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A number of motile functions of macrophages are thought to be mediated by myosin. We have observed that myosin from rabbit alveolar macrophages is heterogeneous with respect to its 20 kDa light chain: two species of 20 kDa light chain are identified by one-dimensional and two-dimensional polyacrylamide gel electrophoresis, in a ratio of 2:1. Native myosin, analyzed on non-denaturing gels, is also composed of two species, in a ratio of 2:1. These results indicate that macrophages contain at least two different myosins, which might have different physiological functions.

Macrophage *Myosin* *Light chain* *Phosphorylation* *Electrophoresis*

1. INTRODUCTION

Myosin from vertebrate macrophages is composed of two heavy chains of M_r 200000, two light chains of M_r 15000 and two light chains of M_r 20000 [1,2]. The structure of macrophage myosin is, by analogy with other vertebrate myosins [3,4], likely to be characterized by two head regions, each of which is composed of a part of the heavy chain in association with one of each of the two classes of light chain. The 20 kDa light chain plays a regulatory role: it must be phosphorylated in order for the myosin to be activated by actin [2]. We now report that two forms of the 20 kDa light chain of macrophage myosin are distinguished by SDS–polyacrylamide gel electrophoresis and by isoelectric focusing. The molar ratio of the two forms is about 2:1. Since the ratio is other than one, at least two isoforms of myosin must exist in macrophages. The presence of two isoforms is directly demonstrated by electrophoretic analysis under non-denaturing conditions.

2. MATERIALS AND METHODS

Myosin was purified from rabbit alveolar macrophages and from cultured cell lines PU5-1R and J774 as in [2]. PU5-1R and J774 cells were kindly provided by Dr Noel Warner and Ms Eva Barry. SDS–polyacrylamide gel electrophoresis was performed as in [5] or [6]. Two-dimensional electrophoresis was performed by a modification of the method in [7], with isoelectric focusing in the first dimension. Electrophoresis of native myosin was performed by a modification of the method in [8].

Phosphorylation of macrophage myosin by endogenous myosin light chain kinase was accomplished by exposing crude actomyosin to [γ-^{32}P]ATP prior to final purification of the myosin, as in [2]. The phosphorylated, purified myosin was analyzed by SDS–PAGE. For detection of radioactivity, the dried gel was exposed to a sheet of Kodak XR5 film, which was subsequently processed according to the manufacturer's recommendations.

Densitometry of the stained gels and autoradiographs was performed on a Zeiss scanning densitometer or on a Zeineh soft laser scanning densitometer (BioMed Instruments, Fullerton CA). On the autoradiographs, the two bands of LC_{20} were not completely separated; therefore it was not possible to obtain separate peak quantities for them. Instead, the relative peak sizes were

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estimated by assuming that each peak was symmetrical (i.e., that the ascending and descending slopes were identical for any one peak) that the ascending shape of LC_{20} represented only LC_{20}-1, that the inflection point of the descending slope represented the apex of the LC_{20}-2 peak, and that the descending slope between the inflection point and the baseline represented only LC_{20}-2. The area of each half-peak was thus determined, and multiplied by a factor of 2 to obtain the area of the whole peak. When tested on a number of clearly separated proteins this method had an error not exceeding 20%.

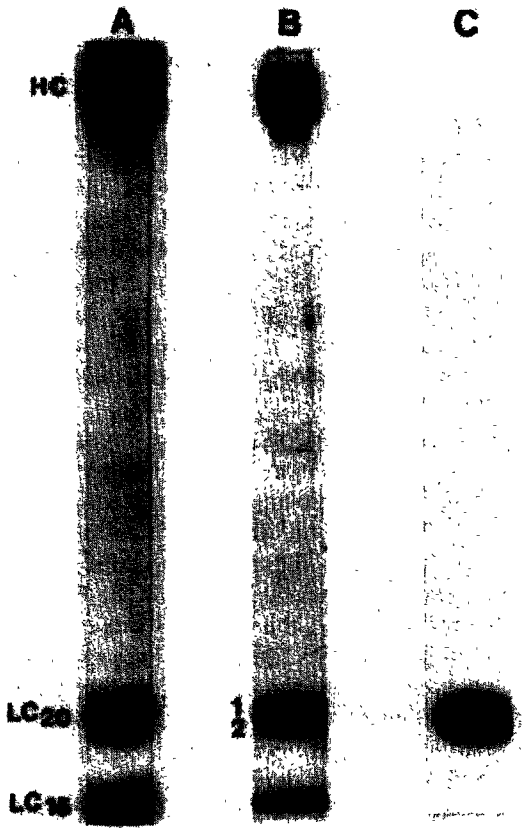

Fig.1. SDS–PAGE of alveolar macrophage myosin: (A) continuous gel (Fairbanks et al.); (B) discontinuous gel (Laemmli); (C) autoradiograph of (B); HC, myosin heavy chain; LC_{20} and LC_{15}, 20 kDa and 15 kDa light chain, respectively; (1,2) the isoforms of LC_{20}.

Table 1

Mass ratios of myosin subunits

Measured ratio	Protein stain
1. LC_{20}-1:LC_{20}-2	1.8 ± 0.6
2. HC: LC_{20}-1 + LC_{20}-2 + LC_{15}	6.3 ± 1.3
3. HC: LC_{20}-1 + LC_{15}	7.9 ± 1.6
4. HC: LC_{20}-1 + LC_{20}-2	10.5 ± 2.0
5. HC: LC_{20}-1	15.9 ± 3.0
Calculated ratio	**Protein stain**
6. HC: LC_{20} + LC_{15}	6.3
7. HC: LC_{20}	11.0

Comparison of the ratios of the subunits of alveolar macrophage myosin derived from densitometric measurements of stained gels and from calculations based on 440 kg HC, 40 kg LC_{20} and 30 kg LC_{15}/mol myosin. The values including LC_{20}-2 (lines 2,4) are closer to calculated value than are those omitting LC_{20}-2 (lines 3,5). The ratio of LC_{20}-1 to LC_{20}-2 is also shown (line 1)

3. RESULTS

Myosin from rabbit alveolar macrophages, when analyzed by continuous SDS–PAGE as described in [5], is composed of 3 polypeptides: M_r 200000, 20000 and 15000 (fig.1A) with a molar ratio of about 1:1:1 [1]. Analysis of the same myosin in the discontinuous buffer system in [6] reveals that the 20 kDa band is composed of two different polypeptides which are separated by a distance equivalent to an M_r difference of about 500 (fig.1B). This separation of the 20 kDa band into two components occurs in gels made of a gradient of acrylamide and in gels with a uniform acrylamide concentration. It is not seen in the 20 kDa light chain of chicken gizzard myosin or of human platelet myosin, regardless of which gel system is used.

We have shown that exposure of crude actomyosin from macrophages to [^{32}P]ATP results in phosphorylation of the 20 kDa light chain up to 1 mol/mol [2]. When such phosphorylated myosin is analyzed by SDS–PAGE using the Tris–glycine buffer system [6], autoradiography of the gel shows that both components of the 20 kDa light

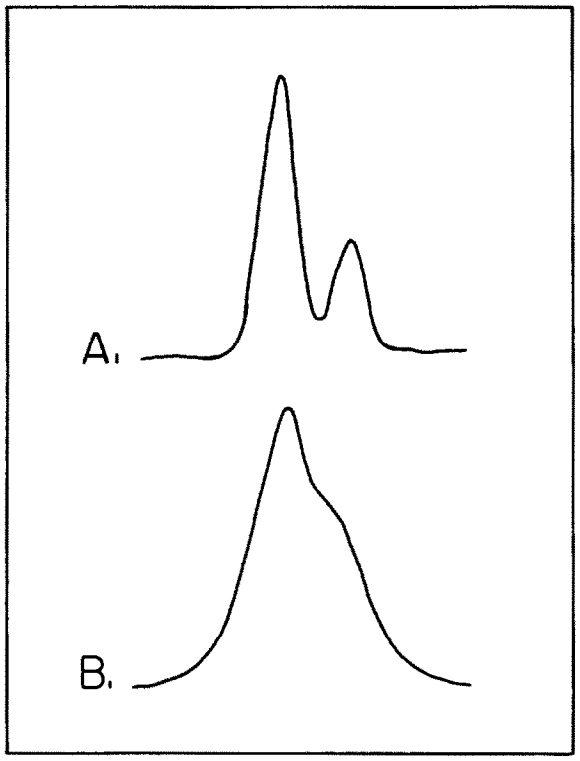

Fig.2. Densitometric scans of SDS–PA gel and autoradiograph of alveolar macrophage myosin. (A) Stained gel; (B) Autoradiograph. Only the LC_{20} region is shown. Top of gel is to the left.

chain are radioactive (fig.1C). Although the photographic process by which these figures have been produced does not permit an accurate estimation of the relative densities of the different bands, it is obvious that the slower-migrating component of the 20 kDa light chain (LC_{20}-1) is more abundant than the faster migrating component (LC_{20}-2) (fig.1B), and that more radioactivity is associated with it (fig.1C). Since accurate determinations have previously shown that up to 1 mol ^{32}P could be incorporated into 20 kg of LC_{20} [2], it is highly likely that LC_{20}-1 and LC_{20}-2 are each phosphorylated up to 1 mol/mol.

In order to determine the relative contribution of LC_{20}-1 and LC_{20}-2 to total LC_{20}, densitometric scans of stained gels and autoradiographs were made and quantitated. The stained bands can be accurately estimated from the scans (fig.2A). The results from 11 separate experiments (table 1) show the ratio of LC_{20}-1 to LC_{20}-2 to be about 2. Quantitative comparison of the radioactive peaks (as seen in fig.2B) by the method outlined above, shows the ratio of radioactivity of LC_{20}-1 to LC_{20}-2 to be about 2 as well. Two-dimensional gel electrophoresis of alveolar macrophage myosin reveals that LC_{20}-1 and LC_{20}-2 differ not only in molecular mass, but also in isoelectric point (fig.3A). Moreover, incubation of myosin with myosin light chain kinase from rabbit skeletal muscle produces a shift in isoelectric point (5.16 $\longrightarrow$ 5.13 for LC_{20}-1; 5.26 $\longrightarrow$ 5.22 for LC_{20}-2), as well

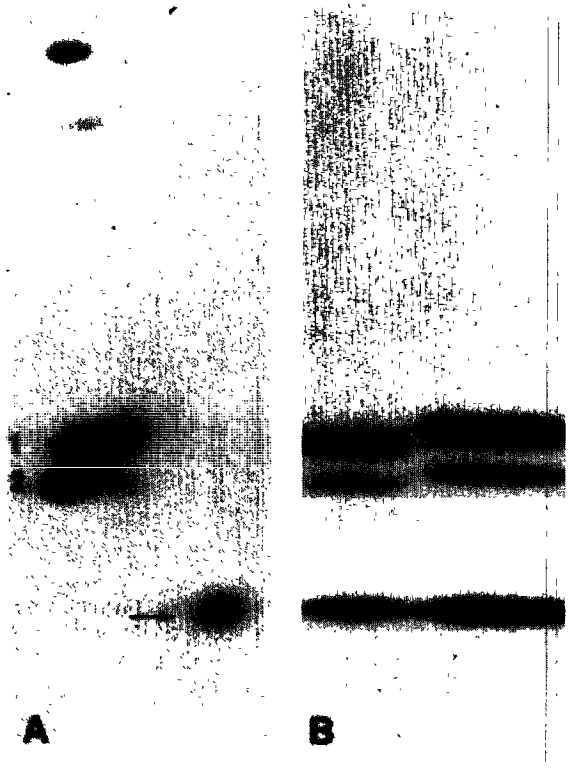

Fig.3. (A) Two-dimensional electrophoresis of macrophage myosin. Isoelectric focusing was performed in the horizontal direction, SDS–PAGE in the vertical direction. LC_{20}-1 and LC_{20}-2 (labeled '1' and '2') are seen to differ by IEP and by app. M_r. Phosphorylation leads to a shift upward and to the right (higher M_r and more acid IEP) of both LC_{20}-1 and LC_{20}-2. LC_{15} (arrow) is unchanged. (B) The shift to a higher app. M_r caused by phosphorylation of LC_{20}-1 and LC_{20}-2 is observed by one dimensional SDS–PAGE. The light chain bands are in register with those in fig.3A. The light chains on the right are phosphorylated, those on the left are unphosphorylated.

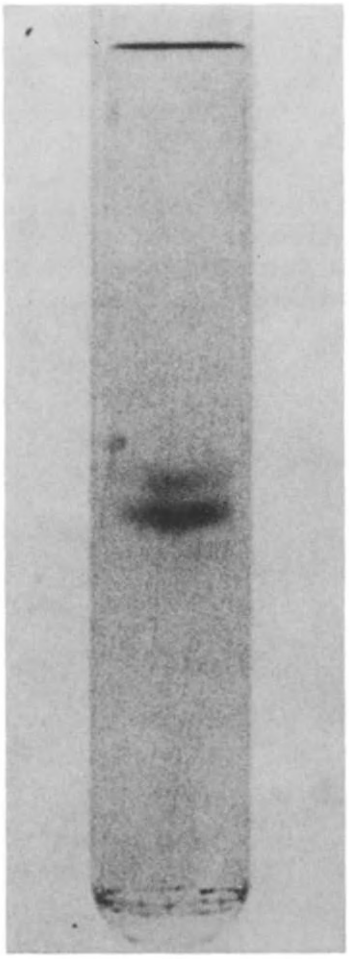

Fig.4. Electrophoresis of macrophage myosin under non-denaturing conditions, in the presence of 40 mM pyrophosphate, shows two species in a ratio of about 2:1. The more abundant species migrate more rapidly toward the anode.

as a small increase in app. M_r. This change in app. M_r is also seen in LC_{20} of myosin phosphorylated by its endogenous kinase (fig.3B).

The observation that all myosin preparations from alveolar macrophages have both LC_{20}-1 and LC_{20}-2, that the ratio is always greater than 1, and that both the components are phosphorylated by endogenous kinase activity, suggests that LC_{20}-1 and LC_{20}-2 are true isoforms of LC_{20}, and that neither is a contaminant that co-purifies with myosin. This idea is strengthened by the further observation that the mass ratios of heavy chain to LC_{20} and to total light chains are close to the calculated values for myosin only if both LC_{20}-1 and LC_{20}-2 are included in the mass value for LC_{20} (table 1). LC_{20}-2 is unlikely to be artifactually produced by proteolysis of LC_{20}-1 since the ratio of the components remains fairly constant in many different preparations, and also because proteolysis of macrophage myosin with papain, which extensively digests the heavy chain, does not change the ratio (not shown). When alveolar macrophage myosin is analyzed by electrophoresis under non-denaturing conditions, two major species are observed. The faster migrating band binds about twice as much Coomassie blue dye as the slower migrating band (fig.4) as determined by quantitative densitometry.

Myosin isolated from two murine macrophage-like cell lines [9], J774 and PU5-1R, shows the same heterogeneity of LC_{20} that is shown by alveolar macrophage myosin (fig.5). Since these

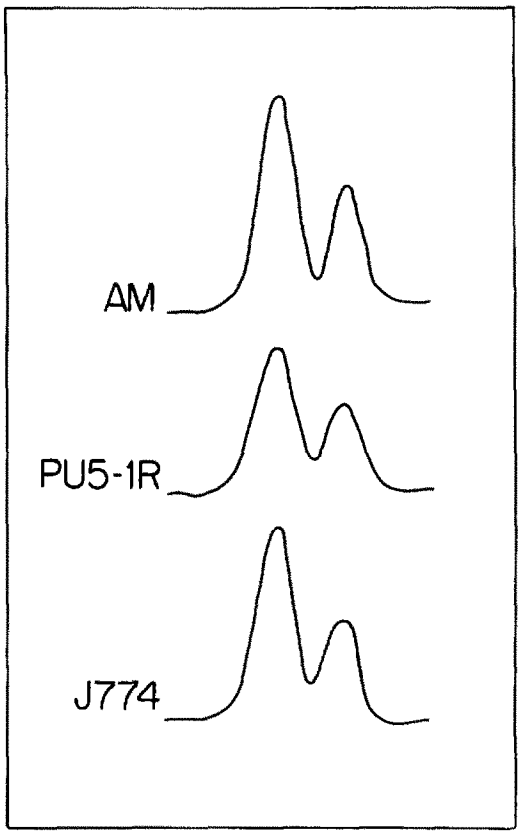

Fig.5. Densitometric scans of the LC_{20}-region of stained SDS–PA gel of alveolar macrophage (AM), PU5-1R and J774 myosins. Top of gel is to the left.

lines are clonally derived from mouse cells, and since it is very unlikely that rabbit alveolar macrophages and the two tumor cell lines would be comprised of the same relative proportions of two different subpopulations of cells, one containing LC_{20}-1 and the other containing LC_{20}-2, it is probable that myosins containing LC_{20}-1 and LC_{20}-2 are present in the same cell.

4. DISCUSSION

Since each two-headed myosin molecule has one LC_{20} per head, the non-integral ratio of LC_{20}-1 to LC_{20}-2 indicates that at least two distinct forms of myosin must exist [10,11]. One of these forms is homodimeric with respect to LC_{20}-1. The remainder of the myosin is either homodimeric, with LC_{20}-2 bound to both heads, or heterodimeric with LC_{20}-1 bound to one head and LC_{20}-2 bound to the other. The existence of two separate myosins is corroborated by the finding of two electrophoretic species of native myosin analyzed on non-denaturing gels. The fact that these isoforms are in a ratio of 2:1 (the same ratio that LC_{20}-1 has to LC_{20}-2) suggests that each myosin is homodimeric with respect to LC_{20}. A precedent for this type of myosin heterogeneity is found in striated muscle, in which it has been shown with respect to the alkali light chains that both homodimers and heterodimers exist [12]. Moreover, in skeletal muscle, individual thick filaments have been shown to be composed of a mixture of the isoforms of myosin [13].

It cannot be determined from these studies whether the myosin isoforms are spatially separated in macrophages, as is the case with the two major species of myosin in *Acanthamoeba castellani* [14], or whether macrophage myosin associates into thick filaments containing more than one isoform. Indeed, the state of association of myosin in non-muscle cells is unknown. However, the presence of more than one type of myosin in individual macrophages strongly implies a functional differentiation; i.e., different myosins might play different physiological roles.

The presence of heavy chain and light chain heterogeneity has been demonstrated in smooth muscle [15] and in brain myosin [15–17], although it has not been determined whether myosin isoforms coexist in the same cells. Myosin from myeloid leukemia cells is heterogeneous with respect to LC_{20} [18] and both LC_{20} variants are phosphorylated by endogenous light chain kinase [19]. It is thus similar to myosin from alveolar macrophages [20,21]. It will be important to re-examine the structure of other vertebrate non-muscle myosins to determine the extent to which isoforms exist. Such a re-examination will have to include an analysis of phosphorylation properties, since myosins from macrophages [21], leukemia cells [19], brain [17] and lymphocytes [22] have been shown to be phosphorylated on the heavy chain as well as on a light chain.

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