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The Control anti- $\alpha\beta 3$ micrograph in the original version of Fig. 5 A was a duplicate of the Control micrograph in Fig. 6 A. The authors have indicated that this was due to a clerical error during figure preparation. A corrected version of Fig. 5 A is shown below.

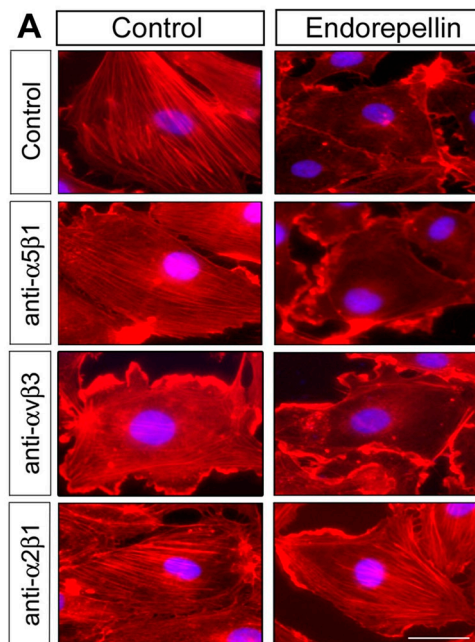


Figure 5. (A) ER action is blocked by function blocking antibody against $\alpha 2\beta 1$ integrin, but not by antibodies against other integrins. The cells were processed as in the legend to Fig. 3, after 10-min incubation with saturating concentrations (10 $\mu\text{g}/\text{ml}$) of specific mAbs \pm ER. Bar, 10 μm .

In addition, the original version of Fig. 9 B showed a composite panel for Total Erk1/Erk2 that contained three duplicated lanes. The authors have indicated that the wrong panel was inserted due to a clerical error during figure preparation. A corrected version of Fig. 9 B is shown below.

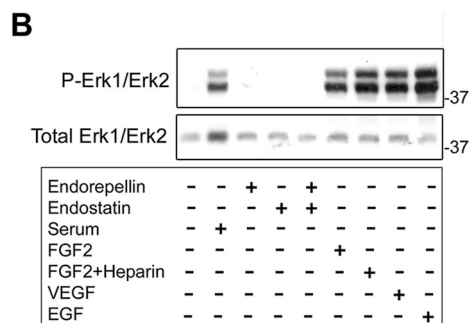


Figure 9. (B) Detection of Erk1/Erk2 MAPK phosphorylated at T202 and Y204 (P-Erk1/Erk2) and total Erk1/Erk2 MAPK upon stimulation of serum-starved HUVECs with 10% FCS, ER (100 nM), endostatin (100 nM), ER and endostatin (100 nM each), FGF2 (75 ng/ml) \pm heparin (100 ng/ml), VEGF (75 ng/ml), or EGF (75 ng/ml), as indicated.

The html and pdf versions of this article have been corrected. The errors remain only in the print version.

Endorepellin causes endothelial cell disassembly of actin cytoskeleton and focal adhesions through $\alpha 2\beta 1$ integrin

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Endorepellin, the COOH-terminal domain of the heparan sulfate proteoglycan perlecan, inhibits several aspects of angiogenesis. We provide evidence for a novel biological axis that links a soluble fragment of perlecan protein core to the major cell surface receptor for collagen I, $\alpha 2\beta 1$ integrin, and provide an initial investigation of the intracellular signaling events that lead to endorepellin anti-angiogenic activity. The interaction between endorepellin and $\alpha 2\beta 1$ integrin triggers a unique signaling pathway that causes an increase in the second messenger cAMP; activation of two proximal kinases, protein kinase A and focal adhesion

kinase; transient activation of p38 mitogen-activated protein kinase and heat shock protein 27, followed by a rapid down-regulation of the latter two proteins; and ultimately disassembly of actin stress fibers and focal adhesions. The end result is a profound block of endothelial cell migration and angiogenesis. Because perlecan is present in both endothelial and smooth muscle cell basement membranes, proteolytic activity during the initial stages of angiogenesis could liberate antiangiogenic fragments from blood vessels' walls, including endorepellin.

Introduction

Angiogenesis, the process of new blood vessel formation from existing blood vessels, allows tumors to expand beyond a few millimeters by providing oxygen, nutrients, and waste disposal at distances greater than allowed by diffusion (Folkman, 2002). Therefore, several antiangiogenic compounds have been characterized as potential cancer therapies. The mechanisms of action of these compounds are gradually being elucidated. Increasingly, these studies have demonstrated the importance of integrins in angiogenesis. For example, endostatin, a COOH-terminal fragment of collagen XVIII, interacts with $\alpha 5\beta 1$ integrin, a major fibronectin receptor in endothelial cells (Rehn et al., 2001), and causes disruption of focal adhesions and actin stress fibers by down-regulating RhoA (Wickström et al., 2002, 2003). Tumstatin, a portion of collagen IV, binds to $\alpha v\beta 3$ integrin and triggers a signaling cascade that leads to inhibition

of endothelial protein synthesis and proliferation (Sudhakar et al., 2003).

Perlecan, a heparan sulfate proteoglycan expressed in all vascular basement membranes (Iozzo, 1998), plays a significant role in vasculogenesis and tumor angiogenesis (Iozzo and San Antonio, 2001). Lack of perlecan in mice causes intra-pericardial hemorrhage and death at embryonic day 10.5 when vasculogenesis is prominent (Arikawa-Hirasawa et al., 1999; Costell et al., 1999), as well as a high incidence of cardiac outflow tract malformations and transposition of great vessels (Costell et al., 2002). Perlecan is secreted by adult endothelial cells and plays an essential role in regulating the vascular response to injury *in vivo* (Nugent et al., 2000).

We recently discovered that the COOH-terminal domain V of perlecan inhibits several aspects of angiogenesis, including endothelial cell migration, collagen-induced capillary morphogenesis, and blood vessel growth in the chorioallantoic

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Key words: perlecan proteoglycan; angiogenesis; endothelial cell; collagen; LG module

Abbreviations used in this paper: Ad, adenovirus; AP, human placental alkaline phosphatase; [cAMP]_i, intracellular cAMP; ER, endorepellin; Hsp27, heat shock protein 27; HUVEC, human umbilical vein endothelial cell; LG, laminin G-like; MEC, human microvascular dermal endothelial cell; NMuMg, normal murine mammary gland epithelial cells; PKA, protein kinase A; SPR, surface plasmon resonance spectroscopy.

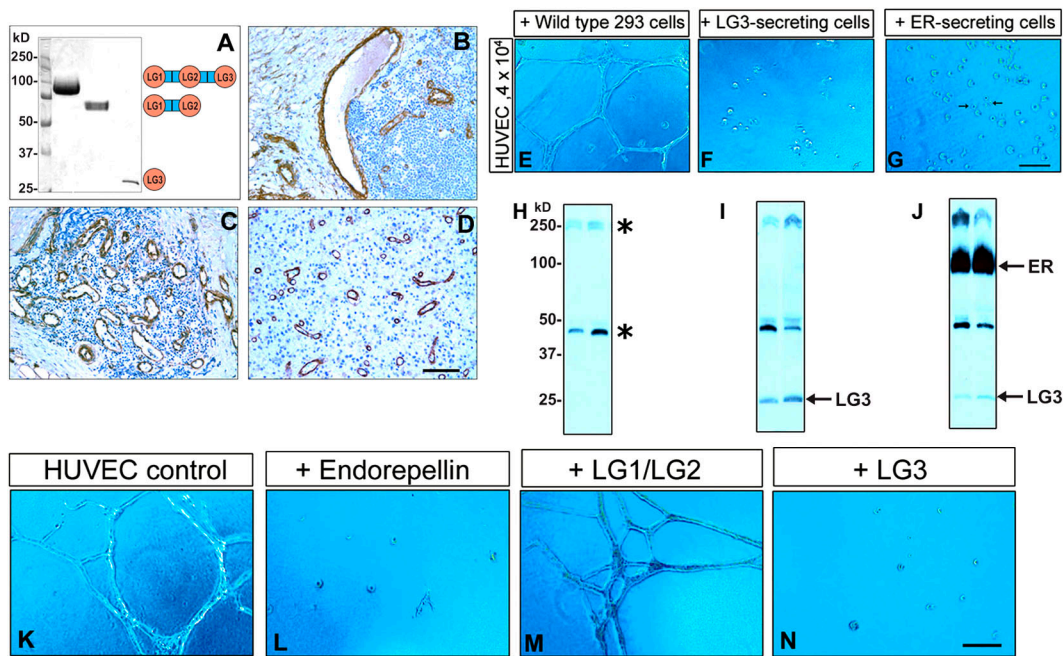


Figure 1. ER and its COOH-terminal LG3 module disrupt capillary morphogenesis in Matrigel. (A) Purification of ER and two fragments encompassing the LG1/LG2 and LG3 domains, respectively, as schematically shown in the right margin. The picture represents a Coomassie-stained SDS-PAGE of purified recombinant proteins. (B–D) Light micrographs of normal breast (B), breast carcinoma (C), and astrocytoma (D) showing specific labeling of blood vessels using the anti-ER antibody. (E–G) Capillary morphogenesis after coculture of 293-EBNA cells (within Matrigel) and HUVECs (on top of Matrigel) as indicated. (H–J) Immunoblotting of conditioned media from the corresponding cocultures shown in E–G. Notice the presence of LG3 and ER (arrow) in the coculture with the LG3- and ER-secreting 293-EBNA cells. The nonspecific bands derived from serum proteins are labeled by asterisks. (K–N) Inhibition of HUVEC capillary morphogenesis on Matrigel by recombinant ER and LG3, but not by LG1/LG2 (150 nM, each, for 24 h). Bars, 100 μ m.

membrane and in Matrigel plug assays (Mongiat et al., 2003). We named it endorepellin (ER) to signify its anti-endothelial activity. In this work, we investigated the molecular mechanism by which ER alters endothelial cell function. We found that ER, and one of its laminin G-like (LG) components, LG3, in a calcium-dependent but heparan sulfate-independent fashion, disrupted actin stress fibers and focal adhesions via interaction with the collagen receptor α 2 β 1 integrin. This interaction led to a rapid rise in intracellular cAMP ([cAMP]_i) levels, concurrent activation of cAMP-dependent protein kinase A (PKA), sustained activation of FAK, and transient activation of p38 MAPK and heat shock protein 27 (Hsp27), which ultimately culminated into disassembly of actin stress fibers and focal adhesions.

Results

Recombinant ER and its COOH-terminal LG3 module disrupt capillary morphogenesis in Matrigel or collagen overlay assays

Using 293-EBNA embryonic kidney cells, we generated ER and two deletion fragments encompassing the two LG modules and the EGF repeats (LG1/LG2) and the terminal LG3 module (Fig. 1 A). Polyclonal antibodies against ER showed specific labeling of blood vessels in normal (Fig. 1 B) and neoplastic tissues (Fig. 1, C and D).

Next, we investigated the antiangiogenic properties of ER and its fragments using a coculture system within Matrigel.

To this end, we cultured for 24 h wild-type 293-EBNA cells or clones expressing ER and its fragments within a three-dimensional Matrigel environment. After 24 h, human umbilical vein endothelial cells (HUVECs; $\sim 10^4$) were seeded on the top and cocultured for another 24 h. Thus, the HUVECs did not have direct physical contact with the 293-EBNA cells, which are embedded in the Matrigel, and any effects on tube formation would be due to secreted factors. The presence of LG3- (Fig. 1 F) and ER-secreting (Fig. 1 G) 293-EBNA cells prevented the formation of capillary morphogenesis obtained when HUVECs were cultured with either wild-type cells (Fig. 1 E) or alone (not depicted). No effects were obtained with LG1/LG2 secreting cells (unpublished data). The conditioned media were analyzed by Western immunoblotting and showed the presence of LG3 and ER (Fig. 1, I and J, respectively), in addition to two cross-reacting bands present in the control samples (Fig. 1 H, asterisks). In further experiments, we found that the two cross-reacting bands were due to serum proteins because they were not detected when cells were cultured in serum-free media. To further corroborate the specificity of these effects, we tested recombinant ER and its fragments (50–150 nM each for 24 h) on capillary morphogenesis on Matrigel. The results showed abrogation of capillary morphogenesis by ER and LG3 but not LG1/LG2 (Fig. 1, K–N). Similar results were obtained using collagen overlay assays (unpublished data). Thus, these data suggest that COOH-terminal module LG3 might contain most of the biological activity of ER.

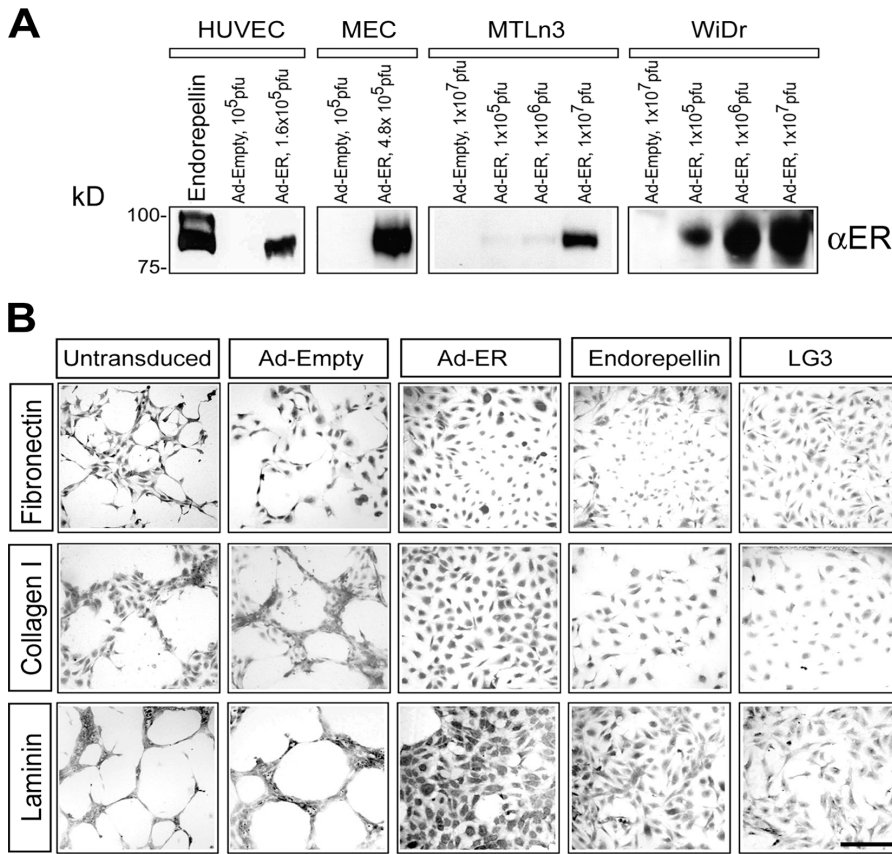


Figure 2. Transduction of endothelial cells with Ad-ER prevents capillary morphogenesis in collagen overlay assays. (A) Western immunoblotting of media from various cells transduced with control adenoviral vector (Ad-Empty) or Ad-ER at the designated plaque forming unit (pfu) using anti-ER antibody (α ER). About 4×10^4 HUVECs and 6×10^4 MECs cells were transduced with indicated amounts of Ad-Empty or Ad-ER for 48 h. 2×10^4 rat breast carcinoma MTLN3 cells and 2×10^4 human colon carcinoma WiDr cells were transduced with Ad-Empty or increasing amounts of Ad-ER as indicated. Conditioned media were harvested after 48 h. (B) Wells were precoated with $5 \mu\text{g}/\text{cm}^2$ human fibronectin or laminin, or with $10.5 \mu\text{g}/\text{cm}^2$ collagen I; 3×10^4 HUVECs were plated and transduced with 4.8×10^6 pfu of Ad-Empty or Ad-ER for 48 h. Control wells were left untransduced or treated with 150 nM ER or 50 nM LG3 for 20 min in serum-free conditions. Collagen gels were added to the cells for 24 h, to induce capillary morphogenesis. At the end of the incubation, collagen gels were removed and cells fixed and stained. Bar, $100 \mu\text{m}$.

Transduction of endothelial cells with adenovirus-containing ER (Ad-ER) prevents capillary morphogenesis

Next, we determined whether or not the endogenous secretion of ER by endothelial cells via Ad-ER would disrupt capillary morphogenesis in a dominant-negative fashion because endothelial cells synthesize perlecan (Iozzo, 1998). First, we determined the effective transduction of several cells with diverse histogenetic backgrounds, including HUVECs, human microvascular dermal endothelial cells (MECs), rat MTLN3 breast carcinoma cells, and WiDr human colon carcinoma cells. These cells showed a significant and dose-dependent synthesis of ER (Fig. 2 A). Next, we plated HUVECs and MECs on fibronectin, collagen I, or laminin for 24 h, and then we transduced them with Ad-ER for 48 h. A collagen gel was then added to the cells to induce tube formation. Untransduced and HUVECs transduced with control adenovirus (Fig. 2 B, Ad-Empty) formed tubes (Fig. 2 B), whereas Ad-ER transduced HUVECs failed to do so. Similar effects were also obtained using recombinant ER or LG3 (Fig. 2 B), as well as when MECs were used (not depicted). Thus, inhibition of capillary morphogenesis is induced by both endogenous synthesis of ER and exogenous recombinant protein, and these effects occur in endothelial cells from umbilical vein and dermal microvasculature.

ER and LG3, but not LG1/LG2, reversibly disrupt endothelial cell actin stress fibers and focal adhesions

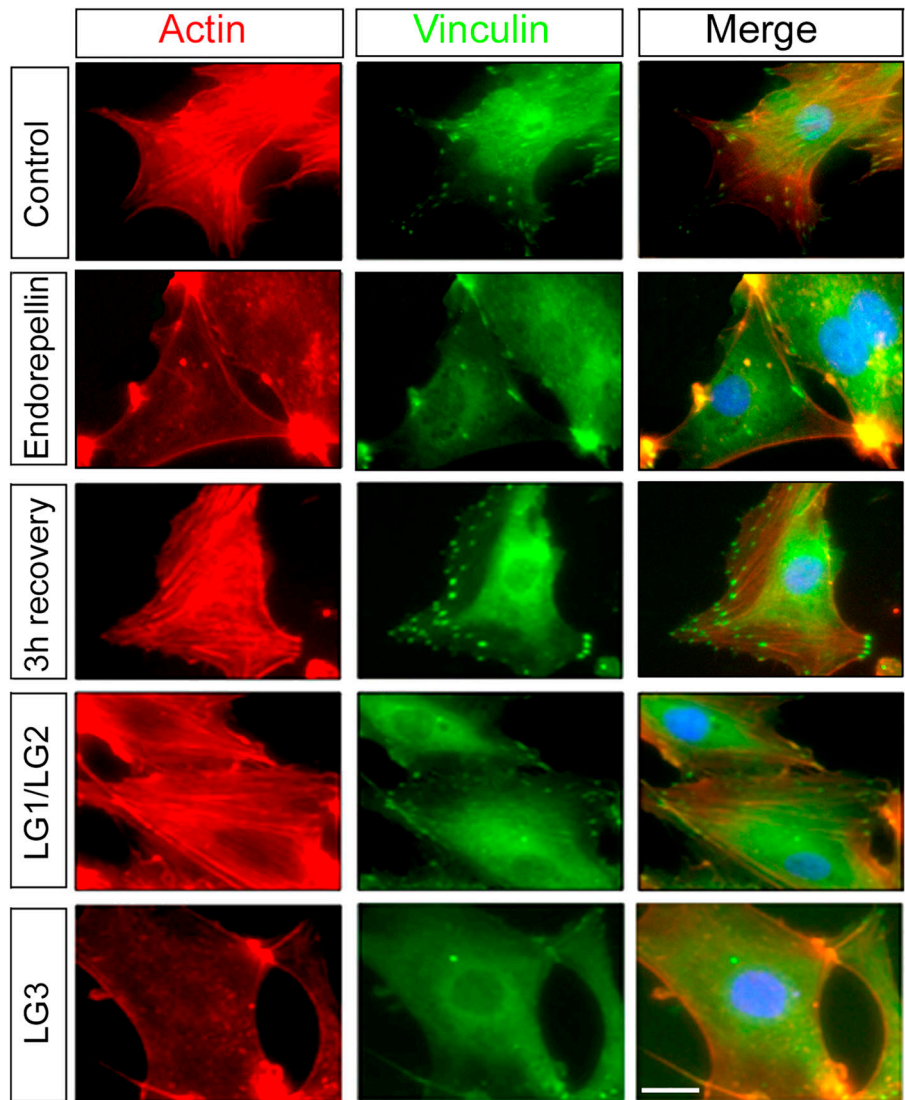
Next, we investigated if ER and its fragments could interfere with the cytoskeleton and focal adhesions. HUVECs and

MECs were grown to subconfluency on collagen I and treated with ER, LG1/LG2, or LG3 for 10 min. Actin stress fibers and focal adhesions were visualized with rhodamine-phalloidin or antivinculin antibody, respectively. Both ER and LG3 caused significant disassembly of actin stress fibers and focal adhesions, which was fully reversible after 3 h (Fig. 3). In contrast, recombinant LG1/LG2 did not have any appreciable effects (Fig. 3). Quantization of the data showed a 75–80% ($P < 0.001$) loss of stress fibers in the ER- and LG3-treated HUVECs (Fig. S1 A, available at <http://www.jcb.org/cgi/content/full/jcb.200401150/DC1>). Interestingly, ER and LG3 had nearly identical effects on MECs (Fig. S1 B); however, no effects on the actin stress fibers of either fibroblasts or Saos2 osteosarcoma cells grown on collagen I were detected (Fig. S1 B), indicating a degree of cell specificity. Thus, ER and LG3 induce a profound reorganization of actin cytoskeleton and focal adhesions in endothelial cells derived from either large or small blood vessels.

ER causes colocalization of $\alpha 2\beta 1$ integrin with collapsed actin and supports cell attachment

Because ER inhibits the attachment of endothelial cells to a collagen I substrate (Mongiati et al., 2003), we hypothesized that ER and LG3 might exert their effects through an integrin-mediated mechanism. To test this hypothesis, we first immunostained ER- or LG3-treated HUVECs for $\alpha 1\beta 1$, $\alpha \nu \beta 3$, $\alpha 5\beta 1$, and $\alpha 2\beta 1$ integrins. HUVECs did not show detectable levels of $\alpha 1\beta 1$ (Fig. 4 B), but showed significant levels of all the other integrins tested. ER and LG3 treatment resulted in a specific clustering of $\alpha 2\beta 1$ and concomi-

Figure 3. ER and its components cause disassembly of HUVEC actin stress fibers and focal adhesions. HUVECs were treated with the various recombinant proteins (25–150 nM) for 10 min as indicated, fixed, permeabilized with 0.1% Triton X-100, and stained to visualize actin stress fibers with rhodamine-phalloidin, focal adhesions with anti-vinculin mAb and appropriate FITC-conjugated secondary antibody, and counterstained with DAPI. In recovery experiments, after the 10-min exposure, ER was removed and the cells were incubated for an additional 3 h in serum-free media. Bar, 10 μ m.



tant colocalization with collapsed lateral bundles of actin (Fig. 4, G–I). In contrast, there was no colocalization with α v β 3 (Fig. 4, D–F) or α 5 β 1 (not depicted).

Next, we determined whether or not there was any functional relationship between the colocalization of α 2 β 1 with collapsed actin and the activity of ER. We pretreated HUVECs for 10 min with saturating concentrations (10 μ g/ml) of a function-blocking antibody against the α 2 β 1 integrin, and then added the same concentration of α 2 β 1 antibody supplemented with either ER or LG3 for an additional 10 min. Treatment with anti- α 2 β 1 integrin antibody resulted in complete preservation of the actin stress fibers in both the ER- and LG3-treated cells (Fig. 5, A and B). None of the other function-blocking antibodies against α 1 β 1 (not depicted), α 5 β 1, and α v β 3 integrins prevented ER or LG3 effects (Fig. 5 A).

Next, we determined if ER itself could support endothelial cell attachment and spreading, and if this attachment and spreading could be inhibited by α 2 β 1 integrin-blocking antibody. Cells readily attached and spread on the collagen substrate, but this process could be completely (>98%) blocked with α 2 β 1 antibody (Fig. 5 C). HUVECs also readily attached to ER but appeared to be less spread as com-

pared with collagen I, i.e., they showed fewer and shorter processes per cell (Fig. 5 C). HUVEC attachment to ER-coated dishes was similarly blocked by α 2 β 1 antibody, but not by α 1 β 1, α 5 β 1, or α v β 3 antibodies (unpublished data). Collectively, these results suggest that α 2 β 1 integrin serves as a functional receptor for ER.

ER binds to α 2 β 1 integrin-expressing cells and to the I domain of the α 2 integrin subunit

We fused the coding region of ER to the heat stable human placental alkaline phosphatase (AP), a soluble marker that can be readily detected. Proper folding of ER/AP chimera was tested by measuring enzyme activity in the conditioned medium of 293-EBNA-secreting cells (unpublished data) and by testing various dilutions of it on HUVECs. The ER/AP-containing medium at 1:5 dilution (equivalent to \sim 200 nM) disrupted the HUVEC actin cytoskeleton within 10 min (Fig. 6 A) in a manner identical to the purified recombinant protein. The activity was due to the ER moiety insofar as its biological function was abrogated by heat denaturation (65°C for 45 min) under conditions in which the human AP moiety is fully preserved (Mongiat et al., 2003).

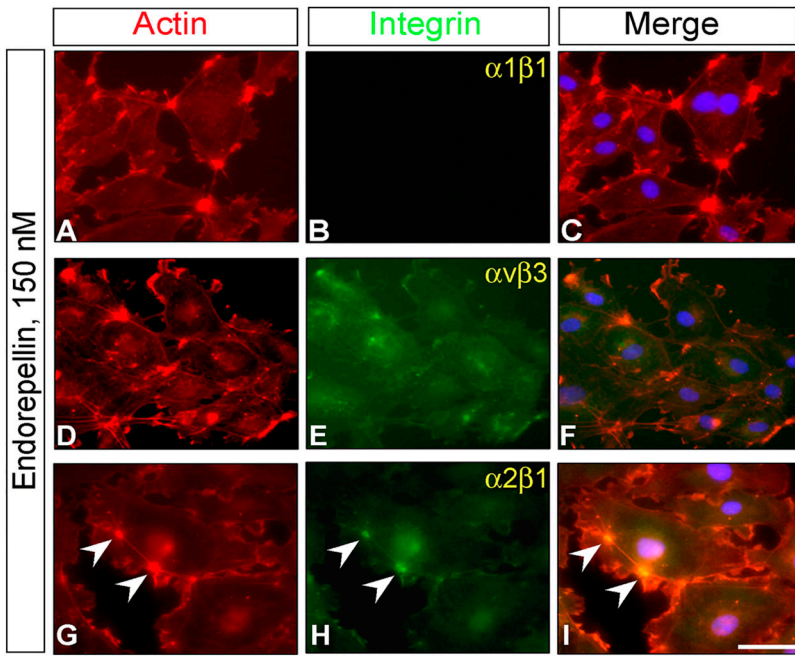


Figure 4. ER causes clustering and colocalization of $\alpha 2\beta 1$ integrin with collapsed actin. HUVECs were treated with 150 nM ER for 10 min followed by labeling of actin (A, D, and G) or immunostaining with antibodies against $\alpha 1\beta 1$, $\alpha 5\beta 3$, or $\alpha 2\beta 1$ integrin (B, E, and H). Identical fields were then merged (C, F, and I). Notice the colocalization of collapsed actin stress fibers with $\alpha 2\beta 1$ epitopes (G–I, arrowheads). Lower ER concentrations (50–100 nM) yielded similar results (not depicted). Bar, 10 μ m.

Next, we tested ER/AP for binding to normal murine mammary gland epithelial cells (NMuMg) that were previously selected for the presence or absence of the $\alpha 2$ integrin subunit (Zutter et al., 1999). The $\alpha 2^-$ cells showed no detectable $\alpha 2$ subunit (Fig. 6 B) and did not bind to collagen I (Zutter et al., 1999). Notably, ER/AP bound only to the $\alpha 2^+$ cells in a dose-dependent and saturable fashion (Fig. 6, C and D). Scatchard analysis revealed a single receptor population with a K_D of 23 nM. Binding of ER/AP to the NMuMg $\alpha 2^+$ cells did not cause disruption of the actin cytoskeleton, indicating that ER might be specific for endothelial cells.

Having established a functional connection between ER and $\alpha 2\beta 1$ integrin, we determined if ER and LG3 could physically interact with the $\alpha 2I$ integrin domain, the major binding site for collagen I (Emsley et al., 2000). Using surface plasmon resonance spectroscopy (SPR), we found a specific binding of ER and LG3 to immobilized $\alpha 2I$ domain (Fig. 6, F and G). Calculation of the kinetics of binding over a range of protein concentrations showed that ER had an affinity for $\alpha 2I$ domain significantly higher than LG3 (K_D of 80 nM vs. 1 μ M, respectively). Binding specificity was determined by using two triple-helical collagen III peptides: one of 2.9 kD containing the $\alpha 2I$ recognition sequence

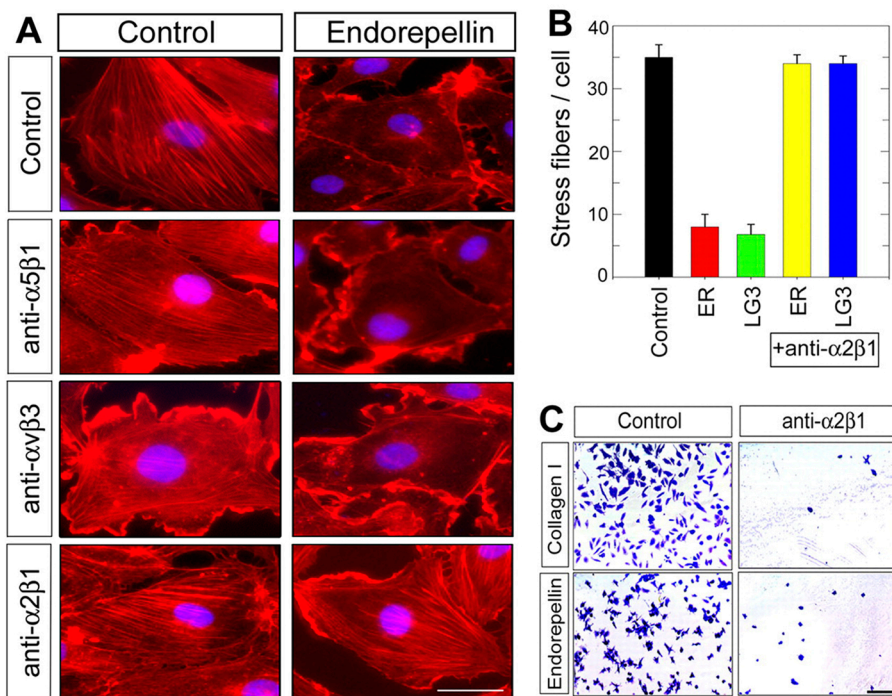


Figure 5. ER supports cell attachment that is inhibited by $\alpha 2\beta 1$ integrin blocking antibody. (A) ER action is blocked by function blocking antibody against $\alpha 2\beta 1$ integrin, but not by antibodies against other integrins. The cells were processed as in the legend to Fig. 3, after 10-min incubation with saturating concentrations (10 μ g/ml) of specific mAbs \pm ER. Bar, 10 μ m. (B) Mean number of visible actin stress fibers per cell was calculated for 100 randomly selected cells for each treatment as indicated. Error bars represent SEM. (C) Light micrographs of HUVECs attached to either collagen I or ER substrata in the presence or absence of anti- $\alpha 2\beta 1$ integrin antibodies. Bar, 50 μ m.

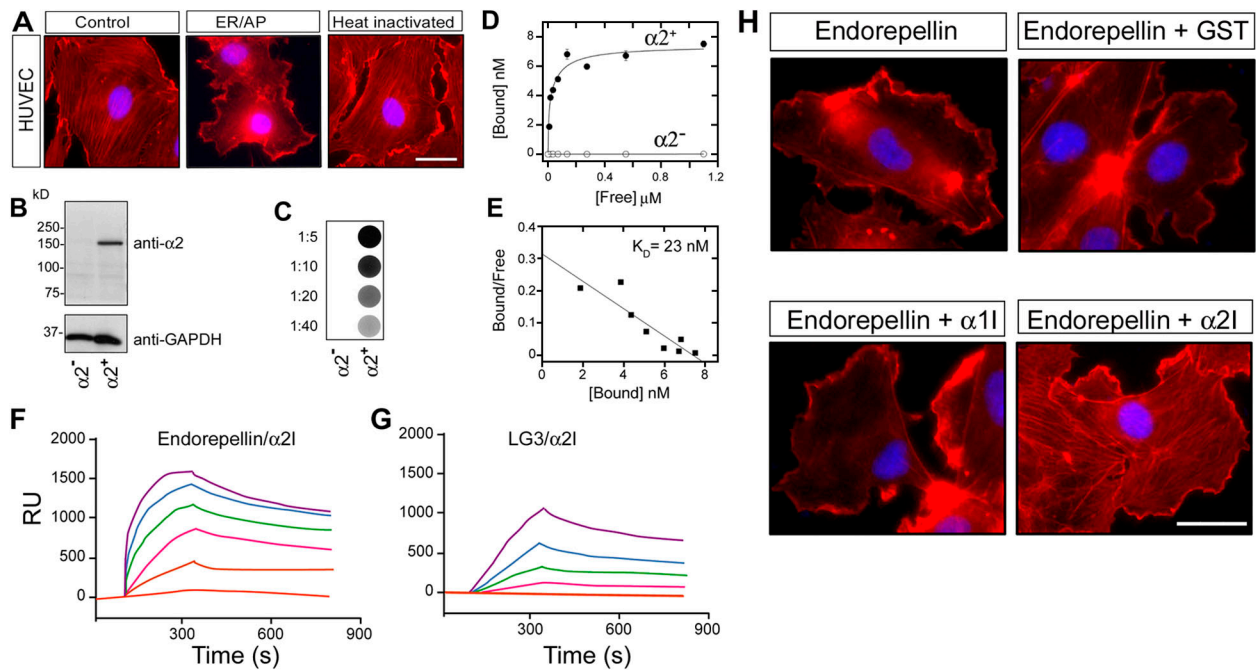


Figure 6. ER binding and activity require the $\alpha 2\beta 1$ integrin. (A) Effects of ER/AP chimera on endothelial cell stress fibers. HUVECs were incubated for 10 min with medium containing ~ 200 nM ER/AP, before and after heat denaturation (65°C , for 45 min). Bar, $10\ \mu\text{m}$. (B) Western immunoblotting of total cell lysates from NMuMg lacking or expressing the $\alpha 2$ integrin subunit, as indicated. (C) Dose-dependent binding of ER/AP-containing media to the $\alpha 2^{+}$ cells. Cells were incubated for 1 h at 25°C with ER/AP media at the indicated dilutions (equivalent to 200, 100, 50, and 25 nM ER/AP), washed and processed for AP chemiluminescence and exposure to an X-ray film. (D) Saturation binding curves using an experimental strategy as in C, with the exception that the values were obtained by measuring the relative light units of total bound ER/AP and by normalizing the results on a standard curve generated with recombinant placental AP. Values represent the mean \pm SEM ($n = 3$). (E) Scatchard analysis shows one single class of surface receptors with relative high affinity ($K_D = 23 \pm 2$ nM). (F and G) Analyses of ER and LG3 binding to the I domain of $\alpha 2$ integrin by SPR. About 3,000 response units (RU) of $\alpha 21$ domain were immobilized on a CM5 chip, and increasing concentrations (0.1–2.0 μM , from bottom to top line) of ER or LG3 were tested as indicated in a buffer containing 1 mM MgCl_2 . The profiles were corrected for response over a reference flow cell containing no immobilized protein. (H) Recombinant $\alpha 21$ but not $\alpha 11$ integrin domain or GST protein, blocks ER effects on HUVECs. Recombinant ER was incubated with $\alpha 21$ -GST or $\alpha 11$ -GST integrin domains or GST alone (at $\sim 1:8$ molar ratio, respectively) for 18 h at 4°C , and then tested on HUVECs as described in Fig. 3. Bar, $10\ \mu\text{m}$.

GER (Emsley et al., 2000; Knight et al., 2000; Xu et al., 2000) and a negative peptide consisting of $(\text{GPO})_{11}$ (where O = hydroxyproline) of 2.94 kD. As expected, collagen III-GER showed a dose-dependent binding to the $\alpha 2\text{I}$ domain, whereas $(\text{GPO})_{11}$ showed no binding at all concentrations tested (Fig. S1 C). Moreover, solid phase binding assays using $\alpha 2\text{I}$ -GST and GST fusion proteins as the bound substrate and ER or LG3 as the soluble ligands showed a specific and high-affinity binding to immobilized $\alpha 2\text{I}$ -GST, with greater affinity for ER as compared with LG3, thus corroborating the results obtained with SPR (unpublished data). Moreover, actin collapse was blocked only by preincubating ER with soluble $\alpha 2\text{I}$ -GST but not with $\alpha 1\text{I}$ -GST or GST alone (Fig. 6 H). Together, these results suggest that the $\alpha 2$ integrin is a major surface receptor for ER and LG3.

ER and LG3 effects on endothelial cells do not require cell surface heparan sulfate proteoglycans

Because endostatin requires cell surface heparan sulfate for its biological activity (Sasaki et al., 1999), we tested if this might also be true for ER. To this end, cells were pretreated with either chlorate (5 or 10 mM for 2 h) or heparinase III (5 mU/ml, for 30 min) to block the endogenous sulfation of heparan sulfate or to degrade cell surface heparan sulfate, respectively,

followed by treatment with ER, LG3, or recombinant human endostatin. Pretreatment with either compound failed to block ER- or LG3-induced stress fiber collapse but did prevent endostatin-induced stress fiber collapse (Fig. 7 A). Independence from cell surface heparan sulfate for ER effects was corroborated by experiments using heparin affinity chromatography. Both ER and LG3 failed to bind to an analytical heparin-agarose column because 72 and 78%, respectively, eluted with ≤ 0.2 M NaCl, whereas $>85\%$ endostatin eluted at higher NaCl concentrations (Fig. 7 B).

ER and LG3 increase $[\text{cAMP}]_i$ levels and activate PKA

Collagen I induces capillary morphogenesis through $\alpha 2\beta 1$ integrin-dependent suppression of $[\text{cAMP}]_i$ and PKA activity (Whelan and Senger, 2003). Because ER's effects mirrored those of collagen I, we determined if ER- and LG3-induced actin depolymerization could involve an increase in $[\text{cAMP}]_i$ and PKA activity, and if these changes might be mediated by $\alpha 2\beta 1$ integrin. Both ER and LG3 caused a rapid (within 5 min) and statistically significant increase in $[\text{cAMP}]_i$ ($P < 0.001$) as compared with control (Fig. 8 A, 10 min treatment shown), and this increase could be blocked by $\alpha 2\beta 1$ antibody. Notably, $[\text{cAMP}]_i$ was also increased in fibroblasts (Fig. S1 D), although no changes in ac-

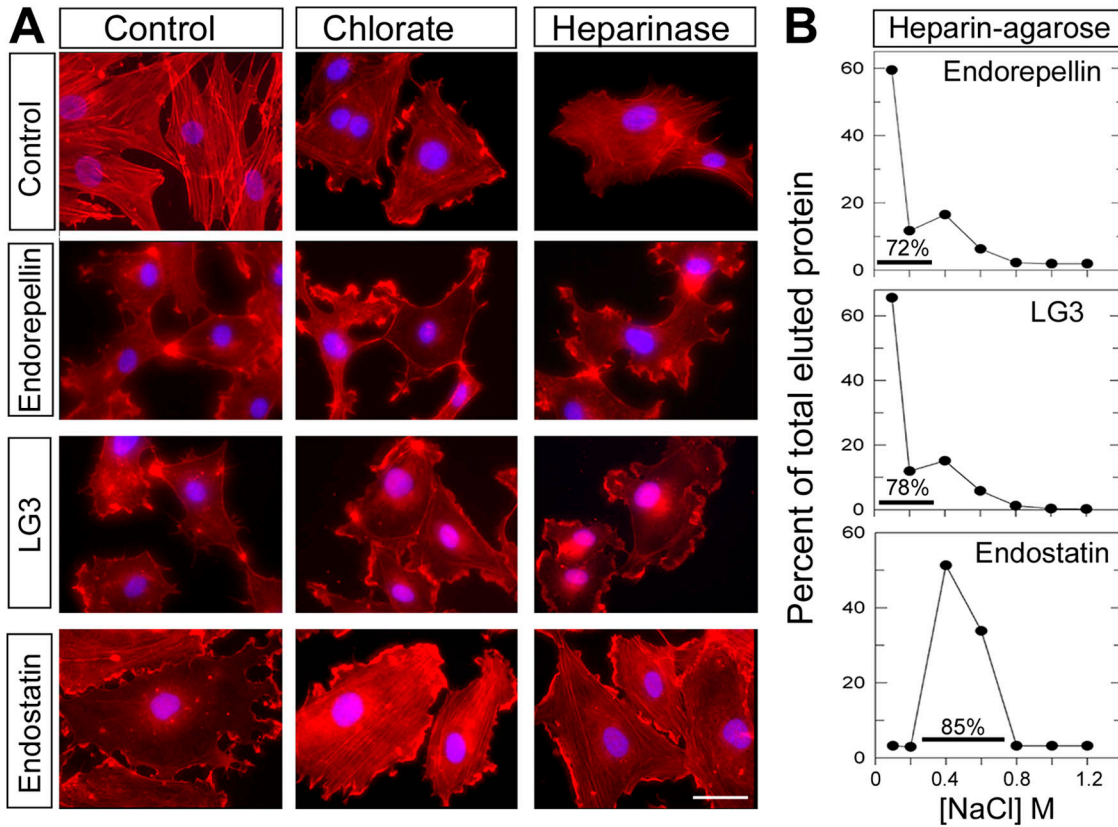


Figure 7. **ER and LG3 effects are independent of cell surface heparan sulfate.** (A) HUVECs were pretreated with either chlorate (5 or 10 mM for 2 h) or heparinase III (E.C.4.2.2.8.; 5 mU/ml, for 30 min) and then exposed to equimolar amounts (50 or 150 nM) of ER, LG3, or human endostatin. Actin fibers were visualized as described in legend to Fig. 3. Bar, 10 μ m. (B) ER and LG3 do not bind to an analytical heparin-agarose column. The majority of ER or LG3 (72% and 78%, respectively) elutes at NaCl < 0.2 M, in contrast to Endostatin, >85% of which elutes at higher NaCl molarities.

tin cytoskeleton were observed (Fig. S1 B), but did not appreciably change in Saos2 osteosarcoma cells (Fig. S1 D), which lack α 2 β 1 integrin (Ivaska et al., 1999).

Next, we discovered that the fraction of active PKA was rapidly (within 5 min) and significantly ($P < 0.001$) increased in cells exposed to ER (Fig. 8 B) or LG3 (not de-

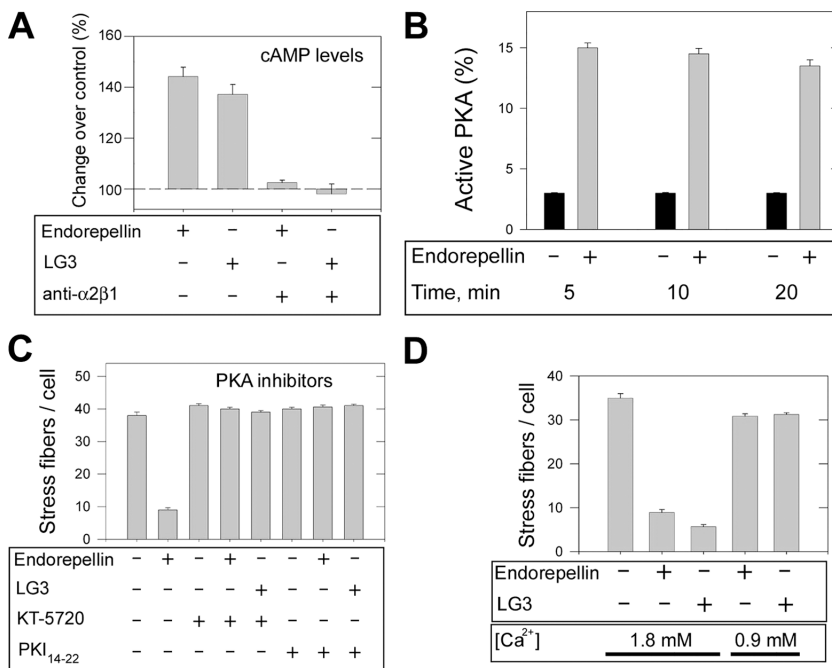
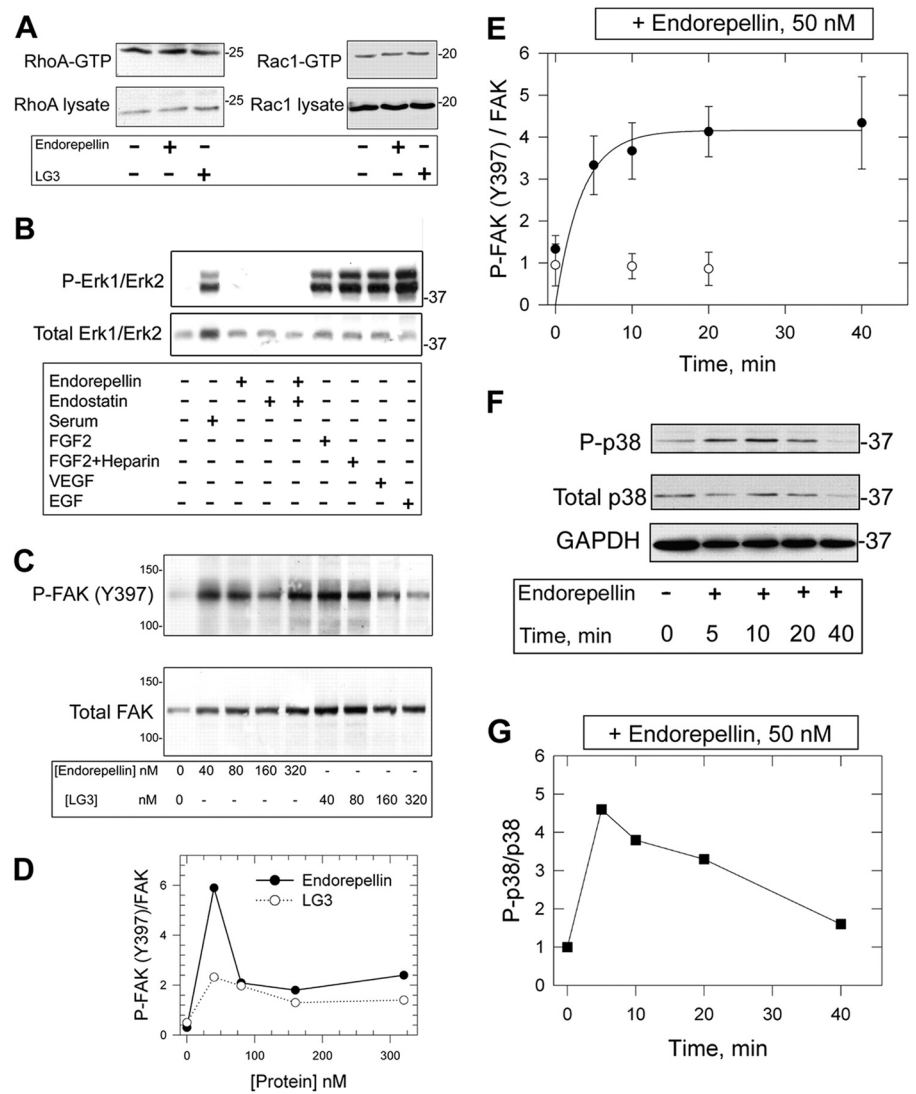


Figure 8. **ER and LG3 increase [cAMP]_i, activate PKA, and require calcium for activity.** (A) HUVECs were treated with ER or LG3 \pm α 2 β 1 blocking antibody (10 μ g/ml) for 10 min. Total cAMP levels were measured with an enzyme-linked immunoassay kit. Results are plotted as percentage of control OD₄₀₅ arbitrarily set to 100%. (B) The percentage of active PKA is significantly enhanced ($P < 0.001$) at 5 min and sustained for at least 20 min by ER, as measured with an ELISA based assay. (C) HUVECs were pretreated with the two cell permeable PKA inhibitors, KT-5720 (10 μ M) or PKI₁₄₋₂₂ (0.41 μ M) for 30 min, and then exposed to ER and LG3 for an additional 10 min using the same concentrations of PKA inhibitors. The mean number of visible actin stress fibers per cell was calculated for 100 randomly selected cells for each condition. Error bars represent SEM. (D) Effects of low Ca²⁺ on ER and LG3 activity. HUVECs were first exposed to normal (1.8 mM) or half-normal (0.9 mM) Ca²⁺ concentrations for 20 min, and then to ER or LG3 for 10 min, followed by staining for actin.

Figure 9. ER activates FAK and p38 MAPK.

(A) Affinity precipitation of RhoA-GTP and Rac1-GTP in HUVECs after a 30-min incubation with PBS, ER, or LG3. The cell lysates were then incubated with Rhotekin Rho binding domain-agarose or PAK-1 p21-binding domain-agarose. The bound proteins were eluted with Laemmli buffer and detected by immunoblotting using mAbs against RhoA or Rac1. Notice that in the RhoA lysate, only 20% of total cell extract was loaded. (B) Detection of Erk1/Erk2 MAPK phosphorylated at T202 and Y204 (P-Erk1/Erk2) and total Erk1/Erk2 MAPK upon stimulation of serum starved HUVECs with 10% FCS, ER (100 nM), endostatin (100 nM), ER and endostatin (100 nM each), FGF2 (75 ng/ml) \pm heparin (100 ng/ml), VEGF (75 ng/ml), or EGF (75 ng/ml), as indicated. (C) Dose response effects of ER and LG3 on FAK(Y397) and total FAK in HUVECs at 1 h. (D) Quantization of the dose response effects of ER and LG3 on activated FAK at 1 h. (E) Time course response of activated FAK after ER treatment (mean \pm SEM, $n = 5$) in the absence (●) or presence (○) of 1 μ g/ml geldanamycin. (F) Time course changes in p38 MAPK phosphorylated at T180 and Y182 (P-p38) and total p38 in response to ER treatment. (G) Quantization of the dose response effects of ER on activated p38 MAPK.



ected). PKA activity remained elevated for up to 20 min. The ER-induced activation of PKA was further confirmed by experiments in which HUVECs were preincubated for 30 min with one of two cell-permeable inhibitors specific for PKA, KT-5720, or PKI₁₄₋₂₂ followed by ER or LG3 treatment. Both PKA inhibitors blocked ER- and LG3-induced actin collapse (Fig. 8 C), indicating that PKA is required for ER action.

Because integrin-mediated signaling is known to be cation dependent (Giancotti and Ruoslahti, 1999), and because ER has putative Ca²⁺ binding sites within its LG domains, we determined if ER and LG3 effects could also be Ca²⁺ dependent. As the complete chelating of these ions with EDTA is readily toxic to cells, we instead exposed the HUVECs to media with either 0.9 or 0.45 mM Ca²⁺ (50 and 25% of the normal Ca²⁺ concentration, respectively). Extracellular Ca²⁺ reduction completely blocked the effects of ER and LG3 on actin stress fibers (Fig. 8 D, 0.45 mM Ca²⁺ not depicted).

Collectively, these results establish that ER induces cAMP-dependent PKA activity in conjunction with elevation of [cAMP]_i, and that PKA activity and Ca²⁺ are required for downstream signaling.

ER activates FAK, and transiently p38 MAPK and Hsp27

Integrin engagement affects the activity of the small GTPases RhoA, Rac1, and Cdc42 (Kjoller and Hall, 1999). These proteins function as key regulators of distinct signaling pathways and affect the microfilament organization of most cells. RhoA affects actin stress fibers, whereas Rac1 and Cdc42 influence lamellipodia and filopodia formation, respectively (Kjoller and Hall, 1999). Because endostatin suppresses RhoA activity via α 5 β 1 integrin-mediated activation of Src tyrosine kinase/p190RhoGAP (Wickström et al., 2003), we first tested the potent Src kinase inhibitor PP1 (Hanke et al., 1996). We found no effects on ER or LG3 activity (Fig. S2, available at <http://www.jcb.org/cgi/content/full/jcb.200401150/DC1>), suggesting that ER engagement with the α 2 β 1 integrin does not activate this signaling pathway. Next, we tested the levels of active RhoA and Rac1 using affinity precipitation with Rhotekin Rho-binding domain-agarose or PAK-1 p21-binding domain-agarose (Ren et al., 1999). We did not find any appreciable changes induced by ER or LG3 in either case (Fig. 9 A). Lack of involvement of the Rho pathway was further demonstrated by

the failure of Y27632, a specific inhibitor of the Rho effector ROCK (Uehata et al., 1997), to block ER or LG3 activity (Fig. S2).

As a readout of ER activity, we investigated the status of extracellular signal-regulated protein kinase 1,2 (Erk1/2) known to be activated by various integrins as well as by cross-talk between integrins and other surface receptors (Giaccotti and Ruoslahti, 1999). As positive controls we used bovine serum, FGF2 with or without heparin, VEGF, and EGF, which are all known to activate this signaling pathway. We found that neither ER nor endostatin activated this pathway, whereas all the positive controls did (Fig. 9 B).

In initial experiments, we noticed a 125-kD protein, which was rapidly phosphorylated by ER as well as by VEGF and FGF2 (unpublished data), suggesting to us that p125 FAK might be involved in ER signaling. We found a prominent activation of FAK at one of its major phosphorylation sites (Y397), which was maximal at 40–60 nM for both ER and LG3 (Fig. 9, C and D). Time course experiments showed a progressive activation of FAK, which was maximal at 40 min (Fig. 9 E) and continued at similar levels for 60 min (note that the dose-response experiments lasted 1 h; Fig. 9, C and D). The requirement for FAK was further corroborated by experiments in which 1-h preincubation with 1 μ g/ml geldanamycin, an antibiotic that blocks FAK phosphorylation in HUVECs (Rousseau et al., 2000), totally blocked ER or LG3 activity on actin stress fibers (Fig. S2), as well as FAK phosphorylation (Fig. 9 E).

Because α 2 β 1 integrin activation is associated with activation of p38 MAPK in other cell types (Ivaska et al., 1999; Klekokta et al., 2001), we tested whether or not p38 MAPK was affected by ER in HUVECs. Indeed, p38 MAPK was transiently activated by ER (Fig. 9 F), with a peak at 5 min and a progressive decline thereafter (Fig. 9 G). In several experiments, total p38 was markedly reduced at 40 min (Fig. 9 F), suggesting that p38 might be targeted for degradation. The requirement for p38 MAPK was further demonstrated by the complete block of ER- and LG3-mediated actin collapse by a 1-h pretreatment with SB202190 (5 μ g/ml; Fig. S2), which blocks the p38-mediated activation of ATF-2 (Jiang et al., 1996). Moreover, ER effects were totally blocked by SB203580 (unpublished data), which blocks the phosphorylation of Hsp27, a well established downstream effector of p38 MAPK (Guay et al., 1997). Therefore, we tested whether or not the phosphorylation status of Hsp27 followed the transient phosphorylation of p38 MAPK. We found that Hsp27 was rapidly phosphorylated at Ser15 by ER treatment and that its activation peaked at 10 min (Fig. S3, A and B, available at <http://www.jcb.org/cgi/content/full/jcb.200401150/DC1>), as also visualized by immunostaining using the same anti-P-Hsp27 (Fig. S3 C). Notably, the total protein content of Hsp27 was also significantly reduced at 40 min, suggesting that both p38 MAPK and Hsp27 might be destined for degradation.

Thus, we conclude that ER and LG3 cause a sustained phosphorylation of FAK and a transient activation of p38 MAPK and Hsp27, both of which are subsequently down-regulated.

Discussion

Having generated recombinant ER and two deletion fragments encompassing the two LG (LG1/LG2) and the terminal (LG3) module, we investigated their antiangiogenic properties using a coculture system within Matrigel. Although the ER-secreting cells were allowed to grow within a three-dimensional matrix, the endothelial cells were seeded on the top and cocultured for another 24 h, during which time they did not have any direct physical contact with the 293-EBNA cells, and any effects on tube formation would be due to secreted factors. The results showed a complete abrogation of capillary morphogenesis induced by ER or LG3, but not by LG1/LG2, indicating that most of the biological activity of ER resides within its terminal globular domain. These findings were further corroborated by using recombinant proteins in both Matrigel and collagen I gel overlay assays. It is notable that we used serum and both FGF2 and VEGF (Mongiat et al., 2003). Thus, ER is capable of overcoming the powerful angiogenic properties of Matrigel, collagen I, and VEGF/FGF2.

We further determined if the endogenous secretion of ER by endothelial cells via Ad-ER would disrupt capillary morphogenesis on different substrata in a dominant-negative fashion because endothelial cells synthesize perlecan. Although the control cells formed tubes, Ad-ER transduced HUVECs and MECs failed to do so. Thus, inhibition of capillary morphogenesis can be reproduced by the endogenous synthesis of ER as well as the addition of exogenous recombinant protein, and these effects occur in endothelial cells derived from umbilical vein or dermal microvasculature.

Reorganization of actin filaments, controlled by actin polymerization–depolymerization events, is essential in adapting the cell shape and its motility to the surrounding microenvironment (Burrige et al., 1997). This process plays a central role in endothelial cell migration and capillary morphogenesis. In this study, we discovered that ER and LG3 exert a profound effect on cytoskeletal dynamics by disrupting actin filaments and focal adhesions within 10 min, in a Ca^{2+} -dependent but heparan sulfate-independent fashion. Ca^{2+} dependence is likely due to Ca^{2+} binding sites found within ER/LG3. The LG1/LG2 construct did not show any significant activity. Furthermore, we were able to inhibit ER activity by first incubating with antibodies against LG3 (unpublished data), which underscores LG3's importance in mediating ER activity. Notably, perlecan LG2 module is the major binding site for collagen XVIII/endostatin (Mongiat et al., 2003) and LG1/LG2 modules are required for high affinity binding to α -dystroglycan, whereas LG3 by itself has much lower affinity (Talts et al., 1999). These data suggest that LG3 is relatively free of high affinity interactions within basement membranes, thereby allowing proteolytic cleavage to occur during the early stages of angiogenesis.

Collagen I regulates endothelial alignment into cords by a specific interaction with α 1 β 1 and α 2 β 1 integrins (Senger et al., 2002). Although microvascular endothelial cells require blocking antibodies to both integrins to abrogate capillary morphogenesis (Whelan and Senger, 2003), HUVECs require only anti- α 2 β 1 blocking antibody for the full effect (Sweeney et al., 2003). Interestingly, we found that ER and

LG3 treatment caused a specific clustering of $\alpha 2\beta 1$ and concomitant colocalization with collapsed lateral bundles of actin. In contrast, there was no colocalization with $\alpha v\beta 3$ or $\alpha 5\beta 1$ integrins. Next, we established a functional relationship between the colocalization of $\alpha 2\beta 1$ with collapsed actin and the activity of ER by showing that a function-blocking antibody against the $\alpha 2\beta 1$ integrin (but not toward $\alpha 1\beta 1$, $\alpha v\beta 3$, or $\alpha 5\beta 1$ integrins) could counteract the ER- and LG3-mediated effects on both HUVECs and MECs. We further discovered that the presence of $\alpha 2\beta 1$ was essential for proper ER binding, as measured by the fully functional ER/AP chimeric protein. Notably, both the NMuMg $\alpha 2^+$ and $\alpha 2^-$ express intermediate to high levels of $\alpha 5$, $\alpha 6$, $\beta 1$, and $\beta 4$ integrin subunits, whereas all lack the $\alpha 1$ subunit (Zutter et al., 1999), further strengthening the concept that ER requires the $\alpha 2$ integrin subunit for its cell surface binding. In agreement with these findings, we discovered that ER and LG3 bound to the $\alpha 2$ I integrin domain, the major binding site for collagen I (Emsley et al., 2000; Knight et al., 2000; Xu et al., 2000), and that ER activity could be counteracted by soluble $\alpha 2$ I-GST but not by $\alpha 1$ I-GST or GST alone. Interestingly, ER had higher affinity for $\alpha 2$ I domain than LG3, suggesting that LG1/LG2 and/or EGF-like motifs might be important secondary binding sites for the integrin.

Collagen I suppresses $[cAMP]_i$ in vascular smooth muscle (Wang et al., 1999) and microvascular endothelial (Whelan and Senger, 2003) cells; and the attachment of endothelial cells to various extracellular matrix constituents, including collagen I, fibronectin, and vitronectin, suppresses PKA activity (Kim et al., 2000). PKA typically becomes activated upon binding to cAMP, and thus agents that elevate $[cAMP]_i$ such as forskolin, dibutyryl cAMP, or isoproterenol can all activate PKA (Schoenwaelder and Burridge, 1999). Pharmacological elevation of $[cAMP]_i$ causes rapid and transient morphological changes that lead to a "cAMP phenotype;" i.e., dissolution of actin cytoskeleton and rounding up of the cells (Dong et al., 1998). In endothelial cells, elevation of $[cAMP]_i$ blocks collagen-induced actin polymerization and capillary morphogenesis in a way that mimics the action of $\alpha 1\beta 1/\alpha 2\beta 1$ integrin-blocking antibodies (Whelan and Senger, 2003). We reasoned that ER might activate $[cAMP]_i$ because its effects were similar to those caused by agents that induce $[cAMP]_i$. Both ER and LG3 caused a rapid increase in HUVEC $[cAMP]_i$ that could be blocked by $\alpha 2\beta 1$ antibody, further supporting a role for this integrin in ER/LG3 signaling. $[cAMP]_i$ was also increased in skin fibroblasts, although no changes in actin cytoskeleton were observed, in agreement with previous results (Whelan and Senger, 2003); however, $[cAMP]_i$ did not appreciably change in Saos2 cells, which lack $\alpha 2\beta 1$ integrin (Ivaska et al., 1999). We further demonstrated a requirement for PKA activity using two cell-permeable inhibitors specific for PKA, KT-5720 and PKI₁₄₋₂₂. Both PKA inhibitors blocked ER- and LG3-induced actin stress fiber collapse, suggesting that both ER and LG3 require PKA activity via $[cAMP]_i$ elevation. Indeed, total PKA activity was significantly increased in ER- or LG3-treated HUVECs.

Activated PKA antagonizes the activity of RhoA through a specific phosphorylation at Ser188 (Lang et al., 1996), which leads to a decreased affinity for its effector ROCK

(Dong et al., 1998). When HUVECs treated with ER or LG3 were probed for changes in GTP-bound (active) RhoA or Rac1, we found no significant involvement of these GTPases in ER signaling. That the Rho pathway was not involved was further shown by the lack of inhibition of ER or LG3 activity by pretreatment of the cells with 1 μ M Y27632, a specific inhibitor of ROCK (Uehata et al., 1997). Because integrin engagement suppresses RhoA activity via a Src-dependent mechanism (Arthur et al., 2000), we tested the potent Src kinase inhibitor PP1 (Hanke et al., 1996), which prevents the activity of endostatin on HUVECs (Wickström et al., 2003). We found no PP1 effects on ER or LG3 activity, suggesting that ER engagement with $\alpha 2\beta 1$ does not activate this signaling pathway. Moreover, we found that ER did not activate the Erk1/Erk2 signaling pathway known to be activated by various integrins as well as by cross-talk between integrins and other surface receptors (Giancotti and Ruoslahti, 1999).

FAK is an important receptor-proximal link between growth factor receptors and integrin signaling (Sieg et al., 2000). Moreover, FAK is required for blood vessel morphogenesis (Ilic et al., 2003), whereas ER prevents capillary morphogenesis. We found a protracted activation of FAK at one of its major phosphorylation sites (Y397). This finding is of interest because FAK phosphorylation is associated with focal adhesion disassembly (Schoenwaelder and Burridge, 1999). In contrast, collagen I inhibits FAK-Y397 in HUVECs (Sweeney et al., 2003), essentially mirroring the effects of ER. Requirement for FAK activation was further proved by the complete block of ER activity by geldanamycin, an antibiotic that blocks the VEGF-induced FAK phosphorylation in HUVECs (Rousseau et al., 2000). A role for FAK in the formation of actin stress fibers is further supported by the finding that this process can be completely abolished by overexpressing FRNK, a noncatalytic COOH-terminal portion of FAK that acts as a negative regulator of FAK (Rousseau et al., 2000). It is noteworthy that exogenous perlecan induces FNRK in smooth muscle cells, thereby suppressing the FAK-mediated activation of Erk1/2 growth signal (Walker et al., 2003).

ER causes a transient activation of p38 MAPK, followed by down-regulation. In contrast, collagen I engagement of the $\alpha 2\beta 1$ integrin in HUVECs causes a sustained (>2 h) phosphorylation of p38 MAPK (Sweeney et al., 2003). Notably, the p38 inhibitor SB202190, which blocks the ability of p38 to phosphorylate the transcription factor ATF-2 (Jiang et al., 1996), inhibits collagen-induced capillary morphogenesis (Sweeney et al., 2003) and completely blocked the action of ER and LG3. In mice lacking p38 α MAPK (Mudgett et al., 2000) and Mekk3 (Hanke et al., 1996), the upstream activator of p38 MAPK, significant defects in placental angiogenesis and blood vessel development were found. These studies have shown that p38 α MAPK is not essential for vasculogenesis, the process by which angioblasts differentiate to form primary capillaries, but rather is required for the vascular remodeling associated with angiogenesis. Our signaling axis is further strengthened by reports establishing a link between activation of $\alpha 2$ integrin subunit and p38 MAPK (Ivaska et al., 1999; Klekokta et al., 2001). Notably, p38 inhibition blocks VEGF-induced migration of

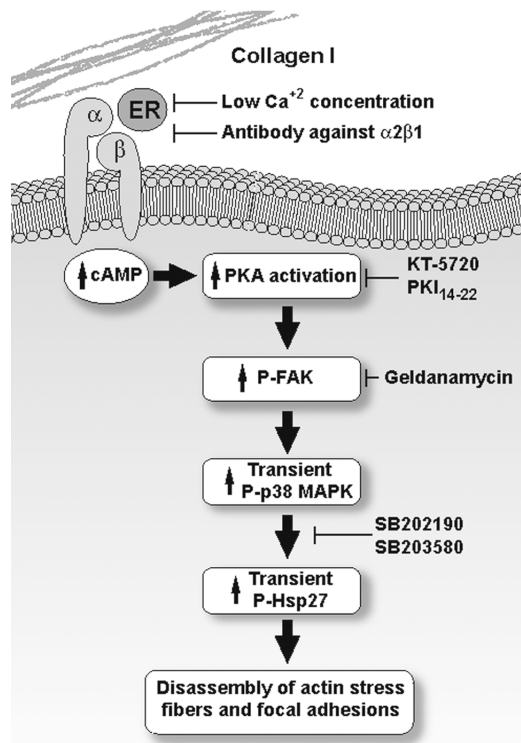


Figure 10. **Schematic model representing the regulation of actin stress fibers and focal adhesions by ER.** The model shows activation of a signaling cascade that involves ER interaction with $\alpha2\beta1$, activation of cAMP, PKA, and FAK, followed by the transient activation of p38 MAPK and Hsp27, and ultimately disassembly of actin stress fibers and focal adhesions. The various blocking agents are also illustrated. For additional details see the text.

HUVECs, whereas inhibition of Erk1/2 is ineffective (Rousseau et al., 1997). Treatment of HUVECs with VEGF causes a transient phosphorylation of p38 MAPK, reorganization of actin into long stress fibers, and vinculin assembly (Rousseau et al., 1997). Therefore, it is possible that several responses associated with actin filament dynamics may be dependent on p38 activation/deactivation events.

ER causes transient activation of Hsp27, a small chaperone protein regulated by p38 MAPK and associated with actin dynamics (Guay et al., 1997; Huot et al., 1997). Interestingly, activation of Hsp27 is usually associated with stress fiber formation in HUVECs (Huot et al., 1997) rather than disassembly. Indeed, HUVECs express high levels of Hsp27 as compared with fibroblasts (6 vs. 1–2 ng/mg⁻¹ total protein), and this might explain why endothelial cells respond differently (stress fiber formation vs. actin fragmentation) than fibroblasts to Hsp27 activation (Huot et al., 1997). This apparent discrepancy with our results suggests that the biology of Hsp27 interaction with actin fibers is more complex than previously thought. Alternatively, it is possible that other, as yet undiscovered, pathways are activated by ER. However, by inhibiting activation of Hsp27 with SB203580, we were able to prevent ER-induced actin disassembly, suggesting that the p38 MAPK/Hsp27 axis is primarily involved. As in the case of p38 MAPK, Hsp27 protein levels subsequently declined. Thus, it is possible that the

down-regulation of p38 MAPK/Hsp27 is a distinguishing feature of ER signaling.

In conclusion, we propose a working model (Fig. 10) where ER mediates its antiangiogenic effects by first interacting with $\alpha2\beta1$ integrin on the endothelial cell surface, resulting in a disruption of the outside-in integrin signaling that normally provides proangiogenic cues. This ER– $\alpha2\beta1$ interaction leads to an increase in [cAMP]; activation of PKA and FAK, which in turn leads to transient phosphorylation of p38 MAPK and Hsp27; and concomitant disassembly of focal adhesions and actin stress fibers. As a result of this reversible and Ca^{2+} -dependent process, ER causes endothelial cells to become refractory to angiogenic cues, both soluble cues, such as FGF2 and VEGF, and static cues, such as collagen and fibronectin. The end result is a profound block of endothelial cell migration and angiogenesis.

Materials and methods

Recombinant proteins and generation of antisera

Recombinant human ER (E3687-S4391), LG1-LG2 (E3687-P4107), LG3 (G4182-S4391), and endostatin were produced in 293-EBNA cells as described previously (Mongiat et al., 2003). Recombinant $\alpha21$ -GST or $\alpha11$ -GST integrin domains were purified as described previously (Dickeson et al., 1997). Purified ER or LG3 (100 μ g) were mixed with complete Freund's adjuvant and injected into rabbits to raise specific antisera. Booster injections were performed at 4 and 8 wk after primary injection.

Preparation of adenovirus vectors and collagen overlay assays

Ad-ER, Ad-LacZ, and Ad-Empty were prepared using the AdEasy™ Adenoviral Vector System (Stratagene). Human embryonic kidney 293 cells were transfected with the Ad-vectors containing the gene of interest (ER or LacZ) using the MBS Transfection kit (Stratagene). Cells were amplified and virus was purified using the Virapur Purification kit (BD Biosciences). The virus was titrated using an end point dilution assay (TCID₅₀), and transduction efficiency was determined by in situ staining with β -galactosidase (Stratagene). Cells were transfected with Ad-ER or Ad-Empty at an MOI of 80–160 for 48 h, and then were overlaid with 100 μ l/cm² of collagen gel. After gel solidification, conditioned media (containing Ad-ER, Ad-Empty, or no virus) were added back to the cells.

Immunofluorescence, cell adhesion, cAMP, and PKA assays

HUVECs (passage 4–8) and MECs (Yale Skin Diseases Research Center) were similarly cultured on collagen-coated flasks in complete media (Mongiat et al., 2003) or EGM-2 MV media (Clonetics), respectively. Fibroblasts and Saos2 osteosarcoma cells were similarly cultured in DME supplemented with 10% FBS (GIBCO BRL). Monoclonal blocking antibody to integrins $\alpha2\beta1$ (mAb 1998Z), $\alpha1$ (mAb 1973Z), $\alpha\beta3$ (mAb 1976), or $\alpha5\beta1$ (mAb 1969), all obtained from Chemicon, were added to the cells. In other experiments, cells were treated with the PKA inhibitors KT5720 (10 μ M) or PKI₁₄₋₂₂ (0.41 μ M; Calbiochem). Cells were pretreated with 5 mU/ml heparinase III (Sigma-Aldrich) for 30 min, or either 5 mM or 10 mM chlorate for 2 h. After serum starving, the cells were treated with serum-free or calcium-modified media containing test compounds as indicated. Routinely, cells were washed with PBS, fixed with 4% PFA, permeabilized with 0.1% Triton-X for 3 min, and incubated with PBS/5% BSA for 30 min. The cells were immunolabeled with mAbs against vinculin (V9131; Sigma-Aldrich) or various integrins as mentioned in this paragraph followed by the appropriate FITC-conjugated secondary antibody (Jackson ImmunoResearch Laboratories). The cells were also stained with rhodamine-phalloidin and DAPI (Sigma-Aldrich). Images were captured with a microscope (model DP12; Olympus) and a SPOT CCD camera and processed with Adobe Photoshop 7.0. Actin stress fibers were assessed from 100 randomly selected cells.

For cell adhesion, 96-well dishes were coated overnight at 4°C with ER (40 μ g/ml), collagen I (100 μ g/ml), or 1% BSA. After blocking with 1% BSA for 30 min, HUVECs were trypsinized, resuspended in serum-free M199 media, and allowed to recover in suspension for 20 min at 37°C. The cells were incubated \pm anti- $\alpha2\beta1$ blocking antibody (10 μ g/ml) for 20 min at 37°C, and then plated for 3 h. The wells were rinsed with PBS, fixed

with 4% PFA, stained with 0.1% crystal violet, solubilized with acetic acid (10% wt/vol), and OD₅₆₀ was measured to quantify the adherent cells.

[cAMP]_i and PKA activity were measured with an enzyme-linked immunosorbent assay kit (Biotechnologies) and a protein kinase assay kit (Calbiochem), respectively.

ER/AP, heparin binding, BIAcore studies, and determination of active RhoA and Rac1

NMuMG, selected for the presence or absence of the $\alpha 2$ integrin subunit, were cultured as described previously (Zutter et al., 1999). ER/AP binding assays were performed as described previously (Mongiat et al., 2003). To assess $\alpha 2$ integrin expression, total cell lysates were immunoblotted with anti- $\alpha 2$ integrin (AB1936; Chemicon) and anti-GAPDH (Immunochemical) antibodies. For heparin binding, recombinant proteins ($\sim 10 \mu\text{g}$ each) were analyzed on an analytical heparin-agarose column (Sigma-Aldrich) and eluted with increasing concentrations (0.1–1.2 M) of NaCl in 50 mM Tris-HCl, pH 7.5, 0.05% BSA, and 0.01% Na₃VO₄. SPR studies were performed on immobilized $\alpha 21$ domain using BIAcore 3000 system and evaluated with the BIAevaluation software (Xu et al., 2000). For determination of active RhoA and Rac1, cells were lysed in Mg²⁺ buffer (MLB; Upstate Biotechnology) containing 25 mM NaF, 1 mM Na₃VO₄, and protease inhibitor (Roche). The lysates were incubated with Rhotekin Rho binding domain-agarose or PAK-1 p21-binding domain-agarose (Upstate Biotechnology) at 4°C for 60 min, washed three times with MLB buffer, solubilized, and detected by immunoblotting using mAbs against RhoA (Santa Cruz Biotechnology, Inc.) or Rac1 (BD Biosciences). Antibodies against Erk1/2 (Promega), P-Erk1/2 (Cell Signaling), FAK (BD Biosciences), P-FAK(Y397) (Upstate Biotechnology), p38 and P-p38 (Cell Signaling), total Hsp27 and P-Hsp27 (Santa Cruz Biotechnology, Inc.) were also used in immunoblottings.

Online supplemental material

Fig. S1 indicates the quantification of ER and LG3 effects on HUVEC actin stress fibers, demonstrates the specificity of ER and LG3 actin effects for endothelial cells (vs. fibroblasts and Saos2 cells), demonstrates that ER causes a significant increase in [cAMP]_i in MECs and fibroblasts (cells that possess the $\alpha 2\beta 1$ integrin) but not in Saos2 cells, which lack this integrin, and demonstrates positive control (triple helical collagen III peptide) and negative control ((GPO)₁₁) interactions with $\alpha 21$ integrin domain by SPR.

Fig. S2 demonstrates that geldanamycin (a FAK inhibitor) and SB202190 (a p38MAPK inhibitor), but not PP1 (a Src inhibitor) or Y27632 (a ROCK inhibitor), block ER and LG3 actin stress fiber collapse as compared with controls.

Fig. S3 indicates that ER causes a transient phosphorylation of Hsp27 maximal after 10 min, followed by a gradual decrease in total Hsp27.

Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200401150/DC1>.

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