Analysis of insulin-receptor phosphorylation sites in intact cells by two-dimensional phosphopeptide mapping

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Insulin stimulates the autophosphorylation of the partially purified insulin receptor initially on tyrosine residues 1146, 1150 and 1151. This is followed by increased autophosphorylation of tyrosine residues 1316, 1322 and two further residues, possibly tyrosine residues 953 and 960 or 972 [Tavaré & Denton (1988) *Biochem. J.* 252, 607–615]. In the present paper we have used two cell lines transfected with insulin-receptor cDNA (CHO.T and NIH 3T3 HIR3.5 cells) to assess which tyrosine residues are phosphorylated on the insulin receptor within intact cells. We show that: (1) insulin causes a rapid increase in phosphorylation of tyrosine residues 1146, 1150 and 1151 in both cell types; tyrosine residues 1316 and 1322 are also phosphorylated, but apparently to a lesser extent in NIH 3T3 HIR3.5 cells; (2) the sites that may correspond to tyrosine residues 953 and 960 or 972 appear to be very poorly phosphorylated in both intact cell types; (3) insulin also promotes a substantial and rapid increase in the phosphorylation of serine and threonine residues on insulin receptors on CHO.T cells; this results in the appearance of two phosphopeptides not evident in the maps of the solubilized receptor preparations autophosphorylated *in vitro*.

INTRODUCTION

The mechanisms by which the interaction of insulin with its receptor result in the diversity of cellular responses elicited by this hormone are poorly understood (Denton, 1986). There is a growing body of evidence suggesting that the tyrosine-specific protein kinase intrinsic to the insulin-receptor β -subunit may be important in some of the actions of insulin (Ellis *et al.*, 1986a; Morgan & Roth, 1987; Chou *et al.*, 1987; Ebina *et al.*, 1987).

Insulin causes a rapid increase in the phosphorylation of tyrosine residues on the insulin receptor in intact cells. This reaches a maximum within 1 min and appears to be followed by a slower rise in serine phosphorylation over the next 10 min (Pang *et al.*, 1985; White *et al.*, 1985; Ballotti *et al.*, 1987). However, on the basis of the separation of labelled phosphopeptides by h.p.l.c., it appears that there may be marked differences in the pattern of tyrosine phosphorylation within intact cells compared with that occurring during autophosphorylation of purified soluble receptors (White *et al.*, 1985). Phosphorylation of the insulin receptor on serine residues appears to inhibit any subsequent phosphorylation on tyrosine residues in response to insulin (Pang *et al.*, 1985; Ballotti *et al.*, 1987).

In a previous study we described a sensitive two-dimensional thin-layer procedure that separates insulin-receptor tryptic phosphopeptides to high resolution (Tavaré & Denton, 1988). The use of this method revealed that the partially purified insulin receptor is phosphorylated on at least seven residues within three distinct domains. One domain (domain 2) is phosphorylated rapidly on three tyrosine residues, namely those at

positions 1146, 1150 and 1151. This is followed by the phosphorylation of tyrosine residues 1316 and 1322 in domain 3, and finally by residues within domain 1 which probably correspond to tyrosine-953 and -960 or -972, which lie in close proximity to the inner face of the plasma membrane.

In the present paper we have made use of two intact cell preparations which express more than half a million insulin receptors per cell to determine which tyrosine residues are phosphorylated on the insulin-receptor β -subunit in intact cells. One cell line, termed 'CHO.T', is derived from Chinese-hamster ovary cells transfected with the human placental insulin-receptor cDNA (Ellis *et al.*, 1986b). The other is a murine NIH 3T3 cell line transfected with the human kidney insulin-receptor cDNA (termed 'NIH 3T3 HIR3.5'; Whittaker *et al.*, 1987).

MATERIALS AND METHODS

Materials

All materials were as given in Tavaré & Denton (1988), except [32 P]orthophosphate (10 mCi/ml), which was from Amersham International, bovine serum albumin, Hepes, SDS, which were from Sigma, sodium pyrophosphate and EDTA, which were from BDH Chemicals, and Triton X-100, which was from Koch-Light.

Tissue culture

CHO.T cells were grown in Ham's F12 medium (Flow Laboratories) and NIH 3T3 HIR3.5 cells in Dulbecco's modified Eagle's medium (Gibco), both media being

Abbreviations used: DNP-lysine, dinitrophenyl-lysine; Tos-Phe-CH₂Cl, tosylphenylalanylchloromethane ('TPCK'); PMSF, phenylmethanesulphonyl fluoride.

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Fig. 1. Phosphorylation and tryptic phosphopeptide mapping of partially purified insulin receptors from CHO.T cells, NIH 3T3 HIR3.5 cells and human placental membranes

Insulin receptors were partially purified from (a) human placenta, (b) NIH 3T3 HIR3.5 cells or (c) CHO.T cells by wheat-germ-lectin–Sephacryl chromatography and incubated with insulin and [γ - 32 P]ATP as described in the Materials and methods section. 32 P-labelled insulin receptor β -subunits were isolated, digested with Tos-Phe-CH₂Cl-treated trypsin and subjected to two-dimensional thin-layer analysis. (a), (b) and (c) are radioautographs of the resulting two-dimensional separations of tryptic phosphopeptides, and (d) is a key to their identification (see the text). The positions of an internal marker, DNP-lysine (⊙), and the origin of sample application (○ or ◐) are shown. Peptides S and T are phosphorylated on serine and threonine residues respectively, and are only observed in insulin receptors phosphorylated within intact cells (Fig. 4). Peptide S is often rather poorly resolved from peptides C1 and C1'.

supplemented with 10% (v/v) foetal-calf serum (Northumbria Biologicals, Cramlington, Northumberland, U.K.) and G418 (400 μ g/ml), streptomycin (100 μ g/ml) and penicillin (100 units/ml) (all from Gibco). Near-confluent cells were subcultured every 3 or 4 days, at a split ratio of approx. 1:10, by trypsin treatment [trypsin (0.5 mg/ml) plus EDTA (0.2 mg/ml) in 'Ca/Mg-free phosphate-buffered saline' (Gibco) for approx. 5 min at room temperature]. Phosphorylation experiments were conducted 2 days after trypsin treatment, when cells were approximately semi-confluent.

Partial purification and phosphorylation of insulin receptors from CHO.T cells, NIH 3T3 HIR3.5 cells and human placental membranes

CHO.T and NIH 3T3 HIR3.5 cells were solubilized as described by Ellis *et al.* (1986b). Insulin receptors were partially purified from these solubilized cell extracts, and from Triton extracts of human placental membranes, by wheat-germ-lectin–Sephacryl chromatography as described by Tavaré & Denton (1988). Receptors were incubated with [γ - 32 P]ATP for 60 min at 0 °C in the presence of 12 mM-MgCl₂, 2 mM-MnCl₂, 1 mM-Na₃VO₄ and 10⁻⁶ M-insulin as described by Tavaré & Denton (1988).

Insulin-receptor phosphorylation in intact cells

Phosphorylation of the insulin receptor in intact cells was carried out essentially as described by Ellis *et al.*

(1986b). Semi-confluent cells in 35 mm-diameter dishes (approx. 10⁶ cells) were washed twice with 2 ml of phosphate-free 'KRBB/BSA (Krebs–Ringer bicarbonate buffer/bovine serum albumin) buffer' (Ellis *et al.*, 1986b) and then incubated for 4 h at 37 °C in 1 ml of the same buffer containing 1 mCi of [32 P]orthophosphate in an atmosphere of CO₂/O₂ (1:9). Incubation was then continued with or without 10⁻⁷ M-insulin for the times indicated in the Figure legends and terminated by aspirating the medium and immediately freezing the cells in liquid N₂. Cells were solubilized on ice in 500 μ l of 50 mM-Hepes, pH 7.6, containing 1% (w/v) Triton X-100, 10 mM-EDTA, 10 mM-NaF, 30 mM-sodium pyrophosphate, 1 mM-Na₃VO₄, 1 mM-PMSF and bacitracin (1 mg/ml). The lysate was centrifuged (10000 g, 2 min), and the supernatant was incubated with 5 μ l of rabbit anti-(insulin receptor) antiserum (Soos *et al.*, 1986) for 60 min at 4 °C, and then also with 3 mg of protein A–Sephacryl for 30 min at 4 °C. The mixture was then centrifuged (10000 g, 1 min) and the pellet washed three times with 1 ml of 50 mM-Hepes (pH 7.6)/1 M-NaCl/0.1% (w/v) SDS/0.5% (w/v) Triton X-100/0.1% (w/v) bovine serum albumin and finally once with 100 mM-Tris/HCl, pH 6.8. Material bound to protein A–Sephacryl was eluted by boiling for 5 min with 100 μ l of electrophoresis sample buffer [62.5 mM-Tris/HCl (pH 6.8)/2% (w/v) SDS/10% (v/v) glycerol/5 mM-EDTA/100 mM-dithiothreitol/0.02% (w/v) Bromophenol Blue]. Proteins were separated by electrophoresis on SDS/7%-(w/v)-polyacrylamide gels.

Fig. 2. Immunoprecipitation of insulin receptors from intact CHO.T and NIH 3T3 HIR3.5 cells incubated with [32 P] P_i

CHO.T (a) and NIH 3T3 HIR3.5 (b) cells were incubated with [32 P] P_i for 4 h and then in the absence (–) or presence (+) of insulin for 5 min. Cells were solubilized and insulin receptors were immunoprecipitated as described in the Materials and methods section. The Figure shows a radioautograph (1 h) of the resulting separation of the immunoprecipitates derived from one dish (approx. 10^6 cells) by SDS/7%-(w/v)-polyacrylamide-gel electrophoresis. The position of the β -subunit of the insulin receptor is marked by an arrow.

Fig. 3. Phosphoamino acid analysis of insulin receptors immunoprecipitated from intact CHO.T and NIH 3T3 HIR3.5 cells

CHO.T (a) or NIH 3T3 HIR3.5 (b) cells were incubated with [32 P] P_i for 4 h and then in the absence or presence of insulin (as indicated) for 5 min. Insulin receptors were immunoprecipitated as described in the Materials and methods section. The 32 P-labelled insulin-receptor β -subunits were isolated, subjected to hydrolysis with 6 M-HCl, and the phosphoamino acids generated were separated by electrophoresis at pH 3.5 with 1 μ g each of carrier phosphoserine (P-S), phosphothreonine (P-T) and phosphotyrosine (P-Y) as markers. The Figure shows radioautograph of the resulting separations; these were for 4 and 21 days in (a) and (b) respectively.

Phosphoamino acid analysis and two-dimensional tryptic phosphopeptide mapping

32 P-labelled insulin-receptor β -subunits, separated by SDS/polyacrylamide-gel electrophoresis, were revealed by radioautography of the untreated wet gel with Kodak X-Omat S pre-flashed film at room temperature for 2 h. They were then electroeluted from gel pieces, digested with Tos-Phe-CH₂Cl-treated trypsin and subjected to either phosphoamino acid analysis or two-dimensional peptide mapping as described by Tavaré & Denton (1988). 32 P-labelled phosphoamino acids or tryptic peptides were revealed by radioautography of thin-layer plates with pre-flashed Kodak X-Omat S film at -80°C for 5–10 days in cassettes with intensifying screens.

Expression of results

The designation of tyrosine residues and tryptic cleavage sites is based on the sequence given by Ullrich *et al.* (1985).

RESULTS

Incubation of partially purified insulin receptors from CHO.T cells, NIH 3T3 HIR3.5 cells and human placental membranes with [γ - 32 P]ATP and tryptic phosphopeptide mapping

Partially purified insulin-receptor preparations from CHO.T cells, NIH 3T3 HIR3.5 cells and human placental membranes were incubated with insulin and [γ - 32 P]ATP, and the resulting 32 P-labelled insulin-receptor β -subunits were isolated and digested to completion with Tos-Phe-

CH₂Cl-treated trypsin as described in the Materials and methods section. Phosphoamino acid analysis revealed that receptors from all three sources were phosphorylated predominantly on tyrosine residues in the presence of insulin (results not shown).

Furthermore, the patterns of 32 P-labelled tryptic phosphopeptides determined by two-dimensional thin-layer analysis (Fig. 1) were similar for all three sources of receptor, with the exception that the apparent relative level of 32 P incorporation into peptides A1 and A2 was higher, and that into B2 and C3 was considerably lower (see Fig. 1d for key) for receptors purified from CHO.T and NIH 3T3 HIR3.5 cells than for those from human placental membranes.

Phosphorylation of insulin receptors in intact CHO.T and NIH 3T3 HIR3.5 cells and phosphoamino acid analysis

Monolayer cultures of CHO.T and NIH 3T3 HIR3.5 cells incubated with [32 P] P_i in the absence or presence of insulin were solubilized, and insulin receptors were immunoprecipitated and analysed by SDS/polyacrylamide-gel electrophoresis and radioautography. Fig. 2 shows that insulin promoted a substantial increase in the phosphorylation of the insulin-receptor β -subunit in both cell types. The maximum effect of insulin was evident after 2–5 min, and was approx. 10-fold in both cell types (Fig. 2), precise quantification being difficult because the level of basal phosphorylation was low and variable between experiments. An elevated level of receptor phosphorylation was maintained over 60 min of

Fig. 4. Two-dimensional phosphopeptide mapping of insulin receptors from intact CHO.T and NIH 3T3 HIR3.5 cells

Intact CHO.T cells (*a-c*) or NIH 3T3 HIR3.5 cells (*d-f*) were incubated with [32 P]P_i for 4 h and then with insulin for 20 s (*a,d*), 2 min (*b,e*) or 5 min (*c,f*). Insulin receptors (from about 5×10^6 cells) were immunoprecipitated, and the β -subunits were isolated and digested with Tos-Phe-CH₂Cl-treated trypsin. The tryptic phosphopeptides were separated by thin-layer analysis and subjected to radioautography for 3 days (*a-c*) and 30 days (*d-f*), as in Fig. 1. The relative migration of DNP-lysine is marked by $\circ$ and the origin of sample application by $\blacktriangledown$.

incubation with insulin. There was an indication in some experiments that the initial rate of receptor phosphorylation was more rapid in CHO.T than in NIH 3T3 HIR3.5 cells, but in both cell types a substantial increase was already apparent after 20 s with insulin (results not shown). The absolute incorporation of 32 P into the insulin receptor tended to be greater in CHO.T than in NIH 3T3 HIR3.5 cells (Fig. 2). Both CHO.T cells and NIH 3T3 HIR3.5 cells appeared to possess similar numbers of insulin receptors per cell as judged by 125 I-insulin binding assays on intact cells and by the relative incorporation of [35 S]methionine into receptor protein (K. Siddle, M. A. Soos & R. Ganderton, unpublished work). Further, the overall incorporation of [32 P]-phosphate into total proteins tended to be lower in NIH 3T3 HIR3.5 cells than in CHO.T cells (R. M. O'Brien & K. Siddle, unpublished work), suggesting that, under the conditions used, cellular ATP specific radioactivity may be greater in CHO.T cells than in NIH 3T3 HIR3.5 cells. However, the possibility cannot be completely ruled out that a greater proportion of insulin receptors were accessible to insulin in CHO.T cells.

The 32 P-labelled insulin-receptor β -subunits were electroeluted from the gels and subjected to phosphoamino acid analysis (Fig. 3). This showed that insulin promoted an increase in tyrosine phosphorylation of the β -subunit in both cell types. There were also marked

increases in the phosphorylation of serine residues (Fig. 3).

Two-dimensional tryptic phosphopeptide mapping of the insulin receptor β -subunit immunoprecipitated from intact cells incubated with [32 P]P_i

Insulin receptors were immunoprecipitated from intact CHO.T and NIH 3T3 HIR3.5 cells that had previously been incubated with [32 P]P_i for 4 h followed by insulin for 20 s, 2 min or 5 min. The 32 P-labelled β -subunits were isolated, digested with Tos-Phe-CH₂Cl-treated trypsin and the products were separated by two-dimensional thin-layer analysis (Fig. 4). Under these conditions there was little detectable 32 P label associated with any of the tryptic peptides derived from insulin receptors of cells incubated in the absence of insulin (results not shown).

Phosphoamino acid analysis of each tryptic phosphopeptide derived from CHO.T-cell insulin receptors (Fig. 5) showed that peptides A1, A2, B1/B2, B3 and C1 were all phosphorylated predominantly on tyrosine residues. Peptide T was apparently phosphorylated exclusively on threonine residues, and peptide S on serine and tyrosine residues. Peptide S was rather poorly resolved and migrated close to peptide C1. This may explain, in part, the presence of phosphoserine in peptide C1 and phosphotyrosine in peptide S. Phosphothreonine was

Fig. 5. Phosphoamino acid analysis of tryptic phosphopeptides from insulin receptors phosphorylated within intact CHO.T cells

CHO.T cells (approx. 6×10^6 cells) were incubated with [32 P] P_i and insulin, and thin-layer analysis of the resulting [32 P]-labelled insulin-receptor tryptic phosphopeptides was performed as described in the legend to Fig. 4(b). The individual tryptic phosphopeptides were recovered from the thin-layer cellulose plates, and phosphoamino acid analysis was performed as described by Tavaré & Denton (1988). The Figure shows a radioautograph (20 days) of the resulting separation of phosphoamino acids at pH 3.5, with the positions of carrier phosphoserine (P-S), phosphothreonine (P-T) and phosphotyrosine (P-Y) indicated.

not well resolved from phosphoserine at pH 3.5, but could be detected as a minor tail running just beneath phosphoserine in the phosphoamino acid analysis of intact β -subunit (Fig. 3). Quantitative interpretation of the relative levels of phosphoserine, phosphothreonine and phosphotyrosine is not possible, owing to their differential acid stability (Cooper *et al.*, 1983).

Phosphoamino acid analysis of NIH 3T3 HIR3.5-cell insulin-receptor β -subunit tryptic phosphopeptides was not possible, owing to the lower level of [32 P]-labelling of insulin receptors in these cells.

DISCUSSION

In a previous paper (Tavaré & Denton, 1988) we described how the two-dimensional thin-layer analysis employed in the present study can resolve three domains of tyrosine autophosphorylation of the partially purified human placental insulin receptor. One domain (domain 2; sequence DIYETDYYRKGGK), phosphorylated at tyrosine residues 1146, 1150 and 1151, was detected as mono- (peptide C1), di- (B2 and B3) and tri- (A1 and A2) phosphorylated peptides cleaved by trypsin at Arg-1143 and either Lys-1153 (A2, B3 and C1) or Lys-1156 (A1 and B2). Another domain (domain 3; sequence SYEEHIPYTHMNGGK), phosphorylated on tyrosine

residues 1316 and 1322, was recovered as a single diphosphorylated tryptic peptide probably cleaved at Arg-1314 and Lys-1329 (peptide B1). A third domain (domain 1), possibly phosphorylated on tyrosine residues 953 and 960 or 972, was recovered as peptides C2 and C3 and as a further peptide which was incompletely resolved from C1 (termed 'C1''; Tavaré & Denton, 1988).

All these species of phosphopeptide are apparent in the tryptic phosphopeptide maps of partially purified CHO.T- and NIH 3T3 HIR3.5-cell insulin receptors incubated with [γ - 32 P]ATP and insulin. The identification of the human placental insulin-receptor tryptic phosphopeptides described above was achieved by a consideration of their net charge at pH 3.5 before and after cleavage by *Staphylococcus aureus* proteinase V8. These conclusions were confirmed by treating each of the tryptic phosphopeptides (in Fig. 1c) derived from partially purified CHO.T-cell insulin receptors with proteinase V8. A pattern of products identical with those observed with the human placental insulin receptor was evident (results not shown). Thus the partially purified insulin receptor appears to be phosphorylated on the same sites regardless of its tissue or cell source. However, the degree of phosphorylation of domain 2 (containing tyrosine residues 1146, 1150 and 1151) appears to be greater in receptors from CHO.T and NIH 3T3 HIR3.5 cells than from human placenta, since a significantly higher proportion of this domain is recovered as the triphosphorylated peptides A1 and A2 (Fig. 1). It can be calculated that approx. 35% of partially purified phosphorylated human placental insulin receptors become triphosphorylated on domain 2 after 60 min incubation, compared with approx. 57% of receptors from CHO.T cells incubated under identical conditions. This may be a reflection of relatively lower levels of protein tyrosine phosphatase activity in preparations of insulin receptor from the two cell types than from human placenta. Another possible difference between the various receptor preparations may be the level of endogenous phosphorylation before incubation with [γ - 32 P]ATP. Such differences may be a partial explanation of the apparently low relative incorporation of [32 P] into phosphopeptides B2 and C3 in preparations derived from both the CHO.T and NIH 3T3 HIR3.5 cells.

Tryptic peptide maps of insulin receptors phosphorylated in both CHO.T and NIH 3T3 HIR3.5 cells (Fig. 4) revealed that, in intact cells, domain 2 is rapidly phosphorylated. In CHO.T cells, maximal labelling of the triphosphorylated species A1 and A2 (relative to B2 and B3) occurred at 2 min, whereas in NIH 3T3 HIR3.5 cells the appearance of peptides A1 and A2 was still increasing, suggesting that this domain is phosphorylated more slowly than in CHO.T cells.

In CHO.T cells there was also a rapid rise in the phosphorylation of tyrosine residues 1316 and 1322 (as peptide B1), again maximal at 2 min (Fig. 4). By contrast, these tyrosine residues appear to be less extensively phosphorylated in NIH 3T3 HIR3.5 cells (Fig. 4). The reasons for these differences in time courses of phosphorylation deserve further investigation.

Phosphopeptides C2 and C3 were barely evident in receptors immunoprecipitated from both intact cell preparations. This apparently poor labelling was not due to their dephosphorylation during the 2 h incubation of cell extracts with antiserum, since identical tryptic phosphopeptide maps of pre-labelled human placental

insulin receptors were obtained before and after incubation with either CHO.T or NIH 3T3 HIR3.5 cell extracts under the conditions used for immunoprecipitation (results not shown).

Ellis *et al.* (1986b), using site-directed mutagenesis, have also suggested that tyrosine residues 1150 and 1151 might be phosphorylated on insulin receptors in intact cells. On the other hand, they did find some residual tyrosine phosphorylation of receptors in which both tyrosine-1150 and -1151 were replaced by phenylalanine (CHO.YF3 mutant). This residual phosphorylation may have been due to the phosphorylation of residues 1146, 1316 and 1322. This group also showed that insulin was still capable of stimulating autophosphorylation of insulin receptors in the CHO.YF3 mutant in intact cells. After the completion of our study, Tornqvist *et al.* (1988) demonstrated by direct sequencing that tyrosine-1146, -1150, -1151, -1316 and -1322 are all phosphorylated after exposure of intact rat hepatoma cells to insulin.

Insulin also causes an increase in serine and threonine phosphorylation of insulin receptors in intact CHO.T cells (Figs. 3 and 4a–4c), which is in general agreement with previous reports (White *et al.*, 1985; Pang *et al.*, 1985; Ellis *et al.*, 1986b; Jacobs & Cuatrecasas, 1986; Ballotti *et al.*, 1987). Our results suggest that the domain of the insulin receptor phosphorylated on threonine residues in response to insulin (tryptic peptide T; Figs. 4a–4c) is quite distinct from those domains phosphorylated on tyrosine residues. It will require improved resolution of peptide S, phosphorylated on serine residues, to establish further its relationship to those domains phosphorylated on tyrosine residues. For NIH 3T3 HIR3.5 cells, insulin also caused a substantial increase in serine phosphorylation (Fig. 3b), and there was also evidence for a rapid, and perhaps transient, appearance of a tryptic peptide which may correspond to peptide T of the CHO.T cells. However, firmer conclusions cannot be made in the absence of phosphoamino acid analysis of the individual tryptic phosphopeptides. Important objectives of future research will be the identification of the serine/threonine kinases which phosphorylate the insulin receptor within intact cells, and the effects of this phosphorylation on insulin-receptor function.

In conclusion, therefore, we have demonstrated that the insulin receptor in intact cells is rapidly phosphorylated within domain 2 (tyrosine-1146, -1150 and -1151) in response to insulin. The degree of phosphorylation of (tyrosine-1316 and -1322) appears to be dependent on the cell type studied. Phosphorylation of domain 1 (tentatively identified as containing tyrosine-953, -960 and -972) was barely detectable. These conclusions are based on the ability of the two-dimensional thin-layer analysis to separate and detect insulin-receptor tryptic phosphopeptides. The technique is of sufficient sensitivity to be applied to the problem of assessing the sites of

tyrosine, serine and threonine phosphorylation on insulin receptors in intact preparations of fat, liver and other tissues that express considerably fewer receptor numbers than do CHO.T or NIH 3T3 HIR3.5 cells.

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