Protease-activated receptor 2 in colon cancer: Trypsin-induced MAP-kinase phosphorylation and cell proliferation is mediated by epidermal growth factor receptor transactivation

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Running title: PAR2 transactivates EGF receptor in colon cancer

The abbreviations used are: AP, activating peptide; BSA, bovine serum albumin; DMEM, Dulbecco’s modified Eagle medium; DMSO, dimethyl sulfoxide; EGF, epidermal growth factor; EGF-R, epidermal growth factor receptor; ELISA, enzyme-linked immunoassay; ERK, extracellular signal-regulated protein kinase; FCS, fetal calf serum; GPCR, G protein-coupled receptor; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; MMP, matrix-metalloproteinase; PAR, protease-activated receptor; PBS, phosphate-buffered saline; TGF-α, transforming growth factor-α; RTK, receptor tyrosine kinase

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Abstract

Several lines of evidence suggest that tumor-derived trypsin contributes to the growth and invasion of cancer cells. We have recently shown that the serine protease trypsin is a potent growth factor for colon cancer cells through activation of the G protein-coupled receptor protease-activated receptor 2 (PAR2). Here, we analyzed the signaling pathways downstream of PAR2 activation which lead to colon cancer cell proliferation in HT-29 cells. Our data are consistent with the following cascade of events upon activation of PAR2 by the serine protease trypsin or the specific PAR2 activating peptide AP2: i) a metalloproteinase (MMP)-dependent release of transforming growth factor (TGF-α) as demonstrated with TGF-α blocking antibodies and measurement of TGF-α in culture medium; ii) TGF-α-mediated activation of epidermal growth factor receptor (EGF-R) and subsequent EGF-R phosphorylation; iii) activation of ERK1/2 and subsequent cell proliferation. The links between these events is demonstrated by the fact that stimulation of cell proliferation and ERK1/2 upon activation of PAR2 is reversed by the metalloproteinase inhibitor batimastat, TGF-α neutralizing antibodies, EGF-R ligand binding domain blocking antibodies and the EGF-R tyrosine kinase inhibitors AG1478 and PD168393. Therefore transactivation of EGF-R appears to be a major mechanism whereby activation of PAR2 results in colon cancer cell growth. By using the Src tyrosine kinase inhibitor PP2, we further showed that Src plays a permissive role for PAR2-mediated ERK1/2 activation and cell proliferation, probably acting downstream of the EGF-R. These data explain how trypsin exerts robust trophic action on colon cancer cells and underline the critical role of EGF-R transactivation.
Introduction

Proteases have been increasingly recognized as important factors in pathophysiology of tumors diseases. Besides their contribution to cancer progression by the degradation of extracellular matrix proteins, there are now substantial evidences that certain proteases serve as signal molecules controlling cell functions through specific membrane receptors, the protease-activated receptors. PARs are seven transmembrane spanning domain G protein-coupled receptors comprising four receptors named PAR-1, PAR2, PAR3 and PAR4 (1, 2). Thrombin is the physiological activator of PAR-1, PAR3 and PAR4 whereas PAR2 is activated by multiple trypsin-like-enzymes including trypsin and mast cell tryptase but not by thrombin. The mechanism of activation of PARs was initially established for PAR1 (3) and seems to be a paradigm for other PARs (1, 2, 4). They are irreversibly activated by a proteolytic mechanism in which the protease binds to and cleaves the amino-terminal exodomain of the receptor. This cleavage generates a new N-terminal sequence that binds intramolecularly to the core receptor and serves as a tethered ligand. Synthetic activating peptides that mimic the tethered ligand domains of PAR1, PAR2, and PAR4 have been developed. The activation of PARs by these synthetic peptides APs is independent of receptor proteolysis (1, 2, 4).

The importance of trypsin, a major serine protease, has been evidenced more recently in many cancers including in digestive tract tumors. Extra-pancreatic production of trypsin was shown in ovarian (5), lung (6), gastric (7), colonic tumors (8) and also colon cancer cell lines (9, 10). In addition, overexpression of exogenous trypsinitogen cDNA in human gastric cancer cells increases their tumorigenicity in nude mice (7). Recently, it has been shown that down regulation of trypsin (11) or serine protease inhibitors suppress carcinogenesis in many different in vivo and in vitro assay systems (12, 13).

We have demonstrated that trypsin acting at PAR2 is a very robust growth factor for human colon cancer cells (14). These data supported the idea that some serine proteases should now be considered as crucial contributors to the development of human colon cancer.
However, the cellular pathways that transmit to the nucleus of colon cancer cells the proliferative signal induced by activation of PAR2 have not been elucidated yet. In this context, the purpose of the present study was to explore the signaling pathways downstream of PAR2 which lead to colon cancer cell proliferation. By using the human colon cancer cell line HT-29 as a model (14, 15), we showed that activation of PAR2 by its physiological activator trypsin or by the synthetic activating peptide AP2 results in a series of events that includes MMP-dependent release of TGF-α, transactivation of the EGF receptor and subsequent activation of ERK1/2. These data represent a breakthrough in the understanding of the role of PAR2 in controlling cell proliferation in colon cancer. They also highlight the role of the EGF receptor as a pivotal downstream integrator in the control of colon cancer cell proliferation by proteinase-activated receptors.
Experimental procedures

Reagents

The reagents were obtained from the following sources: activating peptides AP2 SLIGKV, Neosystem (Strasbourg, France); trypsin (16,000 Unit/mg), Sigma Chemical Co (St Louis, MO); the MEK inhibitor PD98059, the EGF-R tyrosine kinase specific inhibitors Tyrphostin AG1478 and PD168393 and Src inhibitor PP2, Calbiochem (San Diego, CA); batimastat (BB94), British Biotech (Oxford, UK); protein G-Sepharose beads, Amersham Pharmacia Biotech (Les Ulis, France). All other chemicals were from Interchim (Asnière, France).

Antibodies were purchased from the following vendors: phospho-specific antibodies to ERK1/2, New England biolabs (Beverly, MA); polyclonal anti-ERK1/2 antibodies, Santa Cruz Biotec (Santa Cruz, CA); rabbit anti-EGF-R used for immunoprecipitation and for immunoblotting of EGF-R, NeoMarkers (Fremont, CA); monoclonal neutralizing anti-EGF-R and monoclonal anti-phosphotyrosine (4G10), Upstate Biotech, (Lake Placid, NY); monoclonal neutralizing anti-TGF-α, Oncogene (Boston, MA). ELISA kit for TGF-α measurement was from R&D System (Minneapolis, MN).

Cell culture

HT-29 cells were routinely cultured in 25 cm² plastic flasks (Costar, Cambridge, MA) and maintained at 37°C in humidified atmosphere of 5% CO₂/air in DMEM (GIBCO, Grand Island, NY) containing 4.5 g glucose /liter, supplemented with 10% inactivated fetal calf serum (FCS) as described previously (15).

Cell proliferation assay

Determination of cellular proliferation was accomplished by direct cell count. 5,000 cells /well were seeded in 96 cluster wells (Costar) in the appropriate medium and allowed to attach for 3 days. They were starved in serum-free medium for 48 hours. Then 200 µl of a fresh serum-free medium, with or without PAR2 agonists (1 nM trypsin or 100 µM AP2 ) or EGF (6 nM) were added every other day for 4 days. In some experiments, HT-29 cells were
pre-incubated for 60 min with 3 µM AG1478, 2 µM PD168393 or 5 µM PP2, 30 min with 5 µM batimastat or 2 hours with the blocking antibodies for EGF-R or TGF-α prior to cell stimulation with the different PARs activators. After 96 hours of culture, cells were detached from triplicate wells by trypsin/EDTA and counted in a hemacytometer. Cell death was evaluated with trypan blue and the staining of cells treated with the indicated inhibitors revealed 99.9% viability, suggesting that these concentrations are not toxic to HT-29 cells.

**MAP-Kinase Western blots**

For extracellular-regulated protein kinase (ERK1/2) phosphorylation, cells were grown in 6 cluster wells (Costar) at 50% confluency and serum-deprived for 48 hours prior to their activation with 10 nM trypsin or 100 µM AP2 for 5 min. In some experiments, prior to the stimulation with the PAR2 ligands, cells were treated with PD98059 (25 µM), AG1478 (3 µM) or PP2 (10 µM), for 1 hour or with blocking antibodies for 2 hours. Cells were lysed with RIPA buffer (PBS, 1% (v/v) NP40, 0.5% (v/v) sodium deoxycholate and 0.1% (v/v) SDS) containing protease inhibitor cocktail and 1 mM sodium orthovanadate for 30 min at 4°C and lysates were centrifuged at 12,000 g for 15 min. Equal amounts of extracts (50 µg) were separated by SDS-PAGE and transferred to a nitrocellulose membrane which was incubated first with TBS buffer (Tris 20 mM, NaCl 500 mM) containing 5% (w/v) low fat milk and 0.1% (v/v) tween 20 and then with phospho-specific antibodies to ERK1/2 diluted 1:2000 overnight at 4°C that react with the activated, dual threonine and tyrosine phosphorylated forms of p42/p44-MAPK. Then blots were washed and incubated with the anti-IgG-peroxydase linked secondary antibody for 1 hour at room temperature before detection using chemiluminescent detection kit (NEN Life Science, Paris, France) and exposure to X ray films. The same membrane was reprobed with a polyclonal anti-ERK1/2 antibody (diluted 1:1000) which recognizes ERK1/2 regardless of its phosphorylation status and served as loading control.
EGF-R immunoprecipitation assay and Western blot

HT-29 cells were grown in 100 mm dishes at 50% confluency and serum-starved for 48 hours. Cells were then treated with PAR2 agonists (10 nM trypsin or 100 μM AP2) for 5 min at 37°C before being washed with cold PBS and lysed for 45 min on ice in the RIPA buffer containing Tris-HCl (50 mM), pH7.4, NaCl (150 mM), 1% (v/v) NP40, 0.25% (v/v) sodium deoxy-cholate, EGTA (1 mM), Na₃VO₄ (1 mM), NaF (1 mM) and proteases inhibitors. Cell lysates were cleared by spinning at 13,000 rpm for 10 min. EGF-Rs were precipitated overnight at 4°C from lysates with a polyclonal anti-EGF-R antibody coupled to protein G sepharose beads. The precipitates were separated on 7.5% SDS PAGE gel and transferred to nitrocellulose. Phosphorylated EGF-Rs were detected by Western blot with the anti-phospho-tyrosine monoclonal antibody 4G10. The total quantities of EGF-R were detected by Western blot with a polyclonal antibody against EGF-R. In some experiments, prior to the stimulation with the ligands, cells were pre-treated with AG1478 (3 μM), PP2 (10 μM) for 1 hour and were then stimulated with PAR2 agonists or EGF for 5 min.

TGF-α ELISA

HT-29 cells were seeded at 5,000 cells /well in 96 cluster wells (Costar) and allowed to attach for 3 days. They were starved in serum-free medium for 48 hours. To prevent the binding of released TGF-α to EGF-R, we pretreated cells with the anti-EGF-R binding domain antibody (10 μg/ml) for 2 hours prior to cell stimulation with PAR2 agonists. It was hypothesized (16) that this pretreatment increases the measurement of TGF-α in cell culture supernants. Cells were counted and the conditioned medium of different conditions was collected for measurement of TGF-α. ELISA for TGF-α was performed using commercially available Kit, according to the manufacture’s instructions.
Statistical analysis

All results are expressed as means ± S.E.M for a series of experiments. Differences between data were tested by Student’s t-tests for unpaired data. A P value of < 0.05 was considered statistically significant.
Results

Trypsin stimulates cell proliferation in HT-29 cells via ERK phosphorylation pathway

In previous studies we showed that trypsin acts as a growth factor for colon cancer cells through the activation of PAR2 (14). Since ERKs play a pivotal role in the pathway leading to growth factor-regulated proliferation, we investigated the implication of ERKs upon activation of PAR2. As shown in Figure 1A, pre-treatment of HT-29 cells with the MEK inhibitor PD98059 resulted in 80% decrease of cell proliferation induced by activation of PAR2 (10 nM trypsin or 100 µM AP2). Cell counts from control cells treated with PD98059 alone were comparable to those of diluent-treated cells. Further experiments showed that activation of PAR2 with trypsin or AP2 induced a significant phosphorylation of ERK1/2 (Figure 1B). In the presence of the MEK inhibitor PD98059, ERK1/2 phosphorylation induced by PAR2 agonists was completely inhibited (Figure 1B). These results clearly show that PAR2 agonists signal as growth factors for HT-29 cells through the MAPK pathway.

Activation of ERKs and cell proliferation induced by PAR2 agonists are attenuated by EGF-R tyrosine kinase inhibitors

We next determined whether PAR2-mediated ERKs activation is dependent on EGF-R kinase activity. As shown in figure 2, the EGF-R kinase inhibitor tyrphostin AG1478 (3µM) abrogated the effects of PAR2 agonists on ERKs phosphorylation in HT-29 cells. EGF was used as a positive control to show the specificity of the inhibitor (Figure 2). To ascertain the implication of ERGF-R kinase in mitogenic pathway induced by PAR2, we inhibited EGF-R kinase activity with specific inhibitors and measured cell proliferation. As shown in Figure 3A, AG1478 (3 µM) significantly reduced PAR2–mediated cell proliferation. Since inhibition with AG1478 was only partial, we used another inhibitor of the EGF-R tyrosine kinase which was shown to be irreversible and more selective i.e. PD168393 (17). As shown in Figure 3B, this inhibitor completely abolished PAR2–mediated cell proliferation. These data support the
involvement of EGF-R tyrosine kinase activity in ERK activation and subsequent proliferative responses induced by activation of PAR2.

**Activation of PAR2 promotes the EGF-R phosphorylation**

Since PAR2-mediated activation of ERK and cell proliferation could be influenced by inhibitors of the EGF-R tyrosine kinase, we sought to identify whether the activation of PAR2 results in EGF-R tyrosine phosphorylation. HT-29 cells were treated with trypsin, AP2 or EGF for 5 min and the cells were then lysed and assayed for tyrosine-specific phosphorylation of the EGF-R. The EGF-R in HT-29 cells became highly phosphorylated in response to EGF stimulation whereas PAR2 agonist-treatment resulted in moderate EGF-R tyrosine phosphorylation (Figure 4A). Further, we showed that inhibition of the EGF-R tyrosine kinase activity by AG1478 abolished the PAR2-dependent phosphorylation of the EGF-R in HT-29 cells (Figure 4B). As a control we checked that AG1478 also completely inhibited EGF-dependent phosphorylation of the EGF-R. It is concluded that activation of PAR2 leads to tyrosine phosphorylation of the EGF-R.

**Blockade of the EGF-R ligand binding domain inhibits PAR2-mediated effects on cell proliferation and ERK1/2 phosphorylation**

To determine the mechanism by which EGF-R is activated following the challenge of HT-29 cells with PAR2 agonists, we designed experiments to block the binding of EGF-R ligands to the extracellular domain of the EGF-R. We used a monoclonal antibody directed against the extracellular portion of the EGF-R. To avoid the bias of the proteolytic activity of the enzyme on the EGF-R antibodies, we performed these experiments only with PAR2 activating peptide AP2 and not with trypsin. As shown in Figure 5A, pre-incubation of HT-29 cells with the EGF-R blocking antibody reduced significantly AP2-induced HT-29 cell proliferation. In consonance with these data, pre-treating the cells with this antibody also inhibited AP2-stimulated ERK1/2 phosphorylation (Figure 5B). To test the efficiency of the antibody, we showed that the anti-EGF-R also inhibited EGF-induced ERK1/2 phosphorylation and cell proliferation (Figure 5). These results provide strong evidence that
PAR2-induced cell proliferation and signal transduction involve the binding of an EGF-R ligand to the EGF-R.

**A neutralizing antibody for TGF-α blocked PAR2-induced cell proliferation**

The above described data showed that inhibition of EGF-R phosphorylation and blockade of EGF-R ligand binding domain inhibit PAR2–induced ERK1/2 activation and subsequent cell proliferation. We next investigated whether EGF-R transactivation upon activation of PARs is mediated by TGF-α release, the most abundant member of the EGF-R family ligands found in normal colon and colon carcinomas (18). We first analyzed the involvement of TGF-α by blocking its biological activity with neutralizing antibodies. As shown in Figure 6A, treatment of HT-29 cells with TGF-α neutralizing antibodies strongly inhibited the AP2–induced cell proliferation. The TGF-α antibodies also had a small effect on control cells, suggesting that small amounts of TGF-α are constitutively secreted in the absence of PAR2 agonists. We tried to measure TGF-α under conditions in which TGF-α binding to the EGF-R was prevented by pre-incubating cells with EGF-R binding site antibodies (19). Results showed that AP2 significantly enhanced TGF-α release as compared to control cells (Figure 6B). These findings suggest that PAR2 transactivate the EGF-R by causing the extracellular release of TGF-α.

**PAR2-mediated ERK1/2 phosphorylation and cell proliferation involve MMPs.**

A mechanism whereby the TGF-α ligand is released is thought to be the shedding of membrane-bound precursor by metalloproteinases (20). Therefore, we tested the effect of the general metalloproteinases inhibitor batimastat in our model. As shown Figure 7, batimastat abrogated cell proliferation induced by either synthetic agonists of PAR2 (AP2) or by the serine protease trypsin. In contrast, batimastat had no effect on exogenous EGF- or TGF-α-induced cell proliferation (Figure 7A). Batimastat was also tested on ERK1/2 phosphorylation induced by PAR2 agonists (Figure 7B). The MMPs inhibitor dramatically decreased ERK1/2 phosphorylation caused by PAR2 activators (trypsin or AP2). As
expected, batimastat had no effect on EGF-induced ERK1/2 phosphorylation (Figure 7B). These studies suggest that EGF-R transactivation induced by PAR agonists involves MMP-mediated EGF-R ligand cleavage and correlates with our previous experiments which showed a reduction in AP2-induced cell number in the presence the TGF-α blocking antibody.

**Effect of Src inhibitor on PAR agonist-induced EGF-R phosphorylation, ERK1/2 activation and subsequent cell proliferation**

In addition to EGF-R activation, non receptor tyrosine kinases such as Src can be activated following stimulation of GPCRs (21). To examine the involvement of Src in PAR2-mediated responses in HT-29 cells, we utilized the Src tyrosine kinase inhibitor PP2. As shown in Figure 8, pre-treatment of HT-29 cells with 10 µM PP2 did not significantly reduce PAR2 agonist-induced EGF-R phosphorylation (Figure 8A). However, Src inhibition with PP2 significantly blocked ERK1/2 phosphorylation induced by PAR2 agonists in HT-29 cells (Figure 8B). In concordance with the MAPK effect, HT-29 cell proliferation induced by either synthetic agonist of PAR2, AP2 or by trypsin was dramatically decreased in the presence of 5 µM PP2 (Figure 8 C). In contrast, PP2 had only a slight effect, if any, on exogenous EGF-induced EGF-R phosphorylation, ERK1/2 phosphorylation and cell proliferation (Figure 8). Taken together, these results suggest that PAR2 mediate ERKs phosphorylation and cell proliferation by an EGF-R- and Src kinase-dependent pathway.
Discussion

We recently showed that trypsin, acting at protease-activated receptor PAR2, is a potent growth factor for human colon cancer cells (14). In the present study, we expand upon these findings to elucidate the mechanism(s) whereby PAR2 control colon cancer cell proliferation. We found that PAR2 agonists transactivate the epidermal growth factor receptor (EGF-R) through a pathway that includes matrix metalloproteinase-dependent cleavage and release of TGF-α which in turn activates the EGF-R and downstream MAPKs cascade leading to cell proliferation.

The different steps of the pathway leading to cell proliferation after activation of PARs have been analyzed using pharmacological enzyme inhibitors and neutralizing antibodies. Evidences support the involvement of ERK1/2 in PARs-mediated cell proliferation: i) the MEK inhibitor PD98059 abrogated cell proliferation stimulated by PAR2 agonists; ii) activation of PAR2 resulted in ERK1/2 phosphorylation. While the relation between ERKs activation and cell proliferation is well documented (22), the mechanism by which PAR2 activate MAP kinases (ERK1/2) was not known. The important finding of our study was to show that transactivation of EGF-R is an essential link between PAR2 activation and colon cancer cell proliferation. Indeed, blockade of EGF-R tyrosine kinase activity by AG 1478 or PD168393 and blockade of EGF-R binding domain by a specific antibody resulted in robust inhibition of both ERK1/2 phosphorylation and cell proliferation. This transactivation of the EGFR by PAR2 agonists has never been reported before in any cell system. Whether this mechanism can be extended to other cancers such as gastric carcinoma (23), melanoma (24), lung carcinoma (25), pancreatic cancer (26) or glioblastoma (27) in which trypsin promotes cell proliferation remains to be established.

In the present study, the fact that EGF-R tyrosine kinase inhibition and the blockade of extracellular ligand binding domain of the EGF-R result in similar abolition of PAR2-mediated HT-29 cell proliferation suggests that the binding of an extracellular ligand comes into play upon PAR2 activation. Our data show that TGF-α is such as an extracellular EGF-R
ligand. Indeed, incubating HT-29 cells with TGF-α neutralizing antibodies blocks PAR2 agonist-induced cell proliferation. Moreover, we could measure the increase of TGF-α concentration in the culture medium of PAR2 agonist-stimulated HT-29 cells. Amphiregulin, another EGF-R ligand has been shown to be also expressed in colon cancer (28, 29). Its involvement in PAR2-mediated EGF-R transactivation in HT-29 cells is unlikely for the following reasons: i) incubating HT-29 cells with neutralizing antibody against TGF-α in the culture medium completely blocks PAR2-induced cell proliferation; ii) neutralizing antibodies against bioactive amphiregulin did not alter the HT-29 cell proliferation induced by PAR2 agonists (Authors’ unpublished results).

The role of MMP-dependent cleavage of membrane spanning proforms of EGF-R ligands has been shown in GPCRs-mediated EGF-R transactivation (16, 30-34). The involvement of MMPs in the EGF-R transactivation by PAR2 in HT-29 cells is likely since the MMP inhibitor batimastat blocks the PARs-mediated ERK1/2 phosphorylation and cell proliferation. Among the diverse MMPs responsible for the release of EGF-R ligands (20, 35), TACE (Tumor necrosis factor Alpha-Converting Enzyme), a member of the transmembrane metalloproteinase-disintegrin family, has been reported to be a key enzyme for the release of TGF-α (36, 37). This is consistent with the recently described role of TACE in tumorigenesis (38) and GPCRs-induced cell proliferation (39). In our HT-29 cell model which expresses TACE, a selective inhibitor of TACE abrogated the PAR2-induced cell proliferation (Darmoul and Peiretti, personal observation). Further studies are in progress to explore the role of TACE in the progression of colon cancer induced by the activation of PAR2.

Previous reports have implicated Src kinase activity in the cellular responses to transactivated EGF-Rs (40-43). Our data show that PP2, a Src tyrosine kinase inhibitor, completely inhibited ERKS activation and subsequent cell proliferation stimulated by PAR2 agonists. In contrast, PP2 barely affected EGF-R autophorylation induced by PAR2 or EGF. This suggests that Src acts downstream of EGF-R in the PARs-stimulated pathway leading to
cell proliferation and that Src has a permissive role in mediating cell proliferation after EGF-R transactivation by PAR2. This permissive role is not observed when EGF-R is directly stimulated by EGF. Although the involvement of Src in transactivation of the EGF-R by GPCRs has been reported in various cellular models (30, 44-49), depending on the agonists Src may act upstream and/or downstream of the EGFR receptor (reviewed in (41, 43)). The role of Src as a crucial player in the signaling pathway(s) downstream of PAR2 should be considered in future studies.

Recently the role of serine proteases in tumor growth and progression of many cancers including colon cancer has been demonstrated (50). Elevated levels of trypsin, the main endogenous PAR2 activator, have been found in a variety of tumors including colorectal carcinomas (8, 9, 51). Trypsin can originate also from vessels in the vicinity of the tumors (52) or could diffuse from blood to tumour cells. Indeed, elevated trypsin levels were reported in the serum of patients with gastric carcinoma (53) suggesting that serum may be an important source of trypsin in cancerous patients.

Although trypsin expression has been reported in various carcinomas including colon cancers (5, 8, 51, 54-59), its role in colon cancer proliferation remained largely unknown until recently (14). PAR2 upregulation (60) together with trypsin-induced cell proliferation in human colon cancers (14) suggest that trypsin is involved in autocrine/paracrine loops within the colonic tumor resulting in tumor progression. On this basis, it was suggested that the development of selective and potent PAR2 antagonists and/or trypsin inhibitors may be useful for colorectal cancer treatment. In this context, it should be noted that serine protease inhibitors have been reported to suppress carcinogenesis in various organs including colon (13).

On the other hand, selective PAR2 antagonists have not been reported yet. Our present findings identifying the EGF-R transactivation as a central signaling element for trypsin-mediated colon cancer cell proliferation, suggest that EGF-R is a key therapeutical target in colon cancer even for the trypsin-induced colon cancer proliferation. This is in line
with several other observations supporting a central role of EGF-R in mediating colon cancer progression: i) trypsin inhibitor induces a marked decrease of EGF-R tyrosine phosphorylation (61); ii) ligands for two other GPCRs, the prostaglandin E2 receptor (30) and the M3 muscarinic receptor (62), also lead to colon cancer cell proliferation via EGF-R transactivation; iii) upregulation of the EGF-Rs and their ligands is frequent in colorectal tumors (18, 63); iv) inhibition of EGF-R signaling pathway impairs colonic tumor cell proliferation (64, 65).

In summary our data demonstrate that the serine protease trypsin acting on PAR2, triggers mitogenic signaling in colon cancer cells via EGF-R transactivation. This can explain why trypsin exert robust trophic action on colon cancer cells. These findings have potentially important implications regarding blocking EGF-R in colon cancer which are still underway in clinical trials (66).
References


Acknowledgements

We thank Dr. T. Lehy for critically reading the manuscript and Dr Tan Y.V. for illustrations help.
Legends to Figures

FIG. 1. Effect of the MEK inhibitor PD98059 on PAR2 agonist-induced HT-29 cell proliferation and ERK1/2 phosphorylation

Cells grown in serum-free medium were treated for 60 min with vehicle or PD98059 (25 µM) and PAR2 agonists were added to the medium. Cells were stimulated without (control) or with trypsin (1 nM) or AP2 (100 µM). After 96 hours, cells from triplicate wells were counted for each condition. Data are means ± S.E.M. Asterisks represent a significant reduction by PD98059 in trypsin- or AP2-stimulated cell proliferation. ***, p < at least 0.0003. In panel (B) cells were stimulated without (control) or with trypsin (10 nM) or AP2 (100 µM). Cells grown in serum-free medium were treated for 60 min with vehicle or PD98059 (25 µM) and then for 5 min with PAR2 agonists. Cells were stimulated without or with trypsin (10 nM) or AP2 (100 µM). Cell lysates were then directly analyzed by Western blot for ERK1/2 phosphorylation with antiphospho-ERK. Blots were subsequently stripped and reprobed with anti-ERK1/2 to verify equal protein loading between lanes. The blot is representative of three experiments.

FIG. 2. Effect of the EGF-R tyrosine kinase inhibitor AG1478 on PAR2 agonist-induced ERK1/2 phosphorylation in HT-29 cells

Cells grown in serum-free medium were treated for 60 min with vehicle or AG1478 (3 µM) and then for 5 min with PAR2 agonists or EGF. Cells were stimulated without or with trypsin (10 nM), AP2 (100 µM) or EGF (6 nM). Cell lysates were then directly analyzed by Western blot for ERK1/2 phosphorylation with antiphospho-ERK. Blots were subsequently stripped and reprobed with anti-ERK1/2 to verify equal protein loading between lanes. The blot is representative of three experiments.
FIG. 3. Effect of the EGF-R tyrosine kinase inhibitors AG1478 or PD168393 on PAR2 agonist-induced HT-29 cell proliferation

Cells grown in serum-free medium were treated for 60 min with vehicle, AG1478 (3 µM) or PD168393 (2 µM) and then PAR2 agonists or EGF were added in the medium. In Panel (A) cells were stimulated without or with trypsin (1 nM), AP2 (100 µM) or EGF (6 nM) in the presence or absence of AG1478. In Panel (B) cells were stimulated without or with trypsin (1 nM), AP2 (100 µM) or EGF (6 nM) in the presence or absence of PD168393. After 96 h, cells from triplicate wells were counted for each condition. Data are means ± SE. Asterisks represent a significant reduction by inhibitors in EGF-, trypsin- or AP2-stimulated cell proliferation. ***, p < 0.0001.

FIG. 4. Effect of the EGF-R tyrosine kinase inhibitor AG1478 on PAR2 agonist-induced EGF-R phosphorylation in HT-29 cells.

Cells grown in serum-free medium were stimulated without or with PAR2 agonists or EGF in the absence or presence of AG1478. In Panel (A) cells were stimulated for 5 min with trypsin (10 nM) or AP2 (100 µM) or EGF (6 nM). In Panel (B) cells were pre-treated with AG1478 (3 µM) for 60 min prior to 5 min stimulation with AP2 (100 µM), trypsin (10 nM) or EGF (6 nM). Cell lysates were then tested for EGF-R phosphorylation. The EGF-R was immunoprecipitated (IP) with a polyclonal anti-EGF-R and immunoprecipitated proteins were separated on 7.5% SDS PAGE gel and transferred to nitrocellulose. Phosphorylated EGF-Rs were detected by immunoblot (IB) with the anti-phospho-tyrosine monoclonal antibody. EGF-R phosphorylation after EGF treatment was tested as a control. EGF-R total levels after various treatments were then assessed by reprobing the blots with anti-EGF-R polyclonal antibody. EGF-R protein appears to be reduced in EGF-stimulated samples. This may be an artifact due to the immunoblotting steps since EGF-R is detected at similar levels when the immunoblot was directly probed with the anti-EGFR after immunoprecipitation (data not shown). It is suggested that the high signal with the anti-phosphotyrosine antibody prevents
subsequent anti-EGF-R antibody binding to the EGF-R epitope as previously observed (49). The figure shows representative immunoblots.

**FIG. 5. Effect of blocking antibodies against the extracellular binding domain of EGF-R on PAR2 agonist-induced cell proliferation and ERK1/2 phosphorylation**

Cells grown in serum-free medium were treated for 2 h without (control) or with EGF-R binding domain antibodies (10 μg/ml). In Panel (A) cells were then incubated without or with AP2 (100 μM) or EGF (6 nM). After 96 h, cells from triplicate wells were counted for each condition. Data are means ± SE. Asterisks represent a significant reduction by anti-EGF-R in AP2- or EGF-stimulated cell proliferation. ***, p < 0.0001. In Panel (B), cells were stimulated for 5 min with AP2 (100 μM) or EGF (6 nM) in the absence or the presence of the anti EGF-R blocking antibodies. Cell lysates were then directly analyzed for ERK1/2 phosphorylation with anti-phospho-ERK1/2. The blot was subsequently stripped and reprobed with anti-ERK1/2 to verify equal protein loading between lanes. The figure shows a representative immunoblot.

**FIG. 6. Effect of TGF-α neutralizing antibodies on PAR2 agonist-induced cell proliferation and effect of AP2 on TGF-α secretion in HT-29 cells.**

Cells grown in serum-free medium were treated for 2 h without (control) or with TGF-α neutralizing antibodies (10 μg/ml). In Panel (A) cells were then stimulated without or with AP2 (100 μM). After 96 h, cells from triplicate wells were counted for each condition of treatment. In panel (B) cells were pre-treated with an EGF-R binding domain antibody (10 μg/ml) for 2 h, prior to stimulation without or with AP2 (100 μM). Bathing medium were collected and analyzed for TGF-α contents using an ELISA kit. Data are means ± SE. *, p <0.05; ***, p <0.0001.
FIG. 7. Effect of the metalloproteinase inhibitor batimastat on PAR2 agonist-induced HT-29 cell proliferation and ERK1/2 phosphorylation

Cells grown in serum-free medium were treated for 30 min with vehicle or batimastat (5 μM). In panel (A) cells were incubated without (control) or with trypsin (10 nM), AP2 (100 μM), EGF (6 nM) or TGF-α (6 nM). After 96 h, cells from triplicate wells were counted for each condition. Data are means ± SE. Asterisks represent a significant reduction by batimastat in trypsin-, AP2-, EGF- or TGF-α-stimulated cell proliferation. *, p < 0.05; **, p = 0.01; ***, p < 0.0001. In panel (B) cells were stimulated without or with trypsin (10 nM), AP2 (100 μM) or EGF (6 nM). # represents differences between EGF or TGF-α versus control batimastat-treated cells. ###, p <.0001. Cell lysates were directly analyzed for ERK1/2 phosphorylation with anti-phospho-ERK1/2 antibodies. Blots were subsequently stripped and reprobed with anti-ERK1/2 to verify equal protein loading between lanes. The figure shows a representative immunoblot.

FIG. 8. Effect of the Src tyrosine kinase inhibitor PP2 on PAR2 agonist-induced EGF-R phosphorylation, ERK1/2 activation and HT-29 cell proliferation

Cells grown in serum-free medium were treated for 60 min with vehicle or PP2 (10 μM) prior to stimulation with PAR2 agonists or EGF. In panel (A) lysates from cells stimulated without (control) or with trypsin (10 nM), AP2 (100 μM) or EGF (6 nM) were tested for EGF-R phosphorylation. In panel (B) lysates from cells stimulated as in Panel (A) were directly analyzed by Western blot for ERK1/2 phosphorylation with anti-phospho-ERK1/2 antibodies. Total levels of EGF-R and ERK1/2 after various treatments were assessed by reprobing the blots in panel A and panel B with anti-EGF-R and with anti-ERK1/2 antibodies, respectively. In panel (C) cells were pretreated for 60 min with vehicle or PP2 (5 μM) and then stimulated without (control) or with trypsin (10 nM), AP2 (100 μM) or EGF (6 nM). After 96 h, cells from triplicate wells were counted for each condition. Data are means ± SE. Asterisks
represent a significant reduction by PP2 in control, trypsin-, AP2- or EGF-stimulated cell proliferation. *, \( p < 0.05 \); ***, \( p < 0.0001 \).
### A

**IP**: Anti-EGF-R  
**IB**: Anti-phospho-tyr

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### B

**IP**: Anti-EGF-R  
**IB**: Anti-phospho-tyr

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**A**

IP: Anti-EGF-R
IB: Anti-phospho-tyr

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**B**

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**C**

**Cell number/well (x10^3)**

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Significance:

- *: p < 0.05
- **: p < 0.001
Protease-activated receptor 2 in colon cancer: Trypsin-induced MAP-kinase phosphorylation and cell proliferation is mediated by epidermal growth factor receptor transactivation

Dalila Darmoul, Valérie Gratio, Hélène Devaud and Marc Laburthe

J. Biol. Chem. published online March 9, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M401430200

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