Urokinase-dependent Human Vascular Smooth Muscle Cell Adhesion Requires Selective Vitronectin Phosphorylation by Ectoprotein Kinase CK2*

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Urokinase (uPA)- and urokinase receptor (uPAR)-dependent cell adhesion to the extracellular matrix protein vitronectin (Vn) is an important event in wound healing, tissue remodeling, immune response, and cancer. We recently demonstrated that in human vascular smooth muscle cells (VSMC) uPA/uPAR are functionally associated with the ectoprotein kinase casein kinase-2 (CK2). We now asked whether CK2 regulates uPA-dependent cell adhesion to Vn, since the latter is a natural CK2 substrate. We found that Vn is indeed selectively phosphorylated by CK2 and that this phosphorylation is uPA-regulated in VSMC. Vn induces release of ecto-CK2 from the cell surface via a process termed “shedding.” CK2-mediated Vn phosphorylation was decisive for the uPA-dependent VSMC adhesion. Specific inhibition of CK2 completely abolished the uPA-induced cell adhesion to Vn. This effect was specific for cell adhesion to Vn and required participation of both uPAR and αβ3 integrins as adhesion receptors. CK2 localization at the cell surface was highly dynamic; Vn induced formation of clusters where CK2 colocalized with uPAR and αβ3 integrins. These results indicate that the uPA-dependent VSMC adhesion is a function of selective Vn phosphorylation by the ectoprotein kinase CK2 and suggest a regulatory role for Vn phosphorylation in the uPA-directed adhesive process.

The urokinase (uPA)/urokinase receptor (uPAR) system has multiple functions serving as a molecular link between pericellular proteolysis and signaling cascades, regulating cell adhesion, migration, and proliferation (1). Several reports suggest a possible role for uPA/uPAR in these processes independent of the uPA enzymatic activity. For instance, uPA has a nonproteolytic action as a cellular adhesion receptor for vitronectin (Vn). Vn is a multifunctional glycoprotein present in blood, extracellular matrix, inflammatory sites, and in atherosclerotic plaques (2). UPA contains a high affinity Vn-binding site, which is different from the uPA-binding site. This binding site makes uPAR uniquely poised to regulate extracellular proteolysis and the adhesion process (3). Vn has been shown to contain consensus sequences for phosphorylation by various protein kinases, such as cAMP-dependent protein kinase A (PKA), protein kinase C (PKC) and, and as recently described, the protein kinase casein kinase-2 (CK2) (4). Vn can be phosphorylated at specific sites that modulate its conformation and influences its subsequent functions (5–7). However, whether or not Vn phosphorylation plays any direct role in the uPA-directed cell adhesion has not been explored.

We recently demonstrated that in human vascular smooth muscle cells (VSMC), the cell surface CK2 is associated with uPA and might be activated by uPA. Activated CK2 mediates phosphorylation of the cell-surface shuttle protein nucleolin, which translocates then in its phosphorylated form into the cell and regulates mitogenic effects of uPA (8). However, ectoprotein kinases operating on numerous cell types can provide for the phosphorylation of both cell surface proteins and extracellular substrates and are required for the regulation of cell-cell communication, cell-matrix interactions, and signal transduction (9). Moreover, ectoprotein kinases can be set free from the intact cell in a process termed “shedding.” Thus, ectoprotein kinases may play a role in cell-environment interactions over some distances (10). CK2 is an ectoprotein kinase that phosphorylates numerous proteins including endogenous cell surface proteins and soluble protein substrates in the extracellular environment (11, 12). One natural CK2 substrate beyond nucleolin is Vn (7). The purpose of our study was to investigate the probable versatile function of ecto-CK2 in propagation of the uPA/uPAR-related events, in particular to clarify the physiological importance of Vn phosphorylation by CK2 for the uPA-dependent cell adhesion. We found that in human VSMC Vn-selective CK2-mediated phosphorylation determines the outcome of Vn-cell interactions that are highly important for the uPA-induced adhesion process.

EXPERIMENTAL PROCEDURES

Materials—Chemicals were purchased from Sigma, Amersham Biosciences, Inc., Merck, or Serva (Heidelberg, Germany). Radiochemicals and chemiluminescent signal enhancers were obtained from NEN™ Life Science Products, Inc. (Boston, MA). Vectashield mounting media was purchased from Vector Laboratories, Inc. (Burlingame, CA). Vitronectin was from Promega (Madison, WI) and from Life Technologies, Inc., fibronectin was from Life Technologies, Inc., and collagen was purchased from Sigma. Alkaline phosphatase beads were from Sigma. p-Nitrophenyl N-acetyl-β-D-glucosaminide was obtained from Sigma, G66976 and PKI-α were from Calbiochem (San Diego, CA), and 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole (DRB) and K-252b were...
from Alexis (San Diego, CA). uPA was purchased from American Diagnostica Inc. (Greenwich, CT). CK2 was purified from rabbit liver according to the described protocol (13) and was a gift from Dr. A. Vorotnikov. GPD peptide was purchased from Biomol (Plymouth Meeting, PA), a252 peptide was kindly donated by Prof. H. Chapman, and soluble uPA was purchased according to the laboratory protocol after expression in Chinese hamster ovary cells.

Antibodies—Monoclonal integrin anti-human CD61 antibodies were from PharMingen (San Diego, CA). Polyclonal anti-uPAR antibodies were purchased from American Diagnostica Inc., mono- and polyclonal anti-C2-α subunit antibodies were from Calbiochem (San Diego, CA). Cy5-tagged anti-rabbit and anti-goat IgG were purchased from Dianova (Hamburg, Germany). Alexa-488-conjugated goat anti-rabbit and anti-mouse IgG were purchased from Molecular Probes, Inc. (Eugene, OR). Normal rabbit IgG were from R&D system (Minneapolis, MN).

Cell Culture—Human VSMC from coronary artery were obtained from Clonetics (San Diego, CA). The cells were grown in SmGM2 medium (Clonetics) supplemented with 5% fetal bovine serum and were used in passage 7. For the uPA stimulation experiments, cells were cultured for 24 h in serum-free medium and were then treated with uPA as previously described (8).

Release of Ecto-CK2 Activity from Intact Cells and Phosphorylation Assay—The substrate-induced release of ecto-CK2 from intact cells into the supernatant and determination of activity through the phosphorylation of exogenous protein substrate phosphoelastin was carried out as described elsewhere (10) with some modifications. Briefly, subconfluent VSMC were washed once with shedding buffer (150 mM NaCl, 10 mM Tris, 20 mM NaH2PO4/Na2HPO4, 5 mM MgSO4, 0.5 mM EDTA, pH 7.4) prior to the incubation with the same solution containing 1 mg/ml phosphoelastin. After 10 min of incubation at 37 °C the postcellular supernatants were subjected to SDS-PAGE and autoradiography. For the inhibitory studies, the supernatants were preincubated with antibodies (0.1 mg/ml) or different inhibitors (10 μM PKI-α, 100 nM G96976, 1 mM PKI-α, 100 μM G69796) for 30 min at 37 °C before uPA in a final concentration of 25 mM was added. At the end of incubation period, wells were rinsed once with phosphate-buffered saline, and the adhered cells were treated with 50 μl of hexoseaminidase reagent (15 mM p-nitrophenyl N-acetyl-b-glucosaminide, 0.5% Triton X-100, 100 mM NaCl, 100 mM NaF, 0.5% Triton X-100) for 40 min at 37 °C. The reaction was stopped by addition of 100 μl of 0.2 M NaOH in H2O containing 5 mM EDTA, and the absorbance was measured at 405 nm. All experiments were performed in triplicate.

Immunofluorescence Microscopy—Immunofluorescent staining for CK2, uPAR, and α5β1 integrin using Alexa-488- and Cy3-conjugated secondary antibodies and confocal microscopy studies were performed as previously described (14). The degree of protein colocalization was quantified using the Laser Sharp 3.2 and NIH Image 1.62 confocal software.

RESULTS

Ecto-CK2 Is Released by and Phosphorylates Vn—To ascertain the existence of CK2 as an ectoprotein kinase at the surface of VSMC, we used the method of the substrate-induced release or shedding of ectoprotein kinase. Since CK2 has the unique property of accepting both ATP and GTP as phosphate donors, we examined phosphorylation of the specific CK2 substrate peptide by shed kinase in the presence of both donors. As shown in Fig. 1a, phosphoelastin, the substrate used to initiate CK2 shedding, promptly released kinase activity from the intact VSMC. The extent of the phosphorylation was almost the same when [γ-32P]ATP and [γ-32P]GTP were used as co-substrates, indicating that the released ectokinase was CK2-like. To more thoroughly address this conclusion, additional controls were performed. Thus, we used anti-CK2 specific antibody to show immunoblotting that the shed enzyme was CK2 (shown as inset in Fig. 1a). Furthermore, we used specific CK2 inhibitors such as DBR and heparin. Both compounds inhibited the phosphoelastin-released ectokinase activity (Fig. 1b).

We next examined the ability of ecto-CK2 to be shed by and phosphorylate Vn. VSMC were washed and incubated with or without Vn for 10 min. After removal of the cells, CK2 activity and Vn phosphorylation in the supernatants were assayed by adding [γ-32P]ATP. In all Vn phosphorylation experiments, the equal protein loading on the gel was confirmed by Coomassie staining (shown in lower panels). Preincubation with Vn resulted in the release of an ectokinase that phosphorylates Vn (Fig. 1c). No CK2 shedding and Vn phosphorylation was observed when VSMC were preincubated with shedding buffer alone (control lane). Since Vn might serve as a substrate for several protein kinases (4), the specificity of Vn phosphorylation was verified by checking different inhibitors for their ability to block Vn phosphorylation. The results shown in Fig. 1c (right upper panel) demonstrate that neither the PKA inhibitor PKI-α nor the PRC inhibitor G69796 affected the level of Vn phosphorylation. Instead, the CK2-specific inhibitors DBR and K252b completely abolished the Vn phosphorylation. These results strongly suggest that the enzyme specifically shed by Vn and phosphorylating Vn is CK2.

Ecto-CK2 Is Required for the uPA-directed VSMC Adhesion on Vn—CK2 was shown to be required for basal spreading and adhesion on Vn of several cell lines (7, 15). To elucidate the probable requirement of ecto-CK2 for the uPA-directed VSMC adhesion on Vn, several sets of experiments were performed. First, to characterize the adhesion process and to check the specificity of the uPA-induced VSMC adhesion on Vn, different extracellular matrices were used in the adhesion assay. As shown in Fig. 2a, uPA activated cell adhesion on Vn up to

harvested using 5 mM EDTA in phosphate-buffered saline, washed, and resuspended in serum-free medium. Cell suspensions (1.5 × 106 cells/100 μl) were added to each coated or uncoated well for 2.5 h at 37 °C. For inhibitory studies, cells were preincubated with antibodies (50 μg/ml), soluble uPAR (80 nM), peptides (2 mM CK2 substrate peptide, 100 μM a252 peptide, 100 μM random peptide), or inhibitors (44 nM heparin, 1 μM PKI–α, 100 μM G69796) for 30 min at 37 °C before uPA in a final concentration of 25 mM was added. At the end of incubation period, wells were rinsed once with phosphate-buffered saline, and the adhered cells were treated with 50 μl of hexoseaminidase reagent (15 mM p-nitrophenyl N-acetyl-b-glucosaminide, 0.5% Triton X-100, 100 mM NaCl, 100 mM NaF, 0.5% Triton X-100) for 40 min at 37 °C. The reaction was stopped by addition of 100 μl of 0.2 M NaOH in H2O containing 5 mM EDTA, and the absorbance was measured at 405 nm. All experiments were performed in triplicate.
Urokinase-dependent Cell Adhesion Is Regulated by CK2

Fig. 1. CK2 is shed from native VSMC in response to Vn and specifically phosphorylates Vn. 

(a) Time-dependent increase of the released protein kinase activity after incubation of intact VSMC with the CK2 substrate phosphitin was measured using a CK2-specific in vitro kinase assay in the presence of ATP or GTP as phosphate donor. Inset shows identification of shed CK2 in cell-free supernatants using immunoblotting after trichloroacetic acid protein precipitation. 

(b) Phosphitin-induced release of kinase activity was measured as above in VSMC, untreated (light gray bar), and pretreated with CK2-specific inhibitors (dark gray bars). The results are shown as folds of control.

(c) VSMC were treated with Vn as described under “Experimental Procedures.” Vn phosphorylation by the released ectokinase was monitored by SDS-PAGE and autoradiography (upper panels). To reveal the CK2 selectivity in Vn phosphorylation, VSMC were pretreated with anti-CK2 antibody and different inhibitors, as indicated. The Coomassie staining shows equal gel loading (lower panel).

3-fold. The basal cell adhesion corresponded in our experiments to 25–35% of adhered cells and increased after stimulation with uPA up to 50–80% of adhered cells. No uPA-directed cell adhesion was observed when fibronectin or collagen was used. These data are in a good agreement with the results of our experiments on CK2 shedding, demonstrating that CK2 was released from the cell surface exclusively in response to Vn and not to other matrix proteins, such as fibronectin and collagen (Fig. 2b). Thus, CK2 appears to be linked specifically to the uPA-induced VSMC adhesion on Vn. Next, we examined the effects of anti-CK2 antibody and the specific CK2 inhibitors, such as CK2 substrate peptide and heparin, on the basal and uPA-related VSMC adhesion on Vn. Anti-CK2 antibody specifically blocked the uPA-induced VSMC adhesion (Fig. 2c), whereas the basal adhesion remained at the same level. The CK2 inhibitors, heparin and CK2 substrate peptide, completely blocked the adhesive effects of uPA; however, they did not change the basal level of cell adhesion. Both the PKA- and PKC-specific inhibitors, PKI-α and Gö6976, were ineffective. Further evidence indicating that CK2 is necessary for the uPA-mediated cell attachment was obtained in experiments where ecto-CK2 was removed from the cell surface by phosphitin treatment. After phosphitin-induced shedding, VSMC were washed and used for the adhesion assay. The results demonstrate very clearly that CK2 removal from the cell surface almost completely abrogated the uPA-mediated adhesion process (Fig. 2d). Thus, CK2 selectively mediates the uPA-directed VSMC adhesion on Vn.

UPA Regulates Ecto-CK2-mediated Vn Phosphorylation That Is Required for VSMC Adhesion—We recently demonstrated that uPA can activate cell surface-associated CK2 in VSMC (8). To address the question whether this regulation might have relevance for Vn phosphorylation, VSMC were stimulated with different concentrations of uPA. Vn phosphorylation was monitored as described above. Representative results of these experiments are shown in Fig. 3a, upper panel. Already 1 nM of uPA increased 2-fold Vn phosphorylation by shed kinase within 10 min of VSMC stimulation. At higher uPA concentrations this stimulation decreased gradually. The equal protein loading on the gel was confirmed by Coomassie staining (shown in lower panels). In special control experiments it was shown that uPA by itself did not induce any CK2 release (data not shown).

To assess the importance of the ecto-CK2-mediated Vn phosphorylation to the uPA-related adhesion process, we used Vn in its dephosphorylated and phosphorylated form after subjection to dephosphorylation and phosphorylation in vitro. Cytosolic CK2 purified from rabbit liver was used for the Vn phosphorylation (see “Experimental Procedures” for details). The monitored incorporation of 32P in dephosphorylated Vn occurred with a stoichiometry of 0.29 mol of phosphate per mol of Vn (shown as inset in Fig. 3b); the same value was determined for fully phosphorylated Vn by others (7). The use of dephosphorylated Vn resulted in an increase in the uPA-induced VSMC adhesion (Fig. 3b). However, no promotion of cell adhesion in response to uPA was observed when Vn phosphorylated in vitro by CK2 purified from rabbit liver was used, although the basal adhesion was increased when compared with basal adhesion on dephosphorylated Vn (Fig. 3b). These results indicate that Vn phosphorylation exclusively by native cell surface-located CK2 is required for the uPA-directed VSMC adhesion.

UPAR and αβ3 Integrin Are Involved in Promotion of the uPA-induced CK2-mediated VSMC Adhesion on Vn—CK2 may have different functions depending on its subcellular compartmentalization (16). Moreover, the subcellular localization of CK2 appears to be tightly regulated. Therefore, we next sought to investigate the effect of adhesion process on the cell surface CK2 distribution. We relied on fluorescence microscopy as shown in Fig. 4a. Ecto-CK2 underwent displacement on the VSMC surface and formed CK2-enriched clusters when cells were adhered on Vn. In contrast, in VSMC adhering to other matrixes CK2 remained randomly distributed over the entire VSMC body.

Formation of organized adhesive clusters is one of important steps in cell motility that provides functional interaction of different receptors and signaling molecules involved in adhesion process. Redistribution of CK2 on the surface of VSMC upon adhesion on Vn implied its association with other participants involved in regulation of cell adhesion. It has been demonstrated that the uPA-directed adhesion of smooth muscle cells is mediated by uPAR (17). The recent findings of others...
additionally suggest that motility of these cells requires association between uPAR and Vn receptor/β3 integrin (18). To test whether these adhesive receptors were connected with the CK2-mediated uPA-directed VSMC adhesion on Vn observed in our experiments, uPAR- and Vn-directed agents were used. The results of these experiments are shown in Fig. 4b. VSMC treatment with soluble uPAR competing with cell surface uPAR for the uPA binding resulted in abrogation of the uPA-induced cell adhesion on Vn thus confirming the requirement of uPAR for adhesion process. RGD peptide, which blocks Vn binding to its integrin receptor, inhibited VSMC adhesion in response to uPA; however, to a lesser extent than soluble uPAR. To further define the role of supposed association between both adhesive receptors in augmenting VSMC adhesion to Vn the physical association between integrins and uPAR was modulated. For this purpose, the integrin critical binding site for uPAR (residues 424–440) was imitated by a homology peptide, a325. As a control in these experiments, unspecific random peptide (HQLP) was used. In the presence of a325 peptide, the uPA-induced cell adhesion was blocked, whereas control peptide was ineffective. These data essentially confirm the necessity of both uPAR and integrin for mediating the uPA-directed VSMC adhesion on Vn and suggest that the interaction between these adhesive receptors is critical for the adhesion process.

**DISCUSSION**

To our knowledge, this is the first report demonstrating the importance of the cell surface-located ecto-CK2 for the uPA-mediated VSMC adhesion on Vn. Our study has three major findings. First, uPA induces specific phosphorylation of Vn by ecto-CK2. Second, Vn-specific phosphorylation by ecto-CK2 is required for the uPA-related VSMC adhesion. Third, CK2 localization at the cell surface is highly dynamic. When VSMC are exposed to Vn, CK2 forms clusters where it colocalizes with uPAR and αβ3 integrin. Our data indicate that the uPA-

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**FIG. 2.** UPA-induced VSMC adhesion on Vn is CK2-mediated. a, cells were seeded on Vn-, fibronectin-, or collagen-coated wells, stimulated with uPA and subjected to the adhesion assay as described under “Experimental Procedures.” b, CK2 shedding followed by Vn phosphorylation in response to different indicated matrix proteins was performed as in Fig. 1. c, cells were seeded on Vn-coated wells, stimulated with uPA (dark gray bar) or unstimulated (white bar), and treated with anti-CK2 antibody and inhibitors (CK2 substrate peptide and heparin) with the PKC-specific inhibitor PKi69 or PKA inhibitor Go6976 (light gray bars) as described under “Experimental Procedures.” d, cells were treated with phosvitin to release ecto-CK2 and then used for the uPA-induced cell adhesion assay as described above. The result is shown as a direct comparison between phosvitin-treated (dark gray bars) and untreated cells (light gray bars).
induced selective phosphorylation of Vn by cell surface CK2 is a pivotal event in VSMC adhesion and motility. The recent identification of ectoprotein kinases with catalytic properties on the cell surface shed new light on the potential role of extracellular phosphorylation for numerous physiological and pathological processes. Ectoprotein kinases represent the powerful regulatory machinery of protein phosphorylation operating at the cell surface and are critical for intercellular communication and transduction of external stimuli. Remarkably, several cell types were shown to shed ectokinases from their surface in a substrate-induced fashion (19). Shedding of ectokinases, as a specific and immediate cell response to protein substrate, might have a broad role in regulating cell-environment interactions. The stimuli initiating such responses may include cell surface adhesion molecules, growth and coagulation factors, enzymes, and receptors. Although this field of investigation is young, a growing body of evidence suggests that ectoprotein and ectodomain shedding is one widely used mechanism that contributes to various physiological events. These events include host defense, wound healing, arthritis, and Alzheimer’s disease (20–22). However, an understanding of the underlying molecular mechanisms remains largely unknown. CK2-like protein kinases have been reported to be secreted by activated platelets, neutrophils, and endothelial cells in response to various stimuli (23). Furthermore, circulating blood proteins, such as coagulation factors and fibrinogen, were shown to serve as substrates for the platelet-released CK2 (24). The results of our study clearly demonstrate specific ecto-CK2 release from intact human VSMC in response to matrix protein Vn. These data suggest that CK2 might be shed from VSMC surface in response to specific developmental and pathophysiological cues. Apart from these facts, Vn is one major player involved in the uPA/uPAR-mediated cell adhesion, although the question whether or not this process requires Vn phosphorylation has not been previously addressed. We asked whether CK2-mediated Vn phosphorylation might have possible functional consequences for the uPA-regulated VSMC adhesion. Our results provide strong evidence that the uPA-regulated CK2-mediated Vn phosphorylation is a decisive step in the uPA-induced VSMC adhesion. Our findings confirm and extend the pivotal role for...
the CK2-mediated extracellular phosphorylation in regulating several functions of different molecules, such as adhesion, signal transduction, lytic activity, and resistance to proteolytic cleavage (25).

However, several observations require further clarification. Although our results suggest that CK2 shed in response to Vn, they do not indicate that shedding is an obligatory step in Vn phosphorylation. One possibility is a biphasic response in which uPA-activated CK2 may phosphorylate cell surface-bound as well as soluble Vn. The latter process is presumably mediated by CK2 shedding. This hypothesis is in agreement with reports of others demonstrating that ecto-CK2 may regulate protein phosphorylation in its membrane-bound and secreted form (25). VSMC might utilize the uPA-dependent and CK2-mediated Vn phosphorylation in the extracellular environment. This mechanism may be especially effective under certain pathological situations, such as injury, inflammation, or oxygen deficiency, where extracellular ATP required for phosphorylation can reach high concentration (9).

Another puzzling observation was obtained in our experiments using Vn in its dephosphorylated form and after phosphorylation in vitro by CK2 purified from rabbit liver. Contrary to our expectations, no promotion of cell adhesion in response to uPA on fully phosphorylated Vn was observed, although the basal level of cell adhesion was increased. At the same time, uPA did induce VSMC adhesion on the dephosphorylated Vn. These results indicate that the uPA-directed VSMC adhesion on Vn requires its specific phosphorylation by the native cell-specific ecto-CK2. They suggest also that Vn phosphorylation in vivo by cell surface CK2 and its phosphorylation in vitro by cytosolic CK2 of other cellular origin are not identical. In fact, it is hardly to expect that phosphorylations of Vn in vitro and in vivo are the same. In vitro phosphorylation, by using recombinant or purified components of phosphorylation reaction, does not take into account several important parameters. These parameters include the dynamic equilibrium between phosphorylation and dephosphorylation, the level of Vn prephosphorylation, as well as the local concentrations of magnesium and ATP, all of which are decisive for the phosphorylation reaction. Moreover, the CK2 family of protein kinases includes presumably numerous CK2-like enzymes with different, at least in part, properties (26). The existence of more than one isoform of the CK2 subunits may be more widespread than originally thought and opens the possibility that different subunits may confer different functional properties to the enzyme (27, 28).

CK2 has been studied extensively at the biochemical level. However, very little is known about its function in vivo. The definition of this problem is strengthened by the findings of

**FIG. 5.** CK2 and uPAR colocalize in dynamic cell surface clusters upon VSMC adhesion. VSMC were seeded on Vn-coated coverslips, fixed after 1 h (a–c), 2 h (d–f), or 3 h (g–i), and stained for CK2 using anti-CK2 monoclonal antibody and Alexa-488-coupled secondary antibody and for uPAR using polyclonal anti-uPAR antibody and Cy3-coupled secondary antibody, as indicated. In the third column (c, f, i) are superimposed composite figures of CK2 (a, d, g) and uPAR (b, e, h) from the first and the second columns. Yellow color denotes the colocalization of the two proteins. Right panel shows the CK2 and uPAR colocalization patterns in the magnified images (c’, f’, i’) of fields indicated in c, f, i, and the quantification of CK2 and uPAR colocalization. Bar, 50 μm.
others indicating that the actual level of CK2 expression and activity in a given cell type is very specific. Thus, even a small deviation would produce an altered or dysregulated biological response in the cell (29). This conclusion is supported by our results demonstrating that although uPA increased Vn phosphorylation and VSMC adhesion up to 3-fold, the calculated stoichiometry of this reaction was relatively low and corresponded to 0.42 mmol of phosphate per mol of Vn. This corresponds to the reports of others that the stoichiometry of phosphorylation of native Vn in plasma is low (30). Our findings additionally suggest that the Vn phosphorylation event in and of itself is not sufficient to mediate uPA-directed cell adhesion. Most likely, a proper dynamic association of the complex components, such as CK2-uPA-uPAR-Vn-H9251/H9263/H9252 integrin might be decisive to induce adhesive process. These considerations are in agreement with the findings of others that CK2-mediated phosphorylation of and its association with some substrates in vitro and in vivo are different (31). These results indicate that the uPA-directed VSMC adhesion on Vn is a complex and well controlled dynamic interplay of interdependent molecules in which native cell surface CK2 is a prerequisite. Multiple interactions of the involved components probably take place within the dynamic clusters where CK2, uPAR, and αβ3 integrin become colocalized during the adhesion process. Our last finding is consistent with the demonstration that ectokinases are strongly attached to lipid-anchored molecules on the cell surface membrane (32) and that CK2 is functionally associated with integrins (15). This observation may have relevance to a recent study in which uPAR clustering in human neutrophils was coupled to Vn-mediated signaling events (33).

The most important finding of our work is that we demonstrate for the first time the specific phosphorylation of Vn by ecto-CK2 released from the surface of native VSMC, that this process is regulated by uPA, and that the process is required for the uPA-induced cell adhesion on Vn. Of course, it remains to be determined under what physiological or pathophysiological circumstances this mechanism might be triggered and utilized in vivo. One likely situation might be cell damage upon vascular injury or inflammation where local concentration of ATP complexed with magnesium, as well as uPA expression and Vn deposition, are up-regulated. Moreover, Vn produced by VSMC at sites of injury is presumably in its dephosphorylated form and therefore might be effectively utilized in the proposed mechanism. 

Together, these findings highlight an important role of extracellular phosphorylation for the functional regulation of human VSMC and support a hypothetical model in which several possibilities regarding the mechanism and functional consequences of the uPA/uPAR-dependent ecto-CK2 stimulation in VSMC are considered (Fig. 7). VSMC stimulation with uPA in
the presence of Vn (α) leads to the activation of the cell surface, uPAR- and αβ3 integrin-associated ecto-CK2. The activation is most likely due to the conformational changes of this complex resulting from the uPA/uPAR binding and Vn-uPAR-αβ3 integrin association (b). The activated CK2 phosphorylates its natural substrates, such as the shuttle protein nucleolin and matrix protein Vn, which are also associated with uPAR (c). Phosphorylated substrates can contribute to the control of at least two important cellular functions (d). The first pathway addresses the mechanism of uPA-induced mitogenic effects via the CK2-mediated phosphorylation of nucleolin that leads to the nucleolin internalization and cell proliferation control (8). The second pathway encompasses Vn phosphorylation by uPA-activated CK2 that in turn increases Vn adhesive capacity and, correspondingly, the strength of VSMC adhesion mediated by two adhesive receptors, namely uPAR and αβ3 integrin. Under certain conditions, CK2 might be released, at least partially, from the cell surface. This shedding could contribute to phosphorylation of its substrate(s) in the extracellular environment and thereby could lead to cell-cell and cell-matrix interactions. A goal for the future will be to identify the molecular mechanisms of these interactions and their coordination in space and time and to determine how they contribute to regulation of complex events such as cell proliferation and migration.

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