**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
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 \( \alpha_\beta \) integrin in the adhesion of the epidermis to
the underlying dermis (for review see Uitto and Pulkkinen,
1996). Two transmembrane proteins—the integrin \( \alpha_\beta \)
and bullous pemphigoid antigen 2 (BPAG2)\(^1\)—bind to
laminin 5 and collagen type VII, and are linked to cyto-ker-
atin 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
\( \alpha \)-actinin, \( \beta \)-spectrins, and dystrophin, suggesting that an
important function of this class of proteins is to provide
links among the different cytoskeletal filaments (Ruh-
berg and Watt, 1997). Mutations in the genes encoding
these proteins, or autoimmune 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 ex-
tracellular matrix and the cytoskeleton. Thus, mutations in
the extracellular ligands or the \( \alpha_\beta \) 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 hemidesmosomes 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 \( \beta_4 \)
has a much longer cytoplasmic tail than the other \( \beta \) subunits
may make this a specialized case.

To identify additional proteins that are required for in-
tegrin-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 \( \beta \) 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 homozy-
gous mutant for newly generated mutations. The screen is
based on the fact that clones of cells mutant for the PS in-
tegrin 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 hemidesmo-
some components plectin and BPAG1. In contrast to these
proteins, the \( \kakapo \) protein contains motifs from dystro-
phin and the growth arrest protein \( \ GAS \) at its carboxy ter-
minus, 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 plec-
tin (or plakin) family of cytoskeletal linker proteins are
not restricted to linking the unusual \( \alpha_\beta \) integrin to inter-
mediate filaments, but are more widely involved in inte-
grin adhesion events.

**Materials and Methods**

**Drosophila Strains**
The \( \kakapo \) alleles used in this study were 18 of the 19 \( \kakapo \) alleles iso-
lated by Walsh and Brown (1998; one has been lost), and the lethal P-ele-
ment insertions \( l(2)k03010 \) \( \kakapo \) and \( l(2)k03405 \) \( \kakapo \); I. Kiss collection,
Berkeley Drosophila Genome Project). We used two deficiencies for
\( \kakapo \) \( l(2)k08708 \), \( l(2)k04204 \), \( l(2)k06206 \), and \( C-2-29 \) \( \kakapo \) \( ( \) Bloomington Stock Center, Bloom-
ington, IN\). Other \( P \) insertions that were tested and found not to be al-
lelic to \( \kakapo \) were \( l(2)248 \), \( l(2)1105 \), \( l(2)4945 \), \( l(2)5488 \), \( l(2)k0121 \),
\( l(2)k08708 \), \( l(2)k04204 \), \( l(2)k06206 \), and \( C-2-29 \) \( \kakapo \) \( ( \) Bloomington Stock Center
and Berkeley Drosophila Genome Project\). To demonstrate that the
\( \kakapo \) mutations are associated with the \( P \)-element insertions in \( \kakapo \) and
\( \kakapo \) they were jumped out by crossing in \( \Delta \) 3 transposase, outcrossing,
and screening for loss of the \( \wedge \) marker, and then checked for viability
over \( D(f2R)ZX1 \). Both insertions reverted to viability over the defi-
cency 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 \( \mu \)g of each in 1 ml to circularize the
DNA. The DNA was transformed into competent cells, and rescued plas-
mids were selected for by Ampicillin. This procedure yielded genomic
fragments that were used to screen a \( \kakapo \) 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
tron 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, Pam-
pisford, 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 AJ01924 for form A and AJ01925 for form B.
Database analysis was carried out using the BLAST server at Baylor Col-
lege of Medicine (http://kiwi.bcm.tmc.edu:8088/). Alignments of related se-
cquences were carried out using ClustalW and by eye in MacVector. Phylo-
genetic analysis of aligned sequences was carried out using the Dayhof
matrix and ProtDist in PHYLIP3.72 (J. Felsenstein, University of Wash-
ington, Seattle, WA). Accession numbers of the related sequences used

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1. Abbreviations used in this paper: BPAG2, bullous pemphigoid antigen
2. PS, position-specific.
are: human plectin, Z54367; human BPAG1, I39160; mouse ACF7, P12814; human utrophin, U67203; mouse utrophin, A35598; human dystrophin, A35598; human dystrophin, A37702; Drosophila 6-pectrin, A35598; human α-actinin 1, P12814; human α-actinin 2, P35669; 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 (Roche Diagnostics, Lewes, UK) with the primers GCAGGGCCTACATGCATTCTACTG and GGCTTCGCAAATGCCTTGTTAG. 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 mM.

The cell suspension was incubated on ice for 20 min. Sodium deoxycholate was added to 1 mM. The suspension was centrifuged at 15 krpm for 10 min in a Sorvall SS-34 rotor, and the supernatant was discarded. The pellet was gently resuspended in 3 ml of lysis buffer containing 0.5% Triton X-100 and 10 mM EDTA, incubated at 30 °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 antiserum 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 in TBS plus 5% milk, 0.1% Tween-20 overnight at 4°C. Antibody 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.

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 on day 14 to 16 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 antiserum (1:10); mouse mAb DA1B6 anti-Fasiclin 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 CB6G11 anti-β-βx (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-spectrin DM1A (1:50; Sigma Chemical Co.). Secondary antibodies used were FITC-conjugated goat anti–rabbit IgG and biotinylated horse anti–mouse IgG (Vector Labs, 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 Hoyer's reagent as described in Wieschaus and Nüsslein-Volhard (1986). Cuticles were photographed using an Axioskop 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-developed black-and-white film (Eastman Kodak Co.). Embryonic muscles were visualized and photographed using a Nikon polarized light microscope on hand-developed black-and-white film (Eastman Kodak Co.). Secondary antibodies used were FITC-conjugated goat anti–rabbit IgG and biotinylated horse anti–mouse IgG (Vector Labs, 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.).

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 kopupu 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 kakp1 and kakp2. The lethality of both P-element lines can be reverted by jumping out the P-element, as scored by loss of the Polycomb group phenotype (Prout et al., 1997), showing that the kakp mutations are associated with insertion 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 NH2-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 intron.

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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 NH2 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 NH2-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\(^{2+}\)-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 antibodies 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., 1998a). 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).

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-

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**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).
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 kakV168, 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 antisera 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., 1998a). We first detected strong Kakapo expression in these cells at midlate 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 hemiadererens junctions, accumulation of tendon matrix, and increased expression of β1 tubulin (Buttgereit et al., 1991; Prokop et al., 1998a). We do not detect any expression of Kakapo in the muscles (Figs. 2 c, 4 a, and 5), even though comparable hemiadererens 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., 1998a). 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 β1 microtubule based rigidity (Prokop et al., 1998b).

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 hemiadererens junctions (Prokop et al., 1998a). Microtubules extend from these basal junctions to the apical hemiadererens 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
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., 1998b), 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.

**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-βps (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 βps 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.

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.

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**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...
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 early stages of development, 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 β5 integrin subunit on embryos fixed either in methanol (c) or formaldehyde (d). The merged images are shown at the left. 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 mus- cles (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 kakP1 and kakP2. 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, kak165, and the P-alleles kakP1 and kakP2, have a stronger phenotype: in ~15% of the mutant embryos, germband 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. Consis-
tent 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 Fasiciin 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 morph-ogenetic 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-actins. 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 βH-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 αβ4 and intermediate filaments at hemidesmosomes (Ruhrberg and Watt, 1997). However, this simi-
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 hemi-adherens junctions that are present both apically and basally in the epidermal muscle attachment site, as well as some decoration of the intervening cytoskeleton. Basal hemi-adherens 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., 1998a). 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., 1998b). 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 adapter 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 β4 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., 1998a). 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 $\beta_1$ 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., 1998b), 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 adherence proteins in neurons (Prokop et al., 1998b) 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 link 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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