Thrombin Causes a Marked Delay in Skeletal Myogenesis That Correlates with the Delayed Expression of Myogenin and p21CIP1/WAF1

(Received for publication, May 21, 1997, and in revised form, June 27, 1997)

Denis C. Guttridge‡, Alice Lau, Lynn Tran, and Dennis D. Cunningham§

From the Department of Microbiology and Molecular Genetics, College of Medicine, University of California, Irvine, California 92697-4025

Thrombin is a multifunctional serine protease whose activity is regulated in the extravasculature by an extracellular inhibitor, protease nexin-1. Because protease nexin-1 expression has been shown to be regulated during skeletal muscle cell differentiation, we reasoned that thrombin inactivation may be an important requirement for this developmental process. To test this hypothesis, we examined the effects of thrombin on differentiating C2C12 myoblasts. We report here that myogenesis, as scored by myotube formation, is considerably delayed by thrombin. This regulation correlated with delayed expression of myogenin and p21CIP1/WAF1, both considered critical components of the skeletal muscle cell differentiation program. Regulation occurred at the RNA level, indicating that the effect of thrombin is either transcriptional or post-transcriptional. Furthermore, we present evidence suggesting that this regulation is mediated by the thrombin receptor. Although thrombin is mitogenic for certain cell types, we found that delay of myogenesis in C2C12 cells did not involve a mitogenic signal. Taken together, these results imply that inhibition of the serine protease thrombin may be required for proper progression through the myogenic differentiation program. The data point to potentially important roles that thrombin and protease nexin-1 may play during skeletal muscle development.

Thrombin is a multifunctional serine protease that regulates both vascular and extravascular cellular processes. In the extravasculature thrombin is mitogenic for fibroblasts, smooth muscle cells, and astroglia cells (1–3). In addition, thrombin causes neurite retraction in neurons (4) and promotes cell death in certain cell types (5, 6). Most if not all of the actions of thrombin are mediated by a seven-transmembrane domain, G-protein-coupled receptor that is activated by a thrombin cleavage event in the extracellular domain of the receptor (7, 8). Proteolysis generates a new N terminus that acts as a tethered peptide ligand, binding to a site in the receptor, and activating a signal transduction cascade (9).

Thrombin activity is tightly regulated in the extravasculature by the serine protease inhibitor protease nexin-1 (PN-1), also referred to as glia-derived nexin (10, 11). PN-1 expression is relatively high in brain, and because it is regulated during injury it is thought to protect neuronal cells from the potentially harmful effects of serine proteases (12–14). PN-1 may also play a role in synapse formation in skeletal muscle because it is secreted from muscle fibers at the neuromuscular junction where acetylcholine receptors are expressed, and PN-1 blocks the proteolytic action of thrombin, which mediates activity-dependent synapse reduction (15, 16).

Formation of the neuromuscular junction is preceded by the differentiation of skeletal muscle cells, an event morphologically characterized as the fusion of mononucleated myoblasts into multinucleated myotubes. Differentiation is largely controlled by the myogenic basic helix-loop-helix family of transcription factors MyoD, myogenin, myf5, and MRF4 (reviewed in Ref. 17) and the cyclin-dependent kinase (Cdk) inhibitor p21CIP1/WAF1 (18, 19). Recent evidence showed that PN-1 gene expression is strongly regulated during skeletal muscle differentiation (20). Because PN-1 is a potent inhibitor of thrombin, the inactivation of this protease may be required for myogenesis. To test this we examined the effects of thrombin during the differentiation of C2C12 mouse myoblasts. Our investigation revealed that physiological concentrations of thrombin caused a marked delay in myogenic differentiation. Results showed that thrombin-mediated delay of myotube formation correlated with a delay in synthesis of the muscle-specific transcription factor myogenin and the Cdk inhibitor p21CIP1/WAF1 . Furthermore, evidence is presented suggesting that this regulation is mediated through the cognate receptor of thrombin in the absence of a mitogenic signal.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—The murine C2C12 myoblast cell line was obtained from the American Type Culture Collection. Cells were cultured at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 20% fetal bovine serum and antibiotics (Life Technologies, Inc.). To induce differentiation, myoblasts were allowed to grow to approximately 80% confluence and then switched to differentiation medium (referred to here on out as DM, Dulbecco’s modified Eagle’s medium supplemented with 10 μg/ml insulin, 5 μg/ml transferrin, plus antibiotics). Highly purified α-thrombin (specific activity, 6211 NIH units/mg) was obtained from Calbiochem. Unless otherwise indicated, thrombin was added to cells at a final concentration of 100 nM. Antibodies M-19 (anti-p21) and M-225 (anti-myogenin) were purchased from Santa Cruz Biotechnology, whereas antibody MY-32 (anti-myosin) was purchased from Sigma. The thrombin receptor-activating peptide SFLLRN, and the inactive scrambled peptide FSLLRN were synthesized by Chiron Mimotopes Peptide Systems.

Immunofluorescence and Immunoblotting—Immunofluorescence analysis was performed on differentiating myoblasts grown in paranox chamber slides (Nunc Corp.) as described by Prieve et al. (21). Anti-skeletal myosin was used at a 1:500 dilution. Slides were examined and photographed with a Zeiss microscope equipped with phase contrast and UV illumination through a fluorescein isothiocyanate filter. Prep-
Northern Blot Analysis—Total RNA was isolated and fractionated on an agarose gel and transferred onto a nylon filter as described previously (23). p21<sup>CIP1/WAF1</sup> and myogenin mRNA were analyzed using [32P]-c-ATP (NEN Life Science Products) and random prime labeling (Boehringer Mannheim). p21<sup>CIP1/WAF1</sup> and myogenin cDNAs were generated by performing polymerase chain reactions with total RNA prepared from differentiated C2C12 cells. Oligonucleotides 5′-AGCTGCTGCAGG-3′ (forward) and 5′-CAGACACAGAATGTCAGGG-GCTA-3′ (reverse) were used for the mouse p21<sup>CIP1/WAF1</sup> gene (24), whereas the oligonucleotides 5′-TATGACGCTGACTGACGCT-3′ (forward) and 5′-CAGATGGCAGCATTCTCAGG-3′ (reverse) were used for the mouse myogenin gene (25). Detection of the mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene to standardize for RNA loading was performed by generating an antisense riboprobe from the pTRI-GAPDH template (Ambion).

Measurement of DNA Synthesis—C2C12 cells were grown in 6 well plates to 70–80% confluency and then induced to differentiate in DM or DM + thrombin for up to 24 h. At indicated times cells were incubated with [3H]thymidine (2 μCi/ml) for 1 h. Following labeling reaction cells were rinsed twice with phosphate-buffered saline, and DNA was subsequently purified using QIAamp columns (Qiagen). Aliquots of [3H]-labeled DNA samples were diluted in a scintillation mixture and counted. Obtained values were standardized to total DNA concentrations.

RESULTS AND DISCUSSION

Thrombin Markedly Delays Skeletal Muscle Cell Differentiation—Recently, PN-1 gene expression was shown to increase during skeletal muscle cell differentiation (20). Because PN-1 is such a potent inhibitor of thrombin, we reasoned that the inactivation of thrombin may be a requirement for myogenesis and that unregulated thrombin activity could disrupt this differentiation process. To test this, we induced C2C12 myoblasts to differentiate in the absence or the presence of thrombin. In the absence of thrombin (DM alone), cells proceeded through the normal, morphologically well characterized stages of differentiation involving the fusion of myoblasts into contractile myotubes (Fig. 1, panels A–C) (26). In sharp contrast, thrombin-treated myoblasts (DM + thrombin) were severely delayed in their differentiation (Fig. 1, panels D–F). Compared with control cells, which formed myotubes by 48 h post-induction, virtually 100% of thrombin-treated myoblasts remained undifferentiated at 48 h, assessed by scoring for myotube formation from three independent experiments (compare panels B and E). However, by 72 h approximately 20% of thrombin-treated cells had formed myotubes, although they were considerably smaller in size compared with control cells (panels C and F). The addition of fresh thrombin at 24 and 48 h post-induction completely inhibited myotube formation even at 72 h.2 Thus, it appeared that the addition of thrombin given only when differentiation conditions were initiated caused a marked delay in myotube formation, but this was not sufficient to completely block skeletal myogenesis. Previous findings have shown that C2C12 myoblasts secrete low levels of PN-1 (27). Because it has been established that PN-1 binds tightly to the surface and extracellular matrix of many cell types, where it maintains its ability to inhibit thrombin (28), we speculate that the transient effect of thrombin on skeletal differentiation results from its inactivation by PN-1 present on C2C12 cells.

Experiments were also performed to address whether the observed morphological effects on myogenic differentiation were specific to the serine protease thrombin. To test this, myoblasts were treated with the serine protease urokinase, whose activity is also tightly regulated by PN-1 (29). Results showed that urokinase had no effect on C2C12 differentiation, suggesting that delay of skeletal muscle cell differentiation does not result from a general proteolytic mechanism but rather is specific to the activity of thrombin. Results further imply that PN-1 expression is regulated during skeletal muscle differentiation to specifically inhibit thrombin.

We next examined if thrombin could affect the regulation of a specific protein marker of skeletal muscle cell differentiation. The myosin heavy chain (MHC) gene is tightly regulated during skeletal differentiation, and MHC is a major component of the contractile apparatus in muscle fibers. During normal differentiation we detected the expression of the MHC protein throughout myotube structures (Fig. 1, panels G and H). In sharp contrast, we were not able to detect the expression of myosin in thrombin-treated myoblasts, which had been blocked from forming myotubes (Fig. 1 panel I and J). These results showed that the delay in myotube formation caused by thrombin correlated with the delayed expression of a myogenic pro-

2 D. C. Guttridge, A. Lau, L. Tran, and D. D. Cunningham, unpublished observations.
Thrombin Delay of Skeletal Myogenesis

**Thrombin Negatively Regulates p21<sup>CIP1/WAF1</sup> and Myogenin**—Successful progression through the skeletal muscle cell differentiation program is marked by two temporally regulated events. The first is the activation of the myogenic basic helix-loop-helix family of transcription factors, which include MyoD, myogenin, mrf5, and MRF4 (17). The second is the irreversible withdrawal of myoblasts from the cell cycle, an event largely controlled by the Cdk inhibitor p21<sup>CIP1/WAF1</sup> (18) (hereafter referred to as p21). Once a muscle cell has initiated both events it is cleared to express the last series of genes, such as the myosin heavy chain, that give cells their contractile phenotype (30). We demonstrated above that thrombin-treated myoblasts inhibited from forming myotubes also did not express the myosin heavy chain. This implied that thrombin can block gene expression late in the myogenic program. We next investigated whether thrombin could also affect the expression of genes, such as p21 and myogenin, that are upstream regulators in the skeletal muscle cell differentiation program. In untreated cells (DM), results from Western blot analyses were consistent with previous reports (18) (hereafter referred to as p21). Once a muscle cell has initiated both events it is cleared to express the last series of genes, such as the myosin heavy chain, that give cells their contractile phenotype (30). We demonstrated above that thrombin-treated myoblasts inhibited from forming myotubes also did not express the myosin heavy chain. This implied that thrombin can block gene expression late in the myogenic program. We next investigated whether thrombin could also affect the expression of genes, such as p21 and myogenin, that are upstream regulators in the skeletal muscle cell differentiation program. In untreated cells (DM), results from Western blot analyses were consistent with previous reports (18, 30) showing that although very little p21 or myogenin protein was expressed in proliferating myoblasts, there was a steady-state increase in the expression of both proteins as myoblasts differentiated into myotubes (Fig. 2A). In contrast, thrombin-treated myoblasts (DM + thrombin) displayed a decrease in steady-state levels of p21 and myogenin. For both proteins, maximum reduction by thrombin was observed at 24 h post-induction. Interestingly, Western blot analysis with the myogenin antibody displayed a band that migrated slightly above the myogenin protein and was also subject to thrombin regulation (Fig. 2A). Studies are currently ongoing to investigate whether this band represents a post-translationally modified form of myogenin or a separate protein that cross-reacts with the myogenin antibody.

The above results were obtained when cells were treated with thrombin at a concentration of 100 nM. Although this concentration is within physiological range, we performed a dose experiment to examine the sensitivity of thrombin in regulating muscle-specific genes. Western blot analysis revealed that concentrations as low as 1 nM significantly reduced the amount of myogenin protein compared with control levels (Fig. 2B). It is noteworthy that 1 nM thrombin was also sufficient to delay differentiation of myoblasts into myotubes. Therefore, these results show that thrombin is a potent modulator of skeletal muscle cell differentiation. Results also indicate that thrombin regulation of proteins like the myosin heavy chain, which are expressed late in the skeletal differentiation program, most likely occurs through the regulation of p21 and myogenin.

To examine the mechanism of thrombin regulation of p21 and myogenin, Northern blot analyses were performed. In differentiating conditions (DM) p21 and myogenin mRNA expression were similar to what we had observed at the protein level (Fig. 3). Likewise, when cells were treated with thrombin (DM + thrombin) both p21 and myogenin mRNA levels were reduced for up to 48 h, but by 72 h levels appeared to recover relative to control conditions. Interestingly, the timing of this delayed regulation correlated quite well with the time at which myoblasts were inhibited in forming myotubes (Fig. 1). Results from Northern blots therefore demonstrate that thrombin regulates p21 and myogenin either through a transcriptional or post-transcriptional mechanism, suggesting that thrombin-mediated delay of skeletal myogenesis may result from the delayed expression of these two genes.

**Thrombin Regulation of Myogenin Is Receptor-mediated**—Thrombin mediates its cellular effects through a G-protein-coupled receptor. Thrombin activates its receptor by cleaving a...
portion of the extracellular domain thus forming a new N terminus, which acts as a tethered peptide ligand activating a signal transduction cascade (9). Peptides corresponding to the newly generated N terminus can activate the thrombin receptor to fully mimic the intracellular signals of thrombin. The sequence specificity of these activating peptides is highly stringent. Simply switching the order of the first two amino acids at the N terminus blocks receptor activation. We utilized a 6-amino acid thrombin receptor-activating peptide with the sequence SPLLRRN to determine if the effects of thrombin on myogenesis were mediated through its cognate receptor. When myoblasts were induced to differentiate in the presence of the activating peptide, myogenin levels were significantly decreased compared with levels from untreated cells (Fig. 2C). Maximum reduction of myogenin was observed with 100 μM peptide concentration. In contrast, no decrease was seen when myoblasts were treated with the highest concentration of the inactive thrombin receptor peptide, FSLLRRN. These results indicate that the delay in myogenic differentiation induced by thrombin is mediated through the thrombin receptor.

**Thrombin Regulation of Myogenin Does Not Involve a Mitogenic Signal**—Proliferating myoblasts grown in serum or mitogen-rich medium are inhibited from differentiating (17). Cause thrombin is a component of serum and in some cells acts as a mitogen, we examined the possibility that the delay in myogenesis may have resulted from a mitogenic signal elicited by thrombin through its receptor. To test this, myoblasts were induced to differentiate in the absence of the presence of thrombin. At indicated times DNA synthesis was measured by [%H]thymidine incorporation. Using our differentiation conditions we observed a substantial decrease in [%H]thymidine incorporation in both treated and untreated cells shortly after cells had been induced to differentiate (Fig. 4). Results over a 24-h period showed that thrombin does not increase DNA synthesis compared with untreated myoblasts. Furthermore, we did not observe an increase in DNA synthesis in thrombin-treated cells even when experiments were extended out to a 72-h period. We conclude from these results that thrombin does not induce a mitogenic signal in C2C12 cells and that thrombin-mediated delay of myogenesis must therefore occur through an alternative signal transduction pathway.

The results presented in this report demonstrate the potential importance of regulating thrombin activity during the differentiation of skeletal muscle cells. Although the source of thrombin in muscle has not yet been evaluated, thrombin mRNA has been detected in neurons of the central nervous system (31). In an analogous manner, thrombin may be produced by peripheral neurons as they extend toward their muscle targets. The previous finding that PN-1 is expressed from skeletal muscle (15), together with current evidence that these cells express the thrombin receptor, suggests that a delicate balance between the protease and its inhibitor is necessary during this developmental process.

**REFERENCES**

Thrombin Causes a Marked Delay in Skeletal Myogenesis That Correlates with the 
Delayed Expression of Myogenin and p21 CIP1/WAF1

Denis C. Guttridge, Alice Lau, Lynn Tran and Dennis D. Cunningham

doi: 10.1074/jbc.272.39.24117

Access the most updated version of this article at http://www.jbc.org/content/272/39/24117

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 31 references, 14 of which can be accessed free at
http://www.jbc.org/content/272/39/24117.full.html#ref-list-1