Expression of a type I insulin-like growth factor receptor with low affinity for insulin-like growth factor II

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We investigated the binding properties of the type I insulin-like growth factor (IGF) receptor expressed in NIH-3T3 fibroblasts transfected with a human type I receptor cDNA. Cell surface receptors bound IGF-I with $K_D = 1$ nM as predicted. Although recent studies have suggested that IGF-I and IGF-II bind to type I receptors with near-equal affinity, the receptors in this system bound IGF-II with much lower affinity ($K_D = 15$ – 20 nM). When type I receptors from the transfected cells were solubilized and immunopurified, however, both ^{125}I -IGF-I and ^{125}I -IGF-II bound to the purified receptors with extremely high and relatively similar affinities ($K_D = 8$ and 17 pM respectively). Thus the immunopurified receptors had higher affinity but lower specificity for the two ligands. The monoclonal antibody $\alpha\text{IR-3}$ effectively inhibited IGF-I binding to cell surface receptors ($75 \pm 10\%$), but did not inhibit IGF-II binding. In the purified receptor assay, $\alpha\text{IR-3}$ also inhibited IGF-I binding more effectively than IGF-II binding ($38 \pm 7\%$ versus $10 \pm 4\%$). We conclude that the products of this cDNA can account for the binding patterns that we previously observed in receptors immunopurified from human placenta. The differential effect of $\alpha\text{IR-3}$ on IGF-I versus IGF-II raises the possibility that these homologous growth factors bind to immunologically distinct epitopes on the type I receptor.

INTRODUCTION

The insulin-like growth factors (IGFs), also known as somatomedins, initiate their biological effects by binding to specific cell surface receptors. IGF-II binds with highest affinity to the type II IGF receptor (Rechler *et al.*, 1980; Massague & Czech, 1982; Czech, 1989), a single-chain structure that has no tyrosine kinase activity and was recently shown to be identical with the cation-independent mannose 6-phosphate receptor (Morgan *et al.*, 1987; MacDonald *et al.*, 1988; Kiess *et al.*, 1988). Although the role of the IGF-II/mannose 6-phosphate receptor has not been fully defined, it does not appear to mediate the growth-promoting activities of IGF-II *in vitro* (Kiess *et al.*, 1987; Roth, 1988). Thus IGF-II may induce its growth-promoting effects by binding to the type I IGF receptor or to a homologous receptor such as the recently described insulin-receptor-related receptor (Shier & Watt, 1989).

The type I IGF receptor is a disulphide-linked heterotetramer, with an extracellular ligand-binding domain and an intracellular tyrosine kinase domain typical of several other growth-factor receptors (Ullrich *et al.*, 1986; Czech, 1989). Affinity-cross-linking studies and competitive binding experiments originally indicated that the human type I receptor had a 10–20-fold higher affinity for IGF-I than for IGF-II (Rechler *et al.*, 1980; Massague & Czech, 1982). More recent studies, however, showed that IGF-II binds the type I receptor with much higher affinity than previous estimates, suggesting that this receptor might mediate the growth-promoting activities of IGF-II as well as IGF-I (Casella *et al.*, 1986; Steele-Perkins *et al.*, 1988). We have previously reported that type I IGF receptors immunopurified from human placenta have high affinity for both IGF-I and IGF-II (Casella *et al.*, 1986). Furthermore, the monoclonal antibody $\alpha\text{IR-3}$ (Kull *et al.*, 1983) inhibited IGF-I binding, but had a minimal effect on IGF-II binding to the type I receptor. Although

we speculated that IGF-I and IGF-II may bind to immunologically discrete sites on a common receptor, we could not exclude the possibility that IGF-II was binding to homologous receptors or to heterotetrameric hybrid molecules consisting of $\alpha\beta$ insulin-receptor heterodimers linked to $\alpha\beta$ type I IGF-receptor heterodimers (Soos & Siddle, 1989; Moxham *et al.*, 1989; Treadway *et al.*, 1989). More recently it was demonstrated that transfected Chinese-hamster ovary (CHO) cells, which expressed a human type I receptor cDNA, bound IGF-I and IGF-II with very similar affinities (Steele-Perkins *et al.*, 1988). This cDNA (Steele-Perkins *et al.*, 1988) appeared indistinguishable from one previously isolated (Ullrich *et al.*, 1986) in the 80% of the clone that was sequenced, but complete identity has not yet been determined.

In this study, we compared the binding of IGF-I and IGF-II to NIH-3T3 fibroblasts (clone 7, i-24) that overexpressed the type I IGF receptor (Ullrich *et al.*, 1986; Lammers *et al.*, 1989). These fibroblasts have a very low level of expression of endogenous type I receptors, type II receptors and insulin receptors, and are thus an excellent tool for studying binding of the human type I receptor. Because our results with intact cells appeared discordant with previous studies, we proceeded to study type I receptors that were immunopurified from the transfected cells. We also determined the effect of $\alpha\text{IR-3}$ on binding of the two ligands, using this system in which there should be minimal interference from related receptors.

MATERIALS AND METHODS

Materials

Receptor-grade recombinant IGF-I (Mallinckrodt, Paris, KY, U.S.A.) was used for competitive binding studies. Culture-grade recombinant IGF-I (Mallinckrodt) was used for non-specific binding. Recombinant IGF-II was purchased from Bachem

Abbreviation used: IGF, insulin-like growth factor.

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(Torrance, CA, U.S.A.) and human recombinant insulin (Velosulin) was purchased from Nordisk-U.S.A. (Rockville, MD, U.S.A.). ^{125}I -labelled IGF-I (2000 Ci/mmol) was obtained from Amersham (Arlington Heights, IL, U.S.A.). Recombinant IGF-II was iodinated to a specific radioactivity of 2000 Ci/mmol by a lactoperoxidase method and purified by h.p.l.c. on a μ Bondpak (Waters-Millipore) C_{18} reverse-phase column. The monoclonal antibody $\alpha\text{IR-3}$ (Kull *et al.*, 1983), purified from ascites by precipitation with Na_2SO_4 , was a gift from Dr. Judson Van Wyk, University of N. Carolina at Chapel Hill.

Clone 7, i-24 is a stably transfected NIH-3T3 fibroblast cell line that overexpresses a human type I receptor cDNA (Lammers *et al.*, 1989). The cells were propagated in Dulbecco's Modified Eagle's Medium with 10% (v/v) fetal-calf serum, 2 mM-L-glutamine and antibiotics. Passages 6–13 were used for all binding studies. All cell-culture materials were purchased from BRL/Gibco (Grand Island, NY, U.S.A.).

Cell surface binding assays

Confluent monolayers of the transfected NIH-3T3 fibroblasts (clone 7, i-24) were suspended after incubation in phosphate-buffered saline (0.14 M-NaCl/8 mM- Na_2HPO_4 /2.7 mM-KCl/1.5 mM- KH_2PO_4 /5.6 mM-D-glucose, pH 7.2) containing 25 mM-EDTA for 1 h at 37 °C. The cells were washed once with phosphate-buffered saline, then resuspended in Hepes binding buffer (0.1 M-Hepes, 0.12 M-NaCl, 5 mM-KCl, 1.2 mM- MgSO_4 , 8 mM-glucose, 10 mg of BSA/ml, pH 8.0). Approx. 4.25×10^5 cells were added to each tube containing the appropriate concentration of unlabelled ligand and 100 000 c.p.m. (60 pM) of radiolabelled ligand in a final volume of 500 μl . After a 3 h incubation at 15 °C, the cell receptor–ligand complexes were pelleted in a refrigerated centrifuge (1200 g for 10 min), and washed with cold Hepes binding buffer. The bound radioactivity was then measured in a γ -radiation spectrophotometer with an efficiency of 80%. Non-specific binding was determined by parallel incubations in the presence of 1 μM unlabelled IGF-I.

Purification of type I receptors

Confluent monolayers of clone 7, i-24 fibroblasts were washed twice with Hanks Balanced Salt Solution, then solubilized *in situ* by exposure to 2% (v/v) Triton X-100 (Sigma) in TBS (50 mM-Tris/150 mM-NaCl, pH 8.0) for 30 min at room temperature. The preparation was diluted with TBS to obtain a final Triton concentration of 0.5%. The cell extract was centrifuged at 40 000 g for 30 min, then incubated with $\alpha\text{IR-3}$ -linked Sepharose 4B beads overnight. After extensive washing with TBS, immunopurified receptors were eluted from the column with 100 mM- $(\text{NH}_4)_2\text{CO}_3$ (pH 10.3) containing 0.1% Triton X-100. Receptors were stored in elution buffer at 4 °C for as long as 7 days with no loss of binding activity. A typical preparation of seven 150 cm² flasks, containing approx. 5×10^7 cells total, yielded 3.5 pmol of IGF-I-binding activity.

Solubilized receptor binding assays

Each 200 μl reaction mixture contained TBS with 0.1% Triton X-100, the appropriate concentration of unlabelled ligand, 10 μl of purified receptor (approx. 5 fmol of binding activity), and 20 000 c.p.m. (30 pM) of radiolabelled ligand. After incubation overnight at 4 °C, γ -globulin was added to give a final concentration of 0.1%, and poly(ethylene glycol) (25% in TBS) was added to give a final concentration of 12.5%. The tubes were vortex-mixed vigorously, left at 4 °C for 30 min, then centrifuged (1200 g for 10 min) and decanted. The pellets were resuspended in 0.6 ml of 12.5% poly(ethylene glycol), and the tubes were again centrifuged and decanted. Precipitated radioligand was measured in a γ -radiation counter. Non-specific binding was

defined as that measured in the presence of 1 μM cell-culture-grade IGF-I.

To determine the affinity constants, 2 μl samples (approx. 1 fmol of binding activity) of the purified receptor were incubated at radioligand concentrations of 1.5–75 pM. Non-specific binding was determined at each radioligand concentration by parallel incubations with 10 nM receptor-grade IGF-I or IGF-II.

RESULTS

Cell binding assays

Cell surface receptors of the transfected NIH-3T3 fibroblasts (clone 7, i-24 cells) were able to bind ^{125}I -IGF-I with high avidity, incorporating up to 56% of the available ligand (Fig. 1). IGF-II was an effective inhibitor of ^{125}I -IGF-I binding, but at concentrations that were approx. 20-fold higher than with unlabelled IGF-I. Insulin was a much weaker competitor, yielding $62 \pm 9\%$ inhibition (mean $\pm$ S.E.M. in eight experiments) at a concentration of 10 μM -insulin. These data are all consistent with binding to the classic type I IGF receptor. The Scatchard plot of these data was linear, with an estimated K_D of 1 nM and R_0 of 8×10^5 receptors/cell (Fig. 2), in good agreement with previous studies (Lammers *et al.*, 1989). The binding was almost exclusively to the human receptor, because native NIH-3T3 fibroblasts expressed only 3.4×10^3 type I receptors/cell (Lammers *et al.*, 1989). Under our experimental conditions, native NIH-3T3 cells bound only 1.4% of the radiolabelled IGF-I.

In order to determine the affinity of IGF-II directly, identical studies were performed with ^{125}I -IGF-II. Interpretation of these results was complicated, because specific binding of the radioligand was only 6% in basal conditions and increased to 12% in the presence of 10 nM-unlabelled IGF-II. Higher concentrations of IGF-II produced a typical inhibition of ^{125}I -IGF-II binding. The paradoxical increase in ^{125}I -IGF-II binding at low concentrations of unlabelled IGF-II was similar to that attributed to interference from IGF-binding proteins (Clemmons *et al.*, 1986; De Vroede *et al.*, 1986). The binding sites in our system, however, appeared to be type I receptors, because IGF-I was a more potent inhibitor than IGF-II and because high doses of insulin blocked binding ($75 \pm 10\%$ inhibition at 10 μM). Affinity cross-linking to i-24 monolayers confirmed that ^{125}I -IGF-II was binding

Fig. 1. Binding of ^{125}I -IGF-I to transfected NIH-3T3 fibroblasts (clone 7, i-24)

Transfected NIH-3T3 fibroblasts (clone 7, i-24) that overexpress the human type I IGF receptor were incubated with 100 000 c.p.m. of ^{125}I -IGF-I and graded amounts of unlabelled IGF-I (●) and IGF-II (▲), as well as 10 μM -insulin (▼) and 1 μM anti-receptor antibody $\alpha\text{IR-3}$ (■) for 3 h at 15 °C. The bound radioligand was measured as described in the Materials and methods section and expressed as a ratio of specific radioactivity bound to the specific radioactivity bound in the presence of radioligand alone (B/B_0). $B_0 = 56\%$; non-specific binding = 4.4%. Each point represents the mean $\pm$ S.E.M. for duplicate tubes in three experiments.

Fig. 2. Scatchard plots of IGF binding to transfected NIH-3T3 fibroblasts (clone 7, i-24)

Binding of IGF-I (●) and IGF-II (▲) was determined as described in Fig. 1, and plotted as the bound/free ratio versus the concentration bound. Each point represents the mean of duplicate tubes in a representative assay. The slope and intercepts were calculated by linear regression of the data. For IGF-II, the ascending portion of the curve was not included in the calculation of the slope.

to a 130 kDa moiety that was indistinguishable from the type I α subunit (results not shown). The addition of proteinase inhibitors and the use of longer incubation times did not affect the paradoxical increase in IGF-II binding. Furthermore, the binding curves could be reproduced by using suspended i-24 cells that were fixed in 2% paraformaldehyde (results not shown). Thus the 'hook' curve could not be attributed to failure to reach equilibrium, degradation of receptors or ligand, or the secretion of binding proteins into the media. The unusual binding curves produced an upward slope in the early portion of the Scatchard plot, followed by a linear decline at higher concentrations of bound ligand (Fig. 2). Linear regression of the descending portion of the curve suggested that the minimum dissociation constant (highest affinity) was 15 nM. The estimated 15-fold lower affinity for IGF-II is in good agreement with that estimated from the competition curves. The R_0 for IGF-II was 16×10^5 receptors/cell, somewhat higher than the estimated receptor number for IGF-I. The difference in receptor number should be interpreted with caution, however, given the relatively weak binding of IGF-II and the non-linear plot. The higher receptor number was not the consequence of endogenous (mouse) receptors, because we did not observe any specific binding of 125 I-IGF-II to native NIH-3T3 cells.

The lower affinity of IGF-II was surprising, because of the report that the human type I receptor expressed in transfected Chinese-hamster ovary cells had almost equal affinity for IGF-I and IGF-II (Steele-Perkins *et al.*, 1988). Furthermore, our previous studies of receptors purified from human placenta had predicted a much higher affinity for IGF-II (Casella *et al.*, 1986). We therefore examined the binding characteristics of solubilized type I receptors extracted from the transfected cells. This enabled us to immunopurify the human type I receptor and thereby eliminate the remote possibility of interference from binding proteins or endogenous receptors.

Purified receptor binding assays

Cell monolayers were solubilized in 2% Triton X-100 and the immunoreactive protein was isolated on an α IR-3 immunoaffinity

Fig. 3. Binding of 125 I-IGF-I to purified type I receptors

Purified type I receptors were incubated with 20000 c.p.m. of 125 I-IGF-I and graded amounts of unlabelled IGF-I (●) and IGF-II (▲), as well as 10 μ M-insulin (▼) and 1 μ M anti-receptor antibody α IR-3 (■) overnight at 4 °C. The receptor-ligand complexes were precipitated by poly(ethylene glycol) and quantified as described in the Materials and methods section. B_0 = 61 %, non-specific binding = 1.4 %. The data plotted represent means $\pm$ S.E.M. of duplicate tubes in three separate experiments using a common preparation of receptors.

Fig. 4. Binding of 125 I-IGF-II to purified type I receptors

Binding to solubilized receptors was determined as described in Fig. 3. The data plotted represent means $\pm$ S.E.M. of duplicate tubes from three separate experiments using a common preparation of receptors. B_0 = 69 %, non-specific binding = 2.3 %. Symbols as for Fig. 3.

column. The antibody is highly specific for the human type I receptor, so this process excluded endogenous (mouse) type I receptors as well as type II and insulin receptors (Kull *et al.*, 1983; Casella *et al.*, 1986). Affinity cross-linking of the immunopurified receptors revealed that both 125 I-IGF-I and 125 I-IGF-II bound to a 130 kDa protein, consistent with binding to the type I receptor α subunit (results not shown). The immunopurified receptors, assayed by poly(ethylene glycol) precipitation, had extremely high affinity for both IGF-I and IGF-II. Maximal binding was 61 % for 125 I-IGF-I, and 50 % maximal displacement (IC_{50}) occurred in the presence of 45 pM unlabelled IGF-I or 330 pM-IGF-II (Fig. 3). The binding of 125 I-IGF-II was 69 %, and half-maximal displacement occurred with 100 pM unlabelled IGF-II or 27 pM-IGF-I (Fig. 4). The relative affinities of the two ligands could not be inferred from the IC_{50} , however, because the receptor concentration was not significantly lower than the estimated K_D in these experiments (Chang *et al.*, 1975). Furthermore, we could not extrapolate the data on a plot of bound versus bound/free, because the estimated K_D approached the radioligand concentration (30 pM). The dissociation constants for each peptide were calculated by saturating the receptors with

Fig. 5. Scatchard plots of IGF binding to purified receptors

Purified type I receptors ($2\ \mu\text{l}$) were incubated with $1.5\text{--}75\ \text{pM}$ radioligand overnight at 4°C in the absence or presence of $10\ \text{nM}$ unlabelled receptor-grade ligand. Bound radioligand was quantified after poly(ethylene glycol) precipitation as described in the Materials and methods section. The receptor concentration was one-fifth of that used in the displacement curves (Figs. 3 and 4), to ensure complete saturation. The data plotted represent individual tubes in a representative experiment. Key: $\bullet$, IGF-I; $\blacktriangle$, IGF-II.

radioligand. This technique allowed us to use low concentrations of receptor ($3\text{--}6\ \text{pM}$) and to measure the bound and free fractions directly. The estimated dissociation constants were $8 \pm 3\ \text{pM}$ and $18 \pm 3\ \text{pM}$ (mean $\pm$ S.E.M. in five experiments) for ^{125}I -IGF-I and ^{125}I -IGF-II respectively. A representative plot is shown in Fig. 5. Thus IGF-I appeared to have higher affinity than IGF-II, but the relative differences were much less striking than in the intact-cell binding studies. The relative potencies of the two ligands were comparable with that which we observed in receptors purified from human placenta, but the absolute values of dissociation constants were much higher ($200\ \text{pM}$ and $300\ \text{pM}$ for IGF-I and IGF-II respectively) in the earlier study (Casella *et al.*, 1986). This difference may be due to the use of recombinant h.p.l.c.-purified radioligands, and the rapid purification used in the present study.

Effect of $\alpha\text{IR-3}$

The monoclonal antibody to the type I receptor, $\alpha\text{IR-3}$, effectively inhibited ^{125}I -IGF-I binding to cells ($75 \pm 10\%$ inhibition at $1000\ \text{nM}$ in 8 experiments). The antibody did not, however, significantly inhibit IGF-II binding to the cell surface receptors ($B/B_0 = 1.05 \pm 0.15$ at $1000\ \text{nM}$ in 4 experiments). In the solubilized system, $\alpha\text{IR-3}$ was somewhat less effective at inhibiting ^{125}I -IGF-I binding to purified receptors ($38 \pm 7\%$ inhibition at $1000\ \text{nM}$), but had a small effect on IGF-II binding ($10 \pm 4\%$ inhibition). The differential effect of $\alpha\text{IR-3}$ on IGF-I and IGF-II binding is identical with that which we previously observed in receptors purified from human placenta (Casella *et al.*, 1986).

DISCUSSION

We have demonstrated that the receptor encoded by a cDNA isolated from human placenta has binding characteristics similar to those of the classical type I IGF receptor. In the intact cells, there is a $15\text{--}20$ -fold higher affinity for IGF-I than for IGF-II. These results contrast with those described previously of a

human type I receptor cDNA expressed in transfected CHO cells that had almost equal affinity for IGF-I and IGF-II (Steele-Perkins *et al.*, 1988). Part of the discrepancy may be due to the fact that, as noted by the authors, the native CHO cells expressed high levels of endogenous IGF-II receptors (Steele-Perkins *et al.*, 1988). Alternatively, subtle changes in post-translational processing might explain the differences between our studies using NIH-3T3 fibroblasts and those using a CHO-cell transfection system (Steele-Perkins *et al.*, 1988). A more intriguing possibility is that the cDNA used in the CHO system (Steele-Perkins *et al.*, 1988) is different from the cDNA that we examined (Ullrich *et al.*, 1986). Several studies have suggested that subtypes of the type I receptor exist (Morgan & Roth, 1986; Garofalo & Rosen, 1989; Alexandrides & Smith, 1989; Yee *et al.*, 1989; Kellerer *et al.*, 1990), and at least one of these variants is thought to be the consequence of alternative splicing resulting in a three-base-pair deletion (Bueno *et al.*, 1990). Since only 80% of the cDNA examined in the CHO cells has been compared (Steele-Perkins *et al.*, 1988), it is possible that the two cDNAs encode variants of the type I receptor with different specificities.

Although these results appeared to conflict with our earlier studies using immunopurified receptors, the differences can probably be attributed to the method of preparation. The binding characteristics of the purified solubilized receptors extracted from the transfected fibroblasts were significantly different than those in intact clone 7, i-24 cells. In particular, the relative specificity of the receptor changes such that IGF-I bound with only $2\text{--}3$ -fold higher affinity than IGF-II, in close agreement with our earlier findings (Casella *et al.*, 1986) and those more recently described in CHO cells (Steele-Perkins *et al.*, 1988). The affinity constants for both IGF-I and IGF-II were alone much higher in solubilized receptors. It is possible that detergent solubilization altered the structure of the receptor such that it had higher affinity, but lower specificity. Alternatively, it has been noted (Cuatrecasas, 1972) that Triton solubilization of liver and adipocyte membranes yielded more insulin-binding activity than that originally present in the intact membranes, and has suggested that this phenomenon may be due to the release of internal receptor pools. The release of partially processed receptors with different binding characteristics might contribute to the differences that we observed after solubilization.

The fact that $\alpha\text{IR-3}$ blocks IGF-I binding, but only weakly affects IGF-II binding, to the type I receptor was first noted in receptors purified from human placenta (Casella *et al.*, 1986), and also has been observed in human T-lymphocytes (Kozak *et al.*, 1987). During the preparation of this manuscript, it was reported that $\alpha\text{IR-3}$ does not inhibit IGF-II binding to transfected CHO cells that express a human type I receptor cDNA (Steele-Perkins & Roth, 1990). We have now confirmed the differential effect of $\alpha\text{IR-3}$ on IGF-I versus IGF-II binding in this highly homogeneous receptor system. Our results cannot be attributed to IGF-I/insulin hybrid tetramers (Moxham *et al.*, 1989; Soos & Siddle, 1989; Treadway *et al.*, 1989), because the native NIH-3T3 cells contained few endogenous insulin receptors, compared with the huge number of expressed type I receptors (Lammers *et al.*, 1989). The antibody was a more effective inhibitor of the relatively low-affinity ($K_D = 1\ \text{nM}$) IGF-I binding observed in intact cells, compared with the extremely high-affinity binding ($K_D = 8\ \text{pM}$) measured with purified receptors. This may be because the affinity of the antibody for the receptor, previously reported as $K_D = 0.3\ \text{nM}$ (Van Wyk *et al.*, 1985), may not be enhanced by solubilization. Alternatively, solubilization may alter the conformation such that there is a less stringent fit of the IGF-I molecule that is not completely impaired in the presence of the antibody. In both situations, however, $\alpha\text{IR-3}$ inhibited IGF-I binding much more than that of IGF-II. Thus the

differential effect of α IR-3 cannot be attributed to interference from hybrids or homologous receptors. It is possible that α IR-3 induces a conformational change that preferentially affects IGF-I binding, or the antibody may mask epitopes on the receptor that interact with IGF-I but are not required for IGF-II binding. If there are two immunologically distinct sites, as we have speculated in the past, they must be interactive, i.e. binding of one ligand must preclude binding of the other. Whatever the mechanism, these data strongly suggest that IGF-II has a unique interaction with the type I receptor. It was recently demonstrated (Hauguel-de Mouzon & Kahn, 1991) that IGF-I stimulation of Madin-Darby Canine Kidney cells resulted in induction of *c-fos* gene expression at concentrations as low as 0.1 nM. IGF-II, however, was only 25% as effective as IGF-I in stimulating type I receptor autophosphorylation, and did not significantly induce *c-fos* gene expression at concentrations as high as 100 nM. Thus the unique interaction of IGF-II with the type I receptor ligand-binding domains may have important implications in transmembrane signalling of the type I receptor.

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