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Development transitions of thin filament proteins in rat extraocular muscles

Carole L. Moncman^{1,*}, Miguel E. Andrade², Andrea A. McCool¹, Colleen A. McMullen², and Francisco H. Andrade²

¹Department of Molecular and Cellular Biochemistry, University of Kentucky, Lexington, KY 40536

²Department of Physiology, University of Kentucky, Lexington, KY 40536

Abstract

Extraocular muscles are a unique subset of striated muscles. During postnatal development, the extraocular muscles undergo a number of myosin isoform transitions that occur between postnatal day 10 (P10) and P15. These include: 1) loss of embryonic myosin from the global layer resulting in the expression restricted to the orbital layer; 2) the onset of expression of extraocular myosin and the putative tonic myosin (myh 7b/14); and 3) the redistribution of nonmuscle myosin IIB from a subsarcolemma position to a sarcomeric distribution in the slow fibers of the global layer. For this study, we examined the postnatal appearance and distribution of α -actinin, tropomyosin, and nebulin isoforms during postnatal development of the rat extraocular muscles. Although sarcomeric α -actinin is detectable from birth, α -actinin 3 appears around P15. Both tropomyosin-1 and -2 are present from birth in the same distribution as in the adult animal. The expression of nebulin was monitored by gel electrophoresis and western blots. At P5–10, nebulin exhibits a lower molecular mass than observed P15 and later during postnatal development. The changes in α -actinin3 and nebulin expression between P10 and 15 coincide with transitions in myosin isoforms as detailed above. These data point to P10–P15 as the critical period for the maturation of the extraocular muscles, coinciding with eyelid opening.

Introduction

The extraocular muscles follow a different developmental program than the somitic skeletal muscles. The extraocular muscles are derived from two mesodermal cell patches; one anterior to the pharyngeal arches associated with the neural crest cells and another close to the first pharyngeal arch [1–3]. Their origin and development likely influence the unique pattern of expression of cardiac and skeletal muscle myofilament proteins in the extraocular muscles [4]. This includes the presence of at least ten different sarcomeric myosin heavy chains in adult extraocular muscle, including the embryonic isoform and the tissue-specific extraocular muscle myosin [5–7]. These muscles exhibit very low levels of the fast isoform of myosin binding protein C, thus resulting in seemingly uncoordinated distribution of slow myosin binding protein C with fast myosin isoforms [8].

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*Corresponding author: cmonc2@uky.edu, Phone: 859-257-6776, Fax: 859-323-1037.

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Developmental transitions of myosin isoforms in both the rat and mouse extraocular muscles have recently been described [9, 10]. The studies demonstrated that the onset of extraocular myosin and slow, tonic myosin protein expression occurs between postnatal days 10 and 15 (P10 and P15) [9–11]. Embryonic myosin is the predominant isoform in the extraocular muscles at birth; its expression diminishes in the global layer between P10–15, restricting it to orbital layer fibers in the adult extraocular muscle [9, 10]. There is also a redistribution of nonmuscle myosin IIB from a subsarcolemmal to intra-sarcomeric distribution that occurs during P10–15 in development. These data suggest that this period is important for extraocular muscle development. In the present study, we examined whether developmental transitions in thin filament/Z-line associated proteins also take place during this period, and found a transition in the expression of α -actinin 3 and a nebulin isoform. These data further document that important developmental changes occur during the P10–15 window.

Materials and methods

Animals and tissue collection

The Institutional Animal Care and Use Committee at the University of Kentucky approved this study. Adult timed pregnant Sprague-Dawley rats were purchased from Harlan (Indianapolis IN) and time of delivery was monitored. Tissue was collected from pups at postnatal days 1 through 20 (in 5 day intervals), from weaned littermates at post-natal day 30, from purchased 45-day old rats, and from the dams. The animals were euthanized by CO₂ asphyxia followed by pneumothorax. Whole orbits and gastrocnemius muscle samples were collected, placed in 2 M sucrose in phosphate-buffered saline (PBS) with 10 mM EDTA and frozen in 2-methyl butane cooled to its freezing point by liquid nitrogen. Whole extraocular muscles, gastrocnemius, diaphragm and heart samples were also dissected and flash frozen in liquid nitrogen.

Antibodies used in this study

Isoform specific anti-sera to α -actinin 2 (AV41463), α -actinin 4 (AV42202), tropomyosin-1 (AV41392) and tropomyosin-2 (AV48224) were purchased from Sigma (St. Louis, MO). Anti-serum specific for α -actinin 3 was purchased from Abcam, Inc (Cambridge, MA). Monoclonal antibodies for α -actinin (EA-53 and BM75.2) and tropomyosin (CH1 and TM311) were also obtained from Sigma, Inc. A monoclonal antibody against LIM and SH3 protein 1 (LASP1; MAB8991) was purchased from Millipore, Inc. A description of anti-nebulin monoclonal antibodies can be found in Wright et al. [12]. Myosin antibodies were from DSHB: embryonic (F1.652), fast2A (2F7), neonatal slow/fast2A (N2.261), adult slow (A4.951), adult slow (A4.840) and all fast isoforms (MF20 and F59). For immunofluorescence labeling, all of the anti-sera were diluted 1:200 and all the monoclonal antibodies were diluted 1:500. For immunoblot analysis, all of the antibodies were diluted 1:1000.

Immunoblot analysis

For this study, pooled muscles from two adult rats were used per sample; for younger animals, we used muscles from 8 animals for P5, 6 for P10, 4 for P20, 2 for P30 and 45 per sample. Tissues were flash frozen and pulverized under liquid nitrogen. Approximately 100 mg were plunged into pre-warmed 2% SDS, 10 mM Tris-Cl, pH 7.5. The protein concentration of these extracts was assayed using the bicinchonic assay method (Thermo Scientific, Rockford, IL) and the protein concentrations were adjusted to 0.5–1.0 $\mu\text{g}/\mu\text{l}$ with a total of 10 $\mu\text{g}/\text{lane}$ loaded onto the 10–20% polyacrylamide gradient gels (Bio-Rad, Hercules, CA) or 15% polyacrylamide gels using a Tris-glycine buffer system, or 2–12% polyacrylamide gradient gels using Fairbanks buffer [13]. Gel replicas were generated using a semi-dry system to transfer proteins to either nitrocellulose or PVDF [14]. The gel replicas

for the developmental muscle samples were probed with the anti- α actinin, anti-tropomyosin, anti-nebulin or anti-LASP1 antibodies, followed by secondary antibodies coupled to horseradish peroxidase and enhanced chemiluminescence detection (Kirkegaard and Perry, Gaithersburg, MD) or with Alexa Fluor 680-conjugated goat anti-rabbit secondary antibody (1:7500, Invitrogen). Both primary and secondary antibodies were incubated for 1 hr at room temperature. Membranes were finally rinsed with PBS and scanned using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln NE) or exposed to X-ray film.

Immunofluorescence microscopy

Ten- μ m thick orbital cross sections were cut using a MICROM HM525 cryostat and collected on Superfrost plus slides. Whole orbits were sectioned completely through the block. Every 200 μ m, a section was stained with hematoxylin to determine the position of the muscle within the blocks.

Immunolabeling was performed as described previously [15]. Briefly, cryostat sections were treated with 1% Triton X-100 in PBS for 15 min at 4°C, washed and then blocked in 1% normal goat serum in PBS for 1 hr at 4°C. Primary antibodies were diluted in the blocking solution and all incubations were performed at 4°C for 12 hrs. Following washes after the primary antibody, secondary antibodies diluted in blocking solution were applied and incubated at room temperature for 2 hrs. Secondary antibodies, minimal cross-reacting antibodies--absorbed against both mouse and rat serum proteins were purchased from Jackson ImmunoResearch and all were diluted to 1:300 in 1% normal goat serum. After washing the samples, SloFade Gold (Molecular Probes, Invitrogen, Carlsbad, CA) was applied to the sections and cover slips were sealed with black nail polish. Samples were visualized on an Axiovert 200M equipped with an Orca ER camera.

Data analysis

Image collection and fiber counts were performed using OpenLab (Perkin Elmer, Waltham, MA). Fibers were scored as positive when containing an α -actinin isoform specific to the probe or negative when not. The percent of positive fibers was calculated as the positives divided by the total number of fibers in the field. All statistical analyses were performed using KaleidaGraph (Synergy Software, Reading, PA). All statistical analyses are reported as the mean $\pm$ the standard error of the mean.

All gels and films were scanned at 600 dpi and ImageJ was used to measure the distance in pixels between the nebulin and myosin bands for the developmental analysis of nebulin from 4 separate blots. ImageJ was also used to analyze the intensity of the signals for the LIM-nebulette immunoblot for 4 independent blots. The signal for LIM-nebulette in the left ventricle P10 was set as 1 and all other samples were normalized to this value for each blot. The data represents the mean normalized intensity $\pm$ the standard error of the mean.

Results

We have previously shown that sarcomeric α -actinin is present in the developing extraocular muscles using a monoclonal antibody, EA-53 [16]. At least two α -actinin isoforms are known components of the striated muscle Z-lines, α -actinin 2 and α -actinin 3. Based on microarray analysis of message levels, α -actinin 3 is more abundant in global layer fibers than in the orbital layer [17, 18]. We used isoform specific polyclonal antisera against α -actinin 2, 3 and 4 to examine their distribution within the extraocular muscles from adult orbits. As expected, the polyclonal antiserum to α -actinin 2 labeled all of the extraocular muscles in both the global and orbital layers in a manner identical to that of the

anti-sarcomeric monoclonal antibody used previously (Figure 1A). The anti- α -actinin3 antiserum only recognized $39.9 \pm 1.46\%$ fibers in the global layer of the extraocular muscles; whereas the anti- α -actinin 4 detected punctate staining external to the muscle fibers suggesting it recognizes the nonmuscle components (fibroblasts, satellite cells, blood cells, etc...) of the tissues (Figure 1B and C).

Myosin isoforms vary between and within extraocular muscle fibers [5–7]. To determine if α -actinin 3 also varied in longitudinal distribution, adult orbits were cut in serial sections and cryostat sections from the distal, medial and proximal portions of the orbits were stained with the anti- α -actinin3 antiserum (Figure 2). Alpha-actinin3 was present in fibers throughout the length of global layer.

We next examined if α -actinin3 was found in specific fiber types by staining for both α -actinin3 and myosin isoforms (Figure 3). There is no overlap between embryonic myosin and α -actinin3, as would be expected since this myosin isoform is restricted to orbital layer fibers in adult extraocular muscles (Figure 3A). Fibers containing myosin 2A have α -actinin 3 (Figure 3B, C). In rats, the monoclonal antibody that detects the neonatal slow myosin isoform also weakly detects the fast 2A myosin isoform. The fibers that exhibit the strongest staining with this antibody are presumably the fibers containing the neonatal myosin isoform and are not positive for α -actinin 3 (Fig. 3B). Of the fibers positive for α -actinin 3, $84.2 \pm 1.3\%$ of the fibers stained positive for fast myosin 2A (Fig. 3C) and $21.8 \pm 2.0\%$ were positive for extraocular myosin (not shown). Alpha-actinin 3 was not present in fibers positive for the adult slow myosin isoform (Fig. 3D). These data indicate that the presence of α -actinin 3 in the global layer fibers coincides with the adult fast myosin isoforms.

We next examined the abundance of α -actinin 3 during postnatal development in the rat extraocular muscles by western blot analysis. Whole extraocular muscle lysates were compared to gastrocnemius, diaphragm and left ventricle. Probing the western blots with antiserum raised against α -actinin 2 resulted in a single band of 95 kDa from P5 through P45 in the extraocular muscles, gastrocnemius, diaphragm and left ventricle (Figure 4). In contrast, α -actinin3 was not detected in the extraocular muscles at P5 and 10, but was present at P20 and later (Figure 4). Alpha-actinin 3 was present throughout the time course in gastrocnemius and diaphragm and was not detected in the ventricle at any time point (Figure 4). To verify these results, we stained cryostat sections of postnatal whole orbits with anti- α -actinin 3 anti-serum and counterstained with a monoclonal antibody to sarcomeric α -actinin (Figure 5). At P1–10, only the accessory extraocular muscles, retractor bulbi and levator palpebrae superioris, contained significant number of α -actinin3 positive fibers (note arrow to retractor bulbi is shown at P10 in Fig 5). A few fibers in the extraocular muscles exhibited a weak, but detectable signal for α -actinin 3 at P10. α -actinin 3 was more abundant in the extraocular muscles by P15 (Fig. 5B).

We next examined the tropomyosin content of the developing extraocular muscles by western blot analysis (Figure 6). The monoclonal antibodies, CH1 and TM311, detect both tropomyosin 1 and 2 in extraocular muscles, gastrocnemius and diaphragm and only tropomyosin 1 in the left ventricle. Polyclonal antisera raised against peptides specific for tropomyosin 1 or 2 were also used in this analysis. We were unable to detect a signal with the anti-tropomyosin 1 antiserum in this assay; however the anti-tropomyosin 2 recognized the 38 kDa tropomyosin 2 band in the extraocular muscle, gastrocnemius and diaphragm. By immunofluorescence, the antisera and the monoclonal antibodies showed the same distribution: tropomyosin 1 and 2 are found throughout the extraocular muscles at all time points tested (P10 is shown in Figure 7).

Nebulin has developmental isoforms that vary in molecular weight due to alternative splicing [19] [20]. Thus, we identified the nebulin isoforms present in the developing extraocular muscles by gradient gel electrophoresis and western blots (Figure 8). In the extraocular muscles, the nebulin bands exhibit a lower molecular weight at P5–10. By P20, the nebulin band is found at the same molecular weight as in adult extraocular muscle. In comparison, nebulin in P10 gastrocnemius and diaphragm (earliest time point for these muscles) has the same molecular weight as in the adult muscles. In cardiac muscle, the anti-nebulin antibodies did not detect anything in the molecular weight range of nebulin.

In addition to nebulin, skeletal muscles contain LIM-nebulette (LASP2) [21]. A commercial monoclonal antibody raised against LASP1 also recognizes LIM-nebulette, nebulin and nebulette [22]. As LASP1 is not expressed in striated muscles [23], we used this antibody to probe 15% polyacrylamide gel replicas to examine the abundance of LIM-nebulette (Figure 8B). The anti-LASP1 antibody detected a 31 kDa band corresponding to LIM-nebulette in all the samples. Interestingly, in the gastrocnemius and the diaphragm LIM-nebulette is abundant at P10 and decreases in concentration throughout development (Figure 8C). In both the extraocular muscles and heart, the expression level remains constant throughout the time course analyzed.

Discussion

We demonstrated previously that developmental transitions in embryonic myosin, nonmuscle myosin IIB and extraocular myosin occur between P10 and P15 in rat extraocular muscles. In this analysis, we examined postnatal changes in the expression of proteins associated with thin filaments. We find that α -actinin 3 is not detected until P15 and that there is a transition in the mass of nebulin that occurs during this same period.

Mutations in the gene encoding α -actinin 3 result in lack of expression of this isoform in 18% of the human population; genetic analysis of elite athletes suggest a correlation between expression of α -actinin 3 and muscle power. [24, 25]. In humans, the distribution of α -actinin 2 and 3 completely overlap [26]. In mouse models of α -actinin3 deficiency and in humans, α -actinin 2 compensates for the lack of α -actinin 3 suggesting a redundancy of functions between the two isoforms [26]. In the α -actinin3 deficient mouse, there is also remodeling of the Z-lines and smaller fiber size for the fast fibers that normally express α -actinin 3 [27, 28]. However, a recent study in α -actinin 2 deficient zebra fish demonstrate severe muscle defects that cannot be rescued by α -actinin 3, suggesting that α -actinin 2 and 3 have distinct functions [29].

In limb skeletal muscles, α -actinin3 is found in fast, glycolytic fibers [26]. The extraocular muscles have the fast isoform of α -actinin only in the fast fibers, specifically those containing fast myosin 2A and the extraocular myosin. In the extraocular muscles, α -actinin 3 is enriched in the global layer fibers [17, 18]. Our data are in agreement with this observation. From P15 through adult, α -actinin3 is found within the global layer of rat extraocular muscles. We also demonstrate that α -actinin3 is present in fibers throughout the length of the global layer. Further work on the role of α -actinin 3 is necessary to understand its compartmentalization in the global layer.

Although α -actinin 3 is expressed from P1 in the retractor bulbi, levator palpebrae superioris and the somitic derived skeletal muscles, it was first detected in the extraocular muscles at P15, further evidence that the P10–P15 window has a special significance for the ocular motor system: opening of eyelids, appearance of the extraocular muscle myosin, and sarcomeric redistribution of nonmuscle myosin IIB [9, 10, 30]. It is interesting to note that the accessory muscle in rat [10] and mouse [9] do not express the extraocular myosin

isoform nor do they exhibit a sarcomeric distribution for nonmuscle myosin IIB. These differences in protein content further set the extraocular muscles as separate from the accessory muscles.

Previous studies on the distribution of troponin isoforms suggested a compartmentalization of troponin T isoforms between the endplate, orbital, and global layers in rabbit extraocular muscles with unique expression patterns of rare troponin T isoforms [31]. The same may apply to tropomyosin isoforms. Laser capture and microarray analysis of global and orbital layer mRNA predict that tropomyosin 1 is more abundant in the global layer [18]. By western blot analysis, we found that both tropomyosin 1 and 2 are present in the extraocular muscles throughout our time course. Using polyclonal antibodies specific to TPM1 and TPM2, we did not observe differences in the distributions from those observed using monoclonal antibodies that recognize both isoforms. We did not observe a compartmentalization of the tropomyosin isoforms, in contrast to the microarray analysis [18].

Nebulin undergoes developmental transitions in mass due to alternative splicing [20, 32]. Due to the repetitive nature of the primary structure, the best way to examine developmental nebulin isoforms is by gel electrophoresis. Buck et al. [33] compared developmental isoforms of nebulin in a variety of skeletal muscles using P1 neonatal and 100-day adult mice. This study demonstrated that each muscle undergoes a unique developmental pattern. In soleus and diaphragm, the adult nebulin is larger than the neonatal form, while in gastrocnemius and extensor digitorum longus, the neonatal form is larger [33]. In our current study, we did not observe changes in the mass of nebulin for the gastrocnemius or the diaphragm. This may be due to different species (rat vs. mouse) used in each study or the age of the animals (P10 vs. P1). Our results may also reflect that the transition to the mature isoform has already occurred in these muscles. We did, however, observe a change in nebulin's mass in the extraocular muscles with a lower molecular weight protein expressed at P5–10 and a larger isoform present from P20-adult. Significantly, this change occurs between P10 and P15.

Microarray comparisons of extraocular muscles and gastrocnemius indicate that higher levels of the nebulin are found in the extraocular muscles [30]. In our previous analysis of nebulin isoforms in the extraocular muscles, we failed to detect nebulin by western blot analysis [15]. In this study, we used a monoclonal antibody that recognizes four nebulin family members: nebulin, nebulin, LASP1 and (LASP2) and determined that LIM-nebulin, a splice variant of the nebulin locus, is indeed expressed in the extraocular muscles. In the somitic derived muscles, LIM-nebulin expression decreases throughout the time course analyzed. In both the extraocular muscles and the ventricular sample the expression for LIM-nebulin remained constant.

Concluding Remarks

The extraocular muscles undergo a unique differentiation and development being derived from a cranial mesodermal primordia compared to the somitic derived skeletal muscles. In some respects the extraocular muscle exhibit qualities of cardiac muscle such as maintenance of high levels of LIM-nebulin throughout development (this report) and expression of cardiac actin and myosin [34–37]. In other respects, including cellular morphology, expression α -actinin3, and nebulin isoform content, these muscles exhibit characteristics of skeletal muscle. Their distinctive pattern of differentiation is also evident by the identification of a window between P10 and P15 in which a number of transitions in myofibrillar proteins occurs. In addition to transitions of nebulin isoforms, translocation of nonmuscle myosin IIB, onset of expression of α -actinin3 and extraocular myosin occur during this window. Microarray analysis performed over the same developmental time

course also indicate that significant changes in message levels in the extraocular muscles with P14 serving as an inflection point [30] These data suggest that key developmental cues for the extraocular muscles change within this postnatal window. Further work is necessary to define them.

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Figure 1.

Comparison of the distributions α -actinin 2, 3, 4 vs. sarcomeric α -actinin. Cryostat sections of adult rat whole orbits were probed with a monoclonal antibody (EA-53) for sarcomeric α -actinin (red in panels) and counterstained with polyclonal antisera raised against α -actinin 2 (A), 3 (B), and 4 (C). The polyclonal antiserum staining is green in all panels. A. Anti- α -actinin2 recognizes an identical distribution to that detected with EA-53. B. Anti- α -actinin3 recognizes a subset of fibers that are restricted to the global layer. C. Anti- α -actinin4 labels small nonmuscle cells, which are most likely blood cells, satellite cells and fibroblasts within the tissue. Images in each panel are oriented with the orbital layer to the top of the images. Scale bar=10 μ m.

Figure 2.

Analysis of the longitudinal distribution of α -actinin 3. Serial sections of adult rat whole orbits were collected and stained for the distribution of α -actinin3 (green in all panels) compared with phalloidin stained actin (red in all panels). Sections chosen from the proximal, medial and distal portion of the medial rectus demonstrate the presence of α -actinin3 throughout the length of the muscle. Scale bar = 10 μ m.

Figure 3.

Comparison of the distribution of α -actinin3 with various myosin isoforms. Cryostat sections of whole orbits were examined for the distribution of α -actinin3 (green in all panels) compared to isoform specific myosins (red in all panels). A. Comparison of the distribution of α -actinin3 with embryonic myosin demonstrates no overlap with the embryonic myosin restricted to the orbital layer and α -actinin3 restricted to the global layer. B. Staining with a monoclonal antibody recognizes neonatal slow and fast 2 myosin and the α -actinin3 antisera exhibits partial overlap between the myosin positive fibers and the α -actinin3 distribution in the global layer. C. Comparison of the staining with antibodies directed at the fast 2A myosin isoform with the α -actinin3 staining again demonstrates partial of overlap of the fast α -actinin3 with the fast myosin. D. Staining with an antibody specific for the adult slow myosin isoform and the α -actinin3 antisera indicate that there is no overlap between α -actinin3 and the slow fibers. Arrow in panels B and C indicate fibers that are positive for both α -actinin3 and fast 2A myosin. Bar =10 μ m.

Figure 4.

Western blot analysis of α -actinin isoform expression during postnatal development. Whole cell lysates were separated on 10–20% polyacrylamide gradient gel, transferred to PVDF and the gel replicas were probed with antibodies specific for α -actinin2 and α -actinin3 isoforms. Although α -actinin2 is present at all time points analyzed in all tissues, α -actinin3 was not present in the extraocular muscles at P5 and P10 or in the left ventricle at any time point.

Figure 5.

Immunofluorescence analysis of α -actinin3 in the developing extraocular muscle. Cryostat sections of postnatal orbits, collected as outlined in the materials and methods, were stained for the distribution of α -actinin3 and sarcomeric α -actinin. P10 and 15 are shown. Throughout the time course all of the extraocular and accessory muscles were positive for the anti-sarcomeric α -actinin antibody. The anti- α -actinin3 sera stained the retractor bulbi and levator from P1–P30, but positive staining in the extraocular muscles was not observed until weak, patchy staining was detected at P10 (arrowhead points to a fiber positive for α -actinin3 in the lateral rectus). By P15, all of the extraocular muscles exhibited strong staining of fibers within the global layer of the muscles. Bar = 10 μ m.

Figure 6.

Western blot analysis of tropomyosin content of the extraocular muscles. Whole muscle lysates of the extraocular muscles, gastrocnemius, diaphragm and left ventricle were separated on 10–20% polyacrylamide gradient gels and transferred to PVDF. The gel replicas were probed with monoclonal antibodies, CH1 and TM311 that recognize both tropomyosin 1 and 2, and polyclonal antisera that specifically detect either tropomyosin 1 or 2. Throughout the time course analyzed, the monoclonals, CH1 and TM311 detected the presence of tropomyosin 1 and 2 and using the polyclonal anti-sera specific for tropomyosin 2 resulted in detection of this specific isoform throughout the time course.

Figure 7.

Developmental analysis of the distribution of tropomyosin isoforms. Cryostat sections for whole orbits were collected for a postnatal developmental time course and analyzed by immunofluorescence microscopy using polyclonal antisera directed at tropomyosin 1 or tropomyosin 2 and counterstained using a monoclonal antibody that recognizes both isoforms. The tropomyosin 1 and 2 isoform specific antibodies stained all of the fibers throughout both layers at all time points. P10 muscles are shown. Bar= 10 μ m.

Figure 8.

Western blot analysis of nebulin family members. A. Whole cell lysates of the developmental time course were separated on 2–12% polyacrylamide gradient gels using Fairbanks buffer and transferred to nitrocellulose. The gel replica was probed with nebulin monoclonal antibodies (N101 and N107), which recognize the amino terminal portion of the molecule. The antibodies detected nebulin at each time point with the nebulin band of the extraocular muscles exhibiting a lower molecular weight isoform at P5 and P10. The average distance in pixels $\pm$ the standard error of the mean between the anti-nebulin positive band and the myosin band is given for each lane. The India ink shows proteins in the area of myosin of the same blot demonstrating the presence of lysate in each lane. B. The same lysates were separated on a 15% polyacrylamide gel, transferred to nitrocellulose and the gel replica was probed with an anti- LASP1 monoclonal antibody that also recognizes nebulin, nebulette and LIM-nebulette. This antibody recognized a 31 kDa band in all the muscles. Since nebulin and nebulette are much larger and LASP1 is not present in striated muscles, the 31 kDa band is LIM-nebulette. LIM-nebulette appears to be more abundant in the extraocular muscles and heart than in the diaphragm and gastrocnemius. The India ink below represents the staining of the same blot demonstrating total protein content in the area of the actin band. C. Quantitation of the signal intensity from four separate immunoblots for LIM-nebulette supports the abundance of LIM-nebulette throughout the developmental time course of the extraocular muscles and the gradual decrease in concentration for the gastrocnemius. Diaphragm samples are stippled bars, extraocular muscle are given by white bars, gastrocnemius in black bars and the left ventricle samples are in grey. Error bars represent the standard error of the mean.