

## Glycosylation of human proteinase-activated receptor-2 (hPAR<sub>2</sub>): role in cell surface expression and signalling

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We have analysed the role of N-linked glycosylation in regulating human proteinase-activated receptor-2 (hPAR<sub>2</sub>) expression and function. Epitope-tagged wild-type hPAR<sub>2</sub> (wt-hPAR<sub>2</sub>) or hPAR<sub>2</sub> that lacked glycosylation sequons (following site-directed mutagenesis) in either the N-terminus [hPAR<sub>2</sub>N30A (Asn<sup>30</sup> → Ala)], extracellular loop 2 [ECL2; hPAR<sub>2</sub>N222Q (Asn<sup>222</sup> → Gln) or hPAR<sub>2</sub>N222A (Asn<sup>222</sup> → Ala)] or both (hPAR<sub>2</sub>N30A,N222A or hPAR<sub>2</sub>N30A,N222Q) were expressed in the Chinese-hamster ovary (CHO) fibroblast cell line, Pro5. Western blot analysis of wt-hPAR<sub>2</sub> showed mature wt-hPAR<sub>2</sub> to have a molecular mass of 55–100 kDa, and 33–48 kDa following *N*-glycosidase F deglycosylation. FACS analysis and immunocytochemistry of the wt-hPAR<sub>2</sub> and PAR<sub>2</sub> mutant cell lines revealed that removal of both glycosylation sequons decreases (50% of wt-hPAR<sub>2</sub>) cell surface expression. Western blot analysis indicated that both N-linked sites are glycosylated. In functional studies, hPAR<sub>2</sub>N30A displayed a selective and significant increase in sensitivity towards trypsin. Interestingly,

hPAR<sub>2</sub>N222A displayed a loss in sensitivity towards all PAR<sub>2</sub> agonists tested. However, further analysis revealed receptor sensitivity to alanine mutations in this domain, as the more conservative substitution hPAR<sub>2</sub>N222Q displayed no change in response to PAR<sub>2</sub> agonists. hPAR<sub>2</sub>N30A,N222Q displayed increased sensitivity towards trypsin, but a loss in sensitivity towards trypsin and the synthetic peptide SLIGRL-NH<sub>2</sub>, although this loss in sensitivity towards trypsin and SLIGRL-NH<sub>2</sub> was secondary to changes in cell-surface expression. Finally, expression of sialic-acid-deficient wt-hPAR<sub>2</sub> in the CHO Lec2 glycosylation-deficient mutant cell line, showed a 40 kDa loss in molecular mass, in addition to a marked and selective increase in sensitivity towards trypsin. We conclude that hPAR<sub>2</sub> N-linked glycosylation and sialylation regulates receptor expression and/or signalling.

**Key words:** inflammation, protease, sialylation, trypsin.

### INTRODUCTION

Proteinase-activated receptors (PARs) are a novel transmembrane G-protein-coupled receptor (GPCR) family activated by serine proteases [1–3]. Proteolytic cleavage of the PAR N-terminal exodomain exposes a tethered ligand sequence that subsequently binds to and activates the receptor. Synthetic peptides (PAR-activating peptides or PAR-APs), representing the first five or six amino acids of the tethered ligand can activate these receptors independently of proteolysis and are therefore useful pharmacological tools. Currently, four members of the human PAR (hPAR) family exist, PARs 1, 2, 3 and 4 [4–9]. hPAR<sub>1</sub>, hPAR<sub>3</sub> and hPAR<sub>4</sub> are activated principally by thrombin, whereas hPAR<sub>2</sub> is activated by trypsin, mast cell tryptase, matriptase (or membrane-type serine protease 1) and Factor Xa [5,10–12]. Interestingly, the ability of mast cell tryptase to activate PAR<sub>2</sub> varies considerably [10,13–15], and it has been proposed that differential glycosylation of hPAR<sub>2</sub> may be responsible for the variation observed [16]. However, a detailed study investigating the degree to which hPAR<sub>2</sub> is N-linked glycosylated and the impact of glycosylation on receptor signalling has not been performed.

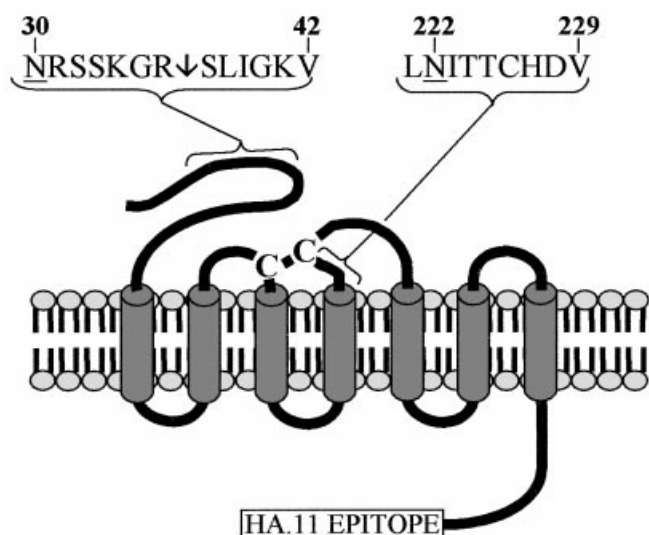
Glycosylation is a common post-translational feature in the GPCR family. Classically, glycosylation has been thought to regulate cell-surface expression of receptors. However, more recently, various studies [17–19] have pointed towards a role for

glycosylation in direct regulation of receptor function. As PARs are activated via a novel proteolytic mechanism, we hypothesized that receptor glycosylation might play a unique role in the activation and function of this receptor family. All four PARs possess a number of potential N-linked glycosylation sequons (Asn-Xaa-Ser/Thr, where Xaa is any amino acid except proline [20]) on their extracellular domains. To our knowledge, no direct evidence exists pertaining to the glycosylation state of PARs 2, 3 and 4. However, one report has convincingly demonstrated that hPAR<sub>1</sub> is an N-linked glycosylated protein, with glycosylation contributing to approx. half of its molecular mass [21], although the involvement of glycosylation in receptor function was not explored. hPAR<sub>2</sub> possesses two N-linked glycosylation sequons (Figure 1), one on extracellular loop 2 (ECL2; Asn<sup>222</sup>Ile<sup>223</sup>Thr<sup>224</sup>) and the second on the receptor N-terminus (Asn<sup>30</sup>Arg<sup>31</sup>Ser<sup>32</sup>). There is mounting evidence suggesting that ECL2 has an important role in PAR<sub>2</sub> signalling. For example, placing ECL2 from PAR<sub>2</sub> into PAR<sub>1</sub> confers PAR<sub>2</sub>-specificity on the receptor, suggesting a major role for ECL2 in ligand recognition [22]. In addition, by mutating in rat-PAR<sub>2</sub> (rPAR<sub>2</sub>) the corresponding residues in ECL2 that were found to be of importance for PAR-AP activation of hPAR<sub>1</sub>, rPAR<sub>2</sub> responsiveness towards PAR<sub>2</sub>-APs, but not trypsin, is significantly compromised [23]. Therefore, we suspected that N-linked glycosylation of ECL2 might affect ligand-mediated receptor activation. It is noteworthy that the proteinase-revealed tethered ligands of

Abbreviations used:  $\alpha$ -MEM,  $\alpha$ -modified essential medium; BAPNA, *N*<sup>2</sup>-benzoyl-DL-arginine *p*-nitroanilide; CHO, Chinese-hamster ovary; ECL2, extracellular loop 2; GPCR, G-protein-coupled receptor; hPAR<sub>2</sub>, human PAR-2; lo-wt-hPAR<sub>2</sub>, wt-hPAR<sub>2</sub> cells with lower receptor expression; PAR, proteinase-activated receptor; PAR-AP, PAR-activating peptide; rPAR<sub>2</sub>, rat PAR-2; STI, soya trypsin inhibitor; wt, wild-type.

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**Figure 1** Representative model of hPAR<sub>2</sub>, displaying the potential N-linked glycosylation sequons and the location of the haemagglutinin epitope tag

Human PAR<sub>2</sub> possesses two potential N-linked glycosylation sites: one located on the receptor N-terminus at Asp<sup>30</sup> and the second located on ECL2 at Asp<sup>222</sup>. Note the close proximity of the N-terminal glycosylation sequon to the cleavage-activation site of the receptor (↓). Amino acid numbering is for hPAR<sub>2</sub>. The haemagglutinin epitope (YPYDVPDYA) was fused to the C-terminal tail of hPAR<sub>2</sub> to enable efficient Western blot analysis of the expressed receptor using the monoclonal antibody HA.11 (see the Materials and methods section).

PARs 1 and 2 appear to bind differently to the receptor body than do the soluble PAR-APs [23,24]. Thus conventional ligand-binding studies using soluble radiolabelled peptide probes are not appropriate for assessing the functional properties of the PARs. Hence, the use of proteinases to unmask the tethered ligand, that then generates a receptor-triggered signal, represents the most direct approach for evaluating receptor binding/activation. This ligand-recognition function can be assessed further by the use of PAR-APs that also stimulate receptor signalling. The role of the N-terminal glycosylation sequon has been proposed to regulate tryptase activation of hPAR<sub>2</sub> [16]. However, no direct evidence in this preliminary study was provided to demonstrate that hPAR<sub>2</sub> is indeed glycosylated; and no results are available to assess the potential role of glycosylation for PAR<sub>2</sub> signalling. Therefore the purpose of this study was to determine the glycosylation state of hPAR<sub>2</sub> and the role of such glycosylation on receptor membrane expression and function. To this end, we expressed epitope-tagged wild-type (wt) or glycosylation-deficient mutant hPAR<sub>2</sub> receptors in Chinese-hamster ovary (CHO) fibroblasts and sought to determine: (i) whether hPAR<sub>2</sub> is N-linked glycosylated, (ii) the influence that deleting glycosylation sequons has on cell surface expression, (iii) which glycosylation sequons are glycosylated, (iv) the role that each glycosylated sequon may have in regulating receptor function, and (v) the role that receptor sialylation has in regulating hPAR<sub>2</sub> function.

## MATERIALS AND METHODS

### Materials

*N*<sup>ε</sup>-benzoyl-DL-arginine *p*-nitroanilide (BAPNA), heparin agarose, Sephacryl S200, leupeptin, soya trypsin inhibitor (STI), porcine pancreatic type IX trypsin (13000–20000 units/mg),

sulphinpyrazone, sodium fluoride, sodium orthovanadate and calcium ionophore (A23187) were all purchased from Sigma (St. Louis, MO, U.S.A.). Foetal calf serum (FCS), Dulbecco's modified Eagle's medium,  $\alpha$ -modified essential medium ( $\alpha$ -MEM, with ribonucleosides and deoxyribonucleosides), non-enzymic cell-dissociation solution, penicillin, streptomycin, amphotericin B, sodium pyruvate, and PBS (without calcium and magnesium) were from GibcoBRL (Gaithersburg, MD, U.S.A.). Synthetic sialic acid was purchased from Calbiochem (San Diego, CA, U.S.A.). All oligonucleotides were synthesized by the University Core DNA and Protein Services (University of Calgary, Calgary, AB, Canada). All peptides were synthesized by the Peptide Synthesis Facility (University of Calgary, Calgary, AB, Canada). Stock solutions of peptides in 25 mM Hepes, pH 7.4, were standardized by quantitative amino acid analysis to confirm peptide concentration and purity. The monoclonal antibody HA.11 (clone 16B12) was purchased from Berkeley Antibody Company (Richmond, CA, U.S.A.) and the anti-mouse FITC-conjugated antibody was purchased from Caltag Laboratories (Burlingame, CA, U.S.A.). *N*-Glycosidase F was purchased from New England Biolabs (Mississauga ON, Canada).

### Purification of tryptase

Human lung tryptase was purified as described previously [25] and stored in 2 M NaCl/20 mM Mes buffer, pH 6.1, at  $-80^{\circ}\text{C}$ . Human lung was obtained according to procedures approved by the University of Calgary, Faculty of Medicine ethics committee. One unit of tryptase activity was defined as the amount of tryptase required to hydrolyse 1  $\mu\text{mol}$  of BAPNA per min at  $25^{\circ}\text{C}$ . Tryptase purity was assessed by specific activity ( $> 2.5$  milliunits/ $\mu\text{g}$  of protein) and SDS/PAGE on a 12% gel wherein tryptase was identified by Western blot analysis using the tryptase-specific monoclonal antibody, AA5 (a gift kindly provided by Dr. Andrew Walls, Respiratory Cell and Molecular Biology, University of Southampton, U.K.). The identity of the tryptase was confirmed further by amino-acid-sequence analysis of protein recovered from Western blot transfer (Alberta Peptide Institute, University of Alberta, Edmonton, AB, Canada). Tryptase was used in the absence of heparin, unless otherwise stated, and concentrations (nM) used in all experiments were calculated on the basis of the molecular mass (134 kDa) of the tryptase tetramer.

### Generation of epitope-tagged cDNAs encoding wt and glycosylation-deficient mutant hPAR<sub>2</sub>

The wt-hPAR<sub>2</sub> cDNA used in this study has been described in detail previously [26]. Using a PCR approach, a hPAR<sub>2</sub> cDNA possessing a 12CA5 haemagglutinin epitope (YPYDVPDYA) at the intracellular C-terminus was generated (Figure 1). Commercially available antibodies to this epitope can be used to visualize the receptor by Western blot analysis [27]. A previous study has demonstrated that the presence of this epitope on the C-terminus of hPAR<sub>2</sub> does not influence receptor signalling [28]. All site-directed mutations were generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.), according to the manufacturer's instructions. To obtain a hPAR<sub>2</sub> cDNA devoid of either an N-terminal or ECL2 glycosylation sequon, Asp<sup>30</sup> or Asp<sup>222</sup> was replaced with alanine or glutamine [hPAR<sub>2</sub>N30A (Asn<sup>30</sup>  $\rightarrow$  Ala), hPAR<sub>2</sub>N222A (Asn<sup>222</sup>  $\rightarrow$  Ala) and hPAR<sub>2</sub>N222Q (Asn<sup>222</sup>  $\rightarrow$  Gln)]. A PAR<sub>2</sub> cDNA deficient in both glycosylation sequons was generated either by replacing both Asp<sup>30</sup> and Asp<sup>222</sup> with alanine (PAR<sub>2</sub>N30A,N222A), or by replacing Asp<sup>222</sup> with glutamine (hPAR<sub>2</sub>N30A,N222Q). The

mutant PAR<sub>2</sub> clones were subsequently sequenced to confirm the engineered mutations using fluorescence-based automated cycle sequencing by the University Core DNA and Protein Services (University of Calgary, Calgary, Alberta, Canada).

### Cell Culture

The CHO fibroblast cell lines, Pro5 and Lec2 (American Tissue Type Culture Collection, Bethesda, MD, U.S.A.), that permanently expressed either wt or mutant hPAR<sub>2</sub>, were grown in  $\alpha$ -MEM containing 10% FCS, 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin, 250 ng/ml amphotericin B, and 1 mg/ml geneticin. The Pro5 cell line is the parent clone for the Lec2 mutant line [29]. The Lec2 cell line displays a substantial loss in ability to attach sialic acid to the terminal positions on oligosaccharides. This cell line contains a genetic mutation within the CMP-sialic acid transporter such that its cellular proteins possess a substantial loss of sialic acid [29,30]. All cell lines were propagated using non-enzymic cell dissociation solution in 95% air/5% CO<sub>2</sub>, at 37 °C.

### Transfection

Semi-confluent cells (40–60%) in 60 mm diameter Petri dishes were transfected using the LipofectAMINE® method, according to the manufacturer's protocol (GibcoBRL). Transfected CHO cells (Pro5 and Lec2) were subcloned in geneticin (1.0 mg/ml) containing medium ( $\alpha$ -MEM containing 10% FCS, 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin and 250 ng/ml amphotericin B). To obtain permanent receptor-expressing cell lines, cells expressing high levels of PAR<sub>2</sub> were isolated by FACS using the B5 anti-PAR<sub>2</sub> rabbit polyclonal antiserum [23,31]. Clones with matched receptor expression, as assessed by FACS analysis, were selected for functional studies. Where it was not possible to match receptor expression between the wt-hPAR<sub>2</sub> cell line and a cell line expressing a mutant glycosylation receptor, the wt-hPAR<sub>2</sub> was used at higher cell confluence in order to decrease receptor expression (see below) and thus match the cell-surface expression of the mutant cell line. Diminished cell-surface expression of the wt-hPAR<sub>2</sub> cell line was subsequently confirmed by FACS analysis.

### Flow cytometry

Semi-confluent cells (approx. 40% confluence) in 75 cm<sup>2</sup> culture flasks were rinsed with PBS (without calcium and magnesium) before harvesting with non-enzymic cell dissociation solution. Pelleted cells were resuspended in fresh medium ( $\alpha$ -MEM containing 10% FCS, 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin, and 250 ng/ml amphotericin B) and placed on ice for 10 min before the addition of anti-PAR<sub>2</sub> antiserum (B5, 1 in 300 dilution [23,31]) for 1 h. Cells were washed before an incubation with the anti-rabbit FITC-conjugated antibody for a further 1 h. Following a further wash sequence, cells were analysed for PAR<sub>2</sub> cell-surface expression by FACS (Becton Dickinson, Franklin Lakes, NJ, U.S.A.).

### Immunocytochemistry

Empty-vector-transfected Pro5 cells, wt-hPAR<sub>2</sub> and glycosylation-deficient PAR<sub>2</sub>-expressing cell lines were dispersed on glass slides using a Shandon Cytospin (Pittsburgh, PA, U.S.A.). A 3,3'-diaminobenzidine substrate immunocytochemistry protocol was utilized as described in detail previously [32], except a mouse monoclonal antibody HA.11 (1 in 1000 dilution) was

employed as the primary antibody and a biotinylated goat anti-mouse antibody (1 in 100 dilution) as a secondary antibody.

### Calcium signalling assay

Calcium signalling was performed as described previously [26]. Harvested cells were incubated in 1 ml of  $\alpha$ -MEM, 10% FCS and 0.25 mM sulphinyprazole, 22  $\mu$ M Fluo-3 acetoxymethyl ester (Molecular Probes Inc., Eugene, OR, U.S.A.) for 25 min at room temperature (25 °C) with mild shaking. Cells were then washed and resuspended in calcium assay buffer, pH 7.4 (150 mM NaCl, 3 mM KCl, 1.5 mM CaCl<sub>2</sub>, 10 mM glucose, 20 mM Hepes and 0.25 mM sulphinyprazole). Fluorescence measurements were performed on a PerkinElmer fluorescence spectrometer 650-10S (Norwalk, CT, U.S.A.), with an excitation wavelength of 480 nm and emission recorded at 530 nm. Cell suspensions (1 ml) in 4 ml cuvettes were mixed with a magnetic stirrer and maintained at 24 °C. The signal produced ( $E_{530}$ ) by the addition of a test agonist was measured as a percentage of the fluorescence peak height, yielded by the addition of 2  $\mu$ M calcium ionophore (A23187).

### Cell membrane preparation

Crude cell membrane extracts were prepared by hypotonic cell shock. Cells were seeded into 75 cm<sup>2</sup> culture flasks and harvested at low confluence (approx. 40%) before washing once with PBS (without calcium and magnesium) and then once with distilled water for 30 s, before the addition of membrane buffer (5 mM Tris, pH 7.5, 0.5 mM EDTA, 1  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml STI, 1 mM orthovanadate and 50 mM NaF). Swollen cells were disrupted by passing through a 26 gauge needle (Becton Dickinson), and nuclei were removed by centrifugation at 500 *g* for 10 min at 4 °C. The nuclei-depleted supernatant was then centrifuged at 20000 *g* for 30 min at 4 °C to pellet the crude membrane fraction, which was subsequently resuspended in membrane buffer. Protein concentration in the membrane pellets was assessed by the Bradford assay (Bio-Rad, Hercules, CA, U.S.A.) and aliquots were frozen at –80 °C. To assess the degree of N-linked glycosylation of hPAR<sub>2</sub>, crude wt-hPAR<sub>2</sub> membrane preparations (20  $\mu$ g) were incubated with *N*-glycosidase F (2500 units/reaction), according to the manufacturer's conditions, except that samples were incubated overnight at 4 °C.

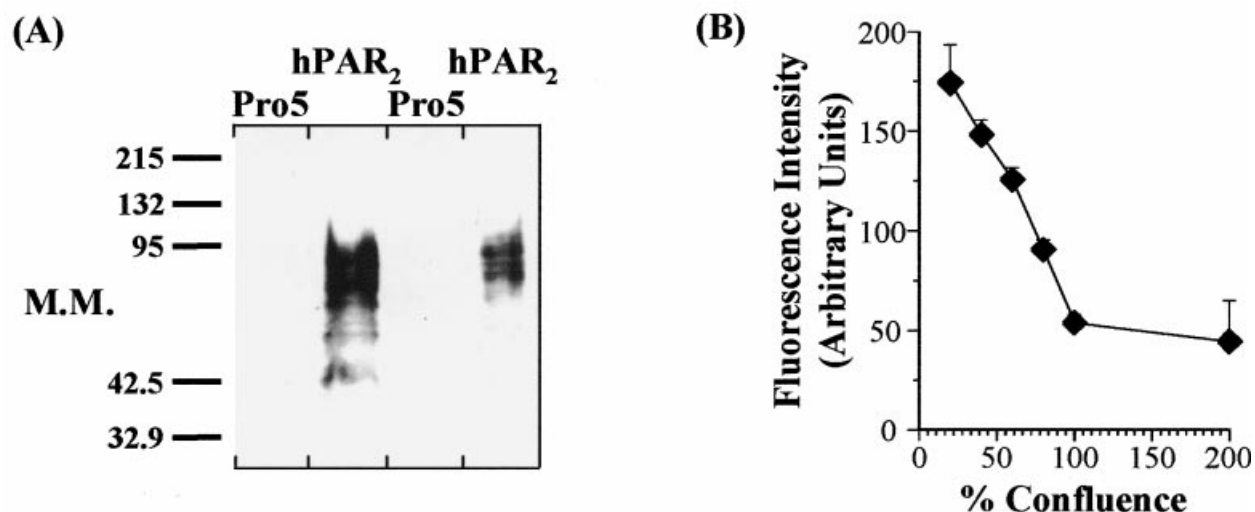
### Immunoblotting

Crude membrane aliquots (20  $\mu$ g) were separated on a 10% SDS/PAGE gel before transfer to Hybond C PVDF membrane (Amersham Biosciences, Little Chalfont, Buckinghamshire, U.K.). The membrane was subsequently blocked with PBS containing 5% non-fat milk before incubation overnight at 4 °C with the mouse monoclonal HA.11 antibody [1 in 1000 dilution in PBS/Tween-20 (0.1%) containing 2% non-fat milk]. Blots were washed with PBS/Tween 20 (0.1%) for 1 h, replacing buffer every 15 min, before a final incubation with the peroxidase-conjugated goat anti-mouse IgG for 1 h. Following a further wash sequence in PBS/Tween 20 (0.1%) for 1 h, the epitope-tagged hPAR<sub>2</sub> receptor was visualized using the enhanced chemiluminescence (ECL®) detection system (Amersham Biosciences).

## RESULTS

### Human PAR<sub>2</sub> is an N-linked glycosylated receptor

We initially attempted to visualize hPAR<sub>2</sub> by Western blot analysis of whole cell lysates using an anti-PAR<sub>2</sub> polyclonal



**Figure 2** Expression of wt-hPAR<sub>2</sub> in Pro5 cells

(A) Western blot analysis of epitope-tagged wt-hPAR<sub>2</sub> expressed in Pro5 cells. Crude membrane preparations of epitope-tagged wt-hPAR<sub>2</sub> and Pro5 membranes were resolved by SDS/PAGE (on 12% gels) and immunoblotted using the HA.11 monoclonal antibody (see the Materials and methods section). Pro5, membranes from non-transfected cells; hPAR<sub>2</sub>, wt-hPAR<sub>2</sub> membranes; M.M., molecular mass. The hPAR<sub>2</sub> membrane preparation on the right was harvested from cells at higher confluence (and therefore at lower receptor density) than the hPAR<sub>2</sub> preparation on the left. Results are representative of three separate experiments. (B) Effect of cell confluence on the cell surface expression of wt-hPAR<sub>2</sub> expressed in Pro5 cells. Cells expressing wt-hPAR<sub>2</sub> were grown to varying stages of confluence, then harvested with non-enzymic cell-dissociation solution before cell-surface expression was assessed by FACS analysis using the B5 antiserum. Results are expressed as means  $\pm$  S.E.M. for three separate experiments. Full (100%) confluence was taken as the cell density achieved at the point at which the cells just covered the surface of the flask. Cells then 'overgrew' the monolayer to twice the cell density (200% confluence).

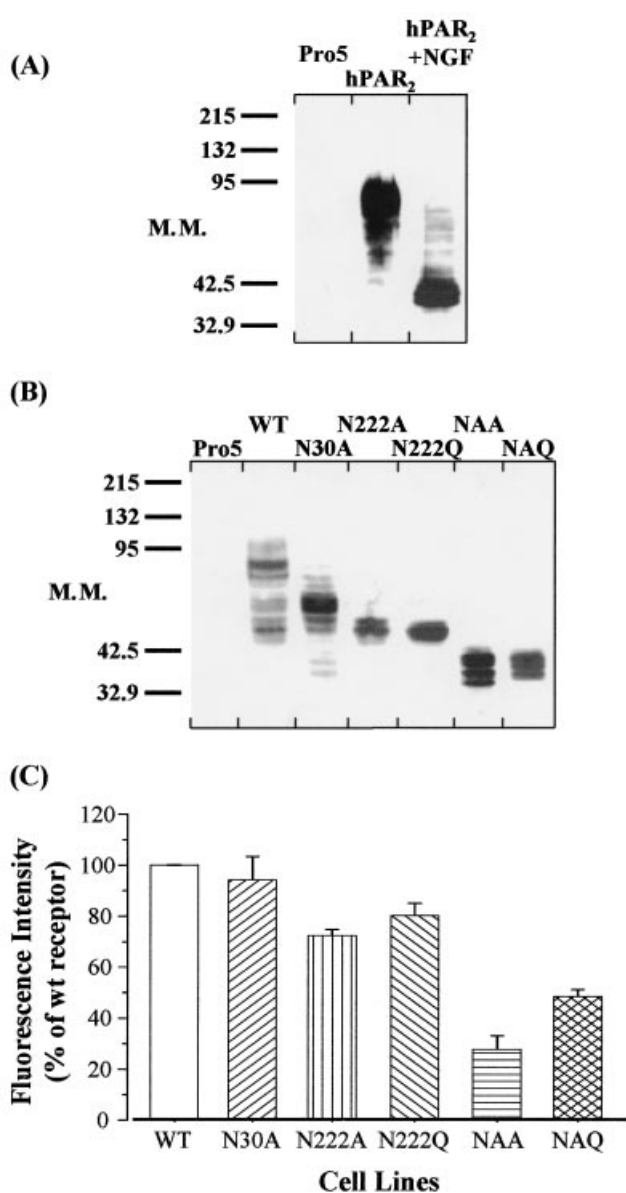
antibody (B5) [16]. However, it was not possible to identify the receptor clearly using B5, because a number of bands in non-transfected, as well as receptor-transfected, preparations were observed. Therefore, in this study we utilized an epitope-tagging strategy where an influenza haemagglutinin epitope (YPYDVPDYA) was fused to the C-terminus of hPAR<sub>2</sub>, which can subsequently be identified by the commercially available monoclonal antibody, HA.11. The extreme specificity of the antibody allows unambiguous identification of the targeted epitope [27], and hence the receptor. However, initial studies employing whole cell lysates for Western blot analysis resulted in little or no staining using the HA.11 antibody. Therefore we prepared crude membrane preparations in order to raise the sensitivity of the technique. Western blot analysis using the HA.11 antibody clearly identified the epitope-tagged wt-hPAR<sub>2</sub> receptor in membranes from permanently transfected Pro5 cells (Figure 2A). Epitope-tagged wt-hPAR<sub>2</sub> migrated as multiple bands from approx. 55 to 100 kDa, with the majority of the receptor being observed from 65 to 100 kDa. A number of minor bands that may represent either incompletely glycosylated receptor species or proteolytic degradation products were routinely observed in the 50–65 kDa range. In non-transfected Pro5 crude membrane preparations, no bands were detected, confirming the specificity of the antibody used. During the propagation of the receptor-expressing cell lines, we found that receptor expression was sensitive to cell confluence (Figure 2A, right-hand lane and Figure 2B). As the cells grew to confluence, the cell-surface-receptor density decreased approx. 3-fold; the reason for this change in receptor density is not clear. Therefore, for all experiments, cells were used at a confluence of approx. 40%, where cell-surface expression was near maximum (Figure 2B).

As the molecular mass of wt-hPAR<sub>2</sub>, based on its amino acid sequence, is predicted to be 44 kDa [5], we hypothesized that the

large heterogeneous molecular mass of the receptor observed by Western blot analysis was probably due to differential glycosylation. Therefore, to test this hypothesis, we incubated wt-hPAR<sub>2</sub>-bearing crude membrane extracts with *N*-glycosidase F to remove such complex sugars. Results are shown in Figure 3(A). The size of the epitope-tagged wt-hPAR<sub>2</sub> from membrane extracts treated with *N*-glycosidase F was markedly decreased to a molecular mass of approx. 36–46 kDa. Since the molecular mass of *N*-glycosidase F-treated wt-hPAR<sub>2</sub> corresponded to the mass predicted on the basis of the amino acid composition of wt-hPAR<sub>2</sub>, it would appear that there was little if any O-linked glycosylation on wt-hPAR<sub>2</sub>, although we did not test this possibility directly.

#### Human PAR<sub>2</sub> is glycosylated on both potential N-linked glycosylation sequons

Since wt-hPAR<sub>2</sub> possesses two N-linked glycosylation sequons, one on the N-terminus (Asn<sup>30</sup>Arg<sup>31</sup>Ser<sup>32</sup>) and the other on ECL2 (Asn<sup>222</sup>Ile<sup>223</sup>Thr<sup>224</sup>), we generated hPAR<sub>2</sub> constructs that were devoid of either one or both of these sequons (hPAR<sub>2</sub>N30A; hPAR<sub>2</sub>N222A and hPAR<sub>2</sub>N30A,N222A). As hPAR<sub>2</sub> is particularly sensitive to mutations within ECL2 [26], additional glycosylation-deficient mutant constructs, hPAR<sub>2</sub>N222Q and hPAR<sub>2</sub>N30A,N222Q were generated to confirm any functional changes observed in either hPAR<sub>2</sub>N222A or hPAR<sub>2</sub>N30A,N222A. Permanently expressing receptor cell lines were generated for all constructs. Western blot analysis of crude membrane preparations from the wt-hPAR<sub>2</sub>, hPAR<sub>2</sub>N30A, hPAR<sub>2</sub>N222A, hPAR<sub>2</sub>N222Q, hPAR<sub>2</sub>N30A,N222A and hPAR<sub>2</sub>N30A,N222Q cell lines was performed in order to establish the degree of glycosylation associated with each glycosylation site. Results are shown in Figure 3(B). All of the glycosylation-deficient receptors displayed a loss in molecular mass with relative sizes:



**Figure 3** Determination of N-linked glycosylation on wt-hPAR<sub>2</sub>

(A) Determination of N-linked glycosylation on epitope-tagged wt-hPAR<sub>2</sub> by digestion with *N*-glycosidase F. Crude membrane preparations from wt-hPAR<sub>2</sub>-transfected Pro5 cells were incubated overnight at 4 °C either with or without 2500 units/50  $\mu$ l of *N*-glycosidase F before separation by SDS/PAGE (12% gel) and immunoblotted using the HA.11 monoclonal antibody. Pro5, membranes from non-transfected cells; hPAR<sub>2</sub>, wt-hPAR<sub>2</sub> membranes; hPAR<sub>2</sub> + NGF, wt-hPAR<sub>2</sub> membranes following pre-treatment with *N*-glycosidase F; M.M., molecular mass. Results are representative of three separate experiments. (B) Western blot analysis of glycosylation-deficient mutant hPAR<sub>2</sub> receptors expressed in Pro5 cells. Crude membrane preparations from cell lines expressing HA-tagged hPAR<sub>2</sub> devoid of one (hPAR<sub>2</sub>N30A, hPAR<sub>2</sub>N222A or hPAR<sub>2</sub>N222Q) or both (hPAR<sub>2</sub>N30A,N222A or hPAR<sub>2</sub>N30A,N222Q) of the potential N-linked glycosylation sequons were analysed by SDS/PAGE (12% gel) and immunoblotted using the HA.11 monoclonal antibody. Owing to the low cell-surface expression of hPAR<sub>2</sub>N30A,N222A, the blot was exposed for an extra 3 min to facilitate visualization of the mutant receptor. Pro5, membranes from non-transfected cells; hPAR<sub>2</sub>, wt-hPAR<sub>2</sub> membranes; N30A, hPAR<sub>2</sub>N30A membranes; N222A, hPAR<sub>2</sub>N222A; N222Q, hPAR<sub>2</sub>N222Q; NAA, hPAR<sub>2</sub>N30A,N222A; NAQ, hPAR<sub>2</sub>N30A,N222Q membranes; M.M., molecular mass. Results are representative of three separate experiments. (C) Cell-surface expression of the wt-hPAR<sub>2</sub> and glycosylation-deficient mutant hPAR<sub>2</sub> receptor cell lines. Cells at approx. 40% confluence were harvested and incubated with the B5 antiserum before incubation with an anti-rabbit FITC-conjugated antibody. Cell surface expression was assessed by FACS analysis. Results are expressed as a percentage of the mean fluorescence obtained with wt-hPAR<sub>2</sub> cells. The bars represent means  $\pm$  S.E.M. of measurements for three separate experiments.

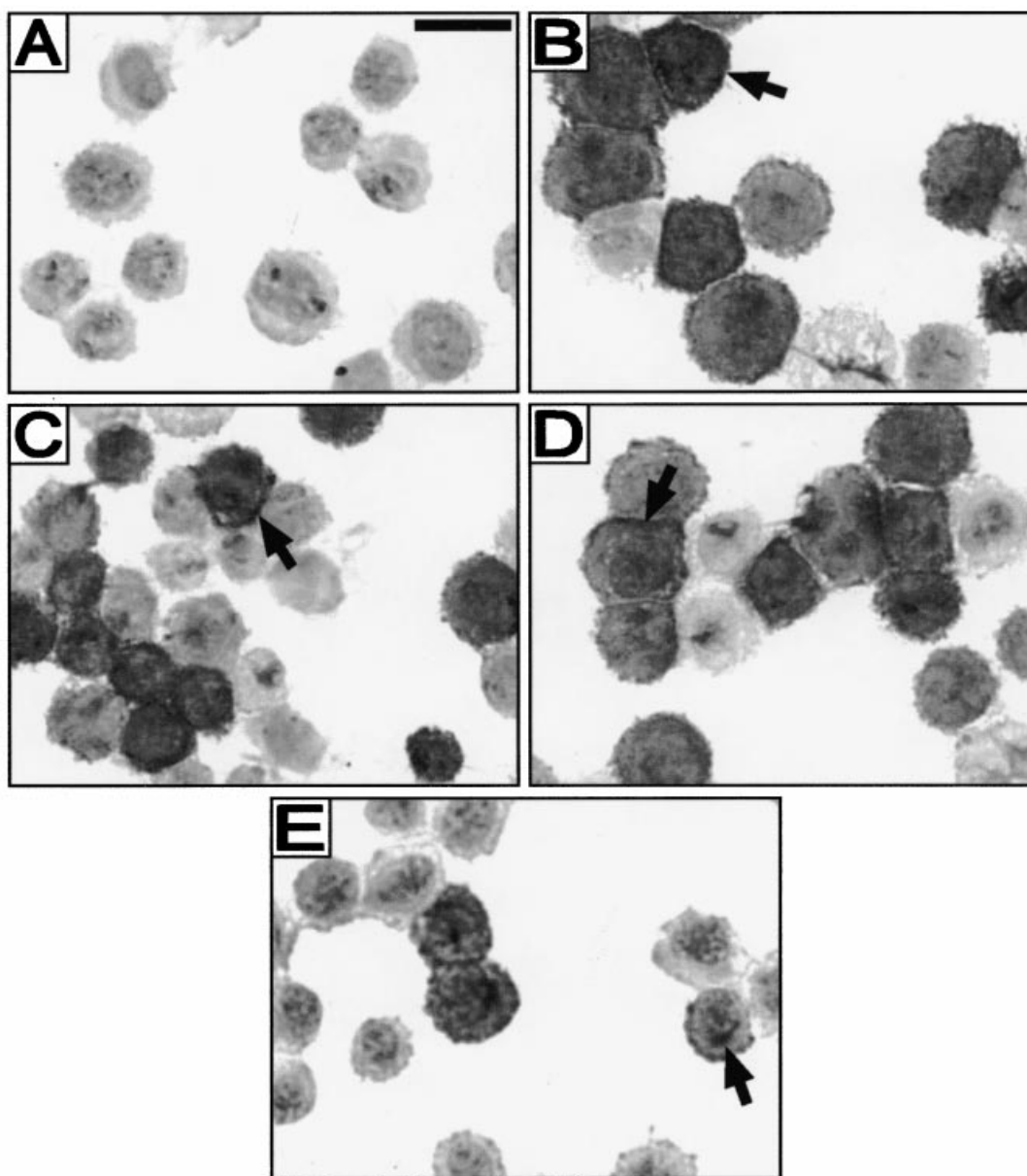
wt-hPAR<sub>2</sub> > hPAR<sub>2</sub>N30A > hPAR<sub>2</sub>N222A = hPAR<sub>2</sub>N222Q > hPAR<sub>2</sub>N30A,N222A = hPAR<sub>2</sub>N30A,N222Q. The hPAR<sub>2</sub>N30A receptor migrated further than wt-hPAR<sub>2</sub> with an apparent molecular mass of 55–80 kDa, showing a loss in molecular mass of > 20 kDa compared with wt-hPAR<sub>2</sub>. The hPAR<sub>2</sub>N222A and hPAR<sub>2</sub>N222Q receptors migrated with an approx. molecular mass of 43–55 kDa, displaying a loss in molecular mass of > 45 kDa compared with wt-hPAR<sub>2</sub>. As there was less hPAR<sub>2</sub>N30A,N222A receptor expressed at the cell surface, the exposure time of the blot with the photographic film was extended to aid visualization of the receptor. The hPAR<sub>2</sub>N30A, N222A and hPAR<sub>2</sub>N30A,N222Q receptors migrated with a molecular mass of approx. 33–43 kDa and 36–43 kDa respectively, notably similar to that obtained with wt-hPAR<sub>2</sub> pre-treated with *N*-glycosidase F (36–46 kDa, Figure 3A). Thus wt-hPAR<sub>2</sub> may possess up to approx. 55 kDa of N-linked oligosaccharide, of which approx. twice as much is associated with ECL2 compared with that on the N-terminal glycosylation site.

#### At least one N-linked glycosylation sequon on hPAR<sub>2</sub> is required for efficient cell-surface expression

FACS analysis was performed to assess the receptor densities of the individual cell lines. Results are shown in Figure 3(C). hPAR<sub>2</sub>N30A, hPAR<sub>2</sub>N222A and hPAR<sub>2</sub>N222Q displayed receptor expression that was comparable with that of wt-hPAR<sub>2</sub> [percentage relative to wt-hPAR<sub>2</sub>  $\pm$  S.E.M.: 94  $\pm$  9; 72  $\pm$  2 and 80  $\pm$  5 ( $n$  = 3) respectively]. Using these four cell lines with comparable cell-surface-expression densities, it was possible to determine structure activity relationships for PAR<sub>2</sub> agonists (see below). In contrast, hPAR<sub>2</sub>N30A,N222A and hPAR<sub>2</sub>N30A,N222Q displayed a substantial loss in cell-surface-receptor expression [27  $\pm$  5 and 48  $\pm$  6% ( $n$  = 3) respectively compared with wt-hPAR<sub>2</sub> expression]. To confirm the FACS results, we performed immunocytochemistry employing the epitope-tagged HA.11 antibody. Results are shown in Figure 4. No detectable staining was observed in the empty vector transfected Pro5 cells (Figure 4A), confirming the specificity of the primary antibody. For wt-hPAR<sub>2</sub>, hPAR<sub>2</sub>N30A and hPAR<sub>2</sub>N222Q (Figures 4B, 4C and 4D respectively) prominent ring staining was observed (see arrows). Results are not shown for hPAR<sub>2</sub>N222A. For hPAR<sub>2</sub>N30A,N222Q (Figure 4E) and hPAR<sub>2</sub>N30A,N222A (results not shown), there appeared to be little staining, with most of the staining localized to one side of the cell (see arrow). Thus deletion of both, but not one, of the glycosylation sequons in hPAR<sub>2</sub> decreased the efficiency of cell surface expression, but nonetheless permitted significant expression at the cell membrane of the hPAR<sub>2</sub>N30A,N222Q mutant.

#### Deletion of the N-terminal glycosylation sequon on hPAR<sub>2</sub> enhances susceptibility to activation by trypsin, but not to trypsin or the selective PAR<sub>2</sub>-AP, SLIGRL-NH<sub>2</sub>

Having established that wt-hPAR<sub>2</sub> displays glycosylation at both N-linked sites, we sought to determine the role that these sites may have in receptor function by constructing concentration-effect curves for trypsin, trypsin and SLIGRL-NH<sub>2</sub> in the wt-hPAR<sub>2</sub>, hPAR<sub>2</sub>N30A, hPAR<sub>2</sub>N222A and hPAR<sub>2</sub>N222Q cell lines. Results are shown in Figure 5. For wt-hPAR<sub>2</sub>, mast cell trypsin stimulated a small, but observable, concentration-effect curve from 3 to 100 nM (Figure 5A), although the maximum response achieved was substantially lower than that

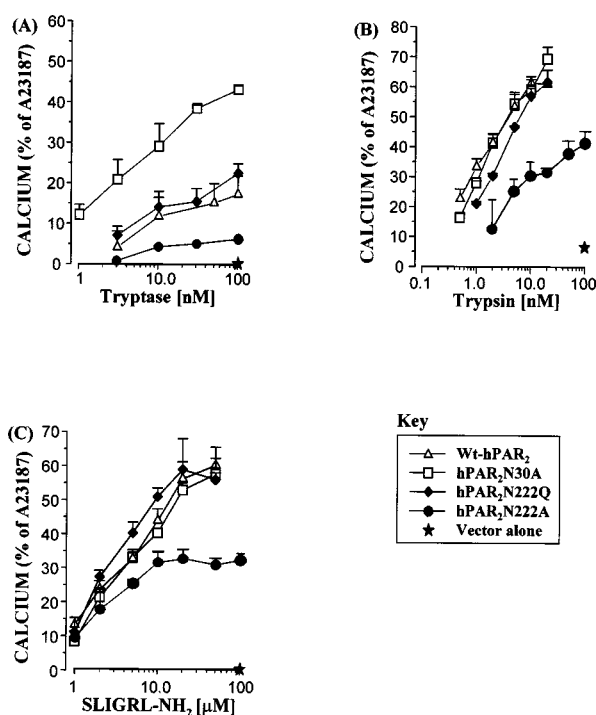


**Figure 4** Immunocytochemistry of wt-hPAR<sub>2</sub> and glycosylation-mutant PAR<sub>2</sub> receptors

Immunocytochemistry employing the epitope-targeted HA.11 monoclonal antibody for non-transfected Pro5 cells (A), wt-hPAR<sub>2</sub> (B), hPAR<sub>2</sub>N30A (C), hPAR<sub>2</sub>N222Q (D), and the non-glycosylated hPAR<sub>2</sub>N30A,N222Q (E) cell lines. Arrows in (B), (C) and (D) show cell-surface immunoreactivity, whereas the arrow in (E) shows staining localized to one cell region.

obtained with either trypsin or SLIGRL-NH<sub>2</sub> (approx. 25% of the maximal responses of trypsin and SLIGRL-NH<sub>2</sub>, see Figures 5B and 5C). Trypsin stimulated a calcium response from 0.5 to 20 nM, reaching a maximal response at 10 nM (Figure 5B). The selective PAR<sub>2</sub>-AP, SLIGRL-NH<sub>2</sub> stimulated a calcium signal from 1 to 50 μM, reaching maximal response at 20 μM (Figure 5C). For hPAR<sub>2</sub>N30A, tryptase was approx. twice as potent in activating hPAR<sub>2</sub>N30A compared with wt-hPAR<sub>2</sub>, stimulating a calcium response from 1 to 100 nM (Figure 5A). Significantly, relative to wt-hPAR<sub>2</sub>, hPAR<sub>2</sub>N30A displayed a 4-fold increase in the maximal response to tryptase, which was comparable with the response obtained with trypsin and the PAR<sub>2</sub>-AP, SLIGRL-NH<sub>2</sub> for the wt-hPAR<sub>2</sub> and hPAR<sub>2</sub>N30A cell lines (see Figures

5B and 5C). Trypsin displayed no observable difference in activating hPAR<sub>2</sub>N30A compared with wt-hPAR<sub>2</sub>, stimulating a calcium signal from 1 to 20 nM (Figure 5B). Similarly, SLIGRL-NH<sub>2</sub> displayed no observable shift in potency or maximum response toward hPAR<sub>2</sub>N30A compared with the wt-hPAR<sub>2</sub> receptor, stimulating a calcium signal from 1 to 50 μM (Figure 5C). Neither tryptase (Figure 5A, 100 nM) or SLIGRL-NH<sub>2</sub> (Figure 5C, 10 μM) stimulated a calcium response at the concentrations tested in the empty vector-transfected Pro5 cell line. Trypsin (Figure 5B, 100 nM) stimulated a very small, but detectable, response in empty-vector-transfected cells, as has been observed previously with KNRK (Kirsten sarcoma virus-transformed rat kidney epithelium) cells [31].

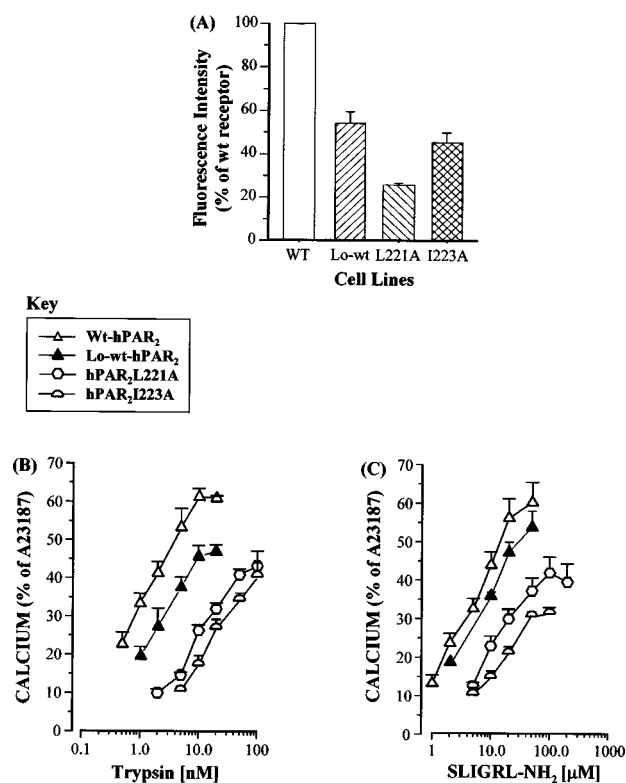


**Figure 5** Calcium signalling in wt-hPAR<sub>2</sub> and the glycosylation-deficient mutants hPAR<sub>2</sub>N30A, hPAR<sub>2</sub>N222A and hPAR<sub>2</sub>N222Q, in response to trypsin, trypstate and SLIGRL-NH<sub>2</sub>

Concentration–effect curves are shown for (A) trypstate, (B) trypsin and (C) SLIGRL-NH<sub>2</sub>. Cells were harvested with non-enzymic cell-dissociation solution and loaded with Fluo-3 acetoxymethyl ester (22  $\mu$ M) before incubation for 25 min at room temperature (25 °C). Cells were challenged with different concentrations of PAR agonists and responses were monitored by fluorescence spectrophotometry (excitation wavelength 480 nm, emission wavelength 530 nm). Responses were normalized to the peak height obtained with 2  $\mu$ M calcium ionophore. Results are represented as means  $\pm$  S.E.M. for three or four separate experiments, each comprising measurements obtained with duplicate cell suspensions. Where error bars are smaller than symbols, they are not shown.

### In ECL2, glycosylation is not required for full agonist/receptor activity of hPAR<sub>2</sub>, but alanine replacements compromise receptor efficacy

For hPAR<sub>2</sub>N222Q, no measurable change in receptor function was observed for all of the PAR<sub>2</sub> agonists tested compared with wt-hPAR<sub>2</sub> (Figures 5A, 5B and 5C). However, hPAR<sub>2</sub>N222A that displayed a receptor density comparable with that of hPAR<sub>2</sub>N222Q (Figure 3C) showed a marked loss in sensitivity for all of the PAR<sub>2</sub> agonists tested (Figure 5). Mast cell trypstate also displayed a significant loss in the maximal response achievable for hPAR<sub>2</sub>N222A compared with wt-hPAR<sub>2</sub> (Figure 5A). Trypsin displayed an approx. 10-fold decrease in potency for hPAR<sub>2</sub>N222A compared with wt-hPAR<sub>2</sub> receptor, stimulating a calcium response from 2 to 100 nM (Figure 5B). The response to the selective PAR<sub>2</sub> agonist SLIGRL-NH<sub>2</sub> displayed only a small difference in the magnitude of response for hPAR<sub>2</sub>N222A at low concentrations (1–5  $\mu$ M) compared with that obtained for wt-hPAR<sub>2</sub> (Figure 5C). However, the maximal response to SLIGRL-NH<sub>2</sub> for the hPAR<sub>2</sub>N222A that was observed from 10  $\mu$ M and above (Figure 5C), was substantially lower (45%) than the maximal response obtained with SLIGRL-NH<sub>2</sub> for the wt-hPAR<sub>2</sub> (Figure 5C).

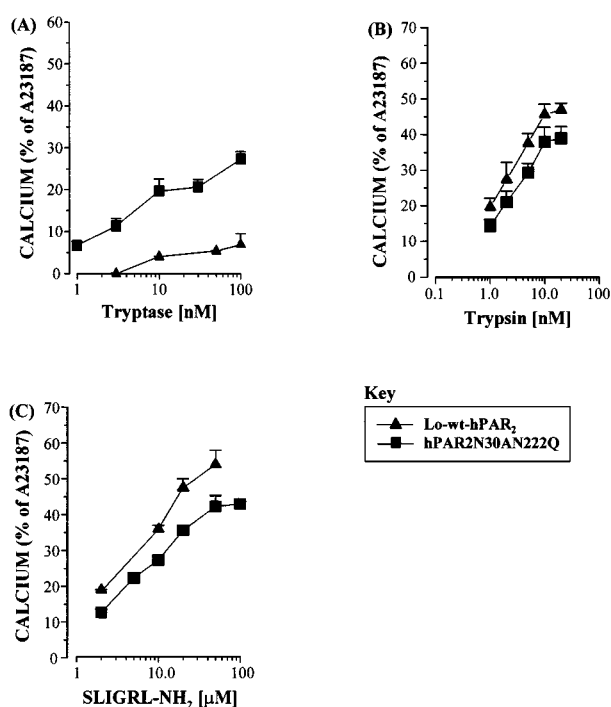


**Figure 6** Cell-surface expression and calcium signalling of wt-hPAR<sub>2</sub>, lo-wt-hPAR<sub>2</sub>, hPAR<sub>2</sub>L221A and hPAR<sub>2</sub>I223A receptor cell lines

(A) Cell-surface expression of lo-wt-hPAR<sub>2</sub>, hPAR<sub>2</sub>L221A and hPAR<sub>2</sub>I223A receptor cell lines as assessed by FACS analysis. Cells, at approx. 40% confluence (except for lo-wt-hPAR<sub>2</sub> cells, which were grown to 100% confluence in order to lower receptor expression to match that of hPAR<sub>2</sub>I223A) were harvested and incubated with the B5 antiserum before incubation with an FITC-conjugated anti-rabbit antibody. Cell surface expression was assessed by FACS analysis. WT, wt-hPAR<sub>2</sub>; lo-wt, lo-wt-hPAR<sub>2</sub>; L221A, hPAR<sub>2</sub>L221A; I223A, hPAR<sub>2</sub>I223A. Results are expressed as a percentage of the mean fluorescence obtained with wt-hPAR<sub>2</sub> cells. Results represent means  $\pm$  S.E.M. of measurements for three separate experiments. (B) and (C) Calcium signalling in wt-hPAR<sub>2</sub>, lo-wt-hPAR<sub>2</sub>, hPAR<sub>2</sub>L221A and hPAR<sub>2</sub>I223A, in response to trypsin and SLIGRL-NH<sub>2</sub>. Concentration–effect curves are shown for trypsin (B) and SLIGRL-NH<sub>2</sub> (C). Lo-wt-hPAR<sub>2</sub> cells were grown to higher confluence in order to match cell surface expression levels with hPAR<sub>2</sub>I223A. Cells were harvested with non-enzymic cell-dissociation solution and loaded with Fluo-3 acetoxymethyl ester (22  $\mu$ M) before incubation for 25 min at room temperature (25 °C). Cells were challenged with different concentrations of PAR agonists and responses were monitored by fluorescence spectrophotometry (excitation wavelength 480 nm, emission wavelength 530 nm). Responses were normalized to the peak height obtained with 2  $\mu$ M calcium ionophore. Results are represented as means  $\pm$  S.E.M. for three separate experiments, each comprising measurements from duplicate cell suspensions. Where error bars are smaller than symbols, they are not shown.

### Human PAR<sub>2</sub> is functionally sensitive to alanine substitution of amino acid residues within, and neighbouring the ECL2 glycosylation sequon domain

The results with the hPAR<sub>2</sub>N222A mutant compared with the hPAR<sub>2</sub>N222Q mutant (see above and Figure 5) suggested that the changes in receptor function were not owing to a lack of glycosylation in ECL2, but rather to the functional sensitivity of the receptor to small perturbations in ECL2, resulting from the alanine substitution strategy. To clarify whether the changes in hPAR<sub>2</sub>N222A function might also result from residues in and around the ECL2 glycosylation sequon being sensitive to alanine substitution, we constructed two additional receptor mutants [hPAR<sub>2</sub>L221A (Leu<sup>221</sup>  $\rightarrow$  Ala) and hPAR<sub>2</sub>I223A (Ile<sup>223</sup>  $\rightarrow$  Ala)] that possessed an alanine substitution either before or after



**Figure 7** Calcium signalling in hPAR<sub>2</sub>N30A,N222Q and lo-wt-hPAR<sub>2</sub> in response to (A) tryptase, (B) trypsin and (C) SLIGRL-NH<sub>2</sub>

Concentration–effect curves are shown for tryptase (A), trypsin (B), and SLIGRL-NH<sub>2</sub> (C). Lo-wt-hPAR<sub>2</sub> cells were grown to higher confluence in order to match cell surface expression levels with hPAR<sub>2</sub>N30A,N222Q. Cells were harvested with non-enzymic cell-dissociation solution and loaded with Fluo-3 acetoxymethyl ester (22 µM) before incubation for 25 min at room temperature (25 °C). Cells were challenged with different concentrations of PAR agonists and responses were monitored by fluorescence spectrophotometry (excitation wavelength 480 nm, emission wavelength 530 nm). Responses were normalized to the peak height obtained with 2 µM calcium ionophore. Results are represented as means ± S.E.M. for three separate experiments, each comprising measurements from duplicate cell suspensions. Where error bars are smaller than symbols, they are not shown.

Asn<sup>222</sup> respectively, and subsequently performed FACS analysis to determine cell-surface expression and concentration–effect curves for trypsin and SLIGRL-NH<sub>2</sub>. Results are shown in Figure 6. FACS analysis showed a dramatic loss in cell-surface expression for hPAR<sub>2</sub>L221A and hPAR<sub>2</sub>I223A [Figure 6A, 25.6 ± 1 and 46 ± 4% (*n* = 3) respectively, compared with wt-hPAR<sub>2</sub>]. To enable a meaningful comparison between the concentration–effect curves obtained for these mutant receptor cell lines and the wt-hPAR<sub>2</sub> cell line, wt-hPAR<sub>2</sub> cells with lower receptor expression (lo-wt-hPAR<sub>2</sub>) were employed using preparations that had been grown to higher confluence (Figure 2B). The cell surface expression level of lo-wt-hPAR<sub>2</sub> was comparable with hPAR<sub>2</sub>I223A (Figure 6A). For the lo-wt-hPAR<sub>2</sub>, trypsin displayed a loss in maximum response (approx. 30% compared with cells that highly expressed wt-hPAR<sub>2</sub>), stimulating a response from 1 to 20 nM, reaching maximal response at 10 nM (Figure 6B). The lo-wt-hPAR<sub>2</sub> cells were slightly less responsive towards SLIGRL-NH<sub>2</sub> at lower concentrations (2–10 µM) but exhibited a comparable maximal response at 50 µM (Figure 6C). For hPAR<sub>2</sub>L221A that had a decreased cell surface expression of receptor, lower than that of lo-wt-hPAR<sub>2</sub>, trypsin displayed a considerable loss in potency and maximal response, even compared with lo-wt-hPAR<sub>2</sub>, stimulating a response from 2 to 100 nM (Figure 6B). SLIGRL-NH<sub>2</sub> also displayed a loss in potency and maximal response towards

hPAR<sub>2</sub>L221A (Figure 6C). For hPAR<sub>2</sub>I223A that had a level of receptor expression comparable with lo-wt-hPAR<sub>2</sub>, trypsin also displayed a considerable loss in potency and maximal response, stimulating a response from 5 to 100 nM (Figure 6B). Likewise, SLIGRL-NH<sub>2</sub> displayed a considerable loss in potency and maximal response towards hPAR<sub>2</sub>I223A, stimulating a response from 5 to 100 nM (Figure 6C).

#### Removal of both glycosylation sequons increases sensitivity to tryptase but not to trypsin and SLIGRL-NH<sub>2</sub>

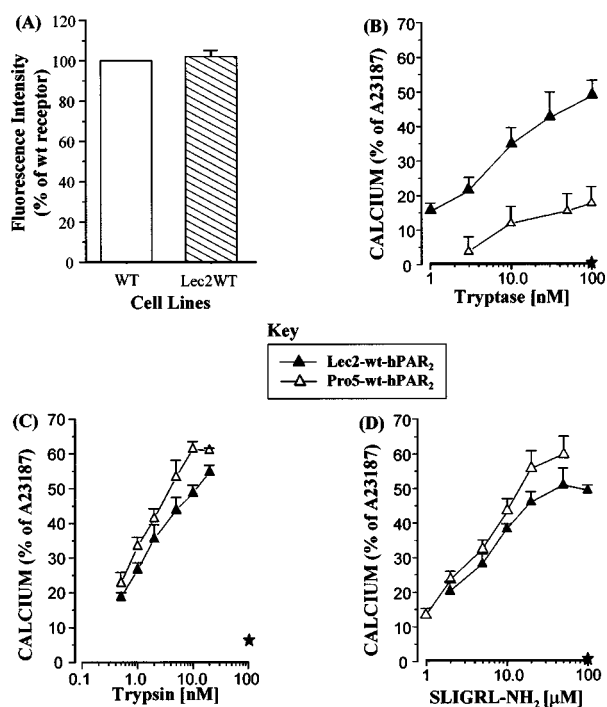
Following the findings that constructing alanine mutations in ECL2 dramatically altered receptor function, we decided to construct concentration–effect curves using hPAR<sub>2</sub>N30A,N222Q instead of hPAR<sub>2</sub>N30A,N222A. Since hPAR<sub>2</sub>N30A,N222Q displayed a loss in cell surface expression compared with wt-hPAR<sub>2</sub> (approx. 50%, see Figure 3C), concentration–effect curves obtained with hPAR<sub>2</sub>N30A,N222Q were compared directly with those obtained with lo-wt-hPAR<sub>2</sub>, as receptor densities for the two cell lines were identical. Results are shown in Figure 7. For lo-wt-hPAR<sub>2</sub>, tryptase stimulated a small calcium response from 10 to 100 nM (Figure 7A). For hPAR<sub>2</sub>N30A,N222Q, tryptase displayed a significant increase in maximal response compared with lo-wt-hPAR<sub>2</sub>, stimulating a response from 1 to 100 nM (Figure 7A). In contrast, in the hPAR<sub>2</sub>N30A,N222Q cells, trypsin stimulated a response from 1 to 20 nM (Figure 7B), similar to lo-wt-hPAR<sub>2</sub> (Figure 7B). This result notwithstanding, in the hPAR<sub>2</sub>N30A,N222Q cells, SLIGRL-NH<sub>2</sub> displayed a significant decrease in maximal response (Figure 7C) compared with lo-wt-hPAR<sub>2</sub> (Figure 7C), stimulating a response from 2 to 100 nM.

#### Tryptase, but not trypsin or SLIGRL-NH<sub>2</sub>, displays increased potency and maximal response towards the sialic-acid-deficient wt-hPAR<sub>2</sub> expressed in the Lec2 mutant cell line

We next sought to investigate whether expressing sialic-acid-deficient wt-hPAR<sub>2</sub> in the Lec2 mutant cell line would influence receptor activation by the PAR<sub>2</sub> agonists. FACS analysis indicated that receptor densities between the Pro5-wt-hPAR<sub>2</sub> and Lec2-wt-hPAR<sub>2</sub> cell lines were comparable (Figure 8A). Concentration–effect curves are shown for tryptase, trypsin and SLIGRL-NH<sub>2</sub> in Figures 8(B), 8(C), and 8(D) respectively. The tryptase concentration–effect curve for Lec2-wt-hPAR<sub>2</sub> (Figure 8B) showed that the enzyme was twice as potent compared with Pro5-wt-hPAR<sub>2</sub> (Figure 8B), stimulating a robust calcium signal from 1 nM and reaching near maximal response at 100 nM. Similar to the observations with hPAR<sub>2</sub>N30A, tryptase exhibited > 3-fold increase in maximal response in Lec2-wt-hPAR<sub>2</sub> compared with that observed for Pro5-wt-hPAR<sub>2</sub>. Trypsin displayed no significant difference in ability to activate Lec2-wt-hPAR<sub>2</sub> (Figure 8C), compared with Pro5-wt-hPAR<sub>2</sub> (Figure 8C), stimulating a calcium signal from 0.5 to 20 nM. Similarly, SLIGRL-NH<sub>2</sub> displayed no significant difference in ability to activate Lec2-wt-hPAR<sub>2</sub> (Figure 8D), although there was a slight loss in the maximal effect compared with that obtained in the Pro5-wt-hPAR<sub>2</sub> (Figure 8D).

As wt-hPAR<sub>2</sub> expressed in the Lec2 cell system yielded a receptor that was deficient in sialic acid, but with an increased sensitivity to tryptase, we wondered if sialic acid itself might affect tryptase function. Therefore a simple substrate assay was performed using BAPNA to determine whether sialic acid was a possible inhibitor of tryptase. Concentrations of synthetic sialic acid from 100 nM to 1 mM had no observable effect on the





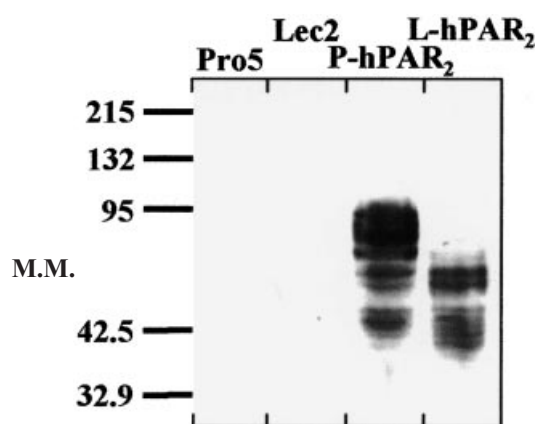
**Figure 8** Cell-surface expression and calcium signalling in Lec2-wt-hPAR<sub>2</sub> cells

(A) Comparison of Lec2-wt-hPAR<sub>2</sub> and Pro5-wt-hPAR<sub>2</sub> cell-surface expression. Cells at approx. 40% confluence were harvested and incubated with the B5 antiserum before incubation with an FITC-conjugated anti-rabbit antibody. Cell-surface expression was assessed by FACS analysis. WT, Pro5-wt-hPAR<sub>2</sub>; Lec2WT, Lec2-wt-hPAR<sub>2</sub>. Results are expressed as a percentage of the mean fluorescence obtained with Pro5-wt-hPAR<sub>2</sub> cells. Results represent means  $\pm$  S.E.M. for three separate experiments. (B), (C) and (D) Calcium signalling in response to tryptase, SLIGRL-NH<sub>2</sub> and trypsin in wt-hPAR<sub>2</sub> expressed in Lec2 cells. Concentration-effect curves are shown for tryptase (B), trypsin (C) and SLIGRL-NH<sub>2</sub> (D). ★, response to each agonist in non-transfected Lec2 cells. Cells were harvested with non-enzymic cell-dissociation solution and loaded with Fluo-3 acetoxymethyl ester (22  $\mu$ M) before incubation for 25 min at room temperature (25 °C). Cells were challenged with different concentrations of PAR agonists and responses were monitored by fluorescence spectrophotometry (excitation wavelength 480 nm, emission wavelength 530 nm). Responses were normalized to the peak height obtained with 2  $\mu$ M calcium ionophore. Results are represented as means  $\pm$  S.E.M. for three separate experiments each comprising measurements from duplicate cell suspensions. Where error bars are smaller than symbols, they are not shown.

ability of tryptase [100 nM, either with heparin (2:1 w/w) or without heparin] to cleave the substrate BAPNA, as described above (results not shown). Furthermore, addition of sialic acid (1 mM) to wt-hPAR<sub>2</sub>-expressing Lec2 cells in the calcium assay also had a negligible effect on the ability of tryptase to activate the receptor (results not shown).

#### Sialic-acid-deficient wt-hPAR<sub>2</sub> displays a considerable loss in molecular mass

To determine the contribution of sialic acid to the molecular mass of wt-hPAR<sub>2</sub>, we performed Western blot analysis for epitope-tagged Lec2-wt-hPAR<sub>2</sub> alongside the epitope-tagged Pro5-wt-hPAR<sub>2</sub>. Results are shown in Figure 9. The molecular mass of Lec2-wt-hPAR<sub>2</sub> ranged from 33 to 60 kDa, 40 kDa smaller compared with the molecular mass of wt-hPAR<sub>2</sub> that was expressed in Pro5 cells. No bands were detected in the non-transfected Lec2 or Pro5 cell membranes.



**Figure 9** Western blot analysis of wt-hPAR<sub>2</sub> expressed in Lec2 cells: comparison of wt-hPAR<sub>2</sub> expressed in Pro5 and Lec2 cell lines

Crude membrane preparations of wt-hPAR<sub>2</sub> expressed in either Pro5 or Lec2 cells, were analysed by SDS/PAGE (12% gels) and immunoblotted with the HA.11 monoclonal antibody. M.M., molecular mass; Pro5 and Lec2, membranes from non-transfected Pro5 and Lec2 cells respectively; P-hPAR<sub>2</sub>, wt-hPAR<sub>2</sub> expressed in Pro5 cells; L-hPAR<sub>2</sub>, wt-hPAR<sub>2</sub> expressed in Lec2 cells. Results are representative of at least three separate experiments.

#### DISCUSSION

The present study shows conclusively, for the first time, that hPAR<sub>2</sub> contains considerable N-linked glycosylation on both glycosylation sites (Asn<sup>30</sup> and Asn<sup>222</sup>), that at least one of the glycosylation sites is required for optimal expression of cell surface hPAR<sub>2</sub>, and that N-terminal sialylation of hPAR<sub>2</sub> regulates sensitivity towards tryptase. Using an epitope-tagging strategy, we demonstrated that hPAR<sub>2</sub> has a molecular mass of 55–100 kDa, where up to 50% of the receptor mass can be attributed to N-linked glycosylation on both glycosylation sites. In calcium-signalling assays, the mutant N-terminal glycosylation-deficient receptor (hPAR<sub>2</sub>N30A) displayed increased sensitivity towards tryptase, but not to trypsin and the PAR<sub>2</sub>-AP, SLIGRL-NH<sub>2</sub>. Although the ECL2 glycosylation-deficient receptor (hPAR<sub>2</sub>N222Q) displayed little change in receptor function, it was found that alanine mutations in this domain of the receptor (e.g. L221A or I223A), including the glycosylation site (N222A), significantly decreased the ability of trypsin and SLIGRL-NH<sub>2</sub> to evoke a calcium signal. Furthermore, it was found that the hPAR<sub>2</sub>N30A,N222Q non-glycosylated mutant receptor showed increased sensitivity towards tryptase, but decreased sensitivity towards trypsin and SLIGRL-NH<sub>2</sub>. However, this loss in sensitivity towards trypsin and SLIGRL-NH<sub>2</sub> was probably because of the loss in receptor cell-surface expression, suggesting a low proportion of 'spare receptors' in this cell system [33]. Finally, we now show that it is not glycosylation itself, but sialylation of the N-terminal oligosaccharide chain, that restricts hPAR<sub>2</sub> activation by tryptase. We propose that N-linked glycosylation of hPAR<sub>2</sub> affects expression at the cell surface and that glycosylation may, along with receptor sialylation, govern receptor sensitivity to tryptase.

In our initial evaluation of the role of glycosylation in PAR<sub>2</sub> function, we were unsuccessful in demonstrating receptor glycosylation directly, because our B5 antibody could not, with certainty, localize the receptor on Western blots, in contrast with studies of the receptor in other systems, where a constituent of 43 or 76 kDa was thought to represent the receptor [31,34]. Nevertheless, by epitope-tagging wt-hPAR<sub>2</sub>, we unambiguously

identified PAR<sub>2</sub>, as shown in Figure 2(A). The molecular mass observed for wt-hPAR<sub>2</sub> (< 100 kDa) was much larger than predicted according to its amino acid composition (44 kDa; [5]). However, treatment of wt-hPAR<sub>2</sub> cell membranes with *N*-glycosidase F decreased the molecular mass of the tagged receptor to approx. 36–46 kDa, which is close to the predicted molecular mass. These results indicate that the additional mass of the mature receptor is due to glycosylation and that most, if not all, of the glycosylation is N-linked.

FACS analysis and immunocytochemistry of the wt-hPAR<sub>2</sub> and mutant cell lines revealed that hPAR<sub>2</sub>N30A,N222Q possessed less receptor at the cell surface. In addition, although it is difficult to differentiate objectively between cell-surface and cytoplasmic localization using the immunocytochemistry technique employed in our study, the staining pattern of hPAR<sub>2</sub>N30A,N222Q is consistent with that of receptor that has been retained in the cytoplasm. Nevertheless, because considerable (50% of wt cells) cell-surface expression was observed for the hPAR<sub>2</sub>N30A,N222Q mutant, we can conclude that N-linked glycosylation facilitates, but is not essential for, cell-surface expression of hPAR<sub>2</sub>.

Western blot analysis of the glycosylation-deficient mutant receptors revealed that hPAR<sub>2</sub> appears to be glycosylated on both N-linked sites. Interestingly, in a recent survey of multi-span membrane glycoproteins [35] it was found that, of the 229 receptors surveyed, over 92% (211/229) were exclusively N-linked glycosylated only on the N-terminal glycosylation site. Interestingly for hPAR<sub>2</sub>, both glycosylation sequons are glycosylated. Given the presence of fewer bands for hPAR<sub>2</sub>N222Q compared with hPAR<sub>2</sub>N30A (Figure 3B), the results suggest a greater heterogeneity of glycosylation on Asn<sup>222</sup>. The appearance of bands for hPAR<sub>2</sub>N30A,N222Q of a molecular mass lower than that predicted for hPAR<sub>2</sub> (44 kDa), may represent proteolysis breakdown products of the receptor. Notwithstanding, the results provide compelling evidence that wt-hPAR<sub>2</sub> is not only a predominantly *N*-linked glycosylated receptor, but is unusually glycosylated on both of the potential N-linked glycosylation sites, in contrast with many other multi-span membrane glycoproteins [35].

The selective increase in potency of tryptase towards hPAR<sub>2</sub>N30A is in agreement with our previous report [16] and suggests that this proteinase obtains greater access to the cleavage/activation site of the receptor. In contrast, it appears that the actions of trypsin and SLIGRL-NH<sub>2</sub> are not affected by the lack of glycosylation on the N-terminus. In addition to an increase in potency, tryptase was able to cause a marked increase in the maximal response by hPAR<sub>2</sub>N30A. This increase in maximal response to tryptase was intriguing, as this implies that, in Pro5 cells expressing wt-hPAR<sub>2</sub>, tryptase can gain access only to a limited number of receptors within the receptor pool. We conclude that N-terminal glycosylation selectively regulates receptor susceptibility to tryptase, but not trypsin or SLIGRL-NH<sub>2</sub>.

Deleting the glycosylation sequon from ECL2 by either mutating Asn<sup>222</sup> → Ala or Asn<sup>222</sup> → Gln had disparate effects on the agonist activity of the proteinases and the PAR<sub>2</sub>-AP, SLIGRL-NH<sub>2</sub>. No significant change in receptor function was observed for hPAR<sub>2</sub>N222Q, although a small loss in cell surface expression was observed (approx. 20%), thus suggesting a role for glycosylation on ECL-2 in enhancing receptor expression but not in regulating receptor signalling. However, hPAR<sub>2</sub>N222A, having a cell surface expression comparable with hPAR<sub>2</sub>N222Q, displayed a significant loss in sensitivity to all the agonists tested. Furthermore, substituting alanine for either of the residues Leu<sup>221</sup> or Ile<sup>223</sup> that neighboured Asn<sup>222</sup>, not only decreased cell-

surface expression below that of hPAR<sub>2</sub>N222Q or hPAR<sub>2</sub>N222A, but, more significantly, altered the responsiveness of PAR<sub>2</sub> to agonists. Thus it would appear that this region of ECL2 is highly sensitive to even neutral mutations such as alanine. Furthermore, the hPAR<sub>2</sub>I223A mutant, when compared with wt cells that express a comparable cell-surface expression of receptor, showed a marked loss in responsiveness both to trypsin and SLIGRL-NH<sub>2</sub>. These results indicate the importance of ECL2 Ile<sup>223</sup>, not only for receptor expression, but also for agonist efficacy. The reason for the sensitivity of this region of the receptor to mutations may lie in the close proximity of Cys<sup>226</sup>, which is believed to form a disulphide bridge with Cys<sup>148</sup>. Mutations within this domain that confer minor shifts in receptor conformation may hinder the ability of these cysteine residues to form a disulphide bridge and hence dramatically alter receptor function. Nevertheless, the finding that hPAR<sub>2</sub>N222Q displayed little alteration in receptor function, signified that receptor conformation had not been compromised by the Asn<sup>222</sup> → Gln mutation, and provided confidence that any changes in receptor expression were due to alterations in the glycosylation state of the receptor. The non-glycosylated receptor, hPAR<sub>2</sub>N30A,N222Q, displayed a loss in sensitivity towards trypsin and SLIGRL-NH<sub>2</sub>, but an increase in sensitivity towards trypsin. The increase in sensitivity towards trypsin was expected, as this is indicative of a loss of glycosylation on the hPAR<sub>2</sub>N30A,N222Q N-terminus. However, the loss in hPAR<sub>2</sub>N30A,N222Q sensitivity towards trypsin and SLIGRL-NH<sub>2</sub> was secondary to the loss in cell-surface expression as wt-hPAR<sub>2</sub>-expressing cells, with identical cell-surface expression to hPAR<sub>2</sub>N30A,N222Q, displayed similar shifts in receptor sensitivity to trypsin and SLIGRL-NH<sub>2</sub>. Thus there is surprisingly low 'spareness' in the PAR<sub>2</sub> system compared with other GPCRs [33].

The ability of tryptase to activate sialic-acid-deficient wt-hPAR<sub>2</sub> with a potency and magnitude of response comparable with trypsin, provides compelling evidence of a role for sialic acid in regulating tryptase activation of hPAR<sub>2</sub>. The fact that we found no evidence for sialic acid as an inhibitor of tryptase enzymic activity in a simple biochemical assay suggests that only sialic acid on the oligosaccharide chain can inhibit tryptase activation of the receptor. Remarkably, Western blot analysis revealed that wt-hPAR<sub>2</sub> expressed in Lec2 cells had a molecular mass that was up to 40 kDa lower than that observed for wt-hPAR<sub>2</sub> expressed in the parent Pro5 cells. Presumably this significant loss of 40 kDa is due to the loss of receptor-associated sialic acid. Thus we provide genetic evidence that PAR<sub>2</sub>-oligosaccharide sialylation plays a key role in specifically regulating tryptase, but not trypsin or SLIGRL-NH<sub>2</sub>, activation of wt-hPAR<sub>2</sub>.

Differential N-linked glycosylation and sialylation of proteins provides a subtle, but important, post-translational mechanism for modifying protein function. Human PAR<sub>2</sub> does appear to exist in various glycosylated forms within a single cell type, as confirmed by the multiple bands observed by Western blot analysis of wt-hPAR<sub>2</sub> expressed in Pro5 cells. Whether or not cell-type-specific glycosylated forms of hPAR<sub>2</sub> exist remains unknown. However, it is well established that the terminal glycosylation (sialylation) of proteins is heavily regulated by differential expression of various sialyltransferases [36]. These findings support the concept that hPAR<sub>2</sub> may be expressed in various cell- or tissue-specific glycosylated forms. Therefore, as sialic acid appears to play such a central role in regulating tryptase activation of hPAR<sub>2</sub>, the variability reported in the ability of mast cell tryptase to activate PAR<sub>2</sub> [10,13–15] may be due, in part, to the cell-specific expression of particular sialyltransferases. Whether or not the functioning of hPAR<sub>2</sub> is altered

in disease settings that display modifications in the expression of the glycosylation machinery (e.g. rheumatoid arthritis [37]) merits further study.

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## REFERENCES

- Hollenberg, M. D. and Compton, S. J. (2002) International Union of Pharmacology. XXVIII. Proteinase-activated receptors. *Pharmacol. Rev.* **54**, 203–217
- Macfarlane, S. R., Seatter, M. J., Kanke, T., Hunter, G. D. and Plevin, R. (2001) Proteinase-activated receptors. *Pharmacol. Rev.* **53**, 245–282
- Dery, O., Corvera, C. U., Steinhoff, M. and Bunnett, N. W. (1998) Proteinase-activated receptors: novel mechanisms of signaling by serine proteases. *Am. J. Physiol.* **274**, C1429–C1452
- Vu, T. K., Hung, D. T., Wheaton, V. I. and Coughlin, S. R. (1991) Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* **64**, 1057–1068
- Bohm, S. K., Kong, W., Bromme, D., Smeeckens, S. P., Anderson, D. C., Connolly, A., Kahn, M., Nelken, N. A., Coughlin, S. R., Payan, D. G. and Bunnett, N. W. (1996) Molecular cloning, expression and potential functions of the human proteinase-activated receptor-2. *Biochem. J.* **314**, 1009–1016
- Nystedt, S., Emilsson, K., Larsson, A. K., Strombeck, B. and Sundelin, J. (1995) Molecular cloning and functional expression of the gene encoding the human proteinase-activated receptor 2. *Eur. J. Biochem.* **232**, 84–89
- Ishihara, H., Connolly, A. J., Zeng, D., Kahn, M. L., Zheng, Y. W., Timmons, C., Tram, T. and Coughlin, S. R. (1997) Protease-activated receptor 3 is a second thrombin receptor in humans. *Nature (London)* **386**, 502–506
- Xu, W. F., Andersen, H., Whitmore, T. E., Presnell, S. R., Yee, D. P., Ching, A., Gilbert, T., Davie, E. W. and Foster, D. C. (1998) Cloning and characterization of human protease-activated receptor 4. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 6642–6646
- Kahn, M. L., Zheng, Y. W., Huang, W., Bigornia, V., Zeng, D., Moff, S., Farese, Jr, R. V., Tam, C. and Coughlin, S. R. (1998) A dual thrombin receptor system for platelet activation. *Nature (London)* **394**, 690–694
- Molino, M., Barnathan, E. S., Numerof, R., Clark, J., Dreyer, M., Cumashi, A., Hoxie, J. A., Schechter, N., Woolkalis, M. and Brass, L. F. (1997) Interactions of mast cell tryptase with thrombin receptors and PAR-2. *J. Biol. Chem.* **272**, 4043–4049
- Takeuchi, T., Harris, J., Huang, W., Yan, K. W., Coughlin, S. R. and Craik, C. S. (2000) Cellular localization of membrane-type serine protease 1 and identification of protease activated receptor-2 and single-chain urokinase-type plasminogen activator as substrates. *J. Biol. Chem.* **275**, 26333–26342
- Camerer, E., Huang, W. and Coughlin, S. R. (2000) Tissue factor- and factor X-dependent activation of protease-activated receptor 2 by factor VIIa. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 5255–5260
- Steinhoff, M., Vergnolle, N., Young, S. H., Tognetto, M., Amadesi, S., Ennes, H. S., Trevisani, M., Hollenberg, M. D., Wallace, J. L., Caughey, G. H., Mitchell, S. E., Williams, L. M., Geppetti, P., Mayer, E. A. and Bunnett, N. W. (2000) Agonists of proteinase-activated receptor 2 induce inflammation by a neurogenic mechanism. *Nat. Med.* **6**, 151–158
- Huang, C., De Sanctis, G. T., O'Brien, P. J., Mizgerd, J. P., Friend, D. S., Drazen, J. M., Brass, L. F. and Stevens, R. L. (2001) Evaluation of the substrate specificity of human mast cell tryptase beta I and demonstration of its importance in bacterial infections of the lung. *J. Biol. Chem.* **276**, 26276–26284
- Schechter, N. M., Brass, L. F., Lavker, R. M. and Jensen, P. J. (1998) Reaction of mast cell proteases tryptase and chymase with protease activated receptors (PARs) on keratinocytes and fibroblasts. *J. Cell. Physiol.* **176**, 365–373
- Compton, S. J., Renaux, B., Wijesuriya, S. J. and Hollenberg, M. D. (2001) Glycosylation and the activation of proteinase-activated receptor 2 (PAR(2)) by human mast cell tryptase. *Br. J. Pharmacol.* **134**, 705–718
- Zhang, Z., Austin, S. C. and Smyth, E. M. (2001) Glycosylation of the human prostacyclin receptor: role in ligand binding and signal transduction. *Mol. Pharmacol.* **60**, 480–487
- Pang, R. T., Ng, S. S., Cheng, C. H., Holtmann, M. H., Miller, L. J. and Chow, B. K. (1999) Role of N-linked glycosylation on the function and expression of the human secretin receptor. *Endocrinology* **140**, 5102–5111
- Zhou, A. T., Assil, I. and Abou-Samra, A. B. (2000) Role of asparagine-linked oligosaccharides in the function of the rat PTH/PTHrP receptor. *Biochemistry* **39**, 6514–6520
- Opendakker, G., Rudd, P. M., Ponting, C. P. and Dwek, R. A. (1993) Concepts and principles of glycobiology. *FASEB J.* **7**, 1330–1337
- Vouret-Craviari, V., Grall, D., Chambard, J. C., Rasmussen, U. B., Pouyssegur, J. and Obberghen-Schilling, E. (1995) Post-translational and activation-dependent modifications of the G protein-coupled thrombin receptor. *J. Biol. Chem.* **270**, 8367–8372
- Lerner, D. J., Chen, M., Tram, T. and Coughlin, S. R. (1996) Agonist recognition by proteinase-activated receptor 2 and thrombin receptor. Importance of extracellular loop interactions for receptor function. *J. Biol. Chem.* **271**, 13943–13947
- Al-Ani, B., Saifeddine, M., Kawabata, A. and Hollenberg, M. D. (1999) Proteinase activated receptor 2: Role of extracellular loop 2 for ligand-mediated activation. *Br. J. Pharmacol.* **128**, 1105–1113
- Blackhart, B. D., Ruslim-Litrus, L., Lu, C. C., Alves, V. L., Teng, W., Scarborough, R. M., Reynolds, E. E. and Oksenberg, D. (2000) Extracellular mutations of protease-activated receptor-1 result in differential activation by thrombin and thrombin receptor agonist peptide. *Mol. Pharmacol.* **58**, 1178–1187
- Compton, S. J., Cairns, J. A., Holgate, S. T. and Walls, A. F. (1998) The role of mast cell tryptase in regulating endothelial cell proliferation, cytokine release, and adhesion molecule expression: tryptase induces expression of mRNA for IL-1 beta and IL-8 and stimulates the selective release of IL-8 from human umbilical vein endothelial cells. *J. Immunol.* **161**, 1939–1946
- Compton, S. J., Cairns, J. A., Palmer, K. J., Al-Ani, B., Hollenberg, M. D. and Walls, A. F. (2000) A polymorphic protease-activated receptor 2 (PAR2) displaying reduced sensitivity to trypsin and differential responses to PAR agonists. *J. Biol. Chem.* **275**, 39207–39212
- Kolodziej, P. A. and Young, R. A. (1991) Epitope tagging and protein surveillance. *Methods Enzymol.* **194**, 508–519
- Bohm, S. K., Khitin, L. M., Grady, E. F., Aponte, G., Payan, D. G. and Bunnett, N. W. (1996) Mechanisms of desensitization and resensitization of proteinase-activated receptor-2. *J. Biol. Chem.* **271**, 22003–22016
- Deutscher, S. L., Nuwayhid, N., Stanley, P., Briles, E. I. and Hirschberg, C. B. (1984) Translocation across Golgi vesicle membranes: a CHO glycosylation mutant deficient in CMP-sialic acid transport. *Cell* **39**, 295–299
- Eckhardt, M., Gotza, B. and Gerardy-Schahn, R. (1998) Mutants of the CMP-sialic acid transporter causing the Lec2 phenotype. *J. Biol. Chem.* **273**, 20189–20195
- Kong, W., McConalogue, K., Khitin, L. M., Hollenberg, M. D., Payan, D. G., Bohm, S. K. and Bunnett, N. W. (1997) Luminal trypsin may regulate enterocytes through proteinase-activated receptor 2. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 8884–8889
- Saifeddine, M., Al-Ani, B., Sandhu, S., Wijesuriya, S. J. and Hollenberg, M. D. (2001) Contractile actions of proteinase-activated receptor-derived polypeptides in guinea-pig gastric and lung parenchymal strips: evidence for distinct receptor systems. *Br. J. Pharmacol.* **132**, 556–566
- Nickerson, M. (1956) Receptor occupancy and tissue response. *Nature (London)* **178**, 697–698
- Napoli, C., Cicala, C., Wallace, J. L., de Nigris, F., Santagada, V., Caliendo, G., Franconi, F., Ignarro, L. J. and Cirino, G. (2000) Protease-activated receptor-2 modulates myocardial ischemia-reperfusion injury in the rat heart. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 3678–3683
- Landolt-Marticorena, C. and Reithmeier, R. A. (1994) Asparagine-linked oligosaccharides are localized to single extracytosolic segments in multi-span membrane glycoproteins. *Biochem. J.* **302**, 253–260
- Kitagawa, H. and Paulson, J. C. (1994) Differential expression of five sialyltransferase genes in human tissues. *J. Biol. Chem.* **269**, 17872–17878
- Axford, J. S. (1999) Glycosylation and rheumatic disease. *Biochim. Biophys. Acta* **1455**, 219–229

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