

Inclusion body myositis

Expression of extracellular signal-regulated kinase and its substrate

S. Nakano, MD, PhD; A. Shinde, MD; S. Kawashima, MD, PhD; S. Nakamura, MD, PhD; I. Akiguchi, MD, PhD; and J. Kimura, MD.

Article abstract—*Objective:* To assess abnormal intracellular signal transduction in inclusion body myositis (IBM). *Background:* Mitogen-activated protein kinases (MAPKs) play pivotal roles in intracellular signal transduction and regulate cell growth and differentiation. Upon their activation, MAPKs translocate from the cytoplasm into the nucleus. *Design/methods:* The authors investigated the localization of several forms of the MAPK family—extracellular signal-regulated kinase (ERK), c-Jun N-terminal protein kinase (JNK), and p38 MAPK (p38)—in 10 patients with sporadic IBM and in 52 control subjects. The relationship between the localization of immunopositive deposits and nuclei was tested with bis-benzimide. *Results:* Vacuolated fibers in IBM displayed very strong focal immunoreactivity of ERK, but not of JNK or p38. The ERK-positive deposits in these vacuolated fibers colocalized with the nuclear substrate of ERK, Elk-1. ERK- and Elk-1-positive deposits were located frequently on the surface of the nuclei in vacuolated fibers in IBM. Similar findings to those of sporadic IBM were observed in three patients with distal myopathy with rimmed vacuoles, but not in eight normal or the other 41 disease controls. *Conclusion:* There is evidence for impaired molecular transport to the nucleus from the cytoplasm in the vacuolated fibers in IBM. This could be due to cytoplasmic aggregation of ERK and Elk-1 or to abnormal nuclear pore machinery involved in the transport of ERK and its substrate upon ERK activation.

NEUROLOGY 2001;56:87–93

Sporadic inclusion body myositis (s-IBM) is the most common muscle disease in patients older than 50 years. In addition to primary endomysial inflammation, muscle biopsies show vacuolated muscle fibers containing tubulofilaments in the cytoplasm and nuclei. Conversely, hereditary inclusion body myopathy (h-IBM) encompasses hereditary progressive muscle diseases with a pathologic process similar to that of s-IBM, except for a lack of lymphocytic inflammation.^{1,2} It includes myopathy with autosomal dominant inheritance of predominantly proximal weakness and autosomal recessive quadriceps-sparing myopathy.¹ Distal myopathy with rimmed vacuoles, which is frequently observed in Japan, and the autosomal recessive h-IBM may be allelic or may be the same disorder.^{3,4}

The vacuolated fibers in IBM express a variety of proteins that are expressed normally at the neuromuscular junction and lesions in neurodegenerative diseases.⁵ The ectopic expression of such proteins should result from abnormal intracellular events and altered transcription in IBM. Because protein kinases play pivotal roles in regulating intracellular signal transduction and transcription,^{6,7} studies of protein kinases in IBM may help to clarify the changes of protein expression in this disease. Accumulation of phosphorylated protein, which is immunoreactive for an antibody directed against phosphorylated neurofilament proteins (SMI-31) in

vacuolated fibers in IBM,⁸ could indicate abnormal intracellular signal transduction.⁹

Extracellular signal-regulated kinase (ERK) belongs to the mitogen-activated protein kinase (MAPK) family, and plays a central role in transducing extracellular signals to the nucleus. After attachment to their receptors, hormones, growth factors, and cytokines evoke several intracellular signal transduction cascades, thereby regulating transcription in the nucleus.^{6,10} In one of the major branches of the cascades, these external stimuli induce tyrosine kinase activity at the cell surface and transient formation of Ras-guanosine 5' triphosphate (GTP). This in turn activates Raf kinase at the membrane, followed by sequential phosphorylation and activation of MAPK/ERK kinase (MEK) and ERK. Activated ERK phosphorylates various cytoplasmic molecules and translocates into the nucleus to phosphorylate a transcription factor called Elk-1.^{6,10} The activated form of Elk-1, together with serum response factor (SRF), binds to the serum response element (SRE) of the promoter region of immediate early genes, including c-fos (figure 1A).¹¹ Conversely, environmental stresses and proinflammatory cytokines induce other phosphorylation cascades. In these stress-activated cascades, p38 MAPK (p38) and c-Jun N-terminal protein kinase (JNK), two subclasses of the MAPK family, take the equivalent position to ERK in the ERK cascade (see figure 1B).^{10,12}

From the Department of Neurology, Graduate School of Medicine, Kyoto University, Kyoto, Japan.

Received March 24, 2000. Accepted in final form October 10, 2000.

Address correspondence to Dr. Satoshi Nakano, Department of Neurology, Graduate School of Medicine, Kyoto University, 54 Shogoin Kawara-cho, Sakyo-ku Kyoto 606-8507 Japan; e-mail: snakano@isola.kuhp.kyoto-u.ac.jp

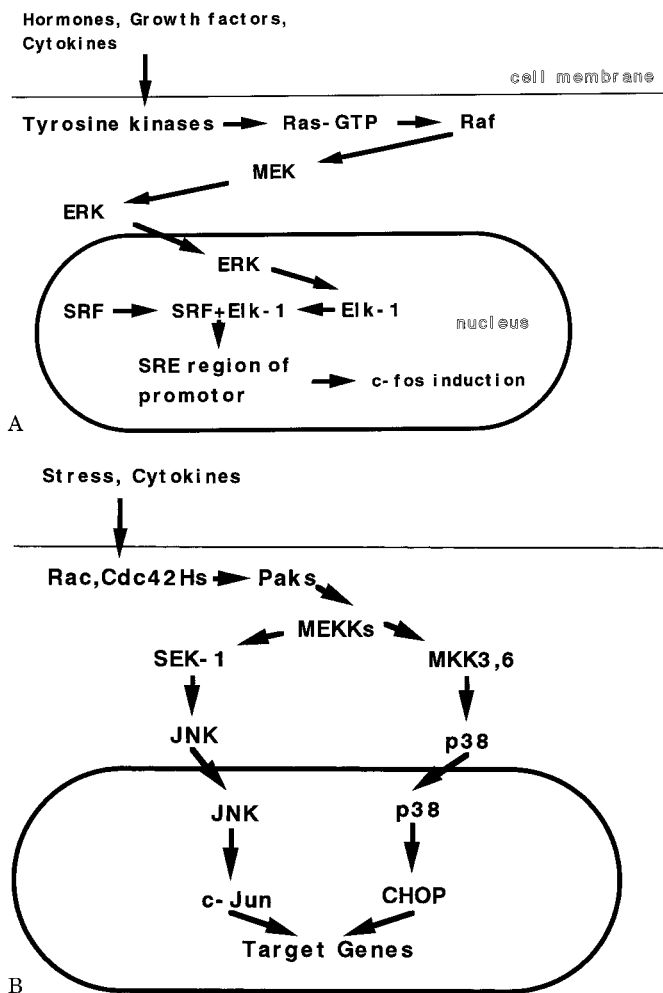


Figure 1. Schematic diagram of the (A) extracellular signal-regulated kinase (ERK) pathway and the (B) stress-activated mitogen-activated protein kinase (MAPK) pathways. MEK = MAPK or ERK kinase; Elk-1 = the nuclear substrate of ERK; SRF = serum response factor; SRE = serum response element; MEKKs = MEK kinases; MKK3,6 = MAPK kinase; JNK = c-Jun N-terminal protein kinase; p38 = p38 MAPK; SEK-1 = SAPK/ERK kinase-1. For details, see references 10 and 12.

These complicated phosphorylation systems provide for a finely tuned, rapid regulation of signals at each level of the cascade, with cross-talk with other intracellular transduction cascades.

We previously reported abnormal focal accumulation of CDK5 in vacuolated fibers in IBM.⁹ Like CDK5, MAPKs belong to the family of proline-directed protein kinases, which phosphorylate serine or threonine followed by proline in the amino acid sequences of substrate proteins. Furthermore, like CDK5, ERK translocates into the nucleus and positively regulates muscle fiber differentiation.^{13,14} These observations prompted us to examine the localization of ERK and its association with the nucleus in vacuolated fibers in IBM.

Materials and methods. *Patients.* Limb muscle specimens from 10 patients (aged 43 to 76 years; nine men, one woman) with sporadic IBM were studied. Each muscle specimen contained endomysial inflammatory cell exudates, rimmed vacuoles, and congophilic inclusions. Therefore, the diagnosis of each patient was definite IBM.¹ Four patients with oculopharyngeal muscular dystrophy (OPMD), three with distal myopathy with rimmed vacuoles, one with colchicine myopathy,¹⁵ and one with acid maltase deficiency were studied as non-IBM vacuolar myopathies. Eight muscle specimens deemed free of neuromuscular disease were used as normal controls. Fourteen patients with polymyositis, eight with dermatomyositis, five with Duchenne muscular dystrophy, four with mitochondrial myopathy, and four with neurogenic muscular atrophy served as other disease controls. The diagnoses were based on conventional criteria.¹⁶ Regenerating fibers were determined by examination of serial hematoxylin-eosin (H-E) sections.¹⁷

Primary antibodies. The table lists the characteristics of the primary antibodies against ERK and other subclasses of MAPKs and against transcription factors used in this study, and the concentrations at which they were applied. The anti-ERK antibody reacts with ERK1 and ERK2.¹⁸ The anti-JNK antibody binds to JNK1 and other isoforms of JNK.¹⁹ The anti-Elk-1 antibody has been well characterized.²⁰ SMI-31 was obtained from Sternberger Monoclonals (Baltimore, MD) and used at 1,000-fold dilution.

Table Primary antibodies against mitogen-activated protein kinases (MAPKs) and transcription factors

Antigen	Immunogen	Antibody	Clone/ID	Source	Concentration or dilution
ERK	C-terminus of ERK1	MMAB	ERK-7D8	Zymed (San Francisco, CA)	2 µg/mL
p38	N-terminus of p38	RPAB	sc-728	Santa Cruz (Santa Cruz, CA)	5 µg/mL
JNK	C-terminus of JNK1	RPAB	sc-474	Santa Cruz (Santa Cruz, CA)	5 µg/mL
Elk-1	379 to 392 of Elk-1*	RPAB	9182	New England Biolabs (Beverly, MA)	1:75
c-Jun	56 to 67 of c-Jun†	RPAB	9261	New England Biolabs (Beverly, MA)	1:75

* Phosphorylated at Ser383.

† Phosphorylated at Ser63.

ERK = extracellular signal-regulated kinase; p38 = p38 mitogen-activated protein kinase; JNK = c-Jun N-terminal protein kinase; MMAB = mouse monoclonal antibody; RPAB = rabbit polyclonal antibody.

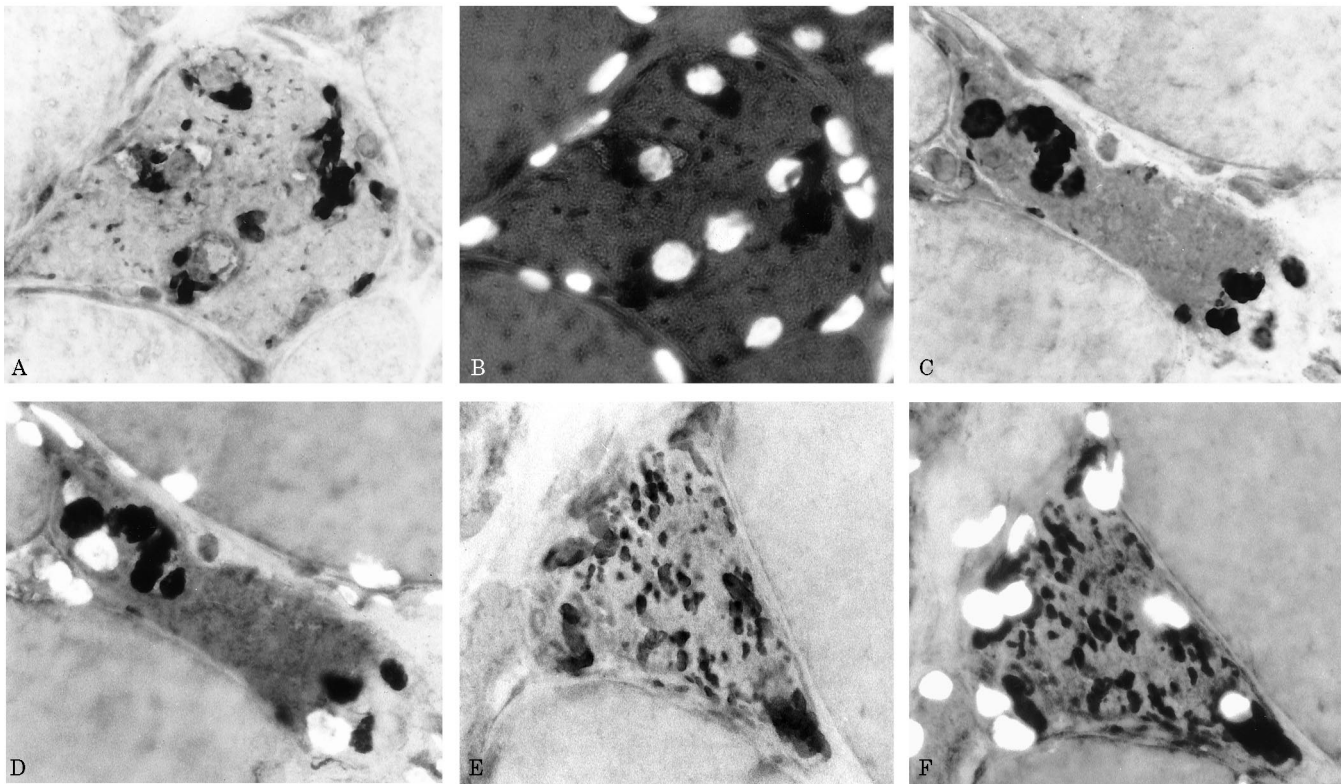


Figure 2. Extracellular signal-regulated kinase immunostaining (A, C, E) in vacuolated fibers in inclusion body myositis, and nuclear DNA staining with Hoechst 33258 in the same sections (B, D, F). The bright spots represent nuclei ($\times 680$ before reduction).

Immunostaining. Consecutive or nonconsecutive 7- μ m transverse cryostat sections were fixed in cold acetone and incubated first in phosphate-buffered saline (PBS) containing 2% bovine serum albumin and 5% nonimmune serum from the species in which the secondary antibody was raised. Second, the sections were incubated overnight at 4 $^{\circ}$ C in the blocking solution containing the primary antibody. After incubation with a biotin-labeled second antibody, the sections were developed by the avidin–biotin complex (ABC) immunoperoxidase method (Vectastain ABC kit, Vector, Burlingame, CA). For double fluorescence staining, the sections were treated with 1) anti-ERK antibody and anti-Elk-1 antibody or 2) SMI-31 and anti-Elk-1 antibody at 4 $^{\circ}$ C overnight, followed by incubation with second antibodies consisting of rhodamine-conjugated goat antimouse immunoglobulin G (IgG) (Protos Immunoresearch, San Francisco, CA) and fluorescein isothiocyanate conjugated (FITC)-labeled swine anti-rabbit IgG (Dako, Carpinteria, CA). The slides were mounted with Vectashield (Vector), and viewed with an Olympus photomicroscope (Tokyo, Japan) equipped for epifluorescence.

Control experiments included omission of the primary antibody as well as substitution of nonimmune rabbit or mouse IgG in place of the primary antibody.

To assess the relationship of ERK- and Elk-1–positive deposits with nuclei, the immunostained sections were further stained with 1 μ g/mL Hoechst 33258 (Molecular Probes, Eugene, OR), a DNA-binding dye. The mounted sections were examined under fluorescent microscopy.⁹

Results. *Localization of MAPKs and Elk-1 in s-IBM.* In s-IBM, 60 to 96% of vacuolated fibers displayed very

strong vacuolar and cytoplasmic immunoreactivity of ERK (figure 2, A, C, and E). The ERK-positive deposits were round or irregular. In vacuolated fibers, no or few deposits positive for p38 or JNK were observed.

Diffuse moderate ERK, p38, and JNK immunoreactivity was observed in regenerating fibers and in some degenerating fibers.

In vacuolated fibers in IBM, there were strong Elk-1–immunopositive deposits (figure 3, A, C, and E). Their localization and contours were identical with those of ERK-positive deposits. We confirmed this in a double immunofluorescence study (figure 4, upper row). We next examined the relationship between the Elk-1–positive deposits and those of SMI-31, a marker of IBM filaments.⁸ The distribution and configuration of the Elk-1–positive deposits were the same as those of SMI-31 (see figure 4, lower row) in vacuolated fibers. Although SMI-31 also stained axons of IM nerves, as described previously,²¹ anti-Elk-1 did not.

There were no immunoreactive deposits for c-Jun, the nuclear substrate of JNK, in vacuolated fibers in IBM.

Comparison of ERK- or Elk-1–positive deposits with the localization of nuclei in vacuolated fibers in s-IBM. ERK- or Elk-1–positive deposits were often observed on the external surface of the nuclei, but were sometimes also present in the cytoplasm unrelated to the nuclear localization (see figure 2, B, D, and F, and figure 3, B, D, and F). There were sometimes overlaps of the positive deposits and nuclei. In rare fibers, protrusions of the positive deposits into nuclei were observed.

A quantitative study of the relationship between ERK-

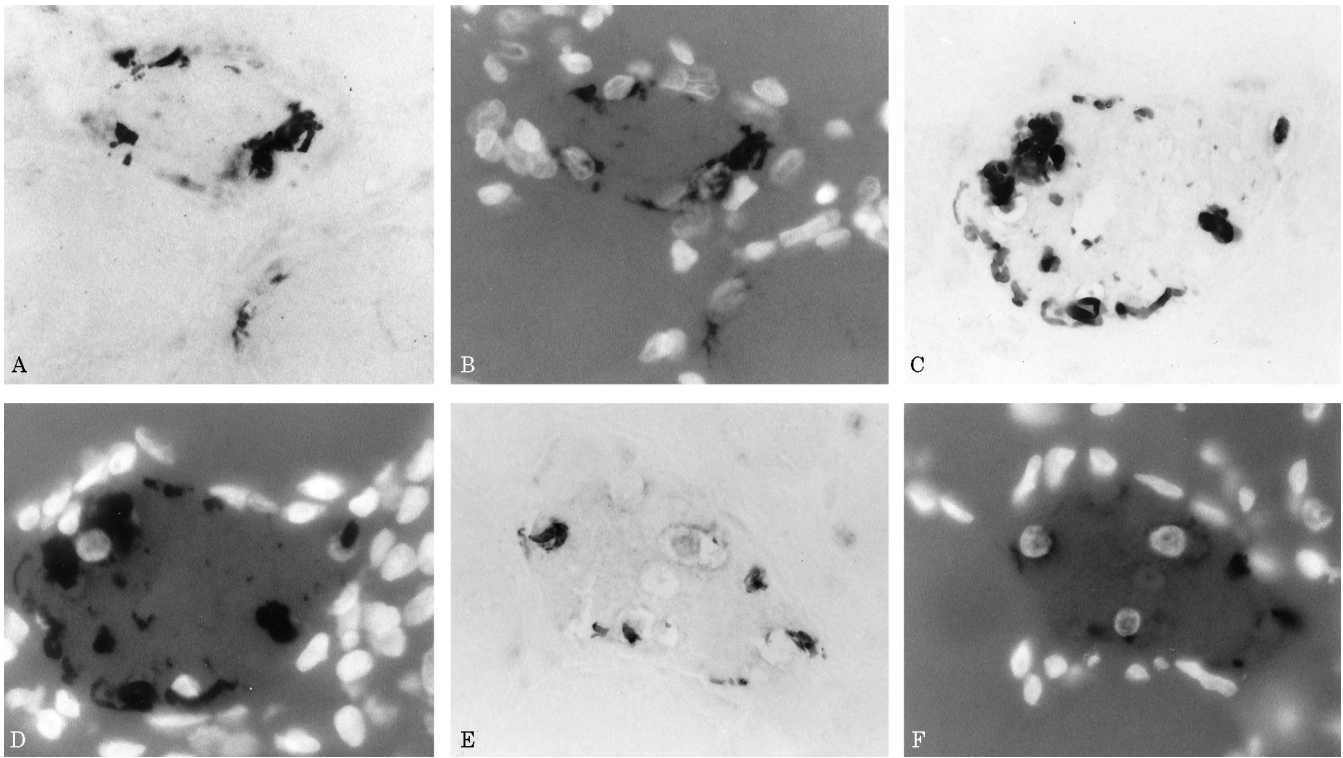


Figure 3. *Elk-1* immunoreactivity (A, C, E) and nuclear staining (B, D, F) in the same sections in vacuolated fibers in inclusion body myositis ($\times 680$ before reduction).

positive deposits and 275 nuclei in 61 randomly selected and photographed ERK-positive fibers indicated that 78.2% of the nuclei were closely associated with the deposits; 3.2% of the 275 nuclei had ERK-positive deposits occupying more than half of their area, and 75.0% of the nuclei

were touched, penetrated, or partially covered by the deposits.

Findings in other vacuolar myopathies. Distal myopathy with rimmed vacuoles. As in s-IBM, strong focal immunoreactivity of ERK and Elk-1, but not of p38, JNK, or c-Jun were observed in vacuolated fibers (figure 5, A and B). As in s-IBM, 28% of the nuclei in ERK-positive fibers were closely associated with the immunopositive deposits.

Acid maltase deficiency. Immunoreactivity for MAPKs was observed on the boundaries of vacuoles (see figure 5C). Elk-1 and c-Jun were negative.

Oculopharyngeal muscular dystrophy. No ERK-positive dots or few small ERK-positive dots were found in vacuolated fibers (see figure 5D).

Colchicine myopathy. Focal cytoplasmic deposits containing ERK, p38, JNK, Elk-1, and c-Jun were observed in a fraction of vacuolated and nonvacuolated fibers (see figure 5E).

Findings in other control subjects. Specimens without pathologic findings were negative or weakly positive for MAPKs and the transcription factors. No focal deposits were observed. Target formations showed strong activity for MAPKs, Elk-1, and c-Jun (see figure 5F). Regenerating/degenerating fibers showed positive immunoreactivity for MAPKs and Elk-1 (see figure 5, G and H). These fibers were variably positive for c-Jun.

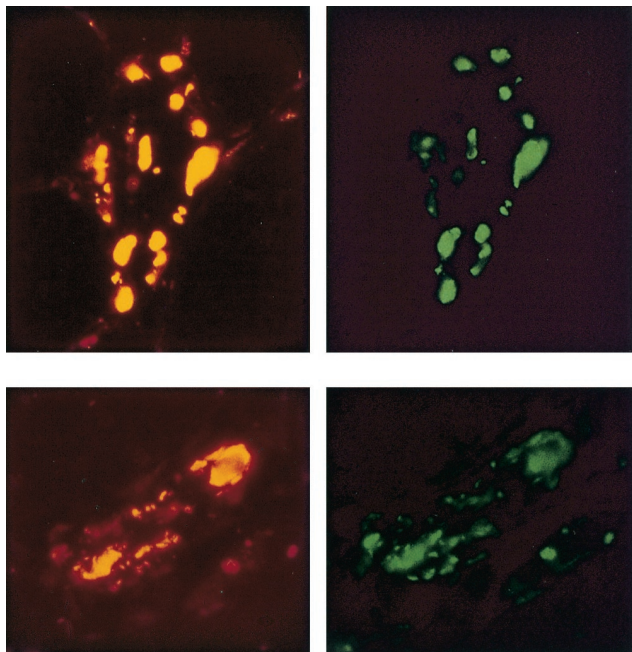


Figure 4. Two-color immunofluorescence. Localization in the same sections and fibers. Upper row: extracellular signal-regulated kinase (orange) and Elk-1 (green). Lower row: SMI-31 (orange) and Elk-1 (green) ($\times 400$).

Discussion. Several growth factors and hormones have been shown to be associated with the growth and differentiation of muscle cells.²² However, until recently, little was known about the consequent intracellular mechanisms that finally activate the

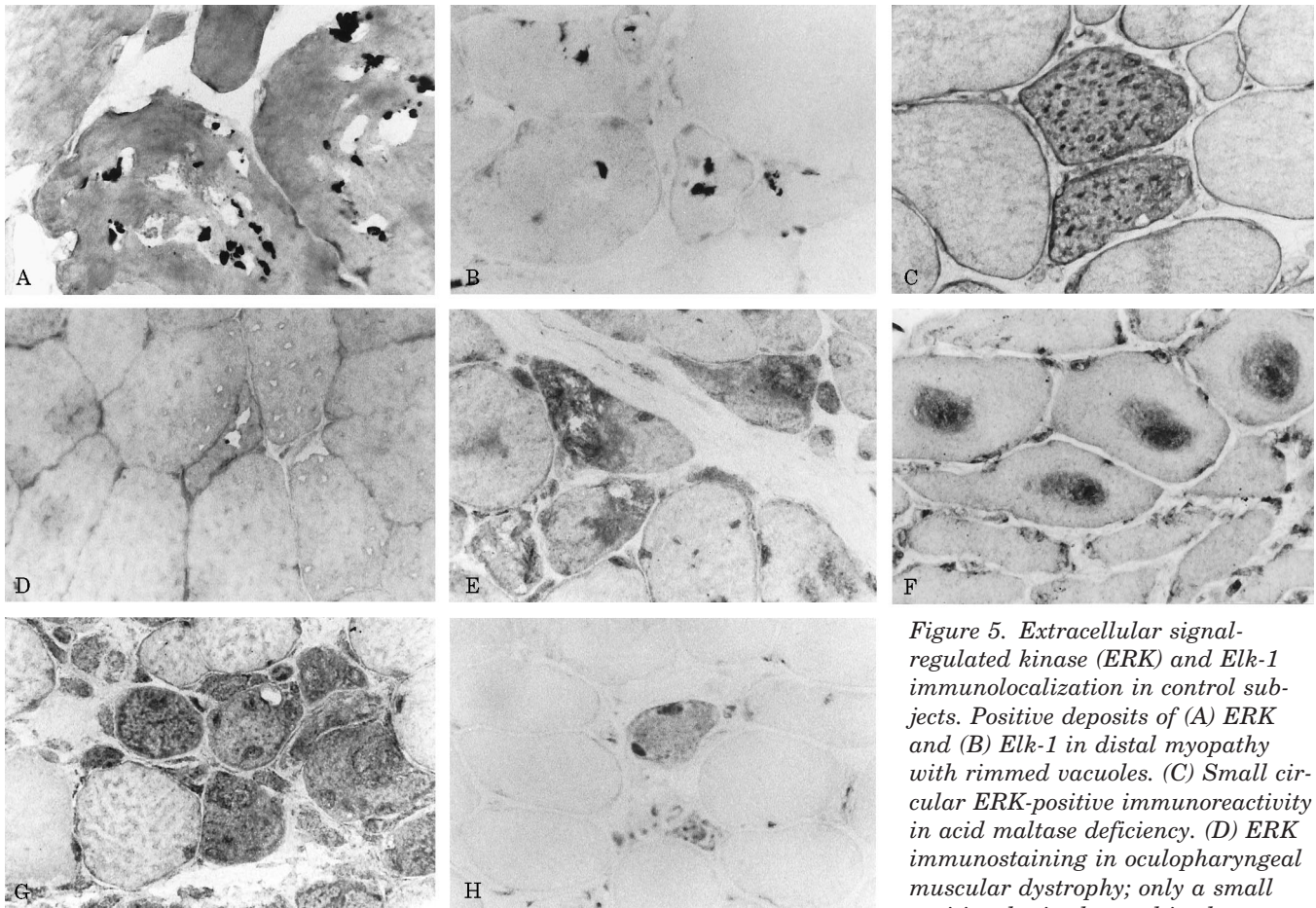


Figure 5. Extracellular signal-regulated kinase (ERK) and Elk-1 immunolocalization in control subjects. Positive deposits of (A) ERK and (B) Elk-1 in distal myopathy with rimmed vacuoles. (C) Small circular ERK-positive immunoreactivity in acid maltase deficiency. (D) ERK immunostaining in oculopharyngeal muscular dystrophy; only a small positive dot is observed in the vacu-

oles. (E) Cytoplasmic ERK-positive deposits in colchicine myopathy. (F) ERK-positive target formations in neurogenic muscular atrophy. (G) ERK and (H) Elk-1 in regenerating fibers in dermatomyositis. Elk-1 shows strong nuclear reactivity ($\times 340$ before reduction).

MyoD family of transcription factors, which regulate differentiation of muscle cells. Recent studies have indicated involvement of MAPKs in the control of the myogenic transcription factors. In a study of C2 muscle cell cultures, ERK was activated during muscle fiber terminal differentiation, and it positively regulated the activity of MyoD, while the high JNK activity of myoblasts was downregulated.¹⁴ Also, p38 may play a positive role in muscle fiber differentiation earlier than ERK via another myogenic transactivation factor, MEF2.²³ Regenerating fibers consist of replicating myoblasts and differentiating myotubes.¹⁷ Thus, the presence of MAPKs in regenerating fibers in diseased muscles may correlate with the findings of the cell culture studies. Because SRF, the partner of activated Elk-1 in the nucleus (see figure 1A), is also essential for muscle fiber differentiation,²⁴ ERK probably takes part in myogenesis via phosphorylation of Elk-1.¹⁴

Whereas ERK-positive immunoreactivity was not found in normal fibers and was diffuse in the cytoplasm of regenerating fibers, very strong immunoreactive deposits of ERK were found in vacuolated fibers in IBM. These deposits were round or irregular in shape, and were present in the cytoplasm or in

vacuoles. A fraction of the ERK-positive deposits was closely associated with the nuclei of the vacuolated fibers, and was frequently located on the surface of the nuclei. The abnormal cytoplasmic condensation of ERK protein may result from an abnormality of the ERK molecule itself or of another molecule that prevents abnormal aggregation of ERK. Because the nuclear transcription factor Elk-1 showed similar cytoplasmic aggregation and perinuclear localization, we suspect a defect in a chaperone-like molecule involved in the folding and nuclear transport of Elk-1 and ERK.

Both cytoplasmic and nuclear proteins, including Elk-1, are synthesized in the endoplasmic reticulum. Newly synthesized proteins destined for the nucleus or some activated enzymes, such as MAPKs, are transported through the nuclear membrane. This import is a selective and mediated process in which sets of chaperone molecules, vehicle proteins, and energy suppliers take part. Inhibition of any component of the nuclear transport system results in cytoplasmic and perinuclear accumulation of karyophilic proteins.²⁵ For example, heat shock cognate protein 70 (HSC70), which belongs to the family of heat shock proteins, is a carrier protein in nuclear transport. In

human cell cultures, cytoplasmic injection of antibodies against HSC70 strongly inhibits the nuclear import of several karyophilic proteins and causes their cytoplasmic aggregation.²⁶ In yeast cells, HOG1 MAPK translocates into the nucleus upon activation. Mutations in transport proteins result in impaired cytonuclear transport and cytoplasmic accumulation of HOG1 MAPK.²⁷ We found cytoplasmic and perinuclear accumulation of ERK in vacuolated fibers of IBM, but not of p38 or JNK, the two kinases which show strong activity in regenerating fibers. ERK is the last MAPK that becomes activated during muscle fiber differentiation.^{14,23} Therefore, the defect that causes abnormal ERK accumulation may occur in the late phase of differentiation, after JNK and p38 activities have declined. Chaperone proteins or heat shock proteins show regulated expression during differentiation and development²⁸; a chaperone protein that is specifically involved in this phase of differentiation might be responsible for the abnormality in the vacuolated fibers in IBM. The same abnormal mechanism may also cause the perinuclear accumulation of Elk-1.

We showed focal deposits of Elk-1 colocalized with SMI-31 immunoreactivity in vacuolated fibers in IBM. SMI-31 has been developed for phosphorylated epitopes of neurofilament proteins. It can also react with other proteins such as microtubule-associated protein (MAP) tau and MAP-2.²⁹ Because SMI-31 stains IBM filaments,⁸ ERK and its target Elk-1 may be associated with the filaments or might be components of the filaments. Electron microscopic studies indicated that IBM filaments are largely cytoplasmic, and only occasional nuclei contain those filaments. Rarely, electron micrographs revealed a disruption of nuclear membranes by filamentous inclusions.³⁰ These observations are in accord with the results of the current study of double staining of ERK- or Elk-1-positive deposits and nuclei; in this study, 3.2% of the nuclei in the ERK-positive fibers harbored or were overlaid by immunopositive deposits, and few nuclei were penetrated by the deposits. Conversely, ERK- and Elk-1-positive deposits were often larger than nuclei, and they sometimes occupied almost entire vacuoles. Therefore, these molecules do colocalize with IBM filaments, but they may also be present in other neighboring regions, as has been indicated in SMI-31 immunoreactivity.³⁰

In distal myopathy with rimmed vacuoles, the localization of ERK and Elk-1 was similar to that seen in s-IBM in vacuolated fibers, though the association of ERK-positive deposits with nuclei was weaker than that in s-IBM (28 versus 78%). Nevertheless, a relevant mechanism related to that in s-IBM may operate in this disease and possibly in its allelic disorder, autosomal recessive h-IBM.^{3,4}

Acid maltase deficiency showed immunopositivity for MAPKs on the vacuolar boundaries, but not for their nuclear substrates. The substrates for MAPKs on the boundaries could be cytoskeletal proteins,

such as dystrophin, which is present on the vacuolar boundaries³¹ and can be phosphorylated by MAPKs.³²

In vacuolated fibers in OPMD, only a few focal deposits of ERK were observed. Therefore, the mechanism of formation of inclusions in OPMD may be different from that in IBM. In OPMD, a short expansion of triplet repeats in the poly(A) binding protein-2 gene may cause intranuclear accumulation of tubulofilamentous inclusions.³³

Colchicine disrupts microtubular networks, thereby preventing intracellular vesicular trafficking.¹⁵ Microtubular blocking agents induce stress-activated protein kinases.³⁴ Furthermore, MAPKs and transcription factors are physically associated with microtubules.^{18,35} Cytoplasmic accumulation of MAPKs and transcription factors in colchicine myopathy may result from a combination of these effects.

Target formations showed the same immunopositivity as colchicine myopathy. This result suggests that MAPKs in the center of the fibers induced by denervation stress are stagnated in the original position. The impairment of molecular trafficking might be more proximal in colchicine myopathy and in target formations than the cytonuclear transport in IBM.

In a previous report, we showed CDK5-positive deposits in vacuolated fibers in IBM. A high proportion of the CDK5-positive deposits abutted on the nucleus, like ERK in the current study.⁹ CDK5 transiently appears in the nucleus during the terminal differentiation and promotes the process.¹³ Thus, two protein kinases, both normally activated and translocated into the nucleus during some phase of differentiation, accumulate in the cytoplasm and around the nuclei in vacuolated fibers in IBM. We suspect that the induction of ERK and CDK5 is part of the intrinsic program of muscle fiber differentiation, and that the abnormally high concentration of these enzymes results from their aggregation in the cytoplasm and inability to enter the nucleus. The study of molecules involved in molecular folding³⁶ and nuclear translocation of ERK and CDK5 during differentiation should clarify the molecular mechanism that underlies IBM.

A recent study showed abnormal expression of α B-crystallin, which belongs to the small heat shock protein family, in IBM.³⁷ Because α B-crystallin may play a role during myogenesis,³⁸ this molecule might be the defective chaperone protein we postulated above or it might be induced to compensate for the function of another chaperone protein.

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Neurology 2001;56;87-93

DOI 10.1212/WNL.56.1.87

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