Abstract

Objective: The congenital disorders of glycosylation (CDGs) are progressive multisystemic disorders characterized by a heterogeneous deficiency of the carbohydrate moieties in various structural and circulating glycoproteins, representing a natural model for glycoprotein hormone studies. Here, we studied the carbohydrate moiety of circulating glycoprotein hormones in four patients with a clinical suspicion of CDGs.

Methods: The diagnosis of CDG-I was confirmed in two out of the four cases by transferrin isoelectrofocusing (IEF) and/or carbohydrate-deficient transferrin (CDT) test. The carbohydrate moiety of serum endocrine-related glycoproteins was investigated by means of Ricin (immunopurified thyrotropin (TSH)) and Concanavalin A (Con-A) (TSH, follicle-stimulating hormone, alpha-subunit and thyroglobulin) lectin affinity chromatography measurement.

Results: CDT concentrations were very high in the two patients with CDG-I and moderately enhanced in the remaining two. In the two CDG-I patients, Ricin analysis of immunopurified TSH showed a severe impairment of lectin binding, both before and after neuroaminidase treatment, indicating a nearly complete lack of terminal sialic acid and galactose residues. In these two cases, Con-A analysis showed a significant prevalence of firmly bound isoforms with poorly processed carbohydrate chains. In the remaining two cases with unknown CDG classification, TSH binding pattern to Ricin was modestly affected and Con-A analysis showed the prevalence of weakly bound glycoprotein isoforms.

Conclusions: The results of Ricin analyses in all four patients were consistent with the CDT test and/or serum transferrin IEF. The severe alteration of TSH binding pattern to Ricin seems to be characteristic of CDG-I. Nevertheless, TSH biological properties are not severely altered, as normal thyroid function was found in both cases.

Introduction

The sugar moiety has a major impact on the stability, folding, secretion, function and clearance of glycoprotein hormones (1–3). The glycosylation of one glycoprotein is a multi-step process involving the activity of numerous enzymes located in the rough endoplasmic reticulum and Golgi (4). These enzymes provide the synthesis of the lipid-linked oligosaccharide precursor and its attachment to the protein backbone, as well as the processing of the protein-bound oligosaccharide. This process leads to progressive maturation of the carbohydrate chains, from the high-mannose to the complex type and the circulating pool of one glycoprotein hormone consists of a mixture of several isoforms differing in structure and the degree of maturation of their carbohydrate moiety (1–3, 5–7) (Fig. 1).

Congenital disorders of glycosylation (CDGs) (8), previously known as carbohydrate-deficient glycoprotein syndromes (9–11), are genetic multisystemic recessive disorders of unknown prevalence caused by defects in the attachment of carbohydrate to protein. These disorders are caused by mutations affecting the pathway for N-glycosylation resulting in the hypoglycosylation of a large number of circulating and structural glycoproteins, including transport proteins, enzymes, coagulation factors, receptors and hormones (12). In the diagnosis of CDG, defective glycosylation is currently revealed by the altered isoelectric focusing (IEF) patterns of transferrin (9–11, 13). Different classifications of CDGs have been proposed over the years (12–19), but very recently, the CDG syndromes have been divided into two types (I and II) depending on the underlying genetic and enzymatic defects (8.
CDGs are characterized by various clinical manifestations, usually including mental and psychomotor retardation, dysmorphism, retinitis pigmentosa, blood coagulation defects and dysfunctions of other tissues and organs, such as liver and endocrine glands (15, 23, 24). In contrast, defects causing CDG-II occur later in the pathway of N-glycosylation affecting the trimming of protein-bound oligosaccharide or the addition of sugars to it.

CDGs are characterized by various clinical manifestations, usually including mental and psychomotor retardation, dysmorphism, retinitis pigmentosa, blood coagulation defects and dysfunctions of other tissues and organs, such as liver and endocrine glands (15, 30–32). In particular, some authors have described the altered secretion of several hormones in CDGs (21, 23, 24). In contrast, defects causing CDG-II occur later in the pathway of N-glycosylation affecting the trimming of protein-bound oligosaccharide or the addition of sugars to it.

20–29). CDG-I is characterized by defects impairing the initial steps of the glycosylation process, such as assembly of lipid-linked oligosaccharide precursor and its transfer to asparagine residues. Five subtypes of CDG-I (Ia to Ie) have been described so far, depending on the altered gene (8, 22, 23, 25–27). About 70% of the patients with CDG-I have inactivating mutations of the gene encoding phosphomannomutase 2 (PMM2) (23) and are classified as having CDG-Ia, the most common form of this syndrome. PMM2 activity is needed for the synthesis of GDP-mannose, an essential compound in nascent N-linked oligosaccharides (21, 23, 24). In contrast, defects causing CDG-II occur later in the pathway of N-glycosylation affecting the trimming of protein-bound oligosaccharide or the addition of sugars to it.

Several physiological and pathological conditions have been associated with the secretion of glycoprotein hormones with altered carbohydrate branching (2, 3, 5–7) and CDGs may represent a useful model for glycoprotein hormone studies. Therefore, we analyzed by means of lectin affinity chromatography the glycosylation pattern of several endocrine-related glycoproteins circulating in two patients with CDG-I (one with normal and the second with impaired PMM2 activity, indicating the diagnosis of CDG-Ia), and another two patients suspected of having a still undefined CDG syndrome.

Subjects and methods

Case reports (Table 1)

Case 1 This case was previously reported by Di Rocco et al. (38). The male patient is the second-born from non-consanguineous, healthy parents. Since the age of 3 months, the patient was admitted several times to the hospital for acute liver failure episodes that recovered spontaneously. The main clinical findings are summarized in Table 1. The clinical diagnosis of CDG was then confirmed by the finding of elevated serum concentrations of arylsulfatase A (80 nmol/ml/h; normal values (n.v.): 33.6 ± 11.5), but mainly by transferrin IEF showing the typical migration pattern consistent with CDG-I syndrome (a marked increase of asialo- and distialo-transferrin). The diagnosis of CDG-Ia was definitely obtained by the reduced PMM activity in the fibroblast culture (0.33 mU/mg protein; n.v. 2.2–6.4; assay performed by J Jaeken at Universitaire Ziekenhuizen, Leuven, Belgium). The blood sample for this study was obtained at the age of 4 years.

Case 2 This is the second child of unrelated parents, born at term after an uneventful pregnancy. Mild intrauterine growth retardation was noted at the seventh month of gestation. The main clinical findings are summarized in Table 1. Blood tests showed increased levels of transaminases (GOT 70–83 IU/l, GPT 108–122 IU/l), while the other tests of liver function and ultrasound imaging were completely normal. Electroencephalogram showed asymmetric activity (slower in the right cerebral hemisphere). A brain computed tomography (CT) scan showed moderately dilated lateral ventricles and diffuse atrophy, more evident in the frontal lobe, right temporal insular region and cerebellar vermis. Cerebral magnetic resonance imaging (MRI) did not show any structural brain abnormalities, but confirmed hypoplasia of the cerebellar vermis and mild dilatation of lateral ventricles. The patient was then referred to the 2nd Pediatric Clinic of Milan University, for further investigations. At admission, the clinical examination showed

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mild dysmorphies (Table 1). Specific laboratory tests showed normal levels of very long chain fatty acids and persistent elevation of transaminases (5- to 10-fold higher than normal values). No X-ray skeletal abnormalities were evident; heart and kidney ultrasound imaging was normal, as was nerve conduction velocity. The diagnosis of CDG syndrome was established at the age of 2.5 years by IEF of serum transferrin showing a migration pattern consistent with CDG-I. PMM activity was normal in fibroblast culture (4.2 mU/mg protein; assay performed by J. Jaeken). The parents of the four patients gave their informed consent to the study.

**Case 3** This girl was born at term after an uneventful pregnancy from unrelated parents. The main clinical findings are summarised in Table 1. The child was referred to us for evaluation of short stature at the age of 1.4 years, her height was 70 cm (<3rd centile) and weight was 7200 g (<3rd centile). Familial history revealed that the elder sister presented a psychomotor delay, with a CT scan documenting cerebellar atrophy. All clinical investigations were normal apart from a raised thyrotropin (TSH) (9.1 mU/l) with normal serum free thyroxine (FT4) and free tri-iodothyronine (FT3) levels, a feature consistent with subclinical hypothyroidism. Anti-thyroid antibodies were absent and a thyroid scan with 99mTc showed a decreased uptake of the tracer. She was treated with a daily dose of 50 μg L-T4 that led to a significant augmentation of growth velocity. Nine months later, however, she noted, despite L-T4 treatment. Full endocrinological and insulin stimulation tests (<4.3 ng/ml; n.v. >10). GH therapy improved growth velocity, with a growth pattern along the 50th centile.

**Case 4** This was the second child of unrelated parents, born at term by cesarean section, due to feto-pelvic disproportion, after an uneventful pregnancy. The main clinical findings are summarised in Table 1; infections are excluded. At the age of 5 months he was hospitalized for several cyanotic crises and respiratory infection; hepatomegaly, growth and psychomotor delay were observed. Blood tests showed an increase of transaminases (GOT=80–316 UI/l, GPT=34–161 UI/l), while the other tests of liver function were normal, as was liver ultrasound imaging. A CT scan was performed showing the complete agenesia of the corpus callosum. At the age of 7 months, clinical examination showed mild dysmorphies (Table 1). No skeletal abnormalities were seen; heart and kidney ultrasound imaging was normal. The patient is now 1 year old with growth (weight of 6500 g and length 63 cm) and psychomotor delay, hepatomegaly and persistent elevation of transaminases (3- to 6-fold over normal values). These clinical manifestations were thought to be consistent with CDG-Ie (29), but such diagnosis was denied by the normal pattern of serum transferrin IEF (performed by Dr Rita Barone, Clinica Pediatrica I, Catania, Italy).

The parents of the four patients gave their informed consent to the study.

The control groups for Concanavalin-A (Con-A) and Ricin lectin affinity chromatography consisted of nine and eleven children (aged 2–9 years) respectively. Blood samples were obtained due to suspicion of other disease, which was subsequently excluded. The exception was represented by the control group for alpha

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**Table 1** Biochemical and clinical features of the four studied patients.

<table>
<thead>
<tr>
<th></th>
<th>Case 1</th>
<th>Case 2</th>
<th>Case 3</th>
<th>Case 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age/sex</td>
<td>4 years/male</td>
<td>3 years/male</td>
<td>4 years/female</td>
<td>1 year/male</td>
</tr>
<tr>
<td>Pregnancy</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Birth weight</td>
<td>2800 g</td>
<td>2680 g</td>
<td>2700 g</td>
<td>3230 g</td>
</tr>
<tr>
<td>Hypopituitar/hypopituitarism</td>
<td>Present/present</td>
<td>Present/present</td>
<td>Present/present</td>
<td>Present/present</td>
</tr>
<tr>
<td>Visual/visual fixation</td>
<td>Convergent squint</td>
<td>Diffuse atrophy</td>
<td>Diffuse atrophy</td>
<td>Normal</td>
</tr>
<tr>
<td>Brain MRI/CT scan</td>
<td>Diffuse atrophy</td>
<td>Diffuse atrophy</td>
<td>Cerebellar atrophy</td>
<td>Normal</td>
</tr>
<tr>
<td>Nerve conduction</td>
<td>Reduced</td>
<td>Severe</td>
<td>Reduced</td>
<td>Moderate</td>
</tr>
<tr>
<td>Psychomotor delay</td>
<td>Impaired</td>
<td>Impaired</td>
<td>Severe</td>
<td>Milder impaired</td>
</tr>
<tr>
<td>Somatic growth</td>
<td>Reduced</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Antithrombin III</td>
<td>Raised</td>
<td>Raised</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Transaminases</td>
<td>Reduced (CDG-Ia)</td>
<td>Normal</td>
<td>Not performed</td>
<td>Not performed</td>
</tr>
<tr>
<td>Transferrin IEF</td>
<td>CDG-I</td>
<td>CDG-I</td>
<td>CDG-I</td>
<td>Normal</td>
</tr>
<tr>
<td>CDT (n.v. &lt;25)</td>
<td>192 U/l</td>
<td>212 U/l</td>
<td>56 U/l</td>
<td>113 U/l</td>
</tr>
<tr>
<td>PMM activity (n.v. 2.2–6.4)</td>
<td>Microcephaly, facial dysmorphism, modest hepatomegaly, cryptorchidism</td>
<td>Microcephaly, facial dysmorphism, modest hepatomegaly, truncal distribution of subcutaneous fat</td>
<td>Microcephaly, facial dysmorphism, modest hepatomegaly, truncal distribution of subcutaneous fat</td>
<td>Normal</td>
</tr>
<tr>
<td>Dysmorphism</td>
<td>Normal</td>
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<td>Normal</td>
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<td>Microcephaly, facial dysmorphism, modest hepatomegaly, truncal distribution of subcutaneous fat</td>
<td>Normal</td>
</tr>
</tbody>
</table>

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subunit (α-SU) Con-A analysis which was made up of adult patients (39).

**Immunometric assays**

Serum concentrations of TSH, follicle-stimulating hormone (FSH), luteinizing hormone (LH), thyroglobulin (Tg), FT₄, FT₃, TBG and sex hormone binding globulin (SHBG) were evaluated by means of specific immunofluorometric assays using Delfia technology (Wallac, Turku, Finland). Serum α-SU concentrations were measured by means of specific IRMA (Biocode, Sclesin, Belgium). Circulating levels of carbohydrate deficient transferrin (CDT) were measured by RIA method (CDTect, Amersham Pharmacia Biotech Italia, Cologno Monzese, Italy).

**Ricin lectin affinity chromatography**

*Ricinus communis* is available in two forms differing on the basis of molecular weight (MW): RCA 60 (MW 60 kDa) and RCA 120 (MW 120 kDa). We used the latter which has specific affinity for the β-D-galactose terminal residues.

**Serum TSH immunopurification** Ricin lectin affinity chromatography is reliably performed using purified materials; immunopurification and concentration of circulating TSH was performed by means for plastic tubes coated with a monoclonal antibody directed against a conformational epitope of the glycoprotein hormone, as previously described (6, 7). Serum samples were incubated overnight at 4 °C in the coated tubes (0.75 ml/tube). Bound TSH was eluted from the antibody by washing the tubes with guanidine-HCl (2 mol/l, pH 3.2; 0.75 ml) and immediately buffered with PBS and hypotonic Hank’s balanced salt solution (HBSS, pH 7.5). About 90% of serum TSH was extracted. Immunopurified TSH was then recovered in hypotonic HBSS+0.4% BSA after dialysis and concentration by filtration (Centriprep, Amicon, Beverley, MA, USA; cutoff: 10 kDa), and final recovery ranged between 50 and 65%.

**Ricin analysis of immunopurified TSH** Ricin specifically binds N-acetylglucosamine (GlcNAc) and galactose (Gal) terminal residues, and the difference of lectin-bound glycoprotein before and after neuraminidase (NAase) treatment gives an estimation of the sialylation degree of the molecule (6, 7). Columns containing 1 ml Ricin lectin bound to agarose (RCA 120, Sigma, Aldrich SRL, Milan, Italy) were equilibrated with PBS+0.05% BSA, pH 7.4. Specimens (25 µl immunopurified samples and 100 µl phosphate buffer, pH 6.6) were loaded into the columns before or after treatment with NAase (10 mU; 4 h at 37 °C). Unbound (UB) TSH was recovered by washing the column with the equilibration buffer (1 ml×9 times) after 1 h incubation at room temperature. Bound (B) TSH was eluted from the column after incubation (1 h at room temperature) and extensive washing (1 ml×9 times) with the buffer supplemented with 20 mmol/l galactose. Final recovery was always >88%.

**Con-A lectin affinity chromatography**

Con-A has specific affinity for terminal residues of α-D-mannose and α-D-glucose and requires Ca²⁺ and Mn²⁺ for correct binding to the oligosaccharides (6). One milliliter Con-A Sepharose (Amersham Pharmacia Biotech, Italy) was added to each column and equilibrated with a specific buffer, 10 mmol/l Tris–HCl, 150 mmol/l NaCl, 1 mmol/l MgCl₂, 1 mmol/l MnCl₂ and 1 mmol/l CaCl₂ (pH 8.0). Then, centrifuged serum (0.5 ml) was loaded in the column and incubated with the lectin for 1 h at room temperature. The unbound (UB) glycoproteins were collected by repeated centrifugations (10 times with 1 ml buffer). Weakly and firmly bound fractions (WB and FB respectively) were obtained by the same procedure and the same buffer supplemented with 10 mmol/l α-methylglucopyranoside or 300 mmol/l α-methylmannopyranoside respectively. The different sample fractions were then concentrated and glycoprotein concentrations were evaluated with the methods illustrated above. Final recovery ranged between 78 and 90%.

**Statistical analysis**

Data were analyzed by Student’s t-test and analysis of variance (ANOVA), as appropriate. P values <0.05 were considered statistically significant. Results are expressed as means±standard deviation (s.d.).

**Results**

Cases 1, 2 and 4 had normal thyroid function tests (TSH=1.2–1.7 mU/l, n.v. 0.24–4.0; FT₄=10.9–16.1 pmol/l, n.v. 9–20; FT₃=5.2–8.7 pmol/l, n.v. 4–9), while case 3 was studied during i-T4 replacement therapy (TSH=1.34 mU/l). Serum TBG concentrations were reduced in the two patients with CDG-I (94.3 and 86.8 nmol/l; n.v. 50–150) were in the normal range, as well as FSH (0.32–2.37 U/l, n.v. 0.1–4.0). LH was undetectable in all cases (<0.10 U/l) (Table 2).

CDT concentrations were definitely elevated in all the cases, but higher levels were found in patients 1 and 2 (Fig. 2).

TSH concentrations in immunopurified samples were 5.3, 4.8, 2.2 and 19.4 mU/l for cases 1, 2, 3 and 4 respectively. Ricin analysis of immunopurified TSH
Table 2 Basal serum hormone levels of the four studied patients.

<table>
<thead>
<tr>
<th>Case (sex/age)</th>
<th>TSH (mU/l)</th>
<th>Tg (mg/l)</th>
<th>TBG (nmol/l)</th>
<th>α-SU (U/l)</th>
<th>FSH (U/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (M/4 years)</td>
<td>1.20</td>
<td>39.6</td>
<td>94.3</td>
<td>0.54</td>
<td>0.34</td>
</tr>
<tr>
<td>2 (M/3 years)</td>
<td>1.70</td>
<td>18.0</td>
<td>57.6</td>
<td>0.71</td>
<td>0.49</td>
</tr>
<tr>
<td>3 (F/4 years)</td>
<td>1.34a</td>
<td>4.3</td>
<td>155</td>
<td>0.40</td>
<td>2.37</td>
</tr>
<tr>
<td>4 (M/1 year)</td>
<td>1.70</td>
<td>16.1</td>
<td>132</td>
<td>0.20</td>
<td>0.32</td>
</tr>
<tr>
<td>Controls</td>
<td>0.24–4.0</td>
<td>&lt;30.0</td>
<td>125–350</td>
<td>&lt;1.0</td>
<td>0.1–4.0</td>
</tr>
</tbody>
</table>

* On L-T₄ therapy.

Discussion

This study provides new data on the alteration of the carbohydrate moiety of pituitary or thyroid...
glycoprotein hormones in a series of patients with various types of CDGs. The almost complete lack of sialylated TSH and a decreased amount of mature isoforms of several glycoproteins were observed in the sera from two patients with CDG-I (one with normal and the other with reduced PMM activity). In the other two patients, the demonstration of a less severe defect of glycosylation at CDT test and lectin affinity chromatography supported the diagnosis of a different CDG subtype.

In both cases with strong evidence of CDG-I, Ricin analysis of immunopurified TSH showed the nearly complete lack of sialic acid and Gal/GlcNAc (very low binding both before and after NAs) terminal residues in this pituitary glycoprotein hormone. In a previous study (35) the glycosylation pattern of serum glycoproteins, including TSH, was evaluated by means of IEF in CDG-I patients at different ages (ranging from 4 months to 43 years). The authors reported that the syndrome does not affect the terminal charged carbohydrate residues in pituitary glycoprotein hormones, as well as in erythropoietin, indicating that the glycosylation defect may not be a generalized feature of the disease. Inter-individual (among different patients) and intra-individual (at different ages) variations in the expression of the glycosylation defect (37) appear to be the major factors accounting for this discrepancy. In fact, our findings were obtained during infancy, when the clinical and biochemical expression of the glycosylation defect is usually more pronounced (11, 30, 37). Moreover, our data indicate that a similar alteration preventing the formation of carbohydrate chains of the complex type may be present in CDG-I patients with normal or reduced PMM activity. However, the absence of the usual terminal sugar residues in complex TSH oligosaccharide chains does not prevent normal hormone bioactivity, as demonstrated by normal thyroid function testing in both patients. This TSH binding pattern has never been observed in other clinical and pathological conditions (6, 7) and it appears to be characteristic of CDG-I. The other two patients showed an intermediate content of Gal/GlcNAc residues between the CDG-I patients and normal subjects, as demonstrated by the amount of immunopurified TSH bound to Ricin after NSase treatment. Transferrin IEF and CDT tests also showed intermediate results between the former two cases and normal values. The exact classification of the glycosylation defect in the latter two patients has not yet been made.

Con-A specifically binds mannose exposed residues and allows the separation of glycoproteins in three different fractions on the basis of the degree of maturation of their carbohydrate moieties. Mature isoforms with sugar chains of the complex type, as well as carbohydrate deficient isoforms, may constitute the UB fraction; isoforms with biantennary or truncated hybrid sugar chains at an intermediate step of the maturation process constitute the WB fraction; poorly mature isoforms with high mannose sugar chains constitute the FB fraction (2, 3, 6). When pituitary (TSH, FSH) and thyroid (Tg) glycoproteins are considered, a significant increase of the less mature isoforms (WB and FB in particular) was observed in the two CDG-I patients. Whatever is the underlying genetic defect in these two patients, it does not prevent the sufficient glycosylation of subunit protein backbones required for their assembly and correct formation of pituitary glycoprotein heterodimers (2, 3). In contrast, the other two patients showed a generalized increase in the UB and especially WB fractions, if compared with the other two cases and to the normal controls. Interestingly, Kim et al. (28) recently demonstrated a similar Con-A binding pattern for transferrin in patients with dolichol-phosphatemannose synthase 1 (DPM1) gene mutations and classified as CDG-Ie, suggesting that these two patients may be affected with the same defect. Finally, free α-SU showed a peculiar binding pattern to Con-A with a modest increase of the UB fractions in the two CDG-I patients, and of the FB fractions in the other two patients. These findings could be related to the presence of the supplementary O-linked chain at Thr39 in free vs TSHβ-bound α-SU (3, 39). Since CDG-Ia was ruled out in these two patients, we cannot exclude the possibility of a defect also in the O-linked glycosylation pathway.

Taken together the data collected in the two patients with CDG-I, the lack of TSH binding to Ricin coexisting with a decrease, but not absence, of UB TSH at Con-A analysis may be interpreted as the presence of TSH isoforms either with a complete carbohydrate deficiency or with sulfated, but not sialylated, sugar chains.

Furthermore, we confirmed that CDG should be considered among the situations characterized by partial TBG deficiency (Table 2) (30, 37). TBG in CDG patients was demonstrated to have normal immunoreactivity and normal affinity for T4, T3 and reverse T3 (34). Moreover, patients with CDG are, in general, biochemically euthyroid and the reduction in serum total iodothyronine concentrations may be explained by low TBG levels and possibly by the interference with T3/T4 binding to TBG by an unidentified substance.

In conclusion, our results confirm that the glycosylation of circulating glycoprotein hormones is similarly altered in two CDG-I patients, with normal or reduced PMM2 activity. Moreover, the availability of a reliable technique for serum TSH immunopurification allowed the observation of a characteristic binding pattern to Ricin of circulating TSH from CDG-I patients, and the presence of a less severe glycosylation defect in the remaining two cases.

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