Copyright © 2000 by the Genetics Society of America

The *tricornered* Gene, Which Is Required for the Integrity of Epidermal Cell Extensions, Encodes the Drosophila Nuclear DBF2-Related Kinase

Wei Geng, Biao He, Mina Wang and Paul N. Adler

Biology Department and Cancer Center, University of Virginia, Charlottesville, Virginia 22903

Manuscript received June 5, 2000
Accepted for publication August 23, 2000

ABSTRACT

During their differentiation epidermal cells of Drosophila form a rich variety of polarized structures. These include the epidermal hairs that decorate much of the adult cuticular surface, the shafts of the bristle sense organs, the lateral extensions of the arista, and the larval denticles. These cuticular structures are produced by cytoskeletal-mediated outgrowths of epidermal cells. Mutations in the *tricornered* gene result in the splitting or branching of all of these structures. Thus, *tricornered* function appears to be important for maintaining the integrity of the outgrowths. *tricornered* mutations however do not have major effects on the growth or shape of these cellular extensions. Inhibiting actin polymerization in differentiating cells by cytochalasin D or latrunculin A treatment also induces the splitting of hairs and bristles, suggesting that the actin cytoskeleton might be a target of *tricornered*. However, the drugs also result in short, fat, and occasionally malformed hairs and bristles. The data suggest that the function of the actin cytoskeleton is important for maintaining the integrity of cellular extensions as well as their growth and shape. Thus, if *tricornered* causes the splitting of cellular extensions by interacting with the actin cytoskeleton it likely does so in a subtle way. Consistent with this possibility we found that a weak *tricornered* mutant is hypersensitive to cytochalasin D. We have cloned the *tricornered* gene and found that it encodes the Drosophila NDR kinase. This is a conserved ser/thr protein kinase found in *Caenorhabditis elegans* and humans that is related to a number of kinases that have been found to be important in controlling cell structure and proliferation.

EPITHELIAL cells typically contain distinct apical and basal/lateral membrane domains that differ both biochemically and structurally. A common structural feature of epithelial cells is the presence of microvilli or other cellular projections on their apical surface (Eaton 1997). In some cell types, such as those in the vertebrate intestine, there are large numbers of apical projections. In others the number is small and tightly regulated. The epidermal cells of the Drosophila pupal wing are good examples of cells that form a single projection from their apical surface (Wong and Adler 1993). This pupal "prehair" develops into the adult cuticular hair. In wild-type cells the prehair is assembled at the distalmost vertex of the cell and extends away from the cell. This leads to the distally pointing adult hair.

The prehair contains both actin filaments and microtubules (Eaton *et al.* 1996; Turner and Adler 1998). The pharmacological antagonism of either of these cytoskeletons leads, depending on dose, to slowed or blocked prehair growth (Turner and Adler 1998). In addition to this shared response there are also differences in the ways that pupal wing cells respond to actin- and microtubule-specific drugs. The disruption of the microtubule cytoskeleton by drugs such as vinblastine (VB) leads to prehair initiation no longer being restricted to the vicinity of the distal vertex (Turner and Adler 1998). This leads to many cells forming more than one prehair. Multiple independent wing hairs are also caused by mutations in downstream components of the *frizzled*-based tissue polarity pathway such as *inturned* (*in*; Wong and Adler 1993). In addition to inhibiting prehair elongation the inhibition of actin polymerization by cytochalasin D (CD) treatment results in split or branched hairs (Turner and Adler 1998). This phenotype is also seen in wing cells mutant for *crinkled* (*ck*; Turner and Adler 1998), which encodes a nonmuscle myosin (Ashburner *et al.* 1999; D. Kiehart, personal communication), and when a dominant-negative Dcdc42 protein is expressed in the wing (Eaton *et al.* 1996). This small G protein has been found to regulate the actin cytoskeleton and the formation of filopodia in many cell types (Nobes and Hall 1995; Tapon and Hall 1997; Castellano *et al.* 1999). When a CD-treated or *ck* cell forms a single unbranched hair it is still shorter and fatter than normal (Turner and Adler 1998). This stunting seems likely to be a consequence of the disruption of actin cytoskeleton function.

A third distinct type of multiple hair cell phenotype is seen in cells that are mutant for the *tricornered* (*trc*) gene. A mutation in this gene was originally recovered more than 20 years ago (Ferrus 1976). It is an organismal recessive lethal mutation but it is cell viable, and

Corresponding author: Paul Adler, Biology Department, University of Virginia, Gilmer Hall, Rm. 245, Charlottesville, VA 22903.
E-mail: pna@virginia.edu

has been used as a gratuitous cuticular marker in a number of genetic mosaic studies (Gubb and Garcia-Bellido 1982; Vinson and Adler 1987). In this article we describe a set of related phenotypes of *trc*, which affect cellular extensions of epidermal cells. Cells mutant for *trc* have a dramatic multiple-hair-cell phenotype forming on average almost six hairs per cell. The multiple hairs produced by *trc* mutant cells are tightly clustered and in some cases they are branched distally. Our observations on both pupal wing cells and adult wings suggest that they are produced by the splitting of a single prehair. When a *trc* cell produces a single hair it appears relatively normal in length and shape and in this way differs from hairs produced by *ck* cells.

The cuticular hairs formed by epidermal cells are not the only examples of cellular projections found in Drosophila. The shaft of sensory bristles is another example of a polarized cell extension. The trichogen cell that forms the shaft is polyploid and the bristle shaft is many times larger than the epidermal hairs. The shaft contains a small number (7–11 in the thoracic microchaete) of large bundles of actin filaments located at the cell periphery and microtubules located centrally (Tilney *et al.* 1995). We found that disruption of actin polymerization by CD or latrunculin A (LAT A) treatment led to branched, stunted, and deformed bristles. Thus, the actin cytoskeleton appears to be important for the integrity, shaping, and growth of bristles. The importance of the actin cytoskeleton for bristle elongation was reported independently by Tilney *et al.* (2000a). Mutations in a number of genes that encode actin-bundling proteins, such as *singed* (*sn*) (fascin; Cant *et al.* 1994), produce bristles that are deformed but only rarely split. Interestingly, in *sn forked (f)* double-mutant bristles the large bundles of actin filaments are lost; however, such cells are still able to form bristles (Tilney *et al.* 1996). These bristles are shorter than normal and have an abnormal, bent, and sometimes twisted shape. Branched bristles, however, are infrequent in *sn f* double mutants. Thus, the large actin bundles are not absolutely required for either bristle growth or integrity, although they appear to be important for the development of proper shape and length. In our experiments we found that *trc* mutations resulted in branched bristles, but had only modest, if any, effects on length and shape. When we examined the actin cytoskeleton in developing *trc* bristles we found that the large actin filament bundles appeared normal and that splitting appeared to occur between neighboring bundles.

Hairs and bristles are not the only types of elongated structures produced by Drosophila epidermal cells. The larval cuticle is decorated with denticles that are produced by projections of larval epidermal cells. The ventral denticles are pigmented and very prominent in cuticle preparations. These denticles are triangular in shape and are relatively shorter and wider than hairs or bristles. We found frequent split denticles in the cuticle of *trc* homozygous larvae; thus *trc* is essential for the normal morphogenesis of denticles. The terminal segment of the antenna is called the arista. It contains a central core and a series of lateral side branches. These lateral branches are formed from outgrowths of individual central-core epidermal cells (B. He and P. N. Adler, unpublished results) and are similar in many ways to epidermal hairs. In *trc* mutants the laterals are frequently split. Thus, *trc* mutations affect the morphology of at least four different types of cellular extensions in analogous ways.

The ability of drugs that inhibit actin polymerization to partially phenocopy the branched hairs and bristles found in *trc* mutants suggested that *trc* might interact with the actin cytoskeleton. Consistent with this possibility we found that a weak *trc* mutant was hypersensitive to CD. The differences between the *trc*-mutant- and drug-induced phenotypes, however, suggested that *trc* was simply not required for actin polymerization. Rather it seems more likely that *trc* interacts with the actin cytoskeleton in a subtle way.

We have used a P insertion allele as a tag for the molecular cloning of *trc*. We found that this *P* element is inserted into the first intron of the Drosophila NDR (nuclear DBF2-related) kinase (Millward *et al.* 1995). Further evidence that *trc* encodes the fly NDR protein kinase was obtained by sequencing of *trc* point mutations and by transformation rescue with a cDNA clone and the GAL4/UAS system (Brand and Perrimon 1993). Two of the *trc* point mutations were found in conserved kinase domain amino acids, arguing that the kinase activity is essential for *trc* function in morphogenesis. In support of this we found an enhanced phenotype when we injected stauroporine, a wide-spectrum ser/thr kinase inhibitor into pupae carrying a weak *trc* allele.

The NDR kinase family is a conserved ser/thr kinase described in humans, worms, and flies (Millward *et al.* 1995). However, until the identification of *trc* as the Drosophila NDR kinase there was no evidence for a specific biological function for any family member. The NDR family is related to a broader family of kinases that includes the myotonic dystrophy kinase of humans, the Rho-activated kinase ROCK (Leung *et al.* 1996), the Drosophila WARTS/LATS kinase (Justice *et al.* 1995; Xu *et al.* 1995), the yeast KNQ1 kinase, the Schizosaccharomyces ORB6 kinase (Verde *et al.* 1998), and the Neurosopora COT1 kinase (Yarden *et al.* 1992). A common feature of many of these kinases is a connection to cell morphology or polarity.

MATERIALS AND METHODS

Fly culture and strains: Flies were grown on standard media. Many mutant and Deficiency-containing stocks were obtained from the stock center at Indiana University. This research was greatly aided by the generous gift of *trc* mutations by Dr. James Kennison.

Cytological procedures: The process of wing hair morphogenesis was studied by confocal microscopy and phalloidin

staining as described previously (Turner and Adler 1998). The morphogenesis of other body regions and cell types was studied in a similar manner. In experiments where we examined *trc*[8]/*Df* pupae and adults the animals were often raised at 29° as the *trc* phenotype is stronger at this temperature. Adobe Photoshop was used to compose bitmap figures and Corel Draw was used to compose line drawings. Confocal images were obtained using a confocal microscope (Bio-Rad, Hercules, CA) at the Keck Center for Biological Imaging at the University of Virginia. Other images were obtained using a Spot digital camera (National Diagnostics) on an Axioskop microscope (Zeiss, Thornwood, NY). For measurements of bristle elongation digital images of phalloidin-stained bristles from carefully timed pupae were obtained with a Spot camera (National Diagnostics) and the length measured using Adobe Photoshop. Unfortunately, we were not able to accurately measure the rate of hair or bristle elongation in *trc*[8]/*Df* pupae as these pupae are quite sick and all aspects of development are variably delayed.

Generation of genetic mosaics: Mosaic clones were generated either by gamma irradiation or by using the FLP/FRT system (Xu *et al.* 1995).

Scoring of mutant wings: Wings were mounted in Euparal (Asco Labs) and examined under bright-field microscopy using approaches described previously (Wong and Adler 1993).

Biochemical procedures: Western blot analysis of pupal wings was done as described previously (Park *et al.* 1994).

Statistical analysis: The Sigma Stat program (Jandel) was used for comparing different genotypes or treatments.

Inhibitor injections: Aged pupae were washed, dried, and then attached to a microscope slide with double-stick tape. The pupal case was opened up anteriorly and inhibitor solutions were injected with a fine glass pipette. Pupae are internally under positive pressure and this often leads to material leaking out and contributes to the varying response of pupae to the inhibitors. To assess the volume injected we did control experiments where we injected radioactive buffer. We found we injected on average 3.1 nl (SD 2.9). Despite variation in the amount injected we were able to see increasing responses to increasing inhibitor concentrations. In every experiment control buffer injections were done. After buffer injection into Oregon-R we had 98.6% viability to adulthood with little or no morphological defects. Cytochalasin D, vinblastine, staurosporine, and colchicine were obtained from Sigma (St. Louis). Latrunculin A was obtained from Molecular Probes (Eugene, OR).

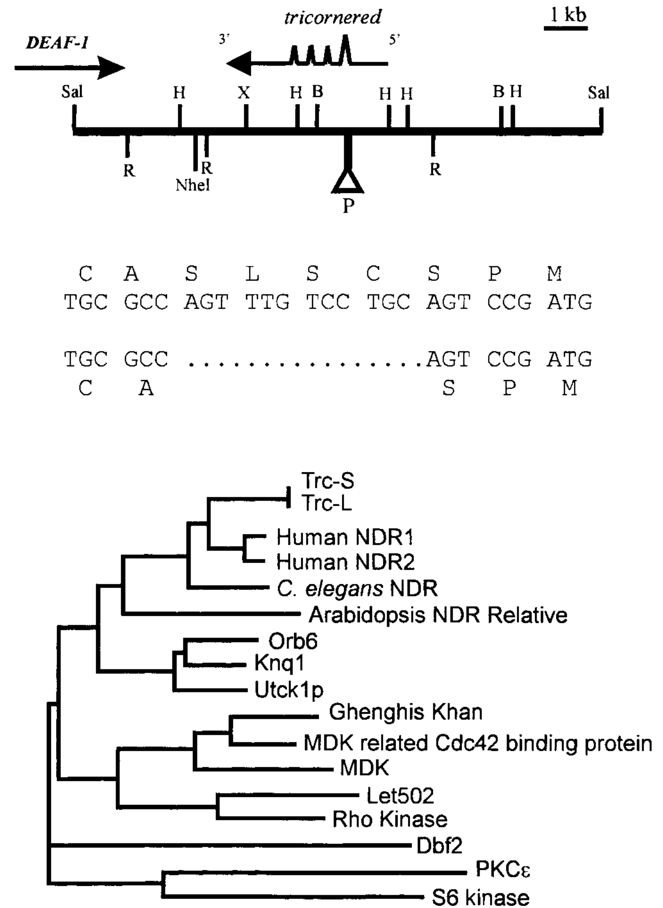

Figure 1.—The top shows a restriction map of the *trc* genomic gene, a cartoon showing the splicing pattern of the primary transcript, the location of the P insertion in *trc*[7], and the location of the nearby *Deaf-1* gene. The middle shows the two splice forms of the *trc* mRNA and the resulting four-amino-acid difference. The bottom shows a Clustal-generated tree showing the relative relationship among *trc*, the human and *C. elegans ndr* genes, and a selected group of other kinases.

RESULTS

Genetics of *trc*: The *trc* gene maps to 76C on the polytene chromosomes and is uncovered by *Df(3L)kto2* (abbreviated *Df* in this article). There exist six EMS-induced alleles, all of which are recessive lethal mutations that produce a strong, multiple-hair phenotype in clones in adult wings. A P insertion allele (*trc*[7]) was found (Deak *et al.* 1997) that mapped to this region and failed to complement *trc* point mutations. This mutation reverted in the presence of P transposase, indicating that the P insertion was the cause of the *trc* mutation on this chromosome. In addition to complete revertants we also obtained a partial revertant that is viable over a Deficiency for the region. This hypomorphic allele (*trc*[8]) has proven useful in phenotypic studies. A more complete description of the properties of these alleles will be presented elsewhere.

Cloning of *trc*: We used plasmid rescue of the *trc*[7] allele to obtain a *trc* genomic clone. The sequencing of the rescued plasmid showed that it contained part of the Drosophila NDR kinase gene. We found that this DNA fragment was next to the *deaf-1* gene (Li *et al.* 1999), and by sequencing both genomic (accession no. AF247814) and cDNA clones [isolated by the BDGP (clone LD15101, accession no. AF238490)] we found that the putative *trc* gene contained four small introns. The *P* element responsible for the *trc*[P] mutation was inserted into the middle of the first intron (Figure 1). To confirm that we had indeed identified the *trc* gene we sequenced genomic DNA from several *trc* point mutants and identified mutations in five EMS-induced *trc* alleles (Table 1). Three were missense mutations, one was a nonsense mutation that would produce a truncated protein, and the fifth was a splice junction mutation that should prevent the normal processing of the *trc* mRNA. The missense mutations argue strongly that the kinase

TABLE 1

Molecular changes associated with *trc* mutations

Allele	Molecular lesion	Comment
trc[1]	Arg^{395} → Pro	This Arg is conserved in all NDR proteins
trc[2]	Intron 1 splice donor site GT → AT	Prevent synthesis of mature mRNA
trc[3]	Not determined	
trc[4]	Gly^{236} → Glu	Conserved Gly in kinase superfamily (DFG)
trc[5]	Trp^{316} → TGA stop codon	Truncated protein
trc[6]	Gly^{100} → Glu	Conserved Gly at ATP-binding site in kinase superfamily
trc[7]	*P* insertion in first intron	From Szeged collection (0669/17)
trc[8]	Partial revertant of *trc*[7]	Alteration in *P* element (details not determined)

activity of *trc* is essential for the gene's activity. The conserved regions of kinase superfamily members are typically broken up into 10 subdomains (Hanks and Hunter 1995). One missense mutation resulted in the change of gly^{100} to glu. This gly is part of the very highly conserved ATP-binding site in kinase subdomain I. A second resulted in the change of gly^{236} to glu. This gly is part of the very highly conserved DFG triplet found in kinase subdomain VII. These two sites are among the 12 sites generally recognized as being invariant (or almost invariant) in the kinase superfamily (Hanks and Hunter 1995). Thus, these sites are strongly implicated as playing important roles in kinase function and it is very likely that these *trc* mutations eliminate or substantially reduce kinase activity. This argues that the kinase activity is essential for the function of *trc*. The third missense mutation resulted in the change of arg^{395} to pro. This arg is not conserved in all kinases, but it is a site that is conserved in all the NDR proteins. This site is in kinase subdomain X.

A comparison of our cDNA sequence, our genomic sequence, and the published NDR sequence (Millward *et al.* 1995) revealed a couple of changes that we suspected were due to either sequencing mistakes or naturally occurring polymorphisms. One change that would result in a four-amino-acid difference did not seem likely to have such an explanation. This change was located at an intron/exon border and suggested the possibility of the use of an alternative splice site. To test this we sequenced this region in four additional cDNA clones (GH16329, GH24041, LD37189, and LP06419) and found examples of both splicing patterns (Figure 1). We refer to the short form as *trc-S* (accession no. AF239171) and the long form as *trc-L* (accession no. AF238490). Three of the cDNA clones were the long form (LD15101, GH16329, and GH24041) and two were the short form (LD37189 and LP06419). Due to the small difference in size we have not determined the relative abundance of the two forms. This four-amino-acid difference is located provocatively in between subdomains VII and VIII, just five amino acids from the sequence identified as a nuclear localization sequence in the human NDR (Millward *et al.* 1995). The region between kinase subdomains VII and VIII (and the C- and N-terminal ends of these regions) has been found to play a major role in the recognition of peptide substrates in other kinases and this region is also a site of an activating phosphorylation (Hanks and Hunter 1995; Newton 1997; Millward *et al.* 1999). Based on the location of this four-amino-acid difference we suggest that there may be functional differences between the two forms of Trc.

P rescue with UAS *trc*: We subcloned the wild-type *trc*-L cDNA into the pUAST vector and obtained germline transformants. We found complete rescue of the wing hair phenotype and bristle phenotypes of a *trc* null genotype when expression of the transgene was driven by an *actin-GAL4* transgene (Brand and Perrimon 1993). The animals died as normal-looking pharate adults. The lack of complete rescue to viability by the *trc*-L cDNA might be due to subtle functional differences between the *trc*-S and *trc*-L mRNAs.

We examined the expression of the *trc* mRNA by Northern blots and found it to be expressed at a similar level at all stages examined (Figure 2C). We also examined the expression of *trc* in the pupal wing and found it to be expressed in all regions (Figure 2A). These data, taken together with the phenotypic rescue by driving expression with actin-GAL4, suggest that the developmental role of *trc* may not be dependent on changes in transcription.

The wing hair phenotype of *trc*: Somatic clones homozygous for strong/null *trc* alleles produced an extreme multihair phenotype (Figure 3). Individual cells produced as many as 16 hairs, but the number was quite variable. This variability was seen when we scored cells from 10 different *trc*[7] clones in the wing. We found clones where there were as few as 3.5 hairs per cell and others where there were an average of >7 hairs per cell. Much of the difference appeared to be related to *trc* cells in more distal and peripheral regions of the wing having a less severe phenotype than cells in other regions. Some *trc* cells produced one relatively normal-sized hair and several very small hairs, while others produced a number of short hairs. Some hairs appeared to be split along the shaft, but in the majority of cases

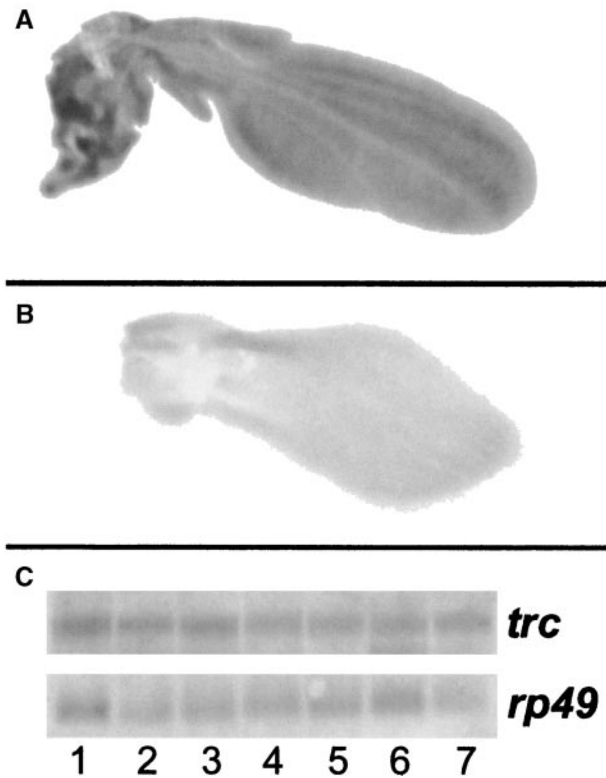

FIGURE 2.—(A) An *in situ* hybridization with a *trc* antisense probe to a pupal wing 30 hr after white prepuae formation. (B) A control from the same experiment where a *trc* sense probe was used on an equivalent pupal wing. (C) The results of probing a developmental Northern with a *trc* or *rp49* probe (loading control). Approximately 50 μg of total RNA was isolated from a variety of developmental stages, fractionated by gel electrophoresis, and blotted to Nytran. The blot was probed with the insert of the *trc* cDNA LD1510, and then the same blot was reprobed with RP49 as a loading control. Lane 1, 0- to 24-hr embryo RNA; lane 2, third instar larval RNA; lane 3, 0- to 1-day pupal RNA; lane 4, 1- to 2-day pupal RNA; lane 5, 2- to 3-day pupal RNA; lane 6, adult female RNA; lane 7, adult male RNA.

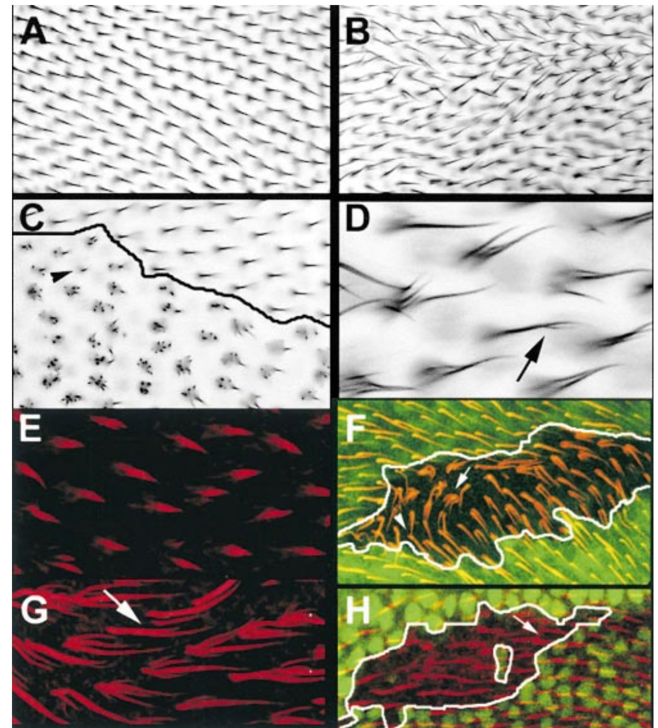

FIGURE 3.—The wing phenotype of *tricornered*. (A) A bright-field image of a wild-type adult wing and (B) the same region of a trc^8/trc^1 wing. A weak multiple-hair-cell phenotype is obvious. (C) Part of the border between a trc^7 somatic clone and neighboring wild-type cells. The clone border is marked in black. The mutant clone cells show a strong multiple-hair-cell phenotype. The hairs formed by the clone cells lay at a larger angle to the wing surface and appear almost like dots in the micrograph. This is common for cells in large *trc* clones. The arrowhead points to a likely *trc* cell (based on its location and orientation) that formed a single hair. (D) A high-magnification image of a trc^8/trc^1 wing. The arrow points to a hair that is split distally. Note the apparent clustering of hairs. (E and G) Confocal images of trc^8/Df pupal wings stained with rhodamine phalloidin (Molecular Probes). Note the weaker phenotype in the younger hairs (E *vs.* G). The arrow in G points to a hair that is split distally. (F and H) Pupal wings, stained with rhodamine phalloidin, which contain trc^7 clones marked by the loss of a transgene where the ubiquitin promoter is driving expression of the green fluorescent protein (GFP). The clone border is outlined in white. The arrows in F and H point to split hairs. The arrowhead in F points to an unbranched normal-length hair produced by a *trc* cell. Note that there is no difference in prehair length or shape between the clone and nonclone cells. Also note the relatively weak phenotype of the prehairs in the clones (compare C and F or H as these clones are of the same genotype). The Ubi-GFP transgene used in these experiments does not express at a high level and we do not always see clear nuclear localization, but it is adequate to identify the clone. In examining these panels remember that the prehair is formed at the distal end of a cell and extends over the neighboring distal cell.

the multiple hairs extended to the cuticular surface formed by the apical surface of the pupal wing cells. The hairs were routinely clustered close together. In some cases *trc* hairs were oriented almost orthogonally to the plane of the wing. In those cases they appeared as dots in bright-field microscopy (Figure 3C). In large clones (>100 cells) we sometimes saw evidence that cell shape was altered as hairs and hair clusters were less evenly distributed than in a wild-type wing. We also occasionally found that the wing was distorted in the region of the clone. Small clones (6–20 cells) in all regions of the wing showed a multihair phenotype, arguing that *trc* must function in all wing cells. This was consistent with our *in situ* hybridization results, which showed the gene was expressed in all wing cells.

The hypomorphic trc^8/Df phenotype was a milder version of the phenotype seen in clones with stronger alleles. A majority of the cells in the more central and proximal regions of trc^8/Df wings produced multiple hairs. In a region we have used in the past for quantifying multiple-hair-cell phenotypes (WONG and ADLER 1993) we found an average of 2.3 hairs per cell. This is substantially less than we saw in clones of the strong *trc* alleles.

It is also less than we saw for *multiple wing hair* mutants (3.7 hairs/cell; Wong and Adler 1993), but more than we saw for *in*, *fy*, or *frtz* mutants (from 1.7 to 1.9 hairs per cell; Wong and Adler 1993). At high magnification we could see that the trc^8/Df wings had some hairs that were split distally, but in most cells the multiple hairs appeared to be separate but clumped closely together (Figure 3D). It is possible that in addition to splitting, some extra hairs might result from independent prehair initiations. When a trc^8/Df cell formed a single hair it appeared normal in shape and length. The wings of trc^8/Df flies had a texture that was similar to that seen in *ck* mutants and the mutant wings often showed a mild curve upward.

We examined developing wing hairs in trc^8/Df pupal wings and in trc^7 clones in pupal wings. Early in hair morphogenesis in trc^8/Df pupal wings the multiple hair phenotype appeared milder than in adult wings. As prehair extension proceeded the multihair phenotype became progressively stronger (Figure 3, E and G). In confocal images some hairs were split along the extending shaft while others appeared to be separate down into the cell. We suggest that in *trc* multiple hairs arise by the splitting of individual hairs and that in at least some cases the splitting occurs distally and is propagated back to the apical surface of the cell. We also found that epidermal cells in other body regions of trc^8/Df animals formed extra trichomes. When we examined pupal wing clones homozygous for trc^7 we found that only mutant cells (marked by the loss of green fluorescent protein expression) showed a *trc* phenotype. The mutant cells had on average a weaker phenotype than clones of the same genotype in adult wings (Figure 3, compare C and F and H). A substantial number of *trc* clone cells did not have a multiple-hair phenotype in the pupal wing clones. This was surprising, as we have only rarely seen hints of such *trc* cells in adult wings. We suspect that this was due to the *trc* phenotype continuing to get stronger as differentiation proceeded. It is important to note that when *trc* pupal wing cells had only a single hair it was the same length as the neighboring wild-type hairs. This argued that *trc* mutations did not delay prehair initiation or slow prehair elongation.

Bristle phenotype: We have principally examined the bristle phenotype of *trc* in trc^8/Df flies (Figure 4). We found bristles with obviously split shafts in all regions of trc^8/Df adult flies, although they were more frequent on the abdomen than in other body regions. There was substantial fly-to-fly variation in the phenotype. In some flies 50% of the bristles in abdominal segment 3 or 4 were split while in some siblings <3% were split. Unbranched bristles in *trc* mutants typically appeared normal in shape (Figure 4). Most bristles showed a single split, but the phenotype was variable and examples of multiply split bristles were seen. The branches typically diverged at various points along the bristle shaft, while infrequently the split originated at the proximal end of the shaft. In general, when a bristle was split both branches were thinner than the segment just proximal to the split. In addition, the thicker branch was routinely longer than the thinner branch. In many cases one of the shaft segments was quite thin and was easily detected only in the compound microscope. We also examined trc^8/Df pupae and were able to detect split bristles using either a fluorescent phalloidin to stain F-actin or the pan-neural 22C10 monoclonal antibody (Zipursky *et al.* 1984). In some cases we saw continuous actin bundles that extended from an unbranched region into branched regions (Figure 4H). We saw no evidence for abnormalities in the actin bundles *per se* or in the level of phalloidin staining and most *trc* bristles were of normal shape, suggesting that *trc* affects neither general actin polymerization nor bundling. In addition to the split-bristle phenotype the polarity pattern of posteriorly pointing abdominal bristles was altered. Many bristles in such abdomens had abnormal polarity, frequently pointing toward the dorsal midline. This suggested the possibility that *trc* might regulate the orientation of mitotic spindles as this has been linked to bristle polarity (Gho and Schweisguth 1998). The phenotype seen in clones homozygous for strong *trc* alleles was similar to that seen for the hypomorphic genotype.

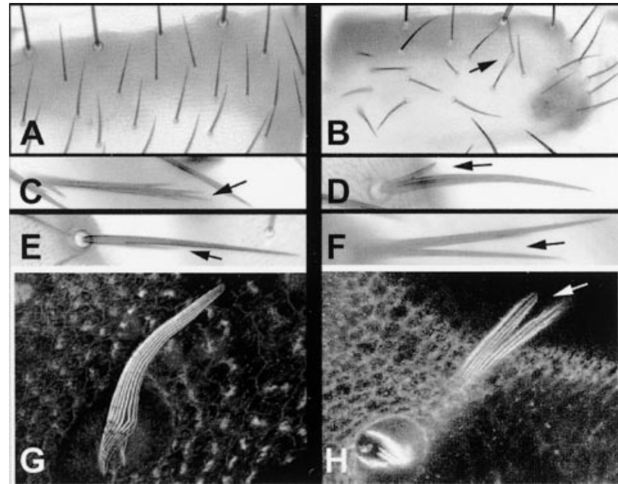

Figure 4.—The bristle phenotype of *tricornered.* (A) A section of a wild-type abdomen. (B) An equivalent region of a trc^8/Df abdomen. Note the abnormal polarity of the abdominal bristles. An arrow points to a split bristle. (C–F) High-magnification images of mutant (trc^8/Df) adult abdominal bristles. Note the range of mutant phenotypes. Arrows point to the split(s). (G) A confocal image of a wild-type bristle stained with rhodamine phalloidin. Note the prominent bundles of F-actin. (H) A split bristle from a trc^8/Df abdomen. Note how the split appears to occur between neighboring actin bundles.

Larval denticles: The denticles seen on first instar *trc* larvae were typically normal in morphology; however, second and third instar larvae showed denticle abnormalities with varying penetrance and expressivity. The pattern of denticles was often abnormal with the fairly

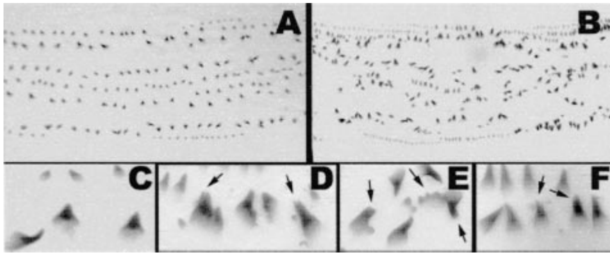

Figure 5.—The denticle phenotype of *tricornered*. (A) A light micrograph of part of the sixth abdominal segment from a wild-type larva. Note the well-organized rows of denticles. (B) An equivalent segment from a trc^1/Df larva. (C–F) High-magnification images of denticles. C shows wild-type denticles while D–F show denticles from trc^1/Df larvae. Arrows point to the split denticles in the mutant.

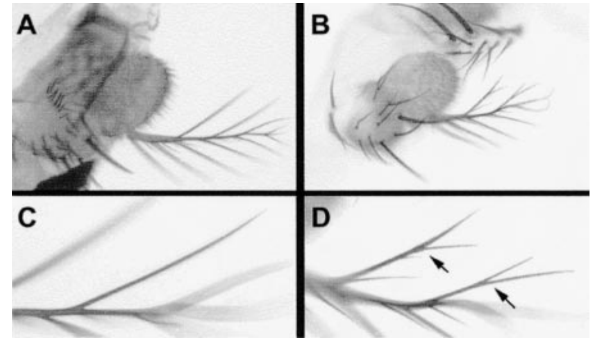

Figure 6.—The arista phenotype of *tricornered*. (A) A wild-type antenna, (B) a trc^8/trc^1 antenna. (C and D) Blowups of part of A and B that give a clear view of the lateral extensions of the arista. The arrows point to split laterals in the mutant arista.

precise rows of denticles being replaced by a more chaotic arrangement (Figure 5). At the level of the individual denticle the predominant abnormality was splitting. More than 30% of the denticles in some denticle bands showed such a phenotype. We suspected we did not see a phenotype in first instar larvae due to maternal rescue. Consistent with maternally provided *trc*-RNA-rescuing embryonic functions, we found extensive embryonic lethality when we injected *trc* dsRNA into embryos, which was not seen with control double-stranded RNAs (Kennerdell and Carthew 1998).

Arista phenotype: The arista is the terminal segment of the antenna, and in a wild-type fly it consists of a number of lateral extensions extending from a central core (Figure 6). In *trc* hypomorphic mutants that survived until adulthood we routinely saw that one or more of these lateral extensions was branched. These appeared to be at least superficially similar to the branches seen in hairs and bristles.

The actin cytoskeleton and bristle splitting: Previous data showing that the disruption of the actin cytoskeleton resulted in split and stunted hairs and bristles came from experiments where we treated pupal wings cultured *in vitro* with CD (Turner and Adler 1998). This system supported the extension of hairs and bristles, but not the differentiation of the normal cuticle. Most of the observations in those experiments were on hairs with only limited examination of bristle morphogenesis. To determine the extent of similarity between the *trc*- and inhibitor-induced bristle phenotypes we reexamined the effects of inhibitors on bristle morphogenesis. To do this we used a recently developed procedure for injecting material into pupae. We found that the injection of either CD or LAT A, both of which inhibit actin polymerization (Spector *et al.* 1999), resulted in frequent split bristles (Figure 7, Table 2). The strongest response was when 1 mm LAT A was injected, which resulted in 61.8% of the postvertical bristles on the head being split. A few of the abnormal bristles seen after CD or LAT A injection resembled those seen in *trc* mutants; however, the drug treatments routinely caused bristles to be shorter and bent or twisted. The effects of actin polymerization inhibitors on bristle morphogenesis were quite similar to those obtained for hair morphogenesis using the pupal wing *in vitro* culture system (Turner and Adler 1998).

We used phalloidin staining to examine developing bristles in pupae injected with CD several hours prior to bristle differentiation (Figure 7). We found many examples of abnormal bristles with multiple shafts. In many cases the branching was at the proximal end of the shaft; indeed the shafts appeared independent. Typically, the longest shaft had relatively normal actin filament bundles while the actin filament bundles appeared disorganized in the smaller shafts. The disorganization consisted of bundles that were irregularly spaced, bundles that did not appear to extend all the way to the proximal end of the shaft, and bundles of varying thickness. In some cases it appeared that a smaller shaft "budded" off of the longest shaft, but in contrast to what we saw with *trc* this "budding" often did not result in a reduction in shaft diameter distal to the "bud." In the most severely affected bristles the overall actin staining level was reduced, which we suggest represents a gross alteration in actin filaments due to the CD. We suggest that CD treatment produces multiple bristles in part by causing the formation of ectopic actin filament bundles.

On the basis of the differences between the mutant and drug-induced phenotypes we concluded that *trc* did not cause split hairs or bristles by a general inhibition of actin polymerization. However, the induction of split bristles after the injection of CD and LAT A did suggest that *trc* might have a functional connection to the actin cytoskeleton. If this was the case we predicted that a weak *trc* mutant would be hypersensitive to low doses of CD. We tested this hypothesis by injecting a low dose (100 μM) of CD into trc^8 pupae. This dose of CD caused only 11.9% of the postvertical bristles in Oregon-R to

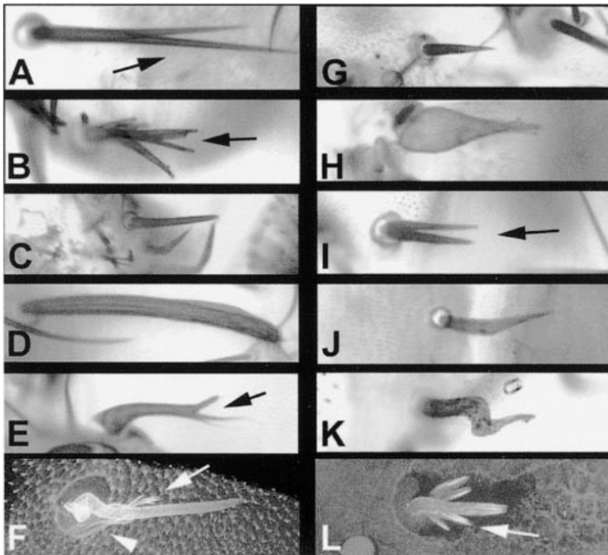

FIGURE 7.—Inhibitor-induced bristle abnormalities. (A–C) The range of abnormalities induced by CD or LAT A injection into pupae. While occasional bristles are split and have a normal shape (A) more frequently we see abnormal shapes as well as splitting (B and C). (D and E) Examples of the abnormalities seen after injection of stauroporine. While some splitting is seen the most notable effect is the abnormal shape and length. (G–I) The range of phenotypes seen after injection of VB or colchicine. High doses routinely yield grossly shortened and bloated bristles as the one in H. (J and K) Examples of sn^3 f^{36a} bristles from the abdomen and head, respectively. (F and L) Rhodamine-phalloidin-stained bristles from pupae that had been injected with 200 μM CD. (F) Two bristle shafts emerge from the cell body. One appears relatively normal. The other shows diffuse and disorganized actin filament bundles (arrowhead) and split ends (arrow). (L) The phalloidin staining is weak, presumably due to the effects of CD. The arrow points to a branch that appears to "bud" from the main shaft without reducing the diameter of the main shaft.

be split, and after buffer injection into trc^8 pupae we found 20% of the bristles were split. When we injected a low dose of CD into trc^8 pupae, we found 40.9% of the bristles were split (Table 2). This was a significant enhancement over either trc^8 with buffer injection (*z*-test, $P = 0.014$) or Oregon-R injected with CD (*z*-test, $P = <0.001$). These results are consistent with *trc* mutations perturbing or interacting with the actin cytoskeleton in some way.

In the experiments where we examined the effects of CD on wing hair morphogenesis we observed that CD treatment resulted in a several-hour delay in the appearance of hairs as well as branching and slowed growth (Turner and Adler 1998). To see if CD produced similar effects on developing bristles we measured the length of postvertical bristles at various times after the injection of a low dose of CD. In this experiment, as in others, we injected the drug at ∼28 hr after white prepupae formation, which was a couple of hours prior to the beginning of overt bristle differentiation. We found a delay in bristle differentiation that was similar to the delay we had seen earlier for wing hair differentiation (Figure 8). We did not see a major reduction in the rate of bristle elongation in this experiment, which used a low dose of CD. Tilney and colleagues recently found that CD and LAT A decreased the rate of bristle elongation in cultured pupal thoraces (Tilney *et al.* 2000a).

Bristle morphogenesis and other inhibitors: The injection of the microtubule antagonist vinblastine resulted in many dramatically stunted and bloated bristles, bent and/or barbed bristles, as well as some split bristles (Figure 7, Table 2). Similar results were obtained in less extensive experiments where we injected colchicine into pupae. The collection of abnormal bristles after VB injection was very different from that seen in *trc* mutants, thus we concluded that it was unlikely that *trc* produced a bristle phenotype by a general inhibition of microtubule function. The ability of VB injection to cause very short bristles suggested that the microtubule cytoskeleton is of major importance in bristle elongation. A likely explanation for this is that the microtubule cytoskeleton is required for the transport of "cargo" to the growing bristle tip. We also examined the consequences of VB injection on the rate of bristle elongation (Figure 8). The injection of VB resulted in both a delay in initiation and a reduced rate of elongation. Surprisingly, Tilney and colleagues did not see any affect of microtubule inhibitors on bristle elongation (Tilney *et al.* 2000a). Their experiments differed from most of ours in that they applied the drug after the initiation of bristle elongation. However, we saw effects from VB and colchicine in the limited experiments where we treated developing bristles after initiation in pupal wing cultures (Turner and Adler 1998) or after injection of inhibitors into pupae (data not shown). In our experiments the effects on bristle morphology were less dramatic when the injection was after initiation.

In a recent article on function of the actin cytoskeleton in bristle development Tilney and colleagues reported that the general kinase inhibitor stauroporine caused the large bundles of actin filaments to be released from their close association with the plasma membrane (Tilney *et al.* 2000b). The authors did not note if this loss of actin organization affected the integrity of bristles. To determine this we injected stauroporine into pupae. A variety of phenotypes were seen in the resulting bristles, presumably due to the broad activity of stauroporine resulting in the inhibition of several different kinases and pathways. The most common phenotype was shorter and fatter bristles. This phenotype resembled that found after vinblastine injection although it was less severe. Perhaps this effect was due to an effect on the microtubule cytoskeleton and not to alterations in the arrangement of actin bundles. We also found a substantial number of bristles with "barbed" ends that resemble those sometimes seen in *f* and/or

TABLE 2

Bristle phenotypes induced by drug injections

Genotype	Inhibitor (and dose)	No. of bristles scored	Fraction of split bristles (%)	Fraction with other gross abnormalities (%)	Fraction of injected pupae that showed a phenotype on the head (%)
oreR	Buffer	60	0.0	0.0	0.0
oreR	CD (100 μM)	42	11.9	14.3	38.1
oreR	CD (200 μM)	16	18.8	25.0	57.1
oreR	CD (250 μM)	38	23.7	31.6	63.2
oreR	LAT A (500 μM)	30	13.3	16.7	46.7
oreR	LAT A (1000 μM)	34	61.8	70.6	76.5
oreR	Stauroporine (250 μM)	42	0.0	2.4	9.5
oreR	Stauroporine (400 μM)	35	8.6	16.7	38.9
oreR	VB (500 μM)	26	7.7	34.6	53.8
oreR	VB (1000 μM)	28	10.7	57.1	78.6
trc^8	Buffer	60	20.0	33.3	63.3
trc^8	CD (100 μM)	32	46.9	50.0	75.0
trc^8	CD (200 μM)	24	50.0	54.2	83.3
trc^8	Stauroporine (250 μM)	40	35.0	37.5	65.0
trc^8	Stauroporine (400 μM)	38	54.5	58.7	85.0
sn^3f^{36a}	Buffer	22	4.5	NR[b]	27.3[a]
sn^3f^{36a}	CD (100 μM)	22	27.3	NR	63.6[a]

[a] Refers to the fraction of injected pupae that showed a split bristle phenotype anywhere on head.
[b] Not relevant.

sn bristles. This could be due to the loss of bundle organization reported by Tilney *et al.* (2000b). At a low frequency we saw split bristles that were reminiscent of *trc* bristles. To see if this could be due to stauroporine inhibiting the activity of Trc *in vivo* we asked if trc^8 pupae were more sensitive to low doses of stauroporine than wild-type pupae. We found this was the case as the injection of 400 μM stauroporine significantly increased the frequency of split bristles in trc^8/trc^8 flies (Table 2) compared to trc^8/trc^8 injected with buffer (z-test, $P = <0.001$) or Oregon-R injected with stauroporine (z-test, $P < 0.001$). This is consistent with the kinase activity of Trc being functionally important for bristle morphogenesis.

The elegant studies of Tilney *et al.* (1995, 1998) have shown that in *sn f* double mutants the large bundles of actin filaments are lost, although a few actin filaments are still found juxtaposed to the plasma membrane. In such double mutants the bristles are shorter than normal and have an abnormal morphology (Figure 7). In our experiments only 2% of the postvertical bristles in sn^3 f^{36a} flies were split, suggesting that the striking arrangement of large bundles of actin filaments is not essential for bristle integrity. The injection of CD into sn^3 f^{36a} pupae was still able to induce bristle splitting (Table 2), thus the large bundles of actin filaments are also not required for bristle splitting. We also did experiments where we injected VB into sn^3 f^{36a} pupae. We found that bristle elongation in sn^3 f^{36a} was much more sensitive to VB than in wild-type pupae. Even low doses routinely completely blocked bristle elongation. We suggest that this enhanced sensitivity is due to partial redundancy between the actin and microtubule cytoskeletons for the transport of cargo to the tip of the growing bristle. The actin-based system is presumably impaired in *sn f* double mutants, hence the dramatic consequences from disrupting microtubules.

***trc* does not alter the expression of actin or tubulin:** Actin and tubulin are major cytoskeletal elements in growing hairs and bristles and as noted above the antagonism of either of these cellular systems can result in split bristles. To test if *trc* could be producing a split hair and bristle phenotype by altering the expression or balance between these two cytoskeletal elements we carried out Western blot analysis of actin and tubulin in wild-type and mutant *trc* pupal wings. No differences were seen, hence it is unlikely that *trc* mutations affect the expression or stability of actin or tubulin (data not shown).

DISCUSSION

NDR kinase family: The NDR kinases are members of the AGC kinase group (they are closest to the AGC-VII subfamily; Hanks and Hunter 1995). Among the well-studied members of the AGC group are the cyclic-AMP-dependent protein kinase (PKA) subfamily and the Ca^{2+}-dependent protein kinase C (PKC) subfamily. The NDR kinase was originally identified in the *Caenorhabditis elegans* genome by sequence analysis and fly

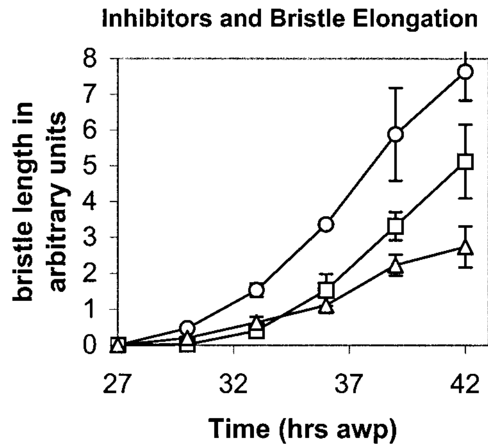

Figure 8.—Bristle length is plotted as a function of time. Inhibitor (or buffer) was injected into pupae at 28 hr awp (after white prepupae) and at various times pupae were sacrificed, fixed, stained, and examined by fluorescence microscopy. Bristle lengths were measured and the data were assembled using Excel. Points for buffer-injected pupae are circles, points for pupae injected with 100 μm CD are squares, and points for injected pupae injected with 200 μm VB are triangles. For some time points the error bars were smaller than the symbol and they are not visible.

and human genes were then cloned on the basis of homology to the worm gene (Millward *et al.* 1995). Further progress in genome project sequencing has resulted in the identification of a second human NDR family member. These proteins are quite similar and likely to be orthologs. The 463-amino-acid Trc-L protein is 68% identical and 81% similar to the human NDR protein over a 444-amino-acid stretch. The Trc-L protein is almost as closely related to the *C. elgans* NDR. Blast *E* values of e^{-150} or less are obtained in comparisons of the individual NDR kinases. Blast searches with Trc find the next most related group of proteins are fungal proteins such as the ORB6 protein of Schizosacchromyces (*E* values of $\sim e^{-119}$; Verde *et al.* 1998) and a number of plant kinases identified in sequencing projects. The Trc-L and Orb6 proteins are 49% identical and 66% similar over 451 amino acids. Somewhat more distant members of this kinase subfamily are the COT-1 protein of Neurosopora (Yarden *et al.* 1992) and the Drosophila WARTS/LATS protein (Justice *et al.* 1995; Xu *et al.* 1995). More distantly related, but still closer to Trc than most kinases are proteins such as the mammalian myotonic dystrophy kinase (Brook *et al.* 1992; Fu *et al.* 1993), the Genghis Khan kinase of flies (Luo *et al.* 1997), the ROCK (Rho-activated kinase of mammals; Leung *et al.* 1996), and the DBF2 protein of yeast (Komarnitsky *et al.* 1998) [note that DBF2 was described previously as being in AGC-VII (Hanks and Hunter 1995)]. Mutations in the genes that encode many of these kinases result in phenotypes that cause alterations in cell morphology or polarity. For example mutations in *orb6* of *Schizosacchromyces pombe* cause a loss of polarized growth and delayed entry into mitosis (Verde *et al.* 1998). Mutations in *cot1* of Neurosopora result in branched hyphae (Yarden *et al.* 1992) and mutations in *warts/lats* from flies results in tumors and altered cell morphology (Justice *et al.* 1995; Xu *et al.* 1995). Given the common links to cellular morphology it seems reasonable to suggest that these kinases might have similar targets (*e.g.*, actin cytoskeleton). Hence, it is somewhat surprising that some of these proteins are nuclear [NDR (Millward *et al.* 1995), DBF2 (Komarnitsky *et al.* 1998)] and others cytoplasmic [ORB6 (Verde *et al.* 1998), ROCK (Leung *et al.* 1996)]. Perhaps this group of kinases has both nuclear and cytoplasmic functions and depending on context they are found primarily in one of these two cellular compartments. A sequence in the human NDR was identified by Millward *et al.* (1995) that acts as a nuclear localization signal and this sequence is conserved in the fly and worm NDRs. Interestingly this sequence is also conserved in Orb6, which was found to be cytoplasmic (Verde *et al.* 1998). We have confirmed that the Trc is primarily nuclear in cultured mammalian cells, although the situation appears more complicated in the fly (W. Geng and P. N. Adler, unpublished results). This raises the possibility that Trc functions in cellular morphogenesis by regulating the expression of target genes that encode cytoskeleton-interacting proteins and not via the direct modification of the cytoskeleton.

How does *trc* affect the integrity of cellular extensions? Mutations in *trc* result in the splitting of epidermal hairs, the shafts of sensory bristles, larval denticles, and the lateral branches of the arista. Mutations in *trc* do not however cause dramatic effects on hair or bristle shape or length. Nor do we see evidence for *trc* delaying prehair initiation or slowing prehair elongation. Based on the similar phenotypes it seems likely that the Trc protein has a similar target(s) in all of these cell types. In comparing the *trc*-induced phenotypes to the effects of various inhibitors the actin cytoskeleton was identified as a candidate target as we found that the inhibition of actin polymerization with CD or LAT A resulted in frequent split hairs, bristles, and arista lateral branches (Turner and Adler 1998; B. He and P. N. Adler, unpublished results). However, the CD- and LAT A-induced phenotypes differed substantially from those found in *trc* mutants in that the drug treatments had major effects on the shape and length of hairs and bristles. Because of this we suggest that *trc* mutations do not inhibit actin polymerization. Consistent with this, *trc* mutant hairs, bristles, and arista laterals routinely stained strongly for F-actin. We also found that *trc* prehairs in clones developed and grew at the same rate as their wild-type neighbors. The data also argue that *trc* is not needed for the bundling of actin filaments in developing bristles as these looked normal in trc^8/*Df* pupae.

The split hairs and bristles that are found in *trc* mu-

tants appear normal except where they are split. In this way the *trc* phenotype differs profoundly from the phenotypes found in cells mutant for genes such as *f*, *sn*, or *ck*, which encode proteins that are functional components of the actin cytoskeleton (Bryan *et al.* 1993; Cant *et al.* 1994; Petersen *et al.* 1994; Tilney *et al.* 1995; Ashburner *et al.* 1999; D. Kiehart, personal communication). In these cases the mutant phenotype is displayed all along the length of the bristle or hair. We suggest that this is because these proteins are part of the machinery that is directly involved in the morphogenesis of hairs and bristles and hence are needed throughout morphogenesis. This suggests the possibility that *trc* has a regulatory as opposed to structural function.

A possible hypothesis is that *trc* functions to repress the initiation of outgrowths and that splitting is a consequence of ectopic outgrowths. This hypothesis can account for some of our data, but it does not easily accommodate our finding that in splitting bristles we do not see evidence for new ectopic large bundles of actin filaments. Rather, existing neighboring bundles appear to separate as the segments split apart. Our observations on *trc* differ from those on bristles after CD treatment where we did see evidence for ectopic bundles of actin filaments.

We suggest that what is important for maintaining the integrity of hairs and bristles is coordinating the growth of actin filaments and/or other cellular components. When some filaments or bundles grow more rapidly than others splitting might occur. We suggest that this mechanism contributes to the split hairs and bristles seen after CD and LAT A treatment. If this is the case then we might expect that developing bristle cells would monitor the polymerization of actin filaments and try to prevent splitting by slowing down the polymerization of filaments or bundles that are growing more rapidly than others (or vice versa). This could be thought of as being equivalent to a morphogenetic checkpoint (McMillan *et al.* 1998). The *trc* gene could be a component of a signal transduction pathway that mediates the monitor function. Such a monitor function might involve regulating gene expression, which could explain the nuclear location of NDR (Millward *et al.* 1995). A hint that such a monitor/checkpoint might exist comes from our finding a delay in bristle or hair morphogenesis after treatment with low doses of CD (Turner and Adler 1998). Further experiments will be needed to determine if such a monitoring function exists and if *trc* encodes a component of it. Several other plausible models exist to explain *trc* function in maintaining the integrity of cellular extensions. The Trc protein could function in the assembly of the cellular components that organize the outgrowth of these cellular extensions. Defects in properly assembling an organizing center might result in the splitting of the extensions at later stages in elongation. The Trc protein could also function to mechanically strengthen cellular extensions in some way to insure their integrity, *e.g.*, by increasing the degree of cross-linking of the membrane cytoskeleton.

This work was supported by a grant from the National Institutes of Health (GM53498).

LITERATURE CITED

Ashburner, M., S. Misra, J. Roote, S. E. Lewis, R. Blaszej *et al.*, 1999 An exploration of the sequence of a 2.9-Mb region of the genome of *Drosophila melanogaster*: the Adh region. Genetics **153:** 179–219.

Brand, A. H., and N. Perrimon, 1993 Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development **118:** 401–415.

Brook, J. D., M. E. McCurrach, H. G. Harley, A. J. Buckler, D. Church *et al.*, 1992 Molecular basis of myotonic dystrophy: expansion of a trinucleotide (CTG) repeat at the 3′ end of a transcript encoding a protein kinase family member. Cell **68:** 799–808.

Bryan, J., R. Edwards, P. Matsudaira, J. Otto and J. Wulfkuhle, 1993 Fascin, an echinoid actin-bundling protein, is a homolog of the Drosophila *singed* gene product. Proc. Natl. Acad. Sci. USA **90:** 9115–9119.

Cant, K., B. A. Knowles, M. S. Mooseker and L. Cooley, 1994 Drosophila *singed*, a fascin homolog, is required for actin bundle formation during oogenesis and bristles extension. J. Cell Biol. **125:** 369–380.

Castellano, F., P. Montcourrier, J. C. Guillemot, E. Gouin, L. Machesky *et al.*, 1999 Inducible recruitment of Cdc42 or WASP to a cell-surface receptor triggers actin polymerization and filopodium formation. Curr. Biol. **9:** 351–360.

Deak, P., M. M. Omar, R. D. C. Saunders, M. Pal, O. Komonyi *et al.*, 1997 P-element insertion alleles of essential genes on the third chromosome of *Drosophila melanogaster*: correlation of physical and cytogenetic maps in chromosomal region 86E-87F. Genetics **147:** 1697–1722.

Eaton, S., 1997 Planar polarization of Drosophila and vertebrate epithelia. Curr. Opin. Cell Biol. **9:** 860–866.

Eaton, S., R. Wepf and K. Simons, 1996 Roles for Rac1 and Cdc42 in planar polarization and hair outgrowth in the wing of Drosophila. J. Cell Biol. **135:** 1277–1289.

Ferrus, A., 1976 Aislamiento y caracterización de mutantes morfogeneticos en *Drosophila melanogaster*. Thesis (in FlyBase). Universidad Autonoma de Madrid, Spain.

Fu, Y.-H., D. L. Friedman, S. Richards, J. A. Pearlman, R. A. Gibbs *et al.*, 1993 Decreased expression of myotonin-protein kinase messenger RNA and protein in adult form of myotonic dystrophy. Science **260:** 235–238.

Gho, M., and F. Schweisguth, 1998 *Frizzled* signalling controls orientation of asymmetric sense organ precursor cell divisions in Drosophila. Nature **393:** 178–181.

Gubb, D., and A. Garcia-Bellido, 1982 A genetic analysis of the determination of cuticular polarity during development in Drosophila melanogaster. J. Embryol. Exp. Morphol. **68:** 37–57.

Hanks, S. K., and T. Hunter, 1995 The eukaryotic protein kinase superfamily: kinase domain structure and classification. FASEB J. **9:** 576–596.

Justice, R. W., O. Zilian, D. F. Woods, M. Noll and P. J. Bryant, 1995 The Drosophila tumor suppressor gene *warts* encodes a homolog of human myotonic dystrophy kinase and is required for the control of cell shape and proliferation. Genes Dev. **9:** 534–546.

Kennerdell, J. R., and R. W. Carthew, 1998 Use of dsRNA-mediated genetic interference to demonstrate that *frizzled* and *frizzled 2* act in the *wingless* pathway. Cell **95:** 1017–1026.

Komarnitsky, S. I., Y.-C. Chiang, F. C. Luca, J. Chen, J. H. Toyn *et al.*, 1998 DBF2 protein kinase binds to and acts through the cell cycle regulated MOBI protein. Mol. Cell. Biol. **18:** 2100–2107.

Leung, T., X.-Q. Chem, E. Manser and L. Lim, 1996 The p160 RhoA-binding kinase ROKa is a member of a kinase family and

is involved in the reorganization of the cytoskeleton. Mol. Cell. Biol. **16:** 5313–5327.

Li, X., A. Veraska and W. McGinnis, 1999 A sequence motif distinct from hox binding sites controls the specificity of a hox response element. Development **126:** 5581–5589.

Luo, L., T. Lee, L. Tsai, G. Tang, L. Y. Jan *et al.*, 1997 *Ghenghis Khan* (*Gek*) as a putative effector for *Drosophila* Cdc42 and regulator of actin polymerization. Proc. Natl. Acad. Sci. USA **94:** 12963–12968.

McMillan, J. N., R. A. L. Sia and D. J. Lew, 1998 A morphogenesis checkpoint monitors the actin cytoskeleton in yeast. J. Cell Biol. **142:** 1487–1499.

Millward, T., P. Cron and B. A. Hemmings, 1995 Molecular cloning and characterization of a conserved nuclear serine(threonine) protein kinase. Proc. Natl. Acad. Sci. USA **92:** 5022–5026.

Millward, T. A., D. Hess and B. A. Hemmings, 1999 Ndr protein kinase is regulated by phosphorylation on two conserved sequence motifs. J. Biol. Chem. **274:** 33847–33850.

Newton, A. C., 1997 Regulation of protein kinase C. Curr. Opin. Cell Biol. **9:** 161–167.

Nobes, C. D., and A. Hall, 1995 Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. Cell **81:** 53–62.

Park, W. J., J. Liu and P. N. Adler, 1994 The *frizzled* gene of Drosophila encodes a membrane protein with an odd number of transmembrane domains. Mech. Dev. **45:** 127–137.

Petersen, N. S., D. H. Lankenau, H. K. Mitchell, P. Young and V. G. Corces, 1994 Forked proteins are components of fiber bundles present in developing bristles of *Drosophila melanogaster*. Genetics **136:** 173–182.

Spector, I., F. Braet, N. R. Shochet and M. R. Bubb, 1999 New anti-Actin drugs in the study of the organization and function of the actin cytoskeleton. Microsc. Res. Techn. **47:** 18–37.

Tapon, N., and A. Hall, 1997 Rho, Rac and Cdc42 GTPases regulate the organization of the actin cytoskeleton. Curr. Opin. Cell Biol. **9:** 86–92.

Tilney, L. G., M. S. Tilney and G. M. Guild, 1995 F actin bundles in Drosophila bristles. I. Two filament cross-links are involved in bundling. J. Cell Biol. **130:** 629–638.

Tilney, L. G., P. Connelly, S. Smith and G. M. Guild, 1996 F-actin bundles in Drosophila bristles are assembled from modules composed of short filaments. J. Cell Biol. **135:** 1291–1308.

Tilney, L. G., P. S. Connelly, K. A. Vranich, M. K. Shaw and G. M. Guild, 1998 Why are two cross-linkers necessary for actin bundle formation in vivo and what does each cross-link contribute? J. Cell Biol. **143:** 121–133.

Tilney, L. G., P. S. Connelly, K. A. Vranich, M. K. Shaw and G. M. Guild, 2000a Actin filaments and microtubules play different roles during bristle elongation in Drosophila. J. Cell Sci. **113:** 1255–1265.

Tilney, L. G., P. S. Connelly, K. A. Vranich, M. K. Shaw and G. M. Guild, 2000b Regulation of actin filament cross-linking and bundle shape in Drosophila bristles. J. Cell Biol. **148:** 87–100.

Turner, C. M., and P. N. Adler, 1998 Distinct roles for the actin and microtubule cytoskeletons in the morphogenesis of epidermal hairs during wing development in Drosophila. Mech. Dev. **70:** 181–192.

Verde, F., D. J. Wiley and P. Nurse, 1998 Fission yeast *orb6*, a ser/thr protein kinase related to mammlian rho kinase and myotonic dystrophy kinase is required for maintenance of cell polarity and coordinates cell morphogenesis with the cell cycle. Proc. Natl. Acad. Sci. USA **95:** 7526–7531.

Vinson, C. R., and P. N. Adler, 1987 Directional non-cell autonomy and the transmission of polarity information by the *frizzled* gene of Drosophila. Nature **329:** 549–551.

Wong, L. L., and P. N. Adler, 1993 Tissue polarity genes of Drosophila regulate the subcellular location for prehair initiation in pupal wing cells. J. Cell Biol. **123:** 209–221.

Xu, T., W. Wang, S. Zhang, R. A. Stewart and W. Yu, 1995 Identifying tumor suppressors in genetic mosaics: the Drosophila *lats* gene encodes a putative protein kinase. Development **121:** 1053–1063.

Yarden, O., M. Plamann, D. J. Ebbole and C. Yanofsky, 1992 *cot-1*, a gene required for hyphal elongation in *Neurospora crassa*, encodes a protein kinase. EMBO J. **11:** 2159–2166.

Zipursky, S. L., T. R. Venkatesh, D. B. Teplow and S. Benzer, 1984 Neuronal development in the Drosophila retina: monoclonal antibodies as molecular probes. Cell **36:** 15–26.

Communicating editor: T. Schüpbach