CDP-glycerol inhibits the synthesis of the functional O-mannosyl glycan of α-dystroglycan

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Running title: CDP-Gro inhibits O-mannosyl glycan synthesis

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ABSTRACT

α-Dystroglycan (α-DG) is a highly glycosylated cell surface lamin receptor. Defects in the O-mannosyl glycan of an α-DG with laminin-binding activity can cause α-dystroglycanopathy, a group of congenital muscular dystrophies. In the biosynthetic pathway of functional O-mannosyl glycan, fukutin (FKTN) and fukutin-related protein (FKRP), whose mutated genes underlie α-dystroglycanopathy, sequentially transfer ribitol phosphate (RboP) from CDP-Rbo to form a tandem RboP unit (RboP–RboP) required for the synthesis of the lamin-binding epitope on O-mannosyl glycan. Both RboP- and glycerol phosphate (GroP)-substituted glycoforms have recently been detected in recombinant α-DG. However, it is unclear how GroP is transferred to the O-mannosyl glycan or whether GroP substitution affects the synthesis of the O-mannosyl glycan. Here, we report that in addition to having RboP transfer activity, FKTN and FKRP can transfer GroP to O-mannosyl glycans by using CDP-glycerol (CDP-Gro) as a donor substrate. Kinetic experiments indicated that CDP-Gro is a less efficient donor substrate for FKTN than is CDP-Rbo. We also show that the GroP-substituted glycoform synthesized by FKTN does not serve as an acceptor substrate for FKRP and that therefore further elongation of the outer glycan chain cannot occur with this glycoform. Finally, CDP-Gro inhibited the RboP transfer activities of both FKTN and FKRP. These results suggest that CDP-Gro inhibits the synthesis of the functional O-mannosyl glycan of α-DG by preventing further elongation of the glycan chain. This is the first report of GroP transferases in mammals.

α-Dystroglycan (α-DG) is a component of the dystrophin glycoprotein complex, which serves as a transmembrane linker to connect the extracellular matrix and intracellular cytoskeleton. α-DG is highly glycosylated with N- and O-glycans, including O-mannosyl glycans, a type of O-glycan in which the reducing terminal mannose (Man) is attached to proteins via Ser or Thr residues. The structure of the O-mannosyl glycan is crucial to its interaction with several extracellular matrix components, such as laminin, and aberrant O-mannosyl glycans of α-DG are the cause of α-dystroglycanopathy, a group of congenital muscular dystrophies...
with neuronal migration defects (1-3). There are three types of core structures of O-mannosyl glycans, namely, core M1 [GlcNAcβ1-2Man], core M2 [GlcNAcβ1-2(GlcNAcβ1-6)Man] and core M3 [GalNAcβ1-3GlcNAcβ1-4Man] (1,2). Recently, the entire structure and the biosynthetic pathway of core M3-type glycan were revealed, and the structure, [(3GlcAβ1-3Xylα1)_n-3GlcAβ1-4Xylβ1-4Rbo P-1RboP-3GlcNAcβ1-3GlcNAcβ1-4(phospho o-6)Manal], contains ribitol phosphate (RboP), a recently identified glycan constituent in mammals (4,5). Basically, all glycosyltransferases involved in the biosynthesis of core M3-type glycan are the causative gene products of α-dystroglycanopathy, indicating that defective core M3-type glycans are the cause of α-dystroglycanopathy (1-3).

The biosynthetic pathway of core M3-type glycan is initiated in the endoplasmic reticulum by protein O-mannosylation via POMT1/POMT2 (6), and the phospho-core M3 unit [GalNAcβ1-3GlcNAcβ1-4(phospho-6)Man] is subsequently formed by the sequential action of POMGNT2, B3GALNT2 and POMK (7). Then, in the Golgi, the first RboP is transferred by fukutin (FKTN) from CDP-Rbo to the C3-position of GalNAc, and the second RboP is transferred by fukutin-related protein (FKRP) from CDP-Rbo to the C1-position of the first RboP to form a tandem RboP-RboP unit (4). Then, a GlcAβ1-4Xylβ1-4 unit is formed by the sequential action of RXYLT1/TMEM5 and B4GAT1 (8,10), and finally, the (-3GlcAβ1-3Xylα1-) repeating unit, which is required to bind laminin, is formed by LARGE (11).

Recently, Yagi et al. reported that there was a glycerol phosphate (GroP)-substituted phospho-core M3 unit in addition to a RboP-substituted phospho-core M3 unit based on their analysis of the glycoforms of a truncated recombinant α-DG expressed in HEK293T cells and HCT116 colon cancer cells (12). The same group also reported that GroP-modifications as well as RboP-modifications were not observed in FKTN-deficient cells, implying that FKTN may be involved in transferring not only RboP but also GroP to the phospho-core M3 structure. However, whether FKTN itself has GroP transferase activity and whether GroP-substitution affects the synthesis of the functional core M3-type glycan remain unclear.

In gram-positive bacteria, several GroP transferases are known to transfer the GroP moiety from the activated precursor CDG-glycerol (CDP-Gro) in the biosynthesis of teichoic acid, a major cell wall component (13). However, these GroP transferases are not conserved in mammals, and to date, no GroP transferases have been identified in mammals. In the present study, we report that FKTN and FKRP have GroP transferase activity using CDP-Gro as a donor substrate. We also demonstrate that GroP-substitution of a phospho-core M3 unit by FKTN inhibits further elongation of the outer glycan chain. Furthermore, we show the inhibitory effect of CDP-Gro on the RboP transferase activity of FKTN and FKRP. The present data suggest the inhibitory function of CDP-Gro in the synthesis of α-DG glycan.

**RESULTS**

*sfKTN* has *Gro* transfer activity using *CDP-Gro as a donor*

To determine whether FKTN has GroP transferase activity as well as RboP transferase activity to the phospho-core M3 unit, we prepared a soluble form of FKTN with a His/myc-tag at the N terminus (sfKTN) and a synthetic phospho-core M3 peptide (AT[*]PAPVAIGPK) modified with phospho-core M3 at the Thr[*] as an acceptor substrate. sfKTN was expressed in HEK293T cells and immunoprecipitated from the culture medium using an anti-c-Myc antibody. The immunoprecipitated sfKTN was found to be of the appropriate molecular weight by western blotting (Figure 1A) and the purity of sfKTN was checked by Coomassie brilliant blue staining (Figure S1A). First, to confirm the enzymatic activity of sfKTN, sfKTN was incubated with CDP-Rbo (Figure 1B) and the phospho-core M3 peptide, and the products were separated by HPLC.
chromatogram, shown in Figure 1C, a small peak of the acceptor substrate (phospho-core M3 peptide, indicated as S) and a large peak of the RboP-transferred product (RboP-phospho-core M3 peptide, indicated as P1) were detected as previously reported (4). Because CDP-Gro is a known donor substrate for GroP transferase in bacterial teichoic acid biosynthesis (13), we examined whether sFKTN has GroP transferase activity using CDP-Gro as a donor substrate. Commercially available CDP-Gro (Sigma) is the mixture of enantiomers CDP-1-Gro and CDP-3-Gro (Figure 1B), and these isomers can serve as the donors for sn-glycerol-1-phosphate (Gro1P) and sn-glycerol-3-phosphate (Gro3P), respectively. Because whether the GroP on the core M3-type glycan of recombinant α-DG (12) is Gro1P or Gro3P remains unclear, we first used the mixture of CDP-Gro enantiomers (mix-CDP-Gro). sFKTN was incubated with mix-CDP-Gro and the phospho-core M3 peptide, and the products were analyzed by HPLC. As shown in Figure 1D, a new small peak was detected in the presence of sFKTN (indicated by P2, lower panel) but not when sFKTN was absent (upper panel). The fraction containing peak P2 was isolated and analyzed using MALDI-TOF-MS. The m/z value of P2 was 1935.9, which corresponds to the [M + H]+ ion of the GroP-transferred phospho-core M3 peptide [GroP + GalNAc-GlcNAc-(P)Man peptide] (Figure 1E) estimated based on the molecular weight of each component, with GroP: 172.0, GalNAc or GlcNAc: 221.2, Man: 180.1, phosphoric acid: 97.9, peptide: 1132.6, and H₂O: 18.0. To confirm the components of P2, the peak at m/z 1935.9 was subjected to MS/MS analysis. As shown in Figure 1F, four fragment ions were detected at m/z 1781.8 [GalNAc-GlcNAc-(P)Man-peptide], m/z 1578.7 [GlcNAc-(P)Man-peptide], m/z 1375.7 [(P)Man-peptide] and m/z 1133.6 [peptide], indicating that the peak at m/z 1935.9 is the GroP-substituted phospho-core M3 peptide. The peak at m/z 1763.8 corresponds to the dehydrated form of m/z 1781.8. There were no obvious peaks in the MS/MS spectrum containing GroP, which suggested that GroP is attached to the terminal GalNAc of the phospho-core M3. The position to which the GroP is transferred is further analyzed below.

**sFKTN transfers GroP to the terminal GalNAc of phospho-core M3 peptide**

To determine the position on the phospho-core M3 peptide to which sFKTN transfers GroP, we used exo-β-N-acetylgalactosaminidase (HexNAcase) from jack bean. Because HexNAcase can remove non-reducing, terminal β-linked HexNAc (GlcNAc and GalNAc) residues but cannot cleave modified HexNAc (non-terminal HexNAc), HexNAcase can be used to determine whether the terminal GalNAc is modified with GroP. First, to confirm the enzymatic activity of HexNAcase toward β-1-3-linked GalNAc, the phospho-core M3 peptide was treated with HexNAcase, and the product was analyzed by HPLC and MS. A new peak (indicated by S') was observed in the HPLC chromatogram after HexNAcase treatment (Figure 2A), and the fraction containing the substrate (S) and product (S') were subjected to MS analysis. In addition to the substrate (S) peak at m/z 1781.9 [GalNAc-GlcNAc-(P)Man-peptide], two major peaks at m/z 1578.7 [GlcNAc-(P)Man-peptide] and m/z 1375.7 [(P)Man-peptide] were observed after HexNAcase treatment (Figure 2B), demonstrating that the terminal GalNAc was removed and that the resulting terminally exposed GlcNAc was also removed. Under these experimental conditions, the GroP-substituted phospho-core M3 peptide (P2) was treated with HexNAcase, and the product was analyzed by HPLC. The intensities of peak P2 with or without HexNAcase treatment were comparable (Figure 2C), although a very small product peak S' was observed after HexNAcase treatment in Figure 2C, because the P2 fraction contained a small amount of phospho-core M3 peptide (S) as a contaminant during the isolation of the GroP-phospho-core M3 peptide synthesized from the phospho-core M3 peptide by sFKTN. MS analysis of the eluates around the peak P2...
showed that the substrate (P2) peak is the only GroP-containing peak regardless of HexNAcase treatment (Figure 2D), indicating that the GroP substituent on the phospho-core M3 peptide was not cleaved by HexNAcase digestion. These results show that sFKTN transferred GroP to the terminal GalNAc of the phospho-core M3 peptide, and this result is consistent with the previous report that GroP is linked to the terminal GalNAc of the phospho-core M3 unit on recombinant α-DG (12).

Both CDP-1-Gro and CDP-3-Gro serve as donors for sFKTN

Next, to determine which enantiomer, CDP-1-Gro or CDP-3-Gro (or both), serves as the donor for sFKTN, we synthesized both isomers using glycerol phosphate cytidyltransferase (GCT), which catalyzes the production of CDP-Gro from CTP and GroP in microorganisms (14). For the GCT reaction, we used a GCT from the bacterium Aquifex aeolicus, AQ1368, which is shown to synthesize CDP-1-Gro and CDP-3-Gro using Gro1P and Gro3P, respectively (Figure S2A) (15). We expressed His-tagged recombinant AQ1368 in E. coli. The purified His-tagged AQ1368 (Figure S2B) was incubated with CTP and either Gro1P or Gro3P. The reaction products were separated by HPLC, and the peak corresponding to CDP-Gro was collected (Figure S2C). The purities of CDP-1-Gro and CDP-3-Gro were verified by HPLC (Figure S2D). HPLC-MS analysis was used to confirm the production of CDP-Gro (Figure S3). Then, sFKTN was incubated with the phospho-core M3 peptide and CDP-1-Gro or CDP-3-Gro, and the products were analyzed by HPLC. As shown in Figure 3A and B, a new small peak (indicated by an open triangle) was detected in the chromatograms after the reaction with CDP-1-Gro or CDP-3-Gro, as is the case with mix-CDP-Gro (Figure 1D). The fractions containing the reaction product with CDP-1-Gro or CDP-3-Gro were subjected to MS analyses, and the m/z values of the products were 1936.1 or 1936.0, respectively, corresponding to the [M + H]^+ ion of the GroP-transferred phospho-core M3 peptide [GroP + GalNAc-GlcNAc-(P)Man peptide] (Figure 3C and D). These results indicate that sFKTN transfers both Gro1P and Gro3P from CDP-1-Gro and CDP-3-Gro, respectively.

Kinetic analysis of sFKTN with CDP-Rbo, CDP-1-Gro and CDP-3-Gro

To compare the efficiency of CDP-Rbo, CDP-1-Gro and CDP-3-Gro as donor substrates for sFKTN, kinetic experiments were performed using increasing concentrations of each donor and 100 µM of phospho-core M3 peptide. As shown in Figure 4A, the transfer reactions of RboP, Gro1P or Gro3P in the presence of CDP-Rbo, CDP-1-Gro or CDP-3-Gro, respectively, followed typical Michaelis-Menten kinetics. Analysis of the results using a Lineweaver-Burk plot (Figure 4B) yielded $K_m$ values for CDP-Rbo, CDP-1-Gro and CDP-3-Gro of 30.8 ± 5.5, 39.4 ± 3.7, and 27.3 ± 1.6 µM, respectively, and $V_{max}$ values for CDP-Rbo, CDP-1-Gro and CDP-3-Gro of 5.53 ± 0.15, 1.01 ± 0.19, and 0.45 ± 0.06 µM/hr, respectively (Figure 4C). The relative catalytic efficiency ($V_{max}/K_m$) values of sFKTN for CDP-Rbo, CDP-1-Gro and CDP-3-Gro were 0.184 ± 0.034, 0.026 ± 0.007, and 0.017 ± 0.003, respectively (Figure 4C), indicating that the transfer efficiency of RboP from CDP-Rbo is approximately ten times higher than those of Gro1P and Gro3P from CDP-1-Gro and CDP-3-Gro, respectively. Based on these results, CDP-1-Gro and CDP-3-Gro are less efficient donor substrates for sFKTN than CDP-Rbo.

Although CDP-1-Gro appears to be a better substrate for sFKTN than CDP-3-Gro, both CDP-1-Gro and CDP-3-Gro serve as significant donor substrates. In addition, when a mixture of equal amounts of CDP-1-Gro and CDP-3-Gro was used as the donor substrate, medial GroP (Gro1P/Gro3P) transfer activity was observed (Figure S4). This result suggests that CDP-1-Gro and CDP-3-Gro do not affect the Gro3P and Gro1P transfer activities of sFKTN, respectively. Therefore, we used mix-CDP-Gro (Sigma) in subsequent experiments, and hereafter, we refer to
mix-CDP-Gro simply as CDP-Gro except where otherwise indicated.

**GroP-phospho-core M3 peptide cannot serve as an acceptor substrate for sFKRP**

Because sFKTN can transfer both RboP and GroP, we were curious to determine the effect of GroP-modification of phospho-core M3 on the synthesis of core M3-type glycan. To this aim, we examined whether the GroP-phospho-core M3 peptide serves as an acceptor substrate for FKRP, the enzyme immediately downstream of FKTN. A soluble form of FKRP (sFKRP) was expressed in HEK293T cells and immunoprecipitated from the culture medium. The immunoprecipitated sFKRP was confirmed to have the appropriate molecular weight by western blotting (Figure 5A) and the purity of sFKRP was checked by Coomassie brilliant blue staining (Figure S1B). To confirm the enzymatic activity of sFKRP, sFKRP was incubated with CDP-Rbo and the RboP-phospho-core M3 peptide, which is the usual acceptor for FKRP, and the products were analyzed by HPLC. As shown in Figure 5B, an sFKRP product (RboP-RboP-phospho-core M3 peptide) was detected in the chromatogram (peak P3), and the content of the substrate (P1, RboP-phospho-core M3 peptide) was markedly reduced by the presence of sFKRP (lower panel). MS analysis of the fraction containing peaks P1 and P3 confirmed the production of RboP-transferred RboP-phospho-core M3 peptide (P3) (Figure 5C). Then, sFKRP was incubated with CDP-Rbo and the GroP-phospho-core M3 peptide, and the products were analyzed by HPLC. As shown Figure 5D, two peaks were detected in both the absence and presence of sFKRP, the large peak (P2) corresponds to the GroP-phospho-core M3 peptide and the small peak (S) corresponds to the phospho-core M3 peptide, which is present as a contaminant during the isolation of the GroP-phospho-core M3 peptide. The peak pattern in the chromatogram after the reaction with sFKRP was almost entirely the same as that of the reaction in the absence of sFKRP, and no new peaks were detected (Figure 5D, lower panel), demonstrating that RboP was not transferred to the GroP-phospho-core M3 peptide by sFKRP. MS analysis of the eluate around the peak P2 (Figure 5D, lower panel) confirmed that RboP-transferred GroP-phospho-core M3 peptide (calculated m/z value: 2149.9), was not produced (Figure 5E). These results indicate that the GroP-phospho-core M3 peptide cannot serve as an acceptor for sFKRP.

**sFKRP also has GroP transfer activity using CDP-Gro**

Both FKTN and FKRP have RboP transfer activity using CDP-Rbo as a donor substrate, and the catalytic domain of FKRP shows significant sequence similarity to that of FKTN (16), indicating that FKRP might also have GroP transfer activity using CDP-Gro. Therefore, sFKRP was incubated with CDP-Gro and its usual acceptor, the RboP-phospho-core M3 peptide, and the products were analyzed by HPLC. As shown in Figure 6A, a new small peak (indicated by P4) was detected in addition to the substrate peak (P1, RboP-phospho-core M3 peptide) in the chromatogram after the reaction in the presence of sFKRP (lower panel) but not in that after the reaction without sFKRP (upper panel). The fraction containing peaks P1 and P4 was subjected to MS analysis, and the results showed that the m/z value of P4 was 2149.8, which corresponds to the [M + H]+ ion of the GroP-transferred RboP-phospho-core M3 peptide [GroP + RboP-GalNAc-GlcNAc-(P)Man peptide] (Figure 6B). The components of P4 were further confirmed by MS/MS analysis of the peak at m/z 2149.8. As shown in Figure 6C, five fragment ions were detected at m/z 1995.8 [RboP-GalNAc-GlcNAc-(P)Man-peptide], m/z 1781.8 [GalNAc-GlcNAc-(P)Man-peptide], m/z 1578.8 [GlcNAc-(P)Man-peptide], m/z 1375.7 [(P)Man-peptide] and m/z 1133.7 [peptide], indicating that the peak at m/z 2149.8 is the GroP-substituted RboP-phospho-core M3 peptide. No obvious peaks containing GroP were detected in the MS/MS spectrum, suggesting that GroP is attached to the terminal RboP. These results
indicate that sFKRP also has GroP transfer activity using CDP-Gro as a donor. In addition, the HPLC data show that the GroP-transferred product peak (P4 in Figure 6A, lower panel) is smaller than the RboP-transferred product peak (P3 in Figure 5B, lower panel) using the same enzyme assay conditions, suggesting that CDP-Gro is a less efficient donor for sFKRP than CDP-Rbo, which is the case with sFKTN.

We further examined whether sFKRP can transfer GroP to the GroP-transferred phospho-core M3 peptide. sFKRP was incubated with CDP-Gro and the GroP-phospho-core M3 peptide, and the products were analyzed by HPLC. As shown in Figure 6D, the substrate peak (GroP-phospho-core M3 peptide, indicated by P2) and the associated peak (phospho-core M3 peptide, indicated by S) were detected, as shown in Figure 5D, but no new peaks were detected, indicating that sFKRP cannot transfer GroP to the GroP-transferred phospho-core M3 peptide. MS analysis of the eluate around the peak P2 confirmed that GroP-transferred GroP-phospho-core M3 peptide (calculated m/z value: 2089.9), was not produced (Figure 6E). The above results demonstrated that the GroP-transferred phospho-core M3 unit cannot be further elongated with RboP or GroP by sFKRP, and a tandem GroP-GroP unit cannot be formed on the core M3 glycan.

**RboP transfer activities of sFKTN and sFKRP are inhibited by CDP-Gro**

Because both RboP- and GroP-modified O-mannosyl glycans of recombinant α-DG were observed in cultured cells (12), it is conceivable that CDP-Rbo and CDP-Gro (CDP-1-Gro or CDP-3-Gro or both) coexist in the cells. Kinetic data of sFKTN (Figure 4) suggest that sFKTN exhibits comparable affinity for CDP-Rbo and CDP-Gro, but the GroP transfer rate is noticeably lower than the RboP transfer rate, suggesting that CDP-Gro may competitively inhibit RboP transfer from CDP-Rbo. Then, we examined the effect of the coexistence of CDP-Rbo and CDP-Gro on the RboP transfer activity of sFKTN. Because the RboP-phospho-core M3 peptide and GroP-phospho-core M3 peptide, both of which are produced by sFKTN, are eluted at almost the same HPLC retention time, it is impossible to monitor only the RboP transfer activity in the presence of both CDP-Rbo and CDP-Gro by HPLC analysis. Therefore, we used [3H]-labeled CDP-Rbo (CDP-[3H]Rbo) to determine only the RboP transfer activity. sFKTN was incubated with 100 µM phospho-core M3 peptide, 500 µM CDP-[3H]Rbo and 0, 500 or 2,500 µM CDP-Gro (molar ratio of 1:0, 1:1 or 1:5 CDP-Rbo to CDP-Gro). The reaction mixtures were analyzed with HPLC, and the radioactivities incorporated into the acceptor peptide were measured. As shown in Figure 7A, the product peak (indicated by an open triangle) became small as the molar ratio of CDP-Gro to CDP-Rbo increased. In accord with this observation, the RboP transfer activity decreased with an increasing molar ratio of CDP-Gro to CDP-Rbo (Figure 7B). The RboP transfer activity decreased to 63% (CDP-Rbo:CDP-Gro, 1:1) or 30% (CDP-Rbo:CDP-Gro, 1:5) of that without CDP-Gro (CDP-Rbo:CDP-Gro, 1:0). The reduction in the RboP transfer activity may not be due to the concomitant increase in the GroP transfer activity, because of the lower amount of the total reaction product generated in the presence of CDP-Gro (Figure 7A), and the low GroP transfer activity of sFKTN using CDP-1-Gro or CDP-3-Gro (Figure 4). These results indicate that the RboP transfer activity of sFKTN is inhibited by CDP-Gro. In addition, we observed that the RboP transfer activity of sFKTN was not inhibited by ribitol, RboP or Gro3P (Figure S5), suggesting that the CDP-moiety is important for the inhibition of sFKTN.

We also examined whether the presence of CDP-Gro also affects the RboP transfer activity of sFKRP. sFKRP was incubated with 50 µM RboP-phospho-core M3 peptide, 250 µM CDP-[3H]Rbo and 0, 250 or 1,250 µM CDP-Gro (molar ratio of 1:0, 1:1 or 1:5 CDP-Rbo to CDP-Gro). The product mixtures were subjected to HPLC analysis, and radioactivities incorporated into the acceptor peptide were determined. As was the case with sFKTN, in the HPLC chromatograms,
the product peak (indicated by an open triangle) became small as the molar ratio of CDP-Gro to CDP-Rbo increased (Figure 7C). In accord with this observation, the RboP transfer activity also decreased with an increasing molar ratio of CDP-Gro to CDP-Rbo (Figure 7D). The RboP transfer activity decreased to 65% (CDP-Rbo:CDP-Gro, 1:1) or 17% (CDP-Rbo:CDP-Gro, 1:5) of that without CDP-Gro (CDP-Rbo:CDP-Gro, 1:0). These results indicate that the RboP transfer activity of sFKRP is inhibited by CDP-Gro much like it is for sFKTN.

**DISCUSSION**

In the present study, we showed that FKTN can transfer GroP from CDP-Gro, in addition to RboP from CDP-Rbo, to the core M3 glycan. These results are consistent with the previous report that the GroP-substituted phospho-core M3 unit is not found in FKTN-deficient cells (12), and the present study provides direct evidence that FKTN itself is the GroP transferase. In addition, we showed that FKRP also has GroP transfer activity using CDP-Gro as well as RboP transfer activity. To date, no GroP transferases have been reported in mammals, and therefore, this is the first report of mammalian GroP transferases using CDP-Gro as a donor substrate.

Based on the kinetic data of sFKTN (Figure 4), the $K_m$ values for CDP-Rbo, CDP-1-Gro and CDP-3-Gro are similar, suggesting that glycerol being shorter than ribitol does not substantially affect its affinity for sFKTN. Meanwhile, the $V_{max}$ value for CDP-Rbo is much higher than those for CDP-1-Gro and CDP-3-Gro, suggesting that the larger size of ribitol is required for efficient transfer. These kinetic properties indicate that CDP-Rbo is a preferred substrate for FKTN. The $V_{max}$ value for CDP-1-Gro is higher than that for CDP-3-Gro. Because the orientation of the hydroxyl group at the $\beta$-carbon of the phosphate group is the same in CDP-Rbo and CDP-1-Gro but not in CDP-3-Gro, the orientation of this hydroxyl group may affect the transfer activity of FKTN to some degree. On the other hand, when CDP-Gro (CDP-1-Gro or CDP-3-Gro or both) and CDP-Rbo coexist in the cells, CDP-Gro may act as a competitive inhibitor of the RboP transfer activity of FKTN due to its substantial affinity for FKTN. Actually, CDP-Gro inhibits the RboP transfer activity of sFKTN from CDP-Rbo in vitro (Figure 7A, B). A similar inhibitory effect of CDP-Gro was also observed in the case of sFKRP (Figure 7C, D), suggesting that FKRP and FKTN have similar kinetic properties in their reactions with CDP-Rbo and CDP-Gro.

How CDP-Gro (CDP-1-Gro or CDP-3-Gro or both) is synthesized in mammalian cells remains unclear. In microorganisms, GCT is known to synthesize CDP-Gro from GroP and CTP (14) as mentioned above. However, GCT is not conserved in mammals. In addition, CDP-Rbo is synthesized from RboP and CTP by ISPD (4,5,17), but GroP does not serve as a substrate for ISPD (18), and ISPD-deficient cells also contain the GroP-substituted core M3 unit (12). These reports indicate that ISPD does not synthesize CDP-Gro. Therefore, mammals may have a different enzyme that catalyzes the synthesis of CDP-Gro.

The data presented herein showed that CDP-Gro inhibits the synthesis of core M3-type glycan by two mechanisms. First, when FKTN transfers GroP from CDP-Gro to the phospho-core M3 peptide, the GroP-transferred product does not then serve as a substrate for FKRP, and further elongation of the outer glycan chain cannot occur. In fact, further glycosylation of the GroP substituent was not observed in the glycoform of a truncated recombinant $\alpha$-DG (12), which is consistent with our results. Second, CDP-Gro inhibits the RboP transfer activities of FKTN and FKRP. This result also suggests that potential CDP-Rbo-using enzymes other than FKTN or FKRP are commonly inhibited by CDP-Gro. However, it remains unclear whether CDP-Gro actively acts as a stop signal of core M3-type glycan synthesis or if FKTN and FKRP erroneously recognize CDP-Gro, and both of these mechanisms result in the inhibition of core M3-type glycan synthesis. The following
issues will be key to revealing whether CDP-Gro functions as a regulator of core M3-type glycan synthesis: 1) the amount of CDP-Gro and the ratio of CDP-Rbo to CDP-Gro in the cells; 2) the biosynthetic machinery of CDP-Gro; and 3) the regulatory mechanisms of the production and/or clearance of CDP-Rbo and CDP-Gro in the cells.

Loss of α-DG glycosylation is observed not only in α-dystroglycanopathy but also in a variety of cancers (19-21). Hypoglycosylation of α-DG hinders interactions between the cells and extracellular matrix, and it can affect intracellular signaling, resulting in invasive and proliferative phenotypes in cancer cells (19,21). In several cancers, reduced expression levels of enzymes involved in the glycosylation of α-DG, such as LARGE in breast cancer (22), have been reported. Since CDP-Gro may also induce hypoglycosylation of α-DG, CDP-Gro may be involved in cancer progression.

**EXPERIMENTAL PROCEDURES**

**Materials**

CDP-Rbo, [3H]-labeled CDP-Rbo (CDP-[3H]Rbo, [ribitol-1-3H]), and RboP were synthesized as described previously (4). CDP-Gro (mixture of CDP-1-Gro and CDP-3-Gro), Gro1P, Gro3P and ribitol were obtained from Sigma.

**Cell Culture and transfection**

HEK293T cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Nakalai Tesque, Kyoto, Japan) supplemented with 10% fetal calf serum, penicillin (100 U/mL) and streptomycin (100 μg/mL). Transfection of the plasmid DNA into cells was performed using Lipofectamine 3000 (Life Technologies Japan, Tokyo, Japan) according to the manufacturer’s protocol.

**Preparation of the phospho-core M3 peptide and RboP-phospho-core M3 peptide**

The synthesis of the core M3 peptide (H-Ala-Thr(Man-GlcNAc-GalNAc)-Pro-Ala-Pro-Val-Ala-Ala-Ile-Gly-Pro-Lys-NH2) was performed via the Fmoc solid-phase peptide synthesis (Fmoc-SPPS) using a glycosylated threonine derivative, that was synthesized as shown in the Supporting Information. In Fmoc-SPPS, each Fmoc-amino acid was coupled by 1,3-diisopropylcarbodiimide (DIPC) and N-hydroxybenzotriazole hydrate (HOBT·H2O). The Fmoc group was deprotected by treatment with 20% (v/v) piperidine/ N-methylpyrrolidone (NMP) for 15 min. Acetic anhydride (2 mL) and pyridine (4 mL) were used for the acetyl capping. After elongation of the peptide, the resin was washed with NMP, dichloromethane, methanol and diethyl ether and then dried in vacuo. To cleave the peptide from the resin and deprotect it, the resin was treated with a mixture of TFA/triisoproylsilane/H2O (95/2.5/2.5) at room temperature. After being stirred for 2 hr, the resulting mixture was filtered. The filtrate was concentrated under reduced pressure, and then diethyl ether was added to give a white precipitate. The resulting solid was separated by decantation, washed with diethyl ether, and then dried under reduced pressure to give the crude, O-acetyl protected glycopeptide. To a solution of the crude glycopeptide in acetonitrile/H2O (1:3, 4 mL), the aqueous solution of tetrabutylammonium hydroxide (TBAOH, 40%/v, 258 µL) was added at 0 °C. The resulting mixture was stirred at room temperature. After 5 hr, TFA (50 µL) was added, and purification by preparative HPLC (column: Inertsil ODS-3, gradient: CH3CN/H2O = 15/85 to 30/70) gave the core M3 peptide (21.1 mg, 39%) as a white powder. Low-resolution MALDI-TOF MS: m/z: calculated for [M+H]+: 1701.9; observed: 1700.9. The phospho-core M3 peptide was synthesized from the core M3 peptide using soluble POMK as described previously (4). The RboP-phospho-core M3 peptide was synthesized as described previously (4).

**Enzyme assay for sFKTN and sFKRP**

The sFKTN or sFKRP expression plasmids (4) were transfected into HEK293T cells. The recombinant proteins were immunoprecipitated from the culture supernatant with anti-c-Myc antibody-agarose (rabbit polyclonal, Sigma). The proteins
bound to the agarose were used as the enzyme sources. sFKTN and sFKRP protein expression levels were determined by Coomassie brilliant blue staining and western blotting as described previously (10). sFKTN enzymatic reactions were performed on 20 µL of solution containing 100 mM MES (pH 6.5), 500 µM donor substrate, 100 µM acceptor peptide, 10 mM MnCl\(_2\), 10 mM MgCl\(_2\), 0.5% Triton X-100, and 5 µL of the enzyme-bound agarose at 37 °C for 15 hr, except where otherwise indicated. For the kinetic experiments on sFKTN, 12.5, 25, 100, 250 and 500 µM CDP-Rbo, CDP-1-Gro or CDP-3-Gro, and 100 µM acceptor peptide were used, and the mixture was incubated for 4 hr. For the sFKTN enzymatic reactions in the presence of both CDP-Rbo and CDP-Gro, 0 or 500 or 2,500 µM mix-CDP-Gro (Sigma), 500 µM CDP-[\(^3H\)]Rbo (50,000 dpm/nmol) and 100 µM phospho-core M3 peptide were used, and the mixture was incubated for 4 hr. For sFKTN enzymatic reactions in the presence of both CDP-Rbo and ribitol, RboP or Gro3P, 500 µM CDP-Rbo, 5,000 µM ribitol or RboP or Gro3P and 100 µM phospho-core M3 peptide were used, and the mixture was incubated for 2 hr. sFKRP enzymatic reactions were performed on 20 µL of solution containing 100 mM MES (pH 6.5), 250 µM CDP-Rbo or mix-CDP-Gro (Sigma), 50 µM acceptor peptide, 10 mM MnCl\(_2\), 10 mM MgCl\(_2\), 0.5% Triton X-100, and 5 µL of the enzyme-bound agarose at 37 °C for 2 hr, except where otherwise indicated. For sFKRP enzymatic reactions in the presence of both CDP-Rbo and CDP-Gro, 0, 250 or 1,250 µM mix-CDP-Gro (Sigma), 250 µM CDP-[\(^3H\)]Rbo, 100 µM RboP-phospho-core M3 peptide and 0.8 µL of the enzyme-bound agarose were used, and the mixture was incubated for 15 min. Because the expression level of sFKTN in the HEK293T culture supernatant was lower than that of sFKRP, and the amount of immunoprecipitated sFKTN from the culture supernatant was smaller than that of sFKRP, FKTN assays were performed with longer incubation time and FKRP assays were done with shorter incubation time. For kinetic experiments of sFKTN (Figure 4) and enzymatic reactions of sFKTN and sFKRP in the presence of both CDP-Rbo and CDP-Gro (Figure 7), we confirmed that the amounts of the reaction products showed the linearity with the incubation time. Each product was separated by reversed-phase HPLC with a Mightysil RP-18GP Aqua column (4.6 x 250 mm) (KANTO CHEMICAL, Tokyo, Japan). Solvent A was 0.085% TFA in distilled water, and solvent B was 0.085% TFA in acetonitrile. Peptides were eluted at a flow rate of 1 mL/min using a linear gradient of 0–40% solvent B. Peptide elution was monitored by determining the absorbance at 215 nm. Each enzyme activity was calculated from the product peak area except where otherwise indicated. Additionally, the separated product peak was collected and lyophilized for MALDI-TOF-MS/MS analysis. To determine the enzyme activity in the presence of both CDP-Rbo and CDP-Gro, the radioactivity of each fraction (1 mL) was measured using a liquid scintillation counter.

**Preparation of CDP-1-Gro and CDP-3-Gro**

CDP-1-Gro and CDP-3-Gro were produced as described previously with slight modifications (15). Briefly, a synthetic codon-optimized gene aq_1368, a GCT encoding gene from *Aquifex aeolicus*, was cloned into the Ndel/BamHI sites of pET19b to generate pET19b_aq1368. *E. coli* BL21(DE3) harboring pET19b_aq1368 were grown at 37 °C in LB medium with ampicillin (50 µg/mL) and were induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 hr. The cells were harvested and resuspended in 10 mM Tris-HCl (pH 7.4) containing 5 mM MgCl\(_2\) and a protease inhibitor cocktail (Nakalai Tesque), and the cells were disrupted by sonication. The homogenate was centrifuged at 20,000 g for 15 min, and the resulting supernatant was collected. His-tagged AQ1368 was purified by using His SpinTrap (GE Healthcare) and dialyzed with 50 mM Tris-HCl (pH 8.6) containing 150 mM NaCl and 5 mM MgCl\(_2\). The purity of His-tagged AQ1368 was verified by SDS-PAGE followed by Coomassie brilliant blue staining. Then, CDP-1-Gro and
CDP-3-Gro were produced by heating 20 µL of solution containing 50 mM Tris-HCl (pH 8.6), 5 mM MgCl₂, 50 µg of His-AQ1368, 10 mM CTP and 10 mM Gro1P or Gro3P at 37 °C for 5 min. Each product was separated using reversed-phase HPLC with a COSMOSIL 5C18-AR-II column (4.6 x 250 mm) (Nakalai Tesque) by isocratic elution with 20 mM acetic acid-triethylamine (pH 7.0). Product elution was monitored by determining the absorbance at 260 nm. The separated CDP-1-Gro or CDP-3-Gro was collected, repurified on the same HPLC system, and collected again. The purities of CDP-1-Gro and CDP-3-Gro were verified by HPLC analysis, and the production of CDP-Gro was confirmed by HPLC-MS analysis.

**MALDI-TOF MS/(MS) analysis**

The peptides were desalted using GL-Tip SDB (GL Sciences, Tokyo, Japan) and reconstituted in 10 µL of Milli-Q water prior to MS/(MS) analysis. 2,5-Dihydrobenzoic acid was dissolved in 50% acetonitrile (MeCN) containing 0.1% TFA; this solution was used as the matrix solution. The peptide solution was mixed with an equal volume of the matrix solution, and 2 µL of this mixture was dropped onto a µFocus MALDI plate 700 µm (Hudson Surface Technology, NJ, USA) and left at room temperature to dry. MS/(MS) analysis was performed on an AB SCIEX TOF/TOF 5800 system (AB SCIEX) operated with TOF/TOF Series-Explorer software version 4.1 (AB SCIEX). For each spot, MS spectra were acquired in the positive ion mode between m/z 800 and 4,000 and accumulated from 1,000 laser shots in a random raster. MS/MS spectra were acquired using the following parameters and methods: acceleration voltage, 1 kV; CID control, OFF; laser shots, 4,000; precursor mass window, 200 resolution (FWHM); and metastable suppression, ON.

**Exo-β-N-acetylatedhexosaminidase (HexNAcase) treatment**

Exo-β-N-acetylatedhexosaminidase (HexNAcase) digestion was performed on 25 µL of solution containing 100 mM citrate-phosphate (pH 3.5), 20 µM peptide, and 20 U/mL HexNAcase from jack bean (GKX-5003, Prozyme, CA, USA) at 37 °C for 22 hr. The product was analyzed by HPLC and MALDI-TOF-MS as described above.

**Statistics**

All experiments were performed at least three times with comparable results. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by a Tukey test (SPSS Statistics version 22.0, IBM, Armonk, NY).

**Conflict of Interest:** The authors declare that they have no conflicts of interest with the contents of this article.

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**FOOTNOTES**

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The following abbreviations are used: α-DG, α-dystroglycan; B3GALNT2, β1,3-N-acetylgalactosaminyltransferase 2; B4GAT1, β1,4-glucuronyltransferase 1; CDP-Gro, CDP-glycerol; CDP-1-Gro, CDP-1-glycerol; CDP-3-Gro, CDP-3-glycerol; CDP-Rbo, CDP-ribitol; CID, collision-induced dissociation; core M1, GlcNAcβ1-2Man; core M2, GlcNAcβ1-2(GlcNAcβ1-6)Man; core M3, GalNAcβ1-3GlcNAcβ1-4Man; FKTN, fukutin; FKRP, fukutin-related protein; GalNAc, N-acetylgalactosamine; GCT, glycerol phosphate cytidylyltransferase; GlcA, glucuronic acid; GlcNAc, N-acetylgalactosamine; GroP, glycerol phosphate; Gro1P, sn-glycerol-1-phosphate; Gro3P, sn-glycerol-3-phosphate; HexNAc, N-acetylgalactosamine; HexNAcase, exo-β-N-acetylgalactosaminidase; ISPD, Isoprenoid synthase domain containing; LARGE, like-acetylgalactosaminyltransferase; Man, mannose; POMGNT2, protein O-mannose N-acetylgalcosaminyltransferase 2; POMT, protein O-mannosyltransferase; POMK, protein O-mannosyl kinase; RboP, ribitol phosphate; RXYLT1, Ribitol xylosyltransferase 1; TMEM5, transmembrane protein 5; Xyl, xylose.
Figure 1

A

kDaA

E

F

GroPGalNAcGlcNAc(P)Man

1200 Mass (m/z)

1935.9

1578.7

1133.6

1781.8

1000 1400

150sFKTN(+)

1763.8

100sFKTN(-)

0

0

V absorbance (mV)

Retention time (min)

15 20 25

15 20 25

15 20 25

CDP-Gro + phospho-core M3 peptide

CDP-Rbo + phospho-core M3 peptide

sFKTN (+)

S

P1

sFKTN (+)

S

P2

P2

150sFKTN(+)

Retention time (min)

15 20 25

Figure 1. GroP transfer activity of sFKTN using CDP-Gro as a donor. A, immunoprecipitation of the Myc-tagged sFKTN. sFKTN in the culture supernatant was immunoprecipitated with anti-Myc-agarose (rabbit polyclonal) and subjected to western blotting with an anti-Myc antibody (goat polyclonal). B, the structures of CDP-Rbo and CDP-Gro. Two enantiomers of CDP-Gro (CDP-1-Gro and CDP-3-Gro) are shown. CDP-Rbo is defined as CDP-5-D-Rbo. CDP-1-Gro and CDP-3-Gro are defined as CDP-1-L-Gro and CDP-3-L-Gro, respectively. C, enzymatic activity of sFKTN with CDP-Rbo and the phospho-core M3 peptide. The products formed after 15 hr of reaction at 37 °C were analyzed by HPLC. S, unreacted acceptor substrate (phospho-core M3 peptide). P1, product of the reaction of sFKTN with CDP-Rbo. D, enzymatic activity of sFKTN with mix-CDP-Gro (Sigma) (mixture of CDP-1-Gro and CDP-3-Gro) and the phospho-core M3 peptide. The products formed after 15 hr of reaction at 37 °C were analyzed by HPLC. Upper, without sFKTN. Lower, with sFKTN. P2, product of the reaction of sFKTN with CDP-Gro. The fraction containing P2 (eluate with the retention time indicated by the dotted line) was subjected to MS analysis (E). E, MALDI-TOF-MS spectrum of P2. Asterisks, fragment ions of P2 formed during the MS experiment. F, MS/MS spectrum of P2 (m/z 1935.9).
Figure 2. sFKTN transfers GroP to the terminal GalNAc of the phospho-core M3 peptide. A, the phospho-core M3 peptide was treated with or without HexNAcase, and the products were analyzed by HPLC. S, unreacted substrate (phospho-core M3 peptide). S’, product of the reaction with HexNAcase. Eluates with the retention times indicated by dotted lines were subjected to MS analysis (B). B, the MALDI-TOF-MS spectra of S without HexNAcase (upper) and S and S’ in A with HexNAcase (lower). C, the GroP-transferred phospho-core M3 peptide was treated with or without HexNAcase, and the products were analyzed by HPLC. P2, the GroP-phospho-core M3 peptide. S, the phospho-core M3 peptide; a small amount of this peptide was present in the P2 fraction as a contaminant. S’, product of the HexNAcase reaction with S. Eluates with the retention times indicated by dotted lines were subjected to MS analysis (D). D, the MALDI-TOF-MS spectra of the eluates in C without HexNAcase (upper) and with HexNAcase (lower). Asterisks, fragment ions of P2 and S formed during the MS experiment. The m/z 1375.7 and 1578.8 peaks (lower) also contain the HexNAcase reaction products with S.
Figure 3. Both CDP-1-Gro and CDP-3-Gro serve as the donors for sFKTN. A, B, enzymatic activity of sFKTN with CDP-1-Gro (A) or CDP-3-Gro (B) and the phospho-core M3 peptide. After 4 hr of reaction at 37 °C, the products were analyzed by HPLC. S, unreacted acceptor substrate (phospho-core M3 peptide). Open triangle, product of the reaction of sFKTN with CDP-1-Gro (A) or CDP-3-Gro (B). Eluates with the retention times indicated by dotted lines were subjected to MS analysis (C, D). C, MALDI-TOF-MS spectrum of the sFKTN product in A. D, MALDI-TOF-MS spectrum of the sFKTN product in B. Asterisks, fragment ions formed during the MS experiment.
Figure 4. Kinetic analysis of sFKTN with CDP-Rbo, CDP-1-Gro and CDP-3-Gro. A, the enzymatic activity of sFKTN with various concentrations of CDP-Rbo, CDP-1-Gro or CDP-3-Gro (12.5, 25, 100, 250 and 500 μM) and 100 μM phospho-core M3 peptide. The assay was conducted as described in the “Experimental procedures”. RboP transfer activities using CDP-Rbo, open squares. Gro1P transfer activities using CDP-1-Gro, open circles. Gro3P transfer activities using CDP-3-Gro, closed circles. B, Lineweaver-Burk plot of 1/V versus 1/[donor] to obtain the kinetic constants ($K_m$, $V_{max}$) for CDP-Rbo, CDP-1-Gro and CDP-3-Gro. C, Kinetic parameters of sFKTN for CDP-Rbo, CDP-1-Gro and CDP-3-Gro. Data represent the mean ± standard deviation (SD) of triplicate measurements. $^aP<0.001$ versus the value of CDP-Rbo. $^bP<0.01$ versus the value of CDP-1-Gro.

|         | $K_m$ (μM) | $V_{max}$ (μM/hr) | Catalytic efficiency ($V_{max}/K_m$) (hr$^{-1}$) |
|---------|------------|-------------------|-----------------------------------------------|
| CDP-Rbo | 30.8 ± 5.5 | 5.53 ± 0.15       | 0.184 ± 0.034                                  |
| CDP-1-Gro | 39.4 ± 3.7 | 1.01 ± 0.19       | 0.026 ± 0.007                                  |
| CDP-3-Gro | 27.3 ± 1.6 | 0.45 ± 0.06       | 0.017 ± 0.003                                  |
Figure 5. The GroP-phospho-core M3 peptide cannot serve as an acceptor substrate for sFKRP. A, immunoprecipitation of the Myc-tagged sFKRP. sFKRP in the culture supernatant was immunoprecipitated with anti-Myc-agarose (rabbit polyclonal) and subjected to western blotting with an anti-Myc antibody (goat polyclonal). B, enzymatic activity of sFKRP with CDP-Rbo and the RboP-phospho-core M3 peptide. Upper, without sFKRP. Lower, with sFKRP. The products formed after 2 hr of reaction at 37 °C were analyzed by HPLC. P1, unreacted acceptor substrate (RboP-phospho-core M3 peptide). P3, product of the reaction of sFKTN with CDP-Rbo. Eluate with the retention times indicated by dotted lines were subjected to MS analysis (C). C, MALDI-TOF-MS spectrum of P1 and P3. Asterisks, fragment ions of P1 and P3 formed during the MS experiment. D, no enzymatic activity was observed for sFKRP with CDP-Rbo and the GroP-phospho-core M3 peptide. The products formed after 2 hr of reaction at 37 °C were analyzed by HPLC. Upper, without sFKRP. Lower, with sFKRP. P2, unreacted acceptor substrate (GroP-phospho-core M3 peptide). S, the phospho-core M3 peptide; a small amount of this peptide was in the P2 fraction during the HPLC fractionation process. Eluates with the retention times indicated by dotted lines were subjected to MS analysis (E). E, MALDI-TOF-MS spectrum of P2 and S. Asterisks, fragment ions of P2 and S formed during the MS experiment.
Figure 6. sFKRP has GroP transfer activity using CDP-Gro. A, enzymatic activity of sFKRP with CDP-Gro and the RboP-phospho-core M3 peptide. The products formed after 2 hr of reaction at 37 °C were analyzed by HPLC. Upper, without sFKRP. Lower, with sFKRP. P1, unreacted acceptor substrate (RboP-phospho-core M3 peptide). P4, product of the reaction of sFKRP with CDP-Gro. The fraction containing P1 and P4 (eluate with the retention time indicated by a dotted line) was subjected to MS analysis (B). B, MALDI-TOF-MS spectrum of P1 and P4. Asterisks, fragment ions of P1 and P4 formed during the MS experiment. C, MS/MS spectrum of P4 (m/z 2149.8). The peak at m/z 1763.8 corresponds to the dehydrated form of m/z 1781.8. D, no enzymatic activity was observed for sFKRP with CDP-Gro and the GroP-phospho-core M3 peptide. The products formed after 2 hr of reaction at 37 °C were analyzed by HPLC. P2, unreacted acceptor substrate (GroP-phospho-core M3 peptide). S, phospho-core M3 peptide; a small amount of this peptide was in the P2 fraction during the HPLC fractionation process. Eluates with the retention times indicated by dotted lines were subjected to MS analysis (E). E, MALDI-TOF-MS spectrum of P2 and S. Asterisks, fragment ions of P2 and S formed during the MS experiment. Mix-CDP-Gro (Sigma) was used.
Figure 7. RboP transfer activities of sFKTN and sFKRP are inhibited by CDP-Gro. A, enzymatic activity of sFKTN with CDP-[^3]H]Rbo, various concentrations of CDP-Gro (molar ratio of 1:0, 1:1 or 1:5 CDP-Rbo to CDP-Gro) and the phospho-core M3 peptide. The products formed after 4 hr of reaction at 37 °C were analyzed by HPLC. S, unreacted acceptor substrate (phospho-core M3 peptide). Open triangles, sFKTN reaction products. B, RboP transfer activity of sFKTN in the presence of various concentrations of CDP-Gro. The content of the[^3]H]RboP-labeled product was measured via liquid scintillation counting. C, enzymatic activity of sFKRP with CDP-[^3]H]Rbo, various concentrations of CDP-Gro (molar ratio of 1:0, 1:1 or 1:5 CDP-Rbo to CDP-Gro) and the RboP-phospho-core M3 peptide. The products formed after 15 min of reaction at 37 °C were analyzed by HPLC. P1, unreacted acceptor substrate (RboP-phospho-core M3 peptide). Open triangles, sFKRP reaction products. D, RboP transfer activity of sFKRP in the presence of various concentrations of CDP-Gro. The content of the[^3]H]RboP-labeled product was measured via liquid scintillation counting. Mix-CDP-Gro (Sigma) was used. Vertical bars represent the range of values obtained from duplicate experiments.
CDP-glycerol inhibits the synthesis of the functional $\alpha$-mannosyl glycan of $\alpha$-dystroglycan

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