A novel C-domain-dependent inhibition of the rainbow trout CMP-sialic acid synthetase activity by CMP-deaminoneuraminic acid

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Abstract:

The CMP-sialic acid synthetase (CSS) activates free sialic acid (Sia) to CMP-Sia using CTP, and is prerequisite for the sialylation of cell surface glycoconjugates. The vertebrate CSS consists of two domains, a catalytic N-domain and a non-catalytic C-domain. Although the C-domain is not required for the CSS enzyme to synthesize CMP-Sia, its involvement in the catalytic activity remains unknown. First, the real-time monitoring of CSS-catalyzed reaction was performed by ^{31}P-NMR using the rainbow trout CSS (rtCSS). While a rtCSS lacking the C-domain (rtCSS-N) similarly activated both deaminoneuraminic acid (Kdn) and *N*-acetylneuraminic acid (Neu5Ac), the full-length rtCSS (rtCSS-FL) did not activate Kdn as efficiently as Neu5Ac. These results suggest that the C-domain of rtCSS affects the enzymatic activity, when Kdn was used as a substrate. Second, the enzymatic activity of rtCSS-FL and rtCSS-N was measured under various concentrations of CMP-Kdn. Inhibition by CMP-Kdn was observed only for rtCSS-FL, but not for rtCSS-N, suggesting that the inhibition was C-domain-dependent. Third, the inhibitory effect of CMP-Kdn was also investigated using the mouse CSS (mCSS). However, no inhibition was observed with mCSS even at high concentrations of CMP-Kdn. Taken together, the data demonstrated that the C-domain is involved in the CMP-Kdn-dependent inhibition of rtCSS, which is a novel regulation of the Sia metabolism in rainbow trout.

Key words: CMAS, CMP-sialic acid synthetase, deaminoneuraminic acid, *N*-acetylneuraminic acid, rainbow trout, sialic acid

Footnote:

[1]Abbreviations: CMP-Sia, CMP-sialic acid; CTP, cytidine 5'-triphosphate; CSS, CMP-sialic acid synthetase; Kdn, 2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid or deaminoneuraminic acid; ManNAc, *N*-acetylmannosamine; Man, mannose; Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; Sia, sialic acid.

Introduction

Sialic acid (Sia[1]) is a family of nine carbon-sugars with a carboxylate group at position C-1 [1]. Sias are negatively charged at physiological pH and have a vast diversity of structures, all of which are derived from *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc), and 2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid (Kdn) [2-5]. Kdn is a sialic acid bearing a hydroxyl group at position C-5, which was first identified in rainbow trout egg polysialoglycoproteins in 1986 [6]. Kdn is considered as a ubiquitous component of vertebrate glycoconjugates [5-10] and bacterial capsular polysaccharides [11]. In rainbow trout, Kdn is highly expressed in ovary and testis, but not in liver and other somatic tissues tested [9,12]. In mammals, Kdn is expressed in lung carcinoma cells [13], and in rat fetal, but not adult lung [14]. Recently, the level of the urinary Kdn was shown to increase in patients suffering from end-stage renal disease [15]. Thus, the expression of Kdn in vertebrates is regulated during normal development and tumorigenesis.

It was first demonstrated that Kdn was transferred to the termini of glycan chains of human transferrin from cytidine monophosphate (CMP)-Kdn by the common α2,6-sialyltransferase [16]. It has since been considered that Kdn-glycoconjugates can be synthesized in place of Neu5Ac-glycoconjugates, once CMP-Kdn is provided to common sialyltransferases. The first CSS that can activate Kdn to CMP-Kdn was reported in rainbow trout [17], and the cDNA for the rainbow trout CSS (rtCSS) was cloned from the testis [18]. The recombinant rtCSS was found to exhibit high activity to synthesize both CMP-Kdn and CMP-Neu5Ac. These results indicate that the recombinant rtCSS can be used to prepare both CMP-Kdn and CMP-Neu5Ac [18]. Most vertebrate CSSs consist of two domains, a catalytic N-domain and a non-catalytic C-domain. The N-domain is known to conserve the CSS activity without the C-domain. Interestingly, the CSS full-length (CSS-FL), containing both N- and C-domains, has been reported to show lower activity than the corresponding truncated enzyme exhibiting only the N domain (CSS-N) [19]. However, the reasons behind such a difference in kinetic parameters in Sia activation performed by CSS-FL and CSS-N remain elusive. Whether the conversion efficiency from Sia to CMP-Sia was different between Kdn and Neu5Ac is also a relevant question that is yet to be answered. In this study, we thus sought to establish whether the C-

domain affects the efficiency and substrate specificity of the CSS-catalyzed reaction using rtCSS-FL and rtCSS-N as enzymes and Kdn and Neu5Ac as substrates.

Materials and Methods

Materials

Neu5Ac, CMP-Neu5Ac, isopropyl-1-β-D-galactopyranoside (IPTG), aprotinin, and leupeptin were from nacalai tesque (Kyoto, Japan). Neu5Gc was from Tokyo Chemical Industry Co. (Japan). KDN was prepared as described previously [16]. 1,2-dimethylenedioxybenzen (DMB) was purchased from Dojindo (Kumamoto, Japan). Pre-stained Mw marker was obtained from Bio-Rad (Hercules, CA). Bicinchoninic acid (BCA) protein assay kit was purchase from Thermo Scientific Pierce (Rockford, IL). The prokaryotic expression plasmid pET32b(+) (Novagen, Madison, WI) comprising the cDNA for the N-domain (amino acid (aa) 1-255), C-domain (aa 256-432), or the full-length (aa 1-432) of rainbow trout CSS was prepared according to a previous report [18]. The pET32b(+) plasmids encoding the cDNA for the N-domain (aa 1-267) or the full-length (aa 1-432) of mouse CSS (Acc# AJ006215) were prepared in the same way.

Preparation of the recombinant enzymes

Expression in BL21(DE3)pLysS and affinity-purification of the recombinant enzymes were performed as described previously [18]. For purity check, SDS-PAGE/Coomassie brilliant blue (CBB) staining was carried out as described previously [18]. The amount of recombinant enzymes was determined by the BCA protein assay.

Assay for the CSS activity

Twenty microliters of recombinant enzyme were added to the pre-mixture, which contained 100 mM Tris-HCl (pH 9.0), 1.0 mM sialic acid (Kdn, Neu5Gc, or Neu5Ac), 2.0~5.0 mM CTP, 20 mM $MgCl_2$, and 0.1 mM Na_3VO_4 (which prevents dephosphorylation of CTP). After incubating at 25 °C for 10 min, excess CTP was digested by alkaline phosphatase treatment, followed by ethanol precipitation [18,20]. Fifty microliter of supernatant was subjected to high-

performance liquid chromatography (HPLC) analysis on a JASCO HPLC system, as described [10,18,20]. CMP-Neu5Ac, CMP-Neu5Gc, and CMP-Kdn in the reaction mixture were quantified using CMP-Neu5Ac as a standard. Relative *in vitro* activity was obtained as the amount of CMP-Sia per that of recombinant enzyme [20].

^{31}P Phosphorus nuclear magnetic resonance (^{31}P NMR)

The ^{31}P NMR monitoring experiments were performed using a Brüker 400 MHz SB Advance II spectrometer equipped with a BBO probe (BB/1H/2H). The procedure described below, adapted from a previous report [21], was realized into standard borosilicate 5 mm NMR tubes. The desired sialic acid (5 µmoles) and CTP disodium salt (10 µmoles) were dissolved into 100 mM Tris-HCl (pH 8.8) containing 20 mM $MgCl_2$ and 10 % of D_2O. ^{31}P NMR experiments were acquired using the zg pulse sequence with the following parameters: d1 (relaxation time)= 3 s; NS (number of scans)= 64; TD (resolution of the spectrum)= 8192; SW (spectral window)= 100; O1P (center of the spectral window)= 0; temperature= 298K. After temperature calibration of the probe, a first ^{31}P NMR spectrum (T0) was recorded before addition of the enzyme. Upon addition of the relevant rtCSS construct, series of 64-scan experiments were acquired in sequence, providing quantitative data of the conversion to CMP-Sia at each time-point of the reaction. rtCSS-FL and rtCSS-N activity monitoring experiments were achieved with 100 µg of purified protein per experiment and at 298 K, which was the optimal condition for rtCSS activity. The final total volume of solution per tube was 650 µL. The duration of each individual NMR experiment (corresponding to each point of the obtained curves) was 3 min 43 s (3'43'').

Effect of CMP-Sia on CSS activity

To test the effect of CMP-Kdn on the CMP-Neu5Ac synthesizing reaction, 0, 0.125, 0.25, 0.5 and 1.0 mM of CMP-Kdn was added into reaction solution, which contained 2 mM CTP, 1 mM Neu5Ac, 20 mM $MgCl_2$, 100 mM Tris-HCl (pH 8.0) and 120 ng of purified CSS protein. All the reactions were performed at 25 °C for 1 h. The effect of CMP-Neu5Ac on the CMP-Kdn synthesizing reaction was evaluated in the same way, except that 1 mM Kdn and 0-1.0 mM CMP-Neu5Ac were used instead of 1 mM Neu5Ac and 0-1.0 mM CMP-Kdn. To test the effect of CMP, CDP and cytidine on the CMP-Neu5Ac synthesizing reaction, 12 mM of CMP, CDP,

cytidine and CMP-Kdn was added into the CMP-Neu5Ac-synthesizing reaction solution (see above). Finally, the synthesized CMP-Sia amounts were quantified by HPLC using DMB derivatization method as described [9,10,22].

Statistics

All values were expressed as the mean ± SE (n is three) and p-values were evaluated by the Student's t-test.

Results

The *in situ* time-resolved monitoring of the rtCSS reaction

To understand how the C-domain of rtCSS and mCSS affect the enzymatic activity, the recombinant rtCSS-FL, rtCSS-N, rtCSS-C, mCSS-FL and mCSS-N proteins were purified by Ni^{+} column and the purity of each recombinant protein was confirmed by CBB staining (Fig. 1). We first performed the *in situ* time-resolved monitoring of the CSS reaction by ^{31}P-NMR, which is useful to assess the kinetic properties of CSS enzymes [21], for the enzyme reactions catalyzed by rtCSS-FL and rtCSS-N using Neu5Ac and Kdn as substrates (Fig. 2). rtCSS-N showed the similar time course profiles of the CSS reaction for Kdn and Neu5Ac (Fig. 2B). On the other hand, under the same conditions, rtCSS-FL showed different time course profiles for Kdn and Neu5Ac, in which a lower initial rate of the reaction was observed for Kdn, compared with Neu5Ac (Fig. 2A). Thus, while rtCSS-N lacking the C-domain similarly activated Kdn and Neu5Ac, rtCSS-FL did not activate Kdn as efficiently as Neu5Ac. These results suggest that the presence of C-domain is involved in the inhibition of the CMP-Kdn production more than the CMP-Neu5Ac production.

The substrate concentration dependency of the rtCSS reaction

Since the substrate concentration (10 mM) of Kdn and Neu5Ac used in the *in situ* monitoring was much higher than the reported K_m values for rtCSS-FL, which are 3.0 mM and 2.8 mM for Kdn and Neu5Ac, respectively [18], we then investigated whether the substrate concentration-dependency of the reaction was different between Kdn and Neu5Ac using the recombinant

rtCSS-FL and rtCSS-N (Fig. 2C,D). For rtCSS-FL, the CMP-Kdn synthesis reach saturation at 10 mM, while the CMP-Neu5Ac synthesis did not reach saturation (Fig. 2A). On the other hand, for rtCSS-N, both of the CMP-Kdn and CMP-Neu5Ac syntheses proceeded with the same reaction efficiency and were not saturated up to 20 mM Kdn and Neu5Ac (Fig. 2D). These results suggest that the CMP-Kdn synthesis is down-regulated at lower substrate concentrations than the K_m value compared to CMP-Neu5Ac synthesis, and that this substrate concentration-dependent suppression happens only for rtCSS-FL, but not for rtCSS-N. Thus, the C-terminal domain appears to suppress the rtCSS-catalyzed reaction for Kdn much more prominently than for Neu5Ac. It is of note that the CMP-Kdn-synthesizing activity of rtCSS-FL was 50% of the CMP-Neu5Ac-synthesizing activity when 10 mM Kdn or Neu5Ac was used as a substrate, consistent with the results obtained from ^{31}P-NMR measurements (Fig. 2A).

Effects of the product CMP-Sia on the rtCSS activity

Based on these results, we hypothesized that rtCSS-FL, but not rtCSS-N, could be strongly inhibited by CMP-Kdn. To demonstrate this hypothesis, we examined the effect of CMP-Kdn on the rtCSS-catalyzed CMP-Neu5Ac synthesis (Neu5Ac + CTP → CMP-Neu5Ac + PPi), using rtCSS-FL and rtCSS-N (Fig. 3A,B). For rtCSS-N, the CMP-Neu5Ac synthesis was inhibited by CMP-Kdn, with an observed 40%-inhibition at 1.0 mM CMP-Kdn. On the other hand, rtCSS-FL was also inhibited by CMP-Kdn, more strongly than what was measured for rtCSS-N, with a 70%-inhibition at 1.0 mM CMP-Kdn. It appears that the inhibition by CMP-Kdn was strengthened in the presence of C-domain. To understand how the C-domain was involved in the strong inhibition, an inhibition profile by CMP-Kdn was examined using an equimolar mixture of rtCSS-C lacking the N-domain and rtCSS-N. The addition of rtCSS-C had no effect on the inhibition profile, which was the same as that of rtCSS-N only, showing that the C-domain-dependent strong inhibition only happened in rtCSS-FL. The IC_{50} value of CMP-Kdn for the inhibition of rtCSS-FL was 0.4 mM. Since rtCSS-FL catalyzes the synthesis of CMP-Neu5Ac as well as CMP-Kdn, we then assessed if CMP-Neu5Ac similarly inhibits the rtCSS-catalyzed CMP-Kdn synthesis (Kdn + CTP → CMP-Kdn + PPi). As shown in Fig. 3B, in both rtCSS-FL and rtCSS-N cases, the CMP-Kdn synthesis was not strongly inhibited in presence of CMP-Neu5Ac, and only a 20%-inhibition was observed at 1.0 mM CMP-Neu5Ac.

Thus, C-domain-dependent inhibition could not be observed with CMP-Neu5Ac. Furthermore, we investigated the effects of CDP, CMP, and cytidine on the rtCSS-FL-catalyzed CMP-Neu5Ac synthesis (Fig. 3C). No inhibitory effect was observed with CDP, CMP, or cytidine at 12 mM, which was an even higher concentration than K_m value of 3.0 mM for CMP-Kdn (Fig. 3C), suggesting that the strong inhibition of rtCSS-FL happens with CMP-Kdn, but not with the dephosphorylated compounds of CTP.

To summarize the results so far, CMP-Kdn inhibits the CSS activity in both N-domain- and C-domain-dependent manners. The N-domain-dependent inhibition is not as strong as the C-domain-dependent one. In contrast, the inhibition by CMP-Neu5Ac is exclusively dependent on N-domain, but not on C-domain. Again, the inhibition by CMP-Neu5Ac is not so strong for rtCSS-FL and rtCSS-N. The N-domain-dependent inhibition may possibly be a product inhibition because both CMP-Kdn and CMP-Neu5Ac are involved. On the other hand, the C-domain-dependent inhibition is CMP-Kdn-specific, and might be different from the N-domain-dependent one in the mode of inhibition.

Effects of CMP-Kdn on the mCSS activity

We then wondered whether CMP-Kdn-specific inhibition is a common property to vertebrate CSSs. We examined effects of CMP-Kdn on the CMP-Neu5Ac synthesis by mCSS-FL and mCSS-N (Fig. 3D). No inhibition was observed for the mCSS-FL- or mCSS-N-catalyzed synthesis of CMP-Neu5Ac with CMP-Kdn even at 2 mM, a much higher concentration than the measured IC_{50} for rtCSS-FL of 0.4 mM. This lack of inhibition suggests that the CMP-Kdn-specific C-domain-dependent inhibition is not common in vertebrates, but rather is specific to rainbow trout.

Discussion

In conclusion, there are two regulatory modes of the rtCSS activity involving the CMP-Kdn. One is the N-domain-dependent product inhibition, which may be related to the active site in the N-domain of CSS. The other is the CMP-Kdn-specific C-domain-dependent inhibition. We show here that 2 mM CMP-Kdn inhibits the CSS activity by 40% through the N-domain-

dependent product inhibition, and 70% through the C-domain-dependent inhibition (Fig. 3A,B). Considering that the intracellular CMP-Sia resides at the 0.1-1 mM level [9,23,24] the C-domain-dependent regulation of the rtCSS activity may effectively work under physiological conditions.

In mammals, it has been reported that the intracellular level of Neu5Ac is highly regulated by the bifunctional enzyme UDP-GlcNAc 2-epimerase/ManNAc kinase (GNE) in the Sia synthetic pathway (Fig. 4). GNE synthesizes ManNAc 6-phosphate (ManNAc-6-P), a precursor of free Neu5Ac, from UDP-GlcNAc, undergoes feedback inhibition by binding of CMP-Neu5Ac to its allosteric site [25,26]. At a concentration of 0.16 mM CMP-Neu5Ac, the activity of GNE was inhibited, suggesting a sophisticated mechanism of feedback inhibition in the sialic acid biosynthetic pathway [27]. This allosteric inhibition by CMP-Neu5Ac affects the epimerase activity of GNE, and regulates the intracellular concentration of free Neu5Ac under normal conditions. The congenital disorder sialuria is diagnosed by the detection of significantly increased concentrations of free Neu5Ac in urine and the cytoplasm of cultured fibroblasts, and is caused by a heterozygous missense mutation in the allosteric site of GNE, which impairs the feedback inhibition by CMP-Neu5Ac [28]. Notably, however, the feedback regulation of GNE by CMP-Kdn is not plausible in mammals, because the level of CMP-Kdn is negligible under normal conditions due to very low or no activity of the mammalian CSS toward Kdn [24,29]. The CMP-Kdn-specific inhibition mechanism might be unnecessary in mammals.

In contrast, in non-mammalian and Kdn-expressing cells, excess CMP-Kdn could be synthesized from Kdn and CTP by Kdn-activating CSSs such as rtCSS, and a regulatory mechanism for the production of CMP-Kdn might be required. Kdn is synthesized from mannose 6-phosphate (Man-6-P) and phosphoenolpyruvate (PEP) by the Sia 9-phosphate synthase (SPS) and the Sia-9-P phosphatase [23,24,29]. It should be noted that Man-6-P is not provided by GNE [23], but by other Man-metabolic pathways [29]. As described above, CMP-Kdn is unlikely to be an allosteric inhibitor for the GNE activity in mammals [29]. Therefore, the GNE-mediated regulatory mechanism for the intracellular level of Kdn is not necessary. Instead, the CMP-Kdn-specific, C-domain-dependent regulation must work to decrease the intracellular level of CMP-Kdn. In the case of Kdn metabolism, CSS, but not GNE, might be a

key enzyme for regulation of the intracellular level of Kdn (Fig. 4). In rainbow trout, Kdn is enriched in glycoconjugates in gametic cells and tissues, such as sperm, testis, eggs, and ovarian fluid [7,12,30], while very little or no Kdn is detected in glycoconjugates in non-gametic cells [9]. Therefore, the CMP-Kdn-specific C-domain-dependent regulation might be functional in the gametic tissues, but not in the non-gametic tissues in rainbow trout.

The C-domain-dependent inhibition by CMP-Kdn does not occur in mCSS (Fig. 3D), showing that it is highly specific to the C-domain of rtCSS. Since mCSS has high activity to Neu5Ac, and very little or no activity to Kdn, the C-domain-dependent inhibition may be related to the Sia species specificity of CSS. Interestingly, there are two CSSs in zebrafish (*Danio rerio*), dreCSS1 (dreCMAS1) and dreCSS2 (dreCMAS2) [31]. dreCSS1 has very high activity toward Neu5Ac, while dreCSS2 exclusively prefers Kdn. dreCSS2, but not dreCSS1, might show the CMP-Kdn-specific, C-domain-dependent inhibition. Many questions regarding the CMP-Kdn-specific, C-domain-dependent inhibition remain to be clarified. Does the CMP-Kdn binding site exist in the C-domain of rtCSS? How does the CMP-Kdn binding affect the N-domain-mediated CSS activity?

Finally, our finding that the C-domain regulates the CSS activity is novel. Although it has been shown that the C-domain affects the physicochemical properties, such as the control of oligomerization [19] and solubility of CSS [22], this study could for the first time demonstrate the significance of the C-domain in the regulation of sialic acid enzymatic activation.

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Legends to figures

Fig. 1. Preparaion of the rainbow trout CSS (rtCSS) and the mouse CSS (mCSS). A. Schematic drawings of rtCSS-FL, rtCSS-N, rtCSS-C, mCSS-FL, and mCSS-N. Amino acid residue numbers are indicated. The N-domain and C-domain are colored by blue and gray, respectively. **B.** SDS-PAGE-CBB staining of the affinity-purified CSS proteins. The molecular mass of each enzyme is indicated at the bottom of the lane.

Fig. 2. in situ Monitoring of the CSS-catalyzed synthesis of CMP-Sia by ^{31}P-NMR (A, B) and substrate concentration-dependent synthesis of CMP-Sia (C, D). A, B. Time course of the CMP-Sia synthesis by rtCSS-FL (A) or rtCSS-N (B). The reaction mixture containing 10 mM Neu5Ac or Kdn, 20 mM CTP, 20 mM $MgCl_2$, and 10% D_2O in 100 mM Tris-HCl (pH 8.8) was incubated at 25 ^oC with 100 μg each of purified rtCSS-FL (A) or rtCSS-N (B). The curve represents the amount (μmol) of the produced CMP-Neu5Ac (○) and CMP-Kdn (●) as a function of time. Representative data are shown. **C, D.** The relative activity of rtCSS-FL (C) and rtCSS-N (D) with 1~20 mM of Neu5Ac (□) or KDN (■) and 20 mM CTP as substrates. The activity with 1 mM Kdn and 20 mM CTP as substrates is set to 1.0. The bars represent standard deviations obtained from three independent experiments. *, $p < 0.01$.

Fig. 3. Effect of CMP-Sia on the CMP-Sia-synthesizing reaction by rtCSS and mCSS. A. Effects of various concentrations of CMP-Kdn on the CMP-Neu5Ac synthesis by rtCSS-FL (FL), rtCSS-N (N), and a mixture of rtCSS-N+rtCSS-C (N+C). The CMP-Neu5Ac synthetic activity without CMP-Kdn is set to 1.0. **B.** Effects of various concentrations of CMP-Neu5Ac on the CMP-Kdn synthesis by rtCSS-FL (FL) and rtCSS-N (N). The CMP-Kdn synthetic activity without CMP-Neu5Ac was set to 1.0. **C.** Effects of 12 mM each of CMP-Kdn, CDP, CMP, and cytidine on the rtCSS-FL-catalyzed CMP-Neu5Ac synthesis. None, no compound added. The reaction mixture consisted of 20 mM CTP, 10 mM Neu5Ac, 20 mM $MgCl_2$, 100 mM Tris-HCl (pH 9), and 100 μg of purified rtCSS-FL. **D.** Effects of various concentrations of CMP-Kdn on the CMP-Neu5Ac synthesis by mCSS-FL (FL) and rtCSS-N (N). The CMP-

Neu5Ac synthetic activity without CMP-Kdn was set to 1.0. The bars represent standard deviations obtained from three independent experiments. *, $p < 0.01$.

Fig. 4. Hypothetical view of regulation of *de novo* synthetic pathways of Sia by CMP-Sia. In mammal, the biosynthetic pathway of Neu5Ac is regulated by excess CMP-Neu5Ac, a product of CSS, through the feedback inhibition of GNE activity. On the other hand, in non-mammalian and Kdn-synthesizing organisms like rainbow trout, CMP-Kdn is synthesized not from ManNAc-6-P, a product of GNE, but from Man-6-P. In this case, as this study suggests, surplus CMP-Kdn regulates the level of itself by inhibiting CSS activity in the C-domain-dependent manner.

Fig. 1

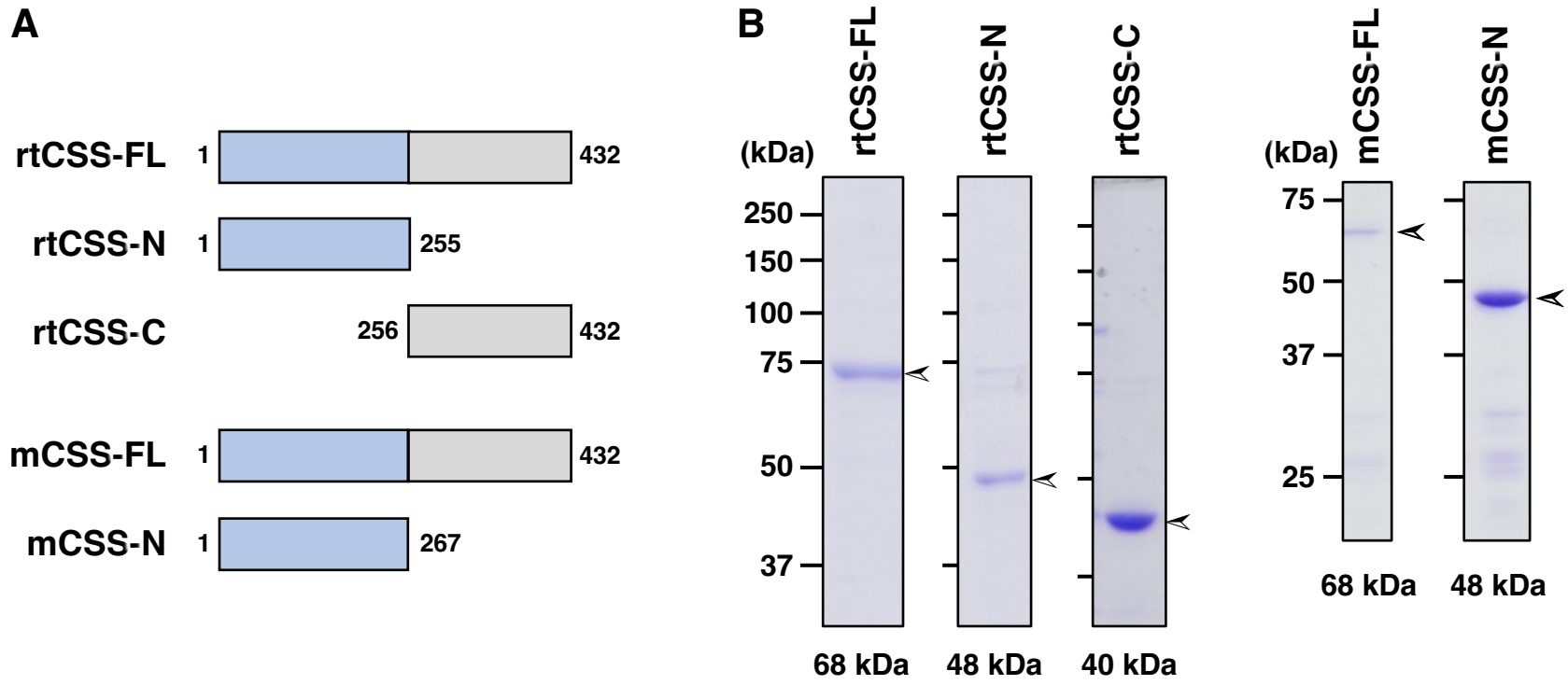

A
rtCSS-FL
1
432
rtCSS-N
1
255
rtCSS-C
256
432
mCSS-FL
1
432
mCSS-N
1
267
B
(kDa)
rtCSS-FL
rtCSS-N
rtCSS-C
250
150
100
75
50
37
68 kDa
48 kDa
40 kDa
(kDa)
mCSS-FL
mCSS-N
75
50
37
25
68 kDa
48 kDa

Fig. 2

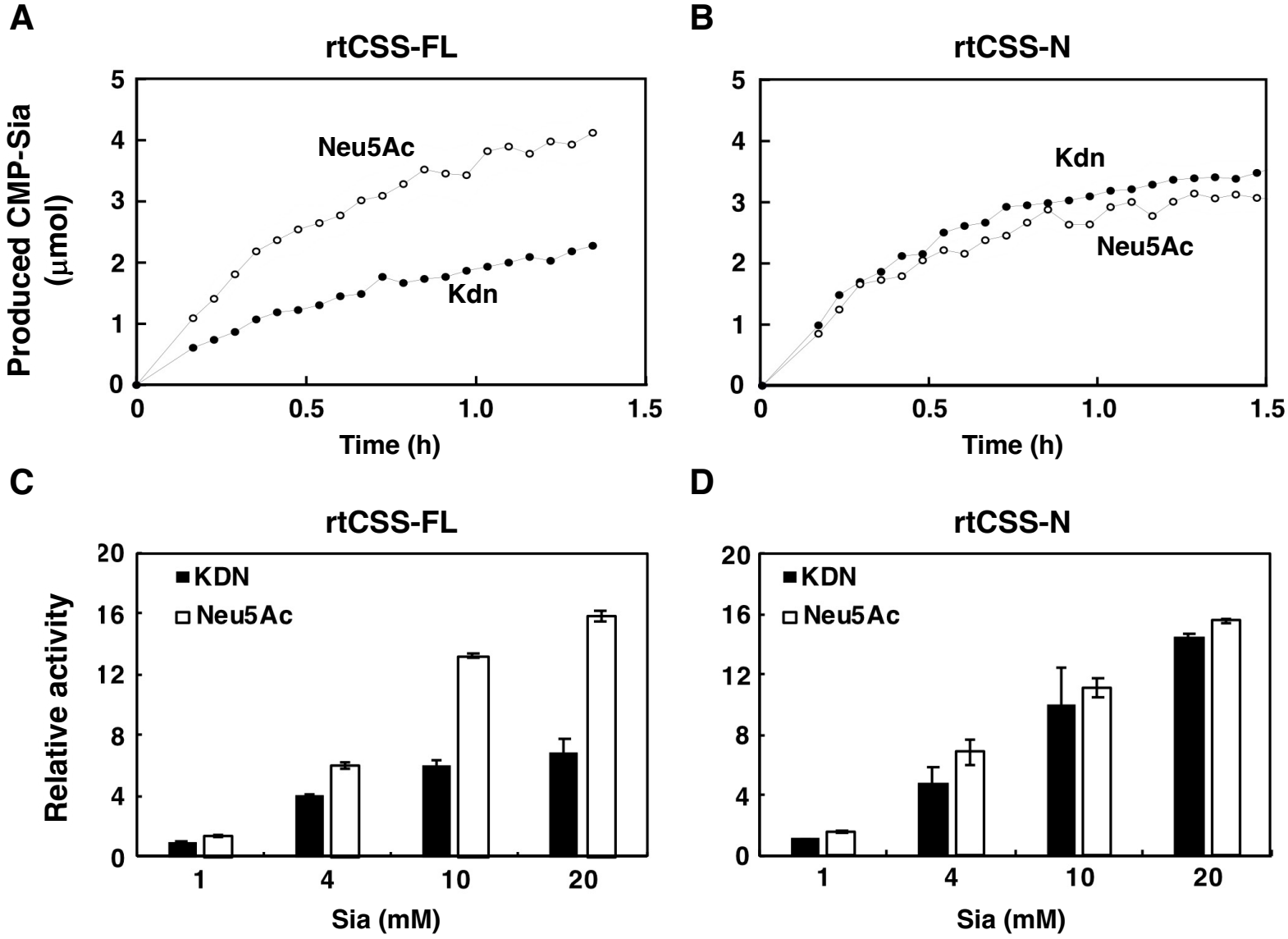

A
rtCSS-FL
Produced CMP-Sia (μmol)
Neu5Ac
Kdn
Time (h)
B
rtCSS-N
Kdn
Neu5Ac
Time (h)
C
rtCSS-FL
Relative activity
KDN
Neu5Ac
Sia (mM)
D
rtCSS-N
KDN
Neu5Ac
Sia (mM)

Fig. 3

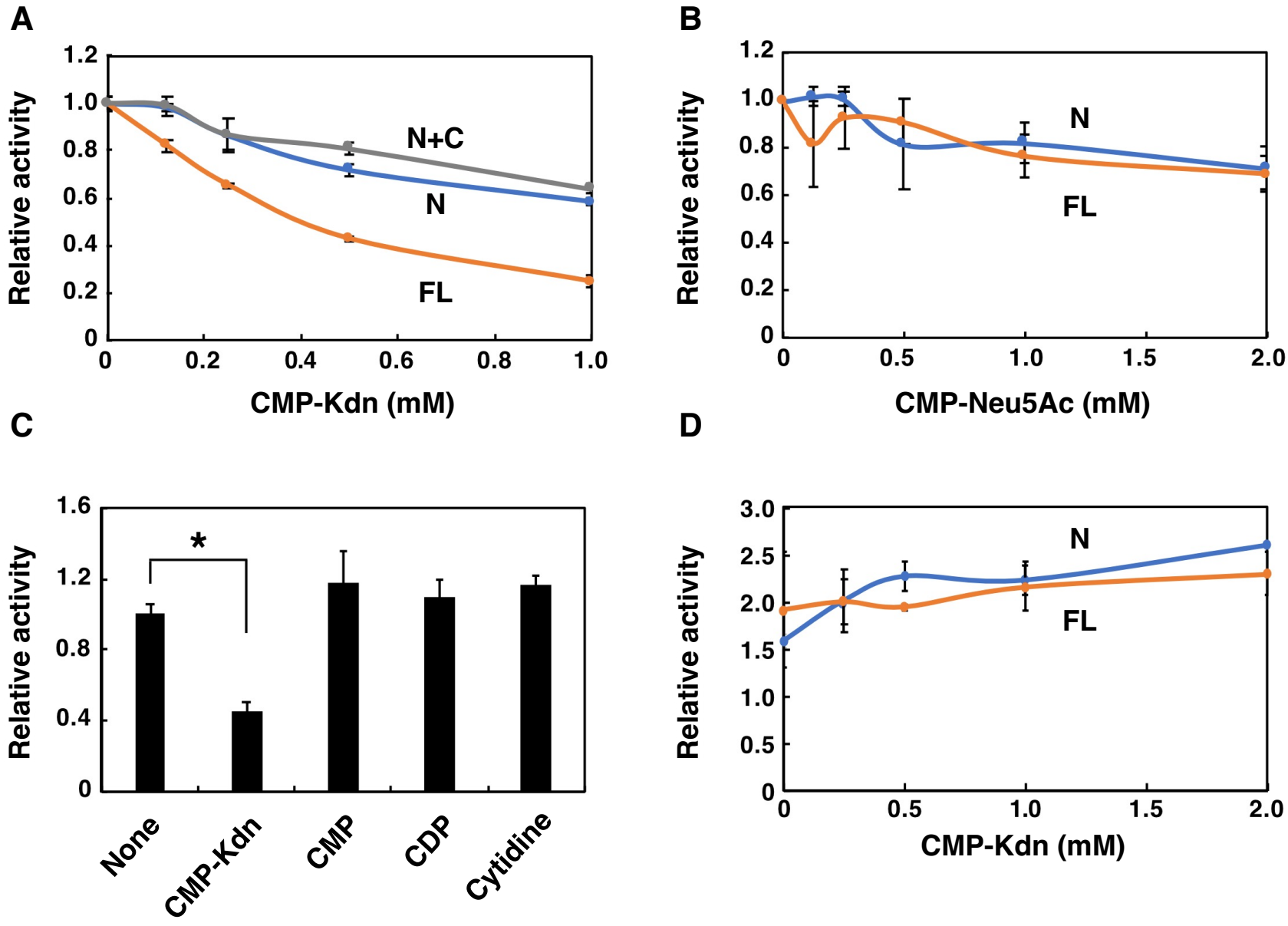
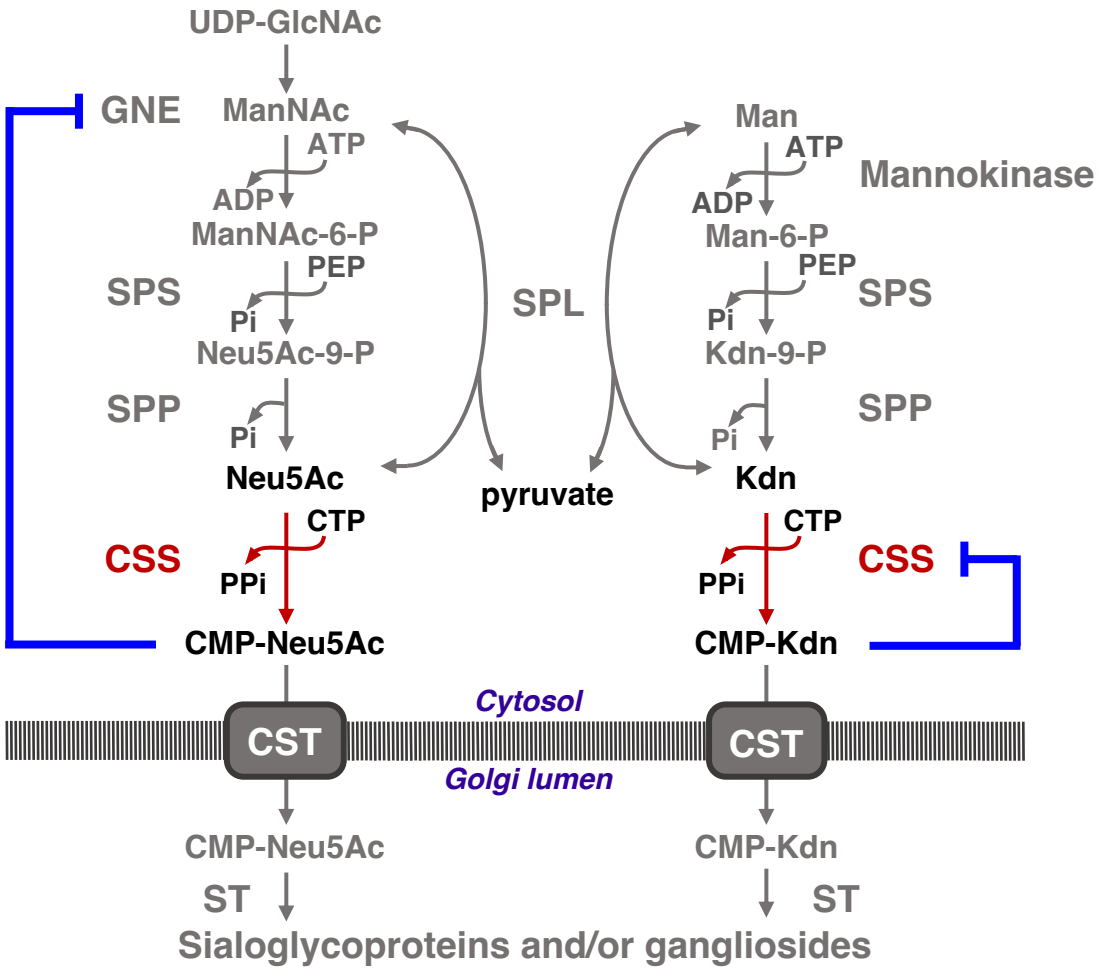

A
Relative activity
1.2
1.0
0.8
0.6
0.4
0.2
0
0
0.2
0.4
0.6
0.8
1.0
CMP-Kdn (mM)
N+C
N
FL
B
Relative activity
1.2
1.0
0.8
0.6
0.4
0.2
0
0
0.5
1.0
1.5
2.0
CMP-Neu5Ac (mM)
N
FL
C
Relative activity
1.6
1.2
0.8
0.4
0
*
None
CMP-Kdn
CMP
CDP
Cytidine
D
Relative activity
3.0
2.5
2.0
1.5
1.0
0.5
0
0
0.5
1.0
1.5
2.0
CMP-Kdn (mM)
N
FL

Fig. 4


UDP-GlcNAc
GNE
ManNAc
ATP
ADP
ManNAc-6-P
SPS
PEP
Pi
Neu5Ac-9-P
SPP
Pi
Neu5Ac
CTP
CSS
PPi
CMP-Neu5Ac
SPL
pyruvate
Man
ATP
Mannokinase
ADP
Man-6-P
PEP
SPS
Pi
Kdn-9-P
SPP
Pi
Kdn
CTP
CSS
PPi
CMP-Kdn
Cytosol
CST
CST
Golgi lumen
CMP-Neu5Ac
CMP-Kdn
ST
ST
Sialoglycoproteins and/or gangliosides