

The SHIP phosphatase becomes associated with Fc γ RIIB1 and is tyrosine phosphorylated during ‘negative’ signaling¹

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Abstract

Immune-complex mediated co-ligation of antigen and Fc receptors on B-cells leads to abortive antigen receptor (BCR) signaling and provides a mechanism for feedback regulation of the immune response. A phosphotyrosine-containing 13 amino acid sequence (ITIM) found in the Fc γ RIIB1 cytoplasmic tail mediates this inhibition and specifically associates with the phosphotyrosine phosphatase SHP1. In vitro binding studies demonstrate that the phosphorylated ITIM binds unidentified proteins of 70 and 160 kD in addition to SHP1. Here we report the identification of p70 as SHP2 and p160 as the SH2 containing phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase SHIP. SHIP is inducibly tyrosine phosphorylated following BCR-Fc γ RIIB1 co-ligation. Further, we observe SHIP association with tyrosine phosphorylated Fc γ RIIB1 in intact cells following BCR-Fc γ RIIB1 co-ligation. To a much lesser but significant degree, tyrosine phosphorylation of SHIP is also observed upon BCR ligation. These observations suggest that SHIP may play an important role in Fc γ RIIB1 dependent and independent regulation of BCR signaling. © 1996 Elsevier Science B.V. All rights reserved

Keywords: B-lymphocytes; Cell signaling; Cellular activation; Fc receptors; Signaling molecules

1. Introduction

The low affinity receptor for the Fc region of IgG expressed on B-cells (Fc γ RIIB1) consists of a single polypeptide chain containing two Ig like domains involved in Fc binding and a cytoplasmic domain with properties consistent with signal transduction. This receptor binds IgG in immune complex form and negatively regulates B-cell activation both in vivo and in vitro. The ability of Fc γ RIIB1 to inhibit B-cell activation is strictly dependent on its co-ligation with membrane immunoglobulin (mIg) as is likely to occur during the late phase of immune responses with the formation of antigen-IgG complexes [1]. For these reasons, co-ligation of BCR and Fc γ RIIB1 is thought to

provide a physiological mechanism for feedback inhibition of antibody production.

A series of recent studies indicate that Fc γ RIIB1 co-ligation aborts B-cell activation at the level of BCR signaling. BCR signaling is mediated by the rapid activation of Src and Syk family tyrosine kinases [2,3], augmented protein tyrosine phosphorylation [4], phosphoinositide hydrolysis and elevation of cytoplasmic free calcium concentration [Ca²⁺]_i, resulting in B-cell proliferation, differentiation and antibody secretion [5,6]. The co-ligation of BCR and Fc γ RIIB1 leads to premature termination of inositol triphosphate (IP3) production [7], inhibition of the extracellular Ca²⁺ influx [8,9], and blockade of blastogenesis and proliferation [10].

Mutational analysis of the Fc γ RIIB1 has revealed that a 13 amino acid sequence, immune-receptor tyrosine-based inhibitor motif (ITIM), within its cytoplasmic domain is required for negative signaling [11,12]. Further, the ITIM has been shown to mediate the

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inhibitory signaling when expressed in a different receptor context [13]. Importantly, Fc γ RIIB1-BCR co-ligation leads to phosphorylation of tyrosine 309 in the ITIM an event which has been shown to be necessary for Fc γ RIIB1-mediated inhibition [13]. In a previous report we used a tyrosine phosphorylated peptide containing the ITIM sequence (pITIM) to affinity purify binding proteins, which could represent putative transducers of Fc γ RIIB1 signaling. Following this approach we identified the phosphotyrosine phosphatase SHP1 (PTP1C), [14–16] as one of three major pITIM binding proteins, and defined a role for SHP1 in Fc γ RIIB1 mediated inhibition of B-cell activation [17].

In the present report we define the identity of the two other major pITIM binding proteins in B-cells, p70 and p160; p70 is defined as SHP2, a ubiquitous phosphotyrosine phosphatase, and p160 as SHIP, a 5'-phosphatidylinositol phosphatase.

2. Materials and methods

2.1. Cell culture and stimulation

The murine B-lymphomas Fc γ RIIB1 expressing A20, the Fc γ RIIB1 negative variant IIA1.6, and the plasmacytoma J558 cell lines were cultured in IMDM supplemented with 5% heat inactivated fetal bovine serum (Hyclone), 50 U/ml penicillin, 50 mg/ml streptomycin at 37°C with 5% CO₂.

For stimulation of A20 and IIA1.6, the cells were harvested, washed once in phosphate buffer saline (PBS), resuspended at 10⁸/ml in PBS and cultured in the presence or absence of intact (80 mg/ml) or F(ab')₂ (50 mg/ml) rabbit anti-mouse immunoglobulin heavy and light chain (Zymed) for 1 min at 37°C.

2.2. Peptides and antibodies

Synthetic non-phosphorylated or tyrosine phosphorylated (p) peptides with the Fc γ RIIB1 ITIM sequence EAENTIT(p)YSLKHK were purchased from Macromolecular Resource Center, Fort Collins, Colorado USA. The peptides were directly conjugated to CNBr activated Sepharose beads (Pharmacia) at 1 mg/ml, 2.4G2 monoclonal antibody was affinity purified from hybridoma supernatant on a protein G column. Rabbit anti-SHP1 antibodies were prepared as previously described [17]. Rabbit anti-SHP2 was obtained from Andrius Kazlauskas (Schepens Eye Institute, Boston), and anti-SHIP was obtained from Gerald Krystal.

2.3. [³⁵S]methionine labeling

For [³⁵S]methionine labeling, A20, IIA1.6 and J558 cells were washed twice in methionine free DMEM

(Sigma), resuspended at 10⁶/ml in methionine free DMEM supplemented with 5% dialyzed FBS and incubated for 4 h in the presence of 0.5 mCi or immunoprecipitation of [³⁵S]methionine (Dupont NEN) at 37°C in 5% CO₂. The cells were subsequently washed twice in PBS and subjected to further treatment and then lysed as described.

2.4. Cell lysis, immunoprecipitations and adsorptions

Stimulated or unstimulated A20 or IIA1.6 cells were lysed in 1% NP-40 lysis buffer: 1% NP-40, 10 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 2 mg/ml each of aprotinin, leupeptin, a-1 antitrypsin, 10 mM NaF, 2 mM Na₃VO₄ and incubated for 15 min on ice. Lysates were centrifuged in eppendorf tubes for 5 min at 14 000 rpm. Cleared lysates were incubated with various peptide or antibody coupled Sepharose beads as described in figure legends.

Cell lysates (20 × 10⁶ cells in 0.5 ml) were precipitated for 1 h at 4°C using the various synthetic peptides (3 mg) directly coupled to CNBr activated Sepharose beads (6 ml of 50% v/v) (Pharmacia). Fc γ RIIB1 immunoprecipitations from lysates of unstimulated or stimulated A20 cell (1.5 × 10⁸ cells in 0.5 ml) were performed using 2.4G2 monoclonal antibody (50 mg) directly coupled to CNBr Sepharose beads (20 ml of a 50% v/v) by incubating 45 min at 4°C. Adsorbates and immunoprecipitates were quickly washed two or three times with lysis buffer and eluted with reducing Laemmli sample buffer.

2.5. SDS-PAGE and Western blotting

Adsorbed and immunoprecipitated proteins were boiled in reducing Laemmli sample buffer and fractionated by SDS-PAGE. Fractionated proteins were transferred on Immobilon-P membranes (Millipore, USA) and blocked using Tris buffered saline containing 5% bovine serum albumin for 2 h. For immunoblotting the following antibodies were used: monoclonal anti-phosphotyrosine (Ab2, Oncogene Science), rabbit anti-SHP1, SHP2, or SHIP. Detection was performed using horseradish peroxidase conjugated Rat anti-mouse-IgG1 (Biorad) (for anti-pTyr) or horseradish peroxidase conjugated Protein A (Amersham) (for anti-SHP1, SHP2, and SHIP) and ECL. For detection of [³⁵S]methionine labeled proteins, the transfers were exposed 1–3 days for autoradiography.

3. Results

3.1. Binding proteins p160 and p70 are SHIP and SHP2 phosphatases, respectively and bind to phosphorylated, but not nonphosphorylated, ITIM

To identify and characterize putative effectors of

Fc γ RIIB1 mediated signaling, we affinity purified pITIM binding proteins from [^{35} S]methionine labeled A20 cells. After incubation of cell lysates with peptide coated Sepharose beads, tightly bound proteins were eluted, fractionated by SDS-PAGE and transferred on Immobilon-P membranes. Autoradiography revealed the presence of three major proteins of 65, 70 and 160 kD which associated with the phosphorylated ITIM (pITIM), but not with the nonphosphorylated ITIM. Subsequent sequential Western blotting of the membranes revealed corresponding bands at 65, 70, and 160 kD, which blotted with antibodies for SHP1, SHP2, and SHIP, respectively, and which also associated with the phosphorylated, but not the nonphosphorylated, ITIM (Fig. 1). These data indicate that p160 and p70 may be recruited to Fc γ RIIB1 by virtue of their interaction with the tyrosine phosphorylated ITIM and that they are SHIP and SHP2, respectively.

3.2. SHIP is tyrosine phosphorylated upon BCR-Fc γ RIIB1 co-ligation

Given the fact that many signal transducing proteins become tyrosine phosphorylated upon receptor engagement we wished to determine the tyrosine phosphorylation state of SHIP following BCR-Fc γ RIIB1 co-ligation. In order to investigate tyrosine phosphorylation of SHIP we initially compared the patterns of tyrosine phosphorylation induced by BCR Fc γ RIIB1 co-ligation or BCR ligation in A20 cells. After stimulation of A20 cells with intact rabbit anti-mouse Ig (mIg) or F(ab') $_2$ rabbit anti-mIg, the cells were lysed and the lysates were precleared with either nonphosphorylated or phosphorylated ITIM peptide coupled Sepharose

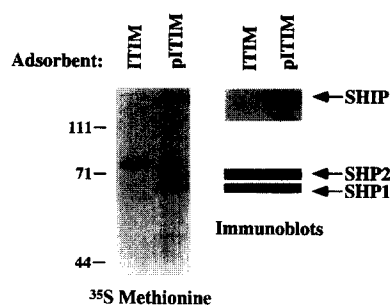


Fig. 1. The p160 and p70 that bind the phosphorylated ITIM of Fc γ RIIB1 can be identified as SHIP and SHP2, respectively. Cleared detergent lysates from [^{35} S]methionine labeled 2×10^7 A20 cells were adsorbed with nonphosphorylated and phosphorylated (pITIM) peptide coupled Sepharose beads. Adsorbed material was boiled in reducing Laemmli sample buffer, fractionated by 10% SDS-PAGE and electrophoretically transferred on Immobilon-P membrane. Peptide-binding [^{35} S]methionine labeled proteins were detected by autoradiography. Sequential immunoblotting of the same membrane was performed using anti-SHP1, SHP2, and SHIP antibodies, which were detected with horseradish peroxidase conjugated Protein A and ECL.

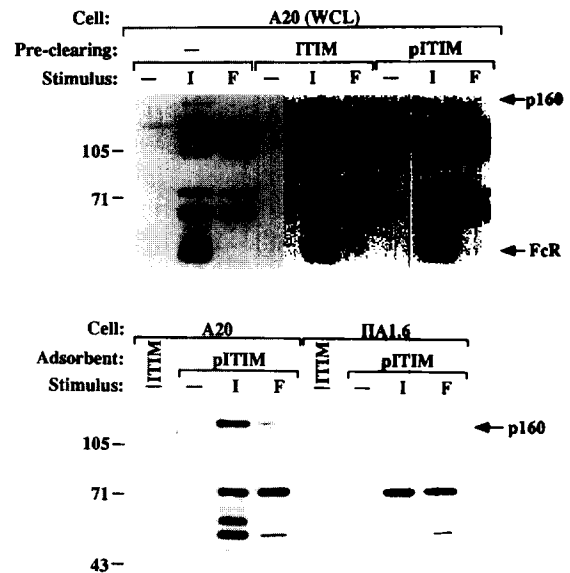


Fig. 2. Tyrosine phosphorylation of SHIP upon BCR ligation and BCR-Fc γ RIIB1 co-ligation. Anti-phosphotyrosine immunoblotting (anti-pTyr) of membrane transferred, 10% SDS-PAGE fractionated, whole cell lysate and peptide adsorbed material from cleared detergent lysate of unstimulated and intact or F(ab') $_2$ rabbit anti-mouse IgG A20 and IIA1.6 cells. (A) Cleared whole cell lysates from 2×10^6 A20 (WCL) were precleared by incubation for 30 min with nonphosphorylated ITIM or phosphorylated pITIM peptide conjugated Sepharose beads and subsequently boiled in reducing Laemmli sample buffer. (B) Cleared lysates from 2×10^6 A20 or IIA1.6 cells were adsorbed with nonphosphorylated ITIM or phosphorylated pITIM peptide conjugated Sepharose beads. The tightly bound material was eluted by boiling in reducing Laemmli sample buffer. Detection was performed with an horseradish peroxidase conjugated Rat anti-mouse IgG1 monoclonal antibody followed by ECL. Migration of p160 and Fc γ RIIB1 are indicated by arrowheads. The position of molecular size markers are indicated on the left.

beads. The precleared cell lysates were then fractionated by SDS PAGE, transferred and the transfers were subjected to anti-phosphotyrosine immunoblotting. Analysis of the patterns of tyrosine phosphorylation revealed that BCR Fc γ RIIB1 co-ligation induced tyrosine phosphorylation of Fc γ RIIB1 and a 160 kD protein in addition to other proteins also phosphorylated upon BCR ligation (Fig. 2A). The 160 kD tyrosine phosphorylated protein previously identified as SHIP was effectively precleared by incubation of the lysate with phosphorylated ITIM, but not by incubation with nonphosphorylated ITIM conjugated to Sepharose beads (Fig. 2A). These data demonstrate that SHIP is tyrosine phosphorylated upon BCR-Fc γ RIIB1 co-ligation. To confirm and extend these findings, we subsequently performed pITIM adsorptions from lysates of A20 and IIA1.6 cells which were cultured without stimulus or cultured with either intact or F(ab') $_2$ rabbit anti-mIg. Anti-phosphotyrosine immunoblotting of membrane transferred, SDS-PAGE

fractionated material, revealed tyrosine phosphorylation of SHIP following BCR-Fc γ RIIB1 co-ligation (Fig. 2B). Surprisingly, weaker but significant tyrosine phosphorylation of SHIP was also detected upon BCR ligation in either A20 and IIA1.6 cells (Fig. 2B and C). Further, the observed equivalent degree of SHIP tyrosine phosphorylation in intact or F(ab')₂ stimulated IIA1.6 indicates that the different extent of tyrosine phosphorylation of SHIP following BCR ligation or BCR-Fc γ RIIB1 co-ligation (Fig. 2A and B) is not due to a difference in the receptor cross-linking ability of intact and F(ab')₂ anti-mIg.

These data demonstrate that SHIP is tyrosine phosphorylated upon BCR-Fc γ RIIB1 co-ligation and further suggest that SHIP may be recruited, via pITIM association, into the BCR-Fc γ RIIB1 complex and subsequently phosphorylated by BCR associated kinases. The observed tyrosine phosphorylation of SHIP upon BCR ligation suggest that p160 may also associate with the BCR complex independently of Fc γ RIIB1 co-ligation. Finally, these findings indicate that SHIP can bind the phosphorylated ITIM regardless of its phosphorylation state.

3.3. SHIP associates with tyrosine phosphorylated Fc γ RIIB1 in intact cells

Having established tyrosine phosphorylation and pITIM association of SHIP, we wished to determine whether the association of p160 with the tyrosine phosphorylated Fc γ RIIB1 could be detected in intact cells. We therefore immunoprecipitated Fc γ RIIB1 from lysates of unstimulated and intact or F(ab')₂ rabbit anti-mIg stimulated A20 cells and looked for coprecipitation of SHIP with the tyrosine phosphorylated Fc γ RIIB1 by anti-phosphotyrosine immunoblotting. This experiment showed that the tyrosine phosphorylated p160 can be coprecipitated with the tyrosine phosphorylated Fc γ RIIB1 following BCR co-ligation (Fig. 3A). Subsequent anti-Fc γ RIIB1 immunoblotting shows that equivalent amounts of Fc γ RIIB1 are immunoprecipitated from all samples (Fig. 3B). These findings confirm that SHIP can associate with the tyrosine phosphorylated Fc γ RIIB1 following BCR-Fc γ RIIB1 co-ligation.

3.4. SHIP associates with tyrosine phosphorylated ITIM independently from the binding of SHP1

Since we reported previously that SHP1 associates with pITIM and plays a role in mediating Fc γ RIIB1 'negative' signaling, we wished to determine whether SHIP binds pITIM via SHP1. To address this issue we performed the pITIM peptide adsorption from cell lysate of a variant of the plasmacytoma cell line J558, which does not express detectable SHP1 (data not

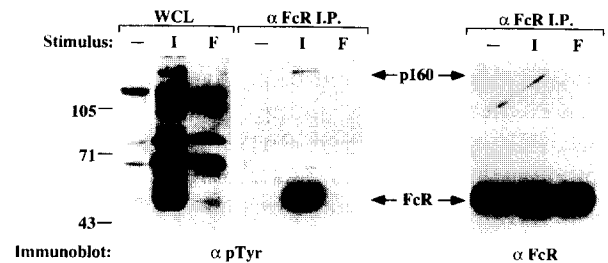


Fig. 3. The protein p160 associates with the tyrosine phosphorylated Fc γ RIIB1 upon BCR-Fc γ RIIB1 co-ligation. Immunoblotting of membrane transferred, SDS-PAGE fractionated, whole cell lysate and 2.4G2 immunoprecipitated material from cleared detergent lysates of unstimulated and intact or F(ab')₂ rabbit anti-mouse IgG A20 cells. Cleared whole cell lysates from 2×10^6 A20 (WCL) and Fc γ RIIB1 immunoprecipitates from 15×10^7 A20 cells were boiled in reducing Laemmli sample buffer and fractionated by 10% SDS-PAGE, subsequently transferred on Immobilon-P membranes which were then subjected to anti-phosphotyrosine immunoblotting (anti-pTyr). The same membrane transfer was stripped and subjected to immunoblotting with anti-Fc γ RIIB1 monoclonal antibody (anti-FcR). Detection was performed by ECL using an horseradish peroxidase conjugated Rat anti-mouse IgG1 for anti-pTyr and horseradish peroxidase conjugated goat anti-rat IgG for anti-FcR. The migration of Fc γ RIIB1 and p160 is indicated by arrowheads. The position of molecular size markers is indicated on the left.

shown). A20 and J558 cells were [³⁵S]methionine labeled, lysed and incubated with phosphorylated and nonphosphorylated Sepharose beads coupled ITIM peptide. The precipitated material was fractionated by SDS-PAGE and transferred. Autoradiography of the transfer showed equivalent binding of SHIP to pITIM in either A20 or J558 cell lysates (Fig. 4A). Subsequent immunoblotting with anti-SHP1 antibody of the same transfer confirmed binding of SHIP1 to pITIM in A20

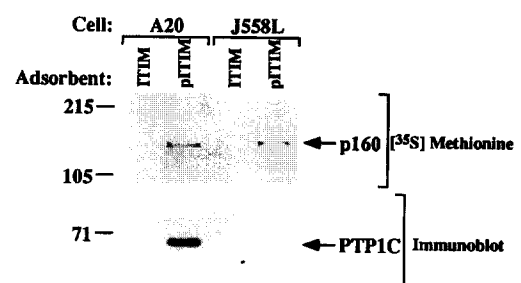


Fig. 4. The p160 association with pITIM does not require SHP1 (PTP1C). Cleared detergent lysates from [³⁵S]methionine labeled 2×10^7 A20 or J558 cells were adsorbed with nonphosphorylated and phosphorylated pITIM peptide coupled Sepharose beads. Adsorbed material was boiled in reducing Laemmli sample buffer, fractionated by 10% SDS-PAGE and electrophoretically transferred on Immobilon-P membrane. A, Autoradiography of membrane transferred, [³⁵S]methionine labeled peptide-binding proteins from A20 or J558. The same transfer was subsequently subjected to immunoblotting with anti-SHP1 antibody and detection was performed using horseradish peroxidase conjugated Protein A and ECL. Migration of SHIP and SHP1 is indicated by arrowheads. The position of molecular size markers is indicated on the left.

cell lysate and no detectable SHIP binding in J558 cell lysate (Fig. 4B). These data indicate that p160 association with pITIM does not require binding of SHP1.

4. Discussion

B-cell activation by antigen receptor aggregation can be modulated by engagement of accessory molecules, via positive or negative cooperation at the level of receptor signal transduction. Fc γ RIIB1 is known to negatively cooperate with BCR leading to abortive B-cell activation by virtue of immune-complex mediated co-ligation of Fc and antigen receptor [18]. The inhibitory effects of Fc γ RIIB1 are strictly dependent on its co-ligation and the independent crosslinking of Fc γ RIIB1 does not effect BCR signaling [10]. Although Fc γ RIIB1 is thought to provide for feedback inhibition of antibody production, the molecular basis of 'negative' signaling is not well understood.

Earlier studies have shown that tyrosine phosphorylation of a 13 amino acid sequence named ITIM is necessary and sufficient for 'negative' signaling [13]. More recently we reported that the phosphorylated ITIM can bind the phosphotyrosine phosphatase SHP1 and two other unidentified proteins of 70 and 160 kD [17]. Analysis of Fc γ RIIB1 function in the SHP1 deficient motheaten mice [17] revealed a deficit in 'negative' signaling, implicating SHP1 in negative regulation of BCR signaling. However, the potential role of p70 and p160 in Fc γ RIIB1 mediated inhibition of B-cell activation remained unexplored.

The present study was aimed at characterizing the role of p70 and SHIP in Fc γ RIIB1 function. Isolation of SHIP based on pITIM binding and immunoblotting revealed that SHIP associates with phosphorylated, but not nonphosphorylated ITIM. Analysis of the tyrosine phosphorylation state of SHIP revealed that it is inducibly tyrosine phosphorylated following Fc γ RIIB1-BCR co-ligation. Surprisingly, we also observed a lesser degree of tyrosine phosphorylation of SHIP following BCR ligation. Interestingly, analysis of BCR-Fc γ RIIB1 co-ligation induced tyrosine phosphorylation in whole cell lysate indicated that SHIP is a major substrate of tyrosine phosphorylation in addition to Fc γ RIIB1.

These observations, together with the demonstrated association of SHIP with the tyrosine phosphorylated Fc γ RIIB1 in intact cells, suggest a model in which phosphorylation of the Fc γ RIIB1 ITIM leads to the recruitment of SHIP and subsequently to its tyrosine phosphorylation presumably by BCR associated Src or Syk family kinases [19]. Since phosphorylated ITIM also mediates the association of SHP1 with Fc γ RIIB1 it is possible that the association of SHIP may depend on SHP1 binding. Analysis of SHIP binding to pITIM in lysates of J558 cells lacking detectable expression of

SHP1 indicates that the association of SHIP does not require binding of SHP1. However, the role of another pITIM binding protein, p70 remained undefined. In parallel studies, we identified p70 as the phosphotyrosine phosphatase SHP2 or PTP1D and immunodepletion of SHP2 from A20 cell lysates did not abolish the association of SHIP with the phosphorylated ITIM (data not shown). In addition, pITIM associated SHIP could be eluted by treatment of the precipitates with the phosphotyrosine analog para-nitrophenyl phosphate confirming the phosphotyrosine dependence of this interaction. These observations suggest that SHIP binds to Fc γ RIIB1 via a direct interaction with the phosphorylated ITIM following BCR co-ligation.

Our findings, describing Fc γ RIIB1 independent tyrosine phosphorylation of SHIP, suggest that SHIP may interact before and/or after receptor ligation with BCR components or BCR associated tyrosine kinases. Although the nature of this interaction is not known, our findings indicate that SHIP may play a more general regulatory function in BCR signaling. The observed tyrosine phosphorylation of SHIP may allow membrane localization and recruitment of additional effectors, in the context of aggregated receptors. Alternatively, tyrosine phosphorylation of SHIP may serve to sequester signaling molecules and prevent their full activation, contributing to the inhibitory function of Fc γ RIIB1. Since both SHP1 and SHIP have been shown to associate with Fc γ RIIB1 via the same phosphorylated ITIM, it is likely that they bind alternatively and may cooperate to regulate BCR signaling.

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