

## Human DNase I Contains Mannose 6-Phosphate and Binds the Cation-Independent Mannose 6-Phosphate Receptor

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**ABSTRACT:** DNase I isolated from human urine (hDNase) or expressed in Chinese hamster ovary (CHO) cells contains mannose-phosphorylated oligosaccharides. hDNase binds to a column of immobilized cation-independent mannose 6-phosphate receptor, with the strongest binding exhibited by the protein bearing diphosphorylated oligosaccharides. The binding is inhibited by 5 mM mannose 6-phosphate, and can be prevented by prior treatment of hDNase with alkaline phosphatase. Phosphorylated high-mannose oligosaccharides were observed at both sites of glycosylation in hDNase by high-performance liquid chromatography–mass spectrometry of a tryptic digest. These results indicate that hDNase, though not an acid hydrolase, may enter the lysosomal trafficking pathway, and may have evolved from a lysosomal enzyme.

DNase I<sup>1</sup> is an endonuclease that, under alkaline conditions, hydrolyzes DNA to oligonucleotides. The enzyme, isolated from bovine pancreas (1–4), has been extensively characterized with respect to its enzymatic activity (5–7), stability (2, 3, 5, 6, 8), amino acid sequence (3, 9, 10), glycosylation (3, 11, 12), and crystal structure (13–17). DNase I has also been isolated and sequenced from sheep (10, 18) and pig pancreas (19). Both bovine and ovine pancreatic DNases I are glycosylated at a single site, asparagine residue 18 (9, 10). Bovine DNase I (bDNase) contains high-mannose and hybrid oligosaccharides, with the latter containing sialic acid (12). The presence of sialic acid, together with a histidine–glutamine polymorphism (20, 21), contributes to charge heterogeneity that is manifested in isoelectric focusing polyacrylamide gel electrophoresis (IEF-PAGE) as a ladder of four bands (22, 23). Similarly, chromatography of bDNase on phosphocellulose yields four major peaks that correspond to sialylated and nonsialylated forms of each of the bDNase proteins containing either histidine or glutamine at residue 118 (20, 21). No unex-

pected post-translational modifications of bDNase have been reported.

Because of its potential as a marker of pancreatic diseases, human DNase has been partially purified and characterized from plasma (24–26), duodenal juice (25–27), and urine (27–34). The equivalence of DNases from these sources has been demonstrated (25, 27). A *pI* in the range of 3.5–4.3 has been reported for the human enzyme (25, 27, 29), and is similar to the range of 4.7–5.2 for the ladder of bands of bDNase in IEF analysis (22). The IEF pattern of desialylated human DNase (hDNase) has been extensively investigated in conjunction with studies of the genetic basis for charge heterogeneity of hDNase isoforms (27–34, 73). The cDNA sequence of human DNase I and characteristics of the recombinant protein expressed in mammalian cells have been reported by Shak et al. (35).

This report describes the evidence for the occurrence of mannose 6-phosphate (Man-6-P) residues in the oligosaccharides on hDNase isolated from urine. Man-6-P is a protein trafficking signal that targets lysosomal enzymes to lysosomes via binding to specific receptors (36–40). Two enzymes are involved in the addition of monoesterified phosphate to high-mannose residues of lysosomal enzymes in the cis-Golgi (41). The first enzyme recognizes a conformational motif (42–44) on proteins destined for lysosomes and transfers *N*-acetylglucosamine 1-phosphate to high-mannose oligosaccharides. The second enzyme then hydrolyzes the ester between the GlcNAc residue and the phosphodiester to leave the monoesterified phosphate. A lysosomal protein can contain more than one Man-6-P residue on a given oligosaccharide (45–50). Glycoproteins can be further processed in the Golgi following addition of phosphate to high-mannose structures, as deduced from the occurrence of Man-6-P-containing hybrid oligosaccharide structures in lysosomal enzymes, as well as from the occurrence of nonphosphorylated complex structures on

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<sup>1</sup> Abbreviations: DNase I, deoxyribonuclease I; CHO, Chinese hamster ovary; DNA, deoxyribonucleic acid; IEF-PAGE, isoelectric focusing polyacrylamide gel electrophoresis; bDNase, bovine DNase I; Man-6-P, mannose 6-phosphate; CI-MPR, cation-independent mannose 6-phosphate receptor; MES, 2-(*N*-morpholino)ethanesulfonic acid; HEPES, (hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PEI, polyethyleneimine; MS, mass spectrometry; hDNase, human urine DNase I; rhDNase, recombinant human DNase I; HIC, hydrophobic interaction chromatography; TEMED, *N,N,N',N'*-tetramethylethylenediamine; EGTA, [ethylenbis(oxyethylenenitrilo)]-tetraacetic acid.

proteins that contain phosphorylated oligosaccharides (45–49). Two receptors have been described (51–53) that target enzymes to lysosomes. These receptors bind Man-6-P residues under physiological conditions and release the bound oligosaccharide at low pH. The receptors are distinguished by their requirement for cations, and the Man-6-P affinities of both have been characterized (54–56). The Man-6-P receptors show no binding of complex oligosaccharides or hybrid structures, and a higher affinity for diphosphorylated carbohydrate structures than for monoesterified structures. In addition to its role in protein sorting from the Golgi to lysosomes, the cation-independent Man-6-P receptor (CI-MPR), which is also the insulin-like growth factor II receptor (57), is found on the cell surface and may mediate the uptake of secreted lysosomal enzymes and their transport to lysosomes (59, 60).

Three nonlysosomal proteins have been described that contain Man-6-P residues: the transforming growth factor  $\beta$  precursor (61–63), proliferin secreted by cultured Chinese hamster ovary cells (64), and the epidermal growth factor receptor (65). The biological significance of Man-6-P in these nonlysosomal proteins, apart from the potential involvement of lysosomes in their catabolism, is unknown. In this study, we show that human DNase I contains Man-6-P residues and selectively binds the CI-MPR, even though it is not an acid hydrolase. We also confirm the earlier findings that no such residues are present on bovine pancreatic DNase I.

## EXPERIMENTAL PROCEDURES

**Materials.** Recombinant human DNase I (35) was produced in CHO cells and purified at Genentech. The cation-independent mannose 6-phosphate receptor was a generous gift from S. Kornfeld of the Washington University Medical School (St. Louis, MO). 2-(*N*-Morpholino)ethanesulfonic acid (MES) was obtained from ICN Biochemicals (Cleveland, OH). Calcium chloride ( $\text{CaCl}_2$ ), sodium chloride (NaCl), hydrochloric acid (HCl), sulfosalicylic acid, trichloroacetic acid, and acetic acid were obtained from Mallinckrodt (Paris, KY). HPLC-grade methanol was from Burdick and Jackson (Muskegon, MI), and ethanol was from J. T. Baker (Phillipsburg, NJ). Isoelectric focusing markers were from Pharmacia. Bovine DNase I and TPCK-treated bovine trypsin were obtained from Worthington Biochemical (Freehold, NJ). *Clostridium perfringens* neuraminidase (type X), *Escherichia coli* alkaline phosphatase (type III), all other reagents were from Sigma (St. Louis, MO).

**Isolation of Human Urine DNase I.** Twelve liters of urine was collected from two individuals and stored at  $-20^\circ\text{C}$ . The urine was allowed to thaw at room temperature and centrifuged at 5500 rpm to remove precipitate. The supernatant was ultrafiltered and diafiltered against 25 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES) and 1 mM  $\text{CaCl}_2$  (pH 7.0) using a 10 kDa molecular mass cutoff membrane stack. The UF/DF pool was concentrated to 400 mL and loaded onto an 83 mL DEAE Fast Flow (Pharmacia) column equilibrated with the same buffer. The column effluent was monitored at 280 nm. The column was then washed with a 35 mM MES saline buffer (pH 5.0) having a total ionic strength of 210 mM. A second elution was carried out using the same buffer that had been adjusted

to pH 3.0 with HCl. Fractions were collected and assayed for DNase by ELISA. The DEAE pool was loaded onto a 2.15 mm  $\times$  150 mm TSK-Phenyl column (TosoHaas) equilibrated with a buffer containing 0.6 M sodium citrate, 50 mM Tris-HCl, and 1 mM  $\text{CaCl}_2$  (pH 7.0). The column was eluted with a linear gradient of citrate to 0 concentration over the course of 35 min at a flow rate of 6.5 mL/min. Fractions were collected and assayed for DNase by ELISA. The HIC pool was concentrated and desalted using a Centriprep 10 (Amicon) device and loaded onto a 4.6 mm  $\times$  50 mm Lichrospher 1000  $\text{SO}_3$  column (EM Separations) equilibrated with a buffer containing 10 mM sodium acetate (pH 4.5) and 1 mM  $\text{CaCl}_2$ . The column was eluted with a linear gradient to 580 mM NaCl over the course of 36 min. The human DNase pool was concentrated and desalted using a Centriprep 10 device and stored at  $-20^\circ\text{C}$ .

**Isoelectric Focusing Gel Electrophoresis.** A 0.4 mm thick gel was poured onto a plastic Gel Fix backing (Serva, Heidelberg, Germany), using an ultrathin layer horizontal casting tray (Bio-Rad, Richmond, CA). The solution contained 4 mL of a 29:1 acrylamide/bisacrylamide mixture (Bio-Rad), 24.25 mL of water, 300  $\mu\text{L}$  of pH 3–10, 600  $\mu\text{L}$  of pH 3–4, and 600  $\mu\text{L}$  of pH 3–5 Ampholytes (Serva), 200  $\mu\text{L}$  of 10% ammonium sulfate (Bio-Rad), and 25  $\mu\text{L}$  of TEMED (Bio-Rad). hDNase samples (20  $\mu\text{g}$ ) were loaded, and the gel was focused in a model 2217 Ultrophor (LKB) electrofocusing unit for 2 h at 1600 mV with a 10 W limit. Following electrophoresis, the gel was fixed with 10% trichloroacetic acid and 5% sulfosalicylic acid prior to staining in 0.1% R250 Brilliant Blue dye (Bio-Rad), 0.05% copper sulfate, and 10% acetic acid for 30 min. The gel was then soaked in 40% methanol, 0.5% copper sulfate, and 10% acetic acid for 30 min and further destained in 20% ethanol and 8% acetic acid until the desired background was obtained.

**Anion Exchange HPLC.** Samples of 0.02–0.5 mg of protein were loaded onto an Interaction Chemicals, Inc., HP-PEI column (7.8 mm  $\times$  100 mm) using a Hewlett-Packard 1090M HPLC system. The column was equilibrated at a flow rate of 1 mL/min with a buffer consisting of 100 mM NaCl, 10 mM HEPES, and 1 mM  $\text{CaCl}_2$  (pH 7.0). Following injection of the sample, a linear gradient to 1.5 M NaCl over the course of 47 min was started. The absorbance of the column effluent was monitored at 280 nm.

**Digestion with Trypsin.** hDNase (10  $\mu\text{g}$ ) in a volume of 50  $\mu\text{L}$  was diluted 1:1 with 40 mM Bis-Tris and 10 mM EGTA (pH 6.0) and the solution incubated at  $37^\circ\text{C}$  for 1 h. After incubation, the sample was exchanged using a Microcon-10 (Amicon) device into 100 mM Tris-HCl (pH 8.0). The samples were then incubated at  $37^\circ\text{C}$  for 4 h with additions of 0.1  $\mu\text{g}$  of TPCK-treated trypsin, at 0 and 2 h. Samples were frozen at  $-20^\circ\text{C}$  until they were analyzed by HPLC.

**HPLC–Mass Spectrometry.** The tryptic digest mixture was analyzed with a 320  $\mu\text{m}$  (inside diameter)  $\times$  150 mm capillary HPLC column (LC Packings, San Francisco, CA) packed with Nucleosil C18 resin and interfaced with a Sciex API III mass analyzer (PE Sciex, Toronto, ON). The column inlet was directly plumbed to the injection port of a Rheodyne 8125 low-dispersion injector fitted with a 5  $\mu\text{L}$  injection loop. The solvent flow from a Hewlett-Packard 1090M HPLC pump was split prior to the column using an Accurate

Table 1: Recovery of hDNase from Urine

column step	recovery ( $\mu$ g)	recovery (%)
DEAE load	860	100
DEAE pool	465	54
HIC pool	385	44
TCX pool	407	47

microflow processor (LC Packings) and the solvent delivered to the injector at a flow rate of 3  $\mu$ L/min. The column effluent was infused directly into the electrospray interface of the mass spectrometer.

**Neuraminidase Digestion.** Samples were exchanged into digest buffer consisting of 200 mM sodium acetate and 2 mM  $\text{CaCl}_2$  (pH 5.6) using an Amicon Microcon-10 ultra-filtration device. Neuraminidase was added to a ratio of 0.3 unit/mg of rhDNase. Samples were incubated at 37 °C for 18 h and immediately subjected to digestion with alkaline phosphatase.

**Alkaline Phosphatase Treatment.** rhDNase samples were exchanged into digest buffer consisting of 50 mM Tris-HCl and 2 mM  $\text{CaCl}_2$  (pH 8.2) using a Microcon-10 (Amicon) device. Alkaline phosphatase (Sigma) was added to a ratio of 7.4 units/mg of rhDNase. Samples were incubated at 37 °C for 6 h and frozen at -20 °C to stop digestion.

**Immobilization of the Man-6-P Receptor.** Epoxy-activated silica (1.1 g) (Rainin, Woburn, MA) was suspended in 3 mL of coupling buffer, consisting of 0.1 M HEPES, 80 mM  $\text{CaCl}_2$ , and 0.05% Triton X-100 (pH 7.0). One milliliter of a 1 mg/mL suspension of cation-independent mannose 6-phosphate receptor (CI-MPR) was added to the epoxy-silica suspension. The suspension was mixed end over end at 4 °C and allowed to couple overnight. The immobilized CI-MPR was slurry packed in the coupling buffer into a 4.6 mm  $\times$  50 mm stainless steel HPLC column (Alltech).

**Chromatography on the CI-MPR Column.** The column was equilibrated for 20 min at a flow rate of 0.5 mL/min with 10 mM HEPES, 5 mM  $\beta$ -glycerophosphate, 2 mM  $\text{CaCl}_2$ , 0.04% C12E8 nonionic detergent, and 150 mM NaCl (pH 7.0) (buffer A). Aliquots (20  $\mu$ g) of bovine or human DNase I were injected, and the column was run isocratically at 100% A for 15 min, followed by a gradient to 100% buffer B (buffer A and 5 mM mannose 6-phosphate) for 10 min. The column was maintained at 10 °C, and the eluate was monitored at 214 nm.

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).** A 12.5% SDS-PAGE gel was run with an increased salt modification (66) to the Laemmli procedure (67). Gels were poured and run using a Mini-Protean II (Bio-Rad) apparatus. All reagents were purchased and the protocol was obtained from Bio-Rad.

## RESULTS

**Purification and Characterization of Human Urine DNase I.** Approximately 800  $\mu$ g of DNase I was isolated from a pool of 12 L of urine collected from two individuals. Table 1 shows the column steps employed to purify hDNase with a yield of 47%. The Lichrospher 1000 SO3 "tentacle" cation exchange column (68) was included in the purification scheme since it has been shown (69) to selectively bind DNase I in a manner that mimics that of DNA. Figure 1 shows a sodium dodecyl sulfate-polyacrylamide gel elec-

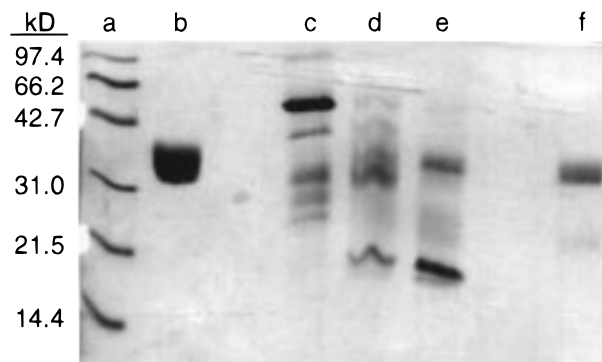


FIGURE 1: Coomassie-stained gel from SDS-PAGE of aliquots of pools of the column steps involved in purification of hDNase from urine: (a) molecular mass markers, (b) recombinant human DNase I, (c) DEAE Fast Flow pool, (d) TSK-Phenyl pool, (e) TSK-Phenyl pool following buffer exchange, and (f) Lichrospher 1000 SO3 pool.

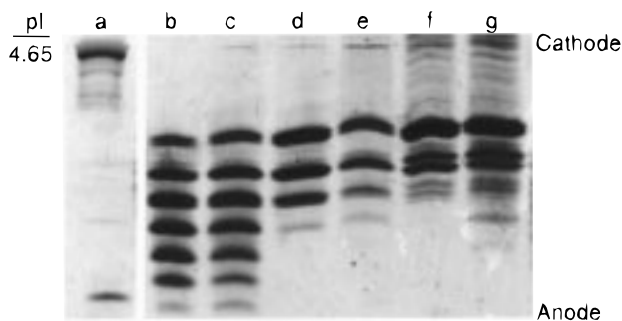


FIGURE 2: IEF-PAGE analysis of rhDNase and hDNase samples: (a) pI standards, (b) rhDNase, (c) hDNase, (d) rhDNase after treatment with neuraminidase, (e) hDNase after treatment with neuraminidase, (f) rhDNase after treatment with neuraminidase and alkaline phosphatase, and (g) hDNase after treatment with neuraminidase and alkaline phosphatase.

trophoresis (SDS-PAGE) analysis of aliquots of the pools of DNase I obtained after each step in the purification process, and illustrates the high purity of hDNase isolated by this procedure. The  $M_r$  of hDNase assessed by SDS-PAGE is approximately 34–38 kDa, consistent with that observed for recombinant human DNase I (lane b).

Tryptic mapping by HPLC with on-line analysis by electrospray mass spectrometry (data not shown) confirmed the identity of the protein as human DNase I with a sequence identical to that reported by Shak et al. (35). Along with the main sequence, the tryptic map provided evidence for a low level of an Arg-Gln polymorphism at residue 222 in the sequence.

**Isoelectric Focusing Polyacrylamide Gel Electrophoresis.** Figure 2 shows the ladder of bands with a pI from 3.5 to 4.3 obtained by IEF-PAGE analysis of (lane b) recombinant human DNase I and (lane c) hDNase isolated from urine. The six main bands in the pattern reflect the different extents of sialylation and phosphorylation of the enzyme isolated from either source, as shown by the simplification effected by serial treatment with neuraminidase (lanes d and e) and alkaline phosphatase (lanes f and g). DNase is prone to deamidation at asparagine residue 74 (69) under alkaline conditions such as those used in the phosphatase treatments for the samples shown in lanes f and g. Deamidation accounts for the appearance of the minor bands intercalated among the major bands in these samples. Both rhDNase



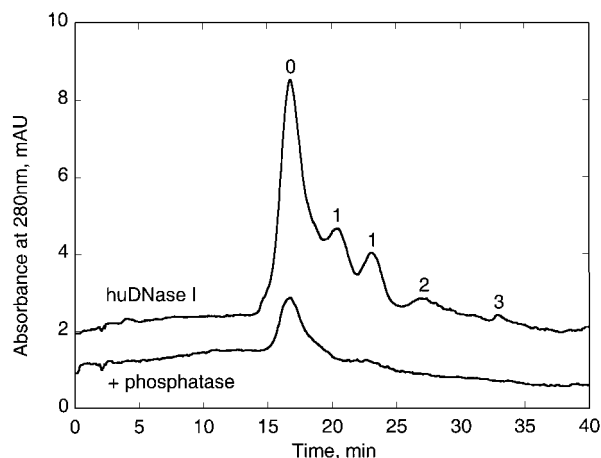


FIGURE 3: (Top trace) Separation of isophosphorylates in 20  $\mu$ g of hDNase by PEI anion exchange HPLC. (Bottom trace) Analysis by PEI anion exchange HPLC of 2  $\mu$ g of hDNase after treatment with alkaline phosphatase.

and hDNase yield similar patterns in IEF analysis in each case, suggesting that the enzyme from either source is similarly phosphorylated and sialylated.

**Anion Exchange HPLC of hDNase.** Separation of hDNase isolated from urine on a polyethyleneimine (PEI) HPLC column yields a chromatogram containing four peaks, as shown in Figure 3 (top trace). Digestion of hDNase with alkaline phosphatase collapses the chromatogram to a single peak, with an elution position that corresponds to that of the most basic peak in the untreated sample (Figure 3, bottom trace). Digestion with neuraminidase does not reduce the number of peaks in the chromatogram, but effects only a small change in the earlier elution profile (data not shown). Hence, anion exchange HPLC on the PEI column is selective for phosphate residues on the protein. The four peaks in the chromatogram of rhDNase contain either zero (peak I), one (peaks II and III), or two (peak IV) phosphate monoesters (70). Peaks II and III differ in the location of the monophosphorylated oligosaccharide at either Asn-106 or Asn-18, respectively. Integration of the chromatogram reveals that hDNase contains approximately 0.4 mol of phosphate per mole of hDNase.

**HPLC-MS.** Digestion with trypsin yields two peptides that contain the two sites of glycosylation of hDNase. One peptide, T3, includes residues 16–31 and the glycosylation site at Asn-18. The second, T9–10, includes residues 80–117 and the site at Asn-106. The sequences of the two peptides are shown in Table 2. On-line HPLC-electrospray mass spectrometry of the mixture of tryptic peptides yields information about the oligosaccharide structures found at the two glycosylation sites. Table 2 shows representative structures of small oligosaccharides found on hDNase along with the mass calculated for the corresponding glycopeptide. Figure 4 shows the reconstructed molecular ion mass spectra obtained for the (A) T9–10 and (B) T3 glycopeptides by this procedure. The molecular ion spectra were deconvoluted from the mass spectra of the multiply charged species by the procedure of Mann et al. (71). The principle ions found for the T9–10 glycopeptides that correspond to structures shown in Table 2 are labeled in the spectrum shown in Figure 4A. The glycopeptides observed in greatest abundance included monophosphorylated penta- (4) and hexamannose

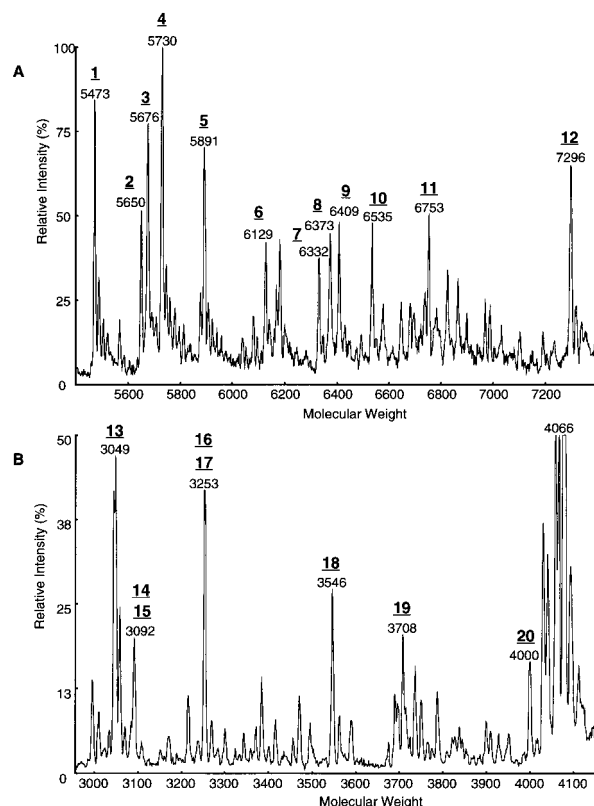


FIGURE 4: (A) Mass spectrometric oligosaccharide map of the T9–10 tryptic peptide comprising residues 80–117 of hDNase. (B) Mass spectrometric oligosaccharide map of the T3 tryptic peptide comprising residues 16–31 of hDNase. The sequences of the peptides and structures assigned to ions in the mass spectrum are shown in Table 2.

(5) structures, along with fucosylated complex and hybrid oligosaccharides. The distribution of ions observed in mass spectrometry is determined in part by the ease with which a particular glycopeptide can be ionized in the electrospray interface, so the spectra shown in Figure 4 probably do not reflect the full gamut of carbohydrate structures on hDNase. Nevertheless, the occurrence of phosphorylated high-mannose and hybrid structures on hDNase is supported by the spectrum of masses of the T9–10 glycopeptides. Figure 4B shows a portion of the relatively more complex deconvoluted mass spectrum observed for the T3 glycopeptide. The T17 peptide that comprises residues 127–157 in the hDNase sequence, and has a mass of 4066, coelutes within the broad peak of T3 glycopeptides in the tryptic map, and hence is seen as a principal peak in the mass spectrum of T3, which has a peptide mass, sans carbohydrate, of 1794 Da. The assignments made in Figure 4B, and shown in Table 2, suggest that the complex and hybrid oligosaccharides attached to hDNase at Asn-18 are not fucosylated to the extent seen at Asn-106 in T9–10. The absence of fucose in these structures hinders the unambiguous assignment of ions in the spectrum to T3 glycopeptides bearing phosphorylated penta- (14) and hexamannose (16) structures, since the masses of these structures differ by only two mass units from those calculated for the diantennary structures 15 and 17 shown in Table 2. The peaks labeled 3092 and 3253, therefore, may indicate the presence of both types of structures at Asn-18 in hDNase.

**Binding of hDNase to the CI-MPR Column.** Figure 5 shows the chromatography of (A) bovine and (B) human

Table 2: Glycopeptide Structures Assigned to Masses Observed during HPLC–MS of the hDNase Tryptic Digest Mixture<sup>c</sup>

## A. Oligosaccharide structures assigned at Asn-106

Identifier	Structure	Glycopeptide Mass <sup>a</sup>
T9-10	YLFVYRPDQVSABDSYYYDDGCEPCG <u>ND</u> TFNREPAIVR	4434 <sup>b</sup>
<u>1</u>		5473
<u>2</u>		5651
<u>3</u>		5676
<u>4</u>		5731
<u>5</u>		5893
<u>6</u>		6130
<u>7</u>		6333
<u>8</u>		6372
<u>9</u>		6407
<u>10</u>		6534
<u>11</u>		6753
<u>12</u>		7297

## B. Oligosaccharide structures assigned at Asn-18

Identifier	Structure	Glycopeptide Mass
T3	MSNATLVSYIVQILSR	1794
<u>13</u>		3052
<u>14</u>		3091
<u>15</u>		3093
<u>16</u>		3253
<u>17</u>		3255
<u>18</u>		3547
<u>19</u>		3709
<u>20</u>		4000

<sup>a</sup> The calculated glycopeptide mass is the sum of the polypeptide and carbohydrate masses using the following values for the monosaccharide residues: *N*-acetylglucosamine (GlcNAc), 203.2; fucose (Fuc), 146.1; mannose (Man), 162.1; galactose (Gal), 162.1; *N*-acetylneuraminic acid (NeuAc), 291.3; and phosphate (P), 79.9. <sup>b</sup> Calculated mass of the unglycosylated peptide. <sup>c</sup> Peptide structures are denoted by the single-letter code, with the site of N-linked glycosylation underlined.

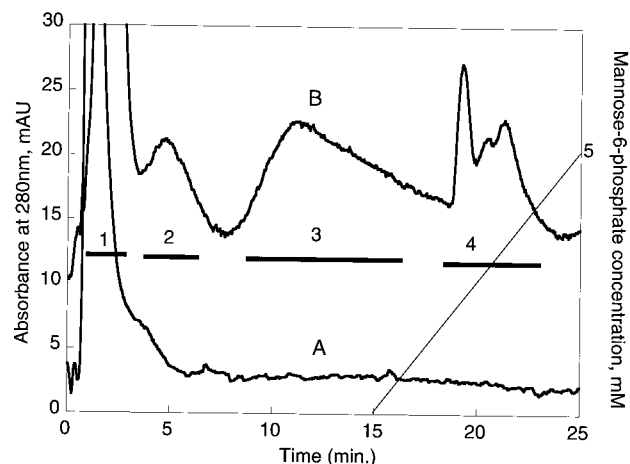


FIGURE 5: Human, but not bovine, DNase I binds to the Man-6-P receptor and can be eluted with mannose 6-phosphate. (A) Eluent profile of 20  $\mu$ g of bovine DNase I on the CI-MPR column. (B) Eluent profile of 20  $\mu$ g of human urine DNase I on the CI-MPR column. The arrow at 15 min marks the start of the Man-6-P gradient. The four fractions collected for subsequent analysis are indicated.

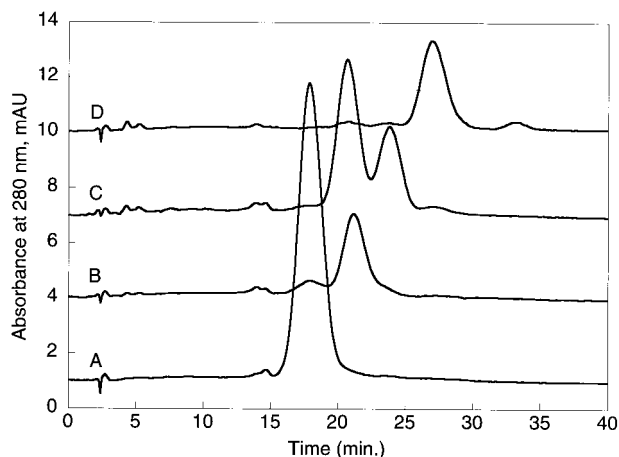


FIGURE 6: hDNase that binds with high affinity to the CI-MPR column contains two phosphomonoesters. Analysis by PEI anion exchange HPLC of (A) rhDNase unretained by the CI-MPR column, (B and C) weakly retained rhDNase collected as fractions 2 and 3, and (D) strongly bound rhDNase that elutes in the Man-6-P gradient.

DNases I on the Man-6-P receptor (MPR) column. bDNase, which has been shown to contain predominantly high-mannose and hybrid oligosaccharides at its single site of glycosylation (11, 12) with no evidence of phosphorylation, does not bind to the column. hDNase binds to the column, and can be eluted with a Man-6-P gradient, as shown in Figure 5B. The most strongly retained species, which elute within the Man-6-P gradient, consist principally of diphosphorylated hDNase, as shown by rechromatography of fractions on the PEI column (Figure 6). Four fractions were collected as shown in Figure 5B that represented (1) unbound, (2 and 3) weakly bound, and (4) strongly bound hDNase. Fraction 1 was shown by rechromatography on the PEI column to lack phosphate (Figure 6A). Fractions 2 and 3 were shown to be monophosphorylated (Figure 6B,C), and fraction 4 was shown to have 2 mol of phosphate per mole of hDNase (Figure 6D).

## DISCUSSION

hDNase isolated from urine exhibits considerable charge heterogeneity on IEF-PAGE analysis, even after removal of sialic acid residues by neuraminidase digestion. This residual heterogeneity has been attributed to genetic polymorphism (27–34). Several mutations have been identified, with a mutation of Gln to Arg at position 222 resulting in polypeptide sequence heterogeneity with a charge shift (73). We have isolated hDNase from urine pooled from two individuals to assess contributions to this heterogeneity. The purification procedure employed anion exchange chromatography, hydrophobic interaction HPLC, and tentacle cation exchange HPLC (68), a technique that exploits the biomimetic interaction between DNase I and the polyanionic ligand that is an analogue of DNA (69) to provide high selectivity. The purified hDNase yielded a single band on SDS-PAGE (Figure 1). Tryptic mapping (data not shown) confirmed the sequence of the protein as human DNase I (35) and the absence of other proteins at appreciable concentrations. Hence, the characterization of carbohydrate structures reported here pertains to oligosaccharides associated with hDNase and not with other contaminating proteins. Along with the expected sequence, hDNase with a Gln residue substituted for Arg-222 was also detected, and was the only polymorphism observed. Although accurate quantification of the proportions of the two sequences could not be achieved by HPLC-MS of the tryptic digests, the Gln-222 isoform probably represented  $\leq 25\%$  of the total.

hDNase yields a ladder of six or more bands on analysis by IEF-PAGE (Figure 2), as reported previously (25, 27, 29). A portion of the charge heterogeneity is due to sialylation of the enzyme, as shown by the simplification of the pattern effected by digestion with neuraminidase (Figure 2). Subsequent treatment of desialylated hDNase with alkaline phosphatase further narrows the distribution of bands seen by IEF-PAGE (Figure 2), although an acidic band arises during treatment at elevated pH that can be attributed to deamidation of the labile Asn-74 residue in DNase I (69). The residue on the carboxyl-terminal side of Asn-74 is serine, and Asn-Ser sequences are known to rapidly undergo cyclization and deamidation (72) under alkaline conditions. Deamidation, since it converts a neutral side chain to an acidic side chain, is manifested in IEF-PAGE and can multiply the charge heterogeneity of hDNase without careful sample handling. Both recombinant and urine-derived hDNases show similar banding patterns on IEF-PAGE in the untreated form as well as after treatment with neuraminidase and alkaline phosphatase, thus demonstrating the close similarity of the enzymes from the two sources.

Ion exchange HPLC on a PEI column, shown in Figure 3, provides a convenient method for quantitative estimation of the extent of phosphorylation of hDNase samples, as well as a means of preparing isophosphorylates for further characterization (70). hDNase derived from urine can be separated by this column into four major peaks that differ in the extent of phosphorylation of the enzyme. Treatment with alkaline phosphatase alone is sufficient to simplify the chromatogram to a single peak (Figure 3B) with the same retention time as nonphosphorylated hDNase. Integration of peak areas of the four peaks in the untreated sample, and weighting of the areas with respect to the number of

phosphomonoesters, yields an estimate of 0.4 mol of phosphate per mole of hDNase. Hence, the PEI column analysis demonstrates that hDNase is partially phosphorylated and contains both mono- and diphosphorylated isoforms, both features that have been described for lysosomal enzymes (45–50).

Tryptic mapping of hDNase with on-line detection by electrospray mass spectrometry permits the localization of phosphate to carbohydrate structures (Figure 4). The two Asn residues that are glycosylated in hDNase occur in two different peptides in the tryptic digest mixture, so HPLC–MS can be employed to separately identify the carbohydrate structures appended at either site. Assignment of molecular ions (Table 2) suggests that Asn-106 carries both phosphorylated penta- and hexamannose residues, along with fucosylated complex and hybrid structures, and that the latter can also be phosphorylated. Chromatography on the PEI column suggests that Asn-18 also carries phosphorylated oligosaccharides, although as noted above the absence of fucose on the complex oligosaccharides makes the assignment of masses ambiguous compared to those for the structures at Asn-106. The occurrence of similar structures in bona fide lysosomal acid hydrolases has been reported (45–50). Hence, hDNase undergoes post-translational modification similar to that characteristic of enzymes that are targeted to lysosomes.

Man-6-P residues on lysosomal enzymes bind to the CI-MPR to initiate their targeting to lysosomes. The occurrence of phosphate in high-mannose residues of hDNase suggests that this enzyme, which is not an acid hydrolase, may bind to the CI-MPR. The selective binding of hDNase to immobilized CI-MPR was demonstrated by coupling the receptor to an epoxy-derivatized column, loading an hDNase sample, and monitoring the UV profile obtained on elution of the column with 6-phosphorylmannose (Figure 5B). The most strongly retained species on the column were shown to be diphosphorylates of hDNase by rechromatography on the PEI anion exchange column (Figure 6). These findings are consistent with the reported higher affinity for the CI-MPR of oligosaccharides bearing two phosphomonoesters (54, 55). Hence, hDNase contains the lysosomal targeting signal and selectively binds the CI-MPR. Hence, hDNase, a secreted enzyme, may be trafficked to lysosomes, at least in part. Since lysosomes contain both proteolytic and glycolytic enzymes hDNase targeted to lysosomes would be degraded, although a portion of the phosphorylated hDNase escapes the trafficking pathway and is secreted. Capture and hydrolysis of phosphorylated hDNase species by lysosomes would be expected to lower the average phosphate content of the pool of hDNase isolated from urine so that it would not reflect the levels of this post-translational modification present on the newly synthesized protein.

Bovine DNase I, which does not contain phosphorylated oligosaccharides, does not bind to the CI-MPR (Figure 5A). The post-translational modification of human DNase I differs markedly from that described for the homologous bovine enzyme. bDNase has a sequence 78% identical with that of hDNase, but differs in that (i) Asn-106 in bDNase is not glycosylated although it occurs in an Asn-Asp-Ser sequence while the same residue occurring in an Asn-Asp-Thr sequence in hDNase is glycosylated, (ii) only hybrid and high-mannose oligosaccharides are found on bDNase, while

mass spectrometric data presented here are consistent with a broader array of structures appended to hDNase, including complex oligosaccharides, and (iii) mannose 6-phosphate residues are found in the glycans attached to hDNase but not bDNase. Recent studies (74) demonstrate the occurrence of phosphomannose residues on bDNase expressed in COS-1 cells in vitro. Hence, the absence of these structures on bDNase isolated from bovine pancreas may be due to tissue- and species-specific differences in post-translational modification of the homologous protein.

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