

Heparan Sulfate/Heparin N-Deacetylase/N-Sulfotransferase

THE N-SULFOTRANSFERASE ACTIVITY DOMAIN IS
AT THE CARBOXYL HALF OF THE HOLOENZYME*

(Received for publication, June 26, 1998, and in revised form,
July 22, 1998)

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Glycosaminoglycan *N*-acetylglucosaminyl *N*-deacetylases/*N*-sulfotransferases are structurally related enzymes that play an important role in the biosynthesis of heparan sulfate and heparin. They are dual catalytic, single membrane-spanning polypeptides of approximately 850–880 amino acids that catalyze the *N*-deacetylation of *N*-acetylglucosamine of glycosaminoglycans followed by *N*-sulfation of the same sugar. On the basis of homologies of these proteins with other *N*-acetylglucosaminyl *N*-deacetylases involved in the biosynthesis of chitin and putative deacetylases from bacteria, we have constructed two soluble chimeras between protein A and the amino- and carboxyl-terminal halves of the above mastocytoma holoenzyme. The carboxyl-terminal chimera half (amino acids 479–880) was able to catalyze the *N*-sulfation of glucosamine of heparan sulfate with a similar affinity for its two substrates, adenosine 3'-phosphate 5'-phosphosulfate and heparan sulfate, as the holoenzyme. However, the reaction only occurred at 30 °C and not at 37 °C, both temperatures at which the holoenzyme was active. The V_{max} of the chimera was 10–20-fold slower than that of the holoenzyme. Soluble chimeras between protein A and amino acids 43–521 and 43–680 of the holoenzyme were unable to catalyze the *N*-deacetylation of the bacterial *N*-acetylglucosaminyl-glucuronic acid polymer K5 under conditions where the holoenzyme was active. The recent appearance in genome data banks of homologs to the *N*-sulfotransferase domain and now the direct demonstration that this domain catalyzes this reaction raises the possibility that both *N*-deacetylation and *N*-sulfation activities of the holoenzyme might have emerged as gene fusions during evolution.

Glycosaminoglycan *N*-acetylglucosaminyl *N*-deacetylases/*N*-sulfotransferases are enzymes that play a pivotal role in the biosynthesis of heparan sulfate and heparin; they are dual catalytic, single polypeptides that catalyze the *N*-deacetylation of *N*-acetylglucosamine of glycosaminoglycans followed by *N*-

sulfation of the same sugar. Such an enzyme was first purified and cloned from rat liver (1, 2), and subsequently isoforms were cloned from mouse mastocytoma cells (4), the mouse tumor (5), as well as human tissues (6). Experiments *in vitro* and *in situ* have shown that a single polypeptide chain of approximately 900 amino acids mediates the above two reactions without any additional proteins or cofactors (3, 4, 7).

Following the enzymatic action of the above two activities, epimerization of glucuronic acid to iduronic acid as well as *O*-sulfation of these uronic acids and glucosamine can occur, leading to the biosynthesis of the glycosaminoglycan moieties in both heparan sulfate and heparin (8).

We are interested in determining the domains of this monomeric, dual catalytic protein mediating the *N*-deacetylation and *N*-sulfation reactions. Such knowledge could be important for (a) mechanistic studies of both reactions for which one would prefer a shorter protein with only one activity; (b) understanding and studying regulators of this important pathway; (c) crystallographic studies of both catalytic domains; and (d) studying the function of homologs to this protein, which have begun to appear in data bases from the genomes of *Caenorhabditis elegans* (9) and *Drosophila melanogaster* (10). In the latter case, transposon deletion mutants in the putative coding regions of this enzyme have been obtained with a cuticle phenotype similar to that of wingless null mutants (11). Whether or not these "structural" homologs have this particular enzymatic activity could be important in understanding the roles of these activities during development in *C. elegans* and *Drosophila*.

We have now found that a soluble chimera between protein A and the carboxyl-terminal half of the mastocytoma *N*-acetylglucosaminyl *N*-deacetylase/*N*-sulfotransferase expresses *N*-acetylglucosaminyl *N*-sulfotransferase activity. The truncated protein has a very similar affinity for its substrates, PAPS¹ and heparan sulfate, as the holoenzyme; because the activity can only be demonstrated at 30 °C and not at 37 °C, both temperatures at which the holoenzyme is active, we speculate that folding of the truncated protein at 37 °C is incompatible with its catalytic activity.

MATERIALS AND METHODS

Expression Constructs—Expression constructs were designed so that the truncated proteins were secreted as protein A fusions as described (3) by cloning into pRK5F10 protein A vector (3). Primers used were (*Sal*I sites are underlined): MS1, ggT gTC gAC CAg gCC AAg gAA CCC TTg CCT CTg CC; MS2, ggt gTC gAC TCA Cgg ATT AAg CAg CAC TgT CAg AAA g; MS3, ggT gTC gAC gTg CTC CCC Cgg CAA ACA TgT ggC; MS4, ggT gTC gAC TCA gCC CAC ACT ggA ATg TTg CAA TTC; MS5, ggT gTC gAC TCA CTg gTA CCA ggA gTA ggC CCT gTC.

Mastocytoma *N*-acetylglucosaminyl *N*-deacetylase *N*-sulfotransferase DNA (4) was used as template. Products from polymerase chain reaction amplification with MS1/MS2, MS3/MS4, and MS1/MS5 were digested with *Sal*I and cloned into a *Sal*I site of the pRK5F10 protein A vector (3). Orientation of the inserts was assessed by restriction analysis, and for each primer pair a positive (correct) and negative (incorrect) orientation was selected. All positive constructs were sequenced to verify that fusions were in frame with protein A.

Transfection of COS7 Cells—Cells were transfected at 40–50% confluence using DEAE/dextran/chloroquine and 10 μg of the corresponding plasmid DNA, in the plus or minus orientation, as well as the

* This work was supported by National Institutes of Health Grant GM34396. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviation used is: PAPS, adenosine 3'-phosphate 5'-phosphosulfate; MST, mastocytoma-derived cells; 5'-PSB, 5' phosphosulfate binding; 3'-PB, 3'-phosphate binding.

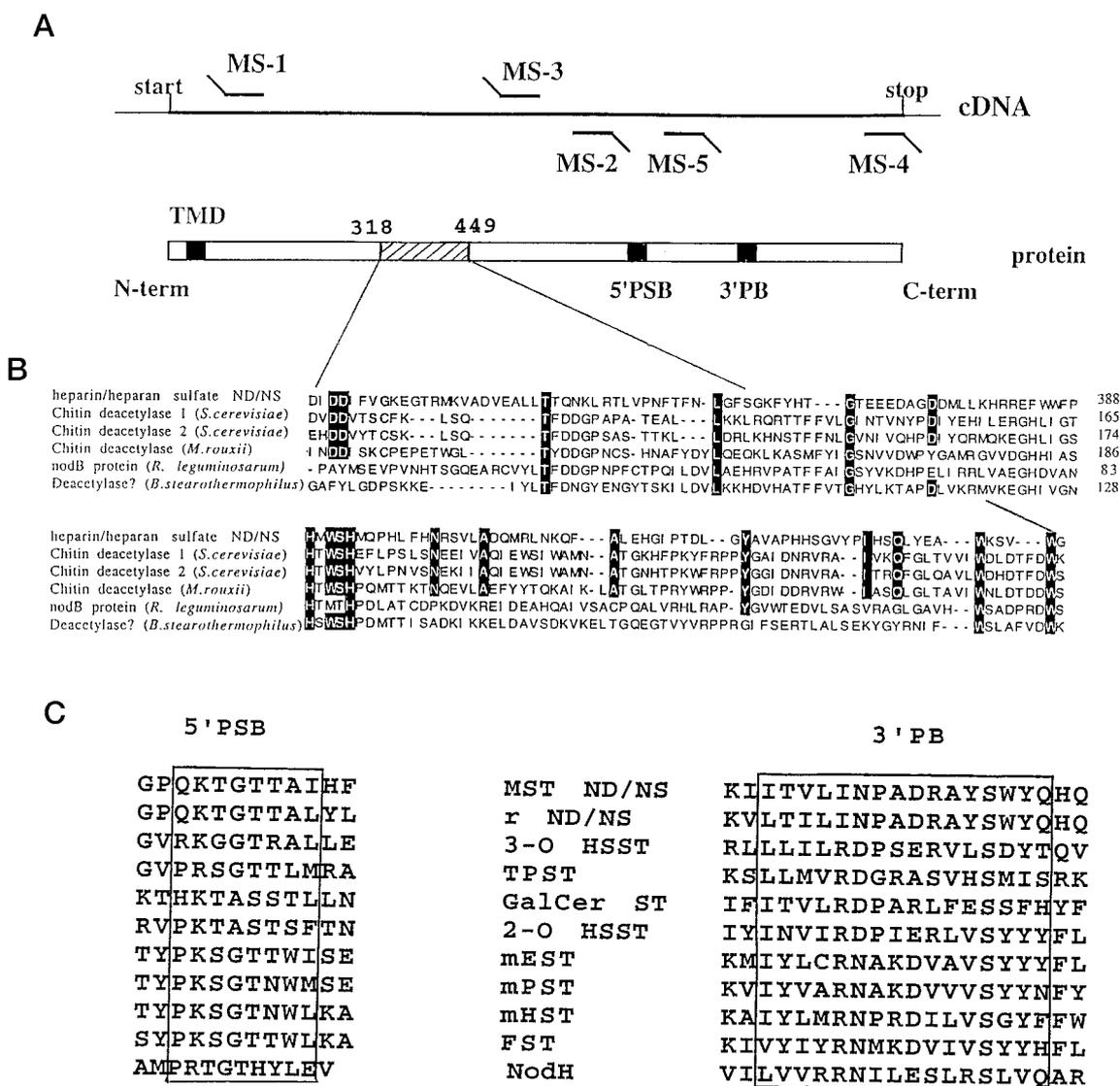


FIG. 1. A (top), position of primers used in the construction of protein A chimeric truncates of the MST heparan sulfate/heparin N-deacetylase/N-sulfotransferase. A (bottom), black boxes indicate position of the transmembrane domain (TMD) and PAPS binding sites (5'PSB and 3'PB (16)) of the holoenzyme; hatched area indicates region of consensus of different N-deacetylases. B, multiple sequence alignments of N-deacetylases. C, multiple sequence alignments of 5'-PSB and 3'-PB regions of membrane and soluble sulfotransferases as published by Kakuta *et al.* (16) plus heparan sulfate 3-O-sulfotransferase (AF019385) and tyrosyl protein sulfotransferase (AF03800).

holoenzyme protein A fusion. After 4 h at 37 °C, a Me₂SO shock (10% Me₂SO in phosphate-buffered saline for 2 min) was performed, and cells supplemented with Dulbecco's modified Eagle's medium/10% fetal calf serum were placed at 30 °C or 37 °C for 4–7 days. Culture media containing the secreted, recombinant protein were incubated at 4 °C with IgG-agarose beads at 4 °C for 8–16 h. Beads were washed with 20% glycerol, 10 mM Tris-HCl, pH 7.5, and stored at –70 °C or assayed immediately.

Detection of expressed protein was performed by immunoblot. Briefly, an aliquot of secreted protein bound to IgG-agarose beads was suspended in SDS-polyacrylamide gel electrophoresis loading buffer and boiled for 4 min; proteins were fractionated by SDS-10% polyacrylamide gel electrophoresis and electrotransferred to an Immobilon P membrane. Monoclonal anti-protein A (Sigma) in a 1:2000 dilution was used followed by horseradish peroxidase-conjugated anti-mouse IgG and enhanced chemiluminescence detection.

Enzymatic Assays—N-Sulfotransferase activity of the IgG-bound soluble proteins was measured by determining the incorporation of radiolabeled sulfate from radiolabeled PAPS into heparan sulfate for 15 min (1). N-Deacetylase activity of the same fractions was measured as the release of [³H]acetate from N-³H-acetylated polysaccharide from *Escherichia coli* K-5 capsular polysaccharide (19).

RESULTS

Our initial rationale for constructing the soluble chimeras between protein A and the amino and carboxyl halves of the N-acetylglucosaminyl N-deacetylase/N-sulfotransferase encompassing separate putative N-deacetylase and N-sulfotransferase activities is outlined in Fig. 1, A–C. As can be seen (Fig. 1, A and B), between amino acids 318 and 449 of the rat liver heparan sulfate N-deacetylase/N-sulfotransferase there are several amino acids that appear to be conserved in different N-acetylglucosaminyl N-deacetylases; these include chitin deacetylases from *Saccharomyces cerevisiae* (12), from *Mucor rouxii* (12, 13), the nod B protein (12, 14), a putative deacetylase from *Bacillus stearothermophilus* (12), as well as all the heparin/heparan sulfate N-deacetylases/N-sulfotransferases and sulfotransferases cloned so far (15–17).

It has been shown in several protein families that although the sequence of the introns shows variability in sequence and length, the intron positions are highly conserved (20). The selection of the boundaries of the truncates was based on the described intron/exon

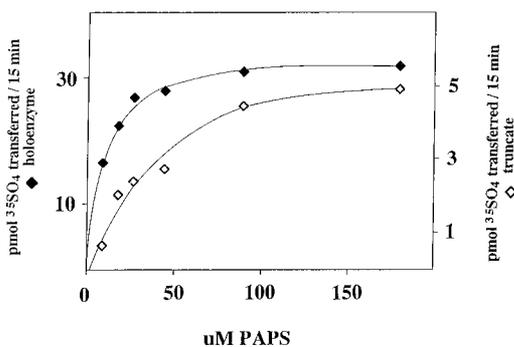


FIG. 2. Heparan sulfate *N*-sulfotransferase activity versus PAPS concentration of the complete mastocytoma *N*-acetylglucosaminyl *N*-deacetylase/*N*-sulfotransferase (MST holoenzyme) and the carboxyl-terminal half (holoenzyme amino acids 479–880; truncate). COS7 cell transfection and assays were performed as described under “Materials and Methods.” Reactions were carried out for 15 min at 30 °C using 50 μ g of heparan sulfate as acceptor. Activities are expressed as picomoles of 35 S transferred in 15 min by immobilized soluble enzyme derived from one 10-cm transfected plate.

boundaries for the human *N*-acetylglucosaminyl *N*-deacetylase/*N*-sulfotransferase gene (6) and the assumption that the splice positions are conserved within the *N*-acetylglucosaminyl *N*-deacetylase/*N*-sulfotransferase family.

As our experiments were almost completed, two reports in the literature appeared that used protein sequence homologies to suggest that the *N*-sulfotransferase activity domain may be toward the carboxyl half of the *N*-acetylglucosaminyl *N*-deacetylase/*N*-sulfotransferase (Fig. 1C). One report is the sequence similarity comparison between the above rat liver *N*-deacetylase/*N*-sulfotransferase and the heparin 3'-*O*-sulfotransferase cloned by Rosenberg and colleagues (17). The other, more recent report using new algorithms suggested regions of homology between different soluble and membrane-bound sulfotransferases, all of which use PAPS on substrates (15, 16). Thus, amino acids 621–628 (QKTGTAL) and 703–718 of the holoenzyme are probably regions interacting with PAPS (Fig. 1C).

We constructed the protein A chimeras (MSA, holoenzyme amino acids 43–521 in the plus or minus orientation; MSB, holoenzyme amino acids 479–883 in the plus or minus orientation; and MSC, holoenzyme amino acids 43–680) using primers shown in Fig. 1A and under “Materials and Methods.” Protein expression was obtained by transient transformation of COS7 cells, which secreted the recombinant protein A fusion proteins into the culture medium. Concomitantly, the complete *N*-acetylglucosaminyl *N*-deacetylase/*N*-sulfotransferase protein A fusion protein was expressed as control and assayed for *N*-deacetylase or *N*-sulfotransferase activity using [3 H]acetyl K5 polysaccharide or 35 S-labeled PAPS and heparan sulfate as substrates. In every case, a protein of the expected mobility (molecular mass) was detected by Western blots with anti-protein A antibodies in the culture medium of COS7 cells transfected with the positive constructs and not with the negative constructs. As shown in Fig. 2, the chimera encompassing amino acids 479–880 of the mastocytoma *N*-acetylglucosaminyl *N*-deacetylase/*N*-sulfotransferase had *N*-sulfotransferase activity with a K_m apparent for PAPS very similar to that of the holoenzyme. The activity, however, could only be detected when cells were grown at 30 °C after transfection and not at 37 °C even though the holoenzyme is active at both temperatures. The truncated protein, although having the same K_m as the holoenzyme, appears to have a V_{max} approximately 5-fold lower than that of the holoenzyme (Fig. 2). The affinity of the truncated enzyme for heparan sulfate, its other substrate, also

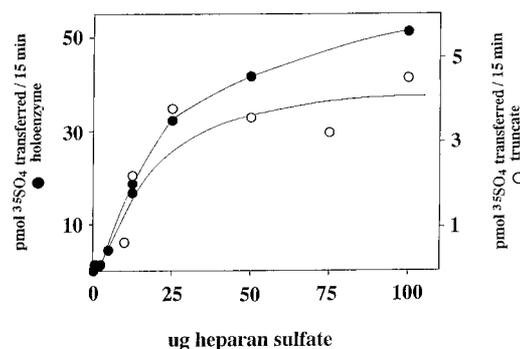


FIG. 3. Heparan sulfate *N*-sulfotransferase activity versus heparan sulfate concentration of the complete mastocytoma *N*-acetylglucosaminyl *N*-deacetylase/*N*-sulfotransferase (MST holoenzyme) and the carboxyl-terminal half (holoenzyme amino acids 479–880; truncate). Reactions were carried out for 15 min at 30 °C using 100 μ M 35 S-labeled PAPS (specific activity, 2300 cpm/pmol). Activities are expressed as picomoles of 35 S transferred in 15 min by immobilized soluble enzyme derived from one 10-cm transfected plate.

appears to be very similar to that of the holoenzyme even though its V_{max} is considerably lower (Fig. 3).

Attempts to demonstrate that Protein A chimeras encompassing amino acids 43–521 and 43–680 of the holoenzyme contain the *N*-deacetylase activity have not been successful even though a protein A recombinant of the correct size was secreted into the medium and assays were done at 30 and 37 °C in the presence of putative stabilizing agents such as polyethylene glycol and trimethylamine *N*-oxide.

DISCUSSION

We have found that a chimera between protein A and amino acids 479–883 of the rat liver *N*-acetylglucosaminyl *N*-deacetylase/*N*-sulfotransferase catalyzes the *N*-sulfation of glucosamine of heparan sulfate *in vitro*. The affinity of this chimeric enzyme for the substrates of the holoenzyme, PAPS and heparan sulfate, is very similar to that of the holoenzyme. The V_{max} is 10–20-fold slower than that of the holoenzyme; enzyme activity could only be demonstrated at 30 °C and not at 37 °C, even though the holoenzyme is active at both temperatures. We speculate that this apparent lack of activity of the truncate at 37 °C may be the result of partial folding or unfolding of this protein at this temperature, which renders it catalytically inactive; attempts to stabilize this putative unstable conformer with previously used stabilizing protein agents such as polyethylene glycol or trimethylamine *N*-oxide were unsuccessful.

While these studies were under way, two reports appeared suggesting that the *N*-sulfotransferase activity domain may be toward the carboxyl half of the *N*-acetylglucosaminyl *N*-deacetylase/*N*-sulfotransferase. One was sequence comparison between the above enzyme and the heparin 3'-*O*-sulfotransferase cloned by Rosenberg and colleagues (17). The other, a more recent report, used new algorithms that indicated regions of homology between soluble and membrane sulfotransferases, all of which use PAPS as substrates (15, 16). Our above results indicate that the heparan sulfate/heparin binding region of the holoenzyme must be in its carboxyl half.

We were unable to demonstrate *N*-acetylglucosaminyl *N*-deacetylase activity in chimeras of protein A and amino acids 43–521 and 43–680 of the holoenzyme even when cells were grown at 30 or 37 °C and assayed at both temperatures. Nevertheless, we still speculate that this region of the holoenzyme is involved in *N*-deacetylation and that, most likely, folding of the protein chimeras results in an inactive protein. This latter result further illustrates the importance of demonstrating di-

rectly enzyme catalysis of putative homolog regions of the enzymes. A hypothesis resulting from these studies, in a manner analogous to the demonstration with the PAPS synthetase (18), a multifunctional enzyme containing ATP sulfurylase and adenosine 5'-phosphosulfate kinase activity, is the possibility that the *N*-deacetylase and *N*-sulfotransferase activities evolved from separate genes in bacteria, fungi, yeast, and plants and only became one gene later in evolution.

It will be interesting to determine the effect of expression of the above *N*-sulfotransferase chimera on the *in vivo* sulfation of proteoglycans and whether or not chimeras encompassing the putative *N*-deacetylase activities have biological activities *in situ*.

Acknowledgment—We thank Karen Welch for excellent secretarial assistance.

REFERENCES

1. Brandan, E., and Hirschberg, C. B. (1988) *J. Biol. Chem.* **263**, 2417–2422
2. Hashimoto, Y., Orellana, A., Gil, G., and Hirschberg, C. B. (1992) *J. Biol. Chem.* **267**, 15744–15750
3. Wei, Z., Swiedler, S., Ishihara, M., Orellana, A., and Hirschberg, C. B. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 3885–3888
4. Orellana, A., Hirschberg, C. B., Wei, Z., Swiedler, S., and Ishihara, M. (1994) *J. Biol. Chem.* **269**, 2270–2276
5. Eriksson, J., Sandback, D., Ek, B., Lindahl, U., and Kjellen, L. (1994) *J. Biol. Chem.* **269**, 10438–10443
6. Gladwin, A. J., Dixon, J., Loftus, S. K., Wasmuth, J. J., and Dixon, M. J. (1996) *Genomics* **32**, 471–473
7. Mandon, E., Kempner, E. S., Ishihara, M., and Hirschberg, C. B. (1994) *J. Biol. Chem.* **269**, 11729–11733
8. Lindahl, U., Feingold, D. J., and Roden, L. (1986) *Trends Biochem. Sci.* **11**, 221–225
9. Wilson, R., Ainscough, R., Anderson, K., Baynes, C., Berks, M., Bonfield, J., Burton, J., Connell, M., Copsey, T., and Cooper, J. (1994) *Nature* **368**, 32–38
10. Power, P. A., and Ganetzky, B. (1991) *Genetics* **129**, 133–144
11. Cumberledge, S., and Reichsman, F. (1997) *Trends Genet.* **13**, 421–423
12. Christodoulidou, A., Bouriotis, V., and Thireos, G. (1996) *J. Biol. Chem.* **271**, 31420–31425
13. Kafetzopoulos, D., Thireos, G., Vournakis, J. N., and Bouriotis, V. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 8005–8008
14. John, M., Rohrig, H., Schmidt, J., Wieneke, U., and Schell, J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 625–629
15. Kakuta, Y., Pedersen, L. G., Carter, C. W., Negishi, M., and Pedersen, L. C. (1997) *Nat. Struct. Biol.* **4**, 904–908
16. Kakuta, Y., Pedersen, L. G., Pedersen, L. C., and Negishi, M. (1998) *Trends Biochem. Sci.* **23**, 129–130
17. Shworak, N. W., Liu, J., Fritze, L. M. S., Schwartz, J. J., Zhang, L., Logeart, D., and Rosenberg, R. D. (1997) *J. Biol. Chem.* **272**, 28008–28019
18. Rosenthal, E., and Leustek, T. (1995) *Gene (Amst.)* **165**, 243–248
19. Bame, K., Reddy, R., and Esko, J. (1991) *J. Biol. Chem.* **266**, 12461–12468
20. deSouza, S. J., Long, M., and Gilbert, W. (1996) *Genes Cells* **1**, 493–505

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J. Biol. Chem. 1998, 273:25556-25559.
doi: 10.1074/jbc.273.40.25556

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