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Amorphin Is Phosphorylase; Phosphorylase Is an Alpha-Actinin-Binding Protein

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In a study of myofibrillar proteins, Chowrashi and Pepe [1982: *J. Cell Biol.* 94:565–573] reported the isolation of a new, 85-kD Z-band protein that they named amorphin. We report that partial sequences of purified amorphin protein indicate that amorphin is identical to phosphorylase, an enzyme important in the metabolism of glycogen. Anti-amorphin antibodies also reacted with purified chicken and rabbit phosphorylase. To explore the basis for phosphorylase's (amorphin's) localization in the Z-bands of skeletal muscles, we reacted biotinylated alpha-actinin with purified amorphin and with purified phosphorylase and found that alpha-actinin bound to each. Radioimmune assays also indicated that phosphorylase (amorphin) bound to alpha-actinin, and, with lower affinity, to F-actin. Negative staining of actin filaments demonstrated that alpha-actinin mediates the binding of phosphorylase to actin filaments. There are several glycolytic enzymes that bind actin (e.g., aldolase, phosphofructokinase, and pyruvate kinase), but phosphorylase is the first one demonstrated to bind alpha-actinin. Localization of phosphorylase in live cells was assessed by transfecting cultures of quail embryonic myotubes with plasmids expressing phosphorylase fused to Green Fluorescent Protein (GFP). This resulted in targeting of the fusion protein to Z-bands accompanied by a diffuse pattern in the cytoplasm. Cell Motil. Cytoskeleton 53: 125–135, 2002.

Key words: muscle; sarcomere; Z-band; enzyme; thin filaments; McArdle's Disease; Green Fluorescent Protein

INTRODUCTION

In striated muscle cells, the Z-bands mark the boundaries of sarcomeres. They are the sites of alpha-actinin concentration and the location where three of the major myofilaments: titin, nebulin, and actin, are anchored [see reviews by Vigoreaux, 1994; Sanger and Sanger, 2001b]. These filaments, together with myosin filaments, provide the framework through which the contractile activity of the muscle functions. Many less abundant proteins localized in the Z-band have been identified recently by virtue of their binding to either alpha-actinin (e.g., ALP, cypher, myotilin, and palladin), or titin (e.g., telethonin) [see reviews by Faulkner et al., 2001 and by Sanger and Sanger, 2001b]. One previously reported Z-band protein, zeugmatin, has turned out to be a proteolytic fragment of titin [Ayoob et al., 2000; Sanger and Sanger, 2001a].

Twenty percent, by weight, of the proteins in skeletal muscle cells are readily extractable by low salt solutions [Scopes, 1970; Masters, 1984]. Termed sarcoplasmic proteins [Scopes, 1970; Masters, 1984], they include the metabolic enzymes required for myocyte function. Two glycolytic enzymes, aldolase and glyceraldehyde-3-phosphate dehydrogenase, comprise 40% of

In memoriam: This paper is dedicated to the memory of Barbara Drucker, a valued member of the Pepe laboratory where this project began.

Prokash Chowrashi and Balraj Mittal contributed equally to this paper.

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these sarcoplasmic proteins. These two (and some other enzymes as well) bind thin filaments via actin and tropomyosin-troponin binding sites [Doelken et al., 1975; Marquetant et al., 1986; Masters, 1984; Mejean et al., 1989; Pagliaro and Taylor, 1988; Stewart et al., 1980]. The localization of the enzyme, phosphorylase, has been more controversial: reportedly localizing, by immuofluorescent stainings, in the cytoplasm between the myofibrils, in Z-bands or in M-bands [Dvorak and Cohen, 1965; Trinick and Loewy, 1977; Heizmann and Eppenberger, 1978; Maruyama et al., 1985].

The current report re-examines the Z-Band protein, amorphin [Chowrashi and Pepe, 1982]. On the basis of its chromatographic separation and similar total amino acid composition, amorphin was suggested to be the glycolytic enzyme phosphorylase [Maruyama et al., 1985]. We have sequenced several trypsin fragments of purified amorphin and confirmed the suggestion of Maruyama et al. [1985] that amorphin is indeed phosphorylase. We also demonstrate that phosphorylase is an alpha-actinin binding protein, consistent with amorphin's reported ability to associate with the Z-Bands of isolated skeletal myofibrils [Chowrashi and Pepe, 1982]. To address the controversy about the localization of phosphorylase in skeletal muscle cells, a method independent of antibodies was used. Myotubes transfected with Green Fluorescent Protein (GFP) plasmids encoding phosphorylase b, showed localization of GFP-phosphorylase in Z-bands of living cells. Part of this work was presented in a preliminary form at the American Society for Cell Biology meeting [Mittal et al., 2000].

MATERIALS AND METHODS

Construction of GFP-Phosphorylase b cDNA

Full-length human skeletal muscle glycogen phosphorylase cDNA (GenBank NM_005609.1) was obtained by RT PCR from total human skeletal muscle RNA (Stratagene, La Jolla, CA), using Superscript one-step RT-PCR for long template (Gibco-BRL, Rockville, MD). Primers TCTCGAGATGTCCCGGCCCCTGTCAGACCAA and TTGTCGACTGATGGCCTCATCCGGGGCTGG, with Xho1 and Sal1 restriction sites on forward and reverse primers, respectively, were used for directional cloning of phosphorylase cDNA into pEGFP-C3 expression system (Clontech, Alto, CA). The reading frame and sequence of the insert was confirmed by DNA sequencing. The alpha-actinin-GFP probe, used as a control for Z-band localization, was constructed as previously reported [Dabiri et al. (1997]. Myotubes were also transfected with pEGFP vector alone to serve as a further control.

Preparation of Muscle Cells and Light Microscopic Imaging

Myoblasts were isolated from the breast muscles of 10-day-old quail embryos using procedures previously described [Dabiri et al., 1999; Ayoob et al., 2001]. On the second day of culture, the cells were transfected with FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, IN) and phosphorylase b-GFP plasmid DNA at a ratio of 3:1 FuGENE/DNA according to the manufacturer's guidelines. After 24 h. the medium was replaced with fresh muscle medium. Images of the live, contracting quail myotubes were taken on a Nikon Diaphot 200 inverted fluorescence microscope with a phase 100× Planapochromat objective. Images were acquired with a liquid-cooled CCD (C 4742-95, Hamamatsu, Bridgewater, NJ). Photographic images were assembled using Image Pro Plus (Media Cybernectics, Silver Spring, MD) and Adobe Photoshop (Adobe, Mountain View, CA).

Preparation of Proteins

The protein designated amorphin was purified from fresh chicken pectoralis muscle according to the procedures described by Chowrashi and Pepe [1982]. This protein ran as a single band with an estimated molecular weight of 85,000 Daltons. Chicken phosphorylase was purified and crystallized from breast muscles using the methods of Fischer and Krebs [1958]. Actin, alpha-actinin, and the z-repeat region of titin were prepared as previously described [Turnacioglu et al., 1998; Ayoob et al., 2000; J.M. Sanger et al., 2000]. Purified rabbit phosphorylase a and b as well as bovine serum albumin were purchased from Sigma (St. Louis, MO). The fusion protein containing the six-z-repeat domains of titin was prepared using methods described previously [Ayoob et al., 2000; Freeman et al., 2000]. The concentration of the six-repeat fusion protein was 1 mg/ml. Alpha-actinin was biotinylated for binding studies [Huang et al., 2002] by dialyzing the purified protein overnight in PBS (Phosphate-buffered saline, pH 7.2), and reacting 100 μl (5 mg/ml) with 10 μl biotin-ester (Sigma) in 0.1 M sodium phosphate (pH 7.2). After incubation for 2 h at 4°C., free biotin was removed by gel Sephadex-G-50 spin columns (Amersham Pharmacia, Piscataway, NJ).

Preparation of Myofibrils and Electron Microscopy

Fibers of the adult chicken pectoralis muscle were tied to sticks using string and placed in a cold (4°C) 1:1 mixture of glycerol and low salt solution (0.1 M KCl, 0.01 M Phosphate buffer, 1 mM $MgCl_2$, pH 7.0). After 24 h, the mixture was replaced with fresh mixture and fibers were stored at −20°C for 2–4 days. Myofibrils were prepared from the glycerinated fibers in an Omni-

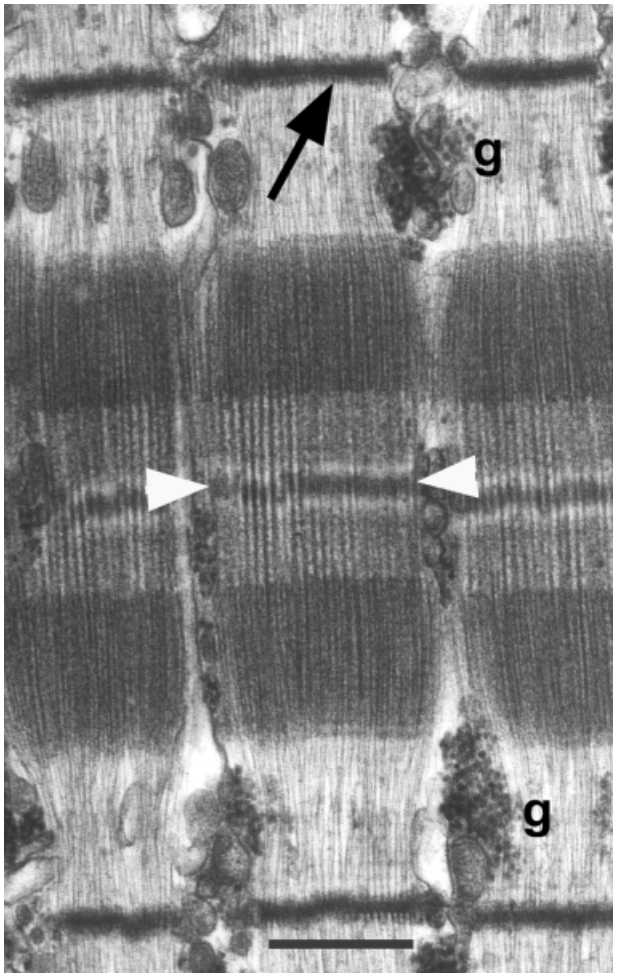

Fig. 1. Longitudinal section of control, glycerinated myofibrils from chicken pectoralis muscle. The proteins associated with the Z-band (*arrow*) and the middle of the thick filaments (M-band) (*arrowheads*) produce the electron density in these regions. Glycogen (g) is localized on either side of the Z-bands and along the sides of the sarcomeres. Bar = 0.5 μ.

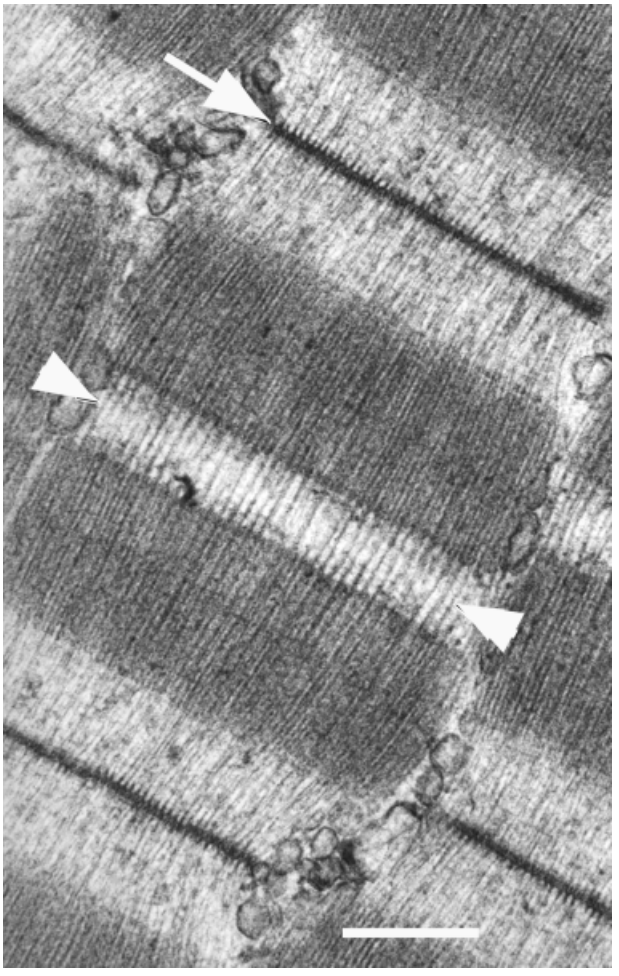

Fig. 2. Longitudinal section of glycerinated myofibrils that were extracted to isolate amorphin. Note that the Z-band (*arrow*) has lost much of the dense amorphous material observed in the unextracted muscle Z-band illustrated in Figure 1 revealing the fishnet substructure of actin filaments. The proteins normally associated with the M-Bands (*arrowheads*) have also been extracted. Gels of this extract reveal that several proteins including a 165-kD protein (M-Band protein) and a 85-kD protein (amorphin) are in the extracts [Chowrashi and Pepe, 1982]. Bar = 0.5 μ.

mixer and then rinsed with the low salt solution to remove the glycerol. Samples of these myofibrils were fixed for electron microscopy as controls (Fig. 1). A second sample of myofibrils was extracted with 0.25 M sucrose, 50 mM Tris, 1 mM EDTA, I mM NaN_3, pH 8.0 to remove amorphin. Samples of these extracted myofibrils were then processed for electron microscopy (Fig. 2). Purified amorphin was added back to another sample of amorphin-extracted myofibrils and processed for electron microscopy (Fig. 3).

In preparation for electron microscopy, the myofibrils were fixed in 5% glutaraldehyde in a low salt solution for 30 min. The fixed samples were rinsed several times in the low salt solution and post-fixed in 1% osmic acid, dehydrated in an ethanol series, and embedded in araldite and sectioned as previously described [Chowrashi and Pepe, 1982].

In order to examine amorphin binding to individual actin filaments in the electron microscope, actin filaments were prepared from chicken muscle according to the procedure of Pardee and Spudich [1982]. For naked actin filaments, a drop of the filaments (formed from monomer actin at 0.5 mg/ml) was placed on carbon-coated grids and the unattached filaments removed by several rinses of a low salt buffer (0.1 M KCl, 0.01 M imidazole buffer, pH 7.0). A few drops of 1% uranyl acetate were placed on the grid and slowly removed with filter paper. To test the binding of a single protein to the actin filaments, a drop of amorphin or alpha-actinin (both at 0.5 mg/ml)

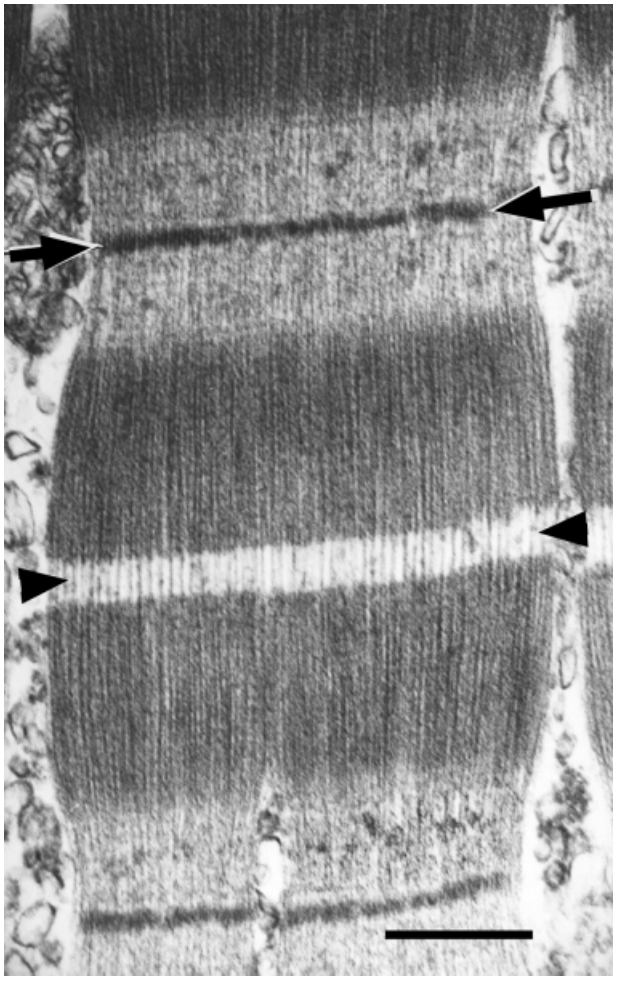

Fig. 3. Longitudinal section of glycerinated myofibrils that were extracted and then incubated with purified amorphin (85 kD). Note that the Z-Bands regained some of the dense amorphous structure seen in control myofibrils (Fig. 1), but the M-Band region of the sarcomeres did not regain density. These results indicate that purified amorphin will bind to the extracted Z-Bands but not to the M-line. Bar = 0.5 μ.

was added to a drop of actin filaments and allowed to bind for 5 min before the sample was rinsed with the low salt buffer and negatively stained with uranyl acetate. To test the binding of amorphin to alpha-actinin-decorated F-actin, first drops of actin and alpha-actinin were added to a grid and incubated for 5 min. A drop of amorphin (0.5 mg/ml) then was added and allowed to incubate on the grid for 5 min before the preparation was rinsed and negatively stained with uranyl acetate. The four different preparations were subsequently photographed using the electron microscope.

Binding of Biotinylated Alpha-Actinin

Alpha-actinin binding to phosphorylase and amorphin was analyzed after the selected proteins were electrophoresed on an 8% polyacrylamide gel and transferred to nitrocellulose membranes. The blots were blocked with 0.2% gelatin plus 3% BSA (bovine serum albumin), and incubated for 2 h at 4°C with biotin-alpha-actinin (1 μg/ml). After washes with TBST (TRIS buffered saline containing 0.05% Tween 20), the membranes were reacted with peroxidase-labeled extra-avidin (Sigma) at a dilution of 1:4,000 for 30 min. The binding was visualized by chemiluminescence (ECL Western blotting analysis system, Amersham Pharmacia).

Western Blots With Amorphin Antibody

Purified proteins were separated by SDS PAGE, transferred to nitrocellulose membranes, and then blocked with 5% non-fat milk in PBS. Polyclonal anti-amorphin antibodies [Chowrashi and Pepe, 1982] were used at a dilution of 1:200, and peroxidase labeled goat anti-rabbit antibody was used at a dilution of 1:2,000. The bands were visualized with chemiluminesence (ECL Western blotting analysis system. Amersham Pharmacia).

Affinity Purification of the Amorphin Antibody

Chicken muscle phosphorylase was coupled to CNBr-activated Sepharose 4B beads according to the manufacturer's recommendations (Pharmacia, Piscataway, NJ). with repeated washings of the Sepharose column to remove unbound phosphorylase. The amorphin polyclonal antibodies (6 ml, 1.3 mg/ml) were placed on the phosphorylase affinity column, and the sample that flowed through was collected and reapplied to the column. This process was repeated 4 to 5 times yielding a sample of non-specific antibodies. At the final step, the entire 6-ml antibody solution was collected from the column by passing 3 ml of the low salt solution. The column was then rinsed several times with the low salt solution prior to regeneration of the column. The bound antibodies then were released from the column by elution with 4 M $MgCl_2$. The $MgCl_2$ was washed from the column with a low salt solution, resulting in a regenerated column to which the non-specific antibody sample was applied for the removal of any specific antibody that had not bound to the phosphorylase column in the first series of applications. This sample was reapplied five times, and the final sample that passed through was used at full strength as a primary antibody for Western blotting of rabbit and chicken phosphorylase and amorphin. The protein concentration of the final non-specific antibody sample was 0.17 mg/ml.

Protein Sequencing

The Wistar Protein Microchemistry/ Mass Spectroscopy Facility at Wistar Institute, Philadelphia, PA, carried out the sequencing. Purified amorphin was electrophoresed on an 8% SDS-polyacrylamide gel, trans-

ferred to Sequi-Blot PVDF membrane (Bio-Rad), and briefly stained in amido black. After three 5-min washings in 5% acetic acid, the membrane was rinsed several times with MilliQ water and air dried. For trypsin digestion, amorphin was electrophoresed on an 8% gel, stained with Commassie blue and destained for 1 h. In situ trypsin-digested fractions were separated by HPLC and selected fractions analyzed by mass spectroscopy (MALDIMS). Two of the larger peptides were N-terminally sequenced.

Radioimmune Assay

Solid-phase radioimmune assays were used to measure the binding of amorphin to actin and alpha-actinin using procedures previously described for other proteins [Langone, 1980; Walliman and Szent-Gyorgyi, 1981; Wachsberger et al., 1983]. Briefly, the proteins, actin and alpha-actinin, each at a concentration of 10 mg/ml, were added to separate wells of a 96-well dish, and allowed to bind for 15 min. Wells without protein served as controls. All wells were rinsed three times with a wash solution of sheep gamma-globulin (5 mg/ml) dissolved in 0.1 M KCl, 5 mM Tris-HCl, pH 7.6. Amorphin was added at varying concentrations to each well and allowed to bind for 15 min before the wells were rinsed three times with the wash solution. Polyclonal anti-amorphin antibody (0.3 mg/ml) then was added to all the wells for 30 min. followed by three rinses with the wash solution. Secondary goat anti-rabbit $I\text{-}^{125}$ antibody, was added to each well for 30 min, and the unbound antibody then rinsed with three changes of wash solution. When the samples were dry, the bottoms of the plastic wells were cut out and the radioactivity of each well was measured in a gamma counter. Each combination of binding interactions was done in triplicate.

RESULTS

Electron Microscopy of Skeletal Muscle

A comparison of unextracted sarcomeres of adult chicken pectoralis muscle in longitudinal section (Fig. 1) with muscle extracted with 0.25 M sucrose, 50 mM Tris, 1 mM EDTA, 1 mM NaN_3, pH 8.0, to remove amorphin (Fig. 2), illustrates the loss of electron dense material in the Z-bands as well as in the M-bands. The typical fishnet network of actin filaments and interconnecting alpha-actinin linkers are easy to see in the extracted muscle (Fig. 2). Gels of similarly extracted muscle reveal two prominent bands of 165 kD (M-Band protein) and 85 kD (amorphin) in the extracts, as previously reported by Chowrashi and Pepe [1982]. When purified amorphin (85 kD) was added back to the extracted myofibrils, the Z-Bands regained some of the dense amorphous structure seen in control myofibrils and the network of actin filaments and alpha-actinin seen in the extracted myofibrils were no longer visible (Fig. 3). In contrast to the added density in the Z-bands, electron density is not restored to the M-Bands.

Sequence of Amorphin Peptides

Trypsin digestion of purified chicken amorphin yielded several peptides that were purified with HPLC. Two of the largest peptides were N-terminally sequenced, and the sequences compared with known sequences in GenBank using Blast software for matches. Rabbit, sheep, and human phosphorylase b proteins emerged as near identical matches. The sequence of chicken phosphorylase b was not found in any data banks. The homologous rabbit phosphorylase b peptides are indicated in Figure 4. The smaller of the amorphin peptides was 17 amino acids long with 16 of the 17 amino acids positive and 14 amino acids identical with rabbit phosphorylase (Fig. 4). The larger, 20-amino acid peptide had 20 positive and 18 amino acids identical with rabbit phosphorylase (Fig. 4). The near identity of these amorphin peptides with the homologous sequences of rabbit, sheep, and human phosphorylase b (843 amino acids) strongly suggests that amorphin is indeed a phosphorylase b.

Amorphin Antibody Reactivity

Amorphin antibodies made by Chowrashi and Pepe [1982] were used to test for reactivity against purified rabbit phosphorylase b. Figure 5 shows a Western Blot illustrating the reactivity of amorphin antibodies with amorphin and rabbit phosphorylase b but not with alpha-actinin or the six z-repeats of cardiac titin**,** supporting the sequencing data that amorphin is phosphorylase. When the amorphin antibody was affinity purified against chicken phosphorylase, the non-specific antibody fraction (see Materials and Methods) did not stain chicken phosphorylase, rabbit phosphorylase, or amorphin (Fig. 6).

Binding of Alpha-Actinin to Phosphorylase

To determine if phosphorylase b bound to Z-bands via interactions with alpha-actinin, the major component of Z-bands, alpha-actinin, was biotinylated and reacted with phosphorylase. Purified rabbit phosphorylase b, chicken amorphin, bovine serum albumin, and a peptide comprising the six-z-repeats of titin were run on a polyacrylamide gel and transferred to nitrocellulose. Incubation of biotinylated-alpha-actinin with the blot showed binding of alpha-actinin to amorphin and phosphorylase b as well as to the titin peptide (Fig. 7). The alpha-actinin probe did not bind to bovine serum albumin (data not shown).

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Fragment # 112

WLDTQVVLALPYDTPVPGYR (chicken amorphin)
W+DTQVVLA+PYDTPVPGYR
WVDTQVVLAMPYDTPVPGYR (rabbit phosphorylase,215-234)

Identities =18/20 (90%), Positives = 20/20 (100%)

Fragment # 109

LKQEYFVVAATLHDVVR (chicken amorphin)
LKQEYFVVAATL D++R
LKQEYFVVAATLQDIIR (rabbit phosphorylase,294-310)

Identities = 14/17 (82%), Positives = 16/17 (93%).
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Fig. 4. Proteolytic fragments 109 and 112 of amorphin (85 kD MW) and their amino acid identity with that of rabbit phosphorylase b (843 amino acids).

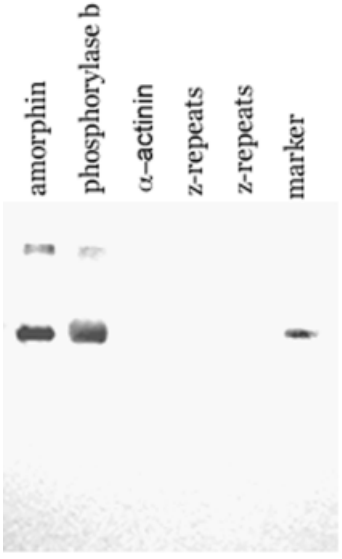

Fig. 5. Western Blot illustrating the reactivity of amorphin antibodies with amorphin and phosphorylase b but not with alpha-actinin or two different concentrations of the six z-repeats of cardiac titin.

To confirm the above binding results, we used a radioimmune assay to measure the binding of amorphin to purified alpha-actinin molecules and actin filaments (Fig. 8). In this assay, amorphin binds with higher affinity to alpha-actinin than to F-actin. At concentrations of amorphin where no actin binding is detected, strong binding of alpha-actinin remains.

We also tested the ability of naked and alpha-actinin-decorated actin filaments to bind amorphin using the techniques of negative staining and electron microscopy (Fig. 9A–D). We detected almost no amorphin protein associating with actin filaments (Fig. 9 B), whereas alpha-actinin-decorated actin filaments readily bound the added amorphin molecules along their lengths (Fig. 9D).

Localization of Phosphorylase in Skeletal Muscle Cells

To examine phosphorylase localization in live myocytes, we cloned phosphorylase b cDNA, derived from human skeletal muscle RNA, into a GFP expression plasmid, and transfected quail skeletal myocytes on day two of culture. Fluorescence was detected within two days diffusely distributed in the myotubes and localized in Z-bands (Fig. 10a,b). After 4 days post-transfection, over-expression of the GFP-phosphorylase led to small dots of fluorescence clustered at the Z-bands (data not shown). Neither the expression nor the over-expression of GFP-phosphorylase affected the spontaneous contractions of the living cultured myotubes. Transfection of the myotubes with GFP-alpha-actinin led to the localization of GFP-alpha-actinin in the Z-bands of the myotubes (Fig. 10c,d). Transfection of myotubes with pGFP alone

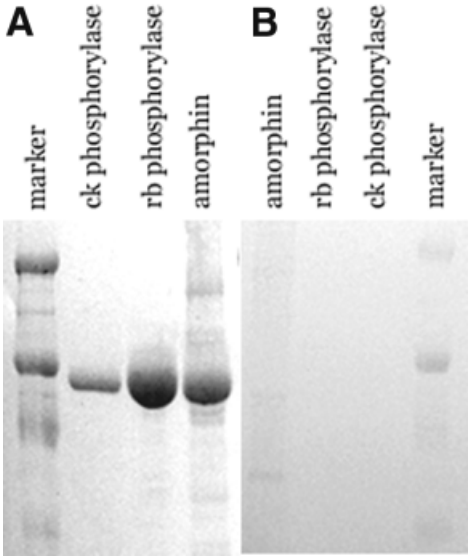

Fig. 6. **A:** Polyacrylamide gel stained with Ponceau S showing bands of chicken (ck) phosphorylase, rabbit (rb) phosphorylase b, and amorphin. **B:** Western blot of the gel in A reacted with affinity purified amorphin antibodies shows no antibody reactivity. The amorphin polyclonal antibodies were affinity purified on a column of Shepahrose agarose beads linked to chicken phosphorylase, the unbound antibodies in the eluant did not react with amorphin.

Fig. 7. Demonstration that alpha-actinin binds amorphin and phosphorylase. Rabbit phosphorylases, amorphin, and the z-repeat region of titin were run on a polyacrylamide gel and transferred to nitrocellulose. Incubation of biotinylated-alpha-actinin with the blot showed binding of alpha-actinin to amorphin and phosphorylase a and b as well as to the known alpha-actinin binding fusion protein like the z-repeats of titin.

led to diffuse fluorescence throughout the myotube (data not shown). There was no localization of GFP with any part of the myotube; similar results were reported when GFP was expressed in cardiac muscle cells [Ayoob et al., 2000].

DISCUSSION

The sequence identity of amorphin peptides with the published sequences of phosphorylase b confirms that amorphin is phosphorylase as was first suggested by Maruyama et al. [1985] based on chromatographic behavior, molecular weight, and total amino acid composition of the proteins. The sequencing data is supported further by the specific binding of amorphin antibody [Chowrashi and Pepe, 1982] to rabbit phosphorylase b and purified chicken phosphorylase (Figs. 5 and 6). Amorphin, thus, joins zeugmatin [Turnacioglu et al. 1996, 1997] as a protein now known to be identical to another previously described Z-band protein.

Several results in this paper support the idea that phosphorylase localization in myofibrils is at the Z-band. The conflicting immunolocalization results of phosphorylase b in I-bands [Doelken et al., 1975], Z-bands and M-bands [Heizman and Eppenberger, 1978; Maruyama et al., 1985], weak Z-band stainings [Trinick and Lowey, 1977], or no myofibrillar immunostaining [Dvorak et al., 1974] could be attributed to different methods of specimen preparation. Phosphorylase can bind creatine kinase that is bound to an affinity matrix [Khakimova et al., 1995] and it binds actin weakly (Fig. 8) [Marquetant et al., 1986]. It is also easily extracted from muscle [Scopes, 1970], although addition of phosphorylase (amorphin) to extracted myofibrils leads to the return of electron den-

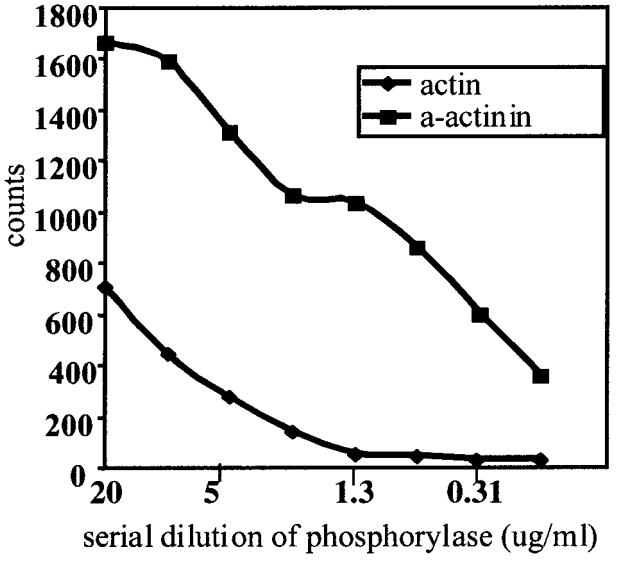

Fig. 8. Radioimmune assay demonstrating the binding of amorphin to alpha-actinin and actin. The upper curve demonstrates that the binding of amorphin to alpha-actinin is much stronger than its binding to F-actin.

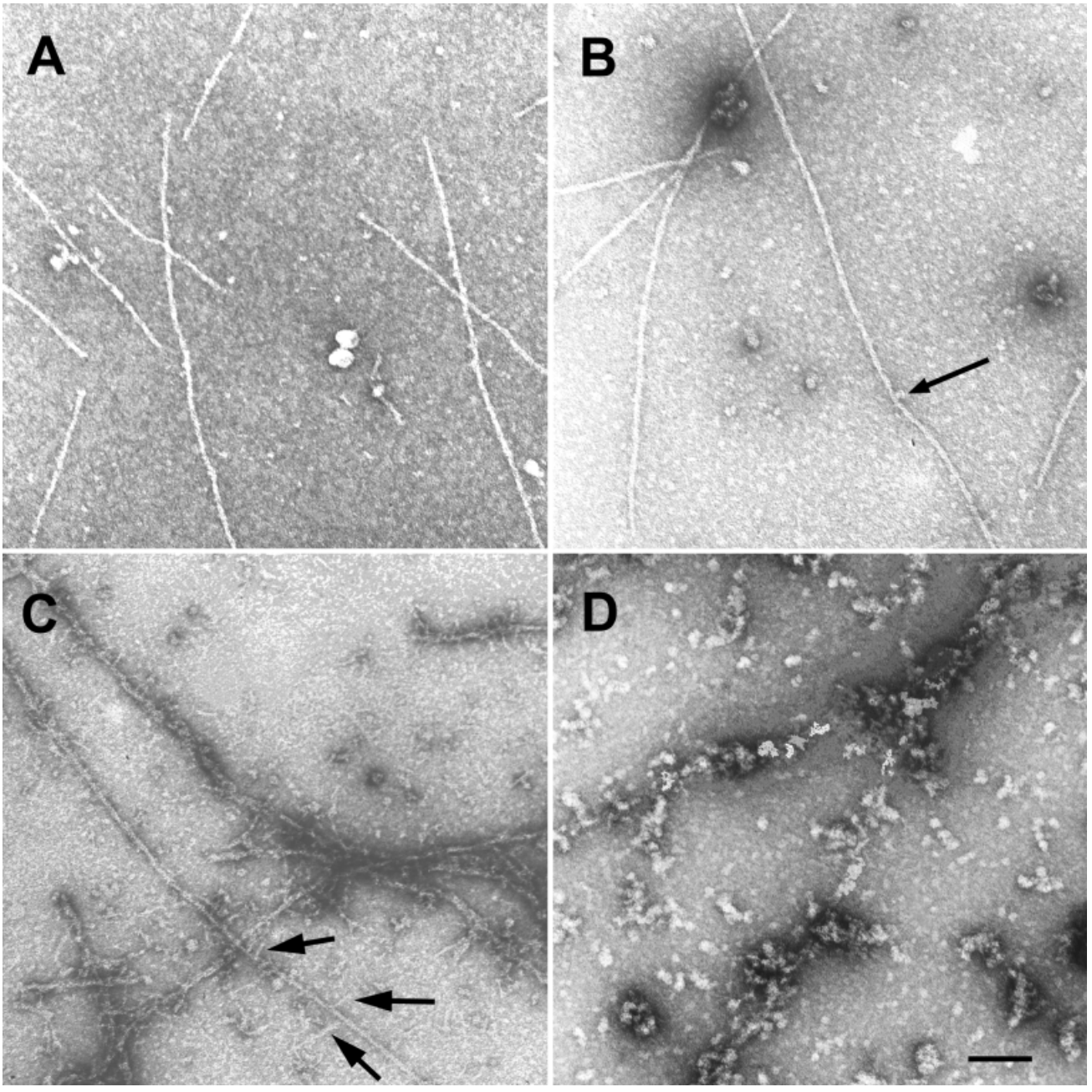

Fig. 9. Negatively stained actin filaments on formvar-coated grids. **A:** Naked actin filaments. **B:** Actin filaments incubated with amorphin before negative staining. Few amorphin molecules (*arrow*) were detected in association with actin filaments. **C:** Actin filaments incubated with alpha-actinin molecules (*arrows*). **D:** Actin filaments previously exposed to alpha-actinin and then incubated with amorphin molecules before negative staining. The actin filaments are coated with both alpha-actinin and phosphorylase molecules. Scale = 100 nm.

sity only to the Z-band (Fig. 3) [Chowrashi and Pepe, 1982].

The evidence that phosphorylase can bind to naked F-actin, F-actin-tropomyosin, F-actin-tropomyosin-troponin, and isolated myofibrils [Marquetant et al., 1986] suggests that it could bind native thin filaments in myofibrils. That we did not detect any localization to the thin filaments in myofibrils may be due to interference from native proteins on the thin filaments, such as nebulin. A similar case is observed with alpha-actinin, which can bind along the length of F-actin filaments unless tropomyosin is added, limiting the binding of alpha-actinin to

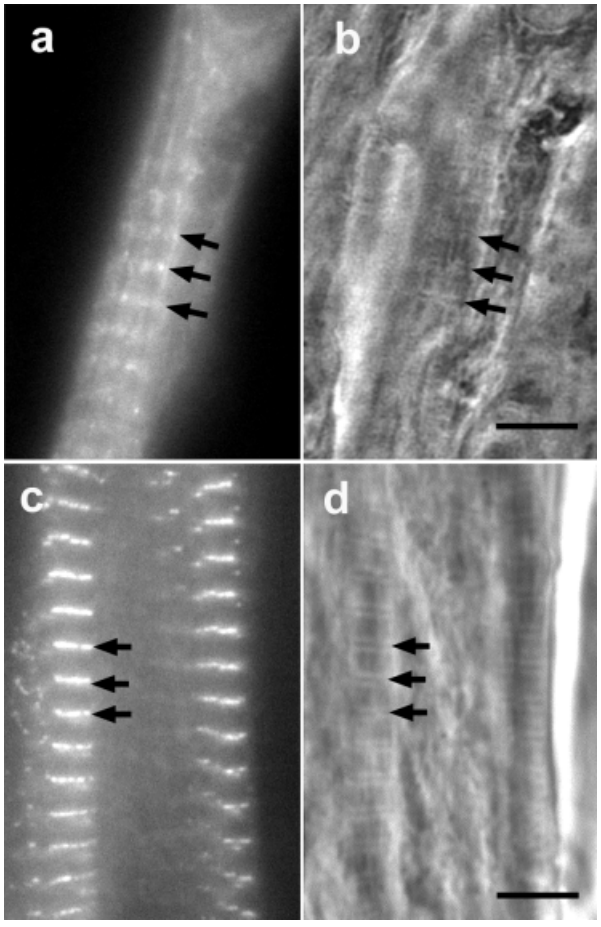

Fig. 10. Living quail myotubes previously transfected with (**a,b**) GFP-phosphorylase b and (**c,d**) GFP-alpha-actinin. (a) Fluorescence (a) and phase contrast (b) images of the same transfected myotube demonstrating the localization of the GFP-phosphorylase b in the Z-bands (*arrows*). c,d: Similar Z-band fluorescence in a myotube expressing GFP-alpha-actinin (c). Arrows point to Z-bands in the fluorescence (c) and phase contrast (d) microscopic images. Scale = 5 μ.

the barbed ends only of the isolated actin-tropomyosin filaments [Goll et al., 1972]. If actin-binding proteins are extracted from isolated myofibrils, fluorescently labeled alpha-actinin will bind all along the naked actin filaments, but if unlabelled tropomyosin is added to the extracted myofibrils before the addition of labeled alpha-actinin, the fluorescent probe will be detected only at the barbed ends of the actin filaments in the Z-bands [Sanger et al., 1984].

Transfection of cells with fluorescent probes allows localization to be seen without fixation and potential problems of epitope availability or extraction and relocalization of a protein [Dabiri et al., 1999; Sanger et al., 2000, 2002]. GFP-phosphorylase concentrated at the Z-bands with diffuse fluorescence in the rest of the cell. We never saw GFP-phosphorylase concentrated in the M- (or A-) bands. In cells with high levels of GFP-phosphorylase, fluorescent aggregates of the protein associated with the Z-bands. We have not seen this type of over-expression pattern in any other transfected cells expressing pGFP-linked Z-band proteins [Dabiri et al., 1997; Ayoob et al., 2000].

Increasing numbers of new myofibrillar proteins are being identified through their binding affinities for major structural components of sarcomeres [see review by Sanger and Sanger, 2001b]. These newly identified proteins are in low abundance and their roles in muscle cells are yet to be determined. Several of the proteins, actinin-associated LIM protein or ALP [Xia et al., 1997], cypher/ZASP [Zhou et al., 1999; Faulkner et al., 1999], myotilin [Salmikangas et al., 1999; Hauser et al., 2000], and palladin [Parast and Otey, 2000] are localized in Z-bands via their binding to alpha-actinin, a protein that comprises 1–2% of the myofibrillar proteins and is localized exclusively in Z-bands [Goll et al., 1972]. Other newly identified Z-band associated proteins: telothonin/Tcap [Mues et al. 1998; Gregorio et al., 1998] and obscurin [Young et al., 2001] bind titin.

In contrast to the roles of these recently discovered sarcomeric proteins, the functions of sarcoplasmic enzymes like phosphorylase are well known. Phosphorylase is present at 0.3% of wet skeletal muscle weight in rabbits [Fischer and Krebs, 1958] and 0.045% in chicken skeletal muscles [Heizmann and Eppenberger, 1978]; actin by comparison represents about 2.5% of skeletal muscle weight [Yates and Greaser, 1983]. Phosphorylase b dimerizes when phosphorylated by phosphorylase kinase, to form phosphorylase a, which catalyses the cleavage of the terminal glucose from glycogen, releasing it as glucose-1-phosphate. The experiments of Marquetent et al. [1986] indicate that 12% of the monomer (b) and 22% of the dimer (a) bind to isolated myofibrils. The absence of muscle phosphorylase in human skeletal muscles leads to McArdle's Disease, a myopathy characterized by rapid exercise fatigue [Bartram et al., 1995].

Phosphorylase is the first sarcoplasmic enzyme reported to bind alpha-actinin. There are several glycolytic enzymes that bind actin, e.g., aldolase, phosphofructokinase, and pyruvate kinase [Masters, 1984; Pagliaro and Taylor, 1988; Mejean et al., 1989] and/or the tropomyosin-troponin complex: aldolase, glyceraldehyde-3-phosphate dehydrogenase [Stewart et al., 1980]. The partitioning of glycolytic enzymes between soluble and bound states has been shown to be an effective method for the regulation of their catalytic activity [Masters, 1984]. Perhaps a similar mechanism of regulation occurs with phosphorylase in muscle cells. The release of glucose-1-phosphate from glycogen by phosphorylase located in the Z-bands would place the metabolite near

many glycolytic enzymes bound to the adjacent thin filaments [Brooks and Storey, 1991].

The importance of the sarcomeric localization of certain glycolytic enzymes in the normal functioning of Drosophila's flight muscles has been demonstrated in mutants of glycerol-3-phosphate dehydrogenase (GPDH) [Wojtas et al., 1997]. GPDH is normally detected in Z- and in M-bands of the flight muscles of wild type flies. Two other enzymes, aldolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), are also localized there due to their binding to GPDH. These three enzymes are lightly bound to the myofibrils, as their levels decrease as single myofibrils are isolated from the muscle cells [Wojtas et al., 1997]. Neither mutant flies lacking GPDH nor transgenic flies expressing a mutant form of GPDH lacking the Z- and M-band targeting domain, could fly, even though normal levels of aldolase and GAPDH and the mutant GPDH were present in the cytoplasm [Wojtas et al., 1997].

Summary

Amorphin is phosphorylase and it is localized in the Z-bands of skeletal muscle cells. This protein can be removed from the Z-bands with the concurrent loss of amorphous electron-dense material in the Z-band. Addition of purified amorphin to the extracted myofibrils results in its localization to the Z-bands and the reappearance of denser Z-bands. Biotinylated alpha-actinin binds both amorphin and phosphorylase. Amorphin antibodies bind purified amorphin, purified chicken phosphorylase, and rabbit phosphorylase b. Partial sequencing of amorphin peptides reveals identity with phosphorylase. Transfections of quail skeletal muscle cells with GFP-plasmids encoding phosphorylase leads to the incorporation of some of the fluorescent probe into Z-bands of the living myocytes. There is also unincorporated GFP-phosphorylase in the myocytes.

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