Effects of preincubation of primary monolayer cultures of rat hepatocytes with low- and high-density lipoproteins on the subsequent binding and metabolism of human low-density lipoprotein

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1. There are two distinct binding sites (Site 1 and Site 2) for human low-density lipoprotein (LDL) on rat hepatocytes in monolayer culture [Salter, Saxton & Brindley (1986) *Biochem. J.* **240**, 549–557]. 2. Binding of ^{125}I -LDL to Site 1, but not to Site 2, is up-regulated between 20 and 44 h in culture by preincubation of the cells with human high-density lipoprotein 3 (HDL₃). 3. A similar preincubation with HDL₂ had no significant effect on binding to either site. 4. Preincubation with human LDL led to a partial down-regulation of subsequent binding of ^{125}I -LDL to Site 1. Since binding after incubation with LDL was measured at 37 °C, binding to Site 2 could not be distinguished from LDL that had been internalized by the cells. 5. Hepatocytes were shown to degrade ^{125}I -LDL, resulting in the accumulation of [^{125}I]iodotyrosine in the medium. Evidence was found that iodotyrosine may be further degraded by deiodinase produced by the cells. 6. Regulation of binding to Site 1 by preincubation with LDL or HDL₃ was found to lead to a parallel regulation of LDL degradation. 7. It is concluded that rat hepatocytes not only bind but also metabolize human LDL and that these processes are under metabolic regulation.

INTRODUCTION

We have previously characterized the binding of human ^{125}I -LDL to two sites on rat hepatocytes in monolayer culture [1,2]. Binding to Site 1 was characterized by: (a) being displaced by sulphated polysaccharides; (b) being dependent on Ca^{2+} ; (c) having a K_d of about 15 μg of protein/ml; and (d) increasing with time in culture when newborn-calf serum was present in the medium. By contrast, binding to Site 2: (a) was not displaced by sulphated polysaccharides; (b) was only partially Ca^{2+} -dependent; (c) had a K_d of about 30 μg of protein/ml; and (d) changed little with increasing time in culture. Site 1 appears analogous to the LDL receptor described on cultured fibroblasts and in a number of other systems in which it is involved in receptor-mediated endocytosis [3]. Site 2 may be part of a distinct system whereby the cell recognizes LDL, and which has been demonstrated with monolayer cultures of human hepatocytes of patients with homozygous familial hypercholesterolaemia [4,5]. It also may be relevant that ligand blotting has demonstrated there to be more than one protein on plasma membranes from rat liver that can bind LDL [6].

In the present study we have investigated the control of the binding of human ^{125}I -LDL to Sites 1 and 2 and its subsequent degradation in rat hepatocytes preincubated with human LDL, HDL₂ and HDL₃.

EXPERIMENTAL

Animals and materials

The sources of rats and most of the materials have been described elsewhere [1,7,8]. 3-Iodo-L-tyrosine was obtained from Sigma (London) Chemical Co.

Preparation and incubation of hepatocytes

The preparation of hepatocytes was as previously described [7]. In early experiments hepatocytes were attached, without the use of collagen, to Primaria tissue culture dishes (Falcon) in a modified [9] Leibovitz L-15 medium containing 10% (v/v) newborn-calf serum. Incubations under serum-free conditions were performed in Leibovitz L-15 medium supplemented with 2 g of fatty-acid-poor bovine serum albumin/l. Because of problems in obtaining an even monolayer, later experiments were performed on cells attached to collagen-coated plates [10].

Lipoprotein isolation and iodination

Human lipoproteins were prepared by sequential ultracentrifugation of freshly isolated plasma [11] taken from healthy young adult males. LDL, HDL₂ and HDL₃ were isolated within the density ranges 1.02–1.06, 1.075–1.125 and 1.125–1.210 g/ml respectively. Lipoproteins were characterized and LDL was iodinated as previously described [1].

Abbreviations used: HDL, high-density lipoprotein; LDL, low-density lipoprotein.

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¹²⁵I-LDL binding and degradation assays

The binding of ¹²⁵I-LDL to hepatocytes at 4 °C was performed in serum-free Leibovitz L-15 medium supplemented with 2 g of fatty-acid-poor bovine serum albumin/l as previously described [1]. ¹²⁵I-LDL released by dextran sulphate was defined as that bound to Site 1, and that remaining associated with the cell as that bound to Site 2. Non-specific binding was measured in parallel incubations in the presence of at least a 30-fold excess of unlabelled LDL. Specific binding was calculated by subtracting non-specific binding from the total.

The degradation of ¹²⁵I-LDL was determined as described by Goldstein *et al.* [12]. After the stated period of time in culture, cells were washed and incubated with ¹²⁵I-LDL at 37 °C. Parallel incubations were performed with plates containing no cells to determine the cell-independent degradation of ¹²⁵I-LDL. After the incubation 1 ml of medium was taken from each plate and added to 1 ml of ice-cold 20% (w/v) trichloroacetic acid. This was left at 4 °C for at least 15 min, and then precipitated proteins were sedimented by centrifugation (bench) at 4 °C. Samples of the supernatant (200 µl) were taken to determine the total acid-soluble ¹²⁵I radioactivity. A further 1 ml of the supernatant was mixed with 10 µl of 40% (w/v) KI, followed by 40 µl of 30% (v/v) H₂O₂. Free ¹²⁵I was then extracted into 2 ml of chloroform. After 15 min at room temperature, tubes were centrifuged for 5 min in a bench centrifuge, and 0.75 ml of the upper aqueous phase was taken for determination of peroxidation-resistant radioactivity (assumed to be [¹²⁵I]iodotyrosine). Control values of total acid-soluble ¹²⁵I radioactivity and [¹²⁵I]iodotyrosine which were obtained in the absence of cells were subtracted from the values obtained in the presence of cells. Non-specific degradation was measured in parallel incubations in the presence of excess unlabelled LDL.

Cells from these experiments were then washed and treated with dextran sulphate as previously described [1] to assess binding to Site 1. ¹²⁵I-LDL remaining associated with the cells was taken to be that bound to Site 2 plus that internalized but not degraded.

RESULTS

Effect on ¹²⁵I-LDL binding of preincubating with human HDL₂, HDL₃ and LDL

We have previously shown up-regulation of human ¹²⁵I-LDL binding to Site 1, but not Site 2, on rat hepatocytes between 24 and 48 h in culture in the presence of 10% newborn-calf serum [1]. Table 1 extends these findings. After 4 h, binding to Site 1 was approximately equal to that to Site 2, but by 44 h it is almost 4 times higher. Binding to Site 1 and Site 2 increased by 3.6- and 2.2-fold respectively between 4 and 20 h in culture. Between 20 and 44 h there was no further significant binding to Site 2, whereas that to Site 1 increased, to 5.6 times the value at 4 h. We have suggested that this up-regulation of Site 1 may be partly mediated by HDL in the calf serum [1]. To test this hypothesis, hepatocytes were incubated for 20 h in the presence of 10% newborn-calf serum, followed by a further 24 h in serum-free medium supplemented with 2 g of bovine serum albumin/l with or without various concentrations of HDL₂ or HDL₃. After this incubation, binding of ¹²⁵I-LDL was measured at 4 °C. HDL₃ caused a marked increase in binding to Site 1, but had little effect

Table 1. Effect of time in culture on the binding of ¹²⁵I-LDL to rat hepatocytes

Rat hepatocytes were maintained in culture for the length of time indicated. They were then incubated with ¹²⁵I-LDL (10 µg of protein/ml) in the presence or absence of excess unlabelled LDL. Specific binding to Site 1 and Site 2 was determined as described in the Experimental section. The results are means ± S.E.M., with the numbers of individual experiments in parentheses: * indicates statistical difference of *P* < 0.05 as measured by paired *t* test.

Time in culture (h)	Specific binding (ng of LDL protein/mg of cell protein)	
	Site 1	Site 2
(i) 4	4.6 ± 1.5 (n = 4)	4.4 ± 1.1
(ii) 20	14.4 ± 4.6 (n = 6)	8.3 ± 1.8
(iii) 44	22.7 ± 4.4 (n = 6)	6.3 ± 1.1
	(i) versus (iii)* (ii) versus (iii)*	(i) versus (ii)*

on Site 2 (Fig. 1). In three independent experiments, preincubation with 100 µg of HDL₃ protein/ml resulted in a 2.5 ± 0.7-fold (mean ± S.E.M.) increase in binding compared with preincubation in medium without HDL. Preincubation with HDL₂ had no significant effect on

Fig. 1. Effect of preincubation with HDL₂ or HDL₃ on the subsequent specific binding of ¹²⁵I-LDL to rat hepatocytes

Rat hepatocytes were maintained in monolayer culture for 20 h in the presence of 10% newborn-calf serum. They were then incubated for a further 24 h in serum-free medium supplemented with various concentrations of human HDL₂ (▲, △) or HDL₃ (●, ○). Plates were then cooled to 4 °C, washed with serum-free medium and incubated with 10 µg of ¹²⁵I-LDL protein/ml for 4 h at 4 °C. Non-specific binding was measured in parallel incubations in the presence of 300 µg of unlabelled LDL protein/ml. This was subtracted from total binding to give the specific binding shown. Binding to Site 1 (○, △) and Site 2 (●, ▲) were determined as described in the Experimental section. The results were reproduced in two further independent experiments.

Fig. 2. Effect of preincubation with HDL₃ on the kinetics of subsequent specific binding of ¹²⁵I-LDL to Site 1 on rat hepatocytes

Rat hepatocytes were maintained in monolayer culture for 20 h in the presence of 10% newborn-calf serum. They were then incubated for a further 24 h in either serum-free medium (○) or serum-free medium supplemented with 200 μg of HDL₃ protein/ml (●). Plates were then cooled to 4 °C, washed with serum-free medium and then incubated for 4 h at 4 °C with various concentrations of ¹²⁵I-LDL in the presence or absence of excess unlabelled LDL. Specific binding to Site 1 was determined as described in Fig. 1, and is shown in Fig. 2(a). Fig. 2(b) shows the analysis of specific binding by the method of Scatchard [13]. Results were reproduced in a further independent experiment.

binding to Site 1 or Site 2. Full dose-response curves of ¹²⁵I-LDL binding were performed after 20 h in serum-containing medium followed by 24 h in serum-free medium supplemented with albumin with or without 100 μg of HDL₃ protein/ml. Fig. 2 demonstrates an increase in $B_{\max}$ of binding after incubation with HDL₃, with little change in K_d .

Hepatocytes were also incubated for 20 h in medium containing 10% newborn-calf serum followed by a 24 h incubation in serum-free medium supplemented with 2 g of bovine serum albumin/l with or without unlabelled LDL (Fig. 3). Binding of ¹²⁵I-LDL was then measured at 37 °C rather than 4 °C. This ensured that all binding sites were available and not blocked by unlabelled LDL, which would presumably be internalized at 37 °C. This means that ¹²⁵I-LDL bound to Site 2 could not be determined as that left associated with the cells after dextran sulphate treatment. This includes that bound to Site 2, and also any that had been internalized but not degraded. The mean extent of inhibition of binding to Site 1 after preincubation with 100 μg of LDL protein/ml was 24 ± 12% (mean ± S.E.M. for four independent experiments). This was not statistically significant.

Fig. 3. Effect of preincubation with LDL on the subsequent specific binding of ¹²⁵I-LDL to Site 1 on rat hepatocytes

Rat hepatocytes were maintained in monolayer culture for 20 h in the presence of 10% newborn-calf serum. They were then incubated for a further 24 h in serum-free medium supplemented with various concentrations of human LDL. Plates were then washed with serum-free medium and incubated with 10 μg of ¹²⁵I-LDL protein/ml, in the presence or absence of excess unlabelled LDL, for 4 h at 37 °C. Specific binding to Site 1 was determined as described in Fig. 1. Results are shown relative to binding after preincubation in the absence of LDL and represent means ± S.E.M. for four independent experiments.

Degradation of ¹²⁵I-LDL

The metabolism of ¹²⁵I-LDL was determined by measuring: (a) total acid-soluble ¹²⁵I and [¹²⁵I]-iodotyrosine in the medium, (b) ¹²⁵I-LDL released by dextran sulphate, and (c) that remaining associated with the cells (Fig. 4). Binding to Site 1 remained relatively constant throughout the experiment. Cell-associated ¹²⁵I-LDL, which includes both that bound to Site 2 and internalized ¹²⁵I-LDL, increased rapidly over the first 2 h and then more slowly thereafter. The total acid-soluble radioactivity in the medium was consistently higher than the non-chloroform-extractable acid-soluble. For example, after 24 h incubation, total acid-soluble radioactivity was the equivalent of 598 ± 327 (mean ± S.E.M.) ng of LDL protein/mg of cell protein, as compared with 80 ± 24 for acid-soluble radioactivity not extractable with chloroform. However, because of the relatively high radioactivity in the total acid-soluble fraction obtained from the medium of control incubations in the absence of cells, which was frequently in excess of 90% of that when cells were present, variability was high and results were inconsistent. In contrast, measurements of [¹²⁵I]-iodotyrosine were reproducible, and control values in the absence of cells were usually less than 50% of the total. In the four experiments shown, ¹²⁵I-LDL was degraded to [¹²⁵I]iodotyrosine at the rate of 1.39 ± 0.21 (mean ± S.E.M.) ng of LDL protein/h per mg of cell protein. It has been suggested that [¹²⁵I]iodotyrosine is specifically degraded to free ¹²⁵I by deiodinase produced by the hepatocytes [14,15,16] and that this might explain the relatively high amounts of chloroform-extractable acid-soluble ¹²⁵I produced. To explore this possibility, in two of the experiments parallel incubations were performed in the presence of 5 mM-monoiodotyrosine, to inhibit the breakdown of [¹²⁵I]iodotyrosine by deiodinase [16]. Monoiodotyrosine increased the amount of [¹²⁵I]-

Fig. 4. Binding, uptake and degradation of ^{125}I -LDL by rat hepatocytes

Rat hepatocytes were maintained in monolayer culture for 20 h in the presence of 10% newborn-calf serum. They were then washed with serum-free medium and then incubated at 37 °C for the length of time indicated with 10 μg of ^{125}I -LDL protein/ml in the presence or absence of excess unlabelled LDL. Fig. 4(a) shows specific binding to Site 1 (○), and ^{125}I -LDL that remained associated with the cells after dextran sulphate treatment (●). Fig. 4(b) shows the total (○) and specific (●) accumulation of peroxidation-resistant ^{125}I in the medium, which was estimated as described in the Experimental section. Results represent means $\pm$ S.E.M. for four independent experiments.

iodotyrosine produced from 1.3 ± 0.4 to 6.7 ± 0.74 ng of LDL protein degraded/h per mg of cell protein. This suggests that measurement of [^{125}I]iodotyrosine may give an underestimation of degradation rate. However, Fig. 5 shows that a strong relationship exists ($r = 0.95$, $P < 0.001$) between the extent of binding to Site 1 and the amount of [^{125}I]iodotyrosine produced. Binding to Site 1 was altered by preincubating the hepatocytes for 24 h in the presence of various concentrations of LDL, HDL₂ or HDL₃.

DISCUSSION

We previously showed [1] that primary monolayer cultures of rat hepatocytes specifically bind human ^{125}I -LDL to two sites (Site 1 and Site 2). We now confirm our findings of a progressive increase in binding to Site 1 with time in culture. Between 4 and 20 h we find increases in binding to Site 1 and Site 2. Major changes in both the morphology [17] and the metabolism [18] of the cells

Fig. 5. Correlation between specific binding to Site 1 and specific degradation of ^{125}I -LDL by rat hepatocytes after pre-incubation with LDL, HDL₂ and HDL₃

Rat hepatocytes were maintained in monolayer culture for 20 h in the presence of 10% newborn-calf serum. They were then incubated for a further 24 h in serum-free medium supplemented with LDL (○), HDL₂ (△) or HDL₃ (▲) at the concentrations (μg of protein/ml) indicated in parentheses. Plates were then washed with serum-free medium and incubated with 10 μg of ^{125}I -LDL protein/ml in the presence or absence of excess unlabelled LDL. Specific binding was estimated as described in Fig. 1, and specific degradation as in Fig. 4(b). Similar results were obtained in three further independent experiments.

occur during the first hours of culture. Since it is not known whether these changes would affect LDL binding, it was decided to use incubations between 20–44 h, when the cells are more stable, to look at the effects of preincubation with lipoproteins. During this period, in the presence of 10% calf serum binding to Site 1 continues to rise, whereas that to Site 2 remains relatively constant. This up-regulation of binding to Site 1 may represent regeneration of receptors which are damaged during hepatocyte isolation, or it may reflect a low extent of binding *in vivo*, with activity increasing in response to culture conditions. The present study shows that binding to Site 1 can be regulated by the lipoprotein composition of the incubation medium. Preincubation with human HDL₃ increases binding to Site 1: HDL₂ has little effect, and LDL partially down-regulates binding. However, HDL₃ and HDL₂ had no significant effect on binding to Site 2. The effect of preincubation with LDL on binding to Site 2 could not be measured, since binding had to be estimated at 37 °C, as opposed to 4 °C. Under these conditions cell-associated ^{125}I -LDL included both that bound to Site 2 and that which was internalized.

The up-regulation of LDL binding, by preincubation with HDL, has been described in several other cell types. Incubation of fibroblasts or smooth-muscle cells with

either HDL₃ or very-high-density lipoprotein ($\rho = 1.21\text{--}1.25$ g/ml) increases both binding and degradation of ^{125}I -LDL [19]. HDL_{2b} ($\rho = 1.063\text{--}1.100$ g/ml) and HDL_{2a} ($\rho = 1.100\text{--}1.125$ g/ml) had little effect on either of these parameters. Human 'heavy' HDL ($\rho = 1.16\text{--}1.20$ g/ml) up-regulates the binding of human ^{125}I -LDL to the human hepatoma cell line Hep G2 [20]. As in our study, this up-regulation was shown to be caused by an increase in the number of receptors rather than a change in affinity. In contrast, 'light' HDL ($\rho = 1.09\text{--}1.13$ g/ml) had no significant effect. Thus results from fibroblasts, smooth-muscle cells and Hep G2 cells agree well with our findings with rat hepatocytes. HDL ($\rho = 1.12\text{--}1.16$ g/ml) from pigs up-regulates degradation of pig ^{125}I -LDL by monolayer cultures of pig hepatocytes, but apparently does not affect uptake. Binding was not measured directly in that study [16]. In contrast, simultaneous incubation of rabbit hepatocytes with rabbit ^{125}I -LDL and a 50-fold excess of rabbit HDL ($\rho = 1.063\text{--}1.21$ g/ml) led to a time-dependent down-regulation of LDL metabolism [21].

The up-regulation of LDL binding by the denser HDL fractions probably relates to the ability of HDL to promote cholesterol efflux from the cells. Preincubation of fibroblasts with HDL₃ increases the rate of sterol synthesis and decreases the rate of cholesterol esterification [22]. Whereas the actual changes in cellular cholesterol were small, the changes in synthesis and esterification were taken as indicators of enhanced cholesterol efflux induced by the presence of HDL₃. HDL₂ had no significant effect on sterol synthesis, and it stimulated esterification. In Hep G2 cells [20] the stimulation of LDL binding mediated by 'heavy' HDL was associated with a 2–4-fold increase in the activity of 3-hydroxy-3-methylglutaryl-CoA reductase, again suggesting cholesterol depletion in the cells. However, as yet, we have failed to demonstrate any inhibition of cholesterol esterification in rat hepatocytes after incubation with human HDL₃ (A. M. Salter, S. C. Fisher & D. N. Brindley, unpublished work). A possible explanation for this might be that in hepatocytes the pool of cholesterol associated with acyl-CoA:cholesterol acyltransferase may be specifically protected from depletion via efflux in HDL.

Preincubation of fibroblasts with as little as $20\text{ }\mu\text{g}$ of LDL/ml for 24 h leads to a 75% decrease in LDL binding [23]. We find that incubation of hepatocytes with $100\text{ }\mu\text{g}$ of LDL/ml decreased subsequent binding to Site 1 by only 24%. Preincubation of Hep G2 cells with a similar concentration of LDL decreased subsequent binding by 43%, whereas in parallel experiments with human fibroblasts binding was decreased by 83% [20]. Human hepatocytes in culture have also been shown to be less responsive to preincubation with LDL than are fibroblasts [4].

This relative lack of response to LDL may reflect the role of the liver in removal of cholesterol from the circulation and its ultimate excretion in the bile. Two possible explanations for this decreased sensitivity are (1) that this level of regulation is absent in hepatocytes, enabling them to accumulate LDL cholesterol even in the presence of high LDL concentrations, and (2) that cholesterol derived from LDL is rapidly mobilized and re-secreted either in the bile or as newly synthesized lipoprotein. Edge *et al.* [4] claimed that, after a 6 h incubation with $200\text{ }\mu\text{g}$ of LDL protein/ml, the chole-

sterol content of both human hepatocytes and fibroblasts approximately doubled. Despite this, the degree of down-regulation of subsequent LDL binding was considerably less in the hepatocytes. In preliminary experiments we have seen a 15% increase in total cellular cholesterol content after preincubation of rat hepatocytes with $200\text{ }\mu\text{g}$ of human LDL protein/ml for 48 h, compared with parallel incubations in albumin-containing serum-free medium (A. M. Salter, S. C. Fisher & D. N. Brindley, unpublished work). Cholesterol ester content increased by 70% over the same period. Davis *et al.* [24] failed to demonstrate any changes in cellular cholesterol content or bile acid secretion by rat hepatocytes after preincubation with rat LDL. However, these cells were incubated in serum-free medium from 4 h in culture. In our system, binding to Site 1 would be very low under these conditions and the uptake of LDL may have been retarded.

Whereas rat hepatocytes in monolayer culture are known to secrete both VLDL and HDL components [25,26,27], little evidence is available concerning the effects of LDL uptake on such secretion. However, stimulation of the formation of cholesterol esters within the hepatocyte leads to a secretion of VLDL particles rich in cholesterol ester [28,29]. We have found an increased rate of esterification in rat hepatocytes of about 2-fold after preincubation with $100\text{ }\mu\text{g}$ of human LDL protein/ml for 24 h (A. M. Salter, S. C. Fisher & D. N. Brindley, unpublished work).

Deiodinase activity towards iodotyrosine is relatively high in liver [14,15,16]. The measurement of ^{125}I -iodotyrosine could therefore underestimate the degradation rate. We found that measurement of total acid-soluble radioactivity (both iodotyrosine and any ^{125}I released by deiodinase), though consistently giving higher values than measurements of ^{125}I -iodotyrosine alone, was not a reliable measure of degradation. This was because the amount of acid-soluble radioactivity from parallel incubations in the absence of cells was frequently in excess of 90% of that in the presence of cells. Incubating cells with ^{125}I -LDL and 5 mM-mono-iodotyrosine increased the amount of ^{125}I -iodotyrosine recovered by about 5-fold. Possibly the measurement of ^{125}I -iodotyrosine production in the presence of unlabelled iodotyrosine might provide a more accurate estimation of specific degradation rates of ^{125}I -LDL. However, it remains to be established whether the iodotyrosine in any way interferes with the metabolism of the cell. So far this procedure has not been used as a routine method. Even in the presence of iodotyrosine the rate of degradation is considerably lower than that reported for fibroblasts [12]. This agrees with studies which have directly compared degradation by either human hepatocytes [4] or Hep G2 cells [18] with fibroblasts.

To investigate whether degradation was directly related to binding, cells were preincubated in media containing different concentrations of LDL and HDL for 24 h. There was a strong correlation between binding to Site 1 and the ^{125}I -iodotyrosine produced. Although the measurement of ^{125}I -iodotyrosine (in the absence of unlabelled iodotyrosine) may under-estimate the total rate of degradation, it must be highly correlated with it. This degradation appears to depend on binding to Site 1, but as yet we have no direct evidence of degradation by Site 2. However, part of the residual degradation observed in

Fig. 5 when binding to Site 1 is extrapolated back to zero may be mediated through binding to Site 2, together with other less specific processes.

The present study shows that rat hepatocytes not only bind, but can also degrade, human ^{125}I -LDL. Binding to Site 1 appears to be regulated in a manner analogous to that shown with human hepatocarcinoma cells [18] and monolayer cultures of normal human hepatocytes [4,5]. This re-affirms our view that the interaction of human lipoproteins with rat hepatocytes *in vitro* provides a useful model for studying of hepatic lipoprotein metabolism. Binding to Site 2 was not regulated between 20 and 44 h in culture by the lipoproteins added to the medium. Site 2 may represent a separate pathway of LDL uptake, such as that which has been demonstrated in human hepatocytes taken from patients suffering from homozygous familial hypercholesterolaemia [4,5].

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