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Reactivation of the Chloroplast CF_1-ATPase β Subunit by Trace Amounts of the CF_1 α Subunit Suggests a Chaperonin-like Activity for $CF_1\alpha$*

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Incubation of tobacco and lettuce thylakoids with 2 M LiCl in the presence of MgATP removes the β subunit from their CF_1-ATPase ($CF_1\beta$) together with varying amounts of the CF_1 α subunit ($CF_1\alpha$). These 2 M LiCl extracts, as with the one obtained from spinach thylakoids (Avital, S., and Gromet-Elhanan, Z. (1991) *J. Biol. Chem.* 266, 7067–7072), could form active hybrid ATPases when reconstituted into inactive β-less *Rhodospirillum rubrum* chromatophores. Pure $CF_1\beta$ fractions that have been isolated from these extracts could not form such active hybrids by themselves, but could do so when supplemented with trace amounts (less than 5%) of $CF_1\alpha$.

A mitochondrial F_1-ATPase α subunit was recently reported to be a heat-shock protein, having two amino acid sequences that show a highly conserved identity with sequences found in molecular chaperones (Luis, A. M., Alconada, A., and Cuezva, J. M. (1990) *J. Biol. Chem.* 265, 7713–7716). These sequences are also conserved in $CF_1\alpha$ isolated from various plants, but not in $F_1\beta$ subunits. The above described reactivation of $CF_1\beta$ by trace amounts of $CF_1\alpha$ could thus be due to a chaperonin-like function of $CF_1\alpha$, which involves the correct, active folding of isolated pure $CF_1\beta$.

The reversible F_0F_1 H^+-ATP synthase-ATPase couples a transmembrane proton flow to ATP synthesis and hydrolysis. Its water-soluble F_1 sector is by itself an active ATPase, composed of five different polypeptide subunits with a stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$ (1–4). The catalytic sites of the F_1-ATPase reside most likely on its β subunit, although various isolated $F_1\beta$ subunits show less than 0.1% of the corresponding F_1-ATPase activities (5–11). These isolated $F_1\beta$ subunits have, however, a reconstitutive capacity. An earlier developed method for the specific removal of the $F_1\beta$ subunit from *Rhodospirillum rubrum* chromatophores leaves behind β-less chromatophores that could neither synthesize nor hydrolyze ATP (6, 12, 13). The purified Rrβ,[1] which showed by itself no measurable ATPase activity, could reconstitute these inactive β-less chromatophores and restore all their lost ATP-linked activities. A similar reconstitutive capacity has also been shown for $F_1\beta$ subunits isolated from dissociated spinach and *Escherichia coli* F_1-ATPases (8, 14). It enabled Ecβ and $CF_1\beta$ to bind to the β-less *R. rubrum* chromatophores and form active hybrid membrane-bound F_0F_1-ATPase complexes.

The method developed for removal, by 2 M LiCl extraction, of Rrβ from *R. rubrum* chromatophores (6) has recently been applied to spinach thylakoids and found to release not only the $CF_1\beta$ but also 70–80% of $CF_1\alpha$, together with trace amounts of $CF_1\gamma$, -δ, and -ϵ (15). Anion-exchange FPLC revealed in this spinach 2 M LiCl extract two separate fractions, containing either only $CF_1\beta$ or equal amounts of $CF_1\alpha$ and $CF_1\beta$ (11). The purified $CF_1(\alpha\beta)$ complex was by itself an active ATPase and could also reconstitute a hybrid F_0F_1-ATPase with β-less *R. rubrum* chromatophores. The purified $CF_1\beta$ showed no ATPase activity, and although it did bind to β-less chromatophores, it could not form an active hybrid ATPase (11). The main difference between this inactive $CF_1\beta$ and the active $CF_1\beta$, earlier isolated from dissociated spinach CF_1-ATPase (8), was traced to the presence of low amounts (less than 5%) of $CF_1\alpha$ (8, 11) and $CF_1\delta$ (11) in the active $CF_1\beta$ preparation.

These surprising observations led us to investigate whether 1) the release of $CF_1\alpha$ as well as $CF_1\beta$ by 2 M LiCl is a general feature of higher plant chloroplasts and 2) the pure $CF_1\beta$ that binds in an inactive manner to β-less chromatophores can be reactivated by traces of $CF_1\alpha$.

EXPERIMENTAL PROCEDURES

Preparations—Thylakoids were isolated from greenhouse-grown spinach and tobacco (*Nicotiana tabacum* line 92 (16)) or from market lettuce as described by Avital and Gromet-Elhanan (11). Control and β-less *R. rubrum* chromatophores were prepared as previously described (12, 13). Incubation of the thoroughly washed thylakoids with 2 M LiCl in the presence of 4 mM MgATP and fractionation of the resulting 2 M LiCl extracts by anion-exchange FPLC were carried out as outlined in Ref. 11.

Assays—Reconstitution of β-less *R. rubrum* chromatophores was carried out at 35 °C for 1 h using chromatophores equivalent to 3–5 μg of bacteriochlorophyll and varying amounts of the different thylakoid 2 M LiCl extracts or their purified $CF_1\beta$ and $CF_1\alpha\beta$ preparations in a final volume of 0.2 ml containing 50 mM Tricine, pH 8.0, 25 mM $MgCl_2$, 4 mM ATP, 1 mM dithiothreitol, and 7.5% glycerol. The reconstituted chromatophores were centrifuged as described (8), the pellets were resuspended in 0.22 ml of 50 mM Tricine, pH 8.0, and duplicate samples of 0.1 ml were assayed for restored ATPase activity. They were preincubated in 0.6 ml of 50 mM Tricine, pH 8.0, and 20 mM Na_2SO_3 (8) for 10 min at 35 °C, and then the reaction was started by adding 0.1 ml of a solution giving a final concentration of 4 mM ATP and 2 mM $MgCl_2$. It was stopped after 20 min by 0.1 ml of 2 M trichloroacetic acid, and the released P_i was measured after centrifugation as described in Ref. 17.

Protein was determined by the Bradford method (18) using bovine serum albumin as the standard. Chlorophyll was measured according

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[1] The abbreviations used are: CF_1, the soluble F_1-ATPase from chloroplasts; $CF_1\alpha$, $CF_1\beta$, Ecβ, and Rrβ, α and β subunits of the F_1-ATPase of chloroplasts, *E. coli*, and *R. rubrum*; $CF_1(\alpha\beta)$, a complex containing equal amounts of α and β subunits of CF_1; $CF_1\alpha\beta$, a complex containing unequal amounts of α and β subunits; Bchl, bacteriochlorophyll; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; FPLC, fast protein liquid chromatography.

to Ref. 19 and Bchl according to Ref. 20. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out on a linear 10–20% polyacrylamide gradient according to the Laemmli procedure (21), but with high concentrations of Tris (22). Staining was performed by the silver stain procedure (23).

RESULTS AND DISCUSSION

Incubation of thoroughly washed tobacco thylakoids with 2 M LiCl in the presence of 4 mM MgATP resulted in the release of $CF_1\beta$ as well as $CF_1\alpha$ (Fig. 1, *lane 5*). But the amount of $CF_1\alpha$ that this treatment released from tobacco thylakoids was lower than from spinach thylakoids (11, 15), amounting to only one-fifth to one-third of the level of released $CF_1\beta$. Fractionation of the tobacco 2 M LiCl extract on an FPLC Mono Q column revealed here, as in spinach (11), two separate fractions. One contained $CF_1\beta$ without any trace of $CF_1\alpha$ and showed no reconstitutive capacity (Fig. 1, *lanes 1* and *2*). The other contained both $CF_1\alpha$ and -β subunits, but its content of $CF_1\alpha$ was always much lower than $CF_1\beta$ (Fig. 1, *lanes 3* and *4*). This $CF_1\alpha\beta$ fraction showed a 3–4-fold higher reconstitutive activity than the 2 M LiCl extract, and its activity increased when the α/β ratio decreased (compare *lanes 3*, *4*, and *5* in Fig. 1).

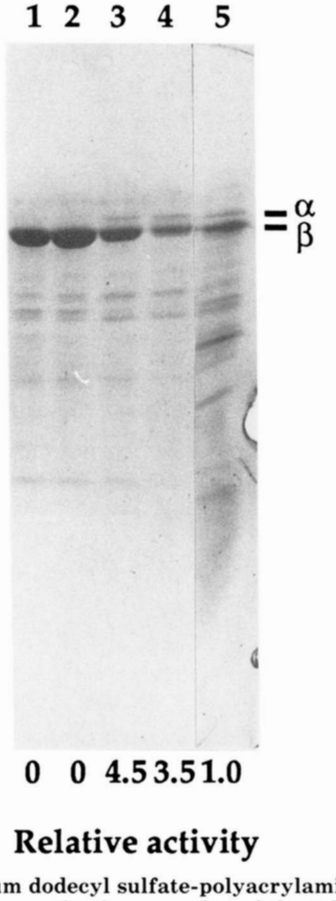

FIG. 1. **Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and reconstitutive capacity of the tobacco 2 M LiCl extract and two fractions of FPLC Mono Q chromatography.** Gels were run and stained as described under "Experimental Procedures." 3 μg of protein was applied to each lane. *Lanes 1* and *2*, the first fraction eluted from the Mono Q column at 150 mM NaCl; *lanes 3* and *4*, the second fraction eluted at 250 mM NaCl; *lane 5*, unfractionated 2 M LiCl extract. The positions of α and β subunits of the CF_1-ATPase are shown on the *right*. The indicated activity reflects the rate of ATP hydrolysis restored to β-less *R. rubrum* chromatophores by their reconstitution with the various tobacco protein fractions at a 1:1 ratio of protein to Bchl. A relative activity of 1.0 equals a rate of 56 ± 5 μmol of P_i released/h-mg of Bchl, which was obtained with the unfractionated 2 M LiCl extract under these reconstitution conditions.

A similar pattern of 2 M LiCl-released CF_1 subunits was also obtained with lettuce thylakoids, except that it was more similar to the one found in spinach than in tobacco. Thus, incubation with 2 M LiCl released in lettuce about 0.5 $CF_1\alpha$ per 1.0 $CF_1\beta$, and fractions containing equal amounts of $CF_1\alpha$ and -β subunits were obtained by anion-exchange chromatography of this 2 M LiCl extract (not shown).

Protein fractions from both lettuce and tobacco, which contained $CF_1\beta$ together with varying amounts of $CF_1\alpha$, were all reconstitutively active. Fig. 2 illustrates the reconstitutive capacity of the lettuce $CF_1(\alpha\beta)$ complex, containing equal amounts of both α and β subunits, and a tobacco $CF_1\alpha\beta$ preparation containing a ratio of one $CF_1\alpha$ to five $CF_1\beta$. Incubation of increasing amounts of these lettuce and tobacco preparations with a fixed concentration of inactive β-less *R. rubrum* chromatophores led to a concentration-dependent restoration of the chromatophores' ATPase activity. But, as was earlier observed with the spinach $CF_1(\alpha\beta)$ complex (11), no restoration was observed when the reconstitution was carried out with less than 0.5 μg of either plant protein (Fig. 2).

These results demonstrate that the release of $CF_1\alpha$ together with $CF_1\beta$ by 2 M LiCl is indeed a general feature of higher plant thylakoids. The ratio of released $CF_1a/CF_1\beta$ does, however, vary from 0.2–0.3 in tobacco (Fig. 1) to 0.5 in lettuce and 0.7–0.8 in spinach (15). Furthermore, the released subunits appear in two discrete fractions, one containing pure $CF_1\beta$ and one containing both $CF_1\alpha$ and -β subunits. Interestingly, none of the isolated pure $CF_1\beta$ preparation was able to form active hybrids with β-less *R. rubrum* chromatophores (Figs. 1, 3, and 4), whereas all the $CF_1\alpha\beta$ preparations did form such active hybrids (Fig. 2) (11). These reconstitutively

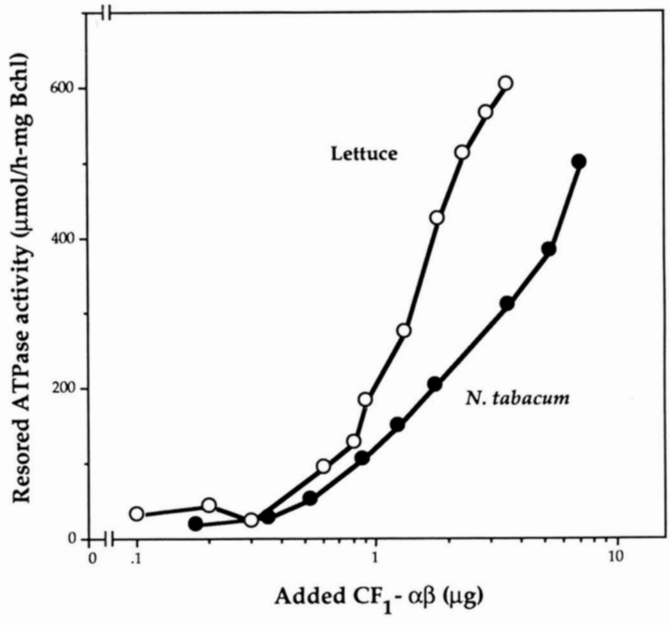

FIG. 2. **ATPase activity restored to β-less *R. rubrum* chromatophores by their reconstitution with varying amounts of $CF_1\alpha\beta$ preparations from lettuce and tobacco.** The reconstitution was carried out with a fixed amount of β-less chromatophores containing 5 μg of Bchl and the indicated amounts of lettuce or tobacco proteins. The resulting hybrid chromatophores were assayed for restored ATPase activity as described under "Experimental Procedures." The lettuce $CF_1(\alpha\beta)$ complex contained equal amounts of α and β, whereas the tobacco preparation contained a α/β ratio of 0.2.

active $CF_1\alpha\beta$ preparations had $CF_1\alpha/CF_1\beta$ ratios varying between 1.0 in the spinach (11) and lettuce (Fig. 2) $CF_1(\alpha\beta)$ complexes, 0.2 in the tobacco $CF_1\alpha\beta$ (Fig. 1, *lane 3* and Fig. 2), and 0.05 in the active preparations of enriched $CF_1\beta$, isolated from dissociated spinach CF_1 (8) or the spinach 2 M LiCl extract (11). It is, therefore, possible that the reconstitutive capacity of $CF_1\beta$ is dependent on the presence of trace amounts of $CF_1\alpha$.

A direct demonstration of the requirement of $CF_1\alpha$ for the reconstitutive activity of a $CF_1\alpha\beta$ preparation was obtained by immunocompetition experiments. For these experiments, protein A-antibody pellets were formed by incubation of 10% Sepharose-protein A with either normal or anti-α serum. The washed pellets were used for immunoprecipitation of antigen-antibody complexes from the tobacco 2 M LiCl extract. The supernatant fractions obtained after immunoprecipitation with normal or anti-α serum lost 18 and 93%, respectively, of their original reconstitutive activity. Immunoblots of these supernatant fractions exhibited a clear correlation between their decreased reconstitutive capacity and the loss of $CF_1\alpha$ (not shown).

We have earlier found that the pure spinach $CF_1\beta$ is able to bind to β-less *R. rubrum* chromatophores, but unlike with $CF_1(\alpha\beta)$, its binding does not lead to restoration of their ATPase activity (11). It was therefore most interesting to examine whether added traces of $CF_1\alpha$ could also reactivate such isolated pure inactive $CF_1\beta$. Unfortunately, no isolated $CF_1\alpha$ is as yet available for such tests. However our observation (Fig. 2) (11) that, when the reconstitution of active $CF_1\alpha\beta$ preparations into β-less chromatophores was carried out at low (below 0.1) ratios of reconstituted protein to Bchl, no restoration of ATPase activity was obtained enabled us to use these low amounts of $CF_1\alpha\beta$ as a source of $CF_1\alpha$. Fig. 3 illustrates the dramatic stimulation of the reconstitutive activity of pure spinach $CF_1\beta$ in the presence of 0.4 μg of the spinach $CF_1(\alpha\beta)$ complex and 4 μg of Bchl. With increasing amounts of this $CF_1\beta$, a concentration-dependent restoration of the chromatophores' ATPase activity was obtained. It saturated at about 4 μg of $CF_1\beta$, and since the 0.4 μg of $CF_1(\alpha\beta)$ contained equal amounts of both subunits, this saturation occurred with a ratio of 0.05 $CF_1\alpha/CF_1\beta$.

The stimulation of the reconstitutive activity of $CF_1\beta$ by trace amounts of $CF_1\alpha$ seems to be a general feature of the plant CF_1 subunits. As is shown in Fig. 4, traces of a lettuce $CF_1(\alpha\beta)$ complex could even stimulate a tobacco $CF_1\beta$. So the various pure $CF_1\beta$ preparations, which bind to the β-less chromatophores in an inactive form, are not defective in any irreversible manner since they can be reactivated by traces of $CF_1\alpha$.

The above experiments have clearly demonstrated that removal of $CF_1\alpha$ inhibits, and its addition stimulates, the capacity of $CF_1\beta$ to bind to β-less *R. rubrum* chromatophores in an active manner. These results by themselves could not specify what is the exact functional role of $CF_1\alpha$ in the reactivation process of $CF_1\beta$. But, together with some very unexpected results reported in two recent publications (24, 25), they do suggest a rather specific function for $CF_1\alpha$. These reports have shown that the mitochondrial $F_1\alpha$ subunit is a heat-shock protein (24) that appears in rat liver peroxisomes unaccompanied by any $F_1\beta$ subunit (25). They have also found that two amino acid sequences of 12 and 13 residues, which are present in $F_1\alpha$, but not -β subunits, of various mitochondrial and chloroplast F_1-ATPase complexes, show a highly conserved identity with amino acid sequences found in molecular chaperones (24). The heat-shock chaperonin class of proteins are known to be involved in the correct folding and assembly of various protein complexes both *in vivo* and *in vitro* (26–30). It is thus plausible that the reactivation of $CF_1\beta$ by $CF_1\alpha$ reflects such a chaperonin-like function of $CF_1\alpha$.

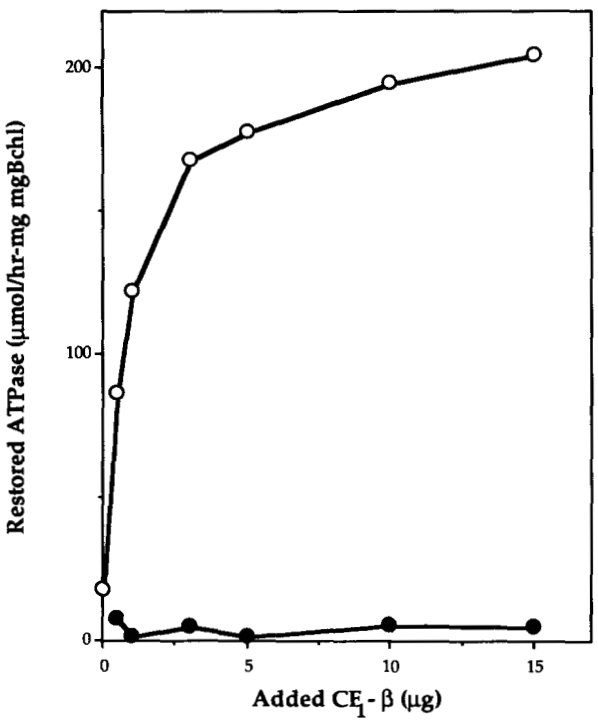

FIG. 3. **Activation of the reconstitutive capacity of pure spinach $CF_1\beta$ by a fixed low amount of pure spinach $CF_1(\alpha\beta)$.** The reconstitution was carried out with β-less chromatophores containing 4 μg of Bchl and the indicated amounts of pure spinach $CF_1\beta$ without (●) or with (○) 0.4 μg of spinach $CF_1(\alpha\beta)$. The resulting hybrid chromatophores were assayed for restored activity, as described in Fig. 2.

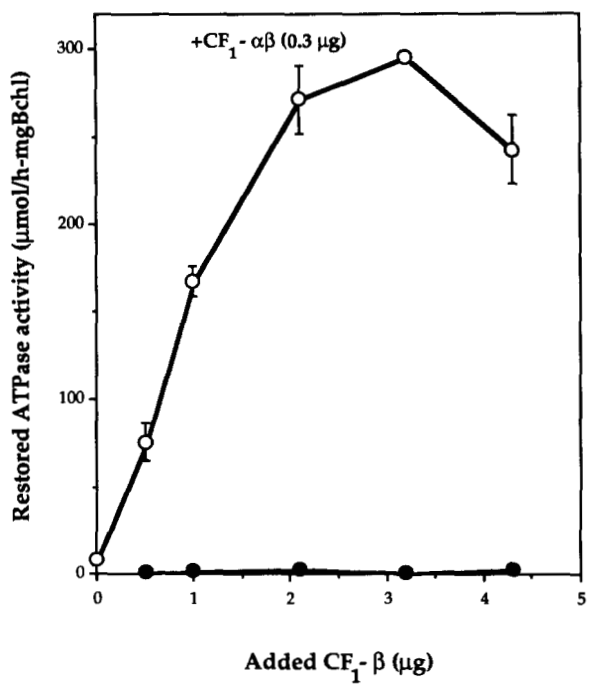

FIG. 4. **Activation of the reconstitutive capacity of pure tobacco $CF_1\beta$ by a low amount of pure lettuce $CF_1(\alpha\beta)$.** The reconstitution was carried out with β-less chromatophores containing 5 μg of Bchl and the indicated amounts of pure tobacco $CF_1\beta$ without (●) and with (○) 0.3 μg of lettuce $CF_1(\alpha\beta)$.

We therefore propose that $CF_1\alpha$ facilitates the correct folding of isolated $CF_1\beta$, thus enabling its assembly into the β-less chromatophores in an active state that results in formation of a hybrid F_0F_1-ATPase. It is clear from the above results and those earlier presented (11) that, as long as the pure $CF_1\beta$ remains in the soluble state, it needs the presence of $CF_1\alpha$ to keep it in the active configuration. But it is not clear yet what happens to $CF_1\alpha$ once this correctly folded $CF_1\beta$ binds to the β-less chromatophores that have their assembled $F_1\alpha$ subunit (6, 13). The fact that even trace amounts of about 0.05 $CF_1\alpha/CF_1\beta$ are enough for keeping the $CF_1\beta$ in the active configuration required for binding with restoration of ATPase activity (Figs. 3 and 4) (11) might indicate that $CF_1\alpha$ does not bind to the β-less chromatophores together with $CF_1\beta$. It would thus remain in solution and be available for reactivation of additional molecules of pure $CF_1\beta$. This suggestion is now under investigation.

It might also be possible that the almost 100-fold increase in soluble ATPase activity observed when isolated $F_1\beta$ subunits are assembled into $F_1(\alpha\beta)$ complexes (9–11) is due to a similar chaperonin-like function of the $F_1\alpha$ subunit. But in this case, unlike in the reconstitution with the β-less chromatophores, there is no source of assembled $F_1\alpha$ subunit, and therefore, equivalent amounts of the $F_1\alpha$ subunit are required to keep the $F_1\beta$ subunit in its active configuration. They thus form an unstable soluble $F_1(\alpha\beta)$-ATPase complex (9–11) that becomes stabilized upon addition of the $F_1\gamma$ subunit (9, 10). When such a $CF_1(\alpha\beta)$ complex, containing equal amounts of both subunits (Fig. 2) (11) binds to the β-less chromatophores, it might dissociate, enabling the $CF_1\beta$ to bind and leaving $CF_1\alpha$ in solution. It is, however, also possible that the whole $CF_1(\alpha\beta)$ complex will bind to the β-less chromatophore. In this case, a special type of hybrid will be formed containing $CF_1\alpha$ and -β subunits and $RrF_1\alpha$, -γ, -δ, and -ϵ subunits. This possibility is supported by some previous observations that restoration of ATP synthesis could be achieved in the 2 M LiCl-extracted β-less *R. rubrum* chromatophores in two ways: 1) by their very efficient reconstitution with $Rr\beta$ (6, 12, 13), and 2) by a much less efficient reconstitution with high concentrations of the whole RrF_1 (31, 32). We are now investigating both possibilities.

The above results suggest that $CF_1\alpha$ belongs to a class of molecules that function in a chaperonin-like manner but can also become an integral part of the protein complex they help to assemble.

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