Characterization of Heparin Affin Regulatory Peptide Signaling in Human Endothelial Cells*

Heparin affin regulatory peptide (HARP) is an 18-kDa secreted growth factor that has a high affinity for heparin and a potent role on tumor growth and angiogenesis. We have previously reported that HARP is mitogenic for different types of endothelial cells and also affects cell migration and differentiation (12). In this study we examined the signaling pathways involved in the migration and tube formation on matrigel of human umbilical vein endothelial cells (HUVEC) induced by HARP. We report for the first time that receptor-type protein-tyrosine phosphatase βζ (RPTPβζ), which is a receptor for HARP in neuronal cell types, is also expressed in HUVEC. We also document that HARP signaling through RPTPβζ leads to activation of Src kinase, focal adhesion kinase, phosphatidylinositol 3-kinase, and Erk1/2. Sodium orthovanadate, chondroitin sulfate-C, PP1, wortmannin, LY294002, and U0126 inhibit HARP-mediated signaling and HUVEC migration and tube formation. In addition, RPTPβζ suppression using small interfering RNA technology interrupts intracellular signals and HUVEC migration and tube formation induced by HARP. These results establish the role of RPTPβζ as a receptor of HARP in HUVEC and elucidate the HARP signaling pathway in endothelial cells.

Heparin affin regulatory peptide (HARP),1 also known as pleiotrophin or heparin-binding growth-associated molecule, is an 18-kDa growth factor that has a high affinity for heparin. HARP is highly conserved among species and shares 50% homology with midkine and the avian analogue of midkine retinoic-induced heparin-binding protein. The above proteins constitute a relatively new family of growth factors with high affinity for heparin (1).

HARP has been originally purified from perinatal rat brain as a molecule that induces neurite outgrowth (2). HARP is also expressed in the uterus (3), cartilage (4), and bone extracts (5). Several reports have established a strong correlation between HARP expression and tumor growth and angiogenesis (6–8). High levels of the protein were found in many human cancers and cell lines derived from human tumors (9, 10). HARP has been reported to be mitogenic for different types of endothelial cells (11) and to be angiogenic in vivo and in vitro (12).

HARP exerts its biological activity through interactions with cell surface proteoglycans, such as N-syndecan (13) or through binding to more specific cell surface receptors. Receptor-type protein-tyrosine phosphatase βζ (RPTPβζ) and its secreted variant phosphacan (14), as well as anaplastic lymphoma kinase (15), have been recently reported to bind HARP and to be implicated in its signaling. HARP has been shown to activate both the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase (PI3K)-Akt signaling axes (16, 17), and inhibitors of Erk1/2 or PI3K inhibit DNA synthesis stimulated by HARP on bovine epithelial lens cells. Additionally, an analysis of tyrosine-phosphorylated proteins following HARP stimulation showed induction of Shc and Erk1/2 phosphorylation (15). Nevertheless, the signals from specific receptors to PI3K or MAPK are still not well documented, although it has been hypothesized that Src is involved in the process (18, 19).

In the present work we examined the effect of HARP on the migration and tube formation on matrigel of human umbilical vein endothelial cells (HUVEC) and investigated the signaling pathway induced by HARP during this process. We report that HARP induces migration of endothelial cells through binding to RPTPβζ leading to activation of Src, focal adhesion kinase (FAK), PI3K, and Erk1/2.

EXPERIMENTAL PROCEDURES

Cell culture reagents were from BiochromKG (Seromed, Germany). Expression of human recombinant HARP was induced in *Escherichia coli* BL21 pLys cells transformed with the human HARP-pETHH8 plasmid, as described previously (11). Antibodies against RPTPβζ (sc-1110), Tyr(P) (PY20), FAK (sc-932), and HARP (sc-1394 and sc-1395) were purchased from Santa Cruz Biotechnology, Inc. Antibodies against non-phospho-Src (np-src) on Tyr417 and phospho-44/42 MAPK on Thr202/Tyr204 were purchased from Cell Signaling. Anti-MAPK1/2 (number 06-182), anti-Src (GD11, number 05-184), and anti-PI3K p85 (number 06-497) antibodies were from Upstate. Anti-β-actin (MAB1501) was purchased from Chemicon International, Inc. All secondary horseradish peroxidase-conjugated antibodies, chondroitin sulfate-C (CS-C), sodium orthovanadate and protein A-agarose (P-1406) were purchased from Sigma. PP1, wortmannin, LY294002, and U0126 were purchased from TOCRIS. All other reagents were of analytical grade and were purchased from Sigma.

Cell Culture—HUVEC were isolated from human umbilical cords, cultured as described previously (11), and used at passages 1–2. The cells were grown as monolayers in medium M199 supplemented with 15% fetal calf serum, 150 μg/ml endothelial cell growth supplement, 5

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1 The abbreviations used are: HARP, heparin affin regulatory peptide; RPTPβζ, receptor-type protein-tyrosine phosphatase βζ; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; HUVEC, human umbilical vein endothelial cells; FAK, focal adhesion kinase; Erk, extracellular signal-related kinase; np-src, non-phospho-Src; CS-C, chondroitin sulfate-C; BSA, bovine serum albumin; TBS, Tris-buffered saline; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; SH, Src homology.

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units/ml heparin sodium, 100 units/ml penicillin-streptomycin, and 50 μg/ml gentamycin. Cultures were maintained at 37 °C, 5% CO2, and 100% humidity. When HUVEC reached 80–90% confluence, they were serum-starved for 18 h before performing any further experiments.

Migration Assay—Migration assays were performed as described previously (12) in 24-well microchemotaxis chambers (Costar, Avon, France) using uncoated polycarbonate membranes with 8-μm pores. Serum-starved HUVEC were harvested and resuspended at a concentration of 10⁶ cells/0.1 ml in M199 containing 0.25% bovine serum albumin (BSA). The bottom chamber was filled with 0.6 ml of M199 containing 0.25% BSA and the tested substances. The upper chamber was loaded with 10⁶ cells and incubated for 4 h at 37 °C. After completion of the incubation, the filters were fixed and stained with 0.33% toluidine blue solution. The cells that migrated through the filter were quantified by counting the entire area of each filter, using a grid and an Optech microscope at a 20× magnification.

Matrigel Tube Formation Assay—The matrigel tube formation assay was performed as described previously (12). Briefly, Matrigel™ that was growth factor-reduced was used to coat the wells of 96-well tissue culture plates (0.04 ml/well) and was left to polymerize for 1 h at 37 °C. After polymerization, 15,000 cells suspended in 0.15 ml of M199 were added to each well. HARP was added directly in the medium. After 6 h of incubation at 37 °C, the medium was removed, the cells were fixed and stained, and the total area of the wells was measured in the total area of the wells, as described previously (12).

RNA Interference—RNA digonucleotide primers were obtained from Ambion, Inc. The following sequences were used as described previously (20): RPTPβ/ζ sense, 5′-AAAAUGGCAUUCCAAUACGCCGU-3′; RPTPβ/ζ antisense, 5′-AACGCUUUAGGAUUCGCAUU-3′. The annealing of the primers was achieved according to Ambion’s instructions. Cells were seeded and grown to a confluence of 40% in medium without antibiotics. The transfection of cells was performed in serum-free medium for 4 h using annealed RNA at the concentration of 50 μm and jetSI-ENDO (Polyplus-Transfection, France) as transfection reagent. Cells were incubated for another 24 h in serum-containing medium and serum-starved before further experiments. The transfection efficiency was evaluated using Silencer™ β-actin siRNA control (Ambion). Double-stranded negative control siRNA from Ambion was also used in migration and differentiation assays.

Immunoprecipitation Assay—Cells were grown to confluence in 145-mm dishes. The medium was aspirated, cells were washed twice with ice-cold phosphate-buffered saline and lysed with 2 ml of ice-cold radioimmunoprecipitation assay buffer (1× phosphate-buffered saline, 1% Triton X-100, 0.1% SDS, 20 mg sodium orthovanadate, 1 μg/ml aprtatin, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA). The lysates were cleared of debris by centrifugation at 12,000 rpm for 10 min at 4 °C. The supernatant was transferred to a new Eppendorf tube and incubated with 30 μl of protein A-agarose beads for 60 min at room temperature. Beads were collected by centrifugation, and the supernatants were transferred to new Eppendorf tubes. After this first pre-cleaning step, supernatants were further incubated with 500 ng of the primary antibody for 60 min at room temperature. At the end of this period, 80 μl of prepared suspension of protein A-agarose beads were added, and samples were incubated for 1 h at 4 °C. Protein A beads and bound proteins were collected by centrifugation (1,000 × g, 4 °C), washed three times with ice-cold cell lysis buffer. The pellet was resuspended with 60 μl of 2× SDS loading buffer (100 μl Tris-HCl, pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, 0.1 μl dithiothreitol) and kept at 4 °C until use. When ready for electrophoresis, samples were heated to 95–100 °C for 5 min and centrifuged, and 50 μl of the supernatant were analyzed by Western blotting.

Western Blot—Proteins were analyzed by SDS-PAGE and transferred to Immobilon P membranes. Blocking was performed by incubating the membranes with Tris-buffered saline (TBS), pH 7.4, containing 3% BSA (in the case of HARP, RPTPβ/ζ and phosphorytrosine) or TBS containing 5% nonfat dry milk (in all other cases) for 2 h at room temperature. Membranes were further incubated in primary antibodies for 1 h at room temperature or for 18 h at 4 °C under continuous agitation. The following antibodies were used: PY20 at 1:5,000 dilution in TBS containing 0.1% Tween 20 (TBS-T) and 1% BSA, anti-FAK antibody at 1,600 dilution in TBS-T containing 3% nonfat dry milk, anti-RPTPβ/ζ antibody at 11,000 dilution in TBS-T containing 1% BSA, anti-HARP antibody at 15,000 dilution in TBS-T containing 1% BSA, anti-np-src antibody at 15,000 dilution in TBS-T containing 5% BSA, anti-Erk1/2 antibody at 1:5,000 dilution in TBS-T containing 3% nonfat dry milk, anti-PI3K p85 antibody at 1:1,000 dilution in TBS-T containing 3% nonfat dry milk, and anti-β-actin antibody at 1,500,000 dilution in TBS-T. Membranes were washed three times in TBS-T and incubated in secondary antibodies (1:12,500 dilution in TBS-T for anti-rabbit and anti-goat antibodies and 1:7,500 dilution in TBS-T containing 3% nonfat dry milk for anti-mouse antibody) for 1 h at room temperature under continuous agitation. Membranes were washed three times with TBS-T and twice with TBS. Detection of immunoreactive bands was performed using the ChemiLucent detection kit (Chemicon) according to the manufacturer's instructions. The protein levels that corresponded to the immunoreactive bands were quantified using the ImageJPC image analysis software (Scion Corporation, Frederick, MD).

Total RNA Isolation and Reverse Transcriptase-PCRs—Total HUVEC RNA was isolated using Nucleospin® RNA II (Macherey-Nagel) according to manufacturer's instructions. Reverse transcriptase-PCRs were performed using the Access Reverse Transcriptase-PCR system (Promega). The sequences of the primers used for RPTPβ/ζ were 5′-GCGTTAAGAGATCATAATG-3′ (sense) and 5′-TCTCCGACTAATTA-CAC-3′ (antisense) and for β-actin were 5′-GGACACTTACCGGTTGTGTTG-3′ (sense) and 5′-AGGCCTACGGAGGATCGACCAGCTCGGATAG-3′ (antisense).

Statistical Analysis—The significance of variability between the results from each group and the corresponding control was determined by unpaired t test. Each experiment included triplicate wells for each condition tested, and all results are expressed as mean ± S.E. from at least three independent experiments.

RESULTS

HARP Stimulates Migration and Differentiation of Serum-starved HUVEC—HARP is known to affect the migration and differentiation of different types of endothelial cells (12). In the present work, we studied the effect of HARP on serum-starved HUVEC. As shown in Fig. 1, human recombinant HARP induced migration of HUVEC in a concentration-dependent manner. The maximum increase was ~40% over the control at the concentration of 100 ng/ml of HARP and was inhibited by sodium orthovanadate and CS-C (Fig. 1), indicating that RPTPβ/ζ may be the phosphatase involved in this process. The significant decrease in the migration of unstimulated HUVEC by sodium orthovanadate could be because of inhibition of phosphatases other than RPTPβ/ζ, which may influence baseline migration independent of HARP stimulation.

HARP also induced tube-like formation by HUVEC on matrigel, and this effect was abolished when cells were treated with sodium orthovanadate or CS-C (Fig. 1). In contrast to its effect on HUVEC migration, sodium orthovanadate had no effect on the tube formation of unstimulated cells on matrigel. Matrigel contains several growth factors and other undefined components involved in cell proliferation or differentiation that may affect the action of sodium orthovanadate, leading to a different response of the same cells cultivated under different conditions.

RPTPβ/ζ Is Present in HUVEC and Associates with HARP—To investigate whether RPTPβ/ζ is found in HUVEC and acts as a receptor for HARP, we cultured cells in 145-mm plates to confluence. Cells were lysed with radioligand precipitation assay buffer, and 400 μg of total protein were subjected to immunoprecipitation against HARP or RPTPβ/ζ. As shown in Fig. 2, RPTPβ/ζ was detected in all immunoprecipitates as one band of molecular mass ~200 kDa, which corresponds to a transmembrane spliced variant of RPTPβ/ζ (20–22). These data suggest that RPTPβ/ζ in HUVEC interacts with HARP.

c-Src Kinase Lies Downstream of RPTPβ/ζ and Links the Receptor with FAK—We investigated whether c-Src kinase is involved in the signaling pathway triggered by the interaction of HARP with RPTPβ/ζ in HUVEC. We used cell lysates from confluent HUVEC and performed immunoprecipitation for...
Immunoprecipitates were analyzed by SDS-PAGE and immunoblotted against c-Src kinase and RPTPα/β/γ. c-Src was detected in RPTPα/β/γ immunoprecipitates, indicating that RPTPα/β/γ can interact with c-Src in presence or absence of HARP. Moreover, it seems that the interaction of c-Src with RPTPα/β/γ is increased in the presence of HARP (Fig. 3, lanes 1 and 2).

Because c-Src is known to bind and further activate FAK (23), we tested whether c-Src binds FAK in HUVEC. In HUVEC lysates we performed immunoprecipitation against FAK and Western blot against c-Src kinase, np-src, and FAK. As shown in Fig. 3 (lanes 3 and 4), c-Src was detected as a band of 60 kDa, indicating the existence of a c-Src-FAK complex, which was increased in the presence of HARP. Moreover, the amounts of np-src interacting with FAK were increased in the presence of HARP (Fig. 3, lanes 3 and 4).

Interestingly, FAK or RPTPα/β/γ was not detected in RPTPα/β/γ immunoprecipitates, respectively (data not shown). Taken together, the above data indicate that c-Src is an intermediate molecule in the transduction of HARP signaling to FAK.

HARP Induces the Association of FAK with PI3K—We also investigated whether PI3K is involved in the HARP signaling

RPTPα/β/γ. Immunoprecipitats were analyzed by SDS-PAGE and immunoblotted against c-Src kinase and RPTPα/β/γ. c-Src was detected in RPTPα/β/γ immunoprecipitates, indicating that RPTPα/β/γ can interact with c-Src in presence or absence of HARP. Moreover, it seems that the interaction of c-Src with RPTPα/β/γ is increased in the presence of HARP (Fig. 3, lanes 1 and 2).

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Interestingly, FAK or RPTPα/β/γ was not detected in RPTPα/β/γ or FAK immunoprecipitates, respectively (data not shown). Taken together, the above data indicate that c-Src is an intermediate molecule in the transduction of HARP signaling to FAK.

HARP Induces the Association of FAK with PI3K—We also investigated whether PI3K is involved in the HARP signaling
pathway in endothelial cells. We used cell lysates from confluent HUVEC and performed immunoprecipitation for RPTPβ/ζ, c-Src, or FAK, and the immunoprecipitates were analyzed by Western blot for the presence of the p85 subunit of PI3K. PI3K was detected only in the FAK immunoprecipitates, suggesting the direct interaction between these two molecules. Lane 1 corresponds to positive antigen control (Upstate, number 12-303).

FIG. 4. PI3K associates with FAK. HUVEC were incubated with (lanes 3, 5, and 7) or without HARP (lanes 2, 4, and 6) for 15 min and then lysed with radioimmune precipitation assay buffer. The cell lysates were immunoprecipitated for RPTPβ/ζ (lanes 2 and 3), c-Src (lanes 4 and 5), or FAK (lanes 6 and 7), and the immunoprecipitates were analyzed by Western blot for the presence of the p85 subunit of PI3K. PI3K was detected only in the FAK immunoprecipitates, suggesting the direct interaction between these two molecules. Lane 1 corresponds to positive antigen control (Upstate, number 12-303).

FIG. 5. Effect of human recombinant HARP (100 ng/ml) on activation of c-Src (dephosphorylation at Tyr527) in serum-starved HUVEC. HUVEC lysates were analyzed by SDS-PAGE, transferred to Immobilon P membranes, and consecutively blotted for np-src and c-Src. Numbers denote the average -fold change of the ratio np-src:src compared with unstimulated cells (set as default = 1) of at least three independent experiments. S.O., sodium orthovanadate 20 nm; CS-C, 100 μg/ml.

FIG. 6. Effect of human recombinant HARP (100 ng/ml) on activation of FAK in serum-starved HUVEC. HUVEC lysates were analyzed by SDS-PAGE, transferred to Immobilon P membranes and consecutively blotted for phosphotyrosine and FAK. Numbers denote the average -fold change of the ratio pFAK:FAK compared with unstimulated cells (set as default = 1) of at least three independent experiments. S.O., sodium orthovanadate 20 nm; CS-C, 100 μg/ml; PP1, 20 nm.

FIG. 7. Effect of human recombinant HARP (100 ng/ml) on activation of Erk1/2. HUVEC lysates were analyzed by SDS-PAGE, transferred to Immobilon P membranes, and consecutively blotted for pErk1/2 and Erk1/2. Numbers denote the average -fold change of the ratio pErk1/2:Erk1/2 compared with unstimulated cells (set as default = 1) of at least three independent experiments. S.O., sodium orthovanadate 20 nm; CS-C, 100 μg/ml; PP1, 20 nm; wortmannin, 100 nm; LY294002, 200 nm; U0126, 20 nm.
levels up to 30 min after HARP application and was inhibited by sodium orthovanadate and CS-C or the c-Src inhibitor PP1 (Fig. 6).

**HARP Induces Phosphorylation and Activation of Erk1/2 through PI3K**—We further tested the effect of HARP on the activation of Erk1/2. HARP induced the activation of both Erk1 and Erk2 within 5 min after the addition of HARP in the medium of HUVEC, with the maximum effect observed after 15 min. HARP-induced Erk1/2 activation was inhibited by sodium orthovanadate and CS-C and PP1, suggesting that RPTPβζ and c-Src may be involved in this process. It was also inhibited by two PI3K inhibitors, wortmannin and LY294002, suggesting that PI3K is involved and lies upstream of Erk1/2 in this signaling pathway of HARP. Finally, the well known inhibitor of MEK U0126 also inhibited HARP-induced Erk1/2 phosphorylation (Fig. 7).

**HARP-induced HUVEC Migration and Tube-like Formation Was Inhibited by Pharmacological Inhibition of c-Src, PI3K, and MEK**—PP1, wortmannin, LY294002, and U0126 completely inhibited HARP-induced HUVEC migration, implying the involvement of c-Src, PI3K, and MAPK in the chemotactic activity of HARP. Furthermore, treatment of HUVEC with the
same inhibitors inhibited HARP-induced tube formation on matrigel (Fig. 8).

**RPTPβζ Knockdown by RNA Interference Interrupts HARP Signaling and HARP-induced Migration and Differentiation of HUVEC—**To further establish that RPTPβζ is the receptor of HARP in HUVEC and that the biological activity of HARP is mediated by binding to RPTPβζ, we performed the same set of experiments in HUVEC after down-regulation of RPTPβζ by RNA interference (20). As shown in Fig. 9, 48 h following transfection, a significant reduction of both mRNA and the protein levels of RPTPβζ was achieved in HUVEC, which led to a significant reduction of HARP-induced c-Src activation, as well as FAK and Erk1/2 phosphorylation (Fig. 10).

Finally, we examined whether RPTPβζ knockdown inhibits HARP-induced HUVEC migration and tube formation. As shown in Fig. 11, the biological effect of HARP was abolished when RPTPβζ expression was down-regulated. It is noteworthy that RPTPβζ knockdown results in the reduction of unstimulated cell tube formation, which is in contrast to the lack of effect of sodium orthovanadate on unstimulated cells (Fig. 1). Methodological differences or the nonspecific actions of sodium orthovanadate could be responsible for this discrepancy.

**DISCUSSION**

HARP is an 18-kDa heparin-binding growth factor that is implicated in cell growth and differentiation and has a potent role in angiogenesis and tumor growth (1). In the present work, we studied the signaling pathway that is activated by HARP in HUVEC and leads to increased cell migration.

Our data suggest that RPTPβζ expressed by HUVEC is involved in HARP-induced HUVEC migration and tube formation. This is supported by the co-immunoprecipitation of the two molecules, as well as the inhibition of HARP activity by sodium orthovanadate or CS-C. These results are in line with previous reports that linked RPTPβζ to cell migration induced by HARP and midkine in neuronal cells (18, 20, 26), where RPTPβζ is considered to be a HARP receptor (19,

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**Fig. 9.** RPTPβζ knockdown in HUVEC was evaluated both at mRNA (A) and protein (B) level. Numbers denote the average -fold change of the ratio RPTPβζ/actin compared with unstimulated cells (set as default = 1) of at least three independent experiments.

**Fig. 10.** RPTPβζ knockdown in HUVEC inhibited HARP-induced signaling. Numbers denote the average -fold change of the ratios np-src:src, pFAK:FAK, and pErk1/2:Erk1/2, compared with unstimulated cells (set as default = 1) of at least three independent experiments.

**Fig. 11.** RPTPβζ knockdown in HUVEC inhibited HARP-induced migration (A) and tube formation on matrigel (B). Results are expressed as percent of the values obtained without stimulation. Data are the mean ± S.E. of at least three independent experiments.
SH2 domain of c-Src kinase is known to recognize and bind RPTPβζ, which has been previously implied as a potential event in the signaling pathway of RPTPβζ. This is the first time that a direct association of RPTPβζ with c-Src was inhibited by sodium orthovanadate (28), which seems to be involved in HUVEC migration and differentiation (Fig. 11). We also showed a decrease in unstimulated HUVEC migration and differentiation in these experiments because of increased basal levels (data not shown), possibly as a result of serum starvation of HUVEC.

The role of RPTPβζ in signaling triggered by HARP in HUVEC is supported further by our results obtained after RPTPβζ knockdown by RNA interference. Our results clearly demonstrate that RPTPβζ down-regulation interrupts HARP signaling (Fig. 10) in HUVEC and abolishes its biological activity on cell migration and differentiation (Fig. 11). Furthermore, it is becoming increasingly recognized that receptor protein-tyrosine phosphatases are implicated in the regulation of integrin-mediated events (38), which seem to play significant role in HUVEC migration and tube formation on matrigel (39). RPTPα-dependent differences in cell spreading on fibronectin and vitronectin substrates are mediated by the αβ3 integrin, and although RPTPα does not modulate integrin-ligand interactions, it seems to regulate the stability of integrin-cytoskeleton bonds (40). Integrin αβ3 uses a complex involving protein kinase C (PKC), RACK1, and PTPα to regulate cadherin-mediated cell-cell adhesion, possibly by modulating tyrosine phosphorylation of β-catenin (41). RPTPβζ seems to participate in a multimolecular complex involving the low density lipoprotein receptor-related protein 6 ectodomain, αβ3-integrin, and αβ3-integrin (42). Whether such a complex is formed in HUVEC and what its possible role might be is not known and is under further investigation. Taken together, our results implicate RPTPβζ in HARP-induced migration and differentiation of human endothelial cells and identify the signaling molecules involved.

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