Inducible Expression and Cellular Localization of Insulin-degrading Enzyme in a Stably Transfected Cell Line*

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Insulin degrading enzyme (IDE) is an evolutionarily conserved, nonlysosomal metalloprotease that has been implicated in the cellular degradation and processing of insulin. However, the site and the mode of the action of this enzyme are unclear. We have addressed these questions by establishing several Ltk" cell lines that can overexpress human insulin-degrading enzyme (hIDE) upon glucocorticoid induction. The level of overexpression of hIDE protein and transcripts in these lines correlates well with an increase in insulin degradation in both cell lysates and intact cells. Comparison of the deduced amino acid sequences of mammalian and Drosophila IDEs reveals a conserved carboxyl-terminal peroxisomal targeting sequence (A/S-K-L), suggesting that IDE may be localized in peroxisomes. To test this possibility, we determined the cellular location of the stably transfected hIDE by both immunofluorescence and immunocytoelectron microscopy. The overexpressed hIDE predominantly colocalized with catalase in peroxisomes, although IDE was also found in the cytosol at a much lower concentration. These results demonstrate that stably transfected IDE catalyzes a rate-limiting step in cellular insulin degradation and is localized predominantly in peroxisomes.

After binding to its receptor on the cell surface, insulin is internalized by receptor-mediated endocytosis; the internalized insulin may be either degraded or released intact. Degradation of insulin (reviewed by Duckworth (1)) is thus important in the termination of signaling and clearance of the circulating hormone. Insulin-degrading enzyme (IDE) (insulin protease, E.C. 3.4.22.11) is an evolutionarily conserved nonlysosomal metalloprotease that plays a crucial role in the degradation of internalized insulin in a number of types of cells (1). Evidence for such a role has come from several kinds of experiments as follows. I) IDE appears to account for the majority of insulin-degrading activity present in homogenates of liver and other tissues (1), and affinity labeling (covalent cross-linking with 125I-labeled insulin) demonstrates the binding of insulin to IDE; no other protease with a comparable affinity for insulin has been identified. ii) IDE cleaves the insulin molecule at multiple specific sites, generating a characteristic pattern of proteolytic fragments. A very similar pattern of cleavage is seen in insulin degraded by intact liver (2) and cultured hepatocytes (3). iii) IDE inhibitors inhibit cellular insulin degradation by L6 myoblasts (4), BC3H1 cells (5), and HepG2 cells (6). Moreover, microinjection of HepG2 cells with monoclonal antibodies to IDE specifically inhibits cellular insulin degradation (7). iv) Expression of transfected IDE in COS cells increases intracellular insulin degradation severalfold, indicating that IDE catalyzes a rate-determining step in cellular insulin degradation (8). We have also obtained similar results in HepG2, NIH3T3 and Ltk" cells.2

Although the evidence for the role of IDE in cellular insulin degradation is strong, it may have other functions as well. In vitro, IDE can degrade other substrates, e.g. transforming growth factor-α (TGF-α) (9), insulin-like growth factor II (10), and atrial natriuretic peptide (11); however, the physiological significance of these findings is not clear. It has also been suggested that IDE degrades oxidatively damaged hemoglobin in erythrocytes (12).

Despite the substantial evidence for the importance of IDE in cellular insulin degradation, controversy over its mode and site of action persists. Degradation of internalized insulin is believed to occur primarily in endosomes, and (in some tissues) in lysosomes. Endosomes are sealed membranous vesicles; the internalized insulin is thus separated from the cytosol by a membrane. IDE has been found to be primarily cytosolic by cell fractionation assays (13, 14). It is therefore unclear whether and how IDE gains access to insulin contained in endosomes. Some reports have suggested that internalized insulin may not be confined to endosomes. Hari et al. (15) observed cytosolic insulin-IDE complexes, and insulin and its receptor have also been reported in the nucleus (16-18).

The elucidation of the sequences for human (19, 20) and Drosophila (21) IDE has suggested another possible subcellular location for IDE. Comparison of the deduced amino acid sequences revealed a conserved carboxyl-terminal peroxisomal targeting sequence A/S-K-L (22, 23), implying that IDE may be localized in peroxisomes. Recently, Baumeister et al. (24) showed that this sequence was also present in rat IDE.

As noted above, the ability to temporarily and selectively increase expression of IDE by transfection has been extremely valuable in demonstrating its role in cellular insulin degradation. However, stably transfected cell lines offer a number of advantages over transient expression systems, including reduced cell stress and expression of the transfected gene in the whole population rather than a limited subset of cells. We report here the creation of stable transfectants carrying an inducible IDE under the control of a mouse mammary tumor virus long terminal repeat (MMTV-LTR) promoter. Induction of IDE expression with dexamethasone results in increased IDE

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The abbreviations used are: IDE, insulin-degrading enzyme; MMTV, mouse mammary tumor virus; LTR, long terminal repeat; TGF-α, transforming growth factor-α; DMEM, Dulbecco's modified Eagle's medium; kb, kilobase(s).

1 W.-L. Kuo, B. D. Gehm, and M. R. Rosner, unpublished observations.

2 W.-L. Kuo, B. D. Gehm, and M. R. Rosner, unpublished observations.
activity in cell extracts and increased insulin degradation by intact cells. The high level of expression in the induced state corresponded with the presence of a peroxisomal targeting sequence, we detectants overexpressing significant levels of IDE constitutively.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dexamethasone, bovine serum albumin, porcine insulin, and HEPES were purchased from Sigma. Trichloroacetic acid was purchased from Sigma. Phosphate-buffered saline (PBS) was used in the experiments containing PBS, 10 mM sodium azide, and 0.2% NaN3.

**DNA and Plasmids**—The construction of plasmids pMMTV-hIDE and pWLneo (20:1) by a standard calcium-phosphate transfection method (27). The control plasmid for the transfection was pLCI, a plasmid expressing chloramphenicol acetyltransferase driven by an MMTV-LTR promoter (28). Colonies resistant to G418 (400 μg/ml) were isolated, and the cells were expanded for analysis and screening. For each clone, an equal number of cells were seeded into two wells of a 12-well plate; one of the wells was treated with 0.1 μm dexamethasone to induce IDE expression. After 18 h, cells were washed once with phosphate-buffered saline and were preincubated in DMEM for 30 min, and 500 μl of binding buffer containing 50 μl 125I-labeled insulin was added to each well. After 45 min at 37°C, duplicates of 100 μl aliquots were removed and treated with trichloroacetic acid to precipitate undegraded 125I-labeled insulin. Degradation was measured by comparing the amount of label in the precipitates and supernatants.

**In Vitro Assay of IDE Activity**—In vitro assay of IDE protein—Preparation of cell extracts, IDE activity assay in vitro, and Western blot analysis of IDE protein were described elsewhere (29). 1 μg of cell extract was used for in vitro assay of insulin degradation and 50 μg of cell extract for Western blot analysis. 2BS is an antisem used for probing IDE protein and was described in Kuo et al. (30).

**Northern Blot Analysis and Labeling of Probes**—The same number of cells from each clone were seeded in 100-mm dishes and grown to 60–70% confluence, and then dexamethasone was added to cells 18 h before RNA extraction. RNA purification and Northern blot analysis were done essentially as described previously (30). Human IDE cDNA fragments (3.0 kb) were isolated from EcoRI digestion of 3.4-kb hIDE cDNA, and β-actin DNA fragments (1.6 kb) from SalI digestion of plasmid pAL-41 DNA. Both DNA fragments were labeled by nick translation (31).

**Immunofluorescence Microscopy**—Cells plated on coverslips were fixed and processed for single or double immunolabeling as described elsewhere (32). All the antibodies were used at a concentration of 10 μg/ml. In double immunolabeling experiments, the specific rabbit antibody was used for IDE (20), and a sheep anti-bovine catalase antibody was applied simultaneously to the cells followed by incubation with the appropriate secondary antibodies. Immunofluorescence microscopy was performed with a Molecular Dynamics Confocal Microscope.

**Immunocytoelectron Microscopy**—Cryoultramicrotomy, immunolabeling with the 2BS antibody to human IDE and IgG fractions of rabbit or sheep antibodies to bovine catalase, and embedding of sections were performed as described elsewhere (32). In single immunolabeling experiments, 10-nm gold aducts on goat antibodies to rabbit IgG were used. In double immunolabeling experiments, 2BS and a sheep antibody to bovine catalase were used followed by 10- and 5-nm gold aducts of donkey antibodies to rabbit IgG and sheep IgG, respectively. The sections were observed without post staining in a Philips CM 12.

**RESULTS**

**Production of Stably Transfected Cell Lines Containing Inducible IDE**—Although we can transiently express high levels of exogenous human or Drosophila IDE in several mammalian cell lines (8, 21), we have not been able to produce stable transfectants overexpressing significant levels of IDE constitutively. The reason for this is not clear; conceivably, constitutive overexpression of IDE may be detrimental to cell growth, because insulin and several growth factors are potential substrates of IDE in cells. To construct a plasmid that can overexpress IDE under the control of an inducible promoter, we attached a full-length (~3.4 kb) hIDE cDNA downstream of an MMTV-pro-
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Fig. 2. In vitro assay of IDE activity and Western blot analysis of IDE protein in KMHL clones. A, selected clones were grown in DMEM plus 10% fetal bovine serum and treated (filled bars) or not treated (open bars) with dexamethasone (DEX) for 18 h before harvesting cells. The control cell line was KMHL28, which did not show dexamethasone-induced IDE activity by the cellular degradation assay. Cell extracts were prepared, and 1 μg of each extract was assayed for IDE activity as described under “Experimental Procedures.” Results are shown as the mean ± S.D. of three determinations. Similar results were obtained in three independent experiments. B, cell extracts from dexamethasone (DEX) treated and untreated clones and the control, Ltk-LC1 cells, were prepared as described for A. 50 μg of each extract was used for Western blotting with antiserum 2BS. IDE bands were visualized by autoradiography of 125I-labeled protein A.

The results showed that in the presence of dexamethasone there was a 20-fold increase in IDE protein in clones KMHL2 and KMHL10, a moderate increase in KMHL36, but no apparent increase in the control cells, Ltk-LC1. These results were consistent with the insulin degradation assays of cell extracts and intact cells.

Human IDE Transcript in KMHL Clones—The dexamethasone-induced expression of hIDE was further confirmed by Northern blot analysis using a labeled hIDE DNA fragment as a probe (Fig. 3, top panel). KMHL clones and Ltk-LC1 cells were treated and analyzed as described under “Experimental Procedures.” Two sizes (6.3 and 3.6 kb) of endogenous mouse IDE transcript were detected and are indicated by arrows; similar results have been reported in rat tissues (24, 30). The exogenous hIDE transcript in the KMHL clones is larger (4.1 kb) than the endogenous 3.6-kb transcript because of the flanking β-globin gene sequences in the pMMTV-hIDE construct (as shown in Fig. 1A); however, the additional α-globin sequences in the pMMTV-hIDE construct are noncoding and did not alter the translated hIDE protein sequence. The level of hIDE transcripts in KMHL2 and KMHL10 cells was greatly increased by induction with dexamethasone, but only a moderate increase was seen in KMHL36 cells. Dexamethasone had no effect on the level of endogenous mouse IDE transcripts in Ltk-LC1 cells. There was at least a 10-fold induction of hIDE transcripts in KMHL2 cells as esti-

Fig. 3. Expression of hIDE transcript in KMHL clones. Total RNA from dexamethasone treated and untreated KMHL clones and Ltk-LC1 (control) cells, was prepared as described under “Experimental Procedures.” 20 μg of total RNA from each sample was separated in a 1% agarose gel containing formaldehyde, blotted onto a Nytran membrane, and hybridized with α-32P-labeled 3.6-kb hIDE cDNA probe (top panel) and α-32P-labeled mouse β-actin cDNA probe (bottom panel), separately. RNA bands were visualized by autoradiography. The endogenous (3.6 and 3.6 kb) mouse IDE transcripts are indicated by arrows.
mated by scanning the Northern blot with an AMBIS radiolabeled imaging system (data not shown). To control for the amount of RNA loaded in each lane, the same membrane was hybridized with a mouse β-actin cDNA probe (Fig. 3, bottom panel). Additionally, the gel was stained with acridine orange before blotting, and the variation in the β-actin signal was resulted from different amounts of RNA loaded in each lane as determined by the intensity of RNA staining. The increased levels of hIDE transcript in induced cells are not attributable to variations in loading.

The magnitude of induction of the hIDE transcripts in both KMHL2 and KMHL10 cells was much greater than the magnitude of induction of IDE activity and IDE protein shown by in vitro assay and Western blotting, respectively. This suggests that expression of IDE might be regulated post-transcriptionally.

Induction of Cellular Insulin Degradation—To confirm that the increased insulin degradation by induced cells was not due to release of IDE into the medium, the rate of cellular insulin degradation by induced and uninduced KMHL2 cells was compared with the rate of degradation by conditioned assay medium (Fig. 4). Although some insulin-degrading activity was observed in the conditioned medium, it was much lower than that observed in the presence of cells. The ~4-fold increase in cellular insulin degradation upon induction is thus not due to increased extracellular IDE.

Optimization of hIDE Expression in KMHL Clones—Dose-response and time course studies were done to find the optimum conditions for dexamethasone induction of IDE activity. As shown in Fig. 5A, KMHL2 and KMHL10 cells are almost identical in their dose response to dexamethasone, with efficient induction of IDE activity at dexamethasone concentrations of 0.1–1 μM. This is comparable with the dose at which induction was observed with dexamethasone in other expression systems (28). In contrast, the IDE activity of Ltk-LC1 control cells was not affected by up to 1 μM dexamethasone. Because both KMHL2 and KMHL10 are highly inducible clones, we used one of these lines, KMHL2, to determine the time course of induction (Fig. 5B). IDE protein expression reached its peak at about 19 h. At longer times, the level decreased but was still significantly elevated over untreated controls even after 74 h of dexamethasone treatment.

Immununolocalization of IDE in KMHL2 Cells—Single and double immunofluorescence microscopy was used to determine the cellular localization of hIDE in the parental nontransfected cells, transfected KMHL2 cells, and transfected KMHL2 after dexamethasone induction. In parental cells, a faint immunofluorescence for hIDE was detected in numerous small vesicular structures scattered through the cytoplasm (not shown). However, in transfected KMHL2 cells these vesicular structures were intensively labeled. The enhanced immunolabeling was without doubt due to a higher expression of hIDE in the transfected cells. In addition to the vesicular structures, immunofluorescence for hIDE was also observed in larger vesicles in nontreated as well as in cells treated for 24 h with dexamethasone (Fig. 6A). Because hIDE contains the typical conserved peroxisomal signal A/S-K-L, we carried out double im-
munolabeling experiments for catalase and IDE. We found that IDE largely colocalized with catalase, a peroxisomal enzyme marker (Fig. 5, B and C). The large vesicles that contained hIDE were also positive for catalase (Fig. 6, B-E). In addition to the peroxisomal labeling, diffuse labeling specific for IDE could also be detected in the cytoplasm.

Immunocytoelecctron microscopy experiments confirmed that a substantial amount of IDE is localized in the peroxisomes of the KMHL2 cells. Transfected cells contained numerous 0.5–1 μm single membrane-bound organelles dispersed throughout the cytoplasm. Gold particles indicating the presence of hIDE were detected over the matrix of these organelles (Fig. 7, A and B). As expected from the immunofluorescence data, the organelles that contain hIDE were indeed peroxisomes as they reacted with antibodies to catalase (Fig. 7, B and C). Close examination also showed that the cytosol contained hIDE but at a substantially lower concentration than in the peroxisomes (Fig. 7B, arrowheads). It is possible that this cytosolic labeling corresponds to hIDE molecules synthesized in the cytosol or on their way to the peroxisomes. Although the vast majority of the organelles contained IDE molecules, a small number of peroxisomes were in fact negative for IDE (Fig. 7D, asterisk). We think that these IDE-negative organelles may be in the process of importing the enzyme, because specific staining for IDE could be detected in the cytosol close to the organelle membrane (Fig. 7D, arrowheads). In the transfected cells treated with dexamethasone for 24 h, we occasionally observed endocytotic vesicles that contained recognizable hIDE-positive peroxisomes in different stages of degradation (Fig. 7E). These endocytotic vesicles likely correspond to the large hIDE- and catalase-positive vesicles seen by immunofluorescence (Fig. 6). The presence of peroxisomes in elements of the endocytotic vesicles is not unexpected. We think that this likely represents an autophagic process. Degradation of organelles within autophagic vesicles is a normal physiological phenomenon, and peroxisomes are frequently observed in endocytotic vesicles of normal rat liver hepatocytes. Taken together, these immunolabeling results indicate that hIDE is predominantly found within peroxisomes of KMHL2. The enzyme is also present in the cytosol and in endocytotic vesicles, although at a much lower concentration.

G. Keller, unpublished observation.
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**FIG. 7.** IDE is mainly localized to the peroxisomes in dexamethasone-treated KMHL2. In single labeling experiments (A, D, and E), thawed cryosections were immunolabeled with the anti-IDE antibody followed by 10-nm gold adducts of goat antibodies to anti-rabbit IgG. A, hIDE, visualized by 10-nm gold particles, was associated with the matrix of organelles characterized by an electron-dense matrix. B and C, In double immunolabeling experiments, IDE colocalized with catalase within the same organelle. C shows a higher magnification of a portion of the matrix of the organelle shown in B. IDE (arrowheads) and catalase (small arrows) were visualized with 10- and 5-nm gold particles, respectively. It should be noted that the cytosol of the cells also contained hIDE although at a much lower concentration than the peroxisomes (B, arrowheads). D, small organelles were sometimes devoid of hIDE (*), although hIDE was detected at the cytosolic face of the membrane (arrowheads). This labeling pattern may correspond to hIDE molecules attached to the membrane before being translocated into the peroxisomes. E, peroxisomes were also sometimes seen in endolysosomes typically consisting of vacuoles enclosing a central lumen (arrow).

**DISCUSSION**

Using a hormone-inducible pMMTV-hIDE vector we have created several stable Ltk− transfectants, which show a range of increased IDE protein expression upon induction with glucocorticoid. These are the first stable lines that can express significant exogenous IDE activity, and the high expression allows us to use simple and rapid IDE activity assays (e.g. trichloroacetic acid precipitation). With these stable lines, we demonstrated the induction of IDE mRNA, protein, and activity. Our results have shown that the increases of IDE activity in cell extracts and intact cells correlated well with the increases of IDE protein and RNA. Thus, it confirmed that IDE is rate-limiting for insulin degradation in these cells. Furthermore, we have shown that most of the IDE is localized to peroxisomes in these cells.

The regulation of IDE expression is an intriguing topic but has not been extensively studied. Developmental regulation of IDE expression has been reported in several biological systems. The level of IDE expression is developmentally regulated from embryo to adult in *Drosophila* (33). An IDE homologue (SZ17) was found in a protozoan parasite, *Eimeria bovis*, and this gene is specifically expressed in the oocyst stage and regulated during sporulation (34). In addition, we have previously shown that IDE is differentially expressed and developmentally regulated in various rat tissues and that IDE or IDE-related transcripts of at least four different sizes could be found in rat tissues (30). These results support a role for IDE or IDE-related genes in regulating cellular growth and development. In this report, we show that the level of hIDE transcripts in KMHL2 and KMHL10 cells was induced at least 10-fold by dexamethasone (Fig. 3), but the IDE protein was induced less than 5-fold. This difference suggests that post-transcriptional regulation is involved in the control of IDE expression.

Although IDE may have substrates other than insulin, it is selective; it does not, for example, degrade proinsulin or epi-
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Moderate methods of cell disruption are employed (41). The leakage from fragile peroxisomes during homogenization may explain why IDE was mainly detected in the cytosolic and other cell fractions (14, 38–40). In a series of preliminary experiments, we immunolabeled cryosections of normal rat liver with the same anti-IDE used in this study and found that labeling specific for IDE was present in both the peroxisomes and, to a lesser degree, in the cytosol. However, the immunolabeling density was so low that it prompted us to use stable transfectants overexpressing IDE to unambiguously identify the cellular localization of the enzyme. Both the immunofluorescence and immunoelectron microscopy indicate that the largest concentration of far IDE is in the peroxisomes of KMH2 cells. Although we cannot absolutely rule out the possibility that enzyme overexpression is influencing its localization in these cells, the predominantly peroxisomal localization is consistent with the fact that IDE from human, rat, and Drosophila contains a peroxisomal targeting motif, A/S-K-L, situated at the extreme carboxy terminus (22). This A/SKL, or a conserved variant, is both essential for peroxisomal sorting and sufficient for directing normally cytosolic or secretory passenger protein to the peroxisomes (22, 41). The conserved carboxy-terminal tripeptide has been identified in a number of peroxisomal proteins and is a ubiquitous topogenic targeting signal to microbodies (42).

However, the results do not imply that IDE occurs and functions only in peroxisomes, because a small portion of IDE was detected in the cytosol. Similarly, the sterol carrier protein-2, a nonenzymatic peroxisomal protein also ending in AKL, is found predominantly in peroxisomes but is also detected in the cytosol (43). Although this cytosolic IDE may represent the enzymes being translocated from its site of synthesis on free poly-somes to the peroxisomes, it is quite possible that the enzyme is functional in this compartment. In cells deficient in peroxi-somes from Zellweger patients, most of the peroxisomal en-zymes that are normally transported into the organelle re-main cytosolic (44). Some peroxisomal enzymes, such as catalase, are stable and biologically active in the cytosolic com-partment (45, 46). IDE-containing peroxisomes were also detected in vesicles that likely represent autophagic processing and endocytic degradation of peroxisomes. Because lysosomes-tropic agents can only partially inhibit insulin degradation (8), we think it is unlikely that IDE in autophagic vesicles plays an important role in insulin degradation. Furthermore, the opti-mal pH for IDE activity is neutral, whereas the elements of the endocytotic vesicles are acidic. The presence of IDE in the peroxisomes of KMH2 cells raises intriguing questions about the role of IDE in insulin degradation and may provide additional insight into its phys-iological function and substrates. Our results demonstrate that IDE catalyzes the rate-limiting step in cellular insulin degra-dation and raise the possibility that IDE may degrade insulin in peroxisomes. However, there is no evidence to date that endocytosed insulin is delivered to peroxisomes, suggesting that peroxisomes may not be the intracellular site of insulin degradation. Our results are also consistent with the possibility that IDE degrades insulin in compartments other than the peroxisomes within the cells. Additionally, as we have proposed previously (30), IDE could be a multifunctional protein that plays an as yet unidentified role in peroxisomes. Although proteo-lytic activity has been detected in plant peroxisomes (47), IDE is the first cloned and characterized protease to be localized to the peroxisomes. The existence of peroxisomal proteases has been postulated in the proteolytic processing of peroxiso-

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