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Short Communication

Preferential selection of Cys-constrained peptides from a random phage-displayed library by anti-glucitollysine antibodies

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Abstract: Phage-displayed peptides recognized by two monoclonal antibodies against glucitollysine were selected. The most prominent feature of the peptide panel was the presence of paired Cys in most of them (21/24 peptides). The availability of a wide variety of peptides having differently spaced paired Cys, as well as truly linear Cys-free peptides, gave the opportunity to explore the role of disulfide bridges in phage selection. Some Cys-containing peptides came from a Cys-flanked cyclic 9-mer library, but most of them (18/21) were derived from a totally random 12-mer library, and hence the presence of Cys was dictated by the selector antibodies. Motifs shared by several peptides (potentially involved in binding) often contained or were flanked by Cys residues. Binding of all Cys-containing phage-displayed peptides was abolished/decreased after a reducing treatment. Screening a random peptide library (without invariant Cys residues) is powerful enough to clearly reveal the need, preferences, and diversity of Cys-mediated structural constraints for recognition. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: phage display; peptide library; disulfide bridges; glucitollysine

INTRODUCTION

Phage display technology [1] opened a new avenue for mapping and mimicking antibody epitopes using peptide libraries [2]. Such techniques are useful to locate epitopes [3], to characterize paratopes [4], and to obtain vaccine candidates [5]. Libraries can display either totally random sequences or structurally constrained peptides that usually have loops due to the formation of disulfide bridges between two invariant Cys [6].

We report the use of two peptide libraries, displaying either random 12-mer [7] or cyclic Cys-flanked 9-mer sequences [8], to isolate peptides recognized by two monoclonal antibodies (Mabs) directed against protein-attached glucitollysine [9]. This antigenic structure is obtained through *in vitro* reduction of the products of nonenzymatic protein glycation (the reaction of free glucose with the epsilon amino group of lysine), a natural modification exacerbated in diabetic individuals [10]. Both Mabs compete for binding to the same or closely related overlapping epitope(s) formed by glucitollysine in the context of protein sequences (independently of the exact amino acid sequence) [9]. Peptides recognized by Mabs directed against nonpeptide epitopes have been used as probes to study the diversity of molecular binding properties of paratopes [11].

RESULTS AND DISCUSSION

From 20 to 60% of the clones obtained after two selection rounds from each library were able to produce phages recognized by each selector Mab in enzyme-linked immunosorbent assay (ELISA) (Figure 1), without cross-recognition neither by the other anti-glucitollysine Mab nor by a control Mab.

Greater than 85% phage binding inhibition by a high concentration (250 μg/ml) of glucitollysine-BSA confirmed that phage-displayed peptides compete for Mabs' paratopes with the natural antigen (Figure 2). Binding of some peptide-displaying phages was similarly inhibited by a tenfold lower natural antigen concentration, whereas others were only partially inhibited (down to 24% inhibition). This can be explained by differences in the affinity/avidity of phages for the selector Mabs, due to the intrinsic affinity and display level of each peptide. The control, reduced nonglycated BSA (at 250 μg/ml), did not produce any inhibition, excluding nonspecific ELISA interference by treated BSA.

The isolation of two nonoverlapping peptide sets, reacting only with the selector Mab, resembles the results with other families of closely related antibodies recognizing small antigens [11]. Peptides could mimic

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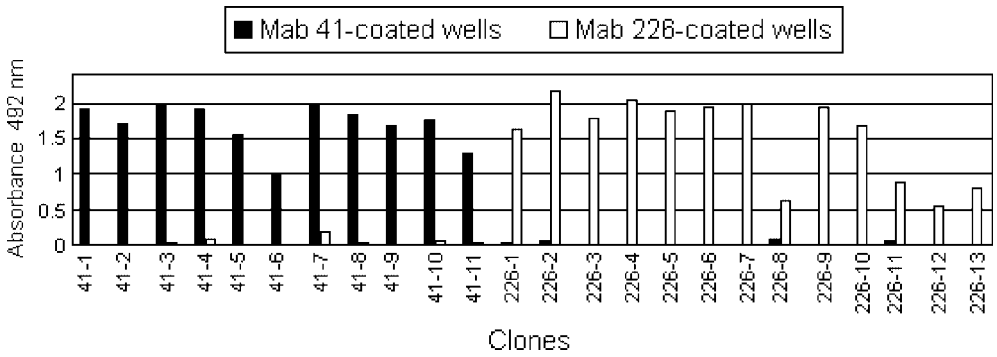

Figure 1 Immunoreactivity of selected phages. Purified phages (2×10^{10} cfu/ml) were tested on ELISA plates coated with anti-glucitollysine Mabs and with an unrelated control Mab. Bound phages were detected with an horseradish peroxidase (HRP)-labeled anti-M13 antibody. Background absorbance levels for each coating antibody (without any phage sample) were subtracted from absorbance values obtained for each clone. All the clones produced absorbances below background level when tested with the control Mab. Clones 41-n and 226-n were selected by Mabs 41 and 226 respectively.

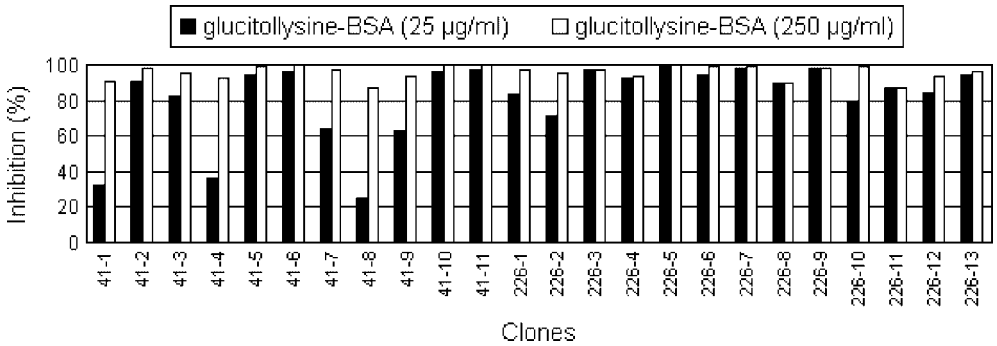

Figure 2 Competition between phage-displayed peptides and the natural antigen. Purified phages (10^{11} cfu/ml) were incubated on ELISA plates coated with the corresponding selector Mab, in the presence or absence of glucitollysine-BSA. Bound phages were detected with an HRP-labeled anti-M13 antibody. Phage binding inhibition by glucitollysine-BSA (%) was calculated.

different structural features of the natural antigen, or they could establish novel interactions with paratopes, not necessarily related to the original antigen–antibody reactions. Positive clones from the cyclic library produced moderate and roughly homogeneous absorbance (Abs) values, whereas the totally random library rendered a wider diversity of binding intensities. Representative clones from both libraries were chosen for further characterization.

After sequencing peptide-coding inserts, recurrent motifs were clearly distinguishable among Mab 41-selected peptides, being Thr–Ser–Arg the most abundant one (Table 1). Strikingly, such TSR sequences are often located between two Cys residues, even in peptides from the random library without deliberately introduced Cys. This finding suggested a role for disulfide bond-mediated internal cycles in recognition. Only two TSR-containing peptides (clones 41-5 and 41-6) do not have Cys residues and seem to be truly linear. TSR sequences are frequently contained into the extended CTSRXC motif, and sometimes located between two more separated Cys. A second Cys-flanked repeated motif is CXHSRC. The single peptide (clone 41-11) repeatedly isolated from the cyclic Cys-flanked 9-mer library includes the TSR motif.

The common motif CYKC was recurrently found among peptides selected by Mab 226 (Table 2). The presence of paired Cys suggested again cycle formation. Another peptide does not strictly contain the motif, but partially resembles it, having a CYK sequence followed by a second Cys separated by four additional aa. Two peptides shared a second common motif (His–Arg–Asn), also located between paired Cys. Only one peptide (clone 226-10) does not contain Cys residues. The cyclic library rendered two additional peptides (clones 226-12 and 226-13), sharing a short Ser–Arg motif.

The most prominent feature of the peptide panel as a whole is the recurrent presence of paired Cys (found in 18/21 unique clones isolated from the 12-mer random library). Similar bias was not found in a sample of the original library. Cys were present in 5/16 unselected clones, with only 1 clone including a pair of them (separated by 10 aa). The abundance of paired Cys in our panel is thus a selection-driven feature. Besides their number, the location of such paired Cys, being included in -or flanking- common motifs shared by several peptides (potentially involved

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Table 1 Phage-displayed peptides recognized by Mab 41

Library	Peptide sequence	Frequency	Identified motif	Representative clone
12-mer	DS**C**TSRT**C**PYLI	7/22	CTSRXC	41-1
	DYTN**C**TSRD**C**AH	6/22	CTSRXC	41-2
	GHY**C**TSRH**C**SII	1/22	CTSRXC	41-3
	RGDN**C**TSRH**C**QA	1/22	CTSRXC	41-4
	FITSRSHPASDA	1/22	TSR	41-5
	FDLTSRSDIPIY	1/22	TSR	41-6
	CVPTSRAD**C**FKS	1/22	TSR	41-7
	VR**C**QETSRVHA**C**	1/22	TSR	41-8
	CVHSR**C**SIQVAW	2/22	CXHSRC	41-9
	GTK**C**MHSR**C**SLH	1/22	CXHSRC	41-10
Cys-flanked 9-mer	**C**VETSRVPVF**C**	6/6	TSR	41-11

Peptide-coding DNA segments from Mab 41-positive clones (22 clones from the 12-mer library and six from the Cys-flanked 9-mer library) were sequenced and aa sequences were deduced. Repeated motifs are underlined. Cys residues are in bold.

Table 2 Phage-displayed peptides recognized by Mab 226

Library	Peptide sequence	Frequency	Identified motif	Representative clone
12-mer	**C**YK**C**DSVGYLDR	2/16	CYKC	226-1
	CYK**C**AKHVNVGL	1/16	CYKC	226-2
	CYK**C**YSSVNFSL	1/16	CYKC	226-3
	CYK**C**MSSDAPMT	1/16	CYKC	226-4
	CYK**C**PQRMSDGG	1/16	CYKC	226-5
	CYK**C**TMGNEVYA	1/16	CYKC	226-6
	SF**C**YKGFGV**C**NP	1/16	CYK (partial)	226-7
	LRET**C**KHRNDI**C**	4/16	HRN	226-8
	CHRNALYD**C**PIT	1/16	HRN	226-9
	RPIYREKDDIRA	2/16	none	226-10
	V**C**GSHHG**C**QRPF	1/16	none	226-11
Cys-flanked 9-mer	**C**PMGRLGSRG**C**	6/7	SR	226-12
	CFSSRPSLPL**C**	1/7	SR	226-13

Peptide-coding DNA segments from Mab 226-positive clones (16 clones from the 12-mer library and 7 from the Cys-flanked 9-mer library) were sequenced and aa sequences were deduced. Repeated motifs are underlined. Cys residues are in bold.

in binding), suggested a role for Cys residues in peptide interactions.

Structural constraints related to the formation of disulfide bonds by deliberately introduced Cys in the so-called cyclic libraries are generally assumed to play a role in phage binding [8]. Despite similar bridges could be formed by selection-driven paired Cys often retrieved from random libraries [12], their role has not always been studied [4]. Selection of paired Cys-containing peptides with anti-HIV antisera (even from a library with low Cys content) highlights the importance of such structural constraints [13]. This result is easy to explain, since the selected motif reproduces a natural Cys-flanked loop of the gp41 HIV antigen.

Our peptide panel, selected by two Mabs against a small nonpeptide antigen, provides a different context to explore the role of disulfide bonds. It includes a wide variety of peptides with differently spaced paired Cys, as well as truly linear Cys-free sequences. Some studies about the role of disulfide bonds in peptide antigenicity have relied on the use of cyclic/linear synthetic peptides [14] or immunoblotting with reduced/unreduced phages [12,15]. Phages directly coated onto ELISA plates (and subsequently reduced or not) have also been used [14]. This is a simple method useful to evaluate multiple samples and conditions. Our approach was similar, except for introducing a phage alkylation step to preclude disulfide bonds re-formation. Recognition of all paired Cys-containing peptides by Mab 41 was virtually abolished (antigenicity was below 7%) by dithiothreitol (DTT)/iodoacetamide treatment of coating phages (Figure 3). A modest antigenicity decrease (more than 65% of antigenicity was preserved) was obtained for

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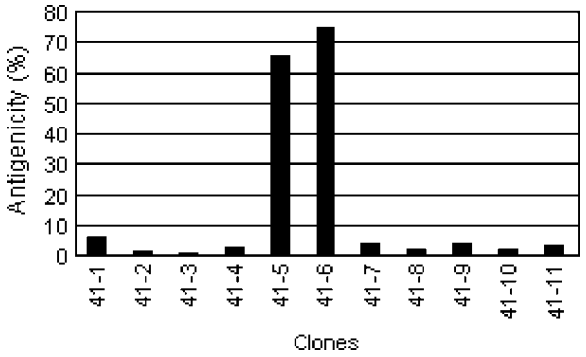

Figure 3 Effect of reducing disulfide bonds on the antigenicity of phage-displayed peptides. ELISA plates coated with purified phages (10^{12} cfu/ml) were treated with DTT and iodoacetamide. Mab 41 was added, and detected with an HRP-labeled anti-mouse IgG antibody. Antigenicity of treated phages (%), as compared with untreated controls, was calculated.

phages displaying linear Cys-free peptides. Since such peptides were not directly modified by the treatment, peptide-independent coating alterations explain the partial effect.

The usefulness of the method described above is not universal, since direct phage coating can produce negative ELISA results, as for most Mab 226-selected phages (data not shown). Phage treatment with DTT and iodoacetamide in solution, prior to evaluation in a classical capture assay on Mab 226-coated plates, can circumvent the problem. The single Cys-free phage-displayed peptide conserved most of its antigenicity (95%), whereas clones displaying paired Cys were severely affected (Figure 4), keeping less than 30% of antigenicity. Recognition of treated phages by a coating anti-M13 Mab was not affected at all, excluding any nonspecific treatment interference in the ELISA. Starting with concentrated phage samples (subsequently diluted in reducing and alkylating solutions, and sample buffer) avoided dialysis steps to eliminate chemicals.

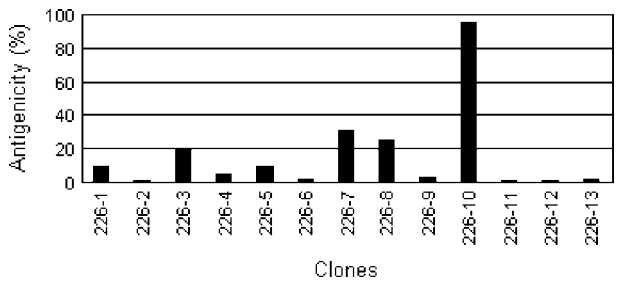

Figure 4 Effect of reducing disulfide bonds on the antigenicity of phage-displayed peptides. Purified phages (2×10^{11} cfu/ml), either untreated or treated in solution with DTT and iodoacetamide, were tested on Mab 226-coated plates. Bound phages were detected with an HRP-labeled anti-M13 antibody. Antigenicity of treated phages (%), as compared with untreated controls, was calculated.

These results showed the importance of structural constraints imposed by disulfide bonds in peptide recognition by both anti-glucitollysine Mabs. A clear-cut distinction between Cys-free peptides and paired Cys-containing peptides was obtained with both methods, which can be used in a high throughput way since the initial phage screening stage. The usefulness of these simple methods could be extended beyond peptide antigenicity in order to explore the importance of similar structural constraints for other interactions.

A critical practical issue is the choice between random 'linear' and Cys-flanked cyclic peptide libraries. It is generally assumed that loops' formation in Cys-constrained libraries would limit mobility and improve binding [8]. Cyclic libraries have been more useful than totally random libraries in certain cases [16]. Nevertheless, the survey of cyclic structures from totally random libraries could be even more complete than the screening of cyclic libraries with an invariant inter-Cys distance.

Our work was not focused on the strict comparison of the performances of the two libraries (cyclic *vs* random) we used, which are also different in terms of peptide insert length and codon composition biases. Cys-flanked 9-mer library was used just as an example of a classical cyclic library, which was designed to offer mainly a single solution (loop formation between the two flanking Cys) to the requirement of structural constraints for peptide recognition. Our results illustrate the potential of totally random libraries to render a wide variety of linear and cyclic peptides (including different cycle lengths and variable cycle-flanking contexts). Combining several cyclic peptide libraries (with differently spaced Cys) is possible, but retrieving such binders' diversity from a single library is very attractive.

If the optimal cycle length for an interaction does not coincide with the lengths defined *a priori* in cyclic libraries, the isolation of shorter/longer cycles formed by internal/external Cys residues (although theoretically possible) is hampered by the display of mixtures of heterogeneous loops formed by three or more Cys. Despite paired Cys separated by two and four aa clearly predominated among peptides selected by Mabs 226 and 41 respectively, no similar short cycles were obtained from the Cys-flanked 9-mer library. The failure to obtain binders from a Cys-flanked 7-mer library using antisera able to select a Cys-flanked 5-mer motif from random libraries was reported [13].

Structural constraints needed for optimal display of different interacting motifs are variable. The use of single cycle length libraries could preclude the identification of some motifs. Screening of the Cys-flanked 9-mer library did not identify two motifs putatively involved in strong interactions with Mab 226 in the context of closer paired Cys. On the other hand, the presence of the same motif in Cys-constrained and

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truly linear peptides of Mab 41-selected panel implies that disulfide bridges' formation is not an obligatory condition for recognition of this motif, but modulates its appropriate display in certain contexts.

Screening a conventional random peptide library (not intentionally Cys-flanked) is powerful enough to clearly reveal the need and/or preferences of Cys-mediated structural constraints for recognition by Mabs. Libraries having a single invariant Cys can render peptides with additional Cys residues (able to form different cyclic structures) [11]. This approach, although less frequently used than paired Cys-containing cyclic libraries, could be a very efficient way to survey disulfide bridge-constrained peptides.

EXPERIMENTAL

Antibodies and Libraries

Anti-glucitollysine Mabs 41 and 226 were supplied by the Center of Genetic Engineering and Biotechnology (Sancti Spiritus, Cuba). Two phage-displayed peptide libraries were used, bearing either 12-mer random peptides [7] or 9-mer random sequences flanked by two Cys residues [8] inserted at the *N*-terminus of the major phage coat protein pVIII. Both libraries contained 10^8 clones.

Microorganisms and Chemicals

Escherichia coli TG1 strain (K12_(*lac-pro*), *sup*E, *thi*, *hsd*D5/F′ *tra*D36, *pro*A + B+, *lac*I^q, *lacZ*_M15) and M13-K07 helper phage were used (Pharmacia, Sweden). Chemicals and culture media were purchased from Sigma (USA), Merck (Germany), and Oxoid (UK).

Preparation of Glucitollysine-BSA

BSA (fraction V) diluted at 5 mg/ml in phosphate buffered saline (PBS) containing 2 mol/l glucose was filter-sterilized, incubated during 15 days at 37 °C, and dialyzed against PBS. The reducing agent sodium cyanoborohydride ($NaCNBH_3$) was added at a final concentration of 100 mmol/l and incubated during 2 h at room temperature (RT), followed by dialysis against PBS. Recognition of treated BSA by anti-glucitollysine Mabs was confirmed by ELISA [9]. Reduced nonglycated BSA was prepared in similar conditions (excluding glucose) to be used as a negative control.

Phage Selection

Peptide-displaying phage particles were rescued from libraries with M13-K07 helper phage and purified [17]. Immunotubes (Nunc, Denmark) were coated overnight at 4 °C with each anti-glucitollysine Mab at 10 μg/ml in PBS. Purified phages [5×10^{12} colony forming units (cfu) for each selection] and coated immunotubes were blocked with PBS containing 2% (w/v) skim powder milk (M-PBS) during 1 h at RT. Blocked phages from each library were added to blocked immunotubes, and incubated 1 h at RT. The tubes were washed twenty times with PBS containing 0.1% (v/v) Tween 20 (PBS-T) and twice with PBS. Bound phages were eluted with 100 mmol/l triethylamine during 10 min at RT, and neutralized with 1 mol/l Tris, pH 7.5. Exponentially growing TG1 cells were used to rescue selected phages [17], in order to start a second selection round in the same conditions.

Phage Screening

After the second selection round, phage particles were produced at a 96 well scale from phagemid-transformed colonies [17]. Medium binding ELISA plates (Costar, USA) were coated overnight at 4 °C with Mabs 41, 226, and the unrelated anti-glycolyl GM3 ganglioside Mab 14F7, at 10 μg/ml in PBS. The plates were blocked with M-PBS during 1 h at RT. Phage-containing supernatants, diluted 1/2 in M-PBS, were added. After incubating 1 h at RT, the plates were washed with PBS-T and an anti-M13 Mab conjugated to horseradish peroxidase (HRP, Pharmacia, Sweden), appropriately diluted in M-PBS, was added. The plates were incubated 1h at RT, washed again, and the substrate solution (500 μg/ml *ortho*-phenylenediamine and 0.015% (v/v) hydrogen peroxide in 0.1 mol/l citrate buffer, pH 5.5) was added. The reaction was stopped 15 min later with 2.5 mol/l sulfuric acid, and absorbances at 492 nm were read. Clones producing absorbances higher than threefold over the background (assessed with an unrelated phage-containing supernatant) were considered to be positive.

Phagemid DNA was purified from 51 positive clones (from both libraries) and sixteen 12-mer random library unselected clones, using Mini Preps kit (Promega, USA). Peptide-coding inserts were sequenced by Macrogen (Seoul, Korea). Positive clones having unique peptide sequences were individually rescued [17] and characterized by ELISA as described above, but adding diluted purified phages (from 10^{12} down to 10^{10} cfu/ml) instead of supernatants.

Competition between Phage-displayed Peptides and Glucitollysine-BSA

Purified phages (10^{11} cfu/ml) were tested by ELISA on Mab-coated plates as described, but including glucitollysine-BSA [10] at 250 and 25 μg/ml in the phage dilution buffer M-PBS in some wells. Control nonglycated BSA (incubated and reduced as glucitollysine-BSA, but without glucose) at 250 μg/ml was used as a competitor in additional wells, to assess nonspecific inhibition effects. The wells incubated with phages without any competitor assessed maximal binding.

Evaluation of the Effect of Reducing Disulfide Bonds on Peptide Antigenicity

Two assays were used. In the first case, ELISA plates were coated overnight at 4 °C with purified phages (10^{12} cfu/ml) in PBS, washed with PBS, and treated with DTT at 100 mmol/l in PBS during 1 h at RT in the darkness. After washing with PBS, iodoacetamide (at 100 mmol/l in PBS) was added and incubated 1 h at RT, to alkylate free sulfhydryl groups. Some coated wells were simultaneously incubated with PBS as untreated phage controls. Plates were washed again and blocked with M-PBS during 1 h at RT. Mab 41 (at 10 μg/ml in M-PBS) was incubated on the plates 1 h at RT. The plates were

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washed with PBS-T and incubated during 1 h at RT with HRP-labeled anti-mouse IgG antibody (Sigma, USA), appropriately diluted in M-PBS. The next steps (from substrate solution addition to Abs measurement) were performed as described for phage screening. Phage antigenicity was calculated as follows:

$$\text{Antigenicity (\%)} = 100 \times \text{Abs treated phage} / \text{Abs untreated phage}$$

The second assay was similar to the phage screening ELISA on Mab 226-coated plates, but with the following sample pretreatment. Purified phages (10^{13} cfu/ml) were diluted 1/5 in PBS with 100 mmol/l DTT, and incubated 1 h at RT in the darkness. Reduced samples were diluted 1/2 in PBS containing 200 mmol/l iodoacetamide, and incubated 1 h at RT. The samples were finally diluted 1/5 in M-PBS and evaluated on Mab 226-coated plates as described. Untreated control phage samples were similarly diluted, but excluding DTT and iodoacetamide. Treated phage antigenicity (%) was calculated. Phage samples were simultaneously evaluated on anti-M13 Mab (Pharmacia, Sweden)-coated plates.

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