Protease-activated receptor signalling, endocytic sorting and dysregulation in cancer

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Summary

Protease-activated receptors (PARs) are G-protein-coupled receptors (GPCRs) that are activated by a unique proteolytic mechanism. PARs play crucial roles in hemostasis and thrombosis, as well as in inflammation and vascular development. Coagulant proteases, which are generated at sites of vascular injury, act mainly through PARs to elicit signalling in a variety of cell types. Since PARs are irreversibly activated signalling must be tightly regulated. Desensitization and trafficking of proteolytically activated PARs control the magnitude, duration and spatial aspects of receptor signalling. Recent studies have revealed novel endocytic sorting mechanisms that regulate PAR signalling. PARs have also been implicated in tumor progression. PARs are overexpressed in several types of malignant cancer, transmit signals in response to tumor-generated proteases and promote tumor growth, invasion and metastasis. Recent work also indicates that matrix metalloprotease 1 (MMP-1) signals through PAR1 to promote tumor growth and invasion. In addition to PAR overexpression, tumor cells display aberrant PAR1 trafficking, which causes persistent signalling and cellular invasion. Thus, a novel type of gain-of-function in GPCR signalling in cancer can be acquired through dysregulation of receptor trafficking.

Key words: Arrestin, GPCR, Thrombin, Trafficking, Coagulant protease

Introduction

Protease-activated receptors (PARs) are G-protein-coupled receptors (GPCRs) that signal in response to extracellular proteases. There are four PARs encoded in the mammalian genome. PAR1, the prototype for this family, transmits cellular responses to thrombin, the main effector protease of the coagulation cascade. PAR3 and PAR4 also respond to thrombin, whereas PAR2 is activated by trypsin-like serine proteases but not by thrombin. PARs have important functions in hemostasis and thrombosis, as well as in inflammatory and proliferative responses triggered by vascular injury, a topic recently reviewed (Coughlin, 2005). The proteolytic nature of PAR activation, which results in irreversible activation, is distinct from most GPCRs. Thus, PAR signalling is tightly regulated by rapid desensitization[†] at the plasma membrane and by receptor trafficking.

PARs are overexpressed in several types of malignant cancer. Coagulant proteases and PARs can promote tumor growth, invasion and metastasis but precisely how PARs contribute to cancer progression is not known. The zinc-dependent matrix metalloprotease 1 (MMP-1), also known as interstitial collagenase, has recently been reported to promote tumor growth and invasion through activation of PAR1 (Boire et al., 2005), providing an important link between tumor-generated metalloproteases and PAR signalling. In addition to PAR overexpression, breast carcinoma cells display aberrant PAR1 trafficking, which causes persistent signalling and cellular invasion (Booden et al., 2004). These studies provide the first example of how aberrant trafficking may cause constitutive GPCR activation to promote tumor cell invasion. Here, we discuss activation of PARs, signal regulation and their dysregulation in cancer.

The PAR family

There are four PAR members, encoded by distinct genes in the GPCR superfamily, the largest family of signalling receptors in the mammalian genome. PAR1 was the first protease-activated receptor discovered: it was found in a search for a receptor that confers thrombin signalling on human platelets and other cell types (Rasmussen et al., 1991; Vu et al., 1991a). Hence, PAR1 was originally dubbed the thrombin receptor. PAR2 was then identified in a mouse genomic library screen using probes homologous to the transmembrane regions of the substance K receptor (Nystedt et al., 1994). The search for other PARs was prompted by studies of PAR1-knockout mice. Fibroblasts derived from PAR1-knockout mice show complete loss of thrombin signalling, whereas PAR1-null platelets respond normally to thrombin (Connolly et al., 1996). These unexpected findings led to the search for and identification of PAR3 and PAR4 and the discovery of species-specific differences in PAR expression in platelets (Ishihara et al., 1997; Kahn et al., 1998; Xu et al., 1998). PAR3 and PAR4 mediate thrombin signalling in mouse platelets, whereas PAR1 and PAR4 are the functional thrombin receptors in human platelets.

PARs have different but overlapping expression patterns. PAR1, PAR3 and PAR4 are expressed primarily by cells in the vasculature and are the major physiological effectors of thrombin signalling in vivo (Coughlin, 2000). However, other proteases can cleave and activate these receptors. PAR2 is

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[†]Desensitization is defined as a loss in the responsiveness of a signalling system

expressed by vascular, intestinal and airway cells and mediates inflammatory and proliferative responses associated with tissue injury. Multiple serine proteases can cleave and activate PAR2, including trypsin (Nystedt et al., 1994), mast cell tryptase (Molino et al., 1997) and coagulation factor (F) VIIa and FXa (Camerer et al., 2000; Riewald and Ruf, 2001) but not thrombin. In many cases, the particular protease that functions as the physiological regulator of PAR activation in a given cellular setting has not been clearly defined.

PAR activation

Proteases activate PARs by cleavage of an N-terminal peptide bond, which results in the formation of a new N-terminus that acts as a tethered ligand and binds intramolecularly to the receptor to trigger transmembrane signalling (Fig. 1) (Coughlin, 1999). Although typically proteolytic cleavage leads to the activation of the same receptor, there is evidence of crosstalk between different PARs. PAR3 binds to and localizes thrombin for activation of PAR4, a receptor that has a low affinity for thrombin (Nakanishi-Matsui et al., 2000). PAR3 is efficiently cleaved by thrombin but does not appear to signal on its own. PAR3 is expressed together with PAR1, but not PAR4, in human endothelial cells, and might modulate the activity of other PARs in certain cell types. Another type of crosstalk has been reported in human endothelial cells: the tethered ligand domain of signalling-defective cleaved PAR1 can transactivate PAR2 (O'Brien et al., 2000), although this mechanism is less efficient than intramolecular activation of the same receptor.

The activation mechanisms of PAR1 and PAR2 have been most extensively studied. PAR1 is activated mainly by thrombin and FXa, which are coagulant proteases formed during vascular injury. PAR3 and PAR4 are also cleaved by thrombin. Thrombin binds to and cleaves PARs with exquisite specificity, whereas other proteases interact with cell surface cofactors to facilitate PAR recognition and cleavage. The PAR1 N-terminal LDPR^{41}-S^{42} sequence, which has a basic residue in the P1 position, is essential for thrombin recognition and cleavage (Fig. 2) (Vu et al., 1991b). A second interaction between thrombin's anion-binding exosite‡ and an acidic region C-terminal to the PAR1 cleavage site increases its affinity for and remarkable potency towards PAR1. This highly acidic region is similar to a sequence present in the leech anticoagulant peptide hirudin (Rydel et al., 1990). Thrombin cleaves PAR1 at the R^{41}-S^{42} peptide bond, exposing the tethered ligand domain. Synthetic peptides that mimic the tethered ligand sequence can activate the receptor independently of proteolytic cleavage. In addition to thrombin, FXa can cleave and activate PAR1 as a monomer or in a complex with tissue factor (TF) and FVIIa (Camerer et al., 2000; Riewald and Ruf, 2001). TF, a single-span membrane protein, is the principal initiator of blood clotting, binds to FVII and supports both its activation and activation of FXa. FXa, together with its cofactor FVa, generates thrombin to initiate coagulation and PAR signalling in response to vascular injury and in thrombotic disease.

PAR1 is also cleaved and activated by the anticoagulant-proteases activated protein C (APC) and plasmin. Protein C,

the precursor of APC, circulates in the blood as a two-chain plasma glycoprotein and is cleaved and activated by thrombin bound to thrombomodulin on the endothelial cell surface. APC bound to its cofactor endothelial protein C receptor (EPCR), an integral membrane protein, can activate PAR1 (Riewald et al., 2002). Although APC and thrombin are both thought to proteolytically activate PAR1 in endothelial cells, they promote anti-inflammatory and pro-inflammatory responses, respectively (Feistritzer and Riewald, 2005). How activation of the same receptor by two different proteases elicits distinct cellular responses is not known. Plasmin cleaves PAR1 at multiple sites, which either activates or incapacitates the receptor, depending on the position of the cleavage site. At high concentrations, plasmin cleaves PAR1 at the R^{41}-S^{42} peptide bond, generating the N-terminal tethered ligand and consequently cellular signalling (Kuliopulos et al., 1999).

Recently, MMP-1 was reported to proteolytically activate PAR1. Boire et al. showed that the addition of purified MMP-1 to cells ectopically expressing PAR1 results in cleavage of the N-terminus and mobilization of intracellular Ca^{2+} (Boire et al., 2005). MMPs generally require hydrophobic residues at the P1′ position and prefer hydrophobic or basic residues at the P2′ position, which are present in the known PAR1 cleavage site (Fig. 2). However, a comprehensive analysis of MMP-1 substrates using peptide libraries failed to identify the PAR1 cleavage site as a compatible substrate (Turk et al., 2001). The ability of MMP-1 to cleave PAR1 may thus be facilitated by interaction with a cell surface cofactor, which could localize the protease to the cell surface and allosterically modulate its activity toward PAR1. Clearly, further research is needed to establish how MMP-1 acts on PAR1 to generate a functional ligand and/or signalling.

PAR2, the only PAR not activated by thrombin, is cleaved and activated by physiological concentrations of trypsin and mast cell tryptase. Cleavage of PAR2 at the N-terminal R^{34}-S^{35} peptide bond is responsible for proteolytic activation and initiation of receptor signalling (Fig. 2) (Nystedt et al., 1994). Synthetic peptides that mimic the tethered ligand domain can bypass proteolytic cleavage to activate PAR2. An N-linked glycosylation site is present in the N-terminus of PAR2 and appears to regulate signalling by tryptase but not trypsin or agonist peptide (Compton et al., 2002), which indicates that posttranslational modifications may confer protease specificity. In addition, cell surface cofactors may orient the protease and allosterically modulate its activity to favor PAR2 cleavage. FVIIa can activate PAR2 either directly in a complex with TF or indirectly through generation of FXa, which may signal more efficiently as a ternary TF-VIIa-Xa complex rather than as a monomer (Riewald and Ruf, 2001). Moreover, a recent study showed that a distinct cryptic form of TF stabilized by cleavage of a disulfide bond interacts with FVIIa but not FXa to elicit signalling through PAR2 (Ahamed et al., 2006). Several other proteases are also capable of cleaving and activating PAR2, as well as other PARs, but whether these proteases function as physiological regulators in vivo awaits validation. In addition, some proteases, including certain MMPs, disable PARs by cleaving downstream of the activation site, resulting in loss of the tethered ligand domain (Ludeman et al., 2004), and thus revealing a potentially important mechanism for regulation of PAR signalling in various cell types.

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‡An exosite is an additional substrate-binding site on a protease distinct from the catalytic core

Fig. 1. PAR1 activation and signalling. PAR1 is a seven-transmembrane-span G-protein-coupled receptor that is activated by a unique proteolytic mechanism. Thrombin (α-Th), a serine protease, binds to and cleaves the N-terminal exodomain of PAR1. The newly unmasked N-terminus of PAR1 then acts as a tethered ligand and binds intramolecularly to the receptor to trigger transmembrane signalling. Synthetic peptides that mimic the tethered ligand domain can activate PAR1 independently of proteolysis. PAR1 couples to $G\alpha_q$, $G\alpha_{12/13}$, $G\alpha_i$ and $G\beta\gamma$ to activate a variety of signalling cascades and cellular responses. The $G\alpha_{12/13}$ subunits bind RhoGEFs, which activate small G-proteins such as Rho. $G\alpha_q$ activates phospholipase Cβ, triggering phosphoinositide hydrolysis, resulting in inositol (1,4,5)-trisphosphate [Ins(1,4,5)P_3] and diacylglycerol (DAG) production and Ca^{2+} mobilization, protein kinase C (PKC) and MAP kinase activation. $G\beta\gamma$ can activate PI-3-kinase, G-protein-coupled receptor kinases (GRKs) and other effectors. PAR1 is uncoupled from G-protein signalling by rapid phosphorylation and arrestin binding.
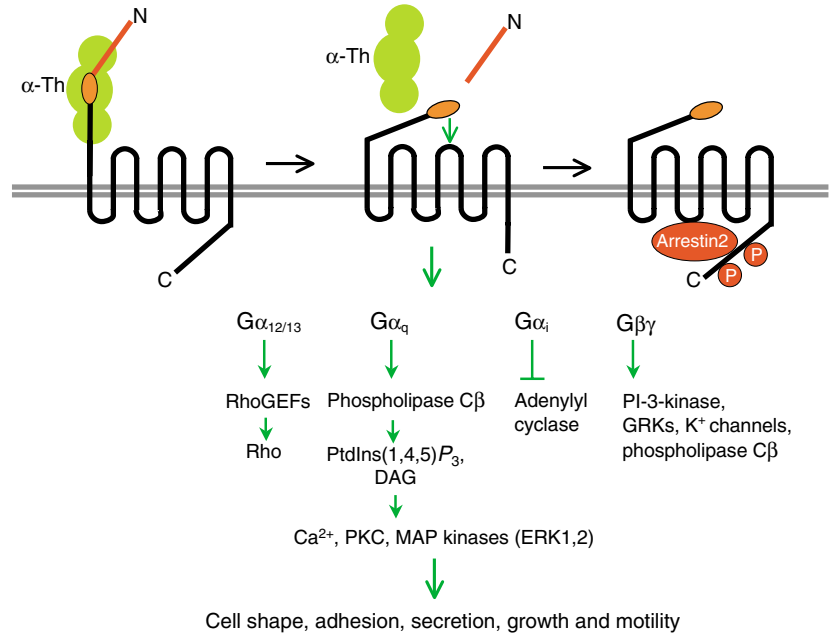
PAR signalling

Once activated, PARs are likely to undergo conformational changes to facilitate coupling to heterotrimeric G-proteins. PAR1 couples to the $G\alpha_q$, $G\alpha_i$ and $G\alpha_{12/13}$ subtypes and induces activation of MAP kinases, mobilization of intracellular Ca^{2+}, RhoGEF-mediated Rho and Rac signalling and regulation of other effectors to promote diverse cellular responses (Fig. 1) (Coughlin, 2005). PAR4 also appears to couple to $G\alpha_q$ and $G\alpha_{12/13}$, but not $G\alpha_i$, at least in fibroblasts (Faruqi et al., 2000), whereas PAR3 has not been shown to signal through G-proteins. Although there is no direct evidence linking PAR2 to G-protein activation, numerous studies show that PAR2-selective agonists increase second messenger responses suggestive of $G\alpha_q$, $G\alpha_i$ and perhaps $G\alpha_{12/13}$ signalling. In addition, activated PAR2 binds to and internalizes with arrestin, a multifunctional adaptor protein (DeFea et al., 2000; Stalheim et al., 2005). The interaction of internalized PAR2 with arrestin is thought to sustain ERK1/2 signalling in the cytoplasm independently of G-protein activation.

Activation of distinct G-protein subtypes and non-G-protein effectors by PARs is crucial for eliciting cell type-specific responses; however, the mechanisms responsible are not known. PAR1 appears to display differential G-protein coupling when activated proteolytically by its tethered ligand versus free peptide agonists (McLaughlin et al., 2005). Several GPCRs display such functional selectivity, and different conformational states of the activated receptor could couple selectively to distinct G-protein subtypes. The compartmentalization of PARs and G-proteins in plasma membrane lipid raft microdomains such as caveolae might also confer PAR–G-protein selectivity. This could explain why APC bound to EPCR is markedly less efficient at stimulating $G\alpha_q$-mediated

responses but promotes ERK1/2 signalling comparable to that elicited by thrombin (Ludeman et al., 2005; Riewald et al., 2002). Clearly, the molecular mechanisms that dictate PAR–G-protein selectivity deserve more research.

PAR signal regulation

The proteolytic activation of PARs is irreversible; thus, signalling must be tightly regulated. Desensitization of activated GPCRs occurs by rapid phosphorylation and arrestin binding, which promotes uncoupling of the receptor from G-protein signalling within seconds. GPCR internalization ensues within minutes and removes activated receptor from G-proteins and signalling effectors at the plasma membrane. Like most GPCRs, activated PAR1 is rapidly desensitized by

Fig. 2. Human PAR N-terminal and C-terminal sequences. Residues flanking the cleavage site specify protease recognition. These are defined as unprimed (P) and primed sites (P′), respectively (i.e. P3-P2-P1/P1′-P2′-P3′, where / indicates the cleavage site). Only transmembrane domain (TM) 1 and TM7 are shown. The number of N-terminal residues omitted before the cleavage site is shown in parentheses and the sequences of the tethered ligand domains are shown in yellow. The hirudin-like sequence in PAR1 and PAR3 responsible for high-affinity thrombin binding is underlined. The potential sites of PAR phosphorylation are shown in red and the PAR1 distal tyrosine-based motif important for µ2 binding is underlined. Asterisks indicate the end of the PAR sequences.
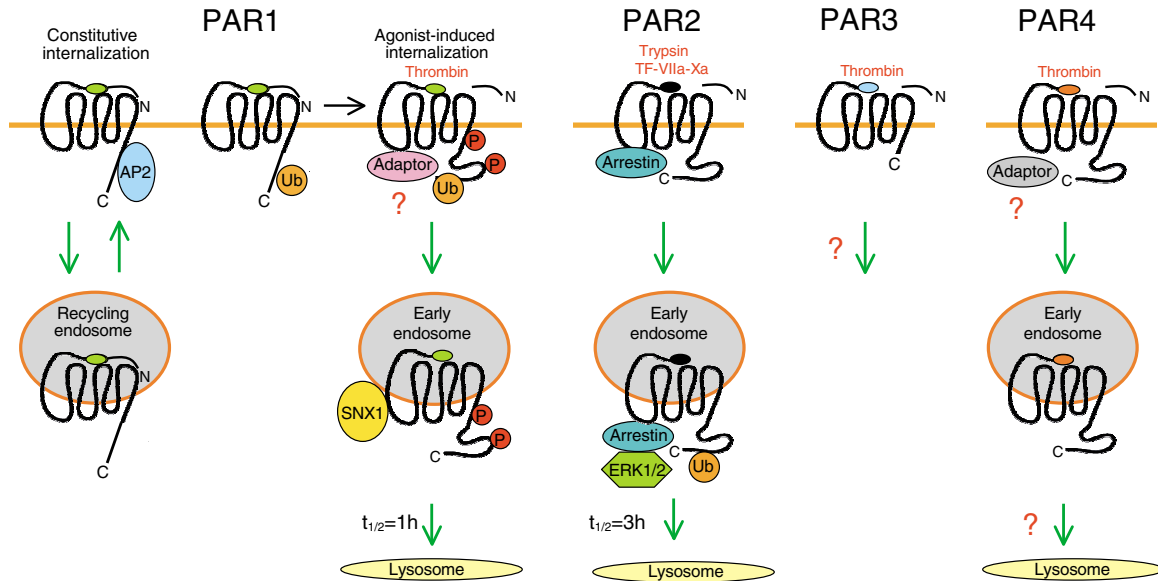
Fig. 3. PAR endocytic sorting. PAR1 displays both constitutive and agonist-induced internalization. Uncleaved PAR1 constitutively cycles between the plasma membrane and an intracellular compartment. The clathrin adaptor AP2, which binds to a tyrosine-based motif in the PAR1 cytoplasmic tail, mediates constitutive internalization. PAR1 is basally ubiquitylated. Agonist-induced internalization of PAR1 requires phosphorylation and ubiquitylation specifies internalization through clathrin-coated pits. The clathrin adaptor that mediates agonist-induced internalization of ubiquitylated PAR1 has not been identified. Internalized PAR1 is sorted from endosomes to lysosomes through a sorting nexin-1 (SNX1)-dependent pathway, which is independent of ubiquitylation and the ESCRT-1 machinery. Arrestins mediate PAR2 internalization. Internalized PAR2 bound to arrestins appear to signal to ERK1/2 from an endocytic compartment. Sorting of PAR2 from endosomes to lysosomes requires ubiquitylation. The sorting mechanism(s) and adaptor proteins that mediate PAR4 internalization and lysosomal sorting are not known, and whether PAR3 redistributes from the cell surface remains to be determined.

phosphorylation and arrestin binding (Fig. 1) (Ishii et al., 1994; Paing et al., 2002). However, unlike most GPCRs, which are internalized and then recycled, activated PAR1 is internalized, sorted directly to lysosomes and rapidly degraded independently of arrestins (Fig. 3) (Hoxie et al., 1993; Trejo and Coughlin, 1999). Internalization of activated PAR1 removes it from signalling effectors, whereas lysosomal sorting prevents it from returning to the cell surface and continuing to signal, terminating signalling (Trejo et al., 1998). Internalization of activated PAR4 also appears to be a mechanism for signal termination, but phosphorylation of the activated receptor has not been detected (Shapiro et al., 2000). A slower internalization rate and/or lack of phosphorylation of activated PAR4 are thought to promote sustained signalling, which is important for thrombin-stimulated platelet aggregation (Covic et al., 2000; Shapiro et al., 2000). The mechanisms that control PAR2 signalling are not clearly defined. On the basis of pharmacological inhibitor studies, a role for phosphorylation in shutting off PAR2 signalling has been suggested but not directly demonstrated, despite the abundance of multiple serine and threonine residues in the cytoplasmic tail (Fig. 2) (Bohm et al., 1996). However, arrestins do mediate desensitization and internalization of activated PAR2 (Fig. 3) (Stalheim et al., 2005). In addition to uncoupling PAR2 from G-proteins, arrestins appear to promote sustained ERK1/2 signalling by activated PAR2 in the cytoplasm (DeFea et al., 2000; Stalheim et al., 2005). The mechanism(s) that regulate signalling by internalized PAR2 are not known.

PAR1 displays two modes of trafficking important for regulation of signalling (Fig. 3). Uncleaved PAR1 cycles constitutively between the cell surface and an intracellular compartment, generating a protected receptor pool that replenishes the cell surface after protease exposure and leads to rapid resensitization independently of de novo receptor synthesis (Hein et al., 1994; Paing et al., 2006). Activated PAR1, by contrast, is internalized, sorted to lysosomes and degraded (Hoxie et al., 1993; Trejo and Coughlin, 1999). Constitutive internalization and agonist-induced internalization of PAR1 are clathrin- and dynamin-dependent (Trejo et al., 2000). However, in contrast to most GPCRs, neither constitutive nor activated PAR1 internalization requires arrestins (Paing et al., 2002). Arrestins interact with clathrin and clathrin adaptor protein complex 2 (AP2) to facilitate internalization of activated GPCRs through clathrin-coated pits (Goodman, et al., 1996; Laporte et al., 1999). Rather than arrestins, AP2 is crucial for PAR1 constitutive internalization and is essential for cellular resensitization to thrombin signalling (Paing et al., 2006). The μ2 subunit of AP2 binds directly to a tyrosine-based motif in the cytoplasmic tail of PAR1 (Figs 2 and 3). This motif and AP2 function are each crucial for maintaining an intracellular pool of receptors that replenish the cell surface with uncleaved PAR1 after protease exposure. Interestingly, internalization of activated PAR1 through clathrin-coated pits is not dependent on arrestins or AP2, which suggests that the two internalization routes are specified by distinct endocytic machineries. The internalization mechanism of activated PAR1 remains to be defined.

Arrestins are required for internalization of activated PAR2 in fibroblasts (Stalheim et al., 2005), a process that is dynamin-dependent (Roosterman et al., 2003). Dynamin, a large GTPase, promotes endocytosis by facilitating release of clathrin-coated pits or caveolae from the plasma membrane. Whether PAR2 is sequestered in clathrin-coated pits or caveolae or both remains to be determined. Interestingly, the recovery of uncleaved PAR2, unlike PAR1, to the cell surface after protease exposure does not involve constitutive cycling but is instead because of movement of naïve receptor from a pre-existing Golgi pool as well as de novo receptor synthesis (Dery et al., 1999). The mechanisms that regulate PAR4 internalization are not known, and whether cleaved PAR3 leaves the cell surface has not been determined.

The best-characterized route for lysosomal degradation of integral membrane proteins involves ubiquitin-dependent sorting by the ESCRT machinery (Katzmann et al., 2002). Ubiquitylation is involved in lysosomal sorting of the β_2-adrenergic and chemokine CXCR4 GPCRs (Marchese and Benovic, 2001; Shenoy et al., 2001). Recent work shows that PAR1 is basally ubiquitylated (B. L. Wolfe, A. Marchese and J.T., unpublished). However, in contrast to the β_2-adrenergic receptor and CXCR4, ubiquitylation of PAR1 is not required for lysosomal degradation since a non-ubiquitylatable PAR1 mutant is degraded comparably to the wild-type receptor in fibroblasts. Moreover, agonist-induced PAR1 lysosomal degradation is independent of Hrs and Tsg101 (Gullapalli et al., 2006), which promote assembly of a multiprotein ESCRT-I complex that sorts ubiquitylated cargo into the involuting membrane of multivesicular endosomes (Raiborg et al., 2003). The novel lysosomal sorting pathway for activated PAR1 does, however, involve sorting nexin 1, a phox homology (PX)-domain-containing protein that binds phosphoinositides and functions in membrane trafficking (Fig. 3) (Gullapalli et al., 2006). Sorting nexin 1 is not responsible for lysosomal sorting of all PARs since ubiquitylation of PAR2 mediates lysosomal sorting and degradation (Jacob et al., 2005) similarly to other GPCRs.

PARs and cancer

PARs elicit cellular responses to coagulant proteases triggered by vascular injury (Coughlin, 2005). In addition, coagulant proteases and PARs have been implicated in several types of malignant cancer. A link between hyperactivation of coagulation and tumor progression is well documented. In fact, tumors have been referred to as 'wounds that do not heal' since similar signalling pathways operate in wound healing and tumor progression (Bissell and Radisky, 2001). In contrast to wound healing, however, in cancer these signalling pathways contribute to tumor growth and metastasis.

Activation of coagulation can support tumor progression at multiple levels. Thrombin activates platelets and endothelial cells and cleaves fibrinogen to generate fibrin (Fig. 4). Tumor cells lodge in vascular sites with platelet and fibrin thrombi, which promotes tumor cell survival and metastasis (Camerer et al., 2004; Gasic et al., 1968; Palumbo et al., 2000; Rickles and Edwards, 1983). Fibrin also serves as a matrix for
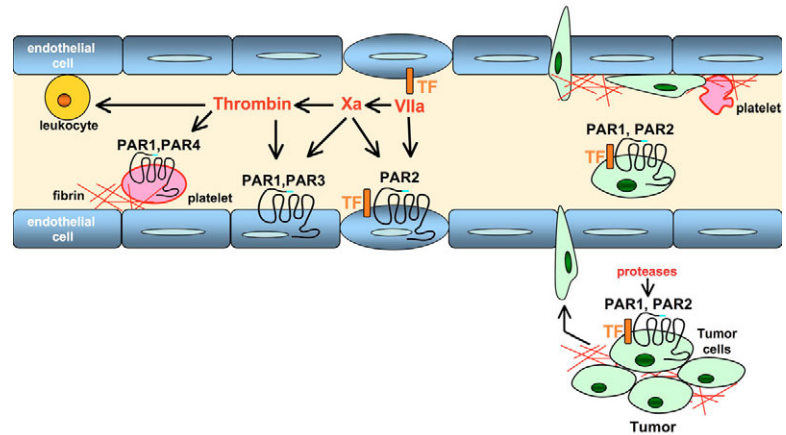

Fig. 4. PARs function in the vasculature and in tumor invasion and metastasis. PAR function is shown in the context of a blood vessel. Thrombin, the main effector protease of the coagulation cascade, activates PAR1, PAR3 and PAR4 to elicit signalling in a variety of cell types. Thrombin activates PAR1 and PAR4 in human platelets and generates fibrin, which is important for thrombus formation and tumor cell survival and metastasis. FXa can also cleave and activate PAR1. Activated endothelial cells express tissue factor (TF) and are represented by an oval shape. PAR2 is activated by TF-bound FVIIa and FXa but not by thrombin. PAR1 and PAR2 are also cleaved and activated by tumor-generated proteases, which contribute to tumor cell growth, invasion and metastasis.

metastatic colony formation as well as for angiogenesis. In addition, thrombin can promote tumor progression by acting directly on tumor cell-expressed PARs. PAR1 is overexpressed in aggressive melanoma (Tellez and Bar-Eli, 2003), colon cancer (Darmoul et al., 2003), prostate cancer (Chay et al., 2002) and invasive breast cancer (Even-Ram et al., 1998). The expression of PAR1 and PAR2 is also increased in stromal fibroblasts of malignant tissues but not those in normal or benign breast tissue specimens (D'Andrea et al., 2001). Overexpression of PAR1 transforms NIH 3T3 fibroblasts (Martin et al., 2001; Whitehead et al., 1995) and induces hyperplasia of mammary gland epithelial cells, an oncogenic phenotype (Yin et al., 2003a). Moreover, targeted overexpression of human PAR1 in mouse mammary glands activates the Wnt and β-catenin pathway, which is crucial for tumor progression in certain types of malignant cancer (Yin et al., 2006).

PARs also have the capacity to transduce signals in response to multiple tumor-generated proteases. Tumors are replete with proteases, including urokinase-plasminogen activator (uPA) and MMPs. Tumor cells upregulate uPA expression, which is associated with poor prognosis (Nicolai and Blasi, 2003). UPA binds to its cell surface receptor uPAR especially at the invasive front (Bissell and Radisky, 2001) and cleaves plasminogen to generate plasmin. Plasmin can proteolytically activate PAR1 (Kuliopulos et al., 1999). Plasmin also degrades extracellular matrix proteins and cleaves and activates MMPs. Reactive stromal cells produce several types of MMPs, including MMP-1, which can activate PAR1 (Boire et al., 2005). TF is also upregulated in many tumor cell types and supports FVIIa and FXa activity (Riewald and Ruf, 2002). TF bound to FVIIa can activate PAR2, whereas FXa can signal through either PAR1 or PAR2 (Fig. 4). In addition, FXa is responsible for proteolytic conversion of prothrombin to thrombin, the main effector

protease of PAR1. A recent study shows that tumor cells are able to synthesize and secrete FVII ectopically, indicating that coagulant proteases can be generated in tumors independently of their production in liver (Koizume et al., 2006).

PAR1 can promote tumor progression through a variety of mechanisms. Thrombin stimulates proliferation of colon cancer and melanoma cells (Darmoul et al., 2003; Tellez and Bar-Eli, 2003). The mitogenic activity of thrombin is mediated by PAR1 and is associated with prolonged ERK1/2 activation (Kahan et al., 1992; Trejo et al., 1996). Sustained ERK1/2 signalling not only promotes cell cycle progression but also contributes to cellular transformation, migration and survival. PAR1 promotes cell motility through a mechanism involving activation of the EGFR in renal carcinoma (Bergmann et al., 2006). In addition, PAR1 activates $\alpha v\beta 5$ integrin in melanoma to promote migration and invasion (Even-Ram et al., 2001). PAR1 may also contribute to tumor cell survival since it can prevent apoptosis of certain cell types (Guo et al., 2004; Zania et al., 2006). However, the role of PAR1 in tumor cell apoptosis has not been firmly established.

Other studies also suggest an important role for PAR1 in tumor growth and invasion. The expression of PAR1 in non-invasive MCF7 breast carcinoma is sufficient to promote growth and invasion in a xenograft nude mouse model (Boire et al., 2005). In addition, antisense- or RNAi-mediated reduction of PAR1 levels diminishes invasiveness of several breast carcinoma cell lines in vitro (Booden et al., 2004; Even-Ram et al., 1998; Morris et al., 2006). The ability of PAR1 to promote tumor growth and invasion could involve its regulation of angiogenesis. Approximately 50% of *Par1*-null mice die at midgestation owing to abnormal vascular development, whereas restoration of PAR1 expression in endothelial cells prevents death (Connolly et al., 1996; Griffin et al., 2001). Thus, PAR1 signalling in endothelial cells is important for blood vessel formation. Moreover, induction of PAR1 expression and signalling in melanoma or prostate carcinoma increases tumor growth, angiogenesis and vascular endothelial growth factor (VEGF) production (Yin et al., 2003b).

PAR1 signalling is also dysregulated in tumor cells. Activated PAR1 fails to be downregulated in highly invasive breast carcinoma, and consequently PAR1 persistently activates ERK1/2 and induces cellular invasion (Booden et al., 2004). One phenomenon contributing to persistent signalling is slowed receptor internalization and/or recycling and a lack of lysosomal degradation. These defects appear to be specific to breast cancer cells, because PAR1 ectopically expressed in normal human mammary epithelial cells displays proper trafficking and signal termination (Booden et al., 2004). Increased PAR1 levels in invasive carcinoma cells may thus be due, at least partially, to defective trafficking. Several other crucial mediators of tumor progression, including EGFR, HER2/ErbB2 and CXCR4, also display defective trafficking in breast carcinoma (Harari and Yarden, 2000; Li et al., 2004). Other mechanisms contributing to increased PAR1 mRNA levels and protein involve changes in gene transcription regulated in part by the transcription factor AP2 (Tellez et al., 2003). Although *Par1* mutations acquired during tumorigenesis could also lead to increased stability of the receptor or constitutive activity, we found no missense mutations in the *Par1* coding sequence in several breast carcinoma cell lines. The mechanisms responsible for defective PAR1 trafficking in invasive breast carcinoma remain to be defined.

There is increasing evidence that PAR2 is an important mediator of tumor progression. The levels of trypsin, a potent and physiological activator of PAR2, are elevated in gastric, colon, ovarian and lung tumors (Ducroc et al., 2002). TF is also linked to cancer progression (Belting et al., 2005). TF-FVIIa and FXa stimulate breast carcinoma cell migration and invasion through activation of PAR2 (Hjortoe et al., 2004; Morris et al., 2006). Induction of breast carcinoma cell migration by activated PAR2 involves arrestin-dependent ERK1/2 activation (Ge et al., 2004). In colon and gastric carcinoma, activated PAR2 stimulates EGFR activity, ERK1/2 signalling and cellular proliferation (Caruso et al., 2006; Darmoul et al., 2004). Interestingly, a TF mutant lacking its cytoplasmic tail domain enhances angiogenesis through a PAR2-dependent mechanism, which suggests that the TF cytoplasmic tail negatively regulates the proangiogenic actions of PAR2 (Belting et al., 2004). Activated PAR2 stimulates protein-kinase-C-mediated phosphorylation of the TF cytoplasmic tail, alleviating this inhibition, and thereby enhances angiogenesis and cell migration (Ahamed and Ruf, 2004; Belting et al., 2004). Thus, one can envision a scenario in which TF-VIIa-mediated activation of PAR2 causes dysregulated signalling and sustained TF cytoplasmic tail phosphorylation, resulting in aberrant tumor cell migration and angiogenesis.

Conclusions and perspectives

PARs are uniquely activated by proteolytic cleavage, which results in irreversible activation, unlike normal ligand-activated GPCRs. Thus, rapid desensitization and receptor trafficking tightly regulate PAR signalling. The temporal and spatial regulation of PAR signalling is crucial for a variety of biological responses, including proper growth and cell migration. Recent studies have advanced our understanding of the molecular mechanisms that control constitutive endocytosis of PAR1 and its importance in control of cellular resensitization. We will now need to explore the mechanism by which activated PAR1 is internalized, which is essential for signal termination. The mechanisms that control signalling and trafficking of other PARs have yet to be fully elucidated and are also vital to our understanding of coagulant protease signalling. Fundamental questions regarding the mechanisms that control PAR–G-protein selectivity also remain.

PARs are implicated in tumor progression but precisely how these receptors contribute to tumor cell growth, invasion and metastasis is not known. Thus, establishing this will be crucial to our understanding of the molecular basis of metastatic disease and for identifying new drug targets for cancer treatment. Proteases are viewed as essential for tumor progression and metastasis, but the development of protease inhibitors has been hampered by lack of specificity and associated toxic effects. Thus, a major challenge in the field is to identify specific protease effectors important for cancer progression. PARs are one class of protease effector implicated in tumor progression that might serve as ideal drug targets.

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