Location and Functional Significance of Retinol-binding Sites on the Serine/Threonine Kinase, c-Raf*  

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Redox activations of serine/threonine kinases represent alternate pathways in which vitamin A plays a crucial co-factor role. Vitamin A binds the zinc finger domain of c-Raf with nanomolar affinity. The retinoid-binding site has been mapped within this structure by scanning mutagenesis. The deduced contact sites were found anchored on Phe-8, counting from the 1st conserved histidine of the zinc finger. These sites agreed with contact amino acids identified by computational docking. The boundaries of a related binding pocket were identified by mutagenesis and partially confirmed by docking trials in the protein kinase Cα C1A zinc finger. They comprised Phe-7, Phe-8, and Trp-22. This trio was absent from the cC1B domain, explaining why the latter did not bind retinol. Reconfiguring at a minimum the two corresponding amino acids of αC1B, Thr-7 and Tyr-22, to conform to αC1A converted this domain to a binder. Deletion of the predicted retinoid-binding site in the full-length molecule created a mutant c-Raf that was deficient in retinol-dependent redox activation but fully responsive to epidermal growth factor. Our findings indicate that ligation of retinol to a specific site embedded in the regulatory domain is an important feature of c-Raf regulation in the redox pathway.

The history of vitamin A research contains a medley of observations concerning widespread physiological roles of retinoids other than the well known functions of retinoic acid in transcription and retinaldehyde in vision (reviewed in Ref. 1). Most convincing for the non-nuclear functions of vitamin A are arguments pointing to the evolution of an elaborate retinoid biochemistry and biology in eukaryotic organisms (2), predating by far the advent of retinoid acidic receptors and retinoid X receptors and the conservation of the vitamin A metabolites, along with the requisite enzymes, from insects to man. Furthermore, essentially all nucleated cells of higher vertebrates store vitamin A in the form of retinyl esters for ready retrieval and conversion to a variety of metabolites. Because these retinoid products, more often than not, exclude retinoic acid, the question arises as to their purpose.

The multitude of defects caused by nutritional vitamin A deficiency, not completely reversible by retinoic acid and ranging from multiple developmental abnormalities (3, 4), to immune defects (5–8), and to male sterility (9, 10), was not explainable by a single non-nuclear target. In fact, multiple molecular targets emerged when the serine/threonine kinases were found by us to harbor high affinity retinoid-binding sites (11). These were encoded within the cysteine-rich domains of several PKC isoforms and c-Raf and overlapped with known structures intimately involved with kinase regulation. This is where lipid second messengers bind and activate the conventional and novel PKC isoforms (12–14) and where, in c-Raf, a crucial half-site is located for recognition of the activating GTP/Ras protein (15–17). Nevertheless, the purpose of retinoid-binding sites remained elusive as the classical receptor tyrosine kinase pathways leading to PKC and c-Raf activation operated independently of vitamin A. With the discovery of the alternate pathway of serine/threonine kinase activation via reactive oxygen species (18, 19), this situation changed. We could show that vitamin A itself served as an essential co-factor in redox activation of both PKCs and c-Raf (20, 21). The hypothesis was developed that the binding of vitamin A to the cysteine-rich domains was required for the controlled oxidation of defined cysteines (22). When absent because of nutritional deficiency or when experimentally displaced by non-functional retinoid antagonists, such as anhydroretinol, PKC, and c-Raf, activation by reactive oxygen species was severely compromised.

Jakob et al. (23) proposed that in bacteria, cysteine-rich domains are organized into a zinc finger fold that functions as a molecular hinge. The mammalian counterpart, although more complex, also forms a composite zinc finger (24, 25). Upon oxidation, the latter has been shown to relinquish the central zinc ions, allowing potentially a conformational change (26, 27). Bound retinol accelerated this oxidation process. Surprisingly, phorbol ester and diacylglycerol also caused the release of Zn\(^{2+}\) from the PKCα zinc finger (27). Thus, the two chemically dissimilar activators, reactive oxygen species and phorbol ester, nevertheless produced the same outcome: disassembly of the zinc finger, and presumably, a conformational shift. Unfolding has long been postulated as a prelude to the release of the auto-inhibition that the regulatory domain imposes on the catalytic domain (28, 29).

Although the role as co-factor in redox regulation presents a conceptual advance to explain vitamin A action, explorations of biological significance have not kept pace. The reason can be traced to the multitude of target molecules (i.e. the PKC and

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1 The abbreviations used are: PKC, protein kinase C; cys, cysteine-rich domain; EGF, epidermal growth factor; GST, glutathione-S-transferase; WT, wild type; GTP\(^{\gamma}\)S, guanosine 5’-3-O-thiotriphosphate; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ELISA, enzyme-linked immunosorbent assay.
Retinol-binding Sites on c-Raf

Sequence of PKC zinc-finger domains, predicted retinol contact sites in bold.

|    | PKCa | ClA | 037 |
|----|------|-----|-----|
|    | PKCa | ClB | 102 |
|    | PKCa | ClB | 231 |
| h  | c-Raf | 139 |

Bacteria Growth and Protein Purification—The c-Raf-cys domain WT and mutants were expressed as GST fusion protein in the BL21/DE3 strain of Escherichia coli (Novagen) (11). Bacteria were initially grown at 37 °C to an optical density (OD) at 600 nm of 0.5, transferred to room temperature. At an OD<sub>600</sub> of 0.7–0.8, protein synthesis was induced by 0.5 mM isopropyl-β-D-thiogalactopyranoside, and the cells harvested 2 h later. Bacteria were passed twice through a French press, and the GST fusion proteins were recovered from the lysates by affinity chromatography on the glutathione-agarose matrix. Purity by Coomassie Blue staining of SDS-PAGE was usually >90% by this protocol.

Retinoid Binding Assay by Quenching of the Endogenous Protein Fluorescence—Quantitative fluorescence measurement of 250 nM GST fusion protein with retinoid titration at 25 nM increments were performed as described (11) in phosphate-buffered saline, purged of oxygen by sparging with helium for 15 min, in a Jasco spectrofluorometer (model FP777). The protein solution was excited at 280 nm, and the protein emission was monitored at 330 nm. Binding constants were calculated by non-linear curve fitting according to the theorem by Norris et al. (31). Qualitative retinol binding assays based on vibronic fine structure determinations were performed as described (22).

Basic-Raf Binding Assay—Quantitative binding of GST-Raf cys WT and mutants to GTP·S/Ras was measured by the ELISA described by Gosh et al. (15).

Computational Biology Methods—Docking of retinoid into c-Raf-1 and PKCa was carried out using the software Autodock3.05 (32) to find potential binding sites of retinoid. Autodock used a Monte Carlo simulated annealing technique for conformational exploration with a rapid energy evaluation using grid-based molecular affinity potentials. It thus combined the advantages of exploring a large search space and a robust energy evaluation. The method has proven to be a powerful approach to the problem of docking a flexible ligand into the binding site of a static protein. Van der Waals interactions were calculated using a Lennard-Jones 12-6 potential, whereas the hydrogen-bonding term was modeled by Lennard-Jones directional 12-10 potential. Electrostatic potential energies were calculated with a distance-dependent dielectric function. Chemical preference terms were also added in its scoring function. The program was tested on a number of protein-substrate complexes, which had been characterized by x-ray crystallography (33) as recommended for docking studies.

c-Raf structure was taken from Protein Data Bank accession number 1FAR (24), and PKCa C1A structure was modeled using IPTQ (25) as template for the program Modeler (34) as part of the molecular modeling software package InsightII from Accelrys. NMR structure coordinates for cC1B were kindly provided by Dr. Marcel Lytten (35). Modeller is known to produce reliable models when high homologous high resolution crystal structures are available as template, which is the case in our study. Quality of the model was checked with modules available in InsightII package.

RESULTS

The GST fusion protein comprising c-Raf amino acids 139–184, spanning the zinc finger domain, encodes one high affinity retinol-binding site (11) and a half-site for GTP/Ras recognition (16, 36). PKCa C1A and C1B zinc fingers comprise amino acids 37–86, and 102–151, respectively. To maintain a unified numbering system between the c-Raf and PKC zinc fingers, the conserved cysteines and histidines were aligned with each other, requiring a gap for c-Raf since this domain lacks the 4-amino-acid phorbol-binding loop. Numbering began at the 1st histidine. (Fig. 1)

To map the retinol-binding site of c-Raf, we converted consecutively all amino acids (except Cys and His of the zinc finger chains) and attendant vitamin A-dependent signal pathways. We have devised a genetic approach that is predicated on the elimination of retinol-binding sites in select signaling molecules. This was accomplished by mutation of critical contact amino acids of the zinc finger domain. As reported here for the example of c-Raf, the impairment of binding for retinol was paralleled by the selective loss of redox regulation, whereas kinase activation by the classic hormone receptor signals was retained. Additionally, introducing three critical contact residues, copied from the PKC zinc finger domains, into the natural non-binding C1B domain conferred retinol binding capacity.

MATERIALS AND METHODS

Reagents—All-trans retinol, GTP·S, bovine serum albumin (enzyme immunoassay grade), p-nitrophenyl phosphate, glutathione-agarose beads, anti-FLAG® M2-agarose affinity gel, anti-rabbit IgG-alkaline phosphatase conjugate, and epidermal growth factor (EGF) were purchased from Sigma. Rabbit antibody to c-Raf C20-terminal peptide was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and anti-glutathione S-transferase (GST) antibody was purchased from Amersham Biosciences.

Cell Culture—COS-7 cells were grown and maintained in Dulbecco’s modified Eagle’s medium high glucose supplemented with 10% fetal calf serum and L-glutamine without antibiotics.

Plasmids and Mutagenesis—The cys domains of c-Raf (124–190), PKCa (137–57), and c-Raf-102–151 were cloned by PCR into the BamHI and EcoRI sites of GEX-2T vector (Amersham Biosciences). Mutagenesis of FLAG-c-Raf full-length (a gift from Dr. R. J. Davis, University of Massachusetts, Worcester, MA) and the cys domains was performed using the QuikChange® site-directed mutagenesis kit (Stratagene). Transfection and Cell Activation—COS-7 cells were transfected by the calcium phosphate method as described (20). To deplete endogenous retinol, cell cultures were first incubated for 30 min with 1 μM anhydroretinol and then cultured with 2 ml of retinoid-free, serum-free, hormone receptor signals was retained. Additionally, introduction of three critical contact residues, copied from the PKC zinc finger domains, into the natural non-binding C1B domain conferred retinol binding capacity.

Kinase Assay—This was carried out as described (31) using cell lysates obtained after exposure to human EGF at 100 ng/ml. Cul-
Head that permitted retinol to dock optimally. Additional con
A groove was identified in the c-Raf zinc finger with Phe-8 at its
modate retinol in headfirst orientation at an energy minimum.
structed to search for a hydrophobic pocket that would accom-
quench method.

Titrations of retinol yielded, after correction for inner filtering, were plotted versus retinol concentration. Affinity constants were computed according to Norris et al. (31). Shown are select examples of peptides listed in Table I.

Because point mutations can cause broad structural disruptions, it was desirable to ascertain the integrity of each mutated protein. Otherwise, the loss of retinol binding might merely reflect a general structural collapse. The zinc finger domain encodes a face where GTP/Ras docks (17). The mutated peptides were tested for retention/loss of GTP/Ras binding capacity by an ELISA devised by Gosh et al. (15). Briefly, GTP/Ras was adsorbed to ELISA plates and incubated with WT and mutant GST fusion proteins. Anti-GST antibody-conjugated phosphatase was used to detect the amount of bound fusion proteins. As shown in Fig. 3 the two retinol-binding loss mutants, F8W and T33W, retained Ras binding capacity comparable with WT.

To experimentally confirm predictions from the docking trials, mutations were introduced into the αC1A domain. As shown in Table II, mutating any of the presumptive contact amino acids (Phe-7, Phe-8, or Trp-22) alone was ineffective, unlike in c-Raf, where single point mutations impaired retinol binding significantly. Double mutations of the presumptive hydrophobic amino acids Phe-7 and Phe-8 believed to contact the head group slightly lowered the binding affinity. However, dual substitutions involving either Phe-7 or Phe-8 in combination with Trp-22 drastically diminished retinol binding. Amino acids Thr-7, Tyr-8, and Tyr-22 are characteristic of the non-binding αC1B domain. When copying this motif into the αC1A domain, retinol binding was abolished, whereas phorbol ester binding was preserved (data not shown), indicating that this triple mutation was structurally permissible.

To test whether αC1B can be converted to a binder, we introduced the reverse set of mutations, T7F/Y22W. Retinol binding assays of the corresponding GST fusion protein by quench of intrinsic fluorescence (Fig. 5A), fluorescence resonance energy transfer, enhancement of retinol fluorescence

![Fig. 2. Determination of binding constants by fluorescence quench method. 250 nM solutions of WT or mutant GST fusion proteins were titrated with retinol added in 25 nM increments. Fluorescence intensity values (excitation, 280 nm; emission, 330 nm), corrected for inner filtering, were plotted versus retinol concentration. Affinity constants were computed according to Norris et al. (31). Shown are select examples of peptides listed in Table I.](Image)

![Fig. 3. Determination of binding capacity for GTP/Ras. Select examples of mutated and WT GST fusion proteins are presented. Binding capacity was measured by the ELISA of Gosh et al. (15).](Image)
emission, and the red-shifted fluorescence excitation spectrum with vibronic fine structure of retinol (Fig. 5B) all indicated strong retinol binding. By titrating retinol and applying non-linear fitting to the quench curve of Fig. 5A, an apparent binding affinity of 15.7 nM was obtained, comparable with the value reported for the αC1A domain (20.7 nM (22)). Docking trials with this in silico mutant yielded a well defined site (Fig. 4, model F).

As reported previously, c-Raf activation by UV was retinol-dependent (20). This was deduced from experiments showing a marked decrease in the endogenous c-Raf kinase activity vis-a-vis MEK, when retinol-deprived cells were activated by UV irradiation, as compared with cells grown in vitamin A-sufficient medium or with cells reconstituted with retinol shortly prior to activation. We tested whether c-Raf lacking a functional retinol-binding site would yield a similarly reduced response as wild-type c-Raf nutritionally deprived of its retinol co-factor. The FLAG-tagged, full-length c-Raf WT, single mutants F146W (F8W), T167 (T33W), or double mutant F146W/T167W (F8W/T33W) were expressed in COS cells. After serum starvation, cultures were reconstituted with retinol 0.5 h prior to activation by UV irradiation. c-Raf phosphotransferase activity was determined in immunoprecipitates using kinase-dead MEK as substrate. As shown in Fig. 6, retinol permitted expression of high kinase activity in response to UV irradiation. The ratio of activity with 10 μM retinol over reference value without retinol was 3.5. The important result was that the loss of either contact site, Phe-8 or Thr-33 of the zinc finger domain, that prevented retinol-binding also diminished kinase activation by roughly 1/3 or 1/2, respectively, indicating that the retinol

| Point mutations | KD (nM) | Ras Interaction |
|-----------------|---------|----------------|
| 2 140 N         | 8.4 +/- 4.8 | ± |
| 2 141 F         | 5.3 +/- 0.2 | ± |
| 4 142 A         | 10.3 +/- 0.7 | ± |
| 5 143 R         | 9.3 +/- 1.0 | ± |
| 6 144 K         | >500 | ± |
| 7 145 T         | 6.7 +/- 1.9 | ± |
| 8 16 F          | >500 | ± |
| 9 147 L         | 20.8 +/- 5.4 | ± |
| 10 148 K        | 7.9 +/- 1.4 | ± |
| 11 149 L        | 8.6 +/- 1.6 | ± |
| 12 150 A        | 9.1 +/- 1.6 | ± |
| 13 151 F        | 6.9 +/- 1.7 | ± |
| 15 153 D        | 15.9 +/- 4.4 | ± |
| 16 154 I        | 4.4 +/- 2.1 | ± |
| 18 156 Q        | >500 | ± |
| 19 157 K        | 16.7 +/- 2.5 | ± |
| 20 158 F        | 9.0 +/- 1.0 | ± |
| 21 159 L        | 14.4 +/- 2.3 | ± |
| 22 160 L        | 12.5 +/- 4.0 | ± |
| 27 161 N        | 12.1 +/- 1.1 | ± |
| 28 162 G        | 11.5 +/- 4.8 | ± |
| 29 163 F        | >500 | ± |
| 30 164 R        | 9.0 +/- 1.2 | ± |
| 32 166 Q        | 19.7 +/- 3.8 | ± |
| 33 167 T        | >500 | ± |
| 34 168 C        | 10.5 +/- 0.5 | ± |
| 35 169 G        | >500 | ± |
| 36 170 Y        | 12.6 +/- 2.1 | ± |
| 37 171 K        | 7.4 +/- 1.7 | ± |
| 38 172 F        | 10.0 +/- 2.7 | ± |
| 40 174 E        | 23.0 +/- 3.4 | ± |
| 41 175 H        | 11.3 +/- 3.9 | ± |
| 42 176 C        | 12.3 +/- 1.4 | ± |
| 43 177 S        | 10.4 +/- 2.8 | ± |
| 44 178 T        | >500 | ± |
| 45 179 K        | >500 | ± |
| 46 180 V        | >500 | ± |
| 47 181 P        | 9.5 +/- 3.6 | ± |
| 48 182 T        | >500 | ± |
| 49 183 M        | >500 | ± |

### Table 1

| Scanning mutagenesis of c-Raf zinc-finger to identify contact residues mediating retinol binding |
|---------------------------------|------------------|----------------|
| Point mutations | * | ** |
| 2 140 N         | W | HNFARKTFLKLAFCDICQKFLINGFCQTCGKYKHFHEICSTKYPTMC |
| 2 141 F         | W | 8.4 +/- 4.8 |
| 4 142 A         | W | 5.3 +/- 0.2 |
| 5 143 R         | W | 10.3 +/- 0.7 |
| 6 144 K         | W | 9.3 +/- 1.0 |
| 7 145 T         | W | >500 |
| 8 16 F          | W | 6.7 +/- 1.9 |
| 9 147 L         | W | >500 |
| 10 148 K        | W | 20.8 +/- 5.4 |
| 11 149 L        | W | 7.9 +/- 1.4 |
| 12 150 A        | W | 8.6 +/- 1.6 |
| 13 151 F        | W | 9.1 +/- 1.6 |
| 15 153 D        | W | 6.9 +/- 1.7 |
| 16 154 I        | W | 15.9 +/- 4.4 |
| 18 156 Q        | W | 4.4 +/- 2.1 |
| 19 157 K        | W | >500 |
| 20 158 F        | W | 16.7 +/- 2.5 |
| 21 159 L        | W | 9.0 +/- 1.0 |
| 22 160 L        | W | 14.4 +/- 2.3 |
| 27 161 N        | W | 12.5 +/- 4.0 |
| 28 162 G        | W | 12.1 +/- 1.1 |
| 29 163 F        | W | 11.5 +/- 4.8 |
| 30 164 R        | W | >500 |
| 32 166 Q        | W | 9.0 +/- 1.2 |
| 33 167 T        | W | 19.7 +/- 3.8 |
| 34 168 C        | W | >500 |
| 35 169 G        | W | 10.5 +/- 0.5 |
| 36 170 Y        | W | >500 |
| 37 171 K        | W | 12.6 +/- 2.1 |
| 38 172 F        | W | 7.4 +/- 1.7 |
| 40 174 E        | W | 10.0 +/- 2.7 |
| 41 175 H        | W | 23.0 +/- 3.4 |
| 42 176 C        | W | 11.3 +/- 3.9 |
| 43 177 S        | W | 12.3 +/- 1.4 |
| 44 178 T        | W | 10.4 +/- 2.8 |
| 45 179 K        | W | >500 |
| 46 180 V        | W | >500 |
| 47 181 P        | W | 9.5 +/- 3.6 |
| 48 182 T        | W | >500 |
| 49 183 M        | W | >500 |
co-factor function normally required for c-Raf activation in the redox pathway decreased markedly (from 3.5- to 2.3-fold \((p < 0.05)\) and 1.8-fold \((p < 0.01)\), respectively. The double mutant also became insensitive to retinol (relative enhancement 1.2-fold with \(p < 0.001\)), but the EGF responsiveness declined as well.

**DISCUSSION**

Systematic scanning mutagenesis is a valid, albeit not conclusive, method of mapping receptor sites. Although pertinent contact sites can be reliably identified, the major drawback is the frequency of false negatives. To the extent that the retinol-binding site likely depends on proper protein folding, amino acid substitutions violating the overall structure may lead to loss of binding. This would be expected for the 6 Cys and 2 His conserved residues needed for zinc coordination (Fig. 1). The respective point mutations were therefore omitted. Other amino acids may be structurally essential in unexpected ways.

### Table II

EFFECT OF MUTAGENESIS OF PREDICTED CONTACT AMINO ACIDS OF PKCα C1A MEDIATING RETINOL BINDING

| Mutated Peptide | Retinol Binding Constant ± S.E. (nM) |
|-----------------|-------------------------------------|
| Wild type       | 28.3 ± 8                            |
| F7T             | 73 ± 54                             |
| F7G             | 69 ± 26                             |
| F8Y             | 13 ± 4                              |
| F8G             | 27.2 ± 6.5                          |
| W22Y            | 30.6 ± 10                           |
| Double          |                                     |
| F7T/F8Y         | 71 ± 26                             |
| F7G/F8G         | 55.8 ± 20                           |
| F7T/W22Y        | >500                                |
| F8Y/W22Y        | >500                                |
| Triple          |                                     |
| F7T/F8Y/W22Y    | >500                                |

Fig. 4. Presumptive retinol-binding grooves on c-Raf and PKCα zinc finger domains. Retinol is illustrated in a green ball-and-stick representation. Presumptive residues in contact with retinol are labeled and space-filled. Model A, nuclear magnetic resonance structure of c-Raf zinc finger domain based on the Protein Data Bank code 1FAR (24). According to the top scoring docking results, Phe-8, Leu-9, and Ala-12 are common residues to hold the \(\beta\)-ionone ring in c-Raf (models B and C) with the lowest energy retinol conformations. The retinol tail is oriented slightly different around residue Cys-34 in model B as compared with model C. A cluster of retinols is shown to bind the same PKCα C1A region as that of the c-Raf zinc finger (model D). Phe-7 and Phe-8 contact the retinol head group. Retinols scattered around αC1B (model E) indicate no preferred binding site, whereas introducing T7F and Y22W mutations into αC1B permits docking of a cluster of retinol in the same region as identified in αC1A and c-Raf (model F).
ditionally, the mutations, S43W, T44W, K45W, V46W, T48W, and M49W, led to retinol binding loss without impacting Ras binding. These amino acids are located on a contiguous stretch on the $\alpha$-helical peptide near the C terminus, form a mildly hydrophilic topology, and represent a potential second retinol-binding region.

Sequence comparisons have not yielded any obvious motif for a retinol-binding site. To narrow the choices among candidate binding sites empirically, ligand-docking experiments were performed, using the published coordinates of the PKC- and c-Raf zinc fingers from X-ray crystallographic (25) and NMR (24, 35) studies. We searched for a hydrophobic groove accommodating the retinol molecule, with the stipulation that retinol bound headfirst. This choice recommended itself because of our previous findings that different retinoid species bound zinc finger domains with similar affinity (11) and that any one of these could displace the other (21). The most likely explanation was that the conserved hydrophobic $\beta$-ionone ring and polyene furnished the contact surfaces, whereas the hydrophilic groups at carbon 15, where most of the chemical differences resided, were less likely to contribute binding affinity. The inferred orientation also accounted for the biological antagonism between the hydroxylated family of retinoids (retinol and 14-hydroxy-retinoic acid) and carboxylated ones (retinoic acids) or non-substituted retinoids (anhydroretinol) (38) since pharmacological mutual reversible inhibition is best explained by competition of a conserved structure for binding to a single receptor.

Based on the highest number of hits in the docking trials with c-Raf, the most likely binding groove was defined by amino acid Phe-8, anchoring the retinol ionone ring, whereas Thr-33 bound the tail end (Fig. 4, model B). These contact sites also stood out in the mutational analysis. Importantly, Phe-8, or a Tyr residue at that position, is conserved among the zinc.
Redox activation of c-Raf obeys the same paradigm that governs PKC activation, although in distinction to PKC, it requires interaction with GTP/Ras. Requirement of intact retinol-binding sites, modification of thiol, and release of zinc as preludes for catalytic activation add up to the notion that serine/threonine kinases can sense and respond directly to changes in the redox microenvironment.

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