Factor VIIa-induced p44/42 Mitogen-activated Protein Kinase Activation Requires the Proteolytic Activity of Factor VIIa and Is Independent of the Tissue Factor Cytoplasmic Domain*

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Signal transduction induced by activated factor VII (FVIIa) was studied with baby hamster kidney (BHK) cells transfected with human tissue factor (TF). FVIIa induced phosphorylation of p44/42 mitogen-activated protein kinase (MAPK) in cells expressing TF, BHK(+TF), but not in wild-type BHK(-TF) cells. BHK(+TF) cells responded to FVIIa in a dose-dependent manner, with detectable phosphorylation above 10–20 nM FVIIa. BHK cells transfected with a cytoplasmic domain-deleted variant of TF also supported FVIIa-induced MAPK activation. Experiments with active site-inhibited FVIIa, thrombin, factor Xa, and Hirudin confirmed that the catalytic activity of FVIIa was mandatory for p44/42 MAPK activation. Furthermore, a high concentration of FVIIa in complex with soluble TF induced p44/42 MAPK phosphorylation in BHK(-TF) cells. These data suggest that TF was not directly involved in FVIIa-induced p44/42 MAPK phosphorylation but rather served to localize the action of FVIIa to the cell surface, potentially to cleave a cell surface receptor. Desensitization experiments with sequential addition of proteases suggested that the p44/42 MAPK response induced by FVIIa was distinctly different from the thrombin response, possibly involving a novel member of the protease-activated receptor family.

The extrinsic pathway of blood coagulation is initiated when factor VIIa (FVIIa) circulating in plasma binds to the integral membrane protein, tissue factor (TF), exposed to the blood upon injury of the vessel wall. TF consists of a 219-residue (1–219) extracellular domain, a 23-residue (220–242) transmembrane domain, and a 21-residue (243–263) cytoplasmic domain. The extracellular part of TF is structured in two fibronectin type III-like domains, which shows structural and sequence homology to the cytokine receptor superfamily. The important role of TF in hemostasis and thrombotic disorders such as atherosclerosis is well established (see (1–5)). Recent findings suggest that TF may participate in biological processes other than hemostasis such as angiogenesis (6), embryonic vascularization (7), and tumor metastasis (8, 9). Furthermore, it has been reported that binding of FVIIa to cell surface FVIIa-induced intracellular Ca2+ oscillations in a number of TF-expressing cells (10, 11), transient phosphorylation of tyrosine in monocytes (12), alteration in gene expression in fibroblasts (13), and enhanced expression of urokinase receptor in pancreatic cancer cells (14). Additional information about FVIIa/TF-induced signal transduction comes from our previous report showing binding of FVIIα to cell surface TF resulted in phosphorylation of p44/42 MAPK (15).

A potential role for the TF cytoplasmic domain in signal transduction was indicated by studies showing that TF expression markedly increased the metastatic potential of melanoma cells (16) and that the pro-metastatic property was critically dependent on this domain (8, 9). This supposition was further substantiated by experiments showing that the cytoplasmic domain of TF can be phosphorylated by a protein kinase C-dependent mechanism (17) and that a synthetic peptide based on the cytoplasmic domain can work as a substrate for cell lysate protein kinase activity (18). Furthermore, cysteine 245 in the TF cytoplasmic domain was shown to be acylated with long chain fatty acids (19).

Although a number of studies, as referred above, suggest that TF is involved in induction of an intracellular activity, it is not clear how signal transduction is mediated across the membrane as a result of binding of FVIIa to TF, just as the role of the cytoplasmic domain of TF in this process is still unclear. The present study explores the effect of removing the TF cytoplasmic domain on p44/42 MAPK signaling and further examines the importance of FVIIa catalytic activity in mediating the activation of the p44/42 MAPK pathway. Our data show that the cytoplasmic TF domain with its putative sites for regulatory modifications is not required for FVIIa-induced p44/42 MAPK phosphorylation. Furthermore, the data provide evidence that specific FVIIa catalytic activity is required and that other serine proteases fail to mimic the response, suggesting that TF/FVIIa may be activating a novel receptor on the cell surface.

**EXPERIMENTAL PROCEDURES**

Cell Culture—The baby hamster kidney cell line BHK-21 tk− ts13 (ATCC CRL 1632) was cultured in Dulbecco’s modified Eagle’s medium containing 10% FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin.

Proteins—Human recombinant FVIIa was expressed and purified as described (20). FFR-FVIIa was prepared as described (21). Human thrombin, human FX and FXα were from Enzyme Research Laboratory Inc. (Lafayette, IN). Recombinant stF was expressed in E. coli and purified as described (22). Hirudin was from Sigma, and trypsin was from Worthington Biochemical Corp. (Lakewood, NJ). Phospho-specific antibody against p44/42 MAPK (Thr-202/Tyr-204) and Western blot detection kit PhosphoPlus™ MAPK was from New England Biolabs Inc. (Beverly, MA).
**Table I**

| Surface exposed TF on transfected BHK cells determined by FVIIa-mediated FXa generation and measurement of $^{125}$I-FVIIa binding |
|---------------------------------------------------------------|
| The amount of total TF was determined by saturation radioligand binding assay. |

| FXa generation* | $^{125}$I-FVIIa binding* |
|-----------------|---------------------------|
| $\text{fA FXa/min cell}$ | $B_{\text{max}}$ (10$^{-6}$ s/micelle$^{-1}$) |
| BHK(+TF) | 2.8 $\pm$ 1.7 ($n = 6$) | 6.3 $\pm$ 0.9 ($n = 3$) |
| BHK(+TF)$_{\text{C245S}}$ | 2.6 $\pm$ 1.5 ($n = 6$) | 5.1 $\pm$ 2.1 ($n = 2$) |
| BHK(-TF) | 3.0 $\pm$ 1.6 ($n = 4$) | 1.9 $\pm$ 0.1 ($n = 3$) |

* FXa generation was measured in the presence of 20 nM FVIIa and 175 nM FX.

**Maximal binding** was determined by curve-fitting to a one-binding-site model.

**Transfection of BHK Cells with TF, TF$_{\text{C245S}}$, and TF$_{\text{C245S}}$**—The complete human TF cDNA was cloned into the mammalian Zen219b expression vector (23). BHK cells were transfected with the TF expression plasmid using the calcium phosphate coprecipitation procedure, essentially as described (24). Cells with stably integrated constructs were selected with 1 $\mu$g methotrexate and cloned by limiting dilution. An expression plasmid for the TF mutant des (248–263) was prepared by a modified version of reverse polymerase chain reaction (a) with the polymerase Pwo (Roche Molecular Biochemicals) and the TF expression plasmid (see above) as a template. The primers CACCTCCTTACCTTCTACTCTTGTTAGAG and TGTAGAAATTGAGTGTTGCCCAGGGTTGAA were used, resulting in introduction of a stop codon after lysine 247. The reverse polymerase chain product was cut with the restriction enzyme DpnI to eliminate template DNA, and competent Escherichia coli were transformed with the reverse polymerase chain product. A single clone containing the introduced stop codon and with no reverse polymerase chain mistakes (confirmed by sequence) was chosen for transfection. BHK cells were transfected with this construct using the Qiafect (Qiagen Inc., Chatsworth, CA) transfection procedure. Transfected cells were selected by 1 $\mu$g methotrexate, and clones were selected by limiting dilution. An expression plasmid for the TF mutant C245S was prepared as described above using the primers ACTCTCTTACTCTTTCTACTTTCTACTTGTTAGAG and TGTTAGAAATTGAGTGTTGCCCAGGGTTGAA, introducing the desired mutation together with a silent HindIII site for easy recognition of mutants. A sequence-confirmed mutant was used for transfecting BHK cells as described above.

**Cell Lysis and Western Blot Analysis**—BHK cells were grown to 70 to 80% confluency in 23.8-cm$^2$ culture dishes and deprived of serum for 24 h before treatment. After specific treatment described under "Results," cells were lysed in 150 $\mu$l of SDS sample buffer (9.2% w/v SDS, 250 mM Tris-HCl, 40% v/v glycerol, 80 mM EDTA, 1.2% w/v bromphenol blue, pH 6.8) supplemented with 3 mM sodium orthovanadate and 24.2 mM diithiothreitol, and 4 $\mu$l of lysate/well was loaded on a 12% polyacrylamide gel. A biotinylated protein marker was loaded on each gel. The proteins separated on the SDS-polyacrylamide gel were transferred to nitrocellulose by electrophoblotting, and p44/42 MAPK was visualized by immunodetection. The total amount of p44/42 MAPK or dually phosphorylated p44/42 MAPK was detected using specific antibodies against phosphorylated MAPK and total MAPK. A detectable increase in phosphorylation over a background of p44/42 MAPK was noted in untreated cells observed in cells treated with 12.5 nM or higher FVIIa concentrations. In additional experiments, we treated BHK(+TF) cells with higher doses of FVIIa (up to 1000 nM) to test whether p44/42 MAPK activation was saturable. FVIIa-induced p44/42 MAPK phosphorylation reached saturation at approximately 200 nM FVIIa (results not shown).

**FVIIa-Induced p44/42 MAPK Phosphorylation in BHK(+TF)$_{\text{C245S}}$ Cells**—BHK cells were stably transfected with a cytoplasmic domain deletion version of human TF, des (248–263)/TF, to examine whether this domain was necessary for FVIIa-induced signal transduction. In addition, it was possible that modification of Cys-245, shown to undergo acylation (19), could be of importance for FVIIa-induced signal transduction. BHK cells were therefore also transfected with a C245S TF mutant. The properties of the resulting BHK cell lines, BHK(+TF)$_{\text{C245S}}$ and BHK(+TF)$_{\text{C245S}}$, in terms of the number of FVIIa binding sites and the FVIIa-mediated FXa generation, were similar to those of the BHK(+TF) cell line (Table I), indicating that these modifications of the cytoplasmic domain of TF did not prevent its cell surface expression or impede its procoagulant activity. As shown in Fig. 2A, treatment of BHK(+TF)$_{\text{C245S}}$ or BHK(+TF)$_{\text{C245S}}$ cells with 100 nM FVIIa for 10 min resulted in marked p44/42 MAPK phosphorylation similar to the FVIIa-induced activation observed in BHK(+TF) cells. The extent of MAPK activation observed with BHK(+TF)$_{\text{C245S}}$ or BHK(+TF)$_{\text{C245S}}$ was quite similar to the extent observed in BHK(+TF) cells. As observed earlier with BHK(+TF) cells, the proteolytic activity of FVIIa was required for p44/42 MAPK phosphorylation in BHK(+TF)$_{\text{C245S}}$ cells, because FVIIa, but not FFR-FVIIa or FXa, was found to induce p44/42 MAPK phosphorylation (Fig. 2C). These data clearly suggested that TF lacking the C-terminal cytoplasmic domain was fully capable of promoting FVIIa-induced signal transduction via the MAPK pathway and also ruled out an obligatory role for Cys-245 acylation in this process.

**Phosphorylation of p44/42 MAPK in BHK(-TF) Exposed to Exogenous sTF/chemp/FVIIa Complexes**—The results obtained so far indicated that FVIIa catalytic activity was a prerequisite for FVIIa-induced phosphorylation of p44/42 MAPK and also...
suggested that this response did not require the participation of the cytoplasmic part of TF. We then asked whether localization of TF on the cell plasma membrane was a necessity for FVIIa-induced p44/42 MAPK phosphorylation and treated BHK(-TF) cells with a soluble complex of sTF-FVIIa or FVIIa alone. As shown in Fig. 3, FVIIa alone failed to activate p44/42 MAPK in BHK(-TF) cells even at a concentration of 1000 nM. In contrast, treatment of BHK(-TF) with a 100 nM preformed sTF-FVIIa complex resulted in a small but consistent response. A more prominent p44/42 MAPK phosphorylation was observed with BHK(-TF) cells treated with 1000 nM sTF-FVIIa complex. No significant signal transduction was observed with 1000 nM FFR-FVIIa.

**Thrombin or FXα Proteolytic Activity Is Not Involved in FVIIa-induced Signal Transduction**—The results of the present and previous studies (15) indicated that the catalytic activity of TF-FVIIa was mandatory for signal transduction, resulting in phosphorylation of p44/42 MAPK and also excluded that the response was induced by an indirect route caused by FVIIa-mediated FXα generation. The involvement of thrombin as an intermediate in signal transduction was also excluded because, as shown in Fig. 4, thrombin at 100 nM did not induce p44/42 MAPK phosphorylation and because hirudin, a specific inhibitor of thrombin, could not inhibit FVIIa-induced signal transduction.

**Desensitization Experiments**—The observed p44/42 MAPK phosphorylation might involve FVIIa-catalyzed proteolysis of a membrane-associated protein, possibly a protease-activated receptor (PAR). Four PARs have so far been identified (25–30). All are G protein-coupled receptors that are activated by thrombin except PAR-2, which is activated by trypsin and trypstatin but not by thrombin (27). So far, soluble FVIIa has not been reported to cleave any of these receptors; however, binding of FVIIa to surface-expressed TF might well be a prerequisite for FVIIa-catalyzed cleavage of known or unknown PARs. To further characterize putative protease-cleavable receptors on BHK(-TF) cells, we compared the p44/42 MAPK phosphorylation response induced by FVIIa with the response obtained with thrombin and trypsin. The data presented in Fig. 5 show that a marked response was induced by exposure of BHK(-TF) cells to FVIIa or trypsin for 10 min, whereas little if any p44/42 MAPK phosphorylation was induced by thrombin. Activation of PARs produces a transient p44/42 MAPK phosphorylation, which renders the cell refractory to a second stimulus for a certain time interval, and such desensitization experiments have been used to obtain indirect evidence for the participation of certain PARs (31–33). BHK(-TF) cells were exposed to control vehicle, FVIIa, thrombin, or trypsin for 40 min before a second 10-min exposure to FVIIa. Preincubation with FVIIa resulted in complete desensitization of FVIIa/TF-induced signaling, whereas pretreatment with thrombin did not prevent the subsequent TF-FVIIa-induced p44/42 MAPK activation. The difference in MAP kinase phosphorylation between FVIIa-pretreated cells and thrombin-pretreated cells was statistically significant (p = 0.038), whereas the difference between control vehicle-pretreated cells and thrombin-pretreated cells was not significant (p = 0.35). Pretreatment of cells with trypsin also did not prevent the FVIIa-induced p44/42 MAPK phosphorylation, although the response was not as marked as observed in either control or thrombin-pretreated cells. The p values for differences in MAPK activation between the groups was as follow. Between trypsin-pretreated cells and FVIIa-pretreated cells, p = 0.033; between trypsin-pretreated cells and control vehicle-pretreated cells, p = 0.27.

**DISCUSSION**

The proteolytic activity of FVIIa and the interaction with TF on the cell surface is of primary importance for initiation of blood coagulation. A number of recent studies suggest that TF...
may also be involved in angiogenesis, cell migration, and embryonic development (7) and in tumor metastasis (8, 9, 16). Additional evidence for the importance of TF in biological functions other than coagulation comes from a recent observation suggesting that TF may act as a true receptor (10–15). Although it was shown that a TF-dependent intracellular activity was induced by binding of FVIIa to TF on the cell surface, it was not clear how signal transduction across the plasma membrane was accomplished. We have shown recently (15) that FVIIa/TF-induced signal transduction leads to p44/42 MAPK phosphorylation. The data of the present study (Fig. 1) show that p44/42 MAPK activation is FVIIa dose-dependent and detectable at physiological levels of FVIIa. The present study confirms that the catalytic activity of FVIIa is required for FVIIa/TF-mediated p44/42 MAPK phosphorylation and also provides further evidence that down-stream coagulation factors FXa and thrombin, which might have been generated as a result of FVIIa activity, are not involved in FVIIa/TF-mediated MAPK activation.

Our studies with TF-transfected BHK cells (Fig. 2) clearly demonstrated that TF mutants lacking the cytoplasmic domain or the acylation site at Cys-245 were fully capable of mediating FVIIa-induced p44/42 MAPK phosphorylation. These data exclude a mandatory role for the cytoplasmic tail TF or its acylation in FVIIa/TF-induced signaling via the MAPK pathway, although we cannot completely exclude the involvement of the tail in modulation of the response. It has been speculated that TF, because of its sequence homology to the cytokine receptors, might be capable of mediating transmembrane signal transduction by receptor dimerization or oligomerization followed by reciprocal phosphorylation of intracellular domains (35). However, our present finding that FVIIa can induce MAP kinase activation in cells expressing truncated TF with no intracellular domain excludes such a mechanism, at least for TF/FVIIa-induced MAP kinase activation. Recent preliminary observations showing that human TF protein lacking the cytoplasmic domain rescues TF−/− mouse embryos (36) provide an indirect support to our present conclusion that the TF extracellular domain can function independently of the cytoplasmic domain in mediating signal transduction. However, one should note that our data do not necessarily rule out a possible involvement of the cytoplasmic tail in other TF-mediated signal transduction events. A requirement for the cytoplasmic domain of TF is suggested from studies on its prometastatic activity (8, 9). Furthermore, the cytoplasmic domain of TF was shown to interact with actin-binding protein 280 (ABP-280), suggesting a role for TF in reactions associated with rearrangement of cellular cytoskeleton elements (37). The exact nature and the importance of these interactions in relation to the p44/42 MAPK pathway remains to be fully unraveled.

The results obtained upon treatment of BHK(-TF) cells with exogenous sTF-FVIIa complex (Fig. 3) are consistent with a pertinent role for membrane-bound TF in FVIIa/TF-mediated signal transduction because of its virtue of creating a localized area of high FVIIa activity on the plasma membrane. The importance of membrane localization was further substantiated by experiments with a truncated version of FVIIa, des(1–44)FVIIa, which lacks its membrane binding γ-carboxyglutamyl acid-containing domain but retains TF-binding and enhanced proteolytic activity. An exogenous complex between this FVIIa derivative and TF, sTF-des(1–44)FVIIa, induced a decreased response compared with sTF-FVIIa when added to BHK(-TF) cells (results not shown) presumably because of the diminished membrane association of this complex because of the absence of the FVIIa-membrane binding domain.

The fact that FVIIa proteolytic activity is required for FVIIa-induced p44/42 MAPK phosphorylation may suggest that signal transduction is mediated by a so-called PAR, which is cleaved when FVIIa binds to TF on the cell surface. The presently known PARs (PAR 1–4) belong to a subgroup of the G protein-coupled receptor family. The thrombin receptor (PAR-1), originally cloned from a human megakaryoblastic cell line (25), is best known and serves as a prototype for protease-activated receptors. The PARs are activated by a mechanism in which a protease recognizes and cleaves an extracellular N-terminal exodomain of the receptor to reveal a new N terminus containing a receptor-activating sequence that serves as a tethered ligand. Intracellular calcium mobilization and inositol phosphate formation are the most commonly reported consequences of PAR activation; however, p44/42 MAPK phosphorylation as a result of protease-catalyzed cleavage has been observed with both PAR-1 (34) and PAR-2 (31). The intracellular reactions following activation of PAR-3 and -4 are not well characterized. The intracellular effects reported, MAPK phosphorylation (15) and calcium mobilization (10), as a result of FVIIa/TF activity are therefore fully consistent with the possible activation of a known or a hereto undiscovered member of the PAR family.

Although a certain protease preference for specific PARs exists, it is well recognized that a given PAR may be cleaved by several proteases and that a given protease may cleave several PARs. This cross-reactivity complicates a functional classification of specific PARs. However, identification clues may be obtained from experiments in which receptor activation by a protease makes this receptor refractory to a second stimulus. We explored this possibility by exposing the cells to FVIIa, thrombin, or trypsin followed by a subsequent exposure to FVIIa. Pre-exposure to FVIIa and not to thrombin resulted in desensitization of the FVIIa-induced response. Exposing the cells to trypsin resulted in partial desensitization of the FVIIa-induced response. These observations indicate that receptors activated/desensitized by thrombin and probably by trypsin were not involved in FVIIa-induced signal transduction. Thrombin activates and is also likely to desensitize PAR-1, -3, and -4 but not PAR-2 (27). Thus the inability of thrombin pretreatment to abolish FVIIa-induced MAP kinase activation can be taken as evidence against the involvement of PAR-1, -3, and -4. Localization of thrombin to the cell membrane (e.g. by
**Fig. 5. Desensitization of protease-stimulated p44/42 MAPK activation in BHK(+TF) cells.** The bars represent results from densitometer scanning of Western blot analysis of phosphorylated p44/42 MAPK. The bands on the autoradiograph were quantified, and the density of the band observed in cells treated with 100 nM FVIIa for 10 min was set to 100%. The bars show the mean and S.D. from three independent experiments. Quiescent BHK(+TF) cells were incubated for 10 min (black bars) or 40 min (open bars) with control medium, 100 nM FVIIa, 100 nM human α-thrombin, or 30 nM trypsin. Gray bars represent quiescent BHK(+TF) cells pretreated for 40 min with control medium, 100 nM FVIIa, 100 nM thrombin, or 30 nM trypsin before subsequently challenged by the addition 100 nM FVIIa for 10 min.

binding to PAR-1) might be a possible prerequisite for its desensitization of PARs 3 and 4, in which a positive identification of PAR-1 on BHK(+TF) cells would be needed to draw conclusions about the putative role of PAR-3 or PAR-4 in TF/ FVIIa-induced signal transduction from our desensitization experiments. However, the observation that BHK cells respond to the PAR-1 agonist, SFLLRN, and thrombin with mobilization of intracellular Ca\textsuperscript{2+} stores\(^2\) suggests that they do indeed express PAR-1 and may not require an additional binding site on the membrane for thrombin desensitization of PAR-3 and PAR-4. The data in Fig. 5 show that trypsin induced a significant p44/42 MAPK activation. The response had not completely returned to the basal level in 40 min, and this may have complicated the desensitization experiment. Nonetheless, FVIIa treatment did enhance MAP kinase activation in trypsin-pretreated cells. Furthermore, treatment of the cells with 300 μM of the murine PAR-2 agonist, SLIGRL, did not induce p44/42 MAPK activation (results not shown) as was expected from PAR-2 activation experiments with rat aortic smooth muscle cells and bovine fibroblasts\(^3\). This raises the possibility that BHK cells may not express PAR-2 and also makes it unlikely that PAR-2 is involved in FVIIa-induced signal transduction. However, the observation of a partly desensitized FVIIa response could indicate that trypsin may, in addition to PAR-2, also cleave the putative receptor activated by FVIIa. So far then, the combined data on characterization of FVIIa-induced signaling seems to exclude the currently known PARs as targets for FVIIa activation, making it possible that a hereto undiscovered member of the PAR family is responsible for FVIIa-induced signal transduction.

The prometastatic function of TF-expressing cells appears to depend on the concerted action of FVIIa/TF activity and certain undisclosed member of the PAR family is responsible for the FVIIa response could indicate that trypsin may, in addition to PAR-2, also cleave the putative receptor activated by FVIIa. So far then, the combined data on characterization of FVIIa-induced signaling seems to exclude the currently known PARs as targets for FVIIa activation, making it possible that a hereto undiscovered member of the PAR family is responsible for FVIIa-induced signal transduction.

**REFERENCES**

1. Camerer, E., Kolsto, A.-B., and Prydz, H. (1996) *Thromb. Res.* 81, 1–41
2. Petersen, L. C., Valentin, S., and Hedner, U. (1995) *Thromb. Res.* 79, 1–47
3. Rapaport, S., and Rao, M. V. L. (1995) *Thromb. Haemostasis* 74, 7–17
4. Semeraro, N., and Colucci, M. (1997) *Thromb. Haemostasis* 78, 759–764
5. Taubman M. B., Fallon, J. T., Schecter, A. D., Giesen, P., Mendlowitz, M., Fyfe, B. S., Marmur, J. D., and Nemerson, Y. (1997) *Thromb. Haemostasis* 78, 200–204
6. Shoji, M., Hancock, W. C., Abe, K., Mimco, C., Casper, K. A., Baine, R. M., Wilcox, J. N., Damave, I., Dillehay, D. L., Matthews, E., Contrino, J., Morrissey, J. H., Gordon, S., Edgington, T. S., Rudnky, B., Kreutzer, D. L., and Rickles, F. R. (1998) *Am. J. Pathol.* 152, 399–411
7. Carmeliet, P., Mackman, N., Moons, L., Luther, T., Gressens, P., Van Vlaenderen, I., Demunck, H., Kasper, M., Breier, G., Evrard, P., Muller, M., Risau, W., Edgington, T., and Collen, D. (1996) *Nature* 383, 75–79
8. Breunig, M. C., Konigsberg, W. H., Madison, J. F., Pawele, A., and Garen, A. (1995) *Proc. Natl. Acad. Sci U. S. A.* 97, 8205–8209
9. Muller, B. M., and Ruf, W. (1997) *J. Clin. Invest.* 101, 1372–1378
10. Rottningen, J.-A., Ender, T., Camerer, E., Iversen, J.-G., and Prydz, H. (1995) *J. Biol. Chem.* 270, 4650–4660
11. Camerer, E., Rottningen, J.-A., Iversen, J.-G., and Prydz, H. (1996) *J. Biol. Chem.* 271, 29034–29042
12. Masuda, M., Nakamura, S., Murakami, S., Koyama, Y., and Takahashi, H. (1996) *Eur. J. Immunol.* 26, 2529–2532
13. Pendurthi, U. R., Alek, D., and Rao, L. V. M. (1997) *Proc. Natl. Acad. Sci U. S. A.* 94, 12598–12603
14. Taniguchi, T., Kakkar, A. K., Tuddenham, E. G. D., Williamson, R. C. N., and Lemoine, N. R. (1998) *Cancer Res.* 58, 4461–4467
15. Poulsen, L. K., Jacobsen, N., Sørensen, B. B., Bergendal, N. C. H., Kelly, J. D., Foster, D. C., Thastrup, O., Ezban, M., and Petersen, L. C. (1998) *J. Biol. Chem.* 273, 6228–6232
16. Mueller, B. M., Reinfeld, R. A., Edgington, T. S., and Ruf, W. (1992) *Proc. Natl. Acad. Sci U. S. A.* 90, 11352–11356
17. Zinocheck, T. F., Roy, S., and Vehar, G. A. (1992) *J. Biol. Chem.* 267, 3561–3564
18. Musy, R. S., and Carlson, S. D. (1997) *Biochemistry* 36, 7869–7875
19. Bach, R., Konigsberg, W. H., and Nemerson, Y. (1988) *Biochemistry* 27, 4227–4231
20. Thim, L., Christensen, M., Sørensen, E. M., Lund-Hansen, T., Pedersen, A. H., and Hedner, U. (1988) *Biochemistry* 27, 7785–7793
21. Sørensen, B. B., Persson, E., Freskgård, P.-O., Kjalke, M., Ezban, M., Willamsa, T., and Rao, L. V. M. (1997) *J. Biol. Chem.* 272, 11863–11868
22. Freskgård, P.-O., Olsen, O. H., and Persson, E. (1996) *Protein Sci.* 5, 1531–1540
23. Busby, S. J., Mulvihill, E., Rao, D., Kumar, A. A., Lioubin, P., Heipel, M., Sprecher, C., Halfpap, L., Prunkard, D., Gambee, J., and Foster, D. C.

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\(^2\) L. C. Petersen, O. Thastrun, G. Hagel, B. B. Sørensen, P.-O. Freskgård, L. V. M. Rao, and M. Ezban, unpublished data.
(1991) J. Biol. Chem. 266, 15286–15292
24. Gorman, C. M. (1985) in DNA Cloning. A Practical Approach (Glover, D. M., ed) Vol. 1–2, pp. 142–165 IRL Press at Oxford University Press, Oxford
25. Vu, T. K. H., Hung, D. T., Wheaton, V. I., and Coughlin, S. R. (1991) Cell 64, 1057–1068
26. Rasmussen, U. B., Vouret-Craviari, V., Jallat, S., Schlesinger, Y., Pages, G., Pavirani, A., Lecoeq, J. P., Pouyssegur, J., and Van Obberghen-Schilling, E. (1991) FEBS Lett. 288, 123–128
27. Nystedt, S., Emilsson, K., Wahlestedt, C., and Sundelin, J. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9208–9212
28. Ishihara, H., Connolly, A. J., Zeng, D., Kahn, M. L., Zheng, Y. W., Timmons, C., Tram, T., and Coughlin, S. R. (1997) Nature 386, 502–506
29. Xu, W.-F., Andersen, H., Whitmore, T. E., Gilbert, T., Ching, A., Davie, E. W., and Foster, D. C. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 6642–6646
30. Kahn, M. L., Zheng, Y.-W., Huang, W., Bigornia, V., Zeng, D., Moff, S., Farese, R. V., Jr., Tam, C., and Coughlin, S. R. (1998) Nature 394, 690–694
31. Belham, C. M., Tate, R. J., Scott, P. H., Pemberton, A. D., Miller, H. B. P., Wadsworth, R. M., Gould, G. W., and Pievin, R. (1996) Biochem. J. 328, 939–946
32. Molino, M., Blanchard, N., Belmonte, E., Tarver, A. P., Abrams, C., Hoxie, J. A., Cerletti, C., and Brass, L. F. (1995) J. Biol. Chem. 270, 1168–1175
33. Renesto, P., Si-Tahar, M., Moniatte, M., Bailey, V., van Dorsellaer, A., Pidard, D., and Chignard, M. (1997) Blood 89, 1944–1953
34. Pages, G., Lenormand, P., L'Allemain, G., Chambard, J.-C., Meloche, S., and Pouyssegur, J. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 8319–8323
35. Bazan, J. F. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 6934–6938
36. Parry, G. C. N., Erlich, J., and Mackman, N. (1998) Circulation 98, Suppl. I, 40 (abstr.)
37. Ott, I., Fischer, E. G., Miyagi, Y., Mueller, B. M., and Ruf, W. (1998) J. Cell Biol. 140, 1241–1253