Sarcospan reduces dystrophic pathology: stabilization of the utrophin–glycoprotein complex

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Introduction

The dystrophin–glycoprotein complex (DGC) is composed of integral and peripheral membrane proteins that span the plasma membrane and connect the extracellular matrix with the intracellular actin cytoskeleton (Campbell and Kahl, 1989; Ervasti et al., 1990, 1991; Yoshida and Ozawa, 1990; Ervasti and Campbell, 1991, 1993). In skeletal muscle, the DGC provides mechanical stability to the sarcolemma during contraction (Petrof et al., 1993). Mutations in the dystrophin gene are responsible for X-linked Duchenne muscular dystrophy (DMD), which is characterized by progressive wasting of skeletal muscles eventually resulting in cardiac and respiratory failure (for review see Durbeej and Campbell, 2002). In DMD patients, loss of dystrophin results in the absence of the entire DGC complex, leading to severe membrane damage and muscle degeneration (for review see Durbeej and Campbell, 2002). mdx mice, which are an established model for DMD, possess a genetic mutation in exon 23 of the murine dystrophin gene (Campbell and Kahl, 1989; Ervasti et al., 1990, 1991; Yoshida and Ozawa, 1990; Ervasti and Campbell, 1991, 1993). In mdx mice, which are an established model for DMD, possess a genetic mutation in exon 23 of the murine dystrophin gene, resulting in loss of dystrophin protein. As a result, the entire DGC is also absent from the sarcolemma, likely because of rapid protein degradation in the absence of a fully assembled complex. Muscles from mdx mice are pathologically similar to DMD patients and display marked membrane disruption as a result of sarcolemmal instability. Akt signaling is hyperactivated in muscles from DMD patients and mdx mice (Peter and Crosbie, 2006), suggesting that the DGC may also play a role in cellular signaling in addition to its role in mechanical stability of the sarcolemma (Judge et al., 2006).

The transmembrane proteins of the DGC serve as important anchorages for the peripheral membrane DGC components. These integral membrane proteins include sarcospan (SSPN), the sarcoglycans (SGs; α-, β-, γ-, and δ-SG), and β-dystroglycan (DG; for review see Michele and Campbell, 2003). The SGs and β-DG are single-pass transmembrane glycoproteins. Dystrophin, an actin-binding protein, is localized adjacent to the sarcolemma by attachment to the intracellular N terminus of β-DG (for review see Michele and Campbell, 2003). On the extracellular face of the membrane, β-DG interacts with α-DG to form a receptor for ligands in the extracellular matrix (Ervasti and Campbell, 1993). The SGs form a tight subcomplex with SSPN (Croisbe et al., 1999; Miller et al., 2007). Together, the SG–SSPN subcomplex functions to anchor α-DG attachment to the sarclemma (Holt and Campbell, 1998). As a whole, the DGC provides a physical linkage across the sarcolemma between the extracellular matrix and the intracellular actin cytoskeleton protecting the membrane from contraction-induced damage (for review see Barresi and Campbell, 2006).

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Abbreviations used in this paper: DG, dystroglycan; DGC, dystrophin–glycoprotein complex; DMD, Duchenne muscular dystrophy; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; H&E, hematoxylin and eosin; SSPN, human SSPN; SG, sarcoglycan; SSPN, sarcospan; Tg, transgene; UGC, utrophin–glycoprotein complex.

The online version of this article contains supplemental material.
It is well established that stable interactions among the integral membrane proteins are critical for DGC function and prevention of muscular dystrophy (for review see Durbeej and Campbell, 2002). Despite their importance, the factors that determine the structural integrity of the DGC are not well understood. The observation that SSPN possesses some sequence homology to the tetraspanin superfamily of proteins raises the possibility that SSPN may serve an important role in mediating and stabilizing protein interactions within the DGC (Crosbie et al., 1997, 1998, 1999). The tetraspanins each possess four transmembrane domains and function to cluster and organize transmembrane protein complexes, thereby controlling a wide range of cellular functions (for reviews see Hemler, 2003; Levy and Shoham, 2005). Using a site-directed mutagenesis approach, we have demonstrated that SSPN exhibits the structural characteristics that define the tetraspanin superfamily of proteins (Miller et al., 2007).

As a first test of SSPN function, we generated SSPN transgenic (SSPN transgene [Tg]) mice with moderate (10-fold) levels of SSPN protein overexpression in skeletal muscle (Peter et al., 2007). Forced elevation of SSPN caused a concomitant increase in DGC protein expression but did not disrupt localization of the complex to the sarcolemma. We found that overexpression of exogenous SSPN dramatically reduced endogenous SSPN to levels that were barely detectable, suggesting that SSPN expression is tightly regulated. 10-fold elevation of SSPN disrupted normal interactions within the SG–SSPN subcomplex, which, in turn, weakened α-DG attachment to the sarcolemma (Peter et al., 2007). As a result, assembly of the extracellular matrix was disrupted, giving rise to severe congenital muscular dystrophy in mice with moderate levels of SSPN overexpression (Peter et al., 2007). Furthermore, membrane instability was not detected in 10-fold SSPN-Tg mice, demonstrating that pathogenetic mechanisms resulting from SSPN overexpression are distinct from dystrophin deficiency. Despite our exhaustive efforts, we were never able to isolate free, unassociated SSPN in 10-fold SSPN-Tg muscle, which strongly supports our conclusion that SSPN’s toxicity is directly related to its association with other molecules within the sarcolemma. SSPN-Tg mice with low levels of SSPN overexpression (two- to threefold) were also characterized (Peter et al., 2007). In contrast to SSPN-Tg mice with 10-fold levels of overexpression, SSPN-Tg mice with low levels of SSPN overexpression did not exhibit detectable signs of muscle pathology. DGC levels were mildly up-regulated in these mice, but interactions with the extracellular matrix were not adversely affected. Endogenous SSPN was not affected by this low level of exogenous SSPN expression. Collectively, these findings lead us to conclude that SSPN levels control DGC structure and function. Furthermore, this work leads to the hypothesis that SSPN may function to orchestrate assembly and stability of the DGC.

Results and discussion

Our previous work has shown that SSPN functions as a tetraspanin to coordinate protein interactions within the DGC (Peter et al., 2007), which led us to rationalize that SSPN expression in dystrophin-deficient muscle might anchor the transmembrane components of the DGC within the sarcolemma. To test this hypothesis, we engineered transgenic mice to overexpress SSPN on the dystrophin-deficient mdx background (SSPN-Tg:mdx). The mdx phenotype is inherited as an X-linked recessive trait, which stems from a premature stop codon in the dystrophin gene, leading to complete absence of the dystrophin protein. The DGC is absent from the sarcolemma of mdx muscle likely because of premature degradation of the protein components in the absence of a fully assembled complex (Ohlendieck and Campbell, 1991). For these experiments, we chose to use low (two- to threefold) SSPN-transgenic (SSPN-Tg) mice, lines 31.6 and 29.1, which displayed normal muscle morphology (Peter et al., 2007). SSPN-Tg mice were engineered to carry the human SSPN (hSSPN) gene under control of the human skeletal muscle α-actin promoter, which limited SSPN protein expression to skeletal muscles (Peter et al., 2007). Although human and mouse SSPN proteins are >90% identical at the amino acid level, antibodies specific to human and mouse SSPN permitted us to distinguish between exogenous (human) and endogenous (mouse) SSPN. SSPN-Tg males were crossed with mdx females to generate dystrophin-deficient mice carrying the SSPN Tg (SSPN-Tg:mdx).

We first investigated whether SSPN overexpression affected membrane association of DGC components in dystrophin-deficient muscle. Indirect immunofluorescence was performed on transverse cryosections of SSPN-Tg:mdx quadriceps muscle to determine the subcellular localization of the other DGC components. In addition to the core components of the DGC, we also analyzed the localization of utrophin, a protein closely related to dystrophin. At the postsynaptic region of the neuromuscular junction in wild-type and mdx muscle, utrophin replaces dystrophin to form an otherwise identical utrophin–glycoprotein complex (UGC; Matsumura et al., 1992). Analysis of wild-type non-Tg and SSPN-Tg muscle served as positive controls, and mdx muscle was used as a negative control. As shown in Fig. 1, DGC expression was robust in positive controls (wild-type non-Tg and SSPN-Tg) but was not detected in negative control samples (mdx and SSPN-Tg:mdx). As expected, staining for exogenous SSPN (hSSPN) was only detected in SSPN-Tg muscle, and utrophin expression was restricted to the neuromuscular junction in wild-type non-Tg and SSPN-Tg muscles (Fig. 1). In agreement with previous results (Matsumura et al., 1992), we found that utrophin expression was slightly up-regulated in mdx muscle at the extrasynaptic sarcolemma. Surprisingly, we found that overexpression of SSPN in mdx muscle broadened the localization of utrophin protein so that it was no longer restricted to the neuromuscular junction but was present throughout the sarcolemma (Fig. 1). Grady et al. (2000) have demonstrated that expression and localization of the UGC is important for proper synaptic formation and clustering of acetylcholine receptors at the postsynaptic membrane. Introduction of utrophin in dystrophin-deficient muscle broadens localization of the UGC to the extrasynaptic sarcolemma, where it ameliorates muscular dystrophy (Tinsley et al., 1996, 1998). The observation that utrophin is localized to the extrasynaptic sarcolemma in SSPN-Tg:mdx muscle raises the possibility that utrophin displays elevated protein levels leading to the observed increased
Figure 1. **SSPN stabilizes the UGC at the sarcolemma.** Transverse cryosections of quadriceps muscle from non-Tg (wild type), SSPN-Tg (wild type), mdx, and SSPN-Tg:mdx mice were stained with the antibodies to dystrophin (Dys), utrophin (Utrn), DGs (α- and β-DG), mouse SSPN (mSSPN), transgenic hSSPN, and SGs (α- and β-SG). Protein staining was visualized by indirect immunofluorescence. Bar, 100 μm.
sarcolemmal localization and possible functional replacement of the DGC. We also found that SSPN overexpression in mdx muscle increased α-/β-DG as well as α-/β-SG levels at the sarcolemma (Fig. 1). These data suggest that SSPN has restored expression of a complete UGC at the sarcolemma in mdx muscle, where it may functionally compensate for the loss of dystrophin and its associated proteins.

To determine how SSPN expression affects UGC protein levels, skeletal muscle lysates from 6-wk-old mdx and SSPN-Tg:mdx mice were analyzed by immunoblotting with antibodies to each of the UGC components. To confirm expression of the Tg, immunoblots were probed with antibodies specific to hSSPN. Exogenous SSPN was abundant in SSPN-Tg:mdx muscle samples, demonstrating that stable SSPN protein was produced from the Tg (Fig. 2 A). Levels of α- and β-DG as well as α-, β-, and γ-SG were dramatically increased in SSPN-Tg:mdx mice relative to mdx controls (Fig. 2 A). DG and SG protein expression in SSPN-Tg:mdx muscle was identical to age-matched non-Tg (wild type) muscle (Fig. 2 A and not depicted). As expected, dystrophin protein was not detected in samples from either mdx or SSPN-Tg:mdx mice. Utrophin protein levels were elevated in SSPN-Tg:mdx muscle relative to mdx muscle (Fig. 2 A). To investigate whether SSPN increased utrophin expression by enhancing transcription, we performed quantitative RT-PCR analysis. Utrophin mRNA levels were similar in SSPN-Tg:mdx and mdx muscle (Fig. 2 B). This finding demonstrates that utrophin up-regulation by SSPN does not involve transcriptional regulation. Furthermore, these data suggest that SSPN maintains utrophin protein at the sarcolemma by providing a tetraspanin scaffold, which stabilizes and promotes protein interactions.

We established that SSPN is a core component of the DGC using rigorous biochemical methods (Crosbie et al., 1997, 1998).
To investigate the functional consequences of increased UGC levels in dystrophin-deficient muscle, we examined SSPN-Tg:mdx muscle for dystrophic pathology. Many necrotic patches are visible in mdx quadriceps. Necrotic fibers were never observed in SSPN-Tg:mdx quadriceps. Boxed regions indicated on H&E staining of quadriceps muscle from mdx and SSPN-Tg:mdx mice are shown at higher magnifications in B. Note the presence of numerous myofibers with central nucleation as well as the variation in fiber size evident in mdx tissue. Muscle from SSPN-Tg:mdx muscle displays reduced central nucleation and improvements in fiber size variation. Bar, 100 μm.

Figure 3. SSPN-Tg:mdx mice display near normal muscle pathology. (A) Transverse cryosections of whole quadriceps muscle from age-matched mdx and SSPN-Tg:mdx mice were stained with H&E to visualize muscle pathology. Many necrotic patches are visible in mdx quadriceps. Necrotic fibers were never observed in SSPN-Tg:mdx quadriceps. Boxed regions indicated on H&E staining of quadriceps muscle from mdx and SSPN-Tg:mdx mice are shown at higher magnifications in B. Note the presence of numerous myofibers with central nucleation as well as the variation in fiber size evident in mdx tissue. Muscle from SSPN-Tg:mdx muscle displays reduced central nucleation and improvements in fiber size variation. Bar, 100 μm.

In normal skeletal muscle, SSPN is enriched at the postsynaptic region of the neuromuscular junction in a utrophin-dependent manner (Crosbie et al., 1999). Our findings that SSPN expression alters utrophin localization and, conversely, that SSPN enrichment at the neuromuscular junction is dependent on utrophin suggest that SSPN functions to regulate utrophin through direct association with the UGC. To biochemically determine whether SSPN is a core component of the UGC, we isolated this complex from skeletal muscle of SSPN-Tg:mdx mice using lectin affinity chromatography followed by ultracentrifugation on 5–30% sucrose gradients. Only proteins that are tightly associated will be maintained as a complex and migrate together during ultracentrifugation (Crosbie et al., 1997, 1998). Fractions were collected and analyzed by immunoblotting with antibodies to components of the UGC. We found that SSPN protein is highly enriched in fraction 4, which contains utrophin and utrophin-associated proteins (Fig. 2 C). We conclude that SSPN regulates utrophin levels in a mechanism that involves direct association of SSPN with the UGC.

To investigate the functional consequences of increased UGC levels in dystrophin-deficient muscle, we examined SSPN-Tg:mdx muscle for dystrophic pathology. mdx pathology is characterized by progressive muscle degeneration, compensatory hypertrophy, and necrosis of damaged muscle fibers. Histological analysis was performed by staining transverse cryosections of quadriceps muscle with hematoxylin and eosin (H&E). Images of whole quadriceps muscle taken from mdx mice revealed numerous patches of necrosis, which were not present in SSPN-Tg:mdx muscle (Fig. 3 A). Central nucleation, a marker of myofiber regeneration, was quantitated by analyzing H&E-stained quadriceps muscle (Crosbie et al., 1999). We found that SSPN-Tg:mdx muscle displays reduced central nucleation and improvements in fiber size variation. Bar, 100 μm.
fibers provides an additional measure of muscle pathology and 
reduction of small, newly regenerating fibers (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb
In addition, expression of SSPN in mdx mice display a threefold reduction in central nuclei compared with mdx age-matched controls. Each value represents mean ± SEM (error bars) of the total quadriceps analyzed [*, P = 6.0 × 10^{-4}]. (B) To examine infiltration of blood serum proteins into damaged muscle fibers, mdx and SSPN-Tg:mdx mice were intraperitoneally injected with Evans blue dye, a marker for membrane instability. mdx quadriceps displays many Evans blue dye–positive fibers (visualized by red fluorescence), which is a marker for membrane damage. Evans blue dye was not detected in muscle from SSPN-Tg:mdx mice, demonstrating that SSPN expression restored membrane stability in dystrophin-deficient muscle. Bar, 20 μm.

In addition, expression of SSPN in mdx mice was associated with a reduction in the number of smaller, atrophic muscle fibers (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb_200808027/DC1). This reduction of small, newly regenerating fibers provides an additional measure of muscle pathology and is similar to the results reported for microdystrophin (Gregorevic et al., 2006).

Loss of the DGC from the sarcolemma causes membrane instability, which is the primary defect of dystrophin-deficient muscular dystrophy (Weller et al., 1990; Menke and Jockusch, 1991; Petrof et al., 1993; Straub et al., 1997). Unrepaired tears in the sarcolemma allow proteins that are normally restricted to the blood serum to freely diffuse across the sarcolemma and accumulate in the sarcoplasm. To further test the functionality of the UGC in SSPN-Tg:mdx mice, we performed a tracer assay with fluorescent Evans blue dye that binds albumin in blood serum (Straub et al., 1997). mdx mice displayed severe sarcolemmal fragility marked by Evans blue dye accumulation in numerous muscle fibers (Fig. 4 B). In contrast, we never observed Evans blue–positive fibers in SSPN-Tg:mdx tissue (Fig. 4 B).

In this study, we provide biochemical evidence that SSPN ameliorates dystrophic pathology in mdx mice by stabilizing the UGC at the sarcolemma through direct interaction with this complex. We demonstrate that SSPN causes redistribution of the UGC to the extrasynaptic sarcolemma, where it functionally replaces the DGC. The primary defect in dystrophin-deficient mice is the loss of sarcolemma integrity, and we demonstrate that SSPN provides stability to the sarcolemma. These data suggest that the actin-UGC–extracellular matrix connection is fully and functionally restored. Future studies will be performed to determine whether SSPN overexpression improves muscle strength in mdx mice.

Tinsley et al. (1996, 1998) have shown that increased expression of utrophin in mdx mice rescues muscular dystrophy by stabilizing the UGC at the sarcolemma in a dose-dependent manner. Complete reduction of mdx pathology to normal wild-type levels (as determined by central nucleation and muscle strength measurements) occurred when utrophin was overexpressed at twice the levels of mdx mice (note that endogenous utrophin levels are already up-regulated in mdx muscle compared with wild-type controls). Their data nicely show that pathology was directly correlated with the level of utrophin overexpression. Lower levels of utrophin expression reduced the pathology, but not completely back to normal levels. Based on these findings, we speculate that increasing the level of SSPN overexpression would have an even greater impact on improving mdx pathology.

Overexpression of DGC proteins, signaling molecules, and compensatory molecules can improve the pathology of muscle fibers in the mdx mouse. Utrophin, dystrophin, integrin, neuronal nitric oxide synthase, and cytotoxic T cell N-acetylgalactosamine transferase have been shown to improve muscular dystrophy. Most of these efforts have been focused on the use of dystrophin and utrophin to restore normal muscle function in mdx mice. Elegant work from Chamberlain’s group has established that full-length (DelloRusso et al., 2002) and internally truncated forms of dystrophin are highly effective at ameliorating mdx pathology in addition to increasing muscle strength (for review see Odom et al., 2007). SSPN is unique and offers numerous advantages over current therapeutic strategies. The SSPN cDNA is under 1 kb, which is well within the range for easy packaging into adeno-associated viral vectors for delivery, circumventing the necessity to generate recombinant forms of SSPN. Because SSPN is expressed in a variety of nonmuscle tissues, even in dystrophin deficiency, increasing the expression of SSPN in skeletal muscle should not pose an immune threat. One therapeutic angle for the treatment of DMD is to identify small molecules that up-regulate utrophin protein expression. Our findings now establish that SSPN regulates utrophin protein levels and localization within the myofiber.

The results in this study were unexpected because, for nearly 10 yr, the precise function of SSPN has remained elusive. Our experiments show that SSPN is a critical mediator of protein
interactions within UGC and raise the possibility that SSPN contributes to the assembly and targeting of the UGC. This is impressive considering that SSPN, the smallest member of the complex, must orchestrate proper structural arrangement of over seven large peripheral and integral membrane proteins. These properties are unique to SSPN and suggest that gene therapeutic strategies targeting SSPN expression may be beneficial for the treatment of DMD.

**Materials and methods**

**Generation of SSPN-Tg:mdx mice**

Transgenic constructs were engineered with the human skeletal actin promoter and the VP1 intron upstream of HSSPN as described previously (Crawford et al., 2000; Spencer et al., 2002; Peter et al., 2007). SSPN transgenic males, line 29.1, were bred with 

**Immunofluorescence**

Quadriiceps from female SSPN-Tg (wild type), female non-Tg (wild type), male non-Tg:mdx, and SSPN-Tg:mdx littersmates were dissected from 6- and 7-μm transverse quadriceps sections were left at RT for 15 min and mounted with Vectashield. Evans blue–positive myofibers were observed in entire quadriceps images were attained under identical conditions using an Axioplan 2 fluorescent microscope and Axiowision 3.0 software (Carl Zeiss, Inc.). The percentage of centrally nucleated fibers and fiber areas were assessed for four SSPN-Tg:mdx and four non-Tg:mdx mice. Data for central nucleation percentages were averaged, and the standard error is represented. Sigma Plot software (Systat Software, Inc.) was used to perform statistical analysis. Entire quadriceps images were attained using a microscope (Axioskop M1, Carl Zeiss, Inc.) and Axiowision Rel. 4.5 software (Carl Zeiss, Inc.).

**Protein preparation**

Male SSPN–Tg:mdx mice were used as controls. Mice were analyzed at 6 wk of age. Transgenic mice used for this study expressed an approximately twofold increase in SSPN protein expression in skeletal muscle compared with non-Tg (wild type) mice (Peter et al., 2007). All mice were maintained in the Life Sciences Vivarium, and all procedures were carried out in accordance with guidelines set by the University of California, Los Angeles Institutional Animal Care and Use Committee.

**Immunofluorescence**

Quadriiceps from female SSPN-Tg (wild type), female non-Tg (wild type), male non-Tg:mdx, and SSPN-Tg:mdx littersmates were dissected from 6- and 7-μm transverse quadriceps sections were prepared using a cryostat (CM 3050S; Leica) and stored on positively charged glass slides (VWR). Sections were stored at −80°C for future analysis. Sections were acclimated to RT for 15 min and blocked using 3% BSA diluted in PBS for 1 h at RT. Sections were then incubated at 4°C for 18 h with antibodies to the following proteins (antibody dilutions are indicated): dystrophin (MANDYS1, 1:10), utrophin (NCL-DRP2, 1:5), Vector Laboratories), α-DG (VP-A105, 1:100; Vector Laboratories), β-DG (MAB361, 1:50; Developmental Studies Hybridoma Bank), α-SG (VP-A105, 1:100; Vector Laboratories), β-SG (VP-B260, 1:50; Vector Laboratories), HSSPN (affinity-purified rabbit 15, 1:25), and mouse SSPN (affinity-purified rabbit 18, 1:25). Polyclonal antibodies to endogenous (mouse) and exogenous (human) SSPN have been described previously (Peter et al., 2007). Primary antibodies were detected by FITC-conjugated anti-rabbit and anti-mouse (Jackson ImmunoResearch Laboratories and Millipore) secondary antibody dilutions at 1:500. Secondary antibodies were incubated for 1 h at RT. To preserve the fluorescence signal, sections were mounted in Vectashield (Vector Laboratories). To determine the level of nonspecific staining, secondary antibodies alone were incubated with sections. Mounted sections were visualized using a fluorescent microscope (Axioplan 2; Carl Zeiss, Inc.) equipped with a Plan Neofluar 40x NA 1.3 oil differential interference contrast objective, and images were captured using Axiowison 3.0 software (Carl Zeiss, Inc.).

**Evans blue tracer assay**

To establish sarcolemmal integrity, mice were injected with 50 μl of Evans blue dye (10 mg/ml in 10 mM of sterile phosphate buffer and 150 mM NaCl, pH 7.4) per 10 g of body weight as described previously (Straub et al., 1997). Peritoneal cavity injection was performed on 6-wk-old SSPN–Tg:mdx and non-Tg:mdx litters. 24 h after injection, quadriicepses were excised and mounted as described in the Immunofluorescence section. 8-μm transverse sections were briefly fixed in ice-cold acetone, washed in PBS, and then mounted with Vectashield. Evans blue–positive myofibers were observed using an Axioplan 2 fluorescent microscope and a Plan Neofluar 40x NA 1.3 oil differential interference contrast objective.

**Histology**

H&E staining was used for visualization of fibrosis, central nuclei, and fiber diameter. 8-μm transverse quadriiceps sections were left at RT for 15 min before beginning the staining procedure. Sections were incubated with hematoxylin for 3 min, washed with water for 1 min, incubated with eosin for 3 min, and dehydrated in solutions of 70, 80, 90, and 100% ethanol. Sections were then incubated in xylene for a total of 6 min. Sections were left at RT briefly to dry before mounting with Permount. All supplies for the H&E staining were purchased from Thermo Fisher Scientific.
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