Protein Kinase A-dependent Phosphorylation of Lutheran/Basal Cell Adhesion Molecule Glycoprotein Regulates Cell Adhesion to Laminin α5*

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Lutheran (Lu) blood group and basal cell adhesion molecule (B-CAM) antigens are both carried by two glycoprotein (gp) isoforms, Lu and Lu(v13), that belong to the Ig superfamily and differ only by the size of their cytoplasmic tail (59 versus 19 amino acids). They interact with the erythroid skeleton through spectrin binding of their common cytoplasmic domain (1). In contrast to Lu(v13), the Lu gp isoform contains a specific dileucine motif responsible for its basolateral targeting in epithelial cells (2) as well as potential phosphorylation sites consistent with a receptor signaling function.

The Lu/B-CAM gps represent the unique red cell receptors for laminin α5 in normal and in sickle red blood cells (3–5). Both Lu and Lu(v13) isoforms bind to soluble and immobilized laminin α5 (3, 4, 6). Laminins are heterotrimeric proteins composed of α, β, and γ chains that associate to form at least 15 heterotrimer proteins that are found in all basement membranes (7–9). Cell adhesion to laminin plays a critical role in proliferation, differentiation, and motility and in the progression of malignant tumors through interaction and activation of specific cell surface receptors (10–12). Laminins α5 (α5β1γ1 and α5β2γ1), which contain the α5 chain, are strong adhesive components for epithelial cells. The major integrin receptors of the laminin α5 are integrins α3β1 (13), α6β1 (14), α6β4 (15), αvβ3 (16), and α2β1 (17, 18). Although these integrins are the coreceptors of several laminins, Lu gps bind only to laminin α5 isoform. Lu/B-CAM gps also act as functional laminin α5 coreceptors with integrin α3β1 in renal epithelial and in smooth muscle cells (11, 19, 20).

Mice lacking laminin α5 chain die during midgestation with multiple morphological abnormalities of several tissues and their compartments (21). In bone marrow, laminin α5 chain is an adhesive substrate to stem cells and progenitor cells and influences progenitor cell migration in vitro (22). In sickle red blood cells, adhesion to thrombospondin, fibronectin, and laminin in the vasculature may dramatically impact vaso-occlusion events (for review, see Ref. 23). Overexpression of Lu/B-CAM antigens on sickle red cells correlates with an increased adhesion to laminin α5 (5, 24) and therefore might participate in the reinforced adhesion of sickle red cells to vascular endothelium. It was recently shown that the physiologic stress mediator epinephrine, acting through the β2-adrenergic receptor, increased the adhesion of sickle red blood cells to laminin α5 via a cAMP and PKA-dependent signaling pathway (25). The authors proposed a classical cascade in which the β2-adrenergic receptor stimulates Gαs proteins that in turn activate adenylyl cyclase, thus elevating the cAMP levels and leading to PKA activation. Lu/B-CAM gps were identified as receptors that mediate the stimulated adhesion of sickle red cells to laminin α5 under continuous flow conditions (26). This signaling pathway may also participate in vaso-occlusion events. However, the authors failed to reveal any target for phosphorylation by...
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PKA that could explain the Lu/B-CAM-mediated cell adhesion to laminin α5.
In this study, we investigated the phosphorylation status of Lu gps in normal and sickle red cells and in transfected erythroid K562 and epithelial Madin-Darby canine kidney cells (MDCK) cells. We demonstrated that Lu gp but not Lu(v13) isoform is phosphorylated by glycoprotein synthase kinase 3β (GSK-3β), casein kinase II (CKII), and PKA, at serines 596, 598, and 621, respectively. The Lu gp phosphorylation is enhanced by forskolin, a major activator of the PKA signaling pathway, in sickle red blood cells and by different stimuli of the PKA-pathway in K562 and MDCK cells. We examined the role of phosphorylation of the Lu gp in regulating the adhesion function to laminin α5 in K562 cells. We demonstrated that PKA-mediated phosphorylation of the Lu gp at Ser-621 positively regulates the adhesion function of Lu gp under flow conditions.

**EXPERIMENTAL PROCEDURES**

**Materials**—Primers used in PCR and mutagenesis experiments were from Eurogentec (Seraing, Belgium). The QuikChange site-directed mutagenesis kit was from Stratagene (La Jolla, CA). The pGEX-5X-3 vector, the protein A-Sepharose CL4B beads, and the glutathione-Sepharose 4B beads were purchased from Amersham Biosciences. The Complete protease inhibitor mixture was purchased from Roche Applied Science. Purified human α5 laminin, epinephrine, butyramine, and PKA catalytic subunit bovine were purchased from Sigma. 

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**Phosphorylation Assays in Transfected Cells**—MDCK or K562 cells were washed twice with ice-cold phosphate-buffered saline and then incubated with 200 μCi of orthophosphate 32P (17 mM) for 2 h at 37 °C in the presence of phosphatase inhibitor (1 μM okadaic acid) and Com-peroxisome proliferator inhibitor mixture. Cells were treated or not with 5 μM br-CAMP for 30 min, 200 μM forskolin for 20 min, or 200 μM epinephrine for 1 min at 37 °C.

**Erythrocyte Phosphorylation Assays**—Phospholabeling of red blood cells was performed on normal and sickle red blood cells, as described by Brunati et al. (29). 400 μl of erythrocytes were preincubated in 3.6 ml of buffer A (150 mM NaCl, 20 mM Tris-HCl, pH 7.5; 10 mM KCl; 1 mM MgCl2; 25 μg/mL chloroquine) for 1 h at 37 °C to deplete endogenous ATP stores. The cells were then centrifuged 750 × g for 3 min and resuspended in 2.1 ml of buffer A containing 300 μCi of orthophosphate 32P (25 mM) for 14 h at 35 °C. Cells were then incubated with 200 μM forskolin 20 min at 35 °C and in the presence of 1 μM okadaic acid (phosphatase inhibitor).

**Anti-Lu/B-CAM Immunoprecipitation**—Cells were lysed for 1 h at 4°C in Triton lysis buffer A for MDCK and K562 cells (150 mM NaCl, 20 mM Tris-HCl, pH 8, 5 mM EDTA) and Triton lysis buffer A for red blood cells (150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 10 mM KCl, 1 mM MgCl2), both containing 1% Triton X-100, 0.2% bovine serum albumin, and phosphatase and protease inhibitors. Lysates were centrifuged at 15,000 rpm for 15 min at 4°C. Aliquots of lysates were mixed with 1 μl of Lu antibody LM342 followed by anti-mouse IgG-coated beads (Dynabeads-M-450, Dynal A.S., Oslo, Norway). Samples were loaded on 8% and 12.5% polyacrylamide gels according to Laemmli and transferred to nitrocellulose membranes. Radioactive proteins were visualized by autoradiography and quantified by PhosphorImager analysis software (Optimas 6.1). The counted cells were then averaged and presented as adherent cells per mm2. For cell activation or inhibition preincubation, cells were washed three times with Triton lysis buffer A, two times with Triton buffer B (500 mM NaCl, 20 mM Tris-HCl, pH 8, 0.5% Triton, 0.2% bovine serum albumin), and once with 50 mM Tris-HCl, pH 8, 0.5% Triton. Immonocomplexes were resuspended and boiled in 1× Laemmli buffer. Samples were loaded on 8% SDS-PAGE gels and transferred to nitrocellulose membranes. Radioactive proteins were visualized by autoradiography and quantified by KODAK Image analysis software.

**Electrophoresis and Western Blot Analysis**—The SDS-PAGE was performed using 8 and 12.5% polyacrylamide gels according to Laemmli and transferred to nitrocellulose membranes. Protein bands were detected using the enhanced chemiluminescence (ECL) system according to the manufacturer’s instructions (Amersham Biosciences).

**Flow Adhesion Assays**—K562 cell adhesion to laminin α5 was measured under physiologic flow conditions using a plate flow chamber as described (30,31). 3.5 μg of the purified human α5 laminin mixture (or purified human fibronectin) were immobilized on clean glass microslides (Camlab, Cambridge, UK) of 3.5 cm2 by incubating overnight at 4°C. Then, microslides were mounted on a microscope stage and viewed by phase-contrast videomicroscopy. One end of the microslide was attached to a Harvard syringe pump, allowing the control of the flow rate across the microslide. The other end of the microslide was attached to a microelectronic valve (Lee Products Ltd., Gerrards Cross, UK), permitting smooth switching between K562 cells suspension and cell-free 0.5% human albumin Hanks’ buffer. Following insertion of the microslide into the flow system, 1 ml of K562 cells (3.10^6/ml) expressing various Lu/B-CAM gps in albumin/Hanks’ buffer were flowed across the microslide at a mean shear stress of 0.5 Pa by increasing the flow rate. Then, adherent cells were washed for 5 min using a flow rate of 0.054, 0.081, 0.108, 0.27, 0.54, 0.81, and 1.08 ml/min, producing shear stresses of 0.2, 0.3, 0.4, 1, 2, 3, and 4 dyne/cm², successively. After each wash, adherent cells were quantified in six representative areas along the centerline of the microslide by microscopy (×10) using a computerized image analysis system (Optimas 6.1). The counted cells were then averaged and presented as adherent cells per mm². For cell activation or inhibition...
Phosphorylation of the Lu gp is stimulated by forskolin in sickle red blood cells. A, amino acid sequence of Lu and Lu(v13) cytoplasmic domains. Black bars indicate consensus motifs potentially recognized by GSK-3β, CKII and PKA. Arrows point to potentially phosphorylated Ser residues (596, 598, and 621). B, Lu but not Lu(v13) is phosphorylated in sickle red blood cells. Normal and sickle red blood cells were radiolabeled with orthophosphate $^{32}$P. Radiolabeled intact normal and sickle red blood cells were incubated in the absence (lanes 1, 2, 4, and 5) or in the presence (lanes 3 and 6) of 200 μM forskolin 20 min at 35°C. Lu/B-CAM gps were immunoprecipitated, and phosphorylation was analyzed by autoradiography and quantified by KODAK ID Image analysis software. Lysates of normal or sickle red blood cells incubated without the F241 anti-Lu/B-CAM mAb overnight (lanes 1 and 4) represent the negative control. Western blots performed with 603 anti-Lu/B-CAM antibody showed equal amounts of Lu gp immunoprecipitated from normal and sickle red blood cells in the presence or absence of forskolin.

Experiments, 1 ml of K562 cells (3.10⁶/ml) were preincubated with 20 nM epinephrine for 1 min at 37°C or 160 μM butoxamine for 20 min at 37°C, centrifuged, and then flowed across the immobilized laminin α5.

Statistical Analysis—A Student’s t test was used to assess the statistical significance between adhesion of each cell line or adhesion of treated versus untreated cells.

RESULTS

Phosphorylation of the Lu gp Is Stimulated by Forskolin in Sickle Red Blood Cells—The amino acid sequence of the cytoplasmic domain of Lu gp isofrom but not of Lu(v13) contains three consensus serine phosphorylation sites for protein kinases GSK-3β, CKII, and PKA at positions 596, 598, and 621, respectively (Fig. 1A). Consensus sequences for these kinases are (S/T)XXX(S/T), SXX(E/D), RX_{-2}S(T)XX, respectively (PhosphoBase, NetPhos). To determine whether Lu/B-CAM gps are phosphorylated in normal and sickle red blood cells, cells were labeled with orthophosphate $^{32}$P, and Lu/B-CAM gps were immunoprecipitated with the F241 monoclonal anti-Lu antibody, which recognizes both Lu and Lu(v13) isoforms. Lu gp isofrom (85 kDa), but not Lu(v13), exhibited a weak level of phosphorylation in both normal and sickle red blood cells (Fig. 1B, lanes 2 and 5). These results suggested that Lu gp is phosphorylated in vivo, presumably by either PKA and/or CKII and/or GSK-3β kinases. To define whether the cytoplasmic domain of Lu gp undergoes PKA-dependent phosphorylation in response to stimulation, Lu gp phosphorylation was tested in the presence of 200 μM forskolin, an activator of adenylyl cyclase known to stimulate Lu gp adhesion to laminin α5 on sickle red blood cells through a cAMP-dependent PKA pathway (25). Stimulation of sickle red blood cells with forskolin resulted in a 2.8-fold increase of Lu phosphorylation (Fig. 1B, lanes 3 and 6), whereas no significant increase in phosphorylation was observed in normal red cells. The different levels of phosphorylation observed in sickle red blood cells before and after treatment with forskolin were significant as similar amounts of Lu gp were immunoprecipitated when tested by Western blot with anti-Lu antibody (Fig. 1B, bottom). These results demonstrated that Lu gp on sickle red blood cells undergoes forskolin-stimulated phosphorylation, presumably by a PKA-dependent pathway.

PKA Phosphorylates Lu gp at Serine 621—To determine whether Lu gp isofrom is a substrate of PKA and which serines are involved, phosphorylation experiments were performed in both erythroid K562 and epithelial kidney MDCK cells stably transfected by the pcDNA3-Lu or pcDNA3-Lu(v13) vectors. K562 and MDCK cells were labeled with orthophosphate $^{32}$P with or without treatment by br-cAMP, a stable, membrane-permeable cAMP analog that activates PKA. Lu/B-CAM gps were then immunoprecipitated as described under “Experimental Procedures.” The Lu gp isofrom (85 kDa) (Fig. 2A, lanes 1 and 7) but not Lu(v13) (Fig. 2A, lanes 5 and 11) was phosphorylated in K562 and MDCK transfected cells. As shown in Fig. 2A (lanes 1 and 2 and lanes 7 and 8), phosphorylation of Lu gp was increased following treatment with br-cAMP (2- and 2.1-fold), indicating that Lu gp might be phosphorylated by PKA in these cells. As expected, Lu(v13) was not phosphorylated after stimulation by br-cAMP in both cell lines (Fig. 2A, lanes 5 and 6 and lanes 11 and 12). Treatment with epinephrine or forskolin, two major activators of adenylyl cyclase, also increased phosphorylation of Lu gp in both cell lines (Fig. 2A, lanes 1, 3, and 4 and lanes 7, 9, and 10). To gain more insights into the role of PKA in Lu gp phosphorylation, we generated Lu mutants S621A and SS596–598AA impaired for the PKA and the clustered CKII/GSK-3β consensus phosphorylation sites, respectively (Fig. 1A). As shown in Fig. 2B (lanes 5 and 6), SS596–598AA mutant maintains enhanced phosphorylation after treatment of K562 and MDCK cells with br-cAMP (1.4- and 1.85-fold, respectively), whereas no significant stimulation was observed for the S621A mutant in both cell types (Fig. 2B, lanes 3 and 4). Differences observed in the level of phosphorylation before and after treatment with br-cAMP were significant as similar quantities of Lu gp were immunoprecipitated in each cell line when tested by Western blot with an anti-Lu antibody (Fig. 2, A and B, bottom). These results indicated that PKA phosphorylates Lu gp at Ser-621 in cellular models.
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GSK-3β and CKII Phosphorylate Lu gp at Serines 596 and 598—We also examined the phosphorylation of Lu gp by CKII and GSK-3β in K562 and MDCK cells. As shown in Fig. 2B, S621A (lane 3) as well as SSS96–598AA (lane 5) mutants remained phosphorylated in the absence of additional stimuli, in both cell types. In addition, phosphorylation was abolished for the triple mutant SSS96–598-621AAA (lanes 7 and 8), indicating that CKII and/or GSK-3β and PKA sites were functional and that no additional phosphorylation sites are involved. Altogether, these results indicated that Ser-596 and/or Ser-598 may be used by GSK-3β and/or CKII.

Since GSK-3β and CKII sites are overlapping and neither specific activators nor inhibitors of these enzymes could be used in our cellular models, we also performed phosphorylation reactions using recombinant fusion proteins GST-Lu, GST-Lu(v13), GST-Lu(596–598), GST-Lu mutants, [γ-32P]ATP, and purified kinases. As shown in Fig. 3A, the cytoplasmic domain of Lu gp (GST-Lu) is clearly a substrate of CKII in contrast to GST-Lu(v13) or GST alone as only GST-Lu is phosphorylated by this enzyme. It is known that GSK-3β requires a primed phosphopeptide for optimal activity with some substrates, i.e. a sequence that carries a phosphate group in the vicinity of the consensus motif (32). As shown in Fig. 3A, phosphorylation of Lu gp by GSK-3β requires the prephosphorylation of Lu gp by CKII since it cannot phosphorylate the cytoplasmic domain of Lu gp when incubated alone with GST-Lu. When serines 596 and 598 were mutated to alanine, no phosphorylation by CKII and GSK-3β was observed, whereas the GST-LuS621A mutant remained phosphorylated (Fig. 3A). Coomassie Blue staining confirmed that equal amounts of GST fusion proteins were used in all assays (Fig. 3B). Together with the experiments performed in cells, these results indicated that the Lu gp isoform is a substrate of CKII and GSK-3β at serines 596 and 598.

Effects of Ser to Ala Substitution on Cell Adhesion to Laminin α5 under Flow Conditions—We investigated the consequences of phosphorylation of Lu gp on cell adhesion to laminin α5 under flow conditions. We used erythroid K562 recombinant cells expressing Lu gp (K562-Lu) rather than epithelial MDCK cells as a cellular model since it was previously reported that Lu gp mediates adhesion of sickle red blood cells to laminin α5 and that epinephrine increased this adhesion (by 1.5–2.5-fold) (25). As control, we also examined adhesion of wild-type K562 (K562-WT) labeled with calcine on the same α5 laminin mixture coated slide. In this assay, a non-fluorescent form of calcine that diffuses into cells is cleaved by intracellular esterases, producing the highly fluorescent calcine and inhibiting its extrusion (“Experimental Procedures”). K562-Lu and fluorescent K562-WT cells were injected together in the flow at 0.2 dyne/cm². Very few K562-WT cells adhered to laminin α5 at shear stresses from 0.2 to 3 dyne/cm², in contrast to K562-Lu cells (Fig. 4A). Although Lu(v13) gp is not phosphorylated (Fig. 2A), an identical level of adhesion was observed for K562-Lu(v13) as compared with K562-Lu (data not shown). Neither Lu/B-CAM nor integrins α2β1, α3β1, α6β1, and α6β4, which represent laminin α5 receptors, are expressed endogenously in K562-WT cells (data not shown), indicating that only Lu or Lu(v13) gp expressed in K562 cells are responsible for the observed cell adhesion to laminin α5. We therefore examined whether the S621A and SSS96–598AA mutants of Lu gp, which impair PKA and CKII/GSK-3β phosphorylation sites, respectively, affect adhesion of K562 cells under flow conditions. K562-Lu and K562-LuSSS96–598AA cells exhibited the same number of adherent cells at shear stresses of 3 dyne/cm² (295 versus 308 adherent cells/mm², p < 0.1), whereas the S621A mutation led to a decrease of adhesion by 200–300-fold (16 adherent cells/mm²), indicating that phospho-epinephrine during 1 min, adhesion of K562-Lu (177 untreated

Epinephrine Stimulates Adhesion of K562-Lu but Not K562-Lu(v13) to Laminin α5 under Flow Conditions—Since Lu gp exhibited enhanced phosphorylation in K562 cells when treated with br-cAMP, forskolin, or epinephrine, we examined the effects of epinephrine on K562 cell adhesion to laminin α5 as it is the major mediator of the physiologic stress response known to elevate cAMP levels (33). When treated with 20 nM epinephrine during 1 min, adhesion of K562-Lu (177 untreated
and 535 treated adherent cells/mm²) but not K562-Lu(v13) cells (1229 untreated and 1026 treated adherent cells/mm²) was increased by 2-fold as compared with untreated cells even at shear stress of 4 dyne/cm² (Fig. 5A). This result indicated that raising cAMP levels by epinephrine in K562 cells, which enhances phosphorylation of Lu isoform, increased adhesion of Lu gp but not Lu(v13) to laminin α5. Differences observed in adhesion before and after treatment with epinephrine were significant as K562-Lu and K562-Lu(v13) cells exhibited similar expression levels of recombinant proteins at the cell surface (67,000 and 75,000/cell, respectively).

Previous studies indicated that the physiologic stress mediator epinephrine acts largely through the β2-adrenergic receptor on sickle red blood cells (33–37). Therefore, we tested whether the β2-selective antagonist butoxamine could inhibit epinephrine-stimulated K562-Lu adhesion to laminin. Fig. 5B shows that butoxamine suppresses the effect of epinephrine as adhesion of K562-Lu (100% of adherent cells/mm²) is no more suppressed by raising cAMP levels by epinephrine in K562 cells, which indicates that β2-adrenergic receptor is activated by epinephrine.

Epinephrine Stimulates Adhesion of K562-LuSS596–598AA but Not K562-LuSS621A Cells to Laminin α5—Since mutation of serine 621 abolished enhanced phosphorylation of Lu gp in br-cAMP-stimulated K562 cells (Fig. 2B) and decreased K562-Lu cell adhesion to laminin α5 under flow conditions (Fig. 4, B and C), we investigated adhesion of K562-LuSS596–598AA and K562-LuSS621A cells to laminin α5 when treated with epinephrine. Adhesion to laminin α5 of K562-LuSS596–598AA cells (Fig. 6A) was increased by 3-fold following treatment with epinephrine (1000 adherent cells/mm²) at shear stress of 3 dyne/cm² as compared with untreated cells (308 adherent cells/mm²). It is noteworthy that the level of increase is comparable with that of K562-Lu cells (295 untreated and 839 treated adherent cells/mm²) (Fig. 6). In contrast, this treatment had no effect on adhesion of K562-LuSS621A cells (16 untreated and 12 treated adherent cells/mm²) (p < 0.01). This result was confirmed at different shear stresses from 0.4 to 3 dyne/cm² (Fig. 6B). This indicated that PKA activation by stimulation of transfected K562 cells with epinephrine positively regulates cell adhesion to laminin α5 and that this activation is mediated specifically by Ser-621 of the Lu gp isoform.

**DISCUSSION**

In this study, we demonstrated that the cytoplasmic domain of the Lu gp, but not of Lu(v13), is weakly phosphorylated in both normal and sickle red blood cells. However, we showed that the phosphorylation of Lu gp was enhanced by forskolin in sickle red blood cells but not in normal red blood cells, implicating a cAMP-dependent PKA phosphorylation mechanism. Phosphorylation of Lu gp was further investigated in recombinant K562 and MDCK cells in which phosphorylation stimulation by epinephrine, forskolin, and br-cAMP was also observed.

Together, *in vitro* and *ex vivo* phosphorylation assays, as well as site-directed mutagenesis, revealed that GSK-3β, CKII, and PKA kinases phosphorylate Lu gp on serines 596, 598, and 621, respectively. We have examined the biological effects of Lu gp phosphorylation in regulating adhesion of K562-Lu cells to laminin α5. Erythroleukemic K562 cells represent immature erythroid cells and provide a good model for studying phosphorylation of Lu gp and adhesion to laminin α5 since these non-adherent cells contain all components of the cAMP/PKA-dependent signaling pathway described in epinephrine-stimulated sickle red blood cell adhesion (25, 38, 39). We showed that phosphorylation of LuSS596–598AA, in which the CKII and GSK-3β phosphorylation consensus sites are mutated, but not of the LuS621A mutant, in which the PKA consensus site is disrupted, remained activated by br-cAMP. It is noteworthy that the stimulation factor of Lu gp phosphorylation after cell treatment by epinephrine is weaker than the one after treatment by br-cAMP or forskolin. This can be due to the fact that incubation time of epinephrine is very short (1 min). Furthermore, stimulation of K562-Lu with epinephrine positively regulates cell adhesion to laminin α5 under flow conditions. This is mediated specifically by Ser-621 phosphorylation since LuSS596–598AA, but not LuS621A mutant, exhibited increased adhesion to laminin α5.

It was previously shown that adhesion of sickle red blood cells to vasculature can be modulated by signaling events (40). Indeed, in the presence of physiological shear stress, integrin-associated protein (CD47) specifically activates sickle red blood cell adhesion to immobilized thrombospondin via a Gα and tyrosine-kinase-dependent pathway. Moreover, Hines et al. (25) demonstrated that sickle red blood cells from about 46% of patients could be stimulated by epinephrine via a cAMP-dependent activating pathway, leading to an increase of Lu/B-CAM adhesion to laminin α5. However, these authors failed to demonstrate Lu/B-CAM gps phosphorylation by PKA and suggested that an unidentified protein may act as an intermediate between PKA and Lu/B-CAM receptor. In contrast, we have clearly identified the missing link by showing in the present
report that Lu gp is phosphorylated by PKA on Ser-621 in sickle red cells and recombinant cell lines and that this phosphorylation regulates K562-Lu cell adhesion to laminin α5. It is noteworthy that forskolin treatment results in similar stimulation factor of Lu gp phosphorylation (our results) and of adhesion of sickle red blood cells to laminin (25). Interestingly, phosphorylation of LW/ICAM-4 by PKA has been recently shown to be an additional mechanism that activates sickle cell adhesion to endothelium through interaction with the endothelial αvβ3 integrin (41). Our current results, together with those from others (25), support the working hypothesis that the phosphorylation of Lu gp may represent one critical factor that modulates the adhesiveness of sickle blood cells to laminin in the vasculature of sickle cell patients. Indeed, laminin α5 is present in endothelial basement membranes and is thus buried beneath endothelial cells that line the vascular wall. However, laminin together with thrombospondin and fibronectin is accessible to circulating blood cells in pathological conditions in which the endothelium is damaged (42). Thus, adhesion of red cells to laminin in the vasculature may dramatically impact the vaso-occlusion events that occur in the sickle cell disease (for review, see Telen (Ref. 23)).

It was previously demonstrated that Lu and Lu(v13) molecules expressed in transfected cells bind to soluble and immobilized laminin α5 equally well and that these proteins both mediate strong adhesion to immobilized laminin α5 in flow adhesion assay (5, 43). Accordingly, we showed that both Lu and Lu(v13) gps strongly mediate laminin α5 binding under flow conditions but that more K562-Lu(v13) cells adhere to laminin as compared with K562-Lu cells (by 1.5–2-fold at shear stresses of 3 and 4 dyne/cm²). Furthermore, we demonstrated that Lu(v13) isoform is not phosphorylated and that stimulation by epinephrine has no effect on K562-Lu(v13) cell adhesion. We suggest that serine 621 phosphorylation may induce
conformational changes of Lu gp, thus increasing its affinity for laminin α5. In contrast, Lu(v13) isomorf might be in an activated conformation and constitutively adheres to laminin α5. Furthermore, Lu(v13) is not the major isomorf in normal and sickle red blood cells since its expression is very low as compared with Lu gp isomorf (Fig. 1B). Indeed, Lu and Lu(v13) expression was quantified on normal and sickle red cells membranes from 8 and 10 individuals, respectively, by Western expression was quantified on normal and sickle red cells membranes from 8 and 10 individuals, respectively, by Western

Recently, Murphy et al. (44) have demonstrated that cAMP signaling can promote sickle red blood cell adhesion to laminin α5 via Lu/B-CAM through two signaling pathways, a PKA- or an Epac-Rap1-dependent pathway. Epac is a widely expressed exchange factor for the small GTPases Rap1 and Rap2 and represents a receptor for cAMP (45). Rap1 represents a molecular switch that cycles between an inactive GDP- and active GTP-bound conformation and promotes the activation of integrin adhesion receptor, thus leading to cellular adhesion (45). Although both Epac and Rap1 are endogenously expressed in

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Effects of epinephrine (epi) and butoxamine (butox) on adhesion of K562-Lu and K562-Lu(v13) cells to laminin α5 under flow. A, effects of epinephrine. Adhesion of K562-Lu (∇) and K562-Lu(v13) (∇) cells to immobilized laminin α5 was measured using the flow adhesion assay after treatment or not for 1 min at 37 °C with 20 nM epinephrine (● and ▲). Adherent cells per mm² are shown after exposure to increasing shear stress of 1–4 dyne/cm². Each result represents a mean of three experiments. Lu and Lu(v13) expression levels were similar as measured by flow cytometry; B, effects of butoxamine. Inhibition of epinephrine-stimulated adhesion of K562-Lu cells to immobilized laminin α5 was measured in the flow adhesion assay. K562-Lu cells were injected in the flow following epinephrine-stimulation (20 nM epinephrine for 1 min) or in the presence of the β2-selective antagonist butoxamine (160 μM for 30 min) followed by epinephrine-stimulation (20 nM epinephrine for 1 min). The histogram represents the percentage of adherent K562-Lu cells treated with epinephrine (+ epi), butoxamine and epinephrine (+ butox + epi), and butoxamine alone (+ butox), at shear stress of 1 dyne/cm², as compared with the 100% of adherent untreated K562-Lu cells. Each result represents a mean of three experiments.

K562 cells (45, 46), we suggest that mainly the PKA-dependent pathway is required for stimulated K562-Lu cell adhesion since substitution of serine 621 into alanine completely inhibits epinephrine stimulation of K562-Lu cell adhesion to laminin.

The Lu/B-CAM antigens are also expressed in epithelial tissues (11, 47). This is consistent with the presence of laminin α5 in these tissues (48). Our current results demonstrated that Lu gp is phosphorylated by GSK-3β, CKII, and PKA in MDCK epithelial cells. It is well known that inside-out regulation of the receptor adhesive properties is frequently promoted by phosphorylation (49–51). For example, adhesion mediated by integrin α4β1 results in increased T cell migration via a phosphorylation-mediated interaction with paxillin, a signaling adaptor protein (52). Moreover, it has recently been demonstrated that Lu/B-CAM is functionally involved in skin tumor cell binding and migration through the laminin α5 containing stroma (47). Since laminin also appears to increase tumor cell adhesion and migration, it will be of interest to further analyze the impact of Lu phosphorylation on these biological events in normal and pathological epithelial cells.

In conclusion, our results supported the view that phosphorylation of the intracytoplasmic domain of Lu gp plays a functional role in PKA-dependent adhesion to laminin α5. Therefore, modulation of the phosphorylation state of Lu gp might be a critical factor for sickle red blood cell adhesiveness to laminin α5 in sickle cell disease.

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