Thrombin Activation of the 9E3/CEF4 Chemokine Involves Tyrosine Kinases Including c-src and the Epidermal Growth Factor Receptor*

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The 9E3/CEF4 gene codes for a chemokine that is highly homologous to human interleukin-8 and melanoma growth-stimulating activity/groα. These chemokines belong to a family of molecular mediators that are importantly involved in inflammation, wound healing, tumor development, and viral entry into cells. On the chorioallantoic membrane the 9E3 protein is chemotactic for monocyte/macrophages and lymphocytes and is angiogenic. In cultured chicken embryo fibroblasts, which have many of the properties of wound fibroblasts, the gene is stimulated by a variety of agents including oncogenes, growth factors, phorbol esters, and thrombin. The strong stimulation of 9E3 by thrombin in culture correlates well with the observation that in young chicks this gene is stimulated to very high levels in fibroblasts upon wounding and remains high throughout wound repair. Activation of 9E3 by thrombin: (i) occurs very rapidly, one minute exposure to thrombin is sufficient to initiate the signals necessary for gene activation; (ii) is independent of mitogenesis; (iii) operates through the proteolytically activated receptor for thrombin (iv) is mediated by tyrosine kinases, including c-src and the epidermal growth factor (EGF) receptor, rather than Ser/Thr kinases such as protein kinase C and protein kinase A. Inhibition of either c-src or the EGF receptor tyrosine kinase inhibits the stimulation of 9E3 by thrombin. We show here for the first time that activation of the EGF receptor through a cell-surface receptor that does not have tyrosine kinase activity can lead to expression of an immediate early response gene which encodes for a secreted protein, a chemokine. This rapidly activated tyrosine kinase pathway may be a general stress response by which in vivo a localized cell population reacts to emergency situations such as viral infection, wounding, or tumor growth.

The avian gene 9E3/CEF4 encodes a secreted protein that is a member of the chemokine superfamily (1–4). Of all the members of this superfamily, the 9E3 protein shows the highest homology to interleukin-8 and also is highly homologous to MOSA1/groα. This gene is constitutively overexpressed in chicken embryo fibroblasts (CEFs) transformed by Rous sarcoma virus, but its expression in normal quiescent cells is tightly regulated (5, 6); serum stimulation of normal CEFs results in transient expression of 9E3 as a consequence of entry into the cell cycle (7). In addition to serum, a wide variety of oncogenes, growth factors, and inflammatory agents also stimulate 9E3 expression in normal quiescent CEFs (5, 6, 8, 9). In vivo, the 9E3 gene is expressed at very low levels in connective tissue, tendon, and bone, but it is highly expressed in the endothelial cells of young blood vessels (10–12). Furthermore, this gene is rapidly overexpressed upon wounding, remains very highly elevated during the inflammatory phase of healing, and 36 h after wounding declines to a plateau of elevated expression that remains constant throughout granulation tissue formation (10). The 9E3 protein is chemotactic for monocyte/macrophages and lymphocytes (12, 13); the high levels of expression shortly after wounding could be responsible for this function. In addition, this protein is angiogenic in vivo (12), which might occur in response to the steady expression of the 9E3 gene during granulation tissue formation. The observations in vivo and in vitro combined with the biochemical properties of the molecule (14) indicate that this gene could play an important role in the inflammatory response and healing of wounds.

We have found that of the growth factors, inflammatory agents, and other molecules released upon wounding, thrombin is the most potent natural activator of 9E3 expression (14, 15). Thrombin is a serine protease that is generated at sites of vascular injury and is known to regulate hemostasis and thrombosis via its serine protease activity by inducing platelet aggregation and clot formation. However, many other actions of thrombin are independent of its thrombogenic activity and suggest that this protease is also important directly or indirectly during the inflammatory response and in the proliferative phase of wound healing (16–19). In these situations, thrombin is known to be chemotactic for monocytes and mitogenic for lymphocytes, fibroblasts, and smooth muscle cells (20–23). Furthermore, thrombin stimulates endothelial cells to express the neutrophil adhesion protein GMP-140 (24–26) and to produce platelet-derived growth factor that stimulates smooth muscle cells to grow (27, 28).

Most of our understanding of signal transduction pathways turned on by thrombin comes from work with platelets (29, 30), Chinese hamster lung fibroblasts (31, 32), and endothelial cells (33). In these systems, upon binding to its seven transmembrane domain receptor, thrombin cleaves part of the N terminus of the receptor, exposing a new peptide that then binds to one of the external loops of the receptor (34). This proteolytically activated receptor for thrombin causes activation of G₁, G₃, and G₁₁ that turn on mitogenic and nonmitogenic events (18). G₁ inhibits adenylyl cyclase and cAMP and reduces the levels of PKA (35), whereas G₁₁ activates phosphatidylinositol turnover, PKC, Na+/H+ antiport, and induces c-fos and c-myc (19, 36–38). In platelets, the G₁₁ can also activate phospholipase
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and leukotrienes, which are often involved in the inflammatory process. More recently, a new proteinase-activated receptor 2 was identified that has 30% homology to proteolytically activated receptor for thrombin but for which the signaling events are not yet worked out (39).

The signaling events activated by thrombin, which are described above, are mediated by Ser/Thr kinases stimulated shortly after receptor activation. However, more recently tyrosine phosphorylation has also been implicated in the signaling events triggered upon thrombin receptor activation. It has been shown that tyrosine phosphorylation in platelets occurs independently of the activation of PKC (40) and that cells that do not undergo mitogenesis in response to thrombin stimulation require tyrosine kinase signals initiated by EGF and platelet-derived growth factor (41) to trigger cell division. In growth-responsive Chinese hamster fibroblasts, thrombin activates the c-src and fyn tyrosine kinases, which provide a link between the thrombin receptor and the downstream events leading to activation of ras and the mitogen kinase cascade (42).

It is now abundantly clear that thrombin is a multifunctional molecule. The fact that thrombin and chemokines are important in the inflammatory response and granulation tissue formation, and the demonstration that thrombin stimulates the expression of chemokines such as MGSAgroa (43, 44), 9E3 (14, 15, 45), and interleukin-8 (46) may indicate that these chemokines could be the mediators of some of thrombin’s effects on healing. Identification and understanding of the signal transduction pathways generated by thrombin leading to activation of chemokines can give insight into ways of manipulating the function of this latter class of molecules. Because the 9E3 gene is stimulated to high levels shortly after wounding and because thrombin is the most potent natural activator of this gene, we are investigating the signal transduction mechanisms by which thrombin activates 9E3 expression. To perform these studies we use primary cell cultures of CEFs. This culture system allows us to make inferences to the situation in vivo because the properties of the fibroblasts in wounded tissue and in granulation tissue resemble those of embryonic fibroblasts (47).

Our results show that the Ser/Thr kinases PKC and PKA are not directly involved in the stimulation of this gene by thrombin, but instead we find that tyrosine phosphorylation is a key event that occurs when CEFs are activated by thrombin to produce 9E3. We show here that the EGF receptor and c-src are two of the tyrosine kinases importantly involved in this process.

MATERIALS AND METHODS

Reagents—Concentrations of activators and inhibitors of signal transduction cited here were selected for efficacy from a wider range of concentrations initially tested. A23187 (1 mM), arachidonic acid (100 mM), and bovine thrombin (9 units/ml) were obtained from Sigma.

Calphostin C (200 nM), cholera toxin (100 ng/ml), genistein (1–25 mM), H-7 (200 nM), ionomycin (1 mM), Asn-Pro-Asn-Asp-Lys-Tyr-Glu-Pro-Phe-OH (Bachem).

Nonmitogenic Receptor-dependent Activation of 9E3 by Thrombin—Addition of serum to CEFs in culture stimulates expression of the 9E3 protein by thrombin occurs via a nonmitogenic pathway. To test this possibility, we plated CEFs in serum-free medium, after starvation for 2 days, and then activated them with 10 units/ml of thrombin and/or treatment of a different batch of cells we used internal positive (thrombin-treated cells) and negative (untreated cells) controls.

Western Blotting—Volumes of the cell culture supernatant corresponding to equal amounts of protein in the cells extracts were loaded on 20% polyacrylamide-gelatin gels and electrophoresed at 16 mA for about 4 h. The concentration of the protein was determined using the Bio-Rad DC-protein kit. Transfer was performed using a semidry transfer apparatus (Mite). After transfer, to check for evenness of protein loading, the upper part of the gel was cut and stained with silver staining for a protein that is ~35 kDa. This protein is present in the supernatant of qCCEF, and its concentration does not vary with treatment of the cells. The efficiency and evenness of transfer of the 9E3 protein was monitored by silver staining the gel after the transfer. The 9E3 protein was detected using polyclonal antibodies raised in rabbit (14) and enhanced chemiluminescence (ECL) reagents (Amersham).

Northern Blot Analysis—CEF were homogenized and total RNA was prepared using TRIzol reagent (Life Technologies, Inc.). RNA samples (20 µg each) were denatured in formamide-formaldehyde buffer containing ethidium bromide and separated on formaldehyde-agarose gel. After electrophoresis, the RNA was transferred to MagnaGraph nylon membrane that were photogated to test the quality of the RNA and confirm equal loading and even transfer. The RNA was UV-cross-linked to the membrane for 2 min and baked at 60 °C for 2 h. Prehybridization was performed for 6–9 h, and hybridization was carried out for 36 h following the procedures described in Ref. 10. Microdensitometry analysis was performed by laser densitometric scanning in a LKB microdensitometer.

The 9E3 gene is expressed in human and bovine dermal fibroblasts and its expression is stimulated by a variety of agents including thrombin, TGF-β, and PDGF. To study the effects of thrombin on 9E3 expression and the effects of inhibitors of signal transduction, we used quiescent confluent CEF (qCEF) cultures and incubated them in serum-free 199 medium containing the specific treatment at various concentrations and for varying times. After the initial experiments, the inhibitors and activators were used at the doses shown above and incubated for 1 h; thrombin was added for another hour when the cells were washed and incubated in serum-free medium for up to 18 h. At the end of the incubation period the supernatant was collected, protease inhibitors were added, and cell debris was removed by centrifugation. Because qCCEF are primary cells rather than a cell line, there are small variations in the basal levels of 9E3 expression from batch to batch of cells. Therefore, for each experiment and/or treatment of a different batch of cells we used internal positive (thrombin-treated cells) and negative (untreated cells) controls. A new proteinase-activated receptor for thrombin but instead we find that tyrosine phosphorylation is a key event that occurs when CEFs are activated by thrombin to produce 9E3. We show here that the EGF receptor and c-src are two of the tyrosine kinases importantly involved in this process.

RESULTS

Here, with the subsequent generation of arachidonate-derived metabolites (29). These lipids, known as eicosanoids, include prostaglandins, thromboxanes, and leukotrienes, which are often involved in the inflammatory process. More recently, a new proteinase-activated receptor 2 was identified that has 30% homology to proteolytically activated receptor for thrombin but for which the signaling events are not yet worked out (39).
Thrombin stimulates 9E3 expression.

With thrombin (Fig. 1), the amount of protein secreted into the supernatant of treated cells was approximately the same for both treatments (Fig. 1B). Our observations at the protein level were confirmed by analysis of the mRNA under the same conditions (Fig. 1C); the level of the protein in cells with the various treatments correlates well with levels of mRNA under the same conditions. To determine if the stimulation of 9E3 by thrombin is via the proteolytically activated receptor for thrombin receptor, we treated qCEFs, which do not express 9E3, with the human thrombin receptor derived peptide (TRDP), which can activate proteolytically activated receptor for thrombin much like thrombin (50). We found that this peptide could stimulate qCEFs to express 9E3 to the same level as those stimulated by thrombin itself (Fig. 1D).

To further determine if the stimulation of 9E3 by thrombin is specific, we performed a dose-dependent study by treating qCEFs with serum-free medium containing increasing doses of thrombin for a period of 24 h. We found maximum accumulation of the protein in the supernatant with a treatment of 9 units/ml or more of thrombin (Fig. 2A). Using a dose of 9 units, we determined that the levels of the 9E3 protein present in the supernatant of treated cells increased with time up to 18 h with saturated levels after this time (Fig. 2B).

The lack of detection of 9E3 early in the time course study (Fig. 2B) could simply be the result of the lack of time to accumulate detectable levels of the protein at the early time point. To determine the time span required for thrombin activation of 9E3, we treated qCEFs with thrombin (9 units/ml) for shorter periods of time, then washed and incubated the cells with serum-free medium for 18 h to allow accumulation of the protein in the supernatant. We found that 1 min exposure of the cells to thrombin was sufficient to turn on the events leading to production of the 9E3 protein (Fig. 2C).

**Additive Effect of v-src and Thrombin on 9E3 Expression**—Our observation that activation of 9E3 by thrombin is independent of mitogenesis, coupled with a previous determination that activation of 9E3 by v-src is via a mitogenic pathway (51) raised the possibility that thrombin and the oncogene might activate 9E3 via different signal transduction pathways. If so, when added together, they should stimulate an additive effect on the expression of 9E3. To test this possibility, we treated tCEFs with thrombin and analyzed them for both protein (Fig. 3A) and mRNA (Fig. 3B) and observed that thrombin and v-src have additive effects. These results suggest that thrombin activates 9E3 by a pathway independent of that turned on by v-src (which involves activation of PKC by the v-src oncogene) (51) but do not preclude the possibility that it could be a superactivation of the same pathway involving PKC.

**Stimulation of 9E3 by Thrombin Is Enhanced by Inhibition of Ser/Thr Kinases**—Thrombin is known to stimulate several signal transduction pathways resulting in activation of a variety of genes and leading to many different biological responses. The pathway stimulated by thrombin that activates PKC involves activation of Gα11 which activates phospholipase Cβ that, in turn, cleaves phosphatidylinositol phosphate into IP3 and diacylglycerol. The latter activates PKC by interacting directly with this kinase, whereas IP3 interacts with its receptor, present in the endoplasmic reticulum resulting in intracellular Ca2+ release. To determine if PKC is involved in the stimulation of 9E3 by thrombin, we treated qCEFs with OAG (oleoyl-2-acetyl-glycerol), an analogue of diacylglycerol that activates PKC directly (52), and found that OAG did not stimulate the production of the 9E3 protein (Fig. 4A). To further test this, we treated qCEFs with calphostin C (Fig. 4B), a specific inhibitor of PKC (53) and found that this treatment did not affect the production of 9E3 by thrombin.
not inhibit stimulation of 9E3 by thrombin but instead it potentiated thrombin stimulation. Furthermore, when we treated qcCEFs with the broad spectrum Ser/Thr kinase inhibitors H7 and staurosporine (53, 54), we found that both inhibitors potentiated the stimulation of 9E3 by thrombin and that staurosporine by itself stimulated 9E3 expression (Fig. 4B). In addition, we also found that an increase in cytosolic Ca\textsuperscript{2+} by treatment with the ionophores ionomycin and A23187 (Fig. 4C) had little effect on the levels of the 9E3 protein and did not enhance that stimulated by thrombin. These results suggest that the signaling pathways activated by G\textsubscript{q,11}, and in particular the activation of PKC, do not play a major role in stimulation of 9E3 by thrombin.

Thrombin is also known to interact with its receptor and activate G\textsubscript{i} or G\textsubscript{s}, and more recently it has also been shown to activate tyrosine phosphorylation (55). We treated qcCEFs with thrombin in the presence of inhibitors or activators of the key players involved in these signaling pathways. Activation of the G\textsubscript{i} pathway leads to inhibition of adenylyl cyclase, decrease in cAMP production, and therefore lack of activation of PKA. To test if G\textsubscript{i} is involved, we used pertussis toxin, which ADP-ribosylates this G-protein, inhibiting it permanently (56, 57). We found that inhibition of G\textsubscript{i} by pertussis toxin failed to inhibit, and in fact potentiated, the stimulation of 9E3 expression by thrombin (Fig. 5A). Treatment of cells with HA1004 and H89, inhibitors of PKA and other Ser/Thr kinases (58, 59), also potentiated the stimulation of 9E3 expression by thrombin (Fig. 5A), suggesting that high levels of PKA might inhibit 9E3 expression. We addressed this possibility by treating qcCEFs with cholera toxin, which specifically activates G\textsubscript{s} and in turn leads to the activation of PKA. This treatment had no affect on the stimulation of 9E3 by thrombin (Fig. 5B), indicating that PKA is not critical for the expression of this gene.

Tyrosine Kinases Are Involved in the Activation of 9E3 Expression by Thrombin—Our findings that Ser/Thr kinases are not significantly involved in 9E3 stimulation by thrombin, led...
us to investigate the role of tyrosine kinases in this process (40–42, 60). We found that genistein, a general inhibitor of tyrosine kinases (61), abolished the expression of 9E3 induced by thrombin both at the protein (Fig. 6A) and mRNA levels (Fig. 6B). To investigate the nature of the proteins phosphorylated on tyrosines, we performed Western blot analysis of extracts prepared from cells treated with thrombin, using antiphosphotyrosine antibodies. A time course study showed that proteins of molecular masses 220, 170, 60, 44, and 42 kDa were phosphorylated on tyrosines in a biphasic manner upon thrombin stimulation of qCEFs with the first peak at 5–7 min and the second at 3–6 h after activation (Fig. 7). By reprobing the same blot or identical blots with specific antibodies, we found that the 170-kDa band was labeled by an antibody to the EGF receptor, the 60-kDa band was labeled by an antibody to the c-src protein and the 44- and 42-kDa bands were labeled by antibodies to ERK1 and ERK2, respectively. In this study we focus on the potential involvement of the c-src and the EGF receptor tyrosine kinases.

To determine if c-src is involved in the stimulation of 9E3 by thrombin we used the specific inhibitor herbimycin A. Treatment of qCEFs with herbimycin A at doses that inhibit pp60c-src only (IC50 = 900 nM) (62) shows that this inhibitor is capable of eliminating 9E3 expression stimulated by thrombin (Fig. 4A). Thrombin stimulation of 9E3 does not involve the Gq or Gs pathways. Effects of Gi inhibitors or activators on 9E3 expression, through Gi (A) and through Gs (B). In all cases the concentration of thrombin was 9 units/ml. 100% Me2SO was used as the solvent for most activators and inhibitors; pertussis toxin (ptx) and chlora toxin (ctx) were used at 100 ng/ml; HA1004-HCl at 20 μM; control consisted of untreated qCEFs. Inhibition of Gi by pertussis toxin and inhibition of PKA by HA1004-HCl and H89 potentiated the expression of 9E3 by thrombin (A), whereas stimulation of Gs by cholera toxin had no effect (B).

FIG. 4. Thrombin stimulation of 9E3 does not involve the Gq or Gs pathways. Western blot analysis of the 9E3 protein produced by cells with various treatments. In all cases the concentration of thrombin was 9 units/ml, and Me2SO was used as the solvent for most activators and inhibitors. A, cells were treated with an analogue of diacylglycerol (OAG; 300 μM), which activates PKC, and were not stimulated to express 9E3. B, cells were treated with the specific inhibitor of PKC, calphostin C (200 nM), and with the broad spectrum inhibitors of Ser/Thr kinases, H7-dihydrochloride (200 nM), and staurosporine (200 nM). Calphostin C potentiated the stimulation of 9E3 by thrombin and so did the broader spectrum Ser/Thr kinase inhibitors, indicating that Ser/Thr kinase inhibitors in general potentiate the stimulation of 9E3 by thrombin. Although the other inhibitors by themselves had no effect on 9E3 expression, staurosporine stimulated it. C, cells treated with the ionophores, ionomycin (1 ng/ml) and A23187 (1 μM), in the presence or absence of thrombin (9 units/ml). The increase in Ca2+ levels triggered by ionomycin or A23187 did not significantly stimulate 9E3.

FIG. 5. Thrombin stimulation of 9E3 does not involve the Gi or Gs pathways. Effects of G, inhibitors or activators on 9E3 expression, through G, (A) and through Gs (B). In all cases the concentration of thrombin was 9 units/ml. 100% Me2SO was used as the solvent for most activators and inhibitors; pertussis toxin (ptx) and cholera toxin (ctx) were used at 100 ng/ml; HA1004-HCl at 20 μM; control consisted of untreated qCEFs. Inhibition of Gi by pertussis toxin and inhibition of PKA by HA1004-HCl and H89 potentiated the expression of 9E3 by thrombin (A), whereas stimulation of Gs by cholera toxin had no effect (B).

FIG. 6. Thrombin stimulation of 9E3 involves the activation of tyrosine kinases. The broad spectrum tyrosine kinase inhibitor genistein (25 μM) inhibits the stimulation of 9E3 by thrombin (9 units/ml) both at the protein (A) and mRNA (B) levels. Controls consisted of untreated qCEFs.
Thrombin Activates the 9E3 Chemokine Via Tyrosine Kinases

8A), indicating that c-src is importantly involved in the signal transduction cascade initiated by thrombin.

To investigate if the EGF receptor is involved in activation of 9E3 by thrombin, we inhibited its tyrosine kinase by using two selective inhibitors, lavendustin A RG14355 (62) and the highly selective and strong inhibitor, tyrphostin AG1478 (62–64).

Lavendustin A RG14355 virtually eliminated the enhancement of production of the 9E3 protein stimulated by thrombin (Fig. 8B), and tyrphostin AG1478 completely inhibited enhanced stimulation by thrombin of both the 9E3 mRNA and protein (Fig. 8C and D). To further investigate the potential involvement of the EGF receptor, we treated qCCEFs with hrEGF in the presence and in the absence of tyrphostin AG1478 and performed similar experiments with hrTGα, which is known to utilize the EGF receptor (65). We found that hrTGα stimulates the production of the 9E3 protein in qCCEFs, confirming our previous findings (8), and that this effect is inhibited by tyrphostin AG1478 (Fig. 8E). hrEGF, which binds less well to this receptor in chickens than does hrTGα (66), also stimulated production of the 9E3 protein but required more than 10 times as much for an effect comparable to that of TGα; tyrphostin AG1478 similarly abolished this stimulation (Fig. 8E).

These results taken together show that the EGF receptor is involved in the activation of 9E3 via direct interactions with its ligands, EGF and TGα, and indirectly by interaction of thrombin with the thrombin receptor.

FIG. 7. Tyrosine phosphorylation of cellular proteins after thrombin stimulation of qCCEFs. A, phosphotyrosine immunoblot after incubation of qCCEFs with thrombin for increasing amounts of time. At least five proteins are differentially phosphorylated showing a maximum at 5–7 min, a minimum at 30 min, and a second maximum at 3–9 h. Using specific antibodies, four of the five bands in the same gel were identified as co-migrating with the EGF receptor (170 kDa), c-src (60 kDa), ERK1 (44 kDa), and ERK2 (42 kDa) proteins. The essentially constant intensity of the bands at 110–120 kDa demonstrate even loading of the gel. Note that ERK1 and ERK2 appear as very well separated bands because we ran 7% SDS-polyacrylamide gel electrophoresis gels rather than 10–12%.

Additional text:...

DISCUSSION

Previous work has shown that serum factors stimulate 9E3 expression (5, 6) and that expression under these conditions is initiated during the G0/G1 transition of the cell cycle and declines in S-phase (8). The results presented here show that for the same number of cells, stimulation of 9E3 by thrombin is 15-fold higher than that stimulated by serum. Furthermore, in all of the other experiments performed here, we used qCCEFs that are contact inhibited; under these conditions only a small number of cells will undergo cell division during 18 h of exposure to serum or thrombin. The reduced amounts of 9E3 in the supernatant of qCCEFs treated with serum is reflective of the small number of cells entering G1 (8), whereas the increased amounts of 9E3 after thrombin treatment must reflect expression by many of the preexisting quiescent cells. Therefore, thrombin stimulation of the 9E3 gene occurs by a pathway that does not involve mitogenesis.

It has been reported previously that when cells are infected with viruses that carry the oncogenes v-fps or v-src, they express the 9E3 gene constitutively; these oncogenes activate PKC, which in turn is responsible for triggering mitogenic events that lead to 9E3 expression (51, 67, 68). Our observation that stimulation of 9E3 by thrombin occurs through a nonmitogenic pathway, coupled with the additive effect with v-src stimulation, suggests that v-src and thrombin operate primarily through different independent pathways. Indeed, thrombin activation of 9E3 does not involve PKC and is potentiated by a variety of inhibitors of Ser/Thr kinases, indicating that inhibition of these kinases in general potentiates the stimulation of 9E3 by thrombin. In support of this proposal are the findings of Ishii et al. (69) who showed that the receptor for thrombin is turned off by Ser/Thr kinases that phosphorylate these amino acids present in the C terminus of the receptor. Thus, inhibition of these kinases might allow the thrombin receptor to act for longer periods and produce greater effects.

We show here that tyrosine kinases are importantly involved in stimulation of the 9E3 gene by thrombin. Five specific proteins (four that co-migrate with the c-src, the EGF receptor, and the ERK 1/ERK 2 kinases) are phosphorylated on tyrosines within 5–7 min of stimulation by thrombin, and this stimulation is synchronous (Fig. 7). This observation, coupled with the rapid activation of the gene (15), suggests direct involvement of these proteins in 9E3 stimulation. Their possible roles in 9E3 activation are the subject of ongoing research in our laboratory.

Our observation that herbimycin A, which preferentially inhibits kinases of the c-src family (62, 70), inhibits 9E3 expression stimulated by thrombin, suggests involvement of c-src in this activation. In support of involvement of c-src is the observation that when chicken hamster fibroblasts are treated with thrombin, the c-src tyrosine kinase is activated (42). Also, staurosporine, a broad spectrum Ser/Thr kinase inhibitor, not only potentiated the action of thrombin but by itself stimulated low levels of 9E3 expression. It has recently been found that staurosporine induces spreading of a human colon cancer cell line (70, 71) and that this effect is inhibited by herbimycin A. One of the proteins phosphorylated on tyrosines upon staurosporine treatment was the c-src tyrosine kinase (70, 71). Therefore, it is possible that the stimulation of 9E3 by staurosporine alone is due to the activation of this kinase by this inhibitor, further supporting the involvement of c-src in the expression of 9E3. It was surprising that c-src is involved in this tyrosine-kinase-dependent pathway of 9E3 activation by thrombin, given that activation of this gene by v-src occurs via a PKC-dependent pathway (51). However, we note that it has been shown (72) that v-src can turn on both PKC-dependent and PKC-independent pathways leading to gene expression, showing that src can participate in multiple signaling pathways.

We have used inhibitors of the tyrosine kinase activity of the EGF receptor to address the involvement of this receptor in the stimulation of 9E3 expression by EGF, TGα, and thrombin. The inhibitors we used have varying specificity for the EGF receptor tyrosine kinase. Genistein inhibits tyrosine kinase (70, 71). Therefore, it is possible that the stimulation of 9E3 by tyrphostin AG1478 is due to the activation of this kinase by this inhibitor, further supporting the involvement of c-src in the expression of 9E3. It was surprising that c-src is involved in this tyrosine-kinase-dependent pathway of 9E3 activation by thrombin, given that activation of this gene by v-src occurs via a PKC-dependent pathway (51). However, we note that it has been shown (72) that v-src can turn on both PKC-dependent and PKC-independent pathways leading to gene expression, showing that src can participate in multiple signaling pathways.

We have used inhibitors of the tyrosine kinase activity of the EGF receptor to address the involvement of this receptor in the stimulation of 9E3 expression by EGF, TGα, and thrombin. The inhibitors we used have varying specificity for the EGF receptor tyrosine kinase. Genistein inhibits tyrosine kinase activity by competing with these enzymes for the ATP binding site and therefore is a broad spectrum inhibitor. Genistein eliminated thrombin stimulation of 9E3 (Fig. 6). Lavendustin A RG14355 is a cell-permeable selective inhibitor for the EGF receptor tyrosine kinase (IC50 = 11 nM) and for pp60src (IC50 = 500 nM) (62). At the concentrations used in our study (100 nM) it is selective for the EGF receptor tyrosine kinase. We found that lavendustin A RG14355 virtually inhibited the stimula-
tion of 9E3 by thrombin (Fig. 8A). We also used tyrphostin AG1478, a more potent and selective inhibitor for this receptor tyrosine kinase (62–64). The evidence that tyrphostin AG1478 is highly specific for the EGF receptor tyrosine kinase is now extensive. Fry et al. (63), in a paper directed specifically to selectivity of inhibition for the EGF receptor, showed that a quinazoline identical to tyrphostin AG1478 except for substitution of bromine for chlorine on the free benzene ring of the molecule is a highly selective inhibitor for the EGF receptor at concentrations from a few nanomolar to a few micromolar. Furthermore, in a comprehensive review of tyrosine kinase inhibition, Levitzki and Gazit (62) showed that tyrphostin AG1478 inhibits the EGF receptor tyrosine kinase in vitro with an IC\textsubscript{50} = 3 nM, whereas the corresponding IC\textsubscript{50} values for inhibition of other tyrosine kinases by this molecule were all greater than 50 \mu M (HER2-neu IC\textsubscript{50} > 100 \mu M; platelet-derived growth factor IC\textsubscript{50} > 100 \mu M). In the present study in vivo, concentrations of 500 nM tyrphostin AG1478 completely obliterated stimulation of 9E3 by thrombin, hrTGF\alpha, and hrEGF. Finally, Daub et al. (64) also showed specificity of tyrphostin AG1478 when studying the stimulation of the fos gene by thrombin via transactivation of the EGF receptor tyrosine kinase in Rat-1 fibroblasts. They confirmed their results by use of a dominant negative mutant of the receptor that is capable of inhibiting events downstream from the receptor by forming signaling defective heterodimers with the wild-type receptor. Both tyrphostin AG1478 and this mutation eliminated the EGF receptor phosphorylation stimulated by thrombin and abolished activation of the fos gene. Experiments using a dominant negative mutant of the EGF receptor, similar to those of Daub et al. (64), are not possible in our system because we are using primary cell cultures, and there are no chicken cell lines to perform such studies. However, use of such a mutant by these authors confirmed the specificity of tyrphostin AG1478, enabling us to infer that the stimulation of 9E3 by thrombin involves analogous transactivation of the EGF receptor as an important step in the 9E3 gene activation cascade triggered by thrombin.

Transactivation of a growth factor receptor by another cell-surface receptor is only a recently recognized phenomenon (64, 73). These two previous studies also involve transactivation of the EGF receptor; the former via the thrombin receptor (dis-
cussed in the previous paragraph) and the latter via the TNFα receptor. In both cases, demonstration of transactivation involved detection of fos gene expression. fos is an immediate early response gene that is activated by phosphorylation of the Elk1 transcription factor by mitogen-activated protein kinases (74). Here we have shown activation of another immediate early response gene, 9E3 (5), which codes for a secreted protein, a chemokine. The promoter of this gene also contains an Elk1 binding site (75) and mitogen-activated protein kinase is also involved in 9E3 gene expression stimulated by thrombin.2 Further work will be required to determine if transactivation of the EGF receptor could represent a general mechanism for turning on immediate early response genes by receptors that do not have an intrinsic tyrosine kinase activity.

The greater stimulation of 9E3 by TGFα than by EGF in this study is explained by differences in affinity for the chicken EGF receptor. Although hrEGF and hrgTFα bind with equal affinity to the receptor in humans, hrTFα binds with 100-fold greater affinity than does hrEGF to the chicken receptor (66), hence EGF requires much higher doses to cause the same effect. Our observation that EGF and TGFα are less efficient than thrombin in stimulating 9E3 expression is consistent with the fact that these growth factors are mitogenic for CEFs (76) and stimulate 9E3 via entry into the cell cycle (8). We previously showed using in situ hybridization that only some cells in qCEF cultures can be stimulated to enter cell division and that in experiments involving growth factors, only the dividing cells express 9E3 (8). On the other hand, thrombin stimulation does not require cell division, hence it will stimulate 9E3 expression in a large fraction of cells resulting in greater integrated expression. An additional factor that could contribute to this difference between these growth factors and thrombin is that growth factors interact with their receptors and down-regulate them, whereas the activation of the EGF receptor by thrombin is intracellular and therefore may stay on for longer periods of time.

At first thought, it might seem surprising that the EGF receptor can function as the entry point to a signal-transduction pathway leading to gene expression through a mitogenic pathway (e.g. for EGF and TGFα) and also serve as a major early step in a nonmitogenic pathway (e.g. for thrombin). However, it is already known that after activation by EGF and consequent dimerization, the EGF receptor autophosphorylates on several tyrosine residues that serve as docking sites for a variety of molecular mediators for different signal-transduction pathways that emanate from this receptor (77). The details of the role played by this receptor in the stimulation of 9E3 by thrombin presumably are different from that for either EGF or TGFα because thrombin activates the EGF receptor intracelularly. Therefore, our results suggest the possibility of still greater complexity of behavior of the EGF receptor than previously recognized.

Our results do not yet clarify whether c-src activation precedes or succeeds EGF receptor activation. However, upon thrombin interaction with its receptor, it has been shown that c-src is rapidly activated by both pertussis toxin-sensitive and -insensitive G-proteins (78). In addition, in Rat-1 fibroblasts, v-src phosphorylates G_{q,11} on tyrosines and increases its activity (79, 80). This is also true for several other G_{q,}proteins (81). Therefore, it appears that upon thrombin activation of its receptor, there is mutual interaction between a G_{q,}protein and c-src (82, 83). Furthermore, the association of c-src with the plasma membrane (84) and its known ability to bind to the EGF receptor (85) suggests that it may be involved in the transactivation event. These and other aspects of the signaling pathways stimulated by thrombin and leading to 9E3 expression are currently being pursued in our laboratory.

In summary, thrombin stimulation of the 9E3 chemokine gene in qCEFs occurs via a nonmitogenic pathway and is accompanied by synchronous, biphasic, phosphorylation of several proteins including c-src and the EGF receptor. Similar stimulation of 9E3 by EGF and TGFα verify that the EGF receptor is functional in these cells, and a series of inhibitors that are highly selective for c-src and the EGF receptor tyrosine kinases eliminate 9E3 stimulation by thrombin. This study is now the third to show stimulation of an immediate early response gene via transactivation of the EGF receptor after initial binding of a ligand to a receptor that does not have an intrinsic tyrosine kinase activity, suggesting that this could be a general mechanism for activation of immediate early response genes by the latter class of receptors.

On a broader scale, the stimulation of the 9E3 gene via entry into the cell cycle may be akin to that occurring in normal tissues where expression is very low and probably represents the result of a small number of cells that are undergoing cell division (8, 10). In contrast, the high levels of 9E3 expression triggered by thrombin could represent a response from all cells to an emergency situation such as wounding, tumorigenesis or viral entry into cells. The signal transduction pathway by which this is accomplished, involving transactivation of the EGF receptor and other tyrosine kinases, offers the opportunity for amplification of the signals and a prolonged response, much like the activation of adenylcyclase by the epinephrine receptor. This type of activation could provide an advantage when a rapid and strong tissue response is needed.

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Thrombin Activation of the 9E3/CEF4 Chemokine Involves Tyrosine Kinases Including c-src and the Epidermal Growth Factor Receptor
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Pradip K. Bandyopadhyay, Clark J. Colledge, Craig S. Walker, Li-Ming Zhou, David R. Hillyard, and Baldomero M. Olivera

Page 5449, Table I: the $K_m$ for FLEEL should read 230.1 ± 6 μM instead of 2301 ± 6 μM.

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Thrombin activation of the 9E3/CEF4 chemokine involves tyrosine kinases including c-src and the epidermal growth factor receptor.

Sucheta M. Vaingankar and Manuela Martins-Green

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