

# An Evolutionarily Conserved Enzyme Degrades Transforming Growth Factor-Alpha as well as Insulin

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**Abstract.** A single enzyme found in both *Drosophila* and mammalian cells is able to selectively bind and degrade transforming growth factor (TGF)-alpha and insulin, but not EGF, at physiological concentrations. These growth factors are also able to inhibit binding and degradation of one another by the enzyme. Although there are significant immunological differences between the mammalian and *Drosophila* enzymes, the

substrate specificity has been highly conserved. These results demonstrate the existence of a selective TGF-alpha-degrading enzyme in both *Drosophila* and mammalian cells. The evolutionary conservation of the ability to degrade both insulin and TGF-alpha suggests that this property is important for the physiological role of the enzyme and its potential for regulating growth factor levels.

GROWTH factors play an important role in cell growth and transformation. Accordingly, the areas of growth factor synthesis, receptor interactions, and signal transduction have been intensively studied. In contrast, with the exception of insulin, little is known about the important area of growth factor degradation and its role in the regulation of growth and transformation.

Transforming growth factor (TGF)<sup>1</sup>-alpha is a polypeptide secreted by a variety of transformed cells that can induce reversible phenotypic transformation of normal mammalian cells in culture and is closely related structurally to EGF (3, 4, 20, 26, 29). Like EGF, TGF-alpha binds to the EGF receptor, leading to activation of the receptor tyrosine kinase and mitogenic stimulation. No receptor specific for TGF-alpha has been identified. Although EGF appears to be degraded through a series of proteolytic cleavages upon binding and internalization but before removal to the lysosomes (19), the enzymes responsible have not been isolated. Less is known about the degradation of TGF-alpha. By contrast, the insulin-degrading enzyme (IDE) has been well characterized as the enzyme responsible for initiating insulin degradation in mammalian cells (5, 12, 23).

One approach that can yield new and relevant insights into the mechanism of action of mammalian growth regulatory proteins is the identification and characterization of homologues of these proteins in lower organisms. Recent work in our laboratory has led to the identification and purification of a growth factor-specific degrading enzyme from *Drosophila* with properties strikingly similar to those of the mammalian IDE (9). The human IDE has recently been cloned and appears to have some homology in limited regions to an *Esche-*

*richia coli* protease (1). Because of their similarities and the fact that both the mammalian and *Drosophila* enzymes cleave porcine insulin at the same major sites (5, 6, 12), we have termed our protein the *Drosophila* IDE. Furthermore, antigenic, physical, and kinetic properties indicate that the *Drosophila* IDE is identical to a previously characterized *Drosophila* growth factor-binding protein (8, 28). This *Drosophila* protein was shown to bind mammalian TGF-alpha, insulin, and EGF with high affinity ( $K_d$  of  $\sim 10^{-9}$ ,  $10^{-7}$ , and  $10^{-6}$  M, respectively) and specificity (8, 28). In this communication we demonstrate that (a) TGF-alpha, like insulin, is degraded by both *Drosophila* and mammalian IDE; (b) both enzymes bind EGF but neither one is able to degrade it under our experimental conditions; and (c) exposure to an excess of one of the growth factors inhibits IDE-mediated degradation. These results raise the possibility of a functional or structural relationship between the insulin and TGF-alpha families that could have profound implications for the coordination of different growth-signaling pathways.

## Materials and Methods

### Materials and Cells

The *Drosophila* Kc cells, obtained from the Cell Culture Center (Massachusetts Institute of Technology, Cambridge, MA) were grown at 25°C in D22 medium supplemented with yeast hydrolysate. Insulin and EGF were purchased from Biomedical Technologies, Inc. (Stoneham, MA). The monoiodinated <sup>125</sup>I-insulin and <sup>125</sup>I-EGF used for degradation assays were purchased from New England Nuclear (Boston, MA). The recombinant TGF-alpha was a gift from Dr. Rik Derynck (Genentech, Inc., South San Francisco, CA), and the synthetic TGF-alpha was a gift from Dr. James Tam (Rockefeller University, New York). Synthetic TGF-alpha was used in all experiments, except for the affinity labeling (Fig. 3) and the inhibition of insulin degradation (Fig. 5) in which the recombinant TGF-alpha was used. Enzymobeads were from Bio-Rad Laboratories (Rockville Centre, NY). In-

1. Abbreviations used in this paper: IDE, insulin-degrading enzyme; TGF, transforming growth factor.

ulin and EGF used for affinity-labeling experiments, as well as synthetic and recombinant TGF- $\alpha$ , were iodinated using enzymebeads as previously described (28) (final specific activity  $\sim 100 \mu\text{Ci}/\mu\text{g}$ ). The anti-human EGF receptor antiserum was described previously (2).

### TCA Precipitation Degradation Assay

Aliquots of purified IDE were diluted into a buffer containing 50 nM insulin, 0.5 mg/ml BSA, 100 mM phosphate, pH 7.2, and 25,000 cpm of monoiodinated insulin (specific activity 80–120  $\mu\text{Ci}/\mu\text{g}$ ). The samples were incubated for 15 min at 37°C and the incubation was stopped by the addition of cold 25% TCA. The relative amount of released radioactivity in the soluble fraction was determined as previously described (9). For  $^{125}\text{I}$ -TGF- $\alpha$  and  $^{125}\text{I}$ -EGF degradation assays, unlabeled insulin was omitted. The extent of specific degradation was evaluated by adding an excess of unlabeled insulin to parallel samples. All concentrations were chosen so that the extent of insulin degradation was linear with time and protein. Competition assays in the presence of EGF and their analyses were carried out as described (9, 10).

### Binding to the EGF Receptor

$^{125}\text{I}$ -EGF or  $^{125}\text{I}$ -TGF- $\alpha$  was incubated with the IDE as above and the

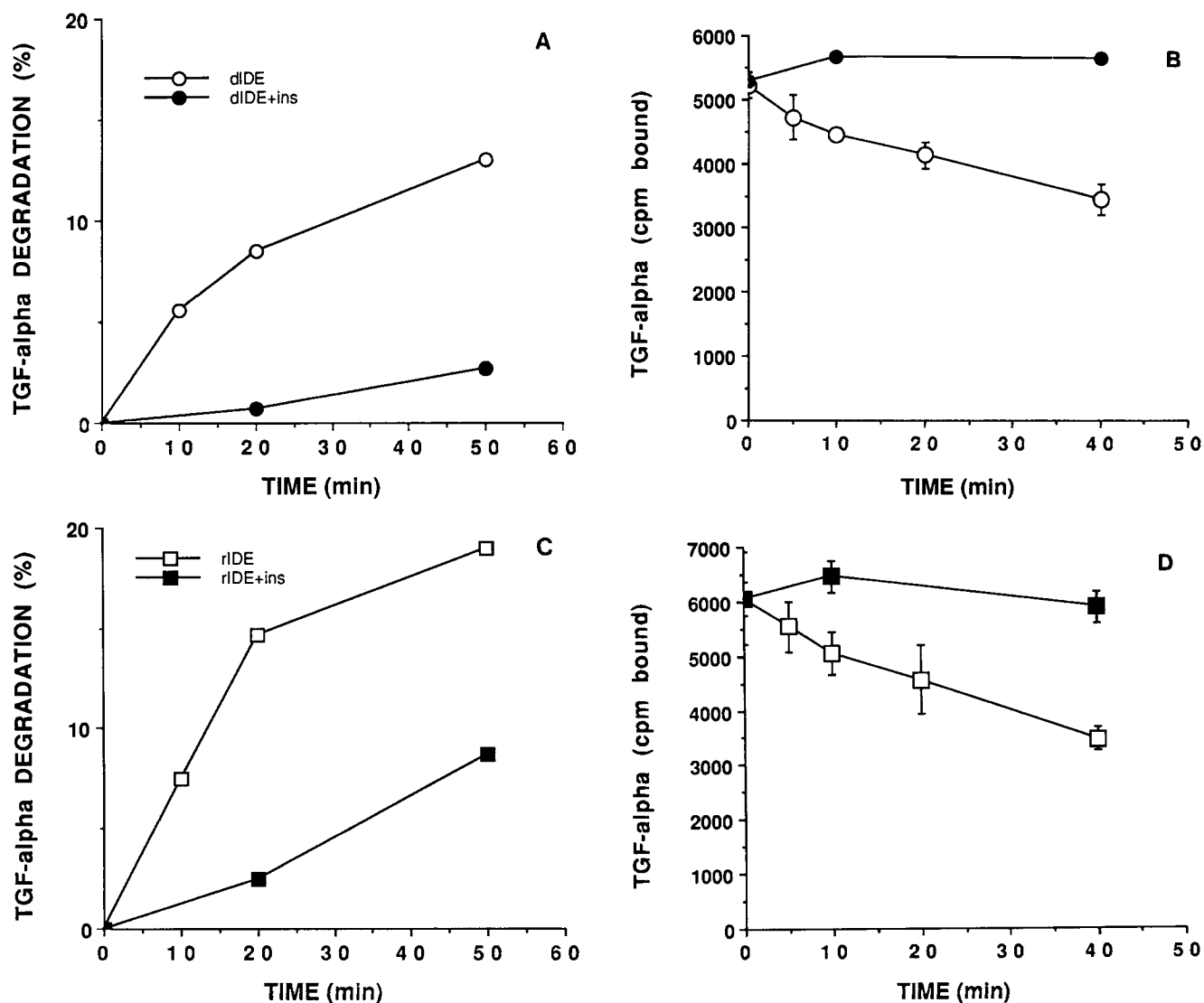
samples were removed at various times. Reactions were stopped by adding excess insulin or by two cycles of freezing and thawing. Samples were then assayed for binding to the EGF receptor as described (7).

### Purification of the *Drosophila* and Rat Liver IDEs

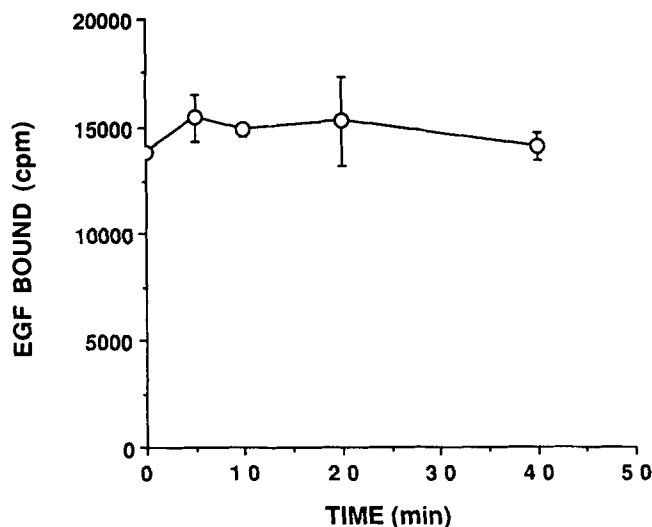
The *Drosophila* IDE was purified and assayed as described (9, 10). The rat liver IDE was isolated as described for the *Drosophila* IDE (9), except that the hydroxyl apatite column step was replaced by a second DEAE Sephadex column and the butyl agarose and chromatofocusing columns were omitted. This partially purified preparation of rat liver enzyme is equivalent to that used for characterization of the properties of the *Drosophila* and rat liver IDEs (9, 24). 1  $\mu\text{l}$  of enzyme (either *Drosophila* or rat liver enzyme preparations) yielded 10% degradation of 0.1 nM  $^{125}\text{I}$ -labeled insulin in 10 min by the TCA precipitation assay. Unless indicated otherwise, all degradation assays were performed with 2  $\mu\text{l}$  of enzyme in a final volume of 50  $\mu\text{l}$ /sample.

### Affinity Labeling

Both rat liver and *Drosophila* IDE (15 and 5  $\mu\text{l}$ , respectively) were incubated on ice with radiolabeled ligand ( $\sim 10^{-9}$  M) in the absence or presence of insulin ( $10^{-5}$  M) or EGF ( $10^{-5}$  M) in a final volume of 50  $\mu\text{l}$  PBS



**Figure 1.** Degradation of TGF- $\alpha$  by the *Drosophila* and mammalian IDEs.  $^{125}\text{I}$ -TGF- $\alpha$  ( $10^{-9}$  M) was incubated with the *Drosophila* IDE (A and B) or the rat liver IDE (C and D) for the indicated times in the absence (○) or presence (●) of unlabeled insulin ( $10^{-5}$  M). The extent of degradation was assayed by TCA precipitation (A and C) or TGF- $\alpha$  receptor binding (B and D). Nonspecific counts per minute determined in the presence of unlabeled EGF ( $7.5 \times 10^{-7}$  M) were subtracted from the final values in the EGF receptor-binding assay. Values represent the mean of three determinations  $\pm$  SD. The standard deviations in B are smaller than the symbols.



**Figure 2.** The rat liver IDE does not degrade labeled EGF.  $^{125}\text{I}$ -EGF (1 nM) was incubated with the rat liver IDE for the indicated times and assayed for EGF degradation by the EGF-binding assay as in Fig. 1. Nonspecific counts per minute determined in the presence of unlabeled EGF ( $7.5 \times 10^{-7}$  M) were subtracted from the final values in the EGF receptor-binding assay. Values represent the mean of three determinations  $\pm$  SD.

for 2 h. At this time, samples were cross-linked with dimethyl suberimidate, separated from labeled BSA, and autoradiographed as described (8, 28).

### Preparation of the Polyclonal Anti-*Drosophila* IDE Antiserum

Partially purified *Drosophila* IDE (80–120  $\mu\text{g}$ ) was loaded to a preparative 6.5% polyacrylamide gel under denaturing conditions as previously described (9). Proteins were visualized by staining with Coomassie blue. The 110,000-D protein band was excised, ground, and injected directly intraperitoneally into New Zealand rabbits at days 1, 21, 35, 51, and 122. Serum samples were obtained at day 1 before the first immunization (control serum) and at days 28, 43, 56, and 128. Antibodies specifically reactive with the *Drosophila* IDE were detected after the second immunization.

### Immunoblotting Analysis

Protein samples (60  $\mu\text{l}$  of enzyme) were separated by PAGE using 6.5% polyacrylamide gels under reducing conditions (8). The samples were then electrophoretically transferred from the gel to a nitrocellulose paper and the paper was probed with rabbit polyclonal antibodies (1:100 dilution) or mouse monoclonal antibodies (10  $\mu\text{g}/\text{ml}$ ) as described (10, 28).

## Results

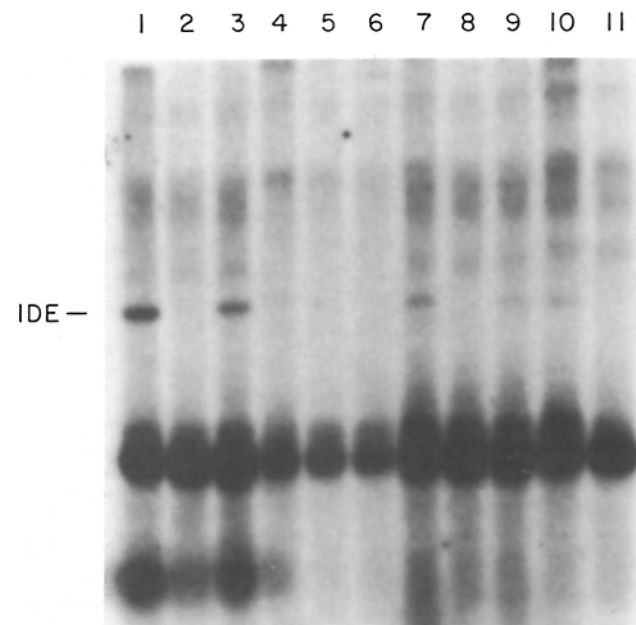
We have recently demonstrated that the *Drosophila* IDE binds TGF- $\alpha$  with higher affinity than insulin (8, 10, 28). Because of this interaction, we determined whether TGF- $\alpha$  could be a substrate for the enzyme. The extent of degradation was measured by TCA precipitation or receptor binding of undegraded TGF- $\alpha$ . As shown in Fig. 1, A and B, the results indicate that the *Drosophila* IDE is able to degrade human  $^{125}\text{I}$ -TGF- $\alpha$  at a physiological ( $10^{-9}$  M) concentration and that TGF- $\alpha$  degradation is specifically inhibited by an excess of unlabeled insulin.

In contrast to the *Drosophila* homologue, the mammalian IDE has been characterized primarily as a degrading enzyme for insulin and related factors. We therefore examined the

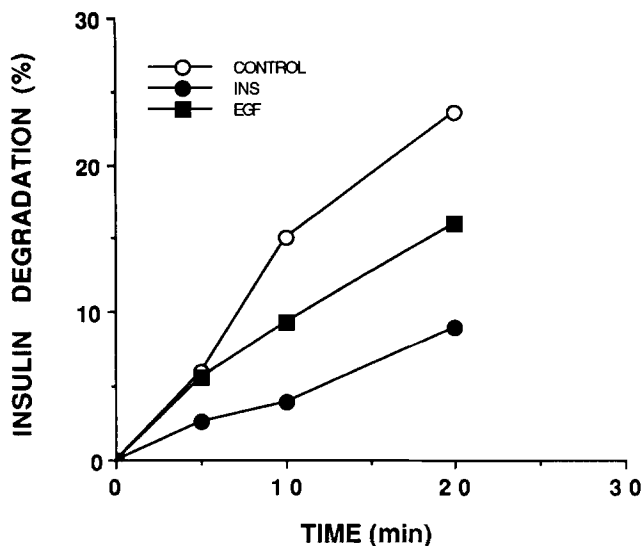
mammalian IDE for TGF- $\alpha$  degradation by the same methods used to characterize the *Drosophila* enzyme. The data presented in Fig. 1, C and D, indicate that the mammalian IDE is also capable of degrading TGF- $\alpha$ . As in the case of the *Drosophila* enzyme, TGF- $\alpha$  degradation could be inhibited by an excess of unlabeled insulin. The extent of TGF- $\alpha$  degradation at mammalian physiological concentrations was also very similar for both enzymes. These results reveal the existence of a single enzyme, both in *Drosophila* and mammals, that is capable of degrading TGF- $\alpha$  and insulin.

Despite the similarity of EGF and TGF- $\alpha$  in terms of structural homology and receptor recognition, neither the *Drosophila* nor the mammalian enzyme was able to degrade EGF under the same experimental conditions. Although several lines of evidence have shown that the *Drosophila* IDE can bind EGF (8, 10, 28), no degradation of EGF was detected by either the TCA precipitation or receptor-binding assays (10). The mammalian IDE was similarly unable to degrade EGF, as shown by the receptor-binding assay (Fig. 2) and TCA precipitation (data not shown). This result serves to illustrate the selectivity of the enzymatic activity for TGF- $\alpha$  and insulin but not EGF, a property of the enzyme conserved through evolution.

To examine if the homology between the *Drosophila* and mammalian enzymes extends to binding properties, we determined whether the rat liver IDE, like the *Drosophila* IDE, could be affinity labeled with either  $^{125}\text{I}$ -EGF or  $^{125}\text{I}$ -TGF- $\alpha$ . As shown in Fig. 3, when  $^{125}\text{I}$ -labeled growth factors were bound and cross-linked to the mammalian IDE, only insulin and TGF- $\alpha$  were able to affinity label the enzyme.



**Figure 3.** Affinity labeling of the rat liver IDE with insulin and TGF- $\alpha$ . The rat liver IDE was incubated with  $^{125}\text{I}$ -insulin (lanes 1–3),  $^{125}\text{I}$ -EGF (lanes 4–6), or  $^{125}\text{I}$ -TGF- $\alpha$  (lanes 7–9) in the absence (lanes 1, 4, and 7) or presence of  $10^{-5}$  M unlabeled insulin (lanes 2, 5, and 8) or EGF (lanes 3, 6, and 9). For comparison, the *Drosophila* IDE affinity labeled with  $^{125}\text{I}$ -TGF- $\alpha$  is also shown in the absence (lane 10) or presence (lane 11) of unlabeled insulin.



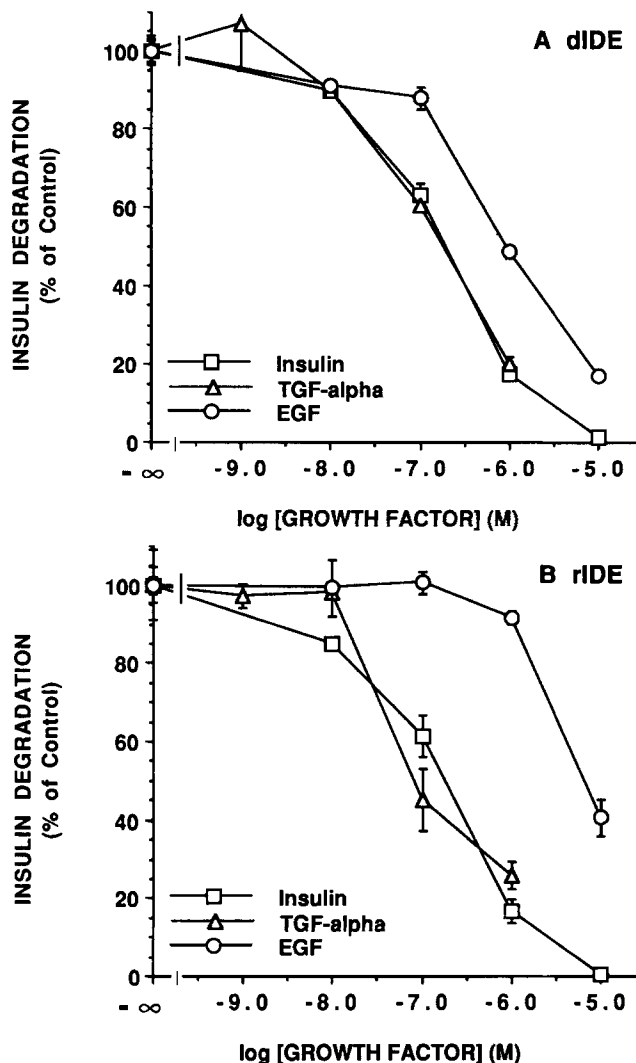
**Figure 4.** Insulin degradation is inhibited by EGF. Rat liver IDE was incubated with  $^{125}\text{I}$ -insulin ( $\sim 1$  nM) in the absence ( $\circ$ ) or presence of unlabeled insulin ( $\bullet$ ) ( $5 \times 10^{-6}$  M) or EGF ( $\blacksquare$ ) ( $5 \times 10^{-6}$  M) and then assayed for insulin degradation by TCA precipitation as in Fig. 1. These results reflect the mean of two determinations, and the error was within 10%.

The greater labeling with insulin than with TGF-alpha may reflect higher efficiency of cross-linking since the affinities of recombinant TGF-alpha and insulin for the enzyme are approximately equal (see below). Unlike the *Drosophila* enzyme, the rat liver enzyme could not be labeled with EGF under our experimental conditions. However, there is evidence to indicate that EGF does bind weakly to the mammalian enzyme. High concentrations of unlabeled EGF partially inhibited affinity labeling by  $^{125}\text{I}$ -TGF-alpha (Fig. 3). Unlabeled insulin had a more potent inhibitory effect, suggesting that the mammalian enzyme has a higher affinity for insulin than EGF. This is in agreement with our observations for the *Drosophila* IDE (8, 28).

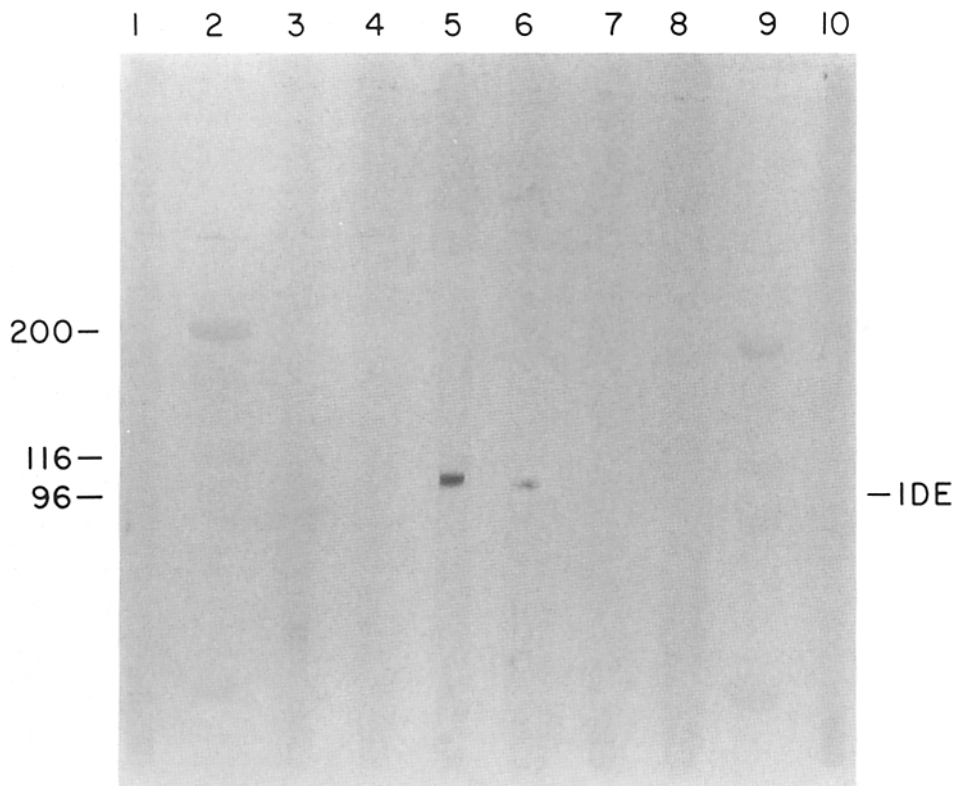
Previous work with the *Drosophila* enzyme showed that EGF inhibited IDE-mediated insulin degradation (10). To test if this inhibition is also a property of the mammalian IDE, the enzyme was incubated with  $^{125}\text{I}$ -insulin in the presence of unlabeled insulin and EGF. Time course studies (Fig. 4) indicated that degradation of  $^{125}\text{I}$ -insulin can be inhibited by EGF and insulin. In experiments using recombinant TGF-alpha, the relative potency of inhibition of  $^{125}\text{I}$ -insulin degradation by the rat liver enzyme was TGF-alpha = insulin > EGF (Fig. 5). As shown, similar results were obtained with the *Drosophila* IDE, further establishing the evolutionary conservation of the enzyme with respect to substrate recognition. In previous experiments (8), *Drosophila* IDE bound synthetic TGF-alpha more tightly than insulin. Direct comparison of synthetic with recombinant TGF-alpha using both IDEs showed the synthetic to be a more potent inhibitor than the recombinant by at least one order of magnitude (data not shown), but the limited availability of the synthetic form precluded its use in comprehensive inhibition studies.

Although most of the IDE properties have been conserved through evolution (9), including limited homology between the human IDE and bacterial protease (1), there are significant immunological differences between the mammalian

and *Drosophila* IDE. Immunoblotting (Fig. 6) revealed a single band for the *Drosophila* IDE when blots were probed with either anti-*Drosophila* IDE (10) or anti-human EGF receptor antiserum (2). A single band for the rat liver IDE was observed when blots were probed with a monoclonal antibody (23) that recognized both the human and rat IDE. The two enzymes differed slightly in molecular weight. However, the antiserum specific for the *Drosophila* IDE and the anti-human EGF receptor antiserum showed no apparent immunological cross-reactivity with the mammalian IDE. Similarly, the anti-human IDE antibodies failed to cross react with the *Drosophila* IDE. These results indicate that certain immunodominant epitopes of the two enzymes have not been conserved.



**Figure 5.** Competitive inhibition of  $^{125}\text{I}$ -insulin hydrolysis by unlabeled insulin, TGF-alpha, and EGF. *Drosophila* (A) and rat liver (B) IDEs were incubated with  $0.1$  nM  $^{125}\text{I}$ -insulin ( $\sim 300$   $\mu\text{Ci}/\mu\text{g}$ ) and the indicated concentrations of unlabeled insulin ( $\square$ ), TGF-alpha ( $\Delta$ ), or EGF ( $\circ$ ). After 10 min, reactions were terminated by the addition of TCA and assayed as described in Materials and Methods. The enzyme aliquots used had an activity of  $0.1$  pmol/min in insulin-degradation assays containing  $50$  nM insulin. Hydrolysis of  $^{125}\text{I}$ -insulin at each concentration is expressed as a percentage of that obtained in the absence of added growth factors. Maximum hydrolysis was  $\sim 30\%$  of total labeled insulin. These results reflect the mean of two determinations  $\pm$  SD.



**Figure 6.** Immunoblot of the rat liver and *Drosophila* IDEs. Rat liver IDE (lanes 1 and 3–5) and *Drosophila* IDE (lanes 6–8 and 10) were probed with preimmune (lanes 1 and 10), anti-*Drosophila* IDE (lanes 3 and 6), anti-human EGF receptor (lanes 4 and 7), and anti-human IDE (lanes 5 and 8) serum. A faint band corresponding to the *Drosophila* IDE in lane 7 is not visible in the photograph. Prestained molecular weight markers are shown in lanes 2 and 9.

## Discussion

Although the biochemical properties of the mammalian IDE have been studied extensively, the enzyme has been characterized primarily as a specific degrading enzyme for insulin and insulin-related factors (5, 25). We now report a novel property of the mammalian enzyme—the ability to bind and degrade TGF- $\alpha$ . This observation reveals a new, growth factor-related class of substrates for the enzyme that could certainly affect the physiological function. Information about nonlysosomal growth factor degradation and its potential role in growth regulation is very limited at present, and these results represent the first description of a nonlysosomal enzyme that selectively binds and degrades TGF- $\alpha$ .

As a degrading enzyme, the IDE has an unusual substrate specificity. Results of the analysis of insulin cleavage suggest that both the *Drosophila* enzyme and its mammalian counterpart display conformational rather than peptide bond specificity (5, 6). The *Drosophila* enzyme cleaves a subset of the insulin bonds that are cleaved by the mammalian IDE (5, 6), a further indication of the evolutionary conservation of the enzyme's substrate specificity. We have also demonstrated previously (8) that the *Drosophila* enzyme binds and cross-links to insulin and EGF-related factors, but not to TGF- $\beta$ , PDGF, nerve growth factor, adrenocorticotrophic hormone, or parathyroid hormone. Thus, the IDE has limited specificity for certain hormones and growth factors.

Previous studies from our laboratory have demonstrated that the *Drosophila* IDE is able to bind both EGF and TGF- $\alpha$ , although the affinities differ by at least two orders of magnitude (8, 28). However, we have not detected significant cleavage of EGF by the *Drosophila* enzyme even at higher EGF concentrations, whereas cleavage of insulin is obtained

at nanomolar concentrations and the  $K_d$  of insulin ( $\sim 10^{-7}$  M) is only 10-fold lower than that of EGF (8). In contrast to the *Drosophila* enzyme, the mammalian IDE homologue does not detectably bind EGF. Therefore, it was striking that the mammalian IDE shares with the *Drosophila* homologue the ability not only to bind but also to degrade TGF- $\alpha$ .

The IDE represents the first example of an evolutionarily conserved protein with a characterized function that is able to discriminate between EGF and TGF- $\alpha$ . In general, the affinities of TGF- $\alpha$  and EGF for the EGF receptor are comparable (8, 28). One exception appears to be the chicken EGF receptor which binds EGF with lower affinity than TGF- $\alpha$  (15). Since no distinct receptor for TGF- $\alpha$  has been identified, it is widely assumed that TGF- $\alpha$  elicits biological responses through activation of the EGF receptor (16). However, there are several reports indicating that TGF- $\alpha$  is more potent than EGF in stimulating a number of physiological responses. These effects, which include calcium mobilization (13), wound healing in skin (22), angiogenesis (21), and cell ruffling (18), are difficult to explain on the basis of EGF receptor interactions alone. Separate degradative pathways for EGF and TGF- $\alpha$  provide a possible mechanism for the differential effects of the two growth factors.

The physiological counterpart of TGF- $\alpha$  degradation by the *Drosophila* IDE is not presently clear. To date, no homologues of TGF- $\alpha$  or EGF have been identified in *Drosophila*, although two genes with EGF/TGF- $\alpha$ -like domains termed Notch (30) and Delta (14) have been characterized and a TGF- $\beta$  homologue has been identified (11). An insulin-like activity in *Drosophila* has also been reported (17). Whether the products of these *Drosophila* genes are substrates for the *Drosophila* IDE remains to be determined.

**Table I. Degradation and Binding Properties of Mammalian and *Drosophila* IDE**

	Mammalian IDE	<i>Drosophila</i> IDE
Growth factor degradation*		
TGF-alpha	+	+
Insulin	+	+
EGF	-	-
Inhibition of insulin or TGF-alpha degradation†		
TGF-alpha	+	+
Insulin	+	+
EGF	+	+
Affinity labeling‡		
<sup>125</sup> I-TGF-alpha	+	+
<sup>125</sup> I-Insulin	+	+
<sup>125</sup> I-EGF	-	+
Inhibition of TGF-alpha or insulin binding§		
TGF-alpha	+	+
Insulin	+	+
EGF	+	+
Immunologic cross-reactivity		
Anti-human IDE	+	-
Anti- <i>Drosophila</i> IDE	-	+
Anti-human EGF receptor	-	+

\* Fig. 1 and references 5, 9, 12, 23, 27.

† Figs. 1, 4, and 5 and references 9, 10, 24, 25.

‡ Fig. 3 and references 8-10, 24, 28.

§ Fig. 6 and references 8, 10, 27, 28.

Cleavage of TGF-alpha and insulin by the mammalian enzyme represents a more physiologically relevant system. Duckworth et al. and Hamel et al. have recently demonstrated that the mammalian enzyme has access to insulin in preacidic endosomal vesicles and appears to account for the majority of initial insulin degradation in hepatocytes (5, 12). Since the EGF receptor and the insulin receptor are both internalized by similar receptor-mediated endocytic mechanisms, these observations suggest that the IDE may be accessible to TGF-alpha as well as insulin in the mammalian cell.

The fact that the IDE degrades factors that regulate growth raises the possibility that the enzyme might also play a regulatory role. We have recently demonstrated that the IDE is developmentally regulated in *Drosophila* (27), suggesting that the enzyme may act at a rate-limiting step and is either detrimental during periods of rapid growth or only required during later stages of differentiation. The novel properties of the highly conserved IDE (summarized in Table I) raise interesting possibilities regarding its potential function as a selective growth factor-degrading enzyme in the cell.

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