GPVI-RBL-2H3-NFAT-Re luciferase cell line: a new cell based system for studying collagen receptor activation

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Introduction

The response of platelets to vessel wall injury is a primary event in arterial thrombosis.
Platelets respond to vessel wall injury via activation of the Collagen surface receptor Glycoprotein VI (GPVI), which accelerates the thrombotic response through release of granule contents and activation of platelet integrins.
GPVI, which represents a relevant antithrombotic pharmacological target, is a platelet membrane protein constitutively associated with the Fc Receptor γ chain (FcR γ). Upon activation by the specific ligand Collagen, GPVI induces a kinase cascade, resulting in the stimulation of PLC-γ2 which finally leads to cytosolic Ca^{++} increase *(Figure 1)*.

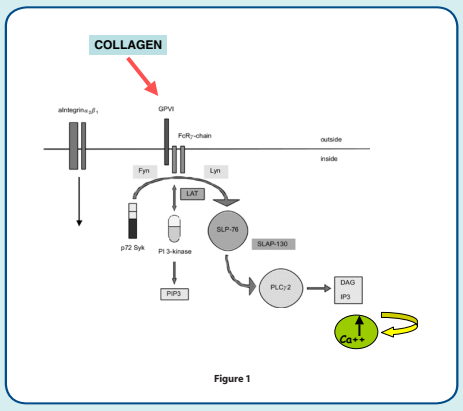


Figure 1

RBL-2H3 cells (a rat basophilic leukaemia cell line) endogenously express the FcRγ chain.
Zheng et al. (.J.Biol.Chem 2001; 276: 12999-06) demonstrated that the heterologous expression of GPVI in these cells is able to confer both signalling and adhesive responses to the high affinity GPVI ligand: convulxin (CVX), a snake venom protein, initially used to bio chemically purify the receptor.

Aim of this study was the configuration of a cell based assay suitable for the identification of specific GPVI antagonists. RBL-2H3 were permanently transfected with a reporter vector expressing Luciferase under the control of NFAT responsive elements (**RBL2H3-NFAT-Re** cell line). The NFAT transcription factors (**N**uclear **F**actor **A**ctivated **T** cells), which play an essential role in immune response gene expression, are in fact activated by stimuli which increase free cytoplasmic Ca^{++} *(Figure 2)*.

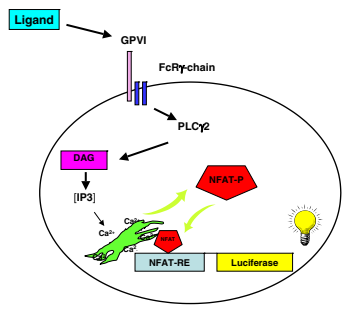


Figure 2

The **RBL2H3-NFAT-Re** cell line was permanently transfected with an expression vector for human GPVI, and a functional clone selection was performed by incubating the **GPVI-RBL2H3-NFAT-Re** cells for five hours in the presence of CVX.
The most responsive clones showed Luciferase stimulation of up 100 fold, with a CVX EC_{50} in the pico-molar range.

This cell based system represents an innovative, reliable, highly sensitive and very fast method for the identification of GPVI antagonists.

Materials and methods

Cloning:

Human GPVI (acc.number AB 035073) full length was amplified from human bone marrow cDNA and was cloned into pIres2-EGFP (Clontech).
The final construct (pIRES2-EGFP-GPVI) was confirmed by sequencing.
The *pNFAT-TA-luc* reporter vector was purchased by Clontech *(Figure 3)*.

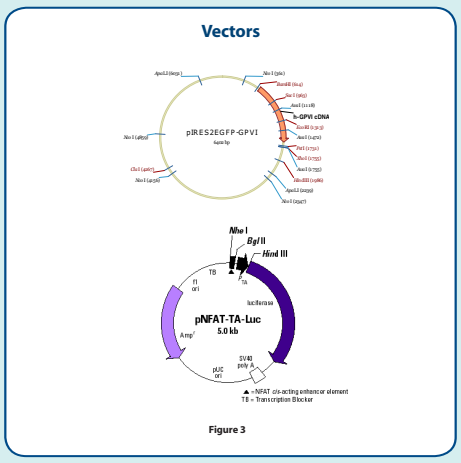


Figure 3

Cell Line

RBL-2H3 cells (rat basophilic leukaemia cell line) were maintained in MEM (EAGLE) with Glutamax and with Earle's salts, 15% FBS; 1% Pen./Strep., 25mM Hepes Buffer Solution, 1.0 mM Sodium Pyruvate; 1% MEM non-essential aminoacids; 1.5 g/L Sodium Bicarbonate; 0.5 mg/ml G418, 0.25ug/ml puromycin.

Cell culture conditions: Split twice a week: $1.0X10^5$ cells/flask T25 (recovering: $4.0X10^6$ cells/T25 flask).

Pre-culture conditions: Cells were seeded for experiments at about 70% confluence.

Ligand

The specific GPVI agonist *convulxin* (CVX), a glycoprotein of 72 kDa isolated from Crotalus durissus terrificus venom, binds to glycoprotein (GP) VI and triggers high affinity intense PLC activation.
It was dissolved in tyrode buffer containing 2mM Ca^{++} at 2 uM and stored in aliquots at -20°C.
The working solutions were freshly made in Serum Free Medium (SFM).

Generation of **RBL2H3-NFAT-Re luciferase cell line** and **GPVI-RBL2H3-NFAT-Re luciferase cell line**

RBL-2H3 cells were stably transfected with the pNFAT-Ta-luc reporter vector Stable **RBL2H3-NFAT-Re** Luciferase clones were selected for luciferase expression after ionomicin incubation *(Figure 4)*.

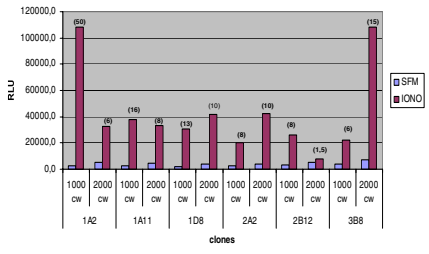


Figure 4
Cells permanently transfected with pNFAT-TAluc were tested 48h after seeding for induced luciferase expression after 5h incubation in serum free medium containing 1mM ionomicin (in parenthesis are indicated the fold of stimulation)

The stable clone showing the best signal to background value was permanently transfected with an expression vector for human GPVI, and first selection was performed for antibiotic (G418) resistance.
G418 resistant cells were isolated after 10 days of selection. Then the cells were diluted at 1 cell/well in 96MTPs (1st limiting dilution) and grown for 10 days.
48 hrs. after plate replication, the positive clones were collected on the basis of the increment of luminescence obtained by a 5 hr incubation with 1nM convulxin, in SFM and measured at CCD lumibox for 60 seconds.
The best final clone was tested for dose/dependent CVX induction at different times after seeding *(Figure 5a, 5b)*.

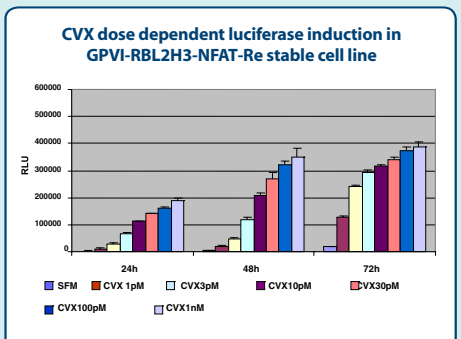


Figure 5a
1500 c/w were seeded in 384MTP.24,48 and 72h after seeding the cells were incubated for 5h with CVX in serum free medium (SFM).
Luciferase activity was detected at the CCD Camera after injection of T/L mix.

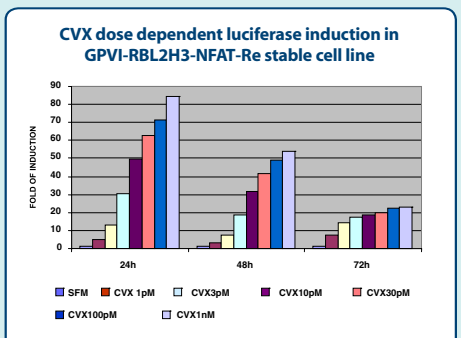
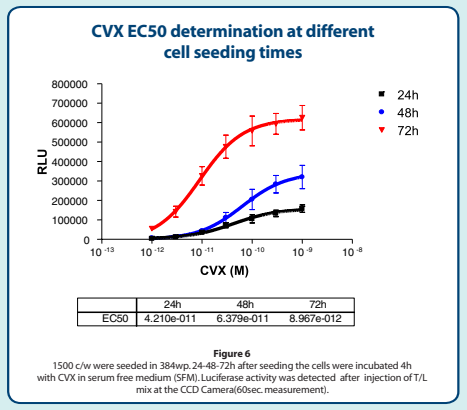


Figure 5b
1500 c/w were seeded in 384wp.24,48 and 72 h after seeding the cells were incubated 5h with CVX in serum free medium (SFM). Luciferase activity was detected at the CCD Camera after injection of T/L mix.

The final clone was completely characterized for CVX EC_{50} *(Figure 6)*.



	24h	48h	72h
EC50	4.210e-011	6.379e-011	8.967e-012

Figure 6
1500 c/w were seeded in 384wp. 24-48-72h after seeding the cells were incubated 4h with CVX in serum free medium (SFM). Luciferase activity was detected after injection of T/L mix at the CCD Camera(60sec. measurement).

Luciferase assay measurement was performed by seeding 1500cell/well in 384 MTPs and by injecting 25 μl of a 1:1 Tryton/Luciferine mix solution on 25 μl medium volume into each well. Measurement was performed at CCD lumibox by recording the signal for 60 seconds at 50-100% sensitivity.

Concluding remarks

In this study we describe the generation of a cell based assay which allows the functional characterization of the human GPVI receptor, by the use of luminescence.
The approach followed is the coupling of the intracellular Calcium mobilization signal, achieved upon specific GPVI stimulation, to NFAT controlled luciferase expression.

The results obtained indicate that this cell based system has properties of high sensitivity, reproducibility and very strong signal to background values and is particularly suitable for the high throughput screening and identification of compounds specifically acting on GPVI as antagonists or agonists.

References

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