Insulin-like growth factor-binding protein-1 (IGFBP-1) regulates the activity of the insulin-like growth factors in early pregnancy and is, thus, thought to play a key role at the fetal-maternal interface. The C-terminal domain of IGFBP-1 and three isoforms of the intact protein were isolated from human amniotic fluid, and sequencing of the four N-terminal polypeptide chains showed them to be highly pure. The addition of both intact IGFBP-1 and its C-terminal fragment to cultured fibroblasts has a similar stimulating effect on cell migration, and, therefore, the domain has a biological activity on its own. The three-dimensional structure of the C-terminal domain was determined by x-ray crystallography to 1.8 Å resolution. The fragment folds as a thyroglobulin type I domain and was found to bind the Fe²⁺ ion in the crystals through the only histidine residue present in the polypeptide chain. Iron (II) decreases the binding of intact IGFBP-1 and the C-terminal domain to IGF-II, suggesting that the metal binding site is close to or part of the surface of interaction of the two molecules.

Insulin-like growth factor-binding protein-1 is a member of a family comprising six secreted proteins (designated IGFBP-1 to IGFBP-6) that can modulate upon binding the availability and thus the biological effects of insulin-like growth factors I and II (IGF-I and IGF-II). Both inhibition and enhancement of the hormone action have been described, and several mechanisms, including binding of IGFBPs to the extracellular matrix, phosphorylation, and proteolysis, have been shown to modulate their affinity for IGFs. Moreover, an increasing number of reports indicate that IGFBPs can regulate cellular functions independently of their ability to interact with IGFs (1, 2).

Each IGFBP polypeptide chain, ranging in length from 216 to 289 amino acids, may be divided into three distinct domains of approximately equal size. The N- and C-terminal portions exhibit a high primary sequence identity across the six IGFBPs and contain spatially conserved cysteine residues that form intra-domain disulphide bonds. The central domain is the least conserved region and in some of the proteins contains post-translational modifications and proteolytic cleavage sites. On the basis of their sequence homology it is assumed that IGFBPs share a common overall fold and have very similar IGF binding pockets. The N-and C-terminal domains are known to be involved in IGF binding (3), and recently the x-ray structure of the ternary complex of the two domains of IGFBP-4 and IGF-I has been reported (4). In this complex the C-terminal domain was partially disordered, and, therefore, a detailed model of this part of the molecule could not be produced. The structure of the N-terminal domain of IGFBP-5, both isolated (NMR data (5)) and complexed to IGF-I (x-ray data (6)), is also known, and NMR spectroscopy has been used to produce a detailed model of the C-terminal domain of IGFBP-6 (7) and to study its interactions with IGF-II (8), but there is no x-ray model available of the C-terminal domain of any member of this protein family, although the C-terminal fragment of IGFBP-4 was crystallized recently (9). Proteolytic cleavage at the mid-region between the two domains of the protein is considered the predominant mechanism for IGF release from all IGFBPs (10), but several studies indicate that the resulting N- and C-terminal fragments still retain the ability to inhibit IGF activity (11). Additionally, recent reports suggest that proteolysis of IGFBP's results in fragments with potential functional properties that differ from those of the intact protein (9, 11).

IGFBP-1 is normally expressed in a tissue-specific manner in the liver, kidney, decidualized endometrium, and luteinizing granulosa cells (12). It is the predominant IGFBP in amniotic fluid (13), a major IGF-binding protein in fetal plasma, and its concentration is increased in maternal circulation during pregnancy. Several studies indicate that the protein plays a relevant role in embryonic growth as a local modulator of IGF's bioavailability at the maternal-fetal interface (14, 15). Moreover, the up-regulation of IGFBP-1 was demonstrated in breast human tumors and was associated with the malignant transformation of breast tissue (16). In addition, IGFBP-1 contains an Arg-Gly-Asp tripeptide that can bind to the recognition site of α5β1 integrin, leading to the stimulation of migration in several cell species (17–20). The mature polypeptide chain of IGFBP-1 is composed of 234 amino acids (the unprocessed precursor is 259 amino acids long) and is phosphorylated at
three serine residues, 126, 144, and 194 (we will follow the nomenclature in the chain).

In this study we describe the isolation of the C-terminal domain of IGFBP-1 from human amniotic fluid and its structural characterization by x-ray crystallography to 1.8 Å resolution. We also show that the C-terminal fragment, which contains the Arg-Gly-Asp tripeptide, retains the capability of the intact protein to stimulate cell migration and that it binds the ferrous ion through the only histidine residue present in the chain.

MATERIALS AND METHODS

Protein Purification and Electrophoretic Analysis—Human amniotic fluid was obtained from discarded amniocentesis samples collected in the weeks 16–18. The pooled fluid (3 liters) was saturated to 90% with ammonium sulfate, and the precipitated proteins, after centrifugation and dialysis, were solubilized in the same buffer containing 6M guanidine HCl and 20 mM pH 7.5, 0.5 M NaCl containing 0.1% Triton X-100, and the protein was dissolved in PBS with 2% bovine serum albumin. After washing 5 times with the Diff-Quick staining kit (Medion Diagnostic GmbH, Switzerland) according to the manufacturer’s instructions. Migration was quantified by counting the stained cells in 10 non-overlapping fields using a light microscope fitted with a grid eyepiece. The data are the means ± S.D. of triplicate wells from at least two different protein preparations.

For the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, cells (200 cells/well) were plated on 96-well plates and grown for 48 h. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) was added to 1 mg/ml for 4 h followed by extraction as reported (20).

Analysis of Cell Migration—Normal human skin fibroblasts (PromoCell GmbH, Germany) were used for cell culture experiments. Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum, 100 units/ml penicillin, 1 mg/ml streptomycin (complete medium) at 37 °C in a 5% CO2 humidified atmosphere. Purified IGFBP-1 and its C-terminal fragment were dissolved in 0.05 M Tris-HCl, pH 7.4 containing 0.15 M NaCl and diluted with serum-free Dulbecco’s modified Eagle’s medium at the indicated concentrations. Cells were plated in 35-mm Petri dishes and allowed to grow to confluency. The confluent monolayers were “wounded” by scraping cells in the center of the culture dishes using a rubber policeman to generate an acellular area (27). After repeated rinses with PBS, the monolayers were examined and photographed under an inverted microscope and incubated for 48 h in serum-free Dulbecco’s modified Eagle’s medium (SFM) with and without 1 µg/ml IGFBP-1 or its C-terminal fragment. At the end of the incubation the cells were fixed in methanol for 15 min at −20 °C, examined, and photographed. The plates were previously marked to ensure that the same area was recorded.

Cell migration was also evaluated in Transwell inserts (8-µm pore size, Nunc, Denmark) 5 × 104 fibroblasts were seeded into the upper wells in 500 µl of SFM alone or with 1 µg/ml IGFBP-1 or its C-terminal fragment. The lower wells contained 500 µl of SFM. Cells were allowed to migrate through the inserts for 48 h; non-migrating cells were removed from the upper surface using a cotton swab, and the cells that had migrated to the bottom surface of the inserts were fixed and stained with the Diff-Quick staining kit (Medion Diagnostic GmbH, Switzerland) according to the manufacturer’s instructions. Migration was quantified by counting the stained cells in 10 non-overlapping fields using a light microscope fitted with a grid eyepiece. The data are the means ± S.D. of triplicate wells from at least two different protein preparations.
with PBST, the microtiter wells were incubated for 1 h with rabbit anti-mouse IgG conjugated to horseradish peroxidase (1:1000 dilution; Dako, Gostrup, Denmark). Finally, the conjugated enzyme was treated with α-phenylethylamine dihydrochloride (Sigma), and the absorbance at 492 nm was monitored with a micro-plate reader (Bio-Rad).

Crystallography and X-ray Data Collection—Screening for crystalization conditions was performed with the hanging drop method at 4 and 20 °C using Hampton Research Screens and mixing 1 μl of the protein solution and the same volume of precipitating solution and equilibrating versus 0.3 ml in the reservoir. Larger diffraction quality crystals could be obtained by using bigger volumes with the sitting drop method. The crystals grow by mixing equal volumes of 35% dioxane, and the protein dissolved in 0.02 M Tris-HCl, pH 7.5, at a concentration of 20 mg/ml. The crystals are orthorhombic, space group P2_1_2_1_2, with unit cell parameters a = 38.54 Å, b = 60.39 Å, and c = 31.24 Å and contain one C-terminal domain of IGFBP-1 in the asymmetric unit.

The diffraction data were collected from crystals frozen to 100 K after a brief immersion in a mixture of 70% of the mother liquor and 30% glycerol. The data for the native crystals and the two heavy atom derivatives were collected at a crystal in mother liquor with the addition of the two compounds at a final concentration of about 1 mM. The data were indexed, integrated, and reduced using the programs MOSFLM (28) and AUTOMAR and Scala (29). The diffraction data statistics are summarized in Table I.

Structure Determination and Refinement—Initial phases to 2.3 Å resolution were determined by multiple isomorphous replacement with the two heavy atom derivatives. The two maximum sites were located in a difference Patterson map (30) and refined using the program MLPHARE (29). The single isomorphous replacement phases were used to locate the most significant mercury site in the difference Fourier map. These two major sites (one site from each of the two derivatives) were used as input for the program autoSHARP (31) that was used to locate the minor sites of the two derivatives and for density modification and electron density map production. The resulting model was subsequently refined with the program REFMAC (33). During the process of refinement, the quality of the model was controlled with the program PROCHECK (34). Solvent molecules were added to the model in the final stages of refinement according to hydrogen-bond criteria and only if their B factors refined to reasonable values and if they improved the Rfree. The final model contains 642 non-hydrogen protein atoms and has very reasonable geometry (see Table I), with 87.5% of the residues in the most favored regions of the Ramachandran plot and the remaining 12.5% in the additionally allowed region. The iron in the co-crystal was modeled into a difference electron density map beyond the low-temperature limit.

The C-terminal domain of IGFBP-1 was included in the asymmetric unit, and the C-terminal portion of IGFBP-1 from a natural source has not been reported before. The C-terminal fragment was submitted in an empty area created by a rubber policeman. The cells, after wounds, were allowed to grow for 48 h in SFM containing 1 μg/ml IGFBP-1 or its C-terminal fragment, and images were taken at 0 and 48 h. Figure 1A shows that in the control plate the cells grow to almost confluency slightly beyond the wounding line, whereas fibroblasts incubated with both the intact protein and the C-terminal fragment are still sparse but have migrated in the denuded area far beyond the wounding line.

Cell migration was confirmed and quantified in a Transwell system using the same protein concentrations and incubation time as in the wounding assay. IGFBP-1 and the C-terminal fragment showed, respectively, a 233 ± 26 and 186 ± 27% increase over the control of the cells migrated to the underside of the filters. The percentage of migrating cells as the average of triplicate wells from different protein preparations is reported in Figure 1B.
The C-terminal Domain of IGFBP-1

Iron (II) Reduces the Binding of IGFBP-1 and Its C-terminal Fragment—Cystals of the C-terminal domain of IGFBP-1 were soaked in 10 mM solutions of the following metal compounds: CaCl₂, FeSO₄, FeCl₃, CoCl₂₆H₂O, NiSO₄, and MnCl₂. X-ray data were collected, and difference Fourier maps were calculated and examined. The crystals soaked in FeCl₃ were found not to diffract at all, whereas the crystals soaked in the other metals did not show any significant extra electron density with only one exception, FeSO₄, which presented a very high peak clearly visible with a cutoff of 7 σ in the $F_{\text{obs}} - F_{\text{calc}}$ map and present in a position of the molecule where it could be easily interpreted in chemical terms. Fig. 3 shows the electron density of the Fe²⁺ ion in the co-crystals of the C-terminal domain of IGFBP-1 in both an $F_{\text{obs}} - F_{\text{calc}}$ and a $2F_{\text{obs}} - F_{\text{calc}}$ maps. The red density of the $F_{\text{obs}} - F_{\text{calc}}$ map is contoured at a 7-σ level, and the blue electron density of the $2F_{\text{obs}} - F_{\text{calc}}$ map is contoured at a 1.5-σ level. The diagram on the right hand of the figure represents the species coordinated to the bivalent ion. The Fe²⁺ ion is coordinated tetrahedral to a nitrogen of the ring of the only histidine present in the polypeptide chain, His-213, and to the oxygen of the side chain of Ser-214. The other two positions are occupied by 2 water molecules that are also bound to the polypeptide chain nitrogens of residues 207 and 215. Of the 4 coordination positions 2 involve side chains, those of His-213 and Ser-214, whereas the other two are dependent on two polypeptide chain nitrogens.

Iron (II) Increases the Binding of IGFBP-1 and Its C-terminal Fragment—The hypothesis that the binding of intact IGFBP-1 and its C-terminal domain to IGF-II, the major growth factor in amniotic fluid, might be affected by the presence of ferrous ion was tested by using an ELISA assay. The
assays were performed in sodium acetate buffer instead of PBS to avoid the precipitation of ferrous phosphate and to maintain a slight acidic pH, which prevents oxidation of the metal ion. The binding experiments showed that the intact protein binds to IGF-II with higher affinity when compared with the C-terminal domain, a result in agreement with the data reported for other IGFBPs (4). In addition, incubation with ferrous ion reduced the binding of both intact IGFBP-1 and its C-terminal fragment with the growth factor, and this inhibition process was abolished by the addition of EDTA (Fig. 4A). The inhibition curve was determined by incubating the intact protein with increasing amounts of ferrous chloride, and the fitting shown in Fig. 4B was obtained assuming that the effect of the ferrous ion is competitive. A similar analysis was not performed with the C-terminal fragment because of its lower affinity for IGF-II. These results suggest that the residues involved in the binding to the ferrous ion are in a region of IGFBP-1 at or near the area of interaction with IGF-II.

**DISCUSSION**

This work describes the structural properties of the C-terminal domain of IGFBP-1 and shows that it maintains the effect of the intact protein on cell motility. The fragment was isolated from human amniotic fluid by conventional chromatographic procedures, and our results indicate that the degradation of IGFBP-1 occurred during the storage or the processing of the fluid that, when examined immediately after sample collection, contains only the intact protein. The polypeptide chain has a unique N-terminal sequence, and therefore, it originates from a specific proteolytic cleavage of the entire protein. IGFBP proteolysis is a well recognized mechanism that controls IGF bioavailability and, although little is known about the susceptibility of IGFBP-1 to enzymatic cleavage in vivo, the protein has been recognized as a potential physiological substrate for matrix metalloproteases (24) such as MMP-11 (stromelysin-3) (37) and MMP-26 (matrilysin 2) (38). Both enzymes cleave in vitro

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**TABLE I**

| Data set          | Native    | K₂PtCl₄   | C₂H₆HgOHPO₂ | Native + FeSO₄ |
|-------------------|-----------|-----------|-------------|----------------|
| Space group       | P2₁2₁2    | P2₁2₁2    | P2₁2₁2      | P2₁2₁2         |
| a (Å)             | 38.54     | 38.92     | 38.63       | 38.21          |
| b (Å)             | 60.39     | 60.40     | 60.71       | 59.60          |
| c (Å)             | 31.24     | 31.42     | 31.40       | 31.06          |
| Resolution range (Å) | 20.1-1.8 | 32.7-2.3  | 32.6-2.3    | 32.1-1.8       |
| Observed reflections | 44,453    | 12,950    | 24,874      | 23,571         |
| Independent reflections | 7,168     | 3,557     | 3,580       | 6,773          |
| Rsym (%)          | 4.1 (27.4)| 6.8 (29.4)| 6.7 (17.3)  | 4.4 (8.5)      |
| Rf (Å)            | 14.2 (2.8)| 9.0 (3.4) | 10.7 (4.4)  | 8.9 (5.6)      |
| Completeness (%)  | 99.5 (98.1)| 99.5 (99.5)| 99.9 (99.2) | 97.0 (98.3)    |
| Sites             | 2         | 2         |             |                |
| Rcrucis (acentric/centric) | 0.718/0.696 | 0.682/0.622 |             |                |
| Phasing power (acentric/centric) | 1.331/1.275 | 1.519/1.627 |             |                |
| Reflections in refinement | 6,801     | 22.36 (32.1)| 21.64 (22.8)| 21.64 (22.8)   |
| Rfree (%) (test set 10%) | 27.50 (27.4) | 27.18 (43.3)|            |                |
| Protein atoms     | 642       | 642       |             |                |
| Water molecules   | 42        | 61        |             |                |
| r.m.s.d. on bond lengths (Å) | 0.008     | 0.010     |             |                |
| r.m.s.d. on bond angles (°) | 1.082     | 1.570     |             |                |
| Planar groups (Å) | 0.004     | 0.005     |             |                |
| Chiral volume deviation (Å³) | 0.073     | 0.086     |             |                |
| Average B factor (Å²) | 23.59     | 24.39     |             |                |
| Protein atoms     | 23.19     | 23.42     |             |                |
| Solvent atoms     | 29.74     | 33.06     |             |                |

**FIG. 2**. Overall structure and folding of the C-terminal domain of IGFBP-1. A, ribbon representation of the C-terminal domain of IGFBP-1. The α-helix is light blue, the four short strands of β structure are green, and coil is red. The three disulfide bridges are shown in yellow. The diagram shows the phosphorylated serine (Ser-194, disordered in the electron density maps), and the RGD motif (Arg-246–Gly-247–Asp-248) as ball and stick models. The Fe²⁺ ion is represented as a gray sphere. The figure was prepared using the program MOLSCRIPT (49). B, stereoview of the Ca chain trace of the domain in approximately the same orientation. This figure was prepared using Dino (www.dino3d.org).
the polypeptide chain of IGFBP-1 between His-165 and Val-166, thus producing a fragment with the same N-terminal sequence that we have found. The observation that the fragment represents a major component of the fluid whereas only small amounts of intact IGFBP-1 are obtained can be ascribed to long term storage of the amniotic fluid in the absence of protease inhibitors. The lack of comparable amounts of the 16-kDa N-terminal domain is likely due to the intrinsic instability of this portion of the molecule. On the contrary, the stability of the C-terminal region suggests that it is a completely very well structured domain of the whole protein, a property that has also been reported for other IGFBPs (11) and is confirmed by our x-ray diffraction studies.

IGFBP-1 stimulates cell migration, and several reports have established that this effect is caused by the binding of its RGD motif to the specific domain of \( \alpha_5 \beta_1 \) integrin present on cell surfaces and have suggested that this interaction results in an intracellular signaling event (20). The requirement of this sequence for a migration promoting effect, initially shown with Chinese hamster ovary (18) and vascular smooth muscle cells (19), has also been demonstrated for placental trophoblast cells (20). In all these models, however, it is clear that not only an intact RGD motif but also some other regions of IGFBP-1 are necessary for binding. Our results indicate that the stable C-terminal domain has the same effect on cell motility as the intact molecule and suggest that proteolytic cleavage of the protein yielding a polypeptide chain with a biological activity on its own might be relevant for the regulatory role of IGFBP-1 on trophoblast migration and invasion.

The C-terminal domain of all the IGFBPs has been recognized on the basis of the disulfide bonding pattern (36) as a member of the thyroglobulin type-I domain (39), which is found in a number of proteins with very diverse function and in different organisms (40, 41). The thyroglobulin type I-fold was first described in the major histocompatibility complex class II-associated p41 Ii fragments bound to cathepsin L (42). More recently, the structure of the C-terminal domain of IGFBP-6 was determined by NMR spectroscopy (7).

The most important element of secondary structure of the C-terminal domain of IGFBP-1 is, as in the other thyroglobulin domains, an \( \alpha \)-helix, which in this case spans residues 175–193, \( i.e. \) it is somewhat longer than in the other two proteins. The \( \alpha \)-helix of the p41 Ii fragment is 9 amino acids long (Thr-195–His-203), and that of the C-terminal domain of IGFBP-6 is 15 amino acids long (Pro-162–Thr-176). Using the program LSQKAB (43), we have superimposed the two sets of coordinates of these thyroglobulin domains (PDB entry codes 1ICF and 1RMJ) to the coordinates of the C-terminal domain of IGFBP-1. Fig. 5A is a stereo diagram that shows the three protein...
The C-terminal Domain of IGFBP-1

**Fig. 5. Comparison of the C-terminal domain of IGFBP-1 model and two related structures.** A, stereo diagram of the three models. The coordinates were superimposed using the program LSQRKAB (43). The model of C-IGFBP-1 is represented in red in the figure. The other two structures are (a) The NMR structure of C-IGFBP-6, represented in blue (Ref. 7, PDB code 1RMJ; the coordinates used for the NMR structure were the first set listed in the PDB file, and the first 27 amino acids were removed from the file) and (b) the x-ray structure of the human major histocompatibility complex class II-associated p41 Ii fragment represented in green (Ref. 42, PDB code 1ICF). B, r.m.s.d. between α-carbon atoms (in Å) of the NMR structure of the C-terminal domain of IGFBP-6 (blue), the x-ray structure of the human major histocompatibility complex class II-associated p41 Ii fragment (green), and the model of the C-terminal domain of IGFBP-1.

**Fig. 6. Sequence comparison of the C-terminal domain of IGFBP-1 and the other 5 members of the human IGFBP family.** The six sequences of the entire proteins were aligned using the program ClustalW (47), but only the C-terminal domains are represented in the figure. The column on the right hand gives the identity percentage of each sequence in the figure and that of the C-terminal domain of IGFBP-1; the disulfide bridges are indicated only for IGFBP-1. The last line has the amino acids identical in all the C-terminal domains of the group, whereas the bottom strip represents the elements of secondary structure of IGFBP-1. An arrow indicates the position of His-213, and the metal binding domain of IGFBP-3 is underlined and in bold.

structures superimposed. Notice that although the essential features of the thyroglobulin type-I domain are preserved in the three models, there is a significant variability in some details like for example the α-helix length and the exact position of the loops defined by the disulfide bonds. The r.m.s.d. in Å of the equivalent α carbons of these two structures and the C-terminal domain of IGFBP-1 are represented in Fig. 5B as a function of the amino acid position using the numbering of IGFBP-1.

Ferrous and other divalent ions, Ni²⁺, Co²⁺, Zn²⁺, Mg²⁺, and Mn²⁺ were found to bind to the C-terminal domain of IGFBP-3, and a 14-amino acid-long metal binding synthetic polypeptide was shown to trigger apoptotic effects as efficiently as intact IGFBP-3 (44). In addition, IGFBP-3 was found to interact with high affinity with the iron-binding proteins transferrin (45) and lactoferrin (46). The hypothesis that the C-terminal domain of IGFBP-1 might bind metal ions as well was tested by immersing crystals of the fragment in sufficiently concentrated solutions of the metals. A total of six ions were tested: Fe²⁺, Fe³⁺, Ni²⁺, Co²⁺, Ca²⁺, and Mn²⁺. Ferric ion was found to completely abolish the diffusion pattern of the crystals, whereas ferrous iron was found to bind in a single very well defined position. Fig. 6 shows the sequences of the C-terminal domains of the six IGFBPs aligned using the program ClustalW (47). The position of His-213 is indicated in the figure with an arrow, and the sequence of the metal binding domain of IGFBP-3 is in bold and underlined. Notice that the domain contains the amino acids aligned with those identified by us as relevant for the binding of Fe²⁺ to the C-terminal domain of IGFBP-1, and therefore, it may be suggested that metal binding in the two proteins could involve equivalent amino acids. The relevant histidine is present only in IGFBP-4, but there is no equivalent to Ser-214 in any of the other members of this protein family, and there is currently no evidence supporting the binding of IGFBP-4 to any metal ion.

On the basis of sequence homology with other IGFBPs, the residues of the IGFBP-1 C-terminal domain that bind the iron ion are located in the region of the protein involved in the binding to IGFs. Photoaffinity-labeling experiments with IGFBP-2 have identified as belonging to the IGF binding site amino acid residues 212–227 but also amino acid residues 266–287 (48). The latter contain Asn-271, which is in a position equivalent to that of His-213 in IGFBP-1 (see Fig. 6). Therefore, our observation that iron (II) reduces the binding of intact IGFBP-1 and of its C-terminal domain to IGF-II is in agreement with a model proposed for another member of this protein family. The fact that metal binding to full-length IGFBP-3 is also inhibited by the presence of IGF-I (44) gives further support to this proposal, but the exact physiological role of metal binding to these proteins will undoubtedly require further work. Taken together, our results indicate that the regulatory action of IGFBP-1 on IGFs and on their functions is more complex than perceived and may involve aspects that had not been identified so far.

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