A disulfide conjugate between anti-tetanus antibodies and HIV (37–72)Tat neutralizes tetanus toxin inside chromaffin cells

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Abstract Conjugates between anti-tetanus F(ab′)$_2$ fragments and the (37–72) fragment of the HIV Tat protein were taken up by chromaffin cells, NG108-15 neurohybridoma cells and Rev-2-T-6 lymphoma cells. The uptake could not be inhibited by competition with (37–72)Tat, but was reduced in the presence of metabolic inhibitors or at low temperature. The disulfide as well as the thioether conjugate were translocated to the cytoplasmic space, but only the disulfide conjugate moderately restored the stimulated transmitter release inhibited by tetanus toxin. Therefore, disulfide conjugates are more promising than thioethers for the neutralization of intracellular antigens. These conjugates provide new tools to study neuroprotection against bacterial neurotoxins.

Key words: Thioether conjugate; Disulfide conjugate; HIV-Tat; Tetanus toxin

1. Introduction

Tetanus toxin (TET), after being taken up by nerve cells [1,2], hydrolyzes synaptobrevin by the enzymatic action of its light chain [3,4]. As a consequence, stimulated exocytotic transmitter release is reduced and may be entirely abolished [5,6]. In vertebrates, inhibitory neurons in the spinal cord are the first to be affected [7]. The ensuing preponderance of excitatory neuronal activity leads to spasms of the skeletal musculature and later on to convulsions [8]. The recovery is very slow. Patients require artificial respiration for about 3 weeks [9]. In chromaffin cells, 7 days after TET intoxication, the inhibition of transmitter release shows no significant reversal [10]. This long action of TET could be due to its very slow degradation in nerve cells [10], so that the TET enzymatic activity continues to hydrolyze the newly synthesized synaptobrevin. Therefore, inactivation of intraneuronal TET with a TET-neutralizing antibody should protect neuronal synaptobrevin from degradation. Indeed, 3 days after introduction of anti-TET antibodies by electroporation into TET-intoxicated chromaffin cells their transmitter release was largely restored [10,14]. Anti-TET antibodies outside neurons had no such effect [11–13], because antibodies cannot pass through the neuronal plasma membrane.

Since electroporation cannot be used in a clinical situation for the delivery of antibodies, we synthesized conjugates between anti-TET antibodies and peptides that are known to be taken up by cells. In this report, results obtained with conjugates of anti-tetanus F(ab′)$_2$ fragments and the (37–72) fragment of the HIV Tat protein are presented. The latter fragment is taken up by cells [15] like the parent (1–86)Tat [16–18], but is easier to synthesize and contains a single Cys (residue 37) for a selective coupling to other molecules via thiol chemistry. Our results show that a thioether as well as a disulfide conjugate of (37–72)Tat and anti-TET F(ab′)$_2$ were taken up by the cells, but only the disulfide conjugate neutralized intracellular TET.

2. Materials and methods

Na^{125}I was obtained from Amersham (Little Chalfont, UK); levo-[ring 2,4,6-^{3}H]norepinephrine was from NEN, 2-iminothiolane (ITH), bis-maleimidohexane (BMH), sulfosuccinimidyl-6-[3′-(2-pyridyldithio)propionamido]-hexanoate (slcSPDP), succinimidyl-4-(*N*-maleimido-methyl)-cyclohexane-1-carboxylate (SMCC), its sulfosuccinimidyl derivative (sSMCC) and Iodogen were purchased from Pierce (Rockford, IL, USA); the fluorophores monosuccinimidyl Cy3 and Cy3.5 were from Molecular Probes (Eugene, OR, USA); all other reagents, solvents and the culture media were of the highest quality available.

2.1. Synthesis of (37–72)Tat

The (37–72)Tat fragment was synthesized on a 433A peptide synthesizer (Applied Biosystems, Foster City, CA, USA) using Fmoc chemistry, and purified as described previously [19]. Purity and molecular weight of the product were determined by HPLC and electrospray mass spectrometry, respectively.

2.2. Anti-TET antibodies and their labeling

Anti-TET F(ab′)$_2$ fragments (nominal 5000 U/ml) were from Behring-Werke (Marburg, Germany). They belong to the batch used by Bartels and Bigalke [10]. The total F(ab′)$_2$ concentration as determined with the Sigma BCA assay was 3×10^{-3} M. The source solution was purified and adjusted to pH 7.0 or pH 8.0 using phosphate buffer or borate buffer, respectively, by spin filtration through Sephadex G25F. Two samples were labeled with ^{125}I by oxidative iodina-

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Abbreviations: BMH, bis-maleimidohexane; DMEM, Dulbecco's modified Eagle's medium; S^{3}HR, stimulated fractional exocytotic ^{3}H release; HPLC, high performance liquid chromatography; ITH, 2-iminothiolane; MCC, 4-(*N*-maleimido-methyl)-cyclohexane-1-carboxyl-; PDP, 6-[3′-(2-pyridyldithio)propionamido]-hexanoyl-; slcSPDP, sulfosuccinimidyl-6-[3′-(2-pyridyldithio)propionamido]-hexanoate; SMCC, succinimidyl-4-(*N*-maleimido-methyl)-cyclohexane-1-carboxylate; TET, tetanus toxin

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tion, using 37 MBq (1 mCi) $Na^{125}I$ and Iodogen. Several samples were labeled with Cy3 or Cy3.5, following the supplier's instructions but using one vial of monosuccinimidyl Cy per 3–6 mg instead of 1 mg of $F(ab')_2$. The labeled fragments were dialyzed against borate buffer (pH 8.5) before use.

2.3. Synthesis of ^{125}I-F(ab′)$_2$-(37–72)Tat using SMCC

65 μl of SMCC (10^{-2} M in dimethylsulfoxide) was added to 250 μl of ^{125}I-F(ab′)$_2$ (5.2×10^{-5} M) in borate buffer (pH 8.5). After 2 h the reaction mixture was centrifuged through Sephadex G25F/phosphate buffer (pH 7.5). The educt MCC-^{125}I-F(ab′)$_2$ was dialyzed against borate buffer (pH 8.5). The protein concentration was 2.88×10^{-5} M, and the MCC/F(ab′)$_2$ ratio was 2:1. 3.57×10^{-8} mol of (37–72)Tat in 35 μl PBS (pH 7.0) was added to 310 μl of this solution. After another 12 h incubation at room temperature, the resulting thioether conjugate was dialyzed against PBS (pH 7.0).

2.4. Synthesis of F(ab′)$_2$-(37–72)Tat and Cy3.5-F(ab′)$_2$-(37–72)Tat using ITH and BMH

2×10^{-2} M ITH in borate buffer (pH 8.5) was added to 2×10^{-4} M F(ab′)$_2$ in borate (pH 8.5) to obtain a 4–5-fold molar excess over the protein concentration. After 2 h incubation at room temperature, the reaction mixture was centrifuged through Sephadex G25F/borate buffer (pH 8.5). A sample was diluted with PBS (pH 7.4), and the thiol concentration was determined with Ellman's reagent. Thiol/F(ab′)$_2$ ratios of about 1.7 were obtained. The thiolated F(ab′)$_2$ was reacted with BMH at a 10-fold molar excess over the thiol concentration at room temperature for 1 h. The reaction mixture was centrifuged through Sephadex G25F/borate buffer (pH 8.5), and (37–72)Tat was added until a 3–4-fold molar excess over the thiol concentration was obtained. After another incubation of 12 h at room temperature, the thioether conjugate in the reaction mixture was purified by dialysis against PBS (pH 7.4). The extinction coefficients at 581 nm and 280 nm were measured to calculate the concentration of Cy3.5 and protein, respectively. The 280 nm extinction was corrected for the Cy3.5 contribution.

2.5. Synthesis of F(ab′)$_2$-(37–72)Tat and Cy3-F(ab′)$_2$-(37–72)Tat with slcSPDP

Solid slcSPDP was added to $0.8–1.5\times10^{-3}$ M F(ab′)$_2$ in borate buffer (pH 8.5), to obtain a 3–6-fold molar excess over the protein concentration. Three hours were allowed for the acylation. The reaction product F(ab′)$_2$-PDP was purified by dialysis against PBS (pH 7.0). The PDP concentration was determined by incubating a sample with an excess of DTT and measuring the thiopyridon extinction at 343 nm. The 280 nm extinction, corrected for the contribution by PDP and the fluorophores, was used to calculate the protein concentration. (37–72)Tat was added to obtain a 3-fold molar excess over the PDP concentration. At least 36 h were allowed for the thiol-disulfide exchange reaction. The resulting solution was purified by dialysis against PBS (pH 7.0). The Cy3 and protein concentrations were determined from the 280 nm and 552 nm extinctions, taking into account the 280 nm absorption of Cy3. The non-fluorescent conjugates and the fluorescent thioether conjugates were stable at 4°C for 3 weeks. The fluorescent disulfide conjugate had to be used within 2 days, thereafter it became turbid.

2.6. Cell cultures

Bovine chromaffin cells were prepared according to an established procedure [13]. They were plated on collagen-coated Petri dishes, allowed 1 day to recover from preparation stress, and were then harvested for experiments. For laser confocal microscopy, they were plated on collagen-coated cover glasses at a density of 10^5 cells/200 mm^2. For electroporation, they were suspended at a density of 5×10^6 cells/ml in DMEM containing TET (1 or 2 nM).

Rev-2-T-6 cells, derived from S-49 mouse lymphoma cells, and NG108-15 neurohybridoma cells were maintained as previously described [20,21].

2.7. Uptake of ^{125}I-F(ab′)$_2$-(37–72)Tat thioether

Rev-2-T-6 cells were suspended in uptake buffer (106 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 0.9 mM NaH_2PO_4, 25 mM HEPES, 25 mM glucose, 0.1% BSA, pH 7.4) to a density of 10^6 cells/ml. ^{125}I-labeled conjugates or ^{125}I-F(ab′)$_2$ were added to 0.5 ml triplicates of this suspension to obtain final concentrations between 10^{-9} M and 3.16×10^{-8} M. The cells were incubated for 1 h, then washed three times with cold (4°C) uptake buffer. The radioactivity of the cell pellets was counted, and the protein concentration of the SDS-solubilized pellets was determined using the Sigma BCA assay.

NG108-15 cells were suspended at a density of $0.5–1.0\times10^6$ cells/ml in uptake buffer (NaCl 109 mM, KCl 5.4 mM, $CaCl_2$ 1.2 mM, $MgCl_2$ 0.4 mM, NaH_2PO_4 0.9 mM, HEPES 12.5 mM, glucose 25 mM, 0.1% BSA, pH 7.4). In the experiments with metabolic inhibitors, glucose and NaCl were isotonically replaced with 25 mM deoxy-D-glucose, 10 mM NaCN and 10 mM NaN_3. The cells were incubated with ^{125}I-F(ab′)$_2$-(37–72)Tat thioether conjugate at concentrations of 1, 3.2, 10, and 32 nM, and at the following conditions: at 37°C, at 37°C in the presence of metabolic inhibitors, at 37°C in the presence of non-labeled (37–72)Tat, and at 4°C.

2.8. Confocal microscopy

Chromaffin cells were incubated for 16 h with conjugates or control substances. They were then washed twice with PBS (pH 7.0), fixed with 4% buffered formaldehyde for 30 min, again washed twice with PBS, covered with glycerol and stored at 4°C. The confocal microscope setup was described earlier [22]. The data presented in this report were obtained using 514 nm excitation, while the emission filter was a 580DF32 (580 ± 16 nm) interference filter. Control experiments with unstained cells showed that autofluorescence was undetectable at the filter and gain settings which were used to record the data. Optical sections were acquired at a spacing of about 0.3 μm. The images were filtered with a 3×3 median filter, and the black level was subtracted.

2.9. Stimulated exocytotic ^{3}H release

Bovine chromaffin cells were electroporated using the method of Bartels and Bigalke [10]. The electroporated cells were plated on 24 well plates (about 2×10^5 cells per collagen-coated well). Two days later, the medium was replaced with DMEM containing either the conjugates (27, 81, or 243 nM) or non-conjugated F(ab′)$_2$ (243 nM). After another 2 days, these media were exchanged for fresh DMEM. The exocytosis experiments were done 3 days after the application of the conjugates. The method described by Bartels and Bigalke [10] was applied with some modifications. Briefly, [^{3}H]norepinephrine was diluted with equilibrated DMEM to 4.4 kBq (120 nCi)/ml, and chromaffin cells plated into the collagen-coated wells of 24 well plates were incubated with 250 μl of this solution at 37°C for 2 h. The solution was replaced with fresh DMEM, and another hour at 37°C was allowed for incorporation of surface-bound radioactivity. The cells were then washed three times with release buffer (125.6 mM NaCl, 4.8 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5.6 mM glucose, 25 mM HEPES, 1 mM sodium ascorbate, 0.2% BSA, pH 7.3). During the next 8 min, the basal release into 450 μl/well of the same buffer was determined. The buffer was exchanged against 450 μl/well of release buffer containing 5×10^{-4} M carbachol. The stimulated ^{3}H release was collected for 8 min. Finally, the cell pellet was suspended in 0.2% SDS. The ^{3}H basal release B, the ^{3}H stimulated release S, and the ^{3}H remaining in the pellet R were counted. The results are given as fractional ^{3}H release $F = 1000\times(S-B)/(B+S+R)$.

2.10. Other methods

For the determination of concentrations by spectrophotometry, the following extinction coefficients were used: E[F(ab′)$_2$ 10^{-5} M in borate buffer (pH 8.5, 1 cm, 280 nm) = 0.977, E[Cy3 10^{-5} M, 1 cm, 552 nm] = 1.3, E[Cy3.5 10^{-5} M, 1 cm, 581 nm] = 1.5, E[pyridyl-2-thion 10^{-5} M, 1 cm, 343 nm] = 0.0808, thiol groups with Ellman's reagent E[chromophore 10^{-5} M, 1 cm, 420 nm] = 0.136. The 280 nm extinction contributed by the fluorophores and by PDP was calculated as follows: E[Cy3, 280 nm] = 0.05×E[Cy3, 552 nm], E[Cy3.5, 280 nm] = 0.24×E[Cy3.5, 581 nm], E[PDP, 280 nm) = 0.63×E[pyridyl-2-thion, 343 nm]. The protein concentration was also determined with the Sigma BCA assay.

2.11. Statistical treatment

A difference between the means of two data samples was tested for significance using the t-test. Multiple differences were tested after one-way analysis of variance with Duncan's, Tukey's and Student-Newman-Keuls' multiple range tests. Non-linear concentration-response data were fitted to several standard curves, and the best-fitting curve was selected for data transformation. Linear regression analysis was then applied to the transformed data. All tests were run at $P = 0.01$.

3. Results

3.1. Uptake of conjugates

The ^{125}I thioether conjugate was taken up at 37°C by NG108-15 cells in a dose-dependent manner without saturation (Fig. 1). The uptake was much lower at 4°C or in the presence of metabolic inhibitors. Each of the four curves in Fig. 1 had a significantly positive slope. An X at a symbol indicates that the corresponding mean is significantly different from the control mean. We assume that the radioactivity associated with the cells at 4°C reflects largely the binding component. The uptake could not be competitively inhibited with non-conjugated (37–72)Tat. On the contrary, the addition of non-labeled Tat resulted in an insignificantly increased uptake of the thioether conjugate. With Rev-2-T-6 cells, analogous results were obtained (data not shown).

3.2. Intracellular localization of the conjugate

In chromaffin cells, the disulfide conjugate labels both the cytoplasm and the nucleus (Fig. 2) with fluorescence. In the controls, autofluorescence was virtually absent. A similar pattern of fluorescence was found with the thioether conjugate, but the nucleus showed less fluorescence (image not shown).

3.3. Restoration of stimulated exocytotic ^3H release

The disulfide conjugate restored the stimulated exocytotic ^3H release from TET-intoxicated cells in a dose-dependent manner. In Fig. 3, bar A shows the fractional stimulated exocytotic ^3H release (S^3HR) from control cells (electroporated in DMEM in the absence of TET), bar B shows the S^3HR from toxin cells (electroporated in DMEM containing 1 nM TET). The S^3HR was significantly inhibited by TET

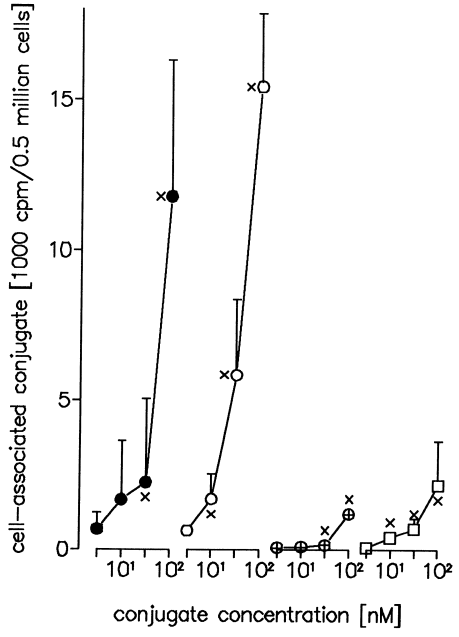

Fig. 1. Uptake and binding of a thioether conjugate of anti-tetanus ^{125}I-F(ab′)$_2$ and (37–72)Tat by NG108-15 cells at 37°C (filled circles), in the presence of 10 μM (37–72)Tat (open circles), in the presence of metabolic inhibitors (crossed circles), and at 4°C (open squares). Experimental conditions: $n=4$ vessels per concentration, 60 min incubation time. An X at a data mean indicates a significant difference between this mean and the mean of the lowest concentration (3.25 nM).

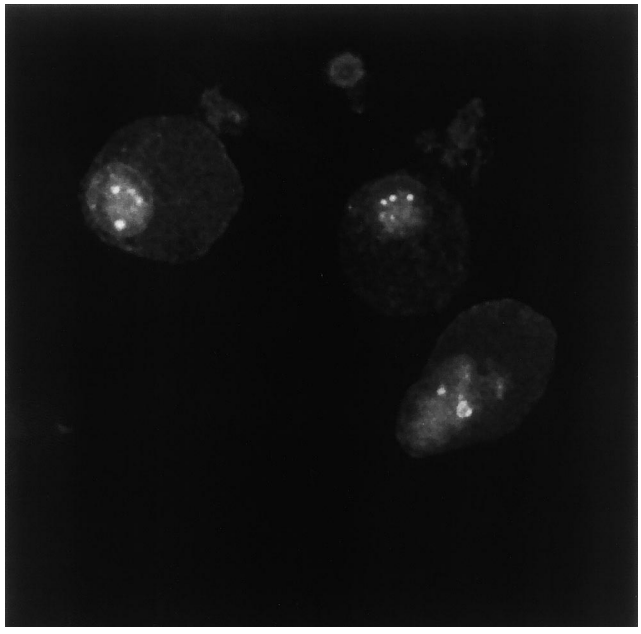

Fig. 2. Detection of a disulfide conjugate of Cy3-labeled F(ab′)$_2$ and (37–72)Tat in chromaffin cells. The cells were incubated with the conjugate (100 nM) overnight. Beginning at the bottom of the cells, confocal sections were acquired using 0.3 μm steps. The image is a maximum value projection of six sections (15–20).

(compare bars A and B), but it was restored by the disulfide conjugate in a dose-dependent manner (bars B, C, D, E). Because the S^3HR were proportional data, their increase was non-linear. For regression analysis, they were transformed using the angular transformation. The slope of the (linear) regression of the transformed data on log concentration was significantly different from zero. In addition, multiple range tests disclosed that the mean response to each of the three doses C, D, E was significantly different from the mean control response B. In other controls, non-conjugated F(ab′)$_2$ had no significant effect on intoxicated cells (compare bars B and F) or on non-intoxicated cells (compare bars A and G), and the disulfide conjugate had no effect on non-intoxicated cells (compare bars A and H). In an additional control experiment, tetanus toxin was reacted with disulfide-linked Tat conjugate to form an immune complex, and the cells were suspended in this solution and electroporated. Their transmitter release was not impaired. This indicates that the neutralizing immune reactivity of F(ab′)$_2$ as a part of the Tat conjugate was not seriously reduced during the conjugate synthesis.

4. Discussion

The restoration of the stimulated ^3H release by the disulfide conjugate confirms our working hypothesis and is the main message of the present study. The uptake of the conjugates resembles the uptake of the parent (37–72)Tat in that it is energy-dependent [18]. This suggests an uptake by vesicular endocytosis, although no evidence was found for a binding of the thioether conjugate to high-affinity receptors. Many natural protein toxins taken up by receptor-mediated endocytosis and many synthesized conjugates with a biological effect contain a disulfide that links the carrier domain with the effector domain. The disulfide bridge is reduced inside the cell, whereupon the effector domain of the molecule leaves the vesicle

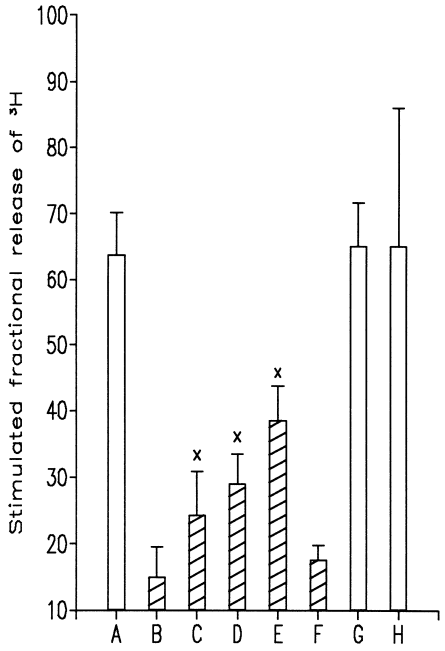

Fig. 3. A disulfide conjugate of (37–72)TAT protein and anti-tetanus $F(ab')_2$ reduces the inhibitory action of tetanus toxin on the stimulated exocytotic fractional ^3H release (S^3HR) from bovine chromaffin cells loaded with [^3H]norepinephrine. Hatched columns: cells electroporated in the presence of 1 nM TET. Open columns: cells electroporated in the absence of TET. The S^3HR of the control cells (A) was decreased by TET (B, intoxicated cells) and restored by the conjugate in a dose-dependent manner (21 nM in C, 81 nM in D, 243 nM in E). An X at a data mean indicates a significant difference between this mean and the mean of B. 243 nM free $F(ab')_2$ had no significant influence on the S^3HR of intoxicated cells (compare B with F) and of control cells (compare A with G), and 243 nM conjugate had no effect on its own on control cells (compare A with H).

and exerts its action. The effect of the protein on cellular components is eliminated or weakened when the disulfide bridge is replaced with a thioether bridge (for instance, [23]). Similarly in our experiments, the reducible disulfide conjugate restored the transmitter release of TET-intoxicated cells, while the non-reducible thioether conjugate was inefficient. This lends further support to the hypothesis that a vesicular process is involved in the uptake of the antibody-Tat conjugates. It should be noted that the thioether (37–72)Tat-enzyme conjugates synthesized by Fawell et al. [15] were investigated for activity on test substrates added to cell extracts, rather than on physiological substrates in living cells. In general, disulfide conjugates are more promising than thioethers for the neutralization of antigens in the cytoplasmic space. Concerning the efficiency of vesicular endocytosis for the translocation of proteins into the neuronal cytoplasm, one should not be misled by the extreme toxicity of clostridial toxins. In clostridial and other toxins, the effector domain of the protein acts as an enzyme. Just a few molecules of this domain may therefore be sufficient to hydrolyze proteins of the synaptobrevin/syntaxin/SNAP25 fusion complex (SNARE) faster than they can be synthesized. Therefore the low efficiency of vesicular endocytosis of the protein or of the translocation of its effector domain may only result in a long latency between the application of the toxin and the onset of the symptoms. In fact, latencies of many days have been observed in vertebrates. However, the low efficiency of translocation of substances into the cytoplasm by vesicular endocytosis may become a limiting factor if the effector domain acts only once. This happens with conjugates containing antibodies, and may explain why, in our experiments, a restoration of transmitter release from TET-intoxicated cells by the disulfide conjugate of (37–72)Tat and anti-TET antibody could be observed only after long incubation at elevated concentrations, and remained incomplete. The conjugates between $F(ab')_2$ and (37–72)Tat prepared and characterized in the present study provide leader pharmaceutical tools to study in vitro and in vivo the neuroprotection against protein toxins.

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