Proteolysis of Platelet Cortactin by Calpain*

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Printed in U.S.A.

(Received for publication, March 4, 1997, and in revised form, May 5, 1997)

Cortactin, a substrate of pp60^src and a potent filamentous actin binding and cross-linking protein, is abundant in circulating platelets. After stimulation of platelet aggregation with collagen, cortactin undergoes a dramatic increase in tyrosine phosphorylation followed by a rapid degradation. The cleavage of platelet cortactin was detected in lysates prepared using either Triton-containing buffer or SDS-sample buffer. However, the degradation of cortactin was not observed in platelets derived from a Glanzmann’s patient, who lacked functional integrin αIIbβ3 (GPIIb-IIIa). In addition, the proteolysis of cortactin was abolished by treating platelets before but not after collagen stimulation with EGTA or calpeptin. Furthermore, recombinant cortactin was digested by µ-calpain in vitro in a dose-dependent manner, indicating that cortactin is a substrate for calpain. We also observed that the calpain-mediated digestion in vitro is dependent on the presence of a sequence containing a proline-rich region and multiple tyrosine residues that are phosphorylated by pp60^src. Tyrosine phosphorylation by pp60^src up-regulates the activity of calpain toward cortactin. Our data suggest that the calpain-mediated proteolysis of tyrosine-phosphorylated cortactin may provide a mechanism to remodel irreversibly the cytoskeleton in response to platelet agonists.

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Cortactin, an F-actin1 binding and cross-linking protein (1, 12), is a major target for tyrosine phosphorylation in response to signaling mediated by fibroblast growth factor (2), epidermal growth factor (3), integrin activation (4), bacteria-mediated phagocytosis (5), and v-src oncogene (6). Overexpression or amplification of the human cortactin gene (also called EMS1) is often associated with human malignancies (7, 8). In v-Src-transformed cells, cortactin has been found to co-localize with Src oncoproteins within podosomes, membrane-substratum contact structures (6). Analysis of cortactin phosphorylation in cells lacking the c-src gene (9) or following overexpression of c-Csk (10), a negative regulator for pp60^src, has provided further compelling evidence that cortactin is an intrinsic sub-

strate for pp60^src.

The protein sequence of cortactin is unique because it contains six and one-half 37-amino acid tandem repeats near the NH2 terminus, and a Src homology 3 (SH3) domain at the carboxyl-terminal end. Between the repeat and the SH3 domain is an α-helix, a proline-rich region, and multiple tyrosine residues. The amino acid sequence of human cortactin within the repeat domain shares nearly 100% identity with the chicken and murine homologues and 70% with HS1, a cortactin-related gene product (11), indicating that the repeat domain plays a fundamental role for cortactin (12). Indeed, the repeat domain has been demonstrated as the binding site for F-actin (12). In contrast, the sequence between the α-helix and the SH3 domain exhibits less than 33% identity to HS1, but the function of this region has not yet been identified.

We recently reported that there is abundant expression of cortactin in megakaryocytes and platelets (13). While tyrosine phosphorylation of cortactin has been described as a major phenomenon in thrombin-stimulated platelets (14, 15), the significance of the tyrosine phosphorylation is unknown. In the present study, we examined the fate of cortactin in platelets stimulated by collagen. We found that cortactin is degraded following tyrosine phosphorylation and that the protease responsible for the cortactin degradation is a calpain-related enzyme, which requires integrin αIIbβ3. Furthermore, we provide in vitro evidence that the sequence containing the proline-rich region and multiple tyrosine residues targeted by pp60^src is required for the calpain-mediated cleavage. Finally, we demonstrated that tyrosine phosphorylation of cortactin by pp60^src dramatically alters its susceptibility to calpain. These data suggest that tyrosine phosphorylation may play a role in the calpain-mediated proteolysis of cortactin.

EXPERIMENTAL PROCEDURES

Antibodies and Chemical Reagents—Monoclonal antibody (mAb) 4F11 was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Monoclonal antibody against phosphorytrosine (RC20) was from Transduction Laboratories (Lexington, KY). The polyclonal antibody against the C-terminal part of murine cortactin was derived from mice immunized with a recombinant protein corresponding to amino acids 323–546. Prostaglandin E1, phenylmethylsulfonyl fluoride, Triton X-100, EGTA, benzamidine, leupeptin, and aprotinin were from Sigma. SDS-PAGE markers were from Bio-Rad. Protein A-Sepharose was from Pharmacia Biotech Inc. Purified µ-calpain derived from pig erythrocytes was obtained from ICN (Costa Mesa, CA). Calpeptin was from Biomol (Plymouth Meeting, PA). Sodium orthovanadate was from Fisher. Type I tendon collagen was from Chrono-Log Co. (Havertown, PA).

Isolation of Human Platelets—Human blood (500 ml) from healthy volunteers was collected into 70 ml of CPD solution, containing 1.84 mg of citrate, 1.78 mg of dextrose, 209 mg of citric acid, and 155 mg of monobasic sodium phosphate. Platelet-rich plasma was obtained by centrifugation at 200 × g for 16 min at ambient temperature. Blood from a Glanzmann patient (female) was kindly provided by Robert Abel (Christina Hospital, Wilmington, DE). Citric acid and prosta-
glandin E, were added to platelet-rich plasma to final concentrations of 4 mM and 1 μg/ml, respectively. The platelet-rich plasma was then centrifuged at 700 × g for 10 min. The platelet pellet was resuspended in washing buffer (4.26 mM NaH2PO4, 7.46 mM Na2HPO4, pH 6.5, containing 5.5 mM dextrose, 125 mM NaCl, 4.77 mM sodium citrate, 2.35 mM citric acid, and 3.5 mg/ml bovine serum albumin) and centrifuged at 700 × g for 10 min. The pellet was then resuspended in a modified Tyrode-Hepes buffer (10 mM Hepes, pH 7.35, containing 136.7 mM NaCl, 5 mM glucose, 2.6 mM KCl, 13.8 mM NaHCO3, 2.35 mM NaH2PO4, and 3.5 mg/ml bovine serum albumin) at 1 × 10^10 platelets/ml.

### Analysis of Cortactin in Triton-solubilized Platelets—Collagen at a final concentration of 2.5 μg/ml was added to the washed platelet samples (6.1 × 10^9 cells/ml) in the presence of 1 mM CaCl2 in an aggregometer cuvette at 37°C for the times indicated. Activated platelets were immediately lysed by adding 200 μl of 4 × Triton lysis buffer (200 mM Tris-HCl, pH 7.2, containing 4% Triton X-100, 20 mM EGTA, 40 μg/ml leupeptin, 40 μg/ml aprotinin, 4 μM phenylmethylsulfonyl fluoride, 4 μM benzamidine, and 4 μM Na3VO4). The lysates were centrifuged at 15,000 × g for 10 min. The pellet (insoluble fraction) was solubilized by adding an equal volume of 2 × SDS sample buffer (18). The soluble fractions were subjected to immunoprecipitation with mAb 4F11 (2.5 μg/ml) as described previously (2). The immunoprecipitates were then washed once with 1 × Triton lysis buffer, resuspended in 2 × SDS sample buffer, and analyzed by immunoblotting analysis with either mAb 4F11 or RC20 as described previously (2).

### Digestion of Cortactin with μ-Calpain in Vitro—Purified recombinant cortactin (3.6 μg) was incubated with μ-calpain for 90 min at different concentrations in 40 μl of reaction buffer (50 mM Tris-HCl, pH 7.36, containing 134 mM KCl, 1 mM MgCl2, 75 μM EGTA, and 75 μM CaCl2). The reaction was terminated by adding an equal volume of 2 × SDS sample buffer, and the proteins were separated by a gradient SDS-PAGE gel (4–20%, w/v). The digested proteins were visualized by either Coomassie Blue staining or immunoblotting with mAb 4F11 or a polyclonal antibody directed against a peptide encoding the amino acid sequence from the α-helix to the SH3 domain.

### RESULTS

**Proteolysis of Cortactin in Collagen-stimulated Platelets**—To evaluate the role of cortactin in platelet aggregation, we examined tyrosine phosphorylation of cortactin in collagen-stimulated platelets. Activated platelets were lysed using a Triton X-100-containing buffer. The soluble fractions were subjected to immunoprecipitation with 4F11, a mAb recognizing the repeat domain of cortactin (6). The pellets were solubilized in SDS sample buffer. Proteins in both fractions were immunoblotted using either a polyclonal antibody against cortactin or a mAb against phosphotyrosine. As shown in Fig. 1A, stimulation of stirred platelets with collagen caused a dramatic increase in the level of tyrosine phosphorylation of cortactin after 15 s and a maximum phosphorylation at 45 s, which was concomitant with platelet aggregation (data not shown). However, the level of phosphorylated cortactin declined slightly after 1 min of stimulation, and this coincided with a decrease in the level of cortactin in the soluble fraction (Fig. 1B).

We examined the possibility that a reduced amount of cortactin in the soluble fraction in response to collagen could be a consequence of the cytoskeletal translocation that has been described previously in thrombin-stimulated platelets (15, 18). Immunoblot analysis of cortactin in the insoluble fraction demonstrated that the stimulation of platelets with collagen enhanced tyrosine phosphorylation of multiple proteins including those that migrated at the positions for cortactin (Fig. 1C). However, the amount of cortactin associated with the insoluble fraction was only transiently increased during the period from 30 to 45 s and diminished afterward (Fig. 1D), suggesting that platelet cortactin, in either the soluble or insoluble fractions, was degraded after collagen stimulation. The degradation of cortactin appears not caused by a nonspecific proteolysis be-
cause pp60c-src associated with the pellets was not degraded under the same conditions even after a prolonged stimulation (Fig. 1D).

To confirm that the apparent degradation of cortactin was not the result of a protease released during the Triton-mediated lysis, we analyzed cortactin in platelet lysates that were prepared by direct lysis in SDS-sample buffer. The results from these experiments were compared with the pattern of cortactin degradation prepared in Triton X-100 buffer. As shown in Fig. 2A, significant amount of degraded cortactin was detected in the SDS-lysed whole platelets after collagen stimulation, although the extent of the degradation, especially at early phases of stimulation (30 and 45 s), appeared to be less than that of Triton-lysed platelets. However, the degradation patterns in both lysates are similar (Fig. 2B).

Calpain-related Protein Is the Major Protease Responsible for the Proteolysis of Cortactin in Platelets—Calpain is a family of calcium-dependent cysteine proteases that are abundantly present in platelets and are activated during platelet aggregation (19, 20). As shown in Fig. 3A, EGTA treatment of platelets significantly inhibited the degradation of cortactin as compared with untreated platelets. Furthermore, treatment with calpeptin, a specific membrane-permeable peptide-derivative inhibitor for calpain (21), resulted in the same reduction of cortactin degradation (Fig. 3B, part a). However, when a lysis buffer containing either EGTA or calpeptin was used to lyse activated platelets, no significant inhibition of cortactin degradation was observed (Fig. 3B, parts b and c). This result further confirms that the degradation of cortactin primarily occurs prior to platelet lysis.

Degradation of Cortactin in Platelets Is Dependent on $\alpha_{IIb}\beta_3$—In platelets, the influx of calcium can be regulated by the activation of $\alpha_{IIb}\beta_3$ (22, 23), a major integrin on the surface of platelets. To evaluate the role of $\alpha_{IIb}\beta_3$ in the proteolysis of cortactin, we examined tyrosine phosphorylation of cortactin in platelets from a Glanzmann’s patient. As shown in Fig. 4, normal platelets exhibited a 60% reduction in the amount of intact cortactin after 3 min of collagen stimulation. In contrast, no significant reduction was found with the Glanzmann’s platelets from a Glanzmann’s patient.

Regulation of Calpain-mediated Proteolysis of Cortactin by Src

FIG. 2. Degradation of cortactin in whole platelets. Washed platelets were stimulated with collagen for the indicated times and lysed by either adding an equal volume of 2 × SDS sample buffer or adding ¼ volume of 4 × Triton lysis buffer and incubated on ice for 30 min. The Triton lysates were further mixed with an equal volume of 2 × SDS sample buffer. The proteins in both lysates were separated by SDS-PAGE (7%, w/v) and analyzed by immunoblotting with 4F11 as described under “Experimental Procedures.” A, a short exposure; B, a longer exposure.

FIG. 3. Degradation of cortactin requires a calcium-dependent protease that can be inhibited by EGTA or calpeptin. A, washed platelets were stimulated with collagen either in the presence or absence of 2 mM EGTA for the indicated times and immediately lysed with Triton X-100-lysis buffer. The proteins in the insoluble fractions were solubilized with SDS sample buffer and immunoblotted with mAb 4F11. B, platelets were pretreated with (a) or without (b) 20 μM calpeptin for 30 s. The treated platelets were then stimulated with collagen and lysed in Triton X-100-lysis buffer in the presence of 10 mM EGTA (a and b) or 80 μM calpeptin (c). Cortactin in the insoluble fractions was identified by immunoblotting with mAb 4F11.

FIG. 4. Proteolysis of platelet cortactin requires $\alpha_{IIb}\beta_3$. Normal platelets and platelets from a Glanzmann’s patient were stimulated with collagen for the times indicated and lysed with an equal volume of 2 × SDS sample buffer. The platelet proteins were analyzed by immunoblotting with mAb 4F11 or mAb against phosphotyrosine.

FIG. 5. In vitro digestion of cortactin by calpain. Recombinant cortactin (1 μM) was incubated with μ-calpain at different concentrations for 90 min and analyzed by SDS-PAGE. The proteins were visualized by Coomassie Blue staining. Lane 1, molecular weight markers; lane 2, without calpain; lane 3–7, with calpain at concentrations of 1.5, 3.1, 6.2, 12.5, and 25 μg/ml, respectively.
Calpain-mediated digestion requires the presence of a sequence containing the proline-rich region and multiple tyrosine residues. A, cortactin was incubated for 90 min in either the absence (lane 1) or presence (lane 2) of 1.5 μg/ml μ-calpain. The digested proteins were immunoblotted with mAb 4F11 (a) or a polyclonal antibody against amino acids 323–546 (b). B, upper part, schematic presentation of cortactin and cortactin mutants. The areas for the repeat (Repeat), the α-helix (Helix), the proline-rich region (P), and tyrosine residues targeted by pp60src (Y) are indicated; lower part, cortactin and its mutants were digested with μ-calpain, and the resultant fragments were analyzed by immunoblotting with mAb 4F11.

Cleavage of Cortactin by μ-Calpain in Vitro Is Dependent on the Presence of a Sequence Containing the Proline-rich Region and Multiple Tyrosine Residues—Purified μ-calpain (calpain-I) digests recombinant murine cortactin in vitro in a dose-dependent manner (Fig. 5A). At a concentration of 6.2 μg/ml of calpain, approximately 90% of the cortactin proteins were digested to multiple fragments. Interestingly, many of the digested fragments were reactive to mAb 4F11, which specifically recognizes the repeat domain of cortactin (12), but not to an antibody directed against the region between the repeat and the carboxyl terminus (Fig. 6A). This implies that the sequence in this region may be more susceptible to calpain. To verify this, we analyzed two cortactin variants, Cort496–546, which lacks the SH3 domain, and Cort375–546, which lacks the sequence from the proline-rich region to the carboxyl terminus (Fig. 6B). As with the wild-type cortactin, the mutant Cort375–546 was efficiently digested by calpain (Fig. 6B, upper part). In contrast, little digestion of the mutant Cort375–546 was detected under the same conditions, indicating that the sequence of amino acids 375–496, which contains the proline-rich region and multiple tyrosine residues, may be involved in the calpain-mediated proteolysis.

Src-mediated Tyrosine Phosphorylation Increases the Susceptibility of Cortactin to Calpain—Amino acids 375–496 contain multiple tyrosine residues that can be targeted for phosphorylation by pp60src. Thus, we performed a calpain digestion of cortactin phosphorylated by pp60src. Fig. 7 shows that most phosphorylated cortactin proteins were digested nearly completely within 2 min. In contrast, significant amounts of nonphosphorylated cortactin remained even after 20 min of digestion under the same conditions. However, when partially digested phosphorylated cortactin was analyzed by SDS-PAGE and compared with nonphosphorylated cortactin, we did not observe any significant difference in the two patterns (Fig. 7). Therefore, it is likely that tyrosine phosphorylation enhances the efficiency of calpain-mediated digestion without altering its cleavage sites.

DISCUSSION

It is unclear whether platelet calpain-mediated proteolysis occurs during lysis of cells or within aggregated platelets (24, 25). Our data indicate that the proteolysis of cortactin occurs within activated platelets. We detected the degradation of cortactin in whole platelets prepared by direct lysis in a SDS-sample buffer (Figs. 2 and 4). In addition, the calpain inhibitors EGTA and calpeptin block the degradation when they are applied before but not after platelet activation. Finally, it appears that the calpain-mediated proteolysis of cortactin is not the result of a nonspecific proteolysis, because pp60src, another substrate for calpain (26), was not degraded under the same conditions that allow cortactin proteolysis (Fig. 1D). However, it should be pointed out that the degree of the proteolysis of cortactin in activated platelets appears to vary depending on the method of lysing platelets. There is more extensive degra-

2 J. Qiu and X. Zhan, manuscript in preparation.
Regulation of Calpain-mediated Proteolysis of Cortactin by Src

Digestion of recombinant cortactin by calpain-mediated digestion. The importance of Src in the digestion of cortactin could be involved in the stimulation (33). These data indicate that the Src-mediated tyrosine phosphorylation of cortactin is dramatically increased by pp60-src (Fig. 3), and the efficiency of the proteolysis of cortactin in vitro (data not shown). Furthermore, a cortactin mutant able to bind to F-actin but lacking the sequence from the proline-rich region to the carboxyl terminus is not efficiently digested by calpain (Fig. 6B). Hence, it is unlikely that the F-actin binding is a rate-limiting step for the calpain digestion.

Many cytoskeleton-associated proteins have been reported to be substrates for calpain. These include actin-binding proteins (27), vitronectin (28), protein-phosphotyrosine phosphatase 1B (29), integrin β3 subunit (30), talin (27), spectrin (31), and protein kinase C (32). As with many of those substrates, proteolysis of cortactin appears to be dependent on α1β3 because it does not occur in Glanzmann’s platelets lacking functional α1β3 (Fig. 4). We have found, however, that the absence of α1β3 does not affect collagen-induced tyrosine phosphorylation of cortactin. Our finding is in agreement with a previous report, which also showed increased tyrosine phosphorylation in thrombin-treated platelets derived from Glanzmann’s patients (15). Furthermore, tyrosine phosphorylation of cortactin can be detected after 15 s of stimulation (Fig. 1A). This is prior to platelet aggregation, which occurs 30–45 s after stimulation. Thus, tyrosine phosphorylation of cortactin is kinetically correlated with the activation of pp60-src, which occurs in the early phase prior to the activation of α1β3 during platelet stimulation (33). These data indicate that the Src-mediated tyrosine phosphorylation of cortactin could be involved in the calpain-mediated digestion. The importance of the Src in the digestion of cortactin is further highlighted by our findings that the digestion of recombinant cortactin by μ-calpain is dependent on the presence of a sequence containing multiple tyrosine residues targeted by pp60-src (Fig. 6), and the efficiency of the digestion of cortactin in vitro is dramatically increased by pp60-src (Fig. 7).

Calpain-digested cortactin in vitro has significantly less F-actin cross-linking activity (data not shown). Interestingly, the F-actin cross-linking activity can be also down-regulated by tyrosine phosphorylation without degradation (1). These dual mechanisms regulating cortactin may be required to ensure the irreversible shape change associated with activated platelets. It is also noteworthy that both mechanisms involve the same structural region between the proline-rich motif and the SH3 domain. This may suggest the importance of this region in the regulation of cortactin function. Since calpain and cortactin are widely expressed in many mammalian cells, future studies using a structure-function approach should reveal the significance of calpain-mediated cleavage of cortactin in cellular cytoskeletal reorganization.

Acknowledgments—We thank Graham Jamieson and Allan Mufson for critical reading of the manuscript and Diana Norman for expert secretarial support.

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