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p42 Mitogen-Activated Protein Kinase and p90 Ribosomal S6 Kinase Are Selectively Phosphorylated and Activated during Thrombin-Induced Platelet Activation and Aggregation

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Human platelets provide an excellent model system for the study of phosphorylation events during signal transduction and cell adhesion. Platelets are terminally differentiated cells that exhibit rapid phosphorylation of many proteins upon agonist-induced activation and aggregation. We have sought to identify the kinases as well as the phosphorylated substrates that participate in thrombin-induced signal transduction and platelet aggregation. In this study, we have identified two forms of mitogen-activated protein kinase (MAPK), p42^{mapk} and p44^{mapk}, in platelets. The data demonstrate that p42^{mapk} but not p44^{mapk} becomes phosphorylated on serine, threonine, and tyrosine during platelet activation. Immune complex kinase assays, gel renaturation assays, and a direct assay for MAPK activity in platelet extracts all support the conclusion that p42^{mapk} but not p44^{mapk} shows increased kinase activity during platelet activation. The activation of p42^{mapk}, independently of p44^{mapk}, in platelets is unique since in other systems, both kinases are coactivated by a variety of stimuli. We also show that platelets express p90^{rsk}, a ribosomal S6 kinase that has previously been characterized as a substrate for MAPK. p90^{rsk} is phosphorylated on serine in resting platelets, and this phosphorylation is enhanced upon thrombin-induced platelet activation. Immune complex kinase assays demonstrate that the activity of p90^{rsk} is markedly increased during platelet activation. Another ribosomal S6 protein kinase, p70^{S6K}, is expressed by platelets but shows no change in kinase activity upon platelet activation with thrombin. Finally, we show that the increased phosphorylation and activity of both p42^{mapk} and p90^{rsk} does not require integrin-mediated platelet aggregation. Since platelets are nonproliferative cells, the signal transduction pathways that include p42^{mapk} and p90^{rsk} cannot lead to a mitogenic signal and instead may regulate cytoskeletal or secretory changes during platelet activation.

Platelets are nonproliferative, terminally differentiated cells that are highly specialized for secretion and adhesion (18, 41, 44, 59). A variety of physiological agonists, such as thrombin, collagen, or ADP, can activate signal transduction pathways which lead to a change in platelet shape, secretion of granules, platelet aggregation, and adhesion (45, 74). It is through these events that the activated platelet can participate in normal as well as pathological processes of hemostasis and thrombosis. Platelets contain exceptionally high levels of tyrosine kinase activity and show many changes in the pattern of protein tyrosine phosphorylation upon agonist-induced activation (21, 22, 24, 26–28, 42, 56, 57, 62, 66, 81). Few details have been worked out concerning the tyrosine kinases and substrates that participate in the activation and aggregation process or the means by which these kinases are activated. Five members of the Src family of tyrosine kinases (Src, Fyn, Yes, Lyn, and Hck) have been identified in platelets (26, 38, 40, 86). p60^{src}, the most abundant, shows an increase in tyrosine kinase activity upon thrombin-induced activation and aggregation (14, 84). During platelet activation, p60^{src} forms a complex with a cytoskeleton-associated substrate, p80/85, which becomes phosphorylated on tyrosine (84). The phosphorylation of p80/85 may participate in secretory or shape changes that occur during platelet activation. In addition, several Src family kinases are found in association with the cytoplasmic domain of a transmembrane adhesion receptor, CD36, suggesting that ligand binding to CD36 could lead to activation of the associated tyrosine kinases (40). In other studies, both Fyn and Src were shown to associate with phosphatidylinositol-3 kinase activity (34, 53), but the significance of this observation to the platelet activation process is unclear.

A subset of the tyrosine phosphorylation events that occur during platelet activation are regulated by adhesion of fibrinogen to the platelet surface integrin, GPIIb-IIIa (22, 27). Recently, a newly characterized protein tyrosine kinase, p125^{FAK}, was identified in platelets (50). p125^{FAK} becomes phosphorylated on tyrosine and exhibits elevated kinase activity upon thrombin and collagen activation (50). The phosphorylation and activation of p125^{FAK} is dependent on platelet activation mediated by GPIIb-IIIa engagement (50).

We have continued the search for kinases and their substrates that function in signaling pathways during platelet activation and aggregation by examining the phosphorylation and activities of mitogen-activated protein kinases (MAPKs) and ribosomal S6 kinases (p70^{S6K} and p90^{rsk}). In other systems, these kinases are rapidly activated upon exposure of cells to phorbol esters or factors that activate tyrosine kinases during cell proliferation (5, 6, 15, 20, 60, 68, 77). Evidence suggests that MAPK and RSK may participate in regulating gene expression, whereas p70^{S6K}, which lies on a distinct signaling pathway from MAPK and RSK, could regulate protein synthesis. These kinases also function in the G_2/M transition during meiotic maturation of *Xenopus laevis* and sea star oocytes (20, 23, 31, 35, 47, 51, 61, 64, 70). This

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report provides evidence that platelet activation by thrombin also proceeds through signal transduction pathways that utilize the activities of a specific form of MAPK and RSK. The data show that platelets express two forms of MAP kinase, $p42^{mapk}$ and $p44^{mapk}$. During thrombin-induced platelet activation, $p42^{mapk}$ but not $p44^{mapk}$ becomes phosphorylated on serine, threonine, and tyrosine. Furthermore, the kinase activity of $p42^{mapk}$ but not $p44^{mapk}$ is elevated during platelet activation. These findings are in contrast to other systems in which where both $p42^{mapk}$ and $p44^{mapk}$ are coactivated in response to various stimuli. Platelets also express two ribosomal S6 kinases, $p90^{rsk}$ and $p70^{S6K}$. The serine phosphorylation and kinase activity of $p90^{rsk}$ but not $p70^{S6K}$ is markedly enhanced during thrombin-induced platelet activation. The enhanced activities of $p42^{mapk}$ and $p90^{rsk}$ are not dependent on platelet aggregation mediated by the integrin GPIIb-IIIa.

MATERIALS AND METHODS

Platelet isolation and activation. Blood was drawn from human volunteers who had not taken aspirin or other drugs for at least 2 weeks prior to blood donation. Washed platelets were isolated as described previously (84) and used for experiments immediately after isolation. The washed platelets were resuspended at a concentration of 5×10^8 to 10×10^8/ml in activation buffer consisting of 137 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.4 mM NaH_2PO_4, 5.6 mM glucose, 0.35% bovine serum albumin (BSA), 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.4), and 0.5 U of apyrase per ml. Platelets were activated by stirring at 37°C in the presence of 1 U of thrombin (Calbiochem) per ml. At the indicated time points, aliquots were harvested by immediate dilution into solubilization buffers. For the experiments shown in Fig. 5 and 7, platelet activation with thrombin was carried out in the presence of either 0.5 mM Arg-Gly-Asp-Ser (RGDS) tetrapeptide or 0.5 mM Arg-Gly-Glu-Ser (RGES) tetrapeptide (Sigma). The peptides were added to platelet suspensions prior to addition of thrombin. For the experiments shown in Fig. 2 and 4C, PC12 cells were treated with nerve growth factor (NGF) for 5 min as described previously (85) and harvested in parallel with the platelet samples. For the experiments shown in Fig. 4D and 5C, NIH 3T3 cells were serum starved and then treated with fresh medium containing serum for 5 min prior to harvest.

Antibodies. A mouse monoclonal antibody directed against phosphotyrosine, 4G10, was purchased from Upstate Biotechnology. 4G10 was used at a 1:500 dilution for Western blots (immunoblots). A rabbit antiserum directed against MAPK, C-1, has been described elsewhere (10). C-1 was used at a 1:250 dilution for Western blots and at 5 μl/200 to 500 μg of protein for immunoprecipitations. Polyclonal antisera against $p90^{rsk}$ and against $p70^{S6K}$ have been described elsewhere (8). Anti-$p90^{rsk}$ was used at a 1:250 dilution for Western blots and at 5 μl/200 to 500 μg of protein for immunoprecipitations. Anti-$p70^{S6K}$ was used at a 1:250 dilution for Western blots.

Immunoprecipitations. Platelet suspensions were harvested at the indicated time points during thrombin activation by dilution with one of three buffer mixes. For denaturing conditions (SDS/BOIL), 20 μl of denaturing buffer (10% sodium dodecyl sulfate [SDS], 10 mM dithiothreitol, 20 mM HEPES (pH 7.4) was added to 0.3 ml of platelet suspension. The sample was heated at 95°C for 5 min and then diluted with 0.8 ml of HNTG buffer (50 mM HEPES [pH 7.4], 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 2 mM EGTA, 10 μg of leupeptin [Sigma] per ml, 10 μg of phenylmethanesulfonyl fluoride [PMSF; Boehringer] per ml, 0.5 mM sodium vanadate [Fisher Scientific]). For selective immunoprecipitation of $p44^{mapk}$, 0.5 ml of platelet suspension was solubilized with 0.5 ml of twofold concentrated radioimmunoprecipitation assay (RIPA) buffer (final concentrations, 50 mM HEPES [pH 7.4], 150 mM NaCl, 1% Nonidet P-40 [NP-40], 1% sodium deoxycholate, 0.1% SDS, 1% aprotinin, 10 μg of ml leupeptin per ml, 10 μg of PMSF per ml, 5 mg of *p*-nitrophenyl phosphate per ml, and 0.5 mM sodium vanadate). For kinase assays, 0.5 ml of platelet suspension was solubilized in 0.5 ml of twofold-concentrated BND buffer (final concentrations, 10 mM KPO_4, 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 0.5% NP-40, 0.1% Brij 35, 0.05% sodium deoxycholate, 10 μg of leupeptin per ml, 10 μg of PMSF per ml, 1.0 mM sodium vanadate, 50 mM β-glycerophosphate, and 2 mM dithiothreitol). The BND extracts were used for selective immunoprecipitation of $p44^{mapk}$, for direct assay of MAPK activity, and for $p90^{rsk}$ immune complex kinase assays. All samples were centrifuged at 4°C in a microcentrifuge for 10 min prior to immunoprecipitation. Clarified extracts were incubated with antibody for 1 to 3 h at 4°C, and then protein A-Sepharose was added for 1 h at 4°C. Immune complexes were collected by centrifugation in a microcentrifuge and then washed three times with 1× solubilization buffer, except for the SDS/BOIL samples, which were washed with RIPA buffer.

SDS-polyacrylamide gel electrophoresis and immunoblotting. Pellets of washed immune complexes were resuspended in Laemmli sample buffer (including 0.5 mM sodium vanadate), boiled for 10 min, and analyzed on an SDS–12% polyacrylamide gel for MAPK and a SDS–7.5% polyacrylamide gel for RSK. For analysis of total platelet protein (Fig. 1), 80-μl aliquots of platelet suspension (approximately 6×10^7 platelets) were mixed with 20 μl of fourfold-concentrated Laemmli sample buffer. Samples were boiled prior to SDS-polyacrylamide gel analysis. For Western immunoblotting with an antibody against phosphotyrosine, proteins were transferred to an Immobilon-P filter (Millipore) at 150 mA for 5 h (Bio-Rad electrophoretic transfer apparatus). The filter was subsequently incubated with 5% BSA in 25 mM Tris (pH 7.2)–150 mM NaCl (Tris-buffered saline [TBS]) for 1 to 3 h at room temperature to block nonspecific protein binding. The filter was next incubated overnight at 4°C with an appropriate dilution of antibody in 5% BSA–TBS and then subjected to a series of washes at room temperature with TBS and TBS plus 0.05% Tween 20 and 0.05% NP-40. The immunoblot was next incubated at room temperature for 1 h with ^{125}I-labeled sheep anti-mouse immunoglobulin G (Amersham) at a final concentration of 0.1 μCi/ml in 5% BSA–TBS. Finally, the immunoblot was washed as described above and exposed to film with an intensifying screen. For Western immunoblots with the antiserum against MAPK, essentially the same method was followed except that nonfat dry milk was used instead of BSA and the TBS detergent wash included 0.05% NP-40 instead of 0.05% Tween 20 and 0.5% NP-40. The antibody was detected by using affinity purified ^{125}I-protein A (Amersham). For Western immunoblots with the antisera against $p90^{rsk}$ and $p70^{S6K}$, the proteins were transferred to nitrocellulose paper (Schleicher & Schull). All incubations and washes were performed in phosphate-buffered saline containing 2% BSA and 0.2% Tween 20, and the antisera were detected by using ^{125}I-protein A (ICN).

^{32}P labeling and phosphoamino acid analysis. Washed platelets were resuspended in labeling medium (10 mM

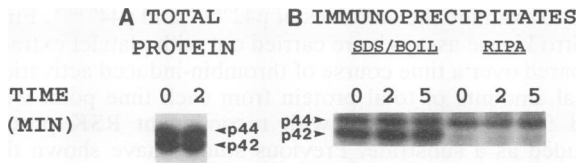

FIG. 1. Western immunoblots of p42^{mapk} and p44^{mapk}. (A) Total protein extracts of either resting (lane 0) or thrombin-activated (lane 2) platelets were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting with the C-1 antibody directed against MAPK. (B) Samples were prepared, by using two different extraction conditions (SDS/BOIL and RIPA), from resting (lanes 0) or thrombin-activated (lanes 2 and 5) platelets. Extracts were immunoprecipitated with the C-1 antibody and analyzed by immunoblotting with the C-1 antibody. The positions of p42^{mapk} and p44^{mapk} are indicated.

HEPES [pH 7.4], 137 mM NaCl, 2.9 mM KCl, 5.6 mM glucose, 1.0 mM $MgCl_2$, 0.25% BSA, 1 U of apyrase per ml) plus 2.5 mCi of $^{32}P_i$ (NEN/Dupont) per ml and incubated for 3 h at 37°C. Next, platelets were gently centrifuged and resuspended in activation buffer. Platelet activation and sample preparation were carried out as described above. Immunoprecipitates of either MAPK or RSK were analyzed by SDS-polyacrylamide gel electrophoresis, after which the proteins were transferred to an Immobilon-P filter for phosphoamino acid analysis as described previously (43).

Protein kinase assays. For Fig. 4A, MAPK activity was measured in unfractionated platelet extracts (BND buffer) by a direct assay using recombinant RSK as a substrate as described previously (11, 13). For Fig. 7A, an immune complex kinase assay was performed with p90^{rsk} immunoprecipitated from platelet extracts as described previously (8). For Fig. 4B and C, an immune complex kinase assay was performed with p44^{mapk}, selectively immunoprecipitated from either platelet extracts or PC12 cells as described above, by using the following method. Washed immunoprecipitates were resuspended in 50 μl of kinase assay buffer (30 mM HEPES [pH 7.4], 10 mM $MgCl_2$, 50 mM β-glycerophosphate, 500 μM sodium orthovanadate, 1 mM dithiothreitol, 500 μg of myelin basic protein [MBP; Sigma] per ml). Ten microcuries of [γ-^{32}P]ATP was added, and the samples were incubated for 15 min at 30°C. Reactions were stopped by addition of 15 μl of 4× Laemmli sample buffer, boiled, and analyzed by SDS–12% polyacrylamide gel electrophoresis. The gel renaturation kinase assays shown in Fig. 4D and 5C were performed as follows. Approximately 40 μg of each platelet extract or NIH 3T3 cell extract was resolved on an SDS–8.5% polyacrylamide gel with MBP immobilized in the gel at a concentration of 0.5 mg/ml. Following electrophoresis, the gel was washed with two changes, 100 ml each, of 20% propanol in 50 mM Tris (pH 8.0) for 1 h at room temperature and then incubated in 250 ml of buffer A (5 mM 2-mercaptoethanol in 50 mM Tris [pH 8.0]) for 1 h at room temperature. The gel was next incubated for 1 h at room temperature in 250 ml of buffer A containing 6 M guanidine HCl and then incubated for 16 h at 4°C in buffer A containing 0.04% Tween 40. Finally, the gel was incubated in kinase buffer (20 mM HEPES [pH 7.2], 10 mM $MgCl_2$, and 3 mM 2-mercaptoethanol) at room temperature for 30 min, after which a kinase reaction was carried out by incubating the gel with kinase buffer supplemented with 20 μCi of [γ-^{32}P]ATP per ml and 50 μM ATP at room temperature for 1 h. The gel was washed extensively with 1% PP_i in 5% trichloroacetic acid. Radiolabeled proteins were detected by autoradiography of the dried gel. Results were quantitated with a PhosphorImager.

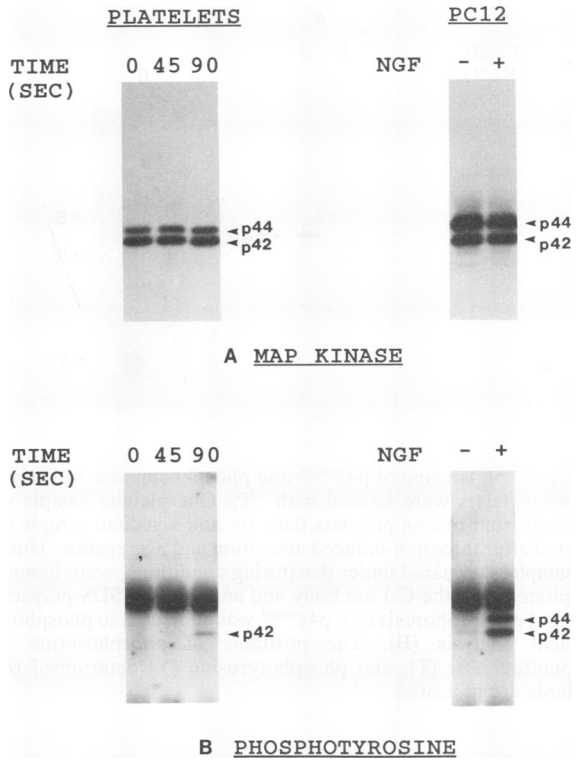

FIG. 2. p42^{mapk} becomes phosphorylated on tyrosine during platelet activation. Extracts were prepared under denaturing conditions from a platelet activation time course and then immunoprecipitated with the C-1 antibody. Immunoprecipitates were divided in half; one portion was analyzed by immunoblotting with the C-1 antibody (A), and the other portion was analyzed by immunoblotting with an antibody against phosphotyrosine (B). For a control, a similar experiment was performed with extracts prepared from PC12 cells in the absence (−) or presence (+) of NGF treatment. The positions of p42^{mapk} and p44^{mapk} are indicated.

RESULTS

Identification of MAPKs in platelets. Platelet extracts were examined by Western blot analysis with an antibody (C-1) directed against a synthetic peptide predicted from the MAPK nucleotide sequence (10). Proteins with molecular masses of approximately 42 kDa (p42^{mapk}) and 44 kDa (p44^{mapk}) were identified in extracts from both resting and thrombin-activated platelets (Fig. 1A). Protein species with similar molecular weights were recognized by two other antibodies directed against MAPK (data not shown). To determine optimal conditions for immunoprecipitation of p42^{mapk} and p44^{mapk}, platelet extracts were prepared by using different solubilization conditions. These extracts were immunoprecipitated with the C-1 antibody, and the immunoprecipitates were subsequently analyzed by immunoblotting with the C-1 antibody. The results demonstrate that under fully denaturing conditions (SDS/BOIL), the C-1 antibody was capable of immunoprecipitating both p42^{mapk} and p44^{mapk} proteins (Fig. 1B), whereas with less stringent solubilization conditions (RIPA), only p44^{mapk} could be immunoprecipitated (Fig. 1B). The levels of both p42^{mapk}

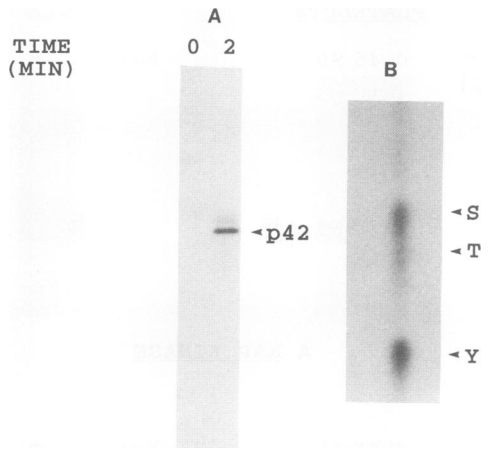

FIG. 3. ^{32}P labeling of $p42^{mapk}$ and phosphoamino acid analysis. Resting platelets were labeled with $^{32}P_i$. One platelet sample was harvested from resting platelets (lane 0), and a second sample was prepared after thrombin-induced activation and aggregation (lane 2). The samples, prepared under denaturing conditions, were immunoprecipitated with the C-1 antibody and analyzed by SDS-polyacrylamide gel electrophoresis (A). $p42^{mapk}$ was subjected to phosphoamino acid analysis (B). The positions of phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) determined from standards are indicated.

and $p44^{mapk}$ remained constant during platelet activation by thrombin.

Phosphorylation of $p42^{mapk}$ but not $p44^{mapk}$ during platelet activation. One of the hallmarks of the activation of MAPK is its phosphorylation on both threonine and tyrosine residues (1, 3, 39, 65). Consequently, we examined immunoprecipitates of the $p42^{mapk}$ and $p44^{mapk}$ proteins by immunoblotting with an antibody directed against phosphotyrosine. The results shown in Fig. 2 illustrate that in resting platelets, neither $p42^{mapk}$ nor $p44^{mapk}$ was recognized by the antibody against phosphotyrosine. Upon platelet activation and aggregation $p42^{mapk}$ but not $p44^{mapk}$ was detected by the antibody against phosphotyrosine (Fig. 2). As a control for these experiments, we examined the phosphorylation of $p42^{mapk}$ and $p44^{mapk}$ in PC12 cells treated with NGF. As in previous studies (7, 30, 32, 54, 67, 72, 80), we found that very little phosphotyrosine could be detected on $p42^{mapk}$ and $p44^{mapk}$ in untreated PC12 cells, but after 5 min of stimulation with NGF, both proteins were clearly recognized by the phosphotyrosine antibody (Fig. 2).

We next confirmed the immunoblot results by labeling platelets with $^{32}P_i$ followed by immunoprecipitation, under denaturing conditions, of both $p42^{mapk}$ and $p44^{mapk}$. As expected, no phosphorylation of either kinase was detected in resting platelets, but upon thrombin-induced activation and aggregation, $p42^{mapk}$ and little if any $p44^{mapk}$ became phosphorylated (Fig. 3A). Phosphoamino acid analysis of the $p42^{mapk}$ revealed the presence of phosphotyrosine, phosphoserine, and a small amount of phosphothreonine (Fig. 3B). Phosphoamino acid analysis of the very faint band seen in the 44-kDa region of the gel revealed only trace amounts of phosphoserine (data not shown).

Elevation of MAPK activity during platelet activation and aggregation. The observation that $p42^{mapk}$ becomes phosphorylated on tyrosine and threonine during platelet activation and aggregation suggests that the activity of this kinase is elevated. To test this possibility, three approaches were used to examine the activities of $p42^{mapk}$ and $p44^{mapk}$. First, in vitro kinase assays were carried out with platelet extracts prepared over a time course of thrombin-induced activation. Equal amounts of total protein from each time point were used for a kinase assay with recombinant RSK protein included as a substrate. Previous studies have shown that this assay is specific for the activity of MAPK in cell extracts (11, 13). No kinase activity was detected in extracts from resting platelets, but upon activation, approximately a 10-fold increase in kinase activity toward the RSK substrate was observed (Fig. 4A). In other experiments, extracts from activated platelets were separated on an anion-exchange column, and the resulting fractions were tested for MAPK activity by using recombinant RSK as a substrate. Only those fractions containing $p42^{mapk}$ and $p44^{mapk}$ showed activity, indicating that one of these two proteins must be responsible for the MAPK activity detected in the unfractionated platelet extract (data not shown).

To determine which form of MAPK was responsible for the kinase activity detected in platelet extracts, $p44^{mapk}$ was first tested in an immune complex kinase assay. Under partially denaturing conditions, $p44^{mapk}$ was selectively immunoprecipitated, and an in vitro kinase assay was performed with MBP as a substrate. No kinase activity was detected in $p44^{mapk}$ immunoprecipitates made from either resting or activated platelets (Fig. 4B). A control experiment in which the $p44^{mapk}$ was immunoprecipitated from PC12 cells demonstrated a significant elevation in activity of this kinase upon NGF treatment of the cells (Fig. 4C). In other experiments, immunoprecipitates of $p44^{mapk}$ from platelets were used for in vitro kinase assays with recombinant RSK added as a substrate. A trace of kinase activity directed against RSK was detected in the $p44^{mapk}$ immunoprecipitates from resting platelets, but this activity was unchanged during platelet activation (data not shown). It was not possible to immunoprecipitate $p42^{mapk}$, with available antibodies, under conditions where it would retain kinase activity.

The data described above indicate that the activity of $p44^{mapk}$ changes very little during platelet activation and suggest that the MAPK activity detected in unfractionated platelet extracts is due to $p42^{mapk}$. This conclusion was substantiated by analysis of MAPK activity with an in-gel renaturation technique. Total platelet protein was prepared from a thrombin activation time course and separated on an SDS-polyacrylamide gel. The proteins were renatured, and a kinase assay was performed in situ with MBP included as a substrate. Extracts from serum-starved as well as serum-stimulated NIH 3T3 cells were analyzed in parallel as a positive control for the increased activity of both $p42^{mapk}$ and $p44^{mapk}$ (11, 13). The results show that very little $p42^{mapk}$ activity is detected in resting platelets, but upon thrombin-induced activation and aggregation, the activity of this kinase increases (Fig. 4D). As predicted, no $p44^{mapk}$ activity is evident in either resting or activated platelet extracts. In contrast, the activities of both $p42^{mapk}$ and $p44^{mapk}$ are elevated in NIH 3T3 cells stimulated with serum (Fig. 4D).

Integrin-mediated platelet aggregation is not required for phosphorylation and activation of $p42^{mapk}$. Platelet activation is a multistep process which culminates in platelet aggregation (18, 41, 44, 45, 59, 74). At least two phases of tyrosine phosphorylation have been defined: those that rapidly follow from agonist-induced activation and others that require platelet aggregation (22, 84). To determine whether activation of $p42^{mapk}$ is dependent on platelet aggregation, the

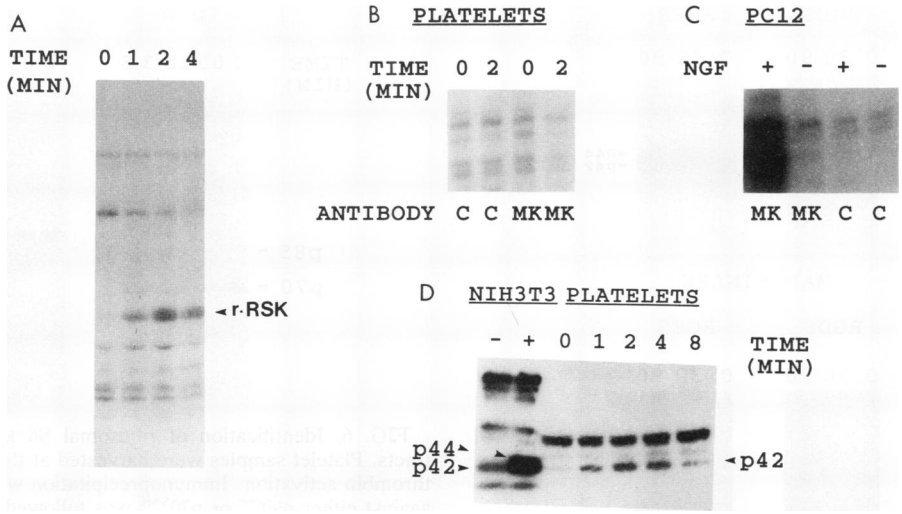

FIG. 4. Protein kinase assays for MAPK activity. (A) A direct assay for MAP kinase activity was performed, with recombinant RSK protein included as a substrate, using BND platelet extracts prepared from a platelet activation time course. The kinase reaction products were analyzed by SDS-polyacrylamide gel electrophoresis. The position of the phosphorylated RSK protein is marked (r · RSK). (B) p44^{mapk} was selectively immunoprecipitated with the C-1 antibody (lanes marked MK) from extracts of either resting (lanes 0) or thrombin-activated (lanes 2) platelets and subjected to an in vitro kinase assay with MBP included as a substrate. Control immunoprecipitates were performed in parallel with preimmune rabbit serum and analyzed by in vitro kinase assay (lanes marked C). The phosphorylated MBPs are shown. (C) A positive control experiment for p44^{mapk} activity was performed by using immunoprecipitates made from PC12 cells either with (+) or without (−) NGF treatment for 5 min. (D) An in-gel renaturation kinase assay for MAPK activity was performed with BND extracts prepared from a platelet activation time course. For comparison, the experiment included samples prepared from NIH 3T3 cells either serum starved (−) or serum stimulated (+). The positions of p42^{mapk} and p44^{mapk} are indicated.

phosphorylation and activity of this kinase were measured during thrombin-induced activation in the presence of RGDS peptide. Under these conditions, thrombin-induced signal transduction is initiated but the platelets are unable to aggregate because the RGDS peptide blocks integrin-mediated adhesive interactions (22, 25, 36, 37, 63). The phosphotyrosine Western blot in Fig. 5B demonstrates that in the presence of RGDS peptide, p42^{mapk} is still phosphorylated on tyrosine after thrombin treatment. Treatment with RGDS peptide alone does not lead to phosphorylation of p42^{mapk}, since no phosphorylation of p42^{mapk} is detected by treating the platelets with RGDS peptide prior to thrombin addition (Fig. 5B, time 0). The background band seen in all lanes of the phosphotyrosine blot does not comigrate with p44^{mapk}. In other experiments, gel renaturation assays performed with extracts of platelets activated by thrombin in the presence of RGDS peptide show an increase in p42^{mapk} activity similar to that in control platelets activated with thrombin in the presence of RGES peptide (Fig. 5C). As expected, p42^{mapk} is phosphorylated on tyrosine in platelets activated in the presence of RGES peptide, which does not block aggregation (Fig. 5B and C).

Identification of p90^{rsk} and p70^{S6K} in platelets. Previous studies have shown that activated MAPK will phosphorylate and activate p90^{rsk} but not p70^{S6K} (11, 13, 75), members of two distinct ribosomal S6 kinase families (3, 4, 8, 12, 20, 76). We next examined platelet extracts for the presence of these two kinases by immunoprecipitation followed by immunoblotting with antisera specific for either p90^{rsk} or for p70^{S6K}. Figure 6 shows that platelets express two forms of p70^{S6K}, p70 (α_2) and p85 (α_1), as well as p90^{rsk}. The levels of these proteins do not change during platelet activation, although p90^{rsk} undergoes a mobility shift, characteristic of this protein when it becomes phosphorylated and activated.

Elevation of p90^{rsk} activity during platelet activation and aggregation. To test directly whether the kinase activities of p90^{rsk} and p70^{S6K} are elevated upon platelet activation, these kinases were immunoprecipitated from platelet extracts derived from a thrombin activation time course and analyzed by in vitro kinase assays. p70^{S6K} showed no activity when isolated from either resting or activated platelets (data not shown). p90^{rsk}, on the other hand, showed a trace of kinase activity when immunoprecipitated from resting platelets and exhibited approximately a 10-fold increase in kinase activity during platelet activation (Fig. 7A).

Integrin-mediated platelet aggregation is not required for activation of p90^{rsk}. Since the increase in p42^{mapk} activity was not dependent on platelet aggregation and since p90^{rsk} is known to be a substrate for p42^{mapk}, we predicted that the increase in activity of p90^{rsk} would also be independent of platelet aggregation. To test this hypothesis, the activity of p90^{rsk} was measured during thrombin-induced platelet activation in the presence of RGDS peptide. The kinase assay shown in Fig. 7B demonstrates that the activity of p90^{rsk} is increased in platelets activated with thrombin in the presence of RGDS peptide, similar to the p90^{rsk} activity seen in control platelets aggregated in the presence of RGES peptide (Fig. 7A). Furthermore, Fig. 7D illustrates that the size shift characteristic of p90^{rsk} activation still occurs when platelet aggregation is blocked with RGDS peptide.

Enhanced serine phosphorylation of p90^{rsk} during platelet activation and aggregation. Next, the phosphorylation state of p90^{rsk} was examined after immunoprecipitation from platelets labeled with $^{32}P_i$. Although p90^{rsk} was phosphorylated when isolated from extracts of both resting and activated platelets, the phosphorylation was more extensive, and the phosphorylated population of p90^{rsk} showed a change in electrophoretic mobility upon platelet activation (Fig. 8A). This shift in mobility is characteristic of p90^{rsk} upon phosphorylation by MAPK (8, 9, 11, 82). Phospho-

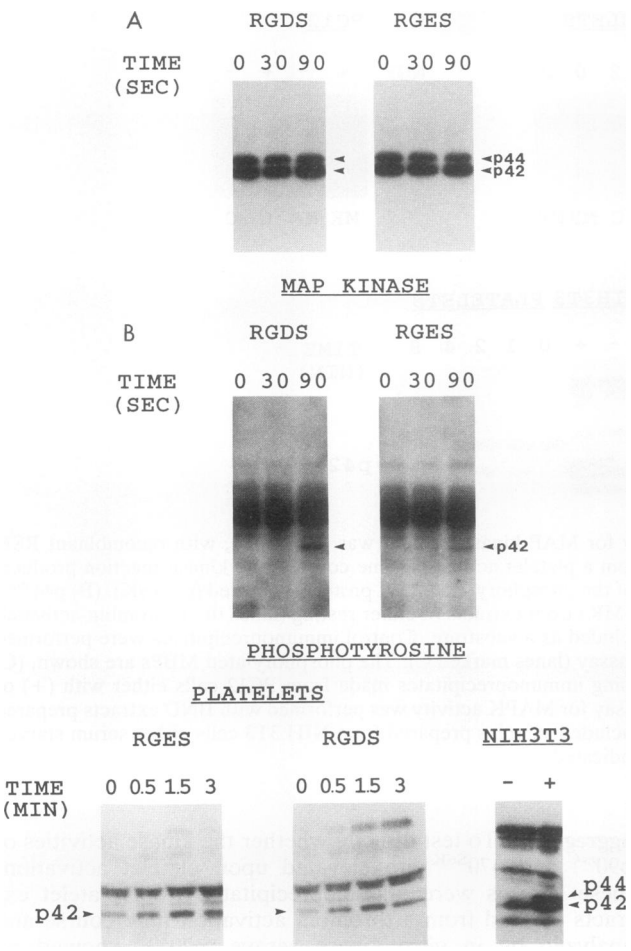

FIG. 5. Thrombin-induced phosphorylation and activation of $p42^{mapk}$ does not require platelet aggregation. (A and B) Platelets were activated by stirring with thrombin treatment either in the presence of RGDS peptide to block aggregation or in the presence of RGES peptide as a control. Samples were harvested under denaturing conditions at the indicated time points and then immunoprecipitated with the C-1 antibody. Immunoprecipitates were divided in half; one portion was subjected to immunoblotting with the C-1 antibody (A), and the other portion was analyzed by immunoblotting with an antibody against phosphotyrosine (B). The positions of $p42^{mapk}$ and $p44^{mapk}$ are indicated. (C) Platelets were activated by stirring with thrombin treatment either in the presence of RGDS peptide to block aggregation or in the presence of RGES peptide as a control. Samples were harvested at the indicated time points in BND buffer and analyzed by an in-gel renaturation kinase assay. For comparison, the experiment included samples prepared from NIH 3T3 cells either serum starved (−) or serum stimulated (+). The positions of $p42^{mapk}$ and $p44^{mapk}$ are indicated.

amino acid analysis showed that $p90^{rsk}$ was phosphorylated exclusively on serine (Fig. 8B).

DISCUSSION

Platelet activation and aggregation is accompanied by the phosphorylation of numerous proteins on tyrosine residues (21, 22, 24, 26–28, 42, 56, 57, 62, 66, 81). In an effort to identify kinases and their substrates that play pivotal roles in platelet signal transduction, we have examined the phosphorylation and activities of MAPKs and ribosomal S6 kinases during thrombin-induced platelet activation. Two forms of

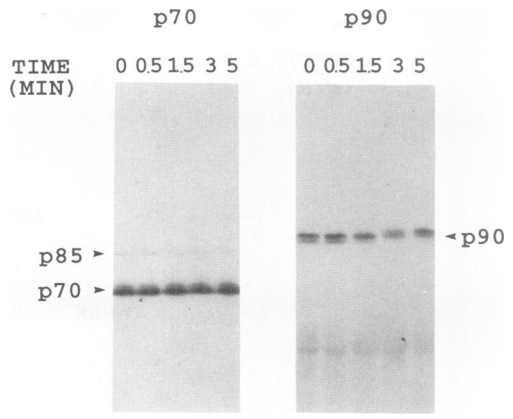

FIG. 6. Identification of ribosomal S6 kinases in platelet extracts. Platelet samples were harvested at the indicated times after thrombin activation. Immunoprecipitation with antibodies directed against either $p90^{rsk}$ or $p70^{S6K}$ was followed by Western immunoblotting with corresponding antibodies. The positions of $p90^{rsk}$ and two forms of $p70^{S6K}$, $p70^{S6K}$ (α_2) and $p85^{S6K}$ (α_1), are indicated.

MAPK, $p42^{mapk}$ and $p44^{mapk}$, were identified in human platelets. Several lines of evidence indicate that the activity of $p42^{mapk}$ but not $p44^{mapk}$ is increased during thrombin-stimulated platelet activation. First, MAPK activity measured directly with platelet extracts is increased up to 10-fold during thrombin activation. Fractionation of platelet extracts on an anion-exchange column followed by a direct kinase assay indicated that all of the MAPK activity was associated with fractions containing both $p42^{mapk}$ and $p44^{mapk}$ (unpublished results). This eliminates the possibility that another, unspecified MAPK family member is responsible for the activity detected in unfractionated platelet extracts. Next, an in vitro kinase assay performed with $p44^{mapk}$, specifically immunoprecipitated from platelets, showed no significant change in kinase activity during platelet activation. Finally, a gel renaturation kinase assay performed with platelet extracts prepared during a thrombin activation time course showed that the kinase activity of $p42^{mapk}$ but not $p44^{mapk}$ was increased two- to threefold. In agreement with our results, the activation of $p42^{mapk}$, independently of $p44^{mapk}$, was recently demonstrated in sheep platelets stimulated with phorbol myristate acetate and platelet-activating factor (69). It is intriguing that the activity of $p42^{mapk}$ is increased independently of $p44^{mapk}$ in platelets, since in other cell systems, both kinases are coactivated by a variety of stimuli (5, 6, 15, 20, 51, 60, 68, 77).

The phosphorylation patterns of $p42^{mapk}$ and $p44^{mapk}$ in platelets are in agreement with the observed activities of these kinases. In resting platelets, no phosphorylation of either kinase is observed. Upon thrombin-induced activation and aggregation, $p42^{mapk}$ but not $p44^{mapk}$ becomes phosphorylated. Phosphoamino acid analysis indicated that serine, threonine, and tyrosine residues were phosphorylated. Other studies have shown that MAPK requires phosphorylation on both threonine and tyrosine for activation (1, 3, 39, 65). The data presented here are in agreement with previous findings. Although serine phosphorylation of MAPK is not apparently required for activation, it is not unprecedented and has occasionally been documented for MAPK in other cell types (7, 19, 29, 31, 72, 79).

In addition to $p42^{mapk}$ and $p44^{mapk}$, platelets also express members of two ribosomal S6 kinase families, $p70^{S6K}$ and

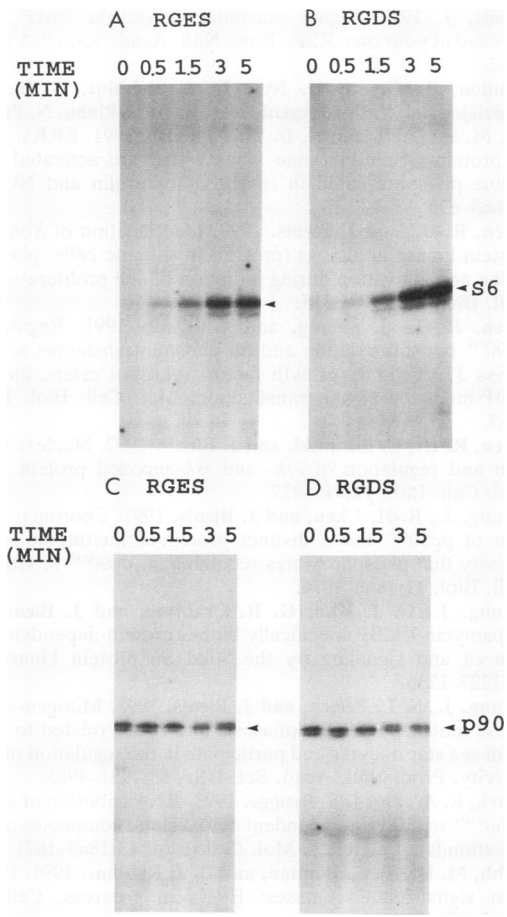

FIG. 7. Kinase activity of p90^{rsk} is increased during thrombin-induced platelet activation and does not require platelet aggregation. Platelets were activated by stirring with thrombin treatment either in the presence of RGDS peptide to block aggregation or in the presence of RGES peptide as a control. Samples were harvested in BND buffer at the indicated time points and then immunoprecipitated with an antibody directed against p90^{rsk}. Immunoprecipitates were divided in half; one portion was subjected to an in vitro kinase assay with S6 protein included as a substrate (A and B), and the other portion was analyzed by immunoblotting with the antibody against p90^{rsk} (C and D). The positions of the phosphorylated S6 protein and p90^{rsk} are indicated.

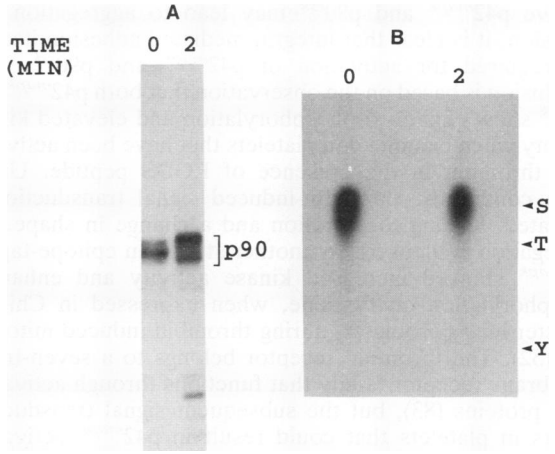

FIG. 8. ^{32}P labeling of p90^{rsk} and phosphoamino acid analysis. Resting platelets were labeled with $^{32}P_i$. One platelet sample was harvested prior to activation (lanes 0), and a second sample was prepared after thrombin-induced activation and aggregation (lanes 2). The samples, prepared under denaturing conditions, were immunoprecipitated with an antibody against p90^{rsk} and analyzed by SDS-polyacrylamide gel electrophoresis (A). p90^{rsk} from both resting and activated platelets was subjected to phosphoamino acid analysis (B). The positions of phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) determined from standards are indicated.

p90^{rsk}. While no change in kinase activity for p70^{S6K} was detected, p90^{rsk} activity was increased during platelet activation. From other studies, it is known that p90^{rsk} is regulated by serine/threonine phosphorylation, in part mediated by MAPK (11, 13, 20, 75). Here we have shown that the activity of p90^{rsk} is elevated at least 10-fold during thrombin-induced platelet activation, coincident with the enhanced activity of p42^{mapk}. Furthermore, during platelet activation p90^{rsk} shows an altered pattern of serine phosphorylation and exhibits a mobility shift characteristic of this kinase when activated by phosphorylation by MAPK (8, 9, 11, 82). While other studies have shown that activation of both p42^{mapk} and p44^{mapk} can lead to phosphorylation and activation of p90^{rsk} (11, 13, 75), the data presented here suggest that p42^{mapk} alone is capable of doing so in platelets. Although p90^{rsk} activity is low in resting platelets, its basal level of phosphorylation is higher than that seen in other unstimulated cell types (8, 76). This observation suggests that protein serine kinases in addition to MAPK participate in the phosphorylation and regulation of p90^{rsk} activity in platelets.

Most studies of MAPKs and p90^{rsk} in other systems have implicated these proteins in mitosis and cell proliferation (5, 6, 15, 20, 60, 68, 77). On the other hand, platelets are terminally differentiated cells, they have no nucleus, and they do not synthesize protein (18, 74). Consequently, the signal transduction pathways that utilize p42^{mapk} and p90^{rsk} in these cells must lead to cellular events other than mitogenesis. This is not unprecedented, however, since a role for MAPKs in neurons, another terminally differentiated cell type, has also been documented (2, 7, 19, 29, 30, 32, 54, 72, 78, 80, 85).

Platelets undergo extensive cytoskeletal reorganization during activation and are highly specialized for secretion and adhesion (41, 44, 59). It is, therefore, reasonable to speculate that p42^{mapk} and p90^{rsk} function in signal transduction pathways that lead to these processes. Aside from p90^{rsk}, the specific substrates for p42^{mapk} during platelet activation are unknown. If p42^{mapk} functions in a pathway that leads to cytoskeletal changes, then it is reasonable to propose that the substrates would include proteins similar to MAP-2, MAP-4, or p220, microtubule-associated proteins that have been identified as substrates for MAPK in other cell types (39, 46, 54, 71, 73, 80). The phosphorylation of these proteins by MAPK reduces their ability to induce tubulin polymerization (39, 73). In mitotic cells, this is thought to contribute to changes in cytoskeletal structure that occur during the transition from interphase to mitosis. Thus, in platelets, MAPK could modulate cytoskeletal protein functions in secretion and adhesion. Another potential substrate for p42^{mapk} in platelets is cPLA$_2$. In other cell types, thrombin stimulates the phosphorylation and activation of cPLA$_2$ by p42^{mapk} (49, 58).

Although the signal transduction pathways in platelets that

involve $p42^{mapk}$ and $p90^{rsk}$ may lead to aggregation and adhesion, it is clear that integrin-mediated adhesion itself is not required for activation of $p42^{mapk}$ and $p90^{rsk}$. This conclusion is based on the observation that both $p42^{mapk}$ and $p90^{rsk}$ show enhanced phosphorylation and elevated kinase activity when examined in platelets that have been activated with thrombin in the presence of RGDS peptide. Under these conditions, thrombin-induced signal transduction is activated, leading to secretion and a change in shape, but aggregation is blocked. In another study, an epitope-tagged $p44^{mapk}$ showed increased kinase activity and enhanced phosphorylation on tyrosine, when expressed in Chinese hamster lung fibroblasts, during thrombin induced mitogenesis (52). The thrombin receptor belongs to a seven-transmembrane receptor family that functions through activation of G proteins (83), but the subsequent signal transduction events in platelets that could result in $p42^{mapk}$ activation have not been identified. We have previously shown that the kinase activity of $p60^{src}$ is rapidly increased during thrombin-induced platelet activation (84). In fibroblasts, $p60^{src}$ has been shown to indirectly activate MAPK (16, 33, 55), but the intervening signal transduction components are not known. In platelets, $p60^{src}$ could also participate in a signaling pathway leading to MAPK, but the link with thrombin receptor activation and the immediate downstream targets for $p60^{src}$ in this pathway are not known.

Several kinases that function immediately upstream of MAPK have been identified in other systems. First, a MAPK kinase (MEK) that can phosphorylate and activate $p44^{mapk}$ has been identified (17). In some cell types, MEK (or MEK activity) can be stimulated by the serine/threonine kinase Raf-1. Raf-1 can be activated by Ras, which, in turn, is activated in response to receptors that have tyrosine kinase activity (6, 60, 68). On the other hand, signals from receptors that are coupled to G proteins, such as the thrombin receptor, are thought to activate MEK through a Raf-independent pathway that utilizes a serine/threonine kinase called MEK kinase (48). Our data suggest the hypothesis that upstream kinases such as MEK may be selectively expressed or modulated in their activities during platelet activation. We are currently examining the possibility that these or similar proteins can lead to the selective activation of $p42^{mapk}$ during thrombin-induced platelet activation.

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