Communication

Thrombin Induces Activation of p38 MAP Kinase in Human Platelets

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In human platelets a proline-directed kinase distinct from the ERK MAP kinases is stimulated by both thrombin and the thrombin receptor agonist peptide SFLLRN and may be involved in the activation of Ca2+-dependent cystosolic phospholipase A2 (Kramer, R. M., Roberts, E. F., Hyslop, P. A., Utterback, B. G., Hui, K. Y., and Jakubowski, J. A. (1995) J. Biol. Chem. 270, 14816–14823). Here we show that this kinase is identical with or closely related to p38 (the mammalian homolog of HOG1 from yeast), a recently discovered protein kinase typically activated by inflammatory cytokines and environmental stress. Further, we demonstrate that activation of this kinase by thrombin is transient (with maximal stimulation at 1 min), is accompanied by tyrosine phosphorylation, and precedes the activation of the ERK kinases. This is the first report to show that p38 kinase is activated by thrombin and to suggest a role for this MAP kinase in the thrombin-mediated signaling events during platelet activation.

We have recently shown that thrombin stimulates the activity of the MAP kinase ERK1 and ERK2 but also activates another proline-directed kinase that is distinguishable from ERK1/2 based on its strong binding to anion exchange resin and the lack of reactivity with anti-ERK1/2 antibodies (1). We further noted that this kinase readily phosphorylates cPLA2 but not the S505A mutant of cPLA2. This observation indicated that the serine residing within the MAP kinase consensus sequence (i.e. Pro-Leu-Ser-Pro) is the target phosphorylation site for this kinase. Significantly, the thrombin receptor agonist peptide SFLLRN also activated this proline-directed kinase but completely failed to stimulate ERK1/2. Nonetheless SFLLRN, like thrombin, mediated activation of cPLA2 by phosphorylation, and we reasoned that this unidentified kinase could play a role in the signal transduction pathways activated through the thrombin receptor. We therefore further characterized the kinase with the goal to determine its identity and define its role in the thrombin-induced signaling events during platelet activation.

EXPERIMENTAL PROCEDURES

Platelet Isolation and Incubation—Fresh human platelets were prepared from platelet-rich plasma of drug-free volunteers in the presence of prostacyclin (10–8 m) and apyrase (0.5 units/ml) as described previously (2), suspended at 1.25 × 108/ml in 140 mM NaCl, 27 mM KCl, 1 mM MgCl2, 2.2 mM CaCl2, 5.5 mM glucose, 0.2 mM EGTA, 10 mM Hepes, pH 7.4, containing 30 mM cytosol, 5 mM MgCl2, 0.5 mM Na3VO4, 5 mM Na2ATP, 0.1% BSA, and 100 μM microcystin-RL (Har-gly-GDP-P-Ne-H2O), (where Mpr is mercaptopropionyl-Gly-Har-Gly-Pen-NH2, Har is homogargincine, and Pen is penicillamine) (3) (kindly provided by Dr. Robert Scarborough, COR Therapeutics, and incubated at 37°C with 5 units/ml α-thrombin (~3500 NIH units/mg Enzyme Research Laboratories). Reactions were terminated by adding (final concentrations) 1% Triton X-100, 5 mM EGTA, 1 mM DTT, 0.2 mM Na3VO4, 100 μM microcystin (Life Technologies, Inc.), 100 μM leupeptin, 0.2 mg/ml aprotinin, 10 μM pepstatin A, 1 mM Pefabloc (Centerchem), and 50 mM β-glycerophosphate, pH 7.5. The suspension was then briefly sonicated, centrifuged for 30 min at 100,000 × g using a Sorvall RC M120E microcentrifuge, and diluted with MonoQ buffer as indicated.

Partial Purification of p38 and ERK Kinases by MonoQ Chromatography—High speed supernatants were subjected to chromatography on a MonoQ HR 5/5 column (Pharmacia Biotech Inc.) at a flow rate of 1.5 ml/min collecting 0.5-ml fractions using two different procedures. In order to partially purify the p38 kinase, the column was first equilibrated in buffer A (1 mM EGTA, 1 mM DTT, 100 mM NaVO3, 50 mM β-glycerophosphate, pH 7.5) containing 150 mM NaCl and subjected to a 30-ml linear salt gradient from 150 to 530 mM NaCl. For rapid partial purification of the p38 and the ERK kinases, the MonoQ column was equilibrated in buffer A containing 50 mM NaCl and then subjected to a step gradient from 50 to 250 mM NaCl (for elution of ERK1/2), followed by a step gradient from 250 to 450 mM NaCl (for elution of p38). MonoQ fractions (0.5 ml) were collected into 10 μl of a mixture providing (final concentrations) 100 mM microcystin, 100 μM leupeptin, 0.1 mg/ml aprotinin, and 1 mM Pefabloc.

Assay for Proline-directed Kinases—Kinase assays were performed as described before (1) using the Thr669 peptide substrate (KRELVE-pSer-Pen-GDWP, where Ser is the known thrombin-activated Thr669 kinase, on the other hand, readily phosphorylated the Thr669 peptide derived from the epidermal growth factor receptor (1). Based upon the distinct chromatographic and immunological characteristics, these kinases could be distinguished and found to consist of the MAP kinases ERK1/2, as well as another unidentified proline-directed kinase. When extracts from control and thrombin-stimulated platelets were applied to MonoQ in buffer containing 150 mM NaCl, ERK1/2 flowed through the column. The unknown thrombin-activated Thr669 kinase, on the other hand, bound tightly to the column and eluted at ~350 mM NaCl (Fig.

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Consequently, the isoelectric point (IEP) of this kinase had to be significantly lower than that of ERK1/2 (6.8). For example, the IEP of cPLA2, that binds similarly to MonoQ eluting with ~400 mM NaCl is 5.1 (4). Only three recently identified proline-directed kinases exhibit calculated IEPs in agreement with the observed chromatographic behavior of the platelet Thr\(^{669}\) kinase. These include JNK2 (p54\(_{62}\)) (5), p38 kinase (6), and an ERK3 homolog (referred to as p9\(^{MAPK}\)) (7) with IEPs of 5.7, 5.6, and 4.8, respectively.

We therefore subjected extracts from thrombin-stimulated platelets and the most active Thr\(^{669}\) kinase MonoQ fractions 34 and 35 (see Fig. 1A) to SDS-PAGE/immunoblotting, probing with antibodies against JNK2, p38, and ERK3. As shown in Fig. 2A, anti-p38 antibodies specifically recognized a protein of ~40 kDa that was enriched in the MonoQ fractions (lane 2) compared with the loaded platelet extract (lane 1). The same ~40-kDa protein strongly reacted with anti-phosphotyrosine antibodies (lane 4). Although ERK1 and ERK2 could be readily detected in extracts (lane 5), they were absent in the MonoQ fractions containing the Thr\(^{669}\) kinase activity (lane 6). As demonstrated in Fig. 2B, no immunoreactivity could be detected in extracts or active MonoQ fractions when probing with anti-JNK antibodies (lanes 8 and 9) or anti-ERK3 antibodies (lanes 11 and 12). By comparison both kinases could be readily seen when standard proteins were tested (lane 7 and lane 10). We confirmed the specificity of two anti-p38 antibodies (anti-p38N and anti-p38C) for the ~40-kDa protein by competition experiments with the respective C- and N-terminal peptides of p38 used for immunization. As demonstrated in Fig. 2C, the reactivity with the ~40-kDa protein was significantly decreased when the immunizing peptides were present during immunoblotting, including the anti-p38N (lane 14 versus lane 13) and the anti-p38C (lane 16 versus lane 15) antibodies. We further examined whether the kinase activity eluting from the MonoQ column (Fig. 1A) paralleled the immunoreactivity of the eluting kinase protein probing with both anti-p38 and anti-phosphotyrosine antibodies. As shown in Fig. 1B (upper right panel), the MonoQ elution of the ~40-kDa protein recognized by the anti-p38 antibodies correlated with the thrombin-induced Thr\(^{669}\) kinase activity. Likewise, coincident with the peak of kinase activity we detected thrombin-induced tyrosine}

![Image](http://www.jbc.org/p38 Kinase Activation in Thrombin-stimulated Platelets)

**Fig. 1.** Partial purification of thrombin-stimulated Thr\(^{669}\) kinase activity. Soluble extracts derived from 7.5 x 10\(^{9}\) platelets incubated for 2 min at 37\(^\circ\)C were subjected to MonoQ column in buffer containing 150 mM NaCl collecting 0.5-ml fractions. A, determination of kinase activity in 8.3-\(\mu\)l aliquots of column fractions as described under “Experimental Procedures.” B, aliquots (10 \(\mu\)l) of loaded extracts (C), flow-through (FT) and selected MonoQ fractions (as indicated) of control platelets (left panels, lanes 1–11), and thrombin-stimulated platelets (right panels, lanes 12–22) were subjected to SDS-PAGE/immunoblotting probing with anti-p38 (C-20, \(\alpha\)p38) antibodies (upper panels) and anti-phosphotyrosine (\(\alpha\)PTyr) antibodies (lower panels) as detailed in Fig. 2. Molecular mass markers are indicated on the left; \(\ast\) designates the fractions with highest kinase activity.

**Fig. 2.** Immunological identification of thrombin-stimulated Thr\(^{669}\) kinase. Soluble extracts (C) from thrombin-stimulated platelets (10 \(\mu\)l), MonoQ fractions containing the Thr\(^{669}\) kinase (K) (10 \(\mu\)l) (as in Fig. 1A), and standard proteins (St) were subjected to SDS-PAGE/immunoblotting probing with different antibodies as detailed under “Experimental Procedures.” A, anti-p38 (C-20) polyclonal antibody at 0.1 \(\mu\)g/ml (Santa-Cruz Biotechnology) (lanes 1 and 2), anti-phosphotyrosine (\(\alpha\)P-Y) monoclonal antibodies 4G10 (Upstate Biotechnology) plus PY20 (ICN) at 1 \(\mu\)g/ml each (lanes 3 and 4), and anti-ERK1/2 polyclonal antibody erk1-CT (Upstate Biotechnology) at 1 \(\mu\)g/ml (lanes 5 and 6). B, anti-JNK2 polyclonal antibody (Santa-Cruz Biotechnology) at 0.1 \(\mu\)g/ml (lanes 7–9) and anti-ERK3 antibody (Transduction Laboratories) at 1 \(\mu\)g/ml (lanes 10–12). The ability of anti-JNK2 and anti-ERK3 antibodies to recognize JNK2 and ERK3, respectively, was verified with purified human JNK2 (Santa-Cruz Biotechnology) (lane 7) and human fibroblast ERK3 (Transduction Laboratories) (lane 10). C, anti-p38 (N-20) antibodies (Santa-Cruz Biotechnology) in the absence (lane 13) and presence of immunizing peptide N-20 (lane 14), and anti-p38 (C-20) antibody in the absence (lane 15) and presence of immunizing peptide C-20 (lane 16). Migration position of molecular mass marker is indicated on the right.
phosphorylation of the same ~40-kDa protein (Fig. 1B, lower panel). Taken together, these data indicate that the proline-directed kinase activated by thrombin is identical with, or closely related to, the p38 MAP kinase.

We determined the kinetics of thrombin-mediated activation of the p38 kinase, resolving it from ERK1/2 by MonoQ chromatography. As shown in Fig. 3A, thrombin induced a transient stimulation of p38 kinase activity that reached a maximum at 1 min and was still detected at the latest time point measured (5 min). The amount of p38 protein purified by MonoQ chromatography was the same for all time points examined, as verified by SDS-PAGE/immunoblotting (Fig. 3B). The appearance and disappearance of p38 kinase activity in thrombin-stimulated platelets temporally coincided with the tyrosine phosphorylation of p38 kinase (Fig. 3C). By comparison, activation of the ERKs was delayed with maximal stimulation at 2 min following thrombin stimulation, as shown by the ability of ERK1/2 to phosphorylate the Thr669 peptide substrate (Fig. 4A) and the decreased electrophoretic mobility of the ERK proteins (Figs. 3C and 4B), indicative of activation (8). The data in Fig. 4 also reveal that activation by thrombin of p38 is more prominent than that of ERK1/2. A similar robust activation of p38 and delayed stimulation of ERK1/2 were observed in aspirinized platelets (where the synthesis of endogenous thromboxane A2 is inhibited), demonstrating that p38 kinase is the target of thrombin and not of the secondary agonist thromboxane A2 that is released from activated platelets.

The p38 kinase belongs to a new subfamily of stress-activated MAP kinases related to the HOG1 gene product, a kinase required for adaptation to osmotic stress in Saccharomyces cerevisiae (9), and has only recently been identified in mammalian cells. Thus, Han et al. (10) first described this novel kinase...
of apparent molecular mass of 38 kDa (therefore referred to as p38) in cells of monocyte lineage, observing that it is rapidly phosphorylated on tyrosine residues in response to endotoxin. Cloning of the p38 kinase revealed that its predicted sequence is 52% identical to the yeast kinase Hog1 (6) and shares with Hog1 the unique sequence TGY comprising the dual phosphorylation site typical of MAP kinases. Lee et al. (11) identified the new kinase CSBP, a target of cytokine synthesis inhibitors, that was found to be identical with p38 kinase. Furthermore, Rouse et al. (12) discovered a stress-activated kinase recognized by antibodies against the Xenopus kinase Mpk2, a kinase closely related to Hog1 from yeast, and Freshney et al. (13) purified an interleukin-1-stimulated kinase from human epidermal carcinoma cells whose biochemical properties closely resembled those of the p38 kinase. Recent studies by Raingeaud et al. (14) showed that p38 kinase is activated not only by osmotic stress and endotoxin but is also stimulated by inflammatory cytokines, particularly tumor necrosis factor, and exposure to UV radiation. In contrast, the p38 kinase was only poorly activated by growth factors, interferon-γ and phorbol ester (6, 10, 14). Here, we report that the serine protease thrombin known to activate a heterotrimeric G protein-coupled receptor causes a marked activation of the p38 kinase. The stimulation of p38 kinase by thrombin not only precedes that of the ERKs but is also more pronounced than that of the ERKs. This suggests that p38, rather than the ERKs, may be involved in early proline-directed phosphorylation events during thrombin-mediated platelet activation. The difference in the temporal pattern of activation is consistent with the notion that the p38 and ERK MAP kinases are independently regulated by distinct signaling pathways (15).

The sequential kinase cascade leading to the activation of the ERK MAP kinases lies downstream of Ras and consists of two protein kinases (Raf and MAP kinase kinase) acting sequentially to activate the ERKs (15). In contrast, the upstream regulatory mechanisms and protein kinases involved in the activation of p38 are not yet fully elucidated. Activation of p38 kinase requires dual phosphorylation on Thr180 and Tyr182 (14). Recently, a MAP kinase kinase referred to as JNKK (16) showed that p38 kinase is activated not as p38, but, unlike thrombin, does not activate ERK1/2 (1). These findings suggest the existence of parallel pathways leading to the activation of either p38 or the ERKs. While both pathways can be stimulated by thrombin, SFLLRN activates solely the signaling pathway causing p38 stimulation. It thus appears that, at least in SFLLRN-stimulated platelets, the p38 kinase pathway is responsible for regulation of cPLA2. While other physiological functions of the p38 kinase remain to be elucidated, p38 is likely to be an integral part of a signaling pathway utilized by the thrombin receptor in platelets, and it will be of great interest to further investigate the role of p38 in platelet function.

CONCLUSION

Thrombin rapidly and potently stimulates the p38 kinase in human platelets, demonstrating that extracellular stimuli other than stress-related events and proinflammatory cytokines can activate this proline-directed kinase. Taken together with our previous findings (1) these observations suggest that in platelets (i) thrombin activates two distinct signaling pathways that result in the activation of either the p38 or the ERK MAP kinases, (ii) the thrombin receptor agonist peptide SFLLRN exclusively signals through the p38 pathway, and (iii) cPLA2 appears to be one of the downstream targets of p38 kinase.

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