Activation of c-Raf-1 Kinase Signal Transduction Pathway in α7 Integrin-deficient Mice

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Integrin α7-deficient mice develop a novel form of muscular dystrophy. Here we report that deficiency of α7 integrin causes an activation of the c-Raf-1/mitogen-activated protein (MAP) 2 kinase signal transduction pathway in muscle cells. The observed activation of c-Raf-1/MAP2 kinases is a specific effect, because the α7 integrin deficiency does not cause unspecific stress as determined by measurement of the Hsp72/73 level and activity of the JNK2 kinase. Because an increased level of activated FAK was found in muscle of α7 integrin-deficient mice, the activation of c-Raf-1 kinase is triggered most likely by an integrin-dependent pathway. In accordance with this, the integrin α7-deficient mice, part of the integrin β1β1 variant in muscle is replaced by the β1A variant, which permits the FAK activation. A recent report describes that integrin activity can be down-modulated by the c-Raf-1/MAP2 kinase pathway. Specific activation of the c-Raf-1/MAP2 kinases by cell-permeable peptides in skeletal muscle of rabbits causes degeneration of muscle fibers. Therefore, we conclude that in α7 integrin-deficient mice, the continuous activation of c-Raf-1 kinase causes a permanent reduction of integrin activity diminishing integrin-dependent cell-matrix interactions and thereby contributing to the development of the dystrophic phenotype.

Integrins are a family of heterodimeric transmembrane receptors consisting of an α- and a β-subunit (1). Each subunit spans the plasmamembrane once, and in both cases the N termini are localized extracellularly binding the ligand, whereas the C termini face the cytoplasm. Integrins interact with proteins of focal adhesions that mediate the attachment of bundles of stress fibers (2). Moreover, numerous proteins involved in signal transduction are concentrated in these regions. Therefore integrins do not merely mediate cell attachment to the extracellular matrix but can also promote physiological processes such as migration and cell invasion. On the molecular level, integrin activation can modulate specific gene expression, cell proliferation, cell cycle progression, or prevention of apoptosis. Integrins can be considered as classic receptors without catalytic activity triggering a variety of signal transduction pathways upon ligand binding. In a simplified model, ligand occupation of integrins leads to the activation of the nonreceptor tyrosine focal adhesion kinase (FAK)1 by its autophosphorylation at Tyr-397 (2, 3). By this tyrosine phosphorylation a binding site (YpAEI motif) for the SH2 domain of protein-tyrosine kinases of the c-Src family is generated. The subsequent Src-dependent phosphorylation at Tyr-576 and Tyr-577 increases the catalytic activity of FAK (4). The Src-dependent phosphorylation at Tyr-925 (YpENV motif) generates a binding site for the SH2 domain of the adapter protein Grb2. The adapter protein Grb2 consists of a middle SH2 domain known to bind to phosphotyrosine residues and N- and C-terminal SH3 domains (5). The SH3 domains of Grb2 are known to interact with SOS proteins (PXXP motif, Ref. 6), which modulate Ras activity, finally resulting in an activation of c-Raf-1 kinase.

Integrins mediate signals both from the extracellular matrix to the cytoplasm (outside-in signaling) and from the cytoplasm to the outside (inside-out signaling) (for a recent review see Ref. 7). Modulation of integrin activity is therefore of major biological significance for a variety of physiological processes like cell migration or differentiation. By the process of inside-out signaling, integrin affinity for its specific ligand is modulated in response to intracellular signals. In a recent report, the Ras/Raf-initiated MAP2 kinase signal transduction pathway was described as a novel transcription-independent modulator of integrin activity (8). Activated MAP2 kinase down-regulates integrin activity without changing the phosphorylation state of the integrin β-subunit. Because ligand binding to certain integrins can result in a Ras/Raf-dependent activation of MAP2 kinase, this novel pathway most probably represents a negative feedback loop in integrin activity.

The αββ integrin, a receptor for the basement membrane glycoprotein laminin, is predominantly expressed in muscle (9, 10). Mice lacking the α integrin subunit develop a novel form of muscular dystrophy (11).

Here we describe an activation of the c-Raf-1/MAP2 kinase signal transduction pathway in muscle tissue derived from α7 integrin-deficient mice. Specific activation of this pathway in rabbit skeletal muscle is involved in degeneration of muscle fibers. Therefore, permanent activation of this signaling cascade might contribute to the manifestation of the dystrophic phenotype by down-modulation of integrin activity.

EXPERIMENTAL PROCEDURES

Animals and Antibodies—Tissues were obtained from mice (11, 12) at several ages ranging from 30 days to 1.5 years. Chinchilla bastard rabbits were used for the injection experiments. Primary antibodies used were rabbit sera, goat sera, or mouse monoclonal antibodies against c-Raf-1 kinase, MAP2 kinase, JNK2, Hsp72/73, FAK, protein-tyrosine, and activated MAP2 kinase, all from Santa Cruz Biotechnology. The integrin β1-specific monoclonal antibody was purchased from

1 The abbreviations used are: FAK, focal adhesion kinase; MAP, mitogen-activated protein; PBS, phosphate-buffered saline.
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Life Technologies, Inc. The integrin β1-specific antiserum was kindly provided by Dr. Staffan-Johansson, and the β1 integrin-specific antiserum was provided by Dr. Engvall. The monoclonal antibodies Q19/10 and HV25-19 specific for the PreS2 domain of the hepatitis B virus surface antigen were kindly provided by Dr. Gerlich and Dr. Mimms.

Protein Purification—The expression plasmid pPreS1/PreS2 for the PreS1/PreS2 protein, the control plasmid pPreS2-38 coding for the mutant harboring a defective translocation motif, as well as the purification procedure were described recently (16).

Protein Analysis—Tissues were lysed in 20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 0.2 mM EDTA, 1 mM EGTA, 10 mM sodium β-glycerophosphate, 50 mM sodium fluoride, 1% Triton X-100, 1 mM sodium orthovanadate, 0.5 mM phenylmethylsulfonyl fluoride (Sigma), 0.15 unit/ml aprotinin, and 2 μg/ml leupeptin by sonication and with a Potter homogenizer. Insoluble material was removed by centrifugation at 20,000 × g at 4 °C for 15 min. Protein concentrations were determined by Bradford assays (Bio-Rad).

For Western blot analysis, proteins were separated by SDS-polyacrylamide gel electrophoresis (13) and transferred onto polyvinylidene difluoride membranes (Millipore, 0.45 μm) and processed as described previously (11). Briefly, after blocking the membranes with 0.05% (v/v) containing 10% nonfat dry milk powder (blocking solution), the blots were incubated at 4 °C under permanent shaking for 15 min. After centrifugation the supernatant was incubated with a specific polyclonal antibody for 90 min at room temperature. After washing, the rabbit polyclonal antibodies were detected using anti-rabbit peroxidase-conjugated antibodies (Amersham Biotech), respectively. Following 45 min of washing, the bands were visualized using the ECL reagent (Amersham Pharmacia Biotech).

Immunoprecipitations—Lysates were prepared as described above. To the lysate containing 500 μg of total protein, 20 μl of a protein A/G-Sepharose suspension (Santa Cruz Biotechnology) were added and incubated at 4 °C under permanent shaking for 15 min. After centrifugation the supernatant was incubated with a specific polyclonal antibody for 90 min at room temperature. After washing, the protein A/G-Sepharose suspension was added, and the incubation was continued for 45 min. The beads were washed twice in 500 mM LiCl, 100 mM Tris-HCl, pH 7.4, followed by a final wash in 10 mM Tris-HCl, pH 7.4. The washed precipitates were resuspended in adequate buffer.

Immunocomplex Assays—For each assay, immunoprecipitated kinases were resuspended in 36 μl of kinase buffer and incubated at 30 °C for 15 min. The reaction was stopped by addition of SDS sample buffer. An aliquot was loaded on a 10% SDS gel and analyzed by autoradiography. In the case of c-Raf-1, the kinase buffer contained 25 mM Tris-HCl, pH 7.5, 25 mM β-glycerophosphate, 10 mM MgCl2, 10 mM ATP, 1 mM dithiothreitol, in the presence of 5 μCi of [γ-32P]ATP and 1 μg of MEK (Santa Cruz Biotechnology) per assay. In the case of MAP2, the kinase buffer contained 25 mM Hepes, pH 7.5, 10 mM MgCl2, 1 mM dithiothreitol, 5 μCi of [γ-32P]ATP, and 2 μg/ml of basic myeloglycoprotein (Santa Cruz Biotechnology) as a substrate per assay. In the case of JNK2, the kinase buffer contained 20 mM Hepes, pH 7.6, 20 mM MgCl2, 10 mM β-glycerophosphate, 2 mM dithiothreitol, 50 μM ATP, 5 μCi of [γ-32P]ATP, and 1 μg of recombinant glutathione S-transferase-Jun (Santa Cruz Biotechnology).

Immunofluorescence—Cryosections of soleus muscle were obtained and processed as described previously (11). Briefly, after blocking the samples with 5% normal goat serum in PBS/Tween for 1 h, incubation with the c-Raf-1 kinase-specific rabbit derived antiserum diluted was performed in 2% normal goat serum in PBS/Tween for 1 h. The slides were washed and incubated with the Cy3-conjugated secondary antibody (Dianova) diluted in 2% normal goat serum in PBS/Tween for 45 min. After final washing, sections were mounted in Vectashield (Vector Lab, Burlingame, CA) and analyzed on an DMR fluorescence microscope (Leica).

Injection Experiments—Protein was injected into the erector trunci muscle in a concentration of 2.5 μmol (100 μl) in PBS in a 1:1 dilution with green ink (Sigma). To minimize individual effects in these experiments, the PreS1PreS2 protein was injected into the left muscle, and the mutant was injected into the right muscle. After 24 h, the equal amount of protein was injected again. The rabbits were sacrificed 24 h later, and the muscle specimen was prepared as described above. The expression plasmid pPreS1/PreS2 was transfected into the right hindlimb muscle in a concentration of 2.5 μg of total protein, 20 mM MgCl2, 1 mM dithiothreitol, 50 mM sodium fluoride, 1% Triton X-100, 1 mM sodium orthovanadate, 0.25 M sucrose, 0.5 mM phenylmethylsulfonyl fluoride (Sigma), 0.15 unit/ml aprotinin, and 2 μg/ml leupeptin by sonication and with a Potter homogenizer. Insoluble material was removed by centrifugation at 20,000 × g at 4 °C for 15 min. Protein concentrations were determined by Bradford assays (Bio-Rad).

In vivo, the activation of c-Raf-1 kinase can be transduced via MEK and results in subsequent activation of MAP2 kinase. Therefore, the activity of MAP2 kinase in lysates of hind limb and diaphragm was determined by immunocomplex assays using recombinant MEK as substrate in lysates derived from skeletal muscle of hind limb and diaphragm (Fig. 1A).

To exclude the possibility that the observed effect was due to an increased expression level of these kinases, the total amounts of c-Raf-1 kinase (Fig. 1C) and MAP2 kinase (Fig. 1D) were determined by Western blot analysis of hind limb-derived lysates. The comparison of integrin α7-deficient mice and controls shows equal amounts of both kinases. Moreover, it was shown by Western blotting that the precipitates that were subjected to immunocomplex assays contained comparable amounts of MAP2 kinase (Fig. 1E) or c-Raf-1 kinase (data not shown). This set of experiments confirms that the observed increase of c-Raf-1/MAP2 kinase activity in integrin α7-deficient mice is indeed due to an activation of the c-Raf-1/MAP2 kinase signal transduction pathway.

Activation of c-Raf-1/MAP2 Kinase Signal Transduction Pathway in α7 Integrin-deficient Mice Is a Specific Process—Activation of c-Raf-1/MAP2 kinases can be mediated by specific pathways that are induced by specific receptor/ligand interactions. Nevertheless, an activation by unspecific cellular stress factors has also been described (14). Therefore, it was investigated whether integrin α7 deficiency gives rise to unspecific cellular stress. Two parameters were chosen for analysis. The level of Hsp72/73, which is known to be elevated by cellular stress, was determined by Western blotting in lysates derived from hind limb or diaphragm, respectively. The comparison of wild type controls and α7 integrin-deficient mice revealed no difference in Hsp72/73 levels (Fig. 2A). In addition, the activity of JNK2 (also designated SAPK for stress-activated protein kinase) was determined by immunocomplex assays. Liver tissue derived from transgenic mice overproducing the LHBs protein of hepatitis B virus (12) served as the positive control. The determination of the JNK2 kinase activity in both tissues (Fig. 2B) confirmed that the loss of α7 integrin does not cause unspecific cellular stress.

Activation of c-Raf-1 Kinase Is a Muscle Cell-specific Process—Skeletal muscle tissue consists of several different cell types. Therefore we wanted to investigate whether the observed activation of c-Raf-1 kinase takes place in muscle cells. Activation of c-Raf-1 kinase is associated with a translocation of the activated kinase to the membrane (Fig. 3A). Cryosections of the soleus muscle were stained with a c-Raf-1 kinase-specific antiserum and analyzed by indirect immunofluorescence. In the case of sections derived from wild type mice, a weak homogenous staining of the muscle fibers was observed characteristic for the inactive state of c-Raf-1 kinase. In contrast, the staining of tissues derived from α7-deficient mice showed an

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One representative assay using 100-day-old mice is shown. The given factors are the mean values of three independent experiments.

The level of MAP2 kinase (lanes 1 and 2) derived from integrin α7-deficient mouse (lane 2), about 4-fold activation of c-Raf-1 kinase versus the age-and sex-matched wild type controls (lane 1) was observed. In the case of the diaphragm lysates (lanes 3 and 4), about 3-fold activation of c-Raf-1 kinase as substrate was observed. The given factors are the mean values of three independent experiments. One representative assay using 100-day-old mice is shown. B, activity of MAP2 kinase was determined by immunocomplex assays using MBP and [γ-32P]ATP as substrates. In the case of hind limb lysates (lanes 1 and 2) derived from integrin α7-deficient mice (lane 2), about 2.2-fold activation was measured, and in the case of the diaphragm lysates (lanes 3 and 4) derived from the integrin α7-deficient mice, about 3.3-fold activation of the MAP2-kinase was determined as compared with the respective wild type controls (lanes 2 and 4). The given factors are the mean values of three independent experiments. One representative assay using 100-day-old mice is shown. C and D, Western blot analysis of lysates derived from hind limb of an integrin α7 knock out mouse (lane 1) and an adequate wild type control (lane 2) using a c-Raf-1 kinase-specific antiserum (C) or a MAP2 kinase-specific antiserum (D) shows that neither the level of the c-Raf-1 kinase (C) nor the level of MAP2 kinase (D) is affected by α7 integrin deficiency. E, hind limb-derived lysates of an integrin α7 knock out mouse (lane 1) and adequate wild type control (lane 2) were immunoprecipitated using the MAP2-specific monoclonal antibody as described above for panel B. The immunoprecipitates were analyzed by Western blotting using the MAP2-specific goat-derived antiserum. The blot shows that comparable amounts of MAP2 were precipitated.

The loss of integrin α7 subunit affects the amount of its heterodimeric binding partner β1. Western blot analysis using β1 integrin-specific antisera was performed. As shown in Fig. 4A, the intensive staining of the subsarcolemmal region, reflecting the translocation of activated c-Raf-1 kinase to the plasma membrane (Fig. 3B). These results indicate that the observed activation of c-Raf-1 kinase occurs within muscle cells.

In the Integrin α7-deficient Mice Part of the Integrin β1, Variant in Muscle Is Replaced by the β1, Variant—The loss of one integrin α subunit could influence the overall expression pattern of integrin subunits. To determine whether the loss of the integrin α7 subunit affects the amount of its heterodimeric binding partner β1, Western blot analysis using β1 integrin-specific antisera was performed.
deficiency of the α7 subunit does not influence the total amount of β1 integrin.

In mice, two different variants of the integrin β1 subunit have been described: β1A and β1D. The integrin β1D variant is strictly muscle-specific, whereas the β1A variant is ubiquitously expressed. Western blot analysis using an antisera specific for the integrin β1D variant revealed that integrin αv deficiency is accompanied with a strong reduction of the β1D level in muscle (Fig. 4B). Because the total amount of β1 integrins remains unchanged, it can be concluded that a decrease in the β1D level can only be adjusted by an increase in β1A. Therefore, in the integrin αv-deficient mice part of the β1D variant in muscle is replaced by the β1A variant.

Tyrosine Phosphorylation of FAK Is Increased in αv Integrin-deficient Mice—Activation of c-Raf-1 Kinase can be initiated by a broad variety of different receptors and signal transduction pathways. In the case of an integrin-dependent activation, the initial step in the cascade is the tyrosine phosphorylation of FAK at Tyr-397 and Tyr-925 (2), finally resulting in the activation of c-Raf-1 kinase.

To test whether the tyrosine phosphorylation of FAK is affected in αv integrin-deficient mice, FAK was immunoprecipitated from lysates derived from the hind limb of wild type and αv integrin-deficient mice. The precipitates were analyzed by Western blotting using a phosphotyrosine-specific serum. The Western blot analysis shows (Fig. 5) a strong increase of an anti-phosphotyrosine reactive band at the expected size of FAK (125 kDa; indicated by an arrow).

Western blot analysis shows (Fig. 5) a strong increase of an anti-phosphotyrosine reactive band at the expected size of FAK (125 kDa). This indicates that the lack of the αv integrin causes an activation of FAK.

The Cell-permeable HBV-derived PreS1/PreS2 Protein Activates the MAP2 Kinase in Rabbit Muscle—In the final set of experiments, it was investigated whether the activation of the c-Raf-1/MAP2 signal transduction cascade per se triggers the degeneration of muscle cells in vivo and therefore the subsequent development of a dystrophic phenotype. To address this question, an activator of the c-Raf-1/MAP2 signal transduction pathway was injected into the erector trunci muscle of rabbits.

For this purpose, the HBV-derived PreS1/PreS2 protein was used. This protein was chosen because it possesses two properties: (i) Because of an amphipatic α helix at the C terminus of the PreS2 domain, the protein is cell-permeable.2 (ii) The PreS2 domain was shown to trigger the activation of the c-Raf-1/MAP2 signal transduction cascade (for an overview see Ref. 16). A protein with mutations in the amphipatic α helix abolishing cell permeability and the activator function (17) served as the negative control in this study. These mutations do not affect the reactivity with the antisera Q19/10 and HBV25-19, which were used to detect these proteins.

For the injection experiments, the protein solutions were mixed with an equal volume of green ink to label the injection channel. Two injections were performed over a time period of 48 h, and the tissues were prepared as described under "Experimental Procedures."

To confirm that the PreS1/PreS2 protein indeed penetrates the muscle cells, immunostaining of cryosections with the PreS2-specific antisera was performed. The microscopic analysis (Fig. 6) shows the injection channel labeled by the green ink (Fig. 6, A and C). In the case of the control, only the intercellular space was labeled, whereas the cells remained unstained (Fig. 6D). In the case of the PreS1/PreS2 protein injection, muscle cells adjacent to the injection channel were stained, indicating that the PreS1/PreS2 protein has entered the cells (Fig. 6B).

In the next set of experiments it was analyzed whether the internalized PreS1/PreS2 protein truly activates the c-Raf-1/MAP2 signal transduction cascade under these conditions. To test this, 10 cryosections covering the injection channel were
The phenotype of the muscle tissue was investigated by histochemical analysis of the cryosections. The Fig. 7 (B and C) are composed of overlapping micrographs and provide an overview at a 100-fold magnification of the tissue close to the injection channel as marked by the green ink. In both samples mild fiber necrosis can be detected because of the injection event. The overview of the control injected tissue (Fig. 7C) as well as the more detailed analysis (200-fold magnification, Fig. 7F) show all normal features of intact muscle fibers. In contrast to this, the PreS1/PreS2 protein injected tissue (Fig. 7B, 200-fold magnification; Fig. 7, D and E) shows severe changes of muscle tissue histology. An increased variability of muscle fiber diameter and many fibers with centrally localized nuclei could be observed. These are typical characteristics of muscle fiber regeneration. Moreover, basophilic degenerated and necrotic fibers as well as infiltration of phagocytizing cells could be detected. This histological analysis of the PreS1/PreS2 protein injected tissue reveals ongoing processes that are typical for muscle fiber degeneration and regeneration resembling early stages of muscular dystrophies. These data demonstrate for the first time that the activation of the c-Raf-1/MAP2 signal transduction pathway can cause the typical symptoms of a muscular dystrophy.

**DISCUSSION**

Integrin $\alpha_7$ deficiency was shown to cause a novel form of muscular dystrophy in mice (11). In accordance, patients lacking the integrin $\alpha_7$ subunit develop a myopathy (18). Integrin $\alpha_7$-deficient mice are viable and fertile, indicating that myogenesis in principle is not affected by loss of $\alpha_7$ integrin. This gives rise to the question of which molecular trigger causes the destruction of the organized muscle architecture, resulting in the dystrophic phenotype.

In a recent report it was described that activated Ras/c-Raf-1 pathway is able to down-modulate the activity of $\beta_1$ or $\beta_3$ integrins via MAP2 kinase in a transcription-independent manner (8). Modulation of integrin activity is of major significance for a variety of biological processes as cell migration, which can be considered as an alternation between a high affinity (attachment) and low affinity (detachment) state. Because integrins are known to activate MAP2 kinase by the Ras/Raf mediated cascade, this pathway most probably represents a negative feedback loop controlling integrin activity.

In this study we demonstrate that $\alpha_7$ integrin deficiency results in a permanent activation of the c-Raf-1/MAP2 kinase signal transduction pathway in muscle cells. In principle, the observed activation of this signaling cascade could be because of an unspecified secondary effect. However, arguing against an unspecified effect are two observations. First, an activation because of unspecified cellular stress was excluded because both tested markers indicating cellular stress, increased activity of JNK2 and increased amount of Hsp72/73, were not affected by $\alpha_7$ integrin deficiency. Second, the activation of c-Raf-1 and MAP2 kinases in $\alpha_7$ integrin-deficient mice is not an age-dependent process, because it was observed at different age stages (between 30 days and 1.5 years; data not shown). The dystrophic phenotype becomes evident in the diaphragm at an age of 2 months (11). Therefore these data indicate that the activation of c-Raf-1/MAP2 kinase signal transduction pathway by the $\alpha_7$ integrin deficiency can be observed before the phenotype of muscular dystrophy becomes evident. Therefore, it can be excluded that the activation of this signaling cascade is a secondary effect caused by the degenerating/regenerating processes of the muscular dystrophy.

In the integrin $\alpha_7$-deficient mice an increased phosphorylation of FAK can be observed, suggesting that the activation of $\beta_1$ integrins is involved in the activation of c-Raf-1 kinase. In a
recent report it was demonstrated that the integrin α7β1 negatively regulates the function of the integrin α5β1 (20). Therefore, the lack of the α7 subunit could result in an activation of the integrin α5β1, resulting in an activation of FAK.

Moreover in the integrin α7-deficient mice a significant change in the composition of the β1 integrin variants occurs. The integrin β1D variant is the only integrin β1 variant that is expressed in the sarcolemma of wild type muscle. In the integrin α7-deficient mice part of the integrin β1D variant is replaced by the integrin β1A variant, resulting in an unaltered amount of β1 integrins. These two variants only differ in their cytoplasmic domains, also affecting their function (21–24). The integrin β1D variant mediates a tighter binding to the matrix, and its activity cannot be regulated (21, 23). Therefore, the reduction of the β1D variant could contribute directly to a diminished tissue stability. In contrast, the integrin β1A variant is well known to be involved in dynamic functions, for example the activation of FAK (21). An interaction of integrin β1D and FAK has not been found in vivo. That might be due to the fact that the in vivo binding motif for FAK only exists in the integrin β1A variant (15). Under the conditions of a strong overproduction, however, a β1D-dependent activation of FAK in vitro was reported (23). Therefore, in the integrin α7-deficient mice the change in the integrin β1 variant composition in combination with the loss of the integrin α7 negative regulatory effect can be causative for the observed activation of FAK and subsequently of the c-Raf-1/MAP2 kinase signal transduction pathway.

As introduced above, activated MAP2 kinase can down-modulate integrin activity (8). That can reduce cell-matrix interactions that are crucial for muscle integrity. To investigate whether activated MAP2 kinase can trigger the destruction of muscle architecture, we established a novel experimental approach: we activated the c-Raf-1/MAP2 kinase signal transduction pathway in muscle fibers by the means of an exogenous protein and investigated whether this activation causes degeneration of muscle tissue.

For this purpose, we used the HBV-derived cell-permeable
PreS1/PreS2 protein. The PreS2 domain triggers an activation of the c-Raf-1/MAP2-kinase signal transduction pathway. These experiments were controlled using a mutant (17)² that displays no activator function and lacks the cell permeability. The reactivity with the antibodies used for the detection of these HBV-specific proteins is not affected by this mutation.

In one set of experiments we determined the reliability of this novel experimental approach. Immunofluorescence microscopy analysis reveals that the PreS1/PreS2 protein indeed enters the muscle cells surrounding the injection channel, whereas the mutant was only found in the intercellular space.

Western blot analysis using an activated MAP-specific antisera confirmed that the internalized PreS1/PreS2 protein triggers the activation of MAP2 kinase in this experimental system, whereas in the case of the control injected animals no activation could be observed, underlining the specificity of this experimental system. This set of experiments demonstrates that the cell-permeable PreS1/PreS2 protein is suitable to trigger specifically an exogenous activation of the c-Raf-1/MAP2 signal transduction cascade.

This novel experimental system provides several advantages. It combines an easy handling with short latency periods. The PreS2 domain triggers highly specifically the activation of the c-Raf-1/MAP2 kinase cascade which has been investigated in detail (16). Furthermore, the existence of the mutant provides the proper control. Because of the spreading capacity of this cell-permeable protein, many cells distant from the injection channel are affected by internalization of the effector protein. This experimental approach provides a model to analyze the direct biological responses of an effector protein in the context of intact tissue.

The physiological effects of the activation of the c-Raf-1/ MAP2 signal transduction cascade on the architecture of the muscle tissue were investigated by histochemical analysis. Only in the case of the PreS1/PreS2 protein injection, severe changes of muscle tissue histology could be observed. Typical characteristics of muscle fiber degeneration like basophilic degenerated and necrotic fibers accompanied with infiltration of phagocytizing cells could be observed. An increased variability of muscle fiber diameter and many fibers with centrally localized nuclei indicated that also muscle cell regeneration occurs. These data demonstrate that the activation of the c-Raf-1/ MAP2 kinase signal transduction cascade per se is sufficient to induce muscle degeneration and regeneration resembling early stages of muscular dystrophies.

In light of these data we conclude that integrin α7 deficiency causes a permanent c-Raf-1/MAP2 kinase activation. The activation of MAP2 kinase triggers muscle cell degeneration and regeneration. Therefore, in the integrin α7-deficient mice the continuous activation of MAP2 kinase contributes to the development of the dystrophic phenotype.

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