Differential Phosphorylation of c-Jun and JunD in Response to the Epidermal Growth Factor Is Determined by the Structure of MAPK Targeting Sequences*§

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MAPK phosphorylation of various substrates is mediated by the presence of docking sites, including the D domain and the DEF motif. Depending on the number and sequences of these domains, substrates are phosphorylated by specific subsets of MAPKs. For example, a D domain targets JNK to c-Jun, whereas a DEF motif is required for ERK phosphorylation of c-Fos. JunD, in contrast, contains both D and DEF domains. Here we show that these motifs mediate JunD phosphorylation in response to either ERK or JNK activation. An intact D domain is required for phosphorylation and activation of JunD by both subtypes of MAPK. The DEF motif acts together with the D domain to elicit efficient phosphorylation of JunD in response to the epidermal growth factor (EGF) but has no function on JunD phosphorylation and activation by JNK signaling. Furthermore, we show that conversion of a c-Jun sequence to a canonical DEF domain, as it is present in JunD, elicits c-Jun activation in response to EGF. Our results suggest that evolution of a particular modular system of MAPK targeting sequences has determined a differential response of JunD and c-Jun to ERK activation.

The AP-1 transcription factor activates the transcription of several genes in response to a variety of external stimuli, including growth factors, tumor promoters, and cytokines. Active AP-1 is a dimeric protein complex whose components are the products of the Jun/AP1 and Fos/Fra gene families (1, 2). The three Jun proteins, c-Jun, JunB, and JunD, share a high degree of sequence homology; however, they display distinct transactivation properties and have different effects on cell proliferation (3). Compared with c-Jun, JunD plays an opposite role in cell growth regulation and transformation; whereas c-Jun behaves as a positive regulator of cell growth and may cause cell transformation, JunD antagonizes both of these effects in immortalized fibroblasts (4, 5). However, JunD-/- primary fibroblasts present an increased sensitivity to p53-de-
main can function independently from the D domain but requires a sustained signal to specify ERK phosphorylation. This appears to be the case with c-Fos (26). c-Jun and JunD display divergent configurations of the D and DEF domains, the former presenting only a D box whereas the latter contains both an N-terminal D motif and a C-terminal DEF domain (Fig. 1A), suggesting that this difference could account for the distinct responses of the two Jun proteins to ERK signaling. Consistent with this hypothesis, Drosophila Jun is a target of the Drosophila ERK type of MAP kinase called Rolled (27) and contains divergent configurations of the D and DEF domains, the former containing an N-terminal D domain and a C-terminal DEF motif. Similarly, the JunD mutant bearing an altered D domain is designated JunD-ATA/ALY (−/−) to indicate that it contains and alanine substitution of the LTL. The JunD form mutated in the DEF motif is designated JunD-ATA/ALY (−/−). The middle and right-hand columns represent sequence configurations of the D and DEF domains of JunD wild-type and mutants.

The expression plasmids for the Gal4-JunD-wt and Gal4-JunD-Δα fusion proteins have been described (13). The expression plasmid for Gal4-cJun was generated by subcloning a DNA fragment corresponding to a Zip-truncated form of human c-Jun (amino acid residues 1–266) into a mammal expression vector in-frame with a ubiquitin promoter-driven Gal4 DNA binding domain (gift of C. Weiss). HA-tagged c-Jun and JunD mutants as well as Gal4-JunD and Gal4-cJun mutants were generated by a PCR-based oligonucleotide-directed mutagenesis system (Stratagene).

Luciferase Assays—Gal4-fused proteins were cotransfected with the reporter plasmid Gal4-luciferase and a Renilla luciferase expression vector, pRL-CMV (Promega), in HEK 293 cells. Transient transfections were performed using the FuGENE-6 reagent as recommended by the manufacturer (Roche Diagnostics). Cells were incubated for 18–20 h, and then luciferase activity was measured with a Dual luciferase kit (Promega) according to the recommendations of the manufacturer. Firefly luciferase activity was normalized to the internal transfection control provided by the Renilla luciferase signal.

Immunoblotting—Cell extracts from HEK 293 cells were prepared in a buffer containing 20 mm Tris HCl (pH 8), 150 mm NaCl, 1 mm NaF, 1 μm Na3VO4, 0.1% SDS, and protease inhibitors (Roche Applied Science). Samples were then boiled in SDS sample buffer for 5 min, and the proteins were separated on 10% SDS-PAGE gels and detected by immunoblotting as described previously (9). HA-tagged proteins were detected by using a mouse monoclonal anti-HA antibody (Santa Cruz Biotechnology). Phosphorylated JunD-HA and c-Jun-HA were detected by using an anti-phospho-specific c-Jun/JunD antibody (New England Biolabs) that recognizes JunD when it is phosphorylated at serine 100 and c-Jun when is phosphorylated at serine 73. Phosphorylated ERK1/2 was detected by using a mouse monoclonal anti-p-ERK (Santa Cruz Biotechnology). Anti-phospho-JNK1/2 was a rabbit polyclonal antibody (Cell Signaling). Endogenously expressed ERK1/2 and JNK1/2 kinases were detected respectively by using rabbit polyclonal anti-ERK2 and anti-JNK1 (Santa Cruz Biotechnology). Antibodies were used according...
to the instructions of the manufacturers. Immune complexes were detected by using a horseradish peroxidase-conjugated secondary antibody followed by ECL (Amersham Biosciences).

**RESULTS**

**EGF-induced Phosphorylation of JunD Is Mediated by D and DEF Motifs**—JunD contains two domains with sequence similarity to the consensus ERK docking sites (R/K)KXXL, referred to as the D domain, and FX(F/Y)P, referred to as the DEF motif (Fig. 1A). We have investigated whether the D and DEF domains present in JunD modulate susceptibility to ERK phosphorylation. To this end we have generated HA-tagged JunD mutants in which the LSL hydrophobic motif of the D domain or the FLYP motif were altered, either separately or simultaneously, as indicated in Fig. 1B. We examined the effect of these mutations on JunD phosphorylation in response to the transient activation of ERK signaling elicited by EGF. HA-tagged JunD proteins were expressed in HEK 293 cells, and JunD phosphorylation was monitored by immunoblot analysis using an anti-phospho-Jun antibody (p-Jun-Ab) that recognizes phosphoserine 100 of JunD. The expression level of JunD-HA proteins was normalized by immunoblotting with HA antibody (Ha-Ab). The ERK1/2 phosphorylation status of ERK1/2 in the various treatments was examined by using anti-phospho-ERK antibodies (p-ERK-Ab) or anti-ERK1/2 antibodies (ERK-Ab) to normalize for the expression level of ERK1/2. Similarly, the phosphorylation status of JNK1/2 was monitored by using anti-phospho-JNK1/2 antibodies (p-JNK-Ab), or anti-JNK1/2 antibodies (JNK-Ab). As positive control of p-JNK-Ab, the extra lane (anis.) contains cellular extract from HEK 293 cells treated with anisomycin (50 ng/ml) for 30 min.

**Fig. 2.** **D and DEF domains sites are required for ERK-dependent phosphorylation of JunD.** A, HEK 293 cells were transiently transfected with HA-JunD-wt. After transfection, the cells were serum starved for 15 h and treated with EGF (100 ng/ml) for the indicated time, either in the absence (−) or the presence (+) of UO126 (10 μM), which was added 15 min previous to the EGF treatments. B, HEK 293 cells were transiently transfected with HA-JunD-wt or HA-JunD mutants as indicated. After transfection, the cells were serum-starved for 15 h and then, where indicated, treated with EGF (100 ng/ml) for 20 min. JunD phosphorylation was examined by immunoblot analysis by using the anti-phospho-specific antibody α-73 (p-Jun-Ab), which recognizes phosphoserine 100 of JunD. The expression level of JunD-HA proteins was normalized by immunoblotting with HA antibody (Ha-Ab). The ERK1/2 phosphorylation status of ERK1/2 in the various treatments was examined by using anti-phospho-ERK antibodies (p-ERK-Ab) or anti-ERK1/2 antibodies (ERK-Ab) to normalize for the expression level of ERK1/2. Similarly, the phosphorylation status of JNK1/2 was monitored by using anti-phospho-JNK1/2 antibodies (p-JNK-Ab), or anti-JNK1/2 antibodies (JNK-Ab). As positive control of p-JNK-Ab, the extra lane (anis.) contains cellular extract from HEK 293 cells treated with anisomycin (50 ng/ml) for 30 min.

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**Fig. 3.** The D domain is required for JNK-dependent phosphorylation of JunD. HEK 293 cells were transiently transfected with HA-JunD-wt or HA-JunD mutants as indicated. 24 h after transfection, cell were treated (+) with anisomycin (Aniso.) (50 ng/ml) for 30 min. Where indicated, cells were treated (+) with UO126 as described in the legend for Fig. 1A. JunD phosphorylation (p-Jun-Ab; top panel) was examined by immunoblot analysis as described in the Fig. 1A legend. The expression levels of JunD-HA proteins were normalized by using an HA antibody (Ha-Ab; second panel from top). The efficiency of the UO126 inhibitor was monitored by analyzing the phosphorylation status of ERK1/2 by using a phospho-ERK antibody (p-ERK-Ab; third panel from top), or an anti-ERK2–1 antibody (ERK-Ab; bottom panel) to normalize for the expression level of ERK1/2.
required for efficient phosphorylation of JunD in response to EGF. Surprisingly, mutations in the D and DEF motifs of JunD had, respectively, modest or no effect on the capacity of JunD to communoprecipitate ERK (Supplementary Fig. 1A, which can be found in the on-line version of this article). However, we found that c-Jun is also able to interact with ERK (Supplementary Fig. 1, A–D), suggesting that c-Jun/JunD-HA heterodimers may contribute to the communoprecipitation of ERK by the HA antibody and thereby compensate for an otherwise greater effect of JunD mutants.

We also investigated the role of the D and DEF domains on in vivo phosphorylation of JunD in response to JNK signaling. HA-tagged JunD-wt or the various Ha-JunD mutants in the D and DEF domains were expressed in HEK 293 cells, and the JNK pathway was induced by anisomycin. To avoid a potential cross-activation of ERK signaling, anisomycin treatments were performed in the presence of the MEK inhibitor U0126. As shown in Fig. 3, phosphorylation of the JunD serine 100 was markedly reduced in the JunD mutant bearing an altered D domain or in the mutant lacking both the D and DEF motifs. In contrast, disruption of the DEF motif did not significantly affect phosphorylation of serine 100, as judged by blotting with phospho-specific antibodies and gel mobility shift. These results indicate that the D domain is sufficient to mediate in vivo phosphorylation of JunD by JNK. Conversely, the DEF motif is not required for JunD phosphorylation by the JNK pathway. Furthermore, analysis of in vivo JunD/JNK interaction, by communoprecipitation experiments, suggests that the effect of a mutated D domain on JunD phosphorylation may be dependent from docking JNK (Supplemental Fig. 1, B–D, found in the on-line version of this article). However these results cannot exclude the possibility that JNK/JunD complexes are unstable.

Regulation of JunD Biological Activity Requires D and DEF Motifs—Next, we investigated the role of the D and DEF domains on transcriptional activation by JunD in response to EGF. JunD-dependent transcription was evaluated in vivo by expressing a Gal4-JunD/Gal4-luciferase reporter system in HEK 293 cells. The Gal4-JunD fusion protein contains the N-terminal region of JunD fused to the DNA binding domain of Gal4 (13). We have generated mutant forms of a Gal4-JunD protein containing the same point mutations indicated in Fig. 1B. Transcriptional activation following EGF treatment was monitored for each of the mutant proteins as well as for a Gal4-JunD mutant bearing alanine substitutions of serine 90, serine 100, and threonine 117 (Gal4-JunD-Ala). As shown in Fig. 4, EGF treatment led to 4.2-fold induction of Gal4-JunD basal activity but had no effect on the Gal4-JunD-Ala mutant, indicating that the enhancement of JunD transcriptional activity was mediated by the phosphorylation of MAPK sites. Similarly, point mutations only in the D domain or in both the D and DEF domain completely abrogate EGF-dependent induction of GAL4-JunD. Disruption of the DEF motif instead reduced to 1.8-fold the effect of EGF on Gal4-JunD activity. These results correlate very well with the effect of the mutations on ERK-dependent phosphorylation of JunD (Fig. 2B) and demonstrate that the D domain is necessary and sufficient for enhancing JunD activity in response to EGF; however, an intact DEF domain is required for maximal JunD activation.

We have shown previously that co-expression of a constitutively active form of ERK2, the kinase fusion protein ERK2-MEK1-LA (31), increases the basal activity of Gal4-JunD but has no effect on Gal4-JunD-Ala (13). Therefore, we examined transcriptional activation of the various Gal4-JunD mutants in response to co-expression of ERK2-MEK1-LA. As expected, ERK2-MEK1-LA increased the basal activity of Gal4-JunD

![FIG. 4. D and DEF domains are required for JunD activation in vivo](image)

HEK 293 cells were transiently transfected with plasmids encoding Gal4 fusion proteins containing a zinc-fingered form of either the wt-JunD protein or JunD mutants together with the reporter plasmid Gal4-luciferase and a Renilla luciferase expression vector, pRL-TK. A, HEK 293-transfected cells were serum-starved and then treated, as indicated, with EGF (100 ng/ml). Where indicated, Gal4-JunD proteins were co-expressed with the ERK-MEK1-LA fusion kinase. B, where indicated, Gal4-JunD-proteins were co-expressed with a plasmid expressing MKK7. For both panels (A and B), firefly luciferase activity was normalized to the internal transfection control provided by the Renilla luciferase. The fold induction represents the ratio of the firefly luciferase activity in the presence (+) or absence (−) of EGF, ERK-MEK1-LA MEK, or MKK7 and was determined by assigning a value of 1 to the basal luciferase activity of Gal4-JunD proteins. Error bars indicate S.D. from four independent experiments, each performed in triplicate.

3-fold but had no effect on GAL4-JunD-Ala. Like EGF treatments, ERK2-MEK1-LA expression had no effect on Gal4-JunD (−/−) or Gal4-JunD (−/−) (Fig. 4A). Conversely, disruption of the DEF domain had only a modest effect on Gal4-JunD activity induced by ERK2-MEK1-LA, suggesting that the DEF motif is less crucial in presence of sustained ERK activation.

A Canonical DEF Domain Leads to c-Jun Activation in Response to EGF—The results shown in Figs. 2 and 4A indicate that the D domain and the DEF motif of JunD function additively to mediate rapid phosphorylation and activation of JunD in response to EGF in an ERK-dependent manner. Conversely, c-Jun phosphorylation and transcriptional activity are not responsive to transient activation of ERK signaling (2, 3). A comparison of JunD and c-Jun sequences flanking the MAPK

![MAPK Targeting Sequences Mediate JunD Phosphorylation](image)
Fig. 5. A canonical DEF domain rescues Jun activation by EGF. A, schematic configuration of the N-terminal domain in wild-type and mutant versions of c-Jun. The D domain is represented as a black box, and the numbers indicate the first and last residue of the domain (upper scheme). The left-hand column indicates the types of c-Jun N-terminal configurations present in HA-tagged proteins or Gal4-cJun fused proteins. The right-hand column represents the sequence configurations that are present in c-Jun wild-type or mutants. Numbers indicate amino acid sequence configurations.
sites shows that c-Jun differs from JunD in the region where the latter contains the DEF motif (Fig. 1A). The FLYP sequence present in JunD contains the consensus phenylalanine and tyrosine residues in positions 1 and 3. The corresponding FLCP sequence present in c-Jun lacks the consensus phenylalanine/tyrosine residue in position 3, which has been shown to be crucial for specifying ERK phosphorylation of either ETS proteins or c-Fos (18, 26). This observation prompted us to explore whether this divergence could account for the differential activation of c-Jun and JunD proteins in response to ERK signaling.

To test this hypothesis, we examined the activity of a Gal4-cJun fusion protein compared with a mutant form, named Gal4-cJun/DEF, where the FLCP sequence of c-Jun was converted to FLYP, as it is in the DEF motif of JunD (Fig. 5A). We also generated a Gal4-cJun/DEF-Ala mutant where the FLYP mutation was inserted into a Gal4-cJun protein bearing the four MAPK phospho-acceptor sites mutated to alanine residues (Fig. 5A). The various Gal4-c-Jun proteins were expressed in HEK 293 cells together with a Gal4-luciferase reporter system, and their relative activities in response to EGF were evaluated. As shown in Fig. 5B, EGF treatments had no effect on the basal activity of Gal4-cJun-wt or Gal4-cJun/DEF-Ala but induced the activity of Gal4-cJun/DEF 3.9-fold, indicating that the insertion of a canonical DEF domain makes c-Jun responsive to EGF in a phosphorylation-dependent manner. To exclude the possibility that the inducibility of the Gal4-cJun-wt protein was constitutively impaired, we evaluated Gal4-c-Jun activity in response to JNK signaling by co-expressing the MKK7 stress kinase (30). As expected, overexpression of MKK7 induced the activity of Gal4-cJun-wt 3.7-fold (Fig. 5B). However, MKK7 had a limited effect on Gal4-cJun/DEF activity, suggesting that conversion of the FLCP motif in the FLYP/DEF domain had a negative effect on c-Jun activation by JNK.

Fig. 6. Distinct configurations of ERK targeting sequences differentiate c-Jun and JunD in their capacity to sense ERK signal duration. Depending on the stimulus, the duration of ERK signaling can be transient or sustained. Transient activation results in phosphorylation and activation of transcription factors as Elk-1, SAP-1, and JunD, which contain an N-terminal D domain and an N-terminal DEF domain flanking the (S/T)P phospho-acceptor sites. Sustained activation leads to phosphorylation of transcription factors such as c-Fos and c-Jun containing only a D domain or a DEF domain. In this way, the quantitative difference of ERK activation is translated into a qualitative difference of transcription factor activation.

position. MAPK sites and mutated amino acids are set in **boldface**. B, HEK 293 cells were transiently transfected with plasmids encoding Gal4 fusion proteins containing a zipper-truncated form of either cJun-wt, cJun-DEF, or cJun-DEF/Ala proteins together with the reporter plasmid Gal4-luciferase and a Renilla luciferase expression vector, pRL-TK (Promega). Transfected cells were serum-starved and then treated, as indicated, with EGF (100 ng/ml) Where indicated, Gal4/cJun-proteins were co-expressed with MKK7. Firefly luciferase activity was normalized to the internal transfection control provided by the Renilla luciferase. The fold induction is the ratio of the firefly luciferase activity in the presence or absence of EGF or MKK7 and was determined by assigning a value of 1 to the basal luciferase activity of Gal4-cJun proteins. **Error bars** indicate S.D. from four independent experiments, each performed in triplicate. C, NIH-3T3 cells were transiently transfected with HA-JunD, HA-cJun-wt, or HA-cJun/DEF as indicated. After transfection, cells were serum-starved for 15 h and then treated with 10% calf serum for the indicated time. JunD and c-Jun phosphorylation was examined by immunoblot analysis by using the anti-phospho-specific antibody α-T3, which recognizes either the phosphoserine 100 of JunD or the phosphoserine 73 of c-Jun (**top panel**). The expression levels of Jun proteins were normalized by immunoblotting with an HA antibody (**bottom panel**).
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Next we evaluated the phosphorylation status of the c-Jun or c-Jun/DEF protein in response to transient activation of ERK signaling. HA-tagged c-Jun-wt, c-Jun/DEF, or JunD proteins were expressed in NIH-3T3, and the phosphorylation of each in response to serum stimulation was monitored by using an anti-phospho-Jun antibody (α-73) that recognizes either c-Jun or JunD only if they are phosphorylated at serine 73 or serine 100, respectively. As expected, phosphorylation of JunD was rapidly induced in response to serum stimulation (Fig. 5C). In contrast, neither 10 nor 30 min of serum stimulation had an effect on the level of c-Jun phosphorylation (Fig. 5B). As expected, phosphorylation of JunD was or JunD only if they are phosphorylated at serine 73 or serine 100, respectively. As expected, phosphorylation of JunD was rapidly induced in response to serum stimulation (Fig. 5C). In contrast, neither 10 nor 30 min of serum stimulation had an effect on the level of c-Jun phosphorylation (Fig. 5B). However, phosphorylation of the c-Jun/DEF mutant was clearly induced after 10 min of the stimulus. Together with the results shown in Fig. 5B, these findings demonstrate that introduction of the DEF motif enables c-Jun to be responsive to transient activation ERK signaling.

DISCUSSION

Extracellular stimuli regulate AP-1 activity by activating MAPK cascades that mediate phosphorylation of distinct substrates (2, 10). Depending on the duration of intracellular signaling, serum and growth factor have been shown to induce AP-1 by distinct mechanisms. Transient activation of ERK, as elicited by EGF, leads to phosphorylation of transcription factors of the ternary complex factor (TCF) family such as Elk-1 and, thus, causes induction of the c-fos gene (10). We have shown previously that AP-1 regulation by ERK signaling may also occur at the level of ERK-dependent phosphorylation and activation of JunD (13). The present study demonstrates that ERK-dependent activation of JunD in response to EGF is mediated by MAPK targeting motifs present in the N-terminal region of JunD. The effect of the MEK-specific inhibitor U0126 (Fig. 1A) on JunD phosphorylation indicates that the EGF-induced phosphorylation of JunD is mediated by ERK activation. Furthermore, the time course of JunD phosphorylation in response to EGF reflects the activation kinetics of ERK rather than of JNK as shown by the analysis of the phosphorylation status of ERK1/2 and JNK1/2 (Fig. 2A). The functional link between EGF-induced phosphorylation and JunD activation is supported by the observation that JunD-dependent transcription was induced by EGF only if the three MAPK phosphoacceptor sites, present in the transactivation domain, were intact (Fig. 3A).

Physiological substrates of ERK have been shown to contain docking motifs, referred to as the D domain and the DEF motif, that, depending on their number and position, regulate the affinity for ERK (17, 18). ERK target proteins containing both motifs, such as Elk-1 and SAP-1, have been shown to have higher affinity for ERK than other substrates containing only a DEF or D motif, such as KRPs proteins (22). We noted that JunD contains both the D domain and the DEF motif flanking the MAPK phosphoacceptor sites in a configuration that is reminiscent of the structures of Elk-1 and SAP-1 (Fig. 1A). Primary sequence requirements for MAPK docking sites have been determined for both the D and the DEF motifs (17, 18). Analysis of JunD mutants bearing alanine substitution of these sequences has allowed us to determine that the D domain mediates JunD phosphorylation by both ERK and JNK (Figs. 2B and 3). Although the D domain was sufficient to induce JunD phosphorylation by JNK signaling (Figs. 3 and 4B), both the D and the DEF motifs were required for efficient phosphorylation and activation of JunD in response to transient activation of ERK, as elicited by EGF stimulation (Fig. 2B and 4A). On the other hand, the DEF domain seems not to be crucial for JunD activation by sustained activation of ERK signaling (Fig. 4A). These observations suggest that the D domain is sufficient to specify both JNK and ERK phosphorylation; however, the simultaneous presence of the D and DEF motifs, by increasing JunD affinity for ERK, allows JunD to be activated in response to EGF. Consistent with this conclusion, c-Fos and c-Jun, containing, respectively, the DEF or the D domain (Fig. 1A), are phosphorylated by extracellular stimuli that cause sustained activation of ERK signaling (12, 26), but not in response to EGF (10, 12). In addition, conversion of the FLCP sequence of c-Jun into the consensus FLYP/DEF motif turned c-Jun into an EGF-responsive transcription factor (Fig. 5). Two lines of evidence indicate that introduction of a DEF motif increased ERK phosphorylation of c-Jun. First, the DEF domain could rescue c-Jun-dependent transcription only if the MAPK sites were intact (Fig. 5B). Second, introduction of the DEF domain elicited c-Jun phosphorylation by transient activation of ERK signaling.

Depending on the cell type, transient or sustained activation of the MEK-ERK pathway has been associated with specific biological outcomes, such as S-phase entry, differentiation, or cell survival (26, 34, 35). Our results indicate that evolution of a distinct configuration of ERK targeting sites has differentiated c-Jun and JunD in their capacity to sense the duration of ERK-signaling (Fig. 6). According to this model, Drosophila Jun, a target of both JNK/Basket and ERK/Rolled pathways (27, 29), contains putative D and DEF motifs (Fig. 1A).

In conclusion, our study suggests that quantitative differences of ERK activation are translated into the qualitative differences of c-Jun and JunD activation. In turn, a differential regulation by ERK signaling may account for the distinct modality through which c-Jun and JunD affect cell proliferation and survival.

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