Review

Cancer Research

Membrane-Anchored Serine Proteases and Protease-Activated Receptor-2–Mediated Signaling: Co-Conspirators in Cancer Progression

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Abstract

Pericellular proteolysis provides a significant advantage to developing tumors through the ability to remodel the extracellular matrix, promote cell invasion and migration, and facilitate angiogenesis. Recent advances demonstrate that pericellular proteases can also communicate directly to cells by activation of a unique group of transmembrane G-protein–coupled receptors (GPCR) known as protease-activated receptors (PAR). In this review, we discuss the specific roles of one of four mammalian PARs, namely PAR-2, which is overexpressed in advanced stage tumors and is activated by trypsin-like serine proteases that are highly expressed or otherwise dysregulated in many cancers. We highlight recent insights into the ability of different protease agonists to bias PAR-2 signaling and the newly emerging evidence for an interplay between PAR-2 and membrane-anchored serine proteases, which may co-conspire to promote tumor progression and metastasis. Interfering with these pathways might provide unique opportunities for the development of new mechanism-based strategies for the treatment of advanced and metastatic cancers.

Introduction

G-protein–coupled receptors (GPCR) are a large family of cell surface receptors that react to extracellular molecules to activate internal signaling pathways, facilitating a wide range of physiologic responses (1). Dysregulation of GPCR functions and their ligands are linked to tumorigenesis, angiogenesis, and metastasis (2). A unique class of GPCRs, known as the protease-activated receptors or PARs, sense and respond to active proteases in the cell microenvironment (3, 4). Uniquely, the proteolytic nature of PAR activation is irreversible, distinct from many other GPCRs. The four PARs found in mammals are activated by various different protease agonists. PAR-1, PAR-3, and PAR-4 are main targets for the coagulation protease thrombin, orchestrating physiologic responses to vascular injury, thrombosis, and inflammation (5–9). PAR-2, on the other hand, is activated by trypsin, several trypsin-like serine proteases (3, 10, 11), and synthetic soluble PAR-2–activating peptides (12), signaling to various downstream pathways that modulate cell proliferation, migration and invasion, cytokine production, stimulation of angiogenesis, and other functions promoting tumor development (2).

This review concerns the roles of PAR-2 and a network of membrane-anchored serine proteases in cancer. There are several excellent comprehensive reviews of PARs in cancer and other diseases (13–16), as well as reviews on membrane-anchored serine proteases in development, tissue homeostasis, and tumor progression (17–21). Here, we focus on recent evidence in support of an interplay between PAR-2 and membrane-anchored serine proteases in proximity on the tumor cell surface that could significantly modulate the magnitude, duration, and nature of PAR-2 signaling, as well as restrict PAR-2 signaling to local membrane microdomains. Their overexpression and dysregulation in tumors have the potential to cooperate to promote aggressive disease through cell–surface interactions, integration of extracellular signals, and induction of intracellular signaling pathways.

Membrane-Anchored Serine Proteases

Unlike trypsin and other secreted, soluble serine proteases, members of the family of membrane-anchored serine proteases, are synthesized as catalytically inactive or near-inactive proenzymes (zymogens) that are converted into active serine proteases by proteolytic cleavage after an arginine or lysine amino acid residue that is positioned in a conserved activation motif within the catalytic domain (22). These proteases possess domains that tether the extracellular catalytic serine protease domain directly to the cell surface, allowing cleavage of cell surface and pericellular substrates (Fig. 1; refs. 19, 20, 22–24). The manner in which they are linked to the cell surface may be through type I or type II single-pass transmembrane domains or linked via glycophosphatidylinositol (GPI)-anchors. The serine protease domains of these enzymes are structurally highly conserved and contain a triad of amino acids (serine, histidine, and aspartate) required for catalytic activity (25). Overexpression of many of the 20 human members of this family has been documented in many cancers, and several membrane-anchored serine proteases have been shown to promote experimental malignant transformation when aberrantly expressed in tumor cells or in *in vivo* tumor models (21, 26). In this review, we will focus on those membrane-

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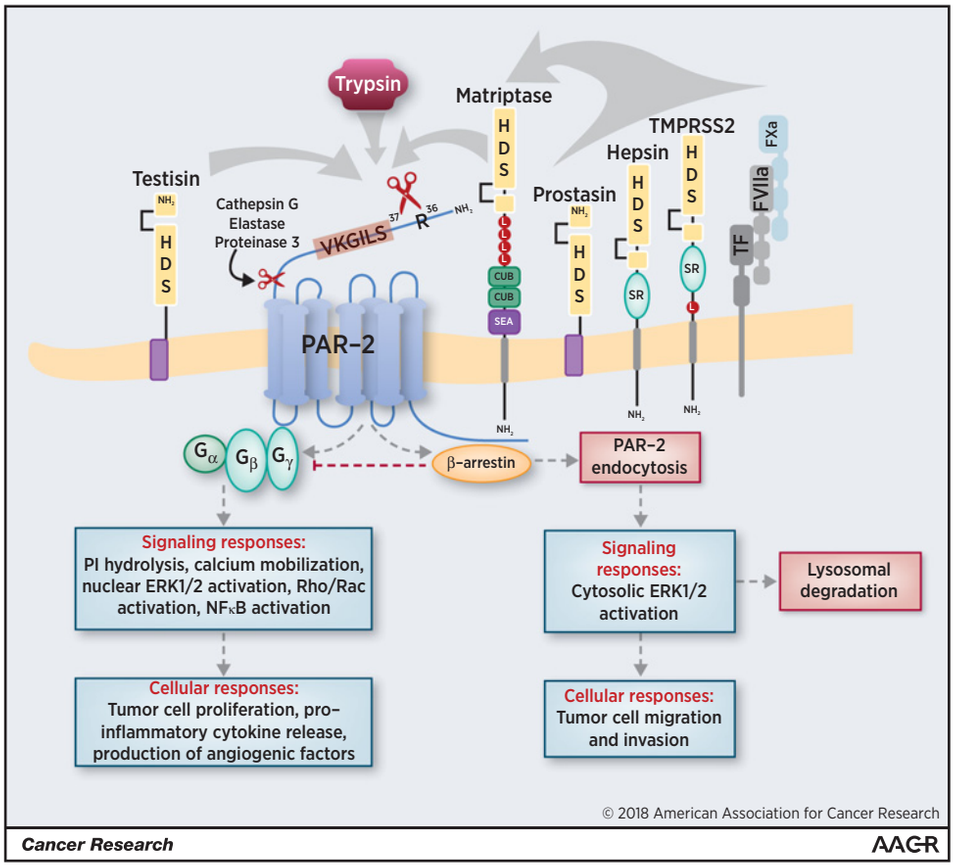

Figure 1.

Activation of PAR-2 by membrane-anchored and secreted serine proteases and implications in cancer. Human PAR-2 is cleaved by its various agonists on the cell surface at the canonical cleavage site, R^{36}, revealing the S^{37}LIGKV peptide sequence as a tethered ligand (red text). Membrane-anchored serine proteases are illustrated with their conserved catalytic domains containing the serine (S), aspartate (D), and histidine (H) residues, their respective extracellular domains [low-density lipoprotein receptor class A domains (indicated by red circles labeled "L"), Cls/Clr, urchin embryonic growth factor and bone morphogenic protein 1 (CUB) domains, sea urchin sperm protein, enterokinase, agrin (SEA), and group A scavenger receptor (SR) domains], as well as their respective membrane-tethering regions. Testisin and matriptase cleave PAR-2 directly at the trypsin cleavage site, whereas prostasin, hepsin, TMPRSS2, and TF:FVIIa/Xa complex have been shown to activate matriptase and thus indirectly activate PAR-2. Upon proteolytic cleavage, PAR-2 can couple to various G-proteins or once phosphorylated, bind to β-arrestin; both outcomes can activate subsequent signaling pathways and influencing tumor cell behavior. It is possible that various membrane-anchored serine proteases are capable of activating similar, overlapping, or distinct signaling responses to induce various cellular responses depending on the context.

anchored serine proteases that have been identified to date to be associated with tumor biology and linked to the PAR-2 signaling axis, namely matriptase, hepsin, prostasin, TMPRSS2, testisin, and the membrane-associated pathway triggered by tissue factor (TF), factor VIIa, and factor Xa (TF:FVIIa/FXa).

PAR-2 Signaling and Cancer

In the majority of studies to date, PAR-2 has been reported to have oncogenic activities, functioning as a positive regulator of tumor growth and/or progression. Initial evidence that PAR-2 may drive tumorigenesis came from experimental studies showing that PAR-2 indirectly enhances thrombin-dependent tumor cell migration and metastasis (27). Increased PAR-2 expression has been reported in a diverse set of human cancers such as breast, ovarian, prostate, and gastric cancer, when compared with normal patient tissue specimens (28–31). In addition, a recent survey of PAR family member expression in human tumor samples of various cancer types from The Cancer Genome Atlas (TCGA) and the Genotype-Tissue Expression projects (GTEx) reveals upregulated PAR-2 in 15 different cancer types compared with normal tissues (16). A global transcriptome array analysis of PAR expression in over 1,000 ovarian cancer and normal tissue samples showed that human epithelial ovarian cancers predominantly overexpress PAR-2, followed closely by PAR-1, with minimal detection of PAR-3 and PAR-4 (32). Consistent with this, increased PAR-2 is associated with poor prognosis and decreased progression-free and overall survival in patients with ovarian, cervical, and hepatocellular carcinoma (30, 33–35). Increased PAR-2 expression and activation is also correlated with the degree of invasiveness exhibited by both primary and metastatic tumors (29, 30, 36). Protumorigenic activities attributed to PAR-2 signaling include chemokinesis, cell proliferation, invasion and migration, inflammatory signaling, and increased angiogenesis (Fig. 1) in several tumor types including breast, oral, renal, pancreatic, gastric, lung, and esophageal cancers (36–39). PAR-2

may also modulate transactivation of other cell surface receptors (i.e., EGF, TGFβ, and Met tyrosine kinase receptors) that are frequently drivers of tumor progression (34, 40–42). In contrast, a few studies have demonstrated tumor-suppressive functions of PAR-2 (43, 44). In a DMBA-induced mouse model of skin carcinogenesis, PAR-2–deficient mice displayed increased tumor number and increased blood vessel infiltration, which was attributed to modulation of tumor-suppressing TGFβ-1 secretion (44). The specific protease agonists associated with the tumor-suppressive and oncogenic roles of PAR-2 in various tumor types are poorly characterized.

Protease-Stimulated PAR-2 Activation and Signaling

PARs are activated by a tethered ligand that is revealed by proteolytic cleavage of an N-terminal sequence and that can bind to an extracellular docking domain to cause a receptor conformational change that triggers intracellular signaling. There are several modes of regulation for PAR-2 activation and signaling. Different ligands can stabilize unique conformations of the cleaved PAR-2 that activate distinct signaling pathways, a phenomenon referred to as biased agonism or functional selectivity (45–47). A study of mutations within the tethered ligand sequence of PAR-2 (48) revealed that the nature of the tethered ligand sequence and the mode of its presentation to the receptor determine biased signaling by PAR-2. In addition, different proteases that cleave PAR-2 at distinct sites activate divergent patterns of receptor signaling and trafficking (reviewed in refs. 42, 47). Signaling outcomes are diverse and can activate pathways leading to release of proinflammatory cytokines and angiogenic factors, increased cell motility and migration, and increased inflammatory responses (Fig. 1; refs. 47, 49).

Activation of PAR-2 by trypsin and other soluble proteases has been most widely studied. Trypsin cleavage of PAR-2 involves hydrolysis at the canonical cleavage site $R^{36}\downarrow S^{37}$, which reveals the tethered ligand SLIGKV (human; ref. 50) or SLIGRL (mouse; ref. 51). The exposed tethered ligand interacts with the second extracellular domain of the cleaved receptor and can trigger MAP kinase/ERK1/2 activation, calcium mobilization via $G\alpha_q$ activation, cAMP formation via $G\alpha_s$ activation, and Rho-kinase activity via $G\alpha_{12/13}$ activation (Fig. 1; refs. 47, 49). The binding of β-arrestin to phosphorylated residues on the PAR-2 C-terminal tail uncouples and terminates G-protein signaling (52, 53), results in endocytosis of the complex (52), and mediates early endosomal signaling via scaffolding complexes containing Raf1 and activated cytosolic ERK1/2 (54).

β-Arrestins are not only active participants in signaling by internalized PAR-2, but can also direct receptor trafficking to regulate the duration and magnitude of PAR-2 signaling. PAR-2 signal termination occurs by direct ubiquitination and trafficking of PAR-2 to lysosomes for degradation by distinct components of the ESCRT machinery, a process that is unique to PAR-2 within the PAR family (42, 55, 56). The pathways that regulate β-arrestin–mediated signaling versus signal termination are incompletely understood.

The specific signaling pathways activated by PAR-2 in the context of cancer *in vivo* have received limited attention and will likely depend on the protease activator(s) and the (patho)biological context. For example, in murine asthma models, disease-promoting PAR-2 proinflammatory signaling is dependent on β-arrestin-2, whereas G-protein–dependent signaling is beneficial (57, 58). Several *in vitro*, *in vivo*, and human patient data suggest that dysregulation of β-arrestin expression, localization, and/or phosphorylation is associated with increased migration and invasion and ultimately poorer outcomes in various types of cancer (59, 60). This may be attributed not only to direct tumorigenic signaling through β-arrestin but also other selective pathways of PAR-2 signaling. The contributions of various G-proteins and β-arrestin signaling downstream of membrane-anchored serine protease activation of PAR-2 *in vivo* are not yet well characterized.

PAR-2 Activation by Matriptase

Numerous *in vitro* and *in vivo* studies identify matriptase (encoded by *ST14*) to be a potent activator of PAR-2. Matriptase (Fig. 1) is a type II single-pass transmembrane serine protease with a unique extracellular stem region containing various conserved protein-binding domains (SEA, CUB, and LDLR-a repeats), which are involved in matriptase activation as well as interaction with its cognate inhibitor hepatocyte growth factor activator (HAI)-1 (61–66) and other proteins. Matriptase is expressed as a precursor or zymogen form that may be proteolytically processed first within the SEA domain, and then activated by further cleavage at a highly conserved R↓VVGG motif by pericellular serine proteases or by autoactivation by matriptase itself (67). Interestingly, the zymogen form of matriptase, unusual among trypsin-like serine proteases, possesses measurable enzymatic activity and was recently shown to be capable of executing the *in vivo* developmental and homeostatic functions of the proteolytically processed protease (68).

Matriptase is widely expressed in normal epithelial tissues, where it plays a critical role in maintaining epithelial barrier homeostasis (69–72). Matriptase was first discovered in breast cancer cell lines (73), and its expression is associated with breast cancer progression (74, 75). Matriptase is also upregulated in many other tumors of epithelial origin, namely prostate, cervical, gastric, esophageal, renal cell, skin, oral squamous cell, ovarian, and cervical carcinomas (reviewed in refs. 76, 77). Upregulation of matriptase expression in many of these cancers is associated with poor outcomes (reviewed in ref. 21).

Substantial molecular and cellular data identify matriptase to be a direct proteolytic activator of PAR-2. Early studies using the human HaCaT transformed keratinocyte cell line, which endogenously expresses PAR-2, showed that treatment with soluble recombinant matriptase (protease domain only) stimulates canonical PAR-2 cleavage ($R^{36}\downarrow S^{37}$) and potent PI hydrolysis (78). In KOLF cells (which do not express endogenous PARs, matriptase, or hepsin), PI hydrolysis in response to recombinant matriptase was observed only upon PAR-2 transfection, indicating direct and specific PAR-2 activation (78). These findings have since been confirmed in several other *in vitro* studies using PAR-2–expressing KOLF or HEK293 cells and soluble or coexpressed matriptase (68, 79–82).

In vivo studies using murine transgenic models of matriptase and PAR-2 deficiencies have provided compelling evidence for a matriptase–PAR-2 signaling axis, specifically during normal embryonic development and placental barrier function (78, 83). Is it possible that a matriptase–PAR-2 signaling pathway regulates global epithelial integrity during homeostasis, and that this pathway becomes dysregulated in cancer? In support of this, PAR-2 has been shown to be critical for matriptase-mediated

tumor progression using several *in vivo* tumor models in which matriptase is overexpressed. In a transgenic mouse model of squamous cell carcinoma (SCC) where matriptase is overexpressed in the epidermis via a keratin-5 promoter (K5-matriptase), animals developed spontaneous multistage SCCs (84) and displayed protumorigenic inflammatory cytokine release that was PAR-2 dependent (81). The downstream effects of matriptase activation of PAR-2 were attributed to selective signaling through $G\alpha_i$ and NFκB-directed cytokine release (81). In this model, matriptase also induced the activation of a HGF/c-Met–dependent pathway, and both c-Met and PAR-2 signaling were independently required for tumor initiation (81).

Matriptase activity seems to be critical for the regulation of inflammatory signaling via the matriptase–PAR-2 axis. Matriptase expression and trafficking, activity, and shedding are controlled by Kunitz-type serine protease inhibitors, specifically HAI-1/*SPINT1* and HAI-2/*SPINT2*, and downregulation of these endogenous matriptase inhibitors increases aberrant matriptase activity (62, 85–88). Interestingly, the specific HAI required for proper matriptase trafficking is cell type dependent (reviewed in ref. 89). The ratio of matriptase to its inhibitors, or the protease–inhibitor balance, is important: loss of or decreased endogenous HAI-1 or HAI-2 enables increased matriptase activity and this has been shown to promote *in vitro* tumorigenesis in several studies (90–96). In human SCCs, increased matriptase expression is correlated with diminished expression of matriptase–HAI-1 complexes and with reduced PAR-2 expression (97), possibly due to PAR-2 overactivation induced by deregulated matriptase activity. The importance of protease–inhibitor balance has also been demonstrated *in vivo* in murine transgenic and xenograft models, where loss or decreased levels of endogenous HAI-1 or HAI-2 and increased matriptase activity promotes carcinogenesis, which can be effectively reversed by increased expression of the inhibitor (84, 98, 99). Recent studies also suggest that matriptase can function in a paracrine manner to activate PAR-2 (100). Pericellular matriptase activity on the surface of oral SCC, caused by insufficient HAI-1, was shown to activate PAR-2 on the surface of cancer-associated fibroblasts (CAF), leading to enhanced CAF migration and infiltration (100).

In human colon cancer, matriptase was originally designated as a tumor suppressor gene due to its loss of heterozygosity (101). Consistent with this, mice with tissue-specific deletion of matriptase in the gastrointestinal tract show increased intestinal permeability, spontaneously develop chronic colitis and ultimately inflammation-induced colon cancer (102). These data suggest that complete loss of matriptase can lead to colon carcinogenesis in the context of inflammation. Although matriptase expression may be downregulated in human colon cancer, additional studies using a specific antibody (A11) that targets active matriptase show that there is increased active matriptase in human colon cancer tissues and in a patient-derived colon cancer xenograft model (92, 103). The presence of increased active matriptase in human colon cancer is also supported by studies on the ratio of matriptase:HAI-1 levels, which suggest that although both matriptase and HAI-1 are downregulated during carcinogenesis, the matriptase:HAI-1 ratio increases during cancer progression, resulting in a population of active matriptase on the cell surface (90, 92). In support of this, the presence of HAI-1 in intestinal epithelium was shown to be protective in two murine models of intestinal carcinogenesis (104). Whether these sequelae are related to matriptase mediated–PAR-2 signaling is an unexplored area.

Activation of the Matriptase–PAR-2 Signaling Axis

Several other members of the membrane-anchored serine protease family were originally reported to functionally activate PAR-2 signaling, namely prostasin (encoded by *PRSS8*), hepsin (encoded by *HPN*), and TMPRSS2 (encoded by *TMPRSS2*). Recent studies indicate that these proteases indirectly trigger PAR-2 activation through the matriptase–PAR-2 axis (Fig. 1). Hepsin and TMPRSS2 are type II transmembrane serine proteases (18), while prostasin is anchored to the plasma membrane via a GPI anchor (105).

Hepsin was shown to indirectly activate PAR-2 and trigger PI hydrolysis only in the presence of matriptase in HaCaT cells. This was attenuated by a specific matriptase-blocking antibody, suggesting that hepsin is capable of activating the matriptase zymogen, which can then activate PAR-2 (78). In the same study, PAR-2 activation induced by recombinant prostasin was only observed in cells that also expressed catalytically active matriptase, indicating that prostasin is capable of functioning as an indirect activator of PAR-2 signaling via activation of the matriptase zymogen (78). TMPRSS2 was originally thought to activate PAR-2 directly, resulting in calcium mobilization in prostate cancer cell lines (106). In a later study, stable overexpression of TMPRSS2 in a variety of prostate cancer cell lines was shown to induce matriptase activation and to increase the metastasis of orthotopic xenografts (107). These results identify matriptase as a possible substrate of TMPRSS2 and indicate that like hepsin, TMPRSS2 activates the matriptase–PAR-2 axis.

The functional interactions between prostasin, matriptase, and PAR-2 activation have been most extensively studied; however, these interactions have proved to be complex and are still incompletely understood. In normal tissues, matriptase and prostasin are ubiquitously coexpressed in epithelial cells, whereas during the progression of multi-stage epithelial carcinogenesis, they are found to be expressed in separate tumor cell compartments, possibly indicating altered regulation or activation requirements during tumor progression (108). Results from several studies highlight the importance of tissue distribution and subcellular localization for the function and regulation of these two membrane anchored proteases in other disease contexts (108–111). It is possible that interactions between their extracellular domains as well as tightly regulated membrane distribution and subcellular localization all contribute to regulating the activation of the matriptase–PAR-2 axis as well. When ectopically expressed in the skin of transgenic mice, prostasin was shown to induce epidermal hyperplasia, ichthyosis, and inflammation, phenotypes that are completely negated when superimposed on a PAR-2–null background, establishing PAR-2 as a pivotal downstream mediator of prostasin inflammatory activity (112). Matriptase may be involved in this activity, because matriptase and prostasin are found to be capable of forming a reciprocal zymogen activation complex stimulating the activation of the zymogen forms of each other (80). The matriptase zymogen, which has a low rate of catalytic activity, has been shown to be capable of activating prostasin (80). Utilizing detailed cell-based analyses and genetically modified animals, Friis and colleagues (68) recently demonstrated that the matriptase zymogen can induce PAR-2 activation in the presence of prostasin, and that this activity requires catalytically active and membrane-anchored prostasin. This finding may indicate that matriptase zymogen–activated prostasin

can execute the activation site cleavage of PAR-2 directly or that the intrinsic catalytic activity of the matriptase zymogen is stimulated effectively by catalytically active prostasin (68).

Hepsin, prostasin, and TMPRSS2 upregulation in epithelial breast, prostate, and ovarian cancer cell lines, mouse models, and patient samples are believed to contribute to increased proliferation, tumor growth, metastasis, ascites formation, and various other invasive processes (107, 113–125). Overexpression of hepsin in prostate epithelium in a prostate tumor model (LPB-Tag mice) resulted in increased basement membrane disorganization and tumor metastasis to distant organs, which did not occur in control mice, indicating that hepsin is capable of promoting prostate cancer metastatic processes (119). Expression of TMPRSS2 is also associated with prostate cancer progression, as knockdown of TMPRSS2 in prostate cancer cell lines results in decreased invasion, tumor size, and incidence in xenograft models (107). In the transgenic adenocarcinoma mouse prostate (TRAMP) model of prostate cancer, mice with TMPRSS2 deficiency exhibited larger tumors but a lower incidence of distant metastasis (125). In contrast, prostasin, like matriptase, is reported to function as a tumor suppressor in colon cancer, with reduced expression correlating with more aggressive clinical stages and shorter patient survival time (114, 116). In many of these cancer contexts, it is not yet known whether the tumorigenic processes attributed to protease expression or activity occur via PAR-2 activation.

In addition to these membrane-anchored serine proteases, the membrane-localized coagulation complex containing TF:FVIIa/FXa was also originally reported to activate PAR-2 directly (126), but was later shown to trigger the activation of matriptase zymogen, which mediates the cleavage and activation of PAR-2 (82) (Fig. 1). In experimental studies using the spontaneous mammary tumor virus (MMTV) promoter-driven model of breast cancer in mice, the PAR-2–deficient phenotype was similar to that of mice with a truncated cytoplasmic domain of TF, suggesting an interplay between TF cytoplasmic domain signaling and PAR-2 in promoting breast cancer progression (127). Interestingly, in this study, PAR-2 deficiency led to a significant delay in the transition from adenomas to invasive carcinoma and was associated with less tumor vascularization and reduced immune cell infiltration. Reconstitution of the PAR-2–deficient tumor cells with PAR-2 mutated at the β-arrestin–binding site restored proangiogenic chemokine induction, tumor growth and increased vessel density (127), demonstrating that the tumor promoting activities of PAR-2 activation in this model were due to G-protein, rather than β-arrestin signaling. The involvement of matriptase in this tumor-associated TF–PAR-2 signaling activity is not known.

Testisin

Testisin (encoded by *PRSS21*) is a GPI-anchored membrane serine protease (128–130) that has been found to induce PAR-2 activation (Fig. 1). Exposure of PAR-2 overexpressing HeLa (human cervical carcinoma) cells to soluble recombinant testisin results in potent intracellular calcium mobilization, ERK1/2 phosphorylation, and NFκB activation (131). Furthermore, coexpression of testisin and PAR-2 results in inflammatory cytokine (IL8, IL6) induction and a decrease in PAR-2 surface expression via receptor internalization (131). The data indicate that testisin is capable of PAR-2 cleavage and activation affecting signaling responses important for tumor cell motility, proliferation, and inflammation. The potential involvement of matriptase or the matriptase zymogen in testisin-mediated PAR-2 activation is not known. Interestingly, testisin shows very limited normal tissue distribution, but is overexpressed in human epithelial ovarian, cervical, and lung carcinomas (132–134). Aberrant overexpression of testisin in epithelial ovarian tumor cells was shown to promote malignant transformation, increase tumor growth, and tumor formation in subcutaneous xenograft models (129). The involvement of PAR-2 activation in the *in vivo* tumor phenotypes is not known.

Other Mechanisms of PAR-2 Activation

Noncanonical PAR-2 cleavage

Cleavage of the N-terminal PAR-2 sequence by membrane-anchored serine proteases at a noncanonical cleavage site has not yet been reported. However, several soluble secreted proteases alter PAR-2 signaling responses via cleavage at noncanonical sites. Cleavage of PAR-2 at residues C-terminal to the tethered ligand domain sequence has the effect of removing the tethered ligand sequence and effectively "disarming" PAR-2 to prevent its activation by trypsin-like proteases and membrane-anchored serine proteases (Fig. 1). The neutrophil proteases cathepsin G and proteinase 3 cleave PAR-2 at $F^{65}\downarrow S^{66}$ and $V^{62}\downarrow D^{63}$, respectively, silencing trypsin-induced calcium mobilization and MAPK signaling, presumably by removing the tethered ligand sequence (135). Neutrophil elastase cleaves PAR-2 at $S^{68}\downarrow V^{69}$, which, unlike trypsin, does not induce calcium mobilization via $G\alpha_q$ activation, recruitment of β-arrestin or receptor internalization (135). On the other hand, neutrophil elastase does activate MAPK signaling independent of the tethered ligand binding, suggesting that it instead stabilizes a unique receptor conformation that favors a distinct signaling profile (135). Thus, in a protease-rich environment, noncanonical PAR-2 cleavage adds another level of complexity in PAR-2 signaling that would be expected to impact the activities of membrane anchored serine proteases in the context of cancer. Whether noncanonical protease activators significantly impact biased signaling that influences tumor cell behavior is an interesting area for future study.

Activation of PAR-2 by PAR-1

O'Brien and colleagues (136) were the first to show that the PAR-1–tethered ligand domain is capable of transactivating PAR-2, indicating a possible role for transactivation of PAR-2 induced by protease-mediated cleavage of PAR-1. Thrombin- and PAR-1–dependent migration of melanoma and prostate cancer cells was found to be dependent on indirect activation of PAR-2. This effect of thrombin on migration and chemokinesis required PAR-1 activation and transactivation of PAR-2 by the PAR-1–tethered ligand, independent of PAR-2 cleavage (27). *In vivo*, PAR-1 transactivation of PAR-2 was shown to contribute to altered responses in late stages of sepsis (137). This mechanism was further characterized and attributed to the formation of PAR-1–PAR-2 heterodimers. Thrombin was shown to cleave PAR-1 at its canonical site to reveal its tethered ligand, which can also bind to and activate PAR-2 (136). A PAR-1 cleavable but nonsignaling variant, with a mutation in the second extracellular ligand-binding domain, was shown to donate its cleaved tethered ligand to wild-type PAR-2, which triggered thrombin-mediated signaling in COS-7 cells. This suggests that transactivation of PAR-2 by the PAR-1–tethered ligand facilitates PAR-1–associated signaling when present as a

heterodimer (136). PAR-1–PAR-2 transactivation can furthermore recruit β-arrestin, cointernalize, and activate nuclear ERK1/2 signaling, all of which are different from the trafficking and signaling in response to PAR-1 activation alone (138). The impact and mechanisms by which PAR-1–PAR-2 heterodimers influence protumorigenic signaling is an understudied area but provides yet another pathway of biased PAR-2 signaling in response to indirect activation by thrombin, and perhaps other PAR-1–activating proteases, such as the cancer-associated protease plasmin and the coagulation protease FXa. To date, no membrane-anchored serine proteases are known to activate PAR-1 directly, but how the coexpression of PAR-2–activating membrane-anchored serine proteases may influence PAR-1–PAR-2 transactivation in these contexts has not been investigated.

Implications and Future Directions

Although considerable progress has been made in our understanding of mechanisms by which proteases and synthetic agonists activate PARs, there is much to learn about the interactions between membrane-anchored and soluble proteases, and specific protease–receptor interactions that influence PAR-2 signaling. This is important because PAR-2 is a signaling receptor with established links to cancer progression and metastasis, and whose protease-dependent activation can signal substantial changes in cell behavior. The emerging data implicate PAR-2 and membrane-anchored serine proteases in proximity on the tumor cell surface as co-conspirators in regulating the nature of PAR-2 signaling responses and as determinants of biased signaling in cancer.

Membrane anchored serine proteases may impact PAR-2 signaling bias in multiple and divergent ways. Biased PAR-2 signaling has mostly been studied with regard to cleavage at different sites by various protease agonists or antagonists. However, although membrane-anchored serine protease activators of PAR-2 identified to date cleave PAR-2 at the trypsin canonical site, other mechanisms that modulate differential PAR-2 activities are likely. The extracellular domains of membrane-anchored serine proteases (Fig. 1) offer unique opportunities for allosteric modulation of PAR-2, potentially by inducing conformational changes that can impart functional selectivity. It is also possible that cell surface and membrane localization may limit signaling responses to specific areas or local microdomains, whereas soluble proteases that cleave and activate PAR-2, such as trypsin, mast cell tryptase, kallikreins, and gingipains, would induce transient activation independent of surface location. In nontransformed cells, matriptase is localized to cell–cell junctions and basolateral surfaces of polarized epithelium (139), prostasin is found on apical membranes (111) and hepsin is associated with desmosomes (121), while testisin is found in lipid rafts (130). The influence of these protease specific localizations on PAR-2 activation and signaling responses is currently unknown. Furthermore, these distributions may be expected to significantly impact and be impacted by epithelial-to-mesenchymal and mesenchymal-to-epithelial transitions associated with tumor progression and metastasis.

PAR-2 present on tumor cells could be considered to act as a "protease sensor" of the tumor cell microenvironment, responding to circulating protease agonists that unmask the tethered ligand as well as circulating proteases that act indirectly as antagonists by "disarming" the receptor. Because membrane-anchored serine proteases are overexpressed and their inhibitors are downregulated in many cancers, these overactive proteases in direct contact with PAR-2 molecules on the cell membrane are poised to induce sustained signaling responses via continual surveillance of the cell membrane, activating PAR-2 present on the surface as well as newly synthesized receptors that repopulate the cell membrane in their vicinity. Such activities would not only affect the type, magnitude, and duration of signaling responses, but would also be expected to modulate canonical activation by soluble proteases in the cell environment. A lack of protease-specific inhibitors, activity assays, and other tools confounds studies aimed at teasing out these mechanistic details. These analyses are further complicated by proteolytic cleavage or "shedding" of protease domains from several of the membrane-anchored serine proteases, potentially releasing protease activity in a soluble form. The emerging evidence for membrane serine protease zymogens with functional activities adds another level of complexity. Undoubtedly, it will be a challenge to understand the factors that determine which proteases cleave PAR-2 in a protease-rich environment, which may be disease or cell-type dependent and will also depend on the local milieu of protease inhibitors present. Membrane-anchored serine protease–mediated allosteric modulation of PAR-2 signaling has not yet been investigated and could potentially facilitate tumor-suppressive or oncogenic activities depending on the tumor cell context.

One approach to interfering with the protumorigenic activities of membrane-anchored serine proteases is the development of specific inhibitors. This strategy has had limited success, likely because there is redundancy and/or overlap in the catalytic specificities and substrates in physiologic and pathologic contexts. An early approach to the development of inhibitors that showed promise was the targeting of selective conformational changes required for protease activity. The human recombinant antibody termed A11, developed against a protease surface loop of matriptase that is less conserved among trypsin-fold proteases, was shown to selectively recognize the active form of matriptase over the zymogen form and is a specific inhibitor of its activity (103, 140). Other strategies to inhibit matriptase activity include treatment with soluble recombinant HAI-1, synthetic small molecules, peptides, and mAbs, all of which have been shown to inhibit matriptase to varying degrees *in vitro* but do not address therapeutic limitations of specificity and optimal serum half-lives *in vivo* (141–144). A recent approach for inhibition of matriptase consists of an engineered variant of natural HAI-1, utilizing a Kunitz-domain 1/Kunitz-domain 2 chimera to replace the less-specific Kunitz-domain 2 of HAI-1, fused to an antibody Fc domain to increase putative binding sites to matriptase (144). This fusion protein was shown to inhibit matriptase activation of pro-HGF and matriptase activity on the surface of cancer cells (144). The activities of membrane-anchored serine proteases have also been exploited as functional biomarkers for imaging tumorigenesis. A11 has been used to visualize aberrant matriptase activity in epithelial tumors *in vivo* (92, 140), demonstrating a potential application in noninvasive tumor imaging and monitoring of disease progression. Fluorescent nanoparticles targeted against hepsin allosteric binding peptides have also been shown to bind specifically to hepsin-expressing LNCaP xenografts (145). Such reagents hold promise for the diagnostic detection and experimental manipulation of protease activities that contribute to disease progression, with potential clinical applications.

An alternative strategy is to potentially manipulate the functional selectivity of PAR-2 to develop novel cancer therapies. In the broader context of GPCR signaling, therapeutic strategies are now being aimed at shifting the signal bias in the appropriate direction to mitigate disease progression (146, 147). It is likely that membrane-anchored serine proteases in proximity to PAR-2 will be a critical consideration in the development of such therapies. Many attempts to develop PAR-2–biased agonists (148) and antagonists (15, 37) have been modeled on analogues of the PAR-2–activating peptide, some of which are capable of preferentially modulating Ca^{2+} or ERK1/2 signaling (148). Other small molecules and antibodies are being developed to target binding pockets and transmembrane regions, as well as allosteric sites on PAR-2, in an effort to prevent conformational changes required for receptor activation upon proteolytic cleavage or to prevent tethered ligand binding to the peptide-binding site (149–151).

Several hallmarks of aggressive cancer are a direct result of proteolytic activity, including tumor cell invasion into the stroma, angiogenesis, and metastasis (152), with the roles of proteases traditionally focused on protein degradation and extracellular matrix remodeling. Advances in our understanding of membrane-anchored serine proteases as modulators of PAR-2 activation and signaling are anticipated to uncover novel avenues for pharmaceutical intervention that could be used to selectively tune receptor activity for the treatment of cancer progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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