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Regulation of the p21Ras-MAP kinase pathway by Factor VIIa

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Abstract

In recent years it has become clear that factor VIIa (FVIIa) is not a passive mediator involved in the linear transduction of the coagulation cascade, but actively engages target cells to induce signal transduction and that this signal transduction fulfills critical functions in angiogenesis, arteriosclerosis and inflammatory processes. The details of coagulation factor-dependent signal transduction are among the least understood in biology and thus we set out to establish the molecular events responsible for MAP kinase activation induced by the interaction of FVIIa with its cellular binding partner tissue factor (TF). We observed that in both BHK and HaCaT cells FVIIa-induced MAP kinase activation correlates with p21Ras activation, and that this p21Ras activation is essential for FVIIa-induced MAP kinase activation. In BHK TF p21Ras activation was mediated by the activation of protein kinase C, whereas stimulation of the Src kinase family mediated FVIIa-dependent p21Ras activation in HaCaT cells. Thus p21Ras activation is instrumental in FVIIa signal transduction and the FVIIa-dependent activation of p21Ras involves either PKC or Src-dependent mechanisms, maybe as a consequence of the cell type-specific expression FVIIa:TF-targeted secondary receptors.
Tissue factor (TF) was originally known for its role in blood coagulation; upon binding of TF to the zymogen FVII, the FVIIa:TF complex induces the formation of a blood clot via the sequential formation of FXa, thrombin and fibrin (1). Research, however, performed in the last decade demonstrated an important role for TF in inflammation, sepsis, angiogenesis and metastasis (2-4). Furthermore, direct correlations between TF expression and advanced stages of malignancy have been observed in breast cancer, colorectal cancer, pancreatic cancer and glioma, suggesting a possible role for TF in tumor development (5-8). It is now believed that FVIIa-induced intracellular signaling on TF expressing cells is important for processes such as angiogenesis and inflammation. Nevertheless, coagulation factor-evoked signal transduction in general and FVIIa-dependent signaling in particular remain among the least understood signaling cascades in physiology. It has become clear, however, that FVIIa:TF interaction results in cytoskeletal rearrangements and in gene transcription (9-11). The latter may lead to synthesis of proteins, such as IL-8, which are potent inducers of inflammation and angiogenesis (12).

One of the major pathways triggered by the FVIIa:TF complex is the Mitogen-Activated Protein (MAP) Kinase pathway. The MAP kinase family consists of several members, among which p42/p44 MAP kinase, p38 MAP kinase and c-Jun-N-terminal kinase are the most prominent. Although not all members of MAP kinase family are activated in the various cells that react to FVIIa-stimulation, p42/p44 MAP kinase activation is consistently observed in all TF-expressing cell types investigated (9,13,14). Furthermore it has been shown that this p42/p44 MAP kinase activation mediates important downstream effects, like e.g. p90 Rsk stimulation (15). Strikingly, the nature of the upstream signal transducers that mediate p42/p44 MAP kinase activation remains unclear; some researchers have suggested the involvement of so-called protease-activated receptors (PARs) (16). These G-protein-coupled receptors which are activated upon proteolytic cleavage of the N-terminal part, consist of four known members; PAR-1 is activated by thrombin and FXa, whereas PAR-3 and -4 are exclusively activated by thrombin. Finally, PAR-2 is activated by both FXa and FVIIa. The involvement of PARs as receptors in FVIIa-induced signaling, however, has been questioned by other groups that have found no evidence for involvement of any of the known PARs in BHK cells stably transfected with TF (17).
Ras is a 21 kDa small G-protein that is mutated in 30% of all human tumors, and therefore it is believed to play a key role in cell transformation. p21Ras activation will typically result in MAP kinase activation, but is not strictly essential for this process. The involvement of p21Ras in FVIIa-induced MAP kinase activation has not been addressed as yet. This prompted us to investigate the potential of FVIIa to induce p21Ras activation in two different cell types. We show a transient activation of p21Ras upon FVIIa-stimulation in BHK\textsuperscript{TF} cells and in spontaneously immortalized keratinocytes (HaCaT). Interestingly, regulation of p21Ras activation differs between these two cell types; whereas p21Ras activation in HaCaT is dependent on Src-like kinase activity, p21Ras activation in BHK\textsuperscript{TF} cells is dependent on Protein Kinase C (PKC) activation and another yet to be identified signal transducer. We hypothesize that this differential activation of p21Ras may reflect the involvement of different receptors in the cell types investigated.
Experimental procedures

Reagents- All phosphospecific antibodies were purchased from Cell Signaling Technologies (Beverly, MA), whereas antibodies against total MAP kinase were from Santa Cruz. The Ras activation kit and the antibody directed against p21Ras was from Upstate Biotech Inc. (Waltham, MA) and the monoclonal HA antibody was from Sigma (St. Louis, MO, USA). PP1 and GF-109203X were from Biomol and LY294002 was from Alexis (Montreal, Canada). Effectene transfection reagent was obtained from Qiagen (Westburg, Leusden, The Netherlands). Recombinant FVIIa was from Novo Nordisk (Bagsvaerd, Denmark). Hirudin was purchased from Calbiochem and TAP was a kind gift from Dr. George Vlasuk (Corvas International, San Diego, CA). Tissue Culture material was from Greiner Bio-one (Alphen a/d Rijn, The Netherlands).

Cell lines- Wild type Baby Hamster Kidney (BHK) cells, BHK cells stably transfected with a construct, expressing full length TF (BHK$_{TF}$; a gift from Dr Lars C. Petersen, Novo Nordisk, Maaloev, Denmark) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco) containing 10% fetal calf serum (FCS), 100 IU/ml penicillin, and 100 µg/ml streptomycin, in a humidified environment at 37°C. HaCaT human keratinocytes were cultured likewise. Prior to experiments, BHK$_{TF}$ cells were serum starved in DMEM for 16-18 hrs, whereas HaCaT cells were starved for 24 hrs. Half an hour before stimulation, cells were treated with the PI3-kinase inhibitor LY294002 (10 µM), the Src-inhibitor PP1 (10 µM) or the PKC inhibitor GF-109203X (10 µM).

Ras activation assay- The p21Ras activation assay was performed according to the protocol supplied by the manufacturer. Briefly, cells, grown in 10 mm culture plates were stimulated with FVIIa and subsequently washed with cold PBS. The cells were lysed in 1 ml lysisbuffer (25 mM HEPES (pH 7.5), 150 mM NaCl, 1% NP-40, 10 mM MgCl2, 1mM EDTA, 10% glycerol) and samples were taken for analysis of MAP kinase phosphorylation. Subsequently, cell debris was spun down at 14,000 rpm for 2 min and the supernatant was incubated with 10 ml 50% agarose beads, coated with the Raf GTP-ras-binding domain for 30 min at 4°C. Subsequently, the beads were washed three times with lysis buffer and the beads were resuspended in denaturing sample buffer (125 mM Tris/HCl, pH 6.8; 4% SDS; 2% b- mercaptoethanol; 20% glycerol, 1 mg/ml bromophenol blue).
Transfection and immunoprecipitation- Cells, seeded in 6-well plates, were transfected with Effectene, according to the manufacturer's protocol. Per condition, 0.2 mg pcDNA3-HA-ERK1 (HA-p44) with either 0.2 mg vector pRSV-RasN17 (a gift from Dr. Paul Coffer, Department of Pulmonary Diseases, Utrecht University) or empty vector was co-transfected. After overnight incubation, the cells were serum starved for 16 h. and subsequently stimulated for the times indicated. The cells were lysed in 0.5 ml lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na₃VO₄, 1 μg/ml leupeptin) and were collected by scraping. Subsequently, the lysate was centrifuged at 14,000 rpm for 2 min, and the supernatant was precleared with 20 ml 50% protein A-sepharose for 1h. The lysate was then incubated overnight with 1 mg HA antibody. The immuno complex was precipitated with 20 ml 50% protein A-sepharose for 1h. The beads were washed three times with lysis buffer and were subsequently resuspended in 40 ml denaturing sample buffer.

Western Blotting- The samples were incubated for 5 min at 95°C, after which 30 ml of the lysates were loaded onto SDS-PAGE and subsequently transferred to a PVDF membrane. The membranes were blocked with Tris-buffered saline (TBS) supplemented with 0.1% tween-20 (wash buffer) and 2% low-fat milk powder, and incubated with primary antibodies over night at 4°C, diluted 1:1000 in wash buffer. Subsequently, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody in wash buffer, containing 2% low-fat milk powder. The bands were visualized, using Lumilight plus® ECL substrate from Roche and a chemiluminescence detector with a cooled CCD-camera (Genegnome) from Syngene. Antibody bands were quantified using Genetools from Syngene.
Results

FVIIa induces p21Ras activation in BHK\textsuperscript{TF} and HaCaT cells- FVIIa mediates activation of MAP kinase, but the molecular pathways mediating this effect are unclear. For instance, the involvement of p21Ras in FVIIa-dependent p42/p44 MAP kinase activation has not been addressed. We investigated the potential of FVIIa to induce p21Ras activation in BHK cells stably transfected with TF. To this end we made use of an experimental system that relies on the specific interaction between the p21Ras-binding domain of Raf and activated p21Ras (18). In this assay the p21Ras-binding domain is linked to agarose beads which are used to precipitate active GTP-bound p21Ras, without co-precipitating the inactive GDP-bound form. BHK\textsuperscript{TF} cells were stimulated with FVIIa for various times and p21Ras activation was analyzed. As evident from Fig. 1A, FVIIa induced a fast, transient activation of p21Ras within 5 min. As a control, also total p21Ras levels were analyzed and these were not affected by FVIIa stimulation (Fig 1A). The transient p21Ras activation correlated with the onset of p42/p44 MAP kinase activation, which was measured in the same samples using a phosphospecific antibody and Western Blotting (Fig 1B). We also investigated FVIIa-induced p21Ras activation in HaCaT cells, a cell type that endogenously expresses TF. As shown in

![Fig 1: FVIIa induces p21Ras activation in BHK\textsuperscript{TF} cells. Cells were stimulated for the times indicated with 100 nM FVIIa. After lysis, the amount of active p21Ras was determined as described in the methods section in BHK\textsuperscript{TF} cells (A) and HaCaT cells (C). Prior to p21Ras analysis, samples were taken for total p21Ras. In the same samples, phosphorylated MAP kinase in BHK\textsuperscript{TF} cells (B) and HaCaT cells (D) was analyzed.](image-url)
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Fig. 1C, also this cell type displays transient p21Ras activation that returns to basal levels after 30 min, and again this correlated with p42/p44 MAP kinase phosphorylation (Fig 1D). We concluded that a p21Ras activation that occurs concomitant with p42/p44 MAP kinase stimulation is a general feature of FVIIa-induced signal transduction.

Fig. 2: FVIIa-induced p21Ras activation is independent of FXa or thrombin generation. BHK^{TF} (A) and HaCaT cells (B) were preincubated with 200 nM Tick Anti-coagulant Protein (TAP) or 25 U Hirudin (Hir) for 30 min. Subsequently, cells were stimulated with 100 nM FVIIa for 5' and 15' respectively. p21Ras activation was assayed as described in the Experimental Methods. Prior to p21Ras analysis, samples were taken for total p21Ras. In the same samples, phosphorylated MAP kinase in BHK^{TF} cells and HaCaT cells was analyzed. (C) To validate the potency of our TAP and hirudin, BHK^{TF} cells were treated with TAP and hirudin as described before and were subsequently stimulated with the indicated concentrations of FXa and thrombin. The Western Blots represent three independent experiments.
FVIIa induces p21Ras activation, independently from FXa or thrombin generation- As FVIIa:TF-mediated FXa and thrombin generation have been known to interfere with FVIIa-induced signal transduction, we determined the requirement for the formation of either FXa or thrombin for FVIIa-induced p21Ras activation. Hence, we pretreated cells with inhibitors to FXa and thrombin, Tick Anti-coagulant Protein (TAP) and hirudin respectively, before stimulating BHK-TF with FVIIa for 5' and stimulating HaCaT cells for 10'. Pretreatment of the cells with the inhibitors, did not influence either FVIIa-induced p21Ras activation or p42/p44 MAP kinase phosphorylation in either BHK-TF (Fig 2A) or HaCaT cells (Fig 2B), whereas TAP and hirudin potently inhibited FXa- and thrombin-induced MAP kinase activation, respectively (Fig 2C). Therefore, FVIIa-dependent FXa and thrombin generation do not contribute to FVIIa-induced p21Ras activation and p42/p44 MAP kinase stimulation in our experimental set up.

Ras activity is essential for FVIIa-induced MAP kinase activation- To address the issue as to whether activation of the MAP kinase pathway is actually dependent on p21Ras or whether this p21Ras activation is a coincidental phenomenon, we co-transfected cells with HA-tagged p44 MAP kinase and p21RasN17, an inactive p21Ras mutant. After stimulation, HA-p44 was immunoprecipitated, using a HA-antibody, and HA-p44 was analyzed for phosphorylation status on Western Blot. Both in BHK-TF (Fig. 3A) and HaCaT cells (Fig. 3B), co-transfection of p21RasN17 resulted in diminished phosphorylation of HA-p44. Therefore, we conclude

![Fig. 3: FVIIa-induced MAP kinase activation requires functional p21Ras. BHK-TF and HaCaT were co-transfected with p21RasN17 and HA-tagged p44 MAP kinase. After serum starvation, BHK-TF cells were stimulated for 10' (A) and HaCaT cells were stimulated for 20' (B), after which HA-tagged p44 was precipitated and analyzed for phosphorylation on Western Blot. Total HA-tagged p44 was determined using an antibody against the HA-tag.](image-url)
that FVIIa-stimulated p42/p44 MAP kinase phosphorylation is dependent on functional p21Ras and thus that the p21Ras activation observed after FVIIa addition to cells is instrumental for p42/p44 MAP kinase stimulation.

**Ras activation in HaCaT cells is mediated by Src-** In A14 fibroblasts, Src-like kinases mediate FVIIa-provoked MAP kinase activation (9). Hence we tested the involvement of Src-like kinases in FVIIa-induced p21Ras activation. As can be seen in figure 4A, in HaCaT cells FVIIa treatment caused substantial activation of Src, but also of PKC-like kinases, allowing a potential role for both kinases in FVIIa-dependent p21Ras activation. In agreement, in HaCaT cells PP1, a specific inhibitor of Src-like kinases, but not the PI3-kinase inhibitor LY294002 inhibited FVIIa-induced p21Ras activation (Fig 4B) as well as activation of p42/p44 MAP kinase to sub-basal levels (Fig 5). Interestingly, although PKC phosphorylation is observed after FVIIa administration, the PKC-inhibitor GF-109203X did not inhibit MAP kinase phosphorylation, excluding a role for PKC-like kinases in this cell type. We conclude that in HaCaT cells this pathway is dependent on the stimulation of Src-like kinases, but not PI3-kinase or PKC.

![Fig 4: Src-like kinases mediate p21Ras activation in HaCaT cells.](image)

*Fig 4: Src-like kinases mediate p21Ras activation in HaCaT cells. (A) HaCaT cells were stimulated with 100 nM FVIIa for the indicated times and the lysates were screened for activated Src-like kinases and PKC isoforms using an antibody against the phosphorylated forms of these proteins on Western Blot. (B) HaCaT cells were pretreated with the indicated inhibitors for 30 min at a concentration of 10 mM and subsequently stimulated with 100 nM FVIIa. p21Ras activation was assayed as described in the Experimental Methods. The lysate was analyzed for equal amounts of Ras on Western Blot. The Western Blots represent three independent experiments.*
Early Ras activation in BHK\textsuperscript{TF} cells is mediated by PKC- We subsequently investigated the upstream components leading to p21Ras and MAP kinase activation in BHK\textsuperscript{TF} cells. Again, phosphorylation of both Src-like kinases and PKC-like kinases was observed in this cell type (Fig 6A). However, whereas PP1-treatment abolishes FVIIa-induced p21Ras and MAP kinase activation in HaCaT, PP1 did not effect either Ras activation (Fig 6B) or p42/p44 MAP kinase stimulation (Fig 7A) in BHK\textsuperscript{TF} cells. In addition, preincubation with the PI3-kinase inhibitor LY294002 did not have any effect, but pretreatment with the PKC-inhibitor GF-109203X, strongly delayed FVIIa-dependent p21 Ras activation (Fig 6A,B) and abolished early p42/p44 MAP kinase activation in BHK\textsuperscript{TF} cells (Fig 7B). These data suggest that PKC activation is important in p21Ras activation in BHK\textsuperscript{TF} cells but not in HaCaT cells. In addition, since PKC inhibition did not completely inhibit p21Ras-MAP kinase signaling, other signal transducers are likely to play a role in p21Ras-MAP kinase signaling in BHK\textsuperscript{TF} cells. Thus, although the activation p21Ras seems to be a general feature of FVIIa-induced signaling, the molecular mechanisms by which this p21Ras activation is achieved shows considerable cell type specificity.
Fig 6: Inhibition of PKC results in diminished p21Ras activation in BHKTT. (A) Activation of PKC and Src-like kinases in BHKTF cells was analyzed on Western Blot using phosphospecific antibodies against several PKC and Src isoforms, after a time-course with 100 nM of FVIIa. (B) Cells were pretreated with 10 mM PPI, GF-109203X or LY294002, before stimulation with 100 nM FVIIa. Subsequently, p21Ras activation in BHKTF was assessed as described in the Experimental Methods. (C) Mock treated cells and cells treated with 10 mM GF-109203X were also analyzed for active Ras in a time-course experiment. The Western Blots represent four independent experiments.
Discussion

Among the major insights into the action of the various coagulation factors in pathology is the insight that such factors are not just passive mediators involved in the linear transduction of the coagulation cascade, but actively engages target cells to induce signal transduction. Many of the details of the thus-induced signal transduction remain obscure. In the present study we addressed the role of p21Ras in the MAP kinase activation induced by FVIIa, the natural ligand for TF. Our results show that p21Ras activation is instrumental in FVIIa signal transduction and the FVIIa-dependent activation, but that the molecular processes by which p21Ras activation is brought about show considerable cell type specificity, involving either PKC or Src-dependent mechanisms. Ras is an important oncogene that is mutated in 30% of all human tumors and excessive p21Ras activity leads to uncontrolled proliferation and transformation. Therefore, TF overexpression observed in cancers and associated with tumor growth, may lead to excessive p21Ras activation. We have however, previously shown that FVIIa does not act as a mitogen (15), excluding a role for TF in proliferation of tumor cells, but it may well be involved in synthesis of proteins that act as promoters of tumor growth.

![Inhibition of PKC kinases results in diminished MAP kinase activation in BHK\textsuperscript{TF} cells.](image)

Cells were pretreated with 10 μM PP1, LY294002 (A) or GF-109203X (B), prior to stimulation with 100 nM FVIIa for the indicated times. Subsequently, MAP kinase phosphorylation in HaCaT cells was assessed using phosphospecific antibodies. Equal loading was determined using an antibody against total p42 MAP kinase.
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In the present study we have shown for the first time that FVIIa induces activation of p21Ras and PKC. Furthermore, we show that in BHK\textsuperscript{TF} cells, this PKC activation is only partially required for p21Ras and MAP kinase activation. In HaCaT cells, however, PKC is not critical for p21Ras and MAP kinase activation. In the latter cell type Src-like kinases seem cardinal regulators of this signaling cassette. Since inhibition of Src-like kinases by their specific inhibitor PP1 also inhibits PKC phosphorylation, Src-like kinase activation is very likely to be one of the main events in FVIIa-stimulated HaCaT cells. The question as to nature of the Src-like kinase activated in HaCaT cells that is responsible for p21Ras activation and subsequent p42/p44 MAP kinase stimulation was not addressed in this study, but attractive candidates are either Yes or possibly a set of different Src-like kinases. We have previously shown that in A14 fibroblasts, FVIIa-stimulation results mainly in Yes activity and to a lesser extent in Lyn and Src activity. Furthermore, Wiiger and co-workers found Yes associated to Pyk2 in FVIIa-stimulated HaCaT cells, in agreement with a principal role for this Src-like kinase in FVIIa signaling (19).

Also the nature of the PKC isoform involved in p21Ras activation in BHK\textsuperscript{TF} cells remains unclear. PKC isoforms can be divided into three classes; 1: classical PKCs that are regulated by calcium- and diacylglycerol-responsive domains, 2: novel PKCs that are regulated by diacylglycerol- but not calcium-regulated domains, and finally 3: the atypical PKCs that lack either domain (20). A role for the classical PKCs, consisting of PKCa, PKC\textbeta{}I, PKC\textbeta{}II and PKC\gamma{} is unlikely, since FVIIa does not induce calcium transients in BHK\textsuperscript{TF} cells. Moreover, making use of phosphospecific PKCa and PKC\beta{} antibodies we were unable to demonstrate enhanced phosphorylation and thus activation of this kinase (unpublished observations). Atypical PKCs, such as PKC\textlambda{} and PKC\zeta{} have a molecular weight of 72-76 kDa in size. PKC activation observed in the present study was detected at a molecular size of approximately 82 kDa, thus involvement of atypical PKCs appears unlikely as well. Thus until more data become available, we hypothesize that the PKC isoform or isoforms that mediate p21Ras activation in BHK\textsuperscript{TF} cells belong to the class of novel PKCs.

It is tempting to speculate on the upstream mechanisms leading to activation of the PKC-Ras cassette and the Src-Ras cassette in BHK\textsuperscript{TF} and HaCaT cells respectively. It is generally assumed that heterotrimeric G-protein coupled to PARs mediate signal transduction events elicited by FVIIa treatment. Since Pertussis Toxin does not impair FVIIa signaling
(unpublished data), Gi does not seem a major mediator in FVIIa-induced p21Ras activation. Also Gs and Gq activation is highly unlikely in view of the absence of FVIIa-induced cAMP generation and, in BHK\textsuperscript{TF} cells, FVIIa-induced calcium signals respectively. Therefore, G12 and G13, G-proteins that are also activated upon thrombin stimulation, at this point of time seem the most likely mediators of FVIIa signaling downstream of PAR activation.

This differential activation of p21Ras in different cell lines might also reflect the different nature of the receptors involved; in HaCaT, FVIIa:TF has been shown to target Protease-activated Receptor 2 (PAR-2), whereas in BHK\textsuperscript{TF} cells involvement PAR-2 and other known PARs (PAR1, PAR3, PAR4) was explicitly excluded. Therefore, it tempting to speculate that this difference in secondary receptors targeted after FVIIa:TF interactions is reflected in the difference of subsequent signal transduction observed with respect to mechanisms upstream of p21Ras activation. Disregarding these unknown details in FVIIa-induced signaling, however, we have demonstrated that FVIIa:TF induces p21Ras activation in BHK\textsuperscript{TF} and HaCaT cells, and the pathways that lead to activation of p21Ras differ in different cell types.

**Fig 8:** Proposed mechanism for activation of the p21Ras-MAP kinase pathway in BHK\textsuperscript{TF} and HaCaT. FVIIa stimulation results in activation of an unknown transmembrane protein or PAR-2 in BHK\textsuperscript{TF} and HaCaT, respectively. In BHK\textsuperscript{TF}, this will result in a partial PKC-dependent Ras and MAP kinase activation. In HaCaT, Src-family members are fully responsible for activation of Ras and MAP kinase.
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