Altered Biogenesis of $\Delta F508$ -CFTR Following Treatment with Doxorubicin

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Key Words

CFTR • $\Delta F508$ • Doxorubicin • Biogenesis • Pharmacology • Anthracyclines

Abstract

Cystic fibrosis (CF) is caused by mutations to the cystic fibrosis transmembrane conductance regulator (CFTR) gene. The most common of these mutations is deletion of a phenylalanine residue at position 508 ($\Delta F508$), which accounts for ~70% of all CF alleles. This mutation interferes with the biogenesis and maturation of $\Delta F508$ -CFTR to the plasma membrane. However, $\Delta F508$ -CFTR can partially function upon proper localization. Thus, pharmacological correction of $\Delta F508$ -CFTR maturation holds promise in CF therapy. Our previous studies indicate that a single non-cytotoxic dose of the anthracycline doxorubicin (Dox) significantly increase $\Delta F508$ -CFTR-associated chloride secretion in MDCK cells by increasing the expression of this protein at the apical plasma membrane. We report here that Dox alters the biogenesis of $\Delta F508$ -CFTR. Treatment with Dox increases the resistance of $\Delta F508$ -CFTR to trypsin digestion, possibly by expediting protein folding. Further, treatment with Dox reduces the amount of polyubiquitinated

$\Delta F508$ -CFTR in cells and prolongs the half-life of this protein. Concomitantly, treatment with Dox decreases the association of $\Delta F508$ -CFTR with HSP70 but does not alter the expression of major HSP70 family members. Based on these results, we propose that Dox expedites the folding and maturation of $\Delta F508$ -CFTR by acting as a pharmacological chaperone, which consequently promotes the functional expression of this protein in MDCK cells.

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Introduction

Cystic Fibrosis (CF) is the most common recessive genetic disorder in Caucasians, occurring in 1 out of 2500 live births. CF, which is typically lethal, is caused by mutations to the gene Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) [1]. While over 1000 mutations to CFTR have been reported to date, the most common mutation, $\Delta F508$ (deletion of phenylalanine residue at position 508) accounts for ~70% of all mutations in patients [1]. This mutant protein is unable to efficiently localize to its correct site of expression - the apical plasma membrane due to a kinetic defect that interferes with

protein maturation [2]. A second problem associated with $\Delta F508$ -CFTR is inefficient channel gating [3, 4]. In spite of these limitations, $\Delta F508$ -CFTR is a target for pharmacotherapy as it can partially function as a chloride channel upon proper localization [5] and because it has been estimated that functional correction of CFTR expression to ~6-10% of wild-type level may benefit CF patients [6]. Efforts are underway to identify compounds that correct $\Delta F508$ -CFTR biogenesis and a few lead structures have been identified [7-9].

CFTR belongs to the ATP-binding cassette (ABC) family of proteins [10]. Other members of this family include P-glycoprotein (Pgp), multidrug resistance associated protein (MRP), and the sulfonyleurea receptor (SUR) [10]. There is evidence of co-regulation of CFTR and Pgp expression in vitro and in vivo [11-14]. Accordingly, our previous work demonstrated that certain Pgp modulators could affect CFTR expression and function [14-16]. In particular, the anthracycline compound doxorubicin (Dox), increased CFTR-associated chloride secretion over two-fold in T-84 cells and partially corrected $\Delta F508$ -CFTR localization, which ultimately resulted in enhanced chloride secretion in an engineered MDCK-C7 cell line [15]. These changes were achieved at a single, non-cytotoxic, pharmacologically relevant dose (0.25 μM). We have performed additional studies to better understand the mechanism of action of Dox on $\Delta F508$ -CFTR. In this paper, we report that $\Delta F508$ -CFTR has an altered pattern of biogenesis in MDCK-C7 cells following treatment with Dox.

Materials and Methods

Cell culture and treatment

An isogenic line of MDCK-C7 cells stably expressing green fluorescent protein (GFP) tagged to the N-terminus of $\Delta F508$ -CFTR (MDCK/ $\Delta F508$) was created in a fashion similar to a cell line expressing green fluorescent protein (GFP) tagged to the N-terminus of CFTR described elsewhere [17]. The cells were a generous donation of Dr. Bruce Stanton (Dartmouth Medical School). Cells were maintained in Minimum Essential Media (Life Tech. Inc., Rockville, MD) supplemented with 10% fetal bovine serum and antibiotics. Additionally, 0.3 mg/ml of G418 Sulfate (Sigma, St. Louis, MO) was added to the media for maintaining selection pressure. For immunoblot analysis following drug treatment, cells were grown in 6-well plates (Costar, Cambridge, MA), washed with PBS and treated with Dox, or vehicle control (water) for 24 hours in fresh media as indicated in the figure legends. For GFP- $\Delta F508$ -CFTR half-life measurements, 100 $\mu g/ml$ cycloheximide (Sigma) was added directly to the media following treatment with Dox and total protein lysates were prepared at various time points.

Immunoblotting and co-immunoprecipitation

For immunoblot analysis, cells were treated with Dox at 70% confluency for 24 hr at which point they were completely confluent, washed once with cold PBS, and scraped in a small volume of lysis buffer (50 mM Tris-HCl, pH 6.8, 150 mM NaCl, 1% NP-40) containing a protease inhibitor cocktail (Roche Biochemicals, Indianapolis, IN). Proteins were extracted from the samples by a 30 min incubation on ice with intermediate vortexing, followed by removal of insoluble materials by centrifugation at 13,000 rpm/4°C for 5 min, and frozen at -70°C. Protein concentration in each sample was quantified by the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 7.5% minigels using 20 μg of protein lysate per lane. Proteins were separated and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) at 100 mA for 1 hour in Towbin's transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol). Following transfer, the blots were blocked and probed sequentially with various antibodies. The highly-specific monoclonal anti-CFTR antibody M3A7 [18] was purchased from Upstate Biotechnology. Various species of $\Delta F508$ -CFTR were identified on the blots representing the different glycosylation states of the protein. In some experiments, small amounts of mature $\Delta F508$ -CFTR were noted, which is in accordance with past data and is not unexpected [19]. All anti-heat shock protein and anti-ubiquitin antibodies were purchased from Stressgen Biotechnologies Corp. (Victoria, Canada). Horseradish peroxidase-labeled anti-rabbit, anti-rat or anti-mouse secondary polyclonal antibodies were used to enable detection of various proteins. Membranes were washed six times in PBST (PBS + 0.3% Tween-20) at room temperature for 10 min per wash between antibodies. The blots were developed with ECL Plus reagent (Amersham, Piscataway, NJ) following the manufacturer's directions and exposed to film.

For co-immunoprecipitation experiments, cells were lysed under non-denaturing conditions as described elsewhere [20] and pre-cleared with Protein A-agarose (Roche Biochemicals, Piscataway, NJ). Typically, 300-500 μg of protein from each sample was immunoprecipitated overnight at 4°C using an appropriate antibody and Protein A-agarose. Total immunoprecipitation volumes were adjusted by addition of lysis buffer. Following three washes in ice-cold lysis buffer, the beads were incubated in sample buffer under reducing conditions at 65°C for 20 min. Proteins were separated and blotted as described above.

Protease digestion assay

Total proteins were isolated from MDCK-C7 cells expressing GFP- $\Delta F508$ -CFTR under non-denaturing conditions (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 0.1% NP-40). Equal amounts of protein (10 mg) from control and Dox-treated cells were digested with Trypsin from bovine pancreas at a concentration of 500 ng/ml (Sigma) at room temperature for the indicated amount of time in equal reaction volumes. Reactions were stopped by rapid addition of 1 mM phenylmethyl sulfonyl fluoride (PMSF) and 10 mg/ml Lima Bean Trypsin inhibitor (Sigma). Samples were mixed with an equal volume of sample buffer and

immediately separated on SDS-PAGE gels under denaturing conditions. Immunoblotting was performed as described above. Following incubation with IgG elution buffer, the blots were re-probed with an anti-vinculin antibody (Sigma) to verify equal protein loading in each well.

Densitometry and statistical analyses

Blots were digitized using a high-resolution scanner and densitometric quantification was performed using NIH Image (National Institute of Health, Bethesda, MD). All statistical analyses were performed using InStat and Prism (Graphpad Software, San Diego, CA).

Results

Altered sensitivity of Δ F508-CFTR to proteolysis upon Dox treatment

Biogenesis of Δ F508-CFTR is an inefficient process [5, 21–23] and unfolded Δ F508-CFTR molecules are rapidly degraded by the ubiquitin-proteasome system in cells. Based on our past data we hypothesized that treatment with Dox would promote the kinesis of Δ F508-CFTR in MDCK/ Δ F508 cells. Specifically, we postulated that Dox would expedite the folding of Δ F508-CFTR and enable a fraction of the mutant protein to correctly localize to the plasma membrane. To test this hypothesis, trypsin digestion of Δ F508-CFTR was performed on extracts of MDCK/ Δ F508 cells followed by immunoblot analyses [24] (Figure 1). Inadequately folded Δ F508-CFTR protein molecules would be vulnerable to trypsin digestion. Conversely, improved kinesis would result in a pool of Δ F508-CFTR molecules that is resistant to digestion [25]. Our results suggest that treatment with Dox increased the resistance of Δ F508-CFTR to protease digestion (Figure 1A) compared to vehicle treated control extracts. As expected, the core glycosylated form of Δ F508-CFTR (Band B) was more susceptible to proteolysis compared to mature Δ F508-CFTR (Band C). A steady increase in the densitometric ratio of detectable trypsin-resistant Δ F508-CFTR in Dox-treated versus control cells (Figure 1B) was noted as well. At 10 min post-addition of trypsin, a statistically significant, >3-fold increase in the ratio was noted (Student's t-test, $p < 0.01$; 95% confidence limit) compared to the initial ratio at the beginning of the digestion experiment. In summary, treatment with Dox resulted in the generation of a trypsin-resistant pool of molecules in cells possibly due to conformational maturation of Δ F508-CFTR through improved kinesis.

Fig. 1. Protease digestion analysis of Δ F508-CFTR. (A) Total proteins from control (-) or Dox (+) treated MDCK-C7 cells (0.25 μ M, 24 hr) expressing Δ F508-CFTR were digested with trypsin as described in Methods and immunoblotted using an anti-CFTR antibody (M3A7). The same blot was stripped and re-probed with a monoclonal anti-vinculin antibody to assess equal protein loading (lower panel). (B) Densitometric ratios of Δ F508-CFTR band intensities through the time-course were calculated from the above blot, corrected for protein loading and plotted. ** Significantly different from 0 min ($p < 0.01$; Student's t-test).

Reduced polyubiquitination of Δ F508-CFTR upon treatment with Dox

Immature Δ F508-CFTR is rapidly degraded in cells by the ubiquitin-proteasome system [22, 23, 26, 27]. Thus in theory, promotion of Δ F508-CFTR biogenesis by Dox would decrease the association of Δ F508-CFTR with ubiquitin and consequent degradation via the ubiquitin-proteasome pathway. Thus, we examined the amount of polyubiquitinated Δ F508-CFTR in MDCK/ Δ F508 cells following Dox treatment using co-immunoprecipitation (Figure 2). An anti-Ubiquitin antibody was used to capture all ubiquitinated proteins on Protein G-coupled agarose. The captured proteins were eluted and subjected to immunoblotting with the anti-CFTR monoclonal antibody M3A7. DeltaF508-CFTR was detected as a smear with a few prominent bands possibly corresponding to

Fig. 2. Effects of Dox on $\Delta F508$ -CFTR ubiquitination. (A) MDCK/ $\Delta F508$ cells were treated with increasing amounts of Dox as indicated for 24 hr and immunoprecipitated using an anti-ubiquitin antibody. The immunoprecipitated proteins were blotted on to solid support and detected using an anti-CFTR monoclonal antibody. Immunoprecipitation with Protein G-agarose alone did not produce a detectable signal (-). (B) Digitized blots were subjected to densitometry to determine the relative amounts of immunoprecipitated $\Delta F508$ -CFTR following treatment with Dox. Data reported as mean $\pm$ standard deviation from three samples per group. (C) Immunoblot analyses of $\Delta F508$ -CFTR expression in MDCK/ $\Delta F508$ cells following treatment with Dox were performed. The core-glycosylated (Band B) and mature (Band C) forms of $\Delta F508$ -CFTR are indicated. The blots were also probed with an anti-Vinculin antibody to establish equal protein loading in each lane.

the various glycosylated forms of the protein in accordance with previous studies [28] (Figure 2A). Treatment with Dox decreased the amount of polyubiquitinated $\Delta F508$ -CFTR in a concentration-dependent fashion (Figure 2A, B). However, some cytotoxicity was noted at concentrations above 0.25 μM as has been previously

Fig. 3. Half-life determination of $\Delta F508$ -CFTR. (A) MDCK-C7 cells were treated with Dox (0.25 μM , 24 hr) or vehicle. At 24 hr post-treatment, cycloheximide (100 $\mu g/ml$) was added to inhibit protein synthesis. Disappearance of $\Delta F508$ -CFTR was followed over 8 hr using immunoblot analyses as described in Methods. A representative blot is shown. (B) Immunoblots were digitized and analyzed by densitometry and values were plotted as % $\Delta F508$ -CFTR remaining post-treatment. Each point represents mean $\pm$ standard deviation determined from three independent samples per group. * Significantly different from control ($p < 0.05$; Student's t-test).

reported with Dox [29]. As expected, concomitant immunoblot analyses of $\Delta F508$ -CFTR expression with M3A7 demonstrated increased expression of $\Delta F508$ -CFTR upon treatment with Dox (Figure 2C). Vinculin was measured as a loading control. Taken together, Dox promoted maturation of $\Delta F508$ -CFTR, which possibly resulted in decreased polyubiquitination of $\Delta F508$ -CFTR in MDCK/ $\Delta F508$ cells.

Treatment with Dox increased the half-life of $\Delta F508$ -CFTR in MDCK/ $\Delta F508$ cells

Promotion of $\Delta F508$ -CFTR maturation should extend the half-life of this protein. We therefore treated these cells with Dox or vehicle and measured the half-life of $\Delta F508$ -CFTR (Figure 3). Following treatment with Dox, cells were treated with the protein synthesis inhibitor cycloheximide and $\Delta F508$ -CFTR expression was monitored over time using immunoblotting. We report that treatment with Dox significantly improved the half-life of $\Delta F508$ -CFTR (Student's t-test, $p < 0.05$; 95% confidence limit) compared to vehicle-treated control cells (Figure

Fig. 4. Effect of Dox on the association of $\Delta F508$ -CFTR to HSP70. (A) Following treatment with Dox ($0.25 \mu\text{M}$, 24 hr), HSP70-associated $\Delta F508$ -CFTR was immunoprecipitated from MDCK/ $\Delta F508$ cells (lane 3) or vehicle treated control cells (lane 2) using a mouse anti-HSP70 antibody ($5 \mu\text{g}/\text{ml}$) and Protein G-agarose. This antibody recognizes both constitutive and inducible forms of HSP70. The immunoprecipitated proteins were immunoblotted with an anti-CFTR antibody (M3A7). Control immunoprecipitation from MDCK-C7 parental cells did not produce a detectable signal (lane 1). (B) Immunoblots were digitized and analyzed as described in Methods. Each bar represents mean $\pm$ standard deviation measurements from three independent samples per group. ** Significantly different from control ($p < 0.01$; Student's t-test).

3A, B). The measured half-life of $\Delta F508$ -CFTR in control cells was in accordance with past data in epithelial cells [30-32]. Thus, Dox promoted the maturation of $\Delta F508$ -CFTR, which increased the half-life of the protein in cells.

Treatment with Dox results in the release of $\Delta F508$ -CFTR from an HSP70 complex

It was previously reported that $\Delta F508$ -CFTR has a prolonged association with the cytosolic chaperone HSP70 compared to the wild-type protein [33], and that this targets the mutant protein for rapid degradation through the 26S proteasomal system [22, 34]. Thus, the association of $\Delta F508$ -CFTR with the cytosolic chaperone HSP70 was examined in MDCK/ $\Delta F508$ cells utilizing a co-immunoprecipitation approach following Dox treatment (Figure 4). Dox reduced the amount of HSP70-associated $\Delta F508$ -CFTR almost three-fold (Figure 4A). A prominent single band with apparent gel mobility of ~ 180 kDa was detected using the co-immunoprecipitation approach. This band represents the core-glycosylated form

Fig. 5. Effect of Dox on protein expression of HSP70 family of chaperones. (A) Immunoblot analyses of MDCK/ $\Delta F508$ cells following Dox treatment. For each lane, $10 \mu\text{g}$ of total protein from control (CRL) or Dox (DOX) treated ($0.25 \mu\text{M}$, 24 hr) samples were separated on SDS-PAGE gels and blotted using a rat monoclonal anti-HSC70 antibody (top panel), a mouse monoclonal anti-HSP70 antibody (middle panel), or a mouse monoclonal anti-HSP90 antibody (bottom panel). Representative lanes are shown in duplicate. (B) Densitometric analysis of chaperone expression MDCK-C7/ $\Delta F508$ cells. Immunoblots from panel (A) were digitized and analyzed by densitometry. Each bar represents mean $\pm$ standard deviation measurements from three independent samples.

of $\Delta F508$ -CFTR (Band B) in agreement with past reports [18, 23, 35]. The higher molecular weight of this band is due to the 30 kDa GFP-tag. Furthermore, no signal was detected from co-immunoprecipitated proteins from parental MDCK-C7 cells (Figure 4, lane 1), which served as a negative control and suggested that the band was $\Delta F508$ -CFTR and not canine CFTR from MDCK-C7 cells. Thus as expected, improved kinesis of $\Delta F508$ -CFTR upon treatment with Dox decreased the association of this protein with HSP70.

Chaperone expression in MDCK/ $\Delta F508$ cells are not altered upon Dox treatment

Dox can alter the expression of HSP70 family of cytosolic chaperone proteins [36] at high doses. The biogenesis of $\Delta F508$ -CFTR is promoted by certain HSP70 family members [37, 38]. Hence, we wanted to investi-

gate whether treatment with Dox resulted in altered expression of the HSP70 family of chaperone proteins. As indicated in Figure 5, Dox treatment did not increase the amount of detectable HSC70, HSP70 or HSP90 species in MDCK/ Δ F508 cells as assessed by immunoblotting using specific antibodies against each chaperone protein. Thus, altered chaperone expression via a general heat-shock type response is not involved in Dox-induced promotion of functional Δ F508-CFTR expression in these cells.

Discussion

The principal problem in CF patients with the Δ F508 mutation is that most of the nascent Δ F508-CFTR molecules do not fold efficiently, aggregate as a result, and are rapidly degraded by the ubiquitin-proteasome system in cells (reviewed in [39]). Correction of this problem through pharmacotherapy may benefit CF patients. We had previously reported that treatment with Dox increased Δ F508-CFTR-associated chloride secretion in MDCK/ Δ F508 cells [15]. In this report, we provide evidence to suggest that Dox promotes the biogenesis of Δ F508-CFTR. We propose that Dox expedites the folding kinetics and maturation of Δ F508-CFTR. This conclusion is based upon our observation that treatment with Dox increased the resistance of Δ F508-CFTR to proteolysis, which was probably caused by restricted access of trypsin to cleavage sites upon improved folding of Δ F508-CFTR (Figure 1). It is indeed possible that Dox may act as a pharmacological scaffold to improve Δ F508-CFTR folding and maturation. Indirect evidence to support our hypothesis comes from several studies. For example, introduction of Δ F508-like mutations to ABC transporters like Pgp and MRP interfere with the kinesis of these proteins (reviewed in [40]). However, these proteins can be rescued upon incubation with ligands or substrates that act as pharmacological chaperones to promote interactions among the various protein domains during the folding process [40]. Recently, Dr. Verkman and colleagues identified anthraquinones, which are structurally related to anthracyclines, along with a few other classes of molecules as high-potency activators of Δ F508-CFTR-associated chloride conductance at nanomolar concentrations [41]. These compounds possibly potentiate Δ F508-CFTR gating by directly interacting with the protein. Given the structural similarities between anthraquinones and anthracyclines, it is plausible that Dox may directly interact with Δ F508-CFTR as well. Additionally, recent stud-

ies by Wang et al. also suggest that certain compounds that act as pharmacological chaperones of Δ F508-CFTR are quite specific in their mode of action [42]. Further, the anthracycline 4'-iodo-4'-deoxy-doxorubicin has been reported to bind to aggregated prion proteins (amyloid fibrils) and promote their resorption in experimental models [43] and clinical trials [44]. Thus, correction of protein aggregation may be a unique property of certain pharmacological compounds belonging to this class. We have initiated additional studies to further substantiate our central hypothesis.

In subsequent experiments, the fraction of polyubiquitinated Δ F508-CFTR was reduced in a dose-dependent manner upon Dox treatment in MDCK cells (Figure 2) as assessed using co-immunoprecipitation with an anti-ubiquitin antibody followed by immunoblot analyses using an anti-CFTR monoclonal antibody. Promotion of Δ F508-CFTR kinesis upon treatment with Dox may cause such an effect by removing a fraction of Δ F508-CFTR molecules originally targeted for degradation via the ubiquitin-proteasome system [45]. This is supported through experiments reported in Figure 2C where increased expression of Δ F508-CFTR was noted in MDCK cells along with a significant increase in half-life of Δ F508-CFTR upon treatment with Dox (Figure 3). However, an alternate possibility also exists. We noted some cytotoxicity above 0.25 μ M in cells upon treatment with Dox. Dox at cytotoxic concentrations may reduce polyubiquitination of certain proteins [46]. Therefore, it is plausible that at the higher concentrations, the effect of Dox on Δ F508-CFTR may be partially related to both genotoxicity and improved kinesis of the protein. It is difficult to conclude at this time whether Dox exclusively promotes maturation of Δ F508-CFTR. However, either outcome is acceptable from a pharmacological standpoint as long as potential "off-target" effects are minimal and/or manageable.

Finally, Dox treatment decreased the amount of Δ F508-CFTR associated with HSP70 (Figure 4). Our findings are consistent with past reports where agents that purportedly interfered with the prolonged association of Δ F508-CFTR with HSP70 promoted chloride secretion [47, 48]. However, Dox had no detectable effect on the expression of HSC70, HSP70 and HSP90 in these cells indicating that altered chaperone expression through a generalized heat-shock type response was not directly involved in promoting Δ F508-CFTR kinesis (Figure 5). However, the "interactome" of CFTR has over 100 proteins [49]. Thus, the possibility that Dox affects one or more of these chaperone proteins at the doses tested must

be entertained eventually. Additionally, a fraction of cellular a dash “508-CFTR” is degraded by an alternate proteolytic system in cells [5]. Therefore, we cannot completely exclude the possibility that Dox affects the proposed alternate degradation pathway for CFTR. Unfortunately, the identity of this protein or family of proteins remains unknown. However, from the standpoint of drug-discovery, a phenotypic anchor and lead structures are sufficient tools to develop pharmacological agents for CF therapy. Thus, identification of Dox as a model compound that can promote Δ F508-CFTR folding and expression at a pharmacologically achievable dose (0.25 μ M) is interesting from a clinical perspective. Since it has been estimated that only a modest increment in chloride secretion to ~10% of wild-type levels may be sufficient to ameliorate the clinical symptoms associated with cystic fibrosis [6, 50], development of pharmacological agents that can promote functional expression of Δ F508-CFTR will be important.

The cytotoxic nature of Dox and other adverse effects associated with chronic use of this compound make this drug unsuitable for clinical use. Traditionally, this class of compounds was designed to maximize cytotoxicity for

cancer chemotherapy. However, it is plausible that other anthracyclines or related compounds can be identified that can correct Δ F508-CFTR kinesis without toxicity. For example, we are intrigued by the recent clinical development of the anthraquinone pixantrone [51-53] for multiple sclerosis. Pixantrone essentially has a heterocyclic ring in place of the A-ring phenyl group of the anthraquinones, resulting in greatly reduced cardiotoxicity and other side effects. Thus, pixantrone is an example where cardiotoxicity (undesirable effect) was separated from myelosuppression (desirable effect) using SAR [51]. A similar project to develop Dox analogs that promote functional expression of Δ F508-CFTR without associated toxicities may provide a clinical benefit in CF.

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