kakapo, a Gene Required for Adhesion Between and Within Cell Layers in *Drosophila*, Encodes a Large Cytoskeletal Linker Protein Related to Plectin and Dystrophin

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Abstract. Mutations in *kakapo* were recovered in genetic screens designed to isolate genes required for integrin-mediated adhesion in *Drosophila*. We cloned the gene and found that it encodes a large protein (>5,000 amino acids) that is highly similar to plectin and BPAG1 over the first 1,000–amino acid region, and contains within this region an α-actinin type actin-binding domain. A central region containing dystrophin-like repeats is followed by a carboxy domain that is distinct from plectin and dystrophin, having neither the intermediate filament-binding domain of plectin nor the dystroglycan/syntrophin-binding domain of dystrophin. Instead, Kakapo has a carboxy terminus similar to the growth arrest–specific protein Gas2. Kakapo is strongly expressed late during embryogenesis at the most prominent site of position-specific integrin adhesion, the muscle attachment sites. It is concentrated at apical and basal surfaces of epidermal muscle attachment cells, at the termini of the prominent microtubule bundles, and is required in these cells for strong attachment to muscles. Kakapo is also expressed more widely at a lower level where it is essential for epidermal cell layer stability. These results suggest that the Kakapo protein forms essential links among integrins, actin, and microtubules.

Key words: integrins • cell adhesion • *Drosophila* • cytoskeleton • extracellular matrix

The integrin family of cell surface receptors was named for its proposed role in integrating the extracellular matrix and the cytoskeleton (Hynes, 1987), which remains one of the crucial functions of this diverse set of receptors. However, the mechanisms by which integrins become connected to the cytoskeleton are not yet clear despite the use of a variety of diverse experimental approaches to address this question.

One of the best-characterized subcellular sites of integrin function is the focal adhesion site, where integrins mediate adhesion to the extracellular matrix and the cytoskeleton becomes organized so that actin stress fibers terminate at the focal adhesions (Burridge et al., 1988; Craig, 1996). Two of the proteins that are concentrated at focal adhesions—talin and α-actinin—have been shown biochemically to interact directly with integrin cytoplasmic tails (Horwitz et al., 1986; Otey et al., 1990), but it is not known whether direct binding of these proteins to the integrins is essential for the integrin–cytoskeletal linkage within the cell. By using anti-integrin antibodies coupled to small beads to cluster integrins, Miyamoto and colleagues (1995*a*) were able to show that before integrins bind to their extracellular ligands, two proteins are associated with the clustered integrins: focal adhesion kinase and tensin. After ligand binding, many additional proteins colocalize with the integrins, including the cytoskeletal proteins talin, vinculin, and actin filaments, as well as many signaling molecules such as Src, Grb2, Csk, and Crk (Miyamoto et al., 1995*b*). A number of groups have succeeded in identifying proteins that can bind directly to integrin tails within the cell using two-hybrid screens in yeast (Shattil et al., 1995; Hannigan et al., 1996; Kolanus et al., 1996). However, these molecules, such as cytohesin and integrin-linked kinase, do not appear to be components of the cytoskeleton, but instead are more likely to function during signaling. Thus, the direct link between integrins and the cytoskeleton is not completely understood, partly because so many proteins colocalize with integrins at focal adhesions that it is difficult to determine which of the molecular connections are essential for this link. This problem is exacerbated by the fact that many of these proteins have multiple binding sites for other colocalized proteins (e.g., Burridge et al., 1992).

Colocalization of signaling molecules with integrins

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raises an alternative possibility: that the role of integrins in linking the extracellular matrix to the cytoskeleton is not a structural one but a signaling one, activating a signaling cascade that leads to linkage of the cytoskeleton to other transmembrane proteins. At present it seems most likely that the integrins perform both a structural and a signaling role, but it is not known what is the relative importance of the two activities at particular sites of integrin function.

Recent progress in identifying the genes associated with hereditary forms of junctional epidermolysis bullosa, a skin-blistering disease, has demonstrated the importance of the $\alpha_6\beta_4$ integrin in the adhesion of the epidermis to the underlying dermis (for review see Uitto and Pulkkinen, 1996). Two transmembrane proteins—the integrin $\alpha_6\beta_4$ and bullous pemphigoid antigen 2 (BPAG2)[1]—bind to laminin 5 and collagen type VII, and are linked to cytokeratin filaments by plectin (also called HD1) and BPAG1. Plectin and HD1 have an intermediate filament-binding domain at their carboxy termini that is similar to that found in the desmosome component desmoplakin. Plectin and some isoforms of BPAG1 also have an actin-binding domain at the NH_2 terminus similar to the one found in α-actinin, β-spectrins, and dystrophin, suggesting that an important function of this class of proteins is to provide links among the different cytoskeletal filaments (Ruhrberg and Watt, 1997). Mutations in the genes encoding these proteins, or automimmune antisera against them, cause skin blistering (reviewed in Ruhrberg and Watt, 1997), and the cellular nature of the defect is consistent with the position of the protein in the link between the extracellular matrix and the cytoskeleton. Thus, mutations in the extracellular ligands or the $\alpha_6\beta_4$ integrin subunits cause detachment of the epidermis from the dermis, whereas mutations in BPAG1 or plectin cause the basal layer of the epidermal cells to break in half, with the basal surface remaining attached via hemidesomosomes to the dermis, and the apical surface remaining attached to the rest of the epidermis by its desmosomal linkage (Guo et al., 1995; McLean et al., 1996). These observations support the model of integrins directly linking the extracellular matrix to the cytoskeleton, although the fact that β_4 has a much longer cytoplasmic tail than the other β subunits may make this a specialized case.

To identify additional proteins that are required for integrin-mediated adhesion, genetic screens have recently been performed in *Drosophila* for mutations with the same phenotype as mutations in the genes encoding the position-specific (PS) integrins (Prout et al., 1997; Walsh and Brown, 1998). The PS integrins are most similar to the vertebrate β_1 family (reviewed in Brown, 1993). These screens used the FLP-FRT method (Golic, 1991; Xu and Rubin, 1993) to generate clones of cells that are homozygous mutant for newly generated mutations. The screen is based on the fact that clones of cells mutant for the PS integrin subunits cause a wing blister in the developing wing because the mutant cells fail to adhere to the opposing layer of wild-type cells in the wing bilayer (e.g., Brower and Jaffe, 1989). Systematic screens for mutations that cause the same defect identified 17 new complementation groups that are likely to encode essential components of integrin-mediated adhesion. Here we show that this screen has successfully identified proteins that are likely to link integrins to the cytoskeleton. We have cloned the *kakapo* locus and found that it encodes a large cytoskeletal protein that is similar at the amino terminus to the hemidesmosome components plectin and BPAG1. In contrast to these proteins, the Kakapo protein contains motifs from dystrophin and the growth arrest protein Gas2 at its carboxy terminus, instead of containing an intermediate filament-binding domain. The pattern of expression of Kakapo and certain aspects of its embryonic phenotype demonstrate that it is required for integrin-mediated adhesion in the embryo as well as in the wing. Thus, members of the plectin (or plakin) family of cytoskeletal linker proteins are not restricted to linking the unusual $\alpha_6\beta_4$ integrin to intermediate filaments, but are more widely involved in integrin adhesion events.

Materials and Methods

Drosophila Strains

The *kakapo* alleles used in this study were 18 of the 19 kakapo alleles isolated by Walsh and Brown (1998; one has been lost), and the lethal P-element insertions *l(2)k03010* (kak^{P1}) and *l(2)k03405* (kak^{P2}; I. Kiss collection, Berkeley Drosophila Genome Project). We used two deficiencies for *kakDf(2R)MK1* (50B3-5 and 50D1-4; a kind gift from V. Hartenstein and T. Volk and *Df(2R)CX1* [Bloomington *Drosophila* Stock Center, Bloomington, IN]). Other *P* insertions that were tested and found not to be allelic to *kak* were *l(2)248*, *l(2)3105*, *l(2)4845*, *l(2)5488*, *l(2)k08121*, *l(2)k08708*, *l(2)k04204*, *l(2)10626*, and *C6-2-29* (Bloomington Stock Center and Berkeley Drosophila Genome Project). To demonstrate that the *kak* mutations are associated with the P-element insertions in kak^{P1} and kak^{P2}, they were jumped out by crossing in Δ2-3 transposase, outcrossing, and screening for loss of the w^+ marker, and then checked for viability over *Df(2R)CX1*. Both insertions reverted to viability over the deficiency at a high frequency (data not shown).

Isolation and Sequence Analysis of cDNA and Genomic Clones

Genomic DNA adjacent to the site of insertion of *l(2)k03010* and *l(2)k03405* was isolated by cutting genomic DNA from heterozygous flies with XbaI or EcoRI and ligating 10 μg of each in 1 ml to circularize the DNA. The DNA was transformed into competent cells, and rescued plasmids were selected for by Ampicillin. This procedure yielded genomic fragments that were used to screen a λ genomic library (a kind gift of R. Blackman, University of Illinois, Urbana, IL) and 12–24 h embryonic and imaginal disc plasmid cDNA libraries (Brown and Kafatos, 1988). The site of both P-element insertions map to the same nucleotide in the second intron of the *kakapo* gene (data not shown). The initial clones ended in intron sequence, so a 3′ end fragment of the cDNA was used to walk toward the 3′ end of the gene. We were greatly assisted in our characterization of the *kakapo* transcript by sharing data with D. Strumpf and T. Volk (see accompanying paper), who were walking from the opposite end. The cDNA clones were sequenced on both strands (Cambridge Biochemistry Department facility) by synthesizing 25 specific primers (Genosys, Pampisford, UK), and were assembled using Sequencher (Gene Codes Corp., Ann Arbor, MI), MacVector, and AssemblyLign (Oxford Molecular Group, Oxford, UK) into a contig of 17,420 bp for the form A transcript. The sequences of the NH_2-terminal portion of the two isoforms of *kakapo* have accession numbers AJ011924 for form A and AJ011925 for form B. Database analysis was carried out using the BLAST server at Baylor College of Medicine (http://kiwi.bcm.tmc.edu:8088/). Alignments of related sequences were carried out using ClustalW and by eye in MacVector. Phylogenetic analysis of aligned sequences was carried out using the Dayhof matrix and ProtDist in PHYLIP3.572 (J. Felsenstein, University of Washington, Seattle, WA). Accession numbers of the related sequences used

1. *Abbreviations used in this paper:* BPAG2, bullous pemphigoid antigen 2; PS, position-specific.

are: human plectin, Z54367; human BPAG1, I39160; mouse ACF7, U67203; *C. elegans* Kakapo, Z93398; human dystrophin, A27605; human utrophin, S28381; mouse utrophin, Y12229; *Drosophila* β-spectrin, Q00963; human β-spectrin, B27016; *Drosophila* β_H-spectrin, A37792; *Drosophila* α-actinin, A35598; human α-actinin 1, P12814; human α-actinin 2, P35609; human filamin, P21333.

Antibody Production and Purification

To generate polyclonal antisera against the Kakapo (Kak) protein, residues 2–341 of Form A were expressed in bacteria as a fusion to maltose-binding protein using the pMALc-2 vector (New England Biolabs, Hitchin, UK). In this fusion, residues 2–143 are unique to Form A, and residues 144–341 are found in both forms of Kakapo protein. To generate this fusion, the 5′ end of a cDNA clone was amplified using *Pwo* high-fidelity polymerase (Boehringer, Lewes, UK) with the primers GCAGGCCTACATCGCATTCCTACT and CGCCTCGACAATGCTCTTAG. This fragment was cut with StuI and BamHI, and was cloned into pMALc-2 cut with XmnI and BamHI in the strain DH5α.

The fusion protein was purified from inclusion bodies as follows. Protein expression was induced in mid log cultures with 0.3 mM IPTG, and after several hours the cells were harvested by centrifugation. 2 g of induced cells were resuspended in 6 ml of lysis buffer (50 mM Tris-HCl pH 8, 1 mM EDTA, 100 mM NaCl) and protease inhibitor cocktail (Sigma Chemical Co., Poole, UK). Lysozyme was added to 0.3 mg/ml, and the cell suspension was incubated on ice for 20 min. Sodium deoxycholate was added to 1 mg/ml, and the suspension was stirred at 37°C until it became viscous. Then, 40 μg DNase was stirred in until the viscosity dropped. This lysate was centrifuged at 15 krpm for 10 min in a Sorvall SS-34 rotor, and the supernatant was discarded. The pellet was vigorously resuspended in 9 ml of lysis buffer containing 0.5% Triton X-100 and 10 mM EDTA, incubated at room temperature for 5 min, and centrifuged at 12 krpm for 10 min, and the supernatant was discarded. The pellet was gently resuspended in 3 ml of denaturing buffer (8 M Urea, 100 mM NaCl, 50 mM Tris-HCl, pH 8, 1 mM EDTA) and gently swirled at 30°C for 1 h. This suspension was centrifuged at 15 krpm for 15 min, and the supernatant was retained. This supernatant contained almost all the induced protein, and was quantitated by Coomassie staining at ~0.5 mg/ml.

Polyclonal antisera were raised against this protein in two rabbits by Eurogentec (Ougree, Belgium) using their standard protocol. To purify specific antibodies, 100 mg of fusion peptide was Western-blotted onto PVDF membrane, blocked with 0.2% Tween-20 in PBS (PBTw) for 1 h, and then 5 ml of the final bleed serum was added and rocked with the membrane for 2 h. Nonbound proteins were removed with several 10-min washes of PBTw followed by 30 min in 0.15 M NaCl and a final wash in PBS. Antibodies were eluted from the membrane strip by adding 360 μl of 0.1 M glycine-HCl, pH 2.6, for 10 min, and this solution was removed from the membrane and neutralized with 40 μl of 1 M Tris-HCl, pH 8.

Embryo lysates for Western blot analysis were made by homogenizing stage 15–17 embryos in PBS plus 8 M Urea, 0.2% Triton X-100, 0.2% Nonidet NP-40, and 0.2× protease inhibitor cocktail (Sigma Chemical Co.). Samples were run on a 4.2% separating gel with a 3.6% stacking gel in 0.2% SDS, 0.6% Tris, 2.88% glycine, and were then transferred to PVDF membrane in 0.3% SDS, 48 mM Tris, 29 mM glycine for 45 min at 100V. Rainbow markers (Amersham, Little Chalfont, UK) were used to indicate mobilities of 250 and 160 kD. The filter was dried, and then blocked in TBS plus 5% milk, 0.1% Tween-20 overnight at 4°C. Anti-Kakapo serum was diluted 1:500 in TBS plus 3% BSA, and was incubated with the filter for 24 h at 4°C before adding biotinylated anti-rabbit at 1:500 and detecting with the Vectastain reagents as recommended (Vector Labs, Burlingame, CA).

Embryo Immunostaining, Cuticle and Muscle Preparations

To get good staining with the anti-Kakapo antibody we found that we had to fix the embryos with methanol rather than the more standard Formaldehyde fixation (see Fig. 5, *e* and *f* for comparison). Embryos were collected from 14 to 16 h after laying, dechorionated in bleach, and fixed in glutaraldehyde-saturated heptane/methanol for 30 min essentially as described in Thomas and Kiehart (1994). After slow rehydration into PBS plus 0.2% Tween-20 (PBTw), embryos were treated with PBS plus 5% Triton X-100 for 1 h to assist in permeabilizing the cuticle. Subsequent incubations with antisera and washes were in PBTw. The primary antibodies used were: affinity-purified rabbit anti-Kak antisera (1:10); mouse mAb DA1B6 anti-Fasciclin III (1:1; Brower et al., 1980); guinea pig anti-Coracle (1:50; the kind gift of R. Fehon; Fehon et al., 1994); mouse mAb CF6G11 anti-β_{PS} (1:100; Brower et al., 1984); mouse mAb19 anti-groovin (1:1; the kind gift of T. Volk; Volk and VijayRaghavan, 1994); mouse mAb 22C10 (1:25; Fujita et al., 1982); mouse mAb anti-moesin (1:1; the kind gift of D. Kiehart); and mouse mAb anti-αTubulin DM1A (1:50; Sigma Chemical Co.). Secondary antibodies used were FITC-conjugated goat anti–rabbit IgG and biotinylated horse anti–mouse IgG (Vector Labs, Inc., Burlingame, CA), both at 1:200, and a streptavidin Texas red conjugate at 1:200 (Amersham, Little Chalfont, UK). Confocal images of embryos were obtained using a MRC1024 confocal microscope (Bio-Rad Laboratories, Hemel Hempstead, UK).

Cuticles of mutant embryos were prepared by aging embryos for 36 h, and then dechorionating on adhesive tape and dissolving soft tissues with Hoyers:lactate as described in Wieschaus and Nüsslein-Volhard (1986). Cuticles were photographed using an Axiophot microscope (Carl Zeiss, Thornwood, NY) on Tech-Pan film (Eastman Kodak Co., Rochester, NY), and were then scanned using a Nikon Coolscan film scanner (Instrument Group, Melville, NY). Embryonic muscles were visualized and photographed using a Nikon polarized light microscope on hand-devitellinized 20–24-h embryos, mounting them in water, and then flattening them by removing excess water. All images were assembled using Photoshop 4.0 (Adobe Systems, Mountain View, CA), and labels and drawings were added using FreeHand 5.0 (Macromedia, San Francisco, CA).

Results

Cloning kakapo

In a screen for mutations affecting processes requiring integrin adhesion, we previously isolated 19 alleles of an embryonic lethal locus that we called *kopupu* (Walsh and Brown, 1998). This number of alleles is much larger than the number found in other genes on the same chromosome arm (2–9 alleles/gene), demonstrating that *kakapo* is highly mutable, and therefore is likely to be a large gene. Complementation testing revealed that our *kopupu* mutants are allelic to the previously named *kakapo* alleles isolated in a similar screen (Prout et al., 1997), so we now refer to this gene as *kakapo*. The *kakapo* (*kak*) locus was originally mapped to *Df(2R)CX1* (Prout et al., 1997; Walsh and Brown, 1998), and using overlapping deficiencies we further narrowed down the cytological interval containing *kak* to 50B3-50D2, as it is still included within *Df(2R)MK1*. We then tested lethal P-element insertion alleles that had been mapped to this cytological interval (see Materials and Methods), and found that two—*l(2)k03010* and *l(2)k03405*—that map to 50C9-10 are allelic to *kak*, and thus we renamed them kak^{P1} and kak^{P2}. The lethality of both P-element lines can be reverted by jumping out the P-element, as scored by loss of the w^+ marker (data not shown), demonstrating that the *kak* mutations are associated with insertion of the w^+ P-element. We recovered the genomic DNA flanking the site of insertion by plasmid rescue, and found that both P-elements are inserted at exactly the same site.

Using the DNA flanking the P-element, five different cDNA clones were recovered (Fig. 1 *a*). Two of the cDNAs extend to putative 5′ ends, since each contains multiple stop codons before an ATG that initiates a long open reading frame. The two cDNAs encode alternate NH_2-terminal sequences of 143 amino acids (form A) and 32 amino acids (form B) before reaching shared sequences. These two cDNAs represent alternative starts of transcription (data not shown), and the P-elements are inserted into the intron that separates the alternate starts from the

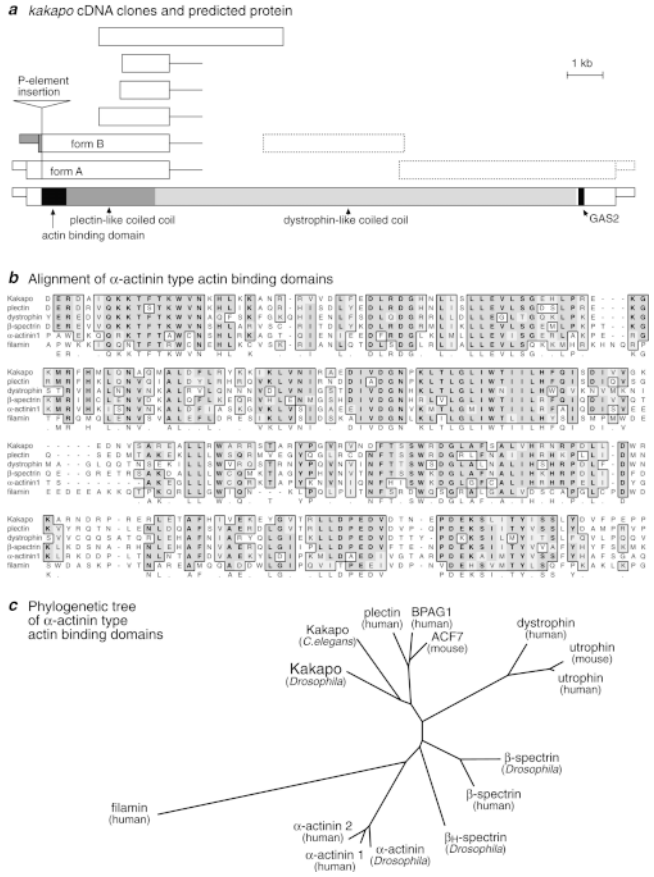

Figure 1. kakapo encodes a large protein containing an actin-binding domain at the amino terminus. (*a*) At the bottom of this panel is a schematic of the *kakapo* form A mRNA, with the open reading frame indicated by the thicker box. The main features of the protein-coding region are marked. Above the mRNA are shown the cDNAs we recovered, with the alternative amino terminus in the form B cDNA indicated in grey, and two of the cDNAs isolated by Strumpf and Volk (1998) shown by dotted lines. The cDNA at the top was isolated in the second round of screening. The P-elements causing the kak^{P1} and kak^{P2} alleles are inserted in intron 2 of both form A and form B transcripts. (*b*) Alignment of the actin domain of Kakapo with human proteins containing a similar domain: plectin, BPAG1, dystrophin, β-spectrin, α-actinin, and the distantly related filamin. Residues common to greater than half of the sequences are boxed; shading and bold type indicate identical amino acids. (*c*) Phylogenetic tree of related actin-binding domains, showing that Kakapo is most similar to plectin, BPAG1, and ACF7. The sequences used are described in Materials and Methods.

shared exons, and therefore are likely to affect both mRNAs by causing premature termination of the transcripts. All five cDNAs have the same 3′ end; however, when we completed the sequence of these clones, we noted that there is a splice donor site just before the end of the open reading frame, and that there is no obvious polyadenylation site close to the poly A tail (not shown). This result suggested that these clones may be copies of incompletely spliced mRNAs that initiate at a run of As in an intron, as previously found in this oligo dT primed library (Brown et al., 1989), and was confirmed by finding a run of As at the end of these clones in the genomic DNA (not shown). We therefore rescreened the libraries with the most 3′ portion of the open reading frame, and recovered one new clone (Fig. 1 *a*, *top*) that, when sequenced, was found to be open throughout its length. At this point, when we had recovered cDNAs and genomic sequence that together encoded a protein of 2,557 amino acids, we exchanged sequences with D. Strumpf and T. Volk and discovered that we were cloning the same gene, and that their cDNA sequence overlapped with ours and extended our cDNA sequence 3′ (see Strumpf and Volk, 1998). Together, the cDNA sequences overlap to give mRNAs of 17,420 nt encoding a 5,497–amino acid protein (form A) and 17,217 nt encoding a 5,385–amino acid protein (form B; Fig. 1 *a*).

The Kakapo Protein is Homologous to Plectin and Dystonin at the NH_2 Terminus, and to Dystrophin at the COOH Terminus

Database searches using the predicted protein sequence of Kakapo revealed striking similarity to three distinct classes of protein. The NH_2-terminal region (residues 135–1200) most closely resembles the plakin class of cytoskeletal cross-linker proteins including plectin, ACF7, and BPAG1/dystonin (Ruhrberg and Watt, 1997). These widely expressed proteins have been implicated in cross-linking actin to intermediate filaments in hemidesmosomes, in stabilizing neuronal structures (Wiche et al., 1991; Bernier et al., 1996; Yang et al., 1996), and when defective, cause epidermal blistering, ataxia, and neurodegeneration (Guo et al., 1995; McLean et al., 1996; Andra et al., 1997; Dowling et al., 1997). The area of strongest similarity is with an actin-binding domain originally defined in α-actinin, but subsequently found in dystrophins and spectrins as well as the plakin family (Dubreuil, 1991). Across this 240–amino acid region, Kakapo shares ~65% amino acid identity with plectin and BPAG1 (Fig. 1 *b*). The high level of conservation suggests that this domain in Kak does bind to actin. We also identified in the database a related sequence from *C. elegans* (CeKak; within cosmid ZK1151) that closely resembles Kak in its actin-binding domain, and phylogenetic analysis of these related actin-binding domains unambiguously places Kak among the plakins or plectin family rather than the spectrin or dystrophin groups (Fig. 1 *c*). The close similarity to plakins continues for the next 1,000 amino acids of Kak sharing ~26% identity with plakins compared with 22% identity with spectrins or dystrophins. This similarity outside the actin-binding domain is to a region in the plakins with no known enzymatic function, but includes a predicted globular head region and much of the subsequent rod domain formed from a coiled coil of alpha helices (Tang et al., 1996). All proteins so far described in the plakin family have a carboxy-terminal domain that binds intermediate filaments, encoded by a single large final exon (Ruhrberg and Watt, 1997). This does not appear to be the case for the Kak protein, which, after residue 1200, has no further sequence similarity with plakins, and instead becomes similar to dystrophin (Fig. 1 *a*).

The central region of Kakapo (amino acids 1,200–4,950) consists of 22 repeats of ~109 amino acids. These repeats are most closely related to those found in the central rod section of the dystrophin family (Koenig et al., 1988), and

are part of the larger family of spectrin repeats (see Strumpf and Volk, 1998 for further analysis). Dystrophin also binds actin, and has been postulated to use the repeat section as a flexible spacer between the cortical actin cytoskeleton and membrane-bound dystroglycan proteins in muscles (Koenig and Kunkel, 1990).

The carboxy terminus of dystrophin contains a cluster of widely conserved domains that bind calcium and mediate interactions with a variety of membrane-bound and regulatory proteins. Kak contains a related but distinct COOH terminus that retains a low level of similarity to the WW domain and Ca^{++}-binding EF hands in dystrophin (see Strumpf and Volk, 1998), but does not have the conserved cysteines or final helices that characterize the dystrophin protein interaction motifs (Brown and Lucy, 1997). Instead, Kak has a region of similarity to Gas2, an actin-associated protein specifically expressed in growth-arrested cultured cells (Brancolini et al., 1992). The region of similarity with Gas2 has not been assigned any specific function, but it is retained in Gas2 deletions or protease cleavage products that give dramatic apoptosis-like rearrangements of the actin cytoskeleton in cell culture (Brancolini et al., 1995). The joining together of segments of the *kakapo* gene that are homologous to different types of protein raised the concern that we had recovered a cDNA from an aberrant transcript that joined exons from adjacent genes. However, in addition to the cDNA we isolated joining the plectin and dystrophin domains, our colleagues isolated a second cDNA that also joins these two regions (Strumpf and Volk, 1998), connecting them at a different position and demonstrating further alternative splicing of this gene. We also recovered two additional cDNAs connecting the Gas2 domain to dystrophin region (data not shown). Thus, we are confident that these diverse domains are linked together in a single protein in *Drosophila*, and like plectin, dystonin, and dystrophin, multiple isoforms are produced (Brown and Lucy, 1997; Ruhrberg and Watt, 1997).

In the Embryo, Kakapo Is Strongly Expressed in the Epidermis at Sites of PS Integrin-mediated Adhesion

Mutations in *kakapo* were isolated because they have the same phenotype as mutations in the PS integrins: when clones of cells lacking these gene products are produced in the developing wing, those cells fail to adhere to the opposing wing layer during pupal development, causing a blister in the adult wing. As a first step in determining whether *kakapo* also has a role in PS integrin-mediated adhesion in the embryo, we wished to determine if they are coexpressed in the embryo. We therefore raised antisera to a fusion protein containing the amino terminus of Kakapo form A (see Materials and Methods). Using affinity-purified anti-Kakapo antisera, we stained embryos and found that Kakapo is strongly expressed in specific epidermal cells (Fig. 2). These are specialized epidermal cells that attach to the muscles, linking the muscles to the exoskeleton (cuticle; e.g., Prokop et al., 1998*a*). Muscle attachment requires the function of the PS integrins, which are strongly expressed at the ends of the muscles and in the epidermal muscle attachment cells (see Brown, 1993).

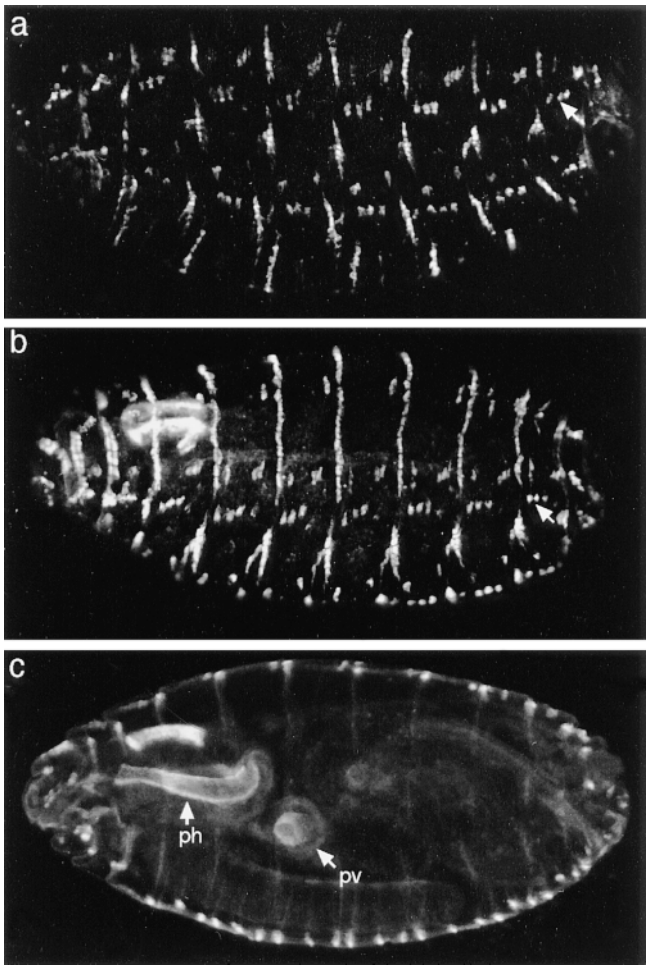

Figure 2. Expression of Kakapo in the embryonic muscle attachment sites and internal tissues. Late stage 16 embryos stained with affinity-purified anti-Kakapo antisera. Lateral (*a*) and dorso-lateral (*b*) surface views of embryos showing expression in the epidermal muscle attachment sites. To help orient the two views relative to each other, the dorsal attachment sites of the transverse muscles in segment A7 are marked in both panels with arrowheads. In *c* the plane of focus is through the interior of the embryo, showing that there is no Kakapo staining in the muscles (see also Fig. 4 *a* and Fig. 5), and that two internal structures, the pharynx (*ph*) and proventriculus (*pv*), express Kakapo (shown in more detail in Fig. 4).

Thus, Kakapo is strongly expressed in the same embryonic cells that express high levels of the PS integrins.

To demonstrate the specificity of our antibody, we tested it on *kak* mutant embryos. Embryos homozygous for the kak^{P2} allele show no staining with our affinity-purified anti-Kakapo antisera, demonstrating that these antibodies are specific for the gene product disrupted by the P-element insertion (Fig. 3, *top*). We confirmed that antibodies could penetrate and label mutant embryos by double staining with mAb19 (Volk and VijayRaghavan, 1994), which still labeled the mutant embryos (Fig. 3, *bottom*). We next used our antibody to determine if the Kakapo protein found in embryos is the size predicted from the cDNA sequence (>600 kD). Western blot analysis of em-

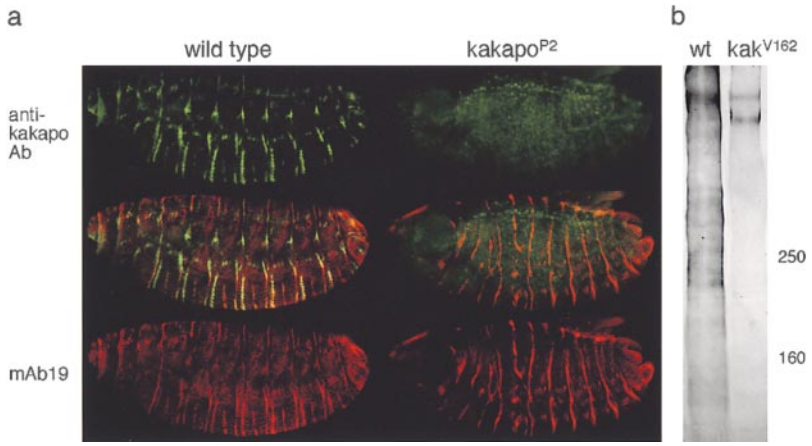

Figure 3. Specificity of anti-Kakapo antisera. (*a*) Affinity-purified anti-Kakapo polyclonal antisera does not stain kakapo mutant embryos. *wild type* (*left*) and *kak*^{P2} mutant (*right*) stage 16 embryos were double-labeled with anti-Kakapo (*green*) and mAb19 (*red*) to confirm that the antibodies penetrate each embryo. The merged channels are shown in the middle, indicating that Kakapo expression is absent in homozygous mutant embryos, while staining of the muscle attachment cells with mAb19 is still observed. (*b*) Western blots using this antiserum detect a high–molecular weight product in wild-type embryo extracts (*wt*). Both wild-type and truncated proteins are observed in extracts from embryos heterozygous for the *kak*^{V168} mutation, confirming that *kakapo* mutants are defective for this gene product, and that the antiserum is specific for Kakapo protein. The position of the 250 and 160-kD marker proteins is shown on the right.

bryo protein shows that the anti-Kak antibody recognizes primarily a single high–molecular weight band in wild-type lysates that migrates much slower than the 250-kD marker (Fig. 3 *b*). Minor amounts of shorter proteins can be seen that may be breakdown products or less-abundant alternative forms. In lysates from embryos heterozygous for *kak*^{V168}, a strong x-ray allele, we detected the wild-type protein and a truncated form (Fig. 3 *b*), showing that the detected protein is modified by a *kakapo* mutation. Thus, the antiserum is specific for Kakapo gene products when used for both immunofluorescence and Western blotting.

Further analysis of Kakapo expression reveals that the protein is present at high levels in all of the epidermal muscle attachment cells (Figs. 2 and 4 *a*): both those that directly attach to the muscles and those that indirectly attach via the tendon matrix (Prokop et al., 1998*a*). We first detected strong Kakapo expression in these cells at mid-late stage 16, which is ~4 h after the muscles first start to attach to the epidermis. During the last two stages of embryogenesis—16 and 17—attachment of the epidermal cells to the muscles or the tendon matrix is elaborated by expansion of the hemiadherens junctions, accumulation of tendon matrix, and increased expression of β1 tubulin (Buttgereit et al., 1991; Prokop et al., 1998*a*). We do not detect any expression of Kakapo in the muscles (Figs. 2 *c*, 4 *a*, and 5), even though comparable hemiadherens junctions, characterized by membrane-proximal electron-dense plaques into which cytoskeletal elements insert, are formed there and the adhesion is also integrin-dependent (Prokop et al., 1998*a*). This difference could indicate that Kakapo has a function that is incompatible with muscle contraction, such as forming stable anchoring structures. Kakapo is strongly expressed in two internal structures as well: the pharynx and the proventriculus (Figs. 2 *c* and 4, *d–h*). In the pharynx, Kakapo is strongly expressed in the endodermal cell layers that attach to the pharyngeal muscles, as seen by comparing Kakapo in Fig. 4 *d* to a Nomarski image of these tissues stained for the muscles at a similar stage (Fig. 4 *e*). As with the epidermal cells that attach to the somatic muscles (see below), Kakapo is found both at the basal surface that contacts the mesoderm and at the apical surface, while the PS integrins are localized just at the basal surface (Leptin et al., 1989). In the proventriculus, Kakapo is expressed in a ring of cells at the anterior margin of the outer layer of this three-layered structure (Fig. 4, *f* and *g*). Expression of the PS integrins is found primarily at the interface between the outer endodermal layer of the proventriculus and the surrounding visceral mesoderm (Fig. 4 *h*). Therefore, in this tissue, the integrins are expressed in more cells than Kakapo. It is not clear why strong expression of Kakapo might be needed at this site, but one phenotype of integrin mutations is that the inner layers of the proventriculus become pulled out (Martin-Bermudo et al., 1997), suggesting that some resistance to mechanical stress is normally necessary to maintain the integrity of the proventriculus structure. We can also detect modest Kakapo expression in the scolopale of the chordotonal organs of the peripheral nervous system (Fig. 4 *c*). A function for the PS integrins in these cells has not been observed to date, but, like muscle attachment cells, it is a site of stabilized β_1 microtubule based rigidity (Prokop et al., 1998*b*).

Kakapo Is Located at Both the Apical and Basal Surfaces of the Epidermal Muscle Attachment Cells

In the epidermal muscle-attachment cells, the PS integrins are localized to the basal surface (Leptin et al., 1989), which contains large hemiadherens junctions (Prokop et al., 1998*a*). Microtubules extend from these basal junctions to the apical hemiadherens junctions, which connect to the exoskeleton (cuticle). The microtubules appear to be serving a similar structural role to that of keratin filaments in the epidermal cells of vertebrates, as intermediate filaments have yet to be identified in *Drosophila*. When we examined the subcellular localization of Kakapo in more detail, we found that it is present at both apical and basal surfaces of the muscle attachment cells (Fig. 5). Kakapo can be seen to be positioned at the termini of the microtubule bundles extending from the apical to the basal surface

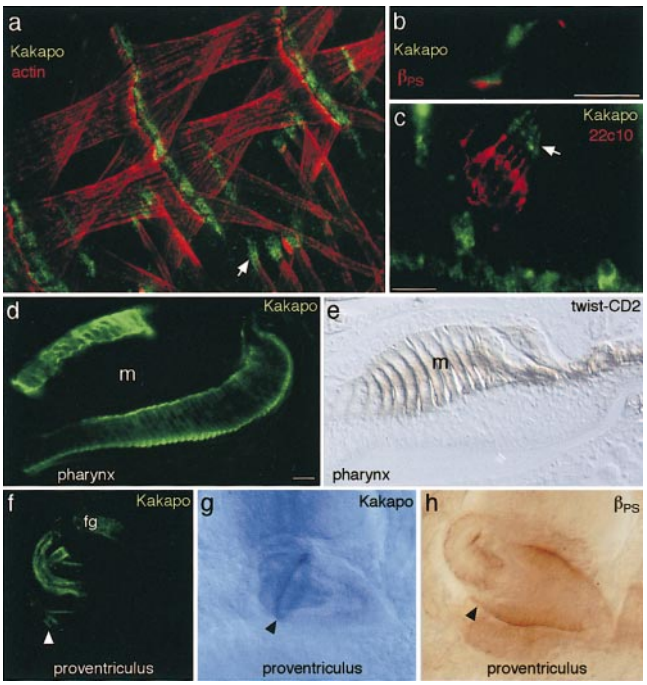

Figure 4. High-magnification views of Kakapo expression. (*a*) The muscles, labeled with rhodamine-phalloidin (*red*), attached to rows of Kakapo (*green*) expressing epidermal attachment cells. The dorsal transverse attachment sites marked by arrows in Fig. 2 are indicated by the arrow. (*b*) Magnified view of a muscle attachment site labeled with anti-Kakapo (*green*) and anti-β_{PS} (*red*) showing the close apposition of these two proteins. (*c*) A chordotonal organ, part of the peripheral nervous system, stained with mAb22C10 to stain the neurons (*red*) and anti-Kakapo (*green*), which stains the scolopale at the end of the dendrite (*arrow*). Kak staining in the pharynx (*d*) is compared with a Nomarski image of a longitudinal section of these structures stained with twist-CD2 (Dunin-Borkowski and Brown, 1995), which outlines the pharyngeal muscles (*e*). The position of the pharyngeal muscles in each panel is marked *m*. (*f* and *g*) Kak expression in the proventriculus is in the most anterior ring of endodermal cells (*arrowhead* in each panel shows one side of this ring). The confocal image (*f*) clearly shows the apical and basal localization of Kak, but has nonspecific staining of the cuticle in the foregut (*fg*). The tissue morphology is seen more clearly by Nomarski optics, where Kak expression (*g*) is shown relative to the expression of the β_{PS} integrin subunit (*h*) at the interface between the visceral mesoderm and the outer layer of the midgut. Anterior is to the left in all panels. Bars, 10 μm. *e* and *h* courtesy of O.M. Dunin-Borkowski and M.D. Martin-Bermudo, respectively.

(Fig. 5 *a*), as well as faintly along their length. This subcellular pattern is distinct from other proteins that share the α-actinin type actin-binding domain, such as β_H-spectrin (Thomas and Kiehart, 1994), demonstrating that this domain does not by itself direct the intracellular localization of proteins containing it. The apical domain of Kakapo expression overlaps with the apical localization of the protein 4.1 superfamily protein Coracle (Fig. 5 *b*). We found reduced staining of Coracle on the lateral surface at this stage compared with earlier stages previously described (Fehon et al., 1994). The 4.1 superfamily of proteins is used to link transmembrane proteins to the cortical cytoskeleton, so the colocalization indicates that Kakapo is found at the cell cortex.

We had difficulty staining for both the PS integrins and Kakapo because the two antibodies require different fixation conditions. However, in spite of this, it is clear that Kakapo expression is adjacent to PS integrin expression (Fig. 4 *b*, Fig. 5 *c*, methanol-fixed embryos; and Fig. 5 *d*, formaldehyde fixed), which consists of expression on the basal surface of the epidermis and at the termini of the attaching muscles. There is no significant expression of Kakapo in the muscles, but the basal expression of Kakapo in the epidermal muscle attachment cells is detected immediately next to, while not overlapping, the epidermal integrin localization (Fig. 4 *b*). Thus, Kakapo is not only present at the basal surface where the PS integrins are located, but also at the apical surface, where as yet no adhesion receptors have been described.

The localization of Kakapo to both ends of the microtubule bundles suggests that Kakapo could have a role in connecting the microtubules to the cortical actin network at the membrane, and may also directly or indirectly link to transmembrane receptors such as the integrins. To test its function in the epidermal muscle attachment cells, we examined *kakapo* mutant embryos. In stage 16 embryos we could not find any penetrant defects in muscle attachment in embryos homozygous for most *kak* alleles (data not shown), although some stronger alleles produce severe disruptions in embryonic morphogenesis (see below). However, in stage 17 embryos mutant for the weaker alleles, we find that the muscles detach from the epidermis, but they stay attached end to end (Fig. 6, *a* and *b*). In the living embryo one can see that although muscle contraction occurs, it is no longer coupled to movement of the exoskeleton (data not shown). The cause of this defect is much clearer in the EM analysis presented in the accompanying paper (Prokop et al., 1998*b*), which shows that the microtubule bundles are no longer attached to the basal membrane, and the epidermal cell rips in half in consequence. This phenotype is highly reminiscent of the BPAG and plectin phenotypes (Guo et al., 1995; McLean et al., 1996), and is distinct from the PS integrin's mutant phenotype where each muscle detaches both from the epidermis and from the other muscles (see Brown, 1993). Thus, Kakapo is required for epidermal attachment to muscles, but not for muscle–muscle attachment, consistent with its expression pattern.

A General Function for Kakapo in Maintaining the Integrity of the Epithelial Cell Sheet

We have shown that Kakapo has a vital role in mediating strong attachment in specific cells within the embryo that require strong mechanical stability, but it may also have more general functions that involve other cell surface receptors in addition to the integrins. This possibility is consistent with the reproducible low level of Kakapo expression we have observed in many cells (such as the epidermal cells shown in Fig. 5) that are not attached to muscles. We therefore examined embryos mutant for Kakapo to see if we could identify more general defects that might be a consequence of this loss of low-level Kakapo expression. Embryos mutant for *kakapo* display a wide range of

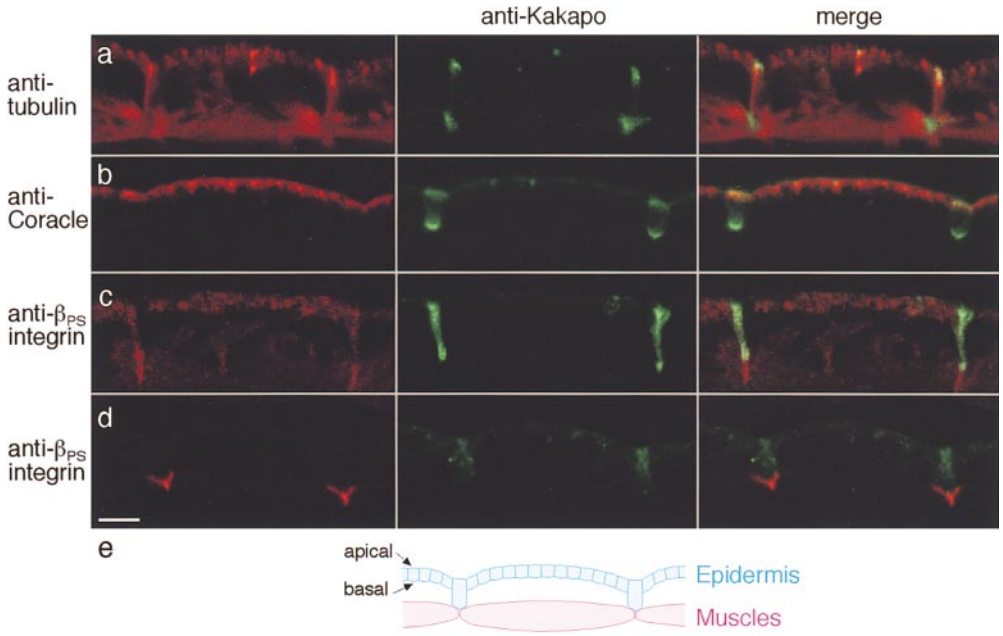

Figure 5. Kakapo is localized at the apical and basal surfaces of the epidermal muscle attachment cells. Each panel shows a horizontal section of a late stage 16 embryo, showing two muscle attachment cells at the segment border as shown in the schematic drawing (*e*). Each panel is stained for Kakapo in green and a second antigen in red: (*a*) tubulin; (*b*) Coracle, a band 4.1 superfamily member; and (*c* and *d*) a β_{PS} integrin subunit on embryos fixed either in methanol (*c*) or formaldehyde (*d*). The merged images are shown at the left. Bar, 10 μm.

phenotypes, from almost normal development to severe morphological abnormalities. This range of phenotypes is also found in embryos deficient for the locus *Df(2R)MK1/Df(2R)MK1* or *Df(2R)CX1/Df(2R)CX1* (data not shown), demonstrating that even the complete absence of the *kakapo* gene does not result in a consistent zygotic phenotype. The variability of the phenotype may be due to a partial redundancy of function between Kakapo and another protein, or variable contribution of maternal protein. We favor the former possibility, since generating germ line clones of one of the *kakapo* alleles did not enhance the phenotype (Walsh and Brown, 1998), and we have not been able to detect Kakapo protein before gastrulation (data not shown).

Consistent with the variability found in embryos deficient for *kakapo*, we find that our *kakapo* alleles also display diverse phenotypes. Most of the alleles isolated in our screen are embryonic lethal as homozygotes, although the two alleles examined previously develop normally through stage 16 (Walsh and Brown, 1998). As shown above, at the

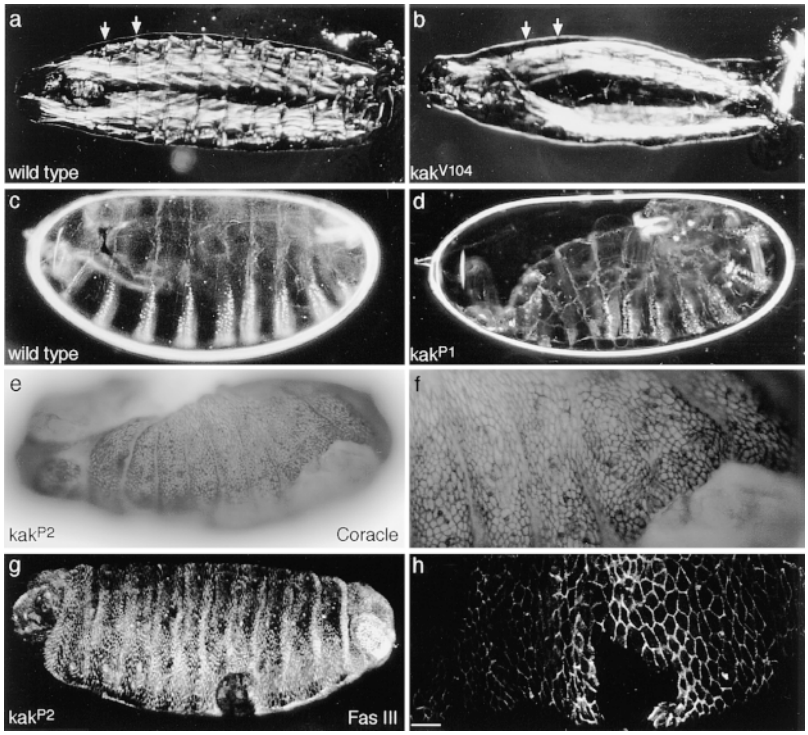

Figure 6. Mutations in Kakapo cause muscle detachment and widespread defects in the epidermis. The first two panels show flat stage 17 embryos with the birefringent muscles and cuticle visualized by polarizing optics: (*a*) wild-type embryo with two arrows marking the close attachment of the muscles to the epidermis. (*b*) In kak^{V104}/kak^{V104} embryos, the muscles pull away from the epidermis, but remain attached to each other (positions on the epidermis equivalent to those marked in *a* are marked by *arrows*). (*c* and *d*) Cuticle preparations, that reveal the underlying pattern of the epidermis: (*c*) wild-type cuticle; (*d*) strongest cuticle phenotype of kak^{P1}/kak^{P1}. (*e–h*) Late-stage 16 kak^{P2}/kak^{P2} embryos stained for the 4.1 homologue Coracle (*e* and *f*; the same embryo magnified) and membrane protein fasciclin III (*g* and *h*; another example magnified), showing ruptures in the epidermis. Bar, 10 μm.

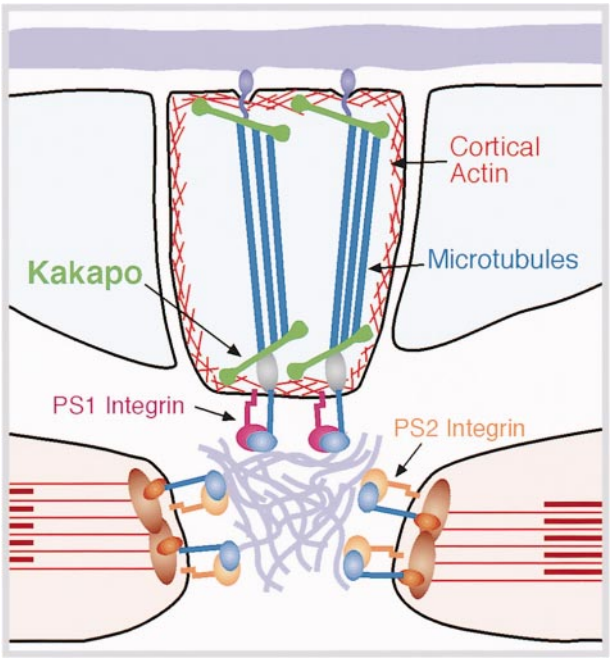

Figure 7. Model for the role of Kakapo in muscle/epidermal adhesion. A schematic of a muscle attachment site is shown with the muscles linked to each other and the epidermis by integrin–extracellular matrix linkage. At the basal surface of the epidermal cell, Kakapo (*green*) links the cortical actin cytoskeleton (*red*) and the microtubule bundles (*blue*) to integrins via an adaptor protein (*grey*). At the apical surface, Kakapo links the cytoskeleton to a currently unidentified receptor (*purple*).

end of embryogenesis (stage 17), the mutant embryos have a phenotype where the epidermis detaches from the muscles (Fig. 6 *b*). By examining the phenotype of the different alleles, we found that some have much stronger phenotypes, indicative of a more general function. We examined the epidermally secreted cuticle, which reflects the pattern of the underlying epidermis, of embryos homozygous for all our different x-ray–induced *kakapo* alleles and the insertion alleles kak^{P1} and kak^{P2}. In the majority of the alleles (17), the homozygous mutant embryos have a normal epidermal pattern, although ~30% of these embryos showed modest cuticular defects (not shown). It should be mentioned that the screen for wing blister mutations may have selected for a particular type of weak allele if more severe alleles cause drastic wing defects. One x-ray allele, kak^{V168}, and the P-alleles kak^{P1} and kak^{P2}, have a stronger phenotype: in ~15% of the mutant embryos, germ band retraction and head involution fail (Fig. 6, *c* and *d*). Approximately 40% of embryos mutant for these alleles have normal-looking cuticles, demonstrating that this phenotype is also not fully penetrant.

Epidermal development was examined in more detail in the embryos homozygous for strong *kakapo* alleles using two markers for epidermal shape (Fig. 6, *e–h*). This examination revealed defects in the integrity of the epidermal cell layer; namely, breaks in the ventral epidermis in the middle abdominal segments of the fully germband-extended embryos (Fig. 6 *e*). These breaks appear to arise at the site of maximum strain during germ band retraction. Consistent with this observation, germband retraction is arrested in some embryos (Fig. 6, *d* and *e*). Other embryos successfully undergo germband retraction, but retain a hole in the epidermis at this position of maximum strain (see Fig. 6, *g* and *h)*, which could account for the disruptions observed in the cuticular denticle belts. The homophilic adhesion molecule Fasiclin III is not present on the surface of the cells bordering the hole (Fig. 6 *h*). Such breaks in the epidermis are not observed in embryos mutant for the PS integrins, suggesting that this Kakapo function involves other cell adhesion molecules. We also observed morphogenetic defects in the internal tissues (data not shown), but because they occur in embryos with severe epidermal defects, it is not yet certain whether this indicates a function for Kakapo in these tissues, or whether the internal defects are a result of the epidermal disruption. The phenotypes in the embryonic epidermis indicate that the low-level general expression of Kakapo is significant, and that Kakapo may play a general role in mediating adhesion, possibly by mediating interactions between transmembrane proteins and the cytoskeleton.

Discussion

Identification of intracellular proteins that are required for integrin functioning is essential for an understanding of how integrins mediate adhesion. In this paper we have described the cloning and characterization of *kakapo*, a gene that was identified in screens for wing blister mutants (Prout et al., 1997; Walsh and Brown, 1998), and we show that it encodes a cytoskeletal adaptor protein related to plectin, BPAG1, and dystrophin. We have demonstrated that Kakapo is expressed in those epithelial cells where stable adhesion is required in the developing embryo; it is required to maintain epidermal adhesion to the muscles, and more generally to maintain cohesion of the epidermal cell layer. From the our characterization of the sequence, pattern of expression, and phenotype of *kakapo* mutations, it appears that Kakapo has a similar function to plectin, and we propose a model in which Kakapo provides links among cortical actin, microtubules, and transmembrane proteins such as integrins (Fig. 7).

The binding of Kakapo to actin is indicated by the presence of a highly conserved actin-binding domain at the amino terminus of Kakapo. This domain is most similar to the equivalent domain in plectin and BPAG1, compared with the domains found in dystrophins, β-spectrins, and α-actinins. This higher sequence conservation may indicate some functional diversity within this domain that is conserved in each subfamily, or it may simply represent the evolutionary history of conservative amino acid replacements. What is clear is that this actin-binding domain does not dictate the intracellular localization of the proteins containing it. For example, the apical and basal localization of Kakapo is distinct from the general cortical localization of both spectrin and the novel β_H-spectrin of *Drosophila* (Pesacreta et al., 1989; Martinez-Arias, 1993; Thomas and Kiehart, 1994).

Kakapo is closely related to plectin and BPAG1, two vertebrate proteins that are required for the link between the integrin $\alpha_6\beta_4$ and intermediate filaments at hemidesmosomes (Ruhrberg and Watt, 1997). However, this simi-

larity does not extend through to the carboxy terminus of these proteins, which contain an intermediate filament-binding domain shared with the desmosome component desmoplakin. As Kakapo does not contain an intermediate filament-binding domain, and so far no intermediate filaments have been identified in *Drosophila*, it is unlikely to have an identical function to plectin and BPAG1 and bind intermediate filaments. In *Drosophila*, stabilized microtubule arrays appear to be used in place of intermediate filaments to hold the cell rigid (Mogensen and Tucker, 1988). This observation is particularly apparent in the adult wing, where transalar parallel arrays of microtubules and microfilaments connect the apical cuticle with the integrin-containing basal junctions (Mogensen and Tucker, 1988; Fristrom et al., 1993), and in the larval epidermal cells that attach to the muscles (Prokop et al., 1998*a*). The fact that Kakapo function is required for cell adhesion in both sets of epithelial cells where stabilized microtubules are found strongly suggests that Kakapo binds to these microtubules rather than intermediate filaments. In contrast to a number of well-conserved actin-binding domains, many microtubule-binding proteins lack a shared microtubule-binding motif so that the lack of similarity between *kakapo* and a known microtubule-binding protein does not contradict our proposal that Kakapo binds to microtubules. It is also possible that Kakapo binds indirectly to microtubules though an interaction with a microtubule-associated protein.

In hemidesmosomes there appears to be a direct molecular interaction between integrins and plectin, since sites on the β_4 cytoplasmic tail have been identified that directly bind to plectin (Niessen et al., 1997). As the PS integrins have a more standard length of β subunit cytoplasmic tail (47 amino acids vs. the 1019 amino acids of β_4), an intervening linker protein may be required (indicated by the grey sphere in Fig. 7) to connect the PS integrins to Kakapo. We might expect this adaptor to be encoded by one of the other loci identified in the screen, such as *rhea*, which has a similar epidermal detachment phenotype (Prout et al., 1997). If this adaptor protein exists, it will be of interest to see if it is similar in sequence to the cytoplasmic tail of β_4.

Kakapo is not only similar in sequence to plectin and BPAG1, it is also similar to dystrophin. However, the pattern of expression of Kakapo is more similar to the expression of BPAG1 than dystrophin, while plectin is expressed almost ubiquitously. Like BPAG1, Kakapo is strongly expressed in epidermal cells, and neither are expressed in the muscles, where dystrophin is strongly expressed. Furthermore, we have shown the subcellular localization of Kakapo to be primarily at the site of the prominent hemiadherens junctions that are present both apically and basally in the epidermal muscle attachment site, as well as some decoration of the intervening cytoskeleton. Basal hemiadherens junctions consist of extensive plaques of electron-dense material where actin and microtubules connect to the extracellular matrix, while at the smaller apical junctions, microtubules are attached to the overlying cuticle (Prokop et al., 1998*a*). This subcellular localization resembles that seen for BPAG1 and plectin, which localize to the inner plaque of hemidesmosomes and decorate intermediate filaments (Wiche et al., 1984; Guo et al., 1995; Svitkina et al., 1996), and is quite distinct from the general cortical staining of dystrophin in the muscles (Brown and Lucy, 1997). The staining we have observed is not completely identical to that seen with an antibody raised against the carboxy terminus of Kakapo, which shows staining earlier in embryogenesis than our antibody to the amino terminus, and looks more cortical (Strumpf and Volk, 1998). The Kakapo gene extends over 70 kb, and there may be additional isoforms of Kakapo yet to be identified. This would be similar to the plectin and BPAG genes that produce alternate isoforms, some of which lack the actin-binding domain or rod sections (Yang et al., 1996; Elliott et al., 1997).

Finally, the phenotype of Kakapo is more similar to BPAG1 and plectin than to dystrophin. Mutations in BPAG1 or plectin cause skin blistering due to rupture of epidermal cells and neuromuscular defects (Guo et al., 1995; Smith et al., 1996). The *kakapo* gene was identified by screening for a related phenotype in *Drosophila*; mutant cells cause blisters in the adult wing (Prout et al., 1997; Walsh and Brown, 1998). In the embryo, *kakapo* mutations cause detachment of the epidermis from the muscles similar to skin blisters, and the ultrastructural phenotype is remarkably similar, where mechanical stress leads to breaking of the cells into apical and basal halves (Guo et al., 1995; Prokop et al., 1998*b*). In contrast, the muscles appear to develop normally, and have normal sarcomeric structure (Fig. 6 and our unpublished results), in contrast to the muscular degeneration that occurs in the absence of dystrophin (Brown and Lucy, 1997). The striking similarity of sequence, expression pattern, and mutant phenotype provide consistent evidence for the functional relationship of Kak with vertebrate hemidesmosomal proteins. Consequently, we propose that Kak plays a homologous role to the vertebrate plakin family in the *Drosophila* hemi-adherens junction, acting as an essential adaptor that distributes the extension stress generated by muscle contraction from membrane-bound receptors into the cortical actin and stabilized microtubule arrays.

The identification of a new cytoskeletal linker protein as the product of a gene required for integrin-mediated adhesion events strengthens the view that integrins do play an important structural role in mediating adhesion. Our current model for the interaction between Kakapo and the PS integrins is that they are connected indirectly through a protein that serves a similar function to the extended cytoplasmic domain of the β_4 integrin subunit (as shown in Fig. 7). However, this result is clearly an incomplete description, because, unlike Kakapo, PS integrins are not essential for the linkage between the plasma membrane and microtubules in the epidermal muscle attachment cells (Prokop et al., 1998*a*). The fact that Kakapo is required for this microtubule–membrane linkage suggests that Kakapo interacts, directly or indirectly, with more transmembrane proteins than just the known integrins. This is also indicated by Kakapo localization at the apical surface, which lacks the PS integrins. Whether these additional adhesion receptors include novel integrins or alternative classes of receptor is an open question, but one that may be resolved by cloning more of the loci identified in recent genetic screens for adhesion defects (Prout et al., 1997; Walsh and Brown, 1998).

The early phenotype we have documented where the integrity of the epidermal cell layer is impaired also indicates the diversity of Kakapo function, because this is not a phenotype found in PS integrin mutants. In addition, the observation that the differentiation of the epidermal muscle attachment cells is perturbed in *kakapo* mutant embryos (Strumpf and Volk, 1998) suggests that Kakapo may be required to allow signaling events leading to cell differentiation. We do not think that the change in differentiation of the epidermal muscle attachment cells is sufficient to account for the epidermal detachment phenotype of Kakapo mutants, since although β_1 tubulin expression is reduced (Strumpf and Volk, 1998), microtubule bundles are still present in those cells, but are detached from the plasma membrane (Prokop et al., 1998*b*), indicating that Kakapo is essential for the link between the two. However, the role of Kakapo in epidermal differentiation combined with its role in determining the subcellular localization of transmembrane adhesion proteins in neurons (Prokop et al., 1998*b*) suggests that one important function of Kakapo is to organize receptors within the plasma membrane. Thus, the Kakapo protein, which from its sequence homology and phenotype seems to be an important component of the cytoskeleton, may potentially affect signaling through the role of the cytoskeleton in organizing membrane-bound receptors into functional signaling complexes.

The *kakapo* gene is the first of the novel genes to be cloned from the screens for mutations required for integrin-mediated adhesion. The fact that kakapo encodes a cytoskeletal linker protein related to proteins that are also implicated in integrin-mediated adhesion demonstrates the success of the genetic screening approach. Therefore, the characterization of the remaining 16 loci isolated from these screens should substantially contribute to our understanding of the cellular machinery required for integrin adhesion.

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