Distinct Substrate Specificities and Functional Roles for the 78- and 76-kDa Forms of μ-Calpain in Human Platelets*

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The intracellular thiol protease μ-calpain exists as a heterodimeric proenzyme, consisting of a large 80-kDa catalytic subunit and a smaller 30-kDa regulatory subunit. Activation of μ-calpain requires calcium influx across the plasma membrane and the subsequent autotryptic conversion of the 80-kDa large subunit to a 78-kDa “intermediate” and a 76-kDa fully autolyzed form. Currently, there is limited information on the substrate specificities and functional roles of these distinct active forms of μ-calpain within the cell. Using antibodies that can distinguish among the 80-, 78-, and 76-kDa forms of μ-calpain, we have demonstrated a close correlation between the autolytic generation of the 78-kDa enzyme and the proteolysis of the non-receptor tyrosine phosphatase, PTP-1B, in ionophore A23187-stimulated platelets. Time course studies revealed that pp60src proteolysis lagged well behind that of PTP-1B and correlated closely with the generation of the fully autolyzed form of μ-calpain (76 kDa). In vitro proteolysis experiments with purified μ-calpain and immunoprecipitated PTP-1B or pp60src confirmed selective proteolysis of pp60src by the 76 kDa enzyme, whereas PTP-1B cleavage was mediated by both the 76- and 78-kDa forms of μ-calpain. Studies using selective pharmacological inhibitors against the different autolytic forms of μ-calpain have demonstrated that the initial conversion of the μ-calpain large subunit to the 78-kDa form is responsible for the reduction in platelet-mediated clot retraction, whereas complete proteolytic activation of μ-calpain (76 kDa) is responsible for the shedding of procoagulant-rich membrane vesicles from the cell surface. These studies demonstrate the existence of multiple active forms of μ-calpain within the cell, that have unique substrate specificities and distinct functional roles.

Calpains comprise a family of calcium-dependent thiol proteases, that are widely expressed in mammalian cells (1–3). There are both tissue-specific and ubiquitous forms of calpain (3, 4), with μ-calpain and m-calpain representing the prototypic members of the latter group. These two enzymes are distinguishable by the concentration of calcium required for their activation in vitro, with μ-calpain requiring micromolar and m-calpain requiring millimolar concentrations of calcium for enzyme activation (3, 5). Both μ- and m-calpain have been identified in human platelets; however, μ-calpain is the predominant isoform in these cells, accounting for more than 90% of total platelet calpain activity (6, 7).

μ- and m-calpains exist inside the cell as inactive proenzymes, composed of two non-identical subunits of approximately 30 and 80 kDa in molecular mass (1). The small subunits of μ- and m-calpain are identical, whereas the larger subunit is unique to each enzyme, suggesting that this subunit confers enzyme specificity (2). A model for calpain activation has been proposed (8), in which the enzyme translocates from the cytosol to the membrane in a calcium-dependent manner, bringing it into close proximity to membrane phospholipids (9). Following translocation, the enzyme undergoes two N-terminal autoproteolytic events leading to the generation of 78- and 76-kDa active forms (8, 10–13). Previous studies have demonstrated that each of these forms of calpain is proteolytically active in vitro (14), although as yet the substrate specificity and the functional role(s) of each of these enzymes have not been established. Subsequent autoproteolytic modification of the N-terminal membrane attachment sequence of the 30-kDa small subunit does not alter the catalytic properties of calpain (8) but leads to the detachment of the protease from the cell membrane (8, 15).

Studies in human platelets have demonstrated that the activation of calpain leads to the limited proteolysis of several key cellular proteins, including the platelet integrin αIIbβ3 (16), the cytoskeletal-structural proteins talin and actin-binding protein (17–19), and several signaling enzymes, including pp125FAK, pp60src, PTP-1B,1 protein kinase C, and phospholipase Cγ (20–22). The ability of calpain to proteolytically regulate various cytoskeletal structural proteins and signaling enzymes has suggested a potentially important role for this protease in modulating cytoskeletal reorganization and calcium-dependent signaling processes within the cell. Consistent with this are recent studies demonstrating that calpain proteolysis of non-receptor tyrosine kinases and phosphatases regulates the level of tyrosine phosphorylation in thrombin and ionophore A23187-stimulated platelets (7). Furthermore, the cleavage of cytoskeletal-associated structural proteins and signaling enzymes leads to the cytoskeletal detachment of integrin αIIbβ3 and relaxed fibrin clot retraction (23). These proteolytic events also result in the dissociation of the actin cytoskeleton from its membrane attachment sites, leading to the release of procoagulant-rich microvesicles from the cell surface (24–29).

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1 The abbreviations used are: PTP-1B, protein-tyrosine phosphatase-1B; mAb, monoclonal antibody; GP, glycoprotein.
In this report, we have investigated the substrate specificity and functional role(s) of the 78- and 76-kDa forms of μ-calpain in human platelets. The studies presented in this article demonstrate that the initial conversion of μ-calpain from the 80- to 78-kDa active form is required for PTP-1B cleavage and for the subsequent inhibitory effects of calpain on the clot retraction process. This is in contrast to pp60c-src, which requires complete autolytic conversion of μ-calpain to the 76-kDa form and is associated with the shedding of procoagulant-rich microvesicles from the platelet surface. These studies suggest unique functional roles for the 78- and 76-kDa forms of μ-calpain in human platelets.

EXPERIMENTAL PROCEDURES

Materials—Calpeptin was obtained from Biomol Research Laboratories, Plymouth Meeting, PA. E-64-d was obtained from the Peptide Institute, Inc., Osaka, Japan. E-64 and calpain inhibitor-I were obtained from Boehringer Mannheim. Ionophore A23187 and purified erythrocyte μ-calpain were from Calbiochem-Novabiochem Pty Ltd, NSW, Australia. All other materials were from sources we have described previously (21, 23, 30, 31).

Antibodies—Anti-PTP-1B polyclonal antibody was obtained from Upstate Biotechnology, Inc., Lake Placid, NY. Anti-pp60c-src mAb 327 was a kind donation from Dr. Joan Brugge (University of Pennsylvania). Anti-GP Ib mAb S222 was purchased from Immunotech, France. Both anti-mouse and anti-rabbit peroxidase-conjugated IgG were from Silenus Laboratories, Victoria, Australia. Rabbit anti-mouse immunoglobulins were purchased from DAKO A/S, Glostrup, Denmark.

Production of Anti-μ-calpain Polyclonal Antibodies—Anti-calpain pre-(80 kDa) and post-autolytic (76 kDa) polyclonal antibodies were produced as described previously (32) (Fig. 1A). The autolytic intermediate form of μ-calpain large subunit (78 kDa) was produced by incubating purified rabbit μ-calpain with 100 μM E-64 in the presence of 5 mM CaCl₂, 5 mM β-mercaptoethanol, and 50 mM Tris-HCl, pH 7.5, at 30 °C for 5 min. The presence of E-64 in these assays inhibits full μ-calpain autolysis, leading to the accumulation of the 78-kDa intermediate form. The cleavage site in μ-calpain to produce the autolytic intermediate was determined by directly sequencing the electrophoretically isolated fragment using a protein sequencer (Applied Biosystems model 492). Based on the information that the cleavage site is located between serine and alanine residues at positions 14 and 15 (see Fig. 1B), a synthetic peptide, AQVQKQKC, was employed as a hapten to produce the antibody specific to the autolytic intermediate form. The haptenic peptide, conjugated to a keyhole limpet hemocyanin (Calbiochem), was used to immunize rabbits. The antibody was affinity-purified and used as described previously (32).

Preparation of Washed Platelets—Human platelets were obtained from healthy volunteers, who had not taken anti-platelet medication in the preceding 2 weeks, and washed as described previously (31). Washed platelets were finally resuspended in modified Tyrode’s buffer (10 mM Hepes, 12 mM NaHCO₃, pH 7.5, 137 mM NaCl, 2.7 mM KC1, and 5 mM glucose), and preincubated with calpeptin (5–100 μM), E-64-d (50–100 μM), or vehicle (0.3% Me₂SO) for 30 min at room temperature prior to use.

Preparation of Platelet Subcellular Fractions—The Triton X-100-soluble fraction was prepared by lysing washed platelets with one volume of 10 × Triton X-100 lysis buffer (10% Triton X-100, 200 mM Tris-HCl, pH 7.4, 10 mM EGTA, 2 mM EDTA, 2 mM sodium vanadate, 250 μg/ml phenylmethylsulfonyl fluoride, 50 μM calpain inhibitor-I) to nine volumes of platelets, and then gently agitated for 60 min at 4 °C. The Triton X-100-soluble fraction was prepared by centrifugation for 4 min at 15,000 × g, as described previously (33). The supernatant containing the Triton X-100-soluble extract was removed and stored at −20 °C. Whole cell lysates were prepared by lysing activated platelets in Laemmli reducing buffer (0.125 M Tris-HCl, pH 6.8, 5 mM EDTA, 20% glycerol, 0.1% SDS, 0.002% bromphenol blue, 10% β-mercaptoethanol) and were boiled immediately for 10 min.

Anti-calpain, pp60c-src, GP Ib, and PTP-1B Immunoblots—Equal quantities of platelet extracts (50 μg) were separated by 7.5% SDS-polyacrylamide gel electrophoresis, under reducing conditions, then transferred to polyvinylidene difluoride membranes. Western blots were performed as described by Towbin et al. (34), using specific primary antibodies, followed by horseradish peroxidase-conjugated secondary antibodies. Blots were developed using ECL according to the manufacturer’s instructions (NEL Life Science Products).

Immunoprecipitation of pp60c-src and PTP-1B from Platelet Lysates—pp60c-src and PTP-1B were immunoprecipitated from the Triton X-100-soluble fraction of resting platelets, prepared as described above. 50 μl of a 50% slurry of Protein A beads was incubated with a protein antibody (8 μg/ml) or anti-pp60c-src mAb 327 (10 μg/ml) in the presence of anti-mouse IgG (1 μg/ml), for 1 h at 4 °C. Excess antibody was removed by washing the Protein A beads three times with 20 mM Tris, pH 7.4, 150 mM NaCl. 400 μg of total protein was added to the Protein A immune complex, and incubated for 2 h at 4 °C. Excess protein was removed by washing the Protein A beads three times with 20 mM Tris, pH 7.4, 150 mM NaCl, containing inhibitors (0.2 mM sodium vanadate, 25 μg/ml phenylmethylsulfonyl fluoride, 5 μM calpain inhibitor-I).

In Vitro Proteolysis of pp60c-src and PTP-1B—In vitro proteolysis experiments were performed using a modified method from Baki et al. (13). Purified erythrocyte μ-calpain (1 μg) was diluted in a buffer containing 10 mM Hepes, pH 7.5, 1 mM EGTA, 1 mM dithioerythritol, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine. In some experiments, EDTA (5 mM) was included in the reaction mixture to prevent calpain activation. For studies using calpain inhibitors, purified calpain diluted in calpain buffer was preincubated with calpeptin (100 μg/ml) or E-64 (100 μM) for 30 min at room temperature. Calpain was activated by incubation with 1 mM CaCl₂ (final) at 37 °C for 1.5 min, in the presence of bovine serum albumin (1 mg/ml). Activated calpain was then added to Protein A beads containing immunoprecipitated pp60c-src or PTP-1B, and incubated for the indicated time of autolysis. Reactions were stopped by the addition of Laemmli-reducing buffer containing 5 mM EDTA, and boiled for 10 min. Reaction mixtures were centrifuged and the supernatant analyzed for substrate cleavage by immunoblot analysis. The PTP-1B immunoblots were performed with a monoclonal antibody to avoid detection of the heavy chain of polyclonal anti-PTP-1B.

Clot Retraction—Clot retraction studies were performed as described (23, 30), with some minor modifications. Washed platelets were pre-treated with calpeptin (100 μg/ml), E-64-d (100 μM), or vehicle (0.3% Me₂SO) for 30 min, prior to activation with thrombin (1 unit/ml) or ionophore A23187 (0.25–1 μM). For studies with ionophore A23187 alone, atroxin (0.1 μg/ml) was added to induce clot formation. In all studies with ionophore A23187, the platelets were left unstirred during the assay. When thrombin was used as the agonist, platelets were stirred for a maximum time period of 45–60 s. Clot retraction was assessed after 60 min of platelet activation. The extent of clot retraction was quantitated by measuring the residual volume of serum (after removal of the fibrin clot) and expressing this as a percentage of the total reaction volume.

Isolation of Platelet Microvesicles—Platelet microvesicles were isolated from platelets according to the method described by Fox et al. (25). Washed platelets were pre-treated with calpeptin (100 μg/ml), E-64-d (100 μM), or vehicle (0.3% Me₂SO) for 30 min, prior to platelet activation. Platelets were treated with thrombin (1 unit/ml) (stirred), ionophore A23187 (0.25–1 μM) (unstirred), or buffer alone, in the presence of 1 mM calcium or 1 mM EGTA and 2 mM MgCl₂, for the indicated time points, prior to the addition of 5 mM EGTA and 10 mM theophylline. The reaction mixture was centrifuged at 15,600 × g for 1 h at room temperature. The platelet pellet was lysed in Laemmli reducing buffer and boiled for 10 min. The supernatant containing platelet microvesicles was centrifuged at 100,000 × g for 2.5 h, and the resultant microvesicle pellet solubilized in Laemmli reducing buffer and boiled for 10 min. Microvesicles were detected by immunoblotting the 100,000 × g pellet with an anti-GP Ib antibody. PTP-1B proteolysis was analyzed by immunoblotting the 15,600 × g platelet pellet with an anti-pp60c-src antibody.

Miscellaneous Methods—SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (35). Fibrinogen was purified from fresh frozen plasma as described previously by Jakobsen and Koerulf (36). Protein concentrations were measured using the Bio-Rad protein assay with bovine serum albumin as a standard.

RESULTS

Activation of the thiol protease, calpain, is one of the many calcium-dependent signaling events that occur as a consequence of platelet activation. Calpain activation is associated with significant autoproteolytic modification of the N termini of the 80-kDa catalytic and 30-kDa regulatory subunits (2). In vitro proteolysis experiments have revealed that the conversion of μ-calpain in human platelets requires complete autolytic conversion of μ-calpain from the 80- to 78-kDa active form is required for PTP-1B cleavage and for the subsequent inhibitory effects of calpain on the clot retraction process. This is in contrast to pp60c-src, which requires complete autolytic conversion of μ-calpain to the 76-kDa form and is associated with the shedding of procoagulant-rich microvesicles from the platelet surface. These studies suggest unique functional roles for the 78- and 76-kDa forms of μ-calpain in human platelets.
**Fig. 1. Autolytic activation of μ-calpain: production of antibodies against the 80-, 78-, and 76-kDa large subunit of μ-calpain.** A, activation of μ-calpain involves the sequential autolytic conversion of the 80-kDa large subunit to a 78-kDa intermediate and a 76-kDa fully autolyzed form, thereby generating unique N termini for the protease. B, peptides were designed to match the N-terminal sequences of pre-, inter-, and post-autolytic μ-calpain. These peptides were used to produce anti-peptide polyclonal antibodies that can distinguish between the different activation states of μ-calpain, as described under “Experimental Procedures” and by Saido et al. (32). C, characterization of pre-, inter-, and post-autolytic μ-calpain antibodies. Purified rabbit μ-calpain was treated with buffer alone (lane 1), 5 mM calcium (lane 2), or 5 mM calcium in the presence of 100 μM E-64 (lane 3), for 5 min at 37 °C, as described under “Experimental Procedures.” Reactions were terminated by the addition of Laemmli reducing buffer and the samples immediately boiled. μ-Calpain autolytic states were monitored by immunoblot analysis using polyclonal antibodies directed against the pre- (Anti-pre-μ), inter- (Anti-inter-μ), and post- (Anti-post-μ) autolytic forms of μ-calpain. Alternatively, μ-calpain autolysis was monitored by electrophoretic mobility shift using a polyclonal antibody that recognizes all autolytic states of μ-calpain (Anti-domain III).

of both subunits to their fully autolyzed forms proceeds sequentially through the generation of a number of intermediate-sized proteins. For example, the 80-kDa subunit is initially converted to a 78-kDa “intermediate” protein, which is further proteolyzed to the 76-kDa fully autolyzed form (Fig. 1A) (12, 37). We have previously raised anti-peptide polyclonal antibodies against the unique N-terminal sequences of the 80-kDa (anti-pre-μ) and 76-kDa (anti-post-μ) large subunits of calpain (32). Using a similar strategy, we have raised an antibody against the N terminus of the 78-kDa (anti-inter-μ) intermediate calpain (Fig. 1B), as described under “Experimental Procedures,” and compared its antigenic specificity against the anti-pre- and anti-post-μ-calpain antibodies. In initial studies, the extent of calpain activation (following the addition of calcium to purified rabbit μ-calpain) was examined using a polyclonal antibody that recognizes all forms of μ-calpain (anti-domain III). In the absence of calcium, only the inactive form of μ-calpain (80 kDa) was detected (Fig. 1C, Anti-domain III, lane 1). With the addition of calcium, full μ-calpain autolysis was observed as demonstrated by the presence of a single protein band with increased electrophoretic mobility (76 kDa) (Fig. 1C, Anti-domain III, lane 2). Pretreating purified μ-calpain with the calpain inhibitor, E-64 (100 μM), prior to the addition of calcium, inhibited full μ-calpain autolysis leading to the accumulation of both the intermediate (78 kDa) and inactive (80 kDa) forms (Fig. 1C, Anti-domain III, lane 3). Immunoblot analysis of these different μ-calpain forms with each of the anti-peptide antibodies revealed that anti-pre-μ selectively recognized 80-kDa calpain (Fig. 1C, Anti-pre-μ, lanes 1 and 3), anti-inter-μ specifically recognized 78-kDa μ-calpain (Fig. 1C, Anti-inter-μ, lane 3), and anti-post-μ only recognized the fully autolyzed form of the protease (76 kDa) (Fig. 1C, Anti-post-μ, lane 2).

**μ-Calpain Activation in Ionophore A23187-stimulated Platelets—**We have demonstrated previously that immunoblot analysis of whole cell lysates with the anti-pre-μ and anti-post-μ antibodies represents a sensitive, specific and direct means of monitoring μ-calpain activation within the cell (32). Using the anti-peptide antibody, anti-inter-μ, we examined the time course for the generation of the 78-kDa form of μ-calpain in ionophore A23187-stimulated platelets. In resting platelets, only the inactive form of μ-calpain was present, as demonstrated by the intact 80-kDa large subunit (Fig. 2). Following treatment with ionophore A23187, the level of the intact 80-kDa subunit decreased in a time-dependent fashion and was no longer present in whole cell lysates after 1 min of platelet activation. The disappearance of the 80-kDa subunit correlated with an increase in the 78-kDa intermediate enzyme, which was apparent within 15–30 s of platelet activation. Beyond 1 min of platelet activation the levels of the 78-kDa enzyme decreased, correlating with the appearance of the fully autolyzed μ-calpain (76 kDa) (Fig. 2). These studies confirm the sequential generation of the 76- and 76-kDa forms of μ-calpain in activated platelets.

We have noted previously that the time course for PTP-1B and pp125Fak cleavage in ionophore A23187-stimulated platelets was considerably more rapid than that of pp60c-src (21). There are a number of factors that may influence the rate of substrate proteolysis in vivo, including differences in substrate susceptibility to proteolysis, proteolysis by distinct forms of calpain, or compartmentalization factors within the cell that may limit substrate accessibility to the protease. It is also
possible that the endogenous inhibitor of calpain, calpastatin, may preferentially modulate the proteolysis of specific calpain substrates. We investigated the possibility that the different forms of active \(\mu\)-calpain (78 and 76 kDa) may cleave distinct substrates. In our initial studies, we compared the time course for pp60\(^{\text{src}}\) and PTP-1B proteolysis in ionophore A23187-stimulated platelets with the irreversible calpain inhibitor, E-64, prior to its activation with calcium inhibits the autolytic conversion of the intermediate form of \(\mu\)-calpain. This was in direct contrast to pp60\(^{\text{src}}\) cleavage, which lagged well behind that of PTP-1B (Fig. 2) and correlated with the appearance of the fully autolyzed 76-kDa form of \(\mu\)-calpain.

We examined in further detail the relationship between the generation of the different forms of active \(\mu\)-calpain and the cleavage of PTP-1B and pp60\(^{\text{src}}\) by pretreating platelets with increasing concentrations of the membrane-permeable calpain inhibitor, calpeptin, prior to ionophore A23187 stimulation. We have previously demonstrated under these same experimental conditions that calpeptin pretreatment of platelets inhibits calpain activation in a dose-dependent manner (23). As demonstrated in Fig. 3, doses of calpeptin (0.5–2.0 \(\mu\)g/ml) which effectively blocked the generation of the 76-kDa form of calpain also blocked pp60\(^{\text{src}}\) proteolysis, but had minimal effect on PTP-1B cleavage. In contrast, higher concentrations of calpeptin (5.0–20 \(\mu\)g/ml) were required to inhibit the generation of the 78-kDa form of calpain and PTP-1B cleavage (Fig. 3). We consistently noted in these experiments an excellent dose-dependent relationship between the amount of 78-kDa calpain generated and the degree of PTP-1B proteolysis. Taken together, these studies suggest that differences in the rates of substrate proteolysis in vitro may be due, at least in part, to differences in the rate of generation of distinct forms of active \(\mu\)-calpain.

In Vitro Time Course for pp60\(^{\text{src}}\) and PTP-1B Cleavage by Purified \(\mu\)-Calpain—Previous in vitro proteolysis experiments comparing the catalytic properties of the fully autolyzed forms of \(\mu\)- and m-calpain have reported no major differences in the substrate specificity of these enzymes (1). While there is evidence suggesting that the intermediate form of \(\mu\)-calpain (78 kDa) may be proteolytically active in vitro (14), it has yet to be established if the catalytic properties of this enzyme differ from that of the fully autolyzed form. To investigate the substrate specificity of the 78- and 76-kDa forms of \(\mu\)-calpain in more detail, we performed in vitro proteolysis experiments using purified \(\mu\)-calpain and immunoprecipitated pp60\(^{\text{src}}\) and PTP-1B. Preincubating purified \(\mu\)-calpain with 1 \(\mu\)M calcium for 90 s resulted in complete conversion of the 80-kDa form of \(\mu\)-calpain to the 76-kDa active enzyme (13). Incubation of activated \(\mu\)-calpain with immunoprecipitated pp60\(^{\text{src}}\) and PTP-1B resulted in rapid proteolysis of both substrates (Fig. 4A), confirming that both of these enzymes are substrates for the 76-kDa form of \(\mu\)-calpain. We adjusted the concentration of \(\mu\)-calpain in these assays to 10 \(\mu\)g/ml to obtain a rate of PTP-1B proteolysis similar to that observed in platelets. Unlike the situation in platelets, however (Fig. 2), the in vitro proteolysis of pp60\(^{\text{src}}\) was only slightly slower than that of PTP-1B (Fig. 4A). While direct comparison of the rates of proteolysis in vitro cannot be directly compared with the results obtained in intact cells, they nonetheless indicate that pp60\(^{\text{src}}\) is not an inherently resistant substrate to \(\mu\)-calpain proteolysis. This contrasts with the rate of casein proteolysis, which occurs approximately 50 times slower than that of microtubule-associated protein 2 in vitro (13).

In further experiments, we examined the substrate specificity of the intermediate form of \(\mu\)-calpain. Consistent with previous studies (14), we have demonstrated that pretreating purified \(\mu\)-calpain with the irreversible calpain inhibitor, E-64, prior to its activation with calcium inhibits the autolytic con-
version of 78-kDa calpain to the 76-kDa form, resulting in the accumulation of the 78- and 80-kDa enzyme (Fig. 1C, Anti-inter and Anti-domain III, lane 3). We consistently found that concentrations of E-64 between 50 and 100 μM inhibited the conversion of the 78-kDa form to the fully autolyzed enzyme (76 kDa) by 90–95% (Fig. 4B, lower panel). These assay conditions were therefore utilized to investigate the ability of intermediate μ-calpain to cleave pp60src and PTP-1B. As demonstrated in Fig. 4B, E-64 (100 μM) dramatically inhibited the cleavage of pp60src by calpain. These assays were performed for 15 min, hence the small amount of pp60src cleavage (<10%) (Fig. 4B, upper panel) observed in this particular experiment was most likely due to the residual amount of 76-kDa calpain present in this assay (Fig. 4B, lower panel). In contrast to pp60src proteolysis, the rate of PTP-1B cleavage was minimally affected by E-64 (100 μM) (Fig. 4, B and 4C). However, pretreating purified calpain with 100 μg/ml calpeptin completely blocked calpain autolysis and abolished both pp60src and PTP-1B cleavage. Our inability to consistently produce pure intermediate μ-calpain precluded detailed kinetic comparison between the 76- and 78-kDa forms of μ-calpain. Nonetheless, the studies presented here, along with our observations in ionophore A23187-stimulated platelets, suggest that pp60src is primarily cleaved by the fully autolysed form of μ-calpain, whereas PTP-1B is cleaved by both the 76- and 78-kDa forms of μ-calpain.

Distinct Functional Roles for the 78- and 76-kDa Forms of μ-Calpain in Human Platelets—Previous studies have demonstrated that the calpain-catalyzed proteolysis of cytoskeletal structural proteins and signaling enzymes regulates two post-aggregation platelet responses, the shedding of procoagulant-rich microvesicles from the platelet surface (24–29), and the retraction of fibrin clots by activated platelets (23). While there is strong evidence supporting a role for calpain in the regulation of these platelet responses, the identity of the calpain forms regulating these responses has not been determined. We therefore performed a series of experiments to examine the relationship between the generation of the 78- and 76-kDa forms of μ-calpain and changes in platelet microvesiculation and clot retraction. In our initial experiments, we correlated pp60src proteolysis (as a marker of full calpain autolysis) with the release of microvesicles from the surface of ionophore A23187-stimulated platelets. For these studies, washed platelets were stimulated with ionophore A23187 (1 μM) for 0, 2, 5, 10, and 20 min, in the absence of stirring. At the indicated time points, microvesicles were separated from intact platelets by centrifugation and the microvesicle content in the supernatant quantitated by immunoblot analysis with an anti-GP IIb antibody. Previous studies have demonstrated that the release of GP IIb from the platelet surface is a reliable quantitative marker for platelet microvesiculation (25). In agreement with previous studies (24), we consistently noted in ionophore A23187-stimulated platelets that the microvesiculation process occurred in two distinct phases. There was an early phase (within 2–5 min of platelet stimulation), which accounted for a minor proportion of microvesicle release (~10%), and a late phase (10–20 min) in which the majority of microvesicles were shed from the platelet surface (~90%) (Fig. 5A). We performed these studies on non-stirred platelets to slow the rate of calpain activation and delay microvesiculation, which allowed for the optimal separation of the two phases. We examined the time course of pp60src proteolysis in these cells and found that cleavage of this kinase correlated well with the late phase of platelet microvesiculation (Fig. 5A), suggesting a potentially important role for the 76-kDa form of μ-calpain in the regu-
tion of this platelet response. Consistent with this possibility, we found that inhibiting the conversion of the 78-kDa form of μ-calpain to the 76-kDa enzyme, by pretreating platelets with 100 μM E-64-d or low concentrations of calpeptin (2 μg/ml), completely abolished pp60<sup>src</sup>-calpain cleavage and the late phase of microvesiculation, but had minimal effect on the early release of membrane vesicles. Increasing the concentration of calpeptin up to 100 μg/ml, which completely abolished the generation of the 78- and 76-kDa forms of μ-calpain, had no inhibitory effect on the early phase of platelet microvesiculation (data not shown). While these results suggest that μ-calpain activation is not essential for early microvesiculation, this platelet response is regulated by calcium, as the chelation of extracellular calcium with EGTA/Mg<sup>2+</sup> results in relaxed clot retraction (23). Consistent with this hypothesis further that the 78-kDa form of calpain in microvesicle release, two lines of evidence suggested a potential role for this enzyme in the regulation of clot retraction. First, the dose-dependent inhibitory effects of calpeptin on the generation of the 78-kDa form of μ-calpain (Fig. 3) correlated closely with the effects of this inhibitor on the clot retraction process (23). Second, we have consistently noted in thrombin-stimulated platelets that assay conditions which primarily favor the generation of the 78-kDa form of μ-calpain, i.e. stirring activated platelets for a maximum time period of 45 s, resulted in significant inhibition of the clot retraction process. Under these assay conditions, we could find no evidence of pp60<sup>src</sup>-proteolysis or full calpain autolysis (data not shown). Pretreating platelets with low concentrations of calpeptin (2 μg/ml) (23) or E-64-d (100 μM) (Fig. 6) completely inhibited the generation of the 78-kDa form of calpain but did not restore normal clot retraction. However, pretreating platelets with higher concentrations of calpeptin (100 μg/ml) abolished calpain autolysis altogether and restored effective clot retraction by both ionophore A23187 and thrombin (Fig. 6).

To strengthen our hypothesis further that the 78-kDa enzyme is responsible for relaxed clot retraction, we performed ionophore A23187 dose-response studies. We found that the addition of low concentrations of ionophore A23187 (0.25–0.5 μM) to unstirred platelets induced the selective formation of the 78-kDa form of calpain (data not shown), leading to PTP-1B proteolysis, whereas higher concentrations of ionophore A23187 (0.75–1.0 μM) stimulated full calpain autolysis, thereby inducing both PTP-1B and pp60<sup>src</sup>-proteolysis (Fig. 7). Activation of platelets with low doses of ionophore A23187 (0.25–0.5 μM) resulted in relaxed fibrin clot retraction but did not induce significant release of membrane vesicles. This is in contrast to the higher doses of ionophore A23187 (0.75–1.0 μM), which could induce both membrane vesiculation and relaxed fibrin clot retraction. Furthermore, in these studies, there was
a strong correlation between the degree of full calpain autolysis, the extent of pp60c-src proteolysis, and the release of membrane vesicles. These studies provide further evidence that the two active forms of μ-calpain in human platelets exhibit distinct substrate specificities and functional roles within the cell.

**DISCUSSION**

The studies presented in this article reveal several novel features regarding μ-calpain-mediated proteolytic events within the cell. First, not all active forms of μ-calpain have the same substrate specificity; second, the autoproteolytic conversion of inactive μ-calpain to intermediate forms represents a potentially novel means of controlling the rate of substrate proteolysis within the cell; finally, different active forms of μ-calpain may have distinct functional roles within the cell.

Previous studies have reported no significant difference in the substrate specificity and catalytic properties of the fully autolyzed forms of μ- and m-calpain (1). While there is strong evidence that calpain activation is associated with the autolytic modification of the large subunit of μ-calpain, generating a number of different active forms (8, 10, 12, 13, 38), the substrate specificity, kinetic properties, and functional roles of these enzymes have not been investigated. Progress in this area has been severely hampered by technical limitations. For example, it remains extremely difficult to consistently control the proteolytic conversion of intact calpain to individual autolytic forms and to maintain them in this state in vitro. Furthermore, unlike the situation with a number of other extracellular proteases, there have been no naturally occurring calpain mutants identified which can be used for kinetic comparisons. Meaningful kinetic comparisons between the different autolytic forms of calpain will require the generation of recombinant calpain mutants, which can be selectively converted to intermediate forms without further autolysis.

While previous studies have suggested that different rates of substrate proteolysis in vivo reflect differences in substrate susceptibility to cleavage, the studies presented here (at least with respect to pp60c-src and PTP-1B) suggest that the degree of protease activation may also play a significant role in controlling the rate of substrate proteolysis. In support of this hypothesis are our studies demonstrating that pp60c-src is rapidly proteolyzed by fully autolyzed μ-calpain in vitro (suggesting that pp60c-src is a sensitive calpain substrate) but is cleaved slowly in platelets activated by a variety of physiological agonists. This contrasts with PTP-1B, which is cleaved rapidly both in vitro and in vivo, even under assay conditions in which fully autolyzed μ-calpain is not detectable. While we cannot exclude the possibility that trace amounts of fully autolyzed μ-calpain may be present in our assays (which remain undetectable by immunoblot analysis with our anti-peptide antibody), we have provided several independent lines of evidence suggesting that the 78-kDa form of calpain is primarily responsible for PTP-1B proteolysis in vivo, whereas full μ-calpain autolysis is necessary for pp60c-src cleavage. First, time-course studies in ionophore A23187-stimulated platelets demonstrated a strong correlation between the generation of intermediate μ-calpain and PTP-1B cleavage, whereas pp60c-src proteolysis correlated with the generation of the fully autolyzed enzyme. Furthermore, low concentrations of ionophore A23187 (0.25–0.5 μM) which activate intermediate μ-calpain induce PTP-1B cleavage, whereas higher concentrations of this agonist (0.75–1.0 μM) were required for full μ-calpain autolysis and pp60c-src proteolysis. Second, dose-response studies with the
calpain inhibitor, calpeptin, demonstrated that low concentrations of this inhibitor (0.5–2.0 μg/ml) abolished full calpain autolysis and pp60src cleavage, whereas significantly higher concentrations (5–20 μg/ml) were required to inhibit the generation of intermediate μ-calpain and PTP-1B proteolysis. Third, weak agonists such as ADP induced the generation of the 78-kDa form of μ-calpain leading to PTP-1B proteolysis, but did not induce full calpain autolysis or pp60src cleavage.2

Fourth, our in vitro proteolysis experiments revealed that PTP-1B could be cleaved by both the 78- and 76-kDa forms of μ-calpain, whereas pp60src was only cleaved by the fully autolysed form of μ-calpain. Importantly, inhibiting full calpain autolysis with E-64 effectively abolished pp60src proteolysis but had minimal effect on the rate and extent of PTP-1B proteolysis. Finally, under each of the experimental conditions examined there was an excellent correlation between the extent of substrate proteolysis induced by calpain and the limited proteolysis of PTP-1B and talin (19–21), and the transcription factors (Fos and Jun) (40). A major outstanding issue regarding calpain pathophysiology is the mechanism by which the proteolysis of these substrates is coordinately regulated. Our observations that intermediate calpains have different substrate specificities than the fully autolysed enzymes, combined with studies demonstrating that the cleavage of the 30-kDa subunit regulates the subcellular distribution of calpains, suggests a high level of complexity by which substrate proteolysis may be regulated in vivo. Given the large number of intermediate calpain forms which can be potentially generated, i.e. homologous proteases in which both subunits are partially autolyzed or heterologous proteases in which the large subunit but not the small subunit is autolyzed and vice versa, the potential exists for a complex hierarchy of proteolytic events in vivo, which can be controlled by the conversion of one active form to another. This may partly explain why the pattern of substrate proteolysis induced by calpain can vary considerably depending on the type of cell stimulation. For example, a number of substrates, such as talin and PTP-1B, are consistently cleaved in platelets stimulated by both strong (e.g. thrombin) and weak agonists (e.g. ADP), whereas other substrates, such as pp60src, are only cleaved in platelets stimulated by strong agonists.2 Based on the studies presented here, the simplest interpretation for these findings is that weak agonists do not induce full calpain autolysis, whereas strong agonists can induce full calpain autolysis and therefore a different pattern of substrate proteolysis.

Our observations that different agonists induce varying degrees of calpain autolysis and substrate proteolysis reconciles several previous inconsistencies regarding calpain activation and membrane vesiculation. For example, it is well documented that ADP stimulation of platelets leads to calpain activation and the limited proteolysis of PTP-1B and talin (22), but membrane vesiculation is not observed in these cells (41, 42). Furthermore, the time course of proteolysis of these substrates occurs relatively rapidly in aggregated platelets (within 1–2 min of stimulation), whereas the majority of membrane vesiculation does not occur until at least 10 min after stimulation (25). Finally, concentrations of calpeptin and E-64 that inhibit membrane vesiculation have no effect on the initial autolysis of calpain or the proteolysis of several calpain sub-

2 S. M. Schoenwaelder and S. P. Jackson, unpublished observations.

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