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Tyrosine Unphosphorylated Platelet SHP-1 Is a Substrate for Calpain

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The platelet phosphotyrosine phosphatase (PTP) SHP-1 is tyrosine phosphorylated during thrombin-induced activation. Stimulation of platelets by the ionophore A23187 in the presence of $CaCl_2$ induced a calpain dependent cleavage of SHP-1. SHP-1 proteolysis was undetectable during thrombin-induced stimulation. When SHP-1 was tyrosine phosphorylated by thrombin, further addition of A23187 failed to induce its cleavage. In the presence of tyrphostin to inhibit thrombin-induced SHP-1 tyrosine phosphorylation, SHP-1 was cleaved. Thus, only the tyrosine unphosphorylated form of SHP-1 was a substrate for calpain. A23187 induced the disappearance of all platelet phosphotyrosine proteins and a two-fold increase in PTP activity, both inhibited by pervanadate, a PTP inhibitor, but unaffected by calpeptin, a calpain inhibitor. The data show that SHP-1 is either tyrosine phosphorylated or cleaved by calpain, and suggest that SHP-1 cleavage does not contribute to A23187-induced PTP activity.

SHP-1 (1), a phosphotyrosine phosphatase (PTP) predominantly expressed in hematopoietic cells (2–5), appears to be a critical signaling molecule. The increase in SHP-1 activity following its binding to the tyrosine phosphorylated FcγRIIB1 receptor leads to inhibition of B cell activation (6, 7). Erythropoiesis is inhibited following binding of SHP-1 to the activated erythropoietin receptor and subsequent dephosphorylation of the Jak2 kinase (8). Mutant phenotypes deficient in SHP-1 activity are characterized by dysregulation in hemopoietic differentiation and immune function (9, 10). On the other hand, SHP-1 plays a positive role in platelets. SHP-1 is rapidly phosphorylated during platelet activation (11–13). Tyrosine phosphorylation increases platelet SHP-1 activity (12) and allows it to bind to the prominent protein tyrosine kinase (PTK) Src (13). Platelet SHP-1 activates Src by dephosphorylating the C-terminal regulatory phosphotyrosine (pTyr) 527 (14).

SHP-1 contains two tandem Src homology 2 (SH2) domains at the N-terminus, a single catalytic domain, and two putative sites of tyrosine phosphorylation (Tyr-536 and Tyr-564) in the C-terminus (15, 16). In its resting form, the PTP activity of SHP-1 is maintained at a basal level by intramolecular autoinhibition (17–20). *In vitro,* truncated mutants of SHP-1 present a higher activity (18).

In the present study, we found that, depending on the agonist used to activate platelets, SHP-1 was either proteolysed or tyrosine phosphorylated. During stimulation by the Ca^{2+} ionophore A23187 when calpain was activated, platelet SHP-1 was cleaved but not phosphorylated. During platelet stimulation by thrombin, SHP-1 was tyrosine phosphorylated and remained uncleaved. Furthermore, tyrosine phosphorylation of SHP-1 protected it from calpain-induced proteolysis.

MATERIALS and METHODS

Antibodies, materials and chemicals. Polyclonal anti-SHP-1 R278 antibody was a gift from Dr. E. R. Stanley (Albert Einstein College of Medicine, New York, USA). Monoclonal anti-SHP-1 and anti-phosphotyrosine (pTyr) PY20 antibodies were obtained from Transduction Laboratories (Interchim, Asnières, F) and monoclonal anti-pTyr 4G10 antibody from UBI (Euromedex, Souffelweyersheim, F). All SDS-polyacrylamide gel electrophoresis (SDS-PAGE) reagents were obtained from Bio-Rad (Ivry sur Seine, F). Metrizamide was obtained from Nycomed (Eurobio, Les Ulis, F) and sodium vanadate from Fisher Scientific (Fair Lawn, USA). Human thrombin was purchased from Diagnostica Stago (Asnières, F). The Ca^{2+} ionophore A23187 (Sigma, Saint Quentin Fallavier, F), and tyrphostin and calpeptin (Novabiochem, France Biochem, Meudon, F), were dissolved in dimethyl sulfoxide as stock solutions. All other chemicals were of the highest available purity.

Platelet preparation and activation. Venous blood from healthy donors was anticoagulated in 0.1 volume of ACD-C. Platelets were isolated using a metrizamide gradient as previously described (13).

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Briefly, platelets were concentrated between 25 and 10% metrizamide phases in 140 mM NaCl, 5 mM KCl, 12 mM trisodium citrate, 10 mM glucose, 12.5 mM sucrose, pH 6, by centrifugation at 1,100*g* for 12 min. This washing procedure was repeated. Finally, platelets were resuspended in 140 mM NaCl, 3 mM KCl, 0.5 mM $MgCl_2$, 5 mM $NaHCO_3$, 10 mM glucose, 10 mM HEPES, pH 7.4, at the concentration of 4×10^8 cells/ml. Stimulation was performed on 400 μl aliquots in the cuvette of a ChronoLog aggregometer (Coultronics, Margency, France) at 37°C under constant stirring (1,100 rpm).

SDS-PAGE, immunoprecipitation and immunoblot. Platelets were solubilized in a SDS-PAGE loading buffer in the presence of 1 mM PMSF, 5 μg/ml leupeptin, 5 μg/ml aprotinine and 1 mM sodium vanadate. After boiling for 5 min, proteins were separated by SDS-PAGE on a 10% polyacrylamide gel and transferred onto a nitrocellulose membrane. The membrane was incubated in a blocking solution (100 mM NaCl, 20 mM Tris/HCl, pH 7.4) containing 5% low fat dry milk before probing with specific antibodies. Detection was performed with an enhanced chemiluminescence system (ECL, Amersham, Les Ulis, France). The molecular weights of the SHP-1 fragments were evaluated using the Image Master software (Pharmacia Biotech, Saint Quentin en Yvelines, France) and the Sharp scanner JX-330. To examine cleavage of actin binding protein-280 (ABP-280), talin and myosin heavy chain (MHC), proteins were separated on a 7.5% polyacrylamide gel. The gel was stained with Coomassie brilliant blue, destained, and dried.

For immunoprecipitation studies, platelets were lysed by incubation with 0.5 volume of 3X concentrated lysis buffer [3% Nonidet P-40 (NP-40), 150 mM Tris-HCl, pH 7.4, 450 mM NaCl, 3 mM EGTA, 3 mM PMSF, 3 mM sodium vanadate, 15 μg/ml leupeptin and 15 μg/ml aprotinine] for 30 min at 4°C. The NP-40 insoluble fraction was sedimented by centrifugation at 16,000 *g* for 10 min. The soluble fraction was incubated with the polyclonal anti-SHP-1 antibody R278 and the immune complex precipitated with protein A-Sepharose. Beads were solubilized in SDS-PAGE sample buffer. After boiling for 5 min, proteins were separated by SDS-PAGE on a 10% polyacrylamide gel and transferred onto a nitrocellulose membrane. The membrane was probed with monoclonal anti-pTyr antibodies or monoclonal anti-SHP-1 antibody, and detection was performed as described above.

For dot-blot studies, 5 μl of whole cell lysates in a SDS-PAGE sample buffer were dropped onto a nitrocellulose membrane. The membrane was probed with monoclonal anti-pTyr antibodies, and detection was performed as described above.

Assay of the whole platelet PTP activity. Phosphatase activity was measured spectrophotometrically with *p*-nitrophenyl phosphate (*p*NPP) as the substrate. Platelets were lysed by incubation with 0.5 volume of 3X concentrated lysis buffer (3% NP-40, 150 mM Tris-HCl, pH 7.4, 450 mM NaCl, 3 mM EGTA, 3 mM PMSF, 15 μg/ml leupeptin and 15 μg/ml aprotinine) for 30 min at 4°C. Activity was measured on 200 μl aliquots of platelet lysates incubated (v/v) with phosphatase buffer (62 mM HEPES, 12.5 mM dithiothreitol, pH 7.5) containing 8 mM *p*NPP. Absorbance was measured at 405 nm using a microplate reader (Dynatech MR5000, Guernesey, IS) and registered for 30 min at room temperature.

RESULTS

SHP-1 is a substrate for calpain following A23187-induced platelet activation. SHP-1 is a protein of 64 kDa. When platelets were activated by 1 μM A23187 in the presence of 1 mM $CaCl_2$, conditions that activate calpain and result in proteolysis of ABP-280, talin and MHC (Fig. 1, lower panel) (21), SHP-1 was proteolysed (Fig. 1, upper panel). Fragments of 63, 60 and 58 kDa appeared twenty seconds after the addition of A23187. SHP-1 proteolysis was prevented by the addition of 1 mM EDTA instead of $CaCl_2$. Moreover, SHP-1 cleavage failed to occur when platelets were preincubated for 30 min with 100 μg/ml calpeptin, a membrane permeable calpain inhibitor, before the addition of A23187, demonstrating that SHP-1 was cleaved by calpain.

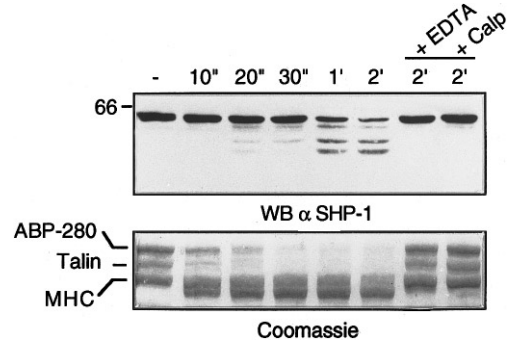

FIG. 1. Calpain-induced cleavage of SHP-1 in platelets stimulated by A23187. Platelets were stimulated with 1 μM A23187 for the indicated times in the presence of 1 mM $CaCl_2$ at 37°C with constant stirring; where indicated, platelets were activated in the presence of 1 mM EDTA instead of $CaCl_2$. Calpeptin (100 μg/ml) (Calp) was added 30 min before stimulation. Upper panel: Proteins from platelet lysates were resolved by 10% SDS–PAGE, transferred to nitrocellulose membrane, and immunoblotted with the monoclonal anti-SHP-1 antibody. Lower panel: Proteins from platelet lysates were resolved by 7.5% SDS–PAGE, and the gel was stained with Coomassie brilliant blue.

Tyrosine phosphorylation protects SHP-1 from calpain. Since SHP-1 is tyrosine phosphorylated during thrombin-induced activation (11–13), we studied the tyrosine phosphorylation state of SHP-1 after A23187-induced stimulation. We also investigated whether the tyrosine phosphorylated SHP-1 could be cleaved by calpain by activating platelets by thrombin for 2 min to phosphorylate SHP-1 before treatment with A23187. Conversely we investigated whether the cleaved SHP-1 could be phosphorylated by activating platelets with A23187 before treatment by thrombin (Fig. 2). In resting platelets, SHP-1 was predominantly found as a single protein (lower panel) lacking pTyr (upper panel). Following activation by 1 μM A23187, SHP-1 was proteolysed (lower panel) and remained unphosphorylated (upper panel). Following thrombin stimulation, SHP-1 was uncleaved (lower panel), and became phosphorylated on tyrosine (upper panel). Proteolysis of SHP-1 was undetectable even at longer times of thrombin stimulation (not shown). When platelets were activated with A23187 before addition of thrombin (A23 + Thr), SHP-1 was proteolysed and remained unphosphorylated. Conversely, when platelets were activated by thrombin before addition with 1 μM A23187 (Thr + A23), only a small amount of SHP-1 fragments was detected (lower panel), and SHP-1 was tyrosine phosphorylated, although to a lesser extent than with thrombin alone (upper panel). Furthermore, in the presence of 100 mM tyrphostin, a tyrosine kinase in-

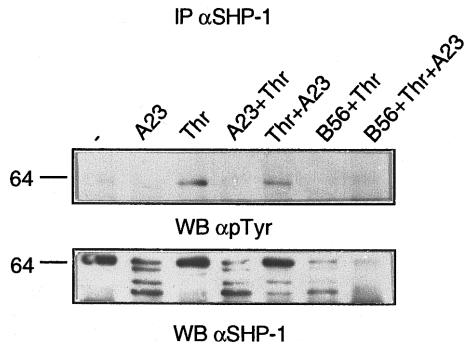

FIG. 2. SHP-1 is either cleaved by calpain or tyrosine phosphorylated. Platelets were stimulated with 1 μM A23187 or 1 U/ml thrombin (Thr) for 2 min, or 2 min of thrombin followed by 2 min A23187 (Thr + A23187), or 2 min A23187 followed by 2 min thrombin (A23187 + Thr), in the presence of 1 mM $CaCl_2$ at 37°C with constant stirring. Where indicated, tyrphostin B56 (100 μM) was preincubated 5 min at 37°C before stimulation. Solubles fraction of platelet lysates were immunoprecipitated with the polyclonal anti-SHP-1 antibody. Beads were resolved by 10% SDS–PAGE, transferred to nitrocellulose membrane, and immunoblotted with monoclonal anti-pTyr antibodies (upper panel) or the monoclonal anti-SHP-1 antibody (lower panel). Consistent results were obtained in three different experiments.

hibitor, to prevent SHP-1 tyrosine phosphorylation (lower panel), thrombin induced SHP-1 cleavage (upper panel). The data suggest that tyrosine phosphorylation and association with other proteins that occur during thrombin-induced platelet activation (13) protect SHP-1 from proteolytic cleavage.

A23187 induces a massive disappearance of tyrosine phosphorylated proteins. Since, *in vitro,* shorter forms of SHP-1 are more active (18), we studied the profile of pTyr proteins after stimulation by A23187 (Fig. 3A). In resting platelets relatively few platelet proteins contain pTyr, among which the major PTK Src (arrow). Following the treatment of platelets with 1 μM A23187, tyrosine phosphorylation diminished in a time-dependent fashion that resulted in the complete extinction of the pTyr signal by 2 min. The dephosphorylation was observed after 10 s, before calpain-induced cleavage of SHP-1 (see Fig. 1). The disappearance of pTyr bands was only weakly prevented by preincubation with EDTA. Calpeptin failed to prevent tyrosine dephosphorylation. Preincubation of platelets with the PTP inhibitor pervanadate allowed an increase in pTyr. Only a combination of calpeptin and pervanadate allowed a significant increase in tyrosine phosphorylation to get a profile comparable to that obtained after thrombin (22). A dot-blot assay was also performed to rule out the possibility that the diminution of a pTyr band resulted from calpain-mediated proteolysis instead of a tyrosine dephosphorylation (Fig. 3B). Platelet lysates were deposited onto nitrocellulose membranes. This assay confirmed that during stimulation by 1 μM A23187 (i) the intensity of tyrosine phosphorylation decreased; (ii) the pTyr signal decrease was unaffected by calpeptin but prevented by 100 μM pervanadate; (iii) A23187 induced an increase in the pTyr signal only in the presence of a combination of calpeptin and pervanadate. The results suggest that 1 μM A23187 induced a strong PTP activity responsible for the diminished pTyr signal.

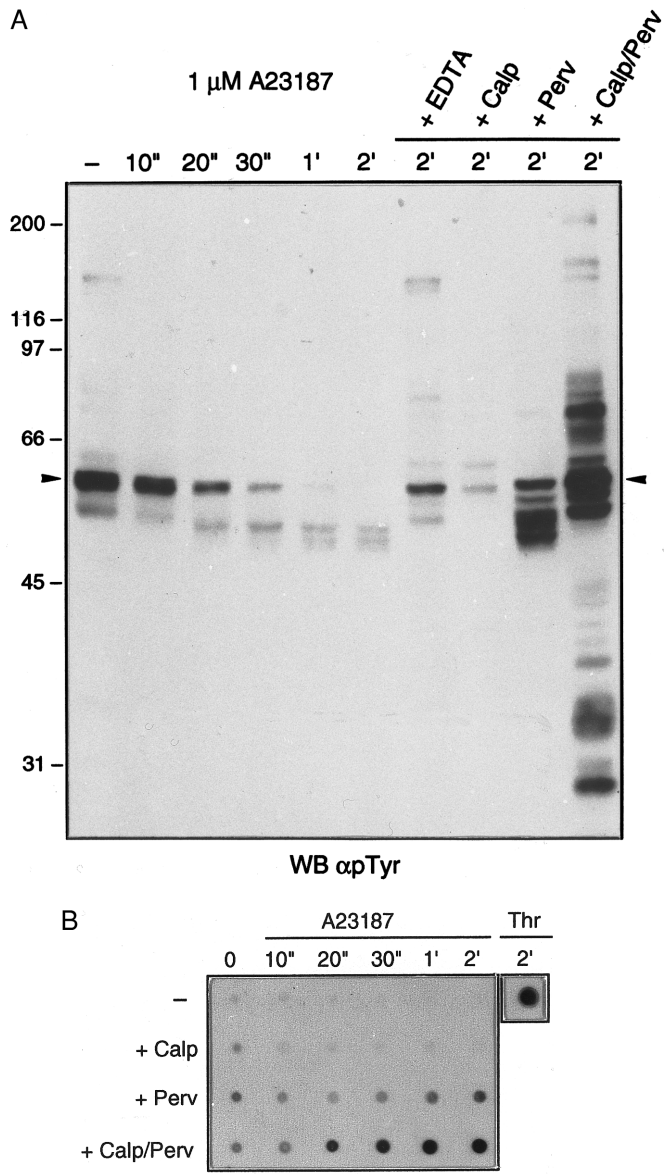

FIG. 3. A23187 induced the disappearance of pTyr proteins. Platelets were stimulated with 1 μM A23187 for the indicated times in the presence of 1 mM $CaCl_2$ at 37°C with constant stirring; where indicated platelets were activated in the presence of 1 mM EDTA instead of $CaCl_2$. Calpeptin (100 μg/ml) (Calp) was added 30 min before stimulation, and pervanadate (100 μM) (Perv) 1 min before stimulation. Platelet lysates were either resolved by 10% SDS–PAGE and transferred to nitrocellulose membrane (3A), or directly dropped on nitrocellulose membrane (3B) and immunoblotted with monoclonal anti-pTyr antibodies. Consistent results were obtained in three different experiments.

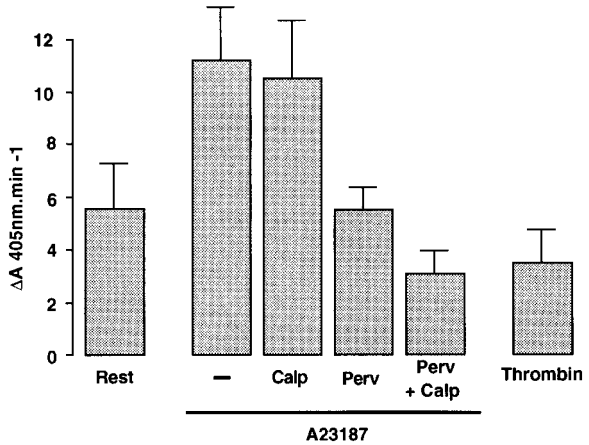

FIG. 4. A23187 induced a significant increase in platelet PTP activity. Platelets were stimulated with 1 μM A23187 or 1 U/ml thrombin for 2 min in the presence of 1 mM $CaCl_2$ at 37°C with constant stirring. Calpeptin (100 μg/ml) (Calp) was added 30 min before stimulation, and pervanadate (100 μM) (Perv) 1 min before stimulation. The ability of platelet lysates to hydrolyze *p*NPP to *p*-nitrophenolate was measured by the absorbance at 405 nm and registered for 30 min. Results are expressed as the variation of absorbance/min and represent the mean ± S.E.M. of seven different experiments.

A23187 induces a phosphotyrosine phosphatase activity independent of calpain. The massive dephosphorylation of pTyr proteins during A23187-induced activation led us to study the PTP activity in platelet lysates. We determined the total platelet PTP activity using *p*NPP as the substrate. In the presence of 1 mM $CaCl_2$, A23187-induced stimulation led to a two-fold increase of the basal PTP activity (Fig. 4). The increase in PTP activity was unaffected by calpeptin but was prevented by preincubation with pervanadate. Worthy of note, a combination of pervanadate and calpeptin more efficiently inhibited the PTP activity than pervanadate alone. By contrast, after 2 min of stimulation by thrombin, the PTP activity was lower than in resting platelets and represented only 60% of the initial PTP activity. Altogether, calpain does not seem to be involved in the PTP activity, suggesting that proteolysis of platelet PTPs is not necessary for the PTP activity induced by A23187.

DISCUSSION

In the present report, we demonstrated that platelet SHP-1 may be differentially implicated during platelet activation. We previously showed that during thrombin-induced activation, SHP-1 is tyrosine phosphorylated thereby allowing its binding to the prominent PTK Src (13); here we show that depending on the stimulating conditions used, SHP-1 was tyrosine phosphorylated or proteolytically cleaved by calpain. Moreover, SHP-1 tyrosine phosphorylation prevented its cleavage.

The cleavage of SHP-1 was (i) observed after activation by A23187, *i.e.* in the presence of a high $[Ca^{2+}]_i$, and (ii) inhibited by calpeptin, suggesting that the cleavage sites were created by a calpain-related enzyme. Calpain belongs to the Ca^{2+}-dependent cysteine protease family of proteins. When activated, calpain undergoes two autoproteolytic cleavages within its N-terminus leading to the generation of two different shorter and active forms (23). Depending on the level of activation, one or two autoproteolytic enzymes of calpain are formed with distinct substrate specificities and functional roles (24). During thrombin-induced platelet activation, calpain is only partially activated; full activation of calpain requires a high increase of the $[Ca^{2+}]_i$, as is the case with a high concentration of A23187 (24). In the present report, we show that SHP-1 was proteolyzed when platelets were activated by A23187, but not after activation by thrombin unless tyrosine phosphorylation was prevented by tyrphostin. In contrast, PTP1B, another platelet PTP not tyrosine phosphorylated, is cleaved during thrombin-mediated platelet activation (11, 25). That the two PTPs, PTP1B (25) and SHP-1 (this report), are proteolysed in different conditions of $[Ca^{2+}]_i$ elevation lends support to the proposal of different levels of calpain activation with different substrate specificities (24). Comparison of the time course of calpain cleavage (24) to that of SHP-1 (present results) during A23187-induced platelet activation, allows us to suggest that proteolysis of SHP-1 does not require the total activation of calpain. Furthermore, we failed to induce the cleavage of SHP-1 when A23187 was added after thrombin, though ABP-280, talin and MHC were proteolysed (not shown). Thus, thrombin treatment did not prevent the cleavage of all calpain substrates, and it is most likely that it is the tyrosine phosphorylation itself that protected SHP-1 from calpain-induced proteolytic cleavage. This proposal is further supported by the fact that SHP-1 was proteolysed after thrombin-induced stimulation when its tyrosine phosphorylation was prevented by tyrphostin. It is noteworthy that a recent study described the opposite for another pTyr protein, *i.e.* cortactin tyrosine phosphorylation up-regulates the activity of calpain towards the protein (26).

There is increasing evidence that calpain activation and protein dephosphorylation are concomitant events in platelets, notably during A23187-induced activation (27, 28) and in a late phase as a post-aggregation event during thrombin-induced activation (25, 27). Here, we observed a massive protein tyrosine dephosphorylation during A23187-induced stimulation. The apparent cleavage of SHP-1 and the decrease of the pTyr bands observed after A23187 treatment cannot be explained by a possible release of proteases or of PTPs during the lysis (29), or by a relocation in the actin cytoskeleton; indeed, cytoskeletal proteins were proteolytically cleaved during A23187-induced platelet activation.

The disappearance of pTyr proteins could result of either inactivation of PTKs, or activation of PTPs, or both. During A23187-induced stimulation, Src is cleaved and inactivated by calpain (30). On the other hand, PTP activity of shorter forms of platelet PTP1B for the N-terminus and of recombinant SHP-1 for the C-terminus is increased (18, 25). Yet we found that calpeptin failed to prevent the disappearance of phosphorylated proteins, suggesting that A23187 activated platelet PTPs independently of calpain and SHP-1 cleavage. Calpeptin had no effect either on the two fold increase in PTP activity induced by A23187. Thus, calpain activation, and hence formation of shorter forms of platelet PTP1B and SHP-1 (24, 28, this report) may not be sufficient to lead to the significant increase in PTP activity observed with A23187.

In conclusion, SHP-1 is an endogenous substrate for calpain, as many other platelet proteins. SHP-1 was not proteolysed when it was tyrosine phosphorylated, and SHP-1 cleavage did not significantly influence the whole PTP activity observed after A23187-induced activation. Future studies are required to establish the functional significance of the calpain-mediated cleavage of SHP-1 in human platelets.

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