Maintenance of the Differentiated State in Skeletal Muscle: Activation of v-Src Disrupts Sarcomeres in Quail Myotubes

L. Castellani,* M. C. Reedy,‡ M. C. Gauzzi,§ C. Provenzano,§ S. Alemà,§ and G. Falcone§

*Dipartimento di Medicina Sperimentale e Scienze Biochimiche, Università di Roma Tor Vergata and Istituto di Neurobiologia, CNR, 00137 Rome, Italy; ‡Department of Cell Biology, Duke University Medical Center, Durham, North Carolina 27710; and §Istituto di Biologia Cellulare, CNR, 00137 Rome, Italy

Abstract. We have used quail skeletal myotubes expressing a temperature-sensitive allele of the v-*src* oncogene to address the issue of the homeostasis of sarcomeric myofibrils in differentiated muscle cells. Reactivation of the v-Src tyrosine kinase by shifting the cultures to the permissive temperature leads within minutes to the formation of F-actin–containing bodies (ABs), that originate in the ventral region of the myotubes and increase in number concomitantly with the dismantling of the I-Z-I complex of the sarcomeres. This process is detailed by confocal and electron microscopy. Indirect immunofluorescence reveals that ABs contain muscle-specific protein isoforms associated with the I-Z-I complexes and vinculin, a component of the cytoskeletal network. Anti-phosphotyrosine antibodies label proteins in ABs and Z-discs. Evidence is presented indicating that this phenomenon specifically depends on the persistent activation of v-Src, rather than on a general increase in phosphotyrosine content such as that induced by vanadate. AB formation is prevented by activation of protein kinase C by phorbol ester or by treatment with the kinase inhibitor 2-aminopurine, without any detectable effect on tyrosine phosphorylation. Taken together these findings indicate that phosphorylation of specific target proteins by v-Src, although necessary, is not sufficient per se to induce AB formation. In addition, the signal transduction cascade that culminates in MAP kinase activation and its nuclear translocation is activated both by v-Src and phorbol ester, and is relatively unaffected by 2-aminopurine. These findings imply that both phorbol esters and 2-aminopurine operate, at least in part, at the level of alternative pathways that may diverge upstream of the MAP kinase and are presumably mediating the early effects of v-Src on the differentiated phenotype.

DIFFERENTIATION of muscle cells involves both repression of subsets of housekeeping genes and induction of muscle-specific gene expression, followed by fusion of mononucleated cells to form multinucleated syncitia. The result is a rapid accumulation of muscle-specific protein isoforms and their assembly into highly ordered arrays, the sarcomeric myofibrils. Of central importance to the functioning of differentiated muscle cells is that the myofibrils must be integrated with one another and with the sarcolemma and the extracellular matrix (Small et al., 1992). Continuity and support within the interdigitated array of myosin thick filaments and actin thin filaments is provided by the thick filament–associated protein, titin and the thin filament-associated protein, nebulin (Wang and Wright, 1988; Trinick, 1991). Enveloping the sarcomeres is the intermediate filament lattice that links the Z-lines to the peripheral cytoskeleton and to the costameres, rib-like structures at the sarcolemma (Pardo et al., 1983; Danowski et al., 1992), thus providing mechanical integration of the myofibrils with the sarcolemma (Granger and Lazarides, 1979; Small et al., 1992).

The cytoskeleton of nonmuscle cells is well known to be reorganized in response to signaling pathways associated with cell growth and motility (Burridge et al., 1988). The cytoskeleton of differentiated striated muscle cells is organized into stable sarcomeric myofibrils, and yet muscles undergo extensive remodeling in response to exercise or injury. Complex regulatory processes therefore allow muscle to simultaneously undergo dynamic remodeling and maintain a highly differentiated state. While the comprehension of the mechanisms that activate the muscle differentiation program has progressed considerably (Olson, 1992; Lassar and Munsterberg, 1994; Weintraub, 1994), the mechanisms underlying the maintenance of the differentiated state are much less understood. Active control, involving an interplay among positive and negative regulatory molecules in different signaling pathways, is hypothesized for both the differentiation process and its maintenance (Blau, 1992). Many signal pathways result in

Please address all correspondence to S. Alemà, Istituto di Biologia Cellulare, CNR, Viale Marx 43, 00137 Rome, Italy. Tel.: 39 6 86 090 356. Fax: 39 6 8273287.

phosphorylation of target proteins, but little is understood on how phosphorylation affects the organization of cytoskeletal proteins, particularly in muscle. One approach to unravel these regulatory mechanisms is the use of differentiating muscle cells in vitro, in which an ectopic tyrosine kinase can be activated at will (for a review see Alemà and Tatò, 1994). It has previously been shown that primary avian myoblasts infected with temperature-sensitive (ts)[1] mutants of Rous sarcoma virus (RSV) display a temperature-dependent transformation and block of differentiation (Holtzer et al., 1975; Boettiger, 1989; Falcone et al., 1985, 1991). Both events are dependent on the expression of the v-*src* oncogene that encodes a 60-kD polypeptide (v-Src) characterized by an intrinsic temperature-sensitive tyrosine kinase activity (Wyke and Stoker, 1987; Parsons and Weber, 1989). Transformed myoblasts shifted to the restrictive temperature withdraw from the cell cycle, assemble myofibrils, and fuse into multinucleated myotubes (Falcone et al., 1985, 1991). Upon shift-down to the permissive temperature, the reactivation of v-Src in postmitotic myotubes causes a selective reduction of contractile protein gene transcripts through transcriptional repression of muscle-specific genes and, presumably, transcript destabilization (Falcone et al., 1991; Alemà and Tatò, 1994).

In the present study we demonstrate that the ts-RSV–transformed myoblasts provide an experimental model suited to study the cellular factors that mediate the selective turnover of specialized structures in muscle cells. We show that reactivation of v-Src in terminally differentiated myotubes reversibly alters the differentiated phenotype and induces major changes in the cellular architecture, involving the disassembly of the contractile filaments from the sarcomere. Globular structures containing filamentous α-sarcomeric actin and muscle-specific and cytoskeletal actin–associated proteins—referred to as actin bodies (ABs)—are observed within minutes from activation of the kinase. The progressive appearance of the ABs after reactivation of v-Src activity matches the dismantling of the I-Z-I sarcomeric segments. Evidence is presented indicating that while AB formation specifically depends on the persistent activation of v-Src, rather than on a general increase in phosphotyrosine content, it is inhibited by activation of protein kinase C (PKC) by phorbol ester and by treatment with the kinase inhibitor 2-aminopurine (2-AP). We also demonstrate that the signal transduction cascade that culminates in mitogen-activated protein (MAP) kinase activation is activated by v-Src in myotubes shifted to the permissive temperature and by phorbol esters in myotubes at the restrictive temperature, and is not inhibited by 2-AP. Taken together these findings suggest that the v-Src–evoked disassembly of the I-Z-I complexes is presumably mediated by signaling pathways that may diverge upstream of the MAP kinase rather than by direct phosphorylation of cytoskeletal proteins operated by v-Src.

1. *Abbreviations used in this paper*: AB, actin-containing bodies; 2-AP, 2-aminopurine; DM, differentiation medium; GM, growing medium; MAP, mitogen-activated protein; PDD, 4α-phorbol-12,13-didecanoate; PKC, protein kinase C; P-Tyr, phosphotyrosine; RSV, Rous sarcoma virus; TPA, phorbol 12-myristate 12-acetate; ts, temperature-sensitive; v-Src, a 60-kD polypeptide encoded by the v-*src* oncogene.

Materials and Methods

Materials

Staurosporine, 2-aminopurine, phorbol 12-myristate 13-acetate (TPA), 4α-phorbol-12,13-didecanoate (PDD) and myelin basic protein were purchased from Sigma Chem. Co. (St. Louis, MO); Calphostin C, bisindolylmaleimide, and HOECHST 33258 from Calbiochem (San Diego, CA) and genistein from Upstate Biotech. Inc. (Lake Placid, NY). Paraformaldehyde and formaldehyde were from Fluka (Buchs, Switzerland). Monoclonal antibodies to α-sarcomeric actin (5C5), vinculin (VIN-11-5), nebulin (NB2), phosphotyrosine (PT-66), and FITC-phalloidin were purchased from Sigma. TRITC-phalloidin was from Molecular Probes (Eugene, OR) and antibody to phosphotyrosine (mAb 4G10) was purchased from Upstate Biotech. Inc. Antibody to skeletal α-actinin (mAb 9A) was kindly provided by Dr. Donald Fischman (Cornell University Medical College, NY) and to ERK2 (#122) by Dr. C. Marshall (Chester Beatty Laboratories, London). Secondary antibodies, TRITC- and FITC-goat-anti–mouse were from Cappel (West Chester, PA), FITC-goat-anti–mouse (Fab/2 fragment) from Sigma Chem. Co., and horseradish peroxidase–conjugated goat anti–mouse and goat anti–rabbit from Bio-Rad Labs (Richmond, CA).

Cell Cultures

Primary cultures of quail myoblasts (*Coturnix japonica*) were prepared as previously described (La Rocca et al., 1989; Falcone et al., 1985). Quail myoblasts were maintained proliferating in DME medium (GIBCO BRL, Gaithersburg, MD) supplemented with 10% FCS, 10% tryptose phosphate broth, and 1% chicken serum (referred to as growing medium, GM) and 5% chicken embryo extract. Differentiation was induced by replacing the GM medium with F14 medium, supplemented with 2% FCS (referred to as differentiation medium, DM). Polyclonal populations of transformed quail myoblasts were established from primary passage cultures infected at high multiplicity with high titer viral stocks of ts-LA29, two temperature-sensitive mutants of RSV, and propagated on collagen-coated dishes in GM at 35°C (permissive temperature). The infected cells were then passaged a few times to allow virus spread. Differentiation was induced by plating 10^5 cells on 35-mm collagen-coated dishes in GM and, the following day, after replacing the medium with DM, by shifting the cultures at 41°C (restrictive temperature) for 3 d. v-Src kinase was reactivated by shifting 3-d-old differentiated cultures to 35°C in DM. All experiments were carried out with cells between passage 9 and passage 16.

Treatments of the Cultures

Orthovanadate and orthovanadate/hydrogen peroxide were added directly to the medium of differentiated myotubes at 41°C. When orthovanadate/hydrogen peroxide was used at 1 mM, however, cells were incubated with orthovanadate/hydrogen peroxide for up to 15 min, and then subjected to a change of medium and left at 41°C for the desired time, as described (Volberg et al., 1992). Treatment of the cultures with protein kinase inhibitors was carried out by adding to myotubes differentiated for 3 d at 41°C genistein (50 μg/ml final), staurosporine (from 0.025 to 1.2 μM), bisindolylmaleimide (from 5.0 to 7.5 μM), calphostin C (from 0.025 to 1.2 μM), and 2-AP (5–7.5 mM) 30 min before temperature shift or stimulation with 5×10^{-7} M TPA or PDD. Equal volume of vehicle (DMSO) was added to controls. Since the activity of calphostin C is light dependent (Bruns et al., 1991), the dishes were kept under fluorescent light during the incubation. Cycloheximide (20 μM final concentration) was added to the medium 1 h before temperature shift to 35°C.

Immunofluorescence

Cultures, after rinsing with PBS (3 × 1.0 ml), were routinely fixed for 10 min with 4% paraformaldehyde in PBS at room temperature and permeabilized with 0.1% Triton X-100 in PBS for 5 min, rinsed with PBS (3 × 1.0 ml), and incubated with primary and secondary antibodies and/or FITC- or TRITC-phalloidin at the appropriate dilutions. Methanol/acetone mixture (1:1, vol:vol; 10 min at −20°C) was sometimes used as fixative for cultures to be processed with antibody to α-actinin. The antibody to α-sarcomeric actin did not label cultures chemically fixed with paraformaldehyde. Fixation with methanol/acetone preserved antibody binding, but induced disordering of the thin filaments, although the alignment of the Z-discs was not affected, as shown by staining with antibody to α-actinin. Of the various fixatives tested for labeling with antibody to α-sarcomeric actin (3% glutaraldeyde; a mixture of 70% ethanol, 5% acetic acid,

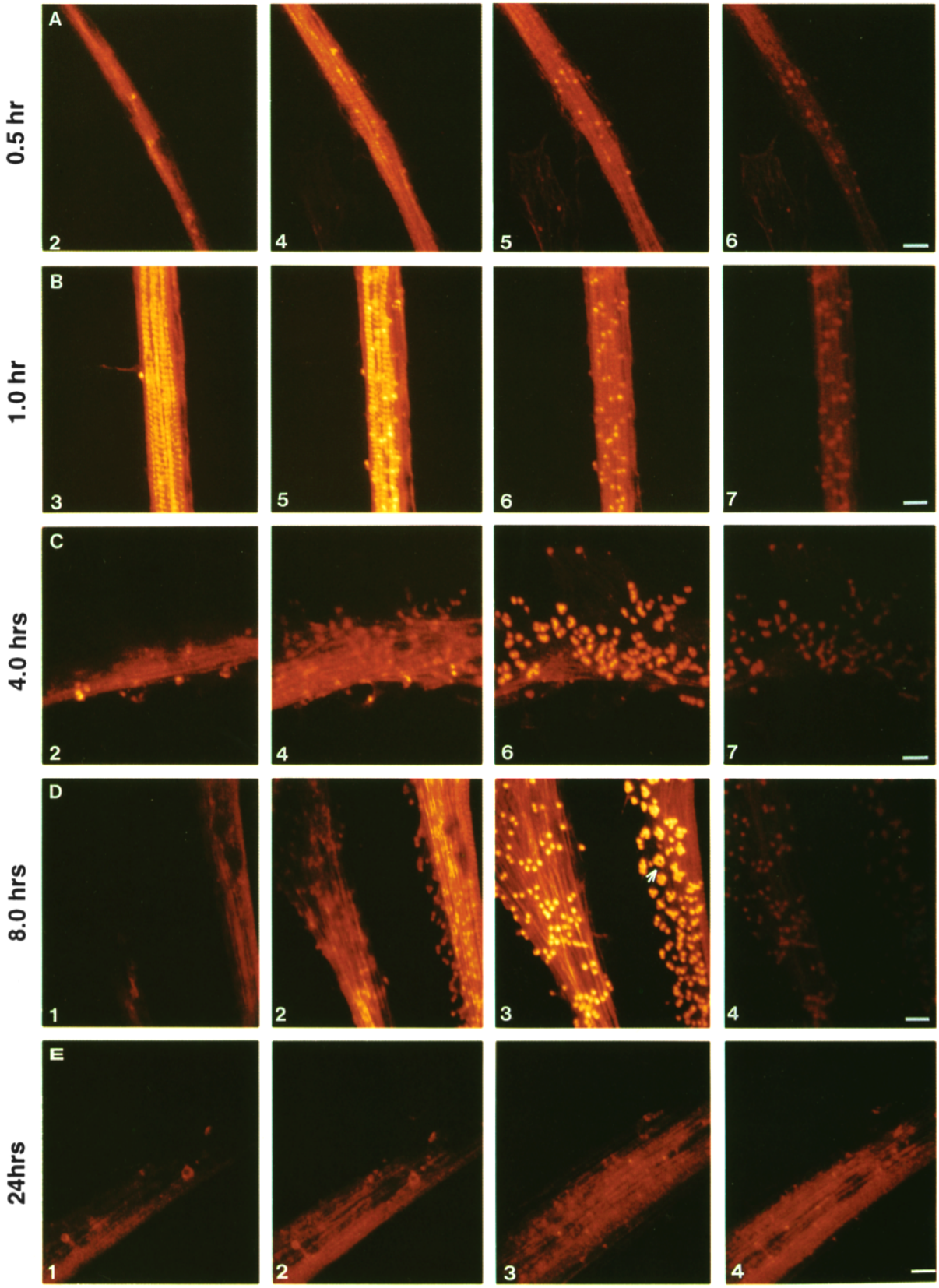
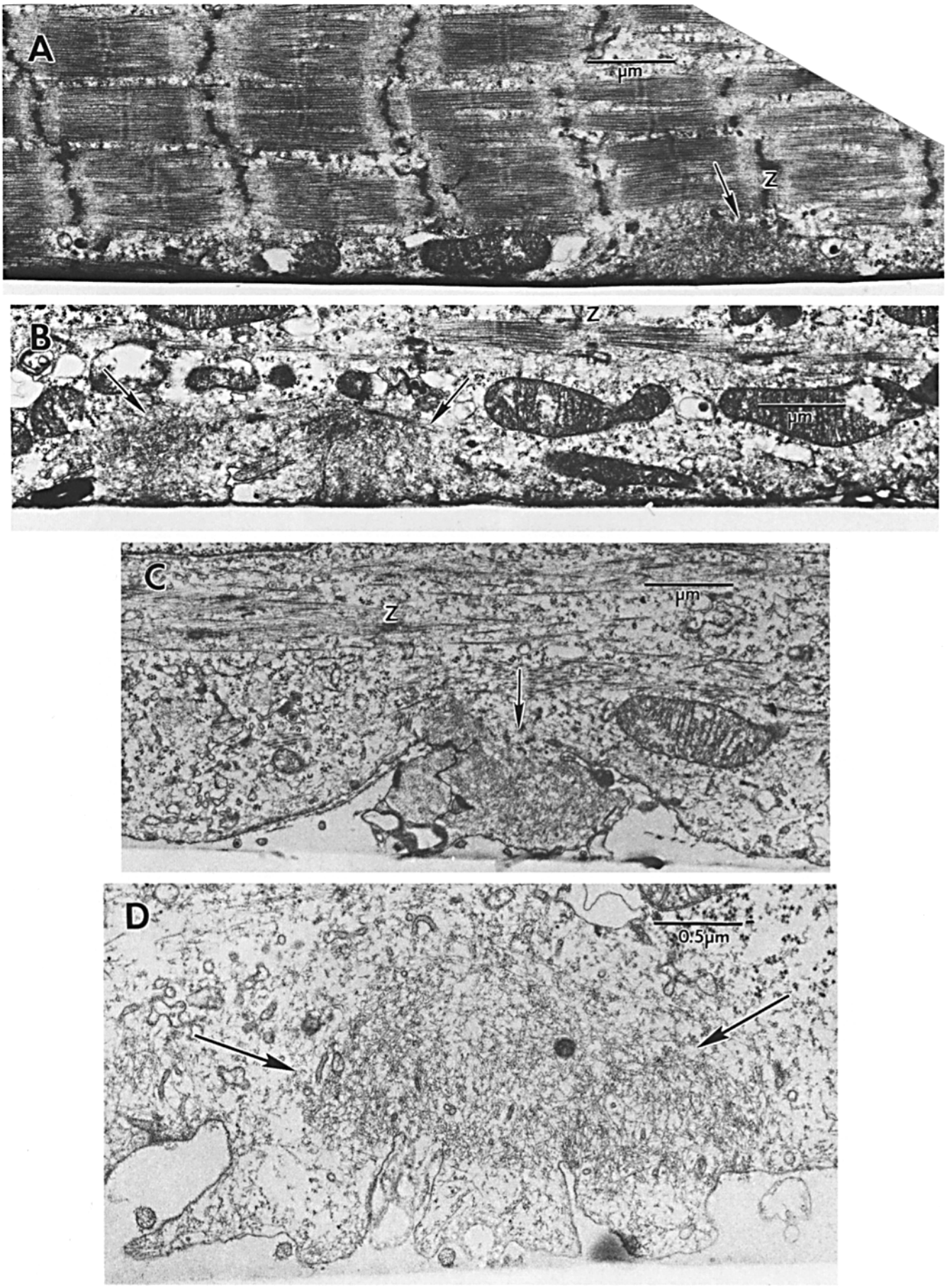


Figure 1. Reactivation of v-Src in fully differentiated myotubes induces the formation of actin bodies (ABs). Optical sections (0.6 μm) of myotubes fixed with paraformaldehyde and stained with TRITC-phalloidin after incubation at 35°C for (*A*) 0.5, (*B*) 1, (*C*) 4, (*D*) 8, and (*E*) 24 h. Images from left to right show selected optical sections (numbered from *1*) from the dorsal to the ventral region of the myotube. After 0.5 and 1 h at 35°C (*A* and *B*), the typical band pattern due to the sarcomeric arrangement of the thin filaments is observed in the dorsal region of the myotube, while the ABs are seen in the more ventral region. At longer incubations (*C* and *D*) the number of ABs increases concomitantly with the disappearance of the thin filaments band pattern. In *D*, two adjacent myotubes are shown, one displaying individual ABs and the other AB aggregates (*arrow*). By 24 h (*E*) only small, ill defined F-actin bodies are observed. Bars, 10 μm.


A
μm
Z
B
Z
μm
C
Z
μm
D
0.5μm

and 3.7% formaldehyde; ethanol:acidic acid [95:5%]), 3% formaldeyde for 10 min gave the best results. For MAP kinase/ERK2 nuclear translocation experiments, myotubes were incubated for 6 h in DM containing 0.2% serum before temperature shift or treatment with PDD. After fixation with 4% paraformaldehyde at 20°C for 15 min followed by permeabilization with 0.2% Triton X-100 for 10 min, the cells were incubated for 2 h with antiserum #122 at 1:200 dilution.

All primary and secondary antibodies were diluted in PBS containing 1 mg/ml bovine serum albumin (RIA-grade, Sigma) and the incubations were carried out at room temperature for 1–2 h with agitation. After a final wash with PBS, cells were incubated with HOECHST 33258 (1 μg/ml) for 5 min before being mounted in Gelvatol. The samples were routinely examined with a Zeiss microscope equipped with 40× and 50× objectives. Confocal analysis was carried out with a Leica TCS 4D system, equipped with 40 × 1.00–0.5 and 100 × 1.3–0.6 oil immersion lenses.

Electron Microscopy

Myoblasts, plated on collagen-coated Thermonox coverslips in 35-mm dishes, were differentiated for 3 d at 41°C and shifted to the permissive temperature (35°C) for increasing lengths of time before fixation. Control cultures were fixed after 3 d of differentiation at 41°C. 3-day myotubes were sampled at 2, 4, 8, 12, 18, and 24 h after shift to 35°C. All myotubes were fixed in situ with primary fixative consisting of 3% glutaraldehyde (TAAB Laboratories Equipment, Aldermaston, Reading), 0.2% tannic acid in 160 mM NaCl, 5 mM $MgCl_2$, 10 mM MOPS, pH 6.8, for 30 min at room temperature. After rinsing with PBS, all specimens were postfixed in ice cold 1% OsO_4 in 10 mM $MgCl_2$, 100 mM PO_4, pH 6.0, for 20 min. After rinsing in water, cultures were block stained in aqueous 2% uranyl acetate for 30 min at room temperature, rinsed in water, and dehydrated in a graded series of ethanols. Specimens were embedded in Araldite 506. Longitudinal sections included both dorsal and ventral-substrate surfaces; distances of 3 mm were sampled along each myotube. Cross and longitudinal sections, ranging in thickness from gray to silver, were cut on a Reichert OMU3 ultramicrotome, picked up on carbon-coated grids and stained with 2% $KMnO_4$ and Sato Lead (Reedy and Reedy, 1985). Sections were photographed on a Philips EM300 on Kodak SO163 EM film.

Polyacrylamide Gel Electrophoresis and Western Blot Analysis

Cells were briefly rinsed with PBS also containing 0.5 mM orthovanadate and collected with 0.2 ml SDS sample buffer (9 M Urea, 0.14 M β-mercaptoethanol, 0.04 M DTT, 2% SDS, 0.075 M Tris-Cl, pH 8.0). SDS-PAGE was carried out according to Laemmli (1970). Equal volumes of sample (5–20 μl/well) were loaded. Western blots were carried out as described by Towbin et al. (1979) using as secondary antibody horseradish peroxidase–conjugated goat anti–rabbit and anti–mouse antibodies and revealed using the ECL chemiluminescence detection system (Amersham, Buckinghamshire, England).

Analysis of MAP Kinase Activation

Western blot analysis of ERK2 was carried out as previously described using polyclonal antiserum #122 (Leevers and Marshall, 1992) in order to detect the slow-migrating, phosphorylated form of ERK2. MAP kinase/ERK2 activation was determined by assaying antiserum #122 immunoprecipitates of lysates prepared from myotubes using an in vitro kinase assay (Leevers and Marshall, 1992). Myelin basic protein was used as exogenous substrate.

Results

Formation of Actin Bodies Upon v-Src Reactivation

Quail skeletal myoblasts infected with the temperature-sensitive mutant of the Rous sarcoma virus ts-LA29 (ts-*src*-myoblasts) exhibit transformation and block of differentiation at the permissive temperature (35°C). When the cultures are shifted to the restrictive temperature (41°C), the cells promptly stop proliferating, initiate the synthesis of muscle-specific products and fuse into multinucleated myotubes (ts-*src*-myotubes) (Falcone et al., 1985, 1991). Full maturation of the myotubes, characterized by organized sarcomeric myofibrils, is attained in 3–5 d, with a fusion efficiency of 85–90%.

Reactivation of the protein tyrosine kinase activity in postmitotic myotubes induced by shifting the cultures to 35°C causes the formation of F-actin containing globular bodies, here referred to as actin bodies (ABs), as early as 30 min after temperature shift. Optical sectioning by laser scanning confocal microscopy (LSCM) of TRITC-phalloidin–labeled myotubes incubated at 35°C for 30 min clearly shows that the ABs are restricted to the ventral region of the cell, within a thickness of 2–3 μm where the sarcomeric staining pattern for F-actin is greatly reduced (Fig. 1 *A*). Throughout the rest of the cell F-actin staining displays the typical sarcomeric band pattern, as indicated by optical sections in the corresponding dorsal region of the myotubes (Fig. 1). This is confirmed by electron micrographs of longitudinal sections that show well-ordered sarcomeres throughout the myotube, with ABs associated with the most ventral myofibrils (Fig. 2). Within 2 h, the vast majority of the myotubes display ABs, which increase in number with time, reaching a maximum in 4–8 h. The formation of ABs is paralleled by a reduction of I-band organized F-actin, as seen both by indirect immunofluorescence (Fig. 1) and EM (Fig. 2). After 4 h at 35°C, the myotubes begin to flatten and the sarcolemma is pushed outwards, especially in the region close to the substrate, displaying festooning with sharp pointed edges often carrying ABs (Fig. 1 *C*). By 24 h most myotubes display only small, ill-defined, phalloidin-labeled structures reminiscent of ABs and a greatly reduced sarcomeric-staining pattern of F-actin (Fig. 1 *E*). The morphology of the ABs varies with time of incubation at 35°C: 30–60 min after temperature shift they appear as individual globules of size varying between 1.6 to 2.0 μm, while after 2 h they reach a diameter of ~2.5–3.0 μm (Fig. 1). After 4–8 h at 35°C, some myotubes show a number of ABs coalescing together to form "flower" and "chain" structures, although individual ABs are still very abundant (Fig. 1, *C–D*). At later times (8–12 h) these aggregates are still observed, but the ABs in the aggregates are not distinguishable individually (*arrow*, Fig. 1 *D*; see also Fig. 2). It should be noted that the small number of mononucleated cells present in cultures of myotubes at 41°C show small 1.0-μm globular phalloidin-labeled structures in the center of the cells, reminiscent of the rosette-like components previously described in fibroblasts, only after 12–24 h at 35°C (Nigg et al., 1982) (not shown).

Figure 2. ABs progressively change shape with increasing time of v-Src kinase activation. *A–D* show electron micrographs of longitudinal sections in the ventral region of the myotubes. (*A*) 2 h after v-Src kinase activation, ABs (*arrow*) can be seen associated with the Z-lines of the most ventral sarcomeres. (*B*) After 4 h, many of the most ventral sarcomeres have been partially disrupted, particularly the I-Z-I segments, and ABs (*arrows*) have increased in size and frequently show indentations of membrane. (*C*) After 12 h, the most ventral sarcomeres have been disrupted, and ABs (*arrow*) project from the ventral surface, tending to lift the myotube away from the substrate. (*D*) A higher magnification view of an AB (*arrows*) in which the actin filament meshwork can clearly be seen. This AB appears to be made of three ABs that have coalesced and are lifting the myotube away from the substrate.

At the gross level, marked morphological changes of the myotubes are observed after 12–24 h at 35°C. They are characterized by widening and flattening of the cells (see for example Fig. 1) and loss of alignment of the nuclei as shown by both light and electron microscopy analysis. The striking alterations in the overall morphology of the myotubes and in the sarcomeric staining for F-actin do not alter the organization of the A-band proteins (skeletal myosin and titin). Disordering of the A-bands becomes noticeable, in fact, after 36–48 h at 35°C (unpublished results). The morphology of myotubes kept at 35°C for 24 h can be reversed by returning the cultures to 41°C. Myotubes shifted to 35°C for 24 h and then returned to 41°C were analyzed using α-actinin as a marker of sarcomere's organization. The regular confinement of α-actinin at the Z-lines, fully disrupted by incubation of the cultures at 35°C, and the overall cylindrical shape of the myotubes is regained after returning the cultures to 41°C for 12–24 h (data not shown).

To investigate whether the formation of ABs requires the persistent activity of v-Src, or is triggered by a "hit and run mechanism," fully differentiated myotubes were incubated at 35°C for 4 h in order to develop ABs, and then returned to 41°C for various lengths of time. The number of ABs is reduced to about half within 15 min from the shift-back to 41°C and their size appears to be slightly reduced (not shown). Although the number of ABs is reduced very rapidly, they disappear completely within 2 h, concomitant with the increase of striations due to the reorganization of the thin filaments into sarcomere throughout the myotube. To determine the relationship between the formation of ABs promoted by the reactivation of v-Src and the initiation of a disassembly process requiring de novo synthesis of protein factors, the effects of v-Src were examined in the presence of cycloheximide, an inhibitor of protein synthesis. Cycloheximide was added to fully differentiated myotubes at 41°C, and then its effect was analyzed after shifting the cultures to 35°C for 2–4 h. Both formation of ABs and disassembly of the I-Z-I complexes proceed with kinetics which are indistinguishable from those of untreated cells (data not shown).

Spatial and Temporal Distribution of the Proteins Assembled in Actin Bodies

The protein constituents and their distribution within the ABs were investigated by indirect immunofluorescence using antibodies to various muscle-specific and cytoskeletal proteins in cultures shifted to permissive temperature for varying lengths of time (Figs. 3 and 4). Differentiated myotubes at 41°C show the typical I-band pattern when labeled with monoclonal antibody to sarcomeric α-actin, as also observed with phalloidin. Upon reactivation of v-Src by temperature shift, the antibody to α-actin reveals structures similar in size and shape to the phalloidin-labeled ABs (Fig. 3 *A*). Furthermore, double-labeled cultures show that sarcomeric α-actin- and phalloidin-labeled ABs coincide (Fig. 3 *B*). The concurrent disappearance of the

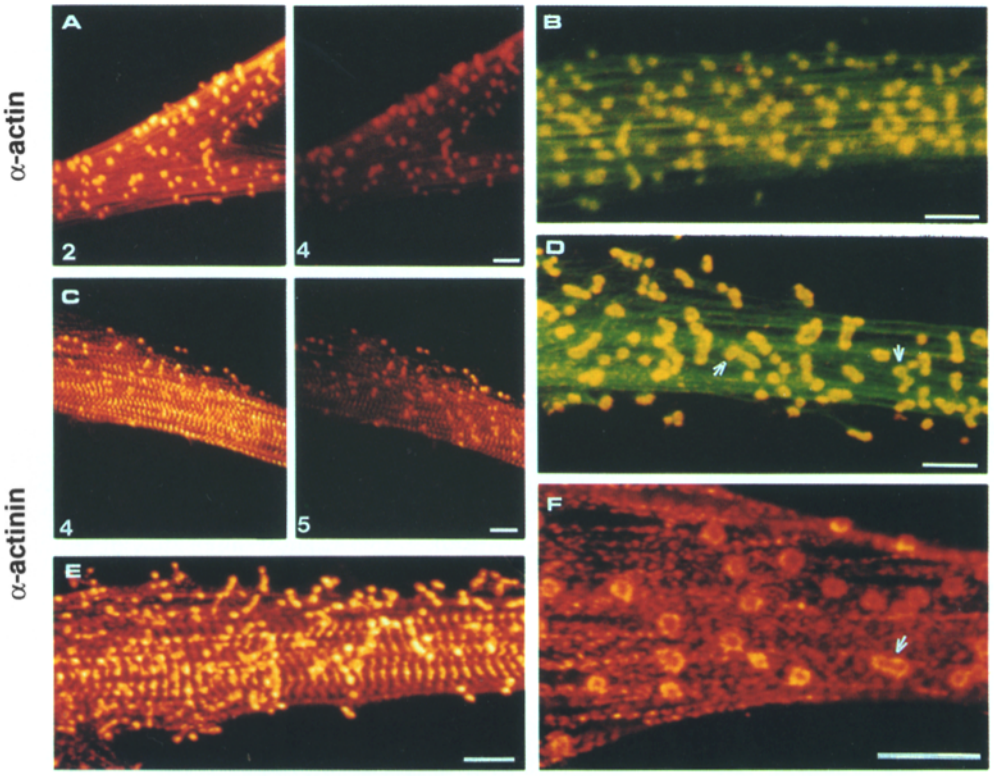


Figure 3. α-Sarcomeric actin and α-actinin are major constituents of the ABs. Confocal immunofluorescence micrographs of myotubes after incubation at 35°C for 3 h labeled (*A*) with antibody to α-sarcomeric actin (revealed with TRITC-conjugated goat anti–mouse antibody) and double labeled (*B*) with anti–α-sarcomeric actin and FITC-phalloidin. Phalloidin and α-sarcomeric actin have the same distribution within the ABs. Myotubes kept at 35°C for (*C*) 0.5 h and (*E*) 1 h and labeled with antibody to α-actinin show the ABs originating in the ventral region of the myotube and, in some cases, lining up with the Z lines, as shown in *E*. Myotube (*D*) incubated at 35°C for 4 h and double labeled with antibody to α-actinin (revealed with TRITC-conjugated goat anti–mouse antibody) and FITC-phalloidin show that the α-actinin staining becomes more pronounced at the edges of the ABs (*arrows*) as incubation at 35°C continues. (*F*) Higher magnification view of a myotube stained for α-actinin after 4 h at 35°C reveals a punctated distribution of stain along the rim of the ABs and of AB aggregates (*arrow*). Optical sections in *A* and *C* are numbered from 1 beginning from the dorsal region of the myotube. The two chromophores are depicted with a green (FITC)/red (TRITC) color scale in *B* and *D* where they are displayed in the same image. (*A–F*) Bars, 10 μm.

thin filament band pattern with the progressive formation of the ABs (see Fig. 1) further confirms that sarcomeric α-actin is a constituent of the ABs. It should also be noted that antibody to sarcomeric α-actin does not label the small phalloidin-labeled globules (~1 μm in diameter) observed in mononucleated cells upon extensive reactivation of v-Src.

A pattern of striations corresponding to the alignment of the Z-discs is observed by immunofluorescence in fully differentiated myotubes labeled with an antibody to skeletal α–actinin. Upon shift of the cultures to 35°C for 30 min, a disruption of the pattern in the region of the cell closer to the substrate is observed simultaneously with the appearance of ABs similar to those observed with phalloidin labeling (Fig. 3 *C*). At early times (30 min to 2 h), the ABs are often seen lining up with the Z-discs stripe pattern, suggesting that the Z-discs may represent sites where these bodies are formed (Fig. 3 *E*). Analysis by EM confirms that ABs are in close contact with the Z-discs of the most ventral myofibrils at 2 h after kinase reactivation (Fig. 2 *A*) and that by 4 h at 35°C both the I- and A-bands of ventral myofibrils are disrupted, while the Z-lines of most other myofibrils have a wavy appearance (Fig. 2). Indirect immunofluorescence of double-labeled cultures clearly shows that the ABs stained by FITC-phalloidin are also labeled by antibody to α-actinin (Fig. 3 *D*). After 4 h at 35°C, in contrast to phalloidin, the α-actinin staining is more pronounced at the edges of the ABs, suggesting a cortical redistribution of this protein (Fig. 3, *D* and *F*). Indeed, optical sections cut through these structures show a ring-like distribution of the α-actinin staining, which, in some cases, shows a punctated appearance (Fig. 3 *F*). In flower- and chain-like aggregates of ABs, typically observed 4–8 h after temperature shift (see above), the α-actinin staining is confined to the cortical region of the aggregates (cf. arrow in Fig. 3 *F*).

To further define the protein composition of the ABs and the spatial and temporal distribution of their components, myotubes were double-labeled with phalloidin and antibodies to nebulin, a protein associated with the thin filaments, and to vinculin, a component of the cytoskeletal network. Fig. 4 shows that nebulin and vinculin are associated with the ABs, although their localization within the ABs differs. Labeling of nebulin, which shows the typical I band pattern in differentiated myotubes at 41°C, reveals globular bodies that closely match the ABs stained by FITC-phalloidin in cultures kept at 35°C from 30 min to 8 h (Fig. 4, *A–C*). Indirect immunofluorescence with antibody to vinculin, a protein typically associated in muscle with costameres and a cytoskeletal network enveloping the myofibrils (Terracio et al., 1990; Massa et al., 1994), shows staining of adhesion plaques and of the cytoplasm in myotubes kept at 41°C. Upon reactivation of v-Src, vinculin is found in the ABs, with a distribution similar to that of α-actinin. While 30 min after temperature shift vinculin labeling matches that of phalloidin in the ABs (Fig. 4 *D*), with longer incubations (2 h and more) it segregates at the edges of the ABs (Fig. 4, *E* and *F*). Serial optical sections through the ABs, in fact, show that the core of the AB is mainly labeled by phalloidin and is surrounded by a ring of vinculin (Fig. 4 *F*). When the ABs begin to aggregate 4 h after temperature shift, strings of vinculin are occasionally seen connecting neighboring ABs and, in the coalesced flower- and chain-like structures, vinculin labeling is segregated at the periphery of the aggregates (Fig. 4 *F*).

Correlation between Phosphotyrosine Containing Proteins and Actin Body Formation

Reactivation of v-Src in postmitotic myotubes causes a rapid and marked increase of phosphotyrosine (P-Tyr) content within minutes from temperature shift (Figs. 5 and 6 *A*). Cell lysates of myotubes incubated at 35°C for various times and analyzed by Western blot using an antibody to P-Tyr show a large number of phosphorylated polypepetides, migrating in SDS-PAGE with an apparent molecular weight ranging from 40 to 200 kD (Fig. 6 *A*). The increase in proteins phosphorylated in tyrosine induced by v-Src correlates well with the appearance of ABs. Fig. 5 shows LSC micrographs of myotubes incubated at 35°C for various lengths of time and double labeled with antibody to P-Tyr and phalloidin or α-actinin. At 35°C the sarcolemma becomes markedly labeled by the antibody to P-Tyr and a regular striped pattern of staining is observed in areas where the sarcomeric band pattern of F-actin is still preserved (Fig. 5 *A*, *B*, and *E*). The periodicity of P-Tyr staining matches that of the Z-discs (Fig. 5 *B*), and disappears with the same time course of the thin filament band pattern. The ABs identified by phalloidin or antibody to α-actinin also show P-Tyr labeling (Fig. 5, *A* and *D*), although the ones closer to the membrane appear to be stained more strongly. After 4–8 h incubation at 35°C, the distribution of P-Tyr staining within the ABs is still clearly visible and matches that of phalloidin, but not that of α-actinin (Fig. 5 *D*).

To establish whether the morphological changes observed in postmitotic myotubes shifted to 35°C are due to the specific activity of v-Src, cultures of differentiated myotubes were incubated at 41°C with 0.1–1 mM vanadate, alone or in combination with 0.1–1 mM hydrogen peroxide and processed for immunofluorescence (Fig. 5 *C*) or analyzed by Western blot using anti–P-Tyr antibody (Fig. 6 *A*). As previously reported for cultured epithelial cells (Volberg et al., 1992), a pronounced increase in P-Tyr level is observed with vanadate, especially when used in conjunction with hydrogen peroxide (Fig. 6 *A*). Vanadate-treated cultures at 41°C double labeled with phalloidin and antibody to P-Tyr, however, do not show ABs, although the sarcomeric arrangement of the actin filaments appears to be disordered (Fig. 5 *C*). A pronounced increase in diffuse fluorescent signal due to P-Tyr is observed, in addition to the regular pattern of striations resembling that noted in myotubes shifted to 35°C (Fig. 5, compare *C* with *B*). Untreated myotubes kept at 41°C show only very weak diffuse staining for P-Tyr (data not shown), consistently with the low level of proteins phosphorylated in tyrosine seen by Western blot (Fig. 6 *A*).

Incubation of differentiated myotubes with protein kinase inhibitors such as genistein, specific for tyrosine kinases (Akiyama et al., 1987), or staurosporine, known to affect v-Src activity (Tamaoki, 1991), for 30 min at 41°C before temperature shift induces a concentration-dependent inhibition of P-Tyr levels, revealed by Western blot analysis (Fig. 6 *C*), paralleled by a reduced number or lack of ABs, monitored in sister cultures processed for immu-

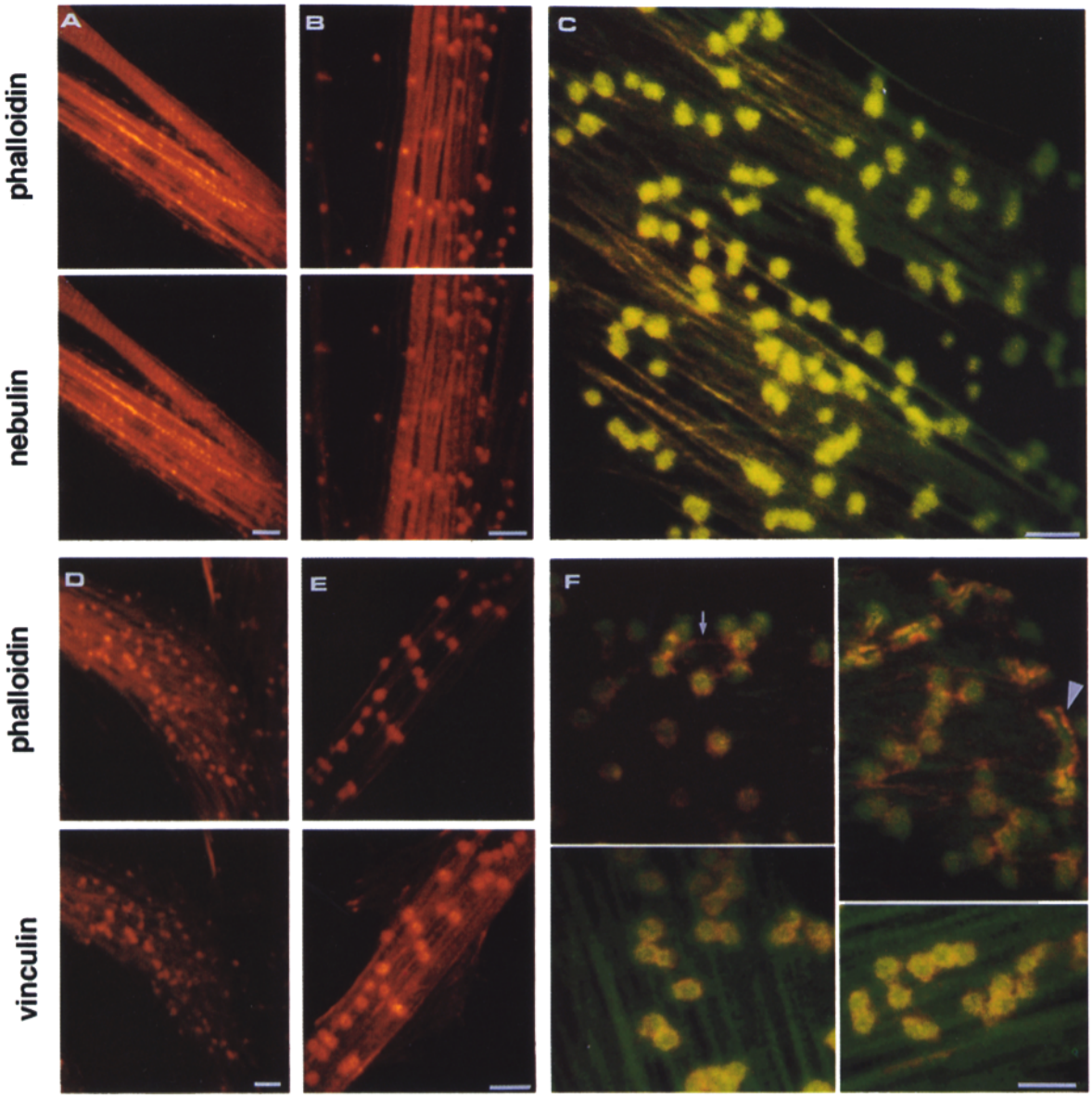


Figure 4. Nebulin and vinculin are present in the ABs from the earliest time of reactivation of v-Src. Confocal micrographs of myotubes incubated at 35°C for 0.5 h (*A* and *D*), 2 h (*B* and *E*), and 8 h (*C* and *F*) and double labeled with FITC-phalloidin and antibody to nebulin (NB2) or to vinculin (VIN-11-5), as indicated. All antibodies were revealed with TRITC-conjugated goat anti-mouse antibody. The two chromophores are shown in *A–B* and *D–E* with the same color scale in separate images and with a green (FITC)/red (TRITC) color scale when displayed in the same image (*C* and *F*). Nebulin colocalizes with phalloidin in the ABs throughout the time of analysis (*A–C*). Vinculin colocalizes with phalloidin as the ABs begin to form in the ventral region of myotubes (*D*), but as the incubation at 35°C continues (*E* and *F*), it assumes a cortical distribution within the ABs. Note that 8 h after temperature shift (*F*) filamentous structures stained by antibody to vinculin are observed connecting neighboring ABs (*arrow*) and AB aggregates show vinculin staining along the periphery (*arrowhead*). Bars: (*A–B*, *D–E*) 10 μm; (*C* and *F*) 5 μm.

nofluorescence with TRITC-phalloidin (Table in Fig. 6 *C*). Incubation with calphostin C, reported to inhibit PKC (Tamaoki, 1991), has no effect at sub-micromolar concentrations, while at higher concentrations modulates the level of P-Tyr content and the formation of ABs in cultures shifted to 35°C (Fig. 6 *C*). In contrast, treatment of myotubes with bisindolylmaleimide (Bm), a specific inhibitor of PKC (Toullec et al., 1991), before temperature shift, affects neither the formation of ABs nor the kinase activity of v-Src in vivo (Fig. 6 *C*).

Activation of PKC Inhibits AB Formation

It has previously been reported that treatment of chicken primary myotubes with the phorbol ester TPA, a well known activator of PKC, induces the formation of actin-containing bodies and the disorganization of myosin thick filaments (Lin et al., 1987, 1989). To investigate the possible role of PKC in the v-Src–evoked disassembly process of the sarcomere and AB formation, myotubes were treated with TPA or PDD for varying lengths of time

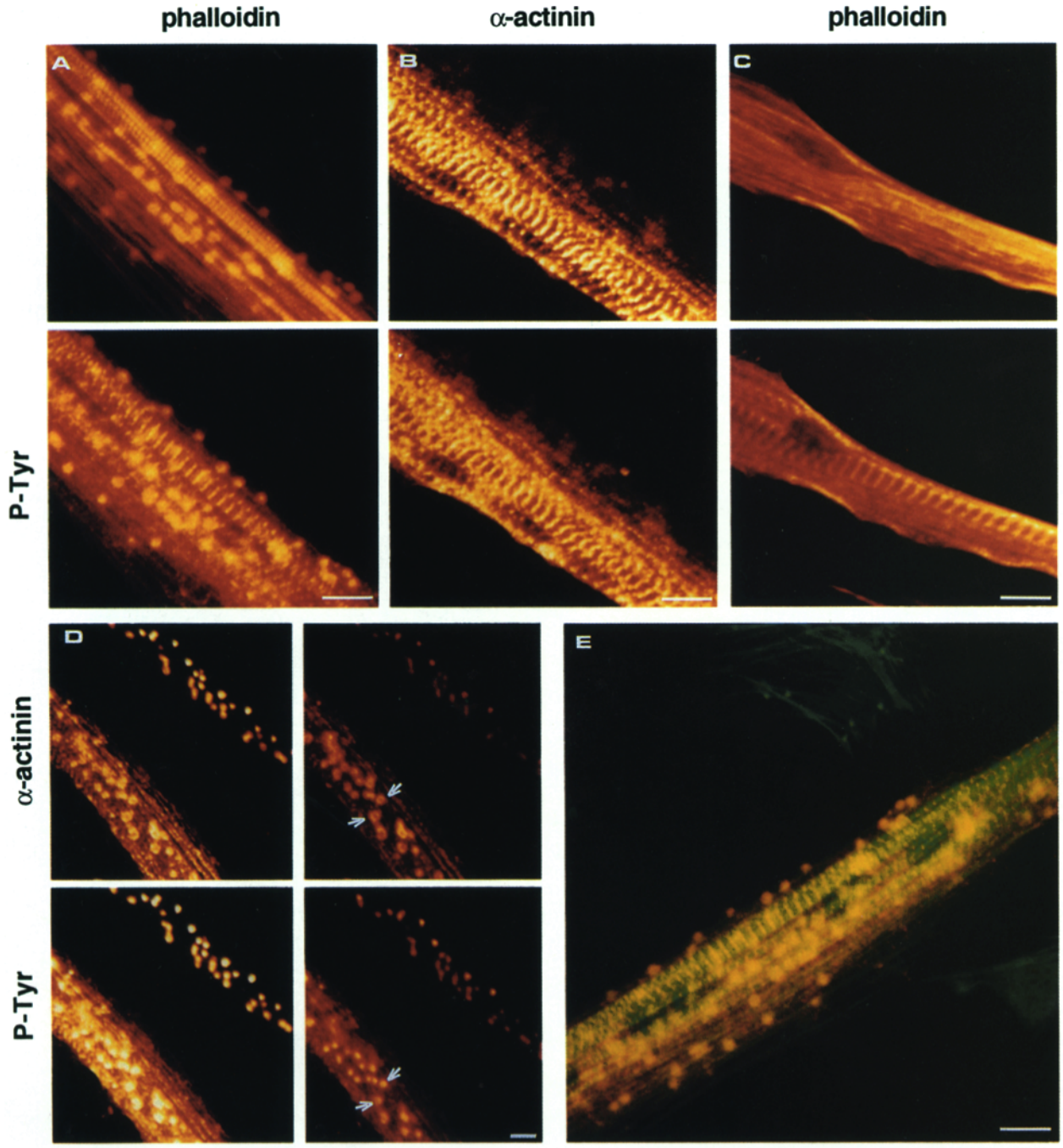


Figure 5. Comparison of the localization of P-Tyr residues in myotubes after reactivation of v-Src at 35°C or treatment with vanadate at 41°C. Immunofluorescence confocal micrographs of myotubes double stained with antibody to P-Tyr (mAb 4G10, revealed with TRITC-conjugated goat anti–mouse antibody) and (*A*, *C*, and *E*) FITC-phalloidin, or (*B* and *D*) antibody to α-actinin (revealed with FITC-conjugated goat anti–mouse Fab/2 fragment). *A* and *B* show myotubes after reactivation of v-Src at 35°C for 2 h. Note that the optical section shown in *A* derives from the middle of the myotube, while the one in *B* from the dorsal region. *E* shows the same myotube seen in *A* with the two chromophores displayed in the same image with a green (FITC)/red (TRITC) color scale. *C* shows a myotube incubated at 41°C with 1 mM vanadate-H_2O_2 for 2 h and *D* shows optical sections of myotubes kept at 35°C for 4 h. Antibody to P-Tyr stains ABs induced by reactivation of v-Src (*A*, *D*, and *E*) and periodic structures that superimpose on the Z-discs both in the dorsal region of myotubes incubated at 35°C for 2 h (*B*), where the sarcomeres are preserved, and in myotubes treated with vanadate at 41°C (*C*). Optical sections through the middle of a myotube at 35°C (*A* and *E*) show that P-Tyr is present both in the periodic pattern and in the ABs, where it colocalizes with phalloidin (*A* and *E*). Adjacent optical sections in the ventral region of a myotube after 4 h at 35°C (*D*) show that labeling for P-Tyr stains the ABs uniformly, while that for α-actinin is more pronounced in the AB cortex (*arrows*). The FITC and TRITC chromophores are shown with the same color scale in all images, except in *E* where they are displayed in the same image. Bars, 10 μm.

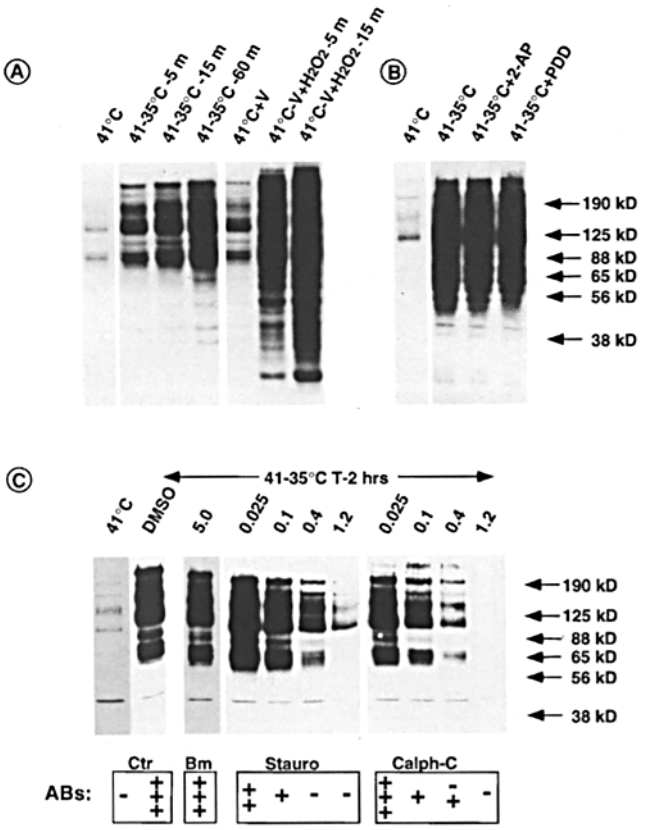


Figure 6. P-Tyr levels in cell lysates of differentiated myotubes after activation of v-Src tyrosine kinase and upon treatments with vanadate, protein kinase modulators, and phorbol esters. (*A*) Western blot with antibody to phosphotyrosine reveals a rapid and marked increase in P-Tyr content after incubation at 35°C. Cultures kept at 35°C from 0 (*41°C*) to 60 min (*m*) are shown. Lysates of differentiated myotubes incubated at 41°C with 1 mM vanadate (*V*) alone and in combination with hydrogen peroxide (H_2O_2) show a dramatic increase in P-Tyr content, more pronounced than that obtained by reactivation of v-Src at 35°C. (*B*) Phosphotyrosine kinase activity of v-Src is not influenced by the serine/threonine protein kinase inhibitor 2-AP and the phorbol ester PDD, added before temperature shift. Myotubes were kept at 35°C for 2 h before harvesting. (*C*) Western blot analysis with antibody to P-Tyr and scoring of ABs with TRITC-phalloidin labeling of myotubes incubated with various protein kinase inhibitors. Bisindolylmaleimide (*Bm*), staurosporine (*Stauro*), and calphostin-C (*Calph-C*) were added to the cultures at 41°C, 30 min before reactivation of v-Src. The cells were harvested for Western blotting or processed for immunofluorescence after 2 h at 35°C. AB formation is reported in arbitrary units. Note the close correlation observed between the number of ABs seen by immunofluorescence and the P-Tyr content revealed by Western blot.

(from 3 to 18 h) at 41°C. Both TPA and PDD stimulate membrane ruffles, but do not induce the formation of ABs in quail myotubes suggesting that activation of phorbol ester–responsive isoforms of PKC is unable to induce ABs in these cells. When myotubes are treated with 0.2 μM PDD (from 15 min up to 2 h) before being shifted to the permissive temperature for v-Src activity, however, the formation of ABs is inhibited up to 90–95%, but the levels of P-Tyr remain unaffected (Fig. 6 *B*). The inhibitory effect of PDD is completely abolished by pretreatment of the cultures with the PKC inhibitor Bm, confirming that the phorbol ester effect is mediated by activation of PKC (not shown). To further assess the role played by PKC activation, quail myotubes were shifted to 35°C for 2 h to induce AB formation, and then treated at 35°C with 0.2 μM PDD for 30 min to 2 h and processed for indirect immunofluorescence with antibody to α-actinin. A nearly complete disappearance of ABs is observed after 30 min incubation with PDD, although myotubes still retain some of the overall characteristics such as festooning of the sarcolemma, typically observed upon reactivation of v-Src (Fig. 7). The effect of PDD at 35°C supports the hypothesis that activation of PKC plays an inhibitory role both on v-Src–evoked AB formation and their maintenance.

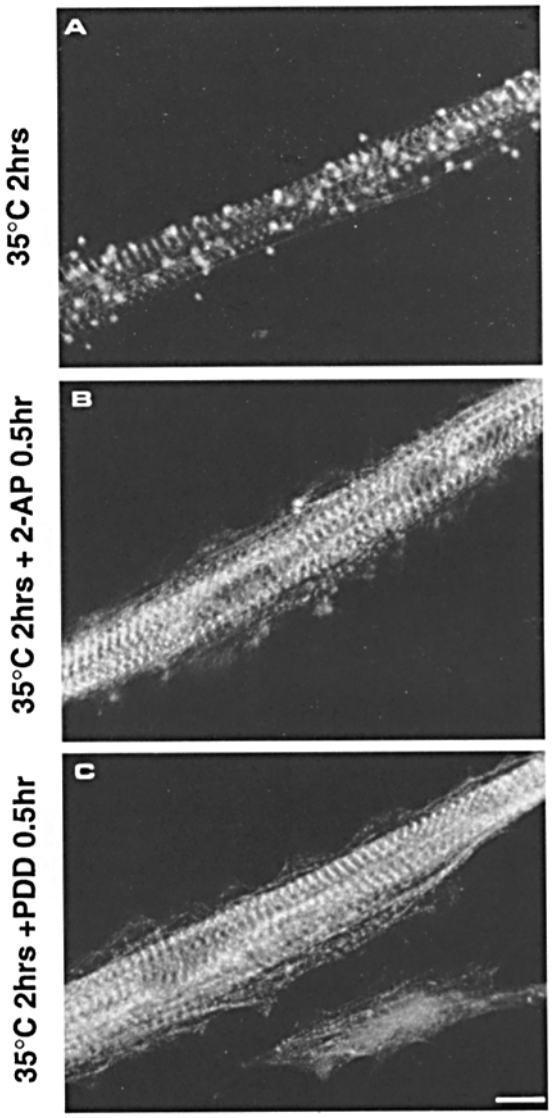


Figure 7. The serine/threonine kinase inhibitor 2-AP and the phorbol ester PDD inhibit AB formation at 35°C. Immunofluorescence micrographs of paraformaldeyde-fixed myotubes stained with antibody to α-actinin, revealed with TRITC-conjugated goat anti–mouse. (*A*) Myotube kept in medium for 2 h displays typical ABs. Cultures incubated at 35°C for 2 h and kept at 35°C for additional 30 min in the presence of (*B*) 7.5 mM 2-AP and (*C*) 0.2 μM PDD show only occasional, poorly defined ABs. Bar, 10 μm.

Regulation of Actin Body Formation by Protein Kinase Modulators

To test the possible role of protein kinase A in regulating AB formation, v-Src–induced responses were investigated in the presence of agents which increase intracellular cAMP levels. The adenylate cyclase activator forskolin (15 μM) or the cAMP analogue dibutyryl-cAMP (1 mM), added 30 min before activation of the tyrosine kinase, do not inhibit or modulate the formation of ABs, monitored after 2 h at 35°C by indirect immunofluorescence (not shown). These results suggest that protein kinase A activation, although influencing the integrity of actin bundles in other systems (Lamb et al., 1988), does not prevent sarcomeric disassembly of the I-Z-I complexes by v-Src.

Cell signaling pathways typically involve phosphorylation events operated by a number of kinases. To understand whether activation of a serine/threonine kinase(s) is a necessary step for the v-Src–evoked AB formation, myotubes were incubated with 2-aminopurine (2-AP), a broad range inhibitor of serine/threonine kinases in vitro, but shown to retain a high degree of selectivity in vivo (Mahadevan et al., 1990). Incubation of myotubes with 7.5 mM 2-AP before reactivation of v-Src inhibits by 80–90% the formation of ABs observed after 2 h at 35°C, without affecting the total P-Tyr content monitored by Western blot (Fig. 6 *B*). In addition, clearance of the ABs is observed as early as 30 min after addition of 2-AP to myotubes already incubated at 35°C for 2 h (Fig. 7). It has been previously reported that there is a constitutive activation of the mitogen-activated protein (MAP) kinase pathway in v-Src transformed cells (Cowley et al., 1994). It was therefore of interest to investigate whether the regulatory cascade of serine/threonine-protein kinases that activates the MAP kinase could be target of the inhibitory action of 2-AP. To determine whether the MAP kinase was activated by v-Src in ts-v-*src*-myotubes, cultures were shifted to 35°C for various lengths of time and analyzed by a Western blot "band shift" assay, in which activation of the MAP kinase is associated with a portion of the protein shifting to a form of lower electrophoretic mobility. Western blot analysis using antiserum 122, raised against murine ERK2 protein (Leevers and Marshall, 1992), of lysates from myotubes shifted to 35°C for 2 h shows activation of the MAP kinase as indicated by the band shift of the ERK2 protein (Fig. 8 *A*). Activation of MAP kinase by v-Src was also detected when immunoprecipitates were analyzed in a kinase assay using myelin basic protein as the exogenous substrate (Leevers and Marshall, 1992) (not shown). Treatment of the myotubes with 7.5 mM 2-AP before temperature shift does not affect the generation of the activated slowly migrating form of the MAP kinase (Fig. 8 *A*). Stimulation of myotubes at the restrictive temperature with 0.2 μM PDD, which inhibits the v-Src–induced AB formation (see above), also leads to a shift in electrophoretic mobility of the MAP kinase protein (Fig. 8 *A*). It has been shown previously that a fraction of the activated MAP kinase enter the nucleus upon treatment of serum-deprived cells with growth factors or phorbol ester (Chen et al., 1992; Lenormand et al., 1993). Indirect immunofluorescence analysis at the confocal microscope was used to examine the distribution of ERK(s) in myotubes at 41°C and after either

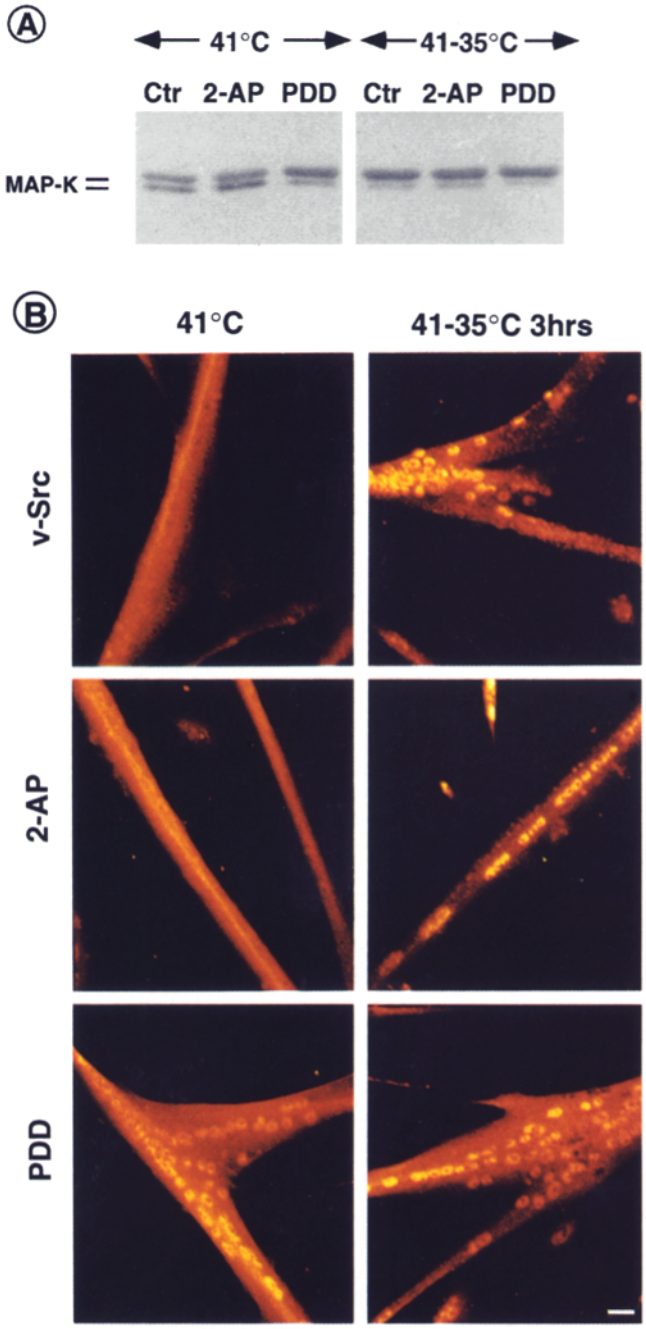


Figure 8. The MAP kinase pathway is activated in myotubes incubated at 35°C, independently of AB formation. (*A*) Western blot analysis with antiserum #122 (anti-ERK2) of cell lysates of myotubes kept at 41°C or shifted to 35°C for 2 h in the absence or presence of 7.5 mM 2-AP or 0.2 μM PDD. A doublet is observed in myotubes at 41°C. Upon activation of v-Src at 35°C or treatment with PDD at 41°C, an increase of the slower migrating form of the MAP kinase (*MAPK*) is observed. Similar results were obtained with antiserum #124 (Leevers and Marshall, 1992). 2-AP does not inhibit the band shift of the MAP kinase induced by temperature shift. (*B*) Confocal micrographs of myotubes labeled with antibody to MAP kinase/ERK2 showing nuclear translocation after v-Src reactivation at 35°C or treatment with phorbol ester (*PDD*) at 41°C. v-Src myotubes were stimulated by the addition of PDD (0.2 μM) or shifted to 35°C for 3 h. Myotubes kept at 41°C show a diffuse staining of the cytoplasm. The nuclei become brightly labeled upon reactivation of v-Src by temperature shift or by incubation with PDD. Note that the serine/threonine inhibitor 2-AP (7.5 mM) does not inhibit ERK2 nuclear translocation. Bar, 20 μm.

treatment with 0.2 μM PDD or shift to 35°C for 45 and 180 min (Fig. 8 *B*). Both phorbol ester and v-Src induce a marked translocation of MAP kinase to the nuclei of myotubes and this phenomenon is not inhibited by treatment with 7.5 mM 2-AP, consistently with the results obtained with the Western blot band shift assay (Fig. 8).

Discussion

During myogenesis, active mechanisms of control involving a dynamic interplay among positive and negative regulatory molecules, have been hypothesized for both activation of the terminal differentiation process and its maintenance (Blau, 1992). We have examined the fate of specialized cytoskeletal structures upon reactivation of a temperature-sensitive tyrosine kinase in fully differentiated quail myotubes. We show that the reactivation of the protein-tyrosine kinase v-Src in terminally differentiated myotubes induces both a rapid increase of P-Tyr levels and a selective disassembly of specialized sarcomeric structures: the I-Z-I segments. As early as 30 min after reactivation of v-Src globular structures made of filamentous α-actin and actin-associated proteins, referred to as actin bodies (ABs), are seen originating in the ventral region of the myotube, while organized myofibrils are maintained throughout the rest of the cell. At these early times of kinase activation, ABs often appear to be closely associated with the Z-discs, both by indirect immunofluorescence and by EM. As their number increases with time of activation of the kinase, the disruption of the sarcomeric arrangement of the thin filaments proceeds from the ventral to the dorsal region of the myotube, reaching completion within 24 h. Taken together, these observations indicate that the ABs originate from the I-Z-I segments of the sarcomeres.

The main protein constituents of the I-Z-I segments of sarcomeres, α-sarcomeric actin, α-actinin and the thin filament–associated protein nebulin, and the cytoskeletal protein vinculin are all present within the ABs. Costameres in muscle and focal adhesions in cultured cells are structural links between the cytoskeleton and the extracellular matrix (Burridge et al., 1988). Specifically, costameres are sites of connection between the Z-discs of the outmost myofibrils and the sarcolemma (Pardo et al., 1983). The presence in ABs of vinculin, typically associated with costameres and adhesion plaques (Small et al., 1992) and their initial formation in the most ventral region of the myotubes suggest that v-Src modifies directly or indirectly the anchoring system of the Z-discs to the sarcolemma. Vinculin remains associated with the ABs also after several hours of activation of the kinase indicating that the components of the ABs originating from the I-Z-I complexes remain associated with components of the cytoskeletal network which envelops the myofibrils (cf. Terracio et al., 1990; Massa et al., 1994). Interestingly, however, desmin, a peripheral constituent of the Z-discs and the main constituent of intermediate filaments in muscle (Small et al., 1992), is not assembled in the ABs (our unpublished data). The disappearance of ABs and I-Z-I complexes within the first 24 h of v-Src activity occurs much earlier than the reduction of the muscle-specific proteins observed 2–3 d after temperature shift (our unpublished data). It is unlikely, therefore, that the loss of ABs occurs through extrusion from the myotubes or by degradation operated by proteases. Interestingly, the loss of the sarcomeric pattern of F-actin and α-actinin staining occurs about 24 h earlier than that of the thick filaments, as indicated by the A-band pattern observed in these myotubes when labeled with antibodies to myosin heavy chain and to titin (our unpublished results).

EM offers a possible explanation for the initiation of disassembly of I-Z-I complexes in ventral myofibrils. The most ventral myofibrils may be affected first because their Z-lines are linked directly to costameres and all myofibrils eventually attach to the substrate in the ventral region of the myotube via a terminal Z-band and a meshwork of actin filaments. Observing myofibrils in longitudinal sections for long distances (2–3 mm) shows that the myofibrils are oriented at a very shallow angle to the long axis of the myotube. Therefore, over a distance of several millimeters, even myofibrils in the most dorsal positions gradually angle down to the ventral region and attach to the sarcolemma and the substrate. The myofibril termini would therefore be staggered along the ventral sarcolemma, without forming a defined region of "myo-substrate junction" comparable to a myotendon junction (Reedy and Beall, 1993). In addition, only the Z-discs of the most ventral myofibrils seem to form direct connections with costameres. It is important to note that myofibrils are not seen terminating at the dorsal sarcolemma and are often separated from the dorsal sarcolemma by ~0.5 μm. Taken together, our observations suggest that the v-Src kinase alters the anchoring to the sarcolemma of the Z-discs and of their equivalents at the myofibril terminus. In this light, it is not surprising that the most ventral myofibrils are affected first. After the termini of all of the myofibrils have been disassembled, the myofibrils become "free-floating" except at their Z-line attachments to the cytoskeleton lattice. The release from these attachments may have other consequences for the maintenance of ordered myofibrils.

In the context of maintenance of the differentiated state, processes such as those described may be relevant to the mechanisms normally used for the turnover of sarcomeric sub-domains. The activation of v-Src in muscle cells would, therefore, initiate the massive disassembly of I-Z-I complexes as the result of the synchronization of a normal process. Interestingly, inhibition of the barbed-end actin-binding protein CapZ during myofibrillogenesis results in a delay in the organization of actin in I-bands and in the formation of F-actin–containing foci, of similar size and intracellular location to actin bodies (Schafer et al., 1995). Moreover, actin-containing bodies have also been described in chicken myotubes after treatment with phorbol esters (Lin et al., 1989). Although the v-Src- and the phorbol ester–evoked myofibrillar disassembly shows overall similarities, the observed differences in the time course of AB formation and in the localization of the protein components within the ABs (Lin et al., 1989) seem to indicate that different mechanisms may be operating in the disruption of the myofibril maintenance program (see below).

The v-Src–evoked myofibrillar disassembly in differentiated myotubes shares some structural and biochemical features with the response of the cytoskeleton in v-*src*–transformed fibroblasts. Previous studies of v-Src in transformed fibroblasts tend to support localization of the ki-

nase at the plasma membrane, associated particularly with residual focal adhesions (Shriver and Rohrschneider, 1981; Kellie et al., 1986; David-Pfeuty and Nouvian-Dooghe, 1990; Resh, 1993), together with other potential regulatory enzymes such as $p125^{FAK}$ tyrosine kinase (Schaller et al., 1992) and PKC (Woods and Couchman, 1992; Jaken et al., 1989). Moreover, several studies have suggested a close relationship between the acquisition of a transformed phenotype, modulation of P-Tyr levels and the state of focal adhesions assembly and integrity (for review see Burridge et al., 1988). v-Src is associated with podosomes and with rosettes at the inner ventral surface of v-*src*–transformed fibroblasts (Nigg et al., 1982; Burridge et al., 1988). Rosettes are clusters of patches composed of an F-actin core with a vinculin rim (Chen, 1989). Podosomes are assumed to be highly dynamic membrane protrusions (~1 μm in diameter) from the ventral cell surface to the substratum and to have a distinctively different structure from focal adhesions (Tarone et al., 1985). These protrusions are filled with filamentous actin and contain vinculin and α-actinin (Marchisio et al., 1987). The similarities between these responses to v-Src strengthen the notion that modifications of the interaction between the plasma membrane and the underlying actin-containing cytoskeleton are an obligatory passage in mediating cell shape changes (for review see Zachary and Rozengurt, 1992). Transformation by RSV is also associated with disruption of focal adhesions and increased tyrosine phosphorylation of focal contact-associated proteins such as talin (Pasquale et al., 1986), vinculin (Sefton et al., 1981), paxillin (Glenney and Zokas, 1989), and β_1 integrin (Johansson et al., 1994). One question to be addressed here is whether the disassembly of the I-Z-I complexes from the myofibrils in ts-*src*-myotubes is due to direct phosphorylation of cytoskeletal proteins operated by v-Src or it results from the activation of a cellular signaling pathway in which several enzymes may be involved. Indeed, antibody to P-Tyr stains the ABs as well as structures that superimpose to the Z-discs. Inhibition of tyrosine-specific phosphatases at 41°C by vanadate, however, although resulting in a highly augmented level of P-Tyr and in the phosphorylation of periodic structures resembling those observed upon reactivation of v-Src, does not induce AB formation. Moreover, treatment of the myotubes with 2-AP, a serine/threonine kinase inhibitor which shows a high selectivity with regard to its in vivo inhibitory action (Mahadevan et al., 1990), does not affect the protein phosphorylation pattern due to activation of v-Src, but results in blockage of AB formation when added before the shift to permissive temperature or in the disappearance of the ABs when added hours after the temperature shift. Taken together, these findings support the hypothesis that cytoarchitecture rearrangements induced by v-Src result from activation of specific signaling pathways, rather than from v-Src phosphorylating components of the myofibril-anchoring network.

Tyrosine protein kinases, such as v-Src, activate multiple signaling systems, that differ in various cell types. It then becomes of interest to establish which of these signal transduction systems is essential for the generation of the cellular response being observed. A constitutive activation of the MAP kinase pathway has been found in v-Src–transformed cells (Cowley et al., 1994). MAP kinases are serine/threonine kinases that are activated in response to a number of signal transduction pathways, including those stimulated by tyrosine kinases and PKC. MAP kinases are phosphorylated and activated by MAP kinase kinases (MEKs), which can phosphorylate both the tyrosine and threonine residues required for activation of MAP kinase (Johnson and Vaillancourt, 1994). Our data show that, even in highly differentiated, postmitotic cells such as myotubes, a membrane-bound tyrosine kinase or PDD/PKC are capable of eliciting a sustained phase of MAP kinase activation, behaving thus as mitogenic stimuli, and that the signals generated at the cell periphery rapidly reach the nucleus. It will be interesting to know whether these signals are responsible for the block of muscle-specific gene transcription by v-Src previously described (Falcone et al., 1991). Irrespective of the mechanism by which the effect of 2-AP is achieved, we have also shown that the formation of ABs can be overcome in the presence of an activated MAP kinase pathway. It is not possible at present to identify precisely the site of action of 2-AP that underlies its ability to inhibit v-Src–induced I-Z-I disassembly, yet, the simplest explanation of our data is that 2-AP acts at the level of a specific effector pathway for the disassembly and clearance of a specialized cytoskeletal structure which may diverge upstream of the MAP kinases.

In chick embryo fibroblasts there is evidence that the activation of ts-v-Src leads within 30–60 min to the inositol trisphosphate-independent elevation of sn-1, 2-diacylglycerol (DAG), a natural activator of PKC (Martins et al., 1989). More recently, however, it was ascertained that in murine fibroblasts v-Src can induce long-term effects such as DNA synthesis (Han et al., 1990), desensitization of the epidermal growth factor receptor (Gray and Macara, 1988) and expression of Egr-1 (Quresi et al., 1991), independently of the diacylglycerol/PKC signal transduction system. In ts-*src*-myotubes a role of PKC as a required positive signal downstream to v-Src seems unlikely, since Bm does not inhibit the formation of ABs induced by v-Src, and acute treatment with phorbol ester before or during reactivation of the tyrosine kinase actually suppresses v-Src–induced AB formation, while activating the MAPK pathway. These data suggest a complex interplay between v-Src and PDD signaling mechanisms and allow two predictions: first, activation of the MAP kinase pathway is either not required or not sufficient to induce the rapid disassembly of the I-Z-I complex and AB formation; second, v-Src and PKC activate separate signaling pathways and the negative effect exerted by activation of PKC on AB formation is presumably exerted on a collateral branch of the v-Src triggered pathway.

The importance of elucidating the tyrosine kinase signaling pathway is highlighted by the necessity for cytoskeletal rearrangements that appear to characterize cell transformation. Highly differentiated muscle cells served as a sensitive assay system for the effects attendant on activating the v-Src signaling pathway. While there is evidence that morphological transformation in fibroblasts can be partly dissociated from mitogenic effects (Weber and Friis, 1979; Frame et al., 1994), our findings provide for the first time a link between events triggered at the cell periphery, as the selective alteration of specialized structures, and block of differentiation. The question arises as to whether

ABs and related ultrastructural changes are involved in transducing signals to the nucleus, leading to altered gene expression. We are exploiting the early events induced by v-Src in muscle cells in an attempt to define downstream effector molecules in the pathways that lead to cytoskeletal changes and to block transcription of muscle-specific genes, and the nature of their possible cross-talk to ensure a fully transformed phenotype. Understanding the cellular signals that influence the formation of ABs and the selective disassembly of sarcomeric domains induced by v-Src, should provide insight into the possible role of cellular tyrosine kinases of the *src* family in the homeostasis of tissue-specific organelles during development.

We thank Chris Marshall (Chester Beatty Laboratories), Donald Fischman (Cornell University), and Franco Tatò (Università di Roma, La Sapienza) for generous gifts of reagents, and Marie Craig, Carmen Lucavecchi, and Shetal Padia for expert photographic assistance. We especially like to thank Maria Teresa Ciotti for generous advice with light microscopy and we are indebted to Franco Tatò for helpful discussions and encouragement. Critical reading of the manuscript by Anna Maria Salvatore and Marco Crescenzi is acknowledged.

This work was supported by grants from Telethon Italy and Consiglio Nazionale delle Ricerche (to L. Castellani), National Institutes of Health (to M. K. Reedy), Progetto Finalizzato Applicazioni Cliniche Ricerca Oncologica, Progetto Finalizzato Biotecnologie e Biostrumentazione and Associazione Italiana Ricerca sul Cancro.

Received for publication 6 December 1994 and in revised form 8 May 1995.

References

Akiyama, T., J. Ishida, S. Nakagawa, H. Ogawara, S. Watanabe, N. Itoh, M. Shibuya, and Y. Fukami. 1987. Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J. Biol. Chem.* 5592.

Alemà, S., and F. Tatò. 1994. Oncogenes and muscle differentiation: multiple mechanisms of interference. *Sem. Cancer Biol.* 5:147–156.

Blau, H. M. 1992. Differentiation requires continuous active control. *Annu. Rev. Biochem.* 61:1213–1230.

Boettiger, D. 1989. Interactions of oncogenes with differentiation programs. *Curr. Topics Microbiol. Immunol.* 147:31–78.

Bruns, R. F., F. D. Miller, R. L. Merriman, J. J. Howbert, W. F. Heath, I. Kobayashi, T. Tamaoki, and H. Nakano. 1991. Inhibition of protein kinase C by calphostin C is light-dependent. *Biochem. Biophys. Res. Commun.* 176:288–293.

Burridge, K., K. Fath, T. Kelly, G. Nuckolls, and C. Turner. 1988. Focal adhesions: transmembrane junctions between extracellular matrix and the cytoskeleton. *Annu. Rev. Cell Biol.* 4:487–525.

Chen, W. T. 1989. Proteolytic activity of specialized surface protrusions formed at rosette contact sites of transformed cells. *J. Exp. Zool.* 251:167–185.

Chen, R-H., C. Sarnecki, and J. Blenis. 1992. Nuclear localization and regulation of *erk*- and *rsk*-encoded protein kinases. *Mol. Cell. Biol.* 12:915–927.

Cowley, S., H. Paterson, P. Kemp, and C. J. Marshall. 1994. Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. *Cell.* 77:841–852.

Danowski, B. A., K. Imanaka-Yoshida, J. M. Sanger, and J. W. Sanger. 1992. Costameres are sites of force transmission to the substratum in adult rat cardiomyocytes. *J. Cell Biol.* 118:1411–1420.

David-Pfeuty, T., and Y. Nouvian-Dooghe. 1990. Immunolocalization of the cellular *src* protein in interphase and mitotic NIH c-*src* overexpressing cells. *J. Cell Biol.* 111:3097–3116.

Falcone, G., F. Tatò, and S. Alemà. 1985. Distinctive effects of the viral oncogenes *myc*, *erb*, *fps* and *src* on the differentiation of quail myogenic cells. *Proc. Natl. Acad. Sci. USA.* 82:426–430.

Falcone, G., S. Alemà, and F. Tatò. 1991. Transcription of muscle-specific genes is repressed by reactivation of $pp60^{v\text{-}src}$ in postmitotic myotubes. *Mol. Cell. Biol.* 11:3331–3338.

Frame, M. C., K. Simpson, V. J. Fincham, and D. H. Crouch. 1994. Separation of v-Src induced mitogenesis and morphological transformation by inhibition of AP-1. *Mol. Biol. Cell.* 5:1177–1189.

Glenney, J. R., and L. Zokas. 1989. Novel tyrosine kinase substrates from Rous sarcoma virus–transformed cells are present in the membrane skeleton. *J. Cell Biol.* 108:2401–2408.

Granger, B. L., and E. Lazarides. 1979. Desmin and vimentin coexist at the periphery of the myofibril Z disc. *Cell.* 18:1053–1063.

Gray, G. M., and I. G. Macara. 1988. The $pp60^{v\text{-}src}$ tyrosine kinase desensitizes epidermal growth factor binding to 3T3 fibroblasts by two distinct protein kinase C-independent mechanisms. *J. Biol. Chem.* 263:10714–10719.

Han, J., J. Gaut, E. Burstein, H. Sadowski, D. Young, and I. G. Macara. 1990. The oncogenic protein $p60^{v\text{-}src}$ has competence activity but does not activate phosphatidylinositol turnover or protein kinase C in Balb/c 3T3 cells. *Oncogene.* 5:467–474.

Holtzer, H., J. Biehl, G. Yeoh, R. Meganathan, and A. Kaji. 1975. Effects of oncogenic virus on muscle differentiation. *Proc. Natl. Acad. Sci. USA.* 72:4051–4055.

Jaken, S., K. Leach, and T. Klauck. 1989. Association of type 3 protein kinase C with focal contacts in rat embryo fibroblasts. *J. Cell Biol.* 109:697–704.

Johansson, M. W., E. Larsson, B. Lüning, E. B. Pasquale, and E. Ruoslahti. 1994. Altered localization of cytoplasmic domain-binding properties of tyrosine-phosphorylated b1 integrin. *J. Cell Biol.* 126:1299–1309.

Johnson, G. L., and R. R. Vaillancourt. 1994. Sequential protein kinase reactions controlling cell growth and differentiation. *Curr. Opin. Cell Biol.* 6: 230–238.

Kellie, S., B. Patel, N. M. Wigglesworth, D. R. Critchley, and J. A. Wyke. 1986. The use of Rous sarcoma virus transformation mutants with differing tyrosine kinase activities to study the relationships between vinculin phosphorylation, $pp60^{v\text{-}src}$ location and adhesion plaque integrity. *Exp. Cell Res.* 165:216–228.

La Rocca, S. A., M. Grossi, G. Falcone, S. Alemà, and F. Tatò. 1989. Interaction with normal cells suppresses the transformed phenotype of v-*myc*-transformed quail muscle cells. *Cell.* 58:123–131.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

Lamb, N. J. C., A. Fernandez, M. A. Conti, R. Adelstein, D. B. Glass, W. J. Welch, and J. R. Feramisco. 1988. Regulation of actin microfilament integrity in living nonmuscle cells by the cAMP-dependent protein kinase and the myosin light chain kinase. *J. Cell Biol.* 106:1955–1971.

Lassar, A., and A. Munsterberg. 1994. Wiring diagrams: regulating circuits and the control of skeletal myogenesis. *Curr. Opin. Cell Biol.* 6:432–442.

Leevers, S. J., and C. J. Marshall. 1992. Activation of extracellular signal-related ERK2 by p21 ras oncoprotein. *EMBO (Eur. Mol. Biol. Organ.) J.* 11: 569–577.

Lenormand, P., C. Sardet, G. Pages, G. L'Allemain, A. Brunet, and J. Pouyssegur. 1993. Growth factors induce nuclear translocation of MAP kinase but not of their activator MAP kinase kinase in fibroblasts. *J. Cell Biol.* 122: 1079–1088.

Lin, Z., J. Eshleman, C. Grund, D. A. Fischman, T. Masaki, W. W. Franke, and H. Holtzer. 1989. Differential response of myofibrillar and cytoskeletal proteins in cells treated with myristate acetate. *J. Cell Biol.* 108:1079–1091.

Lin, Z., J. Eshleman, S. Forry-Schaudies, S. Duran, J. Lessard, and H. Holtzer. 1987. Sequential disassembly of myofibrils induced by phorbol myristate acetate in cultured myotubes. *J. Cell Biol.* 105:1365–1376.

Mahadevan, L. C., A. J. Willis, E. A. Hirst, P. D. Rathjen, and J. K. Heath. 1990. 2-aminopurine abolishes EGF- and TPA-stimulated pp33 phosphorylation and c-*fos* induction without affecting the activation of protein kinase C. *Oncogene.* 5:327–335.

Marchisio, P. C., D. Cirillo, A. Teti, A. Zambonin-Zallone, and G. Tarone. 1987. Rous sarcoma virus–transformed fibroblasts and cells of monocytic origin display a peculiar dot-like organization of cytoskeletal proteins involved in microfilament-membrane interactions. *Exp. Cell Res.* 169:202–214.

Martins, T. J., Y. Sigimoto, and R. L. Erikson. 1989. Dissociation of inositol trisphosphate from diacylglycerol production in Rous sarcoma virus–transformed fibroblasts. *J. Cell Biol.* 103:683–691.

Massa, R., L. Castellani, G. Silvestri, G. Sancesario, and G. Bernardi. 1994. Dystrophin is not essential for the integrity of the cytoskeleton. *Acta Neuropathol.* 87:377–384.

Nigg, E. A., B. M. Sefton, T. Hunter, G. Walter, and S. J. Singer. 1982. Immunofluorescence localization of the transforming protein of Rous sarcoma virus with antibodies against a synthetic peptide. *Proc. Natl. Acad. Sci. USA.* 79:5322–5326.

Olson, E. N. 1992. Interplay between proliferation and differentiation within the myogenic lineage. *Dev. Biol.* 154:261–272.

Pardo, J. V., J. D'Angelo Siciliano, and S. W. Craig. 1983. A vinculin-containing cortical lattice in skeletal muscle: transverse lattice elements ("costameres") mark sites of attachment between myofibrils and sarcolemma. *Proc. Natl. Acad. Sci. USA.* 80:1008–1012.

Parsons, J. T., and M. J. Weber. 1989. Genetics of *src*: structure and functional organization of a protein tyrosine kinase. *Curr. Topics Microbiol. Immunol.* 147:79–128.

Pasquale, E. B., P. A. Maher, and S. J. Singer. 1986. Talin is phosphorylated on tyrosine in chicken embryo fibroblasts transformed by Rous sarcoma virus. *Proc. Natl. Acad. Sci. USA.* 83:5507–5511.

Quresi, S. A., M. Rim, J. Bruders, W. Kolch, U. Rapp, V. P. Sukhatme, and D. A. Foster. 1991. An inhibitory mutant of c-Raf-1 blocks the v-Src-induced activation of the Egr-1 promoter. *J. Biol. Chem.* 266:20594–20597.

Reedy, M. C., and C. Beall. 1993. Ultrastructure of developing flight muscle in *Drosophila*. II. Formation of the myotendon junction. *Dev. Biol.* 160:466–479.

Reedy, M. K., and M. C. Reedy. 1985. Rigor crossbridge structure: tilted single filament layers and flared-X formations from insect flight muscle. *J. Mol.*

Biol. 185:145–176.

Resh, M. D. 1993. Interaction of tyrosine kinase oncoproteins with cellular membranes. *Biochim. Biophys. Acta.* 1155:307–322.

Schafer, D. A., C. Hug, and J. A. Cooper. 1995. Inhibition of CapZ during myofibrillogenesis alters assembly of actin filaments. *J. Cell Biol.* 128:61–70.

Schaller, M. D., B. S. Borgman, B. S. Cobb, R. R. Vines, A. B. Reynold, and J. T. Parsons. 1992. pp125FAK, a structurally unique protein tyrosine kinase associated with focal adhesions. *Proc. Natl. Acad. Sci. USA.* 8:5192–5196.

Sefton, B. M., T. Hunter, E. H. Ball, and S. J. Singer. 1981. Vinculin: a cytoskeletal target of the transforming protein of Rous sarcoma virus. *Cell.* 24:165–174.

Shriver, K., and L. Rohrschneider. 1981. Organization of $pp60^{src}$ and selected cytoskeletal proteins within adhesion plaques and junctions of Rous sarcoma virus–transformed rat cells. *J. Cell Biol.* 89:525–535.

Small, J. V., D. O. Fürst, and L. E. Thornell. 1992. The cytoskeletal lattice of muscle cells. *Eur. J. Biochem.* 208:559–572.

Tamaoki, T. 1991. Use and specificity of staurosporine, UCN-01, and Calphostin C as protein kinase C inhibitors. *Methods Enzymol.* 201:340–347.

Tarone, G., D. Cirillo, F. G. Giancotti, P. M. Comoglio, and P. C. Marchisio. 1985. Rous sarcoma virus–transformed fibroblasts adhere primarily at discrete protrusions at the ventral membrane called podosomes. *Exp. Cell Res.* 159:141–157.

Terracio, L., D. G. Simpson, L. Hilenski, W. Carver, R. S. Decker, N. Vinson, and T. K. Borg. 1990. Distribution of vinculin in the Z-disk of striated muscle: analysis by laser scanning confocal microscopy. *J. Cell. Physiol.* 145:78–87.

Toullec, D., P. Pianetti, H. Coste, P. Bellevergue, T. Grand-Perret, M. Ajakanes, V. Baudet, P. Boissin, E. Boursier, F. Loriolle, et al. 1991. The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. *J. Biol. Chem.* 266:15771–15781.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.

Trinick, J. 1991. Elastic filaments and giant proteins in muscle. *Curr. Opin. Cell Biol.* 3:112–119.

Volberg, T., Y. Zick, R. Dror, I. Sabanay, C. Gilon, A. Levitzki, and B. Geiger. 1992. The effect of tyrosine-specific protein phosphorylation on the assembly of adherens-type junctions. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:1733–1742.

Wang, K., and J. Wright. 1988. Architecture of the sarcomere matrix of skeletal muscle: immunoelectron microscopic evidence that suggests a set of parallel inextensible nebulin filaments anchored at the Z line. *J. Cell Biol.* 107:2199–2212.

Weber, M. J., and R. R. Friis. 1979. Dissociation of transformation parameters using temperature-conditional mutants of Rous sarcoma virus. *Cell.* 16:25–32.

Weintraub, H. 1994. The MyoD family and myogenesis: redundancy, networks, and thresholds. *Cell.* 75:1241–1244.

Woods, A., and J. R. Couchman. 1992. Protein kinase C involvement in focal adhesion formation. *J. Cell Sci.* 101:277–290.

Wyke, J. A., and A. W. Stoker. 1987. Genetic analysis of the form and function of the viral *src* oncogene product. *Biochim. Biophys. Acta.* 907:47–69.

Zachary, I., and E. Rozengurt. 1992. Focal adhesion kinase ($p125^{FAK}$): a point of convergence in the action of neuropeptides, integrins and oncogenes. *Cell.* 71:891–894.