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RNA aptamers that specifically bind to the Ras-binding domain of Raf-1

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Abstract RNA aptamers that bind to the Ras-binding domain (RBD) of a proto-oncogene product, Raf-1, were isolated from a pool of random sequences using a glutathione *S*-transferase-fused RBD (GST-RBD). The RNA molecules bind to the GST-RBD, but not to GST, with dissociation constants of about 300 nM. In contrast, these RNA aptamers do not bind to the Ras-binding domain of the RGL protein, which is also known to be activated by Ras. The aptamers actually compete with Ras for binding to the Raf-1 RBD. The anti-Raf-1 aptamers may be used to specifically inhibit the Ras-Raf interaction in the complicated signaling network in mammalian cells.

Key words: Raf-1; Ras; RNA aptamer; In vitro selection

1. Introduction

Raf-1, the product of a proto-oncogene, *c-raf-1*, is a 74-kDa cytoplasmic serine/threonine protein kinase that regulates mammalian cell proliferation and differentiation. The Raf family kinases (Raf-1, A-Raf, and B-Raf) phosphorylate and activate MAPK/ERK kinases (MEKs), which in turn phosphorylate extracellular signal-regulated kinases (ERKs) belonging to the mitogen-activated protein kinase (MAPK) family [1,2]. The Raf kinases are activated either through interaction with the Ras proto-oncogene product on the plasma membrane [3–8] or by Ras-independent mechanisms [9,10]. Ras belongs to a family of small GTP-binding proteins, and cycles between the GTP- and GDP-bound forms [11,12]. The GTP-bound Ras physically associates with residues 51–131 of Raf-1 (the Ras-binding domain, RBD) [6,13] with a K_d value of 18 nM [14]. In addition to Raf-1, the GTP-bound Ras can bind various downstream effectors, such as the guanine nucleotide dissociation stimulators for the Ral protein, RalGDS and RGL, through the same binding interface as that for the Raf-1 binding [15–18].

The molecular mechanisms of the cellular signaling involving these proteins are therefore complicated, and it is important to elucidate the role of each protein-protein interaction. Accordingly, there have been many efforts to selectively inhibit the Ras-Raf signaling pathway. For instance, dominant-negative Raf-1 mutants, which bind to Ras but lack the kinase activity, prevent the Ras-dependent activation of the wild-type Raf-1 [19]. However, these Raf-1 mutants prevent the GTP-bound Ras from interacting with any downstream effectors. In addition, monoclonal antibodies that bind to an epitope in the Raf-1 kinase domain inhibit both the Ras-dependent and -independent Raf-activation mechanisms [20]. Therefore, it is worth while to develop a selective inhibitor of only the Ras-Raf interaction. Note that the two proto-oncogene products, Ras and Raf-1, are good candidates for targets of anti-cancer drug development.

It is possible to isolate RNA or DNA molecules (aptamers) with high affinity for a given protein by in vitro selection [21,22]. As for proteins related to cellular signaling pathways, aptamers targeting the βII isozyme of protein kinase C and *Yersinia* protein tyrosine phosphatase, for example, have been isolated and demonstrated to inhibit the enzyme activities [23,24]. In the present study, by in vitro selection, we isolated RNA aptamers to the RBD of Raf-1, which actually inhibited the interaction between the two proteins, Ras and the RBD of Raf-1.

2. Materials and methods

2.1. Protein purification

The Raf-1 RBD (amino acid residues 51–131 of human c-Raf-1) fused to glutathione *S*-transferase (GST) (designated GST-RBD) was expressed in *Escherichia coli* (strains BL21 and BL21DE3) and was purified by glutathione-Sepharose 4B (Amersham Pharmacia Biotech) and Poros HQ (PerSeptive Biosystems) column chromatography [25]. The GST-fusion form of the RGL RBD (amino acid residues 632–734 of human RGL) was expressed in *E. coli* BL21, and was purified by glutathione-Sepharose 4B column chromatography and Resource Q FPLC (Amersham Pharmacia Biotech) [26]. The wild-type human Ha-Ras protein was prepared from an *E. coli* overproducing strain, and was purified by DEAE-Sephacel and Sephadex G-75 column chromatography followed by Resource Q FPLC [25,27,28]. The final protein preparations were confirmed to be homogeneous by SDS-PAGE with Coomassie brilliant blue and silver staining.

2.2. In vitro selection

For selection, a pool of RNAs with 60 random nucleotides flanked by defined sequences (Fig. 1) was transcribed from the PCR products of the synthetic DNA templates. The RNAs were heated at 75°C for 3 min, then cooled on ice, and incubated in a binding buffer (phosphate-buffered saline containing 5 mM $MgCl_2$, buffer A) together with the GST-RBD of Raf-1 and a matrix (glutathione-Sepharose 4B) at 4°C for 1 h. The matrix was washed with a buffer (20 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, and 150 mM NaCl, buffer B) and the RNAs were then eluted with distilled H_2O by boiling. Prior to the incubation with the Raf-1 GST-RBD (from the 3rd to the 13th round) and also after the elution (from the 7th to the 13th round), the RNAs were passed through a matrix bearing only GST, to eliminate the RNAs that bind to GST and/or the matrix. The eluted RNAs were reverse-transcribed and amplified by PCR. The RNA pool for the

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Abbreviations: ERK, extracellular signal-regulated kinase; FPLC, fast protein liquid chromatography; GST, glutathione *S*-transferase; MAPK, mitogen-activated protein kinase; RBD, Ras-binding domain; MEK, MAPK/ERK kinase; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate)

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Starting RNA:
5'-GGGAGAUCAGAAUAAACGCUCAA[-N60-]UUCGACAUGAGGCCCCUGCAGGGCG-3'

PCR primer 1:
5'-GCCGGAATTCTAATACGACTCACTATAGGGAGATCAGAATAAACGCTCAA-3'
        EcoRI    T7 promoter

PCR primer 2:
5'-CGCCCTGCAGGGGCCTCATGTCGAA-3'
       PstI
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Fig. 1. Starting RNA pool and the PCR primers used for in vitro selection.

next round was prepared by in vitro transcription from the amplified cDNA. From the 1st to the 13th round, the above selection procedure was applied. After 13 rounds, the selection was continued using a filter-binding method [24]. The renatured RNAs and the Raf-1 GST-RBD were incubated in buffer A at 37°C for 60 min. The RNAs bound to the Raf-1 GST-RBD were separated from the unbound RNAs by filtration through a nitrocellulose filter (Millipore, Bedford, MA, USA), followed by washing with buffer B three times. The RNA molecules were eluted from the filter as described [24]. To eliminate the RNA species that bind to the filter, the RNAs were passed through the filter prior to amplification [29].

2.3. Cloning and sequencing

The PCR product of the 21st round of selection was digested with the *Eco*RI and *Pst*I endonucleases, subcloned into pUC119, and then transformed into *E. coli* MV1190. The plasmid DNA was isolated by alkaline lysis and was sequenced by a dye terminator method (Applied Biosystems) on an automatic sequencer (model 377, Applied Biosystems).

2.4. Nitrocellulose filter-binding assay

The RNAs were transcribed from the PCR products by T7 RNA polymerase (Epicenter Technologies) with [α-^{32}P]UTP (3000 Ci/mmol; Amersham Pharmacia Biotech). The renatured RNA (0.8 μM) and the protein (0.8 μM) were incubated in 60 μl of buffer A at 37°C for 60 min. A portion (50 μl) of the solution was applied to the pre-wetted nitrocellulose filter under gentle vacuum and the filter was washed with 200 μl of buffer B three times.

For the determination of the dissociation constant, K_d, 1.6 nM of the 5′-labeled RNA was incubated with various concentrations (50–1250 nM) of proteins. The amount of radioactivity on the filter was measured with a Bio-imaging analyzer (Fuji BAS 2500). The K_d was calculated by using the Kaleidagraph software (Abelbeck Software, Reading, PA) [24].

2.5. Competition assay

The Raf-1 GST-RBD (25 pmol), in 160 μl of buffer A containing 0.05% Triton X-100, was mixed with 10 μl of the matrix (glutathione-Sepharose 4B) suspended in phosphate-buffered saline (50% slurry). The mixture was incubated at 4°C for 30 min. After brief centrifugation, the supernatant was discarded. Then, the matrix was mixed with Ras (2 pmol), which had been complexed with either GTPγS or GDP as described previously [3], and was incubated with the renatured RNAs (0–12.5 μM) in buffer A (160 μl) at 4°C for 30 min. After the incubation, the matrix was washed with 500 μl of buffer B three times. The proteins were eluted from the matrix by denaturation with Laemmli's buffer and were fractionated by 15% SDS-PAGE. The immunoblots were probed with the anti-Ras antibody RAS004 [30] and were visualized by using the ECL immunodetection system (Amersham Pharmacia Biotech).

3. Results

3.1. Isolation of RNA aptamers to the Raf-1 RBD

To focus on the isolation of aptamers that specifically inhibit the interaction between Raf-1 and Ras, we utilized the Ras-binding domain (GST-RBD) of Raf-1 as a selection target. Since the RBD is a basic protein, it binds to RNAs non-specifically at low ionic strength. The non-specific binding of the RNA pool to the protein decreased by increasing the salt concentration. Consequently, a phosphate buffer containing 137 mM NaCl was used as the binding buffer for selection. In vitro selection was started with a pool of RNAs containing a random 60-nucleotide sequence (Fig. 1). The number of RNA species present in the initial (round 0) pool was esti-

RNA clone[a]	Sequence	RNA Bound(%)[c]
Tight binding clones		
21.01(6)	CUGAUCAAUGGCGUACAAUGGAUUCGUUCUCAUAACCAAAACCCUUACCCCUUGGACUGA	42
21.02	CUGAUCAAUGGCGUACAAUGGAUUCGUUCUCAUAACCAAAACCCUUACCCC-UGGACUGA	
21.03(4)	CUGAUCAAUGGCGUACAAUGGAUUCGUUCUCAUAACCAAAACCCUUACCCCUUGGACUGC	
21.04	CUGAUCAAUGGCGUACAAUGGAUUCGCUCUCAUAACCAAAACCCUUACCCCUUGGACUGC	
21.05	CUGAUCAAUGGCGUACAAUGGAUUCGUUCUCAUAACCAAAACCCUUACUCCUUGGACUGC	46
21.06	CUGAUCAAUGGCGUACAAUGGAUUCGUUCUCAUAACCAAAACCCUUACCCCUUGGACUGU	
21.07	UUGACUCAAUGGCGUACAAUGGAUUCGUUCUCAUAACCAAAACCCUUACCCCUUGGACUG	46
21.08[b]	UUGAAGAUCGUACAAUGGAUUCGAUCAUAACCCGAAGUUUUUAAACACUCUUUACCUGUA	12
21.09	UUUGCCUCGACGGUCUGCGAAUAGAACGCGAACCGUGAUUAGUGUACAA-GGAUUCGGUU	16
Weak binding clones		
21.10	UAUUACCAUAGCCUUGAGGUAAACAAUUUAGCACACCUGAAUACACGAACUAUGAACUCA	
21.11(2)	CUUGAGCCAAUUAAAAGAUUUACAACAAGAACAUGAACGUGACAGCGAUAAUAAUACGA	3
21.12	GCGACAAGCAGCAGAUAAAGUUGAGCGCAACGCCGCUACAGAACCAAAUUAACAUGUAUG	0.2
21.13	UCGAAAGUAAGUCCGAUACAACACAUAACCUAUUAUUUAGCAGCGAUAAUACAAAUAAG	
21.14	GCAGUAAUCCACUUGUAAUUGAAUGUAGAUGCCAUAUAGAGUUAUUAGUAAUCCGAAUUG	
21.15	CGUAGUAGCACACCAUGACCUAUUAAAUCUGCUUCGCAAUGUACCUUAACACAUAAUCAG	
21.16	GAAUGACUAAUAAUUACAACAGAUAACCUUACUCUUGAUAAAUGCUUUGCUUUUGGUUAA	1
21.17	UCUUCGAAGUCCAUGACUGCAAAACCAGAUAGUCCUAAUCUCAAUUAUCAGUCCCAAGUA	
21.18	ACACUCUAAAUUGUGGUACUAAGGGAGUAAGGGCAACUACGAAGACGUGCAAGGAUAAAG	0.5
21.19	UCGAGUCCACGAACAUUACAUAUUUGAACACUUCAGCACCGAACAUGCUUAGUACUAUCC	
21.20	GUCGCAGCAGAAAUAUCAUCGCAAAACCUCAAUUGCAUCUCAUGUAUAUCUAGUCCAA	0.7
21.21	CGAACAUCUGGAGUAAUCAUCUUAAUAACCUCAUUAACCUUUACACUUUCUAAACUA	
21.22	GGGUAAGGGUGAGCAGUUCAAGAUGGUAACUGGCAUUCAUUUGAAGAAAGGUUGGUAGAC	1.3
21.23	GGGUAAGGGUGAGCAGUUCAAGAUGGUAACCGGCAUUCAUUUGAAGAAAGGUUGGUAAAC	
21.24	CUUGGUGUAGUGUUCAAGUGAGAUAUAGUAUAAGGUUAUUGUUGUGCGAACGG	

Fig. 2. Sequences in the random region of the RNAs from the pool in the 21st round. The entire RNA includes the 5′- and 3′-defined sequences, as designated in Fig. 1. The RNA clones including the GUACAA(U)GGAUUCG sequences (underlined) are in the upper group. [a]The number in parentheses indicates the number of the same clone. [b]Clone 21.08 has two point mutations in the defined sequences. [c]The binding ratios of the RNAs to the Raf-1 GST-RBD were measured by a nitrocellulose filter-binding assay.

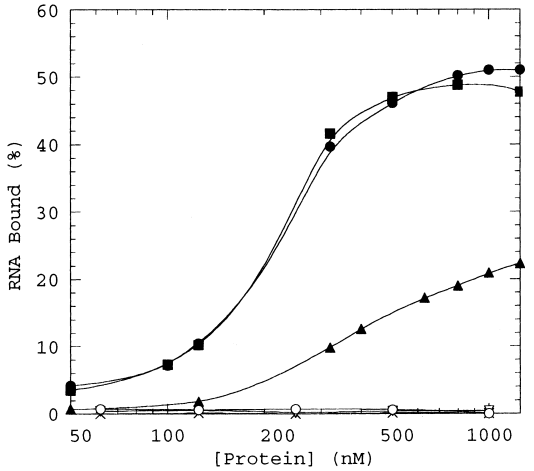

Fig. 3. Binding curves of the cloned RNAs with the proteins (●, ○, ▽, and ×: 21.01 with the Raf-1 GST-RBD, the RGL GST-RBD, GST, and the GTP-bound Ras, ■: 21.07 with the Raf-1 GST-RBD, ▲: 21.11 with the Raf-1 GST-RBD). Data points were obtained by the nitrocellulose filter-binding assay.

mated to be 8×10^{13}, and about 10 copies of each RNA species were included in the pool. Selection was first carried out by immobilization of the protein-RNA complexes on the glutathione-Sepharose 4B matrix. After 13 rounds of selection, the binding ratio of the RNA pool was 0.36%. Accordingly, further selection was continued using nitrocellulose filter immobilization instead of the aforementioned matrix. In addition, we raised the selection temperature from 4°C to 37°C, considering our future plan to use the RNA aptamers as competitive inhibitors of the Ras-Raf interaction in vivo. As a result, the binding ratio of the 21st round pool was sufficiently increased, to 28%.

The sequences of 33 clones isolated from the 21st pool were determined, and 24 different clones were obtained (Fig. 2). The interactions of 10 RNA clones with the Raf-1 GST-RBD were examined by the nitrocellulose filter-binding assay (Fig. 2). The RNA clones were separated into two groups. The binding ratios of clones 21.01–21.09 were over 10%, while the other RNA clones only weakly bound to the Raf-1 GST-RBD ($<3\%$). The K_d values of 21.01 and 21.07 were determined to be 332 ± 93 nM, and that of 21.11 was in the micromolar range (Fig. 3). The 21.01, 21.05, 21.07, 21.08, and 21.09 RNAs did not bind to GST by itself, indicating that these RNAs bind to the Raf-1 RBD moiety rather than the GST moiety. Seven sequences, 21.01–21.07, were probably derived from a single parental clone that accumulated mutations during the PCR amplification. The nine tightly binding clones, 21.01–21.09, were found to have in common a partial sequence of GUACAAUGGAUUCG, except that 21.09 lacks the central U. This conserved sequence, GUACAAUGGAUUCG, was not found in the sequences of the weakly binding clones, 21.10–21.24. The Mulfold program was used to generate possible secondary structures of the RNAs [31]. RNAs 21.01–21.09 extensively form base pairs, involving both the variable and constant regions (Fig. 4). The GUACAA(U)GGAUUCG motif is located in the same internal loop and stem structure (Fig. 4).

3.2. Inhibition of Raf-1 RBD binding to Ras by RNA aptamers

We examined whether these RNA aptamers can interfere with the interaction between Ras and the Raf-1 RBD. Individual RNAs (21.01, 21.07, 21.11, and 21.12), ranging in concentration from 0 to 12.5 μM, and the GTPγS-bound or GDP-bound Ras were incubated with the Raf-1 GST-RBD immobilized on the Sepharose matrix. The binding of Ras to the Raf-1 GST-RBD in the presence and absence of RNAs was detected by immunoblotting with the anti-Ras antibody, RAS004 (Fig. 5, upper panels). RNAs 21.01 and 21.07 effectively decreased the binding between Ras and the Raf-1 RBD (Fig. 5A,B). The RNA concentrations required for the inhibitory effects are consistent with the tight binding of the Raf-1 RBD and the GTP-bound Ras [14]. These RNAs exhibited no affinity for either the GTP-bound Ras (Fig. 3) or the Sepharose matrix (data not shown). In contrast, the weakly binding RNAs, 21.11 and 21.12, had no inhibitory effect on the binding of Ras to the Raf-1 GST-RBD, even when an excess amount was added (Fig. 5C,D). Thus, the inhibitory effects correlate with the binding abilities of these RNA aptamers to the Raf-1 RBD.

For selective inhibition of the Ras-Raf interaction, the RNA aptamers must specifically bind to Raf-1, but not to either the other various downstream effector molecules or Ras. Thus, we examined the interactions of the RNA aptamers (21.01, 21.05, 21.07, 21.08, and 21.09) with another downstream effector, the Ras-binding domain (amino acid residues 632–734) of RGL (RalGDS-like) in the GST-fusion form [26]. The binding ratios of these RNA aptamers were all lower than 0.2% (Fig. 3), indicating that the aptamers sharply discriminate between the two different downstream effectors of Ras. In addition, since these RNA aptamers did not bind to Ras (Fig. 3), they only inhibit the interaction between Ras and Raf-1, with no effects on those between Ras and other effectors.

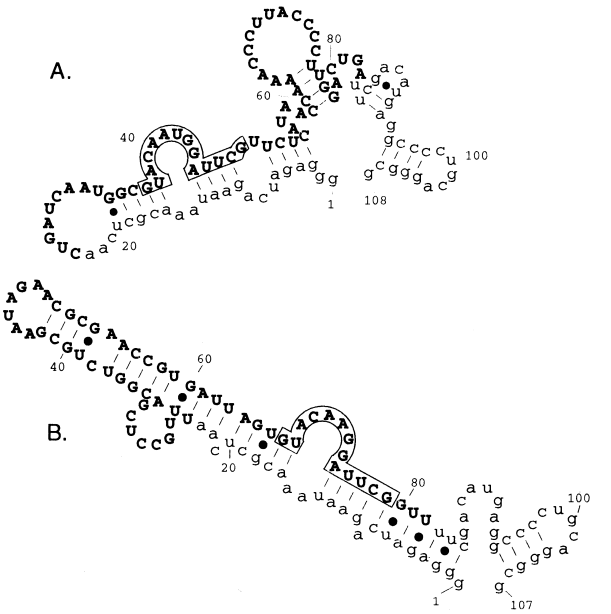

Fig. 4. Predicted secondary structure of 21.01 (A) and 21.09 (B). The 5′- and 3′-defined sequences are shown in lower-case letters. The random regions are shown in bold letters. The conserved sequence is enclosed.

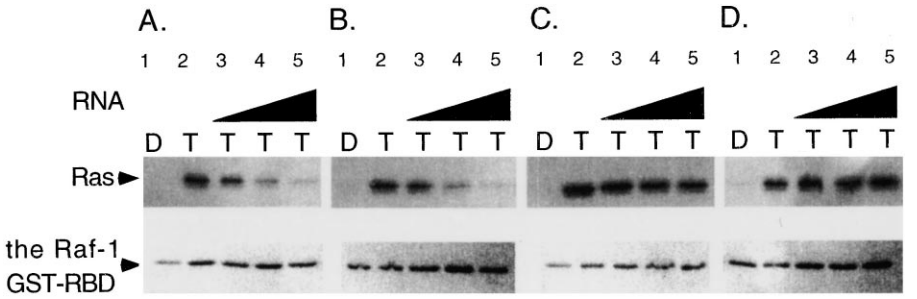

Fig. 5. Inhibition of the interaction between the Raf-1 GST-RBD and Ras by RNA aptamers. A: 21.01, B: 21.07, C: 21.11, and D: 21.12. The amount of Ras binding to the Raf-1 GST-RBD was measured by immunoblotting with the anti-Ras antibody, RAS004 (upper panels). The amount of the Raf-1 GST-RBD was detected by staining with Coomassie brilliant blue (lower panels). Ras (2 pmol), in either the GDP-bound (D) or the GTPγS-bound (T) form, and the Raf-1 GST-RBD (25 pmol) were incubated in the absence and presence of various amounts (lanes 1, 2, 3, 4, and 5: 0, 0, 0.125, 1.25, and 12.5 μM) of the RNAs.

4. Discussion

The RNA aptamers that we isolated bind to the Raf-1 RBD, but not to the RGL RBD, and competitively inhibit the Ras-Raf interaction. The binding site for these aptamers on the Raf-1 RBD is likely to be at or near the binding interface for Ras, although they could possibly interact with other regions of the Raf-1 RBD. The amino acid residues involved in the interface between the Raf-1 RBD and Ras have been identified by mutation analyses [32,33], and also from the crystal structure of the complex between the Ras-like mutant Rap1A and the Raf-1 RBD [34]; several Raf-1 RBD residues, such as Arg-59, Lys-84, and Arg-89, are located in an α-helix and the β-strands of a ubiquitin-like fold [35], and interact with several Ras residues, such as Glu-31, Asp-33, Glu-37, and Asp-38, in the 'effector region'. These RNA aptamers to the Raf-1 RBD discriminate against the RGL RBD with a low sequence homology. On the other hand, the Ras-binding domain of RalGDS (a homolog of RGL) is in the ubiquitin-like fold, which is nearly the same as that of the Raf-1 RBD, and displays similar amino acid residues in the regions corresponding to the Ras-binding interface of the Raf-1 RBD [36–38]. These structural features are well conserved in the RGL RBD (unpublished results). Nevertheless, the slight differences in the interface residue arrangement between the Raf-1 RBD and the RGL and RalGDS RBDs [34,38] are likely to be recognized by the present RNA aptamers. Intriguingly, the Raf-1 RBD and Ras are rich in positively charged residues and negatively charged residues, respectively, with respect to their binding interfaces, as described above. Therefore, the negative charges of the RNA aptamers might be involved in the interaction with the Raf-1 RBD. In this context, it has been reported that RNA aptamers to thrombin, vascular endothelial growth factor, and basic fibroblast growth factor, bind to their target proteins competitively with electronegative heparin, suggesting that these aptamers interact with the electropositive heparin-binding site [39–41].

The aptamers to the Raf-1 RBD may be used to specifically inhibit the Ras-Raf interaction in the complicated signaling network in cells, without affecting other downstream effectors of Ras. The anti-Raf-1 aptamers would be delivered through the cell membrane, or transiently expressed in the cell, as reported for RNA aptamers to HIV Rev [42]. The anti-Raf-1 aptamers may also be potential diagnostic and therapeutic tools targeting the interaction between two proto-oncogene products, Ras and Raf-1, as in the cases of aptamers to human neutrophil elastase used in in vivo imaging of inflammation [43] and in RNA therapy for lung inflammatory injury [44].

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