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# PKB mediates c-erbB2-induced epithelial $\beta_1$ integrin conformational inactivation through Rho-independent F-actin rearrangements

Shahram Hedjazifar[a], Lachmi E. Jenndahl[a], Hiroaki Shimokawa[b], Dan Baeckström[a,*]

[a]Department of Medical Biochemistry, University of Göteborg, Box 440, SE-405 30 Göteborg, Sweden
[b]Department of Cardiovascular Medicine, Kyushu University, Fukuoka, Japan

## Abstract

Signalling from the growth factor receptor subunit and proto-oncogene c-erbB2 has been shown to inhibit the adhesive function of the collagen receptor integrin $\alpha_2\beta_1$ in human mammary epithelial cells. This anti-adhesive effect is mediated by the MAP ERK kinase 1/2 (MEK1/2) and protein kinase B (PKB) pathways. Here, we show that both pathways mediate suppression of matrix adhesion by causing the extracellular domain of the $\beta_1$ integrin subunit to adopt an inactive conformation. The conformational switch was also dependent on rapid and extensive actin depolymerisation. While neither activation nor inhibition of the Rho GTPase affected this rearrangement, Rho was found to be activated by c-erbB2 and to be necessary for conformation-dependent integrin inactivation and, apparently by a different mechanism, a delayed re-formation of stress fibers which did not restore integrin function. Interestingly, the initial actin depolymerisation as well as its effects on integrin function was shown to be mediated by PKB. These results demonstrate how oncogenic growth factor signalling inhibits matrix adhesion by multiple pathways converging on integrin conformation and how Rho signalling can profoundly influence integrin activation in a cytoskeleton-independent manner.
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Keywords: Integrin; Epithelial cells; erbB2; Protein kinase; Cytoskeleton

## Introduction

It has long been recognised that integrins, the cell's major tools for adhering to and sensing the extracellular matrix, are subjected to a complex and refined regulation of their matrix-binding capacity, so-called inside-out signalling

[1,2]. This regulation is thought to occur at several levels, including changes in integrin conformation, cytoskeletal rearrangements and changes in the cell surface distribution of integrins. Recently, striking advances have been made in the elucidation of the structural basis of integrin affinity regulation, demonstrating that transitions between active and inactive states of the integrin heterodimer involve dramatic conformational rearrangements of its highly flexible extracellular domains [3,4].

Less well defined are the intracellular events that trigger the changes in integrin activity. Although the significance of certain integrin-binding proteins (especially talin, [5]) and of components of the intracellular signalling machinery [6] have been firmly established, a coherent picture of the pathways employed by a particular physiological or pathophysiological stimulus in regulating integrin function is in most cases lacking. This is especially true of integrin regulation in epithelial cells, since hematopoietic and, to

some extent, fibroblastic cells have been the preferred systems in most studies. Furthermore, the mechanisms of regulation of the $\beta_1$ family, the largest group of integrins, are considerably less well known as those governing $\beta_2$ or $\beta_3$ integrins.

In this and previous reports, we have investigated how $\beta_1$ integrin-mediated events in human mammary epithelial cells are regulated by signalling from c-erbB2, a growth factor receptor subunit closely related to the epidermal growth factor (EGF) receptor. c-erbB2 has also been identified as a proto-oncogene, the overexpression of which is strongly associated with poor prognosis in breast carcinoma. Indeed, Herceptin (Trastuzumab), a humanised monoclonal antibody against the extracellular domain of c-erbB2, has become an accepted therapeutic agent in the clinical treatment of c-erbB2-positive mammary tumours [7], although far from all patients with such tumours who respond favourably to Herceptin treatment. The oncogenic effects of c-erbB2 overexpression are generally believed to be mediated by ligand-independent c-erbB2 homodimer-isation. We have established a system for studying the effects of c-erbB2 homodimer signalling, consisting of an immortalised human mammary epithelial cell line (HB2, [8]) into which a hybrid "trk-neu" receptor has been introduced [9]. trk-neu was generated by fusion of the extracellular domain of the trkA nerve growth factor (NGF) receptor to the transmembrane and cytoplasmic domains of c-erbB2 [10]. Addition of NGF to cells expressing this construct causes homodimerisation and activation of the intracellular c-erbB2 tyrosine kinase domain, thus mimicking the signalling caused by a c-erbB2 homodimer. In our previous studies, [9,11], we have described how c-erbB2 signalling causes pronounced disruption of morphogenesis in collagen and reduced adhesion and spreading on collagen surfaces, phenomena that depend on the functional inactivation of the collagen-binding integrin $\alpha_2\beta_1$. We have also shown that this c-erbB2-induced integrin inactivation is mediated by several intracellular pathways, including the MAP ERK kinase-extracellular signal-regulated kinase (MEK-ERK) and phosphoinositide-3-kinase-protein kinase B (PI3K-PKB) pathways, acting in a parallel fashion [11]. Here, we show that both these pathways as well as a separate pathway dependent on the Rho GTPase and its effector, Rho-dependent kinase (ROK), all mediate integrin suppression downstream of c-erbB2 by causing the extracellular domains of $\beta_1$ integrins to adopt an inactive conformation. We also show that c-erbB2 induces a striking depolymer-isation of the actin cytoskeleton and that this rearrange-ment is required for the c-erbB2-induced integrin conformational switch and suppression of adhesion. Surprisingly, the actin-destabilising effects of c-erbB2 signalling were not affected by interfering with the function of Rho or ROK; rather, PKB was shown to be the main mediator of the cytoskeleton-dependent integrin inactivation induced by c-erbB2.

## Materials and methods

### Reagents, antibodies and oligonucleotides

Solubilised bovine collagen I (Vitrogen 100) was purchased from Nutacon BV, Netherlands. 2.5 S nerve growth factor (NGF) from mouse submaxillary gland was obtained from Promega. Latrunculin B, dimethyl sulfoxide, the anti-actin monoclonal antibody (mAb) 20-33 and o-nitrophenyl-$\beta$-D-galactopyranoside (ONPG) were from Sigma. PD98059, wortmannin and Y27632 were pur-chased from Calbiochem. Polyethyleneimine (25 kDa) was from Aldrich. The three conformation state-sensitive integrin $\beta_1$ mAbs were obtained as follows: 9EG7 (rat IgG2a) was purchased from BD; B44 (mouse IgG1) and HUTS-4 (mouse IgG2b) were from Chemicon. The integrin $\beta_1$-recative mAbs TS2/16 (ATCC# HB-243) and P5D2 (Developmental Studies Hybridoma Bank, Univer-sity of Iowa) were obtained from hybridoma culture supernatant. The mAb 26C4 against RhoA and antisera against total and Ser-3-phosphorylated cofilin were from Santa Cruz Biotechnology. Antibodies to MEK1/2 and total and Ser-473-phosphorylated PKB were from Cell Signaling Technology. The ILK mAb (clone 3) was from BD. Antibody for detection of the V5 tag was from Invitrogen. Glutathione–Sepharose and reagents for enhanced chemiluminescence (ECL) were purchased from Amersham Biosciences. Alexa Fluor 488-labeled goat anti-mouse antiserum, Alexa Fluor 546-labeled phalloidin and ZenonOne reagents for direct fluorescent labeling of mouse IgG1 were obtained from Molecular Probes. Jasplakinolide (Molecular Probes) was a kind gift from Dr. Margareta Wallin, Department of Zoology, University of Göteborg. Double-stranded oligoribonucleotides for RNAi of PKB and ILK were purchased from Ambion (siRNA id# 42811 and 1461, respectively). Control siRNAs and the Oligofectamine siRNA transfection reagent were purchased from QIAGEN.

### cDNA constructs

cDNA coding for the constitutively activated V12Rac and V14Rho as well as dominant-negative N17Rac and N19Rho (all myc-tagged), originally from Dr. Marc Symons, Picower Institute for Medical Research, Manhas-set, NY, were provided by Dr. Henrik Semb, University of Lund, Sweden. These cDNAs were excised from their original vectors with EcoRI and ligated into pcDNA3.1-based vectors. pCMV5.SNE/PKB$\alpha$ and pCMV5.SNE/PKB$\alpha$(K179A) used for expression of HA-tagged wild-type and dominant-negative PKB$\alpha$, respectively, were from Dr. Brian A Hemmings, Friedrich Miescher Institut, Zürich, Switzerland. Two plasmids containing dominant-negative alleles of the integrin-linked kinase (ILK) were used: pcDNA3.1V5-His/ILK-kd coding for the E359K mutant, provided by Dr. Shoukat Dedhar, University of British

Columbia, Vancouver, Canada; and pMT2/ILK(K220M), provided by Dr. Ian Hiles, Glaxo SmithKline, Stevenage, UK. pECE/HA-MEK1-ca, coding for a constitutively active mutant (S218D/S222D) of hamster MEK1, was a gift from Pär Gerwins, Rudbeck Laboratory, University of Upsala, Sweden. The plasmid pSG5/Myc-p110αCAAX coding for a constitutively active catalytic subunit of phosphoinositide-3-kinase (PI3K) was a gift from Dr. Stefan Wennström, Rudbeck Laboratory, University of Upsala, Sweden. pEF-BOS-myc/RB/PH(TT), a plasmid encoding a dominant-negative form of the Rho-dependent kinase ROK, was a gift from Dr. Hiroaki Shimokawa, Kyushu University, Fukuoka, Japan. pEGFP/mDia1ΔN3(HindIII), a plasmid encoding a GFP-fused dominant-negative form of mammalian homologue of Diaphanous (mDia), was a gift from Dr. Shuh Narumiya, Kyoto University, Japan. The expression plasmid pGEX2T/GST-RBD, coding for the Rho-binding domain of rhotekin fused to glutathione−S-transferase, was a gift from Dr. Martin Schwartz, Scripps Institute, La Jolla, CA. The reporter plasmids pCMVβ coding for LacZ and pEGFP-C1 coding for green fluorescent protein (GFP) were from Clontech. Plasmids were propagated in appropriate *E. coli* strains and purified using the QIAGEN plasmid purification system.

### Cell culture and transfections

The mammary carcinoma cell lines MCF7, T47D and BT-20 were grown in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) containing 10% fetal calf serum (FCS). The HB2/tnz34 cell line [9], a high-expressing trk-neu transfectant derived from the SV40-immortalised human mammary epithelial cell line HB2 [8], was grown in the same medium, supplemented with 5 μg/ml hydrocortisone, 10 μg/ml insulin and 5 μg/ml zeocin (Invitrogen). Transient transfections were performed using the polyethyleneimine method as described [11]. Typically, 6 μg of reporter vector or a mixture of 3 μg of vector coding for the gene of interest and 3 μg of reporter vector were incubated with polyethyleneimine, added to cells in a 6-cm dish and kept for 48 h before analysis. Expression of transfected constructs was analysed by Western blot or flow cytometry. siRNAs were transfected using Oligofect-amine (QIAGEN) according to the manufacturer's instructions. Briefly, 0.5 μg siRNA mixed with 3 μl Oligofectamine in 95 μl EC-R buffer was added to HB2/tnz34 cells grown in 24-well plates (plated at $3 \times 10^4$ cells/well the previous day) in a total volume of 400 μl/well (100 nM siRNA final concentration). Four days later, the cells were harvested and analysed in Western blot or flow cytometry.

### Adhesion assays

Adhesion to serial dilutions of monomeric collagen I immobilised to microtiter plates was measured as described [11], with the following modification: after addition of NGF, cells were immediately dispensed into collagen-coated microtiter wells and incubated at 37°C. This alteration had no effect on the result of the assay as compared to the previously described procedure. Results of adhesion assays were calculated and expressed as $ED_{50}$ values, i.e., the amount of immobilised collagen necessary to obtain half-maximal binding [11]. Total number of adhered cells was detected by crystal violet staining whereas adhesion of transiently transfected cells was selectively quantitated by colorimetric determination of co-transfected β-galactosidase using the chromogenic substrate *o*-nitrophenyl-β-D-galactopyranoside. Differences in transfection efficiencies were compensated for by normalising to β-galactosidase activity in cell suspensions containing an equal number of cells for each transfection.

### Measurement of activation-specific epitopes by flow cytometry

Cells were quickly detached by trypsin treatment, resuspended in serum-containing medium and passed through a syringe to create a single-cell suspension. The cells were then washed and resuspended in serum-free medium containing 5 mM $MgCl_2$ at $10^6$ cells/ml and divided in 1-ml aliquots. After addition of pharmacological substances where indicated (or DMSO only), the cells were kept at room temperature for 1 h. After addition of NGF to 50 ng/ml where indicated, the cells were incubated for an additional 1 h at 37°C. In some experiments, the duration of the NGF treatment was extended; in these cases, adherent cells were treated with inhibitors and NGF before detachment. The samples were then centrifuged, 900 μl of the supernatant was discarded and the cells were resuspended in the remaining 100 μl of supernatant before addition of 1 μg/sample of activation-specific integrin $\beta_1$ monoclonal antibody (9EG7, B44 or HUTS-4). After a 1-h incubation at room temperature, the cells were washed 3 times with cold FACS diluent (i.e., serum-containing medium containing 0.02% $NaN_3$; all subsequent incubations and washes were carried out in this diluent and on ice) and incubated on ice with Alexa Fluor 488-labeled anti-mouse antiserum. After repeated washing, cells were analysed in a FACSCalibur flow cytometer (BD). Binding of antibodies to cells transiently transfected with plasmids was specifically detected by gating for cells expressing co-transfected GFP, whereas cells transfected with siRNA were detected by co-transfection with an Alexa Fluor 488-labeled non-specific dsRNA oligonucleotide (QIAGEN); in these cases, an R-phycoerythrin-labeled secondary antibody was used. In the experiments where the effect of treatment with the conformation-modulating antibody TS2/16 was analysed, the B44 antibody was directly labeled with R-phycoerythrin using the ZenonOne technology (Molecular Probes).

## Rho assays

Immobilised, recombinant rho-binding domain from rhotekin was prepared as described [12] or purchased from Upstate Biotechnology. Active Rho was extracted from cell lysates using the procedure described by Ren et al. [12] and subjected to SDS-PAGE and Western blot using the RhoA antibody 26C4 (Santa Cruz). Bound antibody was detected by horseradish peroxidase-labeled secondary antibody (DAKO Cytomation) and visualised by chemiluminescence.

## Quantitation of actin content

Detergent-soluble and -insoluble actin was fractionated as described [13]. Briefly, cells were lysed with Triton-X100- and phalloidin-containing buffer, followed by perchloric acid precipitation of both the soluble and insoluble materials. Pellets were washed 4 times in 5 mM Tris–HCl, pH 7.5, dissolved in SDS sample buffer and separated by SDS-PAGE. Actin was then detected by Western blot and band intensity was quantitated by densitometric scanning. The ratio of Triton-insoluble to Triton-soluble actin (TI/TS) was calculated from the band intensities in each sample.

## Confocal microscopy

HB2/tnz34 cells were grown on glass slides (ChamberSlides, Nalge Nunc), kept in serum-free medium with or without pharmacological agents for 1 h and treated with or without NGF (50 ng/ml) for different durations before fixation with 4% paraformaldehyde in PBS for 15 min. Cells were then permeabilised in PBS containing 0.1% Triton X-100 for 5 min and blocked with 20% FCS in PBS for 30 min at room temperature. For F-actin staining, cells were incubated with Alexa Fluor 546-phalloidin (freshly diluted 1:40 in PBS containing 5% FCS from a 6.6 μM stock solution in methanol) for 30 min. After washing in PBS, the slides were mounted with Pro-Long antifade kit (Molecular Probes). Confocal images were collected with a Zeiss LSM-510 laser scanning confocal microscope using the alpha-plan-fluor (1.45 numerical aperture) 100× objective lens. Image analysis was performed using the standard system operating software (LSM software release 3.2) provided with the Zeiss LSM-510 series microscope. Image files were assembled and processed using GraphicConverter 3.7.2 (Lemke Software, Peine, Germany). Each group of images of phalloidin-stained cells (i.e., Figs. 3A, 5A, 5C, 7A and 7B) was processed collectively; however, in the case of images of GFP fluorescence as a reporter for transfection in Figs. 5A and 7B, images were adjusted individually with respect to brightness and contrast in order to allow visualisation of weakly transfected cells.

## Results

### c-erbB2 inactivates $\beta_1$ integrins by inducing an inactive extracellular conformation

In a previous study [11], we have demonstrated that c-erbB2 homodimer signalling induced by NGF treatment of HB2/tnz34, a human mammary epithelial cell line expressing the chimaeric trk-neu receptor, causes functional inactivation of the collagen-binding integrin $\alpha_2\beta_1$. We now wished to assess the possible role of integrin conformational changes in this c-erbB2-induced integrin inactivation. The monoclonal antibodies 9EG7, B44 and HUTS-4 bind to epitopes in the $\beta_1$ integrin extracellular domain which are exposed upon integrin activation [14–16]. When measured in flow cytometry, the surface expression of all three epitopes decreased significantly and reproducibly upon NGF-induced c-erbB2 signalling (Fig. 1A; in the following, only results obtained with one of the conformation-specific antibodies will be shown; however, in most cases, the experiments have been performed with at least one more antibody). Total $\beta_1$ integrin expression as determined by binding of the activation state-insensitive monoclonal antibody P5D2 was unaltered in this experiment and under all other conditions tested in this study (maximal deviation from control 2.4%, data not shown). This result indicates that c-erbB2 signalling causes $\beta_1$ integrins or a subset thereof to switch from an active to an inactive extracellular conformation. In order to assess the general validity of this finding, we examined integrin $\beta_1$ activation status of three other mammary cell lines (MCF7, T47D and BT-20) in response to c-erbB2 signalling. As shown in Fig. 1B, conformational inactivation occurred in two of these cell lines when transiently transfected with trk-neu and treated with NGF, suggesting that conformational integrin inactivation may be a common response to c-erbB2 signalling in mammary epithelial and carcinoma cells. Total integrin $\beta_1$ expression was unaltered, as was integrin conformation upon NGF treatment of control transfectants (data not shown).

Next, we wished to assess the significance of the observed conformational change with respect to c-erbB2-induced suppression of integrin $\beta_1$-mediated cell-matrix adhesion. We have previously established that the adhesion of HB2/tnz34 cells to collagen I is dependent on integrin $\alpha_2\beta_1$. The suppression of this adhesion by c-erbB2 can be relieved by forcing the extracellular domain of integrin $\beta_1$ to adopt an active conformation by treatment with the monoclonal antibody TS2/16, suggesting that c-erbB2-induced integrin inactivation indeed is conformational [11]. However, it was conceivable that the observed conformational change induced by c-erbB2 was irrelevant to its anti-adhesive effect and that another, non-conformational mechanism was responsible for integrin inactivation. We reasoned that in such a scenario, TS2/16 would have to induce a higher level of conformational activation than that
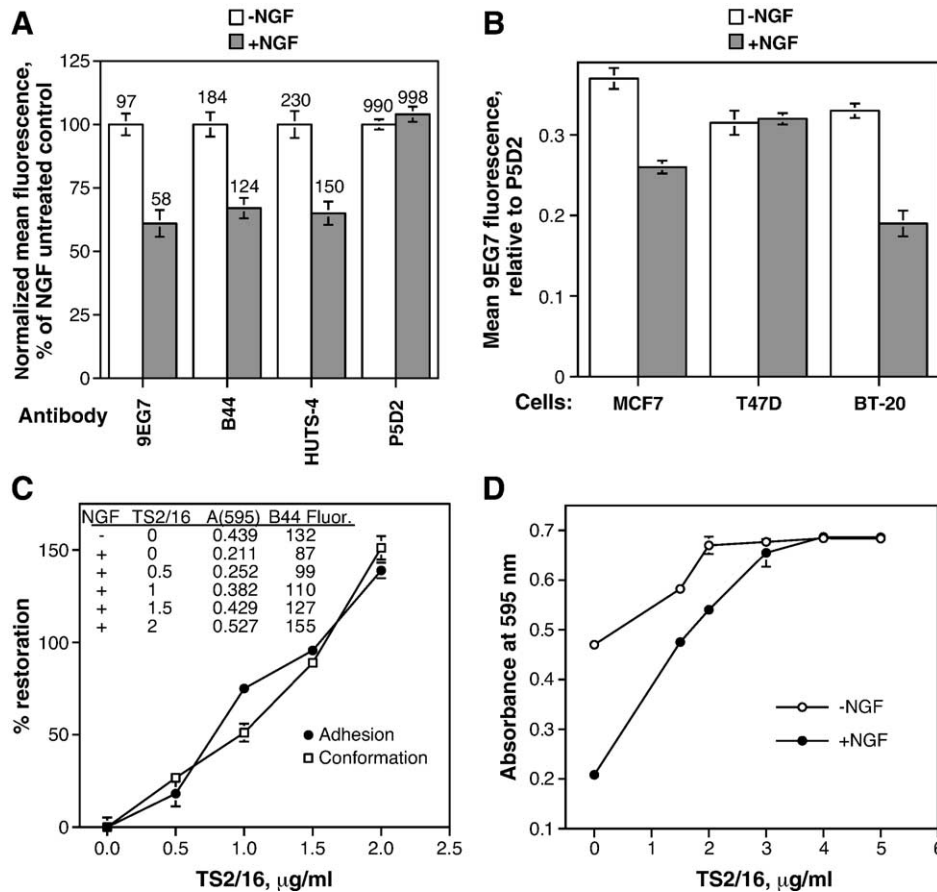
Fig. 1. c-erbB2-induced $\beta_1$ integrin inactivation requires conformational rearrangements of the integrin extracellular domain. (A) Expression of activation-specific epitopes in the integrin $\beta_1$ extracellular domain upon NGF-induced c-erbB2 homodimer signalling. Cells were incubated with 50 ng/ml NGF (grey bars) or left untreated (white bars) for 1 h, stained with the mAbs 9EG7, B44 or HUTS-4 which recognise epitopes in the integrin $\beta_1$ extracellular domain specific for the activated integrin conformation, detected with Alexa Fluor 488-labeled secondary antibody and analysed by flow cytometry. The conformation-insensitive $\beta_1$ integrin mAb P5D2 was included as control for total surface expression. The diagram shows the normalised fluorescence using the value obtained in NGF-untreated controls for each antibody as 100%, whereas the actual mean fluorescence values are displayed as numbers above each bar in the diagram. (B) Binding of the 9EG7 mAb to other mammary cell lines transiently transfected with trk-neu in the absence or presence of 50 ng/ml NGF. Transfected cells were identified by fluorescence of co-transfected GFP, and 9EG7 antibody staining was detected in the GFP-positive population using an R-phycoerythrin-labeled secondary antibody. Results are normalised with respect to total $\beta_1$ integrin expression as measured with mAb P5D2. (C) Quantitative correlation between the degree of restoration of $\beta_1$ integrin conformation and of adhesion to collagen in HB2/tnz34 cells caused by treatment with the monoclonal antibody TS2/16 which induces an active conformation in the integrin $\beta_1$ extracellular domain. Cells were pretreated with the indicated concentrations of TS2/16 for 30 min and subjected to NGF-induced c-erbB2 homodimer signalling before being assayed for adhesion to collagen (●) or binding of the conformation-specific $\beta_1$ integrin monoclonal antibody B44, directly labeled with R-phycoerythrin (□). The restoration values are calculated as $(x_i - x_p) / (x_n - x_p) \times 100$ where $x_i$ is the value obtained at a certain TS2/16 concentration, $x_n$ is the value obtained in control cells (no NGF treatment) and $x_p$ is the value obtained in NGF-treated cells without TS2/16. In the adhesion assays, the absorbance values from crystal violet staining of adhered cells at a collagen coating concentration of 4 ng/well (which is close to the $ED_{50}$, i.e., the collagen amount required for half-maximal binding) for NGF-untreated cells were used for calculations. The actual values used for calculation of restoration are shown in the table inset as follows: TS2/16 concentration is shown in µg/ml; "A(595)" and "B44 Fluor." denote the crystal violet absorbance at 595 nm in the adhesion assay and mean fluorescence in flow cytometry with the B44 mAb, respectively. (D) Effect on adhesion to collagen of saturation with the TS2/16 mAb in the absence (○) or presence (●) of NGF-induced c-erbB2 homodimerisation in HB2/tnz34 cells. Adhesion assays were performed as described above and the results are expressed as crystal violet absorbance. Results are expressed as mean values after background subtraction ± standard deviation of three (A, C) or two (B, D) independent experiments.

seen in cells not subjected to c-erbB2 signalling in order to restore adhesion, since the integrins inactivated by c-erbB2 would not regain activity even if the active conformation were adopted. For the same reason, saturation of NGF-treated cells with TS2/16 would not be able to induce the same degree of adhesion as saturation of NGF-untreated cells. By testing these predictions, we could rule out a non-conformational mechanism for c-erbB2-induced integrin inactivation: first, as shown in Fig. 1C, adhesion and

integrin conformation were restored with a strikingly similar dose-dependence upon TS2/16 treatment, indicating that no excess of conformational integrin activation was required for restoration of adhesion. Second, as shown in Fig. 1D, saturation of the cells with TS2/16 resulted in the same maximal degree of adhesion both in the absence and presence of c-erbB2 signalling, indicating that c-erbB2-induced integrin inactivation cannot persist upon maximal conformational integrin activation. These results strongly

indicate that conformational change is a major mediator of c-erbB2-induced inactivation of the $\alpha_2\beta_1$ integrin.

*Conformational integrin inactivation, like suppression of adhesion, depends on MEK, PI3K and PKB*

Previous studies had shown that c-erbB2-induced integrin inactivation was dependent on the activities of MEK, PI3K and PKB [11]. Furthermore, transfection with dominant-negative integrin-linked kinase (ILK) as well as treatment with rapamycin, which inhibits the mTOR-p70 S6 kinase pathway, also restored adhesion in the presence of c-erbB2 signalling. It was therefore of interest to investigate whether the same kinases were required for the observed c-erbB2-induced integrin conformational changes. When HB2/tnz34 cells were treated with the pharmacological
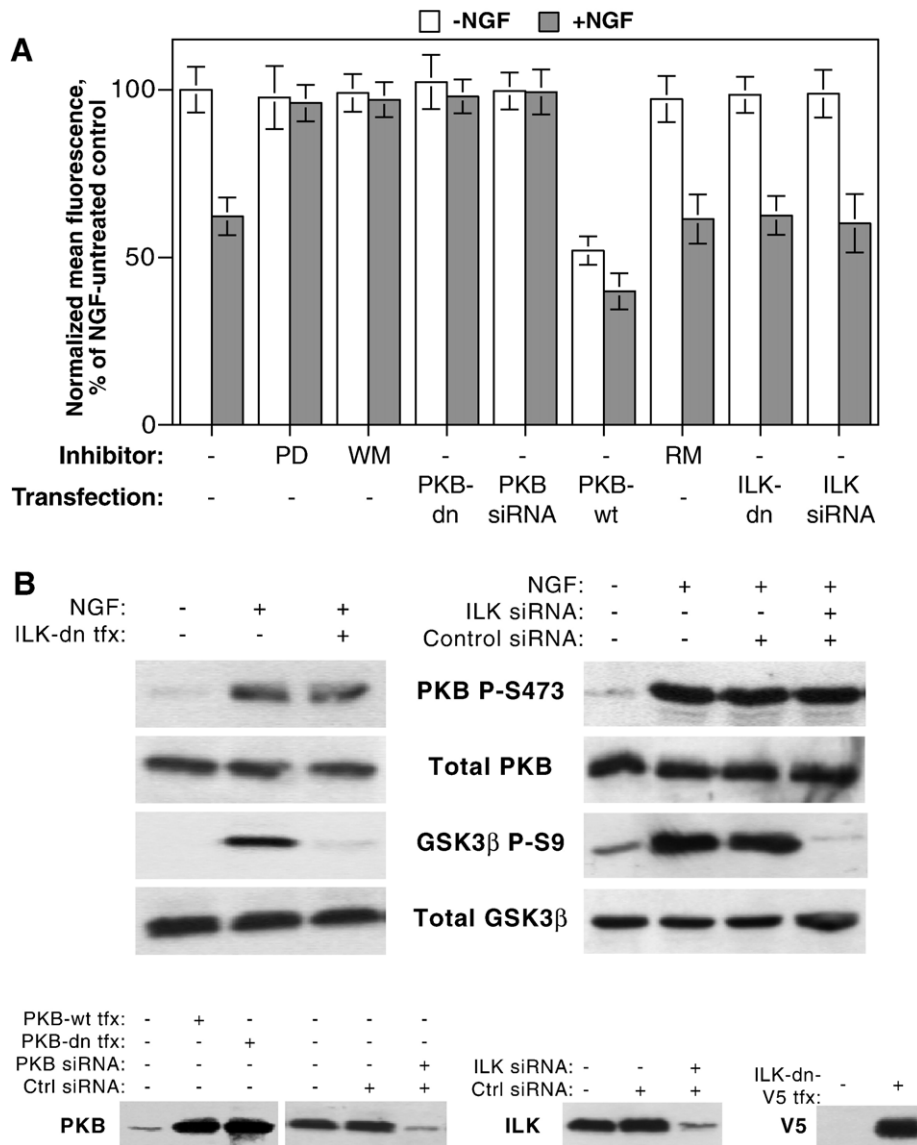


Fig. 2. Dependence of c-erbB2-induced $\beta_1$ integrin conformational rearrangement on signalling pathways known to mediate suppression of adhesion. (A) HB2/tnz34 cells were pretreated with the MEK inhibitor PD98059 (PD, 30 μM), the PI3K inhibitor wortmannin (WM, 0.1 μM), the p70 S6 kinase pathway inhibitor rapamycin (RM, 1 nM) for 1 h or transfected with dominant-negative or wild-type alleles of PKB (PKB-dn and PKB-wt, respectively), dominant-negative ILK (ILK-dn), PKB siRNA or ILK siRNA. The cells were then subjected to NGF-induced c-erbB2 homodimer signalling (grey bars) or left untreated (white bars) for 1 h before staining with the conformation-specific mAb 9EG7. In transfection experiments, transfected cells were identified by fluorescence of co-transfected GFP (plasmid transfections) or Alexa Fluor 488-labeled nonspecific dsRNA (siRNA transfections) and antibody staining was detected using an R-phycoerythrin-labeled secondary antibody. (B) ILK mediates GSK3β phosphorylation at Ser-9, but not PKB phosphorylation at Ser-473 downstream of c-erbB2 in HB2/tnz34 cells. Cells were transiently transfected with dominant-negative ILK or ILK siRNA, serum-starved and then treated with NGF for 1 h or left untreated. Cell lysates were then analysed in Western blot using antibodies to total or Ser-473-phosphorylated PKB or total or Ser-9-phosphorylated GSK3β. Transfection/knockdown controls for both panels (A) and (B) are shown at the bottom. Results shown with dominant-negative ILK were obtained with the V5-tagged E359K mutant; however, use of the K220M mutant yielded similar results (data not shown). Results are expressed as mean values ± SD of three independent experiments (A) or as representative data from experiments performed twice (B).

substances PD98059 or wortmannin (which inhibit MEK and PI3K, respectively), the expression of activation-specific $\beta_1$ epitopes was completely restored (Fig. 2A). Inactivation of PKB by transfection with the dominant-negative K179A mutant or with PKB siRNA showed similar results. Conversely, transfection with wild-type PKB (which strongly suppresses adhesion [11]) resulted in a pronounced conformational inactivation. In contrast, neither rapamycin treatment nor transfection with dominant-negative ILK or ILK siRNA could restore the expression of activation-specific epitopes, suggesting that the mTOR/S6K and ILK pathways downregulate integrin function without contributing to the conformational switch. These results indicate that $\beta_1$ integrin conformational

rearrangements are a necessary but not sufficient step in c-erbB2-induced integrin inactivation and that the conformational inactivation, in turn, is governed by multiple pathways.

Since ILK has frequently been reported to cause activation of PKB by inducing its phosphorylation at Ser-473, the differential effects of ILK and PKB inhibition in Fig. 2A were further investigated. The dominant-negative properties of one of the ILK alleles used by us, E359K, have been disputed [17]. However, similar results were obtained with another reportedly kinase-dead mutant, K220M (data not shown). Furthermore, neither inhibition nor RNAi knockdown of ILK affected c-erbB2-induced PKB phosphorylation at Ser-473 (Fig. 2B), indicating that ILK is
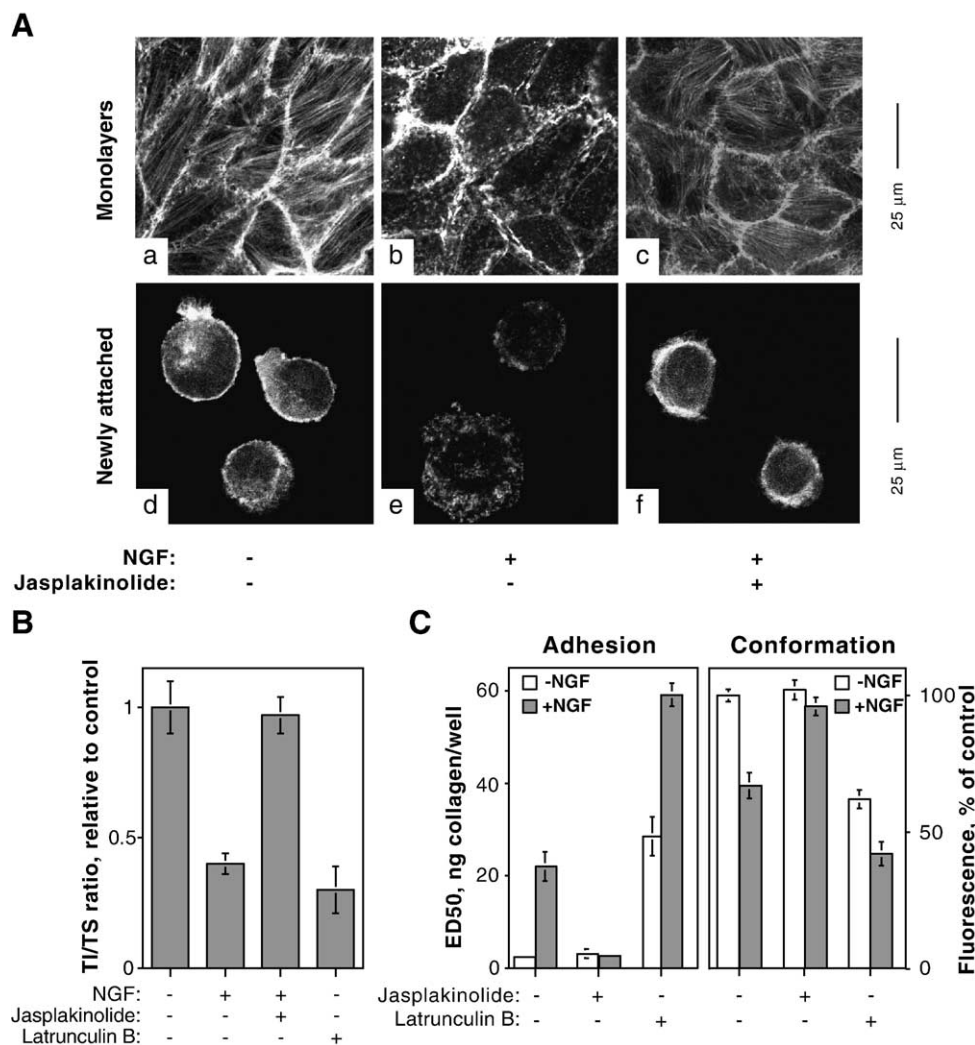


Fig. 3. Effect of c-erbB2 on the actin cytoskeleton and its significance for c-erbB2-induced integrin inactivation. (A) Serum-starved HB2/tnz34 cells were incubated for 1 h with or without NGF, fixed, stained with Alexa Fluor 546-phalloidin and examined in laser scanning confocal microscopy. Prevention of c-erbB2-induced actin reorganisation by pretreatment for 1 h with the actin-stabilising drug jasplakinolide (10 nM) is also shown. (B) Analysis of changes in the ratio of detergent-insoluble to -soluble actin (TI/TS) upon c-erbB2 signalling. Cells were treated with NGF and jasplakinolide as indicated above, separated into Triton X-100-soluble and -insoluble fractions and analysed in Western blot as described in Materials and methods. The TI/TS ratio was normalised relative to untreated control cells. (C) Effects of stabilisation and destabilisation of the actin cytoskeleton using pretreatment for 1 h with jasplakinolide (10 nM) and latrunculin B (100 nM), respectively, on adhesion to collagen and 9EG7 epitope expression in the absence (white bars) or presence (grey bars) of NGF-induced c-erbB2 homodimer signalling. Results in panel (A) show representative fields from one of three repeated experiments; in panels (B) and (C), results are expressed as mean values ± SD of two (B) or three (C) independent experiments.

dispensable for this modification in HB2/tnz34 cells, as has been reported in several other systems [18,19]. In contrast, the same transfections strongly inhibited phosphorylation of Ser-9 in glycogen synthase kinase 3$\beta$ (GSK3$\beta$), another known ILK target [20], indicating that the dominant-negative and siRNA constructs indeed inhibited ILK activity.

### c-erbB2-induced integrin inactivation requires F-actin destabilisation

We have earlier shown that destabilisation of the actin cytoskeleton also can inhibit adhesion of HB2/tnz34 cells to collagen [11]. We now wished to investigate the possibility that cytoskeletal rearrangements could contribute to the integrin-inhibitory effect of c-erbB2. First, the effect on F-actin structures of 1 h of NGF-induced c-erbB2 signalling was analysed using fluorescent phalloidin. This analysis was performed both on cells allowed to grow into monolayers and on cells subjected to the same conditions as in the adhesion assays, i.e., allowed to attach to monomeric collagen for 1 h. In both cases, a very pronounced disruption of F-actin structures was observed: in cell monolayers, stress fibers were almost completely disassembled whereas in recently attached cells (which lacked stress fibers also in the absence of c-erbB2 signalling) as well as in monolayers, the formation of the cortical cytoskeleton was disrupted (Fig. 3A). Both of these cytoskeletal rearrangements were prevented by treatment with the F-actin-stabilising drug jasplakinolide (Fig. 3A). We also quantitated these findings with respect to the relative abundance of detergent-insoluble actin, showing that c-erbB2 signalling indeed significantly decreased the amount of insoluble actin (Fig. 3B). Again, jasplakinolide treatment reversed the effect of c-erbB2.

Next, we wished to determine whether the observed cytoskeletal effects of c-erbB2 were important for integrin inactivation and, if so, whether they affected integrin conformation. The influence of jasplakinolide on suppression of adhesion to collagen and $\beta_1$ integrin conformation induced by 1 h of c-erbB2 signalling was therefore analysed. As shown in Fig. 3C, F-actin stabilisation restored both integrin function and conformation, clearly indicating an important role for cytoskeletal rearrangements in c-erbB2-induced integrin inactivation (note that in this and the following figures, adhesion data are presented as $ED_{50}$ values, i.e., the amount of solid phase-coated collagen required for half-maximal binding; thus, an increase in $ED_{50}$ denotes decreased adhesiveness). Conversely to these results, F-actin destabilisation induced by treatment with the drug latrunculin B, which prevents actin polymerisation, suppressed both adhesion and integrin active conformation (Fig. 3C). These results show that the F-actin destabilisation is induced by c-erbB2 and necessary for its suppressive effect on integrin conformation and function.

### Signalling through Rho and ROK (but not mDia) is required for c-erbB2-induced integrin inactivation

We next wished to investigate the signalling mechanism involved in cytoskeleton-mediated integrin inactivation by c-erbB2. Since the Rho GTPase is known to be a key mediator of cytoskeletal remodelling, we first chose to investigate whether Rho activation is altered by c-erbB2 signalling in HB2/tnz34 cells. In pull-down assays using immobilised Rho-binding domain of rhotekin as bait, we could demonstrate a pronounced activation of Rho upon 1 h of NGF-induced c-erbB2 homodimer signalling (Fig. 4A). Next, we wished to determine the possible role of Rho as a mediator of c-erbB2-induced integrin inactivation. The dominant-negative N19 and constitutively active V14 mutants of Rho were therefore transfected into HB2/tnz34 cells and the effects on adhesion to collagen and on $\beta_1$ integrin conformation were measured in the presence or absence of c-erbB2 signalling. As shown in Fig. 4B, both assays showed that inhibition of Rho caused a reversal of c-erbB2-induced suppression of integrin function. In contrast, introduction of activated V14Rho strongly suppressed adhesion and active $\beta_1$ integrin conformation also in the absence of c-erbB2 signalling. These results indicate that activation of Rho by c-erbB2 is necessary for c-erbB2-induced integrin inhibition to occur.

Fig. 4. Activation of Rho by c-erbB2 and its influence on integrin function. (A) Serum-starved HB2/tnz34 cells were pretreated with inhibitors or lysophosphatidic acid (LPA) for 1 h then incubated with or without 50 ng/ml NGF for another hour. Cells were then lysed and GTP-bound Rho was extracted using a recombinant Rho-binding domain from rhotekin fused to GST and immobilised onto glutathione–Sepharose. The extracted Rho and whole cell lysates were analysed for RhoA by Western blot. Cells treated with LPA (30 $\mu$M) are shown as positive control. LTB, latrunculin B, 100 nM; ca, transfection with constitutively active V14Rho; dn, transfection with dominant-negative N19Rho; WM, wortmannin (0.1 $\mu$M); PD, PD98059 (30 $\mu$M). (B) Effect on adhesion to collagen and on $\beta_1$ integrin conformation of transfection with dominant-negative (N19, Rho-dn) or constitutively active (V14, Rho-ca) Rho in the absence (white bars) or presence (grey bars) of NGF-induced c-erbB2 homodimer signalling. (C) ROK, but not mDia, mediates c-erbB2-induced integrin inactivation downstream of Rho. Effects of inhibition of the Rho effectors ROK and mDia on c-erbB2-induced integrin inactivation as measured by adhesion to collagen and by expression of the 9EG7 epitope. ROK was inhibited by pretreatment with the pharmacological substance Y27632 (1 $\mu$M) or by transfection with a dominant-negative ROK construct (ROK-dn). mDia was inhibited by transfection with a dominant-negative construct fused to GFP (mDia-dn). In adhesion assays, transfected cells in adhesion assay experiments were selectively quantitated using a chromogenic substrate for co-transfected $\beta$-galactosidase. In flow cytometry, transfected cells were identified by fluorescence of co-transfected GFP (with the exception of the dominant-negative mDia construct in flow cytometry which, being a GFP fusion protein, did not require co-transfection with GFP), and 9EG7 antibody staining was detected in the GFP-positive population using an R-phycoerythrin-labeled secondary antibody. Transfection controls for myc-tagged ROK-dn and GFP-tagged mDia-dn using Western blot and flow cytometry, respectively, are shown. Panel (A) shows representative results from one of three repeated experiments; in panels (B) and (C), mean values $\pm$ SD of results from three independent experiments are shown.

Rho is known to exert its effect on the cytoskeleton by binding to and activating downstream effector molecules such as the Rho-dependent kinase (ROK) and the actin nucleation factor mammalian homologue of Diaphanous (mDia). In order to further characterise the Rho-dependent branch of c-erbB2 integrin-inhibitory signalling, these two effectors were inhibited in our adhesion and conformation assays. As shown in Fig. 4C, inhibition of ROK, either using the pharmacological inhibitor Y27632 or by trans-

fecting a dominant-negative mutant of ROK, also restored integrin function in the presence of c-erbB2 signalling, whereas a dominant-negative mDia mutant was without effect in both assays.

We also investigated the possible dependence of the Rho/ROK branch on the other pathways downstream of c-erbB2 known to mediate integrin inactivation. As shown in Fig. 4A, inhibition of MEK or PI3K had no effect on c-erbB2-induced Rho activation. Furthermore, treatment with
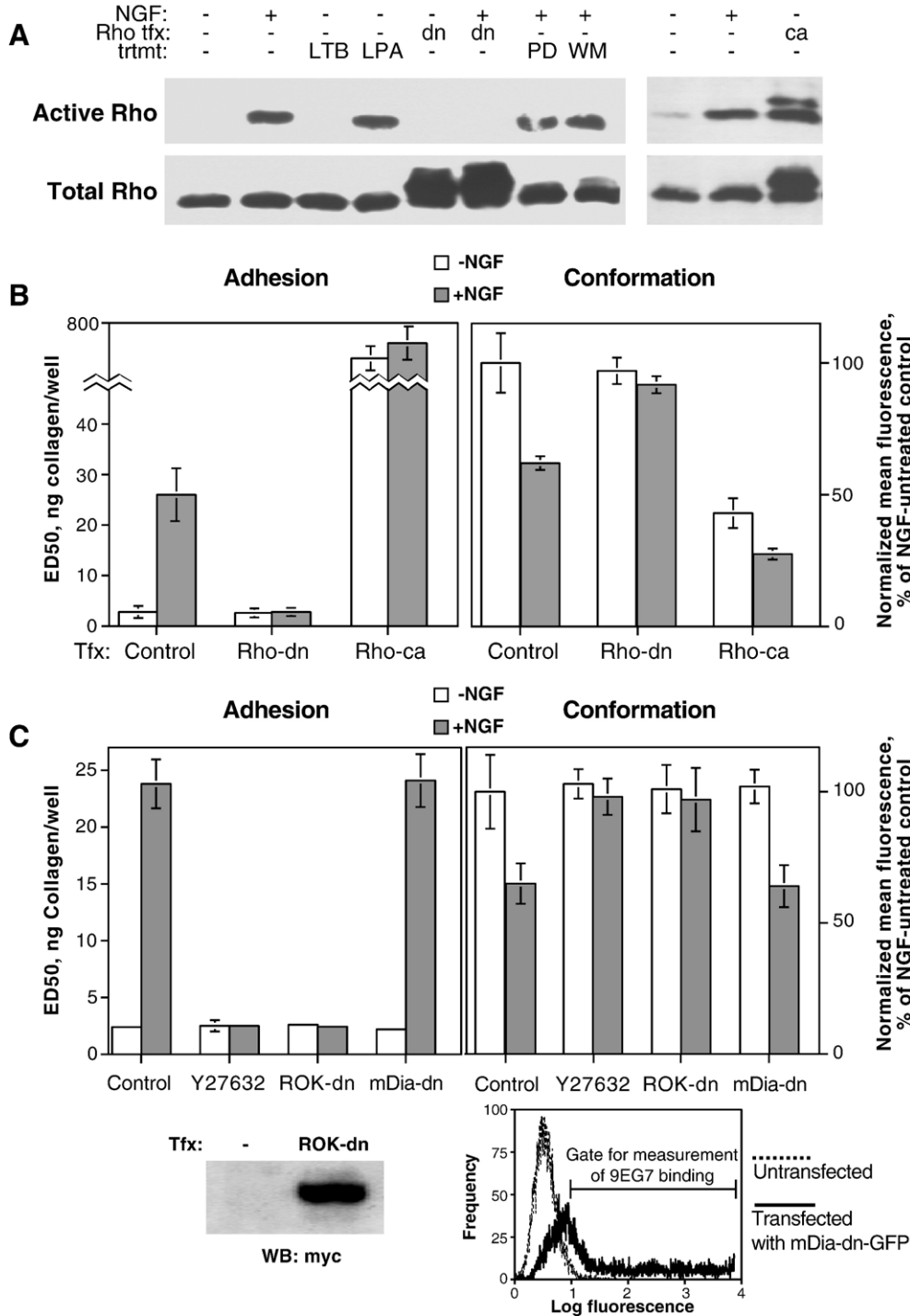
Y27632 had no effect on suppression of adhesion induced by transfection with wild-type PKB or constitutively active MEK1 nor on the activation of MEK or PKB by c-erbB2 (data not shown). We therefore conclude that Rho is activated by c-erbB2 and affects integrin conformation and function in a manner independent of PI3K, PKB and MEK.

### Rho does not mediate c-erbB2-induced F-actin destabilisation, but governs subsequent stress fiber re-assembly

Although Rho is known to cause cytoskeletal rearrangements, our results showing simultaneous activation of Rho and stress fiber disassembly upon c-erbB2 signalling presented an apparent paradox, since Rho has generally been described as an inducer of stress fiber formation. In order to clarify this issue, we measured the effects on the actin cytoskeleton of transfection with constitutively active and of dominant-negative Rho in the absence or presence of c-erbB2 signalling, respectively. Intriguingly, neither construct nor the Y27632 inhibitor had any effect on the structure of the actin cytoskeleton or on the negative influence of 1 h of c-erbB2 signalling on F-actin stability (Fig. 5A). Transfections with dominant-negative or constitutively active forms of Rac were also without effect (data not shown).

The lack of influence of Rho transfections on microfilament structure prompted the question whether Rho-dependent signals responsible for actin rearrangements were somehow blocked downstream of ROK. ROK is known to phosphorylate and activate LIM kinase 2, which in turn phosphorylates and inactivates the actin-severing protein cofilin at serine 3, thereby promoting actin polymerisation. We therefore assayed the phosphorylation at Ser-3 of cofilin in Western blot and found that c-erbB2 induced a pronounced increase in phosphorylation at this site (data not shown). The ROK-dependence of this event was confirmed, as treatment with Y27632 strongly counteracted cofilin phosphorylation.
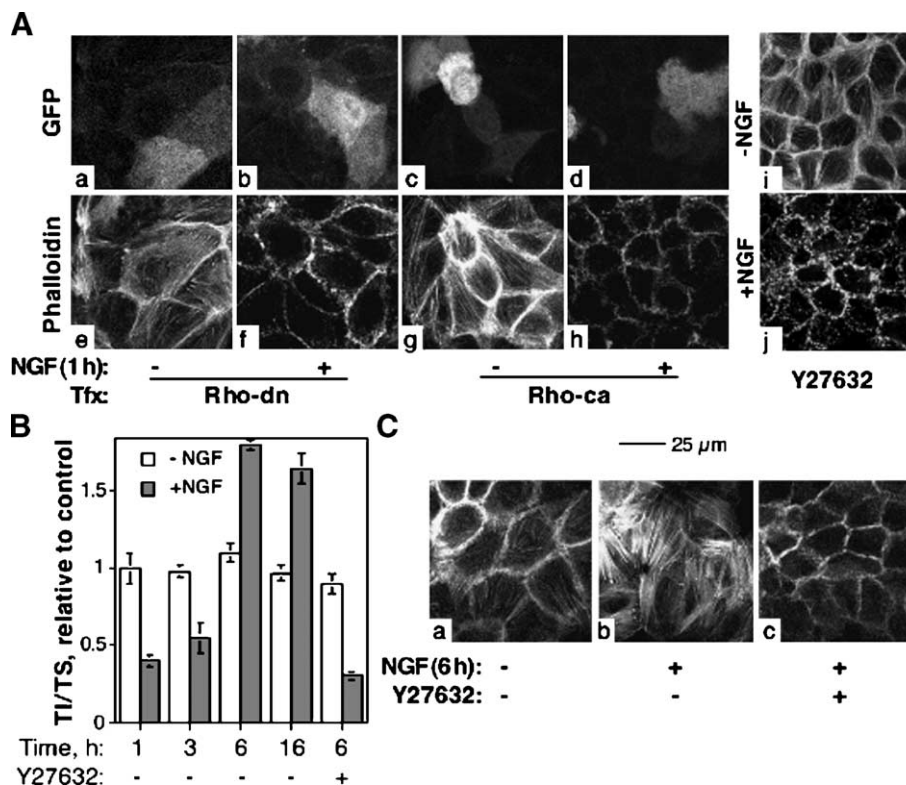


Fig. 5. Role of Rho signalling in c-erbB2-induced actin rearrangements. (A) Transfection with constitutively active (ca) or dominant-negative (dn) alleles of Rho (a–h) or treatment with the ROK inhibitor Y27632 (i–j) is without effect on F-actin structures or on F-actin rearrangements caused by 1 h of c-erbB2 signalling in HB2/tnz34 cells. Transfected cells (a–h) were serum-starved 2 days after transfection, incubated with or without 50 ng/ml NGF for 1 h, then fixed and stained with Alexa Fluor 546-phalloidin. Co-transfected GFP was used as a reporter for transfection. GFP alone had no effect on the F-actin structures (data not shown). Pictures recording green and red light, respectively, were obtained from identical fields. Y27632 (1 μM, i–j) was added to serum-starved cells 1 h before NGF treatment and staining as described above. (B) Time-course analysis of influence on F-actin content of c-erbB2 signalling. Serum-starved cells were treated with NGF for the indicated durations and assayed for soluble and insoluble actin as described in Materials and methods. The TI/TS ratio was normalised relative to untreated control cells. (C) Effect of c-erbB2 signalling for 6 h on F-actin structures visualised by staining by fluorescent phalloidin. Except for the duration of the NGF treatment, conditions were as indicated in the legend to Fig. 3A. Panel (c) shows influence of pretreatment with 1 μM Y27632 on NGF-treated cells. Treatment with Y27632 without NGF for 6 h had no effect (data not shown). Micrographs show representative fields from experiments performed twice (C) or three times (A); data in panel (B) are mean values ± SD of results from two independent experiments.

It was also conceivable that Rho-dependent cytoskeletal responses to c-erbB2 signalling were delayed in relation to the events that caused the initial actin depolymerisation, as has been observed in other systems [21]. We therefore assayed for F-actin content in HB2/tnz34 cells subjected to NGF treatments for varying durations. As shown in Fig 5B, the abundance of F-actin followed a biphasic time-course, with the initial decrease followed by a dramatic increase at 6 h. When cells treated with NGF for 6 h were examined for F-actin structures, stress fibers were observed that were thicker and more abundant than in untreated cells (Fig. 5C, compare panels a and b) whereas cortical structures were virtually absent. This striking rearrangement was apparently Rho/ROK dependent, since it was reversed by treatment with Y27632 (Fig. 5C, panel c) as well as by dominant-negative Rho (data not shown), resulting in a phenotype similar to that of cells treated for 1 h. The re-appearance of stress fibers after 6 h did however not influence integrin function, as adhesion to collagen was still inhibited by c-erbB2 in a manner sensitive to ROK inhibition (data not shown). We have previously shown c-erbB2-induced suppression of adhesion to persist after 24 h of NGF treatment [11].

*c-erbB2-induced actin depolymerisation and cytoskeleton-dependent integrin inactivation are mediated by a PI3K-PKB pathway*

These results raised two questions: were the Rho-induced effects on integrin function indeed independent of cytoske-letal rearrangements and, if so, what other c-erbB2 effector(s) mediated the cytoskeleton-dependent signals required for integrin inactivation? We attempted to answer these questions by transfecting active alleles of known mediators of c-erbB2-induced integrin inactivation into HB2/tnz34 cells and then examine their effects on adhesion and integrin conformation in the presence and absence of the actin-stabilising drug jasplakinolide (which is capable of preventing c-erbB2-induced integrin inactivation, see Fig. 3C). As shown in Fig. 6, transfection with constitutively active forms of MEK, PI3K and Rho as well as wild-type PKB all suppressed adhesion (all, with the exception of MEK, to a higher degree than c-erbB2), but Rho- and MEK-induced suppression of adhesion were completely unaffected by jasplakinolide treatment. In contrast, the anti-adhesive effects of PI3K and PKB were strikingly reversed upon jasplakinolide-induced F-actin stabilisation. The same pattern was observed when the transfectants were analysed for expression of the 9EG7 epitope (Fig. 6).

Since these results strongly indicated that the integrin-inactivating influence of PI3K and PKB was dependent on F-actin destabilisation, it was of interest to establish whether the initial, Rho-independent cytoskeletal effects of c-erbB2 signalling in HB2/tnz34 cells were also mediated by these kinases. We therefore interfered with the c-erbB2-induced activation of PI3K and PKB by wortmannin treatment and dominant-negative PKB transfection, respectively, and studied the effects on F-actin staining. As shown in Fig. 7, c-erbB2-induced stress fiber disassembly was prevented in both cases, demonstrating that the F-actin destabilisation
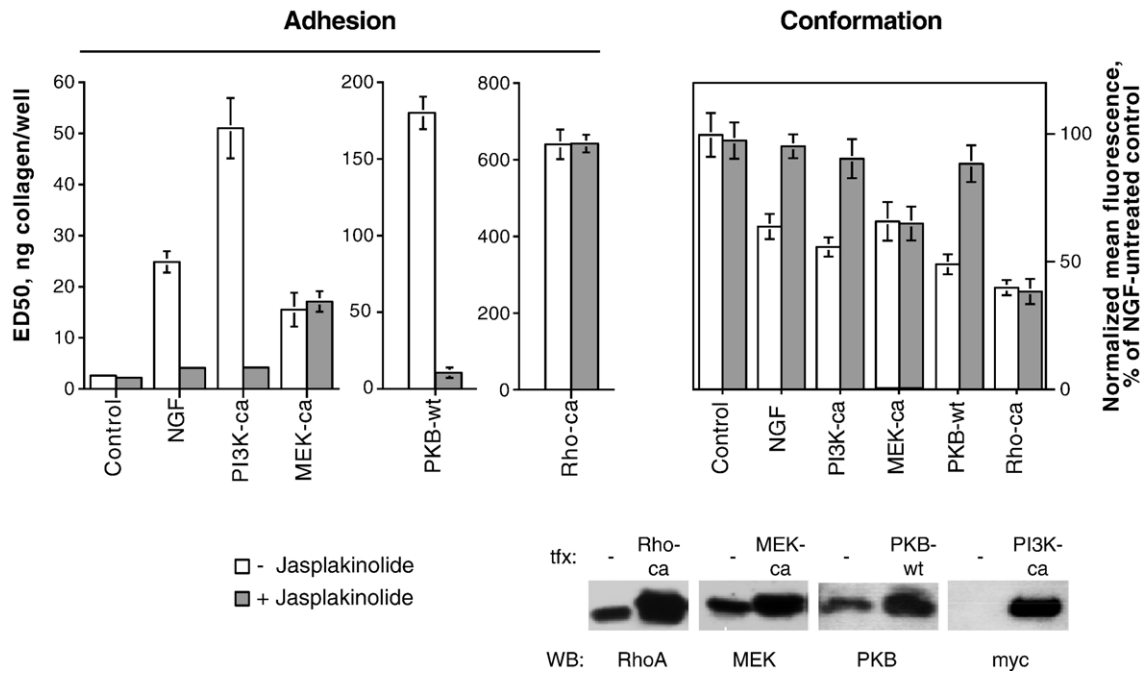


Fig. 6. c-erbB2-induced integrin inactivation mediated by PI3K and PKB, but not Rho or MEK, requires F-actin destabilisation. Cells transfected with constitutively active (ca) forms of PI3K, MEK or Rho or with wild-type PKB (PKB-wt) were incubated in the presence (grey bars) or absence (white bars) of 10 nM jasplakinolide and analysed in collagen adhesion assays or flow cytometric conformation assays using mAb 9EG7 as described above (legend to Fig. 4). Mean values ± SD of results from three independent experiments and transfection controls using Western blot are shown.

Fig. 7. PI3K and PKB, but not MEK, mediate initial c-erbB2-induced cytoskeletal reorganisation. (A, B) Laser scanning confocal micrographs of cells stained with Alexa Fluor 546-phalloidin. (A) Cells pretreated for 1 h with the PI3K inhibitor wortmannin (100 nM) or the MEK inhibitor PD98059 (30 μM) and incubated for 1 h with 50 ng/ml NGF prior to fixation. (B) Cells transfected with the dominant-negative PKB mutant K179A (dn-PKB) subsequently treated with NGF or cells transfected with wild-type PKB (wt-PKB) or a constitutively active PI3K construct (ca-PI3K) without NGF treatment. Co-transfected GFP was used as a reporter for transfection. (C) Time-course analysis of PKB Ser-473 phosphorylation and Rho GTP loading following c-erbB2 signalling induced by treatment with 50 ng/ml NGF for different durations. PKB and Rho activation was analysed as described in legends to Figs. 2B and 4A, respectively. Representative results from experiments performed twice (C) or three times (A, B) are shown.

seen upon c-erbB2 signalling was indeed mediated by PI3K and PKB. This conclusion was further strengthened by the observation that transfection with constitutively active PI3K and wild-type PKB both caused extensive stress fiber disassembly in the absence of c-erbB2 signalling (Fig. 7B). In contrast, inhibition of MEK by treatment with PD98059 (Fig. 7A) of the S6 kinase pathway by treatment

with rapamycin or of ILK by transfection with a dominant-negative construct (data not shown) was without effect, indicating that these kinases are not involved in c-erbB2-induced F-actin destabilisation. In Fig. 7, the cytoskeletal effects have only been shown in cells grown in monolayers; however, the same experiments were performed with cells newly attached to collagen, and c-erbB2-induced F-actin

destabilisation was shown to have the same signalling requirements in these cells as in monolayer cells (data not shown).

Since PI3K-PKB-dependent F-actin disruption was followed temporally by Rho-dependent stress fiber assembly during prolonged c-erbB2 signalling, it was of interest to compare the activities of these pathways at different time points. As shown in Fig. 7C, PKB phosphorylation at Ser-473 peaked at 1–3 h and decreased subsequently, whereas Rho activation increased steadily up to 6 h. These data suggest that the biphasic nature of the cytoskeletal response to c-erbB2 is caused by a shift from PKB- to Rho-dominated signalling.

## Discussion

The aim of our ongoing studies is to understand the molecular mechanisms whereby signalling from c-erbB2 and its downstream effectors negatively affects integrin function in epithelial cells. The focus of the present paper is on changes mediated by rearrangements of integrin conformation and of the actin cytoskeleton. Although it must be strongly emphasised that other mechanisms, not assayed here, may well play major roles in c-erbB2-induced integrin inactivation, the present results allow the following important conclusions:

### c-erbB2-induced integrin inactivation requires changes in $\beta_1$ integrin extracellular conformation

The expression of a number of epitopes located in the extracellular domain of integrin $\beta_1$ has been identified as being associated with an active conformation. NGF-induced c-erbB2 homodimer signalling in HB2/tnz34 cells caused the expression of three activation-specific epitopes to decrease in a significant and reproducible manner (Fig. 1A). Since published mapping studies (reviewed in [22]) have established that 9EG7 recognises an epitope spatially distinct from the region bound by B44 and HUTS-4, it can be concluded that conformational changes consistent with inactivation occur in two discrete regions of the $\beta_1$ extracellular domain upon c-erbB2 signalling. In an earlier study [11], we reported that the signal level in our initial experiments with these antibodies was very low; however, by adopting a revised protocol (see Materials and methods), the quality of the results has been significantly improved. As the $\beta_1$-activating antibody TS2/16 restored integrin conformation and cell adhesion to collagen with highly similar dose–response characteristics (Figs. 1C–D), we also conclude that the c-erbB2-induced conformational changes are necessary for suppression of adhesion. Previously, some cases of correlation between cytokine-mediated $\beta_1$ integrin regulation and changes in expression of activation-specific epitopes have been reported in cells of the immune system [24,23]. However, this is, to our knowledge, the first study

to analyse in detail this type of integrin regulation in epithelial cells and in connection to the $\alpha_2\beta_1$ integrin. Based on measurements of soluble collagen binding, Jung and Moroi [25] have argued that $\alpha_2\beta_1$ activation in platelets is conformational, but in their studies, conformation was never directly assayed. The importance of conformational change in the regulation of $\beta_1$ integrin function has been questioned [22]. However, the recent wealth of structural data overwhelmingly favour the notion that significant conformational changes are a general prerequisite for activation of integrins [4] including $\beta_1$ integrins [26]. We believe that much of the scepticism stems from the expectation that physiological stimuli should induce changes in the binding of activation-specific antibodies as dramatic as those caused by artificial agents such as manganese. The range of physiological conformational changes may be more limited, although still functionally important, as exemplified by the response of integrin $\alpha_4\beta_1$ to cytokines in leukocytes [27]. There may be several reasons why "natural" integrin agonists and antagonists could elicit weaker responses. For instance, artificial stimuli may make the epitopes more exposed or exposed in a kinetically more stable manner. Alternatively, among the population of $\beta_1$ integrins, only a particular subset may be responsive to regulation from a particular physiological stimulus, whereas artificial agents would tend to affect the entire population. In either case, the present study shows that careful attention to apparently more modest changes in integrin conformation can reveal important information on the mechanisms of inside-out signalling.

It should also be noted that, apparently, not all the signalling pathways downstream of c-erbB2 that mediate suppression of adhesion to collagen are involved in integrin conformational rearrangements, since inhibition of ILK or the mTOR-S6K pathway did not restore conformation (Fig. 2A), although these manipulations have been shown to reverse suppression of adhesion [11]. This finding suggests that conformational changes are necessary but not sufficient for c-erbB2-induced integrin inactivation. One may speculate that the pathways controlled by ILK and mTOR-S6K prevent the integrins from reassuming an active conformation when encountering ligand or that they regulate changes that occur after ligand binding. In this context, it is interesting to note that, while the conformation-regulating effectors Rho, MEK and PKB all suppress adhesion in the absence of c-erbB2 signalling when transfected into HB2/tnz34 cells (Fig. 6A), both ILK and S6 kinase are unable to do so ([11] and our unpublished results), suggesting a secondary role for these kinases in integrin inactivation. One may speculate that the common properties of ILK and S6K reflect their presence on a common pathway. Indeed, Tan et al. [28] have found a connection between ILK and mTOR signalling in the induction of HIF-1$\alpha$; however, this connection was PKB-dependent while we have strong evidence against both ILK activation of PKB (Fig. 2B) and PKB activation of S6K [11] in HB2/tnz34 cells.

*c-erbB2 signalling causes radical and time-dependent rearrangements in the actin cytoskeleton*

c-erbB2 signalling for 1 h caused a very striking remodelling of the actin cytoskeleton in HB2/tnz34 cells, leading to a complete loss of detectable stress fiber structures as well as disorganisation of the cortical fibers (Fig. 3A). After 6 h of c-erbB2 signalling and persisting at least to 16 h, the morphology of F-actin structures was dramatically different, showing abundant stress fibers but virtually no cortical fibers. The importance of F-actin structure in integrin regulation was demonstrated by the latrunculin B-induced suppression and jasplakinolide-induced restoration of integrin function (Fig. 3C). However, the effect of latrunculin B cannot be equated with that of c-erbB2, since the cytoskeletal effect of the former but not the latter is sufficient for integrin inactivation. The distinct nature of the latrunculin B effect is also evident from the fact that it produces an additive effect on integrin function when combined with c-erbB2 signalling. Although the changes in stress fiber content were the most conspicuous events in the c-erbB2-induced cytoskeletal rearrangements, they are unlikely to be involved in integrin inhibition, which remained unchanged during both disassembly and delayed re-formation of stress fibers. Instead, integrin inhibition correlated better with disorganisation or loss of cortical fibers, as this effect was seen both in the early and the delayed phases of cytoskeletal remodelling.

*Initial c-erbB2-induced actin depolymerisation is dependent on PI3K and PKB and is required for integrin inhibition*

Using both inhibition and activation of PI3K and PKB, we could demonstrate the absolute dependence on these kinases of the initial cytoskeletal rearrangements (Fig. 7). The role of PKB in actin destabilisation has not been extensively studied. Qian et al. [29] found similar, but less pronounced changes upon ectopic activation of PKB in chick fibroblasts, a phenomenon that was sensitive to rapamycin treatment. In contrast, we found that c-erbB2-induced cytoskeletal rearrangements were insensitive to rapamycin (data not shown) and its target, the mTOR-S6K pathway, was previously shown not to be activated by PKB in HB2/tnz34 cells (although it is activated by c-erbB2 and required for integrin inactivation [11]). The p21-activated kinase PAK1 has also been reported to cause actin destabilisation [21] and to be activated by PKB [30]. However, preliminary results indicate that inhibition of PAK1 cannot restore adhesion or cytoskeletal structure in our system (S. Hedjazifar and D. Baeckström, unpublished data). PKB has also been found to interact directly with the actin cytoskeleton in an activation-dependent manner [31].

Since a strong connection between cytoskeletal integrity and integrin function has long been known, the extensive actin rearrangements mediated by PKB provided a plausible explanation to the striking reversal of c-erbB2-induced integrin inactivation previously observed upon inhibition of PKB [11]. Using the actin-stabilising drug jasplakinolide, we could confirm the dependence on F-actin depolymerisation both of c-erbB2-inuced inhibition of adhesion (Fig. 3C) and, specifically, of the PKB-mediated integrin-inhibitory pathway downstream of c-erbB2 (Fig. 6). The restoration of activation-specific epitope expression by dominant-negative PKB (Fig. 2A) in the presence of c-erbB2 signalling and by jasplakinolide under conditions of wild-type PKB over-expression (Fig. 6) clearly indicates that the PKB-induced cytoskeletal changes affect adhesion in a manner dependent on conformational integrin inactivation.

Our results also have important implications for the role of ILK in c-erbB2-induced integrin inactivation. A number of biochemical studies have implicated ILK as a molecule that activates PKB by (directly or indirectly) causing phosphorylation of the regulatory PKB site Ser-473 [32–34]. This property of ILK can be reversed by mutations which are supposed to abrogate ILK's disputed kinase activity. In contrast, genetic studies in *Drosophila* and mice have strongly indicated that ILK is an essential adaptor protein, linking integrins to the cytoskeleton in a manner insensitive to the kinase-inactivating mutations [18,35]. The present results yield a picture of ILK that is distinct from both of the views described above. On one hand (and contrary to our previous assumptions [11]), the role of ILK in integrin inhibition is clearly not related to PKB activation, since two of the phenomena studied here, downregulation of integrin activation epitope expression and F-actin destabilisation, were dependent on PKB but not on ILK. Moreover, we could directly show that PKB phosphorylation was unaffected by inhibition or knock-down of ILK in HB2/tnz34 cells (Fig. 2B). On the other hand, the reportedly kinase-inactivating ILK mutants E359K and K220M have both previously been shown to efficiently reverse c-erbB2-induced suppression of adhesion [11]. One may speculate that ILK is a multifunctional molecule that performs several distinct cellular tasks, of which only the adaptor role is nonredundant. Further study of the ILK-dependent pathway in integrin inactivation is warranted.

*c-erbB2-induced activation of Rho causes delayed stress fiber assembly as well as rapid and sustained integrin inhibition*

The effects of Rho GTPase signalling downstream of c-erbB2 in HB2/tnz34 cells showed complex and intriguing features. On the cytoskeletal level, the initial PI3K/PKB-dependent phase of actin depolymerisation (measured after 1 h of c-erbB2 signalling) was unaffected by the activation or inhibition of Rho (Fig. 5A), while the subsequent and equally striking stress fiber assembly was absolutely dependent on Rho and ROK (Fig. 5C). The switch from PKB-dependent actin destabilisation to Rho-dependent stress fiber assembly correlated well with the respective kinetics of activation of PKB and Rho: PKB phosphor-

ylation decreased after 1 h of c-erbB2 signalling while Rho activation increased up to 6 h (Fig. 7C). Similarly delayed Rho activation has been observed in studies of opiate signalling in kidney cells [21], although, in that system, initial remodelling was PAK1- and not PKB-dependent. While our available data do not indicate cross-talk between Rho and other pathways involved in c-erbB2-induced integrin inactivation, the inability of transfected active Rho to induce the Rho-dependent stress fiber phenotype seen after 6 h of c-erbB2 signalling (compare Figs. 5Ae and Cb) suggests that additional signalling may be necessary to set the stage for subsequent Rho-mediated actin rearrangements. This nature of this possible interplay deserves further study.

On the adhesive level, the Rho-ROK pathway was required for integrin inhibition after both 1 and 6 h of c-erbB2 signalling, suggesting that the contribution of Rho to the anti-adhesive effects of c-erbB2 is independent of its capacity to cause cytoskeletal rearrangements. This conclusion is strengthened by the inability of jasplakinolide to inhibit Rho-dependent integrin inactivation (Fig. 6). It is also noteworthy that the observed capacity of signalling from Rho to cause integrin conformational rearrangement is contrary to the general consensus [36,37] that Rho-mediated integrin regulation does not influence integrins on the level of affinity. Again, we stress that other mechanisms of integrin regulation, such as changes in post-ligation clustering and cytoskeletal attachment, are likely to be vital to the events studied here; however, the conclusions that Rho signalling can contribute to integrin conformational changes and that these changes are of importance in modulating integrin adhesive function seem difficult to escape.

Taken together, our present knowledge about the signalling requirements for c-erbB2-induced integrin inactivation during its initial phase can be expressed as shown in Fig. 8. The Rho-ROK, MEK-ERK and PI3K-PKB pathways all cooperate in achieving a conformational rearrangement in $\beta_1$ integrins that is unfavourable for ligand binding. Of these, only the contribution by the PI3K-PKB pathway is mediated by cytoskeletal rearrangements. In addition, other events promoted by ILK and the mTOR/S6K pathway (interdependently or in parallel) contribute to integrin inhibition without causing conformational inactivation, perhaps by maintaining the inactive state. This scheme highlights the complexity of integrin regulation induced by "natural" stimuli such as growth factor receptor signalling and contrasts sharply with the apparently straightforward effects obtained when overexpressing or overstimulating a single downstream signalling molecule. It has been a recurring theme in this and our previous study [11] that, while inhibition of only one of the several signalling mediators involved is sufficient to abrogate integrin inactivation induced by c-erbB2, transfection with the corresponding active/activated allele will often suppress adhesion in the absence of stimulation of all other pathways. Thus, on one hand, the inhibition experiments indicate that,
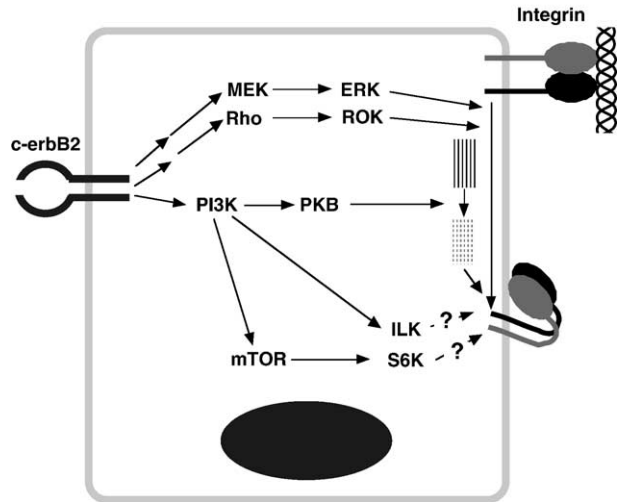


Fig. 8. Hypothetical scheme of the signalling pathways and cytoskeletal events required for c-erbB2-induced integrin inactivation, summarising the present results. c-erbB2 homodimer signalling elicits a number of signalling cascades which are necessary for integrin inactivation. MEK, Rho and PKB act by inducing an inactive integrin conformation (speculatively depicted as bending of the integrin extracellular domains). Of these, only the contribution from PKB requires (presumably cortical) F-actin destabilisation. The ILK- and mTOR/S6 kinase-dependent pathways mediate integrin inhibition without causing conformational inactivation.

among the signalling pathways implicated, each one is necessary but not sufficient for integrin inactivation. On the other hand, the overexpression experiments suggest that signalling from a single mediator may be sufficient (e.g., Rho or PKB). It should however be kept in mind that, when overexpressing active constructs, both the level and the duration of signalling from the pathway affected are elevated in a highly artificial manner. In accordance with this, we have observed that more physiological stimulation of individual pathways, e.g., of PI3K-PKB by insulin or of Rho by LPA, completely failed to induce effects on adhesion (our unpublished results). In light of these considerations, it seems reasonable to conclude that c-erbB2-induced integrin inactivation is governed by a complex signalling network, probably reflecting the complexity of interactions between integrins and the many regulatory proteins surrounding them.

We have previously stated that integrin regulation in epithelial cells is a very sparsely studied subject. This is unfortunate, since the vast majority of all human cancers arise from epithelia and modulation of integrin function is very likely to be important in carcinogenesis, having the potential to give the developing cancer cell new adhesive, migratory and survival properties. c-erbB2 has long been acknowledged as a major causative agent in the development of aggressive breast carcinoma. The emergence of c-erbB2-targeting therapeutic agents such as Herceptin, as well as the problems associated with such treatments in their present form, clearly emphasises the urgent need of a more profound understanding of c-erbB2-induced carcinogenesis. It is our hope that the knowledge gained in this study may

help to facilitate improved therapeutic and diagnostic approaches in the battle against breast cancer.

## Acknowledgments

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