

# An Insulin Epidermal Growth Factor-binding Protein from *Drosophila* Has Insulin-degrading Activity

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**Abstract.** We have recently described the purification and characterization of an insulin-degrading enzyme (IDE) from *Drosophila melanogaster* that can cleave porcine insulin, is highly conserved through evolution and is developmentally regulated. We now report that the IDE is, in fact, an insulin EGF-binding protein (dp100) that we had isolated previously from *Drosophila* using an antihuman EGF receptor antiserum. This conclusion is based upon the following evidence. (a) dp100, identified by its ability to cross-link to labeled insulin, EGF, and transforming growth factor-alpha (TGF-alpha), and to be immunoprecipitated by anti-EGF receptor antisera, copurifies with the IDE activity. Thus, the purified IDE can be affinity labeled with either <sup>125</sup>I-insulin, <sup>125</sup>I-EGF, or <sup>125</sup>I-TGF-alpha, and this labeling is specifically inhibited with unlabeled insulin, EGF, and the insulin B chain. (b) The antiserum

to the human EGF receptor, which recognizes dp100, is able to specifically immunoprecipitate the insulin-degrading activity. (c) The purified IDE preparation contains a single protein of 110 kD which is recognized by both the anti-EGF receptor antiserum and anti-*Drosophila* IDE antiserum. (d) Polyclonal antiserum to the purified IDE, which specifically recognized only the 110-kD band in *Drosophila* Kc cells, immunoprecipitates dp100 cross-linked to <sup>125</sup>I-TGF-alpha and dp100 cross-linked to <sup>125</sup>I-insulin from the purified IDE preparation. (e) EGF, which competes with insulin for binding to dp100, also inhibits the degradation of insulin by the purified IDE. These results raise the possibility that a functional interaction between the insulin and EGF growth factor families can occur which is mediated by the insulin-degrading enzyme.

**I**N vertebrates, growth factors that are members of the insulin and EGF families are integrally involved in the stimulation of mitogenesis and control of cellular metabolism. Further understanding of their biochemical interactions could benefit from identification and investigation of proteins that bind these factors in genetically characterized lower organisms.

One system that appears to be highly conserved between mammals and *Drosophila* is that of insulin and its related proteins. An insulinlike activity in *Drosophila* has been described (10) and *Drosophila* homologues of the human insulin receptor have recently been identified (3, 12-14). In vertebrates, insulin-induced effects are initiated upon binding of insulin to a specific cell surface receptor. However, the actual mechanism by which insulin is degraded within the cell is not entirely clear. While evidence for lysosomal degradation of insulin exists (7-9), a nonlysosomal insulin-specific degrading enzyme has been identified in mammals that may also play a critical role in vivo (2, 18, 19). One approach to resolving which degradative pathway is important is to de-

termine whether these enzymes are present in evolutionarily distant organisms that use insulin-related hormones. In fact, we have recently purified and characterized an insulin-degrading enzyme (IDE)<sup>1</sup> from the cytoplasm of *Drosophila* (5), demonstrating that the nonlysosomal insulin degradation pathway is conserved. We have also demonstrated that the IDE is regulated during *Drosophila* development (20). The fact that such a system is so well conserved during evolution suggests an important role for the IDE.

A second system that has a counterpart in *Drosophila* is the gene for *c-erb*, which codes for the EGF receptor in vertebrates (16). To identify proteins related to the EGF receptor in *Drosophila*, we used polyclonal antisera that recognizes EGF receptors from a variety of species (1). This antiserum reacts with the cytoplasmic domain of the EGF receptor containing the tyrosine kinase region. In the course of this investigation, we identified not only a protein that corresponds to the *Drosophila* EGF receptor but, in addition, a growth factor binding protein for both insulin and EGF (21). Based upon the recognition by anti-EGF receptor antiserum and

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

the affinity labeling with insulin and EGF, it appeared that this latter protein (designated dp100) might be a novel receptor distinct from the *Drosophila* homologues of the EGF and insulin receptors. To explore this possibility further, we characterized the binding spectrum, glycosylation state, and cellular distribution of dp100 (6). The results indicated that dp100 is not a receptor, but a discrete binding protein for insulin and EGF-related factors.

We now report that dp100 is, in fact, the IDE that we had purified from *Drosophila* Kc cells. Since EGF acts as an inhibitor of insulin degradation in this system, but is not degraded at physiological concentrations, it is possible that EGF-like factors in *Drosophila* can influence the degradation and ultimate action of the insulin homologues in this organism.

## Materials and Methods

### Materials and Cells

The *Drosophila* Kc cells, obtained from the Massachusetts Institute of Technology Cell Culture Center, 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 transforming growth factor- $\alpha$  (TGF- $\alpha$ ) was a gift from Dr. Rik Derynck, Genentech, San Francisco, CA and the synthetic TGF- $\alpha$  was a gift from Dr. James Tam, Rockefeller University, NY. Enzymobeads were from Bio-Rad Laboratories, Rockville Centre, NY. Insulin, EGF, and recombinant TGF- $\alpha$ , used for affinity labeling experiments, were iodinated using enzymobeads as previously described (21) (final sp act  $\sim$ 100  $\mu$ Ci/ $\mu$ g). The antihuman EGF receptor antiserum, which was generated against gel-purified EGF receptor, was described previously (1).

### TCA Precipitation Degradation Assay

Aliquots of *Drosophila* extracts, partially purified or 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 (sp act 80–120  $\mu$ Ci/ $\mu$ 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 (5). For <sup>125</sup>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 analysis were carried out as described (5). HPLC analysis indicated that the *Drosophila* IDE degraded insulin at a limited number of cleavage sites similar to those identified for the mammalian IDE (1a).

### Purification of the *Drosophila* Insulin-degrading Enzyme

The IDE was purified by successive chromatography on DEAE cellulose, Sephadex G-200, hydroxylapatite, butyl agarose, and chromatofocusing columns as described (5). This IDE preparation contained a single protein band with molecular mass of 110,000 kD when analyzed by SDS-PAGE. For immunoprecipitation of enzyme activity by the anti-EGF receptor antibody and preparation of the polyclonal anti-*Drosophila* IDE antiserum, the gel filtration and chromatofocusing steps were omitted from the enzyme purification. This latter preparation was used previously to characterize the properties of the *Drosophila* IDE (5).

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

Partially purified *Drosophila* IDE (80–120  $\mu$ g) was loaded to a preparative 6.5% polyacrylamide gel under denaturing conditions as previously described (6). Proteins were visualized by staining with Coomassie Blue. The 110,000-kD protein band was excised, ground, and injected directly in-

traperitoneally into New Zealand rabbits at days 1, 21, 35, 51, and 122. The purity of the gel-purified 110,000-kD protein was confirmed by NH<sub>2</sub>-terminal sequence analysis (5). 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 were separated by PAGE, using 6.5% polyacrylamide gels under reducing conditions (6). The samples were then electrophoretically transferred from the gel to a nitrocellulose paper and the paper was probed with rabbit polyclonal antibodies as described and visualized using a Vectastain ABC Kit (Vector Laboratories, Inc.) (21).

### Immunoprecipitation and Affinity Labeling

Immunoprecipitation was performed with anti-EGF receptor antiserum or anti-*Drosophila* IDE antiserum in 50 mM HEPES buffer pH 7.5 overnight at 4°C followed by addition of protein A-Sepharose and affinity labeled under saturating conditions with <sup>125</sup>I-insulin or <sup>125</sup>I-TGF- $\alpha$  (10 ng) as previously described (6, 21). For immunoprecipitation with the anti-*Drosophila* IDE antiserum, protein samples were incubated in the presence of SDS and at different temperatures before incubation with antiserum.

### Binding to the EGF Receptor

<sup>125</sup>I-EGF was incubated with the IDE as described above and samples removed at various times. Reactions were stopped by adding excess insulin or by two cycles of freeze thawing. Samples were then assayed for binding to the EGF receptor as described (4).

### Two-dimensional Polyacrylamide Gel Electrophoresis

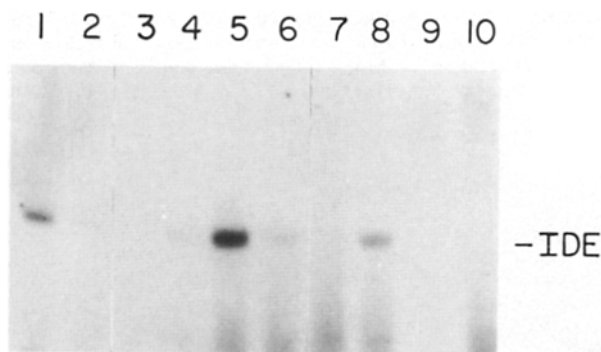
High resolution two-dimensional electrophoresis of protein was performed as described (11). Electrophoresis on a tube gel in an ampholine gradient ranging from pH 3.5–10 was followed by SDS-PAGE on a 7.5% acrylamide slab gel. Protein markers were used to determine the pI of the resulting spots.

## Results

We have previously isolated a protein (dp100) from *Drosophila* Kc cells with the unusual property that it could be affinity labeled with either mammalian <sup>125</sup>I-insulin or <sup>125</sup>I-EGF at physiological concentrations, and in both cases the affinity labeling was inhibited by the addition of an excess of unlabeled insulin or EGF (21). This protein had a molecular mass of  $\sim$ 100–110 kD, and could be immunoprecipitated with an antiserum against the human EGF receptor. The protein was also shown to bind related factors, such as transforming growth factor- $\alpha$  and insulinlike growth factor-II, with a relatively high affinity ( $K_d \sim 10^{-9}$  M) (6). These properties were used as criteria to identify dp100 in studies designed to elucidate its function.

To determine the functional role of dp100, it was necessary to purify the protein. In the course of this investigation, it became apparent that dp100 copurified with an insulin-degrading activity (IDE) that we had identified and purified from *Drosophila* Kc cells (5). The coidentity of these two proteins was illustrated by the fact that the purified IDE could be directly affinity labeled with either <sup>125</sup>I-insulin or <sup>125</sup>I-TGF- $\alpha$  and, in both cases, the labeling was inhibited by the addition of an excess of either insulin or EGF (Fig. 1).

If the two proteins, the IDE and dp100, are in fact the same, they should share antigenic properties. Since the antiserum against the human EGF receptor can also immunoprecipitate dp100, we determined whether this antiserum can selectively



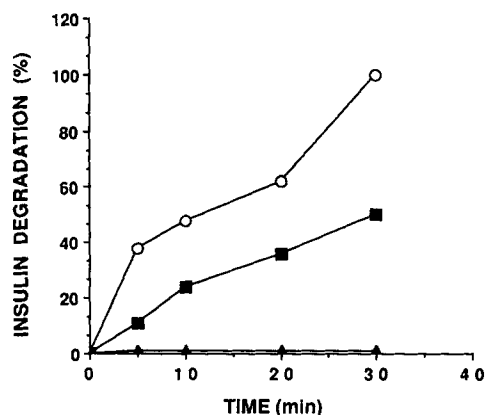
**Figure 1.** Autoradiograph depicting affinity labeling of  $^{125}\text{I}$ -insulin or  $^{125}\text{I}$ -TGF- $\alpha$  to purified IDE. Purified *Drosophila* IDE (0.6  $\mu\text{g}$ ) was affinity labeled with  $^{125}\text{I}$ -insulin (lanes 1-4) or  $^{125}\text{I}$ -TGF- $\alpha$  (lanes 5-8) in the absence (lanes 1, 5) or presence of unlabeled insulin ( $10^{-5}$  M, lanes 2, 6), insulin B chain ( $10^{-4}$  M, lanes 3, 7), or EGF ( $2 \times 10^{-5}$  M, lanes 4, 8), and analyzed as described in Materials and Methods. Labeled  $^{125}\text{I}$ -insulin and  $^{125}\text{I}$ -TGF- $\alpha$  in the absence of IDE are shown in lanes 9 and 10, respectively. The lower bands are nonspecifically labeled BSA which was included in the incubation mixture.

remove the insulin-degrading activity from a partially purified enzyme preparation. As shown in Fig. 2, the anti-EGF receptor antiserum precipitated the insulin-degrading activity while preimmune serum had no effect. Under similar conditions, this antiserum does not immunoprecipitate the *Drosophila* insulin receptor (6). These results suggest that dp100 may have insulin-degrading activity. To explore this possibility further, we also prepared specific antiserum against the purified *Drosophila* IDE.

The 110-kD protein corresponding to the *Drosophila* IDE was excised from polyacrylamide gels and used to prepare rabbit polyclonal antiserum against the IDE. Initial characterization of the resultant antiserum by immunoblotting indicated that the antibody specifically reacted with the IDE in crude cytoplasmic and membrane extracts of Kc cells and with the purified IDE. However, no specific recognition of proteins in the membranes of A431 cells by the anti-IDE an-

**Table I. Similarities between dp100 and the *Drosophila* IDE**

	dp100	IDE
Relative molecular mass (kD)	110	110
Affinity labeling		
Insulin	+	+
EGF	+	+
TGF- $\alpha$	+	+
Immunocross reactivity		
Anti-EGF-receptor antiserum	+	+
Anti- <i>Drosophila</i> -IDE antiserum	+	+
Degradation		
Insulin	+	+
EGF	-	-



**Figure 2.** Immunoprecipitation of insulin-degrading activity with an antihuman EGF receptor antiserum. Partially purified IDE (1  $\mu\text{g}$ ) was incubated with preimmune (8  $\mu\text{l}$ ,  $\circ$ ) or anti-EGF receptor antiserum (4  $\mu\text{l}$ ,  $\blacksquare$ ; 8  $\mu\text{l}$ ,  $\blacktriangle$ ) at  $4^\circ\text{C}$  overnight. The supernatant fractions of the samples were assayed for insulin-degrading activity after removal of the antibodies with protein A as described in Materials and Methods.

tiserum was detected (Fig. 3). A431 cells, which contain large amounts of EGF receptor, were used as a source of EGF receptor to prepare the antihuman EGF receptor antiserum (2, 18, 19) that cross reacts with dp100 (21). Therefore, we conclude that our antiserum is specific for the *Drosophila* IDE and does not cross react with the human or *Drosophila* EGF receptors.

The purity of the 110-kD IDE used for these experiments has previously been established by a number of criteria, including protein sequencing (5). When the purified enzyme preparation was immunoblotted with antiserum against the purified IDE and antiserum against the human EGF receptor, in both cases antigenic reactivity was observed (data not shown), confirming the previous observations. The fact that both antisera were recognizing a single protein was further confirmed by immunoblots of IDE after two-dimensional polyacrylamide gel electrophoresis. When resolved by molecular mass and isoelectric focusing, only a single protein was noted by silver staining at 100-110 kD which reacted strongly with anti-*Drosophila* IDE antiserum and weakly with the antihuman EGF receptor antiserum (Fig. 4). In both cases, no other protein visibly reacted with the antiserum. These results further establish the antigenic cross-reactivity of the *Drosophila* IDE and dp100.

This cross-reactivity was finally illustrated by direct immunoprecipitation studies. Above, we have shown that the antibody that immunoprecipitates dp100 can also remove the insulin-degrading activity. Similarly, we can now demonstrate that antiserum against the *Drosophila* IDE is able to specifically immunoprecipitate purified IDE affinity labeled with TGF- $\alpha$  or insulin (Fig. 5). Nonspecifically labeled proteins, such as BSA in the labeled insulin and TGF- $\alpha$  preparations (Figs. 1 and 6), were not recognized by the anti-IDE antiserum. Unfortunately, the anti-IDE antiserum does not efficiently precipitate the native enzyme; therefore, the addition of a denaturing detergent is required, enabling one to monitor precipitation of the affinity-labeled protein but not the enzymatic activity. In summary, the fact that the purified IDE has the distinctive characteristics of dp100 and

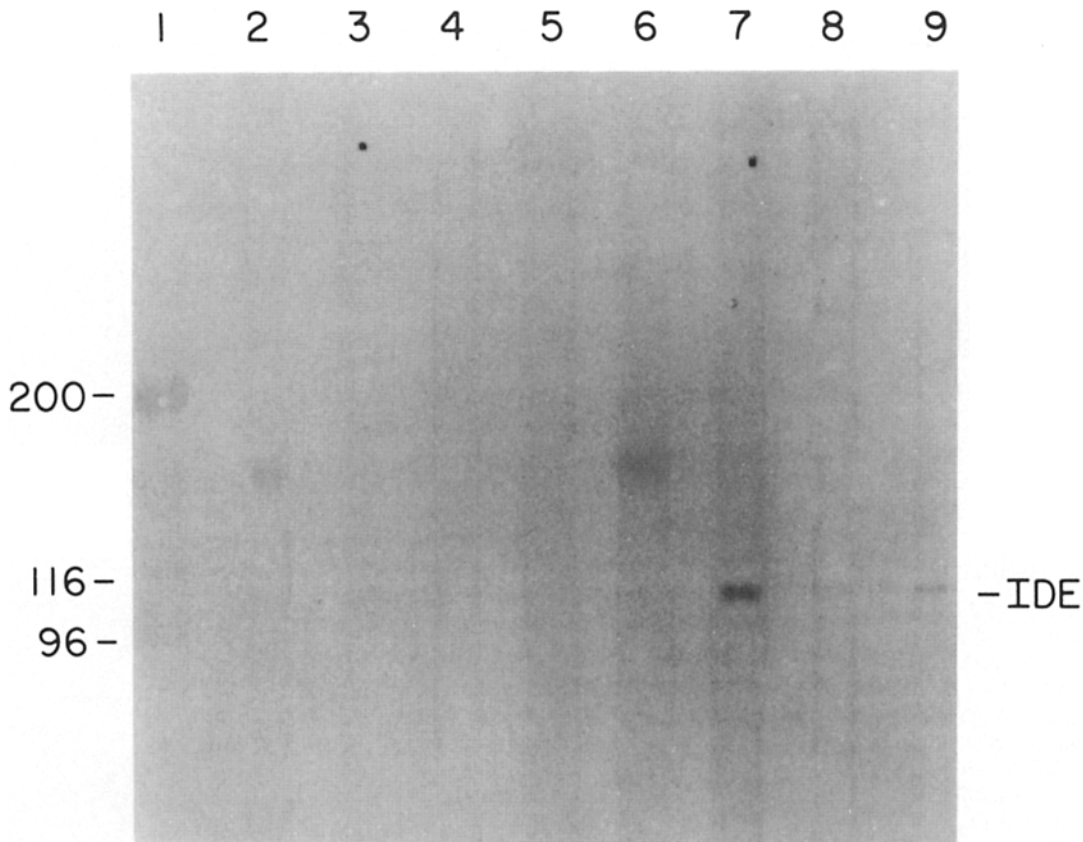


Figure 3. Immunoblot illustrating specificity of antiserum against the *Drosophila* IDE. Samples (1–30  $\mu\text{g}$ ) from purified *Drosophila* IDE (lanes 3, 7), *Drosophila* Kc cell membranes, (lanes 4, 8), and Kc cytoplasm (lanes 5, 9), as well as human A431 cell membranes (lanes 2, 6), were resolved by SDS-PAGE, transferred to nitrocellulose, and blotted with either preimmune serum (lanes 2–5) or anti-IDE antiserum (lanes 6–9) as described in Materials and Methods. Molecular mass markers ( $\times 10^{-3}$ ) are shown in lane 1.

shares physical and antigenic properties argues that the two proteins are, in fact, the same.

Given that the *Drosophila* IDE can bind to EGF, the question arises regarding the physiological reason for this interac-

tion. Therefore, we determined whether EGF might be a substrate for the enzymatic-degrading activity. Two assays were used to assess the state of the growth factors: (a) the TCA precipitation assay which monitors release of small

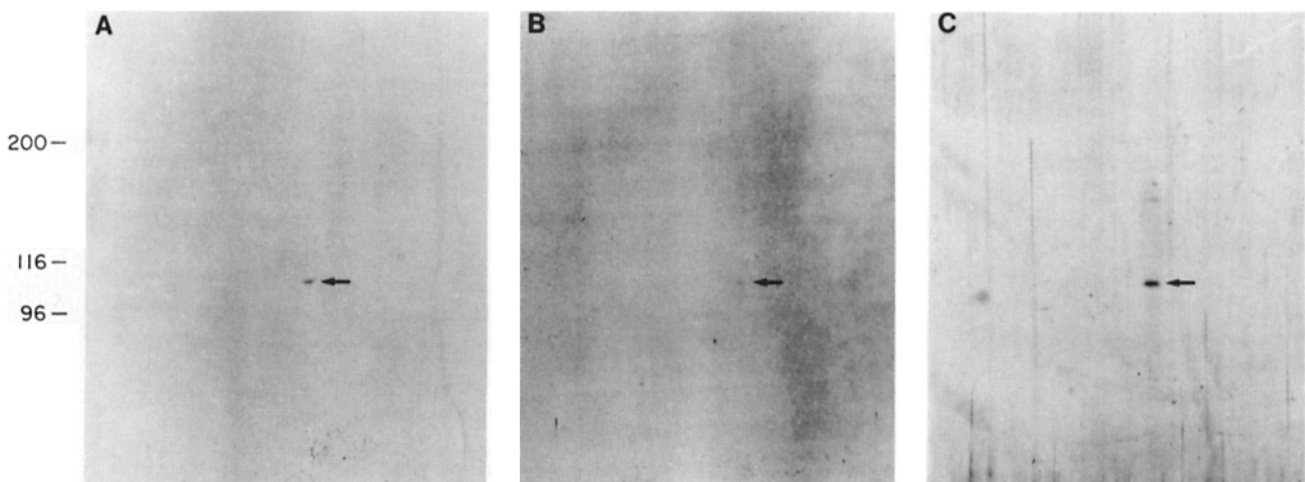
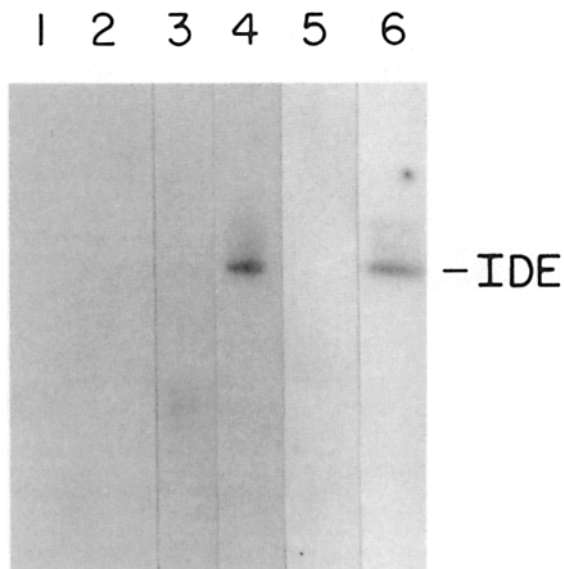


Figure 4. Immunoblot illustrating comigration of the *Drosophila* IDE and dp100 after two-dimensional polyacrylamide gel electrophoresis. Partially purified IDE (5  $\mu\text{g}$ ) was resolved by isoelectric focusing and SDS polyacrylamide gel electrophoresis as described. (A) Immunoblot of IDE reacted with anti-*Drosophila* IDE antiserum; (B) immunoblot of IDE reacted with antihuman EGF receptor antiserum; (C) gel stained with Pierce Gelcode color silver stain. Molecular mass markers ( $\times 10^{-3}$ ) are indicated. The position of the IDE is indicated with an arrow.



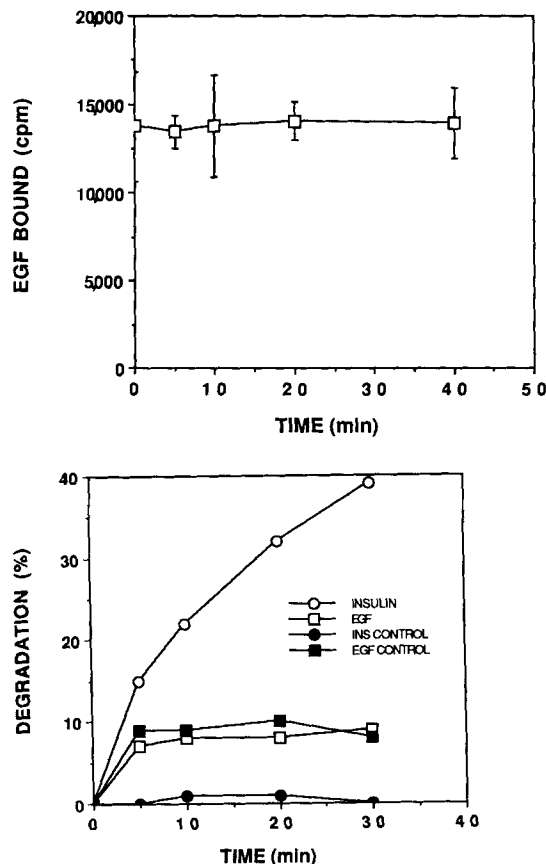
**Figure 5.** Immunoprecipitation of the IDE affinity labeled with insulin and TGF-alpha. Lanes 1-4, 25  $\mu$ l of  $^{125}$ I-insulin-labeled IDE were untreated (lanes 1, 3) or heated for 5 min at 100°C in 0.5% SDS. Samples were then diluted to 0.1% SDS in PBS and immunoprecipitated with 5  $\mu$ l preimmune (lanes 1, 2) or 5  $\mu$ l anti-IDE antiserum (lanes 3, 4). Lanes 5, 6, 20  $\mu$ l of  $^{125}$ I-TGF-alpha-labeled IDE were heated at 100°C for 5 min in 0.5% SDS (lane 5) or 1% SDS (lane 6), diluted to 0.1% SDS with PBS, and then immunoprecipitated with 5  $\mu$ l preimmune (lane 5) or 5  $\mu$ l anti-IDE antiserum (lane 6).

fragments into the non-TCA-precipitable fraction and (b) a receptor-binding assay which measures the remaining fraction of intact growth factor by the ability to bind to the EGF receptor. As demonstrated with insulin, the latter assay is more sensitive to limited cleavage (15). In contrast to the case with insulin, the IDE was unable to degrade EGF (Fig. 6).

Since EGF does not appear to be a substrate for the enzyme, we determined whether it can act as an inhibitor of insulin degradation. EGF and insulin were preincubated with IDE for 30 min at 4°C before addition of labeled insulin. As shown in Fig. 7, both EGF and insulin significantly inhibited degradation of insulin at a concentration of  $5 \times 10^{-6}$  M. Further kinetic analysis of the inhibition by EGF suggests that EGF might act as a competitive inhibitor (data not shown) indicative of a single site for both the substrate and inhibitor (17). These results clearly demonstrate that EGF is able to directly interact with a highly conserved *Drosophila* enzyme and block its ability to degrade mammalian insulin.

## Discussion

The results we have presented here show that the insulin EGF binding protein (dp100) previously characterized by our laboratory is an insulin-degrading enzyme. This identity was established by a number of criteria: First, the insulin EGF binding protein copurified with the insulin-degrading enzyme from Kc cells, and the purified enzyme exhibited binding properties characteristic of dp100. Thus, the purified IDE had the same molecular weight and isoelectric point, could



**Figure 6.** Lack of EGF degradation by the *Drosophila* IDE. (Top) EGF binding assay. Samples containing purified IDE (0.4  $\mu$ g) were incubated with  $^{125}$ I-EGF ( $10^{-9}$  M) for the indicated time periods, and then assayed for binding to the EGF receptor as described. Nonspecific binding (209 cpm), determined by adding  $1.5 \times 10^{-7}$  M unlabeled EGF, was subtracted from the final values. (Bottom) TCA precipitation assay. Samples containing purified IDE (0.4  $\mu$ g) were incubated with either  $^{125}$ I-insulin ( $10^{-9}$  M,  $\circ$ ,  $\bullet$ ) or  $^{125}$ I-EGF ( $10^{-9}$  M,  $\square$ ,  $\blacksquare$ ) for the indicated time periods in either the absence ( $\circ$ ,  $\square$ ) or presence ( $\bullet$ ,  $\blacksquare$ ) of  $5 \times 10^{-6}$  M unlabeled insulin.

be affinity labeled with both EGF and TGF-alpha as well as insulin, and the latter could be inhibited with both EGF and insulin-related factors. Second, the antigenic properties of the two proteins were indistinguishable. The enzymatic activity could be selectively removed by immunoprecipitation with the anti-EGF receptor antibody that recognized dp100. Conversely, antisera against the purified IDE could immunoprecipitate the 100-kD protein affinity labeled with TGF-alpha or insulin. Two-dimensional polyacrylamide gel electrophoresis confirmed that the purified enzyme corresponded to a single band that could be recognized by either antiserum. Finally, the kinetic properties of the IDE reflected the ability of the enzyme to bind EGF-related factors. Thus, although the *Drosophila* enzyme did not degrade EGF, EGF was able to inhibit degradation of insulin. In sum, on the basis of physical, antigenic, and functional criteria, the *Drosophila* IDE is also an EGF binding protein.

One surprising feature of this finding is that, although the IDE is able to recognize EGF, there is, to date, no homologue of EGF that has been identified in *Drosophila*. However, there is evidence to suggest that related factors may exist. For

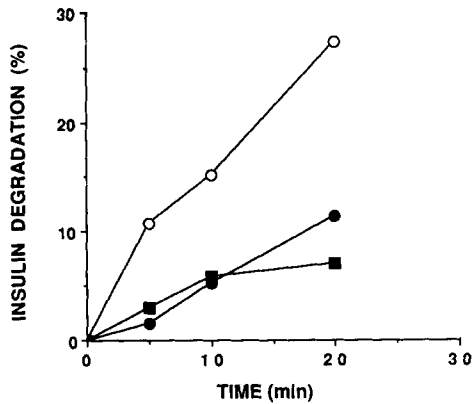


Figure 7. Inhibition of *Drosophila* IDE insulin degradation by insulin and EGF. Samples containing purified IDE (0.2  $\mu$ g) were assayed for  $^{125}$ I-insulin degradation by the TCA precipitation assay in the absence (○) or presence of  $5 \times 10^{-6}$  M unlabeled insulin (●) or EGF (■) for the indicated time periods.

example, the gene coding for the Notch mutation has been sequenced and shown to code for cysteine-rich protein with similar spacing of cysteine as in EGF (22). A similar type of gene has also been located in nematodes (denoted lin). The fact that the IDE is able to bind mammalian EGF may provide further evidence for EGF-like factors in *Drosophila*.

Having established this identity, it is of interest to determine the physiological impact of this relationship between the two growth factor families. Although receptors are not entirely specific, there is no documented case of an insulin receptor responding to EGF, or vice versa. Yet the levels of ligands, as determined in part by the degradation pathway, might influence the extent of receptor-mediated signaling. Thus, the IDE provides one mechanism whereby EGF can influence the extent of insulin degradation. The fact that growth-related factors can regulate the action of the IDE may provide an alternative regulatory mechanism and is consistent with our recent observation that the IDE is developmentally regulated, being present only at low levels in the rapidly growing embryonal stage (20). In the *Drosophila* system, there is no evidence that the IDE can directly degrade mammalian EGF. However, since this is a heterologous system, it is possible that there is an EGF homologue in *Drosophila* that could be a substrate for the enzyme.

One of the most striking features of this work is the high conservation of the IDE between mammals and *Drosophila*. Although we detected the EGF binding to the *Drosophila* IDE, we have now demonstrated that EGF, while not degraded at physiological concentrations, can inhibit insulin degradation by the rat liver IDE in a similar manner (Garcia, J. V., and M. R. Rosner, manuscript submitted for publication). Thus, the existence of a protein that can mediate binding of insulin and EGF at possibly a single site in both mammals and *Drosophila* suggests that this property is an important one in the function of the enzyme and the role it plays in maintaining growth factor levels.

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