Molecular Cloning and Expression of a Third Member of the Heparan Sulfate/Heparin GlcNAc N-Deacetylase/N-Sulfotransferase Family

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N-Deacetylation and N-sulfation of N-acetylglu- coseamine residues in heparan sulfate and heparin initiate a series of chemical modifications that ultimately lead to oligosaccharide sequences with specific ligand binding properties. These reactions are catalyzed by GlcNAc N-deacetylase/N-sulfotransferase (NDST), a monomeric enzyme with two catalytic activities. Two genes encoding NDST isoforms have been described, one from rat liver (NDST1) and another from murine mastocytoma (NDST2). Both isoforms are expressed in tissues in varying amounts, but their relative contribution to heparan sulfate formation in any one tissue is unknown. We now report the identification of a third member of the NDST family, designated NDST3. A full-length cDNA clone (3.2 kilobase pairs) encoding a 873-amino acid protein was obtained from a human fetal/infant brain cDNA library. Human NDST3 (hNDST3) has a nucleotide sequence homologous but not identical to hNDST1 and NDST2. The deduced amino acid sequence shows 70% and 65% amino acid identity to that of hNDST1 and NDST2, respectively. A soluble chimera of hNDST3 and protein A exhibited both N-deacetylase and N-sulfotransferase activity, confirming its enzymatic identity. Northern blot analysis of human fetal brain poly(A)⁺ RNA showed a single transcript of 6.4 kilobase pairs. Reverse transcription polymerase chain reaction analysis revealed more restricted tissue expression of hNDST3 than hNDST1 and NDST2, and high levels in brain, liver, and kidney. Analysis of Chinese hamster ovary cells revealed expression of NDST1 and NDST2, but not NDST3. In a Chinese hamster ovary cell mutant exhibiting reduced N-sulfotransferase activity and reduced sulfation of heparan sulfate (Bame, K. J., and Esko, J. D. (1989) J. Biol. Chem. 264, 8059–8065), expression of NDST1 was greatly reduced, but NDST2 was expressed normally, suggesting that both enzymes are involved in heparan sulfate assembly. The discovery of multiple NDST isoforms suggests that the assembly of heparan sulfate is much complicated than previously appreciated.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF074924.

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Heparan sulfate and heparin are large complex carbohydrate chains that interact with various proteins (e.g. basic fibroblast growth factor, hepatocyte growth factor, antithrombin, and lipoprotein lipase) through unique oligosaccharide sequences. These sequences consist of N-acetylated and N-sulfated glucosamine residues, containing O-sulfate groups and GlcA1 and IdoA in various arrangements (1). The assembly of these sequences occurs by the concerted action of various enzymes that act in three stages: 1) chain initiation and assembly of the linkage tetrasaccharide GlcAβ1,3Galβ1,3Galβ1,4Xylβ1- on serine residues of core protein, 2) chain elongation in which the disaccharide repeat unit of GlcAβ1,4GlcNAcα1,4- are assembled, and 3) chain modification in which N-deacetylation/ N-sulfation of subsets of GlcNAc residues occurs, followed by C5-epimerization of GlcA to l-IdoA, 2-O-sulfation of the uronic acid residues, and 6-O- and 3-O-sulfation of glucosamine residues. GlcNAc N-deacetylation and N-sulfation are prerequisites for all of the other modifications and are catalyzed by a single-chain polypeptide, called GlcNAc N-deacetylase/ N-sulfotransferase (NDST)2 (2–10).

Two different isoforms with high sequence similarities have been identified, one from rat liver (NDST1, Ref. 4) and another from mouse mastocytoma (NDST2, Refs. 7, 9). Human counterparts of NDST1 and NDST2 have been reported, and their chromosomal location was mapped at 5q32–33.1 and 10q22, respectively (11, 12). Both isoforms are expressed in tissues to varying extents (10–12), but their relative contribution to heparan sulfate formation in any one tissue is still unclear. In this report, we describe the identification of a new member of the NDST gene family, designated NDST3. hNDST3 exhibited both tissue-specific expression and enzymatic activities, confirming its identity as a GlcNAc N-deacetylase/N-sulfotransferase and suggesting that it functions as an active NDST enzyme in specific tissues in vivo. In addition, expression of the

1 The abbreviations used are: GlcA, D-glucuronic acid; IdoA, l-iduronic acid; GlcN, unsubstituted glucosamine; NDST, heparan sulfate GlcNAc N-deacetylase/PAPS:GlcN-N-sulfotransferase; hNDST, mNDST, and nNDST, human, rat, and mouse NDST, respectively; CHO, Chinese hamster ovary; PAPS, adenosine 3’-phosphate 5’-phosphosulfate; bp, base pair(s); kb, kilobase pair(s); RT, reverse transcriptase; PCR, polymerase chain reaction; EST, expressed sequence tag; RACE, rapid amplification of cDNA ends.

2 A consensus regarding the abbreviation for the proteoglycan N-sulfotransferases was reached at a recent meeting on proteoglycans. The abbreviation NDST refers specifically to the GlcNAc N-deacetylase/ N-sulfotransferase involved in heparan sulfate and heparin biosynthesis. Species names precede the abbreviation using a single- or double-letter code (e.g. m for mouse, h for human, or hm for hamster) and the different isoforms contain a numeric suffix (e.g. hNDST1 refers to the human isoyme 1). NDST1 refers to the enzyme originally cloned from rat liver (4), NDST2 refers to the one originally described in murine mastocytoma (7, 9), and NDST3 refers to the one described in this paper.
NDST gene family was examined in CHO cells and a mutant, pgsE-606, which exhibited undersulfated heparan sulfate and reduced N-sulfotransferase activity (13–16). The results indicate that both NDST1 and NDST2 participate in heparan sulfate formation in CHO cells.

MATERIALS AND METHODS

Identification and Characterization of Expressed Sequence Tag (EST) Clones—Identification of EST clones that encoded a new member of NDST was performed essentially according to Veugeler et al. (17). Briefly, five clones were found to be similar but not identical to hNDST1 and NDST2 (GenBank accession nos. U36960 and U36961, respectively) by searching the GenBank EST database using the nucleotide sequence of hNDST1 (11, 18, 19). One EST clone (ID 47106), whose terminal sequences were reported as H11065 and H11153, was obtained from Genome System, Inc. The nucleotide sequence was determined using an ABI 373 DNA sequencer with the cycle-sequencing method. The obtained sequence corresponded to the nucleotides 877–3188 of the full-length cDNA sequence.

5′-RACE (Rapid Amplification of cDNA Ends)—5′-RACE was performed using an adapter-ligated library of human fetal brain cDNA (CLONTECH) according to manufacturer’s protocol. Briefly, cDNA was first amplified using a primer consisting of nucleotides 1538–1564 (NDST3-HGS5P, 5′-gggtgctatgctgccacattgaggg-3′) and anchor primer 1, according to the touch-down PCR protocol (30 cycles) using AdvantageTag (CLONTECH). An aliquot (1/250) of the first PCR was subjected to nested PCR (20 cycles) using nucleotides 1132–1158 (NDST3-HGS2P, 5′-ccattgtctgctgccacattgaggg-3′) and primer 2, followed by a second nested PCR (20 cycles) using nucleotides 1120–1147 (NDST3-HGS6P, 5′-atgtcagggaaagactctctcagtg-3′) and anchor primer 1. Southern hybridization was performed using a 5′-labeled DNA fragment representing nucleotides 936–1556 as a probe. The DNA fragment was gel-purified and cloned into vector pCR2.1 (Invitrogen). The nucleotide sequence was determined, and nucleotides 9–931 were further confirmed by analyzing DNA fragments representing nucleotides 9–387 and 366–387 prepared by PCR (40–60 cycles with primers NDST3-AR (5′-tagctctctctcttccctctcttt-3′), nucleotide 9–30) and NDST3-AR (5′-aacagcagctgaacaccacct-3′, nucleotide 366–387) or NDST3-AR (5′-ccagagactctgtaacctct-3′, nucleotide 910–931), respectively, and Pfu polymerase (Stratagene). The fragments were amplified from the initial library.

Northern Hybridization of hNDST3—Human fetal brain poly(A)+ RNA was obtained from CLONTECH. Northern hybridization was performed using ExpressHyb (CLONTECH) essentially according to manufacturer’s protocol. A 32P-labeled DNA fragment (nucleotides 1–1147) was used as a probe. The hybridized band was detected using a PhosphorImager (Molecular Dynamics).

RT-PCR of NDST Gene Family in Human Tissues—Normalized cDNA was obtained from CLONTECH (Human cDNA panel 1 and a fetal cDNA panel). PCR was performed as described above using 32 cycles for hNDST3 and 27 cycles for hNDST1 and NDST2. For the specific amplification of each NDST, six gene-specific primers were used: hNDST1-1F (5′-ccagagactctgtaacctct-3′) and hNDST1-1R (5′-agctctctctcttccctctcttt-3′) for hNDST1 (nucleotides 518–542 and 1124–1148 in AF074924, respectively); hNDST1-1F (5′-ccagagactctgtaacctct-3′) and hNDST1-1R (5′-agctctctctcttccctctcttt-3′) for hNDST2 (nucleotides 3081–3105 and 3499–3523 in U36960, respectively); and hNDST2-1F (5′-tctccgtgaagtttttgcgtg-3′) and hNDST2-1R (5′-atgccagctgtctctcttt-3′) for hNDST3 (nucleotides 2664–2688 and 3065–3089 in U36961, respectively). Each set of oligonucleotides was unique to each NDST isoform, as determined by the program, Amplify. The product was analyzed by 1.2% TAE-agarose gel electrophoresis and detected by ethidium bromide staining.

RESULTS

Cloning of hNDST3—Searching the EST data base using the nucleotide sequences homologous to human NDST1 identified five non-identical EST clones. One was derived from human infant brain (GenBank accession nos. H11065–H11153), two from fetal spleen and liver (AA011263–AA011344 and R07738–R07739) and two from Jurkat lymphoma cells (AA35878 and AA354508). One clone from human infant brain was selected for full sequencing, since it contained the longest 5′-sequence (Fig. 1). The open reading frame was composed of 322 nucleotides and 3931 bases in 1147 cycles for hNDST3 and 27 cycles for hNDST1 and NDST2. For the specific amplification of each NDST, six gene-specific primers were used: hNDST1-1F (5′-ccagagactctgtaacctct-3′) and hNDST1-1R (5′-agctctctctcttccctctcttt-3′) for hNDST1 (nucleotides 518–542 and 1124–1148 in AF074924, respectively); hNDST1-1F (5′-ccagagactctgtaacctct-3′) and hNDST1-1R (5′-agctctctctcttccctctcttt-3′) for hNDST2 (nucleotides 3081–3105 and 3499–3523 in U36960, respectively); and hNDST2-1F (5′-tctccgtgaagtttttgcgtg-3′) and hNDST2-1R (5′-atgccagctgtctctcttt-3′) for hNDST3 (nucleotides 2664–2688 and 3065–3089 in U36961, respectively). Each set of oligonucleotides was unique to each NDST isoform, as determined by the program, Amplify. The product was analyzed by 1.2% TAE-agarose gel electrophoresis and detected by ethidium bromide staining.

RT-PCR of NDST Isozymes in CHO Cells—Culturing of wild-type CHO-K1 (ATCC CCL-61) and the N-sulfotransferase-deficient mutant, pgsE-606, were described previously (13). Poly(A)+ RNA was prepared using Fast Track 2.0 (Invitrogen) and reverse-transcribed into single-strand DNA using the Superscript preamplification system (Life Technologies, Inc.) according to the manufacturer’s instructions. A reaction without reverse transcriptase served as a negative control. PCR analysis (27 cycles for NDST1 and NDST2, 30 cycles for NDST3) was then performed for hamster NDST1 (nucleotides 3104–3544 of rNDST1, M92042) using mNDST1-1F (5′-ccagagactctgtaacctct-3′) and mNDST1-1R (5′-gcttctctctcttccctctcttt-3′), NDST2 (nucleotides 3142–3570 of mNDST2, U02934) using mNDST2-1F (5′-gcttctctctcttccctctcttt-3′) and mNDST2-1R (5′-ccagagactctgtaacctct-3′), and NDST3 (nucleotides 251–609 of hNDST3, AF074924) using mNDST3-2F (5′-gcttctctctcttccctctcttt-3′) and mNDST3-2R (5′-gcttctctctcttccctctcttt-3′).

Enzymatic Activity of a Soluble Chimera of hNDST3—A soluble form of hNDST3 corresponding to amino acid residues 48–873 was fused at its N terminus to a portion of protein A essentially according to Wei et al. (6). Transient expression of the chimera in COS7 cells was achieved with LipofectAMINE (Life Technologies, Inc.) using the manufacturer’s protocol. After 72 h of incubation, cell culture supernatant was recovered and the chimera was collected on IgG-agarose essentially according to Wei et al. (6). Briefly, a portion of the supernatant (10 ml) was mixed with a suspension (100 µl) of rabbit IgG-agarose (Sigma) and rotated end-over-end at 4 °C overnight. The beads were washed twice with 10 ml of buffer A (50 μM Tris-HCl, 20% (w/v) glycerol, pH 7.4) and then suspended in buffer A (200 µl). An aliquot (20 µl) was used for the assay of both N-deacetylation and N-sulfotransferase activity, using [3H-Hepes]/heparosan or de-N-sulfated heparan as a substrate, as described previously (14, 16). [3H]Heparin purified by DEAE-Sephacel chromatography (14) was treated with nitrous acid at pH 1.5 (20) and analyzed by gel filtration high performance liquid chromatography using a TSK G2000SW column (7.5 × 300 mm, LKB) as described by Bame et al. (13). Specific degradation at N-sulfated glucosamine residues by nitrous acid treatment was monitored by counting of radioactivity in each fraction.

3 These primers were based on mouse genomic sequence homologous to hNDST3 cDNA (J. Aikawa and J. D. Esko, unpublished results). Since PCR with this set of primers using mouse brain cDNA as a template produced the 359-bp DNA fragment predicted from the genomic information, the use of this set of primers in PCR should be able to amplify a part of the Chinese hamster NDST3 cDNA.
posed of 714 amino acids with a termination codon (TAG) located similarly to that found in hNDST1 and hNDST2, but it lacked sequence homologous to the N termini of hNDST1 and hNDST2. We therefore prepared the 5'--portion of this clone using 5'--RACE. After two rounds of nested PCR, DNA fragments were obtained that hybridized to a probe containing the 5'--portion of clone 47106. pCR15–1 had the longest insert (1147 bp), and its nucleotide sequence was analyzed (Fig. 1). The sequence of nucleotides 9–931 was further confirmed by using DNA fragments prepared by PCR using high fidelity DNA polymerase (Fig. 1) (see "Materials and Methods"). The combined nucleotide sequence of the EST clone and the 5'--RACE product showed an open reading frame of 2619 nucleotides with 5'-- and 3'--untranslated regions of 403 and 163 nucleotides, respectively. The open reading frame corresponded to 873 amino acid residues. The primary structure of this polypeptide showed a type II transmembrane protein, with 23 amino acids tentatively identified as the transmembrane domain by hydropathy analysis (Fig. 2, underlined sequence) and a 13-residue cytoplasmic domain.

Alignment of this protein with hNDST1 and hNDST2 revealed significant sequence similarities, exhibiting 70% and 65% amino acid identity and 86% and 82% similarity to human NDST1 and NDST2, respectively (10, 11, 18, 19) (Fig. 2). We therefore designated this protein NDST3, a tentative third member of the heparan sulfate heparin GlcNAc N-deacetylase/N-sulfotransferase family of genes.

Enzymatic Properties of hNDST3—To test if NDST3 had GlcNAc N-deacetylase and N-sulfotransferase activities, a soluble form of hNDST3 fused to protein A was constructed and expressed in COS7 cells. A fusion protein was detected in the conditioned medium by SDS-polyacrylamide gel electrophoresis as a 130-kDa polypeptide, the same size as a chimera of rat NDST1 (data not shown; Ref. 6). The fusion protein immobilized on IgG-agarose was tested for enzyme activity. As shown in Fig. 3, protein A-fused hNDST3 exhibited both N-deacetylation and N-sulfation activities. When compared with a comparable construct prepared from rNDST1, the new isozyme had 53% and 62% of the respective activities found in a preparation of the recombinant NDST1. The ratio of N-deacetylase to N-sulfotransferase activities was more like that found in NDST1 (1:1) than in NDST2 (5:1, Ref. 9). The product of the reaction was N-sulfated glucosamine based on nitrous acid treatment at low pH (20) (data not shown).
A single transcript of approximately 6.4 kb for hNDST3 (Fig. 4). The same size transcript was detected in Jurkat cells (data not shown). The expression of hNDST genes was next examined in various tissues using RT-PCR with normalized first-strand cDNA (see “Materials and Methods”). Each PCR yielded a single product with predicted nucleotide lengths of 633, 445, and 428 bp for hNDST3, hNDST1, and hNDST2, respectively (Fig. 5). Expression of hNDST3 was detected in greatest abundance in brain, kidney, liver, and lung of both adult and fetal tissues, adult pancreas and placenta, and fetal spleen and thymus. Expression was not detected in adult/fetal heart and skeletal muscle. On the other hand, expression of hNDST1 and hNDST2 was detected in all the tissues tested, although expression of hNDST1 in adult/fetal skeletal muscle and fetal thymus and hNDST2 in adult skeletal muscle was very low.

**Defective Expression of NDST1 in CHO Cell Mutant, pgsE-606—** The presence of three NDST transcripts in some tissues suggested that they might simultaneously contribute to heparan sulfate biosynthesis. To test this idea, we examined the expression of the three isoforms in wild-type CHO cells and a mutant (pgsE-606) previously shown to be partially defective in N-sulfation of heparan sulfate (13). Primers for rodent NDST1, NDST2, and NDST3 were designed on the basis of rNDST1, mNDST2, and hNDST3 cDNAs. NDST1 and NDST2 derived oligonucleotides amplified specific PCR products in CHO cells (443 and 431 bp, respectively, Fig. 6), but NDST3 did not, even after increasing the number of cycles. RT-PCR analysis of the mutant showed that NDST1 message was much reduced, but normal levels of NDST2 mRNA was present (Fig. 5). This mutant was previously shown to have about 30–40% of wild-type levels of N-sulfotransferase activity and a corresponding decrease in N-sulfation of the chains. These results suggest that the decrease in enzyme activity is most likely due to loss of NDST1 and that the residual sulfation observed in the mutant was due to expression of NDST2.

**DISCUSSION**

The present study describes a new human cDNA clone for N-deacetylase and N-sulfotransferase that resembles NDST1 and NDST2. Two independent findings indicate that this protein encodes a new member of NDST gene family. First, the amino acid sequence showed 65–70% identity and greater than 85% overall homology to the other NDST isozymes. Its structure was a typical type II transmembrane protein, like other Golgi enzymes. Second, production of a protein A-chimera lacking the putative transmembrane domain, but containing the presumptive ectodomain exhibited both N-deacetylase and N-sulfotransferase activity with relative activities similar to rat NDST1. Therefore, we propose to designate this gene product NDST3, and assign it as the third member of heparan sulfate/heparin N-deacetylase/N-sulfotransferase family.

The amino acid sequence of hNDST3 except for the most N-terminal 79 amino acids exhibited significant similarity to...
that of hNDST1 and hNDST2 (74% and 69%, respectively), suggesting that this region directly contributes to enzyme activity. In addition, Shworak et al. (21) have pointed out the similarity between the C-terminal 260 amino acids of NDST1 and NDST2 and the glucosamine 3-O-sulfotransferase (GlcN 3OST1) involved in the formation of the antithrombin binding sequence in heparan sulfate and heparin. Since the homologous region represents ~85% of the entire sequence of GlcN 3OST, this region in NDST could reasonably constitute the actual catalytic domain, or at least a binding site for the heparan sulfate chain. Recent analysis of PAPS binding domains in various sulfotransferases indicates a consensus binding site in the N-sulfotransferases consisting of two domains (Fig. 7). NDST3 contains these consensus sequences, as well. In addition, Berninsone and Hirschberg recently showed that the C-terminal half of NDST2 (amino acids 479–880) was able to catalyze the N-sulfation of glucosamine of heparan sulfate (22).

The most N-terminal 79 amino acid residues are completely different among three NDST isoforms (Fig. 2). In NDST3, this region contains a 13-amino acid cytoplasmic segment, a 23-amino acid transmembrane domain, and an additional 43-amino acids, which may represent a Golgi lumenal stem region by analogy to other Golgi proteins. This region might be critical for the correct localization of this enzyme (23). In the case of α2,6-sialyltransferase, which exhibits the same type II transmembrane topology and resides in the trans-Golgi and trans-Golgi network, its transmembrane and lumenal stem region are required for the correct localization (24). Humphries et al. have suggested that human NDST1 was in trans-Golgi network (11), but the location of NDST2 and NDST3 have not been investigated yet.

Northern hybridization revealed an hNDST3 transcript of 6.4 kb in both fetal brain tissue and Jurkat cells. This differs from NDST1 (8.5–8.0 and 4.2–4.0 kb) (4, 11) and NDST2 (4.0–3.8 kb) (7, 9, 12), suggesting that this transcript is unique to NDST3. A typical polyadenylation signal was not identified in this study. The difference in size of the message and the cDNA clone (3.2 kb) suggest that hNDST3 transcript has an additional ~3 kb of 3'-untranslated portion which was missed in our study. The transcript for NDST1 and NDST2 was detected in all human adult tissues tested, consistent with previous findings (11, 12). However, the expression of NDST1 and NDST2 in liver was different in human and mouse. In mouse liver, abundant message for NDST1 was detected, although NDST2 was not (7, 10). On the other hand, we have detected expression of both NDST1 and NDST2 message in human liver, and their amounts were almost the same. Furthermore, NDST3 is expressed at high levels in human liver. Further study will be required to determine if these transcripts are expressed in the same cell types to the same extent in different species.

Previously, it was proposed that NDST2 was involved in biosynthesis of heparin rather than heparan sulfate, because the enzyme exhibited a higher ratio of N-deacetylation to N-sulfotransferase activity than NDST1 (7). Furthermore, its transcript was abundant in murine mastocytoma that produces heparin, and overexpression of the enzyme in a human kidney cell line resulted in enhanced N-deacetylation and N-sulfation of heparan sulfate (25). However, in this study, we showed that NDST2 was widely distributed in human tissues in agreement with the high frequency of human EST clones (33 human EST clones were found in various tissues including breast, infant brain, and fetal lung and heart). The tissues tested in this study do not produce heparin, but make various amounts of heparan sulfate (26–28), suggesting that NDST2 may play a role in heparan sulfate synthesis as well.

Support for this hypothesis derives from our study of CHO cell mutant pgpE-606, which was originally shown to contain a defect in NDST prior to the discovery of multiple isozymes in the system. This strain exhibits a 3-fold reduction in sulfation of heparan sulfate and a comparable change in NDST activity. Analysis of NDST transcripts in CHO cells indicates that the wild-type expresses both NDST1 and NDST2, but not NDST3, consistent with the more restricted pattern of expres-

4 At the day of submission of this paper, 5 mouse and 2 rat EST clones of NDST2 were also found. A total of 26 human, 5 mouse, and 1 rat EST clones have been reported for NDST1.

\[ \text{hNDST3} \quad \text{hNDST2} \]
sion of the third isozyme. Analysis of the mutant showed that it contains very little transcript forNDST1 (the PCR was adjusted to detect even minute amounts of message), and normal amounts of NDST2 (which under these conditions exceeded the linearity of the measurement). Thus, the residual sulfation of the chains in the mutant most likely arises from the action of NDST2. Inspection of the pattern of sulfation of heparan sulfate in the mutant showed that the loss of enzyme activity decreased the overall sulfation of the chains and reduced the size of the modified sections of the chains. Thus, the data suggest that NDST1 actually works in concert with NDST2 to generate the fully expanded modified tracts characteristic of wild-type CHO cell heparan sulfates.

The detection of a third NDST isoform (NDST3) indicates that the assembly of heparan sulfate and possibly heparin may reflect the concerted action of multiple isozymes expressed in a tissue-specific manner. Further analysis of the involvement of each isozyme in the formation of heparan sulfate will require new tools including better defined substrates to assess the effects of prior modification on further deacetylation and sulfation. Resolving the contribution of each isozyme in the formation of heparan sulfate will require further analysis of the involvement of each isozyme in the formation of heparan sulfate.

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