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A new family of growth factors produced by the fat body and active on *Drosophila* imaginal disc cells

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SUMMARY

By fractionating conditioned medium (CM) from *Drosophila* imaginal disc cell cultures, we have identified a family of Imaginal Disc Growth Factors (IDGFs), which are the first polypeptide growth factors to be reported from invertebrates. The active fraction from CM, as well as recombinant IDGFs, cooperate with insulin to stimulate the proliferation, polarization and motility of imaginal disc cells. The IDGF family in *Drosophila* includes at least five members, three of which are encoded by three genes in a tight cluster. The proteins are structurally related to chitinases, but they show an amino acid substitution that is known to abrogate catalytic activity. It therefore seems likely that they have evolved from chitinases but acquired a new growth-promoting function. The IDGF genes are expressed most strongly in the embryonic yolk cells and in the fat body of the embryo and larva. The predicted molecular structure, expression patterns, and mitogenic activity of these proteins suggest that they are secreted and transported to target tissues via the hemolymph. However, the genes are also expressed in embryonic epithelia in association with invagination movements, so the proteins may have local as well as systemic functions. Similar proteins are found in mammals and may constitute a novel class of growth factors.

Key words: *Drosophila*, Imaginal disc, Fat body, Growth factor, Chitinase, Chitinase-related protein

INTRODUCTION

Despite the enormous progress made in understanding the genetics of *Drosophila* development, this insect has not lent itself to the production of defined systems for the assay of materials with mitogenic or other biochemical activities in vitro. As a result, conventional assays for growth factors have not been applied to *Drosophila* cells, and growth factors similar to those known from mammalian cells have not been identified in *Drosophila*. We suspect that this reflects differences between the experimental systems and the way they have been used, rather than a fundamental difference in cell proliferation control mechanisms between *Drosophila* and mammals.

Some growth factor-like molecules have been identified in *Drosophila* by homology searching and by genetic analysis. Examples include the EGF-related products of the *spitz* (Schweitzer et al., 1995) and *gurken* (Neuman-Silberberg and Schupbach, 1993) genes; and the TGF-beta family member produced by the *dpp* gene (Gelbart, 1989). However, none of these has been shown to have direct mitogenic activity as expected of a growth factor. Some genes encoding secreted proteins including Hh (Capdevila and Guerrero, 1994), Dpp (Pignoni and Zipursky, 1997) and Wg (Neumann and Cohen, 1996) do lead to excess cell proliferation when ectopically expressed, and blocking their function results in growth inhibition (Peifer et al., 1991; Burke and Basler, 1996). However, the effects are quite local and may be indirect results of effects on tissue patterning, rather than to conventional growth-factor activity. Many components of signal transduction pathways that are typically involved in growth-factor responses have been identified in *Drosophila* (Shilo, 1992), but they usually have roles in cell signaling events related to patterning rather than mitogenesis. Experimental results indicate that the fat body secretes mitogenic factor(s) (Davis and Shearn, 1977) but their molecular nature is unknown. Another phenomenon that has no clear analog in *Drosophila* is induction of the transformed phenotype, either by expression of oncogenes or by the action of transforming growth factors. The demonstration of these effects in *Drosophila* cells would open up a wide variety of new experimental approaches to the study of cell regulation.

Well-defined cell culture techniques are needed for identifying growth factors. The first cell cultures made from *Drosophila* were derived from embryos and, although useful for many purposes, they consist of mixed and/or ill-defined cell types which may change over time and have therefore had limited application to experimental studies of proliferation control. More recently, cell lines have been derived from imaginal discs and clonal variants of these lines (e.g. Cl.8+) have been isolated (Currie et al., 1988; Peel and Milner, 1990b). These lines can be transfected and used for assays of proliferation and adhesion (Peel, D. and Kawamura, K., unpublished). However, the cells

do not show the classical contact inhibition seen in mammalian cells, but instead, upon reaching confluence they continue to pile up into large aggregates (Peel et al., 1990a). For these reasons they have not been suitable for conventional assays of growth factors and transformation.

Imaginal disc cell lines are dependent on soluble growth factors for their survival and proliferation, as shown by several findings. First, these cells fail to proliferate in serum-free medium (SFM) but do proliferate when the medium is supplemented by serum, insulin and an extract of adult flies (Cullen and Milner, 1991). Second, when the cells are grown to high density in supplemented complete medium and then allowed to condition SFM, the conditioned medium (CM) supports cell growth and proliferation. In this paper we report the purification and identification of the active factors from this CM. They are the first soluble growth factors to be identified from invertebrates, and surprisingly are related to chitinase enzymes rather than to known growth factors. They appear to correspond to the growth factors that have long been thought to be secreted by the fat body.

MATERIALS AND METHODS

Preparation of conditioned medium

The wing-disc cell line, Cl.8+ (Peel and Milner, 1992), was cultured in Shields and Sang's M3 medium supplemented with 2% fetal bovine serum (Sigma, F3018), 0.125 IU/ml bovine insulin (Sigma, I1882) and 2.5% fly extract (Currie et al., 1988). The cells were replated on 60 mm plastic dishes at a density of 0.5×10^6 cells/ml, and allowed to proliferate for 3-4 days in the same medium until they became subconfluent. The subconfluent cultures were then washed three times with PBS or SFM. In order to ensure thorough washing, they were incubated in the third washing medium for 1 hour. Conditioned medium (CM) was prepared by incubating the cells in SFM for 4 days. The CM was collected after removal of cell debris by centrifugation, and stored under refrigeration. Alternatively, CM was prepared by culture of confluent cells that had been inoculated at high density (4×10^6 cells/ml).

Cell number estimation

After harvesting from the complete medium, cells were washed twice with SFM. They were resuspended in culture medium to be tested at a density of 0.5×10^6 cells/ml. Aliquots (0.5 ml) of suspension were dispensed in 24-well multiplates. Cell number was estimated quantitatively after various periods of culture, using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method (Denizot and Lang, 1986).

BrdU incorporation

Cells were plated on glass cover slips and cultured in SFM or CM for 24 hours. They were then incubated with 10 μM 5-bromo-2′-deoxyuridine (BrdU) for 30 minutes. Labeled cells were stained with anti-BrdU antibody followed by anti-mouse IgG-FITC, using BrdU detection kit I (Boehringer).

Chromatography

The CM was dialyzed against 20 mM phosphate buffer (pH 8.0) and passed through an anion exchange column (2.5×10 cm) of DE 52 (Whatman) equilibrated with the same buffer. The column was eluted with a linear gradient of 0-0.5 M NaCl in the same buffer (4 ml/6 minute/tube). The eluate was monitored for absorbance at 280 nm. Fractionated samples were concentrated by size-exclusion filtration (Centriprep-10, Amicon). Preparative electrophoresis was carried out in the presence or absence of sodium dodecyl sulfate (SDS), using a Model 491 Prep Cell (Bio-Rad, Inc.). The polyacrylamide gel (7%) contained 6 M urea. Each fraction was collected at a constant flow rate (1 ml/minute) of elution buffer. For high performance liquid chromatography (HPLC), a prepacked gel filtration column (Shodex, KW-803) was connected to a Jasco's liquid chromatographic system consisting of an 801-SC system controller, an 880-PU pump and an 875-UV detector (Japan Spectroscopic Co. Ltd). The column was eluted with 50 mM phosphate buffer containing 0.5 M NaCl and 0.05% brij-35 at a flow rate of 0.5 ml/minute.

Protein analysis

In order to assess purity and relative molecular size of proteins, SDS-PAGE was carried out on 10% polyacrylamide gel containing 0.1% SDS in 0.375 M Tris-HCl (pH 8.8); the gel was silver stained (Boehringer), and the amount of protein determined (Lowry et al., 1951). After isolation, the 50 kDa polypeptide was blotted onto PVDF membrane using a sample cartridge (ProSpin, Applied Biosystems). The membrane was washed thoroughly and applied to a gas-phase protein microsequencer (470/900A, Applied Biosystems) to obtain the N-terminal amino acid sequence of the protein.

PCR

cDNA fragments of IDGF genes were amplified by PCR using genomic DNA and an imaginal disc cDNA library as templates. Primers are: IDGF1F, 5′aacgaattcgatttggccctgcagttc3′; IDGF1R, 5′cagtgcaggatccacggaagtcatc3′; IDGF2F, 5′ttggctgaattcaaagtgag-cgccgct3′; IDGF2R, 5′gcactcggatccttgatcagtgcagt3′; IDGF3F, 5′aagctgcctcgagcttagtctgctac3′; IDGF3R, 5′cattgggaagcttagtcaga-ctgagc3′; IDGF4F, 5′tttggccattggtcagaattcccg3′ and IDGF4R, 5′tgctctccagcagggtcagatact3′. PCR cycle was 94°C 1 minute, then 26 cycles of 94°C 30 seconds, 55°C 1 minute, 72°C 2 minutes. Amplified PCR fragments were digested with appropriate restriction enzymes, subcloned into pBluescript SK– (Stratagene) and used for further analysis.

cDNA library screening and sequence analysis

cDNA fragments for each IDGF were ^{32}P radiolabeled by random priming (Stratagene) and used for screening about half a million clones of an imaginal disc and larval cDNA library. Hybridization was performed in 0.5 M sodium phosphate (pH 7.2), 7% SDS and 1 mM EDTA at 65°C. Filters were washed in 0.2× SSC (1×, 150 mM NaCl and 15 mM sodium citrate) and 0.2% SDS at 65°C. After three rounds of screening, positive clones were isolated and subcloned into pBluescript. The nucleotide sequences of IDGF genes on both strands were determined using a ThermoSequenase radiolabeled terminator cycle sequence kit (Amersham). Nucleotide and protein sequences were analyzed using Megalign (DNASTAR, Inc., 1228 South Park Street, Madison, WI 53715).

In situ hybridization

Sense and antisense digoxigenin-labeled RNA were transcribed from linearized pBluescript containing each IDGF cDNA using T3 and T7 RNA polymerase (Promega) and a digoxigenin labeling mixture (Boehringer Mannheim). Dechorionated embryos and partially dissected larvae were fixed and hybridized with RNA probes in 50% formamide, 5× SSC, 250 mg/ml salmon-sperm DNA, 50 mg/ml heparin and 0.1% Tween 20 at 55°C (Theisen et al., 1996). Signals were detected by anti-digoxigenin-AP Fab fragments (Boehringer Mannheim). There were no detectable signals from sense RNA probes.

Production of recombinant IDGF proteins

A full-length cDNA for each IDGF was inserted into the multicloning site of the baculovirus transfer vector, pBlueBac-His2 (InVitrogen) which provides a His tag for affinity purification. The transfer vector and linearized baculovirus DNA were co-transfected overnight into the host cell, sf-9. For lipofection, cellfectin (Gibco, 10362-028) was diluted 1:50 with SF-900II (Gibco, 10902-013). After screening by β-

gal expression and PCR, recombinant baculovirus were purified from positive plaques, cloned and amplified (Webb and Summers, 1990). Three days after transfection with high-titer virus, cells were sonicated in the lysis buffer (20 mM Tris, 0.1 M NaCl, 6 M urea, pH 8.0). His-tagged recombinant proteins were bound to a metal-affinity resin (Clontech) and eluted with 50 mM imidazole. In order to remove the tag, the recombinant proteins were treated with 10 U/ml enterokinase (Biozyme, EK2B) in 70 mM sodium succinate buffer (pH 5.6) for 2 hours at 25°C. The enzyme was heat-inactivated and the protein solution was dialyzed against PBS (pH 7.6) before bioassay.

RESULTS

Medium conditioning by imaginal disc cells

When cultured in supplement-free Shields and Sang M3 medium (SFM, lacking serum, insulin, and fly extract) (Shields and Sang, 1970) cells of the wing-disc cell line, Cl.8+, became flattened within a few hours after plating, but did not form pseudopodia (Fig. 1A). They showed no BrdU incorporation, decreased in number by about 50% over a 4-day period, and underwent apoptosis as indicated by time-lapse video microscopy.

Conditioned medium (CM) was prepared by growing Cl.8+ cells to subconfluence in complete medium and then incubating them in SFM for 4 days. Cl.8+ cells showed a remarkable change of properties when cultured in this CM. Within a few hours after plating, they became bipolar, developed pseudopodia and elongated (Fig. 1B). After 1 day in culture, they formed aggregates and showed enhanced motility as shown by time-lapse video microscopy (data not shown). They did not fragment but rather doubled in number in 2 days (Fig. 2A). After 1 day, they showed a BrdU labeling index of 35.1±9.9% (mean ± s.d.) (Fig. 1C) compared with 0% in SFM. CM prepared from confluent cultures was also effective on cell survival, but it had a weaker effect on cell growth than that from subconfluent cultures (Fig. 2A). These results suggest that Cl.8+ cells secrete factors into the culture medium to stimulate their own growth.

Purification of growth-promoting activity from conditioned medium

After size-exclusion filtration of CM, growth-promoting activity was found in the high molecular-weight (>10 kDa) fraction. When this fraction was separated into seven peaks by anion exchange chromatography (Fig. 3A), fractions 4, 5 and 6 had a positive effect on cell survival, and fraction 5 showed the highest growth-promoting activity. Fraction 5 improved cell survival at a minimal concentration of 0.4 μg/ml, and stimulated cell growth at a minimal concentration of 0.8 μg/ml. Insulin caused a remarkable enhancement of these effects, although it did not show any growth-promoting activity by itself.

After fraction 5 was further purified by gel filtration HPLC, most activity was recovered in the primary peak (Fig. 3B). On SDS-PAGE, the active fractions showed a prominent 50 kDa polypeptide (Fig. 3C, lanes 1-3), which was further purified by preparative electrophoresis in the presence of SDS (Fig. 3C, lane 4).

Five members of the imaginal disc growth factor (IDGF) family

Microsequencing of the purified 50 kDa protein from the N terminus provided a sequence of 32 amino acids (Fig. 4B). Although this sequence did not completely match any protein sequences in available databases, it closely resembled (47% identity over 32 residues) the sequence of the 452aa (47 kDa) glycoprotein DS47 (Kirkpatrick et al., 1995). This protein is abundantly secreted from a *Drosophila* embryo-derived cell line (S2), and is produced in vivo by the fat body and hemocytes and secreted into the hemolymph. It is encoded by the gene *Chit* at 53D, but no mutations in this gene have been

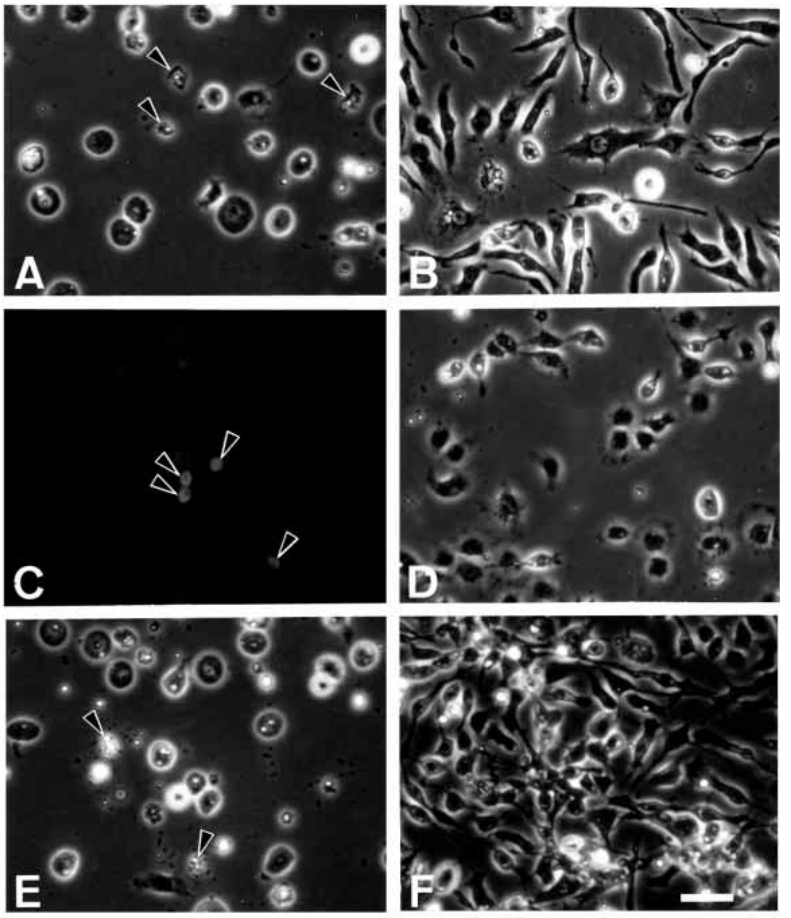


Fig. 1. Effect of conditioned medium and recombinant IDGFs on cell morphology and DNA synthesis in the C1.8+ wing disc cell line. (A) Low-density cells (0.5×10^6 cells/ml) in supplement-free medium (SFM), 2 days after plating. They are round without any pseudopodia. Arrowheads indicate cell fragmentation. (B) Low-density cells in conditioned medium (CM), 2 days after plating. They elongate conspicuously and cell motility is also enhanced. (C) Cells were allowed to grow for 1 day in the CM and then prepared for anti-BrdU immunofluorescence. Labeling index was 35.1±9.9%. Note the labeled nuclei (arrowheads). Cells cultured in serum-free medium showed no nuclear BrdU staining. (D) Cells in SFM plus recombinant IDGF2 (0.2 mg/ml). Most cells have developed lamellipodia. (E) Cells in SFM plus insulin (0.125 U/ml). Most cells are flat and round, and apoptotic fragmentation is occurring (arrowheads). (F) Cells in SFM plus IDGF2 and insulin. Proliferation and elongation are evident. D-F were photographed 4 days after plating. Bar, 25 μm.

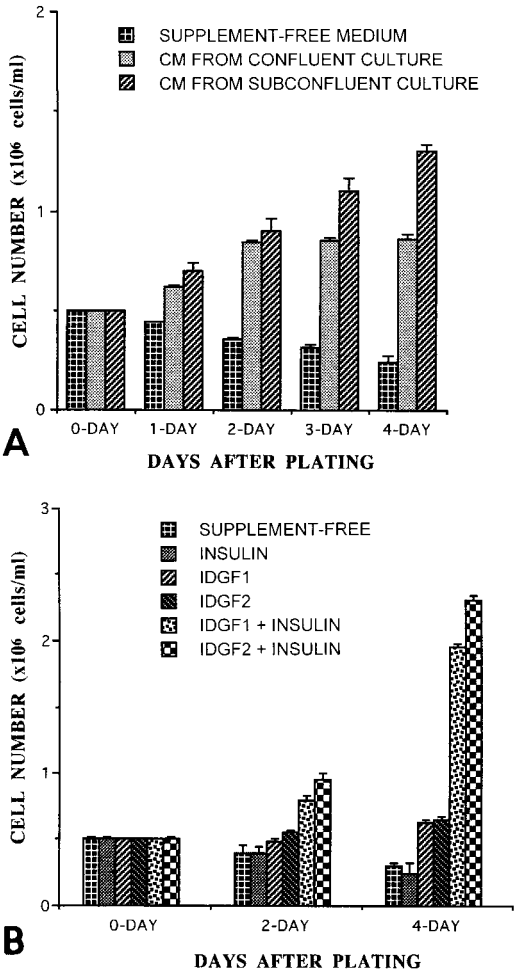


Fig. 2. Effect of conditioned medium and recombinant IDGFs on cell proliferation in the imaginal disc cell line C1.8+. Cells from the wing-disc cell line C1.8+ were plated at 0.5×10^6 cells/ml in 24-well plates and allowed to proliferate for 4 days in the presence of each supplement. Cell number was estimated using the MTT method (Denizot and Lang, 1986). Bars show the standard deviation. (A) SFM, CM prepared from confluent cultures, and CM prepared from subconfluent cultures, (B) SFM supplemented with IDGF1 or IDGF2 (0.2 μg/ml) in the presence and absence of insulin (0.125 U/ml). The results show strong molecular cooperation between IDGFs and insulin in promoting cell growth.

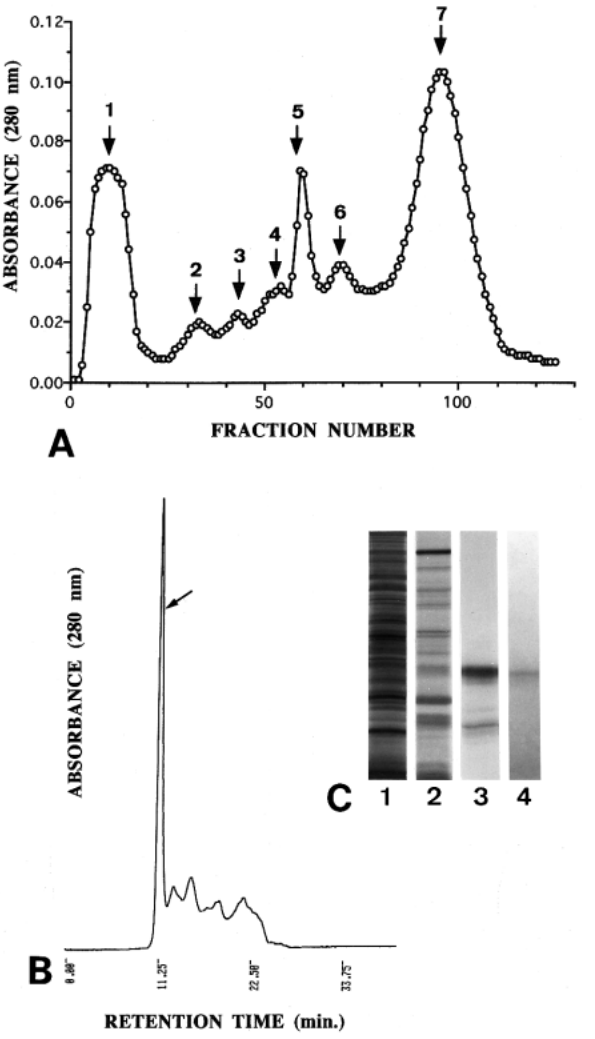


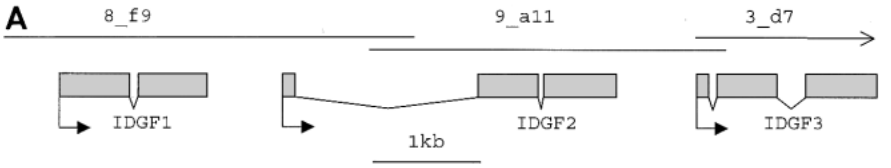
Fig. 3. Fractionation of conditioned medium (CM). (A) Elution profile after anion exchange chromatography. The elution profile of seven peaks (arrows) was reproducible, and the fifth peak had the highest growth-promoting activity. (B) Elution profile after gel filtration HPLC. The primary peak showed growth-promoting activity. (C) SDS-polyacrylamide gel electrophoresis of fractionated samples of CM. Silver staining of 10% gel. Lane 1, crude sample; lane 2, fraction 5 after anion exchange chromatography; lane 3, primary peak after gel filtration HPLC; lane 4, 50 kDa polypeptide purified after preparative electrophoresis.

reported. The N-terminal sequence of our 50 kDa polypeptide shows an even closer match to the amino acid sequences predicted from three putative genes in genomic DNA subclones 8_9f, 9_a11 and 3_d7, all within a 9 kb interval in P1 clone DS02780 from chromosome 2, cytological region 36A2-4 (Fig. 4A; *dachshund* contig; *Drosophila* Berkeley Genome Project, pers. commun.). We refer to these three predicted proteins as IDGF1, IDGF2 and IDGF3 (Imaginal Disc Growth Factors 1, 2 and 3) corresponding to the open reading frames in 8_9f, 9 a11 and 3_d7 respectively. Although the microsequence does not match exactly any of these three sequences, the microsequence data can be entirely explained by assuming that our purified fraction contains a mixture of at least IDGF1 and IDGF3 (Fig. 4B). Since the fractionation was based on molecular weight and the predicted IDGFs are similar in size, there is a good chance that the fraction contained both proteins, and possibly other family members as well.

On the basis of the genomic sequence information, we designed PCR primers for each IDGF gene, and amplified the expected fragments by PCR from genomic DNA and an imaginal disc cDNA library. Using these fragments as probes, we screened an imaginal disc cDNA library and obtained cDNA clones for IDGF1, 2 and 3. The DNA sequences of these clones matched the sequences predicted from the genomic sequence using several gene finder programs.

Database searching revealed six closely related EST clones encoding an additional protein that we named IDGF4. We prepared PCR primers for IDGF4, amplified a fragment and used it as a probe to isolate IDGF4 cDNA from a larval cDNA

library. The predicted IDGF4 protein is 46-50% identical to IDGF1-3. However, the gene is not linked to the other IDGF1-3 genes, but is localized on the X chromosome in band 9A (S. Park and T. Shibata, unpublished). In summary, the evidence suggests the existence of a gene family with five members: the IDGF1-3 cluster, IDGF4, and DS47.



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MICRO  ........................AP....NLVKYYDSQXYQRQGLAKMXMIELXLALQF   32
IDGF1  MRFQLFYILGLLSVTSLTH....AAS....NLICYYDSNSYLRQGLAKMHTNELDLALQF   52
IDGF2  MKAWIWFTF....VACLFAASTEAAS....NLVCYYDSSSYTREGEGKLLNPDLEIALQF   52
IDGF3  MTGSLWLSLAL.SLAVLAQFKVSAAP....NLVCFYDSQGSQRQGLAQFSMIDIELALQF   55
IDGF4  M..KL.YALFSLLVGSLAIGQISAAG..SHHLLCYYDGNSFVREGLSKLILTDLEPALQY   55
DS47   MIIKA.LAIVSLCLASIQASKVGAPQLPKKHLVCYYDSASFVKEGLGKLVIDELEPALQF   59
CASE   MRATL.ATLAVLALATAVQSD......SRARIVCYFSNWAVYRPGVGRYGIEDI..PVEK   51

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IDGF1  CTHLVYGYAGLKSGTLELFSLN....VDLDMFYYKDITALRQKFPQLKILLSVGGDRDVD   108
IDGF2  CSHLVYGYAGLRGENLQAYSMNEN..LDIYKHQFSEVTSLKRKYPHLKVLLSVGGDHDID   110
IDGF3  CTHLVYGYAGVNADNYEMQSINKR..LDLEQRHLAQITSMKERYPHIKFLLSVGGDADTY   113
IDGF4  CTHLVYGYAGINPSSNKLVSNNEKPDLDLGSSLFRQVTGLKRKYPALKVLLSVG GDKDT   115
DS47   CDYLVYGYAGIERDSHKAVSLNQQLDLDLGKGLYRTVTRLKRKYPNVKILLSVGGDKDIE   119
CASE   CTHIIYSFIGVTEGNSEVLIID..PELDVDKNGFRNFTSLRSSHPSVKFMVAVGGWAEG.   108

                                                  * *
IDGF1  EAH.....PNKYVELLEN.RTAQQNFIDSSMILLKRNGFDGLDLAFQLPRNKPRKVHGSL   162
IDGF2  PDH.....PNKYIDLLEGEKVRQIGFIRSAYDLVKTYGFDGLDLAYQFPKNKPRKVHGDL   165
IDGF3  EG.......NQYIKLLESGQQGHRRFIESARDLVRRYNFDGLDLALQLPRNKPRKVHGDV   166
IDGF4  VDPEN....NKYLTLLESSNA.RIPFINSAHSLVKTYGFDGLDLGWQFPKNKPKKVHGSI   169
DS47   LDKDAKELPNKYLELLESPTG.RTRFVNTVYSLVKTYGFDGLDVAWQFPKNKPKKVHSGI   178
CASE   ........SSKYSHMV.AQKSTRMSFIRSVVSFLKKYDFDGLDLDWEYPGAADR......   153

                                                              ▼
IDGF1  GSYWKSFKKLFTGDFVVDPQAEEHKSQFTDLVGNIKNAFRSANLMLSLT.VLPNVNSTWY   221
IDGF2  GLAWKSIKKLFTGDFIVDPHAALHKEQFTALVRDVKDSLRADGFLLSLT.VLPNVNSTWY   224
IDGF3  GSAWKSFKKFFTGDFIVDTESETHKGQVTALIKDLSAALKQNDLLLSLT.VLPNVNSSWY   225
IDGF4  GKFWKGFKKIFSGDHLVDEKAEEHKEAFTALVRELKNAFRPDGYILGLS.VLPNVNSSLF   228
DS47   GNLWKGFKKVFSGDSIVDEKSEEHKEQFTALLRDVKNAFRPDNLLLSTT.VLPNVNSSLF   237
CASE   ..........GGSF.......SDKDKFLYLVQELRRAFIRVGKGWELTAAVPLANFRLM   195

IDGF1  ..FDVPKLHPQFDYINLAAFDFLTPLRNPEEADFTAPIFFQD.EQNRLPHLNVEFQINYW   278
IDGF2  ..FDIPALNGLVDFVNLATFDFLTPARNPEEADYSAPIYHPDGSKDRLAHLNADFQVEYW   282
IDGF3  ..YDAPSIAPSLDFINLGTFDFLTPQRNPEEADFSAPTYEAV.GQNRLGHYNLNFQMEHW   282
IDGF4  ..FDVPAIINNLDYVNLHTYDFQTPERNNEVADFPAPIYE...LNERNPEFNVNYQVKYW   283
DS47   ..YDIPAVVNYLDFVNLGTFDFFTPQRNPEIADYAAPIYE...LSERNPEFNVAAQVKYW   292
CASE   EGYHVPELCQELDAIHVMSYDL..RGNWAGFADVHSPLYKRPHDQWAYEKLNVNDGLHLW   253

IDGF1  LQNHCPGQKLNLGIASYGRAWKLSKGSGLSGAPI.VHETCGVAPGG......GLLSWPEI   331
IDGF2  LSQGFPSNKINLGVATYGNAWKLTKDSGLEGVPV.VPETSGPAPEGFQSQKPGLLSYAEI   341
IDGF3  LLQRVPANKINIGIATYGRSWKMSKDSGDSGMPV.VPSTQGPAPAGPQSKQEGLLNWAEI   341
IDGF4  TGNRAPAAKINVGIATYGRAWKLTKDSGLTGLPP.VAEADGVAPAGTQTQIPGLLSWPEV   342
DS47   LRNNCPASKINVGVATYGRPWKLTDDSGDTGVPP.VKDVKDEAPVGGNTQVPGIYSWPEV   351
CASE   EEKGCPSNKLVVGIPFYGRSFTLSAGNNNYGLGTFINKEAGGGDPAPYTNATGFWAYYEI   313

IDGF1  CSKLSQNASAQYRGELAPLRKVTDLTQKYGNYALRPADDNGDFGVWLSFDDPDFAGIKAV   391
IDGF2  CGKLSNPQNQFLKGNESPLRRVSDPTKRFGGIAYRPVDGQITEGIWVSYDDPDSASNKAA   401
IDGF3  CSLNPNPSNSNARGPNAPVKRVVDPTKRYGSYAFRAADENGDHGLWISYDPPDSASSKAM   401
IDGF4  CAKLPNPANQHLKGADGPLRKVGDPIKRFGSYAYPSADDSGENGVWVGYEDPDTAAIKAE   402
DS47   CALLPNQNNAYLKGANAPLIKVQDPAKRFGSYAYRAADKKGDNGIWVSFEDPDTAADKAG   411
CASE   CTEVDKDDSGWTK.......KWDEQGK..CPYAYKGTQ.......WVGYEDPRSVEIKMN   357

IDGF1  YAKGKGLGGIALFDLSYDDFRGLCTG.QKYPILRSIKY....                     428
IDGF2  YARVKNLGGVALFDLSYDDFRGQCSG.DKYPILRAIKYRL..                    440
IDGF3  YARARNLGGVALFDLTQDDFRGQCTN.DRFPMLRAIKYRLL.                   441
IDGF4  YVKREGLGGIAVVDLSFDDFRGGCTGHDKFPILRQVKSKL..                   442
DS47   YVRTENLGGVALFDLSYDDFRGLCTN.EKYPILRAIKYRLTN                   452
CASE   WIKQKGYLGAMTWAIDMDDFQGLCG..EKNPLIKILHKHMSS (+157 amino acids)  554
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Fig. 4. (A) Genomic map of the region containing IDGF1-3. Top row shows subclones of the P1 clone DS02780 (from FlyBase). The position of sequence tagged sites (STSs) within the P1 clone, compared with the STS framework map, shows that the second-chromosome centromere is to the right of this set of clones. Bottom row shows IDGF1, 2 and 3 transcripts predicted from open reading frames and confirmed by cDNA sequencing. All are transcribed in the same direction, and the right-most intron is in a conserved position relative to the coding sequence. (B) Alignment of the microsequence from the purified fraction ('Micro'), with the predicted sequences of four IDGFs, *Drosophila* DS47 (Kirkpatrick et al., 1995) and authentic gut chitinase (CASE) from *Manduca sexta* (Kramer et al., 1993). Alignment was generated using MegAlign and the shading was added with BoxShade. Dark shading represents identity and light shading indicates similarity with IDGF1 for those residues that are conserved among half or more sequences. Asterisks and star indicate the positions of residues that have been shown to be required for catalytic activity in bacterial chitinase (Watanabe et al., 1993). The first three match the required residues in chitinases, but the fourth (star) is E in chitinases but Q in IDGFs. All four predicted IDGF sequences and DS47 contain an N-terminal signal sequence, and a single consensus motif (arrowhead) for N-linked glycosylation (Kirkpatrick et al., 1995) that is missing in chitinase. Residue no. 6 in the microsequence is not necessarily a mismatch, since cysteines are not detected with the method used. The results from microsequencing can be explained if the active fraction consists of a mixture of at least IDGF1 and 3. GenBank accession numbers: IDGF1: AF102236, IDGF2: AF102237, IDGF3: AF102238, IDGF4: AF102239.

IDGFs are related to chitinases

The amino acid sequences predicted by the IDGF cDNA clones are about 50% identical to each other and to the sequence of DS47 (Fig. 4B). All four predicted sequences contain an N-terminal signal sequence, and a single consensus motif for N-linked glycosylation as previously reported for DS47 (aa 233). They are closely related to (15-25% identity) but distinct from, chitinases (poly N-acetylglucosaminidases), the enzymes that catalyze the hydrolysis of the beta-1,4-N-acetyl-D-glucosamine linkages in chitin polymers of the arthropod cuticle (Hakala et al., 1993). The only other member of this family reported from insects, aside from authentic chitinases, is the Haemocyte Aggregation Inhibiting Protein from the tobacco hornworm *Manduca Sexta* (Kanost et al., 1994).

Recombinant IDGFs cooperate with insulin to promote proliferation of imaginal disc cells

To test whether IDGFs represent the active principle of the Cl.8+ conditioned medium, recombinant IDGF1 and IDGF2 were prepared using a baculovirus protein expression system. At concentrations above 0.2 μg/ml (4 nM) they promoted cell proliferation, depending on dose (Table 1; Fig. 2B). At higher concentrations, the activity was much higher than that of fraction 5 after anion exchange chromatography. From 1 day after plating onward, cells produced lamellipodia and elongated gradually (Fig. 1D), like those treated with fraction 5. IDGF1 and IDGF2 carrying N-terminal His tags were inactive in our assays, indicating that the activity depends on an intact N-terminal structure. Insulin (0.125 IU/ml) caused a remarkable enhancement of the effect of IDGF1 and IDGF2 on cell growth and elongation (Fig. 1F, 2B), but did not show either activity by itself (Fig. 1E).

IDGFs are expressed primarily in yolk cells and fat body

Using PCR fragments as probes, we examined the developmental expression of IDGF1-3 by northern blot analysis. The results showed that each IDGF is expressed at all tested stages but most heavily in mid-larval stages (data not shown). IDGF1 mRNA showed weaker expression than IDGF2 and 3.

We have examined the expression patterns of IDGF1-4 in embryos and larvae by in situ hybridization to whole mounts using digoxigenin-labeled probes. Transcripts of all four genes are detected in the yolk cytoplasm of the early embryo, but are apparently excluded from the blastoderm as it cellularizes (Fig. 5A). During gastrulation, IDGF transcripts are revealed in the basal cytoplasm of cells in rows parallel to the major invaginations including the ventral furrow, the cephalic furrow, the anterior midgut invagination and the posterior midgut invagination (Fig. 5B). The expression patterns of the four IDGFs are very similar to one another but IDGF2 and IDGF4 are expressed more strongly than IDGF1 and IDGF3.

A second phase of IDGF expression appears in late embryos, and again the four IDGF probes reveal similar patterns. The genes are first expressed in yolk cells at stage 11 (Fig. 5C), and at subsequent stages the IDGF staining follows the movements of these cells. The IDGF-positive yolk cells follow the germ band as it retracts, and then are included in the gut after the fusion of anterior and posterior midgut (Fig. 5D,E). The gut itself does not stain with IDGF probes. The formation of constrictions in the gut divides the IDGF-positive yolk cells into four compartments. Then the staining of yolk cells disappears, probably because these cells degenerate. The IDGF probes also strongly stain the fat body and the salivary glands (Fig. 5E,F). At the end of embryogenesis, the tracheal system also shows IDGF expression (data not shown).

In the third-instar larva, as in embryos, all IDGF genes are strongly expressed in the fat body (Fig. 6A). Strong expression is also seen in the ring gland and the lymph glands. All of the IDGF genes also show similar expression patterns in the larval brain (Fig. 6B). They show two parallel lines of strong expression in each optic lobe, and a region of expression in the central brain. In the salivary glands, the four IDGF genes show distinct expression patterns: IDGF1 is expressed in the large

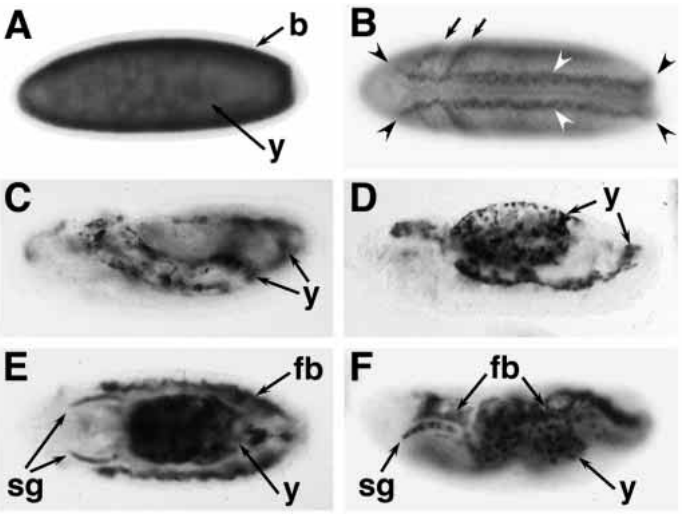

Fig. 5. IDGF4 transcript expression during embryogenesis. Wild-type embryos (anterior to the left) showing in situ hybridization with digoxigenin-labeled antisense RNA probes for IDGF4. (A) Cellular blastoderm stage showing IDGF4 transcripts in the yolk cytoplasm (y) but excluded from the blastoderm cells (b). (B) Ventral view of a stage-7 embryo showing high IDGF expression on each side of the ventral furrow (white arrowheads), the midgut invagination (black arrowheads) and the cephalic furrow (arrows). (C) Lateral view of a stage-11 embryo showing IDGF4 expression in yolk cells (y). (D) IDGF staining in yolk cells follows germ band retraction. (E) Dorsal view of a stage-13 embryo showing IDGF4 staining in yolk cells included in the gut. The IDGF4 probe also detects expression in the fat body (fb) and salivary glands (sg). (F) Lateral view showing strong expression in the fat body.

Table 1. Dose response for the effect of recombinant IDGF2 on cell proliferation in the imaginal disc cell line C1.8+

Protein concentration (nM)	Relative number of cells (%)
20	360.0±4.0
10	220.0±4.0
4	130.0±6.0
2	70.0±4.0

Cells from the wing-disc cell line C1.8+ were plated at 0.5×10^6 cells/ml in 24-well plates in the presence of insulin (0.125 U/ml) and allowed to proliferate for 4 days. Cell number was estimated using the MTT method (Denizot and Lang, 1986).

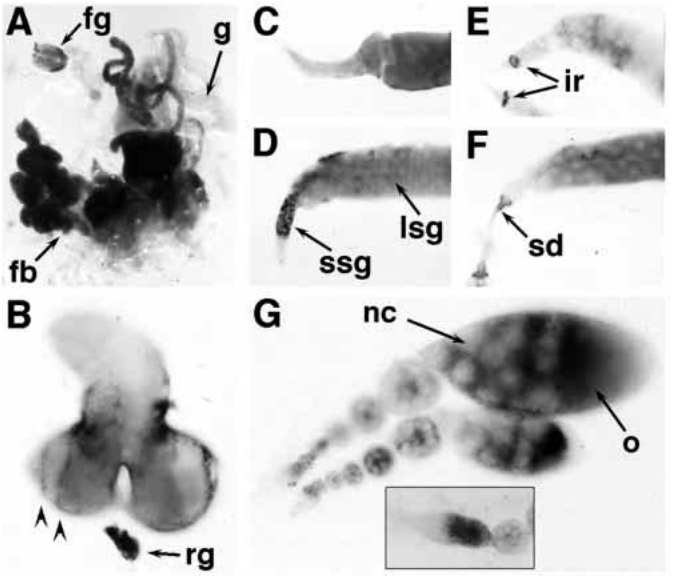

Fig. 6. Larval and adult IDGF expression. (A) Strong IDGF2 expression is detected in the fat body (fb) of the third-instar larva. The gut (g) does not stain except in the foregut (fg). (B) In the larval brain, IDGF4 is expressed in two parallel lines in each optic lobe (arrowheads) and in the central brain. The ring gland (rg) also shows strong IDGF expression. (C-F) The IDGF1 (C), IDGF3 (D), IDGF2 (E) and IDGF4 (F) probes detect specific expression patterns in salivary glands. ir, imaginal ring; lsg, large salivary gland cells; ssg, small salivary gland cells; sd, salivary duct. (G) IDFG2 expression in nurse cells (nc) and oocyte (o) in the adult ovary. In the germarium (box), expression is restricted to the posterior region.

salivary gland cells (Fig. 6C), IDGF3 in the small and large salivary gland cells (Fig. 6D), IDGF2 in the imaginal ring (Fig. 6E), and IDGF4 in the imaginal ring, the salivary duct and large salivary gland cells (Fig. 6F). Finally, all IDGF genes are weakly expressed in variable patterns in the imaginal discs (data not shown).

The IDGF labeling in early embryos suggests the presence of maternal mRNA, so we have analyzed IDGF expression throughout oogenesis. As in embryos, the transcript levels are lower for IDGF1 and IDGF3 than for IDGF2 and IDGF4. IDGF transcripts are detected in the nurse cells and the oocyte, but are absent from follicle cells (Fig. 6G). In the germarium, the staining is localized in the posterior region and is absent from the stem cells (Fig. 6G, box).

DISCUSSION

The mitogenic factors we have identified in conditioned medium from imaginal disc cell lines are not related in amino-acid sequence to known growth factors, but instead show high similarity to chitinase enzymes. However, their activity in the nanomolar range supports the idea that they should be considered as true growth factors and we therefore call them Imaginal Disc Growth Factors (IDGFs).

The cooperation between IDGFs and insulin in stimulating imaginal disc cell growth suggests that the IDGFs might function as cofactors of *Drosophila* insulin or a *Drosophila* insulin-like molecule. Insulin promotes growth of *Drosophila* cell primary cultures (Echalier, 1997) and is necessary for the growth of imaginal disc cells (Cullen and Milner, 1991). An insulin-like molecule has been detected in *Drosophila* larval hemolymph by its cross-reactivity with antibodies against mammalian insulin (Seecof and Dewhurst, 1974; Meneses and De Los Angeles Ortiz, 1975), but the *Drosophila* gene has not been reported. In fact, it is not clear whether the *Drosophila* protein is more closely related to insulin or to one of the insulin-like growth factors (IGFI and II).

The gene encoding the *Drosophila* insulin receptor (DIR) has been cloned and analyzed genetically (Ruan et al., 1995; Fernandez et al., 1995). It is expressed in imaginal discs (Garofalo and Rosen, 1988), and in larvae homozygous for receptor mutations the growth of imaginal discs is inhibited (Chen et al., 1996). DIR is a tetrameric glycoprotein composed of two alpha and two beta subunits (Fernandez-Almonacid and Rosen, 1987). As in mammalian receptor systems, the beta subunit contains the transmembrane domain and a ligand-activated tyrosine kinase domain (Petruzzelli et al., 1986; Nishida et al., 1986). Following receptor activation, the receptor tyrosine kinase is thought to activate the Ras/MAPK pathway (Fernandez et al., 1995). The receptor activity appears to be required in embryonic epidermis and central nervous system (Fernandez et al., 1995). DIR binds mammalian insulin with high affinity but fails to bind to IGFI and II or epidermal growth factor (Petruzzelli et al., 1985). Consistent with this finding, bovine insulin at 50 ng/ml was effective, whereas IGFI was ineffective in inducing cell growth of Cl.8+, even when mixed with IDGFs (our unpublished data).

Although the chitinase-related proteins are closely related to chitinases, two of the family members, *Drosophila* DS47 (Kirkpatrick et al., 1995) and human HC gp-39 (Hakala et al., 1993) have been shown to lack chitinase activity. Chitinases have a consensus sequence [LIVMFY]-[DN]-G-[LIVMF]-[DN]-[LIVMF]-[DN]-x-E, in which E (glutamic acid) is known to be critical for chitinase function (Kirkpatrick et al., 1995) and is thought to be the proton donor for the reaction (Watanabe et al., 1993). This glutamate residue is replaced by glutamine in all of the IDGFs, and this substitution is known to abolish catalytic activity in bacterial chitinase (Watanabe et al., 1993). This suggests that the IDGFs may have evolved from chitinases but acquired a new growth-promoting function that does not require chitinase catalytic activity.

The critical glutamic acid in the catalytic core of chitinases is replaced by leucine in HC gp-39. A similar substitution in the catalytic core of human chitotriosidase has been shown to eliminate catalytic activity; but it also confers strong chitin-binding properties on this protein (Renkema et al., 1998). This suggests that the IDGFs and other chitinase-related proteins might be chitin-specific lectins. This property may be important in explaining the mitogenic activity of the IDGFs, and could also be important in their interactions with insulin since the insulin receptor is already known to bind other lectins (Marin-Hincapie and Garofalo, 1995). However, the affinity of the receptor for chitin-specific lectins has not been tested.

The strong expression of IDGF genes in the fat body is consistent with previous reports showing that the fat body produces mitogenic factors. For example, the conditioning of medium by fat body, or coculture with fat body, allows growth of imaginal discs in vitro by cell proliferation (Davis and Shearn, 1977). These conditions also allow regeneration (Fain

and Schneiderman, 1979; M. J. Fain, personal communication) and transdetermination (Shearn et al., 1980) of imaginal disc fragments, events that are generally thought to require cell proliferation, in vitro. The fat body may therefore be an important source of growth factors that support peripheral tissue growth during insect development. The association between lines of IDGF expression and the boundaries of invaginating cell populations during gastrulation suggest a further function for IDGFs during these early embryonic stages. The invagination movements involve dramatic changes in cell shape (Sweeton et al., 1991), which might represent a cellular response similar to the cell-shape changes we see in vitro after treatment of imaginal disc cell lines with IDGFs.

The IDGFs belong to a family that also includes several mammalian secreted glycoproteins of ill-defined function, none of which had been previously recognized as a growth factor. The family includes the human homolog HC gp-39 (= YKL40; 16-23% identical to IDGFs) which was identified as a major secretory product of articular chondrocytes and synovial cells from patients with arthritis (Hakala et al., 1993) and recently shown to be a candidate autoantigen in rheumatoid arthritis (Verheijden et al., 1997). The pig homolog was reported as the heparin-binding glycoprotein gp38k (18-22% identical to IDGFs) which is produced during differentiation of vascular smooth muscle cells (Shackelton et al., 1995). YKL40 and the closely related YKL39 (15-21% identical to IDGFs) accumulate in chondrocyte conditioned medium (Hu et al., 1996). Additional mammalian chitinase-related proteins include Brp-39 (14-19% identical to IDGFs) secreted by certain murine mammary tumors (Morrison and Leder, 1994) and others that are secreted by the mammalian oviduct (Buhi et al., 1996). All of these proteins show a much higher level of sequence identity over certain regions and show strict conservation of several structurally important residues including proline and cysteine (complete sequence alignments and dendrogram posted at http://mamba.bio.uci.edu/~pjbryant/lab/IDGFs/index.htm). The identification and analysis of the IDGFs should help in understanding the functions of these mammalian family members and their relationships to disease states.

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REFERENCES

Buhi, W. C., Alvarez, I. M., Choi, I., Cleaver, B. D., and Simmen, F. A. (1996). Molecular cloning and characterization of an estrogen-dependent porcine oviductal secretory glycoprotein. *Biol. Reprod.* **55**, 1305-1314.

Burke, R. and Basler, K. (1996). Dpp receptors are autonomously required for cell proliferation in the entire developing *Drosophila* wing. *Development* **122**, 2261-2269.

Capdevila, J. and Guerrero, I. (1994). Targeted expression of the signaling molecule decapentaplegic induces pattern duplications and growth alterations in *Drosophila* wings. *EMBO J.* **13**, 4459-4468.

Chen, C., Jack, J. and Garofalo, R. S. (1996). The *Drosophila* insulin receptor is required for normal growth. *Endocrinology* **137**, 846-856.

Cullen, C. F. and Milner, M. J. (1991). Parameters of growth in primary cultures and cell lines established from *Drosophila* imaginal discs. *Tissue Cell* **23**, 29-39.

Currie, D. A., Milner, M. J. and Evans, C. W. (1988). The growth and differentiation *in vitro* of leg and wing imaginal disc cells from *Drosophila melanogaster*. *Development* **102**, 805-814.

Davis, K. T. and Shearn, A. (1977). In vitro growth of imaginal disks from Drosophila melanogaster. *Science* **196**, 438-440.

Denizot, F. and Lang, R. (1986). Rapid colorimetric assay for cell growth and survival: modification to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Methods* **89**, 271-277.

Echalier, G. (1997). *Drosophila cells in culture.* San Diego: Academic Press.

Fain, M. J. and Schneiderman, H. A. (1979). Wound healing and regenerative response of fragments of the *Drosophila* wing imaginal disc cultured *in vitro*. *J. Insect Physiol.* **25**, 913-924.

Fernandez-Almonacid, R. and Rosen, O. M. (1987). Structure and ligand specificity of the Drosophila melanogaster insulin receptor. *Molec. Cell. Biol.* **7**, 2718-2727.

Fernandez, R., Tabarini, D., Azpiazu, N., Frasch, M. and Schlessinger, J. (1995). The Drosophila insulin receptor homolog: a gene essential for embryonic development encodes two receptor isoforms with different signaling potential. *EMBO J.* **14**, 3373-3384.

Garofalo, R. S. and Rosen, O. M. (1988). Tissue localization of Drosophila melanogaster insulin receptor transcripts during development. *Molec. Cell. Biol.* **8**, 1638-1647.

Gelbart, W. M. (1989). The *decapentaplegic* gene: a TGF-beta homologue controlling pattern formation in *Drosophila*. *Development* **107**, 65-74.

Hakala, B. E., White, C. and Recklies, A. D. (1993). Human cartilage gp-39, a major secretory product of articular chondrocytes and synovial cells, is a mammalian member of a chitinase protein family. *J. Biol. Chem.* **268**, 25803-25810.

Hu, B., Trinh, K., Figueira, W. F. and Price, P. A. (1996). Isolation and sequence of a novel human chondrocyte protein related to mammalian members of the chitinase protein family. *J. Biol. Chem.* **271**, 19415-19420.

Kanost, M. R., Zepp, M. K., Ladendorff, N. E. and Andersson, L. A. (1994). Isolation and characterization of a hemocyte aggregation inhibitor from hemolymph of *Manduca sexta* larvae. *Arch. Insect Biochem. Physiol.* **27**, 123-136.

Kirkpatrick, R. B., Matico, R. E., McNulty, D. E., Strickler, J. E. and Rosenberg, M. (1995). An abundantly secreted glycoprotein from Drosophila melanogaster is related to mammalian secretory proteins produced in rheumatoid tissues and by activated macrophages. *Gene* **153**, 147-154.

Kramer, K. J., Corpuz, L., Choi, H. K. and Muthukrishnan, S. (1993). Sequence of a cDNA and expression of the gene encoding epidermal and gut chitinases of Manduca sexta. *Insect Biochem. Mol. Biol.* **23**, 691-701.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with the phenol reagent. *J. Biol. Chem.* **193**, 265-275.

Marin-Hincapie, M. and Garofalo, R. S. (1995). Drosophila insulin receptor: lectin-binding properties and a role for oxidation-reduction of receptor thiols in activation. *Endocrinology* **136**, 2357-2366.

Meneses, P. and De Los Angeles Ortiz, M. (1975). A protein extract from Drosophila melanogaster with insulin-like activity. *Comp. Biochem. Physiol. A* **51**, 483-485.

Morrison, B. W. and Leder, P. (1994). neu and ras initiate murine mammary tumors that share genetic markers generally absent in c-myc and int-2-initiated tumors. *Oncogene* **9**, 3417-3426.

Neuman-Silberberg, F. S. and Schupbach, T. (1993). The Drosophila dorsoventral patterning gene gurken produces a dorsally localized RNA and encodes a TGF alpha-like protein. *Cell* **75**, 165-174.

Neumann, C. J. and Cohen, S. M. (1996). Distinct mitogenic and cell fate specification functions of *wingless* in different regions of the wing. *Development* **122**, 1781-1789.

Nishida, Y., Hata, M., Nishizuka, Y., Rutter, W. J. and Ebina, Y. (1986). Cloning of a Drosophila cDNA encoding a polypeptide similar to the human insulin receptor precursor. *Biochem. Biophys. Res. Commun.* **141**, 474-481.

Peel, D. J., Johnson, S. A. and Milner, M. J. (1990a). The ultrastructure of imaginal disc cells in primary cultures and during cell aggregation in continuous cell lines. *Tissue Cell* **22**, 749-758.

Peel, D. J. and Milner, M. J. (1990b). The diversity of cell morphology in cloned cell lines derived from Drosophila imaginal discs. *Roux's Arch. Dev. Biol.* **198**, 479-482.

Peel, D. J. and Milner, M. J. (1992). The response of *Drosophila* imaginal disc cell lines to ecdysteroids. *Roux's Arch. Dev. Biol.* **202**, 23-35.

Peifer, M., Rauskolb, C., Williams, M., Riggleman, B. and Wieschaus, E.

(1991). The segment polarity gene *armadillo* interacts with the *wingless* signaling pathway in both embryonic and adult pattern formation. *Development* **111**, 1029-1043.

Petruzzelli, L., Herrera, R., Arenas-Garcia, R., Fernandez, R., Birnbaum, M. J. and Rosen, O. M. (1986). Isolation of a Drosophila genomic sequence homologous to the kinase domain of the human insulin receptor and detection of the phosphorylated Drosophila receptor with an anti- peptide antibody. *Proc. Natl. Acad. Sci. USA* **83**, 4710-4714.

Petruzzelli, L., Herrera, R., Garcia-Arenas, R. and Rosen, O. M. (1985). Acquisition of insulin-dependent protein tyrosine kinase activity during Drosophila embryogenesis. *J. Biol. Chem.* **260**, 16072-16075.

Pignoni, F. and Zipursky, S. L. (1997). Induction of Drosophila eye development by decapentaplegic. *Development* **124**, 271-278.

Renkema, G. H., Boot, R. G., Au, F. L., Donker-Koopman, W. E., Strijland, A., Muijsers, A. O., Hrebicek, M. and Aerts, J. M. (1998). Chitotriosidase, a chitinase, and the 39-kDa human cartilage glycoprotein, a chitin-binding lectin, are homologues of family 18 glycosyl hydrolases secreted by human macrophages. *Eur. J. Biochem.* **251**, 504-509.

Ruan, Y., Chen, C., Cao, Y. and Garofalo, R. S. (1995). The Drosophila insulin receptor contains a novel carboxyl- terminal extension likely to play an important role in signal transduction. *J. Biol. Chem.* **270**, 4236-4243.

Schweitzer, R., Shaharabany, M., Seger, R. and Shilo, B. Z. (1995). Secreted Spitz triggers the DER signaling pathway and is a limiting component in embryonic ventral ectoderm determination. *Genes Dev.* **9**, 1518-1529.

Seecof, R. L. and Dewhurst, S. (1974). Insulin is a *Drosophila* hormone and acts to enhance the differentiation of embryonic *Drosophila* cells. *Cell Differ.* **3**, 63-70.

Shackelton, L. M., Mann, D. M. and Millis, A. J. (1995). Identification of a 38-kDa heparin-binding glycoprotein (gp38k) in differentiating vascular smooth muscle cells as a member of a group of proteins associated with tissue remodeling. *J. Biol. Chem.* **270**, 13076-13083.

Shearn, A., Davis, K. T. and Hersperger, E. (1980). Transdetermination of Drosophila imaginal discs cultured in vitro. *Dev. Biol.* 125-133.

Shields, G. and Sang, J. H. (1970). Characteristics of five cell types appearing during in vitro culture of embryonic material from Drosophila melanogaster. *J. Embryol. Exp. Morphol.* **23**, 53-69.

Shilo, B.-Z. (1992). Roles of receptor tyrosine kinases in *Drosophila* development. *FASEB J.* **6**, 2915-2922.

Sweeton, D., Parks, S., Costa, M. and Wieschaus, E. (1991). Gastrulation in *Drosophila*: The formation of the ventral furrow and posterior midgut invaginations. *Dev.* **112**, 775-789.

Theisen, H., Haerry, T. E., O'Connor, M. B. and Marsh, J. L. (1996). Developmental territories created by mutual antagonism between Wingless and Decapentaplegic. *Development* **122**, 3939-3948.

Verheijden, G. F., Rijnders, A. W., Bos, E., Coenen-de Roo, C. J., van Staveren, C. J., Miltenburg, A. M., Meijerink, J. H., Elewaut, D., de Keyser, F., Veys, E. and Boots, A. M. (1997). Human cartilage glycoprotein-39 as a candidate autoantigen in rheumatoid arthritis. *Arthritis Rheum.* **40**, 1115-1125.

Watanabe, T., Kobori, K., Miyashita, K., Fujii, T., Sakai, H., Uchida, M. and Tanaka, H. (1993). Identification of glutamic acid 204 and aspartic acid 200 in chitinase A1 of Bacillus circulans WL-12 as essential residues for chitinase activity. *J. Biol. Chem.* **268**, 18567-18572.

Webb, N. R. and Summers, M. D. (1990). Expression of proteins using recombinant baculoviruses. *Techn.* **2**, 173-188