The Cloned Thrombin Receptor Is Necessary and Sufficient for Activation of Mitogen-activated Protein Kinase and Mitogenesis in Mouse Lung Fibroblasts

LOSS OF RESPONSES IN FIBROBLASTS FROM RECEPTOR KNOCKOUT MICE*

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The mitogenic activity of thrombin on fibroblasts and smooth muscle cells may contribute to embryonic development and normal wound healing, and it may also play a role in pathological responses to vascular injury. To examine the importance of thrombin signaling in vivo and to define the cloned thrombin receptor's role, we disrupted the thrombin receptor gene (tr) in mice. Platelets from tr−/− mice responded normally to thrombin, but tr−/− fibroblasts showed no thrombin-induced calcium mobilization or phosphoinositide hydrolysis. Thus distinct thrombin receptors act in different tissues. This study focuses on the role of the thrombin receptor in thrombin-induced mitogenesis and mitogen-activated protein (MAP) kinase activation in mesenchymal cells. Thrombin and thrombin receptor agonist peptide both stimulated DNA synthesis and MAP kinase activation in fibroblasts derived from wild-type mice. These responses were selectively lost in fibroblasts from tr−/− mice. Activation of the cloned thrombin receptor is therefore necessary and sufficient for thrombin-induced mitogenesis and MAP kinase activation in mouse lung fibroblasts. The tr−/− mouse thus provides a valuable model for defining the role of thrombin-induced proliferative events in vivo. Because thrombin-induced MAP kinase activation was attributable to a single receptor expressed at natural levels, mouse lung fibroblasts presented an opportunity to define the pathways that normally mediate activation of MAP kinase by the thrombin receptor. Elimination of phorbol-sensitive protein kinase C by prolonged exposure to phorbol ester only partially inhibited MAP kinase activation by thrombin but completely blocked c-Raf kinase activation. Pertussis toxin partially inhibited MAP kinase activation by thrombin but had no significant effect on c-Raf kinase activation. Thus in mouse lung fibroblasts, one thrombin receptor utilizes two pathways for MAP kinase activation: one is protein kinase C- and c-Raf-dependent, and a second is G-dependent and c-Raf-independent.

The ability of thrombin to stimulate proliferation of fibroblasts and smooth muscle cells has led to the hypothesis that thrombin, when generated at sites of vascular injury, may contribute to both normal wound healing and pathological proliferative responses such as restenosis and atherosclerosis. Understanding how thrombin activates mesenchymal cells is, therefore, potentially important for therapeutic development. A recently cloned receptor mediates thrombin signaling in a variety of cell types (1). This receptor is a member of the G protein-coupled receptor family but is activated by a novel proteolytic mechanism. Thrombin cleaves the amino-terminal exodomain of its receptor to unmask a new amino terminus that then functions as a tethered peptide ligand, binding intramolecularly to the body of the receptor to effect activation. A soluble peptide mimicking the first six residues after the cleavage site is an agonist for the receptor. Such "agonist peptides" grossly mimic the actions of thrombin on a variety of cell types including fibroblasts (2–7). However, thrombin is more effective than agonist peptide in activating MAP kinase and stimulating proliferation of CCL-39 hamster lung fibroblasts (8, 9).

A priori, such discrepancies might be caused by differences in signaling by the cloned receptor when activated by thrombin versus agonist peptide (i.e. ligation by tethered versus free agonist might yield different kinetics of ligation or desensitization or different active receptor conformations). Alternatively, a distinct thrombin receptor that cannot be activated by agonist peptide might mediate some of the actions of thrombin. To define the roles of the cloned thrombin receptor in vivo and to answer definitively whether other receptors mediate some cellular responses to thrombin, we disrupted the cloned thrombin receptor gene in mice (10). Platelets from mice homozygous for the thrombin receptor gene deletion (tr−/−) responded normally to thrombin, but tr−/− fibroblasts showed no thrombin-induced calcium mobilization or phosphoinositide hydrolysis. Thus a second thrombin receptor does exist, and distinct thrombin receptors mediate thrombin signaling in different tissues. Defining which thrombin receptors mediate various cellular responses therefore becomes an important biological question and one that is central to therapeutic development. This report focuses on the role of the cloned receptor in thrombin-induced mitogenesis and MAP kinase/extracellular-regulated protein kinase (ERK) activation in mesenchymal cells.

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1 The abbreviations used are: MAP, mitogen-activated protein; ERK, extracellular-regulated protein kinase; MEK, MAP/ERK kinase; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; EGF, epidermal growth factor; bFGF, basic fibroblast growth factor; PMA, phorbol 12-myristate 13-acetate; PDBu, phorbol 12,13-dibutyrate; MBP, myelin basic protein; PIPES, 1,4-piperazine diethanesulfonic acid; WT, wild type.
cells. The MAP kinase pathway is an important route through which a variety of mitogens signal from cell surface receptors to the nucleus to effect transcriptional regulation (11, 12). We report that activation of the cloned thrombin receptor is necessary and sufficient for both thrombin-induced mitogenesis and MAP kinase activation in mouse lung fibroblasts. These cells therefore provide a valuable tool for identifying signaling pathways activated by a single G protein-coupled receptor in an untransformed cell line. In these cells, the thrombin receptor utilizes two pathways to mediate MAP kinase activation: one that is protein kinase C- and c-Raf-independent, and a second that is G-dependent and c-Raf-independent.

EXPERIMENTAL PROCEDURES

Cell Culture—The thrombin receptor knockout mouse was generated as described (10). Lung fibroblast cell lines were derived from wild-type and tr/−− mice by published methods (13). Each primary culture was prepared from four adult mouse lungs. Briefly, tissues were minced, washed with phosphate-buffered saline (PBS), and incubated with 0.05% trypsin/EDTA (0.2 g/liter) for 15 min at 37 °C. After inactivating trypsin with serum, the preparation was triturated, and cells were plated on 100-mm plates using Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS). On every 3rd day, cells were replated at approximately 25% confluency to minimize cell contact. By the 20–25th passage, permanent monomorphic, contact-dependent cell lines were obtained. The MAP kinase pathway is an important route through which growth factor bFGF each increased DNA synthesis to a similar extent (Fig. 1A). Wild-type and tr/−− fibroblasts showed comparable serum- and growth factor-induced increases in DNA synthesis; thus the failure of the tr/−− cells to respond to thrombin was selective. Similar results were obtained with other wild-type and tr/−− fibroblast lines derived from independent primary cultures (data not shown). The ability of both thrombin and thrombin receptor agonist peptide to stimulate DNA synthesis in wild-type fibroblast cultures, and the selectivity of these responses in receptor-deficient cells provide strong evidence that the cloned receptor is both necessary and sufficient for thrombin-induced mitogenesis.

bFGF has been reported to cooperate with thrombin to stimulate DNA synthesis in hamster lung fibroblasts (18). In wild-type mouse lung fibroblast cultures, thrombin and the bona fide growth factor bFGF each increased DNA synthesis to a similar extent (Fig. 1B). When used in combination, the effects of thrombin and bFGF were approximately additive. In tr/−− fibroblast cultures, bFGF stimulated DNA synthesis to a degree comparable with that seen in wild-type fibroblasts, but thrombin plus bFGF did not stimulate DNA synthesis more than bFGF alone. Thus in mouse lung fibroblasts, the thrombin receptor is required for thrombin-induced DNA synthesis even in the presence of bFGF.

We next examined the role of the cloned thrombin receptor in activating MAP kinase, an important mitogenic signaling pathway. Thrombin caused robust activation of MAP kinase in lung fibroblasts derived from wild-type mice (Fig. 2, A and B). The response followed a time course similar to that previously seen with CCL-39 hamster lung fibroblasts (19), with a peak at 5 min followed by a plateau that lasted >120 min (Fig. 2). Mouse thrombin receptor agonist peptide also rapidly activated MAP markers.

RESULTS AND DISCUSSION

To define the role of the cloned thrombin receptor in mediating thrombin-induced mesenchymal cell proliferation, we examined the action of thrombin on fibroblasts cultured from tr/−− mice. Thrombin and thrombin receptor agonist peptide stimulated DNA synthesis in fibroblasts derived from wild-type mice. By contrast, neither agonist caused DNA synthesis in tr/−− mouse lung fibroblast cultures, thrombin and the cloned receptor agonist peptide to stimulate DNA synthesis in hamster lung fibroblasts (18). In wild-type fibroblasts derived from wild-type mice (Fig. 2, A and B). The response followed a time course similar to that previously seen with CCL-39 hamster lung fibroblasts (19), with a peak at 5 min followed by a plateau that lasted >120 min (Fig. 2). Mouse thrombin receptor agonist peptide also rapidly activated MAP markers.
kinase in wild-type mouse fibroblasts, but, as in CCL-39 cells (9), the agonist peptide was less effective than thrombin at elicits prolonged activation of MAP kinase (Fig. 2, A and C). In contrast to the wild type fibroblasts, cells derived from tr
fibroblasts failed to show either early or late MAP kinase activation in response to thrombin (Fig. 3, A and B). Thrombin-induced calcium mobilization and phosphoinositide turnover were also absent in the tr
fibroblasts (10), while activation of MAP kinase by other agents such as the phorbol ester PMA was preserved (data not shown). Similar results were obtained in other independently derived wild-type and tr
mouse lung fibroblast cell lines. These results indicate that the cloned thrombin receptor is necessary for activation of MAP kinase by thrombin in mouse lung fibroblasts.

MAP kinase activation by thrombin receptor agonist peptide was also lost in tr
fibroblasts (Fig. 3, A and C); thus the response to agonist peptide seen in the wild-type cells was mediated by the cloned receptor and not by a second protease-activated receptor. This possibility was not just a formal one because thrombin receptor agonist peptide does activate protease-activated receptor 2 (20). These findings suggest that, despite their differing tempos, MAP kinase activations by thrombin receptor agonist peptide do not occur in wild-type mouse lung fibroblasts and CCL-39 cells. Cells were stimulated with 10 nM α-thrombin or 100 μM SFFLRNPSE for the indicated times. MAP kinase activity was measured as described above. Results are expressed as the fold increase over basal and represent the mean ± S.E. of three experiments. For both B and C, the responses to α-thrombin versus SFFLRNPSE were different when assessed by two-way analysis of variance (p < 0.05), and the responses to α-thrombin versus SFFLRNPSE at late times (120 min) were different (p < 0.05) when assessed using Bonferroni’s t test.
A tide activity was lost during the 2-h incubation shown in Fig. 2, type and effects on MAP kinase activation in lung fibroblasts from wild-type and SFFLRNPSE in wild-type (WT) and tr−/− cells. Cells were treated with either α-thrombin (10 nM) or SFFLRNPSE (300 μM) for the specified times. MAP kinase activity was determined using MBP in an in vitro phosphorylation assay (see “Experimental Procedures”). Data show the time course of agonist-stimulated phosphorylation of MBP from a single experiment. B, comparison of MAP kinase activation by α-thrombin in WT and tr−/− mouse lung fibroblasts. WT and tr−/− cells were exposed to 10 nM α-thrombin for the specified times. MAP kinase activity was measured as described under “Experimental Procedures.” Data are expressed as the fold increase relative to basal and represent the mean ± S.E. of duplicate experiments. C, comparison of MAP kinase activation by SFFLRNPSE in WT and tr−/− lung fibroblasts. Cells were treated with 300 μM SFFLRNPSE for the indicated times. Data are expressed as fold increase in MAP kinase activity over basal and represent the mean ± S.E. of duplicate experiments. In B and C, the responses of WT versus tr−/− fibroblasts to either α-thrombin or SFFLRNPSE were different (p < 0.05) when assessed by two-way analysis of variance. These results were also replicated in independently derived lung fibroblasts from wild-type and tr−/− mice.

Taken together, the observations presented above strongly suggest that the cloned thrombin receptor fully accounts for thrombin-induced MAP kinase activation in mouse lung fibroblasts. The wild-type mouse lung fibroblasts thus provided a unique opportunity to dissect the pathway from receptor to MAP kinase activation in an untransfected cell in which responses can be attributed to a single cloned receptor expressed at natural levels. The pathway from thrombin receptor to MAP kinase activation is potentially complex. The thrombin receptor shows robust coupling to G proteins G1 and Gq (21–23). Downstream signaling molecules that might mediate the actions of these G proteins on MAP kinase include ras, protein kinase C, and c-Raf kinase as well as other MEK (MAP/ERK kinase) kinases (24). c-Raf and other MEK kinases converge on MEK; phosphorylated and therefore activated MEK in turn phosphorylates and activates MAP kinase (see Ref. 25). Toward defining the pathways from thrombin receptor to MAP kinase activation in mouse fibroblasts, we examined the importance of G1-like G proteins and protein kinase C in c-Raf and MAP kinase activation.

Elimination of G1 function by treatment of cells with pertussis toxin partially inhibited MAP kinase activation but had no significant effect on c-Raf kinase activation by thrombin (Figs. 4A and 5A). Pertussis toxin did not inhibit either thrombin-induced phosphoinositide hydrolysis (data not shown) or phorbol ester and EGF stimulation of MAP kinase in these cells (Fig. 4A; data not shown). These data are consistent with pertussis toxin selectively ablating G1 function without nonspecifically inhibiting either thrombin signaling or MAP kinase activation in general. One can also conclude that the thrombin receptor activates phosphoinositide hydrolysis mainly through a pertussis-insensitive, presumably Gq-like G protein (26, 27) in these cells (Fig. 6). Pertussis toxin treatment ADP-ribosylated more than 95% of available substrate in these cells (data not shown) (6); therefore the partial nature of pertussis inhibition of thrombin-induced MAP kinase activation was not due to incomplete action of the toxin. These results show that the cloned thrombin receptor utilizes both pertussis-sensitive and -insensitive G proteins to activate MAP kinase (Fig. 6). Given the receptor’s ability to couple to each (21, 22), G1, and Gq are excellent candidates for mediating such pathways.

Elimination of phorbol-sensitive protein kinase C activation by prolonged PDBu pretreatment of wild-type fibroblasts partially inhibited MAP kinase activation by thrombin but completely blocked activation by phorbol ester (Fig. 4B). PDBu pretreatment had little effect on MAP kinase activation by EGF and no effect on thrombin-induced phosphoinositide hydrolysis (Fig. 4B; data not shown). These data suggest that the thrombin receptor utilizes both protein kinase C-dependent and -independent pathways to couple to MAP kinase.

In contrast to MAP kinase activation, prolonged PDBu pretreatment completely blocked c-Raf kinase activation by thrombin (Fig. 5B). Protein kinase C activation is therefore critical for c-Raf activation by the thrombin receptor in mouse lung fibroblasts. The persistence of thrombin-induced MAP kinase activation in the absence of c-Raf activation in PDBu-pretreated mouse fibroblasts demonstrates a c-Raf independent pathway to MAP kinase activation in these cells.
G protein-coupled receptors have been shown to activate MAP kinase via \( \alpha_q \) or \( \alpha_i \) in both transiently transfected cell systems (28-30) and in stably transfected cell lines (31-34). \( \alpha_q \)-mediated MAP kinase activation involves \( \alpha \) coupling to phospholipase C and protein kinase C and in some cell types involves a c-Raf kinase-dependent pathway (30). In contrast, \( \alpha_i \) mediates MAP kinase activation through an incompletely characterized pathway mediated by the \( \beta \gamma \) subunits released from the \( \alpha_i \) heterotrimer. \( \alpha_r \)-mediated ras and c-Raf kinase activation have been described (31). Our observations suggest that a \( \alpha_r \)-activated c-Raf-independent pathway to MAP kinase also exists and is consistent with previous findings (17).

Taken together, the observations presented above suggest that the cloned thrombin receptor naturally expressed by mouse platelets utilizes two distinct pathways to activate MAP kinase: one via a \( \alpha_r \)-like G protein, protein kinase C, and c-Raf kinase; and a second via a \( \alpha_r \)-like G protein and an unknown MEK kinase (Fig. 6). The use of several pathways by a single receptor cannot represent promiscuity due to receptor overexpression or to expression of the cloned thrombin receptor in an inappropriate cellular context in these untransfected fibroblasts. The biological importance of coupling to multiple pathways for modulation of the MAP kinase response pathway remains to be determined.

In summary, both thrombin and thrombin receptor agonist peptide-stimulated DNA synthesis and activated an important mitogenic signaling pathway, the MAP kinase cascade, in fibroblasts derived from wild-type mouse lung. These responses were selectively lost in fibroblasts derived from tr/− mouse lung. The cloned thrombin receptor is therefore both necessary and sufficient for thrombin-induced mitogenesis and MAP kinase activation in mouse lung fibroblasts. These cells provide an opportunity to define the pathways that the single cloned receptor utilizes to activate the MAP kinase cascade. Our findings indicate that two pathways, one dependent on protein kinase C and c-Raf and a second one dependent on a \( \alpha_r \)-like G protein and c-Raf-independent, both participate in MAP kinase activation by the cloned thrombin receptor (Fig. 6).

The data presented above do not imply that the cloned thrombin receptor is the sole mediator of thrombin responses in all cell types. To the contrary, the tr/− mouse unequivocally demonstrates that mouse platelets utilize a distinct thrombin...

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**Fig. 4. Inhibition of thrombin-stimulated MAP kinase activity by pertussis toxin and phorbol ester pretreatment.**

A. effect of pertussis toxin on thrombin-induced MAP kinase activity. Serum-starved WT lung fibroblasts were incubated in the presence or absence of 100 ng/ml pertussis toxin for 5 h at 37 °C. Cells were then stimulated with DMEM alone (Ctrl) or with DMEM containing 10 ng/mL thrombin or 100 ng/mL PMA for 5 min at 37 °C. MAP kinase activity was measured and expressed as “fold induction” relative to controls. Data shown are the mean ± S.E. of an individual experiment performed in duplicate. The basal activity of MAP kinase was similar in pertussis toxin-treated and untreated cells. Similar findings were observed in three separate experiments, each done in duplicate. B. effect of PDBu treatment on thrombin-stimulated MAP kinase activity. Serum-deprived WT cells were incubated with 0.1% Me2SO (vehicle) or 1 μM PDBu for 18 h and then exposed to DMEM alone (Ctrl) or to DMEM with 10 ng/mL thrombin, 100 ng/mL EGF, or 1 μM PDBu for 5 min at 37 °C. Increases in MAP kinase activity were determined and expressed as fold induction over basal activity. The basal activity was similar in control and PDBu-pretreated cells. Two separate experiments performed in duplicate revealed similar results.

**Fig. 5. Inhibition of thrombin-stimulated c-Raf kinase activity by pertussis toxin but not phorbol ester pretreatment.**

A. effect of pertussis toxin on thrombin-induced c-Raf kinase activation. WT lung fibroblasts were serum-deprived and treated for 5 h at 37 °C with 100 ng/mL pertussis toxin. Cells were then incubated with DMEM alone (Ctrl) or DMEM containing 10 ng/mL thrombin or 100 ng/mL PMA for 5 min at 37 °C. c-Raf was immunoprecipitated, and kinase activity was determined using histone H1 in an in vitro kinase reaction (see “Experimental Procedures”). Data are presented as fold induction relative to controls (mean ± S.E.; n = 2). Basal c-Raf kinase activity was similar in control and pertussis-pretreated cells. These results were replicated in two independent experiments performed in duplicate. B. effect of PDBu pretreatment on c-Raf kinase activation stimulated by thrombin. Serum-starved WT cells were exposed to 0.1% Me2SO (vehicle) or 1 μM PDBu for 18 h. Cells were then exposed to DMEM with 10 ng/mL thrombin, 1 μM PDBu, or to DMEM alone (Ctrl) for 5 min at 37 °C. c-Raf kinase activity was determined as above. Each experiment was performed in duplicate and results expressed as the mean ± S.E. PDBu pretreatment did not alter basal c-Raf kinase activity. These results were replicated in an independent experiment performed in duplicate.
Thrombin Receptor and Mitogenic Signals

Fig. 6. Schematic representation of thrombin receptor signaling to the MAP kinase cascade. Our data suggest that the cloned thrombin receptor accounts for thrombin stimulation of MAP kinase in mouse lung fibroblasts. Even when naturally expressed by these cells, the single receptor appears to utilize two distinct pathways to activate MAP kinase. The cloned thrombin receptor can couple to G\textsubscript{q} and G\textsubscript{i}-like G proteins. Left side pathway, in mouse lung fibroblasts, thrombin-induced phosphoinositide hydrolysis and c-Raf activation are pertussis-insensitive and presumably mediated by a G\textsubscript{i}-like G protein. Protein kinase C (PKC) is activated by phosphoinositide hydrolysis. Elimination of phorbol-sensitive protein kinase C abolished c-Raf kinase activation but caused partial inhibition of MAP kinase activation. This result implies the existence of a c-Raf-independent pathway in these cells. Right side pathway, inactivation of G\textsubscript{q}-like proteins by pertussis toxin (PTX) treatment had no effect on c-Raf activation but caused partial inhibition of MAP kinase activation by thrombin. This second pathway presumably utilizes a MEK kinase distinct from c-Raf.

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