Characterization of a *Drosophila* Protein that Binds Both Epidermal Growth Factor and Insulin-related Growth Factors

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**Abstract.** The identification of a novel protein from *Drosophila melanogaster* that binds both mammalian epidermal growth factor (EGF) and insulin has been reported (Thompson, K. L., S. J. Decker, and M. R. Rosner, 1985, Proc. Natl. Acad. Sci. USA., 82:8443–8447). This 100-kD protein (designated dp100) is also recognized by an antiserum against the human EGF receptor. To further characterize the properties of this protein, we have determined the binding spectrum, glycosylation state, and cellular distribution of dp100. Our results indicate that dp100 binds to other insulin-like and EGF-like growth factors with dissociation constants ranging from $10^{-6}$ to $10^{-8}$ M, and these ligands compete with each other for binding to dp100.

All other ligands tested, including platelet-derived growth factor, transforming growth factor-beta, nerve growth factor, and glucagon, either did not bind or bound with a $K_d > 10^{-6}$ M. Unlike the *Drosophila* insulin receptor, dp100 does not bind to wheat germ agglutinin and is present in a cytoplasmic as well as a membrane-bound form that cannot be differentiated by two-dimensional PAGE. Further, dp100 is the sole transforming growth factor-alpha–binding protein detected by affinity labeling in *Drosophila* Kc cells. These results indicate that dp100 shares properties in common with, but distinct from, the *Drosophila* homologues of the insulin and EGF receptors.

**Identification** and characterization of homologues of mammalian growth regulatory proteins in lower organisms is an approach that can potentially yield new and relevant insights into the mechanism of action of these proteins. In vertebrates, growth factors that are members of the insulin and epidermal growth factor (EGF) families are integrally involved in the stimulation of mitogenesis and control of cellular metabolism (20). Further understanding of their biochemical interactions could benefit from identification and investigation of proteins that bind these factors in genetically characterized lower organisms.

In the interests of developing this approach, we screened *Drosophila melanogaster* cell lines for proteins related to the EGF receptor using a polyclonal antiserum that recognizes EGF receptors from a variety of species (2) but does not cross react with the mammalian or the *Drosophila* insulin receptor (24). In the course of this investigation, we identified in membrane fractions a 190-kD protein that corresponds to the *Drosophila* EGF receptor homologue and a growth factor–binding protein for both insulin and EGF of $\sim 100$ kD (24). This latter protein (designated dp100) is the subject of the present paper.

1. *Abbreviations used in this paper:* dp100, 100-kD *Drosophila* protein; EGF, epidermal growth factor; IGF, insulin-like growth factor; NGF, nerve growth factor; PDGF, platelet-derived growth factor; PTH, parathyroid hormone; WGA, wheat germ agglutinin.

On the basis of the recognition by anti–EGF receptor antiserum and the affinity labeling with insulin and EGF, we proposed that dp100 was a novel receptor (24) distinct from the *Drosophila* homologues of the EGF (13, 21) and insulin receptors (17). To explore this possibility further, we characterized the binding spectrum, glycosylation state, and cellular distribution of dp100. The results indicate that (a) dp100 specifically binds to all intact insulin and EGF-related growth factors tested, which compete with one another for binding; (b) dp100 does not appear to be $N$-glycosylated and is located primarily in the cytoplasm of the cell; and (c) dp100 is the only transforming growth factor-alpha (TGF-alpha)–binding protein detected by affinity labeling in *Drosophila* Kc cells. This new evidence suggests that dp100 is not a receptor, but a discrete binding protein for insulin and EGF-related factors.

**Materials and Methods**

**Materials.** *Drosophila* Kc cells, from the Massachusetts Institute of Technology Cell Culture Center, were grown in D22 media. Human epidermoid carcinoma A431 cells were obtained from the American Type Culture Collection, Rockville, MD. Anti–EGF receptor antiserum was prepared against denatured EGF-R from A431 cells (2). Growth factors were obtained from the following sources: marine EGF (receptor grade and HPLC purified) and porcine insulin (Biomedical Technologies, Inc., Cambridge, MA), $^{125}$I-TGF-alpha and $^{125}$I-TGF-beta (J. Massague), IGF-I (Amgen), IGF-II (M. Czech), synthetic human TGF-alpha (J. Tam), nerve growth factor (NGF)
The Drosophila insulin receptor (17).

Having identified other EGF-like and insulin-like factors that can be cross-linked to dp100, we determined the relative binding affinities of these and other hormones by competitive affinity-labeling experiments. For these experiments, solubilized Kc cell membranes were immunoprecipitated with

Figure 1. Affinity labeling of dp100 with iodinated insulin-like and EGF-like growth factors. Solubilized Drosophila membrane proteins were immunoprecipitated with anti-EGF receptor antisera and incubated with 125I-labeled growth factors (>10⁻⁸ M) in the absence (lanes 1, 3, 5, 7, 9, and 11) or presence of excess (2.5-10 μM) insulin (lanes 4, 8, 10, and 12) or EGF (lanes 2 and 6). Samples were labeled with insulin (lanes 1 and 2), IGF-I (lanes 3 and 4), EGF (lanes 5 and 6), TGF-alpha (lanes 7 and 8), PDGF (lanes 9 and 10), and TGF-beta (lanes 11 and 12). Proteins were cross-linked and analyzed by autoradiography as described in Materials and Methods. The lower band that is not competed by unlabeled insulin is BSA.

Results

In previous work, a 100-kD protein from Drosophila that displayed dual binding specificity for both insulin and EGF was identified (24). This protein (dp100) was initially detected by immunoprecipitation of detergent-solubilized membranes with an antiserum prepared against the human EGF receptor (2) and subsequent cross-linking with 125I-labeled insulin or EGF. dp100 was found in membrane fractions from two different Drosophila cell lines. An excess of either unlabeled insulin or EGF could block binding of both 125I-labeled insulin or EGF to dp100. To further characterize the properties of this protein, we determined the binding specificity, glycosylation state, and cellular distribution of dp100.

To test whether the binding specificity of dp100 extended to other growth factors structurally similar to insulin and EGF (9, 14, 19), we performed immunoprecipitation and affinity labeling with the 125I-labeled ligands (see Fig. 1). The results indicate that dp100 could be affinity labeled with all the insulin-like and EGF-like growth factors tested (TGF-alpha, EGF, insulin, and IGF-I), and that radiolabeled growth factor binding could be inhibited in all cases by addition of excess unlabeled insulin or the specific ligand (Fig. 1). However, neither 125I-TGF-beta nor 125I-PDGF, two structurally unrelated growth factors (3, 4, 27), could be cross-linked to dp100 or any other immunoprecipitated Drosophila protein under the conditions of our experiment. Controls with preimmune serum failed to immunoprecipitate dp100 or any other growth factor–binding protein (data not shown).

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Figure 2. Affinity labeling of dpl00 with ^125I-insulin in the presence of unlabeled growth factors. Drosophila membrane immunoprecipitates were cross-linked with labeled insulin as described in Fig. 1 in the presence of (A) TGF-alpha (lane 1, 1.2 μM; lanes 2 and 3, 0.12 μM; lanes 4 and 5, 0.012 μM; lanes 6 and 7, 1.2 nM; lanes 8 and 9, 0.12 nM; lanes 10 and 11, no addition), or (B) insulin (lane 1, 55 μM; lane 2, 5.5 μM; lane 3, 0.55 μM; lane 4, 0.055 μM; lane 5, 5.5 nM; lane 6, no addition), or EGF (lane 7, 1 μM; lane 8, 0.1 μM; lane 9, 0.01 μM; lane 10, 1 nM; lane 11, 0.1 nM; lane 12, no addition).

Anti-EGF receptor antibody, and the immunoprecipitate was affinity labeled with ^125I-insulin in the presence of different concentrations of unlabeled ligand. The samples were then cross-linked before gel electrophoresis and autoradiography. Autoradiograms illustrating the competition by unlabeled TGF-alpha, insulin, and EGF are shown in Fig. 2. The competitive binding curves (Fig. 3), derived from densitometric scanning of the autoradiograms, were used to calculate the relative binding affinities (Table I). Synthetic human-transforming growth factor-alpha (TGF-alpha) and insulin-like growth factor-II (IGF-II) bound with the highest affinities (K_d of 5 × 10^{-9} M and 1.3 × 10^{-8} M, respectively). EGF, insulin, and insulin-like growth factor-I (IGF-I) bound with dissociation constants ranging from 10^{-6} to 10^{-7} M (Table I). Nerve growth factor (NGF) and glucagon (Fig. 3) as well as pituitary thyroid hormone (PTH) (data not shown) showed some ability to compete but the dissociation constants were higher than 10^{-6} M. No significant binding was detected to the individual A and B chains of insulin at concentrations up to 10^{-3} M (data not shown), similar to results obtained for the mammalian insulin receptor (5). The amino-terminal fragment of urokinase, which binds to the urokinase receptor with an affinity comparable to that of the native enzyme and has cysteine residues spaced similarly to those in EGF (23), did not compete with ^125I-insulin at concentrations up to 3 × 10^{-5} M (data not shown). It should be noted that all the ligands tested were from mammalian sources and thus are not the endogenous ligands for dpl00. The affinity of dpl00 for TGF-alpha and IGF-II is as high as that reported for the binding of porcine insulin to the Drosophila insulin receptor.
Figure 3. Competitive binding of labeled insulin to dp100 in the presence of insulin-like and EGF-like growth factors. Drosophila membrane immunoprecipitates were cross-linked with labeled insulin as described in Fig. 1 in the presence of unlabeled growth factors. Autoradiograms (see Fig. 2 and Materials and Methods) were scanned and the relative amount of labeled dp100 quantitated. Insulin binding is expressed as percent of controls containing no competitor ligand (100%).

(17). These results indicate that dp100 binds to mammalian insulin-like and EGF-like growth factors with a high affinity and specificity for a heterologous system.

dp100 bound to synthetic human TGF-alpha with an affinity three orders of magnitude higher than that for EGF and has the unique ability to bind and differentiate between these two ligands. To ensure that the difference in the binding affinities of EGF and TGF-alpha for dp100 reflected a property of the protein rather than the particular growth factor preparations, we determined the ability of unlabeled EGF and TGF-alpha to compete with 125I-labeled EGF for binding to the EGF receptor in human epithelial carcinoma A431 cells. As shown in Fig. 4, there was no significant difference in the affinities of the EGF and TGF-alpha preparations for the mammalian EGF receptor. The data presented here suggests that dp100 recognizes EGF and TGF-alpha in a different way than the EGF receptor. The fact that dp100 and the mammalian EGF receptor are both able to bind to EGF and TGF-alpha suggests that there might be some common recognition sites in their binding domains. However, specific recognition sites of dp100 and the EGF receptor must differ because only dp100 can differentiate between the two growth factors.

Since the mammalian receptors for EGF and insulin are glycosylated (10, 22), we determined whether the Drosophila dp100 might also contain asparagine-linked oligosaccharides. To test for the presence of oligosaccharides, membrane fractions from Kc cells were applied to a WGA-Sepharose column. This latter column, which binds to N-acetylglucosamine residues, has been shown to retain both the mammalian and Drosophila insulin receptors (10, 17). After application of Triton-solubilized Kc cell membrane fractions to the WGA-Sepharose column, the flowthru was collected, the column washed, and the retained proteins specifically eluted with N-acetylglucosamine. To identify insulin-binding proteins, samples from the flowthru and the eluant were either immunoprecipitated and labeled, or labeled directly, with 125I-insulin. Labeled proteins were then visualized by autoradiography after SDS-PAGE. As shown in Fig. 5, samples that bound insulin and were in the molecular mass range of 100–130 kD were detected in both the flowthru and eluted fractions. However, when dp100 was selectively visualized by prior precipitation with the anti-EGF receptor antiserum and affinity labeling with 125I-insulin, dp100 was only detected in samples from the flowthru. Direct cross-linking with 125I-TGF-alpha was more specific and only labeled proteins in the flowthru, but not the eluate, of the WGA-Sepharose column (Fig. 5). A similar elution pattern for dp100 was obtained when a lentil lectin column, which recognizes mannose and glucose residues, was used (data not shown). Similarly, treatment of dp100 with endoglycosidase H, which cleaves N-asparagine–linked high mannose oligosaccharides, did not result in an electrophoretically detectable loss of oligosaccharide chains under conditions that caused a shift in the electrophoretic mobility of ovalbumin (data not shown). These results indicate that dp100 can be separated

Table I. Dissociation Constants for Ligand Binding to dp100

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<tr>
<th>Ligand</th>
<th>$K_d$ (n)*</th>
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<tbody>
<tr>
<td>TGF-alpha</td>
<td>$5 \times 10^{-4}$ (4)</td>
</tr>
<tr>
<td>EGF</td>
<td>$1.3 \times 10^{-4}$ (4)</td>
</tr>
<tr>
<td>Insulin</td>
<td>$1.1 \times 10^{-4}$ (4)</td>
</tr>
<tr>
<td>IGF-I</td>
<td>$5 \times 10^{-3}$ (2)</td>
</tr>
<tr>
<td>IGF-II</td>
<td>$1.3 \times 10^{-4}$ (2)</td>
</tr>
<tr>
<td>Glucagon</td>
<td>$&gt;10^6$</td>
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<tr>
<td>NGF</td>
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<tr>
<td>PTH</td>
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<td>Insulin A chain</td>
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* (n) = number of determinations.
from the insulin receptor by lectin chromatography and suggest that membrane-associated dp100 is not glycosylated.

The previous studies were conducted with membrane fractions, consistent with a putative role for dp100 as a growth factor receptor. However, to gain more perspective on the nature of the protein, we examined the cellular distribution of dp100. Initially, dp100 was identified by immunoprecipitation with the anti-EGF receptor antiserum and subsequent cross-linking with $^{125}$I-labeled insulin in the presence or absence of unlabeled insulin or EGF. When cytoplasmic and membrane fractions were analyzed by this method, dp100 was detected in both fractions (Fig. 6A) and, in fact, was found predominantly in the cytoplasmic fraction when saturating levels of the antiserum were used (Fig. 6B). Despite extensive washing with PBS, a fraction of the dp100 (~5%) remained membrane associated (data not shown). When intact Kc cells were assayed for binding or cross-linking to radiolabeled insulin or TGF-alpha, minimal binding was detected under conditions where equivalent amounts of the isolated Kc membranes were extensively labeled (data not shown). These results are consistent with the majority of the dp100 being on the inside of the cell in both membrane-associated and cytoplasmic forms. Thus dp100 is not a direct counterpart of the mammalian growth factor receptors that are located in membrane-associated fractions and have their binding domains exposed on the extracellular surface.

To determine whether the cytoplasmic and membrane forms of dp100 differed in any significant respect, several criteria were used. The cytoplasmic dp100 was identified on the basis of molecular weight, recognition by the anti-EGF...
Figure 7. Autoradiograph depicting two-dimensional PAGE of $^{125}$I-insulin-labeled dpl00. Kc cell cytoplasmic fractions (A and B) or membrane fractions (C and D) were immunoprecipitated with anti-EGF receptor antibody and the immunoprecipitates affinity labeled with $^{125}$I-insulin in the presence (B and D) or absence (A and C) of unlabeled insulin. The arrows indicate the spots corresponding to affinity-labeled dpl00.

receptor antiserum, and ability to bind to insulin, EGF, and TGF-alpha. To further test the identity of the cytoplasmic and membrane-associated forms of dpl00, we compared their chromatographic mobility after two-dimensional gel electrophoresis (Fig. 7). Cytoplasmic and membrane extracts of Kc cells were immunoprecipitated with anti-EGF receptor antibody and then cross-linked with $^{125}$I-labeled insulin before separation by isoelectric focusing and SDS-PAGE. The results show identical profiles for both cytoplasmic and membrane samples. The fact that two proteins corresponding to dpl00 that differ in $pI$ ($pI_1 \sim 6; pI_2 \sim 7$) are observed may reflect two different proteins, two forms of the same protein, or may be an artifact of the ligand-cross-linking process. These results indicate that the cytoplasmic and membrane-associated forms of dpl00 are similar, if not identical.

Since dpl00 appeared to be most specific for synthetic TGF-alpha, we determined whether there are any other TGF-alpha-binding proteins in either the cytoplasmic or membrane-associated fractions. When samples that had been directly affinity labeled with $^{125}$I-TGF alpha were compared with samples that had been immunoprecipitated with the anti-EGF receptor antibody before affinity labeling, no dif-
Figure 8. Affinity labeling of cytoplasmic proteins with [125]I-TGF-alpha. Cytoplasmic proteins were labeled with TGF-alpha directly (lanes 4–6) or after immunoprecipitation with anti-EGF receptor antisera (lanes 1–3). Samples were labeled in presence or absence of excess insulin or EGF. After cross-linking, samples were analyzed by electrophoresis followed by autoradiography as described in Materials and Methods. The positions of molecular mass markers in kilodaltons are indicated.

ference was detected (see Fig. 5 for membrane fractions, Fig. 8 for cytoplasmic fractions). These results indicate that, within the limits of our detection system, dpl00 is the only TGF-alpha-binding protein in Drosophila Kc cells and, unlike insulin, TGF-alpha can be used as a specific probe for the protein.

Discussion

Previous studies have identified a 100-kD protein in Drosophila (dpl00) that is recognized by an anti-EGF receptor antisera and binds both mammalian insulin and EGF. We have now demonstrated that dpl00 recognizes not only insulin and EGF but also insulin-like and EGF-like growth factors with dissociation constants ranging from $10^{-6}$ to $10^{-9}$ M. In contrast, hormones and growth factors that are not members of these families do not bind or bind with dissociation constants that are $>10^{-6}$ M. Further, the insulin and EGF-like factors compete with one another for binding to dpl00. These results indicate that insulin-like and EGF-like growth factors can bind with high specificity to a single protein.

Although mammalian ligands were used to probe the binding specificity of a Drosophila protein, we were able to measure dissociation constants that fall within the mammalian physiological range. The binding affinity of IGF-II for dpl00 is comparable to that reported for the binding of mammalian insulin to the Drosophila insulin receptor (17). However, we cannot rule out the possibility that a corresponding Drosophila ligand may bind with higher affinity. Although no Drosophila homologues of mammalian EGF or TGF-alpha have been identified to date, an insulin-like molecule has been found in Drosophila (12, 15). Other potential Drosophila counterparts of mammalian growth factor receptors are the decapentaplegic gene (7), which is homologous to TGF-beta, and the Notch gene (8, 11, 26), which has cysteine repeats similar to those in EGF. It is also possible that other hormones, either of mammalian or Drosophila origin that are not members of the insulin and EGF families, might also be found to bind dpl00.

The results presented here clearly establish that the protein recognized by the anti-EGF receptor antisera, dpl00, is not the alpha subunit of the Drosophila insulin receptor. Like the mammalian counterpart, the adult Drosophila insulin receptor is composed of two insulin binding alpha subunits of 130 kD and two beta subunits of 90 kD that contain the tyrosine kinase domain (17). An embryonal insulin binding subunit of 110 kD has also been reported (18). Both forms of the insulin receptor alpha subunit have been shown to bind to WGA. As illustrated in Fig. 5, we have demonstrated that Drosophila Kc cells also contain an insulin-binding 110-kD protein that is retained by WGA. However, only the insulin-binding proteins that do not bind to the lectin column are cross-reactive with the anti-EGF receptor antisera. This observation, and the fact that the mammalian insulin receptor is exclusively membrane bound whereas dpl00 is also present in the cytoplasm, argues that they are distinct proteins.

dpl00 can be differentiated from the Drosophila EGF receptor homologue on the basis of molecular mass and binding spectrum. Using the same anti-EGF receptor antisera, we have identified a 190-kD protein from Drosophila cells (24) that corresponds to the expected size of the Drosophila c-erbB product (21). Despite the antigenic relationship to the mammalian EGF receptor, the 190-kD protein could not be detected by affinity labeling with either labeled EGF or TGF-alpha. Since no endogenous ligand corresponding to EGF or TGF-alpha have yet been identified in Drosophila, it is possible that this growth factor is less conserved evolutionarily than insulin. However, mammalian EGF is specifically bound by one Drosophila protein, dpl00, which is also the only binding protein for TGF-alpha that we can detect in these cells.

The fact that dpl00 recognizes both insulin-like and EGF-like growth factor families, and that the conserved sequences among the growth factors that bind to dpl00 do include regions of the receptor-binding domains, is consistent with the possibility that the binding domains of dpl00 are evolutionarily related to those of the respective receptors. The primary structures of the human insulin receptor and of both human and Drosophila EGF receptors have been determined (13, 25). Comparison of their extracellular binding domains
shows similarities in the way cysteine residues are clustered along the protein molecule. By contrast, the cysteine residues in the binding domain of the PDGF receptor (28) are less clustered and resemble those for colony stimulating factor-1 receptor. Thus, the receptors for EGF-like and insulin-like growth factors may be more closely related to each other than to other peptide growth factor receptors.

Receptors are not the only proteins that bind these growth factor families. Hormone carrier proteins and hormone modifying enzymes have been well characterized in the literature. The results presented here demonstrate that dp100 has certain properties that distinguish it from mammalian growth factor receptors. Further, no cross-reactivity with other preparations of anti-EGF receptor antisera have been observed, suggesting that the recognition may be limited to one particular anti-EGF receptor antisera (data not shown). Recent evidence suggests that dp100 copurifies with an insulin degrading activity (Garcia, J. V., M. P. Stoppelli, S. J. Decker, and M. R. Rosner, manuscript in preparation) and therefore may have a metabolic function. The possible physiological role in Drosophila of the different insulin and EGF-related growth factors that bind dp100 is an intriguing question that remains to be answered.

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