# The *Drosophila lethal(2)giant larvae* Tumor Suppressor Protein Forms Homo-Oligomers and Is Associated with Nonmuscle Myosin II Heavy Chain

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***Abstract.*** Inactivation of the *Drosophila lethal(2)giant larvae* (*l(2)gl*) gene causes malignant tumors in the brain and the imaginal discs and produces developmental abnormalities in other tissues, including the germline, the ring gland and the salivary glands. Our investigations into the *l(2)gl* function have revealed that the gene product, or p127 protein, acts as a cytoskeletal protein distributed in both the cytoplasm and on the inner face of lateral cell membranes in a number of tissues throughout development. To determine whether p127 can form oligomers or can stably interact with other proteins we have analyzed the structure of the cytosolic form of p127. Using gel filtration and immunoaffinity chromatography we found that p127 is consistently recovered as high molecular weight complexes that contain predominantly p127 and at least ten additional proteins. Blot overlay assays indicated that p127 can form homo-oligomers and the use of a series of chimaeric proteins made of segments of p127 fused to protein A, which alone behaves as a monomer, showed that p127 contains at least three distinct domains contributing to its homo-oligomerization. Among the proteins separated from the immuno-purified p127 complexes or isolated by virtue of their affinity to p127, we identified one of the proteins by microsequencing as nonmuscle myosin II heavy chain. Further blot overlay assay showed that p127 can directly interact with nonmuscle myosin II. These findings confirm that p127 is a component of a cytoskeletal network including myosin and suggest that the neoplastic transformation resulting from *l(2)gl* gene inactivation may be caused by the partial disruption of this network.

RECENT developments have revealed the fundamental role played by cytoskeletal proteins underlaying the plasma membrane in the control of cell proliferation and the development of tumors (Tsukita et al., 1993; Rubinfeld et al., 1993; Su et al., 1993). A causal relationship between the tumorous phenotype and the disruption of the cytoskeleton has been previously inferred from studies showing that the expression of cytoskeletal elements, such as tropomyosin, α-actinin, vinculin, and gelsolin, was profoundly altered in a number of tumor cells (Cooper et al., 1987; Rodriguez et al., 1992; Glück et al., 1993; Vandekerckhove et al., 1990). The data provided so far support the idea that the cytoskeletal defects observed in tumor cells were more the consequence rather than the cause of neoplastic transformation. However, this conception has been recently reversed by the findings that human cancer susceptibility genes and *Drosophila* tumor suppressor genes encode components of submembraneous junctional plaques or elements interacting with these structures (Tsukita et al., 1993).

In humans, the sequence of the tumor suppressor protein of neurofibromatosis type 2 (*NF2*)[1] displays similarities with the sequence of the ezrin-radixin-moesin band 4.1 (*ERM*) proteins (Trofatter et al., 1993; Rouleau et al., 1993). The members of this highly related protein family are known to undercoat the plasma membrane at junctional sites and thought to stabilize the association of the actin network with the plasma membrane (Sato et al., 1992; Algrain et al., 1993). Thus, on the basis that the *NF2* protein shares sequence homology with the *ERM* proteins, it has been concluded that *NF2* tumorigenesis arises from a disruption of the membrane cytoskeleton. Moreover, analysis of the adenomatous polyposis coli (*APC*) gene, in which mutations

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1. *Abbreviations used in this paper*: *APC*, adenomatous polyposis coli; *dlg-1*, *discs large-1*; DMP, dimethylpimelimidate; *ERM*, ezrin-radixin-moesin; *ex*, expanded; *l(2)gl*, *lethal (2) giant larvae*; *NF2*, neurofibromatosis type 2.

predispose an individual to colon cancer, has revealed a direct interaction of the *APC* protein with β-catenin (Rubinfeld et al., 1993; Su et al., 1993), a protein associated with the intracellular domain of the cell adhesion protein E-cadherin (McCrea et al., 1991). This finding suggests that the *APC* protein is a component of junctional structures connecting cell-surface proteins to intracellular cytoskeleton-based receptors and can, thus, transmit information from the adhesion receptors localized at the cell surface to the interior of the cell. Involvement of junctional structures in such a signalling pathway is further strengthened by the similarity shown between the amino acid sequences of β-catenin and the *Drosophila* segment polarity protein, *armadillo* (Peifer et al., 1991, 1994).

Molecular cloning of several tumor suppressor genes in *Drosophila* has also revealed that the encoded proteins may be elements of the cytoskeleton or may contribute to the organization of cytoskeletal networks. The protein encoded by the tumor suppressor gene *lethal(2)giant larvae* (*l(2)gl*), designated as p127, was shown to participate to a cytoskeletal network undercoating the plasma membrane and/or extending in the cytoplasm (Strand et al., 1994). At the cell periphery p127 has been found to be tightly associated with the inner face of the plasma membrane and to form detergent-insoluble aggregates displaying similar characteristics with components of mammalian junctional complexes. Furthermore, examination of the distribution of p127 in a variety of *Drosophila* tissues at various developmental stages has revealed a recurrent enrichment of p127 in regions of cell to cell contact on the plasma membrane. This recurrent pattern suggests that the association of p127 with the plasma membrane may not only be important for establishing and maintaining cell architecture, and particularly cell polarity, but may also play a role in a signaling pathway regulating cell growth.

Involvement of other *Drosophila* tumor suppressors in specialized membrane domains has been essentially assigned by virtue of sequence motifs which have been previously identified in known mammalian junctional proteins. In the case of the *discs large-1* (*dlg-1*) gene whose inactivation leads to neoplastic transformation of the imaginal disc cells, its protein sequence was first found to display similarities with motifs present in guanlyate kinases (Woods and Bryant, 1991); this and other motifs in *dlg-1* are also shared by the ZO-1 protein, a component of mammalian plaque junction (Itoh et al., 1993; Woods and Bryant, 1993; Tsukita et al., 1993). This latest assignment is more in agreement with the immunostaining data showing that *dlg-1* is essentially a peripheral protein associated with septate junctions (Woods and Bryant, 1991). Similarly, the sequence of the protein encoded by the *expanded* gene, in which mutations cause hyperplasia of the imaginal discs, exhibits a series of motifs with limited similarities to sequences present among the members of the *ERM* protein family (Boedigheimer and Laughon, 1993; Boedigheimer et al., 1993). Finally, the *fat* gene, in which mutations lead to hyperplasia of the imaginal discs similar to the *expanded* overgrowth, encodes a putative protein made of 34 tandem cadherin repeats, four EGF-like repeats, a single transmembrane domain and a cytoplasmic domain (Mahoney et al., 1991). These features indicate that the *fat* protein can be a transmembrane protein with a large extracellular domain displaying characteristics of cell adhesion molecules. Analysis of the cellular receptors interacting with the cytoplasmic domain of *fat* may help in identifying new components of the membrane cytoskeleton involved in growth regulation.

The importance of the role played by peripheral membrane proteins in the control of cell proliferation is further enhanced by the findings that an increasing number of growth factor receptors, such as PDGF and EGF receptors (Fazioli et al., 1993; van Bergen en Henegouwen et al., 1992; Ridley and Hall, 1992) and proto-oncogene products, such as Src, Abl, Yes, and Dbl (Kaplan et al., 1990; van Etten et al., 1994; Tsukita et al., 1991; Graziani et al., 1989), are known to be intimately associated with membrane cytoskeletal proteins. Co-localization to membrane cytoskeleton of both tumor suppressor and proto-oncogene proteins suggests that the functions of the proteins may be more interwoven than first thought.

To study the function of the *l(2)gl* gene, we have begun to identify cellular proteins which physically interact with p127. We have chosen a biochemical approach rather than a genetic analysis for two reasons. First, presently available *l(2)gl* mutations consist essentially of large deletions uncovering partially or entirely the gene (Mechler et al., 1985) and are therefore not suitable for identifying interacting genes. Second, hypomorphic mutations, such as temperature sensitive alleles of *l(2)gl*, would only allow the identification of genes whose products bind to the domain of p127 altered by the ts mutation and may thus only define a limited subgroup of all the genes whose products may potentially interact with p127. Moreover, if p127 is a cytoskeletal protein, we would expect that p127 will bind with strong enough affinities to other proteins so that we can purify them by virtue of their direct binding of p127 or through a linker protein. Therefore a biochemical approach may help to define some of the major components interacting with p127 independent of the nature of their respective function. In this study we show that p127 is essentially recovered in high molecular weight complexes made of oligomerized p127 and containing at least 10 additional proteins. One of these proteins with an apparent molecular weight of about 200 kD was purified to homogeneity and, following proteolytic digestion and microsequencing of a series of peptides, was shown to correspond to non muscle myosin II heavy chain, demonstrating that p127 is indeed a component of a cytoskeletal network.

## Materials and Methods

### Gel Sieving Chromatography

Embryonic cells from Oregon R embryos were prepared using discontinuous metrizamide gradients as described in Strand et al. (1994). Cells isolated from ~1 g of embryos were lysed using a tight fitting Dounce homogenizer in 1 ml PBS and centrifuged at 45,000 rpm for 15 min at 4°C in a Beckman TLA45 rotor. The supernatant (0.5 ml) was loaded onto a 1.5 × 35 cm Superose 12 (Pharmacia LKB Biotechnology, Uppsala, Sweden) FPLC column equilibrated in PBS and eluted at a rate of 0.5 ml/min equilibrated in PBS. 0.5 ml fractions were collected from 12 to 20 ml. The proteins contained in each fraction were analyzed by SDS-PAGE and the presence of p127 was detected by Western blotting. The elution profile for each standard protein as well as the void volume (using blue dextran) was determined in separate runs. Gel filtration of protein A–p127 fusion proteins was performed using identical conditions.

### Native Gel Electrophoresis

"Pore exclusion limit electrophoresis" was performed essentially as de-

scribed by Clos et al., 1990. Lysates of *Drosophila* embryos were incubated with anti-peptide antibodies (immunizing peptides derived from COOH or $NH_2$ termini of p127, Strand et al. (1994) and protein A-Sepharose. After washing, the immunoprecipitated p127 complexes were eluted by addition of 100 μg/ml immunizing peptides in PBS and rotation over night at 4°C. The eluate was collected and concentrated into kinase buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, and 1 mM DTT) using a Centricon-100 micro-concentrator (Amicon Corp., Arlington Heights, IL). An aliquot of the eluted sample was analyzed by SDS-PAGE and Western blotting for the presence of either p127 or anti-p127 antibodies released during the elution of p127 complexes. This analysis revealed the presence of p127 and the absence of antibodies in the material eluted following addition of immunizing peptide (data not shown). In the remaining fraction of the eluted material, the p127 complexes were radiolabeled using the endogenous p127-associated kinase by addition of 10 μCi [$^{32}$P-γ]ATP for 30 min at room temperature. After desalting through a Sephadex G-50 column, the labeled proteins recovered in two fractions were electrophoresed on a 4–12% polyacrylamide and 3–10% glycerol gradient gel in 0.5%× TBE buffer for 18 h at 4°C (20 V/cm). High molecular weight calibration proteins (Pharmacia LKB Biotechnology) were electrophoresed in parallel. After electrophoresis, the gel was stained in Coomassie blue, fixed, and dried for autoradiography.

## Expression of Protein A-p127 Fusion Proteins

Portions of the *l(2)gl* sequence derived from the cDNA clone Ecl73 (Jacob et al., 1987) were expressed in *Escherichia coli* as fusion proteins with protein A in the vector pRIT2T using the protein A system (Pharmacia), and purified using affinity chromatography on IgG-Sepharose as recommended by the supplier (Pharmacia LKB Biotechnology). In general, final fusion protein preparations were essentially made of full-length protein, with one minor contaminating bacteria specific protein that eluted as a monomer in the gel filtration assays and did not interfere with the assays.

## Blot Overlays

Proteins from cell extracts were electrophoresed on a SDS-PAGE gel, and transferred to polyvinylidene difluoride (PVDF) membrane using a Multiphor II semi-dry unit (LKB Instruments, Inc., Bromma, Sweden). Before incubation with labeled p127 probes, the protein blots were first washed in 7.5 M guanidine-HCl, 50 mM Tris-HCl (pH 8.3), 2 mM EDTA, 25 mM dithiothreitol for 30 min at room temperature followed by overnight incubation at 4°C in 10 mM Tris-HCl (pH 7.4), 140 mM NaCl, 2 mM EDTA, 1 mM DTE. The blots were then blocked 2 h at 4°C in PBS/0.1% Tween plus 0.2% Tropix I-Block reagent; Serva Feinbiochemica GmbH, Heidelberg, Germany). $^{32}$P-labeled p127 probe was added and the incubation continued overnight at 4°C. After washing in PBS/0.1% Tween twice for 10 min, the blots were dried and autoradiographed.

To $^{32}$P-label p127 probe, p127 complexes from ~$10^7$ Sf9 cells infected with recombinant baculoviruses expressing the entire coding sequence for p127 were immunoprecipitated with 100 μg affinity-purified anti-p127 antibodies and 100 μl protein A-Sepharose (Strand et al., 1994). The immunoprecipitates were washed into 100 μl kinase buffer and labeled using the endogenous p127-associated kinase with 20 μCi [$^{32}$P-γ]ATP (3,000 Ci/mmole; New England Nuclear, Beverly, MA) for 30 min at room temperature. The labeled immunoprecipitates were washed in 500 μl kinase buffer once to remove the excess unincorporated [$^{32}$P-γ]ATP and then heated at 95°C for 5 min in 100 μl 1× SDS loading buffer, and separated by 7% SDS-PAGE using a Midget 2050 electrophoresis apparatus (LKB Instruments, Inc.). Wet gels were autoradiographed for 10–20 s or until strong bands were observed on the film. A gel slice containing the p127 proteins was excised. The $^{32}$P-labeled p127 was electroeluted (4–5 h, 8–10 mA) in a Bio-Rad Laboratories (Cambridge, MA) electroelution apparatus using 50 mM ammonium carbonate, 0.1% SDS buffer. Approximately 80% of the labeled p127, as judged by the amount of radioactivity of the $^{32}$P-labeled p127, was recovered from the gel slices and used as probe for the blot overlays.

## Affinity Chromatography

For immuno-affinity purification of p127 complexes, 5 mg of affinity purified anti-peptide antibodies raised in rabbits against a synthetic peptide derived from the 21 carboxyl-terminal amino acids of p127 (Strand et al., 1994) were adsorbed onto 1 ml protein A-Sepharose beads (Boehringer-Mannheim GmbH, Mannheim, Germany) in econo columns (Bio-Rad Laboratories). The adsorbed antibodies were coupled to the protein A using 20 mM dimethylpimelimidate (DMP) in 0.2 M sodium borate (pH 9.0) for 1 h at room temperature (Harlow and Lane, 1988). The coupling reaction was stopped with 0.2 M ethanolamine (pH 8.0). After 2 h, the coupled antibody beads were extensively washed in PBS followed by three cycles of washes with 5 ml 0.2 M glycine, (pH 2.8) and neutralized with 10 ml PBS. The efficiency of the coupling was controlled by SDS-PAGE and Coomassie blue staining of samples taken before and after addition of DMP and boiled in SDS loading buffer. Routinely, under the elution conditions used in these experiments, no detectable amount of antibodies was released from the column.

Protein extracts of *Drosophila* embryonic cells isolated over metrazimide gradients as described in Strand et al. (1994) were prepared and allowed to bind to the anti-p127 antibody column by overnight rotation at 4°C. The adsorbed extract was removed by settling of the Sepharose beads and washing the column with 20–30 ml PBS. The p127-binding proteins were eluted with 5 ml 0.5 M $MgCl_2$. The columns were regenerated by washing in 2.5 M $MgCl_2$, followed by 0.2 M glycine, pH 2.8, and PBS to release any remaining p127 from the column. The eluted proteins were concentrated using Centricon-100 concentrators (Amicon Corp.) and were analyzed by SDS-PAGE.

For $p127^{his\text{-}tag}$-affinity purification of p127-binding proteins, a fragment of *l(2)gl* cDNA Ecl73 coding for amino acids 306 to 792 (Jacob et al., 1987) was expressed in *E. coli* as a fusion protein with his-tag sequence and purified by affinity chromatography on $Ni^{2+}$ resin. For production of the fusion proteins, the BclI fragment was cloned into the pET15b vector (Novagen Inc., Madison, WI) in the BamHI site such that the his-tag sequence was located upstream of the *l(2)gl* coding sequence. Expressed fusion proteins were purified to near homogeneity using $Ni^{2+}$ affinity chromatography and its chimeric nature confirmed by immunoblotting with antibodies (pEX215) directed against the central domain of p127 (Strand et al., 1994). Approximately, 30 mg of $p127^{his\text{-}tag}$ protein or 30 mg BSA were coupled to 3.5 ml CnBr-activated Sepharose (Pharmacia LKB Biotechnology). Detergent extracts of *Drosophila* embryos were pre-absorbed by passing over the BSA-Sepharose column before rotation overnight at 4°C on the $p127^{his\text{-}tag}$ column. Both the BSA-control and $p127^{his\text{-}tag}$ column were washed extensively with 100 ml 50 mM Hepes (pH 7.0), 0.5% NP-40 before elution with successive 5 ml washes of 0.05, 0.1, 0.25, 0.5 and 1 M NaCl. Fractions were precipitated with 10% TCA before analysis by SDS-PAGE.

## Protein Sequence Analysis

Proteins to be sequenced were separated by SDS-PAGE, transferred to PVDF membranes (Problot; Applied Biosystems, Foster City, OR) and reversibly stained with Ponceau-S stain to visualize protein bands for excision from the membrane. Excised pieces of membrane were digested according to Fernandez et al. (1992) with Endoprotease-lysine C sequencing grade (Boehringer-Mannheim GmbH) overnight at 37°C. The released fragments were separated using a 130 A HPLC separation system (Applied Biosystem) on a Brownlee C18 reverse phase column (220 × 2.1 mm). Selected peaks were chosen for sequencing using a 477 A protein sequencer apparatus from Applied Biosystems with an on-line HPLC system 120 A. Alternatively, samples were transferred to PVDF membranes and amino acid sequences of native amino termini were determined using a Blot-cartridge device (Applied Biosystems).

## Miscellaneous

SDS-PAGE was carried out according to the method of Laemmli (1970). Western blots were developed using the Tropix chemiluminescence (Serva Feinbiochemica GmbH) or Immunogold (Amersham Intl., Buckinghamshire, UK) systems as recommended by the supplier. Cell and *Drosophila* culture were as described in Strand et al. (1994). Antibodies against human non-muscle myosin II heavy chain were obtained commercially from Paesel-Lorei (Frankfurt, Germany). The catalytic subunit of protein kinase A was obtained from Sigma Chemical Co. (St. Louis, MO). The pre-stained molecular weight marker from Sigma Chemical Co. (SDS-7B) was used to calibrate all SDS-PAGE gels.

# Results

## p127 Is Recovered in High Molecular Weight Complexes

Our previous studies showed that p127 can be located in two cytoplasmic regions in *Drosophila* cells (Strand et al. 1994). We found that p127 is, on the one hand, distributed in the

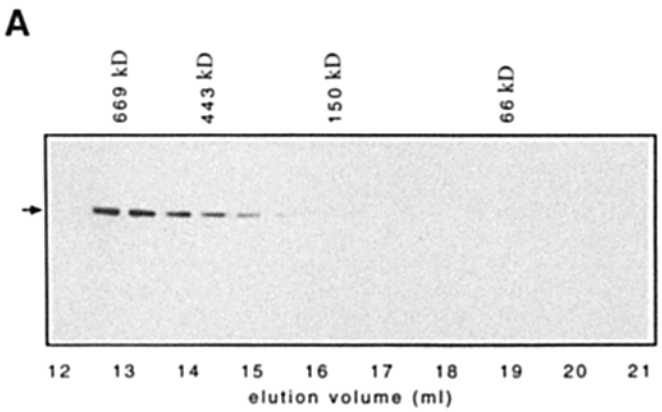



*Figure 1.* Association of p127 in high molecular weight complexes. (*A*) Protein extracts of *Drosophila* embryonic cells were chromatographed through Superose 12 and individual fractions were analyzed for the presence of p127 by SDS-PAGE and Western blotting. Thyroglobulin (*669 kD*), apoferritin (*443 kD*), alcohol dehydrogenase (*150 kD*), and BSA (*66 kD*) were used independently for calibrating the column and eluted as indicated. The arrow indicates the position of p127 in the polyacrylamide gel. (*B*) Pore exclusion limit electrophoresis of p127 complexes. Immunopurified p127 was released from antibody beads by addition of excess p127 peptide against which antibodies have been raised and labeled with [$^{32}$P-$\gamma$]ATP in an in vitro kinase reaction before separation on a non-denaturing 4–12% polyacrylamide and 3–10% glycerol gel for 18 h at 4°C. Before loading, the immunopurified p127 sample was desalted using a G-50 column and lanes *1* and *2* represent the two major fractions in which the desalted material was collected. Lanes *3* and *4* display a longer exposure of the same gel showing the $^{32}$P-labeled protein band migrating with a size of ~130 kD and presumably representing monomeric p127. Markers used (in kD): thyroglobulin tetramer (*1338*), thyroglobulin dimer (*669*), apoferritin (*443*), catalase (*232*), lactate dehydrogenase (*140*), bovine serum albumin (*66*). Bracket indicates the position of p127 complexes and the arrow the position of p127 monomers.

whole cytoplasm and, on the other hand, concentrated along the lateral plasma membrane suggesting a direct association with the plasma membrane. Upon cell fractionation, p127 was accordingly found in two distinct fractions, in a cytosolic fraction and in a membrane-bound fraction. These analyses revealed also that the binding of p127 occurs on the inner face of the plasma membrane and is presumably mediated through a cytoskeletal matrix whose components remain to be defined.

For analyzing the interactions of p127 with other cellular components, we have determined whether p127 is recovered as single monomeric protein or forms oligomeric complexes. In a first series of experiments we have estimated the native molecular weight of p127 extracted from *Drosophila* embryonic cells by using gel filtration and non-denaturing PAGE methodologies. Fig. 1 *A* shows the results of a gel filtration analysis of embryonic proteins fractionated on a superose-12 column. As determined by SDS-PAGE electrophoresis and Western blotting, p127 eluted in the early fractions of the column. No p127 can be detected in fractions where monomeric p127 is expected to be collected. By comparison to the elution profile of a series of globular protein standards used to calibrate the column, the average size of p127 was estimated to reach a molecular weight of ~600 kD. With the assumption that p127 is a globular protein, these results would indicate that in *Drosophila* embryos p127 is essentially present in the form of oligomeric complexes. However, as a component of the cytoskeleton, it is possible that p127 and/or p127 complexes display a more asymmetric shape rather than a globular conformation. If such is the case, the size estimated from the FPLC elution profile may not reflect the actual size of the structures in which p127 is present.

In a separate experiment we determined the size of the p127 oligomeric complexes by non-denaturing polyacryl-

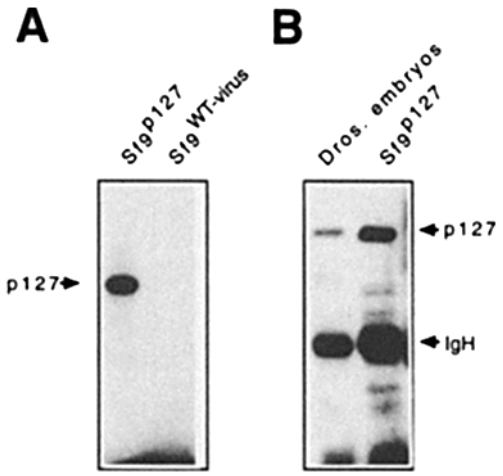



*Figure 2.* p127 binds to itself in a blot overlay assay. (*A*) p127 was immunoprecipitated with C-39 antibody from extracts of Sf9 cells infected with p127 recombinant baculoviruses (*Sf9$^{p127}$*). The immunoprecipitates were incubated with [$^{32}$P-$\gamma$]ATP before separation by SDS-PAGE. Sf9 cells infected with AcNPV (*Sf9$^{WT\text{-}virus}$*) were treated identically as negative control. (*B*) Proteins immunoprecipitated with C-39 anti-p127 antibodies from either *Drosophila* embryos (*Dros. embryos*) or Sf9 cells expressing p127 from baculoviruses (*Sf9$^{p127}$*) were transferred to PVDF membrane after SDS-PAGE. The blot was probed with p127 that was labeled with [$^{32}$P-$\gamma$]ATP in an in vitro kinase reaction as performed in *A*, separated by SDS-PAGE, and electroeluted. IgG heavy chains (*IgH*) from the anti-p127 antibodies which have been used to immunoprecipitate p127 and were released from the protein A-Sepharose beads serve as a positive control for binding of the $^{32}$P-labeled p127 used as probe. For negative controls of proteins that did not bind p127 see Fig. 3, *C* and *D*, (the experiments presented in Figs. 2 and 3 *C* were originally performed on one blot and were separated for the sake of clarity).

amide gel electrophoresis. In this analysis, the proteins are electrophoresed for extended periods of time to the limit of their migration in a non-denaturing polyacrylamide gradient gel. The limit of migration approximates the native size of a given protein or protein complex. For this purpose, we first immuno-purified p127 complexes from extracts of *Drosophila* embryos using anti-p127 peptide antibodies immobilized on Sepharose beads and eluting p127 with an excess of peptides. We concentrated the eluted p127 complexes and in vitro-labeled p127 with [$^{32}$p-$\gamma$]ATP by taking advantage of the presence of a kinase present in the p127 complexes and phosphorylating uniquely p127 (Fig. 2 *A*). The $^{32}$P-labeled p127 proteins were then electrophoresed on a 3–12% polyacrylamide, 5–20% glycerol native gel. As shown in Fig. 1 *B*, the majority of p127 migrated as oligomeric complexes with an estimated molecular weight of 500–800 kD; in addition, some $^{32}$P-labeled material remained near the origin (Fig. 1 *B*, lane *1*), presumably representing very large p127 aggregates. Upon longer exposure of the autoradiogram, we were able to detect a protein band in the size of 130 kD (Fig. 1 *B*, lanes *3* and *4*). Upon shorter exposure, the autoradiogram revealed that the p127 oligomers are distributed in discrete bands with a size difference between each band of ~100–150 kD (Fig. 1 *B*, lane 2). Together with the gel filtration experiments, our results suggest that p127 is able to form high molecular weight complexes with other proteins. However, these data could not formally exclude the possibility that p127 is an asymmetric monomeric molecule and thus we have investigated whether multiple copies of p127 and/or other p127-binding proteins are present in the high molecular weight material containing p127.

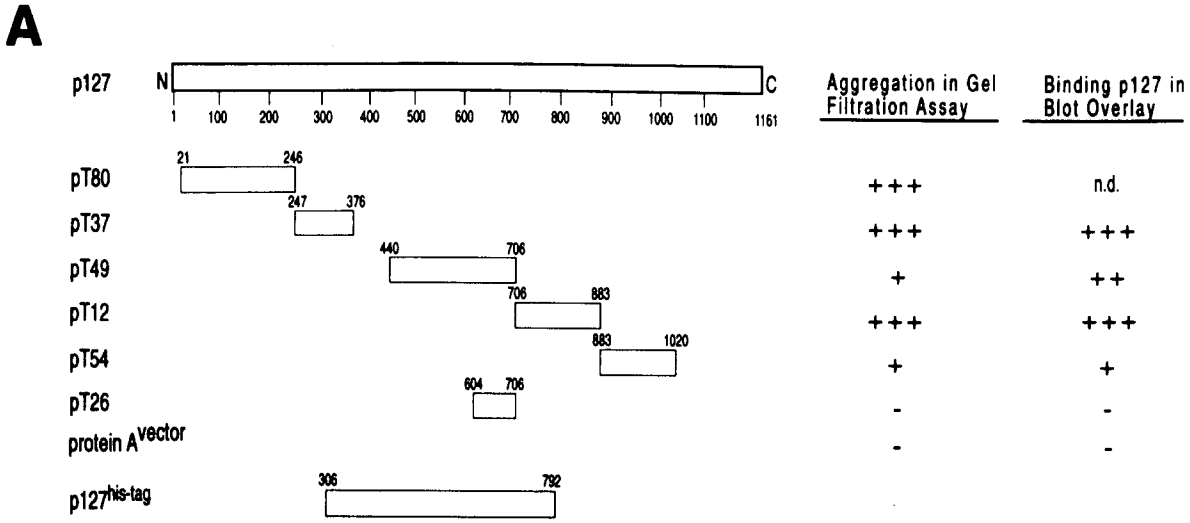
### *p127 Can Be Homo-Oligomerized*

The possibility that p127 may bind to itself was investigated by using a blot overlay procedure. Using this approach we can test whether non-radioactive p127 proteins immobilized on a membrane filter can bind $^{32}$P-labeled p127 proteins. Such an experiment was performed by first immuno-purifying recombinant p127 proteins expressed from baculovirus in *Spodoptera frugiperda* Sf9 cells. The p127 proteins were then in vitro labeled by providing [$^{32}$P-$\gamma$]ATP to the immuno-complexes (Fig. 2 *A*). Similarly to the p127 complexes extracted from *Drosophila* embryos, the recombinant p127 proteins produced in Sf9 cells are associated with a kinase of unknown identity showing the same specificity for p127 as the *Drosophila* kinase (data not shown). To prevent any unlabeled proteins present in the complexes from mediating the binding to p127, we further purified the $^{32}$P-labeled p127 by SDS-PAGE electrophoresis. Then the $^{32}$P-labeled p127 protein was electroeluted and used to probe blots on which immuno-purified p127 proteins from *Drosophila* embryos or Sf9 cells expressing recombinant p127 were immobilized. As shown in Fig. 2 *B*, strong positive signals can be seen at the level of the immobilized p127 protein bands indicating that p127 can bind to itself.

To gain an overview of the domain(s) of p127 which can promote its homo-oligomerization, we have prepared a series of constructs in which distinct portions of the p127 protein were fused to a bacterially expressed reporter protein. We then analyzed whether any of these fusion proteins can form dimers or higher aggregates. Among the available prokaryotic expression systems, we chose to use the pRIT2T protein A gene fusion vector for five reasons. First, protein A behaves as a monomeric protein. Any multimerization of a chimeric protein A should lead to a drastic change in its size. Second, the relatively small size of the portion of protein A expressed in a fused protein, ~28 kD, may result in minimal interference with the folding of the fusion partner into a native configuration. Third, using the pRIT2T protein A gene fusion vector, we found that the bacterially expressed fusion proteins can be recovered under a soluble form in relatively high yields in *E. coli*. Fourth, the IgG-binding domains of protein A provide a rapid, single-step method for purifying the fusion proteins by affinity chromatography on an IgG Sepharose column. Fifth, the same domains can be used for detecting in a one-step immuno-reaction the fused proteins on a Western blot.

As shown schematically in Fig. 3 *A*, a series of fusion proteins made of six p127 polypeptides fused to protein A were expressed in *E. coli*. Each of these fusion proteins was purified by affinity chromatography on an IgG–Sepharose column, eluted and loaded on a Superose 12 column. After gel filtration the distribution of the p127–protein A fusion proteins was determined by SDS-PAGE analysis and Western blotting. As shown in Fig. 3 *B*, the p127–protein A fusion proteins can be separated into three categories: (*a*) those that eluted in fractions where monomers were expected, such as pT26 and the control protein A; (*b*) those that separated as large aggregates, such as pT80, pT37, and pT12; and (*c*) those that were distributed in all fractions from monomeric size to relatively large aggregates, such as pT49 and pT54. Taking into consideration the formation of large aggregates as a reliable measure of protein oligomerization, we can estimate that several domains, at least three, along the p127 protein display strong potential for self-aggregation. Two domains, as revealed by the homo-oligomerization of pT80 and pT37, are located in the $NH_2$-terminal moiety of p127 and the third domain present in pT12 is located in a more COOH-terminal region of p127.

The binding capacities of these fusion proteins were further assessed in a blot overlay assay. Purified p127–protein A fusion proteins were separated by SDS-PAGE electrophoresis, transferred, and immobilized onto PVDF membrane. The blot was further incubated with $^{32}$P-labeled p127 proteins extracted from Sf9 cells. As shown in Fig. 3 *C*, the fusion proteins pT37, pT49 (the intact form as well as a shorter form), and pT12 were able to strongly bind $^{32}$P-labeled p127 proteins, whereas the fusion proteins pT26 and pT54 and the bacterially expressed protein A showed no binding (in this experiment, pT80 was not analyzed). These results reflect those obtained in the previous gel filtration analysis. The fusion proteins exhibiting the highest degree of aggregation were those which were able to bind $^{32}$P-labeled p127. A difference with the gel filtration assay should be however noticed in the case of pT54. Upon gel filtration both fusion proteins pT49 and pT54 displayed a broad elution profile from monomer to high aggregates, indicating the possible presence of weak binding domains, but, in the blot overlay, pT49 bound significantly stronger than pT54 to p127 when the signals are normalized against the amount of proteins loaded (Fig. 3, *C* and *D*). This binding suggests the presence of an additional binding domain in the sequence of pT49. In conclusion, the results of both gel filtration and blot overlay ex-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Aggregation in Gel Filtration Assay
Binding p127 in Blot Overlay
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pT26
604
706
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protein A vector
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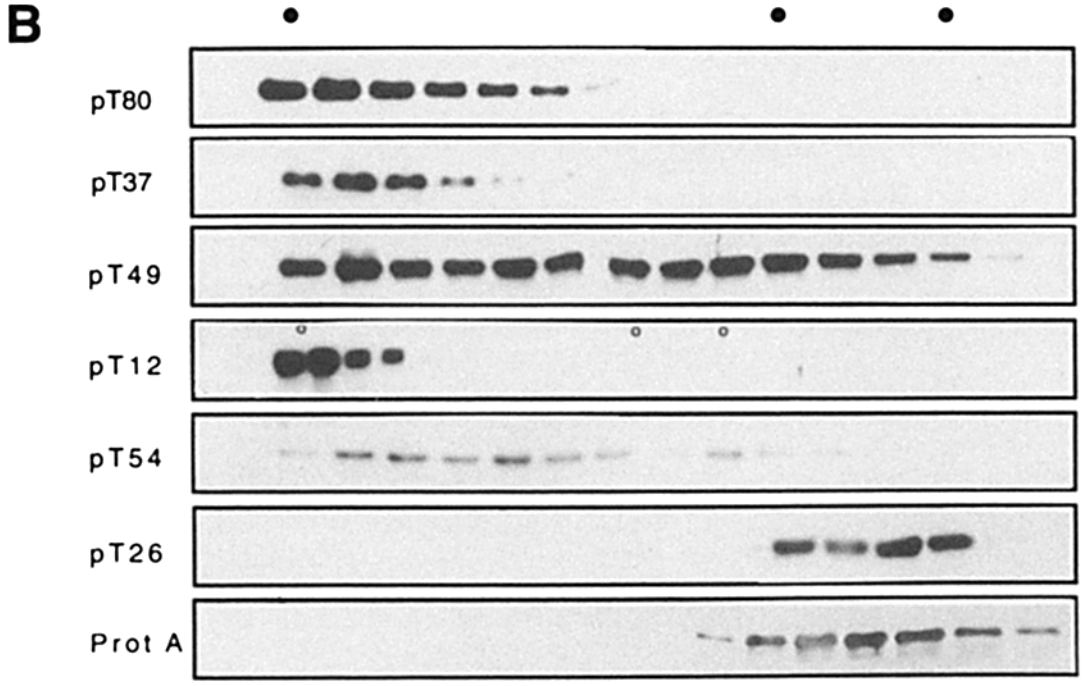
p127 his-tag
306
792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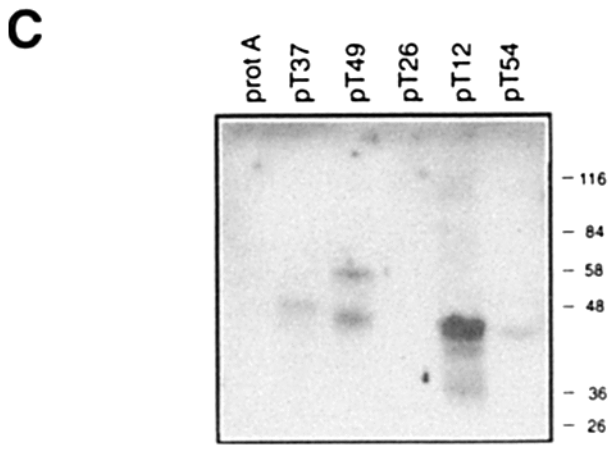
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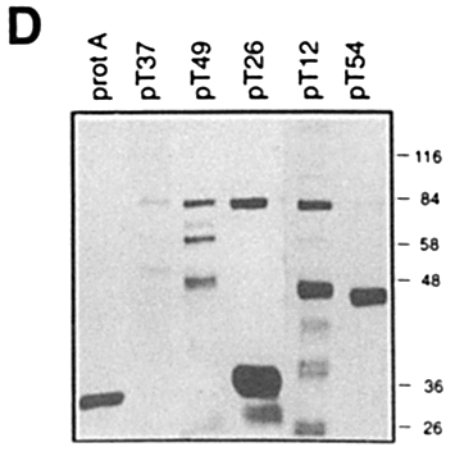
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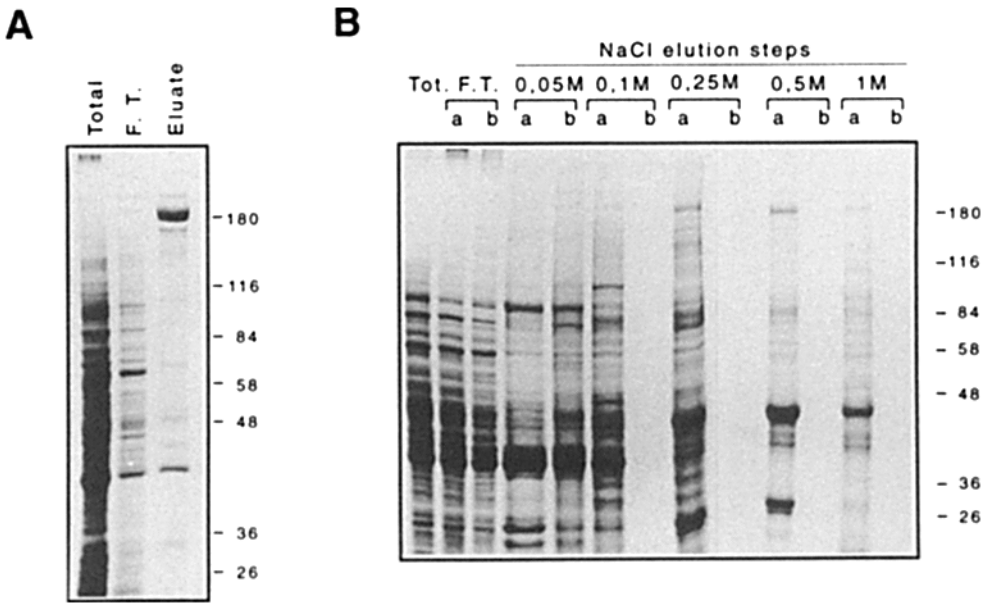


*Figure 4.* Specific sets of proteins are associated with p127 in *Drosophila* embryos. (*A*) Embryonic proteins in starting extract (*Total*), proteins recovered in the flow through (*F.T.*) of the immuno-affinity column, and proteins eluting from the immuno-affinity column with 0.5 M $MgCl_2$ (*Eluate*). Immunoaffinity columns were constructed with 5 mg affinity-purified anti-p127 antibodies coupled to 1 ml protein A–Sepharose. (*B*) Analysis of the proteins recovered in the fractions from the p127 affinity chromatography using (*a*) $p127^{his\text{-}tag}$ (see Fig. 3 *A*) or (*b*) BSA coupled to Sepharose. Detergent extracts of *Drosophila* embryos (*Tot.*) were pre-cleared over the BSA column (*F.T.*, lane *b*) before loading onto the $p127^{his\text{-}tag}$ column. Proteins not bound to the $p127^{his\text{-}tag}$ column are shown in (*F. T.*, lane *a*). After binding both columns were eluted in steps of increasing salt concentration as indicated above the figure. In both *A* and *B*, the proteins separated by 7% SDS-PAGE were stained with Coomassie blue.

periments indicate that p127 is able to form large homo-oligomers resulting from the presence of multiple binding sites dispersed along the p127 molecule.

### *p127-associated Proteins*

To identify the components present in p127 complexes and, more particularly, those interacting directly with p127, we used two different approaches. First, we analyzed the proteins present in large-scale immuno-purified p127 complexes extracted from *Drosophila* embryonic cells and, second, we investigated the *Drosophila* embryonic proteins which can selectively bind to a bacterially expressed p127 protein coupled to a Sepharose matrix. Both approaches provided approximately the same results.

p127 complexes present in detergent extracts of *Drosophila* embryonic cells were first isolated by using affinity purified polyclonal antibodies raised in rabbits against a synthetic peptide corresponding to the 21-carboxyl-terminal amino acids of p127 and coupled to protein A–Sepharose beads. After extensive washes with PBS, the proteins bound to the p127 immunocomplexes were eluted with 0.5 M $MgCl_2$. We used this concentration of $MgCl_2$ because, in other experiments, we have noticed that 0.5 M $MgCl_2$ released the p127-specific kinase present in the p127 complexes but did not affect the binding of p127 to the antibodies (data not shown). Therefore, we dissociated the proteins which specifically interact with p127 in 0.5 M $MgCl_2$ and analyzed them by SDS-PAGE electrophoresis. After Coomassie blue staining of the gel, we detected 10 major bands as well as a series of less intense bands. As shown in Fig. 4 *A*, five of the 10 protein bands with an estimated molecular weight of ~250, 200, 160, 69, and 40 kD, respectively, were present in relatively large amounts. In this large scale preparation, we found that the 200-kD protein band was particularly prominent. The protein contained in this band was selected for further characterization.

In another set of experiments we purified proteins from *Drosophila* cell extracts by virtue of their strong binding affinity to a bacterially expressed p127 fusion protein coupled to a Sepharose matrix. As a source for p127, we constructed a bacterial expression vector in which a large

*Figure 3.* Aggregation and blot overlay assays of protein A-p127 fusion proteins. (*A*) Schematic representation of p127 and six protein A–p127 fusion proteins used in the present assays as well as the length of the $p127^{his\text{-}tag}$ protein used for the affinity chromatography. Numbers correspond to the amino acids from the predicted coding region of the wild type *l(2)gl* gene (Jacob et al., 1987). On the right are summarized the capacities of the p127 domains to homo-oligomerize or to bind p127 in a blot overlay assay as analyzed in *B* and *C*, respectively. (*B*) Purified bacterially expressed protein A–p127 fusion proteins were chromatographed through Superose 12 and individual factions analyzed by SDS-PAGE and Western blotting for the presence of the indicated fusion protein. Dots indicate the elution position of standard proteins: thyroglobulin (667 kD), bovine serum albumin (66 kD), carbonic anhydrase (29 kD) (left to right). The gel slot format used to analyze fractions from the pT12 elution was different. Positions of the standard proteins for this experiment are indicated with open circles. (*C*) Blot overlay assay of protein A–p127 fusions using $^{32}P$-labeled p127 as probe. The binding assay was performed as in Fig. 2 *B*. (*D*) Coomassie blue–stained gel of identical quantities of protein A–p127 fusion proteins used in *C*. The relative molecular weights of the individual proteins are protein A vector (*protA*), 30 kD; *pT37*, 53 kD; *pT49*, 60 kD; *pT26*, 34 kD; *pT12*, 50 kD; *pT54*, 45 kD. We consistently observed an ~84-kD bacterial protein that purifies in our preparations but has not interfered with any of the analyses. The lower molecular weight bands binding in the pT49 and pT12 lanes represent cleaved or shorter forms of these proteins.

portion of the p127 coding sequence (amino acid position 306 to 792) was fused to a his-tag sequence (Fig. 3 *A*, *p127$^{his\text{-}tag}$*). This construct was expressed in bacteria and the p127$^{his\text{-}tag}$ protein was purified to near homogeneity from bacterial lysates. Approximately 30 mg of the recombinant protein were coupled to Sepharose beads in a final volume of 3.5 ml. We have also coupled a similar amount of bovine serum albumin to the same volume of Sepharose beads and have used this material for pre-clearing the protein extracts before loading on the affinity column as well as for making a control column. Proteins extracted from *Drosophila* embryos were prepared, pre-cleared over a BSA column and loaded on, either, the p127$^{his\text{-}tag}$ column or the BSA column. Following extensive washes, the bound proteins were eluted stepwise by increasing NaCl concentrations, desalted, concentrated, and analyzed by SDS-PAGE electrophoresis. As shown in Fig. 4 *B*, a set of proteins was able to bind to the control BSA column but these proteins were totally released from the column following a wash with 0.05 M NaCl. No protein could be detected in the following NaCl elution steps. By contrast, among all the proteins which were bound to the p127$^{his\text{-}tag}$ column, only a subset could be released in the 0.05 M NaCl elution step. Numerous proteins bound with low specificity to p127$^{his\text{-}tag}$ were further released in the two subsequent NaCl washes (0.1 and 0.25 M NaCl). Finally a reduced number of proteins was released in the 0.5 and 1.0 M NaCl elution steps. In these two fractions we could detect approximately 10 major protein bands with three predominant bands in the range of 200, 40, and 23 kD, respectively. These three proteins formed the majority of the eluted material and may correspond to the proteins of similar size present among the proteins released from the immuno-purified p127 complexes. For this reason we decided to further characterize these proteins and, more particularly, the 200-kD protein.

### *Nonmuscle Myosin Type II Can Bind p127*

Our decision to characterize first the 200-kD protein was based on the observation that no other major protein band could be detected on the Coomassie blue-stained gel in the vicinity of the 200-kD protein band (Fig. 4 *B*). Therefore, no other protein in detectable amount could interfere with the isolation and microsequencing of the 200-kD protein. This protein was purified from the 0.5 M NaCl eluate of *Drosophila* embryonic proteins retained on the p127$^{his\text{-}tag}$-affinity column, separated by SDS-PAGE electrophoresis and transferred onto a PVDF membrane. The 200-kD protein band was excised from the membrane and digested in situ with Endoproteinase Lysine C. The released peptides were separated by HPLC and isolated peaks were chosen for amino acid micro-sequencing. Out of seven peaks whose content was sequenced, four were informative. As shown in Table I, the sequencing of the material present in peaks 11 and 13 revealed that each peak is made of a single peptide. By contrast the sequencing of the peaks 18 and 28 showed that each of these peaks contains two peptides. A search in database revealed immediately that the sequences from both unique peptides could be perfectly aligned with the nonmuscle myosin II heavy chain sequence (Ketchum et al., 1990). Comparison of this sequence with the sequences derived from peaks 18 and 28 allowed us to resolve the ambiguities

*Table I. Amino Acid Sequence of Endoproteinase Lysine C Fragments of the 200-kD p127-binding Protein and Their Positions in Cytoplasmic Myosin II Heavy Chain*

| HPLC peak | Sequence | Myosin residue* |
|---|---|---|
| 11 | Lys-Ser-Leu-Glu-Glu-Glu-X-Val-Asn | 1225–1233 |
| 13 | Lys-Try-Leu-Ser-Val-X-X-Asn-Gln-Phe | 58–67 |
| 18 (a) | Lys-Asn-Glu-Leu-Leu-Asp-Ser-Leu-Asp-Thr-Thr-Ala-Ala-Gln-Gln-Glu | 1196–1212 |
| (b) | Lys-Leu-Gln-Gln-Glu-Ala-Glu-Asn-Ile-Thr-Asn-Gln-Leu-Glu-Glu-Ala | 1338–1353 |
| 28 (a) | Lys-Ser-Ala-Ser-Asn-Met-Glu-Ser-Gln-Leu-Thr-Glu-Ala-Gln-Gln-Leu-Leu | 1352–1370 |
| (b) | Lys-Ala-Gln-X-Glu-Leu-Glu-Ser-Gln-Leu-Ala-Glu-Ile-Gln-Glu-X-Leu-X-Ala | 1155–1173 |

* Ketchum et al., 1990

linked to the simultaneous presence of two peptides. For each position of the sequenced peptides we can sort out the relevant amino acid so that the final sequences of both peptides from each peak could be aligned to discrete segments of the sequence of nonmuscle myosin II heavy chain.

To confirm that the 200-kD protein band corresponds to myosin, we probed Western blots of immunoprecipitated p127 and affinity-purified p127 with antibodies raised against human nonmuscle myosin II heavy chain. As shown in Fig. 5 *A*, the 200-kD protein band reacted with the anti-myosin antibodies, corroborating further the identification of myosin

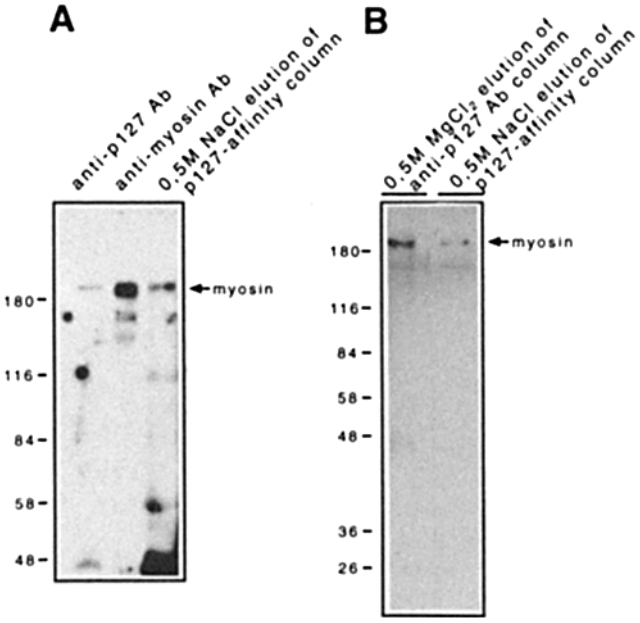


*Figure 5.* Myosin binds p127. (*A*) Blot overlay with $^{32}$P-labeled p127$^{his\text{-}tag}$ protein. Proteins immunoprecipitated with C-39 anti-p127 antibodies (first lane) or anti-myosin antibodies (second lane), and p127 binding proteins released with 0.5 M NaCl from the p127$^{his\text{-}tag}$ column (third lane) were separated by SDS-PAGE and blotted onto PVDF membrane before incubation with labeled p127$^{his\text{-}tag}$. (*B*) Immunodetection of nonmuscle myosin II heavy chain in immuno-affinity-purified p127 complexes (first lane) or in p127 affinity-purified proteins (second lane).

as the 200-kD protein associated with the p127 oligomeric complexes.

We further examined the binding specificity of p127 towards the 200-kD protein, namely myosin, by analyzing whether $^{32}$P-labeled p127 can bind to immobilized myosin on a PVDF membrane. As shown in Fig. 5 *B*, the p127$^{his\text{-}tag}$ protein, labeled in vitro with [$^{32}$P-$\gamma$]ATP with protein kinase A, reacted primarily with the 200-kD myosin band confirming that p127 is able to bind to nonmuscle myosin II heavy chain. These results indicate that the association of p127 and myosin in the isolated p127 complexes results from a direct interaction between these two proteins and is not mediated by the intermediary of another protein acting as a linker between p127 and myosin.

## Discussion

Using a combination of biochemical procedures involving protein purification through gel filtration, immuno-affinity and ligand-affinity chromatography, microsequencing and blot overlay assays, we have found that the *l(2)gl* p127 protein forms high molecular weight complexes, essentially made of homo-oligomerized p127, and interacts physically with nonmuscle myosin II heavy chain. These findings corroborate the previous immuno-histochemical and cell fractionation studies indicating that p127 may be a component of a cytoskeletal network (Strand et al., 1994). In particular, immuno-histochemical analysis has revealed that p127 exhibits a variable pattern of distribution in early embryonic cells, as well as a marked association with the plasma membrane. The intimate association of p127 with the plasma membrane was found to be even more pronounced in certain differentiated cells, such as in the epithelial cells of the larval and adult proventriculus, the midgut and the follicle layer of the ovary. In these cells p127 is essentially localized on the lateral sides of the epithelial cells and absent from the cytoplasm. Furthermore, biochemical investigations have shown that the binding characteristics of p127 to the plasma membrane are similar to those of membrane cytoskeletal proteins in mammalian cells. These observations suggested that p127 may play a dual function participating, on the one hand, to a cytoskeletal network and, on the other hand, to a peripheral membrane matrix.

Peripheral membrane proteins are thought to play a role in signal transduction from adhesion receptors at the cell surface to the transcriptional machinery in the nucleus (Juliano and Haskill, 1991), in the regulation of adhesion molecules at the cell surface (Takeichi, 1991), in the anchoring of the actin cytoskeleton to the plasma membrane (Algrain et al., 1993) and in the structural organization determining cell polarity (Fristrom, 1988). Until recently the majority of the components of the peripheral membrane proteins concentrated at cell junctions have been identified in mammalian cells by means of biochemical procedures, such as protein purification and determination of the amino acid sequence of the purified proteins, either by direct sequencing or by inferring the protein structure from the nucleotide sequence of isolated cDNA. However, recent advances in genetics and developmental biology of *Drosophila* have revealed the existence of insect homologues to a number of vertebrate membrane cytoskeleton proteins, providing new approaches to the study of the role of peripheral membrane proteins in cell cooperation and communication. A series of proteins were shown to contribute to both cell adhesion and signal transduction regulating cell fate during *Drosophila* development, such as the *wingless/wnt-1* cell–cell signaling pathway (Peifer et al., 1991; Oda et al., 1993) and *coracle/Ellipse* interaction involving an EGF-receptor homologue (Fehon et al., 1994), whereas another series of proteins participate in signaling pathways regulating cell proliferation (Merz et al., 1989; Strand et al., 1994; Woods and Bryant, 1991; Tsukita et al., 1993; Boedigheimer and Laughon, 1993).

Among the signaling pathways controlling cell fate, the study of the *wingless* pathway has certainly provided the most information (for review see Peifer et al., 1993). Two junctional components of the *wingless* signaling pathway have been genetically identified, the proteins encoded by the *armadillo* and *disheveled* genes. These two proteins are related to cytoplasmic proteins forming complexes with the intracellular domain of cell adhesion molecules. The *armadillo* protein, which presumably binds to a cadherin homology, is related in sequence to both mammalian $\beta$-catenin (McCrea et al., 1991) and plakoglobin (Peifer and Wieschaus, 1990), which have been defined as components of adherens junctions and desmosomes, respectively. The *disheveled* protein, which is not only required in the *wingless* signaling but also for the polarity of hair and bristles on the body, shares a protein domain with members of the ZO-1 protein family contributing to tight junctions in mammalian cells (Klingensmith et al., 1994; Theisen et al., 1994). In addition, a third protein interacting directly with the *armadillo* protein was found to be related to $\alpha$-catenin, a component of adherens junctions (Oda et al., 1993). In a signaling pathway involving the *Drosophila* EGF receptor, recent genetic studies have revealed that the *Ellipse* mutation, a hypomorphic allele of the EGF receptor gene, can be dominantly suppressed by mutations in the *coracle* gene which encodes a homologue to the vertebrate membrane-skeleton protein 4.1 (Fehon et al., 1994). Originally, the protein 4.1 was identified as a major component of the erythrocyte membrane skeleton linking the spectrin–actin cytoskeleton to the membrane (Marchesi, 1985) and was subsequently found to be expressed in a variety of tissues where it is localized in cellular junctions in the nucleus (Tang et al., 1990; Correas, 1991).

In addition to the p127 protein encoded by the *l(2)gl* gene, two other peripheral membrane proteins involved in the control of cell proliferation have been described so far. One, the product of the *dlg-1* gene (Woods and Bryant, 1991) encodes a protein that shares similarity with the ZO-1 protein (Tsukita et al., 1993) and also contains a domain similar to guanylate kinase. The other protein encoded by the *expanded* (*ex*) gene (Boedigheimer et al., 1993) displays limited similarities to the *ERM* proteins which have been identified as major components of the adherens junctions in mammalian cells (Sato et al., 1992). Both proteins appear to be associated with septate junctions in imaginal disc cells and are necessary for the control of imaginal disc growth, as shown by the disc overgrowth phenotype of *dlg-1* and *ex* mutants. Together, these genetic and molecular experiments indicate that membrane cytoskeletal proteins participate in different intercellular signaling pathways, either, controlling specific cell fate or regulating cell proliferation, and demon-

strate that *Drosophila* and vertebrates have junctional apparatuses composed of essentially identical proteins.

### *pl27 Forms Large Oligomer Complexes*

Using cell fractionation procedures we have previously shown that pl27 can be recovered in both a cytosolic or free fraction and a membrane fraction (Strand et al., 1994). On the membranes we found that a large proportion of pl27 remains resistant to mild detergent extraction indicating that pl27 is forming large aggregates, presumably in association with the membrane cytoskeleton, as suggested by the presence of actin in the pl27 aggregates. In the present study we have first investigated the native size of the cytosolic form of pl27 to determine whether we can establish a precursor/product relationship between the cytosolic and membrane-bound forms of pl27. If the cytosolic pl27 protein were a precursor of the plasma membrane-associated pl27 protein, we would expect to recover cytosolic pl27 proteins as monomers or as moderately oligomerized aggregates. However, we found that the cytosolic pl27 proteins are already assembled in large complexes. No pool of unassembled pl27 molecules could be detected. These results suggest that the association of pl27 with the plasma membrane may depend upon a prior oligomerization of pl27 with itself or with elements of a cytoplasmic skeleton followed by translocation of the pl27 complexes to the cell periphery, or alternatively that the cytoplasmic complex has a separate role of its own.

The findings that: (*a*) pl27 represents the major component of the immuno-affinity isolated complexes; and (*b*) these complexes are forming discrete bands on a native polyacrylamide gel which are separated by a distance corresponding to a size difference of about 130 kD, suggested to us that pl27 is capable of forming homo-oligomers. We explored this possibility further and showed that, in a blot overlay assay, pl27 is indeed able to bind to itself with a high affinity and specificity, despite the relatively harsh conditions used for preparing the pl27 probe and the immobilized pl27 proteins. Further analysis of the oligomerization of internally deleted pl27 proteins has further shown that none of these deletions, each removing approximately a segment of 200–300 amino acids, could prevent the mutated proteins from forming high molecular weight complexes (unpublished data). These results indicated to us that several homo-oligomerizing domains may be scattered along the pl27 protein. By fusing various segments of the pl27 protein to a protein A reporter protein which behaves as a monomer, we were able to show the presence of at least three domains of homo-oligomerization in pl27. Recent investigations have further revealed that the homo-oligomerizing domains could be delimited to three segments of about 50 to 60 amino acids in length, located between amino acid positions 160–213, 247–300, and 706–749, respectively (unpublished data). In blot overlay assays, each of these domains is able to bind to itself and to the other domains suggesting that pl27 can elaborate by itself complex quaternary structures. Additional sites of homo-oligomerization with lower affinity can be detected by gel filtration or binding assays; if these sites are functional they may also contribute to the quaternary structure of pl27. All together our biochemical results show that pl27 can form large molecular weight complexes made of homo-oligomerized pl27 proteins and confirm the histochemical analysis showing that pl27 forms a network of fibrilles and granules in the cytoplasm of various *Drosophila* tissues.

Our analysis of the components of the pl27 complexes isolated by immuno-affinity revealed the presence of 10 other proteins which are consistently detected as Coomassie blue stained bands following polyacrylamide gel electrophoresis. Their size varies between 20 and 240 kD in molecular weight. In the pl27 complexes, these proteins are apparently present in non-equimolar amount with respect to pl27. We always observed an excess of pl27. This indicates that the pl27 complexes are heterogeneous, containing essentially oligomerized pl27 proteins which can bind other proteins in variable amounts. The estimation of the size of the pl27 complex by gel filtration chromatography or native PAGE electrophoresis are critical because we ignore the chromatographic and electrophoretic mobilities of the individual components of the pl27 complex, and in particular of the monomeric form of pl27. Furthermore, the shape of the pl27 complex may also influence its mobility. However, on the basis of the number of different proteins present in the complexes and their molecular ratio and on the findings that both separation procedures gave a similar size for the pl27 complex, we can assume that the estimation of 600 kD corresponds to a good approximation of the size of this complex.

Using an immobilized fragment of pl27 expressed in bacteria we have been able to isolate a series of proteins which bind with high affinity to pl27. Some of these proteins display similar molecular weight to the proteins identified in the immunopurified pl27 complexes. We have isolated a series of these proteins and subjected them to proteolytic digestion and microsequencing of HPLC-purified peptides. This analysis which is not yet completed has already revealed that one of the major components of the pl27 complexes is nonmuscle myosin II heavy chain.

### *Direct Interaction between pl27 and Nonmuscle Myosin II Heavy Chain*

Previous immuno-histochemistry has revealed that during blastoderm formation the pattern of distribution of pl27 displays remarkable similarity with that of skeletal components such as actin, spectrin and myosin (Strand et al., 1994; Young et al., 1991; Dubreuil et al., 1987; Karr and Alberts, 1986). In particular, both pl27 and myosin are concentrated in the cortical periplasm of blastoderm cells, forming cytoskeletal caps over the nuclei. Both proteins move similarly inward into the yolk-free cytoplasm during the cellularization process and are diffusely distributed in the cytoplasm during all the morphogenetic movements. Although the distributions of pl27 and myosin are not always overlapping (for example, the most noticeable discrepancies concern the high concentration of myosin but the absence of pl27 in the contractile ring at the apex of the forming blastoderm cells or in the apical regions of invaginating cells, as well as at the leading edge of cells undergoing dorsal closure), the overall similarity of distribution between both proteins suggests that they may participate to a common cytoskeletal network during early embryogenesis. Our biochemical data demonstrate that myosin can directly interact with pl27. As shown in the blot overlay assay, pl27 binds to myosin with a high

specificity. Although the location and the number of myosin binding sites in p127 have not yet been determined, the use of the bacterially expressed p127$^{his\text{-}tag}$ for isolating myosin from protein extracts of embryos indicates that at least one of the myosin binding sites is located between amino acid positions 306 and 792. The availability of a large series of overlapping segments of p127 fused to protein A will provide tools for determining the location and number of myosin binding sites on p127.

Our work favors the possibility that p127 participates in a cytoskeletal network whose disruption may lead to tissue-specific defects during *Drosophila* development. The malignant transformation of the neuroblasts and ganglion mother cells in the presumptive adult optic centers of the larval brain and the epithelial cells of the imaginal discs is certainly the most dramatic feature of *l(2)gl* gene inactivation. The tumorous cells remain unable to differentiate (Gateff and Schneiderman, 1974) and their morphology is abnormal. In the case of the neuroblasts, the cells remain rounded and invade all areas of the brain hemispheres which, however, at first retain an apparently normal morphology (Hadorn, 1961) before becoming considerably enlarged during the prolonged larval life of the mutant animals. Similarly, the cells in the mutant imaginal discs remain small and cuboidal instead of forming a layer of columnar epithelial cells and lose their apical-basal polarity (Gateff and Schneiderman, 1969; Ryerse and Nagel, 1984). *l(2)gl* tumorous cells are unable to form normal cell contacts and grow as amorphic masses with cell expansion in all directions. All these changes can be interpreted as resulting from the partial disruption of a cytoskeletal network. However this disruption impairs no essential function necessary for cell survival. The *l(2)gl* mutant cells remain viable and capable of performing mitosis and cytokinesis. By contrast, mutations inactivating the *Drosophila zipper* (*zip*) gene, encoding nonmuscle myosin II heavy chain, prevent the completion of embryogenesis (Young et al., 1993). The mutated *zip* embryos fail to complete dorsal closure, indicating that nonmuscle myosin II plays a role in cell movements as well as in maintenance or change of cell shape. The survival of the *l(2)gl*-deficient embryos may result from the perdurance of maternally produced p127 proteins during oogenesis.

Nonmuscle myosin II is thought to participate in cellular activities requiring chemomechanical forces, such as cytokinesis, cell shape change, intracellular vesicle movement, cell surface capping, maintenance of cortical tension, and generation of cell polarity (Spudich, 1989). Although nonmuscle myosin II has been shown to play a critical role in cytokinesis in various organisms (Mabuchi and Okuno, 1977; Kiehart et al., 1982; Knecht and Loomis, 1987), homozygous *zip* embryos display no obvious defects in cytokinesis, presumably due to the maternal contribution of myosin to the embryo. Further genetic and biochemical analyses of nonmuscle myosin II in other organisms have also contributed to the understanding of its function at the cellular level. Disruption of myosin II in *Saccharomyces* reduces considerably the rate of cell growth and division, producing long chains of multinucleated cells which are unable to complete division (Watts et al., 1987), whereas absence of myosin II in *Dictyostelium* essentially blocks cytokinesis (De Lozanne and Spudich, 1987) and impairs locomotion by altering the mechanical properties of the cell cortex (Wessels et al., 1988). These analyses show that myosin II plays a role in a variety of movements and that mutations in different species give rise to distinct phenotypes, reflecting this diversity.

Similarly, mutations in *l(2)gl* and *zip* produce different, if not, opposite phenotypes. *zip* mutations lead to embryonic death, whereas *l(2)gl* mutations produce viable embryos. Furthermore, inactivation of myosin II by antibodies blocks cytokinesis, whereas genetic inactivation of *l(2)gl* results in excessive cell proliferation in larval brain hemispheres and imaginal discs. However, this paradox does not preclude a functional interaction between p127 and myosin. For example, myosin–p127 complexes may exert a stabilizing influence on the newly formed lateral plasma membrane following cytokinesis and may play a role in maintaining cell shape. This is particularly evident in the tumorous *l(2)gl* imaginal disc cells which are abnormal in shape and in which the surface area of lateral cell contact is strongly reduced (Ryerse and Nagel, 1984). The assembly of myosin into filaments and its translocation from the cytoplasm to the cell membrane are thought to be regulated by phosphorylation (Ravid and Spudich, 1989; Pasternak et al., 1989; Ravid and Spudich, 1992; Yumura and Kitanishi-Yumura, 1992). Interestingly, one of the components of the p127 complexes is a kinase which specifically phosphorylates p127 at serine residues and we have recently found that in vitro phosphorylation induces the release of the p127 associated to the plasma membrane (unpublished results). Thus it will be of interest to examine the relationship of this kinase with p127 and myosin II and determine whether p127 may be involved in the regulation of the myosin function.

In conclusion, we describe the physical interaction between the p127 tumor suppressor protein and a known component of the cytoskeletal network, namely, nonmuscle myosin II heavy chain. Future genetic and biochemical works will show whether any of the other components interacting with p127 may participate in a signal transduction pathway regulating cell growth as well as contribute to the organization of the cell architecture. These studies will help to understand the mechanisms leading to neoplastic transformation.

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