Interleukin-9 (IL-9) is a cytokine with pleiotropic effects on mast cell and T cell lines. It exerts its effects through the IL-9R complex consisting of IL-9Rα and the common γc subunit. Here we report functional evidence for receptor heteromerization for efficient signal transduction, and we define minimal requirements in the two receptor subunits for IL-9R function. Tyrosine 336 of the IL-9Rα and the membrane-proximal segment of γc are both crucial for signaling. The activated IL-9R complex employs the Janus kinases JAK1 and JAK3 for subsequent activation of the signal transducer and activator transcription (STAT) factors STAT-1, STAT-3, and STAT-5. This process is independent of Tyk2. We demonstrate further that the activated STAT complexes consist of STAT-1 and STAT-5 homodimers and STAT-1-STAT-3 heterodimers. Finally, we show that IL-9R signaling in a T cell line does not result in detectable mitogen-activated protein kinase activation and leads to unsustained proliferation. Nonetheless, these T cells are efficiently protected from dexamethasone-induced apoptosis. These results further define the molecular architecture of the IL-9R and its specific connections to various biologic responses.

IL-9 expression has been found in patients with Hodgkin’s disease (6) and an autoimmune IL-9 loop for proliferation has been demonstrated in Hodgkin’s lymphoma-derived cell lines (7). Furthermore, it has been shown that IL-9 can protect thymic lymphoma cells and T cell lines from dexamethasone-induced apoptosis (8, 9). These data together suggest a possible role for IL-9 in tumorigenesis.

IL-9 exerts its effects through the functional IL-9R complex, consisting of IL-9Rα (10) and the common γc subunit. The γc subunit is also utilized in the receptor complexes for IL-2, IL-4, IL-7, and IL-15 (11–14). Both IL-9Rα and γc are members of the hematopoietin receptor superfamily (15), which share several common motifs, including four canonically spaced cysteine residues and the WSXWS motif in the extracellular domain, and the Box1 and Box2 motifs in the intracellular domain. The IL-9Rs was shown previously to associate with γc in the presence of IL-9 (16), and evidence for functional interaction of the two receptor chains was derived from studies in which anti-γc antibody treatment abolished IL-9R signaling (17).

Although neither receptor chain has any intrinsic kinase activity, rapid tyrosine phosphorylation of both receptor chains and cellular substrates occurs after receptor engagement. This step is thought to be mediated by the nonreceptor protein tyrosine kinases of the Janus kinase (JAK) family, which are preassociated with the receptor chains (for reviews, see Refs. 18 and 19). Stimulation of the IL-9R was shown to lead to the phosphorylation of JAK1, JAK3, and Tyk2 (20). It remained unclear, however, which of these kinases is necessary for the generation of the signaling response. Phosphorylated tyrosines of the receptor act as docking sites for STAT proteins (signal transducer and activator of transcription), that bind phosphotyrosines via SH2 domains (21, 22). Once bound, they become tyrosine-phosphorylated, dissociate from the receptor, and form homo- or heterodimers with members of their own family via SH2 domain interactions. These activated transcription factor dimers are capable of translocation into the nucleus to bind to specific DNA elements and initiate transcription of target genes. Several studies have suggested that the STATs involved in IL-9R signaling are STAT-1, STAT-3, and STAT-5 (20, 23, 24). However, the specific composition of the STAT-DNA complexes remains unclear.

The major activity of IL-9 on T cells has been proposed to be protection from apoptosis rather than mitogenesis. This has been demonstrated for thymic lymphoma cell lines (8) and T cell lines (9). However, no evidence has been obtained regarding the mechanisms underlying this antiapoptotic activity of the activated IL-9R.

In the present study chimeric receptors between the erythropoietin (EPO) extracellular domain and the IL-9Rα and γc intracellular domains were created and tested, along with several mutants of these chimeras, in COS-7 cells. The role of
JAKs in IL-9R signaling was investigated in the human fibrosarcoma cell lines U4A and U1A that are deficient in JAK1 and JAK3, and JAK3 and Tyk2, respectively. Using these systems, the minimal requirements for IL-9R signaling were defined. Finally, a murine T helper cell line was stably transfected with chimeric receptors, and the effect of receptor stimulation was investigated by assays for STAT-DNA binding, antiapoptotic activity, and proliferation. These data suggest that signaling through the IL-9R blocks dexamethasone-induced activation of components of the cell death machinery, possibly due to activation of the antiapoptotic STAT-3 and STAT-5.

**MATERIALS AND METHODS**

**Cell Lines and Reagents**—The IL-2-dependent murine T helper cell line HT-2 (ATCC) was cultured in RPMI 1640, supplemented with 10% fetal bovine serum, 55 μM 2-mercaptoethanol, 2 mM l-glutamine, and 200 units/ml recombinant human IL-2. Transfections were performed by electroporation (950 μF, 300 V, on a Bio-Rad Gene Pulser II) using 1 × 10^6 cells and 20 mg of DNA; stable transfectants were obtained by selection in G418 (1 mg/ml, Life Technologies, Inc.). Clones isolated by limiting dilution were screened by Northern blot analysis to identify cell lines stably expressing the transfected receptor subunit. Stable transfectants expressing two receptor subunits were derived from cells already expressing either EPOγ or EPOγγ, clones were obtained by selection in G418 and hygromycin B (Boehringer Mannheim) and screened by Northern blot analysis.

COS-7 monkey kidney cells (ATCC) were routinely cultured in Iscove’s medium supplemented with 10% fetal bovine serum and 2 mM l-glutamine. Transfections were carried out in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 2 mM l-glutamine using LipofectAMINE (Life Technologies, Inc.) per the manufacturer’s instructions.

U4A and U1A human fibrosarcoma cells (a kind gift of Dr. G. Stark) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 2 mM l-glutamine using LipofectAMINE (Life Technologies, Inc.) per the manufacturer’s instructions.

Anti–STAT-3 and anti–STAT-5 antisera were obtained from Santa Cruz Biotechnology. Anti–STAT-1 monoclonal antibody was obtained from Transduction Laboratories, antiphosphotyrosine monoclonal antibody (4G10) from Upstate Biotechnology. Recombinant murine IL-9 was purchased from Genzyme; recombinant human EPO was a kind gift of the Chiron Corp.

**Plasmids and Constructs**—All receptor cDNAs were subcloned into the expression vectors pCMV4, pCMV4neo, or pCMV4hygro. Chimeric EPOγ and receptor mutants were generated as described elsewhere (25). The EP09 chimera was constructed in a similar way using a NheI site at the fusion junction between EPOR extracellular domain and the N terminus of EPOR (25). The EPO9 chimera was constructed in a similar way using a NheI site at the fusion junction between EPOR extracellular domain and the N terminus of EPOR (25). The EPO9 chimera was constructed in a similar way using a NheI site at the fusion junction between EPOR extracellular domain and the N terminus of EPOR (25).

**Proliferation Assays**—Conventional 72-h [3H]thymidine incorporation assays were performed using triplicate cultures of 5 × 10^4 cells per sample. Cells were incubated for the indicated time period with the indicated amount of factor. 1 μCi of [3H]thymidine was added for the last 4 h of incubation. Data are expressed as a percentage of [3H]thymidine incorporation of cells treated with 10 nM IL-2.

**Apoptosis Assays**—5–10 × 10^5 cells were incubated for 24 h in medium without IL-2, or stimulated with 10 nM IL-2, 50 units/ml EPO, or 1 μM dexamethasone (Sigma), respectively. Cells were harvested, washed with phosphate-buffered saline, and immediately used for staining. Staining with fluorescein isothiocyanate-conjugated annexin V and propidium iodide was performed using the ApoAlert kit (CLONTECH). Samples were analyzed on a Becton Dickinson FACScan. The percentage of dead (annexin V- and propidium iodine-positive cells) and actively dying cells (annexin V-positive and propidium iodide-negative) was determined by gating on the intact cell population, excluding cellular debris.

**Electrophoretic Mobility Shift Assays (EMSAs)**—20–40 × 10^6 cells were rested in serum-free medium containing 1% bovine serum albumin for 4 h and stimulated as described for 10 min (COS-7, U4A, U1A) or 15 min (HT-2). Cells were lysed, and nuclear extracts were prepared as described previously (26).

DNA binding studies were performed with 1 × 10^5 cpm of probe, 3 μg of poly(dI-dC) (Boehringer Mannheim) and the indicated amounts of nuclear extracts on a nondenaturing 5% polyacrylamide gel. The IgG Fc receptor STAT response element (FcγR1) was end-labeled with [γ-32P]dATP (Amersham Corp.) and polynucleotide kinase (New England Biolabs). Preincubations with different antibodies were performed in the absence of poly(dI-dC) and binding buffer for 45 min on ice prior to initiation of the binding reaction by addition of labeled probe.

MAPK Assay—10–20 × 10^5 cells were rested for 6 h and stimulated as described. In vitro MAPK assays were performed using the MAPK assay kit (New England Biolabs). In short, cells were lysed and activated Erk1/Erk2 MAPK was immunoprecipitated using an antiphospho-MAPK antibody. Kinase assays were performed using an Elk1-glutathione S-transferase fusion protein as a substrate with subsequent immunoblotting for phosphorylated Elk1.

**RESULTS**

**Reconstitution of IL-9R Function in COS-7 Cells**—To evaluate the minimal requirements for efficient signaling in the IL-9R system a chimeric receptor approach was employed in the background of COS-7 cells. Expression plasmids encoding EPOγ (chimeric IL-9Rα subunit containing extracellular domain of EPOR) and EPOγγ (chimeric γc chain containing extracellular domain of EPOR) were cotransfected into these cells together with various components of the signaling machinery and STAT activation was investigated. After stimulation with EPO, nuclear extracts were prepared, followed by EMSA with a STAT-specific binding element (FcγR1). DNA binding activity was observed only when both chimeric receptor chains EPO9 and EPOγ were present (Fig. 1A), and was not detected when of poly(dI-dC) (Boehringer Mannheim) and the indicated amounts of nuclear extracts on a nondenaturing 5% polyacrylamide gel. The IgG Fc receptor STAT response element (FcγR1) was end-labeled with [γ-32P]dATP (Amersham Corp.) and polynucleotide kinase (New England Biolabs). Preincubations with different antibodies were performed in the absence of poly(dI-dC) and binding buffer for 45 min on ice prior to initiation of the binding reaction by addition of labeled probe.

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either receptor chain alone was present. Thus, signaling through the IL-9R apparently depends on heteromultimerization of the two receptor subunits; based on the structure of EPOR these presumably represent dimers, but higher order multimers may exist. Endogenous JAKs and cotransfected JAK3 were necessary and sufficient for the generation of a signal by this receptor complex. In the absence of cotransfected STAT, a single band corresponding to endogenous STAT-1 (as identified by antibody supershift analysis, data not shown) was present; cotransfection of STAT-5 resulted in a second more slowly migrating band, which was identified as STAT-5 by antibody supershift analysis (data not shown). These data show that EPO9 and EPOγ apparently must form heteromers for efficient signal transduction resulting in STAT binding to DNA. This signaling complex requires JAK3 and is able to activate both STAT-1 and STAT-5.

To test which portions of γc are necessary for efficient signaling, several mutants of EPOγ(25) were tested (Fig. 1B). The EPOγ336 mutant, with C-terminal truncation up to the Box2 region, supported induction of both STAT-1 and STAT-5 bands and thus showed no difference compared with the wild-type chain in the EMSA. Further truncation into the Box1-Box2 (EPOγ294) domain and to the TM region (EPOγTM), or internal deletion of either Box1 or Box2, abolished signaling. Since JAK3 has been proposed to bind to the Box1-Box2 domain of γc, these results likely are explained by the inability of those mutants to bind JAK3.

Tyrosine substitution mutants of both receptor chains were tested to elucidate the role of tyrosine residues in the signaling processes (Fig. 1C). Complete substitution of all tyrosines in the EPOγ-chain with phenylalanine (EPOγYF) led to no change in STAT activation. EPO9Y3F was created to verify in this system the recent report that tyrosine 336 of IL-9R is crucial for activation of STAT-1 and STAT-5 (24). Substitution of tyrosine 336 with phenylalanine (EPO9Y3F) in EPO9 led to the loss of both STAT bands following receptor stimulation; based on the structure of EPOR these presumably represent dimers, but higher order multimers may exist. Endogenous JAKs and cotransfected JAK3 were necessary and sufficient for this activity.

Reconstitution of Native IL-9R Function—To confirm these findings and to clarify further the role of the JAKs in IL-9R function, U4A and U1A cells were employed in a similar reconstitution approach. U4A is a somatic mutant of the human 2FTGH cell line that fails to express JAK3 and JAK1 but contains JAK2 and Tyk2 (27). In U4A cells, IL-9-mediated induction of STAT-5 was reconstituted by the simultaneous transfection of IL-9Ra, γc, JAK1, JAK3, and STAT-5 (Fig. 2). No signal was observed when any of these components were omitted from the transfection, indicating that endogenous Tyk2 or JAK2 cannot substitute for JAK1 or JAK3 in this system (Fig. 2). Furthermore, replacement of either wild-type JAK1 or JAK3 with a kinase-inactive mutant that lacks the catalytic lysine (JAK1 K907A or JAK3 K851A, respectively) (28) abolished downstream induction of STAT-5. These findings strongly suggest that the primary kinases required for IL-9R function are JAK1 and JAK3, and that Tyk2 is dispensable for this activity.

To establish definitively whether or not Tyk2 contributes to IL-9R function in this system, similar experiments were performed with the U1A somatic mutant (29) that lacks Tyk2 and JAK3 but retains JAK1 and JAK2. In this cellular context, robust activation of STAT-5 through the IL-9R occurred upon introduction of JAK3 (Fig. 2); Tyk2 evidently was nonessential for this process since these cells lack Tyk2. Transfection with a Tyk2 expression plasmid in place of JAK3 yielded only a very marginal signal (Fig. 2). Therefore, complementary systems (COS, U4A, and U1A) uniformly demonstrated the importance of catalytically active JAK1 and JAK3 and the dispensability of Tyk2 for IL-9R-dependent signaling.

Signaling by a Chimeric IL-9R Induces STAT-1, STAT-3, and STAT-5 in a T Cell Line—The EPO9 and EPOγ (EPO9YF) receptor chains were stably transfected into the IL-2-dependent murine T helper cell line HT-2 to investigate IL-9R signaling in a T cell context. After stimulation of stable transfectants
with EPO, nuclear extracts were prepared and analyzed by EMSA with the FcγR1 probe. DNA binding activity was detected only in cells expressing both chimeric receptor chains, EPO9 and EPOγ (Fig. 3). The resulting triple band vanished when excess unlabeled oligonucleotide was used as a competitor. STAT activation was sustained over a 60-min stimulation period, and the signal became weaker with prolonged stimulation vanishing after 90 min (data not shown). The tripartite band was also observed in cells transfected with vectors encoding EPO9 and EPOγYF (Fig. 3). To determine further the nature of the observed bands, supershift analysis was performed with antibodies against phosphotyrosine and against STAT-1, STAT-3, and STAT-5 (Fig. 3). Isotype-matched antibodies resulted in no change of pattern. Treatment with antiphosphotyrosine antibodies resulted in a complete loss of all three bands, due to prevention of formation of STAT dimers via SH2 domain-phosphotyrosine interactions. When extracts were treated with anti-STAT-1 antibody, the two lower bands were lost, whereas anti-STAT-3 treatment led to selective loss of the middle band and some diminution of the lower band. Anti-STAT-5 antibody resulted in slower migration of the upper band. The anti-STAT-1 and anti-STAT-3 antibodies presumably are blocking antibodies, preventing either STAT-dimer formation or DNA binding, resulting in loss of the band. In contrast, the anti-STAT-5-antibody apparently binds to the STAT-5-DNA complex, which leads to a slower migration of the band on the gel. These data suggest that the STAT complexes formed upon stimulation of the IL-9R consist of STAT-5 and STAT-1 homodimers (upper and lower bands, respectively), STAT-1-STAT-3 heterodimers (middle band), and possibly STAT-3 homodimers (bottom band).

To test whether IL-9R stimulation protected HT-2 cells from glucocorticoid-induced apoptosis, stable cell lines were treated with dexamethasone and evaluated for apoptotic responses by flow cytometry analysis of cells stained with fluorescein isothiocyanate-labeled annexin 5. HT-2 cells were starved of IL-2 for 24 h and treated with either dexamethasone alone or with dexamethasone and EPO. IL-2 withdrawal, as well as additional dexamethasone treatment, led to the onset of apoptosis after ~4 h (data not shown), resulting in a massive number of apoptotic cells after 24 h (Fig. 4). Treatment with dexamethasone increased the percentage of dead and dying cells considerably above the level induced by IL-2 withdrawal. Stimulation of the chimeric receptor complex with EPO led to a complete blockade of apoptosis caused by dexamethasone, but had no influence on the degree of apoptosis caused by IL-2 starvation. EPO-mediated inhibition of dexamethasone-dependent apoptosis was not observed in cells expressing a single receptor subunit (EPOγ or EPO9, Fig. 5, A and B, respectively), but only in cells expressing both EPO9 and EPOγ or EPO9 and EPOγYF (Fig. 4, C and D, respectively).

**The Signal Delivered by the Chimeric IL-9R Does Not Sustain Proliferation in HT-2 Cells**—Since reduction of the percentage of annexin 5-positive cells by treatment with EPO could have been due to an increased overall cell number, the proliferative signal generated by the IL-9R was investigated. HT-2 cells transfected with either EPO9 or EPOγ alone displayed no proliferative response to EPO in the absence of IL-2 as measured by [3H]thymidine incorporation (Fig. 5). Both, EPOγ9 and EPOγYF/9, however, showed vigorous DNA synthesis after 24 h in a dose-dependent fashion. Nevertheless, this response was not sustained and diminished to background levels after 72 h (Fig. 5). Prolonged cultivation in EPO without IL-2 did not lead to generation of EPO-dependent clones.

**Activation of Erk1/Erk2 MAPK Does Not Take Place in Response to EPO**—To test whether the proliferative response after chimeric receptor stimulation with EPO was associated with activation of MAPKs, in vitro kinase assays of immunoprecipitated Erk1/Erk2 MAPK were performed. Unstimulated HT-2 EPOγ9 cells showed no detectable MAPK activity as shown by Western blotting for in vitro phosphorylated Elk1 substrate (Fig. 6). However, upon stimulation with IL-2, strong activation of MAPK was observed. Stimulation of the chimeric EPOγ9 receptor complex with EPO did not result in substantial induction of MAPK activity. These data suggest that the signaling pathways leading to proliferation are different in the IL-2R and IL-9R systems, and apparently are incomplete in the IL-9R system in this cellular context.
trophoresis and immunoblotted with antiphospho-Elk1 antibodies.

The IL-9R and its associated JAK1 and JAK3 apparently activate a specific signaling program involving STAT-1 homodimers, STAT-1-STAT-3 heterodimers and STAT-5 homodimers as shown by antibody supershift analysis of nuclear extracts of stable transfectants. Furthermore, activation of these STAT complexes is linked to tyrosine 336 of IL-9Rα and does not depend upon tyrosines of the γc subunit. Therefore, as in the IL-2, IL-4, and IL-7 receptors, specificity in the signaling program appears to be driven largely through the longer, non-γc subunit of the receptor.

Consistent with earlier studies demonstrating JAK1 and JAK3 activation (20, 23) by IL-9, these kinases were observed to be crucial for generating STAT-DNA complexes in the IL-9R system. In the U4A system, simultaneous transfection of both JAK1 and JAK3 (along with the receptor chains and STAT-5) effectively reconstituted IL-9-mediated STAT-5 induction. Since these cells express Tyk2 endogenously, it is evident that Tyk2 could not replace either JAK1 or JAK3 to support IL-9R function. Additionally, the catalytic integrity of both of these kinases was essential for IL-9R function. These findings are consistent with those observed in the reconstitution experiments, in which endogenous Tyk2 and JAK1 were insufficient to support IL-9R function. Moreover, experiments with the U4A cell line lacking Tyk2 revealed definitively the dispensability of Tyk2 for IL-9R function. Therefore, although Tyk2 may be activated detectably by IL-9 in some cellular contexts, it does not appear to play a major role in IL-9R signaling function.

IL-9R signals through chimeric receptors were found to stimulate short-term DNA synthesis of HT-2 cells. Strong proliferative effects of IL-9 have been observed only in mast cells and activated T-cells. Non-sustained proliferation, as observed here, could be due to a variety of signaling constraints that may be cell context-specific. The failure of the IL-9R to activate Erk1/Erk2 MAPK as measured in the short-term assay in this system is one such example, and other cell-specific pathways also need to be explored.

Perhaps the most important function mediated by IL-9 is prevention of apoptosis, which was shown here to depend upon dimerization of the IL-9Rγ and γc subunits of the IL-9R. The molecular mechanism underlying the antiapoptotic effect remains to be elucidated. STAT-3 and/or STAT-5 may play a role, since they have been implicated in antiapoptotic effects in other signaling systems. For example, it was demonstrated that overexpression of a dominant-negative form of STAT-3 inhibited induction of the antiapoptotic gene bcl-2 upon stimulation of the IL-6R complex (31). Moreover, STAT-5 has been shown to bind to the activated glucocorticoid receptor and to inhibit glucocorticoid receptor-mediated gene transcription in COS-7 cells (32). Further studies are needed to clarify these and other possible mechanisms with regard to the IL-9R. Since apoptosis in general plays an important role in tissue homeostasis and regulation of the immune response (33), clarifying mechanisms by which immune regulatory cytokines exert antiapoptotic activity and proliferative actions remains an important goal. The system presented here should prove useful in addressing these questions.

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**Fig. 5. Induction of proliferation in a T-cell line.** Proliferative response of stably transfected HT-2 cells after stimulation with the indicated doses of EPO. A, cells expressing only a single receptor subunit did not respond to EPO after 24 h of stimulation. The double transfectants EPOγ9 and EPOγYF/9 responded to EPO vigorously in a dose-dependent manner. B, the proliferative response of HT-2 EPOγ9 and HT-2 EPOγYF/9 cells was time-dependent and was not sustained for more than 24 h. Additional dexamethasone treatment completely blocked the proliferative response to EPO (14 units/ml) of these cell lines; the same effects were observed at 10 or 50 units/ml (data not shown). **Error bars** are the standard error of the mean of triplicate measurements; in some cases these error bars are too small to be depicted.

**Fig. 6. Absence of MAPK induction by IL-9 in HT-2 cells.** Total cell lysates of HT-2 EPOγ9 cells were prepared and immunoprecipitated with antiphospho-MAPK antibody. Immunoprecipitates were subjected to in vitro kinase assays with an Elk1-glutathione S-transferase fusion protein, separated by 10% SDS-polyacrylamide gel electrophoresis and immunoblotted with antiphospho-Elk1 antibodies. HT-2 EPOγ9 stimulation with IL-2 yielded a ~40-kDa band (indicated by the arrow) corresponding to phosphorylated Elk1, whereas EPO stimulation did not.

**DISCUSSION**

These studies demonstrate that the functional IL-9R complex consists of heteromers of γc and IL-9Rα, as confirmed in a variety of cellular backgrounds and by various measurements of signal transduction. In a T-cell line, STAT activation, induction of DNA synthesis and protection from apoptosis were observed exclusively upon engagement of both receptor subunits. The data presented here with various mutants of the γc or IL-9Rα cytoplasmic tail are also consistent with the "trigger driver" model proposed for signaling by various chimeric cytokine receptors (30). In this model, the γc chain acts in the IL-2, IL-4, IL-7, IL-9, and IL-15 receptors to initiate the signaling response upon engagement of ligand by conveying a kinase (JAK3) into the receptor complex. Within the IL-9R, the specificity of the resulting signaling response appears to be coupled to tyrosine residues within the IL-9Rα chain.

The IL-9R and its associated JAK1 and JAK3 apparently activate a specific signaling program involving STAT-1 homodimers, STAT-1-STAT-3 heterodimers and STAT-5 homodimers as shown by antibody supershift analysis of nuclear extracts of stable transfectants. Furthermore, activation of these STAT complexes is linked to tyrosine 336 of IL-9Rα and does not depend upon tyrosines of the γc subunit. Therefore, as in the IL-2, IL-4, and IL-7 receptors, specificity in the signaling program appears to be driven largely through the longer, non-γc subunit of the receptor.

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IL-9R Signaling Properties

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