**Drosophila myoblast city Encodes a Conserved Protein That Is Essential for Myoblast Fusion, Dorsal Closure, and Cytoskeletal Organization**

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**Abstract.** The *Drosophila myoblast city* (*mbc*) locus was previously identified on the basis of a defect in myoblast fusion (Rushton et al., 1995. Development [Camb.], 121:1979–1988). We describe herein the isolation and characterization of the *mbc* gene. The *mbc* transcript and its encoded protein are expressed in a broad range of tissues, including somatic myoblasts, cardiac cells, and visceral mesoderm. It is also expressed in the pole cells and in ectodermally derived tissues, including the epidermis. Consistent with this latter expression, *mbc* mutant embryos exhibit defects in dorsal closure and cytoskeletal organization in the migrating epidermis.

**In** vertebrate organisms, a common feature of the myogenic differentiation program of all muscle fibers is the apparent recognition, adherence, and fusion between myoblasts that generate multinucleate syncitia (for review see Fischman, 1972; Wakelam, 1985). Since this process can occur in cultured cells, tissue culture systems have been invaluable in identifying regulators of myoblast fusion. Essential components include cell adhesion molecules, calcium and molecules that are regulated by it, metalloproteases, meltrins, lipids, and others (Yagami-Hirozumi et al., 1995; for reviews see Wakelam, 1985; Knudsen, 1991). Homologues to these vertebrate factors have not yet been shown to function in myogenesis in *Drosophila*. However, morphological studies have established that the differentiated muscle fibers of insects are also syncitial (Ball et al., 1985; Campos-Ortega and Hartenstein, 1985; Bate, 1990, 1993; Doberstein et al., 1997). Therefore, one might anticipate that similar molecules will control fundamental aspects of myoblast fusion in a variety of species. Moreover, the use of genetics to identify critical myogenic regulators and their relationship to other molecules in *Drosophila* seems likely to reveal parallel pathways in vertebrate organisms.

Both the mesodermal and ectodermal defects are reminiscent of those induced by altered forms of Drac1 and suggest that *mbc* may function in the same pathway. MBC bears striking homology to human DOCK180, which interacts with the SH2-SH3 adapter protein Crk and may play a role in signal transduction from focal adhesions. Taken together, these results suggest the possibility that MBC is an intermediate in a signal transduction pathway from the *rho/rac* family of GTPases to events in the cytoskeleton and that this pathway may be used during myoblast fusion and dorsal closure.

While it seems unlikely that all aspects of myogenesis will be analogous between *Drosophila* and vertebrates, many appear to be conserved between these organisms. One example is the apparent conservation of myogenic regulatory molecules. Among these are *nautilus* (Michelson et al., 1990; Paterson et al., 1991), which, like its vertebrate counterparts (for review see Weintraub, 1993), can induce a somatic muscle differentiation program (Keller et al., 1997) and MEF2, an enhancer binding protein that is absolutely required for the induction of muscle-specific structural genes and myogenic differentiation (Lin et al., 1996; for review see Olson et al., 1995). Similarities are also apparent at the cellular level. For example, extensive proliferation of myosin-expressing myoblasts is not observed in either vertebrates (for review see Holtzer et al., 1975a) or *Drosophila* (Campos-Ortega and Hartenstein, 1985; Bate, 1993; Rushton et al., 1995). In addition, although fusion normally occurs before myosin expression in vertebrate cells, it is not an absolute prerequisite for expression of myosin in either system (Holtzer et al., 1975a, and references therein; see also Emerson and Beckner, 1975; Endo and Nadal-Ginard, 1987; Luo et al., 1994; Paululat et al., 1995; Rushton et al., 1995; Doberstein et al., 1997). Finally, recent studies have revealed striking similarities between the ultrastructure of fusing *Drosophila* myoblasts (Doberstein et al., 1997) and fusing vertebrate myoblasts (Engel et al., 1985; for review see Kalderon, 1980).
The above studies suggest several parallels between myogenesis in *Drosophila* and in vertebrate systems. However, little is actually known about the genes regulating myoblast fusion in *Drosophila* and their mechanism of action. Recently, several loss-of-function mutations that exhibit defects in myoblast fusion have been identified, including rolling stone (Paululat et al., 1995), myoblast city (mbc) (Rushston et al., 1995), blown fuse (Doberstein et al., 1997), sticks and stones (Abmayr, S.M., M.R. Erickson, B.A. Bour, and M. Kulp. J. Cell. Biochem. 1994. 18D (Suppl.):474), and singles bar (Maeland, A.D., J.W. Bloor, and N.H. Brown. 1996. Mol. Biol. Cell. 7:39A). The protein coding sequence of blown fuse, the first of these genes to be identified, has not yet provided insight into its function. By comparison, examination of altered forms of the small rho-like GTPase, Drac1, has been useful in understanding myoblast fusion in *Drosophila* (Luo et al., 1994). Drac1 is the *Drosophila* homologue of the vertebrate gene rac1, which has been shown to induce membrane ruffling through reorganization of the actin cytoskeleton (Ridley et al., 1992). Both dominant negative and constitutively active forms of Drac1 have been shown to cause defects in myoblast fusion (Luo et al., 1994). Interestingly, altered forms of Drac1 also disrupt the actin cytoskeleton in the epidermis, causing defects in cell migration and dorsal closure (Harden et al., 1995), and in apical regions of the wing imaginal disc (Eaton et al., 1995).

A role for the cytoskeleton in myoblast fusion in vertebrates has previously been shown using low concentrations of the inhibitor cytochalasin B, which interferes with the formation of actin filaments. In these studies, the fusion of myoblasts in culture was severely limited in the presence of cytochalasin B, and most myotubes contained only two nuclei (Sanger et al., 1971; Sanger and Holtzer, 1972). More recent studies have confirmed that both cytochalasin B and D inhibit myoblast fusion and correlate the lack of fusion with the disruption of actin filaments (Constantin et al., 1995). While the role of the cytoskeleton at this early stage of myoblast fusion remains unclear, it may be related to the formation of lipid-rich domains within the cell membrane. Just before fusion, for example, vertebrate myoblasts have been shown to undergo a topological change that results in the creation of protein-depleted, lipid-enriched membrane domains (Kalderon and Gilula, 1979; Fulton et al., 1981). These lipid-rich domains are believed to be associated with an increase in membrane fluidity (for review see Wakelam, 1985) and may create sites for membrane–membrane fusion. Thus, subcellular structures that organize these lipid-rich domains may be dependent on cytoskeletal rearrangements.

Herein we describe the isolation and characterization of the *mbc* gene. MBC is one of the first proteins identified in *Drosophila* that is essential for myoblast fusion. It is expressed in a broad range of tissues throughout embryonic development, including the presumptive musculature and epidermal cells involved in the process of dorsal closure. Consistent with its expression pattern, *mbc* mutant embryos exhibit defects in dorsal closure and cytoskeletal organization as well as myoblast fusion. These abnormalities are similar to those described above for the small GTPase Drac1, and suggest that (a) *mbc* functions in the same pathway as Drac1 in the epidermis and (b) this pathway is used in the mesoderm for events leading to myoblast fusion. MBC has striking homology to DOCK180, a human gene that was identified on the basis of interaction with the small adapter protein Crk. DOCK180 may be involved in signal transduction from focal adhesions, and results reported herein are consistent with a similar function for MBC. Finally, open reading frames (ORFs) from several genome projects suggest that DOCK180 and MBC define a new gene family.

**Materials and Methods**

**Drosophila Stocks**

All stocks were grown on standard cornmeal medium at 18 or 25°C, as necessary. Balancer and marked chromosomes are described in FlyBase (http://cbbridges.harvard.edu:7081). *Df(3R)mbc-30* has been described (Rushston et al., 1995). *Df(3R)mbc-15A* was created by treating males homozygous for *Prv*<sup>−</sup>, *lacZ/A192.2* (Bloomington Stock Center) with 4,000 rads of γ-rays. Approximately 25,000 chromosomes were screened for the loss of the *lacZ* marker. Three deficiencies, including *Df(3R)mbc-15A*, were recovered.

*Df(3R)CA15* and *Df(3R)CA2* were obtained by imprecise excision of the homozygous lethal P-element insertion *l(3)4684* (Bloomington Stock Center, Bloomington, IN). Mobilization occurred in flies carrying *l(3)4684* over *Sh*, *Delt2-3* *ry*, and excision events were recovered over *MKRS* or *TM2*. 677 excision events were analyzed. 330 imprecise excision events were identified by lack of complementation of *Df(3R)mbc-F5.3/TM3* (see Fig. 1). These were subsequently reevaluated for lack of complementation of *l(3)95Bcd* and *l(3)10152* and complementation of *mbc*<sup>−</sup>. Four deficiencies were obtained, two of which are shown in Fig. 1.

EMS mutageneses to obtain alleles of *mbc* have been described (Rushton et al., 1995). In similar screens, ~11,000 additional chromosomes were analyzed, and 14 new *mbc* alleles were obtained (Fig. 1). *mbc*<sup>−</sup> was later found to be a small deletion. Additional mutations in this region identify other lethal complementation groups. A subset of these are shown in Fig. 1.

**Enriched DNA and Southern Analysis**

DNA enriched for the mutant chromosomes was obtained by mating heterozygous (*mbc*/+) males and females, and collecting the homozygous mutant embryos. Genomic DNA was prepared from unhatched embryos according to Jowett (1986).

Approximately 10 μg of DNA was digested with EcoRI and BamHI, separated on an 0.8% agarose gel, and blotted using the TurboBlotter Rapid Downward Transfer System (Schleicher and Schuell, Inc., Keene, NH). Blots were probed with various genomic fragments from region 95A-C and, as a control for loading, a 2-kb HindIII fragment that includes exons 2, 3a, and 3b from the gene encoding myosin heavy chain (MHC) (Wassenberg et al., 1987). All probes were labeled by random priming (Feinberg and Vogelstein, 1983).

**Library Screens, Northern Blots, and DNA Sequencing**

Cosmids containing genomic DNA in cytological region 95A-C were obtained from the European *Drosophila* Genome Project (EDGP). The P1 clone was isolated by the Berkeley *Drosophila* Genome Project (BDGP) and provided by A. Spradling. Bacteriophage lambda clones were isolated from a *Drosophila* genomic library in Charon 4 (Maniatis et al., 1978). Fragments were subcloned and analyzed using Southern blots of DNA enriched for the deficiency chromosomes (see above). All DNA between the distal breakpoint of *Df(3R)CA15* and the distal breakpoint of *Df(3R)mbc-15A* was recovered. Genomic fragments containing coding sequence were identified by probing Northern blots (Sambrook et al., 1989).

Subclones of genomic DNA that detected transcribed sequences were used to screen an embryonic 9–12-h cDNA library (Zinn et al., 1988), and several independent cDNA clones were obtained. These included ZS (nucleotides [nts] 1–2854), Z1.2 (nts 597–1928), Z10b (nts 4184–7040), and 1.
Total RNA was prepared from mbc isogenic ru st e adults. cDNA was synthesized with Superscript II (GIBCO BRL, Gaithersburg, MD) from various mbc-specific primers. Resulting cDNAs were used to localize mutations within the mbc transcript using the Non-Isotopic RNAScope Assay (NIRCA) of the Mismatch Detect H2a kit (Ambion, Inc., Austin, TX). The mutation in allele 

PCR product. This result was confirmed by amplification of a second region between nucleotides 622 and 1515 (data not shown). To ensure that approximately equal amounts of total RNA were present in each sample, a Northern blot of the original RNA was probed with 

Whole Mount Embryo Analysis

Embryos were collected on apple juice/agar plates for 0–6 h and aged as necessary. The embryonic expression pattern of mbc mRNA was determined as described (Tautz and Pfeifle, 1989; Michelson et al., 1990) using a digoxigenin-labeled cDNA fragment (nts 602–1022). mbc-encoded protein was analyzed using a polyclonal rat antiseraum (Cocalico Biologicals, Reamstown, PA) that was directed against a 286-amino acid fusion protein. It included amino acids 1717–1790 from the COOH-terminal portion of mbc and was purified from inclusion bodies. Before use, the antiseraum was affinity-purified against the original antigen coupled to Affigel 15 (BioRad Labs, Hercules, CA). For confocal studies, anti-MBC was used at a dilution of 1:50, rabbit anti-MEF2 was used at 1:100 (Bour et al., 1995), and monoclonal antiphosphotyrosine (Upstate Biotechnology, Inc., Lake Placid, NY) was used at a dilution of 1:100. For detection, fluorescein-conjugated goat anti–rabbit antiseraum (Vector Laboratories, Burlingame, CA), fluorescein-conjugated goat anti–mouse antiseraum (Rockland Inc., Gilbertsville, PA), and CY-3-conjugated goat anti–rat antiseraum (Rockland Inc.) were used, as appropriate. All were preadsorbed overnight on 0–12-h embryos before use. Colorimetric immunohistochemistry used a monoclonal anti-MHC antibody (D. Keihart) at a dilution of 1:2000 and an anti–Fascinil III monoclonal supernatant (Patel et al., 1987) at a dilution of 1:10. These were detected with biotinylated antiamouse antiseraum and the Vectastain ABC kit (Vector Laboratories). Where necessary, balancer chromosomes were identified by β-galactosidase activity (Klambt et al., 1991) or by colorimetric immunohistochemistry using a mouse monoclonal anti-β-galactosidase antibody (Promega Corp., Madison, WI) at a dilution of 1:1,000. Staining with Texas red–conjugated phaloidin (Molecular Probes, Inc., Eugene, OR) was as described (Ashburner, 1989b). Four independent experiments were conducted. In two of these studies, wild-type and mutant embryos were treated in parallel throughout the entire analysis but kept in separate tubes. In two subsequent experiments, embryos from a wild-type stock and a balanced mbc mutant stock were pooled. As in the first two experiments, balancer containing embryos from the mutant stock were identified using anti-β-galactosidase. An average of 30 unstained embryos were mounted and analyzed in each experiment. The results of all four experiments gave comparable results. In the phosphotyrosine experiments, a total of 30 embryos were analyzed in two independent experiments carried out in parallel in separate tubes.

Results

Genetic Localization of mbc

myoblast city was originally identified in a genetic analysis of cytological region 95, on the right arm of the third chromosome (Rushton et al., 1995). Since this is the location of nautilus (nau), the Drosophila homologue of a conserved family of myogenic regulatory genes (Michelson et al., 1990; Paterson et al., 1991), it was of interest to examine genetic lesions in this region for defects in myogenesis. Alleles of mbc were revealed in this analysis since embryos mutant for mbc are characterized by an absence of differentiated muscle fibers and the presence of a correspondingly large number of unfused myoblasts (Rushton et al., 1995). Overlapping deficiencies and EMS-induced point mutations were therefore generated to refine the location of mbc and establish that it represents a novel gene, independent and separate from nau.

The current genetic map of this region is shown in Fig. 1. Df(3R)mbc-30 has been described (Rushton et al., 1995), Df(3R)mbc-15A was generated by γ-irradiation of a homozygous viable P-element insertion in this region.
Figure 2. MBC sequence and alignment with related open reading frames. The alignment was done using Clustal W and presented using Boxshade. Black boxes indicate amino acid identity, while gray boxes indicate amino acid similarity to MBC. Arrowheads highlight mutations found in mbc alleles. The consensus Crk-binding sites (PPxLPxK) of DOCK180 are underlined. A potential Crk-binding site in MBC is noted with dots. Stars mark essential SH3 consensus residues (Musacchio et al., 1994). The GenBank/EMBL/DDBJ accession numbers are D86964 for the myeloblast-specific cDNA KIAA0209, Z81032 and Z81054 for the C. elegans ORFs, and AA110899 for the mouse expressed sequence tag. mbc sequence data are available from GenBank/EMBL/DDBJ under accession number AF007805.
Df(3R)CA15 and Df(3R)CA2 were isolated by imprecise excision of l(3)04684, a homozygous-lethal P-element insertion. Df(3R)CA15 deletes from the P-element insertion toward the distal end of the chromosome. In contrast, Df(3R)CA2 deletes from the P-element toward the centromere. These deficiencies have been examined for the presence or absence of nau sequences by Southern analysis of DNA from embryos homozygous for the deficiencies. By comparison to Df(3R)mbc-30 and Df(3R)mbc-15A, which completely remove nau, neither Df(3R)CA15 nor Df(3R)CA2 appear to remove any known nau sequences (data not shown). Consistent with this genetic map, recent results have established that nau is located in the region centromeric to the proximal breakpoint of Df(3R)CA2 (Keller, C.A., and S.M. Abmayr, unpublished results). Df(3R)mbc-F5.3 actually represents an EMS-induced deletion. It does not complement mbc, as shown in Fig. 1, but contains all known nau sequences (data not shown). Finally, EMS-induced point mutations reveal several additional complementation groups in this region. Other than the transposable element insert TnM2, only those groups distal to the Df(3R)CA2 breakpoint are shown in Fig. 1.

These deficiencies refined the location of mbc to the region between the distal breakpoint of Df(3R)CA15 and the distal breakpoint of Df(3R)mbc-15A and facilitated the cloning of the mbc gene. Of note, several attempts to isolate a P-element insertion in mbc were unsuccessful (data not shown). Therefore, a molecular walk through this region was initiated using DNA fragments isolated from P1 clones and cosmids that have been mapped to region 95BC. Fragments within this genetic interval were identified by Southern analysis of DNA from embryos homozygous for the various deficiency chromosomes. A representative example is shown in Fig. 1C. The entire region between these deficiency breakpoints is diagrammed in Fig. 1B and spans ~34 kb of DNA. As indicated, the organization of these fragments has been confirmed by isolation of bacteriophage lambda clones containing Drosophila genomic DNA.

**Identification of the mbc Gene**

A single full-length transcript of ~7.5 kb was detected by Northern analysis throughout development using cloned DNA fragments within the 34-kb region described above. Although full-length clones were not obtained, several small overlapping cDNA clones provided most of the coding sequence of this transcript. The sequence of a small region not covered in the cDNA clones was obtained from embryonic mRNA by reverse transcriptase PCR amplification and sequencing. The embryonic transcript is ~7.4 kb, with a coding sequence of 5,910 nt. Untranslated regions at the 5' and 3' ends of the isolated cDNAs are 560 and 906 bp, respectively. While the genomic organization of mbc has not been analyzed completely, a minimum of eight introns have been identified in a genomic region that spans at least 16 kb. The cDNA sequence has been submitted to GenBank and is not reproduced herein. The deduced amino acid sequence is shown in Fig. 2.

To confirm that this transcript encodes the mbc gene, EMS-induced alleles of mbc were analyzed for sequence alterations. To date, 18 independent alleles of mbc have been generated. Four of these have been described previously (Rushton et al., 1995), while the remaining 14 were generated as part of this analysis. Southern analysis of all alleles was performed to reveal visible rearrangements induced by the chemical mutagen. This analysis uncovered a novel band in an EcoRI/BamHI double digest of DNA from mbcF6.4 (data not shown). Sequence analysis confirmed that the BamHI site had been destroyed by a C to T transition. This missense mutation at amino acid 1579 of the coding sequence changes a proline to a leucine in a conserved region of the protein (Fig. 2, arrowhead).

Additional aberrations were uncovered using a procedure for detecting point mutations that is based on the ability of RNase A to cleave at single base pair mismatches. Several regions of the coding sequence were analyzed by this method and apparent alterations in the candidate sequence were found in three EMS-induced mbc alleles (data not shown). Direct sequencing of these alleles revealed that all were GC to AT transitions at single nucleotides, consistent with the most common form of EMS-induced mutations (Ashburner, 1989a). These changes resulted in nonsense mutations at amino acid 492 in mbcF12.7 and at amino acid 97 in mbcD11.2. By comparison, mbcF6.4 is a missense mutation at amino acid 168 where a glycine has been replaced by a glutamic acid (Fig. 2).

**Structural Homologues of MBC**

The size of the mbc-encoded protein is 1,970 amino acids, with a predicted molecular mass of about 226 kD. Database homology comparisons using BLAST (Altschul et al., 1990) aligned the MBC protein with DOCK180, a human protein of 1,866 amino acids, with a predicted molecular mass of 215 kD (Hasegawa et al., 1996). DOCK180 was isolated on the basis of an interaction with Crk, a small adapter protein consisting mainly of SH2 and SH3 domains (Reichman et al., 1992; see Discussion). MBC and DOCK180 have significant homology throughout their entire length. In particular, DOCK180 contains a putative SH3 domain that proceeds from amino acids 11–71 and includes the three essential SH3 consensus residues (Musacchio et al., 1994). These three residues, along with several others within this domain, are identical in MBC. DOCK180 contains two copies of the Crk-binding consensus site PPxF (Knudsen et al., 1994; Matsuda et al., 1996), while MBC has one exact and one slightly divergent copy of this consensus site (Fig. 2). By contrast, the putative ATP-binding site noted by Hasegawa et al. (1996) is not conserved. Several additional blocks of homology are present, notably a region in which 24 of 27 amino acids are identical (residues 1566–1592 of MBC). A lesion in the central proline of this block, identified above in mbcF6.4, resulted in a mutant phenotype identical to that previously described (Rushton et al., 1995). This apparent loss-of-function phenotype is identical to that found in alleles with a severely truncated protein (e.g., mbcD11.2, data not shown).

Subsequent BLAST searches also revealed two ORFs with extensive homology to MBC and DOCK180. The first ORF is from a human myeloid cell line, and the second is from the Caenorhabditis elegans genome project. The predicted myeloblast protein is highly homologous to...
both MBC and DOCK180, while the predicted C. elegans protein is more divergent. Partial sequence from a mouse expressed sequence tag suggests the existence of a murine homologue as well.

Temporal Expression of mbc

Northern analysis revealed that mbc is expressed early in development, in embryos ~0–4 h after egg laying. mbc transcript levels remain relatively high during embryogenesis, with the possible exception of a decline from 8–12 h that may be, in part, an artifact of slightly degraded mRNA (Fig. 3A). Expression was not evident during larval stages, but the transcript does reappear during pupation, suggesting a possible role in adult development. A form of mbc with slightly altered mobility appears late in metamorphosis. This transcript may reflect alternative splicing and is under further investigation (Fig. 3A, lane 9). PCR amplification of two different regions from the mRNA of unfertilized embryos revealed a small but detectable signal, and suggested that the transcript is maternally provided. Finally, the transcript was expressed in adult males and females, as evidenced by PCR analysis of cDNA (Fig. 3B).

Spatial Expression Pattern of mbc mRNA and Protein during Embryogenesis

The earliest expression of the mbc transcript is in the pole
cells (Fig. 4 A). It is later found in lateral portions of the embryo during cellularization (Fig. 4 B) but is not evident at the termini. Surprisingly, the ventral furrow, which will invaginate during gastrulation to form the mesoderm, shows no expression at this time (Fig. 4 C). At germband elongation, expression is still quite strong in the ectoderm (Fig. 4 D). By late stage 12, the mRNA appears to be decreasing in the ectoderm, leaving a pattern of stripes (Fig. 4 E, arrows). mbc is expressed in both the mesoderm and endoderm during stage 12 (Fig. 4 F). Expression decreases in both the epidermal layer and the somatic mesoderm during stage 14 (Fig. 4 G) but remains strong in the vis-

Figure 5. Spatial expression pattern of MBC in wild-type embryos. Anterior is to the left and dorsal to the top in all except A. (A) Stage 13 embryos from the progeny of mbcD11.2/TM3 lacZ-hg stained immunohistochemically for MBC. The embryo to the top left expressed β-galactosidase and therefore carried TM3 lacZ-hg; the embryo to the bottom right (which is barely visible) did not express β-galactosidase (data not shown) and was therefore homozygous for mbcD11.2. As anticipated, no MBC expression is visible in the homozygous mutant embryo, establishing specificity of the antiserum. (B) Wild-type; Lateral view, stage 5. (C) Wild-type; Lateral view, stage 8. (D) Wild-type; Lateral view, stage 14; arrow indicates the visceral musculature (vm). (E and F) Wild-type; Lateral views, stage 16; arrows in E highlight somatic muscles 8, 12, and 19, using the nomenclature of Crossley (1978). Arrow in F marks the dorsal vessel (dv). Bars: (A–D and F) 100 μm; (E) 10 μm.
ceral musculature (Fig. 4 H, arrowheads). Examination of a stage 16 embryo revealed mRNA in both the cardial and pericardial cells of the dorsal vessel (Fig. 4 J). Of note, the mbc transcript is not observed in mature muscle fibers.

The expression pattern of MBC was analyzed by fluorescent immunohistochemistry and confocal microscopy using an antiserum directed against the COOH-terminal portion of the protein. Examination of embryos homozygous for mbc<sup>11.2 D11.2</sup> confirmed that the antiserum was specific (Fig. 5 A) since this allele encodes a severely truncated form of MBC that would not be detected. While slight temporal differences were evident between maximal levels of mRNA (stage 4; Fig. 4 A) and maximal levels of protein (stage 5; Fig. 5 B) in the pole cells, the expression of the protein essentially correlated with that of the mRNA. MBC appeared to be localized in the cytoplasm (Fig. 5 C), consistent with its human counterpart DOCK180. MBC is also present in the visceral musculature (Fig. 5 D, arrow) and the dorsal vessel (Fig. 5 F, arrow). Cross reactivity of the MBC antiserum was observed in the filtzkorper (Fig. 5 F) but does not correlate with the presence of transcript. Although mRNA was not evident in mature muscles, the protein could be detected at a low level (Fig. 5 E).

Fluorescent immunohistochemistry and confocal microscopy were used to confirm that MBC is present in myoblasts. For this analysis, the embryos were hybridized with antibodies to both MBC and MEF2. The mef2 gene encodes a transcription factor that appears to be expressed throughout the mesoderm, including somatic muscle precursors and all muscle fibers (for review see Olson et al., 1995). Nuclei expressing MEF2 were visualized in a late stage 12 embryo in green (Fig. 6 C). By comparison, cytoplasmic expression of MBC was visualized in red (Fig. 6 A). As anticipated from the expression pattern of mRNA, MBC is present in ectodermal and endodermal germ layers. Of note, expression in the ectoderm is concentrated in the epidermal layer and appears to be absent from the underlying neuroectoderm. MBC is also clearly present in presumptive myoblasts, coincident with the MEF2-expressing nuclei (Fig. 6 B).

**Examination of Mesodermal Derivatives in mbc Mutant Embryos**

Given the broad expression pattern of mbc, it was of interest to examine mbc mutant embryos for defects in other tissues. For this purpose we used mbc<sup>F12.7</sup>, since the protein is truncated at amino acid 492, and analyzed embryos that were genetically mbc<sup>F12.7 Df(3R)mbc-30</sup>. These embryos exhibited the severe somatic muscle phenotype previously reported (Rushton et al., 1995) and shown in Fig. 7 B. By comparison, although the visceral musculature appeared to be present, as evidenced by myosin-staining cells, obvious defects in midgut constriction and orientation were observed in ~25% of the embryos (Fig. 7, C and D). However, these defects may be an indirect consequence of the lack of somatic muscles rather than a direct effect of the loss of MBC in either the visceral mesoderm or the endoderm. The overall structure of the heart, which expresses MBC late in development, appeared to be normal at this level of analysis (Fig. 7, E and F).

**Examination of Dorsal Closure and Cytoskeletal Organization in the Epidermis**

Although no epidermal defects had been reported in mbc mutant embryos (Rushton et al., 1995), the early expres-
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sion of mbc in the ectoderm, which persists in the epidermis into stage 14, led us to reexamine mbc mutant embryos for epidermal defects. Visualization of the epidermis with an antibody to Fasciclin III, a glycoprotein on the cell surface (Patel et al., 1987), revealed defects in dorsal closure in ~80% of the mutant embryos (Fig. 8, E and F). The extent of completion of dorsal closure varied from a relatively small opening surrounded by puckered misshapen cells (data not shown) to a very large opening (Fig. 8 E). In the normal course of dorsal closure in a wild-type embryo, the epidermal cells elongate as shown in Fig. 8 B, and the epithelium stretches over the entire circumference of the embryo (Young et al., 1993). In the early stages of dorsal closure in mbc mutant embryos, the cells along the leading edge of the epidermis appeared to be normal (data not shown). As dorsal closure neared completion, however, many cells along the leading edge ceased to be elongated, adopted a rounded shape, and expressed Fasciclin III abnormally along their migrating edge (Fig. 8, B, D, and F).

The cytoskeleton along the leading edge of the epidermis has been implicated in driving the process of dorsal closure (Young et al., 1993). We therefore used fluoro-}

cently conjugated phalloidin, which binds filamentous actin, to examine the mbc mutants for defects in cytoskeletal formation and organization. Both wild-type and mbc mutant embryos displayed some variability in the intensity and organization of staining, the range of which is shown in Fig. 9. As shown, the signal in wild-type embryos (Fig. 9 A, a and c) was always stronger than that in mbc mutant embryos (Fig. 9 A, b and d). While frequently more dramatic in cells along the migrating edge, this reduction in signal was also observed throughout the epidermis, consistent with the observed expression of mbc. In addition, it should be noted that ~20% of the mbc mutant embryos do not exhibit defects in dorsal closure (mentioned above). One might anticipate that these embryos would express relatively normal levels of filamentous actin and exhibit only mild cytoskeletal defects, such as that shown in Fig. 9 A, b. In summary, this analysis suggests that there is a modest but reproducible reduction in cytoskeletal organization in the epidermis of mbc mutant embryos. Unfortunately, examination of the cytoskeletal structure in muscle cells was complicated by the dynamic nature of wild-type muscle cells, making rigorous comparisons with comparable muscle cells in mbc mutant embryos difficult.
DOCK180, the apparent human homologue of mbc, may be involved in Crk-associated signal transduction from focal adhesions (Hasegawa et al., 1996). If mbc functions in a similar signal transduction pathway in Drosophila, we anticipated that it would be downstream of focal adhesions. Examination of putative focal adhesions in the epidermis of mbc mutant embryos was accomplished using a monoclonal antibody directed against phosphotyrosine, as previously described (Maher et al., 1985; Hanks et al., 1992; Harden et al., 1996). In contrast to the cytoskeletal defects described above, comparison of phosphotyrosine staining patterns in the epidermis of wild-type embryos and homozygous mbcF12.7 embryos revealed no apparent difference during dorsal closure (Fig. 9 B, a and b). This observation is consistent with the possibility that MBC, like DOCK180, is downstream of phosphotyrosine-containing complexes in a signal transduction pathway that, in Drosophila, ultimately affects cell migration, dorsal closure, cytoskeletal organization, and myoblast fusion.

Discussion

The results reported here describe the cloning and characterization of myoblast city, a gene that was initially identified on the basis of a defect in myoblast fusion (Rushton et al., 1995). mbc encodes a novel Drosophila protein with a high degree of homology to the human Crk-associated protein, DOCK180 (Hasegawa et al., 1996). Consistent with that of its human counterpart, mbc expression is not restricted to the somatic mesoderm. Early in development, expression is observed in the pole cells and ectoderm but is absent from the mesodermal epithelium. Later in development, expression is most evident in the epidermis and mesoderm but is absent from neural tissues. The latest detectable expression is in mesodermal derivatives that include the heart and visceral musculature. Consistent with this pattern of expression, defects in myoblast fusion are accompanied by abnormalities in the midgut constrictions and in the ability of the epidermal cells to complete dorsal closure. These cells exhibit alterations in shape, migration, and deposition of Fasciclin III, as well as cytoskeletal organization. Previous studies have reported similar defects for Drac1 (Luo et al., 1994; Harden et al., 1995), the Drosophila homologue of the small GTPase rac1, and imply that mbc may function in the same pathway. Finally, ORFs identified from multiple genome sequencing projects may indicate that MBC and DOCK180 are members of a highly conserved gene family.

The Role of mbc in Ectodermally Derived Tissues

Early expression of mbc in the ectoderm and its persistence in the epidermis led us to examine mbc mutant embryos for epidermal defects. Using Fasciclin III as a marker, we observed that mbc mutant embryos were unable to complete the process of dorsal closure. Contractile filaments formed from actin and myosin are thought to provide the driving force for dorsal closure. Consistent with this suggestion, the absence of nonmuscle myosin in zipper

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mutant embryos is likely to be responsible for their failure to complete this process (Young et al., 1993). Similarly, overexpression of a form of Drac1 that disrupts both actin and nonmuscle myosin accumulation at the leading edge of the migrating epidermis also inhibits dorsal closure (Harden et al., 1995). Finally, the dorsal closure defects observed in mbc mutants are accompanied by reduced detection of filamentous actin. These results implicate mbc in cytoskeletal organization and dorsal closure and suggest that it may function in the same pathway as Drac1.

Recent studies have shown that Drac1 is necessary for the presence of phosphotyrosine-containing complexes at the leading edge of the epidermis that have been suggested as focal adhesions (Harden et al., 1996). The rho/ rac family of small GTPases has also been implicated in the formation of focal adhesions and in the organization of the actin cytoskeleton in vertebrates (Ridley and Hall, 1992, 1994; Ridley et al., 1992; Nobes and Hall, 1995; Chrzanowska-Wodnicka and Burridge, 1996; for reviews see Clark and Brugge, 1995; Richardson and Parsons, 1995; Takai et al., 1995). The loss of mbc does not appear to affect the formation of these phosphotyrosine-containing complexes, implying that mbc may function downstream of Drac1. This interpretation is consistent with one possible role of human DOCK180 in mediating a signal from focal adhesions to downstream effectors (Hasegawa et al., 1996). Specifically, DOCK180 was isolated on the basis of interaction with the small SH2-SH3 domain-containing adapter protein, Crk (Reichman et al., 1992). Studies addressing the roles of both c-Crk and its oncogenic counterpart v-Crk have suggested an involvement in signal transduction pathways that include receptor tyrosine kinases, ras and MAP kinase, and focal adhesions (Tanaka et al., 1993; Feller et al., 1994; Hempstead et al., 1994; Matsuda et al., 1994; Schaller and Parsons, 1994; Clark and Brugge, 1995; Richardson and Parsons, 1995; Hanks and Polte, 1997). Recently, several proteins have been identified on the basis of interaction with Crk and are likely to

Figure 9. Accumulation of filamentous actin and phosphotyrosine in the epidermis of mbc mutant embryos. Confocal micrographs of embryos stained with Texas red–conjugated phalloidin (A) or an antiphosphotyrosine antibody (B). Anterior is to the left in all panels. A, a and c, and B, a show stage 14 wild-type embryos. A, b and d, and B, b show stage 14 mbc(D11.2) homozygous embryos. Bar, 10 μm.
be downstream effectors. Among these is the guanine nucleotide exchange factor C3G (Tanaka et al., 1994). Crk may be a critical mediator of signal transduction to events in the nucleus through these molecules. In addition, biochemical evidence has shown that v-Crk and c-Crk can interact with phosphorylated Paxillin (Birge et al., 1993; Schaller and Parsons, 1995), one of the components of the focal adhesion (Turner et al., 1990).

It should be noted that although both mbc loss-of-function and dominant negative Drac1N17 embryos exhibit similar defects in dorsal closure and cytoskeletal organization in the epidermis and fusion of myoblasts in the mesoderm (see below), Drac1N17 also induces defects in the peripheral nervous system. In contrast, both the motor neurons and the peripheral nervous system of mbc mutant embryos appear to be normal (Rushton et al., 1995; Prokop et al., 1996; Erickson, M.R.S., and S.M. Abmayr, unpublished observation), consistent with the minimal level of MBC expressed in neural tissues. The simplest interpretation of this apparent inconsistency is that particular factors mediate different aspects of the Drac1 signal transduction cascade. In support of this hypothesis, several different molecules have been identified on the basis of an interaction with vertebrate Crk (Feller et al., 1995, and references therein).

**The Role of mbc in Myoblast Fusion**

The most apparent mesodermal defect in embryos mutant for the mbc gene is an inability of myoblasts to fuse into muscle fibers, suggesting a role for mbc in the progression of cells from myoblasts to myotubes. This multistep process has been divided into several stages (Knudsen and Horwitz, 1977, 1978; for reviews see Bischoff, 1978; Wakelam, 1985) and includes the acquisition of fusion competence, a time-dependent behavior that may be related to withdrawal from the cell cycle (Bischoff and Holtzer, 1969; Yaffe, 1971; Holtzer et al., 1975), myoblast adhesion, and plasma membrane union.

Several features of the mbc-encoded protein seem somewhat inconsistent with a role in either cell adhesion or membrane fusion itself. First, MBC does not have features reminiscent of cell adhesion molecules and appears to be present throughout the cytoplasm rather than membrane bound. Second, both MBC and its structural homologue, DOCK180 (Hasegawa et al., 1996), are expressed in a wide range of tissues that do not fuse. The potential conservation of MBC and DOCK180 in *C. elegans*, in which the muscle fibers remain mononucleate (Waterston, 1988), is also inconsistent with a direct role for mbc in the fusion process. An alternative possibility is that mbc functions in myoblast differentiation. As mentioned earlier, DOCK180 was identified and subsequently isolated on the basis of interaction with the adapter protein Crk (Reichman et al., 1992). Studies addressing the roles of both c-Crk and v-Crk have implicated these molecules in cell differentiation (Tanaka et al., 1993; Hempstead et al., 1994). Thus, mbc may be essential for a cytoskeleton-related step in differentiation through which, among other things, myoblasts become competent to fuse.

We favor the interpretation that the function of MBC in the mesoderm is analogous to its role in the epidermis and that it functions as an essential intermediate in a signal transduction cascade that also includes the small GTPase Drac1. This pathway could involve tyrosine phosphorylation of complexes that directly modulate events in the cytoskeleton through proteins that include MBC. Alternatively, MBC may function in signal transduction to the nucleus via the ras and MAP kinase pathway and may affect the cytoskeleton only indirectly. Interestingly, while vertebrate studies have not revealed a specific requirement for focal adhesions in myogenesis, they have implicated extracellular matrix components that stimulate focal adhesions, such as fibronectin, in myogenic differentiation (Chen, 1977; Furcht et al., 1978; Menko and Boettiger, 1987; Guan and Shalloway, 1992; Hanks et al., 1992; Enomoto et al., 1993). Additional studies in vertebrates support a role for the cytoskeleton in myoblast fusion. As previously described (see introduction), myoblast fusion is severely limited in the presence of cytochalasin B, an alkaloïd that interferes with the assembly of actin filaments (Sanger et al., 1971; Sanger and Holtzer, 1972). While the role of the cytoskeleton in myoblast fusion remains unclear, it may be involved in the formation of lipid-rich domains within the cell membrane that create sites for membrane–membrane fusion. Alternatively, actin filaments may be required for the formation or organization of vesicles that have been observed under the plasma membrane just before fusion of both vertebrate and *Drosophila* myoblasts (Doberstein et al., 1997; for review see Kalderon, 1980). Interestingly, these vesicles are not observed in mbc mutant embryos, perhaps as a consequence of defects in the actin cytoskeleton (Doberstein et al., 1997).

Additional studies will be necessary to resolve the exact role of mbc in myoblast fusion. In particular, whereas focal adhesions in vertebrates are generally thought to mediate interactions between the cell and the extracellular matrix, no cell–matrix interactions have yet been identified in the *Drosophila* mesoderm (Tepper and Hartenstein, 1994). In addition, on the basis of examination of phosphotyrosine-containing complexes, our studies seem most consistent with a role for mbc downstream of Drac1 in the epidermis. By comparison, Doberstein et al. (1997) place mbc upstream of a constitutively active form of Drac1. As discussed by these authors, however, the analysis of Drac1 is presently limited to targeted expression of altered forms of the protein and is problematic in the absence of a loss-of-function mutation. It may also reflect a second role for Drac1 in myoblast fusion, not inconsistent with the suggestion that GTPases may act downstream of focal adhesions (Schaller and Parsons, 1994; Clark and Brugge, 1995; Hanks and Polte, 1997). One intriguing possibility consistent with our data and that of Doberstein et al. (1997) is an early requirement for activated Drac1, perhaps to facilitate recruitment of paired vesicles to the membrane via the cytoskeleton, followed by an equally important requirement for Drac1 inactivation later, before fusion. One final issue is that genetic studies have not yet revealed a role for integrin subunits, one of the major components of vertebrate focal adhesions, in myoblast fusion. The larval body wall muscles in embryos mutant for the major integrin subunits, βPS, αPS1, and αPS2, do not appear to exhibit defects in fusion (Brown, 1994; Roote and Zusman, 1995; for reviews see Brown, 1993; Gotwals et al., 1994).
However, the number and alternatively spliced forms of integrins identified in Drosophila has continued to increase (Gotwals et al., 1994), and family members that play other roles in myogenesis may yet be isolated. Thus, greater knowledge of GTPases and integrins and the identification of Drosophila homologues to components of vertebrate focal adhesions are likely to refine our working model.

The Role of mbc in Other Tissues

Although mbc is quite highly expressed in the heart and the visceral musculature late in development, these tissues do not appear to be severely affected by the loss of mbc. The visceral musculature does appear to be somewhat defective, as evidenced by the absence of midgut constrictions in a low percentage of embryos, but the heart appears to be relatively normal. One interpretation of such behavior is that another gene, yet to be identified, serves a redundant role in these tissues. Another interpretation is that, while the level of expression observed in unfertilized eggs is quite low, adequate maternally derived MBC protein may be available to embryos lacking zygotic expression of a functional protein. This may be particularly true for the pole cells, which express relatively high levels of MBC early in development.

In summary, we have reported the cloning and characterization of mbc, a novel gene that is essential for events leading to myoblast fusion and dorsal closure. The striking conservation of this molecule with DOCK180, a human gene that may be a target of a signal transduction cascade activated through focal adhesions, suggests the involvement of a signaling cascade in myogenesis, perhaps through organization of the actin cytoskeleton. As a more detailed picture of DOCK180, focal adhesions, and the family of rho/rac like GTPases is revealed, our understanding of the precise role of mbc will grow. Thus, the further identification of common features and homologous genes in different developmental systems may allow us to take advantage of the benefits of each to address the function of a conserved pathway.

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