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# ALPHA-ENOLASE PLASMINOGEN RECEPTOR IN MYOGENESIS

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## TABLE OF CONTENTS



## 1. ABSTRACT

Plasmin is a potent extracellular protease specialized in the degradation of fibrin (fibrinolysis). Active plasmin is generated by proteolytic activation of the zymogen plasminogen (Plg) by urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA). Alpha-enolase, although traditionally considered a glycolytic enzyme, constitutes a receptor for plasminogen on several cell types, serving to localize and promote plasminogen activation pericellularly. Localization of plasmin activity on the cell surface plays a critical role in fibrinolysis and in physiopathological processes involving extracellular matrix remodelling.

Previous studies have unambiguously demonstrated that uPA-dependent plasmin generation is necessary for myogenesis *in vitro* and for muscle regeneration *in vivo*. However, the implication of alpha-enolase plasminogen receptor in myogenesis had never been investigated. This review focuses on the recently reported expression and function of alpha-enolase plasminogen receptor during myogenesis. Skeletal myoblasts express alpha-enolase plasminogen receptor, being its expression greatly induced during the differentiation process *in vitro*. MAb 11G1, a monoclonal antibody against anti-alpha-enolase plasminogen receptor, that inhibits plasmin generation, was able to fully abrogate myoblast fusion and differentiation. Moreover, both plasmin activity and alpha-enolase plasminogen receptor expression were significantly augmented in injury-induced regenerating muscle of wild type mice and in the dystrophic muscle of *mdx* mice, an animal model of Duchenne muscular dystrophy (DMD). Altogether, these results indicate that the plasminogen activation (PA) system is an important component of skeletal myogenesis *in vitro* and *in vivo*. In particular, the expression of alpha-enolase plasminogen receptor may serve to concentrate and enhance plasmin generation on the cell surface of migratory myoblasts contributing to efficient muscle repair.

## 2. INTRODUCTION

Plasmin, the primary fibrinolytic enzyme, has a broad substrate spectrum and is implicated in numerous non-fibrinolytic processes dependent upon proteolytic activity, such as tissue remodeling and cell migration. Active plasmin is generated from proteolytic cleavage of the zymogen plasminogen (Plg) by either urokinase-type plasminogen activator (uPA) or tissue-type plasminogen activator (tPA). Plasmin activity is regulated by its inhibitor, $alpha_2$-antiplasmin, and by inhibitors of plasminogen activators, primarily plasminogen activator inhibitor 1 (PAI-1). The activity of the fibrinolytic system is stimulated by co-localization of plasminogen and plasminogen activators on the substrate, fibrin (1). By an analogous mechanism, co-localization of plasminogen and plasminogen activators on cell surfaces markedly stimulates plasminogen activation (Figure 1), while the formed plasmin is protected from inactivation by $alpha_2$-antiplasmin (reviewed in 2, 3). The proteins serving as plasminogen and t-PA receptors vary among cell types whereas uPA binding is mediated ubiquitously by the well-characterized uPA receptor (uPAR, CD87) (2-10). The plasminogen activation (PA)/plasmin system plays a role in a wide variety of physiopathological processes involving extracellular matrix (ECM) degradation and tissue remodeling, including ovulation, trophoblast invasion, post-lactational mammary involution, neurite outgrowth, wound healing, inflammation, angiogenesis and tumor cell invasion (reviewed in 7, 8, 11).

## 3. THE PLASMINOGEN ACTIVATION SYSTEM IN MYOGENESIS

Recent studies support a novel role for the PA system in myogenic cell function. Myogenesis refers to the formation of skeletal muscle during development and during pathological muscle regeneration. It involves the proliferation, migration and differentiation of myoblasts, followed by their alignment and fusion to form multinucleated myotubes, which will finally give rise to

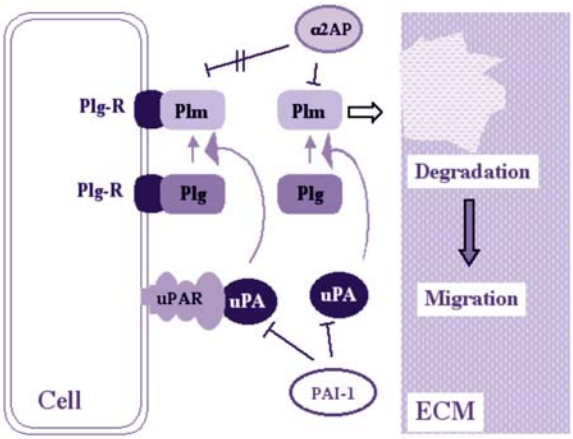


**Figure 1.** The plasminogen activation system. Active plasmin (Plm) is generated from its zymogen plasminogen (Plg) by urokinase-type plasminogen activator (uPA), which, in turn, can be inhibited by plasminogen activator inhibitor (PAI-1). Plasminogen receptor (PlgR) binds plasminogen and plasmin with similar affinity. Localization of plasminogen to the cell surface promotes plasminogen activation by uPA or uPA receptor (uPAR)-associated uPA. Plasminogen receptors may then concentrate plasmin on the cell surface and thereby facilitate localized degradation of specific plasmin substrates. Furthermore, receptor-associated plasmin is protected from its major inhibitors, alpha$_2$-antiplasmin and alpha$_2$-macroglobulin.

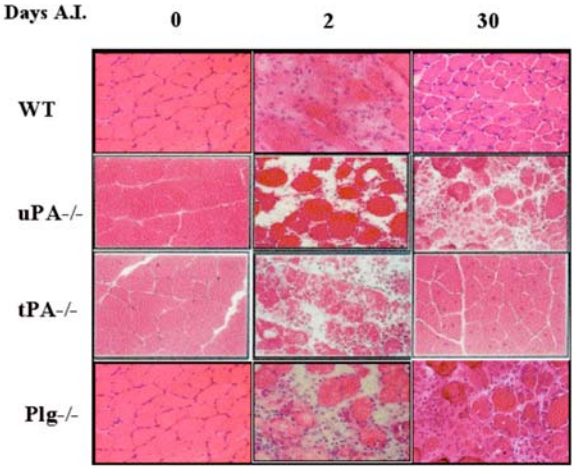


**Figure 2.** uPA- and Plg-deficient mice show a regeneration defect following muscle injury. Experimentally-induced injury results in impaired skeletal muscle regeneration of uPA- and Plg-deficient mice but not tPA-deficient mice. Frozen sections of injured muscles from wild type (WT), uPA-, tPA- and Plg-deficient mice, respectively, were stained with Hematoxilin/Eosin at 2 and 30 days after injury (days A. I.). Contralateral control muscles were also stained with Hematoxilin/Eosin (0 days A.I.). In WT and tPA-deficient mice, regeneration is complete after 30 days, as evidenced by the presence of centrally nucleated myofibers. In uPA- and Plg-deficient mice, however, a regeneration defect is still visible 30 days after injury.

mature myofibers. Despite the complexity of this multistep process, myogenesis can be effectively recapitulated *in vitro* by myoblastic cell culture models. Chicken, mouse, rat and human muscle cells have been shown to express proteins of the plasminogen activation system (12-16). Evidence of a role for the uPAR/uPA/PAI-1 tripartite complex was provided during myogenic migration and cell fusion (15, 16). Furthermore, Quax *et al*. (1992) demonstrated that the amino-terminal fragment of uPA, which retains its ability to bind to uPAR, but has no proteolytic activity, inhibited myogenesis *in vitro*, suggesting than uPA binding to its receptor is necessary for myogenesis (14). Our group (1997) showed that specific inhibition of uPA proteolytic activity abrogated migration, fusion and differentiation of murine myoblasts *in vitro* (17). Moreover, a specific requirement for uPA, but not tPA, proteolytic activity during skeletal muscle regeneration *in vivo* was demonstrated (Figure 2), suggesting that there is no redudancy in plasminogen activation between uPA and tPA in myogenesis (18). Finally, plasmin activity was shown to be necessary for myoblast fusion and differentiation *in vitro* (Figure 2), as well as for efficient skeletal muscle regeneration *in vivo* (Figure 2) (19). Altogether, these studies provide evidence of the implication of the PA/plasmin system in myogenesis *in vitro* and *in vivo*.

## 4. ALPHA-ENOLASE AS A PLASMINOGEN RECEPTOR

Plasmin can be concentrated on the cell surface through its binding to specific receptors. Several candidate receptors have been proposed: alpha-enolase and annexin II, among others (10, 20-24). We and others had previously identified alpha-enolase as a plasminogen receptor on the surface of different cell types including carcinoma cells (21), monocytoid cells (25-27), leukocytic cell lines (28), rat neuronal cells (29) and pathogenic streptococci (30). More recently, using an antibody against alpha-enolase plasminogen receptor (MAb 11G1), we showed that this receptor is expressed by skeletal muscle cells *in vitro* and *in vivo* (see Chapter 5). The binding of plasminogen to the surface of cells has profound profibrinolytic consequences: enhancement of plasminogen activation, protection of cell-bound plasmin from its inhibitor alpha$_2$-antiplasmin, and enhancement of the proteolytic activity of cell-bound plasmin (25, 31). Furthermore, using the monoclonal antibody MAb 11G1, which specifically blocks cell-dependent plasminogen activation (Figure 3), but not fibrin dependent plasminogen activation, we were able to show that □lpha-enolase has a major role in the promotion of plasminogen activation in several cell types (28). MAb 11G1 can thus be used to explore the physiopathological processes that specifically require plasminogen binding as well as pericellular plasmin formation.

In addition to its role as a plasminogen receptor, alpha-enolase has other cellular functions and subcellular localizations that are distinct from its well-established function in glycolysis. Thus, alpha-enolase appears to be a "moonlighting protein", one of a growing list of proteins that are recognized as identical gene products exhibiting multiple functions at distinct cellular and extracellular sites through "gene sharing" (32-34). For example, □lpha-enolase binds to F-actin and tubulin (35) and has been localized to the

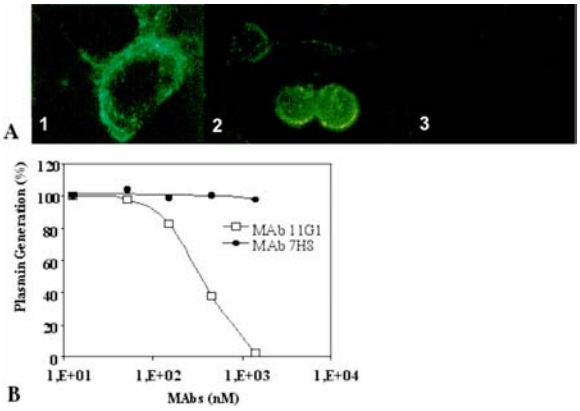


**Figure 3.** Characterization of MAbs against the purified alpha-enolase plasminogen receptor from MCF7-MF cell lysates. **A.** Immunofluorescence studies of MCF7-MF cells incubated with: MAb 7H8 (A1) and MAb 11G1 (A2), and an irrelevant antibody used as negative control (A3). Magnification 100x. **B**. Effect of different concentrations of MAb 11G1 or MAb 7H8 on the plasminogen activation rate, using a chromogenic substrate (S-2251), in the presence of Nalm6 cells (2 x $10^6$ cells/ml), incubated with 500 nM Lys-plasminogen, 50 pM uPA and 0.5 mM S-2251).

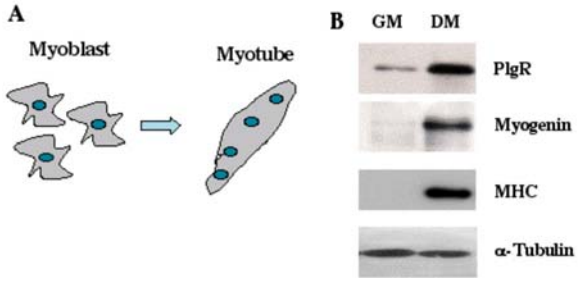


**Figure 4.** Alpha-enolase plasminogen receptor (PlgR) expression is induced during myoblast differentiation. **A**. C2C12 as a model of myoblast differentiation *in vitro*. **B**. C2C12 myoblasts were cultured in growth medium (GM: DMEM + 10 % fetal bovine serum) and then shifted to differentiation medium (DM: DMEM + 2 % horse serum), and PlgR protein expression was determined by Western blotting of cell lysates using the anti-PlgR specific monoclonal antibody, MAb 11G1. C2C12 myogenic differentiation was analyzed by re-probing the same membrane with an antibody against the muscle differentiation markers myogenin and myosin heavy chain (MHC), respectively. An anti-alpha-tubulin antibody was used to confirm equal protein loading in all lanes.

centrosome in HeLa cells (36, 37) and to cell-surface membranes in brain tumor cells (38). Alpha-enolase is a lens crystallin in several vertebrates (39), undergoes axonal transport in guinea pigs (40) and binds *in vitro* to single-stranded DNA in *Saccharomyces cerevisae* (41). Recently, a 35 kDa protein (MBP-1) that acts as a transcriptional regulator of c-myc in HeLa cells has been identified as a C-terminal fragment of alpha-enolase, and it is an alternative translation product of the □lpha-enolase gene (42). It has also been suggested that up-regulation of alpha-enolase contributes to hypoxia tolerance through nonglycolytic mechanisms (42). A fraction of alpha-enolase is also expressed on the plasma membrane (25, 26, 38). Thus, alpha-enolase exhibits enzymatic, structural and receptor functions.

## 5. ALPHA-ENOLASE PLASMINOGEN RECEPTOR IN MYOGENESIS

Muscle cells express alpha- and beta-enolase isoforms. Beta-enolase accounts for more than 90 % of total enolase activity in the adult muscle (43, 44) and is considered one of the earliest markers of myogenic differentiation. However, very little is known about alpha-enolase expression in myogenesis (45). We recently used MAb 11G1 as a tool to undertake the study of alpha-enolase plasminogen receptor expression in skeletal myoblasts as well as its functional role in pericellular plasminogen activation during myogenesis.

C2C12 mouse myoblasts express low levels of alpha-enolase plasminogen receptor, which are greatly increased during the differentiation process (Figure 4). MAb 11G1 reduced cell surface-associated plasminogen activation on differentiated myoblasts, and was able to fully abrogate C2C12 myoblast fusion and differentiation *in vitro* (Figure 5) (46). Moreover, both alpha-enolase expression and plasmin activity were significantly increased in experimentally-induced muscle regeneration and in the dystrophic muscle of the *mdx* mouse, an animal model of human Duchenne muscular dystrophy, DMD (Figure 6), suggesting an important contribution of the fibrinolytic system to pathological muscle regeneration (46). In particular, this study represented the first demonstration that the alpha-enolase plasminogen receptor appeared as an important regulator of skeletal myogenesis, by concentrating and enhancing plasmin generation on the myogenic cell surface.

## 6. REGULATION OF PLASMINOGEN ACTIVATION IN MYOGENESIS

The finding that loss of plasmin activity impedes muscle regeneration (19) raises the broader question of how this proteolytic activity is involved in muscle tissue repair. The uPA/Plg system is specialized in fibrin solubilization (47). Previously published studies showed that uPA- and Plg-deficient mice accumulated fibrin in the extracellular matrix of the degenerating muscle fibers (18, 19). Consequently, we were able to demonstrate that systemic fibrinogen depletion of both uPA- and Plg-deficient mice resulted in a significant amelioration of the muscular regeneration defect. Fibrin accumulation in the extracellular basal membrane of muscle may have deleterious effects, such as the impediment of normal nutrition to the muscle tissue and normal inflammatory cell migration. Inflammation is a process frequently associated with tissue repair, since degenerating tissues are invaded by inflammatory cells. In response to muscle injury, damaged tissue is infiltrated by fibroblasts and inflammatory cells (48). uPA- and Plg-deficient mice presented a severe regeneration defect with decreased recruitment of blood derived monocytes, lymphocytes and macrophages to the site of injury, suggesting that uPA/plasmin activity may

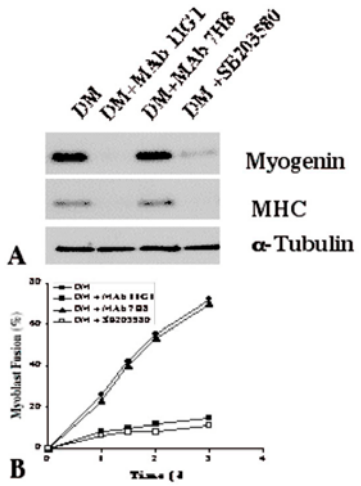


**Figure 5.** Inhibition of cell surface-generated plasmin activity decreases myotube formation and differentiation. C2C12 cells grown in GM were switched to DM for three days to promote differentiation and fusion in the absence or presence of 400 nM MAb 11G1, 400 nM MAb 7H8 or 10 μM SB203580, respectively. A. Inhibition of cell surface-generated plasmin activity by MAb 11G1 decreases myoblast differentiation. Western analysis of 40 microgram total protein from C2C12 myoblasts grown in GM, or switched for 3 days to DM to induce myogenic differentiation, in the absence or presence of MAb 11G1, MAb 7H8, or SB203580, respectively. The antibody used for each Western blot is indicated. An anti-alpha-tubulin antibody was used to confirm equal protein loading in all lanes. B. Inhibition of myoblast fusion by MAb 11G1. Fusion is represented as the percentage of nuclei in myotubes. Values are mean of 3 independent experiments, with similar results.

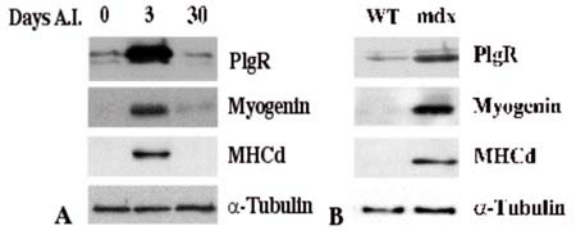


**Figure 6.** Alpha-enolase plasminogen receptor expression is increased during injury-induced skeletal muscle regeneration and in regenerating muscle of mdx mice. **A**. PlgR expression is induced upon cardiotoxin-induced muscle regeneration. Muscle lysates were prepared from the gastrocnemius muscle of WT mice, either non-injured (0) or 3 and 30 days after injury (days A.I.). Western blot analysis was performed to detect expression of PlgR using MAb 11G1. The regeneration process after muscle injury was examined by re-probing the membrane with antibodies against the regeneration markers myogenin and developmental myosin heavy chain (MHCd). An anti-alpha-tubulin antibody was used to confirm equal protein loading in all lanes. **B**. PlgR expression is higher in *mdx* muscle than in muscle of WT mice. Muscle lysates were prepared from the gastrocnemius muscle of WT and *mdx* mice of 4 weeks of age (at the first episode of the disease). Western analysis was performed as in panel A.

have a profound effect on inflammation and inflammation-related muscular disease. In addition to participating in fibrin degradation, plasmin may regulate the expression or/and activity of cytokines involved in inflammatory processes. For example, endogenously produced uPA can amplify tumor necrosis factor alpha neosynthesis by mononuclear phagocytes, representing a novel mechanism by which a phagocyte-derived protease contributes to generating proinflammatory signals (49). In addition, plasmin has been shown to release macrophage derived interleukin-1 and to activate transforming growth factor-beta (50, 51). It is, therefore, interesting to speculate that uPA/plasmin might be playing a similar role in the inflammation caused by muscle injury.

In addition to these potential roles of plasmin, other steps clearly in operation in the *in vitro* models of myogenesis are candidate targets for modulation by plasmin. A number of proteolytic enzymes have been proposed to play a role during muscle regeneration, either in the inflammatory response, and/or in the migration of myoblasts across the basal lamina and in their further fusion to form the terminal muscle fiber (52, 53). Metalloproteinases such as MMP-2 and MMP-9, meltrin-α and cathepsin B seem to be required for myotube formation *in vitro* (53-56). Moreover, the expression of MMP-2 and MMP-9 has also been reported in the degeneration-regeneration process of myofibers *in vivo* (57). Some MMPs can be directly or indirectly activated by cleavage to a lower molecular weight protein by plasmin (58), suggesting that activation of MMP-2 and MMP-9 in regenerating skeletal muscle might also be mediated by plasmin proteolysis. Proteolytic processing of components of the extracellular matrix or adhesion molecules involved in cell-matrix and cell-cell interactions during myoblast fusion are also candidate targets for plasmin activity.

## 7. PERSPECTIVE

The hypothesis that, in general, plasminogen activation facilitates cellular penetration of fibrin-containing matrices fits with a putative scenario occurring in normal skeletal muscle regeneration, with local conversion of plasminogen to plasmin and subsequent fibrin degradation. Plasmin is probably one member of a team of carefully regulated and specialized matrix degrading enzymes, including serine-, metallo-, and other classes of proteases, which together serve in matrix remodeling and cellular reorganization of wound fields. The results shown here support a role for cell-associated plasmin generation in myogenesis *in vitro* and in regeneration of skeletal muscle *in vivo,* either following injury or in congenital dystrophinopathies, such as *mdx* muscular dystrophy. Moreover, they provide the first demonstration that the alpha-enolase-type plasminogen receptor is an important modulator of muscle function. A potential collaboration between alpha-enolase and uPAR in the generation of plasmin activity in this complex biological process can be envisioned. Therefore, delineating the mechanisms by which plasmin and the alpha-enolase plasminogen receptor regulate myogenesis and muscle tissue repair will be the aim of future studies.

The findings here reviewed highlight the importance of the PA/plasmin system in myogenesis, with a special emphasis on the role of alpha-enolase plasminogen receptor-mediated proteolysis. Moreover, they reveal MAb 11G1 as an ideal tool for studying the role of alpha-enolase plasminogen receptor in several physiopathological processes, such as myogenesis, by blocking selectively the plasminogen binding and plasminogen activation enhancement, without interfering with the other activities of this multifunctional protein.

## 8. ACKNOWLEDGMENT

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