFV2genB vector. The corresponding numbers for pSFV2genC were 28 (+), 23 (++), and 42 (+++). Although it is rather difficult to give exact numbers of expression levels based on immunodetection, we estimate that the category of medium expression is in the range of 0.5–1 mg and high expression 1–10 mg/L culture. The targets compatible to structural biology were therefore defined as those belonging to categories of medium (++) and high (+++) expression levels. For pSFV2genB 60.0% and for pSFV2genC 69.9% fell into these categories, suggesting that the biotin tag did not have a negative impact on the expression levels. Taken together, the total number of structural compatible GPCRs was 70.

Expression comparison in different cell lines

Although the majority of the GPCRs were expressed in BHK-21 cells, a cell line frequently used for SFV production and also pharmacological characterization of GPCRs, a number of receptors were also evaluated in CHO-K1 and HEK293 cells (Table 2) and a few in DU-145 and C8166 cells. The majority of GPCRs (71 of 92 tested) showed positive signals in BHK-21 cells, whereas the number of positive signals was lower in both CHO-K1 and HEK293 cells. There are, however, two points to be taken into account: First, 50 GPCRs were tested for expression in CHO-K1 cells and the number in HEK293 cells was 27. Second, those targets that preliminarily were negative in BHK-21 cells were next evaluated in the other two cell lines. In summary, CKR2_HUMAN and ORPD_HUMAN showed positive signals in CHO-K1 cells, but not BHK-21 cells. The MC5R_HUMAN was

Fig. 2. Immunodetection of SFV-based expression of selected GPCRs. Expression was compared for GPCRs expressed from either the pSFV2genB (B) or pSFV2genC (C) vector as indicated. Lane 1, 5H1AHUMAN/B; lane 2, 5H1A_HUMAN/C; lane 3, 5H1B_HUMAN/B; lane 4, 5H1B_HUMAN/C; lane 5, 5H2A_HUMAN/C; lane 6, 5H7-HUMAN/C; lane 7, AA1R_HUMAN/C; lane 8, AG2R_HUMAN/C; lane 9, BRB1_HUMAN/C; lane 10, CB2R_HUMAN/C; lane 11, CCR3_HUMAN/C; lane 12, CKR6_HUMAN/C; lane 13, EDG3_HUMAN/C; lane 14, ETR1_HUMAN/C; lane 15, HH2R_HUMAN/B; lane 16, HH2R_HUMAN/C; lane 17, STE3_YEAST/B; lane 18, STE3_YEAST/C. An anti-FLAG antibody (1:500) was used for detection.

Table 1

Expression evaluation by immunodetection of 101 GPCRs from pSFV2genB and pSFV2genC

	M#	SFVB	SFVC		M#	SFVB	SFVC
ACM1_HUMAN	23	Nd	+++	ET1R_HUMAN	51	-	+
ACM1_MOUSE	24	+++	+++	ACTR_HUMAN	27	+	+
ACM2_PIG	26	Nd	+	ETBR_BOVIN	52	-	+
ACM2_HUMAN	25	+	-	MC3R_HUMAN	64	nd	+
A1AA_HUMAN	104	Nd	+++	MC4R_HUMAN	65	nd	+
A1AA_RAT	14	+	+++	MC5R_HUMAN	66	nd	+
AA1B_HUMAN	15	++	++	NY1R_HUMAN	79	-	+++
A1AB_MESAU	105	Nd	+	NY2R_HUMAN	80	++	nd
A1AD_RAT	106	Nd	+	NY4R_HUMAN	81	++	+
A2AA_HUMAN	107	Nd	+++	OPRD_HUMAN	83	+	+
A2AB_HUMAN	16	Nd	+	OPRD_MOUSE	84	nd	++
A2AC_HUMAN	18	Nd	+	OPRK_HUMAN	85	++	++
B3AR_HUMAN	31	++	+	OPRK_MOUSE	86	++	++
D2DR_HUMAN	43	-	++	SSR1_HUMAN	93	+++	+++
D2DR_MOUSE	44	-	+++	SSR2_HUMAN	94	+++	+++
D3DR_HUMAN	45	+	+++	SSR3_HUMAN	95	+	+++
D4DR_HUMAN	46	Nd	+++	NK1R_HUMAN	72	+++	+
DADR_HUMAN	109	Nd	++	NK1R_RAT	73	+	+
DBDR_HUMAN	110	Nd	+++	NK2R_HUMAN	74	+	+++
HH2R_HUMAN	61	+++	+++	NK2R_RAT	75	+	+++
HH2_RAT	62	+	+++	NK3R_HUMAN	76	+++	++
5H1A_HUMAN	1	+++	+++	V1BR_HUMAN	103	nd	+++
5H1B_HUMAN	2	+++	+++	GALR_HUMAN	54	nd	+++
5H1D_HUMAN	3	Nd	+++	GALT_HUMAN	56	nd	++
5H1E_HUMAN	4	+	++	PAR1_HUMAN	99	-	nd
5H1F_HUMAN	5	+++	++	MCR1_HUMAN	60	nd	++
5H2A_HUMAN	6	+	++	LSHR_RAT	133	nd	+++
5H2A_MOUSE	7	Nd	+	FML1_HUMAN	139	nd	++
5H2B_HUMAN	8	-	+	TSHR_RAT	116	nd	+
5H2C_HUMAN	9	-	+++	PE23_HUMAN	90	nd	++
5H4_HUMAN	144	Nd	+++	AA1R_HUMAN	19	++	++
5H5A_HUMAN	10	+	+++	AA2A_HUMAN	20	nd	++
5H6_RAT	111	Nd	+++	AA2B_HUMAN	21	-	++
5H7_HUMAN	12	-	+++	P2Y2_MOUSE	87	nd	+
5H7_RAT	117	Nd	+	P2Y2_HUMAN	88	nd	+++
AG2R_HUMAN	28	++	+	P2Y6_HUMAN	137	nd	+
BRB1_HUMAN	32	-	+++	P2YR_HUMAN	89	+++	+++
BRB2_HUMAN	33	Nd	+++	CB1R_MOUSE	121	nd	+
FSHR_HUMAN	131	Nd	+	CB2R_HUMAN	37	++	+++
IL8A_HUMAN	63	Nd	++	EDG3_HUMAN	48	+	+++
IL8B_HUMAN	136	Nd	+	CLT1_HUMAN	129	nd	+++
CCR3_HUMAN	39	Nd	+++	CLT2_HUMAN	130	nd	+
CCR4_HUMAN	135	Nd	+++	CALR_HUMAN	34	nd	-
CKR1_HUMAN	40	Nd	+++	GLR_HUMAN	59	nd	++
CKR2_HUMAN	41	Nd	+	MGR2_HUMAN	67	nd	-
CKR6_HUMAN	42	Nd	++	MGR3_HUMAN	68	++	++
CKR7_HUMAN	145	Nd	++	MGR4_HUMAN	69	nd	+++
CKRB_HUMAN	153	Nd	+++	MGR7_HUMAN	114	nd	+
CCKR_HUMAN	38	-	++	STE2_YEAST	96	nd	+++
GASR_HUMAN	57	Nd	++	STE3_YEAST	97	+++	+++
ET1R_BOVIN	50	Nd	-				

M#, MePNet number; nd, not done; SFVB, pSFV2genB; SFVC, pSFV2genC.

Table 2

Comparison of SFV-based expression in various mammalian cell lines

	ID+	ID–	Total
(A)			
BHK-21	71	21	92
CHO-K1	18	32	50
HEK293	11	16	27
	ID+/BHK-21	ID+/CHO-K1	ID+/HEK293
(B)			
BHK-21	—	9	6
CHO-K1	7	—	3
HEK293	4	2	—

(A) The number of GPCRs showing positive (ID+) and negative (ID–) signals evaluated by immunodetection and the total number of GPCRs. (B) The number of GPCRs originally negative in various cell lines (ID–s), which showed positive signals in other cell lines.

expressed both in CHO-K1 and HEK293 cells, but not in BHK-21 cells. Interestingly, LSHR_RAT and GLR_HUMAN were preferentially expressed in DU-145 cells. The CKR7_HUMAN was expressed at high levels not only in C8166 cells, but also in suspension cultures of BHK-21 cells. These findings suggested that the host cell background is very important for achieving high expression levels as previously shown for opioid receptors [43].

Radioligand binding on selected targets

Selected GPCRs expressed from SFV vectors were subjected to radioligand binding assays. In total, 41 GPCRs were analyzed for specific binding, which demonstrated a huge variation in binding values (Table 3). The binding activity levels were divided into three categories, low (< 1 pmol/mg), medium (1–10 pmol/mg), and high levels (> 10 pmol/mg). Only 2 GPCRs showed no specific binding, 10 showed low, 16 medium, and 13 high binding activities. The highest binding activity of 287 pmol/mg was observed for the human adenosine A2A receptor (AA2A_HUMAN).

Comparison of immunodetection and specific binding

The expression levels evaluated by immunodetection were compared to the binding data obtained (Table 2). Interestingly, the rat serotonin 5-HT7 receptor (5H7_RAT), which showed no specific signal in western blotting, resulted in a binding activity of 2.18 pmol/mg. Among the 10 targets in the category of low (+) immunodetection, 2 showed no binding activity, 3 low, 4 medium, and 1 high specific binding. The six GPCRs in the category of medium (++) expression were evenly distributed (2 each) in the binding activity groups. Of the group of 24 high (+++) expressing targets 2 GPCRs showed low, 11 medium, and 11 high binding activity.

Table 3

Comparison of SFV-based expression evaluation by immunodetection and binding activity

	MePNet#	SEV ID	SFV BA
ACM1_HUMAN	23	+++	2.44
ACM1_MOUSE	24	+++	2.93
ACM2_PIG	26	+	22
ACM2_HUMAN	25	+	6.1
A1AA_HUMAN	104	+++	0.25
A1AA_RAT	14	+++	4.7
A1AB_MESAU	105	+	0
A1AD_RAT	106	+	0
A2AB_HUMAN	16	+	0.05
A2AC_HUMAN	18	+	0.65
D2DR_HUMAN	43	+++	29.2
D2DR_MOUSE	44	+++	21.8
D3DR_HUMAN	45	+++	1.8
D4DR_HUMAN	46	+++	3.47
HH2R_HUMAN	61	+++	20
5H1A_HUMAN	1	+++	12.7
5H1B_HUMAN	2	+++	19.2
5H1D_HUMAN	3	+++	11
5H1E_HUMAN	4	+	0.75
5H1F_HUMAN	5	++	0.69
5H2A_HUMAN	6	+	2.62
5H5A_HUMAN	10	+++	31
5H6_RAT	111	+++	1.4
5H7_HUMAN	12	+++	2.2
5H7_RAT	117	-	2.18
BRB1_HUMAN	32	+++	0.19
BRB2_HUMAN	33	+++	7.9
NY1R_HUMAN	79	+++	2.3
NY2R_HUMAN	80	+	1.85
OPRD_HUMAN	83	+	1.85
OPRD_MOUSE	84	++	4
OPRK_HUMAN	85	++	1.6
OPRK_MOUSE	86	+++	1
NK1R_HUMAN	72	+++	28.6
NK1R_RAT	73	+++	68.9
NK2R_HUMAN	74	+++	3.6
NK2R_RAT	75	+++	41.6
NK3R_HUMAN	76	++	26.5
V1BR_HUMAN	103	+++	26.1
AA1R_HUMAN	19	++	0.4
AA2A_HUMAN	20	++	287

M#, MePNet number; SFV ID, SFV-based expression evaluated by immunodetection; SFV BA, SFV-based expression evaluated by radioligand binding assays.

Expression optimization

In attempts to further improve the expression levels of GPCRs in mammalian cell lines, the supplementation of ligands was investigated. Previously, it was demonstrated that the addition of ligand to the cell culture medium, significantly increased the binding activity of the histamine H2 receptor in SFV-infected COS-7 cells [15]. Here, we tested the addition of increasing concentrations of a specific ligand, Atropin, to CHO suspension cell cultures infected with SFV expressing two musca-

Fig. 3. Optimization of SFV-based expression of muscarinic receptors by ligand addition. Increasing concentrations (0–500 μ M) of Atropin was added to the medium of cells infected with the pSFV2genC vector carrying the human M1 (ACM1_HUMAN). Binding assays were carried out applying 24 nM 3 H-QNB (Perkin Elmer) as ligand.

rinic receptors. In this context, the binding activity of the human muscarinic M1 receptor (ACM1_HUMAN) could be enhanced from 0.75 to 5 pmol/mg (Fig. 3). Similarly, ligand binding improved approximately five-fold to 22 pmol/mg (data not shown) for the pig muscarinic M2 receptor (ACM2_PIG). In contrast, ligand addition did not affect the binding activity of the mouse muscarinic M1 receptor (ACM1_MOUSE) (data not shown).

Application of mutant SFV vectors

Several mutant SFV vectors have been engineered with the aim to reduce their inherent cytotoxicity on host cells [13,44]. In addition to the lower cytotoxicity, the SFV-PD vector also showed elevated levels of reporter protein expression [16]. Here, we could demonstrate that the binding activity of the mouse muscarinic M1 was enhanced four- to five- fold when expressed from the SFV-PD vector in comparison to the pSFV2genC vector (Fig. 4). Interestingly, in contrast to the lack of any effect

Fig. 4. Expression comparison of pSFV2genC and SFV-PD vectors in presence of ligand. Increasing concentrations (0–300 μ M) of Atropin was added to the medium of cells infected with the pSFV2genC and pSFV2genC-PD vectors carrying the murine M1 (ACM1_MOUSE) receptor. Binding assays were carried out applying 24 nM 3 H-QNB (Perkin Elmer) as ligand.

on binding levels in the pSFV2genC vector after ligand addition, the SFV-PD generated significantly higher binding activity (4.5 pmol/mg).

Discussion

We have evaluated the expression of 101 GPCRs applying SFV-based expression in mammalian cell lines. In Western blots, 95 of the 101 targets gave a positive signal. The expression levels were divided into three categories—low, medium, and high—and the results indicated that 70 GPCRs were expressed at structural biology compatible levels. Two SFV vectors harboring various tags and other accessory sequences (Fig. 1) were applied for the study. The pSFV2genB and pSFV2genC vectors were otherwise identical except for the C-terminal biotin tag present in the latter. As no major differences in expression levels were observed between the two vectors after analysis of half of the targets, it was decided to concentrate on the pSFV2genC vector for the evaluation of the rest of the GPCRs. Of the six GPCRs that did not show positive signals in western blots, the human dopamine D2 (D2DR_HUMAN), the rat serotonin 5-HT7 (5H7_RAT), and the human metabotropic glutamate R2 (MGR2_HUMAN) receptors have previously been successfully expressed from SFV vectors [12,45]. Also the endothelin 1B receptor has been well expressed before [46]. In a similar way, the human NK1R (NK1R_HUMAN) [13], the human adrenergic α 2B (A2AB_HUMAN) [47], and the rat NK1R (NK1R_RAT) have previously showed 5- to 20-fold higher binding activity in comparison to values obtained in this study [48]. In contrast, the AA2A_HUMAN receptor showed extremely high binding activity (287 pmol/mg) in comparison to the earlier observed 40 pmol/mg [49]. This discrepancy may be at least partly explained by the differences in SFV vector constructs. Particularly, the presence of the signal sequence from influenza HA at the large extracellular domain of the mGluR2 may have presented a negative effect on the expression pattern. Additionally, variation in virus batches and cell culture conditions may have affected the results. Most importantly, in most of the previously published studies at least some optimization of the expression conditions had taken place. In contrast, as described for the 101 GPCRs evaluated in this study, only limited optimization through ligand addition and vector selection was applied. The main purpose of this study was to evaluate the expression levels of a large number of GPCRs in parallel. The introduction of a signal sequence upstream of a GPCR sequence has previously demonstrated improved transport to the plasma membrane and enhanced binding activity in baculovirus-infected insect cells [50]. Here, we have not included transport and localization studies on GPCRs expressed

from the pSFV2genB and C vectors in comparison to standard SFV vectors. However, binding assays suggested no significant superiority of either type of vector and only future localization studies will reveal whether any improvement in receptor delivery to the plasma membrane can be achieved.

The successful expression of many GPCRs of which several showed high specific-binding activity forms a good basis for further studies. It will now be essential to optimize the expression conditions and binding activity for selected GPCRs in attempts to carry out further purification and structural characterization with the ultimate goal of obtaining high-resolution structures of several GPCRs.

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