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The role of alternative splicing and C-terminal amino acids in thromboxane receptor stabilization

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Abstract

The thromboxane receptor has two alternatively spliced isoforms, α and β, which differ only in sequences within the cytoplasmic C-terminal domain. Oxidative stress induced by H_2O_2 in a COS-7 cell model results in stabilization of the thromboxane receptor β isoform by translocation from the endoplasmic reticulum to the Golgi complex, which in turn results in protection of the receptor from degradation. We now report that both the α and β thromboxane receptor isoforms respond identically to oxidative stress. Further, mutagenesis studies indicate that replacing the normal C-terminus with a nonsense sequence also does not alter stabilization behaviour ruling out a role for the distinct C-termini in this process. Further mutagenesis implicates a cluster of arginine residues within the C-terminal domain as involved in oxidative stress-induced stabilization. These data identify a region of the thromboxane receptor that is responsible for responding to oxidative challenge and open the possibility of identification of the molecular machinery underpinning this response.

Keywords: Thromboxane; ER-associated degradation; Isoprostane; Protein turnover; Mutagenesis; Oxidative stress

Thromboxane (Tx) A_2, the primary cyclooxygenase product of arachidonic acid in platelets, is a potent stimulator of platelet shape change, aggregation, secretion, and a constrictor of bronchial and vascular smooth muscle [1]. Synthesis of TxA_2 is increased in a variety of diseases including myocardial infarction, stroke, bronchial asthma, and pregnancy-induced hypertension, all of which are believed to coincide with imbalances either in the levels of TxA_2, its synthesis or its receptor [2]. TxA_2 mediates its actions through interaction with the receptor termed TP [3], a member of the G protein-coupled receptor superfamily that is expressed as two alternatively spliced isoforms transcribed from a single locus, TPα (343 residues) and TPβ (406 residues) that share the first 328 residues [4,5] (Fig. 1). TP engages with the signal transduction machinery via heterotrimeric G proteins, principally G_{q11}, resulting in activation of phospholipase C, release of Ca^{2+}, and stimulation of protein kinase C (PKC). More recent studies point to a highly complex signalling cascade beneath TP [6–13]. Some evidence suggests that endocytosis of the surface pool of TP [14] is involved in signal transduction. It has also been shown that the alternative splicing of the carboxyl terminal plays a crucial role in TP signal transduction and tissue-specific expression [15,16].

We previously demonstrated that one of the isoprostanes, 15-F_{2t}-IsoP ($iPF_{2\alpha}$-III), is a potent coronary vasoconstrictor and a partial agonist at the thromboxane receptor [17], and that the critical determinant of the intrinsic activity of this isoprostane is thromboxane receptor reserve. Isoprostane production is enhanced by oxidative stress and increased levels have been reported in a number of conditions associated with

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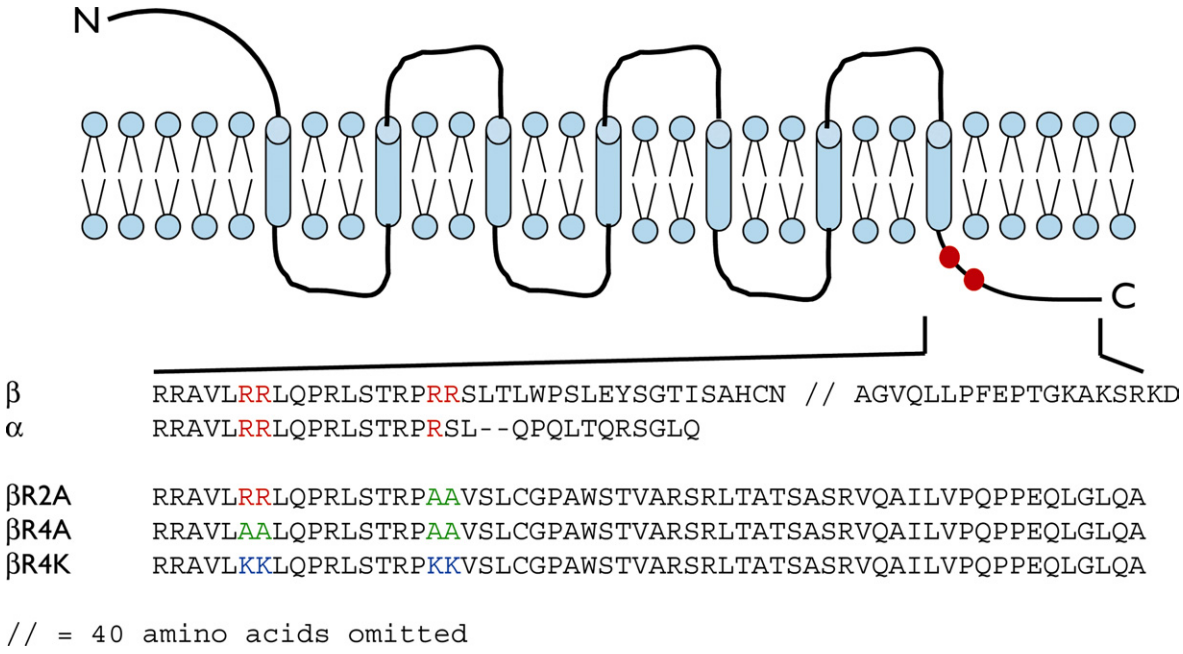

Fig. 1. Topological model of TP showing the differences between TPα and β and the locations of mutations. A model for TP based on topological studies is shown together with red spots indicating the arginine clusters at the carboxyl-terminal domain of the receptor. Transmembrane domains are shown as cylinders. N, N-terminus; C, C-terminus. The amino acid sequences of the TPα and β C-termini are also shown exploded, with the targeted arginine residues highlighted in red. A double forward slash in the β sequence indicates omission of 40 amino acids for clarity. Note that differential splicing alters the primary structure C-terminal to the second arginine pair. TP mutant constructs also use an alternative open reading to that occurring in normal TP (see text for details). Amino acid sequences of the various mutants are also shown. βR2A, construct with one arginine pair replaced with alanine (green). βR4A, construct with both arginine pairs at the carboxyl-terminal domain replaced with alanine. βR4K, construct with both arginine clusters replaced with lysine residues (blue).

oxidative stress, including hypercholesterolaemia [18] and atherosclerosis [19,20]. Therefore, oxidative stress may not only enhance production of the isoprostane 15-F_{2t}-IsoP, but may also upregulate the receptor through which it acts. Clearly, the molecular mechanism that underpins this oxidative stress response is of some importance, not only for understanding the physiological basis of TP regulation, but also for development of potential strategies to manipulate TP signalling.

We recently developed a tissue cell culture model to study the influence of oxidative stress from H_2O_2 on TPβ at the molecular level. Unexpectedly, the vast majority of the receptor was found to be located on internal membranes, mainly of the endoplasmic reticulum, whilst little receptor was located at the cell surface. Degradation kinetics of TPβ following cycloheximide treatment, a protein synthesis inhibitor, suggested not only that TPβ is a short-lived protein predominantly localized to the ER but also that TPβ degradation is modulated in the presence of H_2O_2 with an increase in receptor half-life rapidly following exposure to oxidative stress. Most significantly, the receptor was rapidly translocated from the ER to the Golgi complex, and stabilization could be blocked by treatment with brefeldin A, suggesting that translocation is an essential aspect of TP stabilization [21]. Hence, our results indicate that oxidative stress induces maturation and stabilization of the TPβ protein probably by intracellular translocation. Importantly, these observations also suggest that TPβ levels are modulated by proteosome-dependent ER-associated degradation (ERAD) and controlled by the efficiency of transport to post-ER compartments [21]. Further, biochemical analysis suggested that TPβ degradation was mediated by the unfolded protein response (UPR). Hence, rapid stabilization of TPβ by translocation from a degradative compartment, i.e., the ER, can account for the augmentation of receptor density observed in vitro and may account for the similar augmentation of receptor density observed in vivo during cardiovascular disease [22–24].

The mechanism of this stabilization is not well understood, and in particular the regions of the TP polypeptide required are not known, nor are the protein factors that recognize the TPβ identified. Using truncation and site-directed mutagenesis together with protein turnover analysis, we have investigated the role of the carboxyl-terminal domain on the stability of the TP and demonstrate a role for a cluster of cytoplasmically oriented arginine residues.

Experimental procedures

Materials. Simian kidney (COS-7) cells were obtained from American Type Culture Collection (ATCC). cDNAs encoding TPα

and β isoforms were kindly provided by Dr. S. Narumiya (Department of Pharmacology, Kyoto University Faculty of Medicine, Kyoto), and Drs. J.A. Ware and A.W. Ashton (Albert Einstein College of Medicine, New York), respectively. Mammalian expression vector pcDNA 3.1/CT-GFP, pcDNA 4/CT-myc-his, Dulbecco's modified Eagle's medium (DMEM) supplemented with glucose (4.5 g/liter), foetal bovine serum (FBS), and antibiotic/antimycotic solution were purchased from Invitrogen Life Technologies (Carlsbad, CA). 4′,6-Diamidino-2-phenylindole (DAPI) and anti-Golgin-97 Texas red-conjugated mouse monoclonal antibody were purchased from Molecular Probes (Eugene, OR). Anti-myc mouse monoclonal antibody was purchased from Santa Cruz Biotechnology. DNA *Taq* polymerase was obtained from Stratagene (La Jolla, CA). The Bradford protein assay kit was from Bio-Rad (Hercules, CA). Horseradish peroxidase-conjugated goat anti-mouse IgG and all the other chemicals, analytical grade, were purchased from Sigma Chemical (UK).

Site-directed mutagenesis and subcloning of C-terminal myc-his-tagged human TPα or β cDNA. Human TP cDNA was amplified by PCR. The oligonucleotides used were 5′-CGGGATCCATGTGGC CCAACGGCAGT-3′ and 5′-CGGATATCCTGCAGCCCGGAG CGCT-3′ for the α isoform, and 5′-CGGGATCCATGTGGCCCAAC GGCAGT-3′ and 5′-CGCAGTGATATCCGCCTGTAATCCAG-3′ for the β isoform with *Bam*HI and *Eco*RV sites (underlined). The PCR products were subcloned into pcDNA 3.1/CT-GFP or into pcDNA 4/CT-myc-his at *Bam*HI and *Eco*RV sites. Mutations were introduced by PCR (Quickchange, Stratagene). The correct insertion of the TP cDNA and production of the desired mutations were confirmed by dideoxy DNA sequencing.

Cell culture and expression of the myc-his-tagged human TP. COS-7 cells were maintained in DMEM supplemented with 10% heat-inactivated FBS, and antibiotic–antimycotic mix at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. To create cell lines expressing TPα or β, pcDNA 4/CT-myc-his expression vector containing the cDNAs of the myc-his-tagged TPα or β (pCDNA4/TPα or β-myc-his) was transfected into COS-7 cells using FuGENE 6 Transfection Reagent (Roche Diagnostics, IN, USA) as described [21].

Immunochemistry. Cell lysates were prepared as described [21] and following Bradford protein estimation 20 μg of cell lysate protein per lane was separated on 12% SDS–polyacrylamide gels. In some cases, the proteins were then transferred onto Hybond-ECL nitrocellulose membrane (Amersham Pharmacia Biotech, UK) by wet transfer by standard procedures. Filters were blocked in 5% milk, phosphate-buffered saline, and 0.1% Tween 20 (PBST), probed with primary antibodies (in 10 ml block solution) for 1 h, washed four times with PBST, and then incubated with the appropriate secondary antibody (diluted 1:5000) in blocking buffer for 1 h and washed in PBST. Detection was performed by chemiluminescence and exposure to X-ray film.

Receptor turnover analysis. COS-7 cells were grown and transiently transfected with pCDNA4/TPα or β-myc-his as described above. At 24 h post-transfection, COS-7 cells were treated with cycloheximide (200 μg/ml) by adding the drug to the medium, and then cells were submitted to oxidative stress (H_2O_2, 10 μM, 40 min). The medium was then replaced with fresh medium containing cycloheximide for the duration of the experiment. At various times cells were collected and placed on ice prior to preparation of whole cell lysates. Lysates were fractionated by electrophoresis on SDS–PAGE gels as described above. A loading control was performed using an anti-tubulin mouse monoclonal antibody (TAT-1) (gift from Keith Gull, Oxford) by reprobing of the filters. Data were quantitated by scanning of the X-ray films using a Heidelberg 1200 scanner followed by densitometric analysis with ImageJ (http://rsb.info.nih.gov/ij/).

Immunofluorescence microscopy. Cells were fixed for 10 min in PBS/3.6% paraformaldehyde and permeabilized for 10 min with PBS/0.5% Triton X-100 at room temperature. For colocalization of TPα-myc-his, cells were stained using DAPI and anti-Golgin-97 Cy3 conjugated (*cis*-Golgi marker) monoclonal antibody. Immunofluorescence was visualized under a Nikon E600 immunofluorescence microscope equipped with 100×/1.3 Plan-Fluor oil immersion objective. The digital images were collected using Metamorph (Universal Imaging), merged, and assembled into figures using Adobe Photoshop (Adobe Systems).

Results

We considered the topology and structure of TP (Fig. 1). The protein is predicted to have seven transmembrane domains, three intracellular loops, and a cytoplasmic C-terminal tail, which differs in primary structure between the α and β splicoforms. Investigations have suggested that the cytoplasmic loops are important for ligand binding, and therefore we considered these regions as unlikely to have a major role in response to oxidative stress. Further, the splicoform differences result in distinct interactions with downstream signalling pathways and tissue-specific expression [15], suggesting that the unique regions have very specific functions. In addition, a cluster of arginine residues within the portion of the C-terminus that are not affected by differential splicing were particularly apparent (red in Fig. 1). By contrast, analysis with Prosite (http://ca.expasy.org/prosite/) failed to discover motifs within the cytoplasmic portion of the molecule that could indicate interaction sites with cytoplasmic coat proteins, for example KKXX motifs, nor are there clear regions of sequence conservation between TP and other members of the prostanoid receptor family (data not shown). Taken together, we considered that the cytoplasmic region was the best initial candidate to contain the residues required for response to oxidative stress, and that the conserved arginine residues seemed the most likely site. We chose to test the possible role of this region, and of the arginine cluster in particular, experimentally by first comparing the α and β TP isoforms, followed by deletion and point mutation analysis.

Oxidative stress evokes an intracellular translocation of the TPα

We expressed TPα in COS-7 cells by transient transfection and localized the protein using anti-c-myc antibody. At steady state, immunofluorescence microscopy showed that a large proportion of TPα (green) was localized to the ER (Fig. 2) and no significant colocalization was apparent between TPα and Golgin-97, a resident Golgi membrane protein. By contrast, challenge with H_2O_2 rapidly evoked an intracellular translocation of the TPα from the ER to the Golgi complex inducing a partial colocalization with Golgin-97. These results are similar to our previous observations for the TPβ isoform [21], suggesting that the alternative splicing of the carboxyl terminal of the TP does not interfere with the intracellular translocation mechanism induced by oxidative stress. Therefore, the amino acids downstream of

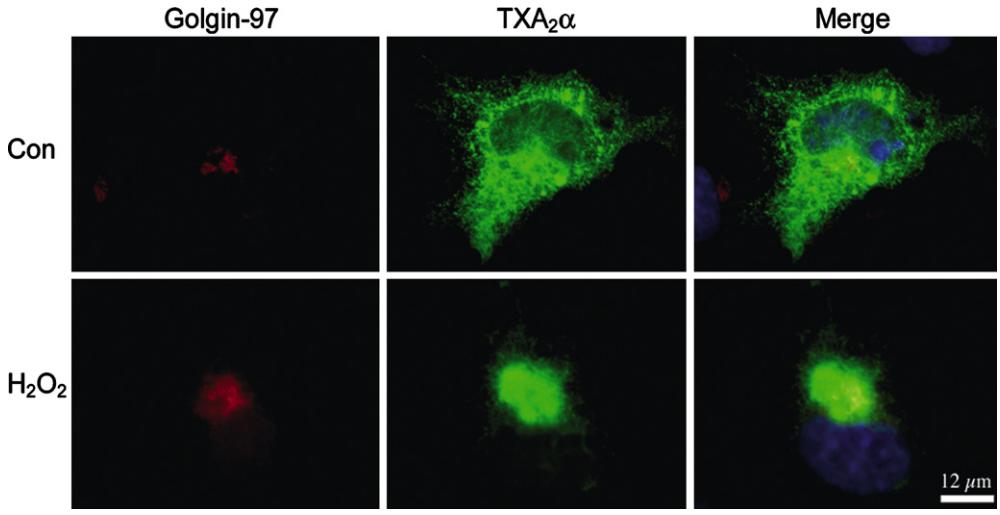

Fig. 2. Immunofluorescence microscopy indicates TPα translocates to the Golgi complex following oxidative stress. COS-7 cells were transiently transfected with TPα-GFP (green), fixed, and the Golgi complex was visualized with anti-Golgin-97 antibodies (*cis*-Golgi marker) and a secondary Texas red-conjugated antibody (red). The nuclei were stained with DAPI (blue). Without H_2O_2 treatment (top), TPα-GFP is detected in reticular structures distributed throughout the cytoplasm consistent with the ER and with limited colocalization with the *cis*-Golgi complex marker. By contrast, in the presence of H_2O_2 treatment (lower), TPα-GFP is localized in a very restricted perinuclear region showing a colocalization with the *cis*-Golgi complex marker (yellow). These results confirm that oxidative stress induces a translocation of TPα to the Golgi complex in a similar manner to the TPβ isoform [21] and excludes a role for the longer C-terminal region of the β-isoform in response to peroxide.

the differential splice site are unlikely to have a role in TP translocation (see Fig. 1).

Oxidative stress is involved in the stabilization of the TPα

We also wished to determine if there was a similar stabilization of TPα in response to H_2O_2 as seen for TPβ, in addition to translocation to the Golgi complex as demonstrated above. At 24 h post-transfection, cycloheximide was used to abolish de novo protein synthesis, and TPα and β protein levels were determined by Western blotting using anti-c-myc antibodies (Fig. 3). In the absence of H_2O_2, the half-life of the TPα and β isoforms was essentially identical within the accuracy of

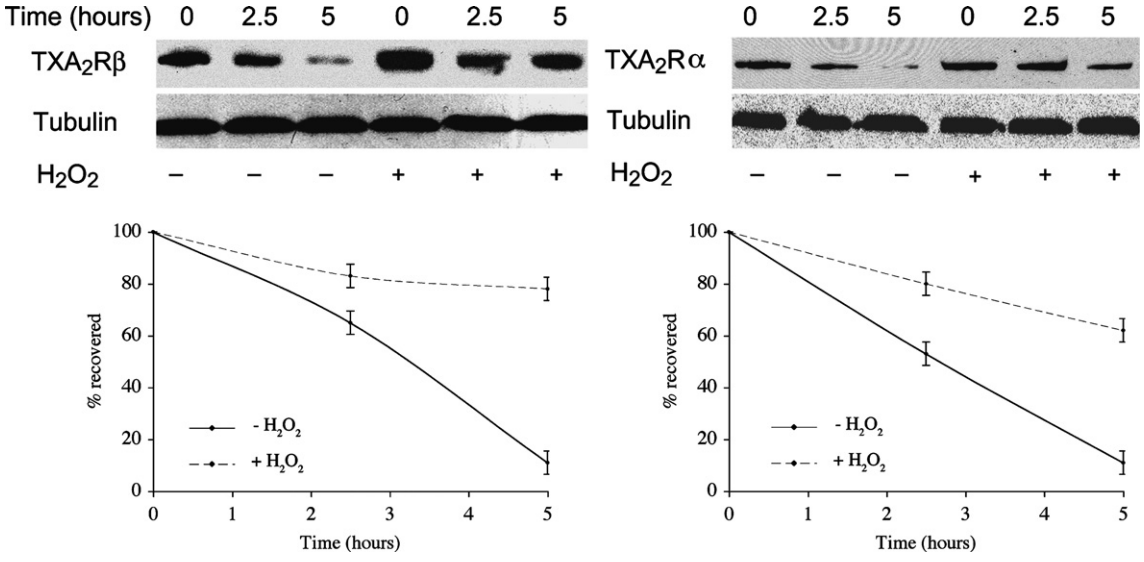

Fig. 3. Oxidative stress induces stabilization for both isoforms of TP. COS-7 cells were transfected with myc-tagged TPα or β isoforms, and cell homogenates were analyzed by immunoblotting using anti-myc antibodies. Kinetic analysis using cycloheximide (200 μg/ml, 2 h) was performed to investigate the effect of a H_2O_2 treatment (H_2O_2, 10 μM, 40 min) on the half-life of the TP isoforms. The upper panels show representative raw data, with tubulin used as a loading control. The lower panels show quantification of the data normalized at $t = 0$–100%. Data suggest that oxidative stress increases the half-life of TP for both isoforms, indicating that alternative splicing at the carboxyl terminal of the protein does not interfere with the receptor stabilization mechanism induced by oxidative stress. The data are representative of three experiments.

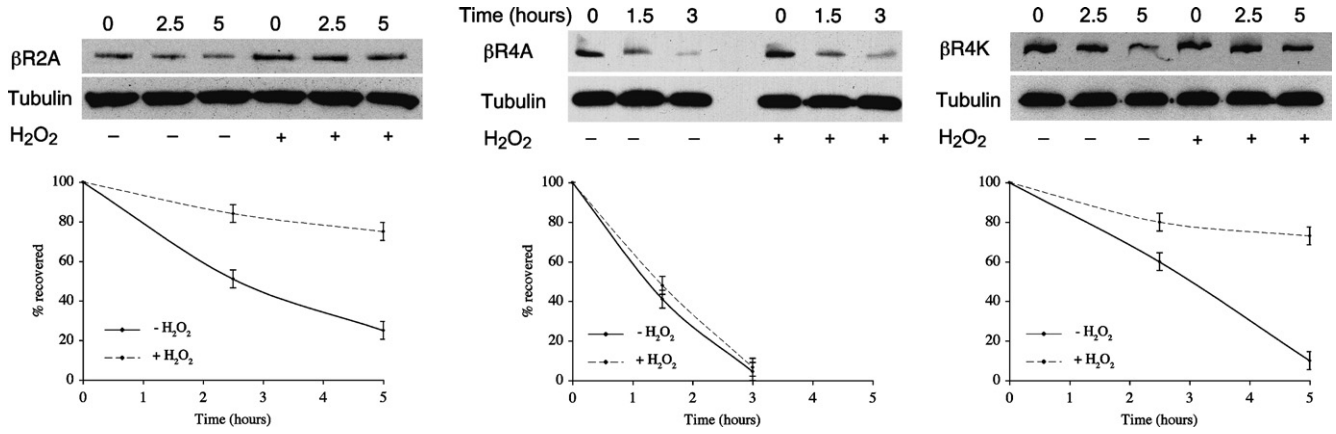

Fig. 4. Effect of mutation at the carboxyl terminal of the TPβ on oxidative stress-induced stabilization. COS-7 cells were transfected with the βR2A construct of TPβ, which has one arginine pair at the carboxyl-terminal domain replaced with alanine, the βR4A construct, which has two arginine pairs at the carboxyl-terminal domain replaced with alanine, and the βR4K construct, which replaces the same residues with lysine. Cell homogenates were analyzed by immunoblotting using anti-myc antibodies. Kinetic analysis using cycloheximide (200 μg/ml, 2 h) was performed to investigate the effect of a H_2O_2 treatment (H_2O_2, 10 μM, 40 min) on the half-life of TPβ. Raw data are represented with tubulin used as a loading control. Quantifications of the data are normalized at $t = 0$–100%. The data suggest that removal of one arginine pair does not affect the process of stabilization of TPβ induced by oxidative stress. The results also show that the presence of positive charged residues at the carboxyl terminal of the protein is crucial for the stabilization mechanism of TPβ. The data are representative of three experiments.

our analysis; 2.8 ± 0.3 h for the α isoform versus 3.2 ± 0.3 h for the β isoform ($n = 3$). In the presence of H_2O_2, TPα and β degradation occurred less rapidly than in the control cells. After 5 h, only $23 \pm 5\%$ and $31 \pm 5\%$ of the α and β isoforms were degraded ($n = 3$), suggesting that oxidative stress is able to mediate a very similar degree of stabilization for both TP isoforms. Hence, these data indicate that the different C-terminal regions of the TPα and β isoforms are unlikely to play a major role in translocation or stabilization of the thromboxane receptor following H_2O_2 challenge.

Charged amino acids within the TP C-terminus are required for oxidative stress-induced stabilization

We next tested the hypothesis that the charged residues in the cytoplasmic segment of the TP play a role in receptor stabilization. By PCR-mediated mutagenesis we created a number of mutants with altered charged amino acids in this region as well as distinct C-terminal tail sequences (Fig. 1). For this we took advantage of the presence of an alternative open reading frame encoded within the C-terminal region of the TP sequence. The resulting constructs therefore did not retain any of the unique features of the TPα or β isoforms but did express a C-terminal extension that was of intermediate length to the two natural splicoforms. We expressed each of these TP mutants in COS-7 cells and analysed their turnover in the presence and absence of oxidative stress (Fig. 4).

The mutagenesis of one pair of arginine residues to alanine (βR2A) did not affect the effect of oxidative stress on turnover. $82 \pm 4\%$ of βR2A remained after 2.5 h in presence of oxidative stress versus $81 \pm 5\%$ for the wild type receptor ($n = 3$) (Fig. 4). This also rigorously ruled out any role for the C-terminal sequences encoded by the natural TP splicoforms. By contrast, mutation of both arginine pairs (βR4A) decreased the half-life of the protein and ablated oxidative stress-induced stabilization. $20 \pm 4\%$ of βR4A remained after 2.5 h in the absence of oxidative stress versus $66 \pm 5\%$ for TPβ ($n = 3$). Further, βR4A was not stabilized following oxidative stress; $22 \pm 4\%$ of βR4A remained after 2.5 h in the presence of oxidative stress versus $83 \pm 4\%$ for TPβ ($n = 3$) (Fig. 4).

To determine whether receptor stabilization was the consequence of the specific presence of arginine residues or whether it was due to the presence of positive charges at the carboxyl-terminal domain, we mutated the four arginine residues to lysine (βR4K) and assessed the influence of these amino acid alterations on receptor turnover (Fig. 4). We observed that the presence of lysine restored the half-life of the protein not only in the absence of oxidative stress ($62 \pm 5\%$ ($n = 3$) of βR4K remained after 2.5 h) but also following oxidative challenge with H_2O_2 ($81 \pm 5\%$ ($n = 3$) of βR4K remained after 2.5 h; Fig. 4).

Discussion

Protein turnover is an essential mechanism for regulation of function, and includes roles in mitosis, transcription, development, and homeostasis. In a previous study, we validated the COS cell system as a model for analysis of TP function; specifically COS cells express low levels of an endogenous TP, and hence these cells are equipped for TP expression and function, and also

the location of the ectopic copy of the receptor is not affected by expression levels, ruling out over-expression artefacts [21]. We demonstrated that TPβ is a short-lived protein mainly located within the ER. Degradation of TPβ is effected by a process related to the UPR and ERAD. Experimental oxidative stress resulted in stabilization of the protein as well as translocation to the Golgi complex; both of these processes were inhibited by brefeldin A, suggesting that translocation was required for stabilization. The consequence of such stabilization is an increase of receptor density in the membrane fraction, indicating that the mechanism is likely of physiological relevance [22–24].

Because the carboxyl terminal appears critical in interactions of the receptor with cytoplasmic factors [15], we have compared the half-life of TPα and β in order to determine if the differential splicing affected behaviour or stability of the protein. Immunofluorescence microscopy revealed that in quiescent cells, TPα was located mainly in the ER compartment, and was translocated to the Golgi complex following H_2O_2 exposure as we have previously observed with the β isoform [21]. Therefore, both receptor splicoforms respond to oxidative stress in a similar manner. Further, complete substitution of the C-terminal sequence with a nonsense C-terminus corresponding to an alternative reading frame also did not affect the ability of TP to be stabilized by peroxide stress. It was initially thought that TPβ was expressed specifically in endothelial cells [5] while platelets expressed both TPα and TPβ [4], however, subsequent studies have shown that both TPα and TPβ are expressed in a wide range of tissues, though the relative expression of each splicoform varies considerably due to extensive differences in the level of TPβ expression [16]. The TP splicoforms also exhibit differences in signalling and patterns of expression, and it is therefore likely that they have distinct physiological or pathophysiological roles [25]. With this in mind, a difference in the response of each splicoform to oxidative stress might have been anticipated, but this was not the case.

We hypothesized that the residues between the last transmembrane region and the C-terminus of TPβ could be essential for the stabilization process, because this region contained a number of positively charged amino acids in a long stretch of uncharged residues. Removal of two arginine residues (βR2A) did not affect receptor stabilization induced by oxidative stress, but by contrast, the mutation of four arginines (βR4A) decreased the half-life of the protein and abolished the stabilization of TPβ induced by oxidative stress. Further, substitution of the arginine residues with lysines, conserving the positive charges in this region of the protein, restored the sensitivity of TPβ to oxidative stress. Hence, the presence of a cluster of four positive charges within the cytoplasmic C-terminal domain of TP is required for the response to H_2O_2. This result accounts for the similar behaviour of the TPα and β isoforms, as whilst the splice site interrupts the second arginine pair (Fig. 1), the conserved region includes six R residues in the β form and five in TPα. Presumably removal of four out of six positive charges is sufficient to disrupt any interaction with the stabilization machinery.

There are some strong parallels between the behaviour of TPβ and HMG CoA reductase [26], a polytopic ER membrane enzyme that catalyses the first committed step in the mevalonate/sterol biosynthetic pathway. The latter protein is subjected to a complex set of regulatory mechanisms that are highly responsive to cellular cholesterol levels. In cells with high cholesterol, HMG CoA reductase is unstable and rapidly degraded via a ubiquitin-dependent ERAD-related mechanism [27]. Under conditions of low cholesterol HMG CoA reductase is stabilized; the region required for stability is restricted to a portion of the molecule close to the C-terminus and including the final transmembrane domain (reviewed in [26]). Stabilization of HMG CoA reductase is also in part due to multimerization [28]. It is possible that TPβ is also stabilized by a similar mechanism; a role for ERAD is clearly established; the potential influence of oligomerization will require considerable further work. Further characterization of the molecular mechanisms of TPβ signalling, the potential for receptor oligomerization, and the mode of degradation may provide a means to manipulate the action of the receptor for therapeutic benefit.

Acknowledgments

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