Evidence of an Additional Myosin Isoform in Turkey Skeletal Muscle

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Abstract

The myosin composition of *posterior latissimus dorsi* (PLD) muscle was examined at different postnatal ages in a turkey strain selected for its rapid growth rate and high body weight (HW) and in a farm turkey strain used as control. The biochemical methods of native gel electrophoresis, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting and peptide mapping were used. The growth characteristics of muscle, in terms of fiber number and size, were studied in parallel. After the fifth week, fiber enlargement in HW PLD was significantly greater than that in control muscle. A fourth fast isomyosin (FMX), differing from FM3, FM2 and FM1 by its heavy chain composition, was expressed earlier in HW turkey muscle than in control muscle (5 weeks *vs* 15 weeks). This isoform, identified for the first time in birds, is a specific marker of postnatal muscle development in the turkey.

Key words: heavy weight turkey, fast muscle, myosin isoforms, postnatal development.

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Various myosin isoforms differing in their heavy (MHC)- and light (MLC)-chain composition are found in vertebrate skeletal muscle. In the chick, two slow isomyosins (SM2 and SM1) containing distinct isoforms of MHC encoded by separate mRNAs have been detected in slow muscle [23, 24, 34], together with three fast isomyosins (FM3, FM2 and FM1) in fast muscle. FM1 represents the MLC3F homodimer, FM2 the MLC1F/MLC3F heterodimer and FM3 the MLC1F homodimer [15, 23]. As chick development proceeds, SM1 predominates in embryonic and SM2 in adult slow muscle [20, 23, 34], whereas decreased FM3 accumulation in fast muscle is associated with reduced MLC1F synthesis and increased FM1 accumulation with greater MLC3F synthesis [2, 20, 23]. The existence of three distinct fast MHC isoforms, referred to as embryonic, neonatal and adult [1, 3, 6, 10, 43], together with two embryonic fast MHC [29, 41] and as many as five embryonic MHCs [22], suggests that other MHC-based isomyosins may be expressed in the myogenetic process.

In postnatal development, modifications in environmental factors induce changes in isomyosin composition [10, 16, 17, 19, 35]. Substantial enlargement of chicken skeletal muscle, induced experimentally by exercise training, tenotomy of synergistic muscles and wing weighting [for review, see 21 and 40], is associated with a change in the myosin isoform pattern [18, 26, 27]. A turkey strain (British United Turkey, BUT9) developed for its commercial food value was selected for the present study because of its rapid growth rate and high body weight (HW). This animal gains about twice as much in muscle weight as the Betina farm strain turkey (Betina) used as a control.

After morphometric analysis, we investigated the isomyosin pattern in fast *posterior latissimus dorsi* (PLD) muscle of posthatched HW and farm turkeys, demonstrating for the first time the existence of four distinct fast isoforms in turkey fast muscles.

Materials and Methods

Animals and muscles

The experiments were performed on male BUT9 turkeys (*Meleagris gallopavo*) selected for their rapid growth rate and HW, and on Betina farm strain turkeys used as controls. At 15 weeks posthatching, HW turkey weight was 2.2 times as high as that of controls (Figure 1). Analyses

were performed on fast PLD muscles at 1, 2, 3, 5, 10 and 15 weeks posthatching.

Histomorphometrical analysis

In each series, samples were removed from the midbelly of the muscle and frozen in liquid nitrogen-cooled isopentane. Cryostat-cut cross-sections (12 µm thick) were stained with hematoxylin and eosin. A computerized image analysis system (VIDS) was used for morphometric quantitative study. The muscle cross-sectional area was measured, and the number of fibers determined per unit area from randomly selected fields (each area analyzed contained a minimum of 500 fibers) to estimate the average values for the number of muscle fibers in muscle. The mean diameter of fibers was determined from a minimum of 100 fibers, as recommended in [13]. Statistical analysis was performed using the parametric Student t-test. For small samples the non-parametric statistical Mann-Whitney U-test was used [38].

Myosin extraction

Preparation of crude myosin was carried out as described in [14].

Electrophoretic analysis of native isomyosins

Nondenaturing pyrophosphate gel electrophoresis was performed at 80 V for 19 h at 4°C. The running buffer contained 20 mM sodium pyrophosphate (pH 8.5), 10% glycerol, 0.01% 2-mercaptoethanol and 2 mM MgCl2. Gels were stained with Coomassie blue, and the ratio of isomyosins was estimated by densitometry.

MLC analysis

Native isomyosins were first separated under non-dissociating conditions as described above, and the bands corresponding to each isoform were excised from multiple gel lanes, pooled and electro-eluted. Electro-elution was carried out overnight in an electro-eluter/concentrator (C.B.S. Scientific Company, Inc) according to the procedure previously described in [25], except that the elution buffer was composed of 25 mM Tris(hydroxymethyl)-aminomethane, 0.1% SDS, 0.192 M glycine pH 8.3. The electro-eluted isomyosins were placed on 15% SDS/polyacrylamide gel for further electrophoresis according to Laemmli [28].

Analysis of native MHC type by immunoblotting

Isomyosins separated on pyrophosphate gels were electrophoretically transferred onto a nitrocellulose sheet at about 200 mA for 4 h in a Trans-Blot cell [9]. After washing in 0.2% Tween, the sheet was incubated for 1 h at room temperature with monoclonal antibodies (mAb) (diluted 1:200 in PBS) raised against MHC isoforms in chicken fast muscles. The specificity of EB165 mAb for embryonic/adult MHC, 2E9 mAb for neonatal MHC and AB8 mAb for adult MHC was previously documented [4, 5, 10]. These mAbs were found to react with turkey MHC isoforms [33]. mAb binding by the Western blot technique

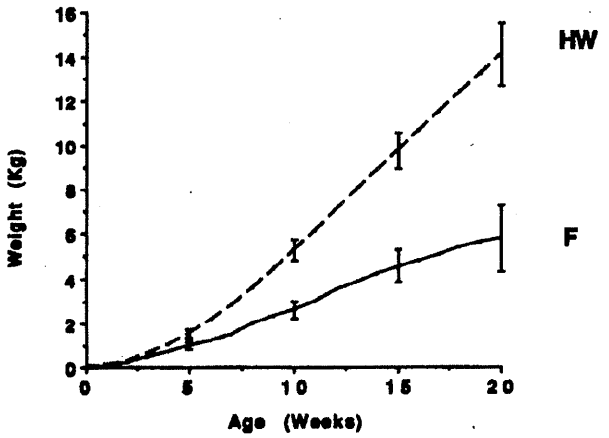

Figure 1. Comparison of turkey growth. Body weight (mean ± SEM) from 1 to 15 weeks posthatching in high-weight (HW) and control farm (F) turkeys.

was visualized using enhanced chemoluminescence (Amersham blotting kit). Tests for immunochemical specificity were performed by omission of primary mAbs and the use of preabsorbed controls.

Analysis of MHC peptide maps

The pyrophosphate gels were briefly stained and destained, and the bands corresponding to FM3, FM2, FM1 and FMX isomyosins were individually excised. To obtain the amount of MHC needed for mapping, about 40 bands from each native isomyosin were combined and electro-eluted. Proteolytic digestion of MHC contained within each isoform was carried out at 37°C for 30 min by addition of *Staphylococcus aureus* V8 protease [diluted in 2.3% SDS, 0.125 M Tris-HCl (pH 6.8), 5% β-mercaptoethanol and 10% glycerol, 0.01% bromophenol blue] according to the procedure of [11]. Proteolysis was stopped by boiling the samples for 5 min. The resulting peptides were separated on 15% SDS-polyacrylamide gels and detected using the sensitive silver-stain method (Amersham).

Results

Postnatal muscle hypertrophy

Data are summarized in Table 1 and illustrated in Figure 2. No significant differences in mean fiber breadth and number were observed between HW and control turkeys during the first 5 weeks. Compared to fiber diameters at one week, the values were doubled in both control PLD and HW PLD at 5 weeks. Between 5 and 15 weeks, the fiber enlargement in HW muscle was significantly greater than in control muscle (an increase of 110% in HW PLD *versus* 59% in control PLD). No significant modification in the number of fibers per section was detected during postnatal development in PLD muscle of either turkey strain.

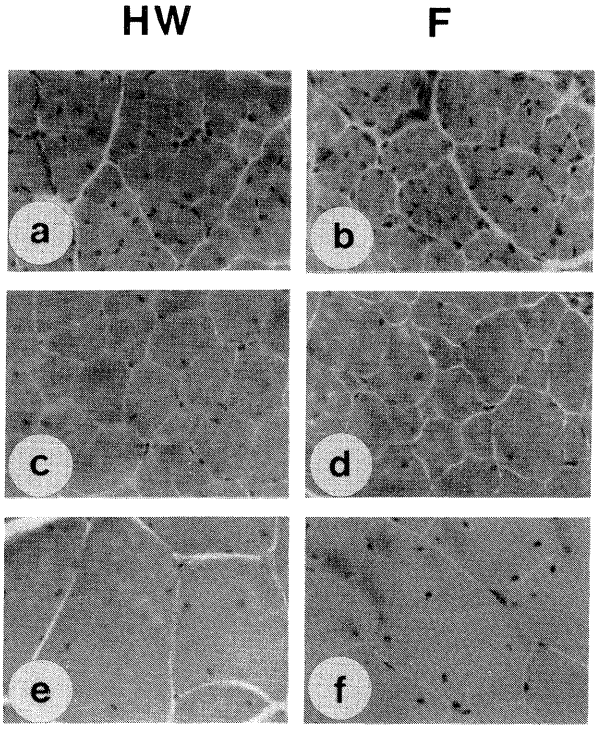

Figure 2. Transverse sections of turkey PLD muscles. Cryostat cross-sections (12 μm thick) of PLD muscle from 1 (a,b)-, 5 (c,d)- and 15 (e,f)-week-old high-weight (HW) and control farm (F) turkeys. Original magnification x 240. Hematoxylin-eosin staining. Myonuclei were often centrally located in PLD muscle fibers as previously reported in avian species.

Changes in native myosin isoforms

At all stages studied in control and HW turkeys, PLD muscle expressed three fast prominent isoforms identified as FM3, FM2 and FM1, considering their increasing mobility. During the first 3 weeks, the percentage of FM1 increased in control and HW PLD muscle, whereas that of FM3 decreased (Figure 3). From 5 weeks onwards, PLD muscle in HW turkey exhibited an additional faster migrating band which was only expressed from 15 weeks onwards in control PLD muscle. The identification of four fast migrating bands in PLD muscle was confirmed by co-electrophoresis of myosins extracted from this muscle and slow ALD muscle. Indeed, six distinct bands were clearly shown: the two slow MHC isoforms SM2 and SM1 contained in ALD muscle and the four fast migrating isoforms present in PLD muscle (Figure 3). The fourth fast migrating isoform, not previously identified in birds, was designated as FMX. It is assumed that the differential mobility of the three fast isomyosins (FM3, FM2, FM1) resulted from the variable composition of the three MLCs. FM3 and FM1 represented homodimers of MLC1F and MLC3F respectively, while FM2 was a heterodimer of MLC1F and MLC2F [15, 31]. These three isoforms are generally considered to consist of the same MHC.

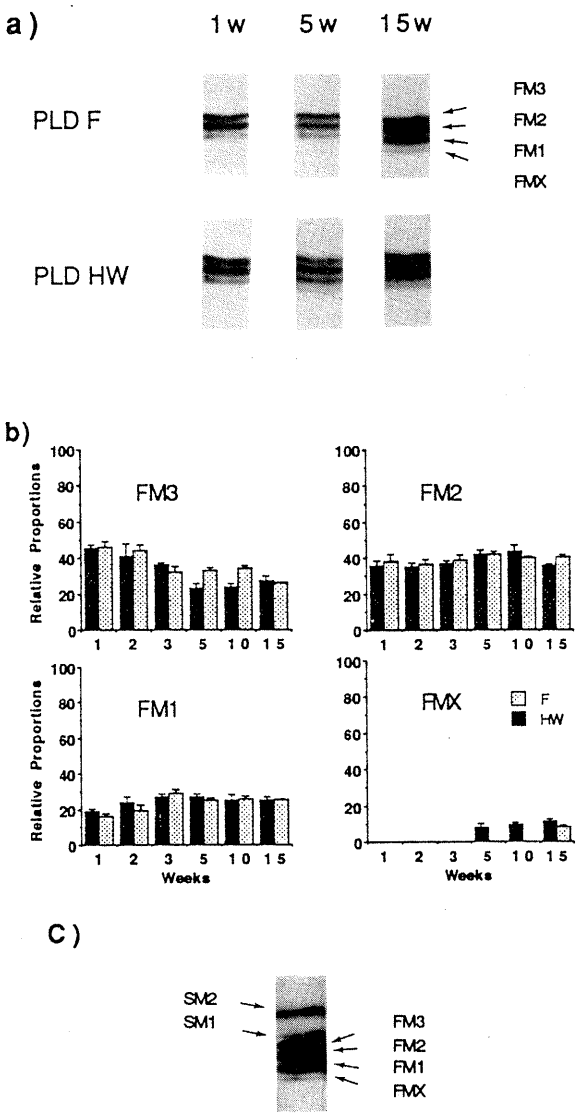

Figure 3. Analysis of native isomyosins. (a): Nondenaturing gel electrophoresis of PLD muscle myosin from 1-, 5- and 15-week-old high-weight (HW) and control farm (F) turkeys. (b): Relative proportions of fast isoform FM3, FM2, FM1 and FMX of PLD muscle from HW and F turkeys during postnatal development. (c): Co-electrophoresis of myosin from ALD and PLD muscles of 5-week-old HW turkey.

Nevertheless, polymorphism into fast myosin isoforms is not limited to MLCs but has been detected for MHCs [1, 3, 6, 10, 29, 41, 43]. Electrophoretic separation of the fast migrating isoforms in their native state could result from differences in MLCs, MHCs, or both. We also screened

*Table 1. Growth characteristics of turkey PLD muscles. fiber breadth and number (mean ± SEM) of muscles from 1-, 5- and 15-week-old high-weight (HW) and control farm (F) turkeys. (n): number of muscles. Student's t-test: ** $p < 0.01$. Mann-Whitney U test: * $p < 0.05$.*

Age (Weeks)	Breadth (µm) F	(n)	HW	(n)	Fiber number F	HW
1	9 ± 0,2	(5)	10 ± 0,5	(4)	30435 ± 2499	31643 ± 4249
5	29 ± 1,6	(6)	30 ± 0,6	(6)	24520 ± 2783	35054 ± 1791
15	46 ± 1**	(6)	63 ± 1,6**	(6)	25937 ± 1684	31417 ± 2374

the subunit content of myosin bands first resolved in non-dissociating conditions.

MLC and MHC composition of FMX

We examined the subunit composition of FMX isolated from PLD of 5- and 15-week-old HW and 15-week-old control turkeys in an attempt to determine why this isomyosin migrated faster than FM3, FM2 or FM1 on pyrophosphate gels. Analysis of the MLC of the three isomyosins corresponding to FM3, FM2 and FM1, isolated from PLD of each turkey strain, confirmed previous findings that they represent respectively the MLC1F homodimer, the MLC1-MLC3F heterodimer and the MLC3 homodimer [15, 23]. The fourth band, FMX, was found to be a MLC3F homodimer like FM1 (Figure 4), which suggests that its faster electrophoretic mobility was due to its MHC composition.

To characterize the MHC type contained in FMX, immunoblots of native myosin isoforms were done on three samples of PLD muscle taken from each turkey. The three native isomyosins (FM3, FM2 and FM1) all reacted respectively with EB165, 2E9 and AB8 mAb, indicating that each isoform contains fast embryonic, neonatal and adult MHC. At a stage of development, when different MHCs are coexpressed, the possibility arises for the existence of heterodimers (myosin molecules containing two different MHCs) or homodimers (myosin molecules with two identical MHCs). It is known that avian developmental MHCs (fast embryonic, neonatal and adult isoforms) are indistinguishable on nondenaturing gels [30]. Therefore, it is to be expected that each band (FM3, FM2, FM1) contains three types of myosin molecules with respect to their MHC composition. Interestingly, Lowey et al. [32] found that myosin isoforms expressed after chicken hatching associate predominantly into homodimeric molecules consisting of identical MHCs. FMX native isomyosin was immunostained with EB 165 and 2E9 mAb and unstained with AB8 mAb (Figure 5). The absence of reactivity of the FMX MHC band to AB8 mAb could have been due to the small amount of this component in total myosin extract. Normally loaded gels (Figure 5d) as well as overloaded gels (Figure 5e) confirmed the absence of adult MHC isoform. FMX appeared to differ from FM3, FM2 and FM1 in its MHC composition. The peptide mapping procedure described in Materials and Methods was used to examine

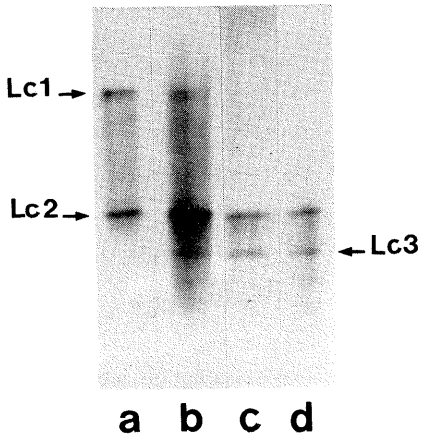

Figure 4. Myosin light chain analysis by re-electrophoresis in SDS-polyacrylamide gels of myosins first separated by electrophoresis in non-dissociating conditions. PLD muscle of 15-week-old high-weight turkey. (a) FM3, (b) FM2, (c) FM1, (d) FMX.

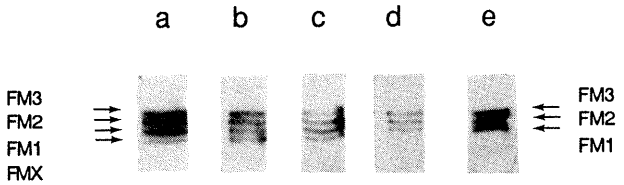

Figure 5. Immunoblots of native myosin isoforms. PLD muscle of 15-week-old high-weight turkey. Isoforms were stained with amido-black (a), EB 165 mAb for embryonic/adult MHC (b), 2E9 mAb for neonatal MHC (c) and AB8 mAb for adult MHC (d, e). In (e), gel was overloaded to increase the amount of FMX band.

MHC in FM3, FM2, FM1 and FMX isolated from control and HW PLD. Figure 6 shows that the patterns of V8 *S. aureus* cleavage products of MHC from FM3, FM2 and FM1 were very similar and differed from that of FMX. In fact, the MHC from FM3, FM2 and FM1 exhibited two peptide fragments not present in FMX digests (Figure 6). A difference in enzymatic digests was also noted between

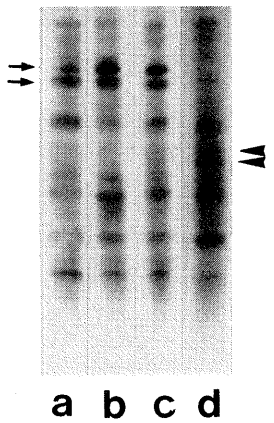

Figure 6. Myosin heavy chain (MHC) peptide maps. Comparison of peptide maps of MHC of the four individual PLD isomyosins of 15-week-old high-weight turkey (a: FM3, b: FM2, c: FM1, d: FMX) first separated under non-dissociating conditions and digested with S. aureus V8 proteinase. Bands were revealed by silver-staining. Differences between the peptide maps of FM3, FM2, FM1 and FMX are indicated by arrows (unique to FM3, FM2, FM1) and arrowheads (unique to FMX).

FMX and the other three isomyosins: the FMX peptide maps contained two peptide fragments that were absent from the FM3, FM2 and FM1 patterns (Figure 6). The peptides characteristic of FMX and of FM3, FM2 and FM1 were present in a region of higher molecular masses than MLC, indicating that they were MHC fragments. This suggests that the primary structure of FMX MHC is different from that of FM3, FM2 and FM1 MHC.

Discussion

This study compared the isomyosin composition of fast-twitch PLD muscle from a turkey strain selected for its high body weight and rapid growth rate with that of a control farm turkey strain characterized by lower body weight and a slower growth rate.

Morphometric analysis showed a similar increase in fiber diameter in fast PLD of HW and control turkeys during the first 5 weeks posthatching, whereas a 1.3-fold increase in mean fiber diameter was noted between 5 and 15 weeks in HW as compared to control PLD. No significant differences were observed between the isomyosin pattern from HW and control PLD until 5 weeks posthatching. The presence of a fourth fast migrating isomyosin (representing about 10% of total myosin) in fast PLD muscle (and also in fast *pectoralis major* muscle; results not shown) was detected from 5 weeks onwards in HW turkey but only from 15 weeks onwards in controls. This myosin isoform, evidenced for the first time in birds, was designated as FMX. Analysis of the type of MLC present in each native myosin demonstrated that FMX, like FM1, is a MLC3F homodimer. During certain periods of muscle maturation, multiple MHC isoforms are simultaneously expressed in fast-type avian muscles [4, 5]. Since nondenaturing gels cannot resolve developmental MHC isoforms [30], this method was combined with immunoblotting and peptide-mapping to search for the possible presence of different MHC types in the native myosin bands. The similarity of the MHC digestion pattern for FM3, FM2, FM1, as reported here, confirms the initial observations of [14, 30]. In addition, the reactivity of FM3, FM2 and FM1 isoforms to EB165, 2E9 and AB8 mAb suggests that each band contains embryonic, neonatal and adult MHC isoforms. This simultaneous identification of adult, neonatal and embryonic MHC isoforms in turkey PLD 15 weeks posthatching corroborates analyses of MHC expression in chicken PLD, demonstrating that the temporal expression of fast MHCs in this muscle is different from that observed in *pectoralis* muscle. Embryonic fast MHC is expressed throughout PLD muscle development, which is not the case for *pectoralis* muscle [4, 12]. The MHC content of FMX, when stained with a mAb specific for embryonic and neonatal MHC but not for adult MHC, appeared different from FM3, FM2 and FM1 isomyosins. These results suggest that the faster electrophoretic mobility of FMX essentially depends on the nature of its MHC. This assumption is supported by peptide-mapping analyses showing that the MHC structure of FMX is different from that of FM3, FM2 and FM1. Some studies have shown differences in the peptide maps of adult fast MHCs from several fast-twitch and mixed chicken muscles. At least three adult fast MHC peptides have in fact been characterized in the chicken [7, 12, 30, 37]. Using high-resolution column chromatography, Rushbrook and coworkers [36] suggested that chicken PLD muscle contains three major MHC isoforms and a fourth minor component. One of the major MHC appears to be identical with the adult *pectoralis* major isoform, the new species do not include the *pectoralis major* posthatch isoform. In view of these results, the possibility also exists that peptides unique to FMX MHC may be specific for another MHC not detected by our mAb repertoire. It appears somewhat surprising that FMX, the latest isoform to be synthesized, is the only one which does not react with adult AB8 antibody. This suggests that FMX may contain a homodimer of another adult MHC (differing or not from the one studied by other researchers) which contains EB165 and 2E9 epitopes but not the AB8 epitope.

The biological significance of FMX has not yet been determined. It has been shown in other studies that Ca^{2+}-activated myosin ATPase activity and maximal muscle-shortening velocity are directly proportional [8], and that the enzymatic activity involved is determined primarily by the MHC composition of myosin [42]. The earliest work indicating that each chicken fast isomyosin (FM3, FM2 and FM1) has the same Ca^{2+}-activated ATPase activities, and that there is no detectable difference in their MHC content [23], is consistent with these data [8, 42]. Thus, it is very likely that the shortening velocity of turkey PLD muscle changes when an additional MHC isoform (FMX) is accumulated. In this respect, it was found that differences in shortening velocities in rabbit skeletal muscle fibers containing a variety of

myosin isozymes were related to the MLC1F/ MLC3F ratio, MHC type, or a combination of both [39]. It is therefore possible that MLC3F-containing FMX isomyosin modulates the rate of muscle contraction in the turkey. However, regardless of the functional significance of FMX, it constitutes a useful marker for analysis of the factors inducing its expression during posthatch fast muscle development in the turkey.

The biochemical methods of native gel electrophoresis, Western blot and peptide mapping used in this study indicate that fast PLD muscle during postnatal development of the turkey expresses more than the three fast MHC isoforms characterized in developing chicken pectoral muscle (embryonic, neonatal and adult MHC). Molecular analysis of MHC mRNA transcripts will ultimately specify the number of MHC isoforms expressed in fast turkey muscle.

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