Developmental regulation of an insulin-degrading enzyme from *Drosophila melanogaster*

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ABSTRACT The precise mechanism by which insulin is degraded in mammalian cells is not presently known. Several lines of evidence suggest that degradation is initiated by a specific nonlysosomal insulin-degrading enzyme (IDE). The potential importance of this insulin protease is illustrated by the fact that there is an IDE in *Drosophila melanogaster* Kc cells that shares both physical and kinetic properties with its mammalian counterpart. We now demonstrate that the IDE is present in other *Drosophila* cell lines and in the embryo, the larvae, the pupae, and adult tissues of the fruit fly. Further, the level of the IDE is developmentally regulated, being barely detectable in the embryo but elevated ≈5-fold in the larvae and pupae and ≈10-fold in the adult fly. The IDE levels in the cell lines are particularly high, at least 10-fold greater than in the adult fly. Analysis of Schneider L3 cells indicates that the addition of the *Drosophila* hormone ecdysone, which induces differentiation of the cells, causes a small but reproducible increase in the level of the IDE and the insulin-degrading activity. These results demonstrate that the IDE is evolutionarily conserved and that its expression is tightly regulated during differentiation of *Drosophila*. The particular pattern of developmental regulation suggests that the IDE plays a specific and critical role in the later stages of the life cycle of the fly.

Although insulin is one of the best characterized effectors of cell growth and metabolism, many aspects of its action need to be clarified. In particular, the questions of how insulin is degraded in the cell and of whether degradation of insulin plays a critical regulatory role in insulin action have not yet been resolved. Although lysosomal degradation may account in part for insulin breakdown (1, 2), a nonlysosomal insulin-degrading enzyme [IDE, formerly termed dp100 (3)] has been characterized that cleaves insulin at sites corresponding to the physiological degradation sites of insulin (4, 5) and appears to influence cellular insulin degradation *in vivo* (6). Identification and characterization of a nonmammalian homologue of the IDE would provide valuable evidence for establishing the importance and functional role of this enzyme in cell growth and metabolism.

The insulin system has been highly conserved during evolution. Insulin-like molecules have been isolated in various invertebrates such as *Drosophila* and are active in bioassays developed for the mammalian systems (7, 8). Homologues for the insulin receptor have also been identified in *Drosophila* (9, 10). We have purified and characterized a nonlysosomal IDE from a *Drosophila* cell line that has both physical and kinetic properties that are strikingly similar to those of its mammalian counterpart (11).

The IDE has a number of interesting properties that suggest it might play an important role in growth and development. In addition to recognizing insulin and insulin-like growth factors, the *Drosophila* and mammalian enzymes

bind transforming growth factor type α (TGF-α) with relatively high affinity (refs. 3 and 12 and unpublished data). Since *Drosophila* is a powerful system for studying differentiation, we determined the expression of the IDE in various *Drosophila* cell lines and during different stages of *Drosophila* development. At least three criteria for assessment of IDE levels were used: affinity labeling of the enzyme, assay of insulin-degrading activity, and immunoblotting with antiserum against the purified *Drosophila* IDE. The results indicate that the IDE is widely distributed in *Drosophila*, and the specific developmental profile favors an active role for the IDE in the more differentiated stages of the organism's life cycle.

METHODS

Materials and Cells. The *Drosophila* Schneider line 3 cells, obtained from M. Pardue (Massachusetts Institute of Technology, Cambridge, MA), were grown in a gassed (5.5% CO_2/94.5% air) humidified atmosphere at 25°C in revised Schneider medium (GIBCO) containing 10% (vol/vol) heat-inactivated fetal bovine serum. The *Drosophila* Kc cells, obtained from the Massachusetts Institute of Technology Cell Culture Center, were grown at 25°C in D22 medium (17) supplemented with yeast hydrolysate. The *Drosophila* flies were grown at 25°C. Insulin and epidermal growth factor (EGF) were purchased from Biomedical Technologies (Stoughton, MA). The monoiodinated ¹²⁵I-labeled insulin used for degradation assays was purchased from New England Nuclear. The recombinant TGF-α was a gift from R. Derynck (Genentech, South San Francisco). Enzymobeads were from Bio-Rad, and ecdysterone was from Behring Diagnostics (San Diego, CA). Insulin and TGF-α were iodinated by using Enzymobeads as described (3) (final specific activity, 100 μCi/mg; 1 Ci = 37 GBq).

Immunoprecipitation and Affinity Labeling. Immunoprecipitation was performed with a 1:10 dilution of the anti-EGF receptor antiserum in 50 mM Hepes (pH 7.5) overnight at 4°C, and affinity labeling under saturating conditions with either ¹²⁵I-labeled insulin or ¹²⁵I-labeled TGF-α (10 ng) was as described (3, 12).

Ecdysone-Induced Differentiation of Schneider L3 Cells. Growing Schneider cells were diluted to 3 × 10⁶ cells per ml and allowed to grow for 1 day before ecdysterone was added to a final concentration of 1 μM. The number, viability, and morphology of the cells in culture were monitored daily.

Preparation of Cell Extracts. Cells were washed twice with phosphate-buffered saline containing 10 mM glucose, 0.1 M sucrose, 1.2 mM Mg SO_4, 1.2 mM Mg Cl_2, and 1 mM $CaCl_2$ and lysed in 50 mM Hepes (pH 7.5), 10% (vol/vol) glycerol, plus 1% Triton X-100 for 1 hr at 4°C. The debris was removed by centrifugation in a Microfuge for 10 min. The protein concentration was estimated by the method of Bradford (13).

Preparation of Extracts from Staged *Drosophila*. Staged *Drosophila* were washed extensively with Ringer's solution (18), frozen, ground in a mortar, resuspended in hypotonic solution, and homogenized as described by Thompson *et al.* (12) for the Kc cell line.

^{125}I-Insulin Degradation Assay. Aliquots of the *Drosophila* extracts were diluted into a buffer containing 50 nM insulin, bovine serum albumin (0.5 mg/ml), 100 mM sodium phosphate (pH 7.2), and 25,000 cpm of monoiodinated insulin (specific activity, 80–120 μCi/μg). The samples were incubated for 15 min at 37°C, and the incubation was stopped by the addition of cold 25% (wt/vol) trichloroacetic acid. The relative amount of released radioactivity in the soluble fraction was determined as described (11). 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 concentration.

Immunoblotting Analysis. The extracts were separated by NaDodSO$_4$/polyacrylamide gel electrophoresis on a 6.5% gel. The samples were then electrophoretically transferred from the gel to nitrocellulose, and the blot was probed with an anti-IDE antibody as described (12). The anti-IDE antibody was prepared by excision of the purified IDE from a

polyacrylamide gel and injection of the gel band into a rabbit. The resultant polyclonal antiserum was shown by immunoblotting to bind specifically to the IDE in crude Kc cell extracts and purified enzyme preparations (J.V.G. and M.R.R., unpublished results).

RESULTS

We have described an IDE from *Drosophila* Kc cells (11) that specifically binds insulin, TGF-α, and related factors (ref. 3 and unpublished data) and crossreacts with an anti-human EGF receptor antibody (3, 12). This crossreactivity appears to be specific to a particular anti-EGF receptor antibody preparation and does not extend to the mammalian IDE. The *Drosophila* 110-kDa protein is able to degrade porcine insulin (11) and, like its mammalian counterpart, is found primarily in the cytoplasm of cells (3). The distinctive binding and antigenic properties of the *Drosophila* IDE were used as the initial criteria for identifying the enzyme and determining its distribution in various cell lines and at various stages of development.

The *Drosophila* IDE was readily detected in all other *Drosophila* cell lines tested. To visualize the *Drosophila* IDE, total cell extracts were concentrated by immunoprecipitation with the anti-EGF receptor antibody and then affinity labeled with ^{125}I-labeled insulin. The results obtained for the Schneider line 3 (Fig. 1) revealed a 110-kDa band that was specifically immunoprecipitated and labeled with ^{125}I-labeled insulin. Both unlabeled insulin and EGF were able to compete with the labeled insulin for binding, and no immunoprecipitation of this band was detected when preimmune serum was
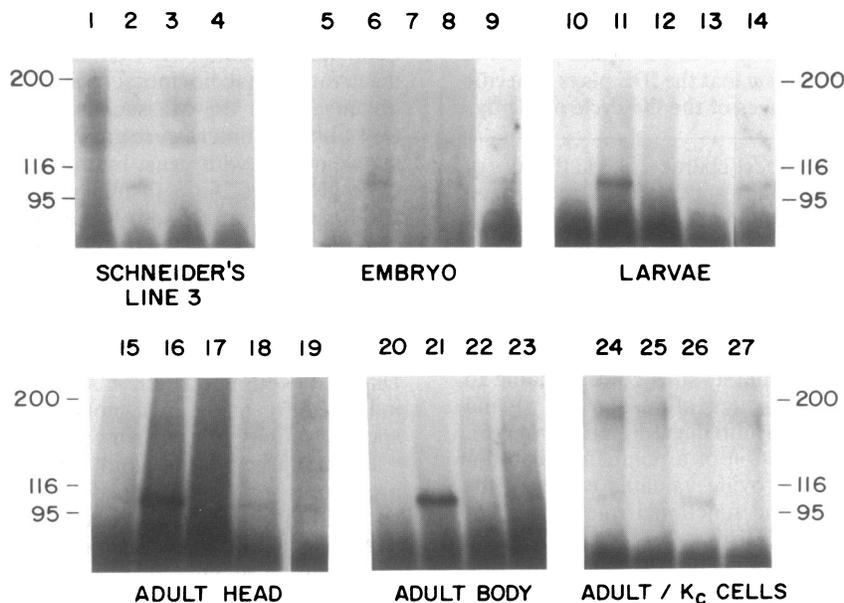
FIG. 1.   Autoradiograph of the IDE isolated from cell lines and various developmental stages of *D. melanogaster*. Schneider L3 cells (50 μg of total cell extracts) were immunoprecipitated with preimmune serum (lane 1) or with anti-EGF receptor antiserum (lanes 2–4) and subsequently labeled with ^{125}I-labeled insulin in the absence (lanes 1 and 2) or in the presence of excess unlabeled insulin (lane 3) or EGF (lane 4). Embryos [400 μg of cytoplasmic (lanes 5–8) or 800 μg of membrane (lane 9) extracts from 0- to 24-hr embryos] were immunoprecipitated with preimmune serum (lane 5) or with an anti-EGF receptor antibody (lanes 6–9) and subsequently labeled with ^{125}I-labeled insulin in the absence (lanes 5, 6, and 9) or in the presence of excess of unlabeled insulin (lane 7) or EGF (lane 8). Larvae [200 μg of cytoplasmic (lanes 10–13) or 500 μg of membrane (lane 14) extracts from first, second, and third instar larvae] were immunoprecipitated with preimmune serum (lane 10) or anti-EGF receptor antiserum (lanes 11–14) and subsequently labeled with ^{125}I-labeled insulin in the absence (lanes 10, 11, and 14) or in the presence of excess unlabeled insulin (lane 12) or EGF (lane 13). Adult heads [400 μg of cytoplasmic (lanes 15–18) or 500 μg of membrane (lane 19) extracts from adult fly heads] were immunoprecipitated with preimmune serum (lane 15) or with anti-EGF receptor antiserum (lanes 16–19) and subsequently labeled with ^{125}I-labeled insulin in the absence (lanes 15, 16, and 19) or in the presence of excess unlabeled insulin (lane 17) or EGF (lane 18). Adult bodies [400 μg of cytoplasmic extracts from bodies of adult flies] were immunoprecipitated with preimmune serum (lane 20) or anti-EGF receptor antiserum (lanes 21–23) and subsequently labeled in the absence (lanes 20 and 21) or in the presence of excess of unlabeled insulin (lane 22) or EGF (lane 23). Adult/Kc cells [a mixture of total extracts from adult flies (1 mg) and Kc cells (0.5 mg)] were incubated with ^{125}I-labeled TGF-α in the absence (lanes 24 and 26) or in the presence (lanes 25 and 27) of excess unlabeled insulin. All samples were then crosslinked and analyzed by NaDodSO$_4$/PAGE. The molecular mass of the IDE is 110 kDa.

Developmental Biology: Stoppelli *et al.*

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used. Similar results were obtained for the *Drosophila melanogaster* Schneider L2 and the *Drosophila hydei* line 15 cells (data not shown).

The ubiquitous distribution of the *Drosophila* IDE became apparent when various developmental stages of the fly were examined (Fig. 1). Cytoplasmic extracts of embryo, larvae, and adult bodies and heads contained a 110-kDa protein corresponding to the IDE that was immunoprecipitated by the anti-EGF receptor antibody and was specifically labeled by ^{125}I-labeled insulin. Similar results were obtained for the pupae (data not shown). As in the case of Kc cells (3), the IDE was also detected in membrane fractions of these organisms, but the relative amount was low compared to that in the cytoplasmic fractions. The specific binding of the IDE to ^{125}I-labeled TGF-α in adult fly and Kc cell extracts is also illustrated in Fig. 1. On the basis of binding specificity, molecular weight, and crossreactivity with the anti-EGF receptor antibody, the IDE identified in the intact fly appears to be the same as that characterized from the *Drosophila* Kc cell line.

The observations that the IDE is highly conserved between *Drosophila* and mammals and that its ability to degrade

insulin is inhibited by other growth factors suggest that the enzyme might be a target for regulation during the developmental process. To explore this possibility, we initially determined the levels of the IDE in *D. melanogaster* Schneider L3 cells, which are induced to differentiate by the molting hormone ecdysterone (14). Upon exposure to ecdysterone, the L3 cells stop growing and change morphology; at the same time there is an increase in the overall protein synthesis. This effect is illustrated in Fig. 2 *C* and *D*. When the level of the *Drosophila* IDE in ecdysterone-treated cells was examined as described above, a small but significant increase of 2-fold over background protein levels was detected (Fig. 2 *B* and *D*). Actin levels, in contrast, decreased over the same time period (Fig. 2*E*). When insulin-degrading activity was examined directly in the cell extracts by a trichloroacetic acid precipitation assay (11), a similar pattern of induction by the hormone was noted (Fig. 3). These results suggest that the IDE can be specifically regulated in the course of differentiation.

To determine the pattern of expression of the IDE during development, the level of the IDE was quantitated in samples from various stages of *Drosophila* development. When the
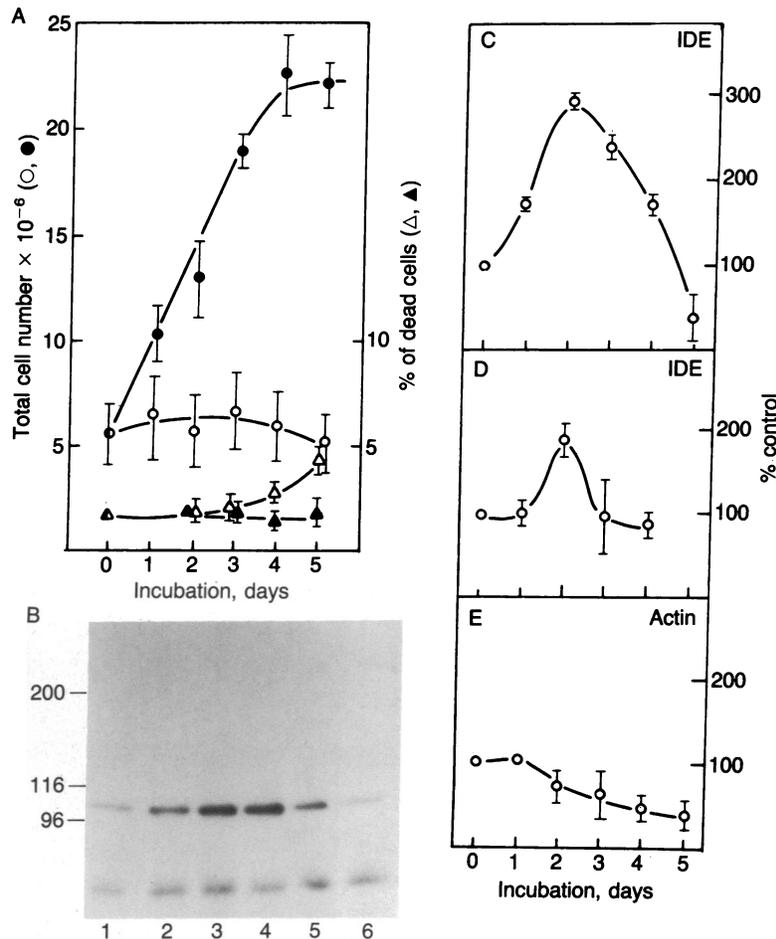
FIG. 2. Ecdysone induces an increase in the level of the IDE in Schneider L3 cells. (*A*) Cell growth and viability of L3 cells after ecdysone treatment. Growing L3 cells were incubated in the absence (●, ▲) or presence (○, △) of 1 μM ecdysone for the indicated times at 25°C. The extent of the ecdysone-induced differentiation was monitored by determining the increase in cell number (○, ●), and the cell viability (△, ▲) was tested by the trypan blue exclusion assay. (*B*) Autoradiograph depicting the time course of ecdysone-induced changes in IDE expression. Cells were treated with ecdysone for 1 day (lane 1), 2 days (lane 2), 3 days (lane 3), 4 days (lane 4), 5 days (lane 5), or 6 days (lane 6). (*C–E*) Ecdysone-treated or control cells (5 × 10⁴ cells per sample), as shown in *A*, were lysed at the times indicated, and the amount of the IDE was quantitated by immunoprecipitation with an anti-EGF receptor antibody and affinity labeling with ^{125}I-labeled insulin, both under saturating conditions. The samples were then resolved by NaDodSO₄/PAGE, and the IDE was quantitated by densitometric scanning of the autoradiograph. (*C* and *D*) Time course of ecdysone-induced changes in the IDE. The amount of IDE was normalized to a constant cell number (*C*) or to a constant cell protein concentration (mg) (*D*). Data are presented as % control. (*E*) Ecdysone-induced changes in actin. Total cell extracts containing equivalent amounts of protein from ecdysone-treated cells and control cells were resolved by NaDodSO₄/PAGE. The prominent 45-kDa actin band was quantitated by densitometry, and data are presented as % control.
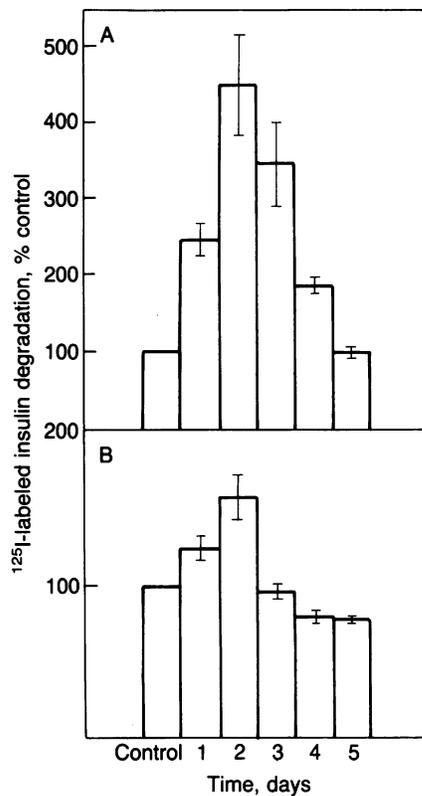
FIG. 3. ^{125}I-labeled insulin degradation in Schneider L3 cells after ecdysone treatment. Total cell extracts prepared as in Fig. 2 were also incubated with ^{125}I-labeled insulin in the presence or in the absence of excess unlabeled insulin to determine the extent of insulin degradation. The data are plotted as % control. (*A*) Data are normalized to cell number. (*B*) Data are normalized to mg of protein.

Table 1. Quantitation of *Drosophila* IDE in extracts from various developmental stages and cell lines

Source	Normalized ^{125}I-labeled insulin binding	Normalized insulin-degrading activity
Embryos	1.00	1.00
Larvae	5.53 ± 1.05	4.59 ± 1.66
Pupae	5.7 ± 1.24	4.1 ± 2.13
Adults	10.7 ± 0.78	8.06 ± 1.62
Kc cells	335 ± 50	23.3 ± 4.49

Samples were prepared as described in the legend to Fig. 4, and the level of the IDE in the autoradiographs was quantitated by densitometry. All results were normalized relative to the amount of ^{125}I-labeled insulin bound to the IDE in the embryo extract. Extracts containing equivalent amounts of protein were prepared and analyzed for insulin degradation activity. All results were normalized relative to the level of insulin-degrading activity in the embryo extract.

embryo to larvae and pupae was ≈5-fold and the increase from embryo to adult approached 10-fold (Table 1). No difference was detected between the IDE protein levels in early-stage embryos (0–8 hr) and late-stage embryos (8–24 or 12–20 hr). The Kc cell line, like the other *Drosophila* cell lines, expressed unusually high levels of the protein (≈30-fold over the level in adult flies), raising the possibility that this enzyme is required for maintaining the cells in culture.

This pattern of differentiation-enhanced expression was confirmed when the IDE level was determined either by direct insulin-degradation assays of the extracts (Table 1) or by immunoblotting analysis of the samples (Fig. 5) with a specific antibody directed against the purified IDE from Kc cells (unpublished data). In both cases, a gradual increase in the level of the IDE from the embryo to the adult stage was observed, with the highest level detected in the Kc cell line. Thus, the primary target of IDE action appears to be in the most fully differentiated state.

relative levels of the IDE were examined, after immunoprecipitation with the anti-EGF receptor antibody and affinity labeling with ^{125}I-labeled insulin under saturating conditions, a gradual increase from the embryo to the adult was observed (Fig. 4). To ensure that the results were quantitative and that the IDE samples were not being selectively degraded, an internal exogenously labeled IDE standard was added to a duplicate set of control organisms. Quantitation of the 110-kDa IDE band indicated that the increase from

DISCUSSION

We have demonstrated that a *Drosophila* IDE is a widely distributed protein that is present in isolated *Drosophila* cell lines and during the various stages of the organism's development. The expression of the IDE is regulated in an
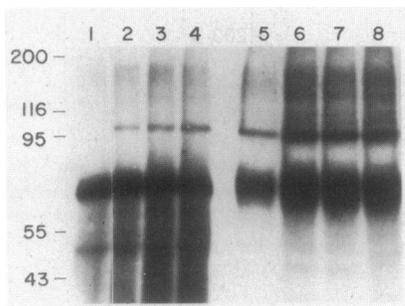
FIG. 4. Autoradiograph illustrating the relative levels of IDE from various stages of *D. melanogaster* development. Equivalent amounts of protein of total extracts from embryo (lane 1), adult (lane 2), Kc cells (lane 3), or Kc cytoplasmic fraction (lane 4) were subjected to immunoprecipitation with anti-EGF receptor antibody and to labeling with ^{125}I-labeled insulin under saturating conditions. Lane 5 contains ^{125}I-labeled insulin IDE that was used as an internal standard to assess degradation. Lanes 6–8 contain the ^{125}I-labeled IDE mixed with embryo, adult, and Kc extracts, respectively, and immunoprecipitated in parallel incubations. Samples were analyzed by NaDodSO$_4$/PAGE. The molecular mass of the IDE is 110 kDa.

FIG. 5. Autoradiograph of immunoblot showing the relative amounts of IDE present in the various *Drosophila* developmental stages. Samples were probed with preimmune antisera (lanes 1–4) or anti-*Drosophila* IDE antibody (lanes 5–8). Lanes: 1 and 7, 275 μg of extract from embryos; 2 and 6, 192 μg of extract from adult flies; 3 and 8, 54 μg from Kc cells; 4 and 5, 275 μg of extract from embryos mixed with purified IDE.

ecdysone-induced cell line and in the intact fly. In both cases, the level of the enzyme directly correlates with the extent of differentiation. These observations, in conjunction with the fact that the physical and kinetic properties of this enzyme are highly conserved between mammals and *Drosophila* (11), suggest that the IDE plays a discrete and important role in the maintenance of the differentiated organism.

Since crude extracts of the cells and tissues were used, we utilized at least three criteria for identifying and quantitating the *Drosophila* insulin-degrading protein. The distinctive features of this enzyme relate to the fact that the protein can bind porcine insulin and human TGF-α with high affinity ($K_d \approx 100$ nM and 1 nM, respectively) and can be isolated through immunoprecipitation with an anti-EGF receptor antiserum (3). Since this assay is more sensitive and more specific than that of insulin degradation, this was the primary method used for identification of the enzyme. We have also prepared a specific antiserum against the purified enzyme that is very effective in immunoblot analysis but less efficient at immunoprecipitating the native enzyme (unpublished data). Therefore, this antiserum was used to verify the relative distribution of the IDE during the various stages of *Drosophila* development.

The significance of the high level of expression of the IDE in the *Drosophila* cell lines is not presently clear. Although the slight elevation of the levels of the IDE in the differentiating *Drosophila* cell line is consistent with the changes observed in the intact organism, the tissue that the cell line corresponds to is not known. The origin of the cell lines assayed is embryonal, but the expression of the IDE in these lines is an order of magnitude greater than that in the intact embryo. Thus, it is possible that expression of the IDE is required for establishment or maintenance of the cells in culture. The fact that the IDE is present in fly heads and bodies is consistent with the wide distribution of the IDE in mammalian tissues (cf. ref. 15).

It is interesting to compare the developmental profile of the IDE to the expression of the *Drosophila* homologues of the insulin receptor. Rosen and colleagues (9, 10, 16) identified in *Drosophila* a counterpart to the mammalian insulin receptor that could be crosslinked to ^{125}I-labeled insulin and reacted with anti-human insulin receptor antisera. The molecular mass of the insulin-bound α subunit present in both embryonal and adult *Drosophila* was 120–135 kDa (10, 16), similar to that of the α subunit of the mammalian insulin receptor. An insulin-binding α subunit of ≈ 100 kDa was also expressed transiently in embryos (10) and correlated with the major insulin-induced tyrosine kinase activity. Our results suggest that the IDE may act in a coordinate fashion with the adult

Drosophila homologue of the mammalian insulin receptor in the differentiated organism.

In summary, these findings indicate that the *Drosophila* IDE, although ubiquitous, is under tightly regulated developmental control, suggesting that the enzyme acts at a rate-limiting step and is either detrimental during periods of rapid growth or only required during the later stages of differentiation.

1. Krupp, M. N. & Lane, M. D. (1982) *J. Biol. Chem.* **257**, 1372–1377.
2. Hammons, G. & Jarett, L. (1980) *Diabetes* **29**, 475–486.
3. Garcia, J. V., Stoppelli, M. P., Thompson, K. L. & Decker, S. J. (1987) *J. Cell Biol.* **105**, 449–456.
4. Duckworth, W. C. & Kitabchi, A. (1981) *Endocr. Rev.* **2**, 210–232.
5. Hamel, F. G., Peavy, D. E., Ryan, M. P. & Duckworth, W. C. (1986) *Endocrinology* **118**, 328–333.
6. Shii, K. & Roth, R. A. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 4147–4151.
7. Meneses, P. & Ortis, M. A. (1975) *Comp. Biochem. Physiol.* **51A**, 483–485.
8. LeRoith, D., Lesniak, M. A. & Roth, J. (1981) *Diabetes* **30**, 70–76.
9. Petruzzelli, L., Herrera, R., Garcia, R. & Rosen, O. (1985) in *Growth Factors and Transformation Cancer Cells*, eds. Feramisco, J., Ozanne, B. & Stiles, C. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), Vol. 3, pp. 115–121.
10. Petruzzelli, L., Herrera, R., Garcia-Arenas, R. & Rosen, O. (1985) *J. Biol. Chem.* **260**, 16072–16075.
11. Garcia, J. V., Fenton, B. W. & Rosner, M. R. (1988) *Biochemistry*, in press.
12. Thompson, K. L., Decker, S. J. & Rosner, M. R. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 8443–8447.
13. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254.
14. Courgeon, A. M. (1972) *Exp. Cell Res.* **74**, 327–336.
15. Shii, K., Yokono, L., Baba, S. & Roth, R. (1986) *Diabetes* **35**, 675–682.
16. Fernandez-Almonacid, R. & Rosen, O. M. (1987) *Mol. Cell. Biol.* **7**, 2718–2727.
17. Echalier, G. (1976) in *Invertebrate Tissue Cultures: Applications in Medicine, Biology and Agriculture*, eds. Kurstak, E. & Maramorosch, K. (Academic, New York), pp. 131–150.
18. Brakel, C. L. & Blumenthal, A. B. (1977) *Biochemistry* **16**, 3137–3143.