Research Article

Overexpression of RhoA-GTP induces activation of the Epidermal Growth Factor Receptor, dephosphorylation of focal adhesion kinase and increased motility in breast cancer cells

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Abstract

Rho GTPases are overexpressed in human tumors and are involved in a variety of cellular processes such as organization of the actin cytoskeleton, cell–cell contact and malignant transformation. EGFR activation plays a key role in the acquisition of motile properties in carcinoma cells, and it has been proposed that downregulation of FAK activity is one of its most relevant consequences. In the present study, using mammary MCF-7 cells, we demonstrated that overexpression of the active form of the small GTPase RhoA induced the activation of EGFR by a phenomenon that depends on the activity of a metalloproteinase (MMP), which presumably cleaves a membrane-bound EGFR ligand. The EGFR tyrosine phosphorylation correlates with ERK1,2 activation and the stimulation of urokinase production. An aggressive mammary cell line (MDA-MB-231) that overexpresses both RhoA and EGFR in their active forms also displayed an MMP-dependent activation mechanism of EGFR. RhoA-GTP-transfected cells showed a cortical array of F-actin, rounded morphology, reduced spreading potential and a dephosphorylation of FAK that was released by integrin-dependent fibronectin adhesion and a specific EGFR tyrosine kinase inhibitor. Our results suggest that the MMP-dependent EGFR activation observed in V14 RhoA cells represents the starting point of a signaling route that promotes cell motility by activation of ERK1,2 and further enhancement of proteases production.

Keywords: Rho A; EGFR; Tumoral invasion; Urokinase

Introduction

In carcinoma cells, particularly in breast cancer cells, Rho GTPases are frequently overexpressed, and it has been proposed that this attribute is suggestive of malignancy and poor prognosis [1]. The specific role of RhoA in the acquisition of motile properties has been studied in both tumoral and non-tumoral cellular systems. Overexpression of the wild type human RhoA in tumoral mesothelial (MM1) cells conferred these cells an invasive capacity in vitro and in vivo [2]. In monocytes, it has been demonstrated that transendothelial migration requires RhoA activity for retraction of the tail at the rear of the cells [3]. More recently, using a 3D model, it was proposed that the mechanisms of cell migration are defined by cell morphologies. Thus, rounded cells migrate by a process that depends on RhoA-Rho Kinase (ROCK) signaling pathway and is associated with the formation of patches rich in F-actin and small membrane blebs. This type of migration does not require pericellular proteolysis. On the contrary, elongated cells do not require Rho or ROCK signaling to migrate [4]. In addition, the size of focal adhesion formed by different cells can predict the behavior of RhoA activity in cell motility. In stationary cells such as fibroblasts, which form large focal adhesions, the activation of Rho and ROCK is related to an inhibition of migration. On the contrary, in motile cells such as leukocytes, which form small focal adhesions, Rho and ROCK tend to stimulate cell motility by limiting membrane protrusions at the leading edge [5].

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Overexpression of the Epidermal Growth Factor Receptor (EGFR) constitutes a hallmark of the epithelial malignancy [6]. A substantial amount of evidence led to EGFR being considered as both a transducer of its own ligand stimulus and a downstream element in signaling pathways triggered by a variety of other stimuli, thus playing a crucial role as a cross-talk mediator of highly heterologous signaling systems [7,8]. Non-EGF like stimuli that are able to transactivate the EGFR include G-protein-coupled receptor (GPCRs) agonists, membrane depolarization, UV light and stressors [7]. There is evidence that a mechanism of EGFR transmodulation, involving metalloproteinase activity that causes the release of HB-EGF from the cell surface, operates for GPCRs, and more recently for uPAR [9,10]. The latter has been proposed by our group as a mechanism to explain the activation of a signaling route that depends on uPA and stimulates the invasive capacity of MCF-7 breast carcinoma cells [11].

It has been proposed that EGFR activation is a potent stimulus to cell migration [12]. In human carcinoma cells that overexpress this receptor, ligand activation of EGFR induces morphological changes and cell detachment, which correlates with tyrosine dephosphorylation and reduced kinase activity of focal adhesion kinase (FAK). Two other proteins, $p130^{cas}$ and paxillin, which are components of focal adhesions and substrates of FAK, were also dephosphorylated upon EGF treatment [13]. In MCF-7 cells, the activation of the IGF-1 receptor promotes migration and invasion by a mechanism that also correlates with dephosphorylation of FAK, $p130^{cas}$ and paxillin, suggesting that this profile of dephosphorylation is a common feature in carcinoma invasion [14].

Up to date, the connection between EGFR activation and the mechanisms of GTP loading on Rho GTPases is still poorly understood. Present data that show a relationship between these two activation processes mainly consider Rho-like proteins as downstream effectors of EGFR activation. Moreover, some authors have proposed that lysophosphatidic acid (LPA) exerts its activation effects on Rho GTPase by a mechanism that is mediated by EGFR [15]. At the transcriptional level, it has been proposed that EGF induces the activation of Rho-like GTPases by a mechanism that requires the function of AP-1 [16]. One of the few studies that showed evidence for the effect of Rho activation on a growth factor signaling pathway was carried out by Sordella et al. They described that cells deficient in p190-Rho GAP that express excessive Rho activity show deficiencies in the IGF-1 signaling pathway [17,18].

Results of the current study show that, in mammary cells, the overexpression of the active form of RhoA (V14 RhoA) induced tyrosine phosphorylation of EGFR, an increased expression of urokinase-like plasminogen activator (uPA) and a higher motility. V14 RhoA cells also displayed a rounded morphology associated with a dephosphorylation of FAK and a lower spreading capacity. We proposed that V14 RhoA cells activate EGFR by a mechanism depending on a membrane-bound substrate because treatment with a metalloproteinase inhibitor restores the degree of EGFR phosphorylation and motility to the level expressed by control cells. Similar results were obtained using the MDA-MB 231 cell line that expressed a high basal level of EGFR phosphorylation and RhoA activation.

Materials and methods

Antibodies and reagents

Monoclonal antibodies to RhoA were from Cytoskeleton (Denver CO), anti- vinculin and β-actin were from Sigma (St. Louis, MO) and anti-p-ERK1,2 was from Santa Cruz Biotechnology (Santa Cruz, CA). 4G10 anti-phosphotyrosine monoclonal antibody was provided as a gift from Dr. M.R. Bono (University of Chile). Polyclonal antibodies against peptides of residues 1176–1186 (anti-C-EGFR) have been previously characterized [19,20]. Anti ERK1,2 and FAK were from Santa Cruz Biotechnology (Santa Cruz, CA).

The metalloproteinase inhibitor GM6001 (Ilomastat) was purchased from Chemicon (Temecula, CA) and the inhibitor of Rho-kinase Y27632 and AG1478 (Tyrphostin) from Calbiochem (San Diego, CA).

Cell culture and transfection methods

Mammary MCF-7 and MDA-MB231 cells were purchased from the ATCC (Manassas, VA) and were grown in a phenol red-free DMEM/F12 plus 10% FCS. V14 RhoA cDNA that encodes for the active form of RhoA was provided by Dr. Senen Vilaró (Universitat de Barcelona, Spain).

To generate stable MCF-7 cell clones that overexpress V14 RhoA, 4×10^5 cells were transfected with 2 μg of the RhoA expression construct using Lipofectamine Plus reagents (Invitrogen Carlsbad, CA). Cells were selected using 1 mg/ml G418 (Invitrogen Carlsbad, CA) for 21 days. After this period, cells were cultured in maintenance doses at 0.2 μg/ml G418.

Affinity precipitation of GTP-bound RhoA

A construct that encodes the Rho binding domain of Rhotekin (RBD) as a glutathione *S*-transferase fusion protein (GST-RBD) was kindly provided by Dr. Keith Burridge (University of North Carolina).

Cells (10^7) were washed once with ice-cold PBS and lysed with 50 mM Tris, pH 7.6, 0.5 mM $MgCl_2$, 500 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, 10 μg/ml of each aprotinin and leupeptin and 1 mM PMSF. Lysates were clarified by centrifugation at 14,000 rpm for 10 min, and the supernatant was incubated on a roller for 1 h with 30 μg of GST-RBD (GST fusion protein containing the RhoA-binding domain [amino acids 7–89] of Rhotekin) bound to glutathione–Sepharose beads (Sigma, St. Louis,

MO). Samples were washed three times with lysis buffer and bound proteins eluted by boiling in sample buffer and then immunoblotted with RhoA monoclonal antibodies. Whole cell lysates were also immunoblotted for RhoA as loading controls. Lysophosphatid acid (LPA) (Sigma, St. Louis, MO) was used in a concentration of 10 μM for 5 min as a control for the activation of RhoA [21].

Immunoflorescence

Cells plated on coverslips were washed once with PBS and fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.25% Triton X-100 during 2 min and incubated with PBS containing 4% BSA for 1 h at room temperature. Primary antibodies diluted in PBS containing 1% BSA (Rockland, Gilbertsville, PA) were used in a dilution of 1/200 for anti-p-Tyr and 1/100 for anti-vinculin and incubated for 1 h at room temperature. Afterwards, antigen–antibody complex was washed and incubated with FITC conjugated anti-mouse IgG (Rockland, Gilbertsville, PA). F-actin was stained with Alexa fluor 594 Phalloidin–rhodamine (Molecular Probes). Fluorescence images were collected on a Zeiss Axioplan microscope and photographed using a 63× immersion objective and the Axiocam camera.

Preparation of Triton-soluble and Triton-insoluble fractions

Cells in 60 mm culture dishes were lysed for 15 min with a buffer containing 50 mM Hepes pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$, 2 mM EGTA, 1% Triton X-100, 10% glycerol, 2 mM PMSF, 2 μg/ml pepstatin, 2 μg/ml leupeptin and 1 mM sodium orthovanadate. The solubilized material (hereafter Triton soluble fraction) was obtained after centrifugation for 10 min at 14,000 rpm. The material that remained adhered to the plastic dish after Triton solubilization was scrapped with a rubber policeman in the presence of RIPA. This suspension was boiled in SDS-PAGE sample buffer to dissociate proteins and centrifuged at 14,000 rpm. This supernatant corresponded to the Triton-insoluble fraction.

Immunodetection of EGFR, FAK and ERK

Cells (10^7 cells per dish) were serum starved in FCS-free DMEM/F12 for 12 h and treated with GM6001 (25 μM) or Y27632 (10 μM) for the same period. EGFR was immunoprecipitated using the anti-C antibody as described [20]. Cells were lysed with buffer B containing 50 mM Hepes pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$, 2 mM EGTA, 1% Triton X-100, 10% glycerol, 2 mM PMSF, 2 μg/ml pepstatin, 2 μg/ml leupeptin and 1 mM orthovanadate at 4°C. Equal amounts of proteins from different treatments were immunoprecipitated, and immune complexes were resolved by SDS-PAGE and analyzed by immunoblotting with antibodies 4G10 for EGFR tyrosine phosphorylation and antibody EGFR984 for the EGFR total mass using the ECL chemiluminescence detection kit (Amersham, Arlington Heights, FL).

In experiments with MDA-MB231 cells, a mild acid treatment to release endogenous ligands was made by incubating with Earle's balanced salt solution EBSS, 10 mM HEPES pH 7.4 for 2 min, 50 mM glycine–HCL, 100 mM NaCl pH 3.0 for 30 min and 0.5 M HEPES, 0.1 NaCl pH 7.5 for 2 min as described. Afterwards, cells were allowed to restore their basal EGFR phosphorylation level by incubation in serum-free medium for 16 h [22].

In experiments in which EV cells were treated with media conditioned by V14 RhoA cells, confluent cultures of the later were incubated 24 h in the absence of serum. The resulting media was added to serum-depleted EV cells for a period of 30 min.

FAK activation experiments were performed as follows: cells (3×10^6 cells per dish) suspended in serum-enriched DMEM/F12 medium were plated onto culture dishes coated with or without fibronectin for 4 h. Then, cells were lysed and immunoprecipitated with an anti-FAK antibody and resolved by SDS-PAGE. Blot was revealed with antiphosphotyrosine (4G10) antibody to evaluate FAK tyrosine phosphorylation and anti-FAK antibody for loading control. In experiments in which FAK activation was modulated by AG1478 (1 μM) or Y27632 (10 μM), the same amount of cells were serum starved in FCS-free DMEM/F12 for 12 h and treated with the agents for 1 h before proceeding, as described above.

ERK1,2 activation and total mass were assessed by immunoblots of cell lysates (30 μg) resolved by 10% SDS-PAGE using either anti-ERK or anti-phosphotyrosine ERK, as described [23].

Gelatinase activity and uPA activity

The expression and activity of soluble uPA and MMPs were measured by immunoblot and zymography on a 10 times concentrated media conditioned for 24 h as previously described [24].

Cell spreading assay

Cells that were detached from the substratum by a brief exposure to Trypsin/EDTA were plated onto fibronectin-coated plates (10 μg/ml) for 90 min. Cell spreading was stopped by pouring off the medium, washing adhered cells once with PBS, fixing them with methanol during 2 min and incubating with 0.2% crystal violet during 5 min [25]. After several washes, spread cells were scored from at least 4 fields of different regions of the dish. Images were captured with a camera Sony DSC through a Zeiss Axiovert 25 inverted microscope.

Migration assay

Migration of MCF-7 was studied using a 6.5 mm Transwell chamber with a pore size of 8 μm. The Transwell membranes were coated with 20% fetal bovine serum for 2 h

at 37°C on the underside only. Both membrane surfaces were blocked with 10 mg/ml BSA for 2 h at 37°C. V14 RhoA cells (10^5 cell/100 μl) in serum-free medium were pretreated with either GM6001 (25 μM) or Y27632 for 15 min and were then added to the upper chamber of each Transwell unit. Cells transfected with empty vector were used without treatment. The lower chamber of the Transwell was filled with a medium solution enriched with 10% FCS. Migration was allowed to occur for 24 h, after which cells of the upper membrane surface were removed by a cotton swab and repeatedly washed with PBS. Migration values were determined by counting five (20×) fields per chamber [26] after fixing the membrane in methanol and staining the migratory cells with 0.2% crystal violet.

Statistical analysis

Statistical significance was determined by Student's *t* test or nonparametric *U* test (Statistica 6.1, Statsoft Inc 1984–2004). $P < 0.05$ value is accepted to be significant.

Results

Basal and overexpressed levels of RhoA-GTP in breast carcinoma cells

To asses whether the expression level of RhoA-GTPase correlates with the invasive capacity of breast carcinoma cells, we determined the basal level of total RhoA in the weakly invasive breast cancer cell line MCF-7 and the strongly aggressive cell line MDA-MB-231. The results indicate that that MDA-MB 231 cells further overexpress RhoA as compared with MCF-7 cells (Fig. 1A). Moreover, a large proportion of the protein detected in MDA-MB 231 cells is present in an active form, as is demonstrated by a pull down assay that showed similar levels of RhoA-GTP between control and LPA-stimulated cells (Fig. 1B). The activation state of RhoA was also evaluated in MCF-7 cells stably transfected with a vector that expresses V14 RhoA, a constitutively active form of RhoA. A pull-down assay of transfected cells showed that they expressed a significantly higher proportion of RhoA as Rho-GTP as compared to either cells transfected with an empty vector (EV) or to cells treated with LPA that were included as positive control (Fig. 1C).

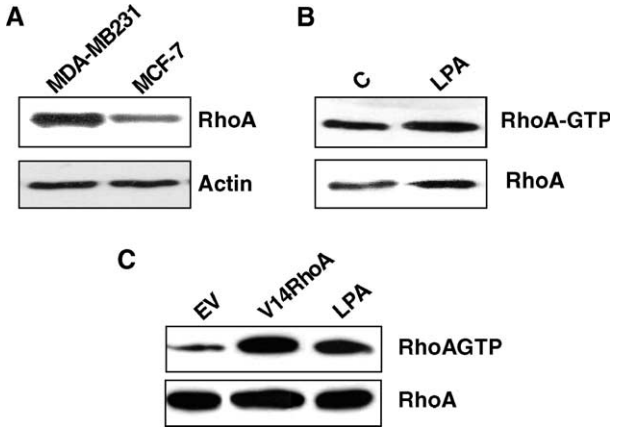

Fig. 1. Basal and overexpressed levels of RhoA-GTP in breast carcinoma cells. Immunoblot of the expression of RhoA protein in MDA-MB 231 and MCF-7 cells (A). Level of activation of RhoA assessed by pull down of the RhoA-GTP form was determined in MDA-MB 231 (B) and MCF-7 cells transfected with empty vector or V14 RhoA (C). In both cases, LPA was used as positive control.

Morphological and functional properties of Rho-GTP expressing cells

Activation of RhoA induced a clear change in cell shape in MCF7 transfected cells. Cell phenotype was characterized by a tight ring of cortical actin and prominent cell-matrix adhesion protrusions which emanate from this actin ring. These actin-containing micro-spikes were enriched in vinculin as is shown in Fig. 2A. In contrast, empty-vector-transfected cells showed a rather smooth surface and a diffuse array of F-actin. This visible difference in the distribution of surface-associated vinculin was not a result of an enhanced expression of the protein as shown by immunoblot analysis (Fig. 2B). Since previous studies demonstrated that Rho-dependent functional focal adhesion induces strong cell attachment to ECM, we determined whether vinculin remained associated to soluble or insoluble fractions after cell lysis. Results of Fig. 2C showed that a larger fraction of vinculin remained attached to the Triton-insoluble fraction as compared to control cells (Fig. 2C), indicating that cell attachment was increased in V14 RhoA cells.

V14 RhoA expressing cells display a basal activation of EGFR and ERK1,2

To analyze whether the activation of RhoA induces changes on the cell signaling machinery that depend on EGFR, we compared the basal levels of EGFR and ERK1,2 activation between V14 RhoA cells and empty-vector-transfected cells. Our results showed that activation of RhoA induced a constant activation of EGFR, a pattern which is maintained through all of the experimental conditions assayed. A similar activation pattern was observed in ERK1,2 (Fig. 3B). We also analyzed whether RhoA-dependent EGFR activation in MCF-7 cells induces functional changes that lead to an increased cell malignancy. In doing so, we evaluated the production of cellular proteases that are relevant to this phenomenon [27]. As is shown in Fig. 3C, media conditioned by V14 RhoA cells contains higher levels of urokinase-type plasminogen activator (uPA), measured by casein reverse zymography or Western blotting. In the same media, we also measured the activity of matrix metalloproteinases (MMPs) by gelatin zymography and found that V14 RhoA cells expressed the highest MMPs activity. This increase was higher for MMP-9 expression level (Fig. 3D). Together, these data strongly

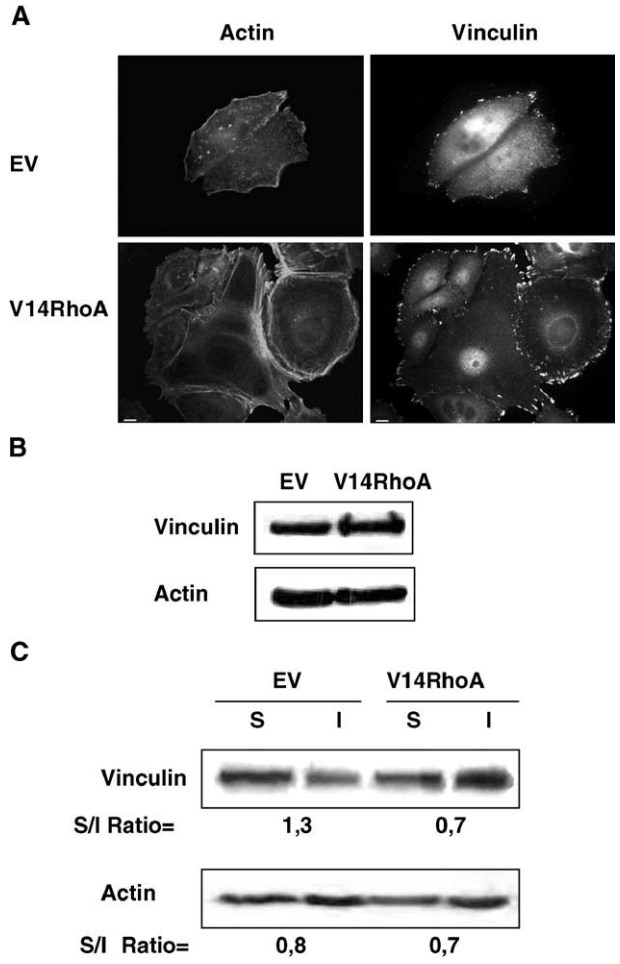

Fig. 2. Overexpression of V14 RhoA promotes focal adhesions. Immunofluorescence analysis for actin cytoskeleton stained with Alexa Fluor 594 rhodamine labeled with Phalloidin or for focal adhesions with anti-vinculin antibody in both cell types (A). Immunoblot for vinculin and actin (B). Distribution of Triton-soluble (S) and Triton-insoluble (I) form of vinculin and actin in MCF-7 cells transfected with empty vector (EV) and V14 RhoA. The S/I ratio was calculated from densitometric analysis of bands (C). Scale bars, 5 μm.

suggest that V14 RhoA cells express higher proteolytic machinery than the empty-vector-transfected cells. To assess the role of EGFR activation in the induction of uPA and MMP-9, we treated V14 RhoA cells with Tyrphostin AG1478, which inhibits the receptor tyrosine kinase activity. Fig. 3E shows that AG1478 treatment strongly blocks either proteolytic activities or the basal level of ERK1,2 activation of V14 RhoA cells (Fig. 3F), demonstrating a major role of EGFR in the acquisition of malignant properties in these cells.

V14 RhoA cells display dephosphorylation of FAK reversed by EGFR inhibitor AG1478 and a lower spreading potential reversed by a ROCK inhibitor

It has been proposed that the acquisition of invasive properties in epithelial cells stimulated by EGF requires the activation of EGFR and the dephosphorylation of FAK as part of a complex phenomenon that includes morphological changes and cell detachment [13]. To evaluate whether activation of EGFR in V14 RhoA cells also induces an inactivation of FAK, we determined the basal tyrosine phosphorylation of FAK in control and V14 RhoA cells and evaluated the effect of the attachment on this phosphorylation pattern via integrins. As is shown in Fig. 4A, overexpression of Rho-GTP induced a dephosphorylation of FAK as compared with empty-vector-transfected cells. Plating both types of cells onto fibronectin for 4 h, to facilitate integrin clustering and to form new cell adhesions, allowed V14 RhoA to overcome the effect of the overexpression of active RhoA on FAK activation.

To test whether this reduced amount of activated FAK in V14Rho cells show a relationship with the activation level of EGFR and with the activity of the Rho-dependent kinase (ROCK), the main downstream effector of Rho, we treated previously attached cells with AG1478 or with the ROCK inhibitor Y27632 for 1 h. A strong inhibition of FAK phosphorylation in both cell types was elicited by Y27632 (Fig. 4B). This effect was accompanied with a disruption of

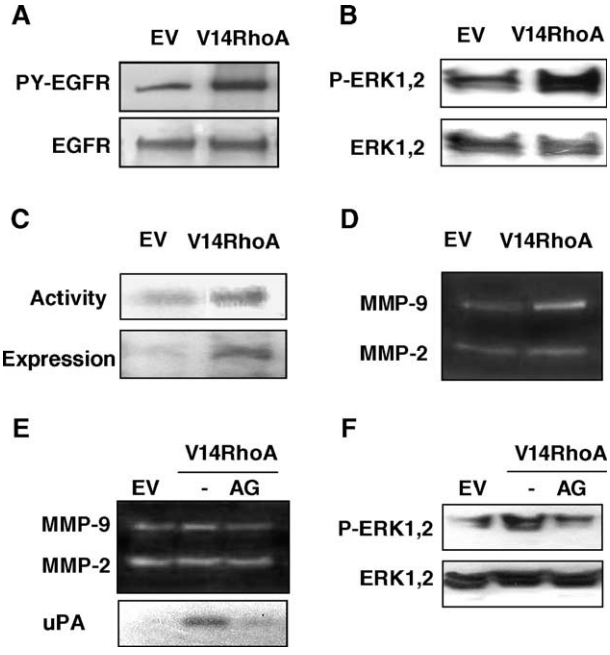

Fig. 3. V14 RhoA cells express an active form of EGFR and ERK1,2 that correlates with the endogenous expression of uPA and MMP-9. Cell extracts of serum-starved EV and V14 RhoA cells were immunoprecipitated with anti-EGFR antibody followed by immunoblotting with anti-phosphotyrosine (PY) antibody and EGFR antibody as loading reference. Immunoblot is representative of at least four separate experiments, $P < 0.021$ (A). ERK1,2 activation, measured by its phosphorylated form (P-ERK1,2), and total ERK1,2, both assessed by immunoblot in an aliquot of the lysate used in panel A (B). Urokinase production in media conditioned by EV and V14 RhoA cells measured by zymogram (upper row) or immunoblot (lower row) (C). Zymographic analysis of MMP-9 production in the same media as in panel C (D). Effect of Tyrphostin AG1478 on MMP-9 and uPA activity and activation of ERK1,2 in V14 RhoA cells (E, F).

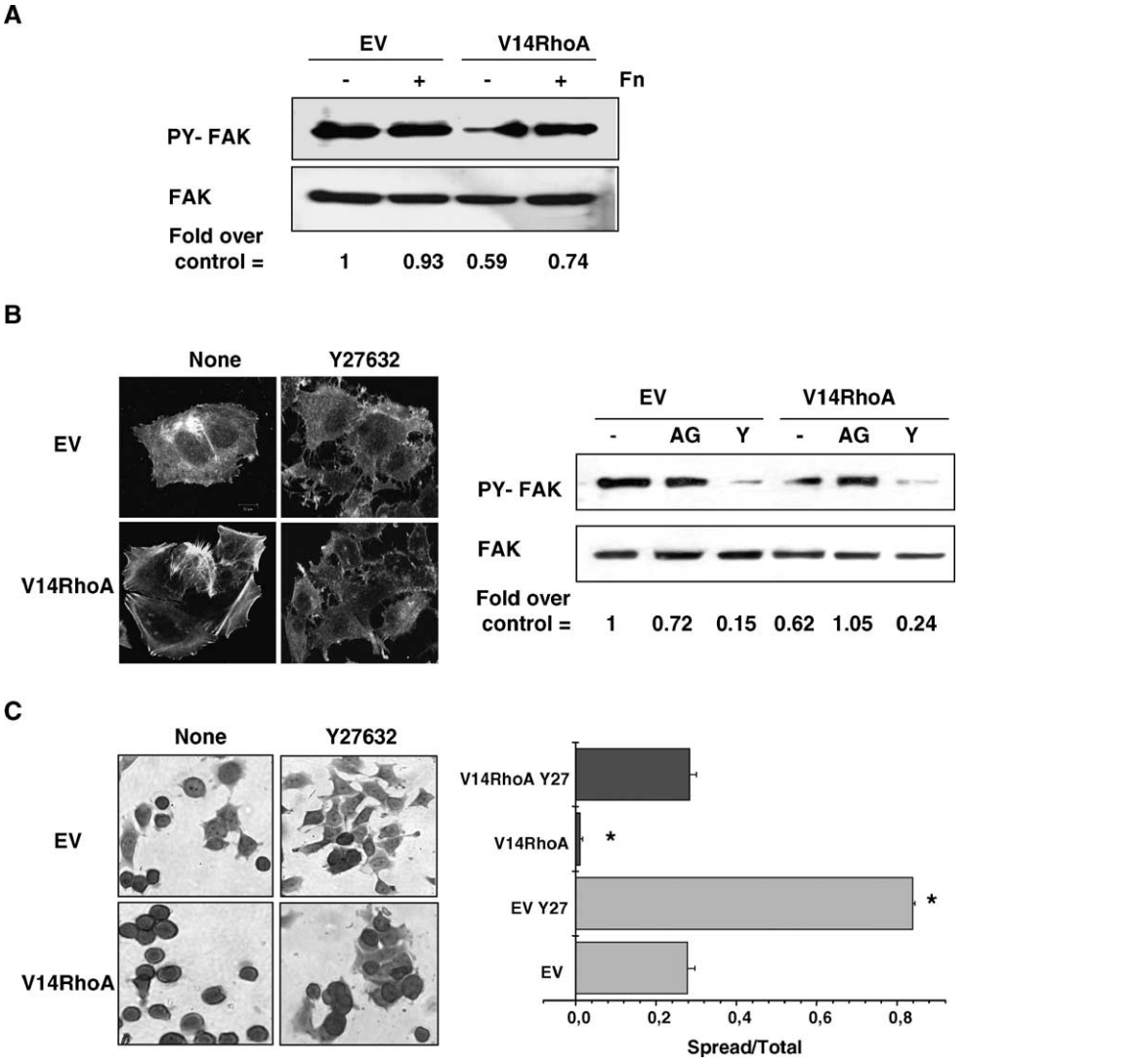

Fig. 4. Active RhoA induces FAK dephosphorylation that is reversed by integrin-dependent adhesion and AG1478. V14 RhoA cells display a rounded morphology that is reversed by Y27632. V14 RhoA and EV cells were seeded onto either plastic or fibronectin-coated plates and allowed to adhere for 4 h. Afterwards, the amount of PY-FAK was measured by immunoprecipitation with anti-FAK antibody followed by Western blot analysis with 4G10 anti-PY antibody. Activated FAK was determined by densitometric analysis of the immunoblots and expressed as percentage of the control (A). V14 RhoA and EV cells were treated with AG1478 (AG) or Y27632 (Y) for 1 h. Next, cells were fixed for F-actin immunofluorescence using Alexa Fluor 594 or lysed for immunoprecipitation. As described in panel (A), FAK activation was evaluated by densitometry analysis and expressed as percentage of the control. Immunoblots of PY-FAK and total FAK from EV and V14 RhoA were representative of at least four separate experiments, $P < 0.021$ (B). Quantitative analysis of cellular spreading with and without Y27632: V14 RhoA and EV cells were plated for 90 min onto Fn-coated plates and stained with crystal violet. Spreading of cells was calculated by counting of at least 4 fields from different regions of the culture dish (C). Data are the mean of values obtained from 4 separates experiments. (*) Significant differences from the EV. Student t test with $P < 0.0001$. Error bars represent standard error.

actin fibers and a more spread cell morphology, as shown in the actin immunofluorescence analysis (Fig. 4B, left panel). Treatment of V14 RhoA cells with AG1478 induced an enhancement of FAK activation, suggesting that FAK dephosphorylation is an EGFR-activation-dependent phenomenon (Fig. 4B right panel). No relevant morphological changes were observed in cells cultured with AG1478 (data not shown).

To quantify whether the morphological changes observed in cells treated with the ROCK inhibitor affect their spreading potential, we plated cells previously treated with Y27632 and untreated cells on fibronectin EV and V14 RhoA. From the bar graph of Fig. 4C, it is possible to observe that, after 90 min, Rho-GTP cells reached a level of spreading that was significantly lower than that of EV cells. Empty-vector-transfected cells show the typical flat polygonal morphology of MCF-7 cells under these conditions. On the contrary in Rho-GTP cells, we observed a clear predominance of rounded cells with no signs of spreading. When these V14 RhoA cells were pretreated for 15 min with Y27632, they recovered the spreading potential of the empty vector non-treated cells. As expected, the addition of ROCK inhibitor enhanced the spreading level of EV cells by nearly 80% (Fig. 4C).

Different levels of reversion of the activated state of EGFR in Rho-GTP-expressing cells by MMP and ROCK inhibitors

We further tested whether ROCK inhibition in Rho-GTP-expressing cells affects the activation level of EGFR, and consequently, the activation of MAP kinase route. For this purpose, we incubated empty vector and V14 RhoA cells overnight with Y27632 and then analyzed the phosphorylation status of EGFR and ERK1,2 as well as the uPA production in both cell types. Fig. 5A shows that ROCK inhibitor does not induced a significant decrease in the phosphorylation level of EGFR in V14 RhoA cells, in spite of some degree of attenuation of ERK1,2 activation was observed. Surprisingly, in empty vector cells, Y27632 promoted an activation of EGFR that is transmitted to ERK1,2, thereby inducing uPA production (compare lanes 1 and 2). Previous studies have reported that Y27632 was able to promote ERK phosphorylation in the absence of other stimuli in MCF-7 cells [28]. The phosphorylation of EGFR by the ROCK inhibitor suggests that the target of this agent localizes upstream from ERK activation and provides a possible explanation for the stimulation of the MAPK route by Y27632.

To emphasize the signaling role of Rho-GTP over-expression, we used immunofluorescence to assay the phosphotyrosine (PY) pattern of cells treated in the same

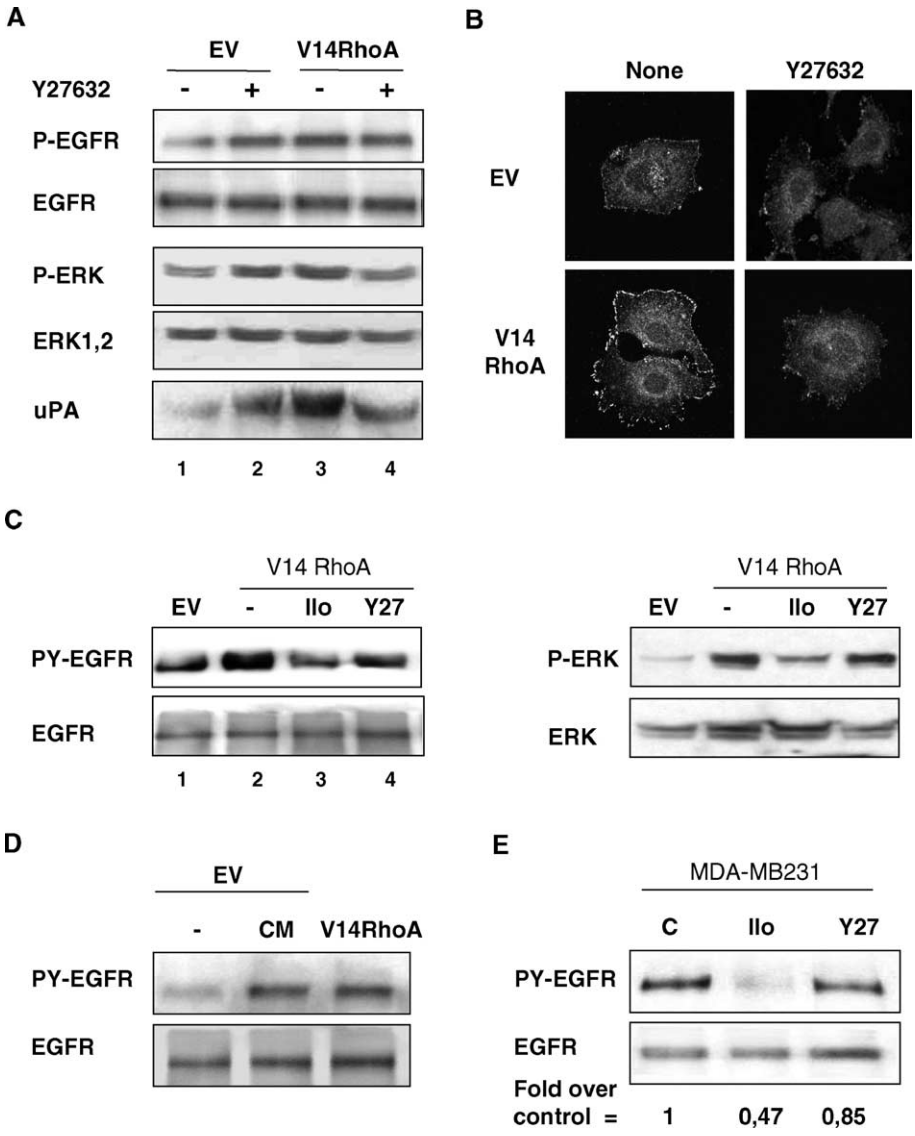

Fig. 5. Differential effects of Y27632 on EGFR, ERK1,2 activation and uPA production in V14 RhoA and EV cells. Inhibition of metalloproteinase activity prevents EGFR and ERK1,2 phosphorylation induced by Rho activation. Serum-starved V14 RhoA and EV cells were incubated with Y27632 for 16 h, and the EGFR and ERK1,2 phosphorylation levels and soluble uPA production were evaluated as described in Fig. 3A. Phosphotyrosine distribution on cells under the same treatment as in panel (A) was evaluated by immunofluorescence (B). V14 RhoA cells were treated with GM6001 (Ilomastat; Ilo) or Y27632, and EGFR and ERK1,2 were evaluated as in Fig. 3C. Phosphorylation level of EGFR from V14 RhoA and EV cells treated or not with media conditioned (CM) by V14 RhoA cells (D). Serum-starved MDA-MB 231 cells, which produce a high amount of endogenous uPA, were first subjected to mild acid treatment to eliminate endogenous ligands and were then incubated with serum-free medium for 16 h as in panel (C), in the presence of 25 μM GM6001 (Ilo) and 10 μM Y27632 (Y27) (E).

experimental conditions as described above. Fig. 5B shows that V14Rho cells concentrated the immunoreactive PY at the cell surface, a property which was lost when cells were treated with Y27632 (Fig. 5B). Transactivation of EGFR by ligands different from EGF has been extensively studied, and a convincing mechanism involving the release of heparin-bound epidermal growth factor like protein (HB-EGF) from the cell surface by metalloproteinase (MMP) activity has been proposed [29]. To evaluate whether this mechanism is present in V14 RhoA cells, we treated these cells with GM6001 (Ilomastat), a potent MMP inhibitor. Fig. 5C shows that the phosphorylation status of EGFR and ERK1,2 in RhoA-expressing cells was diminished down to the level expressed by empty vector cells by treatment with Ilomastat (compare lanes 2 and 3), suggesting that the shedding of a membrane-bound ligand of EGFR possibly explains the high proportion of phosphorylated EGFR and ERK1,2. We included in this figure the result obtained with Y27632 on EGFR phosphorylation in order to compare the effect of both inhibitors. To analyze if the activation of EGFR in V14 RhoA cells is caused by the release of endogenous-produced ligands (or trans-ligands), we incubated for 30 min serum-depleted EV cells that display a low basal level of EGFR activation, with a media conditioned (CM) by V14 RhoA cells. As Fig. 5D shown, after a 30 min incubation with this CM, EV cells increased their EGFR tyrosine phosphorylation reaching a level similar to that of V14 RhoA cells. To test if this pattern was also observed in cells that overexpress EGFR and RhoA, we analyzed the tyrosine phosphorylation of EGFR in MDA-MB-231 cells. In doing so, we first subjected these cells to an acid wash step to reduce background autocrine stimulation and then studied the effect of Ilomastat and Y27632 upon EGFR autophosphorylation after a 16 h recovery period. Fig. 5E shows that Ilomastat completely blocks the recovery of tyrosine phosphorylation after acid treatment, while Y27632 exerts a lesser effect.

V14 RhoA-expressing cells exhibit an enhanced motility that is reversed by Ilomastat

Previous studies of our group and others have described that the transactivation of EGFR correlates with the acquisition of motile properties in MCF-7 cells [11]. To evaluate whether EGFR activation is also associated with motility in V14 RhoA cells, we assayed the migratory potential of empty vector and V14 RhoA cells in the same culture conditions used to assay EGFR phosphorylation. Cell migration assessed using Transwell chambers revealed that V14 RhoA cells express a significantly higher motility than empty vector cells and that this activity is reversed by Ilomastat (Fig. 6). These results are in agreement with the phosphorylation level of EGFR and ERK and support the possible role for the Ras/ERK route in MCF-7 migration [26]. However, when V14 RhoA cells were treated with Y-27632, they expressed the maximum migration level, even above those observed in untreated cells. This result suggests that the relaxation of actin cytoskeleton due to ROCK inhibition, which previously showed to increase the spreading level of transfected cells (Fig. 4C), is a powerful stimulus of migration and probably acts in an EGFR-independent manner.

Discussion

We described here for the first time that the activation of RhoA leads to a metalloproteinase-dependent activation of the Epidermal Growth Factor Receptor (EGFR) in a human mammary cell line. Activation of RhoA induces MCF-7 cells to express some malignant features, such as the EGFR-dependent production of the soluble form of urokinase (uPA) and MMP-9 as well as the acquisition of motile properties. Our results also show that overexpression of V14 RhoA provokes the dephosphorylation of FAK, a phenom-

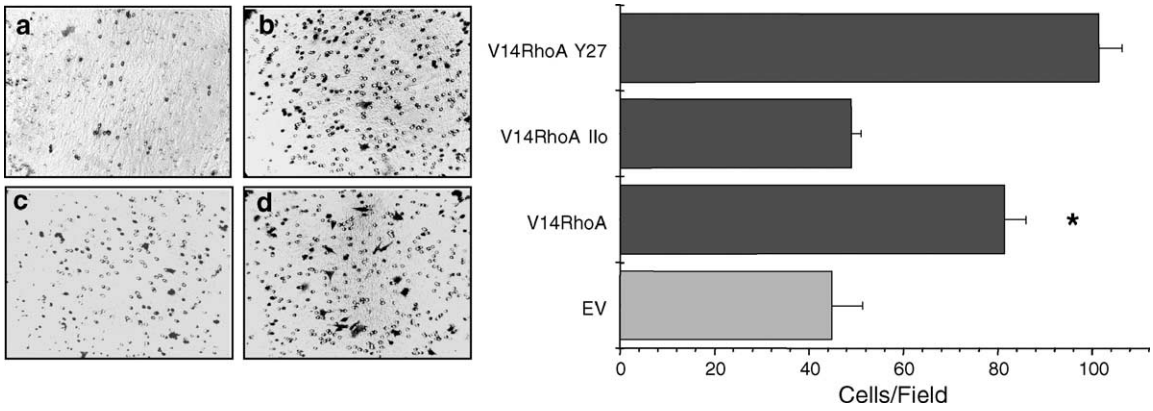

Fig. 6. Differential effects of GM6001 and Y27632 on the migration capacity of V14 RhoA cells. V14 RhoA cells treated with GM6001 or Y27632 or not treated were allowed to migrate for 24 h through a Transwell membrane. Cell migration to the under side of each membrane was determined by counting fixed and stained cells as indicated in Materials and methods. (a) EV cells; (b) V14 RhoA cells; (c) V14 RhoA cells incubated with Ilomastat; (d) V14 RhoA cells incubated with Y27631. Data are the mean of values obtained from 3 separates experiments. (*) Significant differences from the EV (Student t test with $P < 0.002$). Error bars represent standard error.

enon whose dependence on EGFR activation was verified with AG1478, a specific inhibitor of EGFR kinase activity. These results suggest that V14 RhoA cells showed the properties of Rho GTP-expressing cells in that they activate ROCK and display morphological changes and functional characteristics of malignant cells. Based on these results, we proposed a mechanism for cell malignancy and motility that links the activation of RhoA, tyrosine phosphorylation of EGFR and dephosphorylation of FAK.

We proposed the dependence of MMP activity on tyrosine phosphorylation of EGFR in MCF-7 cells in order to explain the uPA-mediated transactivation of the EGF receptor [11]. This finding, along with the higher expression of uPA by V14 RhoA cells (Fig. 3D), suggests the establishment of an autocrine loop that maintains an EGFR-dependent cellular-activated state. This mechanism should depend on the secretion of uPA and/or the endogenous ligands of EGFR. Our finding that the media conditioned by V14 RhoA cells was able to activate EGFR of EV cells strongly supports this proposition (Fig. 5D). Our results with MDA-MB 231 cells that express a constitutively high amount of uPA and maintain EGFR in a permanent activated state are in agreement with this proposal. This suggests that uPA-dependent EGFR autophosphorylation could be a general mechanism to account for the maintenance of the expression of EGFR-dependent malignant properties (Fig. 5E). A correlation between malignancy and uPA production has been demonstrated in breast carcinoma, indicating that enhanced proteolytic activity is a critical element in the acquisition of malignancy [30].

V14 RhoA cells exhibit a rounded morphology with a poor spreading capacity that is characteristic of FAK-deficient cells [31]. This cell shape is most likely a consequence of the cortical array of stress fibers since pretreatment of cells with Y27632, an inhibitor of ROCK, produced a notable reversion of spreading blockage and an increase in cell motility. This finding provides evidence that Rho kinase is a mediator of the morphological changes induced by the overexpression of Rho-GTP (Fig. 4C). Similar results were obtained by other authors in FAK-deficient cells, which reversed the round cell morphology and enhanced their motility after Y27632 treatment [32]. The acquisition of both properties, cell spreading and motility, probably results from the inhibition of the LIM kinase that, in turn, relieves the inhibition of cofilin, an actin-depolymerizing factor [33]. Therefore, the suppression of ROCK activity by Y27632 may reduce the amount of phosphorylated cofilin to an optimal level, thus allowing for the formation of actin-barbed ends required for lamellipodial protrusion and motility [34]. In accordance with this hypothesis, a recent paper by Worthylake and Burridge has shown that THP-1 cells treated with Y27632 express a dephosphorylated version of cofilin that promotes actin remodeling with a consequent enhancement of adhesion and spreading [5]. Our data show that ROCK inhibition that releases V14 RhoA cells from their rounded morphological structure, favoring migration, has an additional effect by inhibiting the level of FAK activation, thereby enlightening the association of FAK inhibition and cell motility (Fig. 4B).

ROCK blockade slightly inhibits EGFR and ERK1,2 activation on V14 RhoA cells, whereas a stimulatory effect was observed in empty vector cells (Figs. 5A and C). Similarly, Jo et al. showed that, in MCF-7 cells, Y27632 promoted a ligand-independent ERK1,2 phosphorylation, which might be the result of an upstream effect of the inhibitor on EGFR activation [28]. The relationship between the activation of EGFR and the polymerization status of actin has been previously analyzed in experiments demonstrating that the binding of EGFR to F-actin deactivates the receptor [35]. This evidence might explain why Y27632, by modifying the level of actin polymerization in empty vector cells, could release F-actin inhibition of EGFR (Fig. 5A). This is not the case for V14 RhoA and MDA-MB 231 cells (Figs. 5C and D) where the ROCK inhibitor induces a partial decrease in the level of EGFR activation. Currently, it is plausible that RhoA-ROCK is able to activate a wide repertoire of molecular targets affecting not only actin cytoskeleton but also proteins engaged in growth factor signaling [18]. Recently, Marinissen et al. demonstrated that RhoA can promote gene expression throughout two different pathways; one is dependent on cytoskeleton remodeling and another promotes c-Jun expression and is independent of cytoskeleton [36]. Our results support the hypothesis that the pharmacological inhibition of ROCK has different consequences depending of the initial level of F-actin content. Therefore, Y27632 produces a stimulus on tyrosine phosphorylated EGFR in EV cells that have almost no stress fibers (Fig. 5A). Meanwhile, in V14 RhoA cells, this inhibitor was able to disorganize both the tight ring of polymerized actin and the easily detected array of cell surface proteins phosphorylated on tyrosine (Fig. 5B), suggesting that treated cells have a lower capacity to elicit proper signaling transduction responses.

Together, our observations suggest that the overexpression of the active form of RhoA in MCF-7 cells generates a cellular phenotype that displays some properties of an Rho GTP-expressing cells, which activate ROCK (i.e. round morphology and a strong cortical actin ring) and also show some functional characteristics of malignant cells. We propose that the MMP-dependent EGFR activation observed in V14 RhoA cells represents the starting point of a signaling route that promotes cell motility by the activation of ERK1,2 and further enhancement of proteases production.

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