Molecular Dissection of the Human $\alpha_2$-Macroglobulin Subunit Reveals Domains with Antagonistic Activities in Cell Signaling*

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Elisabetta Mantuano, Gatambwa Mukandala, Xiaqing Li, W. Marie Campana, and Steven L. Gonias

From the Departments of Pathology and Anesthesiology, University of California, San Diego, La Jolla, California 92093

$\alpha_2$-Macroglobulin ($\alpha_2$M) is a plasma protease inhibitor, which reversibly binds growth factors and, in its activated form, binds to low density lipoprotein receptor-related protein (LRP-1), an endocytic receptor with cell signaling activity. Because distinct domains in $\alpha_2$M are responsible for its various functions, we hypothesized that the overall effects of $\alpha_2$M on cell physiology reflect the integrated activities of multiple domains, some of which may be antagonistic. To test this hypothesis, we expressed the growth factor carrier site and the LRP-1 recognition domain (RBD) as separate GST fusion proteins (FP3 and FP6, respectively). FP6 rapidly and robustly activated Akt and ERK/MAP kinase in Schwann cells and PC12 cells. This response was blocked by LRP-1 gene silencing or by co-incubation with the LRP-1 antagonist, receptor-associated protein. The activity of FP6 was also blocked by mutating Lys$^{1377}$ and Lys$^{1374}$, which precludes LRP-1 binding. FP3 blocked activation of Akt and ERK/MAP kinase in response to nerve growth factor- $\beta$ (NGF- $\beta$) but not FP6. In PC12 cells, FP6 promoted neurite outgrowth and expression of growth-associated protein-43, whereas FP3 antagonized the same responses when NGF- $\beta$ was added. The ability of FP6 to trigger LRP-1-dependent cell signaling in PC12 cells was reproduced by the 18-kDa RBD, isolated from plasma-purified $\alpha_2$M by proteolysis and chromatography. We propose that the effects of intact $\alpha_2$M on cell physiology reflect the degree of penetration of activities associated with different domains, such as FP3 and FP6, which may be regulated asynchronously by conformational change and by other regulatory proteins in the cellular microenvironment.

$\alpha_2$-Macroglobulin ($\alpha_2$M)$^2$ is a 718-kDa homotetrameric glycoprotein, found in the plasma and extracellular spaces, which was first recognized as a broad spectrum protease inhibitor (1). Reaction with proteases induces a major conformational change in $\alpha_2$M so that the protease is physically trapped (2, 3). The same conformational change reveals a cryptic recognition site for low density lipoprotein receptor-related protein (LRP-1) (4, 5). Because of the function of LRP-1 as an endocytic receptor, $\alpha_2$M-protease complexes are rapidly cleared from the bloodstream and probably other sites of generation (6).

In addition to its role as a protease inhibitor, $\alpha_2$M is an important carrier of specific growth factors, including transforming growth factor- $\beta$ (TGF- $\beta$), platelet-derived growth factor- $\beta$ (PDGF- $\beta$), nerve growth factor- $\beta$ (NGF- $\beta$), and neurotrophin-4 (7, 8). $\alpha_2$M-carrier interactions are principally reversible in nature (7). As a result, $\alpha_2$M may inhibit growth factor activity (9, 10) or stabilize the growth factor for potential delivery to cell signaling receptors (11). There also is evidence that $\alpha_2$M, which is "activated" by reaction with proteases, initiates cell signaling by binding to LRP-1 (12–15) or other receptors, such as glucose-regulated protein-78 (Grp 78) (16, 17). However, in studies with intact $\alpha_2$M, the possibility that cell signaling results from growth factors that are carried by $\alpha_2$M must be considered (11, 18).

A structural model of $\alpha_2$M has been developed, based on the crystal structure of complement component C3, which is homologous to $\alpha_2$M (19). This model describes $\alpha_2$M as a modular structure, consisting of multiple independently folded domains. To localize $\alpha_2$M domains that are responsible for various activities, our laboratory generated a library of glutathione S-transferase (GST) fusion proteins, containing overlapping segments of the $\alpha_2$M subunit (20–22). Fusion protein-3 (FP3) includes residues 591–774 and contains the sequence in $\alpha_2$M responsible for binding growth factors. FP6 contains amino acids 1242–1451 and the LRP-1-binding site, in which a single $\alpha$-helix that includes Lys$^{1378}$ and Lys$^{1374}$ plays a central role (23, 24). Assignment of $\alpha_2$M activities to specific fusion proteins, such as FP3 and FP6, has been validated by mutagenesis of full-length recombinant $\alpha_2$M (25, 26). Thus, the $\alpha_2$M fusion proteins provide an opportunity to assess activities assigned to different $\alpha_2$M domains independently.

Given the modular structure of $\alpha_2$M and the important activities assigned to various domains, it is not surprising that $\alpha_2$M gene knock-out mice demonstrate abnormal responses to a variety of exogenous challenges (27–31). It is also not surprising that in vitro studies of $\alpha_2$M and its homologues, using closely related experimental model systems, have yielded differing results. For example, in cultures of rat PC12 pheochromocytoma cells and mouse cortical neurons, $\alpha_2$M and related proteins have been reported to either promote or inhibit neurite outgrowth (13, 32, 33).
In this study, we examined cell signaling in response to \(\alpha_2M\)-peptide fusion proteins, in which different \(\alpha_2M\) activities have been isolated, in Schwann cells and PC12 cells. We also studied neuritogenesis and expression of growth-associated protein (GAP-43), a neuronal differentiation marker (34), in PC12 cells. Our results show for the first time that different regions of \(\alpha_2M\) demonstrate antagonistic activities. How these activities are integrated in the intact protein may be regulated by \(\alpha_2M\) conformational change and by the presence of other regulatory molecules in the cellular microenvironment.

**EXPERIMENTAL PROCEDURES**

**Proteins and Reagents—**\(\alpha_2M\) was purified from human plasma by the method of Imber and Pizzo (35). \(\alpha_2M\) was activated for binding to LRP-1 by dialysis against 200 mM methylamine-HCl in 50 mM Tris-HCl, pH 8.2, for 12 h at 22 °C and then exhaustively against 20 mM sodium phosphate, 150 mM NaCl, pH 7.4. Modification of \(\alpha_2M\) by methylamine in the final preparation (\(\alpha_2M\)-MA) was confirmed by demonstrating the characteristic increase in \(\alpha_2M\) electrophoretic mobility by non-denaturing PAGE (2). The 18-kDa receptor-binding domain (RBD) of \(\alpha_2M\) was generated by treating \(\alpha_2M\)-MA with papain and purified by chromatography, as previously described (4).

Receptor-associated protein was expressed as a GST fusion protein (GST-RAP) in bacteria and purified as previously described (37). GST-RAP binds to LRP-1 and blocks the binding of all other LRP-1 ligands (38). As a control, we expressed GST in bacteria transformed with the empty vector, pGEX-2T. GST-RAP and GST were subjected to chromatography on Detoxi-Gel endotoxin-removing columns (Pierce). Rabbit polyclonal antibodies specific for phosphorylated Akt, phosphorylated extracellular signal-regulated kinase-1/2 (ERK/MAP kinase), total ERK/MAP kinase, and horseradish peroxidase-conjugated secondary antibodies were purchased from Cell Signaling Technologies (Santa Cruz, CA). Monoclonal antibody specific for \(\beta\)-actin and \(p\)-nitrophenyl \(p\)-guanidinebenzoate (pNPG) were from Sigma. NGF-\(B\) was purchased from Invitrogen (San Diego, CA). Recombinant rat PDGF-BB was from R&D Systems (Minneapolis, MN). Recombinant human erythropoietin (Epo) was purchased from Johnson & Johnson (San Diego, CA). Polyclonal \(\alpha_2M\)-specific antibody was from Dako (Carpinteria, CA).

**\(\alpha_2M\) Peptide-GST Fusion Proteins—**Three previously described GST fusion proteins that include segments of the human \(\alpha_2M\) sequence were expressed in BL-21 cells (20–22). These include: FP3 (amino acids 591–774), FP4 (amino acids 775–1059), and FP6 (amino acids 1242–1451). FP3 includes the characterized binding site for growth factors (20, 21, 25). FP6 contains the LRP-1 recognition sequence (22). A construct encoding FP6, in which Lys1370 and Lys1374 are mutated to Ala (FP6(K \(\rightarrow\) A)), was previously described (22). These mutations abrogate \(\alpha_2M\) binding to LRP-1 (23, 24). All of the fusion proteins were partially purified from induced bacterial suspensions by selective detergent extraction (20). FP3, FP4, FP6, and FP6(K \(\rightarrow\) A) were then further purified to homogeneity by chromatography on glutathione-Sepharose. The resulting preparations yielded clearly defined bands, with the correct molecular masses when assessed by Coomassie Blue staining of SDS gels or immunoblot analysis with GST-specific antibody (20, 22). All of the GST fusion proteins were subjected to endotoxin decontamination. Fig. 1 shows the \(\alpha_2M\) sequences encoded by the fusion proteins studied here, and the relationship of these fusion proteins to the predicted domains in \(\alpha_2M\), as described by Doan and Gettins (19). The growth factor-binding site, expressed in FP3, is contained within the MG6 domain and the LRP-1 recognition site, which is encoded in FP6, is part of the MG8 domain.

**Cell Culture—**The rat pheochromocytoma cell line, PC12, was cultured in Dulbecco’s modified Eagle’s medium (DMEM; high glucose; Invitrogen, Grand Island, NY) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 5% heat-inactivated horse serum (Omega Scientific Inc., Tarzana, CA), 100 units/ml penicillin, and 100 mg/ml streptomycin at 37 °C. Schwann cells were isolated from the sciatic nerves of 1-day-old Sprague-Dawley rats and further selected from fibroblasts using fibronectin-specific antibody and rabbit complement, as previously described (39). The final preparations consisted of 95–99% Schwann cells, as assessed by immunofluorescence for S100, which is a specific Schwann cell marker. Primary cultures of Schwann cells were maintained in DMEM containing 10% FBS, 100 units/ml penicillin, 100 \(\mu\)g/ml streptomycin, 21 \(\mu\)g/ml bovine pituitary extract, and 4 \(\mu\)M forskolin (complete medium) at 37 °C under humidified 5.0% CO\(_2\). Schwann cell cultures were passaged no more than four times before conducting experiments.

Murine embryonic fibroblasts (MEFs) that are genetically deficient in LRP-1 (MEF-2 cells), and control LRP-1-expressing MEFs (PEA-10 cells) were obtained from the ATCC. PEA-10 and MEF-2 cells were cloned from the same culture, heterozygous for LRP-1 gene disruption, and selected with the LRP-1-selective toxin, Pseudomonas exotoxin A (PEA) (40). MEFs were cultured in DMEM with 10% FBS.

**LRP-1 Gene Silencing—**A previously characterized rat LRP-1-specific siRNA (L2): CGAGCGACCUCCUAUCUUUUU
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(41) and pooled non-targeting control (NTC) siRNA were purchased from Dharmaco (Chicago, IL). Primary cultures of Schwann cells ($1 \times 10^6$) were transfected with LRP-1-specific siRNA or with NTC siRNA (25 nM) by electroporation using the Rat Neuron Nucleofector kit (Amaxa, Gaithersburg, MD) (41). The degree of LRP-1 gene silencing, at the mRNA level, was 88–95%, 48–72-h post-electroporation as determined by quantitative PCR (qPCR) (41). LRP-1 gene silencing was confirmed by immunoblot analysis. Cell signaling experiments were performed 48 h after introduction of siRNAs.

FP6 Activity Assay—The $\alpha_2M$ sequence in FP6 includes the LRP-1 recognition site (22). To confirm that FP6 binds to LRP-1, FP6 was radioiodinated using Iodobeads, according to the manufacturer’s instructions (Roche Applied Sciences). The specific activity was 2.5 $\mu$Ci/ug. LRP-1-expressing PEA-10 cells and LRP-1-deficient MEF-2 cells were plated in 24-well plates and cultured until almost confluent. The cells then were washed twice with Earle’s Balanced Salts Solution (EBSS) containing 10 mm Hapes, pH 7.4, and 1.0 mg/ml bovine serum albumin (EHB) and equilibrated in the same solution. $^{125}$I-FP6 was incubated with the cells for 4 h at 4°C in the presence and absence of non-radiolabeled FP6 (50 nM) or 0.2 $\mu$M GST-RAP. The cultures were then washed twice with EHB and once with EBSS. Cell extracts were prepared in 0.1 M NaOH and 1.0% SDS. Cell-associated radioactivity was determined in a PerkinElmer Wizard 1470 Automatic Gamma Counter.

Activation of Akt and ERK/MAP Kinase—Primary cultures of Schwann cells and PC12 cells were plated in 60-mm wells at a density of $2 \times 10^5$ cells/well in serum-containing medium and cultured until ~70% confluent. The cultures then were transferred to serum-free medium and maintained for 5 h (PC12 cells) or for 1 h (Schwann cells) prior to adding candidate stimulants, including $\alpha_2M$ peptide-GST fusion proteins, NGF-β, PDGF-BB, or Epo. Incubations with these agents were conducted for 10 min unless otherwise described. The cells then were rinsed twice with ice-cold phosphate-buffered saline. Cell extracts were prepared in radioimmune precipitation assay buffer (phosphate-buffered saline with 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitor mixture, and sodium orthovanadate). The protein concentration in cell extracts was determined by bicinchoninic acid assay. An equivalent amount of cellular protein (40 $\mu$g) was subjected to SDS-PAGE and electrotransferred to nitrocellulose membranes. The membranes were blocked with 4% nonfat dry milk in 20 mM Tris-HCl, 150 mM NaCl, pH 7.4 with Tween 20 and incubated with primary antibodies. The membranes then were washed and treated with horseradish peroxidase-conjugated secondary antibodies for 1 h. Immunoblots were developed by enhanced chemiluminescence (Amersham Biosciences).

Neurite Outgrowth—PC12 cells were plated at $2 \times 10^5$ cells/well in serum-containing medium and cultured until ~60% confluent. The cultures then were transferred to serum-free medium for 5 h prior to adding various reagents, including NGF-β, NGF-β plus FP3 (100 nM), FP3 alone (100 nM), FP6 (100 nM), or vehicle. At the indicated times, DNA-free total RNA was extracted using Trizol (Invitrogen) and treated with Turbo DNA-free DNase. cDNA was synthesized using the ProSTAR first-strand RT-PCR kit (Stratagene). Expression of GAP-43 was determined by qPCR using Taqman primers and probes purchased from Applied Biosystems Inc. The one-step program included 2 min at 50 °C; 10 min at 95 °C followed by 40 cycles of: 95 °C for 15 s; 60 °C for 1 min, using an ABI 7300 instrument. Rat cyclinB1 mRNA was measured in each sample as a housekeeping gene (41). Samples without cDNA were analyzed as “no-template” controls. GAP-43 mRNA levels were measured in duplicate, in four separate experiments, and normalized against the Ct value for the housekeeping gene.

RESULTS

Specific Binding of FP6 to LRP-1—FP6 contains the $\alpha_2M$ sequence known to mediate binding to LRP-1 (22–24). To confirm that FP6 binds to LRP-1, $^{125}$I-labeled FP6 (0.1 nM) was incubated with LRP-1-expressing PEA-10 cells and with LRP-1-deficient MEF-2 cells at 4 °C. Fig. 2A shows that $^{125}$I-labeled FP6 bound mainly to the LRP-1-expressing cells. Unlabeled FP6

![Figure 2](image-url)
inhibited binding of $^{125}$I-labeled FP6 to PEA-10 cells by >85%, indicating that the majority of the binding was specific; the residual binding observed in the presence of unlabeled FP6 was equivalent to that observed with MEF-2 cells. GST-RAP inhibited the binding of $^{125}$I-labeled FP6 to PEA-10 cells by about 85%, confirming that the specific binding was due to LRP-1. GST-RAP did not significantly affect FP6 binding to the LRP-1-deficient MEF-2 cells. These results demonstrate that the LRP-1-binding site is functional in FP6.

By SDS-PAGE, the apparent mass of FP6 was 53 kDa, as anticipated (Fig. 2B). Treatment of FP6 (20 μM) with 0.5 μM active trypsin revealed a single new major band, migrating with an apparent mass of 24 kDa. This mass is consistent with that predicted for the α2M sequence in FP6 as well as isolated GST. Further degradation of the 24-kDa product(s), to form more rapidly migrating species, was not apparent in the gel, arguing against large areas of disordered structure in FP6, which tend to be susceptible to proteolysis. Intact FP6 and the 24-kDa band, generated by trypsin, were both detected by immunoblot analysis using polyclonal α2M-specific antibody; purified GST was immunonegative (results not shown), confirming that a peptide derived from the sequence of α2M was present in the 24-kDa band.

FP6 Activates Cell Signaling in Schwann Cells and PC12 Cells—LRP-1 gene silencing decreases the basal level of Akt activation in Schwann cells, presumably by inhibiting autocrine cell signaling pathways involving endogenously produced LRP-1 ligands (41). In this study, we sought to determine whether α2M activates Akt and ERK/MAP kinase in Schwann cells by binding to LRP-1. Because intact α2M may activate cell signaling by delivering growth factors to the cell, such as PDGF-BB and NGF-β (21), we compared activated α2M-MA with FP6, which lacks growth factor-carrier activity (23). As shown in Fig. 3A, FP6 and α2M-MA activated Akt in Schwann cells. Akt also was activated by Epo and PDGF-BB, as anticipated (42). FP4, which does not carry growth factors or bind to LRP-1 and thus represents a negative control, had no effect on Akt activation. Similar results were obtained when we probed for activation of ERK/MAP kinase. Again, FP6, α2M-MA, Epo, and PDGF-BB activated ERK/MAP kinase in Schwann cells, whereas FP4 did not.

Next, we examined PC12 cells. These cells are frequently studied as a model system to assess neuronal differentiation in response to NGF-β and other growth factors (43, 44). As shown in Fig. 3B, FP6 shows that FP6 activated Akt and ERK/MAP kinase in PC12 cells. α2M-MA also activated these cell signaling proteins, as
did NGF-β, which served as a positive control. FP4 had no effect on Akt or ERK/MAP kinase.

**LRP-1 Is Essential for FP6-initiated Cell Signaling**—Previous studies suggest that activated α₂M may initiate cell signaling independently of LRP-1, by binding to Grp 78 or the NGF receptor, TrkA (16, 32). To test whether LRP-1 is necessary for FP6-initiated cell signaling in Schwann cells and PC12 cells, we applied a number of distinct strategies. First, we assessed cell signaling in response to FP6(K3A). Mutation of two Lys residues in this derivative precludes binding to LRP-1 (23, 24). FP6(K3A) failed to activate Akt and ERK/MAP kinase in Schwann cells (Fig. 4A) and PC12 cells (Fig. 4B). Next, we studied cell signaling in response to FP6 in the presence of the LRP-1 competitive antagonist, GST-RAP. When added to cultures of Schwann cells alone, GST-RAP had no effect on cell signaling (Fig. 4C), confirming results obtained by other investigators (12, 45). However, when added 15 min in advance, GST-RAP blocked cell signaling in response to FP6 in both Schwann cells (Fig. 4C) and PC12 cells (Fig. 4D). GST-RAP also blocked the response to α₂M-MA in PC12 cells.

As a third test of the role of LRP-1 in FP6-initiated cell signaling, we applied LRP-1 gene silencing, which is highly efficient in Schwann cells (41). LRP-1-specific siRNA blocked activation of Akt and ERK/MAP kinase by FP6 in Schwann cells (Fig. 5A). Cell signaling in response to Epo and PDGF-BB were unaffected, demonstrating that the effects of the siRNA on FP6 are specific. In cells that were transfected with NTC siRNA, FP6 activated Akt and ERK/MAP kinase, as did Epo and PDGF-BB (Fig. 5B). Taken together, our results with FP6(K3A), GST-RAP, and LRP-1 gene silencing demonstrate that FP6 activates Akt and ERK/MAP kinase by a mechanism that requires LRP-1.

**FP3 Inhibits Activation of Akt and ERK/MAP Kinase**—Next, we assessed the activity of FP3, which includes the sequence in intact α₂M responsible for binding neurotrophins and other growth factors (20, 21). Availability of the growth factor-binding site in intact α₂M is regulated by α₂M conformational change (7). Fig. 6 shows that FP3 did not independently regulate the activity of Akt or ERK/MAP kinase in response to NGF-β. The ability of FP3 to antagonize the response to NGF-β was specific. FP3 had no effect on cell signaling in response to FP6. ERK/MAP kinase and Akt have been implicated in neuronal differentiation/neurite extension in PC12 cells (46–49). Thus, we examined the effects of FP3 and FP6 on PC12 cell neurite extension. Fig. 7 shows that FP6 robustly supported neuritic outgrowth in PC12 cells maintained in serum-free medium for 48 h. A comparable response was detected in cells that were treated with NGF-β. FP3 did not independently regulate neurite outgrowth but instead, entirely blocked the response to NGF-β. Thus, the effects of FP3 and FP6 on neurite outgrowth paralleled their effects on cell signaling in the PC12 cell culture model system.
Our neurite extension results were supported by separate experiments in which we examined expression of mRNA for GAP-43, a neuronal differentiation marker (34). Fig. 8 shows that FP6 significantly increased GAP-43 expression at 4 and 8 h \( p < 0.05 \). The magnitude of the response was as great or greater than that observed with NGF-\( \beta \). FP3 had no independent effect on GAP-43 expression, but instead completely blocked GAP-43 mRNA expression in response to NGF-\( \beta \).

The 18-kDa \( \alpha_{2}M \) RBD and FP6 Activate Cell Signaling Equivalently—GST fusion proteins may be expressed as dimers (50). For FP6, dimeric structure might allow cell signaling to occur by cross-linking of LRP-1 to a co-receptor such as Grp 78 or by cross-linking of LRP-1 into homodimers. To test whether the \( \alpha_{2}M \) RBD activates cell signaling as a monomer, we isolated the 18-kDa RBD from plasma-purified \( \alpha_{2}M \) (4). Fig. 9A shows SDS-PAGE analysis of the final, purified product. The 18-kDa RBD activated both Akt and ERK/MAP kinase in PC12 cells, mimicking the results obtained with FP6 (Fig. 9B). GST-RAP blocked the response to the 18-kDa RBD, indicating an essential role for LRP-1.

**DISCUSSION**

Although a number of reports indicate that activated \( \alpha_{2}M \) initiates cell-signaling, questions remain regarding this activity. First, in some cell culture model systems, LRP-1 does not appear to be necessary for the response (16, 32). Furthermore, the relationship between \( \alpha_{2}M \)-initiated cell signaling, and its ability to carry growth factors has not been addressed. In this study, we show that the isolated LRP-1-binding domain of \( \alpha_{2}M \), expressed as a GST fusion protein or isolated from the plasma protein, robustly activates Akt and ERK/MAP kinase in

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**FIGURE 7.** FP3 and FP6 have opposite effects on PC12 cell neuritogenesis. PC12 cells were cultured for 48 h in serum-free medium alone (A), or supplemented with 100 nM FP6 (B), 50 ng/ml NGF-\( \beta \) (C), 100 nM FP3 (D), or NGF-\( \beta \) + FP3 (E). Axodendritic process formation was assessed by phase contrast microscopy. Representative fields are shown (\( n = 3 \)).

**FIGURE 8.** FP3 and FP6 differentially regulate expression of GAP-43. PC12 cells were cultured in serum-free medium for 5 h and then treated with vehicle (SFM), NGF-\( \beta \) (50 ng/ml), NGF-\( \beta \) + FP3 (100 nM), FP3 (100 nM), or FP6 (100 nM). Incubations were conducted for 4 or 8 h. Total RNA was isolated. GAP-43 mRNA expression was determined by qPCR (mean ± S.D., \( n = 4 \); * \( p < 0.05 \), one-way analysis of variance with Tukey’s posthoc for each time period).
Schwann cells and PC12 cells. We provide three separate lines of evidence indicating that LRP-1 is necessary. First, the response was blocked by GST-RAP. LRP-1 gene silencing neutralized the response to FP6. Finally, mutating two amino acids in FP6, which are known to function in LRP-1 binding, eliminated the ability of FP6 to trigger cell signaling. The mutation in FP6(K → A) also may block binding to Grp 78 (24); however, the three lines of evidence only may be interpreted as indicating an essential role for LRP-1.

The ability of FP6 and the 18-kDa RBD of \( \alpha_2M \) to trigger robust cell signaling to Akt and ERK/MAP kinase by binding to LRP-1 justifies clustering \( \alpha_2M \) with other LRP-1 ligands, such as tissue-type plasminogen activator and apolipoprotein E, which also activate cell signaling by binding LRP-1 (45, 51). The consequences of these LRP-1-initiated cell signaling events remain to be fully elucidated. We previously demonstrated that in Schwann cells, regulation of the basal level of activated Akt by LRP-1 may be important for Schwann cell survival (41). In peripheral nerve injury, Schwann cell survival is essential for nerve regeneration (42). In this study, we assessed neurite formation in PC12 cells. FP6 robustly promoted neurite outgrowth and increased expression of GAP-43, matching the magnitude of the response observed with NGF-β. This result was consistent with the demonstrated ability of FP6 to activate ERK/MAP kinase and Akt in PC12 cells. These cell signaling factors have been linked to neuronal differentiation (46–49). Because FP6 functions via an LRP-1-dependent mechanism, our results are consistent with a model in which LRP-1-initiated cell signaling may promote neuronal differentiation.

Availability of the LRP-1 recognition site in \( \alpha_2M \) is dependent on the \( \alpha_2M \) conformation (1, 6). In the native form of \( \alpha_2M \), which circulates in the plasma, the LRP-1 recognition sequence is completely cryptic. In the activated conformation, the LRP-1 recognition sequence is fully available. Chemically modified forms of \( \alpha_2M \), which have undergone partial conformational change, bind to LRP-1 but with decreased efficiency compared with \( \alpha_2M \)-MA (6). Thus, as the structure of \( \alpha_2M \) moves toward the fully transformed state, the LRP-1 recognition site is increasingly exposed and available. The effects of \( \alpha_2M \) conformational change on availability of the growth factor-binding site are more complicated. The growth factor-binding site is partially available in the native form of \( \alpha_2M \) (7), accounting for the function of native \( \alpha_2M \) as the principal carrier of TGF-β1 in the plasma (11, 53). Fully activated \( \alpha_2M \) binds most growth factors with increased affinity (7, 8); however, there is evidence that the growth factor binding site may be most available in intermediate \( \alpha_2M \) conformations (52, 54, 55). Thus, penetration of the activities associated with FP3 and FP6 may vary asynchronously in \( \alpha_2M \) conformational change.

In the studies presented here, FP3 demonstrated activities that oppose those of FP6. In the presence of NGF-β, FP3 inhibited activation of Akt and ERK/MAP kinase, blocked PC12 cell neurite outgrowth, and inhibited expression of GAP-43. We propose that the effects of FP3 on cell physiology, demonstrated here, are due to the previously demonstrated ability of FP3 to bind NGF-β (8). FP3 did not antagonize the activity of FP6. In the presence of FP3, FP6-induced cell-signaling to Akt and ERK/MAP kinase was unchanged. We think this result explains why intact \( \alpha_2M \)-MA activates Akt and ERK/MAP kinase similarly to FP6. In the intact protein, in the absence of concomitantly added growth factors, the function of FP6 is dominant. However, our model predicts that if activated \( \alpha_2M \) and NGF-β were added to cultures of Schwann cells or PC12 cells together, the effects would not be additive because of the inhibitory effects of the FP3 region of \( \alpha_2M \) on NGF-β activity. The FP3 region within full-length-activated \( \alpha_2M \) also may regulate growth factors produced endogenously by cells.

Although native \( \alpha_2M \) binds NGF-β with lower affinity than activated \( \alpha_2M \), according to our model, because the LRP-1 recognition site is entirely cryptic in native \( \alpha_2M \), the FP3 site is dominant and the overall effects of intact, native \( \alpha_2M \) are expected to mimic FP3. Similarly, the FP3 region may dominate in \( \alpha_2M \) conformational intermediates, which are reported to exist in vivo (36, 54). Understanding the balance between functional sites contained within different domains of \( \alpha_2M \) is a goal for future investigation.

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