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Methylation of STAT6 Modulates STAT6 Phosphorylation, Nuclear Translocation, and DNA-Binding Activity

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Signal transducer and activator of transcription 6 is a transcription factor important for the development of Th2 cells and regulation of gene expression by IL-4 and IL-13. It has been reported that STAT1 activity is regulated by methylation of a conserved arginine residue in the N-terminal domain. Methylation of STAT6 has not yet been explored. We observed methylation of STAT6 in cells transfected with wild-type STAT6, but not in cells transfected with Arg27Ala mutant, confirming that STAT6 is methylated on Arg27. Transfectants expressing mutant Arg27Ala STAT6 displayed markedly diminished IL-4-dependent STAT6 phosphorylation and nuclear translocation, and no STAT6 DNA-binding activity compared with wild-type STAT6 transfectants. To confirm this, the experiments were repeated using inhibitors of methylation. In the presence of methylation inhibitors, STAT6 methylation was diminished, as was phosphorylation of STAT6 and STAT6 DNA-binding activity. Thus, methylation is a critical regulator of STAT6 activity, necessary for optimal STAT6 phosphorylation, nuclear translocation, and DNA-binding activity. Furthermore, methylation of STAT6 has distinct effects from those reported with STAT1. The Journal of Immunology, 2004, 172: 6744–6750.

Activated STAT6 dimers then translocate to the nucleus, bind specific consensus sequences, and promote transcription of downstream genes. The crystal structure of STAT1-DNA complexes revealed that dimeric interactions between the two respective SH2 domains were critical for the formation of the DNA binding region (13). Nuclear translocation of STAT6 depends on its phosphorylation and dimerization (11, 14); however, the elements responsible for nuclear localization of STAT6 have not been identified.

Although STAT6 activation in response to IL-4 and IL-13 has been well documented, the molecular mechanisms responsible for the regulation of STAT signaling are less well understood. A number of negative regulators of the JAK-STAT signaling pathway have been described, including silencers of cytokine signaling (SOCS) and protein inhibitors of activated STAT (PIAS). SOCS proteins form a negative feedback loop whereby SOCS genes are induced following cytokine stimulation and inhibit cytokine signaling (15, 16). SOCS-1 has been shown to bind and inhibit JAK kinases, but this may not be true of all SOCS family members. A number of PIAS proteins have recently been described with specificity for some STAT family members (17, 18). These proteins bind specifically to phosphorylated STAT6 dimers and prevent them from binding DNA (19).

Recent studies have demonstrated that STAT1 function is modulated by methylation of Arg11 (20). For STAT1, the mechanism of this methylation-dependent modulation appears to be regulation at the level of STAT1-PIAS1 association. PIAS1 binds to STAT1 dimers and prevents STAT1 DNA binding. Methylation of STAT1 does not alter STAT1 phosphorylation, but reduces the ability of STAT1 to associate with PIAS, and thus, increases STAT1 DNA-binding activity (20). No PIAS has yet been identified for STAT6. Several studies have implicated tyrosine phosphatases in the regulation of STAT signaling (21–24). Consistent with this possibility, studies examining STAT1 have shown that activated STAT1 disappears from the nucleus within 60 min and that removal of the activated STAT1 is dependent on a protein tyrosine phosphatase (21). Recently, a nuclear phosphatase, TC45 (also referred to as TcPTP), a nuclear isozyme of T cell protein phosphatase, was identified as the tyrosine phosphatase responsible for STAT1 dephosphorylation in the nucleus (25). Interestingly, another recent study...
demonstrated that dephosphorylation of STAT1 by TC45 (or TcPTP) is regulated by arginine methylation of STAT1, and that this regulation is dependent on PIAS (26). Their data are consistent with a model whereby inhibition of methylation increases association of STAT1 with PIAS with a concomitant decrease in binding of STAT1 to TcPTP, and thus delayed dephosphorylation of STAT1. Arg31 is conserved among the STAT molecules, and methylation is most likely generally important for STAT function.

In this study, we investigated whether STAT6 was methylated on Arg27 and the mechanism by which STAT6 methylation affects its function. Our results demonstrate that STAT6 is methylated on Arg27 and that methylation of STAT6 has a distinct regulatory effect from that reported with STAT1.

Materials and Methods

**Constructs**

Murine STAT6 cDNA was amplified with forward primer 5'-GGGGTAC CACCATGCTCTGGGGCTGAAATTCC-3' and reverse primer 5'- CTCCTGGTCATTGACATCATCTTCATTTAATCCCACTGGGTTGTCCTT AGGTC-3' to add a Flag tag at the C terminus, digested with KpnI and XhoI, and cloned into pBKS. Primers 5'-CTTCCCAACACGC TCTAGGCACTCTCTGCTGCAG-3' and 5'-GTCAGCAGAAGATGC GCTAGGCGTTGTGGAAAG-3' were used in Quickchange mutagenesis (Invitrogen, Carlsbad, CA) to mutate Arg27 to Ala27 according to the manufacturer's instruction. The Flag-tagged STAT6 and the Flag-tagged STAT6-R27A were then cloned into KpnI and XhoI sites of pREP9 (Invitrogen). Murine STAT6 cDNA was amplified with primers 5'-GGGGTACCAACCAT GTTCGTTGTCCTGTACTTC-3' and 5'-CCCTCGAGTCATTTATCATCATCATTTATAATCCCAGCTGGGGTTGGTCCTT-3' to add a His tag at the C terminus, digested with KpnI and XhoI, and cloned into pCEP4 (Invitrogen).

**Transfection**

HEK293 cells were transfected with STAT6-Flag-pREP9 or STAT6-R27A-Flag-pREP9 using Effectene (Qiagen, Valencia, CA). Stable transfected cell lines of STAT6-Flag-pREP9 and STAT6-R27A-Flag-pREP9 were selected in medium containing 400 μg/ml G418. STAT6-Flag-pREP9 cells or STAT6-R27A-Flag-pREP9 stable-transfected cells were further transfected with STAT6-His-pCEP4, and the double transfecteds were selected in medium containing 400 μg/ml G418 and 200 μg/ml hygromycin. Expression of the transfected Flag- or His-tagged protein was determined by Western blot using anti-Flag (Sigma-Aldrich, St. Louis, MO) or anti-His (Roche Molecular Biochemicals, Mannheim, Germany) Abs.

**Immunoprecipitation and immunoblotting**

Immunoprecipitation and immunoblotting were performed, as previously described (27). Briefly, cells were stimulated with 10 ng/ml human IL-4 (PeproTech, Rocky Hill, NJ) or 200 U/ml human IFN-γ (R&D Systems, Minneapolis, MN), or medium for the desired time. For methylation inhibition, cells were pretreated with adenosine, l,l-homocysteine, and N-methyl-2-deoxyadenosine (MDA) (Sigma-Aldrich) (26). Cells were lysed in immunoprecipitation lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 5 mM iodoacetamide, 1 mM sodium orthovanadate, 20 mM NaF, and 1 mM EDTA) and then centrifuged at 20,000 × g for 20 min. The supernatant was precleared with protein A/G plus agarose (Santa Cruz Biotechnology, Santa Cruz, CA), and immunoprecipitated with anti-Flag, anti-His, anti- mono/di-methylarginine (Abcam, Cambridge, U.K.), anti-STAT6 (Santa Cruz Biotechnology), anti-STAT1 (Cell Signaling Technology, Beverly, MA), or isotype control Abs (BD Pharmingen, San Diego, CA) and protein A/G plus agarose. The samples from immunoprecipitation were resolved on 10% SDS-PAGE and analyzed by Western blot using anti-Flag, anti-His, anti-STAT1, anti-phospho-STAT1 (Tyr701) (Cell Signaling Technology), anti-STAT6, or anti-phospho-STAT6 (Tyr641) (Cell Signaling Technology) Abs. The blots were developed with HRP-conjugated secondary Abs (Santa Cruz Biotechnology) and ECL reagents (Amersham Biosciences, Piscataway, NJ).

**EMSA**

Electrophoretic mobility shift assay (EMSA) was performed, as previously described (27).

![FIGURE 1. STAT6 is methylated at Arg27. Flag-tagged wild-type STAT6 or R27A mutant was stably expressed in HEK293 cells. A. Western blot of total cell lysate from untransfected HEK293 cells (10 μg), wild-type STAT6-Flag transfected (1 μg), or STAT6-R27A-Flag transfected (10 μg). B. Immunoprecipitation of total cell lysate from untransfected HEK293 cells, wild-type STAT6-Flag transfected, or STAT6-R27A-Flag transfected using anti-mono/di-methylarginine Ab (anti-MDMA) or isotype control IgG1 and Western blot using anti-Flag Ab.](http://www.jimmunol.org/)
STAT6 (or STAT1) probe (Santa Cruz Biotechnology), as previously described (27). For pervanadate treatment, pervanadate was produced by the equimolar combination of sodium orthovanadate and hydrogen peroxide, and the cells were treated with 1 mM pervanadate for 30 min (28, 29).

**Pulse chase experiments**

Cells were starved in methionine/cysteine/pyruvate/FBS-free DMEM medium (Invitrogen) for 1 h and then incubated in fresh methionine/cysteine/pyruvate/FBS-free DMEM medium containing 10 μCi/ml 35S-methionine (PerkinElmer, Shelton, CT) for 3 h. Complete medium (containing methionine, cysteine, pyruvate, and FBS) with or without IL-4 (10 ng/ml) was added and incubated for desired chase time. Total cell lysate was immunoprecipitated, resolved by 10% SDS-PAGE, and visualized by autoradiography or analyzed by Western blot.

**Immunostaining and confocal microscopy**

Cells were growing on cell culture coverslips (Fisher Scientific, Pittsburgh, PA) in six-well plates. After treatment or stimulation, the cells were washed with cold PBS for three times and fixed with 3% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA) for 10 min and incubated with 15 mM glycine in PBS for 10 min to quench endogenous fluorescence. The cells were incubated with blocking solution (PBS with 3% BSA, 3% cold fish gelatin, 3% FBS, and 0.2% saponin) and then incubated with 15 mM glycine in PBS for 10 min to quench endogenous fluorescence. The cells were then incubated with 50 μl of anti-Flag Ab (5 μg/ml) or isotype control mouse IgG1 (5 μg/ml) for 30 min. The cells on the coverslips were washed three times with PBS with 1% FBS and 0.2% saponin. The cells were incubated with 50 μl of 10 μg/ml FITC-conjugated anti-mouse IgG1 (BD PharMingen) for 30 min at room temperature. The coverslips were washed three times, rinsed with H2O once, and inverted onto slides with Vectorshield (Vector Laboratories, Burlingame, CA), and the edges were sealed with nail polish. The slides were observed under a Leica DM IRBC confocal microscope (Leica Microsystems, Heidelberg, Germany) (27, 30).

**Results**

**STAT6 is methylated at Arg27**

Arg27 at the N terminus of STAT6 is conserved in all STAT family members. Studies on STAT1 have demonstrated that this conserved arginine is methylated and that methylation modulates STAT1 function (20). We first examined whether STAT6 was methylated. HEK293 cells were chosen for stable expression of epitope-tagged STAT6 because this cell line has no endogenous STAT6 activity. These cells were stably transfected with either wild-type STAT6-Flag or Arg27Ala mutant STAT6-Flag. As shown in Fig. 1, wild-type STAT6 was methylated. Notably, STAT6 was methylated at baseline, and IL-4 treatment did not alter STAT6 methylation. However, when Arg27 was mutated to alanine, no methylated STAT6 was detected (Fig. 1) in the presence or absence of IL-4, demonstrating that STAT6 is methylated at Arg27. The expression of transfected wild-type STAT6 was ~10-fold higher than the transfected R27A STAT6 mutant. Even when we corrected for this difference (Fig. 1), we did not observe any methylation of the R27A STAT6.

**Arg27 is critical for the function of STAT6**

Next, we examined whether Arg27 is important for the STAT6 function. We first investigated whether mutation of Arg27 affected IL-4-induced tyrosine phosphorylation of STAT6. As shown in Fig. 2, tyrosine phosphorylation of the R27A STAT6 mutant was nearly abolished compared with wild-type STAT6. We next examined IL-4-dependent STAT6 DNA-binding activity in the transfectants (Fig. 2). STAT6 DNA-binding activity was absent in transfectants expressing the R27A STAT6 mutant compared with wild-type STAT6.

Because DNA-binding activity was completely absent in the R27A mutant cells, we expected that the nuclear translocation of STAT6 would also be impaired in these transfectants. Confocal microscopy of STAT6 following stimulation of IL-4 confirmed that the R27A STAT6 mutant remained in cytoplasm after treatment of IL-4, while wild-type STAT6 translocated into nucleus (Fig. 3).

**MDA treatment inhibits STAT6 methylation and its function**

Our data to date revealed that Arg27, which we have shown to be methylated, was critical for STAT6 phosphorylation, translocation, and DNA-binding activity. In order to confirm that our observations were reflecting the absence of STAT6 methylation rather than a structural problem with the transfected R27A STAT6 protein, we used an inhibitor of protein methylation. It has been shown that treatment of cells with adenosine, d-l-homocysteine, and MDA resulted in complete inhibition of STAT1 arginine methylation (26). Incubation of HEK293 cells expressing Flag-tagged STAT6 with MDA inhibited the methylation of STAT6, as shown in Fig. 4. Under these conditions, both tyrosine phosphorylation and STAT6 DNA-binding activity of STAT6 were nearly abolished, as shown in Fig. 5. This together with our data on the R27A STAT6 transfectants confirm that methylation of STAT6 on

**FIGURE 3.** IL-4-induced nuclear translocation of STAT6 is inhibited by the R27A mutation. Cells were stimulated (or unstimulated) with human IL-4 (10 ng/ml) for 5 or 60 min, and the subcellular localization of Flag-tagged STAT6 or STAT6 R27A mutant was analyzed by confocal microscopy.
Arg27 is important for tyrosine phosphorylation of STAT6 and subsequent nuclear translocation and DNA-binding activity. We compared the effect of MDA treatment on STAT6 and STAT1 in Hela cells for the tyrosine phosphorylation and DNA-binding activity. The MDA treatment resulted in the inhibition of both IL-4-induced tyrosine phosphorylation and DNA-binding activity of STAT6 (Fig. 6A), as seen in STAT6-transfected HEK293 cells. With the MDA treatment, the IFN-γ-induced DNA-binding activity of STAT1 was inhibited, but the tyrosine phosphorylation of STAT1 was not affected (Fig. 6B), which is consistent with the results reported by others (26), suggesting the different role of methylation in activation of STAT6 and STAT1.

Inhibition of tyrosine phosphatases does not restore DNA-binding activity of R27A STAT6

To explore possible mechanisms underlying the inhibition of tyrosine phosphorylation and loss of DNA-binding activity of R27A STAT6, we treated R27A STAT6-transfected cells with pervanadate, an inhibitor of protein tyrosine phosphatases, and then examined the tyrosine phosphorylation and DNA-binding activity. As shown in Fig. 7, the tyrosine phosphorylation of R27A STAT6 was not affected by pervanadate treatment in the presence or absence of IL-4 stimulation, suggesting that the R27A STAT6 undergoes tyrosine phosphorylation at a very low level upon IL-4 stimulation, and R27A mutation mainly affects the phosphorylation rather than dephosphorylation. No STAT6 DNA-binding activity was observed in R27A STAT6-transfected cells after pervanadate treatment and/or IL-4 stimulation. In comparison, the treatment with pervanadate and/or IL-4 stimulation induced significant tyrosine phosphorylation and STAT6 DNA-binding activity in cells transfected with wild-type STAT6. The DNA-binding activity detected in R27A STAT6-transfected HEK293 cells was not STAT6 specific, as it was not supershifted by anti-STAT6 Ab and it was also present in untransfected cells.

The R27A STAT6 is less stable than wild-type STAT6

Because R27A STAT6 had lower expression level compared with wild-type STAT6, we determined their stability by pulse chase experiments with labeling of 35S-methionine and immunoprecipitation. The R27A STAT6 had a shorter $t_{1/2}$ than wild-type STAT6 (Fig. 8). The R27A STAT6 was gradually degraded, while the wild-type STAT6 was stable over time. IL-4 stimulation had no effect on the stability of R27A STAT6 (Fig. 8). When IL-4 was

**FIGURE 4.** MDA treatment inhibits methylation of STAT6. Cells were treated with or without MDA (600 μM) for 6 h and then stimulated or not with human IL-4 (10 ng/ml) for 15 min. Total cell lysate was subject to immunoprecipitation using anti-mono/di-methylarginine Ab (anti-M/DMA) or isotype control IgG1 and Western blot using anti-Flag Ab.

**FIGURE 5.** MDA treatment inhibits IL-4-dependent STAT6 tyrosine phosphorylation and DNA-binding activity in STAT6-transfected HEK293 cells. Cells were treated with MDA (600 μM) for 6 h and then stimulated (or unstimulated) with human IL-4 (10 ng/ml) for 15 min. Total cell lysate from untransfected HEK293 cells, wild-type STAT6-Flag transfected, or STAT6-R27A-Flag transfected was immunoprecipitated using anti-Flag Ab and then immunoblotted using anti-Flag or anti-phospho-STAT6 (Tyr641) Abs. The nuclear extract (5 μg) was used for STAT6 DNA-binding activity by EMSA.

**FIGURE 6.** MDA treatment inhibits IL-4-dependent STAT6 tyrosine phosphorylation and DNA-binding activity, and IFN-γ-dependent STAT1 DNA-binding activity in Hela cells. Hela cells were treated with MDA (300 μM) for 3 h and then stimulated (or unstimulated) with human IL-4 (10 ng/ml) for 15 min (A) or human IFN-γ (200 U/ml) for 30 min (B). Total cell lysate was immunoprecipitated using anti-STAT6 or anti-STAT1 Abs and then immunoblotted using anti-STAT6, anti-phospho-STAT6 (Tyr641), anti-STAT1, or anti-phospho-STAT1 (Tyr701) Abs. The nuclear extract (10 μg) was used for STAT6 or STAT1 DNA-binding activity by EMSA.
added to the chase medium, the stability of R27A STAT6 was not altered. This suggests that arginine methylation not only contributes to the IL-4-induced tyrosine phosphorylation, but also stability of STAT6.

**Effect of unmethylated STAT6 on the function of wild-type STAT6**

We next wanted to examine whether the presence of R27A STAT6, incapable of methylation, affected the function of wild-type STAT6. To examine this, cells were cotransfected with Flag-tagged R27A, and His-tagged wild-type STAT6 were compared with transfectants expressing Flag-tagged wild-type and His-tagged wild-type STAT6. STAT6 DNA-binding activity was not affected in wild-type STAT6 and R27A STAT6 mutant double-transfected cells compared with that in wild-type/wild-type double-transfected cells (Fig. 9). However, the expression of the R27A STAT6 was very low compared with wild-type STAT6, and the effect of unmethylated STAT6 may require a larger presence of unmethylated STAT6. Alternatively, a small percentage of the total STAT6 pool may be activated upon cytokine stimulation, and this may be unaffected by the presence of low levels of R27A STAT6.

**Discussion**

STAT6 is critical for IL-4- and IL-13-dependent gene induction. Although STAT6 activity is known to be regulated by tyrosine phosphorylation, it was previously unknown whether STAT6 was methylated and what role methylation played in STAT6 function. Our data demonstrate that STAT6 activity is regulated by arginine methylation of Arg27. In the absence of methylation, STAT6 phosphorylation was profoundly diminished in response to IL-4, and nuclear translocation and DNA-binding activity were absent. Thus, methylation of STAT6 is critical for its function.

Arginine methylation of STAT1 has been reported previously (20). Arginine methylation has been shown to modulate STAT1 function by affecting its ability to associate with PIAS1 (20). PIAS1 interacts with activated STAT1 dimers and prevents their ability to bind DNA. STAT1 arginine methylation decreases the ability of STAT1 to interact with PIAS, and thus, effectively increases STAT1 activity. A specific PIAS has not been found for STAT6, suggesting that methylation may have a different role in regulation of STAT6 function. It is likely that all STATs are methylated because the arginine residue is highly conserved in the N-terminal domain. However, our data suggest that methylation may play distinct roles in the activities of STAT proteins. Our results support the hypothesis that methylation may play distinct roles in the activities of STAT proteins.
with the Arg27Ala STAT6 mutant establish that STAT6 methylation occurs at this conserved arginine residue. This mutation dramatically decreased the level of tyrosine phosphorylation of STAT6, supporting that this residue contributes to the step of tyrosine phosphorylation in the STAT6 activation cascade. This contrasts with the results from the STAT1 studies, which showed that the methylation of STAT1 is independent of STAT1 tyrosine phosphorylation (20). Fig. 10 schematically summarizes the effect of arginine methylation on STAT6 and STAT1 function. The finding of STAT4 N-terminal amino acid residues contributing to the tyrosine phosphorylation is not unique to STAT6. In the case of STAT4, selective mutations at the N-terminal domain of STAT4 interfere with the IFN-γ-induced tyrosine phosphorylation of STAT4 (31). It is most likely that the N terminus of each STAT family member has a unique function. This is further supported by recent studies examining STAT1-STAT4 chimeric proteins; the N-terminal domains of STAT1 and STAT4 were not functionally interchangeable (31).

Because arginine methylation of STAT1 regulates its dephosphorylation by TcPTP (26), we tested whether tyrosine phosphatases are involved in the inhibited tyrosine phosphorylation and DNA-binding activity of R27A STAT6. Pervanadate treatment did not increase the tyrosine phosphorylation of R27A STAT6, indicating the tyrosine phosphatase activity is not enhanced in R27A STAT6-transfected cells and suggesting that the decreased tyrosine phosphorylation for R27A STAT6 is probably due to defect of the phosphorylation step rather than of the dephosphorylation step. The R27A STAT6 had no DNA-binding activity after pervanadate treatment and/or IL-4 stimulation, supporting that the nuclear translocation of R27A STAT6 is still inhibited. The methylated arginine might be a critical site for recruiting proteins required for STAT6 phosphorylation and nuclear translocation. We also found R27A STAT6 was less stable compared with the wild-type STAT6. The STAT1 mutant in which Arg31 was replaced by alanine is also unstable (20). It has been reported that the dephosphorylation of STAT6 is regulated by not only tyrosine phosphatases, but also proteasome-mediated protein degradation (32). This raises the possibility that the action of proteasome-mediated protein degradation is enhanced for R27A STAT6.

It is not surprising that the nuclear translocation and DNA-binding activity of STAT6 induced by IL-4 stimulation were affected by the R27A mutation or methylation inhibition because the tyrosine phosphorylation is a requirement for the formation of active dimers, which are subsequently translocated into nucleus and bind specific DNA sequence to activate transcription. To test the effect of STAT6 R27A mutant on the activation of wild-type STAT6, we coexpressed R27A STAT6 mutant and wild-type STAT6. We observed no significant difference in the total STAT6 DNA-binding activity between the wild-type/wild-type double transfectant and wild-type/R27A double transfectant after IL-4 stimulation. However, the R27A mutant was expressed at a substantially lower level than the wild-type STAT6. Thus, direct competition or other regulation between the R27A mutant and wild-type STAT6 cannot be ruled out. Furthermore, because we did observe some minimal phosphorylation of R27A STAT6 following IL-4 treatment, it remains possible that heterodimers can form between unmethylated and methylated STAT6 forms. Because much less STAT6 R27A mutant was tyrosine phosphorylated compared with the wild type, it is likely that the R27A STAT6 mutant affects the early step of STAT6 activation by competitively binding proteins required for the tyrosine phosphorylation of STAT6.

As discussed above, arginine methylation of STAT1 regulates its association with PAIS1; likewise, arginine methylation of STAT6 might regulate its association with other proteins, such as proteins involved in STAT6 tyrosine phosphorylation. The amino acid sequence containing Arg27 is LRHLL, which matches the LXXLL motif found within the trans activation domain of STAT6 mediating the interaction with NcoA-1 (33). It is possible that LXXLL motif within the N-terminal domain mediates the interaction of STAT6 with unidentified cytoplasmic proteins, and the methylated arginine in this motif is the key residue. There are several protein arginine methyl transferases (PRMTs) that have
been identified, and STAT1 has been shown to be a substrate for PRMT1 and PRMT3 in vitro (20). It remains unclear how these enzymes are regulated. How arginine methylation is regulated and how methylation of arginine affects the interaction of STAT6 with other proteins remain to be determined.

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