Minireview

# The Grb2 adaptor

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**Abstract Grb2 is an 'adaptor' protein made of one SH2 and two SH3 domains. The SH3 domains bind to prolinerich motifs in the C-terminal part of the ras exchange factor Sos. Binding of the Grb2 SH2 domain to phosphotyrosine motifs on receptors, or other adaptor proteins such as Shc, recruits this Grb2/Sos complex at the plasma membrane where Sos stimulates nucleotide exchange on ras, then ras activates raf and leads to MAP kinase activation. The structure of Grb2, the precise motifs recognised by its SH2 and SH3 domains, the way Grb2 performs its function, a possible regulation of its association with Sos, and its ability to complex with other proteins in vivo, are discussed.**

*Key words:* Grb2; Ras; SH2 domain; SH3 domain; Sos; Tyrosine kinase

## 1. Introduction

The Growth factor binding protein Grb2 is an 'adaptor' protein made of one SH2 domain flanked by two SH3 domains [1] (Fig. 1). The SH2 domain bind to specific phosphotyrosine motifs, whereas the SH3 domains bind to proline rich motifs. The C-terminal part of the ras exchange factor Sos contains several proline rich motifs and binds Grb2 with a very high affinity. This Grb2/Sos complex mediates ras activation in response to growth factors, in many different kinds of cells: fibroblasts, neural cells, lymphocytes, etc. Then ras activates raf and starts the cascade of phosphorylations leading to MAP kinase activation. The precise role of Grb2 to start this pathway is still a matter of debate. Is it only a passive adaptor that plugs the associated Sos on the receptor to activate ras, or does it play an active role in signal transduction? Does Grb2 bind to other proteins than Sos and has other functions?

The structure of complete Grb2 has been solved and Grb2 is now the best characterised member among the growing family of adaptor proteins that also includes Crk or Nck [2].

## 2. Function of Grb2 homologs in nematodes and Drosophila

Sem-5, the Grb2 homolog from *C. elegans*, was discovered as a gene involved upstream of ras in the Let-23 receptor tyrosine kinase signalling pathway that controls vulval development and sex myoblast migration. Several mutants of the SH2 and SH3 domains have been isolated. Loss of function mutants lead to high mortality at the larval stage [3]. The P49L mutation in the N-terminal SH3 domain (N-SH3) mutates a highly conserved proline of the ligand binding site to a leucine and decreases the affinity for proline rich ligands by several orders of magnitude. It is a severe mutation, only 9% of the larva develop to adults and all of them show the 'vulvaless' phenotype. The E89K (Glu $\beta$B8) and S90N (Ser loop B-C) mutations in the SH2 domain are much milder, there's no lethality and only a fraction of the animals develop the 'vulvaless' phenotype. The E89K mutation has only minor effects on the affinity for phosphotyrosine peptides, consistent with the structure of Grb2 SH2 where E89 side chain points to the solvent, thus the structural reason for the biological effects of this mutation is not understood. The G203R mutation in the C-terminal SH3 domain (C-SH3) does not lead to a 'vulvaless' phenotype, it is much milder than the P49L mutation, suggesting that Sem-5 C-SH3 is less important than the N-SH3 for signal transduction, most likely because the G203R mutant of Sem-5 is still able to interact with Sos. In Grb2, the N-SH3 binds proline rich motifs of Sos with a ten times higher affinity than the C-SH3 [4,5] indicating that the N-SH3 contributes more to Sos binding than the C-SH3. However we should keep in mind that the P49L or the G203R mutations greatly decrease the affinity of the corresponding SH3s for proline rich peptides but may not impair all the interactions with the Sos C-terminal (see last paragraph on the complete Grb2 structure).

Drk, the Drosophila homologue of Grb2 makes a complex with Sos [6,7] and Sos is implicated in signal transduction downstream of several tyrosine kinase receptors: the 'sevenless' receptor (Sos stands for Son Of Sevenless, [8]), the Drosophila EGF receptor [9] and Torso (homologous to the PDGF receptor) [10]. As in the nematode, homozygotes for loss of function alleles of Drk die at a larval stage. Two mutants that disrupt signalling downstream of the Sevenless receptor tyrosine kinase have been isolated. The R67H (Arg $\alpha$A2), and H106Y (His $\beta$D4) mutations in the SH2 domain are both affecting directly the phosphotyrosine binding site.

## 3. The Grb2/Sos complex in signal transduction

Since the initial discovery that the Grb2/Sos complex mediates signal transduction from the EGF receptor to ras, in fibroblasts [11–15], this Grb2/Sos complex has been implicated in signal transduction from many other receptor tyrosine kinases in several cell types. The interaction of Grb2 with the insulin receptor involves an additional adaptor: the Shc protein, that also binds Grb2 in a tyrosine phosphorylation dependent manner [16,17]. In myeloid cells too, Shc seems to be important in coupling Grb2/Sos to the M-CSF receptor [18]. A wide vari-

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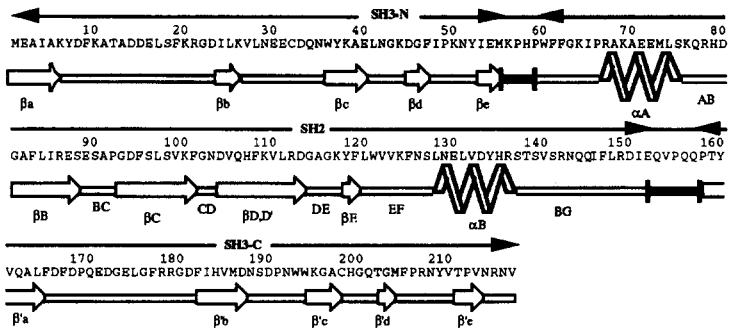


Fig. 1. Grb2 sequence with secondary structure elements. Regions of $\beta$-sheet (arrows) and $\alpha$-helix are indicated below the sequence. SH3 and SH2 domains of Grb2 are connected by linkers (plain line: KPHP sequence for the first and EQVPQQP for the second). This second linker is part of an extended segment (LRDIEQVP) with $\beta$ strand geometry which continues in strand $\beta'$a (QPTYVQAL) of the C-terminal SH3.

ety of stimuli induce tyrosine phosphorylation of Shc, that seems to be mediating ras activation in many cell types. The protein tyrosine phosphatase PTP1D may also be used as an additional adaptor, for instance it is involved in bridging the Grb2/Sos complex to the PDGF receptor [19]. The same role is postulated for its homologue *Corkscrew* and the *Torso* receptor, in Drosophila [10]. Grb2 can also bind to focal adhesion kinase (FAK) when FAK is phosphorylated in response to integrin receptors clustering on extracellular matrix [20], to the receptor protein-tyrosine phosphatase $\alpha$ [21], and to the chimeric oncogene BCR-Abl in human leukemias with a philadelphia chromosome [22]. The involvement of this Grb2/Sos complex in signal transduction in T-cells has been reviewed recently [23]. An alternative splice of Grb2 transcripts leads to the Grb3-3 variant, with a deletion in the SH2 domain, that behaves as a dominant negative protein, suppressing proliferative signals [24]. In NRK cells, EGF or PDGF induce cell division but also actin stress fibers disassembly and membrane ruffles formation (controlled by the small G proteins rho and rac, respectively). Antibodies against Grb2 block both responses, suggesting that Grb2 could also play a role in the activation of small G proteins of the rho/rac family [25].

## 4. Properties of Grb2 SH2 domains

The SH2 domain of Grb2, as other SH2s [26], is made of a central antiparallel $\beta$-sheet flanked by two helices (Fig. 2). The binding site is made of one phosphotyrosine binding pocket conserved in all SH2s (Grb2 R67, R86, S88, S90, H107, K109) and a binding surface for the 3 or 4 residues following this phosphotyrosine that are responsible for the specificity of each SH2 (Q105, F107, H108, L120, W121, Y134, L148). A nice alignment of SH2 domains can be found in [27]. The pioneering work of Songyang et al. [28,29] enabled to define the precise phosphotyrosine motifs recognised by specific SH2 domains. Grb2 SH2 was shown to recognise a YxNx motif. Optimal motifs with a YVNV sequence bind Grb2 SH2 with high affinities ($K_d$ = 10–100 nM). The presence of a lysine at position +4 decreases the affinity by a factor of 2–4 and lowers the tryptophan fluorescence signal, indicating that position +4 may also be involved in the binding to Grb2 SH2 [4]. In Grb2 the preference for asparagine +2 is mainly determined by tryptophan 121 (TrpEF1) [30]. Surprisingly, in the structure of unliganded Grb2 SH2 this tryptophan sits in the ligand binding cleft in a position where it is expected to interfere with binding (Fig. 2) [31], we have to wait for the structure of the SH2-Phosphotyrosine peptide complex to see how the position of this tryptophan is affected by ligand binding. Fluorescence experiments indicate that it moves to a more hydrophobic environment when the ligand is there [4]. Recent results (Cussac et al., in preparation) suggest that the requirement for an asparagine at position +2 is not as strict as previously expected. Nevertheless, the highest affinity binding motifs found so far on Shc, the EGF or HGF receptors, PTP1D, or the BCR-Abl fusion protein, all have a Y(V/E/Q/I) N (V/Q) sequence.

## 5. Properties of Grb2 SH3 domains

Each SH3 domain of Grb2 forms a $\beta$ barrel, as previously described for other SH3s (Fig. 3). Ren et al., were the first to show that at least some SH3 domains bind to short proline rich motifs [32]. Since genetic studies in Nematodes and Drosophila had shown that Grb2 and Sos were located between tyrosine kinase receptors and ras, this result prompted several groups to show that the SH3 domains of Grb2 bind proline rich motifs in the Sos C-terminal [12–15]. A short motif of Sos (VPVPPPVPPRRRP) is sufficient to bind Grb2 [14] and binding can still be detected with shorter proline rich peptides (such as PPPVPPR) which adopt a Polyproline II helix conformation [33]. The highest affinity found so far for a proline rich peptide binding on Grb2 N-SH3 is in the micromolar range [4]. The arginine seems to be essential since substitution of a lysine for this arginine in the EVPVPPPVPPR peptide greatly reduces its binding on Grb2 [15]. We have observed that the presence of a second arginine at the C-terminal end of the proline-rich peptide (PVPPPVPPRR) increases the affinity for Grb2 and gives a two times higher tryptophan fluorescence signal, suggesting that the arginines come close to the conserved tryptophan of the SH3 domain [4]. However, the NMR structure of the isolated Grb2 N-SH3 shows that the second arginine does not bind to the SH3 [34,35], it seems to help this region of the peptide to adopt a helical conformation that places the first arginine in an appropriate position (Fig. 3). In the crystal structure of the Sem-5 C-SH3 [36] the aliphatic portion of the first arginine packs against the side chain of the conserved tryptophan (explaining the tryptophan fluorescence increase observed upon ligand binding) and the guanidino group makes a salt bridge with a conserved carboxylic acid (E16 in Grb2

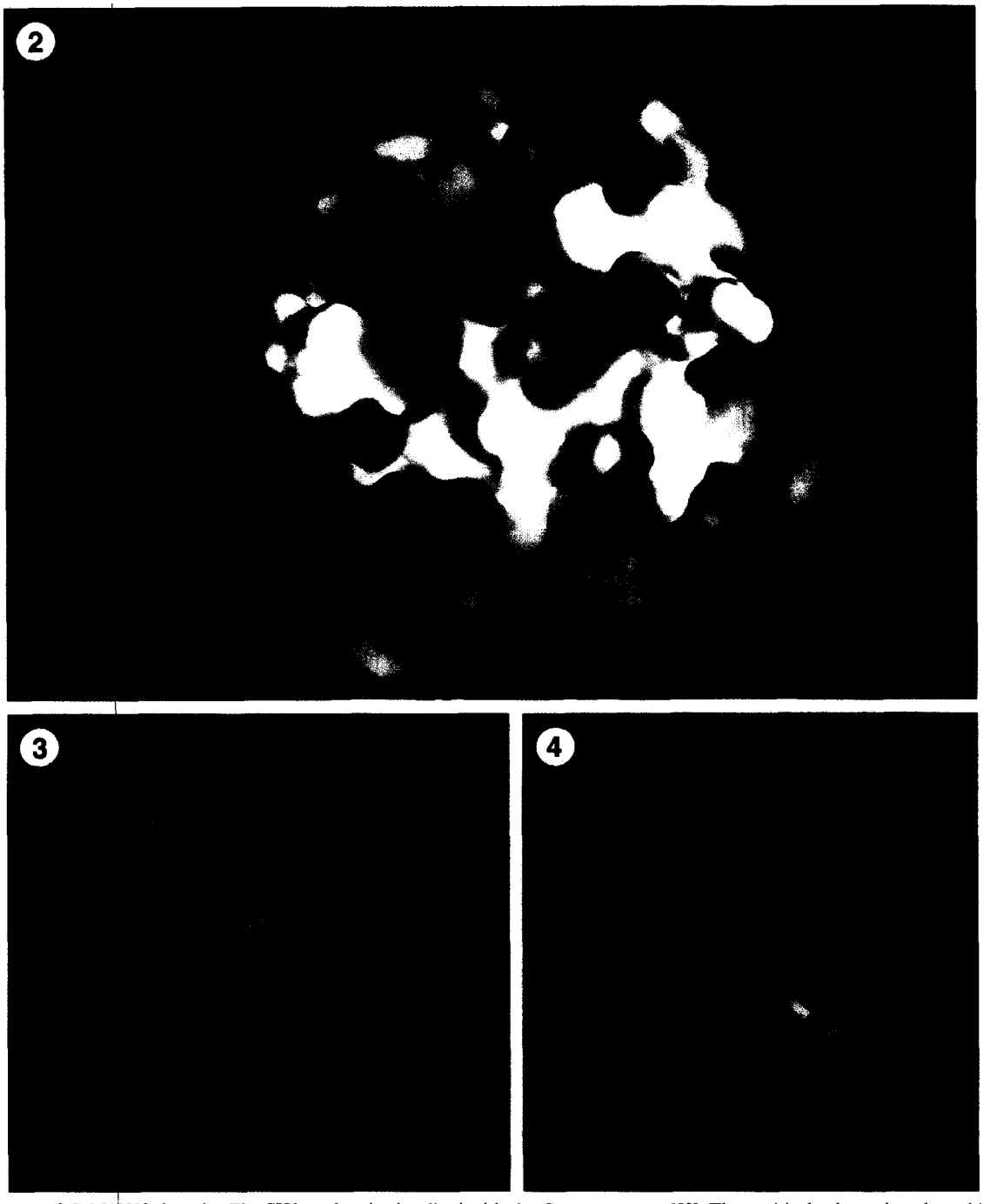


Fig. 2. Structure of Grb2 SH2 domain. The SH2 surface is visualised with the Grasp program [53]. The positively charged pocket which binds the phosphotyrosine (indigo) appears in blue in the front. The peptide is represented in green. Its position is deduced from the structure of the Syp SH2-peptide complex [27] and is only indicative. In this conformation the binding of the peptide would be impaired by tryptophan 121, which is found in the middle of the ligand binding cleft (top), in the structure of unliganded Grb2 SH2.

Fig. 3. Structure of Grb2 N-SH3 domain. The VPPPVPPRR peptide is represented in indigo. The N-terminal proline (P2) is close to tyrosines 7 and 52 on the left, the valine in the 'box' constituted by tyrosine 52, phenylalanine 9 and tryptophan 36 appears in the middle, the conserved arginine which packs against tryptophan 36 and participates in electrostatic interactions with glutamic acid 16 and aspartate 33 is on the right. Reproduced from Goudreau et al. [34].

Fig. 4. Structure of complete Grb2. Ribbon diagram produced with the Molscript [54] and Raster3D [55] showing the secondary structure and relative orientation of the SH2 (green), N-SH3 (white) and C-SH3 (indigo) domains. Linkers are indicated in the same color as the SH2 (green). Peptide ligands are indicated as red ribbons with an arrow at the C-terminal end. These peptides have been modeled from the structures of the individual SH3-ligand complexes [34–36] or SH2-ligand complexes [26,27] and have been added only to indicate the orientation of the binding sites.

N-SH3, E174 in Grb2 C-SH3). This extremity of the binding site is flanked by two negatively charged loops: D14, D15, E16 on one side and E30, E31, D33 on the other side, in the N-terminal SH3. Mutation of the first acidic cluster in the C-terminal SH3 (corresponding to D14, D15, E16 in the N-ter SH3) greatly decreases ligand binding [36]. The acidic cluster around positions 30, 31, 33 of the N-ter SH3 is the most mobile loop of the structure. Surprisingly, it was found that the SH3 domains of Grb2 bind proline rich peptides in the opposite orientation than Abl, PI3Kinase and Fyn [34–36]. This is made possible by the pseudo-symetry of the polyproline II helix. The ligands of the PI3Kinase and Fyn SH3 domains contain an arginine at the N-terminal end, whereas an arginine is present at the C-terminal of Grb2 SH3 ligands. Thus the N-terminal or C-terminal localisation of a positively charged residue seems to be critical to determine the orientation of the ligand, however the other non-proline residues of the ligand also determine the affinity by interacting with specific SH3 residues [37]. For instance the valine of the PPPVPPR motif packs against W191 and Y207 of Sem-5 C-SH3 (W36 and Y52 in Grb2 N-SH3, W193 and Y209 in Grb2 C-SH3).

## 6. Partners of Grb2 SH3 domains

Grb2 and the Sos C-terminal bind with high affinity and make a very stable complex. However several other proteins can bind to the SH3 domains of Grb2: Abl [38], dynamin [39], synapsin [40], 5-Lipoxygenase [41] and Shc (D. Cussac, unpublished). The interaction between SH3 domains and the proline rich tail of dynamin is rather promiscuous: the SH3 domains of PLC$\gamma$, p85, Src and Fgr also bind dynamin [39]. Furthermore, a wide range of proteins containing at least one proline rich motif are able to bind on several GST-SH3 fusions coupled to beads. This kind of experiment strongly suggests that the interaction with an SH3 may play an important role in the function of the protein, but do not characterise which SH3 is the physiologically significant partner. Since SH3 domains appear as rather 'sticky', other functional arguments are needed to sort-out the physiologically relevant interactions.

In the absence of strong experimental data there is still a controversy about the amount of Grb2 relative to Sos in the cell. The rat homolog of Grb2 was cloned independently and named Ash for Abundant Src Homology protein (6 clones out of $10^5$ in a rat cDNA library [42]) whereas Sos seems to be present at lower levels (less than 1 clone out of $10^5$ in several human cDNA libraries [12]). This is a rough estimation, but our experience with anti Grb2 and anti Sos antibodies leads us to similar estimations: at least 3–10 times more Grb2 than Sos, in fibroblasts. This Grb2/Sos ratio could change in different cell types, for instance high levels of Grb2 are found in neural cells [45]. If only a fraction of the Grb2 pool is complexed to Sos, the remaining Grb2 would be free to bind other proline rich proteins such as dynamin or synapsins. These proteins could represent storage compartments for Grb2 or their interaction with Grb2 could play an active role. For instance, the interaction of SH3 domains with dynamin could be involved in the internalisation of growth factor receptors, that rapidly follows activation, as a down-regulation mechanism [43]. However, a pool of free Grb2 that would be able to complex phosphotyrosine motifs through its SH2 domain but would not lead to ras activation is expected to behave as a dominant negative in this transduction pathway. And indeed, it has been observed that excess Grb2 can inhibit signalling to ras [44]. Nevertheless, NIH/3T3 cells expressing 20–30 fold higher levels of Grb2 show higher levels of ras activation in response to EGF than control NIH/3T3 [45] and similar results have been found in HER14 cells overexpressing the EGF receptor [46]. So, the effects of Grb2 overexpression seem to depend on the cell type, but it is likely that the release of free Grb2 has to be tightly controlled. Cell compartmentation could explain this paradox: the pool of Grb2 complexed to other proteins than Sos may not have access to the receptors whereas only the Grb2/Sos complex would be in a favorable position in close proximity of the receptors. When a GST-Grb2 fusion protein is microinjected into the cytoplasm of REF-52 fibroblasts, most of it goes to membrane ruffles (where ras proteins are also preferentially localised) and this localisation requires the integrity of both SH3 domains [47]. However the precise localisation of endogenous Grb2 has still to be determined.

Recent experiments suggest that the amount of Sos bound to Grb2 may be modulated. For instance T-cell stimulation transiently increases the amount of Sos that can be co-immunoprecipitated with Grb2 and this correlates with tyrosine phosphorylation of Shc [48]. In 3T3-L1 adipocytes the amount of Grb2 that can be co-immunoprecipitated with Sos decreases upon prolonged insulin treatment [49]. Activated MAP kinase is able to phosphorylate Sos on serine/threonines, mostly in its C-terminal part, presumably as a feedback mechanism [50]. The consensus phosphorylation sites do not overlap with the proline rich motifs and phosphorylation of the Sos C-terminus does not impair its binding on Grb2 in-vitro, however it correlates with a decrease in the amount of Grb2 bound to Sos in vivo [49,51]. Still, the mechanisms that regulate Grb2/Sos association are not precisely understood.

## 7. Structure of complete Grb2

Grb2 is a small protein of 217 a.a. (25 kDa), its structure has recently been solved [31]. It shows two parts (Fig. 4), the SH2 on one side and the two contiguous SH3s on the other. The SH2 is separated from the SH3s by two long flexible arms (or linkers) the structure of the SH2 not being affected by the adjacent SH3s. Consistent with the observations that the isolated SH2 has exactly the same biochemical properties than when present on complete Grb2, and that the binding of a phosphotyrosine ligand on the SH2 does not affect the binding of proline rich ligands on the SH3s and conversely [4]. The two SH3s make contacts and may not be totally independent. Each SH3 of Grb2 probably binds proline rich motifs following the general rules defined for SH3-polyproline II helix interactions [37], the two adjacent SH3s interacting with adjacent sites in the Sos C-terminal, however the two proteins could make several additional contacts explaining the specificity and the high stability of this Grb2/Sos complex. Alternatively, the rather weak interaction between the two SH3 domains, and the flexibility of the spacers linking the SH3s to the SH2 could enable the SH3s to dissociate and recognize multiple targets with diversely spaced proline rich motifs [31]. Interestingly, it has been shown that Grb2 can interact with the SH3-SH2-SH3 containing protein Vav through dimerization of the Vav N-SH3 and Grb2 C-SH3 domains [52], suggesting that Vav N-SH3 could substitute to Grb2 N-SH3 for binding to Grb2 C-SH3. However most of the

Grb2 N-SH3 residues making contacts with the C-SH3 (E30, C32, D33, Y37, K50, N51, I53, E54, M55) are not conserved in Vav N-SH3, thus the interaction between Vav N-SH3 and Grb2 C-SH3 may involve different regions.

Grb2 crystallises as a dimer, the two molecules being related by a local twofold axis, the SH2 of one molecule contacting the C-SH3 of the other and conversely. The interface between the two molecules in the dimer (4100 $Å^2$) is large, suggesting a possible physiological significance. This dimer can also be observed in solution, at high concentrations of Grb2 (>10 mg/ml), but at lower concentrations Grb2 behaves as a monomer. In the dimer, the binding sites on the two SH2s and the four SH3s are accessible [31]. Since four motifs of the Sos C-terminal are good SH3 ligands it is tempting to speculate that a Grb2 dimer could bind a single Sos molecule. However a detailed understanding of the Grb2/Sos C-terminal interaction awaits crystallisation of this complex.

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