Epitope Spreading and Autoimmune Glomerulonephritis in Rats Induced by a T Cell Epitope of Goodpasture’s Antigen

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An amino-terminal region of \( \alpha_3 \) chain of type IV collagen noncollagenous domain \([\alpha_3(IV)\text{NC1}]\) that induces experimental autoimmune glomerulonephritis (EAG) in rats has been identified. Only recombinant antigens that contain a nine–amino acid (AA) span of \([\alpha_3(IV)\text{NC1}]\), consistent with a T cell epitope, could induce EAG. It was hypothesized that synthetic peptides of this region should induce EAG. Human and rat peptides of this region were synthesized and rats were immunized to define the nephritogenic epitope. A 13-AA rat peptide induced EAG with proteinuria, decreased renal function, and glomerular basement membrane (GBM)-bound deposits in half of the rats. This peptide induces lymph node cell proliferation and development of antibodies to epitopes of \([\alpha_3(IV)\text{NC1}]\) external to the peptide immunogen. Carboxy-terminal extension to 21 amino acids results in all rats’ demonstrating anti-GBM antibody and severe EAG. Asparagine at position 19 is critical for EAG induction. None of the 50 rats that were immunized with peptide that contained human sequence with isoleucine at position 19 developed EAG, whereas rat sequence with asparagine 19 induced EAG. Truncation of amino terminal AA of the peptide aborts EAG induction. These studies demonstrate that a T cell epitope of \([\alpha_3(IV)\text{NC1}]\) induces lymph node cell proliferation, EAG, and intramolecular epitope spreading; that the length of this peptide influences the formation of anti-GBM antibody; and that the presence of asparagine at position 19 of the peptide is critical to disease induction.

Goodpasture’s syndrome is one of the few forms of autoimmune glomerulonephritis in humans for which the antigen has been identified (1,2). Epitope mapping using sera from patients with Goodpasture’s syndrome has identified the \( \alpha_3 \) chain of type IV collagen noncollagenous domain \([\alpha_3(IV)\text{NC1}]\) of the glomerular basement membrane (GBM) as the responsible antigen (3,4). Further mapping has localized the antibody immunodominant region to the amino-terminal third of the \( \alpha_3(IV)\text{NC1} \) (5–9). Although this disease has been considered an antibody-mediated process, there is persuasive evidence to suggest that cellular immunity may play an important role in its induction (10). This is based on phenotypic analysis of kidney biopsies, in vitro evidence of cellular-mediated immunity (CMI), and relatively poor correlation of clinical course with antibody titers (1). Therapeutic response occurs with modalities that remove antibodies as well as those that curb CMI.

Delineation of pathogenic mechanisms is essential to tailoring therapeutic intervention. To that end, we and others have used a model of experimental autoimmune glomerulonephritis (EAG), which recapitulates Goodpasture’s syndrome in humans (11–15). Using this model, it has been demonstrated that antibodies alone and CMI alone can induce disease (16–24).

EAG develops in animals without antibody production, transfer of disease without antibody can be accomplished with cells, and disease in which antibodies are not detectable on the GBM may be induced (12,22,25,26). We have shown that the responsible epitope for EAG in rats is in the same amino-terminal third of \( \alpha_3(IV)\text{NC1} \) as the human antibody epitope (27) and that discrete segments of \( \alpha_3(IV)\text{NC1} \) are responsible for EAG induction (25). A critical amino acid (AA) sequence within the amino-terminal region consisting of nine or fewer AA conveys nephritogenicity to the non nephritogenic \( \alpha_1(IV)\text{NC1} \). Disease induced with this construct is associated with severe glomerulonephritis with crescents and fibrin with proteinuria but with minimal or absent IgG on the GBM in many animals (25). The presence of T cell proliferation in vitro without in vivo antibody binding suggested that this region might contain a T cell epitope that is responsible for induction of glomerulonephritis. We therefore hypothesized that a peptide construct of this region should induce EAG in rats, possibly without antibody formation. The purpose of these studies was to examine this hypothesis.

Materials and Methods

Preparation of Immunogens

GBM was isolated from glomeruli by differential sieving, sonication, and collagenase solubilization (cs) to form csGBM (12,14). NC1 domains were isolated by column chromatography. Recombinant human \( \alpha_3(IV)\text{NC1} \) was prepared and isolated as described (25,27). This protein induces EAG in rats and binds Goodpasture’s serum from patients with Goodpasture’s disease. Sequence analysis was performed by liquid chromatography/mass spectroscopy/mass spectroscopy (LC/MS/MS).
on peptides that were obtained from purified trypsinized recombinant human α3(IV)NC1 using a Thermo Electron LCQ mass spectrometer (28). The published sequences of human and rat α3(IV)NC1 were used to synthesize peptides (29–31). The synthesized peptides are shown in Figure 1. The design of the peptides is addressed in the Results section.

**Experimental Animals and Immunization**

Female WKY rats, 4 to 6 wk of age, were obtained from Harlan (Indianapolis, IN). These rats have been inbred for >60 generations. The protocol was approved by the Animal Care and Use Committee and adhered to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The immunizing protein antigens were prepared and administered using complete Freund’s adjuvant as described previously (14,27). Peptides were administered in PBS by the same protocol. Negative controls were immunized with CFA alone.

**Serum Biochemistries, Urinalysis, and Total Urinary Protein**

Urinary protein was determined using 3% sulfosalicylic acid (14). Urine was also examined for hematuria (0 to 3+; Multistix 10 SG, Bayer Corporation, Elkhart, IN). Serum creatinine and blood urea nitrogen were measured using kits from Sigma (St. Louis, MO; Procedure No. 555). Urine protein was determined weekly. Urea nitrogen and creatinine were quantified every 2 wks after immunization.

**Immunofluorescence Studies and Histologic Examination**

Kidney tissue obtained at death or killing at 12 wk after immunization was stained for rat IgG and fibrinogen (31). The intensity of deposits was graded semiquantitatively in a masked manner from 0 to 4+ (12,14). As little as 6 femtograms of IgG per glomerular section can be detected (12). Tissue fixed in Bouin’s solution was stained with hematoxylin-eosin and examined in a masked manner using a four-point scale (31). mAb 17 was used to identify α3(IV)NC1 (7,25,27).

**Elution of Antibodies from Kidney**

Kidneys were eluted by the glycine method (32). Eluate protein concentration was determined by the BCA method (Pierce, Rockford, IL) and by measuring absorbance at 280 nm.

**Antibody Studies**

Serum from patients with Goodpasture’s syndrome served as a source of human autoantibodies. Horseradish peroxidase–conjugated antibodies, fluorescein-conjugated goat anti-rat IgG and fibrinogen, and antihuman IgG were purchased from ICN/Cappel (Irving, CA). Tandem adsorption of rat anti-p13 antisera was performed using avidin-biotin and Sepharose columns. Biotinylated p13 was bound to an avidin-biotin adsorption column (NeurtrAvidin Biotin-Binding Protein column; Pierce). p13 was conjugated to cyanogen bromide–activated Sepharose 4B gel (Sigma-Aldrich, St. Louis, MO). Sera from rats with reactivity to p13 and chimeric/recombinant proteins in ELISA were adsorbed four times on the avidin-biotin column. Because anti-p13 activity remained, they were adsorbed twice more on Sepharose. The wash-through and eluted antibody to p13 was tested in ELISA versus peptides and proteins.

ELISA assays for antibody to native and recombinant proteins and peptides were performed as described (6,12,14,33). SDS-PAGE and immunoblotting were performed in 12.5% gels under nonreducing conditions (25,27,30).

**Lymphocyte-Proliferation Assay**

Lymphocytes isolated from lymph nodes 2 mo after immunization were stimulated in vitro with different antigens and cultured in 96-well flat-bottom plates (30,31). Data are expressed as the stimulation index, the ratio of stimulated to medium counts per minute. We considered stimulation indices of 2.0 or greater as significant (31).

**Statistical Analyses**

Data are expressed as mean ± SEM. Statistical differences between groups were evaluated by the t test and ANOVA (12).

**Results**

**Design of Peptides**

Our studies using chimeric human proteins and point mutations within these constructs identified a sequence in the amino-terminal portion of α3(IV)NC1 domain responsible for induction of EAG (25,27). The initial human peptide that we synthesized and tested, p21, is shown in Figure 1. A total of 50 rats were immunized with various doses of this peptide under various conditions. When that peptide failed to induce EAG under any conditions, we considered that that peptide length might be too long and affect antigen processing. We elected to use a shorter peptide beginning at the same amino terminal site using rat rather than human sequence. Because the region containing AA “TAIPS” was critical in nephritogenicity using chimeric protein mapping (25), we elected to include this region in the peptide. When the shorter peptide induced EAG, we next shortened the peptide by one AA in a sequential manner from
both the amino- (p12a, p11a, and p10a) and carboxy-terminal ends (p12c and p11c) and tested the ability of these peptides to induce EAG. Finally, we went back to our original human peptide, p21, confirmed the sequence, and investigated the impact of converting a single AA from the human to the rat sequence.

**Induction of EAG**

Twenty-one (81%) of 26 animals immunized with p13 rat sequence developed EAG with a spectrum of disease similar to csGBM (Figure 2A). Animals with EAG developed proteinuria that was comparable but of slightly lesser degree than that in animals immunized with csGBM (Figure 2B), hematuria, and abnormal renal function (Figure 2, C and D). Nephritic rats had no detectable IgG in 59% of animals that were immunized with p13 (Figure 3). Thirty-two percent of p13-immunized animals had 1 to 2+ linear GBM deposits, and 9% had deposits >2+ intensity, comparable to animals immunized with csGBM. Fibrinogen was present in glomeruli of all but one rat. Most rats had fibrinogen of intensity comparable to positive csGBM controls.

**Lymphocyte Proliferation Studies**

Lymph node cells from animals immunized with p13 proliferated in response to p13 peptide (Figure 4). p21 induced low-grade proliferation, whereas p21-N induced strong proliferation. csGBM induced the most marked proliferative response in p13-immunized rats.

**Correlation with IgG Bound to GBM and Histologic Score**

Because some rats had EAG with GBM-bound IgG but others did not, we examined the correlation between GBM-bound IgG and the histologic score by hematoxylin and eosin (Figure 5). Fibrinogen deposits were comparable between csGBM-positive controls and animals immunized with p13 (Figure 3). However, there was no correlation between GBM-bound IgG and histologic score.

**Antibody Studies**

**Serum and Kidney Eluate.** p13-immunized rats developed serum antibody that recognized p13 itself and human \( \alpha_3(IV)NC1 \) in ELISA (Figure 6). The average amount of antibody to recombinant human \( \alpha_3(IV)NC1 \) was low, although some rats had higher levels of reactivity. There was reactivity to human \( \alpha_3(IV)NC1 \) by immunoblot, which was greatly decreased under reducing conditions (data not shown). There was no reactivity against rat glomeruli by indirect immunofluorescence on saline-processed kidney sections. There was, however, antibody binding to human glomerular sections in most animals. Because some animals had linear GBM deposits whereas others were negative, we pooled negative and positive kidneys, respectively, and eluted glomeruli. Eluates were tested by indirect fluorescence on normal rat and human kidney processed in saline and after treatment with glycine/urea to expose cryptic antigens (34). Serum antibodies were also retested on glycine/urea-treated sections. With the use of these methods, neither eluate nor serum of rats that lacked GBM-bound antibody

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**Figure 2.** Histology of rats immunized with collagenase-solubilized glomerular basement membrane (csGBM; positive control) and p13 peptide (A) and total urinary protein (B). Rats that were immunized with p13 and csGBM had significantly decreased renal function as measured by urea nitrogen (C) and serum creatinine (D). Histology at death or killing, creatinine, and urea nitrogen at 7 wk.
fixed to rat GBM. Serum and eluate from rats with GBM-bound IgG, which had absent or barely detectable antibody to GBM on saline-processed kidney sections, were clearly positive on glycine/urea-pretreated human kidney. Kidney eluates but not serum from the GBM-positive rats fixed to rat kidney sections.

Immunosorption Studies. Sera from p13-immunized rats reactive in ELISA to p13 and human α3(IV)NC1 domains were subjected to column immunoadsorption. After multiple adsorptions on p13 columns, antibody activity to domains on human α3(IV)NC1, chimeric proteins of human α3(IV)NC1, and rat NC1 remained despite no anti-p13 activity (Figure 7). Anti-p13 eluted antibody reacted only with p13.

Kidney eluates of positive kidneys were likewise subjected to immunoabsorption. Anti-p13 adherent eluate did not bind rat or human GBM (Figure 8), whereas nonadsorbed kidney eluate fixed in a linear pattern to both rat and human GBM and to essentially all tubular BM in rat sections. The pattern of kidney eluate binding was the same as the distribution of α3(IV)NC1 for both human and rat kidney sections, respectively. These data demonstrate production of antibodies that react with α3(IV)NC1 domains outside the immunizing T cell peptide. These external epitopes were shared between rat and human sequences. Our studies further demonstrate that only antibody to human GBM was detectible in the circulation by indirect fluorescence despite ELISA reactivity to both human and rat GBM antigens, whereas antibody to both rat and human epitopes was present in kidney-bound antibodies.

Nephritogenicity of p21 and Amino/Carboxy-Terminal Truncated Peptides

As noted previously, immunization of multiple rats with human p21 sequence was unsuccessful in inducing glomerulonephritis. Rats did develop high-titer antibody to p21N and p21. Animals immunized with p10a and p11a had no evidence of EAG. Two of five rats immunized with p12a developed EAG, and all rats immunized with p12c and p11c developed EAG (data not shown).

Importance of Isoleucine to Asparagine Substitution in Position 19

Even though the human sequence when contained within the recombinant protein, native GBM, and various human chimeric constructs induces EAG (25,27,35), the human peptide sequence did not. Nonetheless, shorter peptides that contained asparagine at position 19, rat sequence, did induce EAG. Therefore, we re-examined a peptide identical to the original p21 but with a substitution of asparagine for isoleucine in position 19 (p21-N). This is the only position within the nephritogenic amino-terminal region of our recombinant proteins that differs between rat and other mammalian species (36,37). This single substitution resulted in conversion of the nonnephritogenic p21 peptide to a nephritogenic peptide (Figure 9). All animals developed abnormal urinary protein, with linear GBM deposits of IgG, fibrinogen deposits, and proliferative EAG by light microscopy. Like p13- and csGBM-immunized animals, there was no correlation between GBM-bound IgG and histologic score. Mass spectrometric analysis of the recombinant human α3(IV)NC1 confirmed that the AA at position 19 was in fact isoleucine, as predicted.

Discussion

Previous studies identified an amino-terminal immunodominant region of human α3(IV)NC1 responsible for Goodpasture antibody binding and induction of EAG (5,27,38). A segment of this region, consistent with a T cell epitope, induced EAG (25). Point mutations within this critical region abrogated antibody binding and EAG induction. We further explored this region using synthetic peptides. Our studies, presented here, demonstrate that a 13-AA peptide rat T cell epitope induces EAG with development and spreading of antibody response to both rat...
and human epitopes on \(\alpha_3(IV)\)NC1 external to the immunizing peptide. Although a peptide sequence of 13 AA that causes T cell proliferation might be expected to produce a totally non–IgG-associated disease, nonetheless, 41% of the animals had linear IgG on the GBM. The other animals had no IgG present on the GBM despite the presence of EAG by light microscopy. We do not know why some of these highly inbred rats developed antibodies and others did not. This has been reported by others and remains to be explained (22). There was no correlation between development of EAG and IgG deposits, suggesting that CMI induced the EAG in some and perhaps all of the animals. Support for a role for CMI alone in induction of the disease derives from a variety of sources that show that T cells are required for induction of disease, antibody-deficient animals still develop EAG, interruption in T cell activation abrogates disease, the histologic phenotype is consistent with a CMI response, disease may be transferred by cells alone, and intervention that blocks CMI blocks development of disease (12,14,18–21,23,26,39–41).

Wu et al. (22,23,42) also demonstrated lack of concordance among disease induction, antibody deposition, and EAG. They showed that immunization of rats with peptide of the same region as reported here induced EAG (43). In these latter studies, the authors reported that all of their animals developed severe EAG. Even though they were unable to demonstrate the presence of circulating antibody to rat GBM or production of antibody by in vitro techniques, no immunofluorescence findings in the kidneys of rats were reported. Thus, it was not clear whether these animals with peptide-induced EAG also had antibody along the GBM as observed in some of our animals.

They did not use tissue treated to reveal cryptic epitopes,
glomerular eluates, or human kidney substrate (43). In subsequent experiments, they showed that the T cell epitope did indeed induce GBM-bound antibodies in many of their rats (42). Circulating antibody bound only to the immunizing peptide, not GBM, but it was not reported whether human kidney sections were used as a substrate. We observed anti-p13 antibody and low levels of circulating antibody to human \( \alpha_3(IV)NC1 \) (Figure 6) but could detect minimal or no antibody to native GBM in tissue sections until the cryptic epitopes were exposed. Then we could show antibody in the circulation to human but not rat GBM in tissue sections, as described previously (25,30). Positive antibody binding to rat antigens by ELISA but not by indirect immunofluorescence may relate to conformational versus linear epitopes as postulated by others (42) or to epitopes revealed under the conditions of ELISA but not by indirect serum binding to cryostat sections.

We expected that such a short AA sequence that induced T cell proliferation and also EAG would produce antibody-negative EAG. In human glomerulonephritis and in various models of EAG, much evidence points to a purely T cell–mediated process. We were surprised when this short peptide of 13 AA was still associated with antibody formation in many animals. This suggested that several processes may be ongoing in the pathogenesis of EAG. First, antibodies to p13 might be cross-reactive to GBM epitopes. Antibody from serum and urine of rats with EAG and mAb derived from rats with EAG induce EAG (16–18,24). Antibody induces proteinuria within hours, with rapid development of glomerulonephritis comparable to csGBM-induced EAG. However, anti–p13-specific antibody did not bind to GBM. Second, cells alone can induce EAG during native immunization (12,22,25,27) and by transfer of cells from animals with EAG to naive recipients (18,23). In this latter case, EAG requires weeks to develop but is not associated with the formation of anti-GBM antibodies (23). Third, T cell–mediated

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**Figure 8.** Indirect immunofluorescence on normal human (A, C, E, and G) and rat (B, D, F, and H) kidney sections. \( \alpha_3(IV)NC1 \) distribution in human kidney is limited to GBM and Bowman’s capsule (arrow), and distal tubule BM (arrowhead; A) as described previously (34) but is present in essentially all BM of rat kidney (B). Kidney eluate fixes in an “\( \alpha_3 \)” pattern characteristic of human (C) and rat (D) \( \alpha_3(IV)NC1 \). Serum depleted of anti-p13 from p13-immunized rats is positive in an “\( \alpha_3 \)” pattern versus human kidney (E) but negative versus rat kidney (F). Negative control serum demonstrates only nonspecific background staining (G, H). Mesangial staining is a normal pattern for rat glomeruli. Tissue that was treated with glycine/urea to reveal cryptic epitopes (34).
tissue damage might result in release of neoantigens and autoimmunization to multiple kidney antigens, as shown by Wu et al. (42). However, we were able to demonstrate only anti-\(\alpha_3(IV)NC1\) antibodies, not antibodies to diverse GBM antigens. Our results thus suggest that yet another mechanism may be involved: Intramolecular epitope spreading.

We examined the possibility of epitope spreading by fractionating serum and kidney eluate into anti-p13 and non–anti-p13 antibody. Repeated adsorption of serum on p13-adsorbent columns demonstrated that serum contained antibody to the immunogen and also additional antibodies to human and rat \(\alpha_3(IV)NC1\) epitopes by ELISA. p13 is found only in the aminoterminus of \(\alpha_3(IV)NC1\), yet serum from p13-immunized rats, depleted of anti-p13 antibody, recognized determinants in rat NC1 and other portions of human \(\alpha_3(IV)NC1\) and human chimeric proteins, representing intramolecular epitope spreading. We presume that the reactivity of antibody to both rat and human antigen was resultant from cross-reactive antibodies. These findings could occur only by spread of the immune response to antigens external to the p13 immunogen. This provides an explanation for the observation by Wu et al. (42) of antibody on the GBM after peptide immunization and immunoprecipitation of multiple GBM antigens by glomerular eluates. Their studies were consistent with epitope spreading, but the precipitated proteins were not identified. It is important to note that we and Wu et al. both demonstrated antibody activity to both human and rat GBM after immunization with rat peptide. Epitope spreading has previously been described in other experimental models, including ovaritis, encephalomyelitis, multiple sclerosis, and thyroiditis (44–49). To our knowledge, this is the first documentation of the phenomenon of intramolecular epitope spreading in glomerulonephritis. This is illustrated in the chart in Figure 10. We cannot explain the presence of circulating antibody to human but not rat GBM by indirect immunofluorescence. Cross-reactive antibodies were present in eluate by indirect immunofluorescence and by ELISA in serum. It is possible that linear versus conformational epitopes play a role. Circulating antibody might be related to denatured antigens, which would not be present in rat kidney eluate, but then should react with both human and rat kidney sections. It is also possible that there was intermolecular epitope spreading undetectable in the rat because of the different distribution of \(\alpha_3(IV)NC1\) in the rat, i.e., on GBM as well as tubular BM. In this case, we might have expected a non-\(\alpha_3(IV)NC1\) pattern on human kidney (Figure 8E). Different antibody affinities could also play a role in being able to detect anti-\(\alpha_3(IV)NC1\) antibodies on rat versus human kidney sections. Finally, the specific reactivity of circulating antibody in a human \(\alpha_3(IV)NC1\) pattern (Figure 8E) but not to rat antigens suggests an alternative explanation. We believe that this may demonstrate B cell epitope spreading to recruit B cells that produce antibody specific to epitopes on human rather than rat, \(\alpha_3(IV)NC1\).

The fine specificity of the AA sequence in inducing EAG in this model is notable. Immunization of WKY rats with GBM from a variety of species—rat, rabbit, mouse, human, and bovine—induces EAG. Furthermore, recombinant human \(\alpha_3(IV)NC1\) induces EAG (25,27,35). Nonetheless, when synthetic peptides that contained exactly the same AA as full-length protein were used to immunize rats, a T cell proliferative response was induced but no EAG. Although the rat sequence has been reported to be identical to the human sequence in this

![Figure 9. EAG in p21-N–immunized rats. Substitution of asparagine for isoleucine in position 19 converted the nonnephritogenic p21 peptide, which did not induce EAG under any circumstances, to a nephritogenic peptide. All rats that were immunized with p21-N developed abnormal urinary protein, decreased kidney function, associated with linear deposits of IgG along the GBM, intense fibrinogen (FIB) deposits within glomeruli, and severe glomerulonephritis. Serum creatinine in p21-N versus p21 or CFA (\(P < 0.0002\)); blood urea nitrogen in p21-N versus p21 or CFA (\(P < 0.005\)). H&E evaluation was not performed on p21 and CFA rats because of normal kidney function and lack of immunoreactants in tissue sections.](image-url)
region (36), more recent reports demonstrated asparagine rather than isoleucine at position 19 (37). Thus, the rat sequence for this area differs from other mammalian species in one single AA. Substitution of asparagine for isoleucine in position 19 induced florid EAG in the context of both a 13-AA synthetic peptide and a 21-AA peptide. Finally, we confirmed that position 19 in the human \( \alpha_\text{H9251}^3(\text{IV})\text{NC1} \) nephritogenic protein was in fact isoleucine, not asparagine. It is not clear why the human sequence in the intact protein induces EAG but as peptide does not. It is possible that intracellular protein processing results in modification of the isoleucine such that it fits into the MHC of the antigen-presenting cell to allow T cell receptor recognition. This processing may be absent or modified with peptide, thus requiring the specificity of the rat asparagine in the synthetic peptide. Additional study will be needed to clarify how isoleucine 19 in one context but not another can induce EAG.

In summary, we have shown that a rat T cell epitope induces EAG; that for reasons yet to be clarified, isoleucine 19 of \( \alpha_\text{H9251}^3(\text{IV})\text{NC1} \) whole protein is permissive to induction of EAG but asparagine 19 is required for peptide induced EAG; and that intramolecular antibody spreading, possibly with heterologous intramolecular spreading as well, is induced by the T cell epitope. Delineation of peptide epitopes that induce EAG with both antibody-negative and -positive phenotype and antibody epitope spreading provides a basis for further understanding the pathogenesis of glomerulonephritis. The close association of Goodpasture antibody binding to these same epitopes suggests that similar regions may serve as a focus for further insight into the development of the human disease.

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