Characterization of Myosin II Isoforms Containing Insertions of Amino Acids in the Flexible Loop Near the ATP-binding Pocket

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There have been a multitude of myosins discovered to date that fall within at least nine structurally distinct classes. Within one of these classes, the myosin IIs, there are a number of isoforms of each myosin that are the products of different genes and/or alternative pre-mRNA splicing. We have been studying the function and regulation of isoforms of the heavy chain of vertebrate smooth muscle and nonmuscle myosin II that are the products of alternative splicing. The isoforms we are interested in contain either an insertion or deletion of a cassette of amino acids in a region near the ATP-binding pocket. This region corresponds to a part of the molecule that was not resolved in the crystal structure of S1 and is thought to be a flexible loop (Rayment et al., 1993). The sequence and number of amino acids within the insert is different in the smooth muscle and nonmuscle myosin heavy chains (MHCs), but both inserts occur at the exact same site in the primary sequence that begins at amino acid 212. The data below, summarized for both vertebrate smooth muscle and nonmuscle myosin II, suggest that this flexible loop region of the molecule is a functionally important region for generating myosins with different enzymatic activities and/or different regulatory pathways.

There is one vertebrate smooth muscle MHC gene and at least two pairs of alternatively spliced isoforms of the protein. One pair of isoforms is generated by alternative splicing near the 5′ end of the mRNA resulting in MHCs containing either an insertion or deletion of seven amino acids in the flexible loop region near the ATP-binding pocket (Hamada et al., 1990; Kelley et al., 1993; Babij, 1993; White et al., 1993). The insert was found to be present in myosin from intestinal smooth muscle, which is a "phasic" fast-contracting muscle and absent in myosin from the aorta, which is a "tonic" vascular smooth muscle that contracts and maintains tension for long periods with little ATP expenditure. Using an in vitro motility assay, we found that myosin containing the insert moved actin filaments at an average velocity 2.5-fold faster than noninserted myosin. Similarly, the V_{max} of the actin-activated Mg^{2+}-ATPase activity of the inserted myosin was about twofold higher than the noninserted myosin (Kelley et al., 1993). Based on these results, we believe that the insert confers a higher enzymatic activity on the myosin. The proximity of the insert to the ATP-binding pocket suggests that the mechanism by which the insert influences enzymatic activity is to affect the rate of nucleotide binding, hydrolysis, or product release. These results may also partially explain the molecular mechanisms underlying the heterogeneity in contractile properties observed among smooth muscle tissues, but whether it is sufficient to explain the differences between all "phasic" and "tonic" smooth muscles remains to be determined.

There are at least two genes that encode the nonmuscle MHC isoforms, MHC-A and MHC-B (Katsuragawa et al., 1989; Kawamoto and Adelstein, 1991). The MHC-B pre-mRNA can also be alternatively spliced resulting in additional isoforms that contain insertions or deletions of amino acids in a region corresponding exactly to the region of insertions in the smooth muscle isoforms discussed above (Takahashi et al., 1992; Bhatia-Dey et al., 1993). One interesting feature of the nonmuscle myosin insert, which is not true of the smooth muscle myosin insert, is that it contains a consensus sequence for phosphorylation by cyclin-p34 cdc2 kinase. In cultured *Xenopus* XTC cells, we have identified two inserted MHC-B isoforms and a noninserted MHC-A isoform by immunoblotting of cell extracts. When myosin was immunoprecipitated from XTC cells and phosphorylated in vitro with cdc2 kinase, the kinase catalyzed the phosphorylation of the inserted MHC-B isoforms but not the noninserted MHC-A isoform (Kelley et al., 1995). Isoelectric focusing of tryptic peptides generated from MHC-B phosphorylated with cdc2 kinase revealed one major phosphopeptide that was purified by reverse-phase high-performance liquid chromatography and sequenced. The phosphorylated residue was identified as Ser-214, the cdc2 kinase consensus site within the insert near the ATP-binding region. The same site is phosphorylated in intact XTC cells during log phase of growth and in cell-free lysates of *Xenopus* eggs stabilized in second meiotic metaphase but not interphase. Moreover, Ser-214 phosphorylation is detected during maturation of *Xenopus* oocytes when the cdc2 kinase-containing maturation-promoting factor is activated, but not in G2 interphase-arrested oocytes (Kelley et al., 1995). Taken together, these results demonstrate that MHC-B phosphorylation is tightly regulated by cdc2 kinase during meiotic cell cycles: it is unphosphorylated on Ser-214 before entering meiosis, becomes phosphorylated on Ser-214 by cdc2 kinase during meiosis, and is again dephosphorylated at this site in the interphase, which is equivalent to the fertilized egg. Thus, MHC-B phosphorylation by cdc2 kinase, at a site located near the ATP-binding region, is correlated with the cortical reorganization that occurs in meiosis, and dephosphorylation at this site correlates with the cortical contraction that occurs at fertilization, which aids in pronuclear fusion. Further experiments should elucidate the precise function of this modification during early *Xenopus* development.

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