Invited Comment

G protein β3 subunit 825T allele, hypertension, obesity, and diabetic nephropathy

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Abstract The 825T allele of the gene GNB3 which encodes the β3 subunit of heterotrimeric G proteins is associated with enhanced signal transduction via G proteins through the generation of a splice variant termed Gβ3s. It was detected following a classical candidate gene approach using cell lines from patients with enhanced signal transduction and essential hypertension. The high frequency of the 825T allele in ‘old’ ethnicities, e.g. bushmen and Australian aborigines as well as in black populations, together with its strong association with obesity suggests that the 825T allele is a true ‘thrifty genotype’. Development of obesity associated with the 825T allele is strongly influenced by lifestyle, e.g. physical activity, and other exogenous influences like pregnancy. In hypertension the 825T allele is associated with low renin activity and appears to strongly predict the development of left ventricular hypertrophy. In type 2 diabetes the 825T allele was reported to be predispose for end-stage renal disease, whereas this effect has not yet been confirmed for patients with type 1 diabetes.

Keywords: cardiovascular disease, diabetes, hypertension, signal transduction, sodium

Introduction

It is commonly accepted that essential hypertension (HT) which affects up to 30% of individuals in industrialized countries is a multifactorial, polygenetic disorder. The motivation to detect gene alterations causing or contributing to HT is driven by several promises of molecular medicine: Identification of involved loci will lead to a better understanding of the pathophysiological mechanisms leading to high blood pressure and, potentially, to the development of novel drugs for causal treatment. In addition, this will eventually result in the abolition of the term ‘essential’ HT and help to further stratify patients with HT in terms of specific traits or phenotypes associated with a defined allelic variant or a combination of variants. Moreover, genetic tests could be an important tool for preventive medicine in that individuals at risk can be identified before blood pressure increases and eventually escape this ‘fate’ through the implementation of lifestyle changes. Genotyping for common polymorphisms may also help to predict side effects, potential risks or blood pressure-lowering efficacy of existing drugs. In patients with type-1 or type-2 diabetes genetic markers predictive for the development of nephropathy will be highly useful in risk stratification, the early initiation of optimal therapy, and will hopefully improve patient compliance.

A variety of polymorphisms associated with HT have already been found in the genes coding for angiotensinogen, angiotensin-converting enzyme, α-adducin, and the β2-adrenoceptor, to name but a few [1]. The present review will focus on the discovery of the C825T polymorphism in the gene encoding for the G protein β3 subunit (GNB3), evolution of the 825T allele, and its association with obesity and hypertension-related phenotypes.

Enhanced Na/H exchanger activity in HT

Many independent studies have confirmed an increased Na/H exchanger (NHE) activity in a subset of patients with HT [2,3]. This ubiquitously expressed ion transport system mediates an electroneutral exchange of extracellular Na⁺ ions against intracellular H⁺ ions, thereby contributing to intracellular pH homeostasis and [4], potentially, to a rise of cytoplasmic pH following hormonal stimulation of cells [5]. In addition, NHE isoforms play a pivotal role in mediating Na⁺ reabsorption in the proximale tubule. Increased Na/H exchanger activity in HT was found in all cells and tissues examined including erythrocytes, leukocytes, platelets, and skeletal muscle [2,3]. This observation gave rise to two alternative hypothesis linking enhanced NHE activity to pathogenetic processes which could finally precipitate in hypertension. An increased NHE activity in the kidney could result in increased Na⁺ reabsorption and induce a state of
volume expansion. In fact, Diez et al. observed an increased Na⁺ accumulation and suppressed renin concentrations in individuals with enhanced Na/H exchanger activity [6].

Alternatively, it was proposed that elevated NHE activity would efficiently prevent cells from acidosis during proliferation processes and might, therefore, contribute to vascular remodelling of resistance vessels frequently described as media hypertrophy [7]. This hypothesis was supported by findings showing an association between high NHE activity and left ventricular hypertrophy in individuals with HT [8,9]. Despite these highly reproducible findings and correlations the molecular mechanisms underlying enhanced NHE activity in HT remained obscure. Aviv’s group postulated that enhanced NHE activity results from a primary disturbance in cellular Ca²⁺ homeostasis, a higher cytosolic Ca²⁺ in HT being causally related to enhanced NHE activity [10]. In fact, when thrombin-evoked cytosolic free Ca²⁺ and pHᵢ rises were compared in platelets from hypertensive and normotensive subjects, higher pHᵢ rises correlated with higher Ca²⁺ rises in HT [11]. Others claimed a major role for abnormalities in glucose metabolism, e.g. hyperinsulinemia, in enhanced NHE activity. Insulin was found to stimulate the NHE in erythrocytes [12] and, vice versa enhanced NHE activity was found in overweight (potentially hyperinsulinemic) but not lean subjects with HT [13]. Interestingly, antihypertensive treatment with the ACE inhibitor quinapril normalized NHE activity in lymphocytes suggesting again that this phenomenon occurred secondarily to metabolic and/or hormonal changes in HT [14].

In view of this plethora of open questions we decided to use a novel experimental approach in order to find out whether high NHE activity is a ‘true’ intermediate, i.e. inherited, phenotype in HT. To achieve this we immortalized lymphocytes from patients with HT and high NHE activity and from normotensive controls with low NHE activity using Epstein Barr virus. Thus, we established permanently growing lymphoblastoid cell lines which had never been exposed to the ‘hypertensive in vivo milieu’. Such a model has frequently been used to dissect exogenous from inherited phenotypes in a variety of disorders. We could demonstrate that the phenotypes of low or high NHE activity persisted in these immortalized cell lines suggesting that this property was somehow under genetic control [15]. Furthermore, cells with high NHE activity proliferated faster and progressed faster through the cell cycle than those from individuals with low NHE activity [15,16].

Subsequently we sequenced the cDNA encoding for the NHE-1 from various cell lines but failed to detect any mutation [15]. Others used the same experimental setup and essentially confirmed our findings. It was shown that NHE expression was unaltered in immortalized lymphoblasts despite enhanced activity [17], whereas an increased phosphorylation of the ion exchanger correlated with enhanced activity. In line with these findings, the activity of the mitogen-activated protein kinase was increased in these respective cell lines [18]. These findings together with an earlier report, showing that inhibitors of protein kinase C could normalize elevated NHE activity [19], led us to investigate whether signal transduction processes upstream of NHE activity control are altered in HT.

From Na/H exchanger activity to G protein activation—the missing link

Using the established immortalized lymphoblasts we quantified platelet-activating factor (PAF)-mediated rises in cytosolic free Ca²⁺ and inositol phosphate formation and found a significantly increased formation of these second messengers in cells with high NHE activity [20]. PAF receptors in B-lymphoblastoid cell lines are coupled to heterotrimeric G proteins. On pretreatment of these cell lines with pertussis toxin (PTX), which allows to discriminate between the involvement of G proteins belonging to the G₁₁, G₁₂/G₁₃, or G₁₃ family, the originally observed differences in PAF-evoked Ca²⁺ signals were completely abrogated [20]. Moreover, agonist-stimulated binding of GTP to permeabilized lymphoblasts, indicative of G protein reactivity, was consistently enhanced in cells from subjects with HT [20]. These results strongly suggested an enhanced G protein activation as the ultimate reason for enhanced signal transduction, proliferation, and NHE activity in these immortalized cell lines.

Studies on primary skin fibroblasts from the same individuals confirmed these findings [21]. In addition, as skin fibroblasts express receptors for hormones which are also relevant for the cardiovascular system we were able to identify more exactly the type of G protein responsible for enhanced cell responsiveness. On stimulation with agonists which activate receptors coupled to PTX-insensitive G proteins, G₁₃-coupled, e.g. bradykinin and endothelin-1, maximum Ca²⁺ signals were not significantly different in fibroblasts from individuals with HT and controls. However, on stimulation with agonists which at least in part activate PTX-sensitive G proteins, e.g. thrombin and lyso-phosphatidic acid, Ca²⁺ signals and inositol phosphate formation were significantly enhanced in cells from subjects with HT [21]. Interestingly, these differences were completely abrogated on pretreatment of cells with PTX. In accordance with the results obtained from lymphoblasts we observed an increased, PTX-sensitive DNA synthesis in fibroblasts from subjects with HT following stimulation of cells with serum, platelet-derived growth factor, or lyso-phosphatidic acid [21].

Taken together, these congruent findings in immortalized lymphoblasts as well as in primary skin fibroblasts strongly suggested an inherited increased activation of G proteins in cells from subjects with HT and also made it very likely that specifically PTX-sensitive G-proteins were involved.
Structure and function of heterotrimeric G proteins—the G protein activation/deactivation cycle

Heterotrimeric G proteins are ubiquitously expressed mediators of stimuli from heptahelical receptors, but also receptors with intrinsic tyrosine kinase activity (insulin, platelet-derived growth factor, epidermal growth factor), into all cells of the human body [22]. They are composed of α-, β- and γ-subunits, βγ dimers forming a functional monomer. In the non-activated state G protein α-subunits bind GDP (Fig. 1), which upon interaction of the α-subunit with an activated receptor is released in exchange for GTP. GDP release and GTP binding are the steps which initiate G protein activation. Subsequently, G protein α- and βγ-subunits dissociate and both α- and βγ-subunits can activate a plethora of effectors e.g. ion channels, phospholipase C, the MAP-kinase pathway, the adenyl cyclase system, which ultimately results in a cellular response, e.g. hormone secretion, contraction, proliferation, etc. [23].

G protein activation is terminated through the intrinsic GTPase activity of the α-subunit which hydrolyzes bound GTP to GDP. Following this step, G protein α- and βγ-subunits re-associate and are available for a new activation cycle through a G protein-coupled receptor. There exists a huge variety of different α-subunit isoforms, 5 β-subunits, and 13 γ-subunits encoded by different genes, which not only differ with regard to their molecular properties but also their tissue-specific expression [24]. For example, the G protein α-subunits Gzα12, Gzα3, Gzα11, and Gzα4 are widely expressed, whereas Gzα15,16 appear confined to haematopoetic cells, and Gzα9 is predominantly expressed in neuronal tissues. The β1-, β2- and β3-subunits are widely distributed, whereas the expression pattern of β4 is not very well known and β5 is confined to the brain. The different γ-subunits can not freely combine with any of the five β-subunits suggesting a selective preference of certain γ-subunits for specific β isoforms [25,26]. Receptor selectivity of G protein α-subunits is obviously obtained through their differential association with specific βγ-subunit combinations [27–29].

Activation of G proteins can be quantified in isolated cell membranes or permeabilized cells by different experimental approaches. One technique consists of using a radioactive, non-hydrolyzable GTP analog like GTP[S] which upon receptor activation combines with βγ-subunits and forms a rather stable complex as it is not hydrolyzed by the intrinsic GTPase activity of the α-subunit [30]. Thus, quantification of radioactive GTP[S] bound to activated α-subunits is a technically simple way of quantifying G protein activation.

Although the described experiments argued in favour of a molecular alteration in G protein subunits as the major reason for increased signal transduction in cells from subjects with HT. However, the molecular nature of this alteration was unpredictable and, even worse, these functionally studies did not provide any clues as to whether these alterations had to be searched in α- or βγ-subunits. At least, through Western blot analysis an overexpression of G protein α-subunits could be ruled out. Therefore, we systematically sequenced cDNAs encoding for different G protein α- and βγ-subunits from cell lines with ‘normal’ and ‘enhanced’ signal transduction.

The G protein β3 subunit gene (GNB3) and the C825T polymorphism.

Since mutations in the G protein α subunits Gzα12, Gzα3, β1, and β2 had been ruled out [21] we sequenced the cDNA encoding for Gβ3. The coding gene (GNB3) is located on chromosome 12p13 and the β3 protein has been reported to be ubiquitously expressed [31]. The gene consists of 11 exons, the start codon ATG being located in exon 3 and the stop codon TGA in exon 11 (Fig. 2). We detected in all cell lines with increased G protein activation the nucleotide T at position 825 of the cDNA although the published cDNA sequence [32] reported the nucleotide C at this respective position. All cell lines with low G protein reactivity were derived from homozygous C825 allele carriers. Originally this finding was rather confusing as the C825T exchange does not change the encoded amino acid (serine). Subsequently we observed that all individuals with a 825T allele expressed a truncated splice variant of Gβ3, termed Gβ3s, through a mechanism that remains to be completely understood [33]. Alternative splicing of the gene occurs through the use of a cryptic splice site located within exon 9 (Fig. 2), which is apparently activated in individuals carrying the 825T allele. Thus, 825T allele carriers express two gene products from GNB3, the 'wild-type' protein and

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Fig.1. The G protein activation—deactivation cycle heterotrimeric G proteins consist of α- and βγ-subunits. In the resting state, GDP is bound to the α-subunit. On receptor activation by a hormone (H), the receptor undergoes a conformational change, interacts with the G protein, and the α-subunit exchanges GDP for GTP. The α- and βγ-subunits dissociate and can then interact with a variety of targets like ion channels, phospholipase Cβ (PLCβ) isoforms or the mitogen-activated protein kinase (MAPK) pathway. This finally results in a specific cellular function or response. G protein activation is terminated through the hydrolysis of GTP to GDP and re-association of the βγ dimer with the α-subunit.
Fig. 2. Structure of GNB3. The gene encoding the \( \beta \)3-subunit of heterotrimeric G proteins consists of 11 exons, the start codon ATG being located in exon 3 and the stop codon TGA in exon 11. The C825T polymorphism is located within exon 10. Both a regularly but also an alternatively spliced G\( \beta \)3 mRNA is observed in 825T allele carriers. The C-T transition in exon 10 supports alternative splicing of exon 9 through the use of a cryptic splice site located in exon 9 (cDNA position 620). The alternatively spliced mRNA displays a deletion of 123 nucleotides corresponding to 41 amino acids. For further details see reference [33].

Fig. 3. Proposed structures of G\( \beta \)3 and G\( \beta \)3s. G\( \beta \)-subunits form a propeller-like structure with seven so-called propeller blades (top). The deletion resulting in the generation of G\( \beta \)3s is indicated (hatched background; top). This results in a novel G\( \beta \)3 protein with only 6 propeller blades (bottom).

Ethnic distribution of the 825T allele is in accordance with the ‘thrifty genotype hypothesis’

The thrifty genotype hypothesis originally set up by Neel [39], and extended by others [40], in principle proposes that certain genes enhancing salt retention or fat accumulation might have been crucial for the survival of our hunter/gatherer ancestors but may become detrimental in Westernized societies with an unrestricted access to food and salt in combination with a sedentary lifestyle characterized by a lack of physical activity. To study whether the 825T allele could represent such a thrifty genotype we conducted a world-wide co-operative study to determine the frequency of the 825T allele in different ethnicities and in non-human primates [41]. The 825T allele was absent in all non-human primates examined including chimpanzees, orangutan, and gorilla. However, relatively high frequencies of the 825T allele were found in ‘old’ populations such as bushmen, pygmies, and aborigines from Australia and Papua New Guinea [41]. Highest 825T allele frequencies in the range of 80–90% were
determined in all black African populations as well as in black Americans (Fig. 4). Intermediate 825T allele frequencies in the range of 40–60% are found in East Asia (Japan, Korea, China), in some American Indian tribes and in the Saudi Arabian population. Lowest 825T allele frequencies are present in Caucasian populations and average 30%. These findings have some important implications. The geographic distribution of the 825T allele is fully compatible with the ‘Out-of-Africa’ hypothesis of modern humans [42]. Moreover, the high 825T allele frequencies in blacks and in Australian aborigines may explain why individuals from these ethnicities are especially prone to the development of obesity and hypertension upon abandoning their original life style, moving to big cities and adopting the typical Westernized way of life [43-47].

**825T allele and hypertension—weak or strong association?**

Independent case-control and population-based studies in Caucasians have demonstrated an association of the 825T allele with hypertension, the odds ratios for homozygous 825T allele carriers versus homozygous C825 allele carriers being in the range of 1.8 - 2 [33,48,49]. In the study by Brand et al. no such association was found [50]. However, this was obviously due to a misclassification of normotensive controls which included subjects with borderline hypertension, i.e. diastolic blood pressure values between 90 and 94 mm Hg. A stronger association with HT and higher risk associated with a homozygous 825T allele status was reported for blacks living in the UK with odds ratios for HT in the range of 4 [51]. Similarly, high odds ratios for HT associated with the 825T allele were reported for Caucasians with a strong family history of HT [52].

It should be noted that such genetic case-control studies are not very much informative given that HT is a weak phenotype which can result from multiple hormonal and/or cellular alterations. For example, Bianchi’s group has been able to demonstrate that a common polymorphism in a-adducin may not always track with hypertension in association studies, but consistently indicates an enhanced responsiveness of subjects with HT to diuretics [53]. Therefore, it is clinically much more relevant to describe in detail additional phenotypes and risks associated with a certain gene alteration in subjects with HT rather than to repeat association studies which naturally will yield low odds ratios. In the case of the 825T allele these hypertension-associated phenotypes can be easily predicted. The phenotypes associated with high NHE activity have been fairly well characterized in the past and include obesity [13], salt retention and low plasma renin activity [6], as well as left ventricular hypertrophy (LVH) [9]. As enhanced NHE activity is ultimately caused by enhanced G protein activation indicated by the 825T allele it must be expected that these above mentioned traits should strongly accumulate in 825T allele carriers. In fact, independent studies have already shown an association of the 825T allele with obesity in different populations (see below). Likewise, Schunkert et al. described a significantly reduced renin activity in subjects carrying a 825T allele [49], suggesting that the 825T allele is associated with ‘low renin hypertension’. A recent report indicates a highly increased frequency if the 825T allele in hypertensive subjects with LVH [54]. If the reported results were representative, homozygous and heterozygous 825T allele carriers have a 6-fold and 3.4-fold, respectively, increased risk for LVH.

It is expected that upcoming reports will provide further insights into the hypertensive phenotypes associated with the 825T allele and potentially lead to the

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**Fig. 4.** Worldwide distribution of the 825T allele. Figures are 825T allele frequencies (%). See the text and ref. [41] for further details.
definition of a well described entity in ‘essential’ hypertension, i.e. ‘G protein hypertension’. For this hypothetical disorder, odds ratios associated with the 825T allele can be expected to be remarkably high.

825T allele and obesity

Although the cellular phenotype associated with the 825T allele—enhanced G protein activation—is exquisitely well understood, the pathogenetic mechanisms linking increased intracellular signal transduction to the development of high blood pressure remain to be understood. That this mechanism may be rather complex is nicely illustrated by a genetic association study conducted in the Canadian Oji-Cree. In that population-based study the 825T allele was associated with lower rather than increased blood pressure values except in hypertensive individuals in which highest 825T allele frequencies were observed [55]. This latter effect failed to reach statistical significance due to the low number of individuals with HT in the study group. Likewise, others found no association of the 825T allele with HT in Japanese individuals using a case-control study design [56]. On a first glance these apparently contradictory observations are confusing and may give rise to several interpretations and speculations. The predisposing effect of enhanced G protein activation for HT might be restricted to Caucasians due to the fact that genetic background differs drastically from Japanese and Canadian Indians. This argument of heterogeneity of genetic background is frequently used when a lack of association of a single genetic marker with a trait is reported, but what exactly this term describes is hardly defined. In the context of the 825T allele such an assumption appears not supported by published data because a strong association with HT was reported for blacks [57] and a convincing trend for increased 825T allele frequency was seen in Canadian Indians with HT [55]. On the other hand, the 825T allele may become detrimental only in conjunction with specific environmental or behavioral factors, e.g. a certain nutritional patterns or lack of physical exercise. Such an effect was recently shown for a common polymorphism in the gene encoding the β2-adrenoceptor which exerts no effect on body mass index in individuals with regular physical activity but is significantly associated with obesity in individuals who do not exercise regularly [58].

G proteins have been shown to play a key role in adipogenesis. Expression of constitutively active Gz3 subunits or overexpression of Gz3 subunits in stem cells induces an adipogenic conversion [59]. Vice versa, animals in which Gz3 is knocked out are runted and display reduced fat mass [60]. Since the 825T allele predicts enhanced G protein activation, especially of PTX-sensitive G proteins, we investigated whether 825T allele carriers have an increased risk of obesity. A strong association with obesity was found in subjects with HT, odds ratios for homozygous 825T allele carriers being in the range of 3 [61]. A significant association of the 825T allele with increased body mass index was independently observed in young German males, young Chinese, and young black African individuals from Zimbabwe and South Africa [41]. This latter study also demonstrated the enormous contribution of non-genetic factors to obesity. Young blacks from Zimbabwe displayed a high 825T allele frequency in the range of 80%. However, an effect of the 825T allele on body mass index was exclusively observed in individuals living in Zimbabwe’s capital, Harare, whereas no such effect was seen in individuals from rural Zimbabwe despite an identical 825T allele frequency [41]. This again underscores that lifestyle plus genetic predisposition are required for a susceptibility gene to exert its effect on a highly variable trait such as obesity and stays in line with the observation that urbanization is a major risk factor for obesity and hypertension in the African community [45–47]. In a different study design we investigated genetic and non-genetic factors predisposing young healthy Caucasian women for obesity. We observed a strong risk for obesity in homozygous 825T allele carriers following their first pregnancy which was completely counteracted by regular physical exercise [62]. Again, this study highlights the importance of thoroughly characterizing individuals in terms of non-genetic influences when an association between a single allele and a highly variable trait is examined.

Finally, it should be mentioned here that the association between the 825T allele and increased body fat was recently confirmed in Canadian Inuvit [63].

Taken together, it seems that the association between the 825T allele and obesity could be much stronger than that seen with hypertension, at least if unselected individuals not further characterized in terms of specific hypertensive phenotypes are investigated. Nevertheless, such an association would explain why the 825T allele is associated with late-onset rather than early HT [33]. Potentially, HT associated with the 825T allele results from a combination of hyperinsulinaemia and increased sympathetic tone which could also explain Na+ retention and low renin in hypertensive subjects carrying a 825T allele.

825T allele and diabetic nephropathy

Several considerations lead to the hypothesis that the 825T allele should be a good genetic marker for the prediction of onset of nephropathy in both type 1 and type 2 diabetes. As described above, the cellular phenotypes ultimately leading to the identification of the C825T polymorphism in GNB3 were enhanced NHE activity, increased intracellular signal transduction, and enhanced G protein reactivity. The same cellular phenotypes have been described to be associated with nephropathy in type 1 and type 2 diabetes.

Enhanced NHE activity has been demonstrated in platelets [64], erythrocytes [65], leukocytes [66], and skin fibroblasts [67] from patients with type 1 diabetes and nephropathy. Like in HT, this enhanced NHE
activity is preserved in immortalized cells [68,69] and associated with an increased proliferation [67,68]. These abnormalities appear to be caused by enhanced G protein activation especially in patients with both nephropathy and hypertension [70]. Likewise, cultured fibroblasts from patients with type 2 diabetes display enhanced NHE activity [71], as well as increased rises of cytosolic pH and cytosolic free calcium on stimulation with insulin or angiotensin II [72,73]. Finally, as hypertension or a family history of hypertension or cardiovascular events are major risk factors for the development of nephropathy [74], an association of nephropathy with the 825T allele appears more than likely. Bäthnert et al. in fact found a significantly increased frequency of the 825T allele (36.2%) in dialyzed patients with type 2 diabetes as compared to patients without microangiopathy (825T allele frequency = 28.6%). Using the published figures one can calculate an odds ratio of 11.8 for homozygous 825T allele carriers versus homozygous 825C allele carriers for end-stage renal disease [75]. Future studies will have to confirm these findings preferentially in a prospective study design.

For type 1 diabetes the issue remains to be settled. Fogarty et al. described similar 825T allele frequencies in patients with type 1 diabetes with or without nephropathy [76]. It should be emphasized, however, that 65% of 216 patients with nephropathy in their study sample suffered from end-stage renal disease. In addition, patients with nephropathy had significantly longer duration of diabetes, higher values of HbA1c, and the number of patients with hypertension was significantly increased compared to the non-nephropathic group. Unfortunately, genotype distribution for the group of patients displaying concurrently with nephropathy and hypertension was not given. Such studies can be severely confounded by a survivor effect associated with the C825 allele if a patient group with nephropathy comprises individuals with end-stage renal failure on dialysis. It was reported that the 825T allele is associated with an increased mortality in dialysis patients [77].

Again, prospective studies or appropriate retrospective studies are required to resolve the question whether or not the 825T allele is associated with nephropathy in patients with type 1 diabetes. The notion should be kept in mind, that a single nucleotide polymorphism may more likely predict when rather than whether a diabetic patient will develop nephropathy.

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