The myoplasm of ascidian eggs: a plasma membrane skeleton which is modified during evolution

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This paper addresses the molecular organization and evolution of a plasma membrane skeleton in ascidian eggs. The myoplasm of ascidian eggs contains proteins related to ankryin, spectrin and Na^+K^+ATPase, which are present in the plasma membrane skeleton of vertebrate cells. The membrane skeletal proteins co-distribute and segregate with the myoplasm throughout development. These proteins first appear during oogenesis and become restricted to a thin layer under the plasma membrane of unfertilized eggs. After fertilization, they undergo ooplasmic segregation, first accumulating in a cap near the vegetal pole and then in a crescent in the future posterior region of the uncleaved zygote. During cleavage, these proteins are partitioned to presumptive muscle lineage cells and enter the larval tail muscle cells. Some of these proteins also appear de novo in non-muscle cells of the tadpole larva. Whereas eggs of all indirect developing ascidian species contain a myoplasm, this cytoplasmic region has been deleted or reduced in molgulid ascidian species that exhibit direct development. Eggs of indirect developing molgulid species exhibit a myoplasm containing spectrin and Na^+K^+ATPase, but lacking ankryn. Eggs of direct developing molgulid species have lost the myoplasm, as well as ankryin and spectrin, and show a uniform cytoplasmic distribution of Na^+K^+ATPase. Phylogenetic information suggests that the loss of ankryin in an indirect-developing molgulid ancestor preceeded the loss of the myoplasm and spectrin and the modification of Na^+K^+ATPase localization, and was a possible preadaptation to the evolution of direct development. The results suggest that evolutionary changes in the molecular organization of the membrane skeleton of ascidian eggs may generate an alternate mode of development.

Key words: *Ascidian eggs, myoplasm, ooplasmic segregation.*

INTRODUCTION

Nothing in biology makes any sense except in the light of evolution
(Th Dobzhansky, 1973)

Invertebrate eggs contain localized cytoplasmic regions that specify cell fate during embryogenesis (see Jeffery, 1988, for review). One of the best characterized of these regions is the myoplasm of ascidian eggs. The myoplasm is localized in the cortex of unfertilized eggs. After fertilization it is initially translocated into a cap in the vegetal hemisphere and then into a crescent in the posterior region of the uncleaved zygote (reviewed by Jeffery and Swalla, 1990a). During cleavage, the myoplasmic crescent is segregated into blastomeres that give rise to the larval tail muscle cells. The myoplasm has multiple roles in embryonic development; however, one of its major functions is specification of the larval tail muscle cells. Muscle cell specification does not requiere cell interactions (Jeffery, 1993) and is presumably caused by the inheritance of muscle

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determinants from the egg. Two lines of evidence suggest that muscle determinants are present in the myoplasm. First, when myoplasm is redistributed into non-muscle cells during cleavage the recipients later develop some of the properties of muscle (Whittaker, 1980). Second, when anucleate egg fragments containing myoplasm are fused to non-muscle blastomeres, the fusion products attain the ability to develop muscle cell features (Nishida, 1992).

The myoplasm is a cytoskeletal domain consisting of an actin lamina, which is closely associated with the plasma membrane, and an underlying network of filaments, with associated cortical pigment granules, mitochondria, endoplasmic recticulum, and mRNA (Jeffery and Meier, 1983; Jeffery, 1984b; Speksnijder *et al*, 1993). The nature of the filamentous network has not been completely established, although it is likely to be composed of intermediate filaments (Jeffery and Swalla, 1990a). Characterization of isolated crescents indicates that a specific set of polypeptides are present in the myoplasm (Jeffery, 1985). Among these is a 58 kDa protein (p58) detected by NN18 (Swalla *et al*, 1991), a monochlonal antibody against a vertebrate middle molecular-weight neurofilament protein. The protein p 58 segregates with the myoplasm during early development and is highly enriched in the tail muscle cells of the tadpole larva. Recent studies have also shown that the myoplasm contains a protein related to ankryin, the key element linking the cytoskeleton to the plasma membrane in erythrocytes (Bennett, 1990) and other vertebrate cells (Pumplin and Bloch, 1993).

The majority of ascidian species exhibit indirect development in which the egg develops into a tadpole larva. The tadpole larva contains a head, with a brain and a pigmented neural sensory cell, and a tail, with a notochord and flanking bands of striated muscle cells. A few ascidian species have modified or eliminated the tadpole larva, and develop directly into an adult (see Jeffery and Swalla, 1990b, for review). Most of the direct developing species are found in the family Molgulidae (Berril, 1931). The direct developing species do not differentiate the neural sensory cell, notochord, or tail musculature (Whittaker, 1979; Swalla and Jeffery, 1990). Some of the missing larval tissues can be restored, however, when the genome of an indirect developing species is introduced into the egg of a direct developing species by interspecific fertilization (Swalla and Jeffery, 1990; Jeffery and Swalla, 1991; 1992). The restoration of tadpole larval features in the direct developing embryo suggests that changes in zygotic processes are responsible for the evolution of direct development. However, muscle cell differentiation was not rescued in these hybrids, suggesting that maternal changes are also involved in this evolutionary modification. We have shown that the myoplasmic protein p 58 is absent or significantly reduced in quantity in eggs and embryos of several direct developing species (Swalla *et al*, 1991; Jeffery and Swalla, 1992).

Ankryin, spectrin and transmembrane proteins, such as Na^+K^+ATPase, are major components of the membrane skeleton in erythrocytes and other vertebrate cells (reviewed in Bennett, 1990; Pumplin and Bloch, 1993). In this paper, we present evidence that these membrane skeletal proteins are present in the myoplasm of ascidian eggs. The results also suggest that molecular changes in this membrane skeleton are responsible for generating an alternate mode of development during evolution.

METHODS

The ascidian species used in this study were *Ascidia ceratodes* (collected at Bodega Bay, CA), *Molgula occulta* (collected at Roscoff, France), and *Molgula citrina* (collected at Woods Hole, MA). The animals were maintained in running sea water. *A. ceratodes* gametes were obtained from the oviducts and sperm ducts of dissected animals. The eggs were washed several times in a large volume of Millipore filtered sea water (MFSW), treated with acidic MFSW (pH6.0) for 30-60 min to remove the surrounding gelatinous encasement, and washed several times in MFSW before insemination. A drop of dry sperm was diluted in 10 ml of sea water and several drops were used to inseminate eggs suspended in about 40 ml of MFSW.

Preparation of *M. occulta* gametes and insemination was carried out as described previously (Swalla and Jeffery, 1990). Embryos of the oviparous species were cultured in MFSW at 14° C (*A. ceratodes*) or 18° C (*M. occulta*). Eggs and developing embryos were dissected from the brood sacs of *M. citrina*, an ovoviviparous species containing eggs and embryos at different stages of development.

Polyclonal ankryn and spectrin antibodies raised from chicken erythrocytes and polyclonal Na^+K^+ATPase antibody raised from rabbit liver were purchased from East Acres Biologicals (Southbridge, MA). The antibody used to detect the myoplasm was NN18 (ICN Immunobiologicals, Lisle, IL), a monoclonal antibody against the vertebrate middle-molecular weight neurofilament protein which recognizes the ascidian protein p 58 (Swalla *et al*, 1991). Eggs and embryos were collected for immunocytochemistry by centrifugation, the supernatant was decanted, and the pellet was fixed in 100% methanol and then in 100% ethanol, both for 20 min at –20° C, and embedded in polyester wax (Swalla *et al*, 1991). Sections were cut at 8 μm, de-waxed in absolute ethanol, rehydrated, and stained with the anti-ankryin, anti-spectrin, and anti-Na^+K^+ATPase (1:30 dilutions in PBS) or NN18 (1:25 dilution in PBS) antibodies. After staining, the specimens were rinsed in PBS and the immune complexes were detected with fluoroscein-labelled, mouse anti-rabbit IgG (anti-ankryin, anti-spectrin and anti-Na^+K^+ATPase) or rhodamine-conjugated, goat anti-mouse IgG (NN18) (Cappel Laboratories Inc, Downingtown, PA). After rinsing several times in PBS, the specimens were mounted in glycerol, and photographed using a Leitz epifluorescence microscope.

RESULTS

The myoplasm of ascidian eggs contains an actin lamina resembling the membrane skeleton of vertebrate cells (Jeffery and Meier, 1983; Jeffery, 1984a; reviewed by Jeffery and Swalla, 1990a). Therefore, we have used vertebrate antibodies to determine whether antigens related to the membrane cytoskeletal proteins spectrin, ankryin, and Na^+K^+ATPase are present in ascidian eggs. These antibodies react with proteins having a molecular mass similar to vertebrate spectrins, ankryins, and Na^+K^+ATPase in Western blots (Jeffery and Swalla, Ms submitted, 1993; Jeffery, unpublished). Figure 1 shows the results of an experiment in which sections of fertilized *Ascidia ceratodes* eggs were stained with antibodies to the membrane skeletal proteins at the completion of the first phase of ooplasmic segregation. As shown in controls stained with NN18 antibody (Fig 1A), the myoplasm is segregated into the vegetal cortex at this stage of development. Likewise, spectrin (Fig 1B) and ankryin (Fig 1C) antigens were localized in the vegetal cortex of the egg, indicating that they are present in the myoplasm. The Na^+K^+ATPase antigen was also localized in the myoplasm (see Fig 5D). In each of these cases, the ascidian membrane skeletal proteins were localized in a thin layer beneath the egg plasma membrane. This layer probably represents the interface between the myoplasmic actin network and the egg plasma membrane.

Each of the membrane skeletal proteins was localized in the myoplasm of unfertilized eggs and segregated with this region into the future posterior region of the uncleaved zygote. During cleavage, these proteins entered the larval tail muscle cells with the myoplasm. Figures 2 and 3 show myoplasm-containing muscle cells of tailbud embryos containing high concentrations of ankryin (Fig 2B), spectrin (Fig 2C), and Na^+K^+ATPase (Fig 3). During the tailbud stage, some of the membrane skeletal proteins also appear in non-muscle cells. Ankryin (Fig 2B) and Na^+K^+ATPase (Fig 3) are highly concentrated in the apical margins of epidermal cells, and Na^+K^+ATPase is also present in the mesenchyme cells (Fig 3). Whereas the mesenchyme cells probably obtain some of the membrane skeletal protein with the myoplasm (Conklin, 1905), the epidermal cells likely synthesize ankryin and Na^+K+ATPase *de novo* during embryogenesis. The results show that the myoplasm of ascidian eggs contains proteins related to vertebrate ankryin, spectrin, and Na^+K^+ATPase, which are distributed prima-

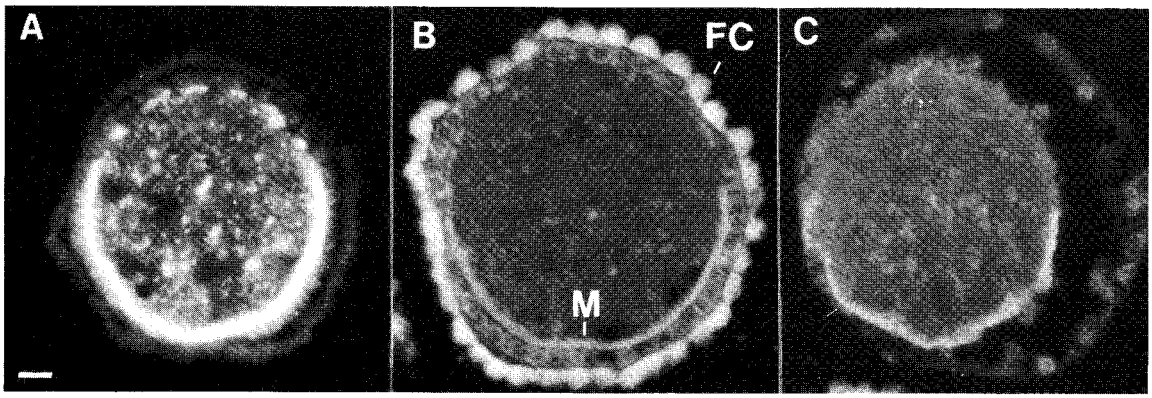

Fig 1: Immunofluorescence microscopy of *A. ceratodes* eggs. Sections of uncleaved zygotes which have completed the first phase of ooplasmic segregation stained with NN18 antibody to show the distribution of the myoplasm (A), anti-spectrin to show the distribution of the spectrin-like protein (B), and anti-ankryin to show the distribution of the ankryin-like protein. The myoplasm (M) contains spectrin and ankryin-like proteins. Anti-spectrin also stains the follicle cells (FC). Scale bar is 10 μm; magnification is the same in each frame.

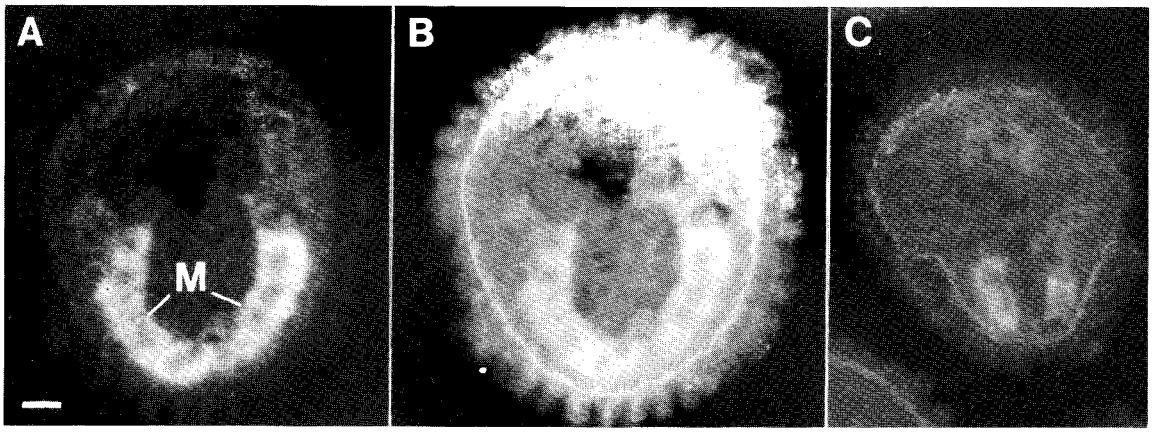

Fig 2: Immunofluorescence microscopy of *A. ceratodes* early tailbud embryos. Frontal sections stained with NN18 antibody to localize the position of the myoplasm-containing tail muscle cells (A), anti-ankryin to show the distribution of the ankryin-like protein (B), and anti-spectrin to show the distribution of the spectrin-like protein (C). The cytoplasm of presumptive tail muscle cells (M) contains myoplasm, ankryin and spectrin. The apical layer of the epidermal cells (thin line surrounding the embryo in B) also contains ankryin. Scale bar is 10 μm; magnification is the same in each frame.

rily to the larval tail muscle cells during embryogenesis.

The indirect-developing ascidian species described above exhibit a myoplasm containing membrane skeletal proteins. In contrast, direct-developing ascidian species have lost or reduced the myoplasm (Swalla *et al*, 1991; Jeffery and Swalla, 1992). Thus, we investigated whether the membrane skeletal proteins are present in eggs and embryos of direct-developing ascidian species. The experiments were conducted with *M. occulta*, a direct developing species in the family Molgulidae (Swalla and Jeffery, 1990). Some clutches of *M. occulta* eggs lack the myoplasm, whereas others show reduced levels of myoplasm (Jeffery and Swalla, 1992). Unfertilized eggs from an *M. occulta* clutch lacking myoplasm were probed with anti-ankryin, anti-spectrin, and anti-Na^+K^+ATPase antibodies. The results are shown in Figure 4. Neither ankryin (Fig 4A) nor spectrin antigens (Fig 4B) could be detected in *M. occulta* eggs Na^+K^+ATPase was detectable in *M. occulta* eggs; however, it was uniformly distributed (Fig 4C) rather than localized in a thin cortical zone, as in eggs of an indirect developing species (see Fig 5D). The membrane skeletal proteins did not reappear in *M. occulta* embryos during

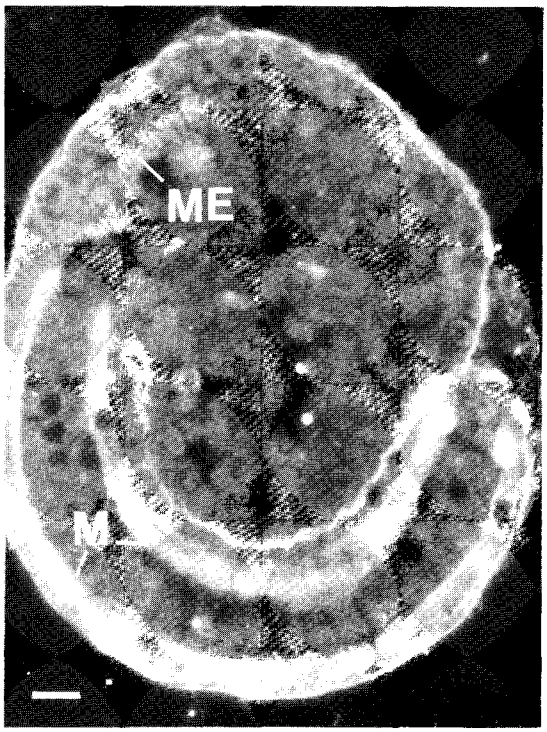

Fig 3: Immunofluorescence microscopy of an *M. citrina* mid-tailbud stage embryo. A parasagittal section stained with anti-Na^+K^+ATPase. Na^+K^+ATPase is present in the tail muscle cells (M), the apical margins of epidermal cells (thin line surrounding the surface of the embryo), and at lower concentrations in the mesenchyme cells (ME). Scale bar is 10 μm.

later development (data not shown). Similar results were obtained with *M. occulta* egg clutches containing low levels of myoplasm, although there was a tendency for the spectrin-like protein to accumulate in the reduced myoplasm in these clutches. These results suggest that the membrane skeletal proteins have been lost or modified in concert with the myoplasm in eggs of the direct developing ascidian *M. occulta*.

The phylogenetic relationships between the ascidian species examined in this paper are shown in Figure 7. The direct developer *M. occulta* is a member of the family Molgulidae, wheareas the indirect developer *A. ceratodes* is a member of the more primitive family Ascidiidae. The family Ascidiidae contains only indirect developing species, whereas the family Molgulidae contains species with indirect and direct development (Berril, 1981; Jeffery and Swalla, 1990b). In order to place our results in the proper evolutionary perspective, we also examined membrane skeletal proteins in *Molgula citrina*, an indirect developing molgulid containing a myoplasm (Swalla *et al*, 1991). Figure 5 shows the distribution of the myoplasm, ankryin, spectrin, and Na^+K^+ATPase in *M. citrina* oocytes or mature eggs. Surprisingly, the myoplasm of *M. citrina* oocytes does not contain the ankryin-like protein, which is instead localized in spherical bodies surrounding the germinal vesicle (Fig 5A, B). Previous studies have shown that these ankryin-containing structures arise in the perinuclear region of previtellogenic oocytes of both indirect and direct developing ascidian species (Jeffery and Swalla, 1993). These bodies may be

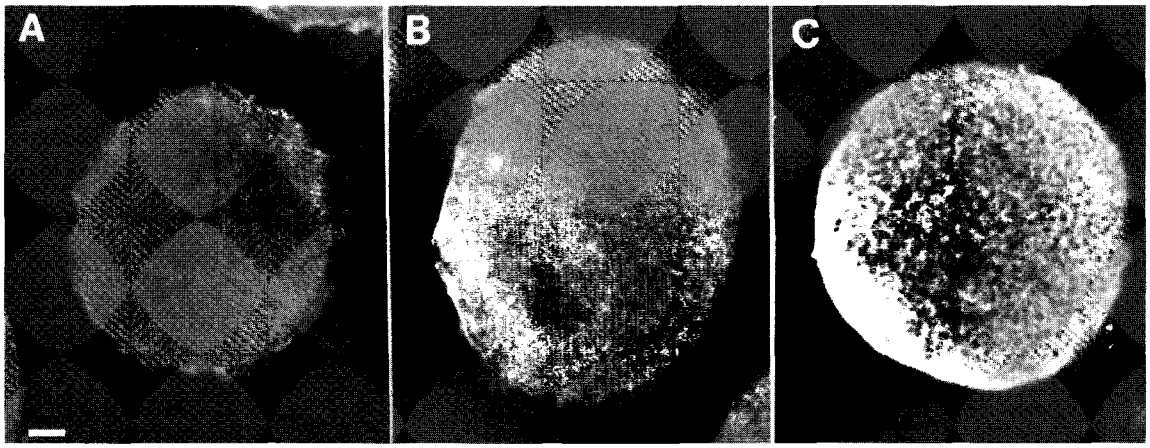

Fig 4: Immunofluorescence microscopy of *M. occulta* eggs. Sections of unfertilized eggs stained with anti-ankryin (A), anti-spectrin (B) and anti-Na^+K^+ATPase (C) antibodies to determine the distribution of ankryin, spectrin, and Na^+K^+ATPase. Ankryin and spectrin cannot be detected in eggs, whereas Na^+K^+ATPase is distributed throughout the cytoplasm rather than being localized in the myoplasm, as it is in eggs of indirect developing species. Scale bar is 10 μm; magnification is the same in each frame.

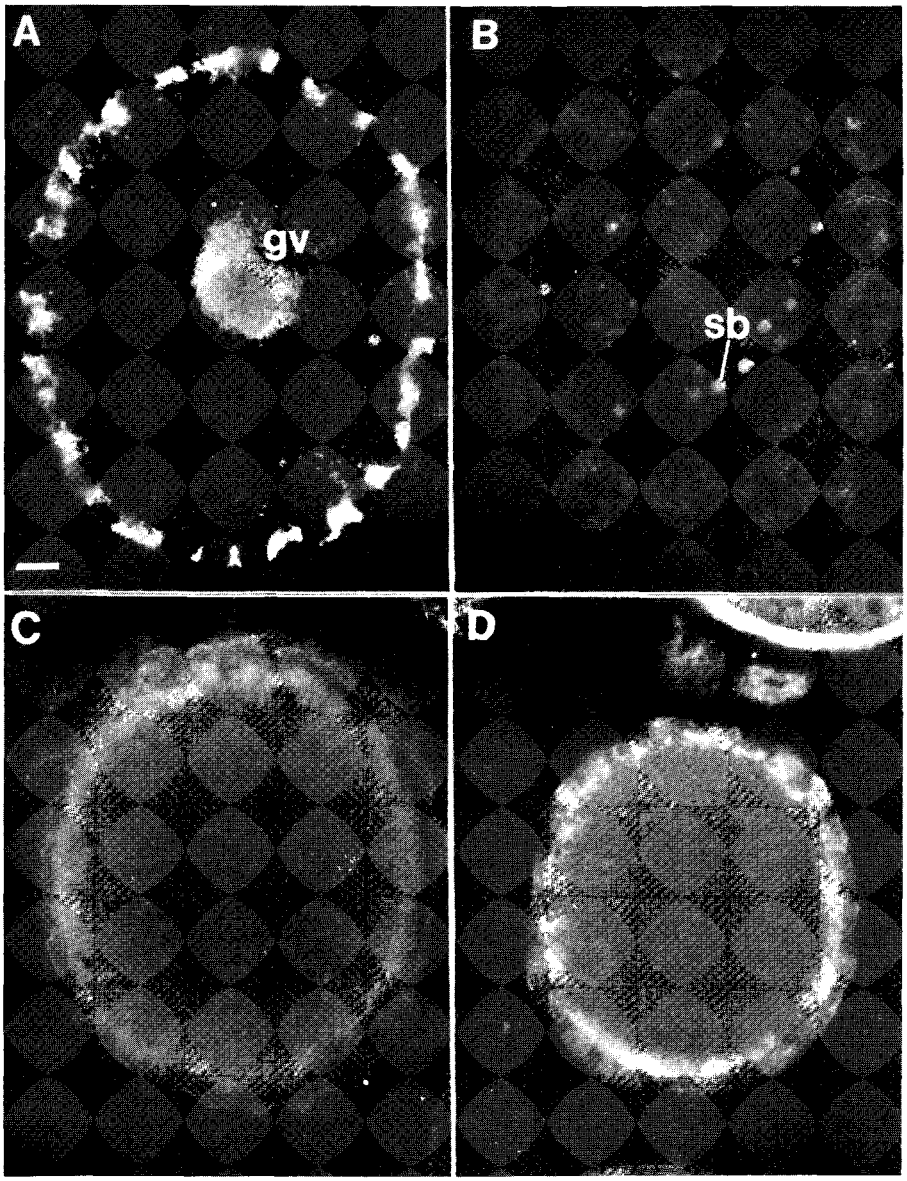

Fig 5: Immunofluorescence microscopy of *M. citrina* oocytes and unfertilized eggs. A, B. A section of a post-vitellogenic oocyte double stained with NN18 to detect the myoplasm (A) and anti-ankryin to determine the distribution of ankryin (B). The myoplasm (A) is localized in the cortex and near the germinal vesicle (gv), whereas the ankryin-like protein (B) is present in spherical bodies (sb) in the internal cytoplasm. C, D. Sections of unfertilized eggs stained with anti-spectrin (C) or anti-Na^+K^+ATPase (D) to determine the distribution of these membrane skeletal proteins. Both spectrin and Na^+K^+ATPase are localized in the myoplasm. Scale bar is 10 μm; magnification is the same in each frame.

vesicles upon which ankryin and other membrane skeletal proteins are assembled during oogenesis (Fishkind *et al*, 1990a). During maturation, the ankryin-containing structures disappear from *M. citrina* eggs, and ankryin does not reappear in embryos later development (Fig 6C, D). Although unfertilized *M. citrina* eggs lack ankryin, they contain cortical localizations of spectrin and Na^+K^+ATPase (Fig 5C, D). Spectrin and Na^+K^+ATPase were later observed in muscle cells of tailbud stage embryos (Fig 6A, B). In summary, the membrane skeletal proteins show different patterns of expression during *M. citrina* development: spectrin and Na^+K^+ATPase are present in the myoplasm

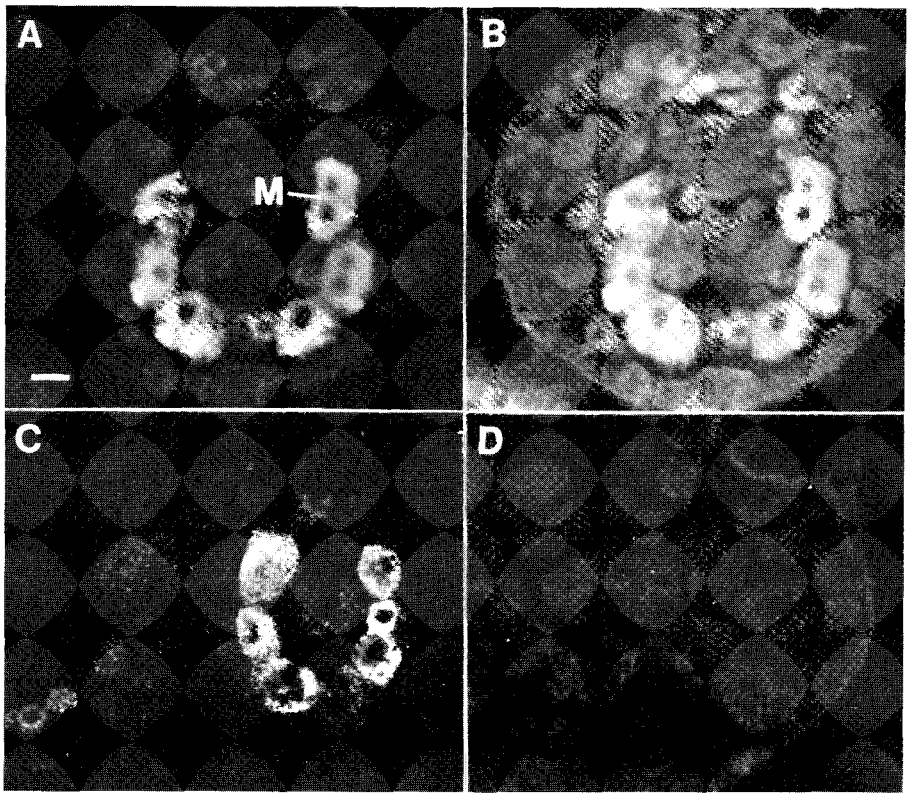

Fig 6: Immunofluorescence microscopy of *M. citrina* tailbud embryos. A, B. Cross-sections through the posterior region of early tailbud embryos showing the spectrin-like and Na^+K^+ATPase-like proteins concentrated in the tail muscle cells (M). C, D. A cross section through the posterior region of a mid-tailbud stage embryo which was double stained with NN18 antibody (C) to detect the location of the myoplasm-containing tail muscle cells and anti-ankryin (D) to determine the position of the ankryin-like protein. The six muscle cells surrounding the central notochord are positive for myoplasm but not the ankryin-like protein. Scale bar is 10 μm, magnification is the same in each frame.

of eggs and embryos, whereas ankryin expression is restricted to oocytes and disappears in mature eggs.

DISCUSSION

The results suggest that ascidian eggs contain a plasma membrane skeleton with proteins related to the vertebrate ankryins, spectrins, and Na^+K^+ATPase. The plasma membrane skeleton is well characterized in avian and mammalian erythrocytes (reviewed in Bennett, 1990). It consists of a reticular network of short actin and spectrin filaments that is localized immediately below the plasma membrane. The vertices of the filamentous network contain ankryin, a key protein which interacts with both spectrin and transmembrane proteins. In some vertebrate cells, these transmembrane proteins include Na^+K^+ATPase. Although few studies have been done on the plasma membrane skeleton of eggs, it has been shown that sea urchin (Fishkind *et al*, 1990b), amphibian (Campanella *et al*, 1990), and mammalian (Reima and Lehtonen, 1985) eggs contain cortically localized proteins that are related to erythroid spectrin. The ascidian egg differs from these examples, however, in that its membrane skeleton is localized in and segregates with the myoplasm throughout development. Thus, the ascidian membrane skeleton could have a role in the localization and function of muscle determinants.

Althought the organization of the membrane skeleton of ascidian eggs in unknown, there are distinct similarities to the erythro-

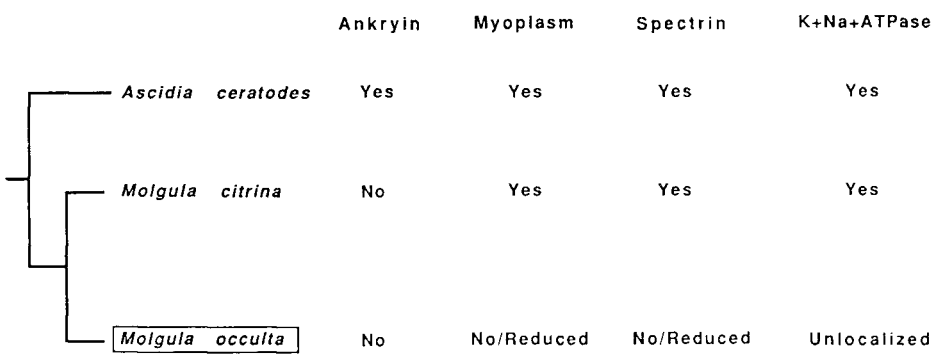

Fig 7: A summary of the results obtained when eggs and embryos of various ascidian species were stainedwith antibodies to determine the presence of the myoplasm and membrane skeletal proteins. The inferred phylogeny of the three ascidian species used in this investigation (after Hadfield *et al*, Ms Submitted, 1993) is shown on the left. The branch lengths are not proportional to evolutionary distance. The columns on the right summarize the expression patterns of ankryin, the myoplasm (as determined by the presence of p 58; Swalla *et al*, 1991; Jeffery and Swalla, 1992), spectrin, and Na^+K^+ATPase in each species. The direct developing species is boxed.

cyte membrane skeleton. The myoplasm of ascidian eggs contains a peripheral lamina of actin filaments (Jeffery and Meier, 1983; Jeffery, 1984a). Several lines of evidence suggest that the peripheral actin network is closely associated with the egg plasma membrane. First, relatively strong centrifugation is required to displace the actin lamina from the egg cortex, although the filamentous region localized deeper in the egg cytoplasm can be dispersed by weaker centrifugal forces (Jeffery and Meier, 1984). Second, fragments of the plasma membrane are present on the outer surface of isolated myoplasmic crescents prepared from ascidian eggs by biochemical methods (Jeffery, 1985). Third, isolated ascidian egg cortices contain actin filaments adjacent to the plasma membrane (Sardet *et al*, 1992). Finally, actin filaments have been detected near the egg plasma membrane by electron microscopy (Sawada and Osanai, 1985). The association of spectrin, ankryin, and Na^+K^+ATPase with the peripheral actin network of ascidian eggs is suggested by their localization in a thin layer of cytoplasm immediately beneath the plasma membrane.

Ankryin plays a pivotal role in linking the membrane skeleton of erythrocytes to the plasma membrane. The same general relationship is proposed to exist at the interface of the myoplasm and the plasma membrane in ascidian eggs. After fertilization, ankryin and the other membrane cytoskeletal proteins segregate with the myoplasm into the presumptive larval tail muscle cells. In contrast to their peripheral localization in eggs, the membrane skeletal proteins are distributed throughout the cytoplasm of muscle cells. This internal distribution suggests that they could play a role in organizing myofilament bundles and sarcoplasmic reticulum as well as the membrane cytoskeleton. A low level of Na^+K^+ATPase was also found in the mesenchyme cells of late stage embryos. The mesenchyme cells are derived from the same part of the cleaving embryo as the muscle cells (Conklin, 1905), and may inherit membrane skeletal proteins with the myoplasm. Na^+K^+ATPase and ankryin are also present on the apical side of the epidermal cells, which inherit little or no myoplasm. Thus, these proteins must be produced *de novo* from the epidermis during embryogenesis. Presumably, the membrane skeleton functions in polarizing the epidermal cells, which may be required for the synthesis and vectorial secretion of the larval test components.

Ascidians are important models systems for studying evolutionary problems in development because closely related species can exhibit either direct or indirect development (Berrill, 1931; Jeffery and Swalla, 1990b). Recent studies have shown that direct development can be explained in part by changes in the organization of the myoplasm (Swalla *et al*, 1991; Jeffery and Swalla, 1992). Spe-

cies of direct developing ascidians that lack or contain reduced levels of myoplasm are unable to complete muscle cell differentiation during embryogenesis. The results of the present investigation show that spectrin, ankryin, and Na+K+ATPase expression is also modified in eggs of a direct developing species. Thus, multiple changes have occurred in the organization of the myoplasm in ascidians with direct development. Figure 7 summarizes the results obtained on the expression of membrane skeletal proteins in the context of ascidian phylogeny. The phylogeny suggests that indirect development, the myoplasm, and an egg membrane skeleton containing ankryin, spectrin and Na^+K^+ATPase, is the ancestral condition in ascidians. An important observation is that ankryin expression has disappeared in mature eggs of both a direct (*M occulta*) and an indirect (*M. citrina*) developing molgulid. Despite lacking ankryin, however, eggs of the indirect-developing species contain a myoplasm with localized spectrin and Na^+K^+ATPase. These features are finally lost in eggs of direct developing species. Loss of ankryin may have preceeded loss of the myoplasm and the other membrane skeletal proteins, thus serving as predaptation to the evolution of direct development in the molgulid ascidians.

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