Nef is a 27-kDa myristoylated protein conserved in primate lentiviruses. In vivo, simian immunodeficiency virus Nef is required in macaques to produce a high viral load and full pathological effects. Nef has at least three major effects in vitro, induction of CD4 down-regulation, alteration of T cell activation pathways, and enhancement of viral infectivity. We have used the yeast two-hybrid system to identify cellular proteins that interact with HIV-1 Lai Nef and could mediate Nef function. A human cDNA was isolated that encodes a new type of thioesterase, an enzyme that cleaves thioester bonds. This novel thioesterase is unlike the animal types I and II thioesterases previously cloned but is homologous to the Escherichia coli thioesterase II. Nef and this thioesterase interact in vitro and are co-immunoprecipitated by anti-Nef antibodies in CEM cells expressing Nef. Nef alleles from human immunodeficiency virus-1 (HIV-1) isolates unable to down-regulate CD4 do not react or react poorly with thioesterase. An HIV-1 NefLai mutant selected for its lack of interaction with thioesterase was also unable to down-regulate CD4 cell surface expression. These observations suggest that this human thioesterase is a cellular mediator of Nef-induced CD4 down-regulation.

The nef auxiliary gene of the human immunodeficiency virus (HIV) is highly conserved in most primate lentiviruses (HIV-1, HIV-2, and simian immunodeficiency virus) (for review, see Refs. 1 and 2). The biological importance of Nef for both high virus load and disease progression has been demonstrated in vivo in SIVmac-infected rhesus monkeys (3) and confirmed in humans by studies on long term non-progressor patients infected by HIV-1 isolates with a deleted or truncated nef gene (4, 5). In cultured cells, Nef facilitates virus replication and enhances the infectivity of virions (6–9).

The best documented biological activity of Nef in vitro is the triggering of CD4 endocytosis, its accumulation in early endosomes, and its subsequent degradation in lysosomes (10–13), by a mechanism that may involve a dileucine endocytosis target motif in CD4 (11). Intracellular sequestration of CD4 in the Golgi apparatus has also been found in nef transgenic mice (14). In addition, we have reported that Nef stimulates the endocytosis of the major histocompatibility complex class I molecules (15).

Conflicting results concerning the effects of Nef on the T cell activation pathways have been reported and seem to depend on its targeted intracellular localization (16–20).

The functions of Nef in HIV-1-infected cells are probably mediated by specific interactions with cellular proteins, and elucidation of the mechanisms of Nef action will require the identification of its cellular partners. We have attempted to identify the cellular proteins involved using the yeast two-hybrid system. The present report describes the identification of a cDNA encoding a Nef-interacting protein that is 42% identical with the Escherichia coli thioesterase II, an enzyme involved in cleavage of thioester bonds. This human thioesterase (hTE) corresponds to a new type of eucaryotic thioesterase, distinct from the two types of animal thioesterases previously described. The Nef-hTE interaction was confirmed in vitro and in CEM cells expressing Nef. There is also a correlation between Nef-hTE interaction and CD4 down-regulation, suggesting that hTE could be one of the cellular proteins involved in Nef-mediated CD4 down-regulation.

Experimental Procedures

Two-hybrid System—Two-hybrid screening was performed as described previously (21) in the yeast reporter strain HF7c, with the NefLai protein fused to the Gal4BD in pGBT10 and a Jurkat cell cDNA library fused to the Gal4AD in pGAD1318. Positive clones were rescued and tested for specificity by retransformation into HF7c with Nef or with the extraneous targets, yeast SNF1 (22), HIV-1 Vpr, and HIV-1 Gag. The cDNA inserts from specific positive clones were sequenced using the Sanger dideoxy termination method adapted to the ABI 373A Automated Sequencer.

Nef sequences from HIV-1 isolates HXB3, A01, D01, and E01 were amplified by polymerase chain reaction and inserted into pGBT10 as described for the nefLai gene (21). The liquid culture assay for quantitative β-galactosidase activity was performed using SFY526 strain as described previously (23). Each assay was performed in triplicate.

Assay of Thioesterase Activity—The hTE coding sequence was subcloned into pGEX-4T3 (Pharmacia Biotech Inc.), and the GST-hTE fusion protein was expressed in Escherichia coli (24). Acyl-CoA thioesterase activity was measured at 22 °C in a spectrophotometric assay (25) using recombinant hTE (rhTE) protein prepared from GST-hTE fusion cleaved by thrombin (24). The purified E. coli TE II (gift from S. Smith) was used as a control. Incubation mixtures contained various
amounts of rhTE in 0.05 m potassium phosphate buffer, pH 8.0, 0.1 m 5,5’-dithiobis(2-nitrobenzoate), 20 μg/ml bovine serum albumin, and 7 different concentrations (between 2 and 80 μM) of acyl-CoA (either C6-CoA, C8-CoA, C10-CoA, C12-CoA, C14-CoA, C16-CoA, or C20-CoA, Sigma), as substrate. The reactions were monitored spectrophotometrically by recording absorbance at 412 nm. A unit of activity is the amount of enzyme required to hydrolyze 1 nmol of substrate/min.

In Vitro Binding Study—The GST-hTE fusion protein was expressed in E. coli and immobilized on GSH-agarose beads (24). Purified recombinant Nef (rNef) was prepared from GST-Nef cleaved by thrombin (24). 14 μg of GST-hTE or GST immobilized on GSH-agarose beads were incubated for 4 h at 4 °C, with 10 μg of rNef in TENN buffer (24). The beads were washed three times with TENNG buffer and once with TENG buffer without Nonidet P-40, and subjected to SDS-polyacrylamide gel electrophoresis. rNef binding was analyzed by Western blot with rabbit anti-NefLai Abs (gift from E. Bahraoui) and the enhanced chemiluminescence system (Amersham Corp.). The band corresponding to the longer cDNA isolated in the two-hybrid screen and encodes the human sequence numbering was according to Ref. 13780.

**FIG. 1.** Specific interaction of Nef with hTE in the two-hybrid system. The HF7c reporter strain expressing the pairs of indicated hybrid proteins fused to the Gal4BD and Gal4AD was analyzed for histidine auxotrophy and β-galactosidase activity. Double transformants were patched on selective medium with histidine (center panel) and on Whatman filter for α-naphthyl acetate assay (right panel). Growth in the absence of histidine and expression of β-galactosidase activity indicate the interaction between hybrid proteins. Clone 20 (lane 2) corresponds to the longer cDNA isolated in the two-hybrid system and screens the full-length hTE, whereas clone 5A (lane 1) corresponds to the smaller cDNA coding for hTE lacking 6 aa at its N terminus. Each patch represents an independent transformant.

**FIG. 2.** Amino acid sequences of human thioesterase (upper sequence) and the E. coli thioesterase II (lower sequence). Identical residues are indicated by dashes and conservative residues are indicated by dots. The E. coli TE II sequence is numbered according to Ref. 25. The human sequence was deduced from the nucleotide sequence of the hTE cDNA, starting at the first in frame methionine residue. The conserved Val-His-Ser motif corresponding to the putative active site of the E. coli TE II enzyme is boxed; the putative peroxisomal C-terminal targeting signal (SKL) in the last three residues of hTE is underlined. The hTE nucleotide sequence will be lined. The hTE nucleotide sequence will be described previously (15, 29). Briefly, P4-2 cells were co-transfected with the expression vector (Invitrogen), and the resultant plasmids containing mutant coding sequences were cloned into the pcDNA3 mammalian expression vector (Invitrogen), and the resultant plasmids (pDNA-NefLai and pDNA-Nef*) were used for transient expression in P4-2 cells (HeLa cell line expressing the human CD4 molecule) as described previously (15, 29).

**Generation and Screening of the NefLai Mutant Library**—The nef gene from the HIV-1Lai isolate was amplified by error-prone polymerase chain reaction according to Ref. 28, using the 3′ primer LexBonR (AATTTCGCGCGAATTAGC) and the 5′ primer GadSeqF (GGATGATGTATATAACTATC) priming into pGBT10. The amplified amplified fragments were double-digested by BamHI and SalI and then cloned into pGBT10 as described (21). The E. coli DH5a strain was transformed and about 5 × 10⁸ transformants were obtained, representing the complexity of the potential mutant library. These transformants were pooled, and the plasmid library was prepared. HF7c yeast strain was cotransformed with the NefLai mutant library and the Gal4AD-hTE hybrid. Plasmids from mutants unable to interact with hTE were rescued and used to retransform the reporter strain with the Gal4AD-β-COP-Cter hybrid (21). A NefLai mutant (Nef*) that did not interact with hTE but did interact with β-COP-Cter was selected and completely sequenced.

**Assay of Nef-mediated CD4 Down-regulation**—NefLai and Nef* mutant coding sequences were cloned into the pcDNA3 mammalian expression vector (Invitrogen), and the resultant plasmids (pDNA-NefLai and pDNA-Nef*) were used for transient expression in P4-2 cells (HeLa cell line expressing the human CD4 molecule) as described previously (15, 29). Briefly, P4-2 cells were co-transfected by electroporation (200 V, 960 microfarads, using 4-mm wide cuvettes in a 25 cm Hepes, pH 7.4, 150 mM KCl, 5 mM EDTA, and 1% Triton X-100. Lysates were incubated overnight at 4 °C with anti-Nef Abs (HIV-1BH10 Nef antiserum obtained from the National Institutes of Health AIDS Research Program, see Ref. 27) diluted 1:1000. Protein A-Sepharose beads were then added, and after extensive washes, immunoprecipitates were analyzed by Western blot with anti-Nef and purified anti-hTE Abs and the enhanced chemiluminescence system (Amersham Corp.). Specific anti-hTE Abs, raised in rabbits by immunization with a synthetic peptide (Gln-304 to Lys-318 of the hTE open reading frame, see Fig. 2), were affinity purified by adsorption on GST-hTE immobilized on a nitrocellulose membrane. The Abs were then eluted in glycine HCl, pH 3.0, and neutralized with 1 m Tris, pH 8.0.

300 310 319
LAVTCQMGSVVRKPKQVESKLR
||| : ||| : |
LVASTYQQSRK
280
RESULTS

cDNA Cloning of a Novel Thioesterase Interacting with HIV-1 Nef—The two-hybrid system was used to identify the cellular proteins interacting with the HIV-1 Nef protein. Two independent overlapping cDNAs with different 5'-ends were isolated. These cDNAs conferred on the yeast reporter strain the ability to grow in the absence of histidine and to express the β-galactosidase activity in the presence of the Gal4BD-Nef hybrid (Fig. 1, lanes 1 and 2) but not with extraneous targets fused to Gal4BD (lanes 3–6).

The longer cDNA clone contained a 1153-base pair insert flanked 3' by a stretch of A residues preceded by a consensus polyadenylation signal 17 base pairs upstream from the poly(A). Northern blot analysis using a probe corresponding to the hTE cDNA revealed a single 1.3-kilobase pair mRNA transcript widely expressed in all human tissues tested (data not shown), indicating that hTE cDNA was close to the full-length mRNA transcript. Starting at the first in frame methionine residue, the cDNA contained an open reading frame encoding a 319-amino acid (aa) long protein with a predicted molecular mass of 35.6 kDa. A data base search revealed a 42% identity with the thioesterase II (TE II) coded by the tesB gene from E. coli (25), suggesting that this cDNA encodes the human homolog (hTE) of the E. coli TE II (Fig. 2). hTE was also similar to the putative TE II homologs from Caenorhabditis elegans, Homo sapiens, and Saccharomyces cerevisiae (37, 38, and 24% identity, respectively).

hTE has no significant homology with either of the two types of animal thioesterases that have been cloned (30), indicating that it encodes a new type of eucaryotic thioesterase. In particular, neither the Gly-Xaa-Ser-Xaa-Gly nor the Gly-Xaa-His motifs conserved in the active site of type I and type II animal thioesterases of the classical serine esterase enzymes are found in hTE or the E. coli enzyme. By contrast, the His-58 residue of the E. coli TE II, which is suspected to be part of the active site of the enzyme (25), is conserved in the human sequence at position 78, together with the adjacent residues Val-77 and Ser-79 (Fig. 2). Interestingly, a search in the Prosite data base revealed a C-terminal peroxisomal targeting signal (Ser-Lys-Leu) in the last three residues of hTE (see Fig. 2), suggesting that this novel thioesterase enzyme is imported in peroxisomes (31).

Recombinant Human Thioesterase Displayed Enzymatic Activity In Vitro—To confirm that hTE cDNA encoded a thioesterase enzyme, a spectrophotometric assay was performed using fatty acyl-CoA as substrates and recombinant hTE (rhTE) obtained from thrombin cleavage of GST-hTE fusion protein. The rhTE catalyzed the hydrolysis of the decanoyl-CoA thioester bond (Fig. 3A). The kinetic properties of rhTE obeyed Michaelis-Menten kinetics with this substrate. The Vₘₐₓ and Kₘ values were calculated by fitting the experimental data to the Michaelis-Menten equation. The values found (Vₘₐₓ 7.1 μmol/min/mg and Kₘ 10.1 μM) were of the same order as those obtained with the purified E. coli TE II (32) used as control in this assay (data not shown). The fatty acyl chain length specificity was determined by measuring the enzymatic activity of rhTE with a panel of acyl-CoAs substrates and comparing them to the specific activity obtained with decanoyl-CoA (Fig. 3B). Clearly, rhTE showed a preference for acyl-CoAs with medium chain length of C10 to C14, with maximal activity with myristoyl-CoA (C14). The enzyme was less active with C6-, C8-, and C10-CoA, and no activity was detected with C18- or C20-CoA as substrates. These results indicate that hTE has, like the E. coli TE II, a relatively broad acyl chain length specificity that extends to medium chain lengths. The influence of Nef on the activity of rhTE in vitro was tested, but there was no evidence for any significant modification of the enzymatic activity (data not shown).
Interaction between Nef and Human Thioesterase in Vitro and in CEM Cells Expressing Stably HIV-1Lai Nef—The interaction between Nef and hTE was confirmed in vitro using recombinant proteins. As shown in Fig. 4A, rNef bound specifically to GST-hTE (lane 3) but not to GST (lane 2). Densitometry scanning of the signals obtained in Fig. 4A indicated that 47% of rNef used in the reaction was bound to GST-TGE (compare lanes 1 and 3). Allowing for the respective quantities of rNef used (10 μg = 3.7 \times 10^{-10} \text{ mol}) and of GST-hTE (14 μg = 2.2 \times 10^{-10} \text{ mol}) immobilized on GSH-agarose beads, approximately 80% of GST-hTE seemed complexed with rNef. These in vitro binding studies demonstrated that Nef and hTE were capable of direct physical interaction independent of any yeast intermediate protein. They also suggest that myristoylation of Nef is not necessary for its association with hTE, since rNef is not myristoylated.

hTE and Nef were also associated in CEM T-lymphocyte cells expressing stably HIV-1Lai Nef. The two proteins were co-precipitated from total cell lysates using anti-Nef Abs. When the immunoprecipitates were blotted and probed with purified anti-Nef Abs (Fig. 4B, upper panel), hTE was detected as a 35-kDa polypeptide only in CEM cells expressing Nef (lane 4) and not in control CEM cells (lane 3). The specific reactivity of the 35-kDa band detected with anti-hTE Abs was demonstrated by its disappearance when Abs were incubated with the peptide used for immunization prior to the Western blot analysis (not shown). The blot was subsequently probed with anti-Nef Abs to check that Nef was indeed co-precipitated with hTE. Therefore, these results confirm that the Nef-hTE interaction is direct and requires no additional viral proteins. They provide strong evidence that Nef interacts physically with hTE in a specific manner within human T-lymphocyte cells expressing Nef.

Interaction of Nef Alleles with Thioesterase and Nef-mediated CD4 Down-regulation—To determine whether interaction with hTE could influence one of the known activities of Nef, we studied the interaction of hTE with Nef proteins from several HIV-1 isolates. NefA01 and NefE01 are proteins from primary isolates that are as efficient as NefLai in promoting CD4 down-regulation (26). By contrast, Nef proteins from the HXB3 laboratory strain and from another primary isolate (NefD01) are unable to down-regulate CD4 despite expression levels comparable to NefLai (16). These four Nef proteins were fused to the Gal4BD and expressed in yeast. Their interaction with Gal4AD-hTE was measured by a two-hybrid quantitative -galactosidase assay. All of these Gal4BD fusion proteins were expressed at comparable levels, as judged by Western blot analysis on yeast extracts (not shown). The results of these experiments are summarized in Table I. NefA01 and NefE01 interacted strongly with hTE and induced more β-galactosidase activity than the NefLai protein, indicating that interaction with hTE is a general property of HIV-1 Nef alleles. By contrast, NefHXB3 interacted very poorly with hTE, with less than 10% of the β-galactosidase activity of the NefLai protein, indicating that interaction with hTE is a general property of HIV-1 Nef alleles. By contrast, NefHXB3 interacted very poorly with hTE, with less than 10% of the β-galactosidase activity (9.6 units) obtained with the NefLai protein (122.5 units), whereas NefD01 did not interact at all with hTE, giving a β-galactosidase activity (1.9 units) equivalent to the background (2.8 units). These results suggest that there is a striking correlation between the ability of Nef alleles to induce CD4 down-regulation and to interact with hTE.

We checked this correlation by selecting a NefLai mutant (Nef*4) that did not interact with hTE (Fig. 5A, lane 1) but still interacted with β-COP-Cter (lane 2) (21) by two-hybrid screening of a Nef random mutant library. NefLai used as a control

### Table I

| Nef allele | β-Gal units | CD4 down-regulation |
|------------|-------------|---------------------|
| NefLai     | 122.5 (100) | +                   |
| NefA01     | 136.9 (112) | +                   |
| NefE01     | 152.8 (125) | +                   |
| NefHXB3    | 9.6 (7)     | −                   |
| NefD01     | 1.9         | −                   |
| Nef*4      | 2.1         | −                   |

*Nef alleles fused to the Gal4BD were analyzed for interaction with Gal4AD-hTE hybrid by quantitative β-galactosidase (β-Gal) two-hybrid assay. The results were compared to the β-galactosidase activity obtained with the Gal4BD-NefLai hybrid.

CD4 down-regulation activity of Nef alleles has been published previously (Skowronski et al. (16) and Schwartz et al. (26)) except for NefD01 and Nef*4 mutant (this study; see Fig. 5B).
were analyzed by Western blot with anti-Nef antibodies. Nef*4
assay. P4-2 cells transiently transfected with either pcDNA-Nef*4
expression in transfected HeLa CD4 cells used in the CD4 down-regulation
encoded by the DNA copy of the hTE gene. We found that hTE
interaction of Nef*4 with hTE giving rise to a
interaction with hTE and their ability to induce CD4 down-regulation. Two HIV-1 Nef alleles (NefHXB3 and NefD01) that
were transiently transfected with pcDNA-NefLaiWT (lane 3)
were unable to down-regulate the cell-surface expression of CD4. These results clearly indicated that the Nef*4 mutant was completely unable to induce CD4 down-regulation and confirmed the correlation between the ability of Nef proteins to interact with hTE and to down-regulate the CD4 cell-surface expression.

**DISCUSSION**

These results provide three lines of evidence that HIV-1 Nef interacts directly with a new thioesterase enzyme. First, Nef interacts specifically with hTE in the yeast two-hybrid system; second, Nef binds to hTE in vitro; and third, co-immunoprecipitation experiments indicate that Nef and hTE are physically associated in Nef-expressing cells.

We also find a correlation between the capacity of Nef alleles to interact with hTE and their ability to induce CD4 down-regulation. Two HIV-1 Nef alleles (NefHXB3 and NefD01) that
were unable to down-regulate the cell-surface expression of CD4 were also unable to interact with hTE. In contrast, the HIV-1 Nef proteins from HIV-1Lai or from primary isolates (NefA01 and NefE01), which can down-regulate the cell-surface expression of CD4, still interact with hTE. These results strongly suggest that the physical interaction between Nef proteins and hTE is required for Nef-induced CD4 down-regulation. In addition, we have isolated a Nef mutant from the Lai isolate (Nef*4) that does not interact with hTE and is also completely unable to down-regulate CD4.

The aa mutations found in the Nef*4 mutant (W57R, F68S, D123G, H166R, and L170Q) are required for the loss of interaction with hTE, but these mutations suppressed Nef-CD4 down-regulation activity without affecting the stability of the protein. The Trp residue in position 57, corresponding to the potential cleavage site of Nef by the HIV-1 protease (33, 34), probably does not play an essential role since the first 57 N-terminal aa of Nef are not necessary for binding to hTE. By contrast, deletion of the 17 C-terminal residues completely disrupts the Nef-hTE interaction (data not shown). These results are in agreement with recent observations indicating that mutations in the C-terminal region of Nef had more deleterious effects on CD4 down-regulation activity than those in the N-terminal region (35). The elucidated conformational structure of Nef (37, 38) should allow the more precise definitions of the structural domain(s) involved in Nef activities. It will be interesting to compare the structures of Nef alleles that down-regulate CD4 with those of Nef proteins unable to do so.

Likewise the E. coli TE II, hTE has no sequence similarity with the previously reported animal thioesterases known to be involved in the chain termination step of fatty acid synthesis (30); the type I enzymes that cleave long chain fatty acyl-CoAs and the type II enzymes that are more active on medium chain length acyl-CoAs. In addition, no similarity was found with the palmitoyl-protein thioesterase (39). All these thioesterases are, like the E. coli TE I, serine esterases. Therefore, hTE is probably the prototype of a new subfamily of eucaryotic thioesterases.

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physiological roles of hTE and its E. coli TE II counterpart are presently unknown. The two enzymes can hydrolyze thioester bonds of fatty acyl-CoAs in vitro and have a relatively broad acyl chain length specificity. Like the E. coli TE II that functions as an homotetramer (25), hTE is able to interact with itself in two-hybrid assay (not shown). Thus, hTE probably functions also as a tetramer suggesting a conserved catalytic mechanism between the two enzymes. The specific cellular substrate(s) of hTE must be identified to elucidate the physiological role of the enzyme.

Nef is a cytoplasmic protein targeted to the inner face of the plasma membrane by myristoylation. This membrane anchor motif is strictly required for its biological functions. The CD4 molecule that is down-regulated by Nef as well as its associated tyrosine kinase p56lck, is also myristoylated (43). Previous reports have shown that palmitoylation can influence the rate of endocytosis of molecules at the plasma membrane (44–46). It has also been reported that the efficiency of protein myristoylation is regulated by the size of the cell acyl-CoA pools (see Ref. 47 for review). hTE could disturb the acylation of these proteins by regulating the intracellular steady-state level of acyl-CoA and so influence the endocytosis rate of the CD4 receptor. The fatty acylation status of Nef, CD4, and p56lck within T-lymphocyte cells expressing Nef must now be analyzed to assess this hypothesis.

Nef can also alter the membrane traffic of the major histocompatibility complex class I molecules (15), suggesting that it interacts specifically with the HIV-1 Nef protein. In the absence of acyl-CoA pools, buds accumulate, and coated vesicles fail to pinch off. In addition, the formation of coated vesicles and transport are blocked by a non-hydrolyzable analogue of palmitoyl-CoA (48), indicating that hydrolysis of long chain fatty acyl-CoA is required for efficient intracellular traffic. Since hTE is able to hydrolyze palmitoyl-CoA, it may be involved in this process.

Several other Nef-interacting proteins, including the Src-related protein tyrosine kinases p60lck and p56lck (19, 49, 50), the β-COP protein (21), and an unidentified protein kinase related to p21-activated kinases (PAK) (51–53) have been reported. However, the multiple independent activities of Nef are compatible with the existence of several cellular partners (13, 49, 54). Although experiments are still in progress to determine whether the Nef-β-COP interaction is required for some Nef functions, the Nef-p60lck interaction seems to be involved in the Nef enhancement of virus infectivity (13, 49), and the Nef-p56lck and Nef-PAK interactions seem to be linked to the alteration of T cell activation pathways (19, 50–54). None of these cellular proteins seems to be involved in the Nef-induced CD4 down-regulation (13, 35, 49, 54).

In conclusion, we have identified a human thioesterase enzyme that interacts specifically with the HIV-1 Nef protein. This thioesterase represents probably the prototype of a new subfamily of eucaryotic thioesterases characterized by similarity with TE II from E. coli. Additional evidence is needed to demonstrate the physiological relevance of this interaction in vivo. Nevertheless, the correlation between the ability of Nef to interact with hTE and to mediate CD4 down-regulation from the cell surface suggests that hTE is a cellular mediator of Nef function in HIV-1-infected cells.
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Binding of HIV-1 Nef to a Novel Thioesterase Enzyme Correlates with Nef-mediated CD4 Down-regulation
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