PAR_2 regulation of PAR_4 trafficking

Margaret Cunningham[1,2], Kathryn McIntosh[1], Graeme Milligan[3], Robin Plevin[1]. *[1]Strathclyde University, 161 Cathedral Street, G4 0RE, UK, [2]Bristol University, Bristol, BS8 1TD, UK, [3]Glasgow University, Glasgow, G12 8QQ, UK*

Proteinase activated receptor-4 (PAR_4) is a class A, G-protein coupled receptor (GPCR), that is activated by means of proteolytic cleavage, by serine proteases such as thrombin and trypsin (Xu *et al,* 1998). Due to the irreversible nature of activation, newly synthesized pools of receptor are required to be mobilized to the cell surface in order for responsiveness to agonist to be retained (MacFarlane *et al*, 2001). However, unlike the other PAR family members, the mechanisms responsible for trafficking of PAR_4 remain unknown. We therefore investigated the mechanisms responsible for trafficking of PAR_4 to the plasma membrane.

Membrane expression of PAR_4 is relatively low in most cell types and therefore proves difficult to detect. This study therefore employed techniques to transiently transfect in fluorescent tagged PAR_4 constructs to study its trafficking in the keratinocyte cell line NCTC2544, Clone G cells (NCTC2544 stably expressing PAR_2) or HEK293s, which have endogenous levels of PAR_2. PAR_4 trafficking and interaction with proteins was assessed using techniques including, Western blotting, co-immunoprecipitation, Fluorescence energy transfer (FRET), and inositol phosphate assays.

Initial experiments demonstrated that PAR_4 was poorly expressed at the plasma membrane and was mainly retained in the endoplasmic reticulum. Immuno-precipitation studies revealed that in PAR_2 deficient cells, PAR_4 interacted with the COPI protein subunit β-COP1, which functions to retain proteins within the ER. Sequence analysis of PAR_4, revealed an arginine-based (RXR) ER retention sequence located within extracellular loop 2, mutation of this sequence ($R^{183}AR$ – $A^{183}AA$), resulted in enhanced membrane delivery of PAR_4. Further experiments revealed that co-expression of PAR_4 with either endogenous or transfected PAR_2 enhanced plasma membrane expression, and PAR_4 was demonstrated to interact with PAR_2 through immuno-precipitation and FRET ($PAR_2/_4$ = 1.883 ± 0.003), however no FRET signal was detected when the fluorescent tags from the constructs were expressed alone (CFP/YFP = 1.101 ± 0.008). Interaction of PAR_2 with PAR_4 facilitated the disruption of the β-COP1 – PAR_4 complex, allowing interaction instead with the chaperone protein 14-3-3-zeta, and the subsequent anterograde traffic of PAR_4 to the plasma membrane. This resulted in enhanced PAR_4 signalling, as measured through inositol phosphate accumulation.

This study identifies a novel regulatory role for PAR_2 in the anterograde traffic of PAR_4. PAR_2 was shown to both facilitate and abrogate protein interactions of PAR_4, impacting upon both receptor localization and cell signal transduction. These studies will contribute greatly to the understanding of PAR_4 receptor pharmacology in both physiological and patho-physiological states.

MacFarlane, SR *et al*. (2001) Pharmacol Rev **53**:245-82

Xu, W *et al*. (1998) Proc Natl Acad Sci USA, **95**:6642-6