Heterologous Expression and Biophysical Characterization of GPCRs Produced in Mammalian Cells Grown in Suspension

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Abstract: We have devised protocols for the reliable and high-level expression of GPCRs using Semliki Forest Virus vectors which are used to infect CHO cells grown in suspension in Wave bioreactors. Additionally, we have developed strategies to manipulate the N-linked glycosylation of overexpressed GPCRs by the use of glycosylation inhibitors or cell lines with impaired N-glycosylation pathway. These improvements would likely facilitate crystallization by increasing the homogeneity of the GPCR samples. In order to characterize the quality of the expressed and purified GPCRs we have developed a fluorescent assay to measure the thermal stability of membrane proteins. The assay measures the thermally induced change in the accessibility of the native cysteines, which are derivatized in real time with a highly reactive maleimide-based fluorescent probe (CPM). We use the thermal stability assay to identify constructs or buffer condition/additives which enhance the thermal stability of the detergent-solubilized GPCRs and thus increase the chances for crystallization. Finally, we have used site-directed mutagenesis and homology modeling to identify and characterize constitutively active mutants of APJ receptor, a class A GPCR.

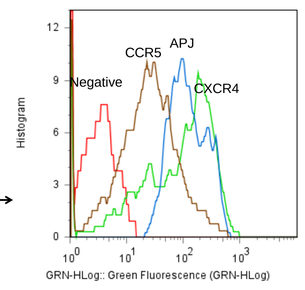
Semliki Forest virus mediated expression: workflow

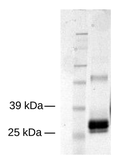
Suspension CHO cells grown in media bottles

Wave bioreactor

Guava flow cytometer

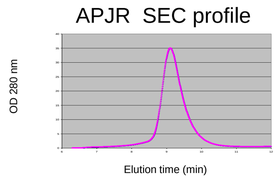
Cell surface or total in-cell expression





SDS-PAGE
Purified and deglycosylated APJR

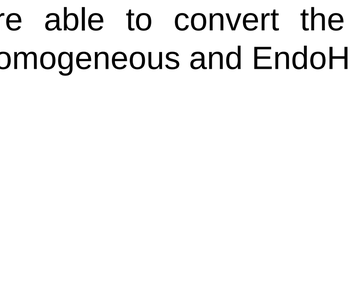
APJR SEC profile



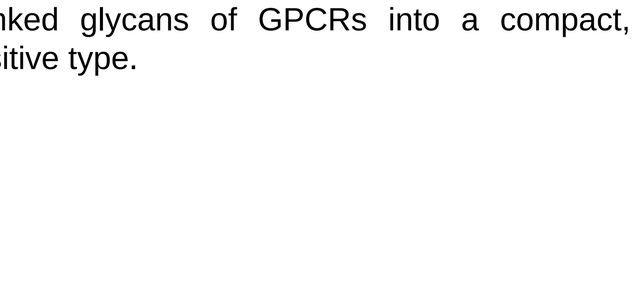
Conclusions: An efficient protocol for overexpression of GPCRs in suspension CHO cells has been established. The protocol combines the excellent scalability and ease of operation of the Wave bioreactors and the quality control capabilities of the Guava flow cytometer to ensure high-level and reproducible protein expression.

Manipulating N-glycosylation of overexpressed GPCRs

SDS-PAGE analysis of APJ receptor



Apelin13 competition binding

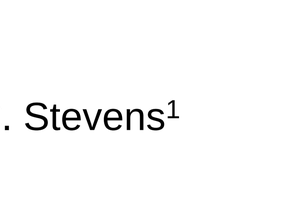


MALDI-TOF analysis of N-glycans linked to APJ receptor in CHO lec1 cells

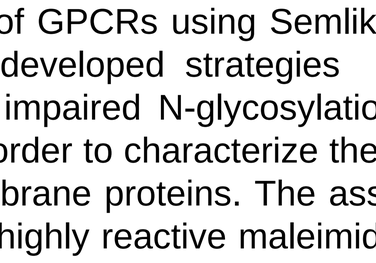
Conclusions: Through the use of glycosylation inhibitors, mainly kifunensine, as well as cell lines with impaired glycosylation capabilities, we are able to convert the N-linked glycans of GPCRs into a compact, homogeneous and EndoH sensitive type.

APJ stability profiling: a primer for class A GPCR

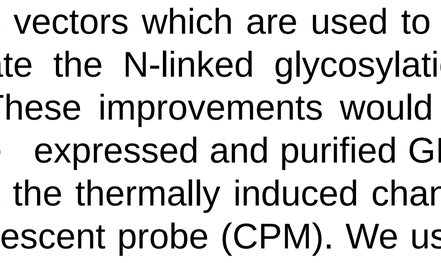
CPM assay of APJ receptor



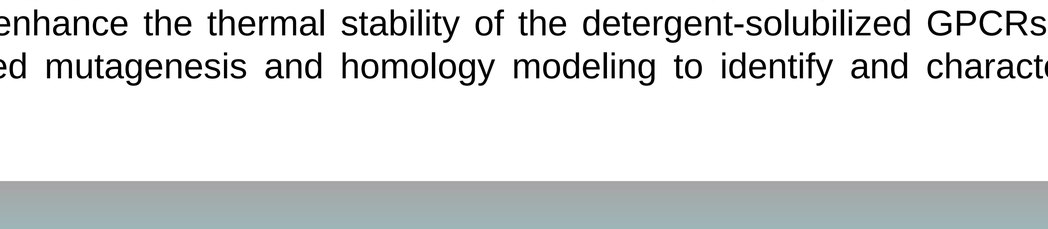
Effect of salt type and concentration



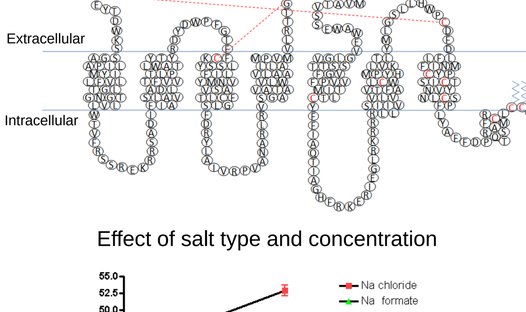
Effect of buffer system and pH



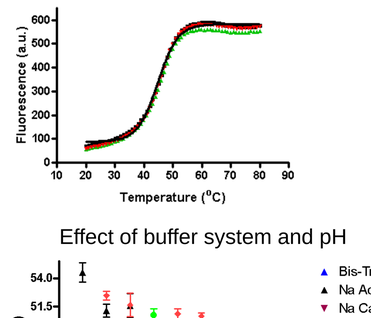
Effect of detergents and small-molecule amphiphiles



Elucidating APJ receptor activation mechanism

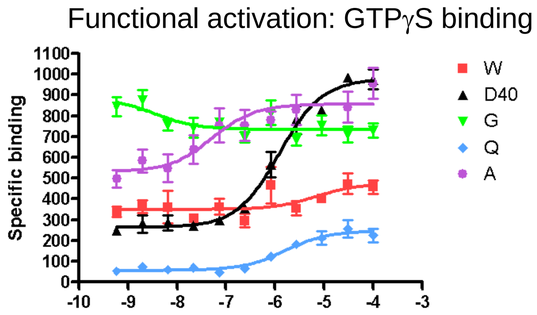


Total in-cell expression: Guava flow cytometry

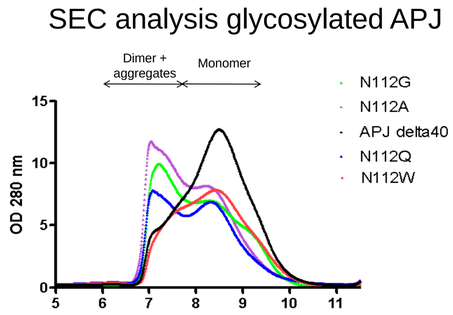


Size of 3.35 residue G<A<N<Q<W

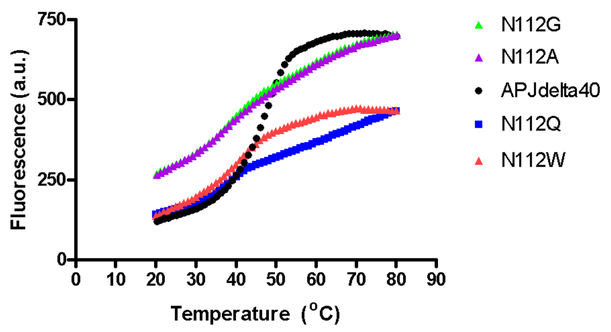
Functional activation: GTPγS binding

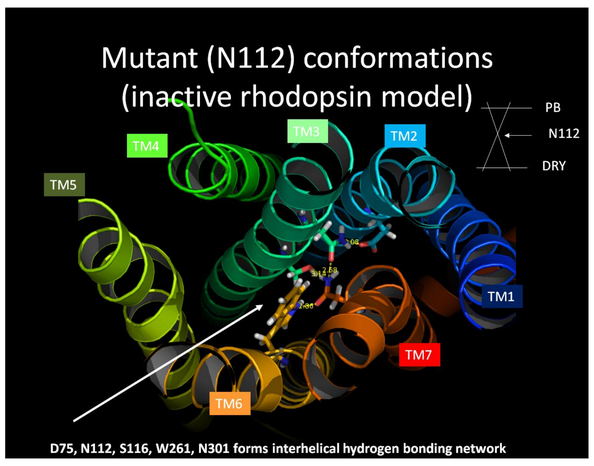


SEC analysis glycosylated APJ



CPM stability analysis





Conclusions: We have identified and characterized, both pharmacologically and biochemically, constitutively active mutants (CAMs) of APJ receptor. Our findings underscore the importance of TM3 and the size of residue 3.35 in the activation mechanism of class A GPCRs.