Identification of Amino Acids That Modulate Mannose Phosphorylation of Mouse DNase I, a Secretory Glycoprotein*

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We have reported that bovine DNase I, a secretory glycoprotein, acquires mannose 6-phosphate residues on 12.6% of its Asn-linked oligosaccharides when expressed in COS-1 cells and that the extent of phosphorylation increases to 79.2% when lysines are placed at positions 27 and 74 of the mature protein (Nishikawa, A., Gregory, W., Frenz, J., Cacia, J., and Kornfeld, S. (1997) J. Biol. Chem. 272, 19408–19412). We now demonstrate that murine DNase I, which contains Lys27 and Lys74, is phosphorylated only 20.9% when expressed in the same COS-1 cell system. This difference is mostly due to the absence of three residues present in bovine DNase I (Tyr54, Lys124, and Ser190) along with the presence of a valine at position 23 that is absent in the bovine species. We show that Val23 inhibits phosphorylation at the Asn18 glycosylation site, whereas Tyr54, Lys124, and Ser190 enhance phosphorylation at the Asn106 glycosylation site. Tyr54 and Ser190 are widely separated from each other and from Asn106 on the surface of DNase I, indicating that residues present over a broad area influence the interaction with UDP-GlcNAc:lysosomal enzyme N-acetylglucosamine-1-phosphotransferase, which is responsible for the formation of mannose 6-phosphate residues on lysosomal enzymes.

In a previous report, we demonstrated that bovine DNase I, a secretory glycoprotein of the pancreas and the salivary gland, acquires mannose 6-phosphate moieties on its Asn-linked oligosaccharides (1), confirming the findings of Cacia et al. (2) with human DNase I. However, the level of oligosaccharide phosphorylation (12.6%) was considerably less than that observed with lysosomal hydrolases (usually 50% or greater). This indicated that DNase I is a weak substrate for UDP-GlcNAc:lysosomal enzyme N-acetylglucosamine-1-phosphotransferase (phosphotransferase), the enzyme that synthesizes the Man-6-P recognition marker for targeting to lysosomes. Phosphotransferase is known to recognize a conformation-dependent protein determinant that is present in lysosomal hydrolases, but absent in most secretory glycoproteins (3). This determinant has been shown to involve a broad surface patch that includes critical lysine residues (4–11). The binding of lysosomal hydrolases to phosphotransferase is followed by the transfer of GlcNAc-P from UDP-GlcNAc to selected mannose residues on the Asn-linked high mannose oligosaccharides of the hydrolases. The N-acetylglucosamine is then removed by N-acetylglucosamine-1-phosphodiesterase-a-N-acetylglucosaminidase to generate the phosphomannosyl recognition determinant that allows binding to the mannose 6-phosphate receptors in the Golgi and subsequent targeting to lysosomes (12). The basal level of phosphorylation of bovine DNase I was shown to be dependent on three lysines (Lys50, Lys124, and Arg27), consistent with the finding that lysine residues are important components of the phosphotransferase recognition determinant on lysosomal hydrolases (1). When Arg27 was replaced with a lysine, oligosaccharide phosphorylation increased to 54%, demonstrating that phosphotransferase prefers lysine residues over arginines. Furthermore, mutation of Asn74 to a lysine also increased phosphorylation to 50.3%, and the double mutant (R27K/N74K) was phosphorylated 79.2%, equivalent to the values observed with authentic lysosomal hydrolases. Interestingly, the lysine at position 27 specifically stimulated phosphorylation of the oligosaccharide at Asn18, whereas the lysine at position 74 selectively stimulated phosphorylation of the oligosaccharide at Asn106.

The R27K and N74K bovine constructs were initially prepared because the murine DNase I sequence has lysine residues at these positions, along with a lysine at position 50 (a glutamate is present at position 124). Yet in preliminary experiments we found that the murine DNase I was phosphorylated only 21% when expressed in the standard COS-1 cell system. We reasoned that this relatively poor phosphorylation might indicate that murine DNase I either lacks amino acids present in bovine DNase I that are necessary for efficient phosphorylation or contains amino acids that impair mannose phosphorylation. We also considered the possibility that both situations might hold. Our first clue that particular amino acid residues may impair phosphorylation came from the finding that substitution of Lys117 with an alanine in the bovine DNase I molecule enhanced phosphorylation by greater than 50% (1). In this paper, we identify five amino acids that modulate the phosphorylation of murine DNase I to a large extent. Two residues (Val23 and Lys117) are present in the murine sequence and impair phosphorylation. The other three residues (Tyr54, Lys124, and Ser190) are found in bovine DNase I but not murine DNase I and stimulate phosphorylation. Together these findings provide new insight into the nature of the phosphotransferase recognition domain.

EXPERIMENTAL PROCEDURES

Materials—COS-1 cells were obtained from ATCC. [2-3H]Mannose was purchased from NEN Life Science Products. QAE-Sephadex, Con-
canavalin A-Sepharose, and the pSVK3 expression vector were from Amersham Pharmacia Biotech. Recombinant endoglycosidase H fused to maltose-binding protein and protein A-agarose were from New England Biolabs and Repligen, respectively. Bovine calf serum, Lipofectin, and Opti-MEM were from Life Technologies, Inc. Other reagents were obtained from Sigma Chemical Co.

Plasmids—Bovine DNase I cDNA lacking a signal sequence (13) was kindly provided by Dr. D. Suck (EMBL, Heidelberg, Germany). This cDNA was inserted into the pSVK3 expression vector behind the signal sequence of human DNase I as described (1). The cDNA encoding murine DNase I was obtained from Dr. Susan Spencer (Genentech). The constructs encoding chimeric proteins between bovine and murine DNase I were prepared using the sequential polymerase chain reaction procedure (14). The expression vectors containing the various mutations in the murine DNase I were also constructed by this procedure. All plasmids used for transfections were purified with the Qiagen plasmid kit.

COS-1 Cell Transfections—COS-1 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% calf serum. The cells were transfected with purified plasmid DNA using the Lipofectin reagent and then labeled with [2-3H]mannose in the presence of 10 mM NH₄Cl as described (1).

Immunoprecipitation and Oligosaccharide Analysis—Rabbit anti-bovine DNase I serum was prepared by immunizing a rabbit with bovine DNase I from Sigma and purified on one additional step on a concanavalin A-Sepharose column. Rabbit anti-murine DNase I was provided by Dr. Jerry Cacia (Genentech). The harvested COS-1 culture medium containing the [2-3H]mannose-labeled secreted proteins was incubated with 2 µl of antiserum overnight with rotation at 4°C. Then, 100 µl of a 50% protein-A agarose bead suspension was added. After 1 h of additional rotation, the bead suspension was transferred to a fresh tube. The beads were washed three times with 100 µl Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, and 10 mCi of [35S]methionine/cysteine mixture (Amersham Pharmacia Biotech, Pro-mix [35S]). Four hours later, 0.5 ml of medium containing methionine/cysteine, 10% calf serum, and 10 mM HN₄Cl was added, and the cells were incubated for an additional 4 h at 37°C. The medium was collected and the DNase I immunoprecipitated as above. One-half of the washed protein-A-agarose beads were used for SDS-PAGE. The immunoprecipitated proteins were eluted in 50 µl of 0.1 M Tris-HCl, pH 7.0, 2% SDS, 10% 2-mercaptoethanol, and 20% glycerol by boiling for 5 min. The eluates were subjected to SDS-PAGE using 13% acrylamide SDS gels. Following electrophoresis, the gels were stained with Coomassie blue R-250 and dried. Autoradiography was then carried out, and the intensity of the bands corresponding to DNase I was quantitated with a scanning densitometer (Molecular Dynamics). The other half of the beads were used to assay DNase I activity following the procedure of Sinicropi et al. (15). Briefly, 500 µl of the reaction mixture was added to the beads and incubated for 4 h at 37°C. The units of DNase I activity were determined by comparing the values to those obtained with a standardized vial of DNase I (Sigma). Cells transfected with an empty plasmid served as control. The specific activity was obtained by dividing the units of activity by the relative intensity units obtained by scanning the autoradiograms.

RESULTS

Basal Phosphorylation of Murine and Bovine DNase I—The phosphorylation of the two Asn-linked oligosaccharides of bovine and murine DNase I secreted by COS-1 cells is shown in Table I. The bovine DNase I had 12.6% of its oligosaccharides phosphorylated, mostly with a single Man-6-P residue, whereas 20.9% of the oligosaccharides on the murine enzyme were phosphorylated, including 7.7% with two Man-6-P residues. Whereas the murine DNase I is clearly better phosphorylated than the bovine enzyme, its level of phosphorylation is much less than that achieved by a bovine enzyme containing a R27K/N74K double mutation (Table I). Because the murine enzyme contains lysine residues at both of these positions (Fig. 1), it was surprising that it was phosphorylated so much less than the mutant bovine enzyme. We considered two possibilities to explain this difference. The first is that the murine DNase I, but not the bovine enzyme, contains amino acids that impair the interaction with phosphotransferase, thereby decreasing the level of phosphorylation. Alternatively, the bovine enzyme could have amino acids that are required for optimal phosphorylation that are absent in the murine form of the enzyme. The difference in the level of phosphorylation of the two forms of the enzyme could also result from both of these mechanisms being operative. The subsequent experiments were designed to test these possibilities.

Phosphorylation of Bovine-Murine DNase I Chimeras—We first constructed a serial of plasmids encoding various chimeras between the R27K/N74K mutant bovine enzyme and the murine enzyme. The R27K/N74K double mutant was used so that all the chimeras had these two stimulatory amino acids. These chimeras were expressed in COS-1 cells and their level of oligosaccharide phosphorylation determined. The results of these experiments are summarized in Fig. 2. It is apparent that when murine sequences derived from either the amino, middle, or carboxyl regions of the protein were substituted into the bovine sequence, a lower level of phosphorylation resulted (compare CP1, CP2, and CP3 to bovine DNase I, Fig. 2). When additional murine sequences were introduced, the extent of phosphorylation decreased further (CP4-CP5). These results indicated that multiple amino acids distributed throughout the linear sequence of murine DNase I influenced the level of phosphorylation.

Effect of Point Mutations on the Phosphorylation of Murine DNase I In the next set of experiments, a number of amino acids in the murine DNase I sequence that differed from those found in the bovine sequence were replaced by the equivalent bovine residues. These were selected on the basis that the two corresponding amino acids differed significantly in structure. The effect of the amino acid substitution on oligosaccharide phosphorylation was then determined using the COS-1 cell expression system. As shown in Fig. 3, six mutations (V22S, E54Y, E54Y, D61N, K117A, E134K, and P190S) resulted in 30% or more enhanced phosphorylation, whereas four mutations (R64H, I191T, V204A, and H208N) had no significant effect on the level of phosphorylation. Interestingly, the V22S mutation primarily enhanced the formation of oligosaccharides with one phosphate, whereas the E54Y and P190S substitutions stimulated the synthesis of oligosaccharides with two phosphates.

The substitutions that enhanced phosphorylation could be
doing so by the removal of an inhibitory amino acid or the addition of a stimulatory amino acid. To distinguish between these possibilities, Val23, Glu54, and Pro190 were replaced with alanine residues (the V23A, E54A, and P190A constructs) and analyzed for the level of phosphorylation (Fig. 3). The E54A substitution did not enhance phosphorylation suggesting that Tyr54 is a stimulatory residue. The V23A construct was phosphorylated to the same extent as the V23S mutant, indicating that Val23 inhibits phosphorylation. The P190A construct was phosphorylated somewhat better than wild type murine DNase I, although not nearly as well as the P190S construct. This suggests that Pro190 may impair phosphorylation to a small extent, whereas Ser190 has a stimulatory effect as well. Because Lys124 has been shown to be a stimulatory residue in bovine DNase I (1), we assume that the enhanced phosphorylation obtained with the E124K substitution is due to the stimulatory effect of the lysine at this position. In addition, the K117A construct showed a markedly enhanced phosphorylation, consistent with Lys117 being an inhibitory residue in the context of the murine sequence, just as it is in the bovine sequence (1).

Effect of Multiple Mutations on Oligosaccharide Phosphorylation—The effect of combinations of mutations on DNase I oligosaccharide phosphorylation is shown in Table II. Construct 1, which has four mutations (V23S, E54Y, E124K, and P190S) was phosphorylated 70.1% compared with 18.9% for the wild type murine DNase I analyzed in the same experiment. Thus mutation of these four residues brings the level of phosphorylation of the murine DNase I close to that obtained with the bovine enzyme containing the R27K/N74K mutations (79.2%). The extent of phosphorylation increased to 84.2% when a fifth residue (Lys117) was mutated to alanine (construct 2).

Effect of Mutations on Phosphorylation of the Oligosaccharide at Asn 18—Murine DNase I, like its bovine counterpart, contains two Asn-linked glycosylation sites, one at position 18 and the other at position 106 (Fig. 1). In our previous study of bovine DNase I, we established that the oligosaccharide at Asn18 primarily acquired a single Man-6-P residue, whereas the oligosaccharide at Asn106 mostly acquired two Man-6-P residues (1). Furthermore, we showed that a R27K substitution selectively stimulated the phosphorylation of the oligosaccharide at position 18. Because Val23 is positioned nearby Lys27, and its substitution with either serine or alanine resulted in a selective increase in oligosaccharides with one Man-6-P residue, we postulated that Val23 was impairing the phosphorylation of the oligosaccharide at Asn18.
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TABLE II

Effect of combinations of mutations on oligosaccharide phosphorylation

COS-1 cells transfected with the various constructs were labeled with [2-3H]mannose, and the secreted DNase I was immunoprecipitated from the medium and analyzed as in Table I. The results are the average of two to four separate determinations. PM, phosphomonoester.

| Construct | Residue | Oligosaccharide phosphorylation | 2 PM:1 PM ratio |
|-----------|---------|---------------------------------|-----------------|
| I. Mwt    | Val Glu Lys Lys Glu Pro 18.9 0.56 |
| 1         | Ser Tyr Lys Lys Lys Ser 70.1 1.72 |
| 2         | Ser Tyr Lys Ala Lys Ser 84.2 2.51 |
| II. Mwt (∆18) | Val Glu Lys Lys Glu Pro 36.6 1.06 |
| 3         | Val Glu Lys Lys Lys Pro 45.9 1.56 |
| 4         | Val Tyr Lys Lys Glu Pro 60.7 2.60 |
| 5         | Val Tyr Lys Lys Glu Ser 72.4 5.07 |
| 6         | Val Tyr Lys Lys Ser 90.9 6.67 |
| III. Bwt (∆18, Lys174) | Ser Tyr Lys Lys Ser 85.9 12.1 |
| 7         | Ser Ala Lys Lys Lys Ser 62.0 4.2 |
| 8         | Ser Ala Lys Lys Lys Ala 23.1 1.5 |

Fig. 4. Effect of amino acid substitutions on phosphorylation of the Asn18 oligosaccharide. Constructs were prepared with Asn106 changed to a Gln, and the other mutations were made as noted. The various constructs were expressed in COS-1 cells, and the extent of phosphorylation of the secreted proteins was determined as described under “Experimental Procedures.” The number of separate determinations is indicated in parentheses.

In a complementary experiment, Tyr54 and Ser190 were mutated to alanines in a bovine DNase I construct containing Lys74 and only Asn106 (Bwt (∆18, Lys54)). As shown in Table II, mutation of Tyr54 to alanine decreased phosphorylation at Asn106 to less than 85.9% (construct 7). The effect of Tyr54 mutation was further decreased in the level of phosphorylation of the secreted proteins with two Man-6-P residues, from 92.4 to 60%.

A Hydrophobic Residue Is Required at Position 54—Our initial experiments using single amino acid changes showed that substitution of Glu54 with tyrosine stimulated phosphorylation, whereas substitution with alanine had no effect (Fig. 2). To determine whether other bulky hydrophobic residues could substitute for tyrosine, constructs were prepared with Glu54 changed to either phenylalanine or methionine. Both residues stimulated phosphorylation at the Asn106 site, with methionine being equivalent to tyrosine and phenylalanine being somewhat less effective (Table III). These findings suggest that it is the hydrophobic character of the residue at position 54 that is most essential for stimulating phosphorylation at Asn106.

Mouse DNase I Mutants Retain Enzymatic Activity—Because phosphotransferase binds to a conformation-dependent protein determinant present on its substrates, the various amino acid substitutions could influence the level of phosphorylation by altering the proper folding of the DNase I. One indication of
Lys50. We reasoned that murine DNase I either lacked other lysines causing the selective phosphorylation of the oligosaccharide at Asn 106. The current study was stimulated by the observation that murine DNase I is phosphorylated at Asn 106, so these two residues may act together.

| Glycosylation site | Construct | Oligosaccharide phosphorylation |
|-------------------|-----------|-------------------------------|
|                   |           | HM + 1 PM | HM + 2 PM | Total |
| Asn106            | Mwt       | 19.6      | 19.9      | 39.5  |
|                   | E54A      | 19.7      | 51.1      | 70.8  |
|                   | E54F      | 22.9      | 38.0      | 60.9  |
|                   | E54M      | 22.4      | 46.8      | 69.2  |

**DISCUSSION**

Our previous study of bovine DNase I identified four lysine residues (Lys27, Lys50, Lys74, and Lys124) that determine high level phosphorylation (79%) by phosphotransferase (1). Among these residues, Lys27 and Lys74 were most important, with Lys27 causing the selective phosphorylation of the oligosaccharide at Asn106 and Lys74 causing the selective phosphorylation of the oligosaccharide at Asn106. The current study was stimulated by the observation that murine DNase I is phosphorylated at 29.9% despite having Lys27 and Lys74 as well as Lys50. We reasoned that murine DNase I either lacked other residues present in the bovine enzyme that are essential for high level phosphorylation or contained inhibitory residues not found in the bovine species. Our experiments show that both postulates are correct. Furthermore, these data provide new insight into the nature of the phosphotransferase recognition domain.

Starting with the analysis of chimeric proteins containing elements of both bovine and murine DNase I and then proceeding to amino acid substitution experiments, we have identified four residues at positions 23, 54, 124, and 190 that account for the majority of the difference in the phosphorylation of the two forms of DNase I. The valine in position 23 of the murine sequence impairs phosphorylation because its replacement with either an alanine or a serine (as in the bovine species) enhances phosphorylation about 30%. Replacement of the glutamic acid at position 124 of the murine sequence with a lysine stimulated phosphorylation to about the same extent. This was expected because this residue was known to facilitate phosphorylation of the bovine DNase I. The unexpected and most dramatic effects were observed when the glutamic acid at position 54 and the proline at position 190 of the murine sequence were replaced with tyrosine and serine, respectively. Each of these substitutions stimulated phosphorylation about 75%. Because substitution of alanines at these positions had little (position 190) or no (position 54) effect, we conclude that Tyr54 and Ser190 function as positive elements. When these four changes were combined the resultant murine DNase I was phosphorylated 70%, close to the 79% value obtained with bovine DNase I containing Lys27 and Lys74. These values are equivalent to that observed with authentic lysosomal acid hydrolases.

The experiments using a mutant DNase I with the Asn106 glycosylation site deleted demonstrated that the effect of Val23 is primarily on the phosphorylation of the oligosaccharide at Asn106. As shown in the space filling model of DNase I (Fig. 6), Val23 is located close to Asn106 and is adjacent to Lys50, which is a key residue in the phosphorylation of the oligosaccharide attached to Asn106. Thus, we predict that Val23 inhibits the interaction of Lys27 with phosphotransferase.

In contrast to Val23, the residues Tyr54, Lys124, and Ser190 function primarily to facilitate the phosphorylation of the oligosaccharide attached to Asn106. Substitution of these three residues increased phosphorylation from 36.6 to 90.9% at this site. In addition, there was a strong enhancement of the formation of oligosaccharidic esters with two Man-6-P residues. The importance of Tyr54 and Ser190 for phosphorylation at the Asn106 site was confirmed by the finding that replacement of these residues with alanines in bovine DNase I resulted in a decline in phosphorylation from 85.9 to 23.1%. These results provide additional evidence that amino acids other than lysines are involved in the generation of the phosphotransferase recognition domain (5, 8, 11). It is apparent from Fig. 6 that Tyr54 and Pro190 are located a considerable distance from each other and from Asn106. Tyr54 is adjacent to Lys74, which has also been shown to stimulate phosphorylation of the oligosaccharide at Asn106 (1), so these two residues may act together.

When these results with murine DNase I are compared with those previously obtained with bovine DNase I, a number of interesting differences emerge with regard to their phosphotransferase recognition domains. The bovine DNase I is missing the two most potent stimulatory residues (Lys27 and Lys74) but also lacks the Val23 inhibitory residue. Murine DNase I, on the other hand, has Lys27 and Lys74, but it also contains Val23.
and is missing the stimulatory residues Tyr154, Lys154, and Ser190. Both species have the stimulatory residue Lys95, as well as the inhibitory residue Lys117. The net result is that both bovine and murine DNase I are relatively poor substrates for phosphotransferase, but each can be converted into an excellent substrate by changing just a few amino acids. It is curious that these two forms of DNase I should differ so much in the manner in which they attenuate oligosaccharide phosphorylation by phosphotransferase. It is also interesting to speculate whether other secretory glycoproteins might contain potential phosphotransferase recognition elements that are either blocked by strategically located inhibitory amino acids or lack key stimulatory residues. This may have been a mechanism for controlling the compartmentalization of such proteins during evolution.

The finding that the optimal phosphorylation of DNase I requires widely spaced residues is consistent with our previous studies of cathepsin D (6–8), as well as the work of others with arylsulfatase A (20). We found that the minimal components of a phosphotransferase recognition patch in the carboxyl lobe of cathepsin D consisted of two noncontiguous primary sequences (Lys203 and amino acids 265–293) that come together in three-dimensional space. However, phosphorylation was enhanced by the presence of other regions of cathepsin D, including amino lobe elements, indicating that the optimal recognition domain involves a larger surface area. Consistent with these findings, Lukong et al. (21) recently reported that peptides derived from several regions of cathepsin D, including one located on the side opposite to the phosphorylated oligosaccharide, inhibit lysosomal enzyme phosphorylation by phosphotransferase. Schieraui et al. (20) came to a similar conclusion using a totally different approach (20). These investigators prepared monoclonal antibodies that bind to at least six different epitopes at different locations on the surface of arylsulfatase A. All of the antibodies were shown to bind outside the lysine-rich recognition domain, yet Fab fragments of these antibodies inhibited interaction of arylsulfatase A with phosphotransferase. The authors concluded that their results support a model in which phosphotransferase interacts with a large surface area of arylsulfatase A. In the lysosomal enzyme aspartylglucosaminidase, three lysine residues and a tyrosine located in three spatially distinct regions of the structure have been found to be necessary for full phosphorylation (11). Mutagenesis of these residues indicates that they function in a cooperative manner, with all three sites being required for optimal phosphorylation.

If one accepts that phosphotransferase interacts with a large surface area on its target glycoproteins, it is still necessary to explain the striking finding that Lys27 and Lys74 selectively stimulate the phosphorylation of different oligosaccharides on DNase I. We have noted previously that Lys27 and Lys74 are located relatively close to the oligosaccharide the phosphorylation of which they affect and a considerable distance from the other oligosaccharide (1). Based on this fact, we suggested that these lysines serve to orient phosphotransferase in a manner that facilitates the phosphorylation of the oligosaccharide in the vicinity of the critical lysine. In the context of the current discussion, we would envisage phosphotransferase interacting with a broad surface of the protein with key lysines serving to orient the catalytic site toward the oligosaccharide.

Sahagian and co-workers (22) have suggested a simpler model for the recognition signal. They have proposed that the signal consists of just two lysine residues spaced about 34 Å apart with a particular orientation relative to the oligosaccharide. They also suggest that each lysine may be situated in specific microenvironments. This proposal is based on the finding that two lysines, spaced 34 Å apart, are necessary for full phosphorylation of cathepsin L and cathepsin D. However, in the case of cathepsin D, mutation of both lysines (Lys203 and Lys295) to alanines inhibits phosphorylation by only 70%. Because 30% of normal phosphorylation remains, other residues must be involved, consistent with the proposal that a large surface area is required for the generation of an optimal recognition determinant.

Phosphotransferase has been shown to be a very large 540-kDa complex composed of disulfide-linked homodimers of 166 and 51 kDa subunits and two 56-kDa subunits (23). As such, it should have the capability to contact a large surface area on its glycoprotein substrates. The ultimate explanation for how phosphotransferase interacts with its substrate may only come with the determination of the tertiary structure of this enzyme bound to its substrate.

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