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The Thrombin Receptor Is Present in Myoblasts and Its Expression Is Repressed upon Fusion*

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Cultured myoblasts derived from limb muscle of newborn rats express thrombin receptor immunoreactivity on their surface. Receptor expression is repressed upon myoblast fusion. This is due at least in part to a decrease in the amount of the thrombin receptor mRNA. Addition of thrombin triggers calcium transients only in mono- but not multinucleated muscle cells. Furthermore, thrombin increases the rate of myoblast proliferation that coincides with an activation of mitogen-activated protein kinase. Northern analysis of thrombin receptor mRNA expression in skeletal muscle showed that the transcript is present at a relatively high level at birth, but is almost undetectable in the adult. By *in situ* hybridization, the mRNA at birth appeared to be present mostly in mononucleated cells grouped in clusters, but not in muscle fibers. Very few nuclei surrounded by a mRNA signal were present on muscle sections of rats 24 days postnatally. These results suggest that the thrombin receptor plays a role in muscle development.

Most of the cellular effects of thrombin are mediated via the thrombin receptor (ThR).[1] This receptor is a member of the G protein-coupled receptor family and is activated by cleavage of an Arg–Ser bond within its N-terminal extracellular domain (1, 2). The new N terminus generated by this proteolytic event acts as a tethered ligand for the receptor (1, 3). Synthetic peptides corresponding to the newly created N terminus mimic the effects of thrombin in mobilizing Ca^{2+} in *Xenopus* oocytes microinjected with ThR mRNA, in causing platelet aggregation (1) and in several other systems (4) including neuronal cells (5, 6). Recently ThR knockout mice were generated (7). Half the mutant embryos die at embryonic day 9–10, whereas the rest continue to develop without any obvious phenotype. However, platelets of the surviving animals degranulate and aggregate in response to thrombin. It was therefore suggested that the ThR plays a role in development and that there is a second receptor which can be activated by thrombin (7).

During the course of muscle development, muscle cell precursors migrate from the somites to the limbs where they continue to proliferate and eventually align and fuse to form multinucleated myotubes that are irreversibly postmitotic. The identity of the factors that control myoblast proliferation and differentiation *in vivo* is not known. From *in vitro* work, however, several agents have been shown to play a role in these processes. For example, fibroblast and epidermal growth factors are mitogenic for myoblastic cells and inhibit their differentiation (8, 9), whereas transforming growth factor-β suppresses only differentiation (10, 11). As muscle differentiation ultimately involves the generation of postmitotic multinucleated cells, it is essential that pathways leading to DNA synthesis are shut down in muscle fibers.

The cellular effects of thrombin have been most intensely studied in blood and blood vessels. Thrombin for example activates platelets (12), chemoattracts neutrophils (13, 14), activates endothelial cells and alters their permeability (15, 16), and is mitogenic for fibroblasts (17, 18) and smooth muscle cells (19, 20). However, ThR expression has been detected in somewhat unexpected places such as the nervous system (21–23), and the activation of the ThR in neural cells triggers neurite retraction (5, 6, 24) and reversal of astrocyte stellation (25–27).

Recent studies have suggested that thrombin may play a role in synapse elimination in muscle (28). We therefore sought to investigate the expression of the ThR on the pre- and postsynaptic cell, starting here with muscle. Previous work has shown that thrombin increases thymidine incorporation and inhibits creatine kinase expression in the muscle cell line BC3H1, indicating that the ThR is expressed by these cells (29). However, BC3H1 cells do not fuse, and they have characteristics of smooth muscle cells (30). Due to this we thought it was necessary to unequivocally study thrombin-mediated responses and the regulation of ThR expression in a different muscle cell system, preferably in primary cells. It is reported here that the ThR is present in skeletal muscle, and its expression is developmentally regulated. Myoblasts but not myotubes express the receptor *in vitro*. ThR activation in myoblasts induces a Ca^{2+} signal and promotes their proliferation probably through the activation of the MAP kinase pathway.

EXPERIMENTAL PROCEDURES

Materials

Human α-thrombin was a generous gift of Dr. Stuart Stone, MRC Center, University of Cambridge, Cambridge, UK. ThR peptides were synthetically prepared, purified by high performance liquid chromatography, their molecular weight ascertained by mass spectroscopy, and their composition verified by amino acid analysis. Insulin, transferrin, lysophosphatidic acid, and phorbol 12-myristate 13-acetate were from Sigma.

Cell Culture

Isolation of Cells—Primary muscle cultures were established from the upper hind limb muscles of newborn rats. The animals were mostly at P0 or P1 but rats at P2 and P3 were occasionally used. Dulbecco's modified Eagle's medium (DMEM) with particular additions was used for dissection, various manipulations, and cell growth. The limb parts were freed from the skin, and the muscles were separated from the bone and other visible tissues. The muscles were minced with fine scissors, trypsinized (0.25% trypsin, Life Technologies, Inc.) for 45 min, and

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[1] The abbreviations used are: ThR, thrombin receptor; MAP, mitogen-activated protein; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

triturated in the presence of 5% horse serum (Amimed) and DNase I (50 μg/ml, Boehringer Mannheim). After filtration through a nylon strainer (70 μm, Falcon), the suspension was spun, and the cells were resuspended in the presence of 10% horse serum and filtered. A 1.5-h preplating step followed (cells derived from one animal/3 ml of medium/10-cm dish). Myoblasts poorly adhere to plastic, and numerous cells of other types are eliminated at this stage. After preplating, the suspensions were combined and centrifuged, and the cells were resuspended (cells derived from one animal/1 ml) in DMEM containing 10% horse serum and 5% chicken embryo extract (Life Technologies, Inc.).

Substratum—Cells were plated on laminin on glass or plastic surfaces that were precoated with poly-L-lysine. Dishes (35 mm, Falcon) or glass coverslips (graded 12 mm, Eppendorf or plain 13 and 22 mm, Chance Proper) were treated with a solution of poly-L-lysine (50 μg/ml, Sigma M_r 70,000–150,000) for 30 min at 37 °C, washed with water, and air-dried in the hood. Laminin (40 μg/ml PBS, Collaborative Biomedical Products) was then added at 0.5 $\mu g/cm^2$ and left for about 2 h in a humidified and 37 °C-heated incubator. The laminin solution was aspirated just before plating.

Plating and Growth—Cells were plated at densities that varied according to the application. Many cells in the final suspension, although they excluded trypan blue, did not adhere and were removed when the medium was changed the following day. Experiments on myoblasts were carried out within the first 2 days after plating. Otherwise, the chicken embryo extract was removed 3 days after plating, and half a day later, the antimitotic drug cytosine arabinoside (10 μM, Sigma) was added. Fusion usually was minimal after 3 days in culture and was extensive by the 4th and 5th day. Some mononucleated cells, mostly fibroblasts, remained after 7 days.

Immunocytochemical analysis for MyoD1 carried out 1–2 days after plating showed that the nuclei of 60–70% of the cells were positive for this muscle-specific transcription factor (31). However, the number of myoblasts is probably higher than that estimated by MyoD1 staining. The level of MyoD1 is known to be decreased by serum (32), and we find MyoD1-negative cells that have a typical myoblast morphology.

Immunocytochemical Analysis

ThR Staining—For the immunodetection of the ThR on the surface of living cells, a rabbit peptide antibody was used (gift of Dr. Stuart Stone) (see also Ref. 33). This antibody was raised against the mouse ThR 17-mer agonist peptide SFFLRNPSENTFELVPL (amino acids 42–58) and affinity-purified against the rat/mouse ThR 8-mer agonist peptide SFFLRNPS. The cells were washed four times during 1 h in DMEM containing 50 μg/ml delipidated BSA and incubated for 20 min on bench with the ThR antibody (4 μg/ml). After a brief wash, the cells were fixed for 20 min with 4% paraformaldehyde, washed in PBS, incubated for 30 min with Cy3-conjugated Fab fragments of goat anti-rabbit immunoglobulins, washed again, and mounted.

MyoD1 Staining—Cells were fixed and permeabilized by a 15-min incubation in 1% paraformaldehyde and 0.2% Triton X-100 in PBS, washed, and blocked for 10 min in 10% serum in PBS. Three 20–30-min incubation steps (with intervening washes) followed: with the anti-MyoD1 antibody (1:50; Novocastra Laboratories Ltd.), with a biotinylated anti-mouse IgG (1:250; Biospa) and with the streptavidin alkaline phosphatase complex (1:200; Biospa). The color reaction was carried out for 3–5 min in the presence of 340 μg/ml nitro blue tetrazolium (Sigma) and 175 μg/ml 5-bromo-4-chloro-indolyl-phosphate (Boehringer Mannheim). Unstained nuclei were visualized with Hoechst dye (5 min in 10 μg/ml bisbenzimide in PBS). For double staining experiments, 4% formaldehyde was used for fixation and a Cy3-conjugated anti-mouse IgG was used for detection of the primary antibody (see below).

MAP Kinase Staining—After the stimulation with various agents, the cells were fixed and permeabilized (4% formaldehyde and 0.2% Triton X-100), blocked in 5% serum. The cells were then incubated for 30 min with a rabbit anti-MAP kinase antibody (1:500; Upstate Biotechnology Inc.), washed, incubated for 30 min with a rhodamine-conjugated goat anti-rabbit IgG (1:100; Jackson ImmunoResearch Laboratories Inc.), and washed again, and the nuclei were labeled with Hoechst dye. For double staining studies, the rabbit anti-MAP kinase antibody was added together with the monoclonal anti-MyoD1 antibody. The secondary antibodies were fluorescein isothiocyanate-labeled goat anti-rabbit IgG and Cy3-conjugated Fab fragments of goat anti-mouse IgG (each at 1:100; Jackson ImmunoResearch Laboratories Inc.).

Changes in Intracellular [Ca^{2+}]

Cells grown on graded coverslips for the specified periods were washed twice, 5 min each, in phenol red-free DMEM (Life Technologies, Inc.) containing 50 μg/ml delipidated BSA and then incubated for 50 min at 37 °C with 1 μM fura-2/AM (Molecular Probes), containing 0.06% w/v Pluronic (Molecular Probes) and 0.3% v/v Me_2SO (Merck) and washed again for 5 min. Thus, altogether the cells were under serum-free conditions for about 70 min before commencing the experiment. The coverslip was placed in the perfusion chamber mounted on an upright microscope (Axioscop FS, Zeiss) equipped with a xenon lamp (XBO 75 watts, Zeiss), double filter wheel (Ludl) within bandpass (340/10 and 380/10 nm) and optical density filters (0.3 and 0.6 OD), a shutter, a quartz fiber optic light guide (Luigs & Neumann), a 475-nm dichroic mirror (Lys & Optic), and a 40 × water immersion objective (Achroplan, Zeiss). Fluorescence was visualized by an intensified video CCD camera (Photonic Science). The video output was directly connected to a Macintosh-based imaging system (IonVision, ImproVision). Eight video frames were averaged at each of the 340- and 380-nm excitation wavelengths. The direct (noncorrected) ratio of the emission intensities (340/380) which is a measure of intracellular Ca^{2+} concentration (34) was displayed on line and for subsequent off line analysis, background autofluorescence was subtracted.

Experiments were carried out at room temperature (21 ± 1 °C). The basic medium used in these experiments was phenol red-free DMEM containing 50 μg/ml BSA and 10 mM HEPES (pH 7.4 ± 0.1). The perfusion system consisted of multiple channels of electric valves and a pump, all controlled through the imaging system. The volume of the perfusion chamber was about 0.6 ml, and the perfusion rate was set to 5 ml/min.

Cell Proliferation Assay

Cells were plated and grown for 24 h in the presence of horse serum and chicken embryo extract as described above. The cells were washed four times during 1 h in DMEM containing 50 μg/ml delipidated BSA and subjected in the same solution to different specified conditions. After 24 h the cells were dissociated by trypsinization and counted in a Coulter counter (Coulter Electronics Ltd.). In some experiments the fraction of cells expressing MyoD1 was determined in parallel cultures before and after the treatment. No appreciable difference in this fraction was seen in response to the tested mitogens.

Northern Blot Analysis and in Situ Hybridization

Northern analysis of ThR expression was carried out as described previously (22) except that the chemiluminescent substrate CSPD (Tropix) was used here. Signals were the result of 10–20-min exposures of X-Omat S films (Eastman Kodak Co.).

In situ hybridization on rat muscle sections was carried out with digoxigenin-labeled riboprobes essentially as described (35). For newborn rats, the upper part of the hind leg was cut out of the animals and was freed from the skin, whereas for the muscle of P14 and P24 rats, the gluteus maximus was dissected out. Tissues were immersed for 5–10 min in 5 mM EDTA in PBS, embedded in Tissue Tek (Miles Inc.), frozen in isopentane at −40 °C, and stored at −80 °C until use. Hybridization was carried out for 24 h at 70 °C with 400 ng/ml hydrolyzed digoxigenin-labeled cRNA probe of the rat ThR (22). The alkaline phosphatase color reaction was carried out for 24 h in the presence of 340 μg/ml nitro blue tetrazolium and 175 μg/ml 5-bromo-4-chloro-indolyl-phosphate. Subsequently, the sections were rinsed in PBS, incubated for 5 min with Hoechst dye (10 μg/ml bisbenzimide in PBS), washed, and embedded in Kaiser's gelatin (Merck).

RESULTS

The ThR Is Expressed by Mono- but Not by Multinucleated Muscle Cells—Myoblasts put into culture and grown under appropriate conditions proliferate and eventually fuse to form postmitotic multinucleated cells. To detect the ThR on the cell surface, immunocytochemical analysis was carried out on live cells using an affinity-purified anti-ThR antibody that can block thrombin-induced responses (33). The great majority (80–90%) of myoblasts appeared to express ThR immunoreactivity on their surface (Fig. 1*a*). The signal was seen on much of the cell surface including the processes and their tips. The staining is competed away if the anti-ThR antibody is preincubated with the mutant peptide FSFLRNPS, which is recognized by the antibody but does not cause receptor activation (Fig. 1*b*). No signal was seen if the cells were pretreated for 20 min with either 0.2 nM thrombin or 100 μM ThR-activating peptide SFFLRNPS (not shown). Such conditions are known to

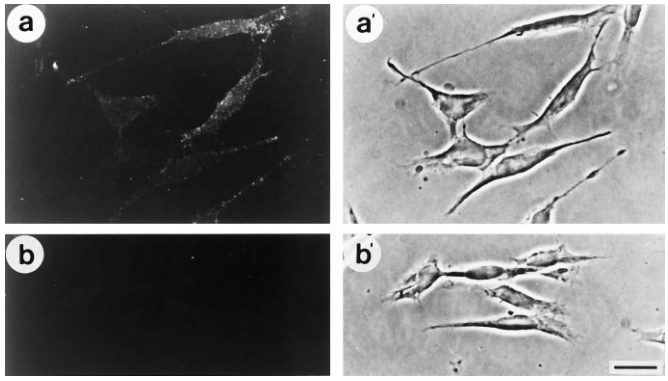

FIG. 1. **ThR immunoreactivity is present on the surface of myoblasts.** Cells derived from the hind leg muscles of P1 rats were grown in culture for 1 day and a density of ~300 cells/mm^2 was attained. The cells were then washed four times during 1 h in DMEM containing 50 μg/ml delipidated BSA, and the ThR immunoreactivity on their surface was detected as described under "Experimental Procedures." The corresponding phase images are presented (*a'* and *b'*). No staining was seen (*b*) if the antibody was preincubated with an excess of the mutant ThR peptide FSFLRNPS, which is recognized by the antibody but does not cause receptor activation. *Calibration bar* represents 20 μm.

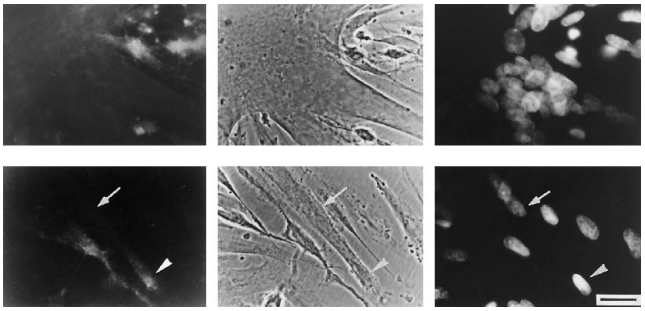

FIG. 2. **The ThR is not detectable on the surface of multinucleated muscle cells.** Muscle cells were grown for 3 days, a stage at which myotubes started to form, washed, and stained for the ThR as described in the legend to Fig. 1. The corresponding phase images and Hoechst nuclear staining are presented. No staining was seen in multinucleated cells (*upper panels* and *arrow* in *lower panels*), whereas a fusing myoblast (*arrowhead* in the *lower panels*) is positive. *Calibration bar* represents 20 μm.

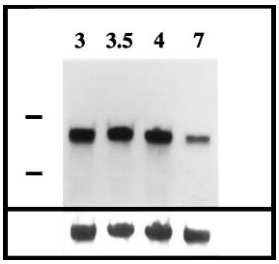

FIG. 3. **Decrease in ThR mRNA in muscle cultures coincides with fusion.** RNA was prepared from cultures of muscle cells taken at the indicated days after plating, and 6-μg samples were processed for Northern analysis as described under "Experimental Procedures." The mobilities of the 28 and 18 S rRNA are marked by *bars* on the left. The methylene blue staining of the 18 S rRNA in the *lower panel* confirms the loading of comparable amounts of RNA. At day 3 after plating, the chicken embryo extract was removed, and the cells were left in 10% horse serum. At day 3.5, cytosine arabinoside was added. A small number of myotubes is seen at this stage. At day 4 after plating, fusion is extensive. At day 7, the culture consists mainly of myotubes and remaining mononucleated cells, mostly fibroblasts. This experiment was repeated, and identical results were observed. From separate experiments we ascertained that ThR mRNA levels were essentially the same whether the cells were grown in 10% horse serum with or without chicken embryo extract.

induce receptor internalization (36, 37).

ThR immunoreactivity was seen regardless whether myoblasts were derived from animals taken at P0 or at P3 or whether they were grown for 1 or 4 days in culture. However, no ThR immunoreactivity could be detected on the surface of multinucleated cells. Fig. 2, *top panel*, shows a large multinucleated cell that has no ThR signal, whereas neighboring cells show the immunoreactivity. We have not seen a multinucleated cell that was positive for the receptor, whether the staining was done on young (3–4-day cultures) or older myotubes (7–9-day cultures). Importantly, myoblasts appear to fuse as ThR-expressing cells (Fig. 2, *lower panel*) and thereafter the receptor immunoreactivity is absent in the differentiated state.

Northern analysis was carried out to determine whether the absence of the ThR from multinucleated cells involves regulation on the level of the transcript. Fig. 3 shows that the relatively high amounts of ThR mRNA, detected at the stage preceding (day 3) and during (day 4) the formation of myotubes, decreased substantially when the myoblasts fused to form the multinucleated cells (day 7). The residual signal seen on day 7 is attributed to remaining mononucleated cells, mostly fibroblasts, which express ThR immunoreactivity on their surface (not shown) and are known to respond to thrombin (17, 18).

ThR Activation Induces Ca^{2+} Signals in Mono- but Not Multinucleated Cells—To ascertain the presence of a functional ThR on the surface of myoblasts, but not on myotubes, the ability of thrombin to elicit Ca^{2+} signals in these cells was investigated. Fig. 4 shows that the addition of 0.6 nM thrombin to a 1-day-old culture triggers an increase in the intracellular Ca^{2+} concentration of myoblasts as seen from changes in the ratio of the emission intensities of the Ca^{2+}-sensitive dye fura-2. Most of the MyoD1-positive cells responded to thrombin (MyoD1 was immunodetected after the experiment, Fig. 4*f*). The Ca^{2+} concentration increased sharply 30–60 s after addition of thrombin and slowly declined thereafter even in the continued presence of the protease. A Ca^{2+} signal similar to that seen with thrombin was also measured upon addition of the ThR-activating peptide SFFLRNPS (not shown). Worth mentioning is a trinucleated muscle cell (indicated with a *white arrow*, Fig. 4, *d* and *f*) that did not show a Ca^{2+} signal in response to thrombin. Furthermore, when thrombin was added to a 4-day-old culture, in which fusion was extensive, only the mononucleated cells responded with a rise in intracellular Ca^{2+}, whereas the myotubes did not (Fig. 5). The responding cells included the remaining myoblasts and MyoD1-negative cells. The Ca^{2+} signal in cells of the 4-day-cultures was generally of lower magnitude and did not have a sharp onset (three experiments) as compared to those of the 1-day cultures (six experiments).

These results not only demonstrate that the ThR present on the myoblast surface activates second messenger systems when cleaved by thrombin, but also confirm that the myotubes lack a functional ThR.

ThR Activation Induces Myoblast Proliferation—A well documented effect of thrombin is the modulation of cell proliferation. It was therefore determined whether this protease affects myoblast proliferation. Fig. 6*a* shows that after 24 h under serum-free conditions, 10 nM thrombin caused a rise in the number of MyoD1-positive cells. On average, 10 nM thrombin increased the number of myoblasts by 40 ± 20% (n = 7) over a 24-h stimulation period. Under the same conditions, thrombin also induced an increase in the proliferation rate of the myoblastic cell lines C_2C_{12} and L6. Similar to the primary cells, the average rise in the number of C_2C_{12} and L6 cells was 48 ± 12% (n = 9) and 50% (n = 1), respectively. The dose-response curve of the effect of thrombin in C_2C_{12} cells was broad and the maximal efficacy was achieved at thrombin concentrations of

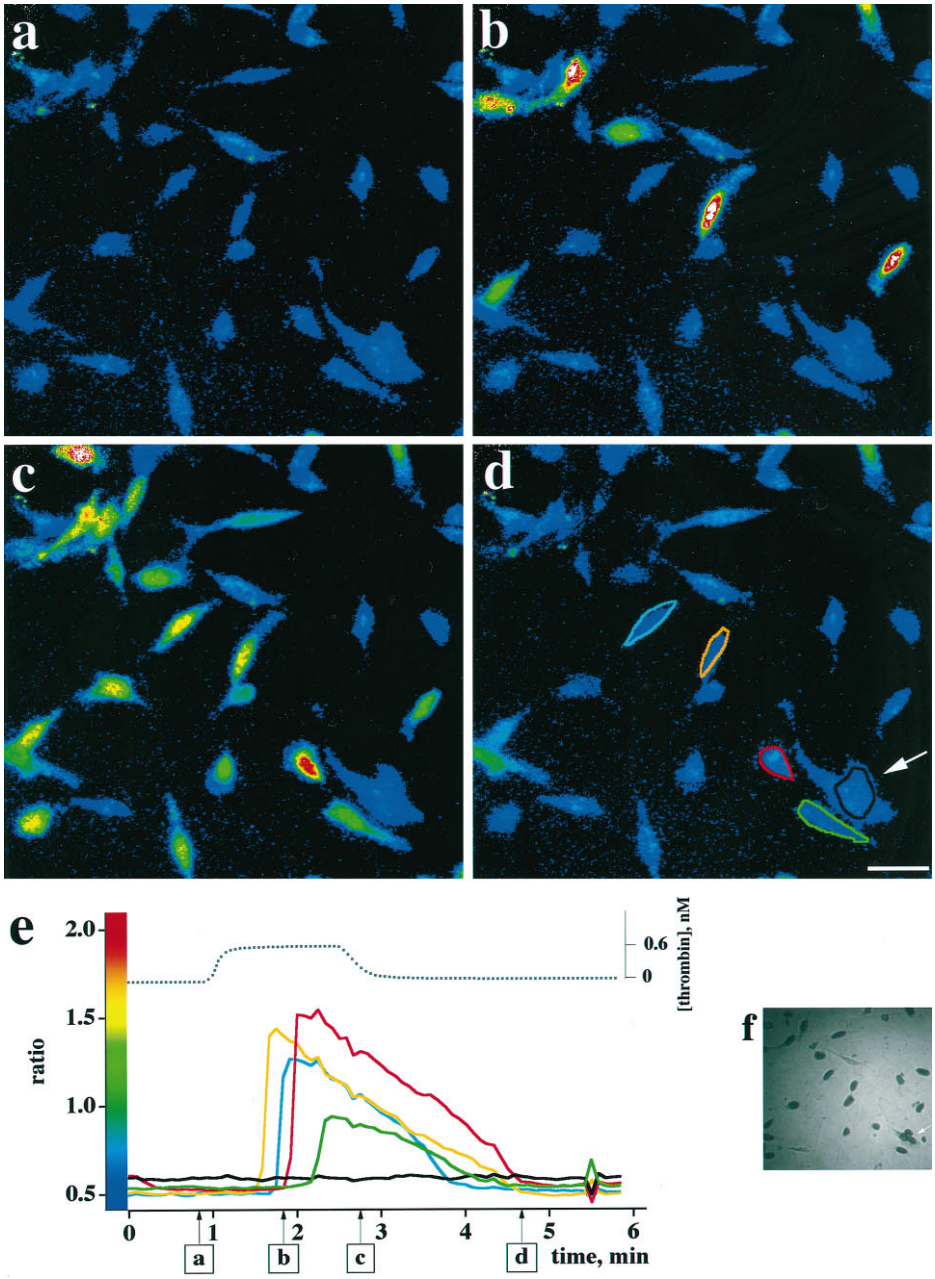

FIG. 4. **Thrombin induces Ca^{2+} signals in myoblasts.** The effect of thrombin on intracellular Ca^{2+} concentrations was examined on cells grown for 24 h in 10% horse serum and 5% chicken embryo extract as described under "Experimental Procedures." Pseudocolor coded images of the cells showing the rise in Ca^{2+} are shown before (*a*), during (*b* and *c*), and after (*d*) stimulation with 0.6 nM thrombin (the *color index bar* is joined to the *y* axis in panel *e*). The time points at which each image was taken is indicated on the *x* axis of panel *e*. The increase in thrombin concentration from 0 to 0.6 nM is represented by the *dashed trace* (*panel e*), which was constructed using a dye under identical perfusion conditions. Five MyoD1-positive cells were selected and color coded (*panel d*), and the rate of the change in their Ca^{2+} concentration is shown (*panel e*). After the termination of the experiment, the cells were fixed and stained for MyoD1 (*panel f*). Some cells seen in the fluorescence images detached during the staining procedure. Note that most cells responded to thrombin, with the notable exception of a trinucleated muscle cell, indicated with a *white arrow* in *panels d* and *f*. *Calibration bar* represents 20 μm.

about 3 nM (not shown). The ThR-activating peptide SFFLRNPS and lysophosphatidic acid, an agent which mimics the effect of thrombin in various systems (38), were also mitogenic both for myoblasts and myoblastic cell lines (not shown) (39).

Horse serum is a strong mitogen for myoblasts. At serum concentrations below 1%, thrombin caused an increase in cell number (Fig. 6*b*). However, when the mitogenic effect of horse serum attained its maximum, thrombin did not cause a further increase in the cell number. The increase in cell number caused by thrombin is smaller as compared to that seen with horse serum. It should be emphasized, however, that serum contains trophic factors which promote cell survival and most probably act in synergy with mitogenic agents to induce a larger increase in cell number.

The mitogenic effect of thrombin in fibroblasts is dependent on the activation of MAP kinases (40, 41). It was therefore determined whether MAP kinase activation in response to thrombin also occurs in myoblasts. Mitogens acting through the MAP kinase pathway are known to cause phosphorylation of the kinases $p44^{mapk}$ and $p42^{mapk}$ (ERK1 and ERK2) and their translocation from the cytoplasm to the nucleus (42, 43). Fig. 7*a* shows that within 1 h thrombin induces a translocation of MAP kinase(s) into the nucleus, indicating that the MAP kinase pathway has been activated. A translocation of MAP kinase(s) could be detected already after 30 min in the presence of thrombin (not shown). The antibody used for the immunoanalysis detects both $p44^{mapk}$ and $p42^{mapk}$. Double staining for MAP kinase(s) and MyoD1 confirms that the responding cells are myoblasts (Fig. 7*a*). The ThR-activating peptide SFFLRNPS and lysophosphatidic acid also induce a translocation of MAP kinase(s) into the nucleus (Fig. 7*b*). The translocation in response to all three agents was also seen in C_2C_{12} cells (not shown). The effect of phorbol 12-myristate 13-acetate, an established activator of MAP kinases, is presented as a positive control in this system (Fig. 7*b*). Taken together, these results demonstrate that thrombin is mitogenic on myoblasts and that this effect is likely to involve an activation of the MAP kinase pathway.

ThR Expression in Skeletal Muscle—In Northern analysis of ThR expression in skeletal muscle, the receptor mRNA was

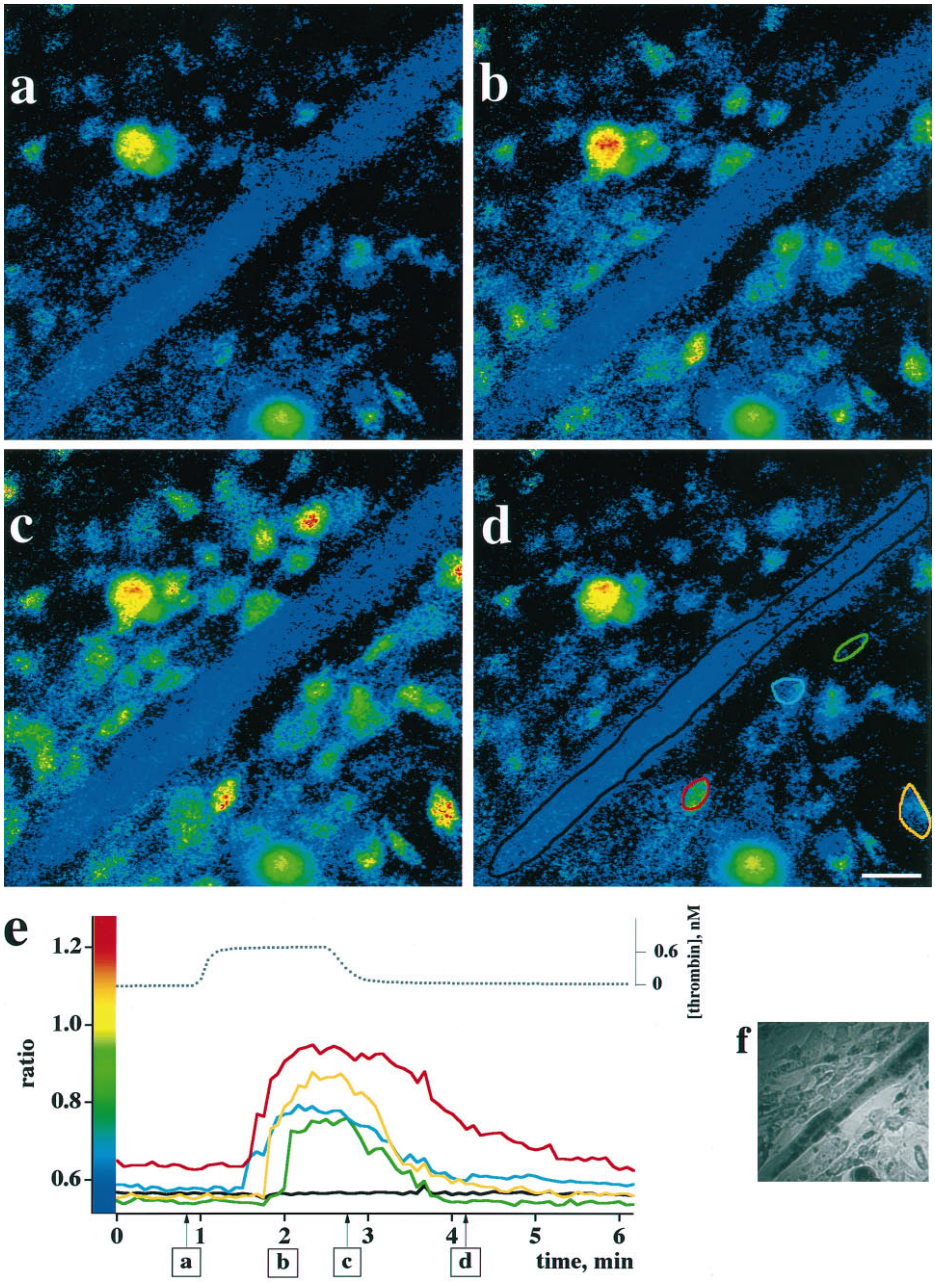

FIG. 5. **Thrombin does not alter Ca^{2+} levels in myotubes.** This experiment was carried out as described in Fig. 4 except that the cells were grown for 4 days in 10% horse serum and 5% chicken embryo extract. For the analysis in *panel e*, five cells were selected: a myotube (*black*), a myoblast (*green*), and three non-MyoD1-expressing cells (*red*, *turquoise*, and *mustard/orange*). No changes were seen in the myotube Ca^{2+} concentration, whereas most other cells responded with a Ca^{2+} signal. *Calibration bar* represents 20 μm.

found at relatively high amounts at birth, but was barely detectable by P24 (Fig. 8*a*). As an increase in muscle fiber number in the rat occurs also postnatally (44–46), the decrease in the ThR signal seen by Northern analysis could be coinciding with the completion of postnatal fusion. *In situ* hybridization studies were therefore carried out, and the cellular distribution of the ThR mRNA was found to be in agreement with the signal pattern seen by Northern analysis. Fig. 8*b* shows that, at birth, the ThR mRNA signal surrounds many nuclei. At higher magnification the majority of these nuclei do not seem to be associated with muscle fibers, which led us to assume that they include mononucleated myogenic cells. This is reinforced by the appearance of the positive cells in clusters, which are thought to contain at most one muscle fiber. At the resolution of the light microscope, it cannot be said with certainty whether the newly formed myotubes are positive. Receptor mRNA expression remained present at P14 (Fig. 8*c*), but only few nuclei surrounded by a mRNA signal were present by P24 (Fig. 8). These results corroborate the *in vitro* observations, namely that ThR expression is regulated by mechanisms associated with fusion, and strongly suggest that this receptor plays a role during muscle development.

DISCUSSION

Removal of the ThR after Fusion—ThR immunoreactivity was detected on the surface of myoblasts, but not on that of myotubes. A strong decrease in the ThR mRNA occurred when most of the myoblasts in the culture fused. It therefore appears that the cessation of ThR expression on the surface is due at least in part to a decrease in the level of the transcript. The removal of the ThR from the surface appears to be rapid because: 1) ThR immunoreactivity was never detected on myotubes and thrombin did not elicit a Ca^{2+} signal in these cells. 2) Myobags, consisting of as few as three nuclei, did not show a Ca^{2+} response upon addition of thrombin. 3) ThR-positive myoblasts were occasionally seen fusing to ThR-negative myotubes. If the turnover of the ThR on the plasma membrane is high, then a rapid decrease in the concentration of the ThR mRNA would be sufficient to cause a decrease in the number of receptor molecules on the cell surface. In such a case, the observed

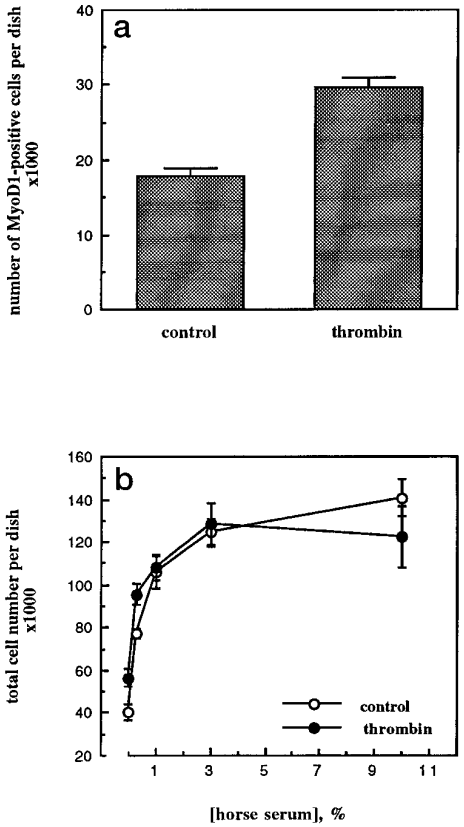

FIG. 6. **Thrombin increases the rate of myoblast proliferation.** Muscle cells were plated and grown for 24 h in the presence of horse serum and chicken embryo extract as described under "Experimental Procedures." The cells which at this stage were at a density of ~20 cells/mm^2 were washed with DMEM containing 50 μg/ml delipidated BSA, and then 10 nM thrombin was added for 24 h without serum (*a*) or in the presence of varying concentrations of horse serum (*b*). In *a*, the number of MyoD1-positive cells was calculated by multiplying the total cell number by the fraction of MyoD1-expressing cells (62 ± 2%) determined from parallel cultures. The total number of untreated cells remains essentially unchanged during the 24-h period after serum removal, thus ruling out that the increase in cell number seen with thrombin was merely due to the promotion of cell survival. These results represent the means of three determinations ± S.D.

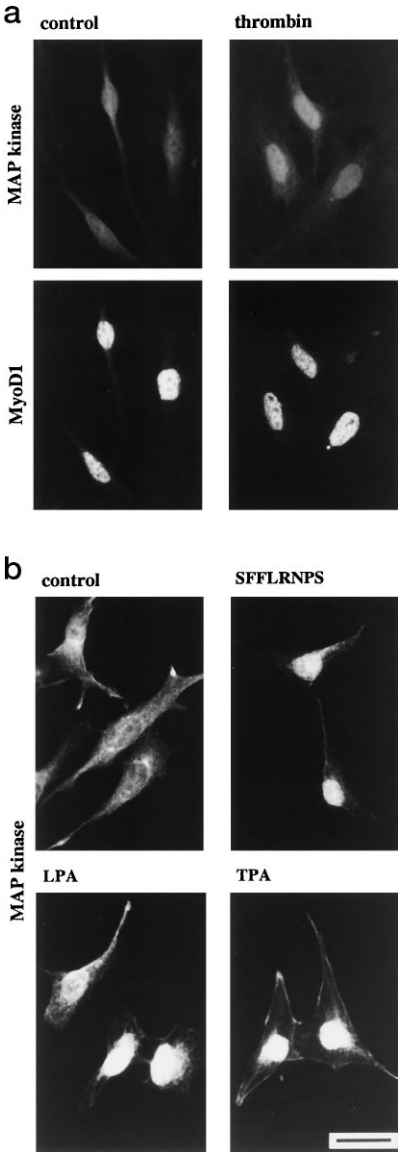

FIG. 7. **ThR activation causes translocation of MAP kinase to the nucleus.** Muscle cells which were grown for 24 h and attaining a density of ~35 cells/mm^2 were washed three times, 5 min each, and left overnight under serum-free conditions in DMEM containing 5 μg/ml of each of insulin and transferrin and 50 μg/ml delipidated BSA. The cultures were then treated for 60 min with DMEM + BSA in the absence or presence of either 10 nM thrombin, 300 μM SFFLRNPS, 10 μM lysophosphatidic acid (*LPA*) or for 30 min with 500 nM phorbol 12-myristate 13-acetate (*TPA*). The cells were then fixed and processed for a double staining of MAP kinase and MyoD1 (*a*) or of MAP kinase alone (*b*) as described under "Experimental Procedures." Thrombin, the ThR-activating peptide SFFLRNPS, LPA, and TPA cause a translocation of MAP kinase to the nucleus. The MAP kinase signal in *a* (fluorescein isothiocyanate) appears weaker as compared to *b* (rhodamine). The rhodamine-conjugated antibody was needed in *a* for the co-detection of MyoD1. *Calibration bar* represents 20 μm. These results were reproduced in three independent experiments.

decrease in ThR mRNA due to repression of transcriptional activity, and/or reduction in mRNA stability, would account for the loss of the ThR from the cell surface. Otherwise, it cannot at present be excluded that fusion activates a mechanism that removes the ThR from the cell surface. Such a mechanism has for example been demonstrated in epithelial cells, where endothelin-1 causes a rapid internalization of the ThR (47).

The results presented here argue that receptor removal is associated with fusion. In the case of transforming growth factor-β, the sensitivity to this growth factor in C_2C_{12} cells appears to be coupled to fusion and not to differentiation *per se*: C_2C_{12} cells that were biochemically differentiated by serum removal but were prevented to fuse by the addition of EDTA still responded to transforming growth factor-β (48). Additional work is needed to determine precisely when the changes in ThR mRNA and protein levels occur and whether they are strictly coupled to fusion.

The Mitogenic Signal of Thrombin in Myoblasts—In the present work we demonstrate that thrombin is mitogenic on primary skeletal muscle cells and that ThR signaling in these cells leads to the activation of MAP kinase as inferred from its translocation to the nucleus. The mitogenic effect of thrombin is associated with MAP kinase activation in several cell types including fibroblasts (43), smooth muscle cells (20), and astrocytes (49). MAP kinase activation could occur directly or indirectly through a thrombin-induced release of a mitogen from the myoblasts or a neighboring fibroblast which will act on the muscle cells in an autocrine or a paracrine manner, respectively. However, our experiments addressing MAP kinase activation were performed with cells at a low density (~35 cell/mm^2), and a translocation of the kinase was detected as early as 30 min after stimulation of the ThR. We therefore consider it unlikely, under the present experimental conditions, that MAP kinase activation and the consequent mitogenic response

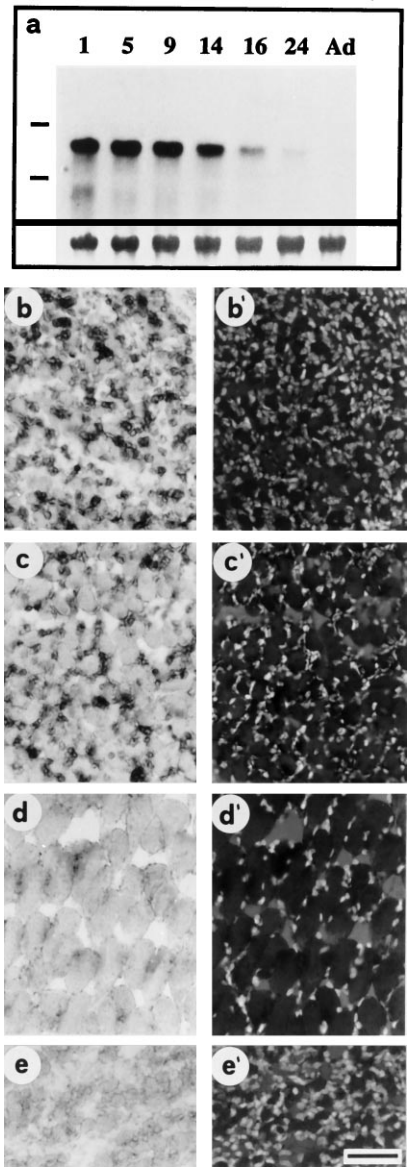

FIG. 8. **ThR mRNA expression in skeletal muscle.** *a*, Northern analysis. RNA was prepared from the gluteus muscle of rats taken at the indicated postnatal days (*Ad* = adult, 2 months old), and 6-μg samples were processed for Northern analysis as described under "Experimental Procedures." The mobilities of the 28 and 18 S rRNA are marked by *bars* on the left. The methylene blue staining of the 18 S rRNA in the *lower panel* confirms the loading of comparable amounts of RNA. The 3.4-kilobase ThR mRNA signal decreases within and after the second postnatal week. Essentially identical results were observed in another analysis carried out on RNA preparations derived form a separate series of animals. *b–e*, *in situ* hybridization. Cryostat sections of P0 hind leg muscle (*b*) and of the glutei sections of P14 (*c*) and P24 (*d*) rats were processed for *in situ* hybridization of the ThR as described under "Experimental Procedures." Control hybridization with a sense probe for the P0 muscle is shown in *e*, and the corresponding Hoechst nuclear staining is presented in *b′*, *c′*, *d′*, and *e′*. *Calibration bar* represents 75 μm.

triggered by thrombin were mediated through the synthesis and release of a secondary growth factor.

Reasons for the Repression of ThR Expression—It is possible that the ThR plays a role in events occurring during muscle development and receptor expression ceases in the muscle fiber because it is no longer needed there. Alternatively, ThR expression may be incompatible with the function of the muscle fiber. The potential of the ThR to promote proliferation and inhibit differentiation is naturally unwanted in a postmitotic cell. The receptors of epidermal and fibroblast growth factors, two mitogenic agents which also inhibit differentiation, were found to be permanently removed from the surface of differentiated MM14 cells (50, 51). Similar is the case of transforming growth factor-β and its cell surface binding sites (48, 52). In contrast, insulin-like growth factors I and II and their receptors, which promote both proliferation and differentiation of myoblastic cells, increase upon myoblast differentiation (see Ref. 11 and references therein). Placed in such a context, ThR removal upon fusion may be associated with its potential to inhibit differentiation rather than that to promote proliferation.

From what is known about ThR-mediated responses in blood and in the nervous system, moreover, there is no absolute requirement for receptor removal when cells become irreversibly postmitotic. For example, the ThR is present in megakaryocytic cells and their progenitors where it exerts multiple effects including modulation of cell proliferation (1, 53), and it is continued to be expressed in platelets, which are devoid of nuclei, where its cleavage by thrombin induces changes in shape, secretion, and aggregation. Furthermore, in platelets (54), as well as in neurons (55, 56), an activation of MAP kinase in response to various agents including thrombin obviously does not initiate a proliferative response.

There are other reasons for which the presence of the ThR may be incompatible with the function of the muscle fiber. One reason may have to do with the ability of the ThR to modulate actin-based contractility and ion channel activity. Modulation of actin-based contractility by thrombin has been seen in a number of cell types, such as smooth muscle cells (57), fibroblasts (58, 59), and neuronal cells (5, 6). Effects of thrombin on ion channel activity and membrane potential have for example been recorded in ventricular myocytes (60), smooth muscle cells (61), and megakaryocytic cells (62). It is therefore plausible that to limit the generation of changes in membrane potential and major actin-based contractility to the cholinergic input, muscle fibers have to repress the expression of the ThR. Another reason may be associated with the ability of thrombin to cause neurite retraction. It has recently been reported that the thrombin inhibitor hirudin attenuates the electrical activity-dependent synapse reduction in a coculture of sympathetic neurons and muscle cells (28), and it was suggested that muscle-derived proteases modulate synapse elimination (63). It is accordingly conceivable that to confine the action of thrombin and similar proteases to the pre- but not postsynaptic site at the neuromuscular junction, ThR expression is shut down in the muscle fiber. Noteworthy here is that the decrease in ThR mRNA in the rat muscle coincides closely with the termination of polyneuronal innervation.

Possible Activators of the ThR in Muscle—The identity of the endogenous agent that has the potential to activate the ThR on myoblasts is unknown as yet. It was recently reported that synthesis of prothrombin mRNA in cultured myotubes and thrombin activity in the medium are augmented by cholinergic stimulation (64). However, we could not detect prothrombin mRNA by Northern analysis of muscle derived either from newborn or adult rats (results not shown). It otherwise may be that the endogenous activator of the ThR in muscle is another serine protease. In neural cells, the ThR can be activated by trypsin or the T cell protease granzyme A (5, 33). Additionally, it cannot at present be excluded that the ThR is endogenously activated by peptides similar to those based on the ThR tethered ligand domain.

By demonstrating the presence of the ThR in myoblasts, our work indicates that during muscle development thrombin and/or other proteases with similar specificity participate in events occurring up to cell fusion. These results predict that an induction of ThR synthesis will occur during neomyogenesis in

the adult, for example in response to muscle overwork or injury.

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