Role for p38 Mitogen-activated Protein Kinase in Platelet Aggregation Caused by Collagen or a Thromboxane Analogue*

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p38 mitogen-activated protein kinase (MAPK) was identified in platelets on the basis of (a) its reactivity with antibodies to C-terminal and N-terminal peptides, and (b) its ability to activate MAPK-activated protein kinase-2, which phosphorylates the small heat shock protein, hsp27. p38 MAPK was activated in platelets by collagen fibers, a collagen-related cross-linked peptide, thrombin, or the thromboxane analogue U46619. A highly specific inhibitor of p38 MAPK (SB203580), a pyridinyl imidazole known as SB203580, inhibited the platelet enzyme in vitro (IC50 = 0.5 μM). At similar concentrations it also inhibited agonist-stimulated phosphorylation of hsp27 in platelets, and platelet aggregation and secretion induced by minimal aggregatory concentrations of collagen or U46619, but not thrombin. Inhibition of aggregation was overcome by increasing agonist dose. SB203580 might act by inhibiting thromboxane generation, but this was only inhibited by 10–20% at low agonist concentrations.

p38 MAPK provides a crucial signal, which is necessary for aggregation caused by minimal concentrations of collagen fibers or U46619. Thrombin or high doses of these agonists generate signals that bypass the enzyme, or render the enzyme no longer rate-limiting.

p38 mitogen-activated protein kinase (MAPK) is a member of a family of enzymes activated by dual phosphorylation upon

The abbreviations and trivial names used are: MAPK, mitogen-activated protein kinase; hsp, heat shock protein; IL1, interleukin 1; JNK, J un-terminal kinase; MAPKAPK-2, mitogen-activated protein kinase-activated protein kinase-2; PMA, phorbol 12-myristate 13-acetate; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl) imidazole; SKF86002, 6-(4-fluorophenyl)-2,3-dihydro-5-(4-pyridyl)-2-thionyl and tyrosyl residues separated by a single amino acid (1–4). The first members of this family to be discovered in mammalian cells were the closely-related p42 and p44 MAPKs, which are activated by a wide variety of growth factors and many other stimuli (5). Subsequently p54 MAPKs (α, β, and γ) (6) and p38 MAPK (1–4) were identified. These latter types of MAPK were found to be activated only weakly by mitogens, but very strongly by stressful stimuli, endotoxin, and the inflammatory cytokines interleukin 1 (IL1) and tumor necrosis factor (2–4, 6, 7).

p42 and p44 MAPK phosphorylate a variety of intracellular proteins including other kinases, transcription factors, and cytoplasmic and cytoskeletal proteins. They are important for proliferative and differentiative responses (5, 8). p54 MAPKs strongly phosphorylate and activate the transcription factors c-jun (they are also known as c-Jun N-terminal kinases or JNK) and activating transcription factor-2 (9–11). Their major function appears to be to control expression of c-jun itself and other genes regulated by activator protein-1 complexes (12).

The function of p38 MAPK is unclear. It is related to the yeast gene product HOG1, which lies on an osmosensing pathway that regulates glycerol synthesis enabling the microorganism to withstand hyperosmolar conditions (13). In mammalian cells p38 MAPK is a potent activator of MAPK-activated protein kinase-2 (MAPKAPK-2), which phosphorylates the small heat shock protein (hsp27) (2, 3). The physiological significance of this is controversial, but it may help cells resist thermal stress (14, 15).

A group of pyridinyl imidazole compounds have recently been found to be highly specific inhibitors of p38 MAPK (4, 16). They were discovered serendipitously as inhibitors of endotoxin-stimulated cytokine production (17). They strongly interacted with one particular 40-kDa cellular protein which was purified, cloned, and identified as the human p38 MAPK (4). The inhibitors are potentially a powerful experimental probe to explore the role of p38 MAPK in physiology.

During platelet activation there is a strong increase in phosphorylation in hsp27 (18). We therefore examined the possibility that p38 MAPK may be causing this and that the enzyme plays a role in platelet responses. Use of a specific inhibitor in this relatively simple cell system could provide clues to the wider functions of p38 MAPK.

EXPERIMENTAL PROCEDURES

Isolation of Human Platelets—For studies of aggregation and thromboxane production, platelets were prepared from freshly drawn blood. Otherwise platelets were obtained from concentrates (supplied within 24 h of collection by National Blood Service, Cambridge, United Kingdom (UK)). Red cells were removed from these by centrifugation for 15 min at 160 × g, and the supernatant was centrifuged for 15 min at 450 × g. Platelets were resuspended in buffered saline (145 mM NaCl, 5 mM KCl, 10 mM glucose, 1 mM MgSO4, 0.5 mM EDTA, 10 mM HEPES, pH 7.4) containing 0.25 units/ml apyrase (Sigma). Platelets were recentrifuged and resuspended at 107/ml in buffered saline (minus apyrase). Procedures were carried out at room temperature.

Western Blotting—Platelets were lysed by addition of an equal volume of lysis buffer (100 mM Tris, 150 mM NaCl, 50 mM NaF, 20 mM pyrophosphate, 2 mM EDTA, 0.2 mM Na3VO4, 2 mM phenylmethylsulfonyl fluoride, 0.2% Nonidet P-40, 0.04% NaN3, 20 μg/ml leupeptin, pH 7.4). Lysates were electrophoresed, alongside recombinant human p42

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MAP and p38 MAPK (expressed in Escherichia coli as glutathione S-transferase fusion proteins, and purified to homogeneity) in SDS-polyacrylamide gels (10% total acrylamide) (19). Proteins were transferred electrophoretically to nitrocellulose membrane. Membranes were blocked in Tris-buffered saline containing 10% bovine serum albumin, then incubated in first antiserum in blocking solution. Antiserum were made in rabbits to p38 C-terminal peptide (ISFPVPLDQEMES) (used at 1/1000) and p38 N-terminal peptide (MSQERFTTYQELNK) (used at 1/5000). Anti-serum 124 to p42 MAPK was a generous gift of Prof. C. J. Marshall (Institute of Cancer Research, London, UK) and used at 1/5000. Second antibody (horseradish peroxidase donkey immunoglobulin in rabbit immunoglobulin from Sigma) was detected on Fuji film by enhanced chemiluminescence.

Stimulation of Platelets—Platelets were stimulated at 30°C by adding indicated stimulating agent in 0.05 ml of buffered saline, except in the case of collagen fibers (see below) to 0.45 ml of suspension with vortexing. Bovine thrombin (catalog no. T4265), and U46619 (9,11dioxy-13a-epoxymethanoprostaglandin F2α) were from Sigma. Native type 1 collagen fibers were given by Ethicon Corp., Somerville, NJ, and dialyzed exhaustively against 0.1 M CH₃COOH and stored at 4°C. They were diluted for use into 0.05 ml 0.01 M CH₃COOH (rather than buffered saline). The collagen-related peptide GCP(6GPP)₃GCP(6P)(6P) (P₆ signifies hydroxyproline) was synthesized, purified, and cross-linked with N-succinimidyl 3-[2-pyridyl]-dithiopropionate (20). After appropriate incubation times, platelets were lysed as described above.

Assay of p38 MAPK—Lysates (0.5 ml) prepared as described above were used. In some cases, after checking that their protein concentration were the same, an equal volume of 2 × immunoprecipitation buffer was added (200 mM Tris, 300 mM NaCl, 20 mM pyrophosphate, 2 mM EDTA, 50 mM β-glycerophosphate, 0.2 mM Na₃VO₄, 2% Nonidet P-40, 1% sodium deoxycholate, 0.2% SDS, pH 7.4). Protein A-agarose beads were coated with antiserum to p38 MAPK C-terminal peptide in immunoprecipitation buffer, washed well, and incubated with lysates (30 μl of 50% slurry in 1 ml) with mixing for 4 h at 4°C. Beads were recovered by centrifugation and washed well. MAPKAPK-2 purified to homogeneity (1) was 3-activated KB cells (3) was immunoprecipitated by treating with phosphatase 2A (300 units/ml for 30 min at 30°C). Phosphatase was inactivated by adding okadaic acid (final concentration 30 μM). Mixtures (-90 μl) were made containing beads, 3.0 milliunits of MAPKAPK-2 (1 unit phosphorylated 1 nmol of hsp27 in 1 min), 15 μl of 6 × kinase buffer (300 mM Tris, 60 mM MgCl₂, 120 mM ATP, pH 7.4) and 4.5 μCi of (γ-³²P)ATP. After incubation at room temperature for 20 min, 1.5 μg of hsp27 was then added and incubation continued for another 20 min. Incubation was stopped by adding SDS sample buffer preheated to 100°C, and products were separated on SDS-PAGE. hsp27 phosphorylation was detected by autoradiography. Results were expressed as % reactivation of MAPKAPK-2. Natural hsp27 was purified from the supernatant of Escherichia coli cells using immunoblotting.

Inhibition of p38 MAPK—4-(4-Fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridyl) imidazole (SB203580), 6-(4-fluorophenyl)-2,3-dihydro-5-(4-pyridyl)imidazole (SKF86002), and 2-(4-methylsulfonylphenyl)-3-(4-pyridyl)-6,7-dihydro-[5H]-pyrrolo[1,2-a]imidazole (SP60012) were synthesized at SmithKline Beecham. Immunoprecipitates of p38 MAPK were preincubated 20 min with indicated concentration of imidazole compound and then assayed on MAPKAPK-2 and hsp27 in the presence of compound as described above.

For studies in intact cells, platelets were incubated with 100 μCi/ml ([³²P]orthophosphate) for 60 min at 30°C, then washed and incubated with imidazole compound or vehicle (MgCl₂) for an additional 30 min. Platelets were lysed, and hsp27 was immunoprecipitated (3). Immunoprecipitates were run on SDS-gel electrophoresis, and dried gels were autoradiographed. hsp27 phosphorylation was measured by scanning autoradiographs.

Platelet Aggregation—Platelets were made from freshly-drawn blood and washed (21), then suspended in Tyrod's buffer to half the original volume. Ca²⁺ (to 1.5 mM) and human fibrinogen (to 1.5 mg/ml) were added. Fibrinogen was omitted when thrombin was used.

Aggregation was measured at 37°C turbidimetrically (20) and agonists were used at the minimum dose needed to elicit a full aggregatory response. In some instances aggregation and secretion of ATP were measured concomitantly in a Chronolog Aggro-Meter (model 550, Coulter Electronics Ltd.) using a bioluminescence technique (20).

Thromboxane B₂, ASSAY—Thromboxane B₂ was measured by an EIA Biotrak kit from Amersham International plc, UK.

Fig. 1. Detection of p38 MAPK in platelets by Western blotting (A) and as immunoprecipitated kinase (B). A. Platelet lysates were run on SDS-gel electrophoresis alongside samples of purified recombinant p38 MAPK and p42 MAPK. Proteins were transferred to nitrocellulose and reacted with the indicated antisera as described under "Experimental Procedures." Bars indicate positions of standard protein molecular mass markers. B. Assay of immunoprecipitated p38 MAPK on MAPKAPK-2. Platelets were stimulated with 20 μg/ml collagen-related peptide for the indicated time and lysate p38 MAPK was immunoblotted as described under "Experimental Procedures." Lanes 1-5 are an experiment with natural hsp27; for lanes 6-12, recombinant hsp27 was used. Lanes 1, 2, and 6 are material from unstimulated platelets; lanes 3-5 are from platelets stimulated for 1 min with collagen-related peptide. For lanes 1 and 3, lysates were treated with preimmune serum; for lanes 2 and 4-10, they were treated with antiserum to p38 MAPK C-terminal peptide. MAPKAPK-2 was omitted from the incubation for lane 5. For lanes 7-10, platelets were stimulated for 15, 45, 120, and 300 s, respectively. Lanes 11 and 12 are samples of MAPKAPK-2 after and before phosphatase treatment.

RESULTS AND DISCUSSION

Detection of p38 MAPK—p38 MAPK was identified in platelets on the basis of its immunological reactivity and substrate specificity (2, 3). Antiser to C-terminal or N-terminal peptides immunoblotted an antigen of similar size to recombinant p38 MAPK (Fig. 1A, middle and right panels). Neither antiser to recombinant p42 MAPK, but both stained some other bands in platelet lysates. An antiser to p42 MAPK failed to react with recombinant p38 MAPK but demonstrated putative p42 MAPK in lysates (Fig. 1A, left panel).

The activity of p38 MAPK immunoprecipitated from lysates was assayed by its ability to activate MAPKAPK-2 as judged by the latter enzyme’s ability to phosphorylate hsp27. Lysates of platelets activated by a cross-linked collagen-related synthetic peptide, found previously to be highly aggregatory for platelets (20), contained increased p38 MAPK activity when compared with lysates from unstimulated cells (Fig. 1B, lane 2 versus lane 4). hsp27 phosphorylation was not seen if preimmune serum was used (lanes 1 and 3), or MAPKAPK-2 omitted (lane 5). Natural hsp27 is partially cleaved and runs as two bands. Recombinant hsp27 was used for assay of immunoprecipitates of platelets stimulated by the collagen-related peptide for increasing times. These showed increasing activity (Fig. 1B, lanes 6-10). Recombinant hsp27 substrate was used for subsequent experiments. The immunological reactivity and substrate specificity (2, 3) of the enzyme identify it as p38 MAPK.

p38 MAPK was rapidly activated in platelets stimulated by thrombin, collagen, collagen-related peptide or the thromboxane analogue U46619. Fig. 2 shows the time course (a–d) and dose dependence (e–h) for each agonist. All increased activity within 45 s; that induced by thrombin, collagen, and U46619 were measured concomitantly in a Chronolog Aggro-Meter (model 550, Coulter Electronics Ltd.) using a bioluminescence technique (20).
Fig. 2. Activation of p38 MAPK by platelet agonists. Platelets were prepared and stimulated for indicated times and doses. p38 MAPK was immunoprecipitated from lysates and assayed for ability to reactivate MAPKAPK-2. Each ligand was tested on at least five different batches of platelets, and representative experiments are shown. Agonists were as follows: a and e, bovine thrombin (1 unit/ml in a); b and f, native type I collagen fibers (10 μg/ml in f); c and g, collagen-related peptide (20 μg/ml in c); d and h, U46619 (0.2 μM in d).

decayed after 2 min. The thrombin receptor-related peptide SFLLRNPNND also activated p38 MAPK (data not shown). Activation was not dependent on aggregation of platelets, since it was unaffected by including the peptide RGDS (which prevents aggregation by blocking α1bβ3) in the suspension at 500 μM. The effects of optimal concentration of collagen and thrombin were not secondary to thromboxane production since they were unaffected by aspirin treatment (data not shown).

Inhibition of p38 MAPK—SB203580, a member of the class of pyridinyl imidazoles that inhibit p38 MAPK, has been shown to be a highly selective inhibitor of the enzyme, since it had no action on a wide range of other kinases, including p42 and p54 MAPKs, and phosphatases (16). We further checked the specificity of SB203580; at 10 μM, it did not inhibit (a) the activation of p42 MAPK by IL-1 or PMA in fibroblasts, (b) the activation of p54 MAPK by IL-1 in fibroblasts, or (c) the activator of p38 MAPK from IL-1-stimulated KB cells. Fig. 3A shows that SB203580 and a related inhibitory compound SKF86002 inhibited (in the 0.1–1 μM range) p38 MAPK that had been immunoprecipitated. SB203580 was the more potent; its IC₅₀ lay between 0.1 and 1 μM, consistent with the published value of 0.6 μM (16).

We then investigated the ability of these compounds to inhibit p38 MAPK in intact platelets. Platelets were metabolically labeled with [32P]orthophosphate, pretreated with inhibitor, and then stimulated with thrombin and lysed. hsp27 phosphorylation was measured as an indicator of p38 MAPK activity. SB203580 inhibited the increase in hsp27 phosphorylation in the range 1–10 μM. SKF86002 was also effective (Fig. 3B). Similar results were obtained with collagen.

These results were consistent with the phosphorylation of hsp27 being regulated by p38 MAPK. MAPKAPK-2 can be activated by p42 MAPK in vitro (2), and p42 MAPK may be activated in platelets (22, 23). However, p38 MAPK is a much stronger activator of MAPKAPK-2 than p42 MAPK (2, 3), so it was not surprising that its inhibition strongly affected hsp27 phosphorylation. We next tested the effect of SB203580 upon platelet aggregation. Platelets were stimulated by the minimum concentration of collagen fibers sufficient to cause full aggregation. Aggregation was strongly inhibited by 1 μM SB203580 (Fig. 4A and B) shows platelets of two different individuals) with concomitant inhibition of secretion (Fig. 4B). The relationship between the concentration of inhibitor and the degree of inhibition was similar to that observed for inhibition of hsp27 phosphorylation (Fig. 3B). IC₅₀ for platelets from 5 individuals varied within the range 0.2–1 μM. SB203580 was also effective. A structurally related compound (SKF105890), which is inactive on p38 MAPK (16), did not inhibit aggregation. The inhibition of aggregation could be overcome by increasing the agonist concentration 3-fold (Fig. 4C). Aggregation induced by a minimum concentration of U46619 was also strongly inhibited. Inhibition was overcome by doubling U46619 concentration (Fig. 4D). However, aggregation induced by a minimum aggregatory concentration of thrombin was not reproducibly inhibited (only one of five platelet samples was inhibitable).

Thromboxane production is necessary for platelet aggregation and might be impaired by SB203580 interfering with cyclooxygenase. Platelets were pretreated with SB203580 then stimulated for 1 min with collagen (10 μg/ml) as described in Fig. 1, in the presence of both EGTA and RGDS peptide (100 μM) to prevent aggregation. Thromboxane released into the medium (measured as thromboxane B₂) rose from 3.6 to 200 ng/ml upon stimulation and was unaffected by concentrations of SB203580 ranging from 0.3 to 10 μM. SB203580 was not acting as an inhibitor of cyclooxygenase.

p38 MAPK appeared to provide a signal necessary for platelet aggregation caused by low concentrations of collagen or U46619. Aggregation caused by low concentrations of collagen fibers is dependent upon production of thromboxane A₂, which causes positive feedback via its own receptor. It was possible...
FIG. 4. Effects of SB203580 on platelet aggregation and secretion. A, aggregation stimulated by native type I collagen fibers at 1 μg/ml (minimal concentration to elicit a full aggregatory response). Platelets were preincubated for 30 min at 37 °C with indicated concentrations of SB203580 or vehicle. Addition of agonist is indicated with an arrow. B, a separate experiment in which secretion was also measured in response to collagen (1 μg/ml). C, inhibition by SB203580 is overcome by increasing collagen fiber concentration. D, inhibition by SB203580 of aggregation induced by U46619 is overcome by increased agonist concentration.

FIG. 5. Effect of SB203580 on thromboxane B₂ generation by collagen-stimulated platelets. Platelets (10⁷/ml) were incubated in suspension buffer containing the peptide RGDS (100 μg/ml) in the absence (Control) or presence of (SB203580) for 15 min. They were then stimulated with indicated concentrations of collagen fibers for 4 min. Suspension buffer was assayed for thromboxane B₂ as described under “Experimental Procedures.” Results are means of duplicate experiments.

that p38 MAPK activation was needed for the generation of thromboxane by low concentrations of collagen fibers. SB203580 had a small but significant (10-20% inhibition) effect on the amounts of thromboxane produced in response to low concentrations of collagen. Fig. 5 shows an experiment representative of five on platelets from different individuals. We estimated that, since a minimum aggregatory concentration of U46619 was 30–40 nM and basal thromboxane levels were ~10 nM, then a 3–4-fold increase over the basal level may be needed to trigger aggregation. SB203580 is therefore as likely to be causing inhibition by interfering with secretion or other aspects of the response, as with thromboxane generation.

The results indicate that p38 MAPK plays a role in platelet aggregation. It enables platelets to respond to low concentrations of collagen or thromboxane. It is not necessary for full sensitivity to thrombin. It is likely that thrombin at low concentration and collagen or thromboxane at high concentrations activate pathways that bypass p38 MAPK, rendering it redundant.

Addendum—As this paper was being prepared for submission, Kramer et al. (24) reported the activation of a p38 MAPK-like enzyme in platelets by thrombin and thrombin receptor peptide.

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