

# Mechanisms of Signaling through Urokinase Receptor and the Cellular Response

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## Introduction

The urokinase-urokinase receptor (u-PA-u-PAR) system seems to play a crucial role in a number of biological processes, including local fibrinolysis, tumor invasion, angiogenesis, neointima and atherosclerotic plaque formation, inflammation, and matrix remodeling during wound healing and development.<sup>1-6</sup> Binding of urokinase to its specific receptor provides cells with a localized proteolytic potential. It stimulates conversion of cell surface-bound plasminogen into active plasmin, which, in turn, is required for proteolytic degradation of basement membrane components, including fibronectin, collagen, laminin, and proteoglycan core proteins.<sup>7</sup> Moreover, plasmin activates other matrix-degrading enzymes, such as matrix metalloproteinases.<sup>8</sup> Overexpression of u-PA/u-PAR correlates with tumor invasion and metastasis formation,<sup>9-13</sup> while reduction of cell-surface bound u-PA and inhibition of u-PAR expression leads to a significant decrease of invasive and metastatic activity.<sup>14</sup> Specific antagonists that suppress binding of u-PA to u-PAR have been shown to inhibit cell-surface plasminogen activation, tumor growth, and angiogenesis both in vitro and in vivo models.<sup>15,16</sup>

Independently of its proteolytic activity, u-PA is implicated in many biological processes that seem to require u-PAR-mediated intracellular signal transduction, such as proliferation, chemotactic movement and adhesion, migration, and differentiation.<sup>17</sup> Data obtained in the late 1980s indicated that u-PA not only provides cells with local proteolytic activity, but might also be capable of transducing signals to the cell.<sup>18-22</sup> At that time, however, the u-PAR has just been isolated, cloned, and identified as a glycosylphosphatidylinositol (GPI)-linked protein and not a transmembrane protein. Signaling via the u-PAR was, therefore, regarded as being unlikely, and the effects of u-PA on cell proliferation<sup>18-22</sup> were thought to be mediated by proteolytic activation of latent growth factors. The assumption of direct signaling via u-PAR was, in fact, considered controversial, until about 10 years later when a physical association between u-PAR and signaling proteins was found.<sup>23</sup> From this report on, several proteins associated with u-PAR have been

identified. Now, u-PAR seems to be part of a large "signalosome" associated and interacting with several proteins on both the outside and inside of the cell.

## Cell Surface Components of the Urokinase Plasminogen Activator "Signalosome" System

### *Urokinase-Type Plasminogen Activator*

The u-PA molecule is synthesized and secreted in a variety of normal and malignant cells as a single-chain zymogen (scu-PA) with little intrinsic plasminogen activator activity. Proteolytically active u-PA is generated by "reciprocal zymogen activation," where u-PA binds u-PAR and interacts with cell membrane-bound plasminogen. This results in initial plasmin generation that, in a positive-feedback manner, leads to the formation of the active two-chain form of u-PA (tcu-PA).<sup>24</sup> This form is susceptible to inhibition by plasminogen-activator inhibitors (PAIs). The receptor-binding domain of u-PA is located within its amino-terminal region (ATF, residues 1-135) and does not involve the protease domain.<sup>25</sup> Besides plasmin, kallikrein is also capable of activating scu-PA to active tcu-PA.<sup>26</sup> Matrix metalloproteinase-3 (MMP-3) can specifically hydrolyze scu-PA and active tcu-PA, yielding a small amino-terminal domain comprising the u-PAR binding site and a carboxyl-terminal fragment containing the protease domain of u-PA, without affecting receptor binding or enzymatic properties of the specific fragments.<sup>27</sup>

### *The Urokinase-Type Plasminogen Activator Receptor*

The receptor for urokinase is a heavily glycosylated GPI-anchored protein of 45 to 65 kDa belonging to the Thy-1/Ly-6 superfamily. It is comprised of three homologous domains of approximately 90 amino acids. The amino-terminal domain (DI) of u-PAR is responsible for high-affinity binding to the epidermal growth factor-like domain of u-PA. The functions of domains DII and DIII are not yet completely elucidated. Removal of DII and DIII does not change the u-PA-DI binding properties, although some reports indicate that signal transduction via u-PAR occurs only when u-PA is bound to more than one domain.<sup>28</sup> There seems to be two forms of cell surface-bound u-PAR, intact and cleaved (DII+DIII). The latter form is generated by the u-PA-catalyzed cleavage of u-PAR.<sup>29</sup> In studies, u-PAR has been predominantly localized in plasmalemmal microdomains rich in glycosphingolipids, sphingomyelin, and

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cholesterol.<sup>30</sup> In addition to the cell surface GPI-anchored u-PAR, a soluble form of u-PAR (su-PAR) that lacks the GPI anchor was identified in ascites and in the serum of patients with ovarian carcinoma and paroxysmal nocturnal hemoglobinuria. It has also been found in lower amounts in normal human plasma and tissues.<sup>31</sup> Other GPI-anchored members of the LY-6/u-PAR family include Sca-2 /TSA-1, RIG-E.<sup>32,33</sup>

#### Vitronectin

Several groups have reported that u-PAR can directly bind vitronectin.<sup>34-36</sup> The vitronectin binding site appears to be distinct from the u-PA binding site. One group of investigators demonstrated that treatment of su-PAR with an antibody directed to the DI-domain prevents its interaction with vitronectin.<sup>37</sup> These data contradict with another observation in which an anti-(DI+DII) antibody was shown to disrupt u-PAR-vitronectin interaction, while an anti-DI antibody showed no inhibitory effect.<sup>36</sup> In any case, vitronectin appears to bind only intact three-domain u-PAR.<sup>37</sup> The affinity of u-PAR to bind vitronectin is increased when u-PAR is occupied by scu-PA. PAI-1 in its active, but not in its latent or cleaved form, inhibits this interaction<sup>35</sup> because binding of vitronectin to u-PAR<sup>34</sup> and PAI-1, respectively, both occur via the amino-terminal somatomedin B domain of vitronectin, which is located in close proximity to the binding sites for  $\alpha v$ -integrins.<sup>38,39</sup> In contrast to the interaction of vitronectin with integrins, binding of vitronectin to u-PAR is not dependent on the RGD motif and does not require divalent cations to stabilize complex formation. The interaction of u-PAR with vitronectin promotes cellular adhesion and migration<sup>40,41</sup> and may direct u-PAR to sites of focal contacts.<sup>42</sup> Recently, it was shown that soluble u-PAR could form a ternary complex with vitronectin and u-PA, and it is in this form that the molecule may bind to the cell surface. Through this mechanism, vitronectin might focus the u-PA/su-PAR complex to cell surfaces and extracellular matrix sites, leading to the increase in local u-PA activity required for cell migration and tissue remodeling.<sup>43</sup>

#### Kininogen

Binding of high molecular weight and the cleaved form of kininogen to human endothelial cells has been shown to occur through a zinc-dependent interaction with u-PAR.<sup>44</sup> The binding site for kininogen seems to be within the DII and DIII domains of u-PAR, since its association to the cell surface is inhibited by vitronectin, anti-(DI+DII) antibodies, and recombinant soluble u-PAR, but not by u-PA. The major physiological role of such an interaction might be an increase in the catalytic efficiency of u-PA activation by forming the multiprotein complex that includes scu-PA, u-PAR, kininogen, and kallikrein.<sup>26,44</sup>

#### Glycoprotein (GP) 130

The transmembrane protein GP130 was recently found to form a complex with u-PAR in a human kidney epithelial cell line, TCL-598.<sup>45-47</sup> This protein is known to function as a co-recep-

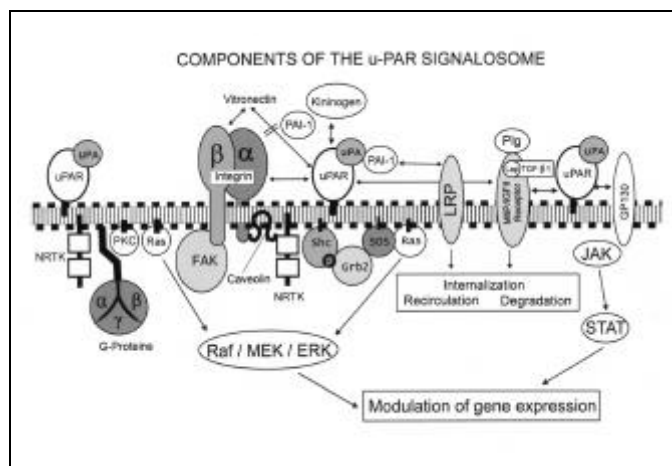


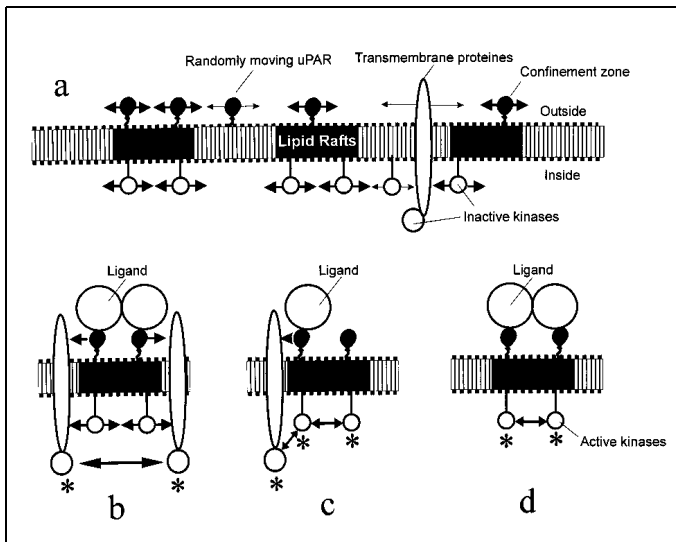
Figure 1. Schematic summary of possible signaling pathways influenced and/or regulated by u-PAR (the "uPAR-signalosome"): u-PAR is found associated or interacting with several components on the outside of cells as well as with signaling molecules. Explanations are given in the text. NRTK = non receptor tyrosine kinase

tor for some cytokine receptors, coupling transmembrane partners, or even GPI-anchored proteins, such as the receptor for the ciliary neurotrophic factor to the Janus kinase (JAK)/signal transducers and activators of transcription (STAT) signaling pathway. The association of GP130 with u-PAR, therefore, links u-PA/u-PAR signaling to the JAK/STAT pathway,<sup>47</sup> and clustering of u-PA occupied u-PAR in fact leads to translocation of STAT to the nucleus and DNA binding.<sup>48</sup>

#### Integrins

Several reports have demonstrated interactions between the u-PAR and integrins. Integrins were found to co-immunoprecipitate with u-PAR. u-PAR seems to be physically associated in a reversible manner with  $\beta 1$ ,  $\beta 2$  and  $\beta 3$  integrins, and the affinity of integrins for their corresponding matrix ligands is modulated by this association.<sup>49</sup> u-PAR was reported to be physically and functionally associated with leukocyte  $\beta 2$  integrins CR3 (Mac-1, CD11b/CD18) and CR4 (CD11c/CD18). CR3 associates with u-PAR on resting cells, whereas CR4 was found to interact with u-PAR at lamellipodia of migrating cells. CR4 molecules bind and release u-PAR in an oscillatory manner coordinating local proteolysis, cell adherence, and migration.<sup>50-52</sup> Neutrophils from leukocyte-adhesion-deficient patients (lacking  $\beta 2$ -integrins) showed impaired  $Ca^{2+}$ -signaling.<sup>53</sup> Moreover, COS transfectants mobilized  $Ca^{2+}$  upon u-PA incubation only when both u-PAR and CR3 were expressed,<sup>53</sup> suggesting that CR3 may act as a transmembrane adapter for u-PAR. Association of u-PAR and  $\beta 2$ -integrins has been found in various myeloid cell lines, human monocytes, and in resting neutrophils.<sup>54</sup>

In non-hematopoietic cells not expressing  $\beta 2$ -integrins, cooperation of u-PAR with  $\beta 1$ - and  $\beta 3$ -integrins has been observed.<sup>55</sup> In a fibrosarcoma cell line, u-PAR was found to be associated with members of the  $\beta 1$ - and  $\beta 3$ -integrin family when the cells were allowed to adhere to the respective specific substrates.<sup>55</sup> The basic effect of u-PAR binding to integrins appears



**Figure 2. Model for signaling via the GPI-linked u-PAR:** In the not activated state, u-PAR on the outside as well as inactive lipid anchored kinases on the inside move either randomly between lipid rafts or remain confined within the rafts; also transmembrane proteins move randomly mainly outside the rafts (a). Upon binding of ligands to u-PAR (b, c, d) the affinity of the ligand u-PAR complex for transmembrane proteins increases (e.g. GPI130, b; integrins, c); thereby kinases linked to transmembrane proteins activate each other (b) or are brought close enough to the kinases on the inside of the lipid raft to allow reciprocal activation (c). Alternatively, cross-linking of u-PAR by ligand binding increases the residence time of u-PAR in the rafts (increased time in the confinement zone) thereby influencing also the residence time of lipid anchored kinases in the inner leaflet of the lipid rafts. The increased residence time of the kinases leads to their reciprocal activation and in turn signal transduction (d).

to be modulation of the normal ligand-binding function of the specific integrin. Expression of u-PAR in a kidney embryonic cell line resulted in complex formation with  $\beta$ 1-integrins and inhibition of the adhesive function of the integrin, suggesting that u-PAR can act as a disintegrin.<sup>56</sup> In other experimental systems, however, integrin-dependent adhesion was reported to be facilitated by u-PAR,<sup>42,57</sup> suggesting that u-PAR may act as an integrin amplifier depending on the expression or availability of additional cellular compounds.

#### Plasminogen Activator Inhibitor-1

PAI-1 was shown to form stable ternary complexes with u-PA-u-PAR. Formation of this complex generates the high-affinity site on PAI-1 for the LRP- $\alpha$ 2-macroglobulin receptor, inducing internalization of the whole complex via endocytosis.<sup>58,59</sup> After internalization, u-PA and PAI-1 are degraded in lysosomes, whereas u-PAR is recycled back to the cell surface. Recent data indicate that LRP might also be involved in signal transduction via a stimulatory heterotrimeric G-protein and a downstream protein kinase A (PKA)-dependent pathway.<sup>60</sup> These data suggest that this complex may also act as a transmembrane adapter that mediates u-PAR-dependent signal transduction. Furthermore, because PAI-1 induces u-PAR endocytosis via low density lipoprotein receptor-related protein (LRP), the number of u-PAR molecules on the cell surface can be modu-

lated by PAI-1 and, in turn, u-PAR activities. By this mechanism PAI-1 can also influence cell adhesion in a positive manner. This mechanism has to be distinguished from the inhibitory effects of PAI-1 on vitronectin-dependent adhesion.<sup>61,62</sup>

#### The Mannose 6-phosphate/Insulin-Like Growth Factor-II Receptor

It was recently demonstrated that the DII and DIII domains of u-PAR are also involved in binding to the cation-independent, mannose 6-phosphate/insulin-like growth factor-II (M6P/IGF-II) receptor.<sup>63,64</sup> This binding is not affected by u-PA or mannose-6-phosphate and leads to internalization and degradation of u-PAR in lysosomes.<sup>63</sup> Whether association of u-PAR with M6P/IGF-II receptor has only clearing function or might contribute to signal transduction is not yet understood. The M6P/IGF-II receptor interaction with u-PAR, however, seems to be involved in the plasmin-dependent generation of TGF- $\beta$  and, thereby, indirectly in signal transduction via u-PA/u-PAR.<sup>64</sup>

#### Intracellular Components of the Urokinase Receptor "Signalosome" System

##### Caveolin

Recent data indicate that u-PAR is localized in caveolae and forms a stable complex with caveolin.<sup>30,36,42,45,50</sup> Caveolin is not a transmembrane protein but is inserted into the membrane as a hairpin with its middle part forming a loop in the inner leaflet of the membrane, while both terminal ends reach into the cytosol.<sup>65</sup> Therefore, the association between u-PAR and caveolin appears to be indirect and might itself depend on additional transmembrane adapters. In light of this, it is of interest that caveolin-1 is physically associated with the  $\alpha$ -chain of integrins and is required for integrin-mediated activation of anchorage-dependent cell growth.<sup>66</sup> Detergent-insoluble caveolin-containing fractions, also called lipid rafts, localize not only u-PAR, but also an array of other signaling molecules. Lipid rafts seem to be important not only for src-kinase signaling but also for the GP130 mediated pathway: Upon clustering of u-PAR, activation of JAK occurred, followed by STAT phosphorylation and redistribution from the caveolae to the nucleus.<sup>47</sup>

##### Intracellular Signaling and Cytoskeleton Proteins

As revealed by co-immunoprecipitation, u-PAR is physically associated, in a number of cell lines with intracellular proteins known to be involved in signal transduction pathways. These include non-receptor tyrosine kinases of the src family (p60<sup>src</sup>, p53/p56<sup>lyn</sup>, p56/p59<sup>hck</sup>, p59<sup>fgfr</sup>),<sup>23</sup> guanine-nucleotide-binding proteins (G-proteins), Jak1, STAT proteins and Tyk2 proteins,<sup>47,48</sup> protein kinase C,<sup>67</sup> and the cytoskeleton proteins vinculin,  $\alpha$ -actinin, and actin.<sup>68</sup> Interestingly and probably of major importance for these signaling processes, all of these proteins are either lipid-anchored or are associated with lipids.

### Signaling via Urokinase-Type Plasminogen Activator Receptor

Earlier studies have identified mitogenic effects mediated by u-PA in a number of cell lines.<sup>19,20,22</sup> Binding of u-PA to u-PAR can, in fact, induce numerous intracellular signaling events. These include serine phosphorylation of cytokeratins 18 and 8 in the epithelial cell line WISH;<sup>67</sup> tyrosine phosphorylation of a 38 kDa protein in the monocytic cell line U937;<sup>69</sup> tyrosine phosphorylation of focal adhesion kinase (FAK), paxillin, and p130<sup>cas</sup> in bovine aortic endothelial cells;<sup>70</sup> activation of mitogen-activated protein kinase MAPK, ERK1 and ERK2 in human fibrosarcoma cell line HT 1080 cells<sup>71</sup> and in bovine aortic endothelial cells;<sup>70</sup> activation of the JAK/STAT pathway in the human kidney epithelial cell line TCL-598<sup>47</sup> and in human aortic smooth muscle cells;<sup>48</sup> de novo synthesis of diacylglycerol;<sup>72</sup> cAMP formation;<sup>73</sup> activation of inositol phosphate turnover; induction of Ca<sup>2+</sup> influx; release of Ca<sup>2+</sup> from intracellular stores,<sup>74</sup> and c-fos gene expression.<sup>71,75</sup> These effects are all mediated via the receptor-binding domain of u-PA and can occur in the absence of proteolytic activity. Treatment of cells with phosphatidylinositol-specific phospholipase C, which cleaves GPI-anchored proteins from the cell surface, blocks u-PA-induced signaling processes, indicating the requirement of an intact u-PAR on the cell surface. Taken together, signals induced by u-PA–u-PAR interaction are not at all uniform and, therefore, cannot easily be explained by one single mechanism or single, uniform transmembrane “adapter” molecule. In fact, all molecules within the “u-PAR signalosome” (Fig. 1) could in some way participate in the signaling events. In the following sections, two possible transmembrane adapter mechanisms, via the integrins and GPI30, will be used as examples for u-PAR signaling. Finally, we will evaluate a model for signaling of GPI-linked protein, such as u-PAR, based upon recently described lipid rafts in the cell membrane.

### Focal Adhesion Kinase Signaling

The amino-terminal fragment of u-PA was recently shown to induce association of u-PAR with  $\beta$ 1-Integrins in a u-PAR-transfected prostate carcinoma cell line when the cells were grown on fibronectin. This, in turn, led to tyrosine phosphorylation of focal adhesion kinase and p130<sup>cas</sup> and enhanced haptotactic motility.<sup>76</sup> FAK is localized in focal adhesion contacts and becomes rapidly phosphorylated and activated upon integrin ligation. FAK interacts with paxillin, localizing src, fyn and PI-3 kinase to focal adhesion contacts. In addition, association of FAK with Grb2 has been reported, suggesting a role for FAK in connecting integrins to downstream signaling pathways, such as the MAP kinase pathway. It is, however, unlikely that u-PAR directly interacts with FAK or paxillin. The binding of u-PA to u-PAR induces clustering of the u-PAR in focal adhesions, where it transduces its signal by activating FAK, paxillin, and p130cas through the cooperation with integrins and promotes cell migration by increasing turnover of focal adhesion contacts. There are other examples for such a mechanism of signal transduction by GPI-linked proteins, such as Ly-6, CD59 and CD55.<sup>70</sup> By the integrin-mediated mechanism u-PA induces association of u-PAR with integrins (Fig. 2c), integrins would, in turn, bring together the associated kinases with src-kinases localized in the inner leaflet of lipid rafts.

### JAK-STAT Signaling

Besides src-kinases, kinases of the JAK-family can also associate with u-PAR in several cell types. u-PAR was found to be associated with JAK1 and STAT proteins in detergent-insoluble membrane fractions in the kidney tumor epithelial cell line TCL-598<sup>47</sup> and co-localized with JAK1 and Tyk2 in the leading edge of the migrating human aortic smooth muscle cells, while JAK2, JAK3 and the Src-PTKs remained mobile in the plane of the plasma membrane.<sup>48</sup> These results link u-PAR to a known signaling pathway mainly utilized by cytokines. This pathway proceeds via dimerization of STAT proteins, their translocation to the nucleus and specific binding to the DNA interferon-gamma activation site (GAS), and interferon-stimulated response elements (ISREs). The u-PA-induced binding of the STAT transcription factors to these promoter elements was also observed in HT-1080 cells after transfection with ISRE sequences fused to a reporter gene.<sup>77</sup> In contrast to signaling via integrins as possible transmembrane adapters, in this case, src kinases in the lipid rafts do not appear to participate. Cross-linking of u-PAR would lead to increased affinity for GPI30 and would induce close contact of GPI30 associated JAK, leading to JAK activation and STAT signaling (Fig. 2b)

### Lipid Rafts, Caveolae, and Signaling via GPI-Anchored Proteins

In general, GPI-anchored proteins are confined in detergent-resistant membrane domains referred to as “lipid rafts,” which are enriched in glycosphingolipids, sphingomyelins, polyphosphoinositides, and cholesterol. These domains have also been shown to contain lipid-anchored tyrosine protein kinases and other cytoplasmic molecules implicated in membrane trafficking, cell adhesion, and signal transduction. Lipid rafts are normally small (less than 70 to 300 nm in diameter) and homogeneously distributed over the plasma membrane.<sup>78,79</sup> Friedrichson and coworkers recently discovered that rafts are present on the plasma membrane of living cells and contain approximately 15 protein molecules.<sup>80</sup> These lipid rafts might represent membrane zones in which GPI-anchored proteins are transiently confined for ~7-9 seconds. After such a confinement time they randomly move outside the rafts to enter a new raft. Approximately one-third of the GPI-anchored proteins are confined within these zones.<sup>81</sup> It is probably likely that similar movement occur in the inner sheet of the plasma membrane where then lipid-anchored signaling molecules would move in a similar fashion and would also be confined in the rafts for a respective time period. This time period, and the density of the signaling molecules, would not be sufficient for reciprocal activation of the kinases.

Cross-linking of the GPI-anchored proteins could either lead to association of these diffusely distributed rafts in larger caveolae, cholesterol-rich invaginated domains of the plasma membrane that participate in signal transduction,<sup>82</sup> or could simply increase the confinement time of GPI-linked proteins within a single raft (Fig. 2d).

By virtue of associated transmembrane adapters or by influencing the fluidity of the rafts in toto, confinement time on the inside of the rafts for lipid-anchored protein kinases would

increase, allowing enough time for reciprocal activation. Such fluidity change induced from the outside to the inside would critically depend on the cholesterol content of the rafts, since a decrease in cholesterol and an increase in the ratio between saturated and non-saturated fatty acids would also reduce signaling via GPI-anchored proteins.<sup>83</sup> Consistent with such a model are findings in some hematopoietic cells that can be stimulated rather non-specifically just by clustering of any cell surface GPI-protein.<sup>84</sup>

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