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Extracellular Matrix Controls Insulin Signaling in Mammary Epithelial Cells Through the RhoA/Rok Pathway

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Cellular responses are determined by a number of signaling cues in the local microenvironment, such as growth factors and extracellular matrix (ECM). In cultures of mammary epithelial cells (MECs), functional differentiation requires at least two types of signal, lactogenic hormones (i.e., prolactin, insulin, and hydrocortisone) and the specialized ECM, basement membrane (BM). Our previous work has shown that ECM affects insulin signaling in mammary cells. Cell adhesion to BM promotes insulin-stimulated tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) and association of PI3K with IRS-1, whereas cells cultured on stromal ECM are inefficient in transducing these post-receptor events. Here we examine the mechanisms underlying ECM control of IRS phosphorylation. Compared to cells cultured on BM, cells on plastic exhibit higher level of RhoA activity. The amount and the activity of Rho kinase (Rok) associated with IRS-1 are greater in these cells, leading to serine phosphorylation of IRS-1. Expression of dominant negative RhoA and the application of Rok inhibitor Y27632 in cells cultured on plastic augment tyrosine phosphorylation of IRS-1. Conversely, expression of constitutively active RhoA in cells cultured on BM impedes insulin signaling. These data indicate that RhoA/Rok is involved in substratum-mediated regulation of insulin signaling in MECs, and under the conditions where proper adhesion to BM is missing, such as after wounding and during mammary gland involution, insulin-mediated cellular differentiation and survival would be defective.

Extracellular matrix (ECM) provides supportive, adhesive, and barrier functions for tissues, and also triggers specific signaling pathways and cytoskeleton reorganization. Various cellular responses, including proliferation, differentiation, migration, and survival are regulated by ECM. As these responses are also controlled by growth factors, cross-talk between ECM- and growth factor-activated signaling pathways has been proposed and documented (Giancotti and Tarone, 2003; Streuli and Akhtar, 2009). In this study, we explore possible mechanisms linking ECM to the insulin signaling pathway, using mammary epithelium as a model.

The breast is a glandular tissue composed of two types of epithelial unit: the collecting ducts, and the alveoli. The latter contain mammary epithelial cells (MECs) that adhere to a specialized ECM, the basement membrane (BM) in vivo. This interaction is necessary for the efficient activation of specific signaling pathways, for example those driven by prolactin and insulin, but not EGF (Edwards et al., 1998; Lee and Streuli, 1999; Wang et al., 2004). Recent findings have further revealed that β1 integrin is required for proper prolactin signaling, and Rac GTPase mediates this effect by decreasing the association of SHP-2 with Jak2 (Naylor et al., 2005; Akhtar and Streuli, 2006).

Culture studies have shown that insulin is critical for MEC differentiation and survival (Rosfjord and Dickson, 1999; Hadsell and Abdel-Fattah, 2001; Marshman and Streuli, 2002). Insulin initiates cascades of signaling via tyrosine phosphorylation of the insulin receptor (IR). The active receptor then recruits and phosphorylates Shc and the insulin receptor substrate (IRS), which contain multiple tyrosine residues and thus serve as docking proteins for downstream signaling molecules (Taniguchi et al., 2006). Several lines of evidence in a variety of cell systems have demonstrated that ECM regulates insulin/IGF-I signaling at different levels, thereby altering the intensity or duration of signaling.

First, ECM modulates the levels of insulin signaling components. In IR-overexpressing CHO-T cells, internalization of IR is decreased in cells adhered to galectin-8 compared to those cultured on fibronectin, collagen, or laminin (Boura-Halfon et al., 2003). IRS-1 expression is also modulated by adhesion; in DA2 fibroblasts this occurs at the transcriptional level, primarily through FAK (Lebrun et al., 2000). Second, integrins can regulate the phosphorylation of insulin/IGF-I signaling components by interacting with them. In 3T3 and Rat1 fibroblasts, adhesion promotes the association of αvβ3 integrin with IR, while in CHO or PC3 cells overexpressing β1 integrin, β1 integrin interacts with IGF-I receptor (IGF-IR) and IRS-1 in an IGF-I-dependent and independent manner, respectively (Schneller et al., 1997; Goel et al., 2004). In addition to αvβ3 and β1 integrin, α6 integrin

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associates with IGF-IR in embryonic lens cells in vivo (Walker et al., 2002). Interestingly, in T47D cells or MDA-MB-435 cells overexpressing β4 integrin, engagement of α6β4 integrin itself induces tyrosine phosphorylation of IRS-1 (Shaw, 2001). Third, tyrosine kinases downstream of integrin may be involved in insulin signaling, since FAK directly associates with IRS-1 in 293 cells overexpressing FAK, and transient expression of FAK together with Src restores insulin-induced tyrosine phosphorylation of IR and IRS-1 in suspended 3T3 cells that overexpress IR (Lebrun et al., 1998; El Annabi et al., 2001). Finally, ECM can prevent the dephosphorylation of insulin/IGF-I signaling components. In smooth muscle cells, αvβ3 integrin prolongs tyrosine phosphorylation of IGF-IR through a control on the rate of SHP-2 recruitment to the activated receptor (Maile and Clemmons, 2002). In pancreatic adenocarcinoma cell lines, MIA PaCa-2 and PANC-1, cell adhesion to fibronectin transactivates IGF-IR by preventing SHP-2 from dephosphorylating IGF-IR (Edderkaoui et al., 2007).

Many of these studies are restricted to overexpression analysis in a variety of cell and cancer lines, and no work has approached the mechanism of this cross-talk in normal epithelia. We have demonstrated that in primary cultures of normal MECs, insulin signaling propagates more effectively in cells plated on BM than in those on a stromal ECM represented by native collagen I or on tissue culture plastic (where cells adhere to fibronectin and vitronectin deposited from serum) (Farrelly et al., 1999; Lee and Streuli, 1999). Greater extents of IRS-1 tyrosine phosphorylation and IRS-1/PI3K association take place in cells cultured on BM; however, levels of tyrosine phosphorylation of IR are comparable in cells on both substrata. We have therefore argued that ECM modulates insulin signaling downstream of the receptor at the level of IRS-1 tyrosine phosphorylation.

In this study we have explored the underlying mechanisms of how ECM regulates insulin signaling, with an emphasis on the RhoA/Rho kinase (Rok) pathway and protein tyrosine phosphatases (PTPs), using primary cultures of MECs isolated directly from mammary gland.

Materials and Methods

Reagents

Murine EGF, bovine insulin, and hydrocortisone were purchased from Sigma (St. Louis, MO). Antibody to Rok was obtained from Becton Dickinson (Bedford, MA). Antibody to phospho-IRS-1 (Ser636/639) was from Cell Signaling (Beverly, MA). Antibodies to IR, RhoA, SHP-2, hemagglutinin (HA) and myosin phosphatase target subunit 1 (MYPT1) were from Santa Cruz Biotechnology (Santa Cruz, CA). Rho activation assay kit, recombinant MYPT1 (654–880) and antibodies to phosphotyrosine (clone 4G10), IRS-1 and phospho-MYPT1 were from Upstate Biotechnology (Lake Placid, NY). Y27632 was obtained from Calbiochem (San Diego, CA).

Substrata and cell culture

Reconstituted BM matrix, Matrigel, was purchased from Becton Dickinson and coated onto dishes at 7 mg/ml. All experiments were performed with first or second passage MECs derived from mid-pregnant ICR mice. Primary epithelial cultures were prepared from isolated mammary alveoli and cultured in nutrient mixture F-12 (Sigma) containing 10% fetal calf serum (Hyclone, Logan, UT), 1 mg/ml fetuin (Sigma), 5 ng/ml EGF and 1 μg/ml hydrocortisone. The cells were plated onto Matrigel, serum-treated tissue culture plastic dishes (where the cells adhere to fibronectin and virtonectin), or 2 mg/ml collagen I renatured from HAc-dissolved rat tail collagen fibrils. After 72 h, cells were serum-starved 6–8 h in Dulbecco's modified Eagle's medium/nutrient mixture F-12 (Invitrogen, Grand Island, NY) containing hydrocortisone, and then subjected to various treatments.

Adenovirus infection

Recombinant adenovirus carrying HA-tagged dominant negative RhoA (N19RhoA) and constitutively active RhoA (L63RhoA) were generated as previously described (Ming et al., 2002). For cells cultured on plastic, recombinant adenovirus was added to cells directly; whereas for those cultured on BM, cells were trypsinized, infected in suspension at 37°C for 1 h, and then plated onto Matrigel-coated dishes (Watkin and Streuli, 2002). After incubated with adenovirus for 18–24 h, cells were serum-starved for 6–8 h, and stimulated with insulin (100 nM) for 15 min.

Immunoprecipitation and Western blot analysis

Untreated or growth factor-treated cells were lysed in lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1 mM Na_3VO_4, 10 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100. Normalization of protein was confirmed by SDS–PAGE, followed by Coomassie Blue staining. Cell lysates containing equal amounts of protein were incubated with 1–2 μg of antibody and 20–50 μl of protein A-Sepharose beads (Zymed Laboratories, Inc., South San Francisco, CA) overnight at 4°C. Immunoprecipitates or whole cell lysates were subjected to SDS–PAGE, transferred to PVDF membrane (NEN), and probed with antibodies to anti-phosphotyrosine (4G10; 1 μg/ml), IR (1 μg/ml), IRS-1 (1 μg/ml), phospho-IRS-1 (Ser636/639) (1:1,000), RhoA (2 μg/ml), HA (1 μg/ml), Rok (1 μg/ml), phospho-MYPT1 (2 μg/ml), MYPT1 (1 μg/ml), and SHP-2 (1 μg/ml). Proteins were visualized using an ECL kit (Cell Signaling). In each of the studies presented, the results shown are typical of three independent experiments.

Rho activity assay

Measurement of RhoA activation was performed according to manufacturer's instructions (Upstate Biotechnology). Briefly, cleared cell lysates were incubated for 1 h at 4°C with GST-Rhotekin Rho-binding domain bound to glutathione-agarose beads to precipitate GTP-bound Rho. Total lysates and precipitates were analyzed by Western blotting using antibody to RhoA.

Rok activity assay

IRS-1 associated Rok activity was measured according to manufacturer's instructions (Upstate Biotechnology) with minor modifications. Briefly, IRS-1 immunoprecipitates were incubated with 500 ng recombinant MYPT1 (654–880) and 200 μM ATP (Cell Signaling) in kinase buffer (Cell Signaling) for 30 min at 30°C. The reaction was stopped by adding Laemmli sample buffer, and then boiled for 5 min. Phosphorylation of MYPT1 was assessed by immunoblotting using antibody to phospho-MYPT1.

In-gel PTP assay

Experimental procedures followed the protocol reported by Burridge and Nelson (1995). Briefly, 1 mg of poly(glutamate/tyrosine) (Sigma) was incubated with 500–1,000 U of v-abl kinase (Calbiochem) and 500 μCi of [γ-^{32}P]ATP in 0.5 ml kinase buffer (30 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM Na_3VO_4, 0.2–1 mM ATP, 10 mM dithiothreitol, 0.05% Triton X-100, 50 mM imidazole, pH 7.2) at room temperature for 18 h, and the reaction was terminated by addition of an equal volume of 20% trichloracetic acid. After 30 min on ice, the precipitated poly(glu/tyr) were sedimentated at 12,000*g* for 10 min at 4°C. The pellet was dissolved in 2 M Tris-base, passed over a G50 Sephadex column equilibrated in 50 mM imidazole, pH 7.2, and collected in 0.5 ml fractions. ^{32}P-labeled substrate was added into the regular polyacrylamide gel mix prior to polymerization at $\sim 10^5$ cpm/ml. Samples were separated by SDS–PAGE. Following electrophoresis,

gels were first incubated in 50 mM Tris–HCl, pH 8.0, containing 20% isopropanol overnight to remove the SDS. Gels were then washed twice in 50 mM Tris–HCl, pH 8.0, containing 0.3% β-mercaptoethanol, and denatured by incubating with the same buffer containing 6 M guanidine hydrochloride and 1 mM EDTA for 90 min. The gels were then incubated three times in renaturation buffer (1 mM EDTA, 50 mM Tris–HCl, pH 8.0, 0.3% β-mercaptoethanol, and 0.04% Tween-40). The gels were finally incubated overnight in renaturation buffer containing 4 mM dithiothreitol. Following this incubation, the gels were stained with Coomassie brilliant blue, destained, and dried for autoradiography. The clear bands in a black background represent where PTPs localize.

Results

MECs cultured on plastic exhibit higher level of RhoA activity

Our previous work demonstrated that the nature of the substratum that MECs adhere to affects insulin signaling downstream of its receptor, at the level of IRS-1 tyrosine phosphorylation (Farrelly et al., 1999; Lee and Streuli, 1999). The aim of this study was to further identify the mechanism for differential IRS activation in MECs grown on two substrata, tissue culture plastic where cells form monolayers and are poorly responsive to insulin, and BM where cells form three-dimensional acini and show robust insulin-activated signaling.

One approach to delineate the mechanism is to find out the differences in signaling pathways triggered by cell adhesion to plastic and BM, and then evaluates the involvement of these pathways in modulating insulin signaling. Among the pathways known to be activated by cell adhesion, we were particularly interested in Rho since its downstream effector Rok has been shown to regulate insulin signaling (Farah et al., 1998; Begum et al., 2002; Furukawa et al., 2005; Kanda et al., 2006; Lim et al., 2007). We found that MECs cultured on plastic displayed higher RhoA activity than those cultured on BM (Fig. 1). In addition, when cells were cultured in monolayers on collagen I, there was a similar level of RhoA activity as in cells cultures on plastic (data not shown).

This result shows that activation of RhoA is differentially regulated by cell adhesion to different substrata.

RhoA activity is detrimental for insulin signaling in MECs

We then went on to examine whether RhoA accounted for the defectiveness of insulin signaling in MECs cultured on plastic. Lowering RhoA activity by expressing dominant negative RhoA (N19RhoA) augmented insulin-induced IRS-1 tyrosine phosphorylation by up to 1.8-fold (Fig. 2B, lane 4 vs. lane 2, and Fig. 2C). In contrast, expression of constitutively active RhoA (L63RhoA) inhibited IRS-1 tyrosine phosphorylation by 52% in MECs cultured on BM (Fig. 2E, lane 4 vs. lane 2, and Fig. 2F). Both treatments had marginal effects on IR tyrosine phosphorylation, but these were not statistically significant (Fig. 2A,C,D,F).

Collectively, these data suggest that culture on plastic, a substratum that is not suitable for optimal insulin signaling, activates RhoA, which in turn blocks insulin signaling in MECs.

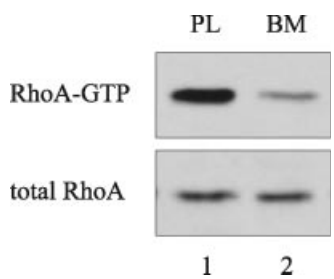

Fig. 1. MECs cultured on plastic exhibit higher RhoA activity. MECs were cultured on plastic (PL) or BM. Cell lysates were incubated with GST-Rhotekin bound to glutathione-agarose beads to precipitate GTP-bound Rho. Total lysates and precipitates were then analyzed by immunoblotting using antibody to RhoA.

Rok binds and phosphorylates IRS-1 in cells cultured on plastic

A number of downstream effectors are activated by RhoA, including Rok. Rok is a serine/threonine kinase and is involved in several cellular responses, such as stress fiber formation, actinomyosin contraction, cell–substratum adhesion, cell–cell interaction, migration, cytokinesis, apoptosis, and gene expression (Riento and Ridley, 2003). One notable function of Rok is to regulate insulin/IGF-I signaling. It has been shown to bind and phosphorylate IRS-1, thereby suppressing tyrosine phosphorylation of IRS-1 (Farah et al., 1998; Begum et al., 2002; Lim et al., 2007). Based on these findings, we examined the extent of association of Rok with IRS-1 in MECs cultured on different substrata. A higher amount of Rok was co-immunoprecipitated with IRS-1 in cells cultured on plastic (Fig. 3A), coinciding with greater Rok activity associated with IRS-1 in these cells (Fig. 3B). The IRS-1-associated Rok activity in cells cultured on plastic was 3.6-fold more than that in cells cultured on BM (Fig. 3C).

Since Rok bound to IRS-1 might directly phosphorylate IRS-1, we then measured IRS-1 phosphorylation at serine residues 307, 612, and 636/639 by immunoblotting. Serine phosphorylation of IRS-1 at residues 307 and 612 was not detected in MECs regardless the substratum. However, the level of serine phosphorylation at residues 636/639 was higher in cells cultured on plastic (Fig. 4A), and this was diminished by the treatment of Y27632, a Rok inhibitor (Fig. 4B).

Our data thus suggest that higher RhoA activity in MECs cultured on plastic leads to greater association of Rok with IRS-1, resulting in phosphorylation at serine residues.

Inhibition of Rok activity by Y27632 increases tyrosine phosphorylation of IRS-1 in MECs cultured on plastic

Serine phosphorylation of IRS-1 exerts a negative impact on IRS-1 tyrosine phosphorylation. Various serine/threonine kinases phosphorylate IRS-1, including PKC, Erk, JNK, IKKβ, and Rok, as well as downstream effectors in the insulin-stimulated PI3K pathway, comprising GSK-3, mTOR, and p70S6K (reviewed by Zick, 2001; Johnston et al., 2003; Taniguchi et al., 2006). Given that greater extent of Rok binds and phosphorylates IRS-1 in MECs cultured on plastic (Figs. 3 and 4), we examined whether inhibition of Rok activity by Y27632 could ameliorate insulin-induced tyrosine phosphorylation of IRS-1. Indeed, inclusion of Y27632 enhanced level of tyrosine phosphorylation of IRS-1 in response to insulin by twofold in these cells (Fig. 5B, lane 3 vs. lane 1), but did not affect tyrosine phosphorylation of IR (Fig. 5A). For cells cultured on BM, no significant effect of Y27632 on insulin signaling was observed (Fig. 5C,D), presumably because it was already maximal.

Taken together, our results suggest that the inefficiency of insulin signaling in MECs cultured on plastic is due to, at least in part, over-activation of RhoA/Rok pathway, leading to IRS-1 serine phosphorylation and thus disruption of insulin signaling propagation downstream of IR. In contrast, MECs cultured on BM exhibit low level of RhoA, thereby acquiring full capacity of insulin signaling.

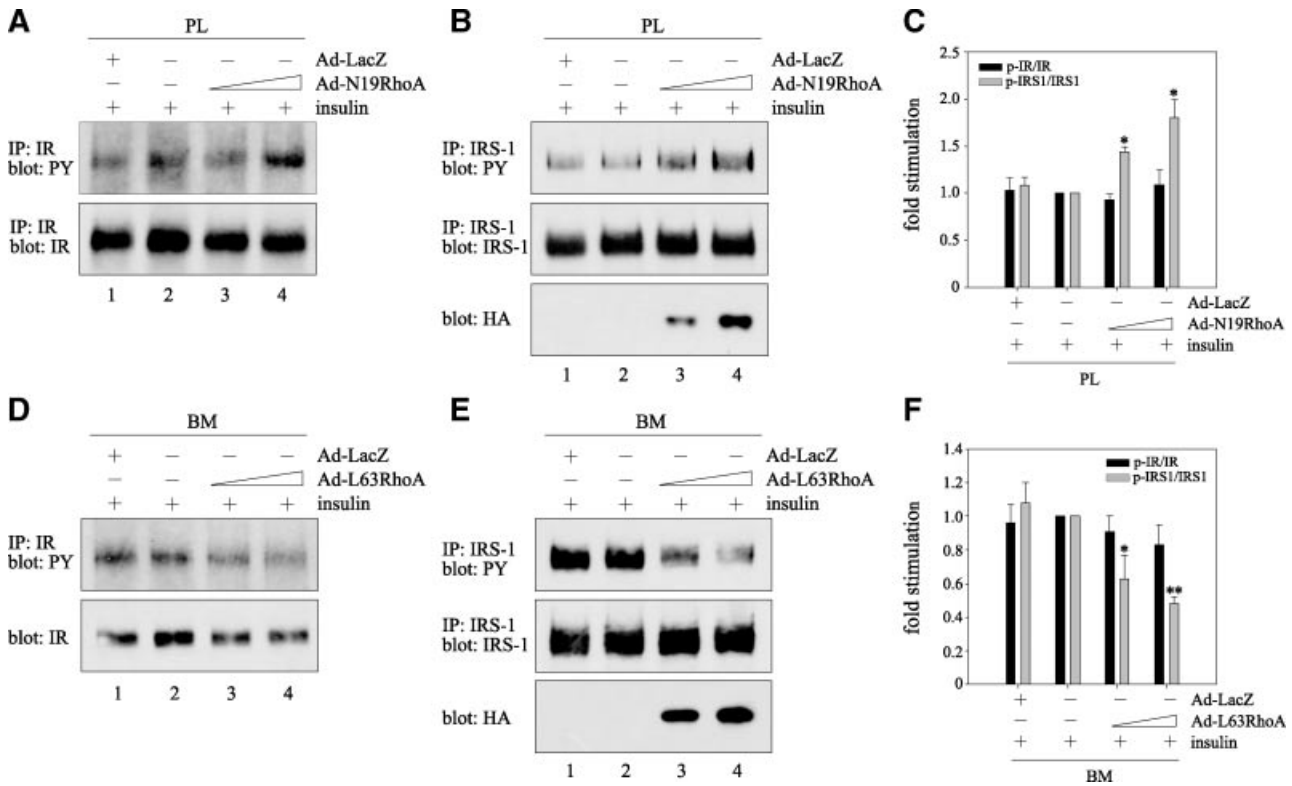

Fig. 2. Expression of dominant negative RhoA improves insulin signaling in MECs cultured on plastic, whereas expression of constitutively active RhoA inhibits insulin signaling in MECs cultured on BM. MECs cultured on plastic were infected with adenovirus carrying dominant negative RhoA (Ad-N19RhoA) or LacZ in situ (A,B). MECs cultured on BM were trypsinized, infected with adenovirus carrying constitutively active RhoA (Ad-L63RhoA) or LacZ, and replated on BM (D,E). After 24 h, cells were serum-starved for 8 h and stimulated with 100 nM insulin for 15 min. Cell lysates were immunoprecipitated (IP) with antibody to IR (A,D) or IRS-1 (B,E), followed by immunoblotting with anti-phosphotyrosine antibody (PY). Blots were stripped and reprobed with the appropriate precipitating antibodies. Expression of HA was monitored by immunoblotting. C,F: Quantification of the effect of N19RhoA (C) and L63RhoA (F) on insulin signaling. Immunoblots from three independent experiments were analyzed by densitometry, and data are expressed as fold stimulation with respect to mock-infected cells. *$P < 0.05$; **$P < 0.01$.

PTPs association with IR and IRS-1

Our previous work revealed that the PTP inhibitor, vanadate, elevates the tyrosine phosphorylation of IRS-1 in MECs cultured on collagen I or plastic, although the mechanism was not determined (Lee and Streuli, 1999). Never-the-less, these data suggest that an additional mechanism for the ECM-mediated modulation of IRS-1 tyrosine phosphorylation

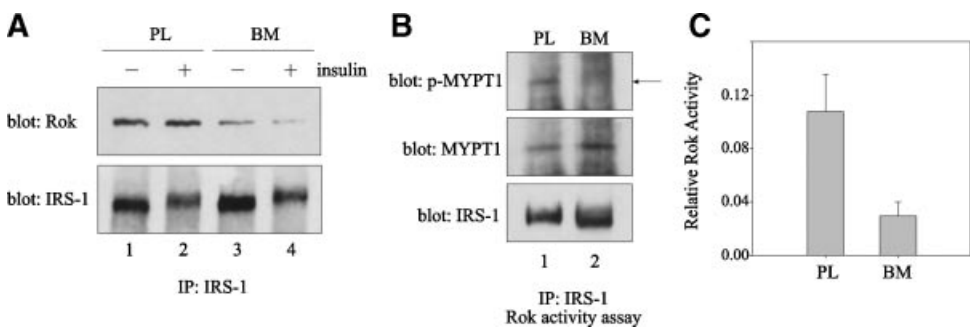

Fig. 3. The amount and activity of Rok associated with IRS-1 are higher in MECs cultured on plastic. A: MECs cultured on plastic (PL) or BM were incubated in the absence or presence of 100 nM insulin for 15 min. Cell lysates were immunoprecipitated (IP) with antibody to IRS-1, followed by immunoblotting with anti-Rok antibody. The blot was then stripped and reprobed with antibody to IRS-1. B,C: Cell lysates were immunoprecipitated with antibody to IRS-1 followed by Rok activity assay using recombinant MYPT1 as a substrate. The extent of MYPT1 phosphorylation was monitored by immunoblotting with anti-phospho-MYPT1 antibody. The blot was then stripped and repobed with antibody to MYPT1. IRS-1 level in immunoprecipitates was also monitored by immunoblotting. C: Immunoblots of p-MYPT1 and IRS-1 were quantified by densitometric analysis. Relative Rok activity associated with IRS-1 was calculated by dividing value of intensity of p-MYPT1 by that of IRS-1. Data are expressed as mean ± SE from three separate experiments.

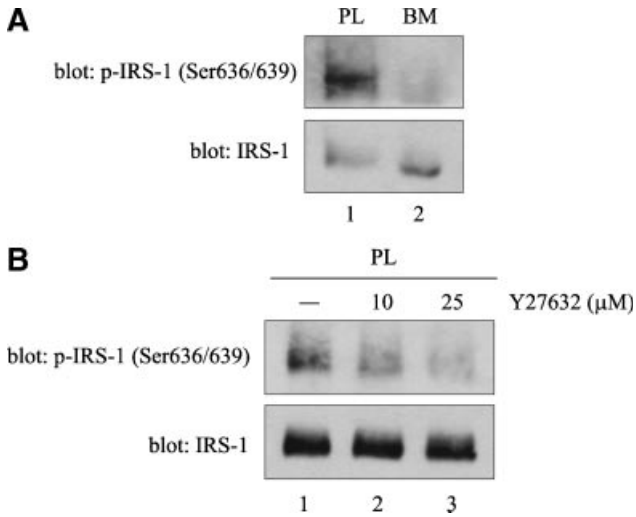

Fig. 4. MECs cultured on plastic exhibit higher extent of IRS-1 serine phosphorylation, and this can be inhibited by Y27632. A: Total cell lysates obtained from MECs cultured on plastic (PL) and BM were analyzed by immunoblotting with antibodies to phospho-IRS-1 (Ser636/639) and IRS-1. B: MECs cultured on PL were pretreated without or with 10 or 25 μM Y27632 for 1 h, and cell lysates were analyzed by immunoblotting with antibodies to phospho-IRS-1 (Ser636/639) and IRS-1.

may involve a PTP. To search for possible PTPs associated with IRS-1 or the receptor complex, an in-gel PTP assay was performed (Burridge and Nelson, 1995). Phosphorylated poly(glutamate–tyrosine) was incorporated into acrylamide gels, and samples were separated by SDS–PAGE. Following electrophoresis, gels were subjected to denaturation and then renaturation, and PTP activity was detected by autoradiography. The clear bands in a black background represent where PTPs localize. This method does not resolve membrane-associated PTPs, but is useful for possible identification of soluble PTPs.

The overall profile of PTPs from total cell lysates using the in-gel PTP assay was initially obtained. Similar patterns but different intensities of PTP were observed in MECs cultured on the two different substrata (Fig. 6). PTPs with a molecular weight of ~130 and ~65 kDa were predominant in cells cultured on plastic (asterisk), whereas those at the size of ~35 and ~40 kDa were detected mainly in cells cultured on BM (circle). Insulin stimulation did not alter the PTP profile. This result suggests that cell–ECM interaction may affect PTP expression or activity in MECs.

We then examined the possibility that adhesion to different substrata influences the association of PTPs with IRS-1 or IR. Thus, IR and IRS-1 immunoprecipitates were subjected to the in-gel PTP assay. Multiple PTPs coprecipitated with IR, but their levels did not change in response to insulin or the substratum (Fig. 7A). Notably, the migration profile of these PTPs on SDS gels is distinct to that of the overall PTP profile (compare Figs. 6 and 7), indicating that they represent a set of PTPs that associate specifically with IR. By contrast, insulin stimulated the association of a ~70 kDa PTP with IRS-1 (Fig. 7B). This occurred

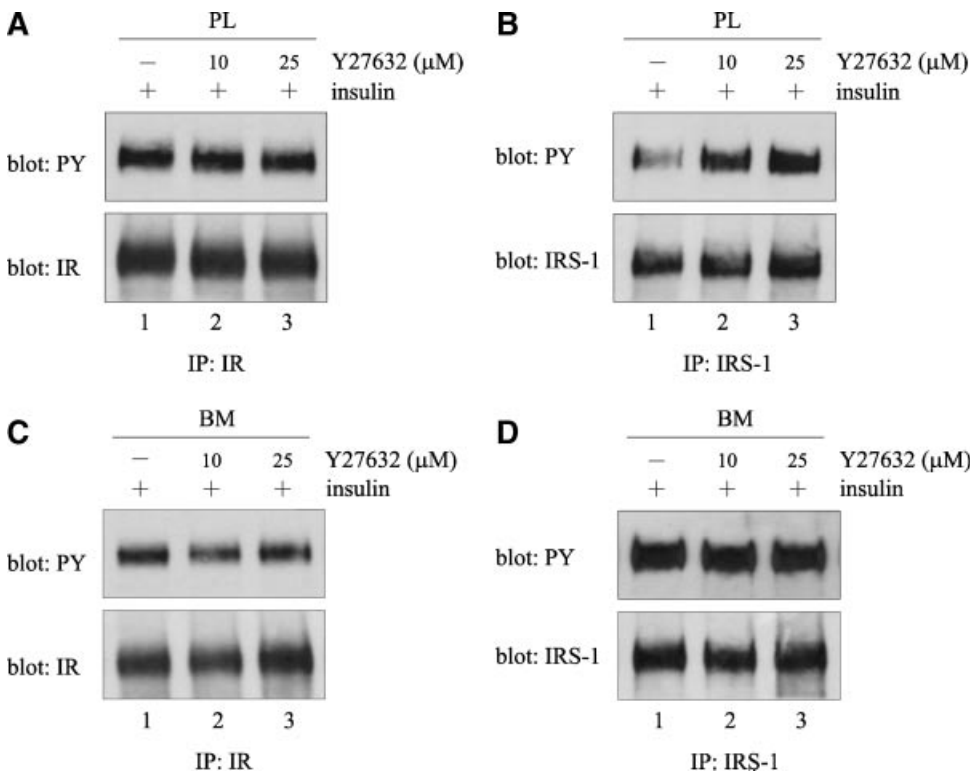

Fig. 5. Y27632 enhances insulin signaling in MECs cultured on plastic. MECs cultured on plastic (PL) (A,B) or BM (C,D) were pretreated without or with 10 or 25 μM Y27632 for 1 h, and then incubated with 100 nM insulin for 15 min. Cell lysates were immunoprecipitated (IP) with antibody to IR (A,C) or IRS-1 (B,D), followed by immunoblotting with anti-phosphotyrosine antibody (PY). Blots were stripped and reprobed with the appropriate precipitating antibodies.

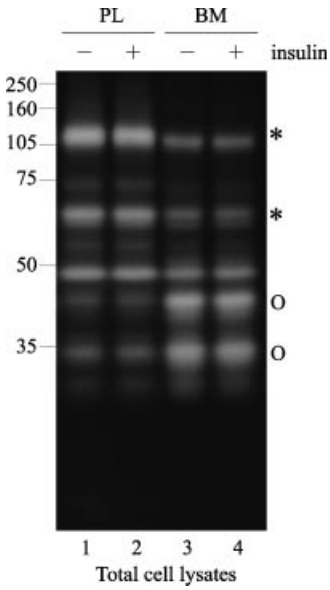

Fig. 6. The overall PTP expression profile is altered according to the substratum that MECs are cultured on. MECs cultured on plastic (PL) or BM were incubated in the absence and presence of 100 nM insulin for 15 min. Total cell lysates were subjected to in-gel PTP assay. PTPs predominantly expressed in MECs cultured on plastic and BM are denoted by * and °, respectively.

in cells on both substrata, but the presence of this band was greater in cells cultured on BM (Fig. 7B, lane 2 vs. lane 4). This PTP may correspond to SHP-2 since it migrates at approximately the same size as the major band from SHP-2 immunoprecipitates (data not shown). To confirm this, immunoprecipitation of IRS-1 followed by immunoblotting with SHP-2 antibody was carried out. In agreement with Figure 7B, a higher level of SHP-2 was associated with IRS-1 upon insulin stimulation in cells cultured on BM (Fig. 7C).

The recruitment of SHP-2 to IRS-1 requires IRS-1 to be tyrosine phosphorylated first. We therefore hypothesized that more SHP-2 binds to IRS-1 in MECs cultured on BM because of its higher level of tyrosine phosphorylation.

To test this possibility, we determined the effect of RhoA/Rok on insulin-induced recruitment of SHP-2 to IRS-1. Overexpression of constitutively active RhoA in MECs cultured on BM resulted in a decline in both IRS-1 tyrosine phosphorylation and the extent of IRS-1/SHP-2 association (Fig. 8A). The converse experiment of including Y27632 in MECs cultured on plastic caused elevation of both tyrosine phosphorylation and SHP-2 binding (Fig. 8B). These results suggest that the recruitment of SHP-2 to IRS-1 is not an upstream event controlling IRS-1 tyrosine phosphorylation, but rather it is secondary to IRS-1 tyrosine phosphorylation.

Thus, Rho/Rok exerts a negative effect on insulin signaling via a mechanism that does not involve SHP-2. Here we infer that SHP-2 might have a positive role in insulin signaling as reported by others (Maegawa et al., 1999).

Discussion

In MECs, insulin potentiates prolactin-induced milk protein gene expression and promotes cell survival (Farrelly et al., 1999; Lee and Streuli, 1999). These effects are apparent in cells cultured on BM, a substratum that is permissive for insulin signaling, but not in cells cultured on plastic dishes. We previously discovered that ECM regulates insulin signaling at the level of IRS-1 tyrosine phosphorylation, and here we explore the mechanism of IRS regulation (Lee and Streuli, 1999). We demonstrate that cell adhesion to plastic activates RhoA and the downstream effector Rok, which then binds and phosphorylates IRS-1 on serine residues, leading to inhibition of IRS-1 tyrosine phosphorylation.

This differential activation of insulin signaling according to ECM contact is conceptually important because it explains in part why MECs survive and differentiate on BM, but are unable to do so when cultured on stromal ECM. Moreover, it has

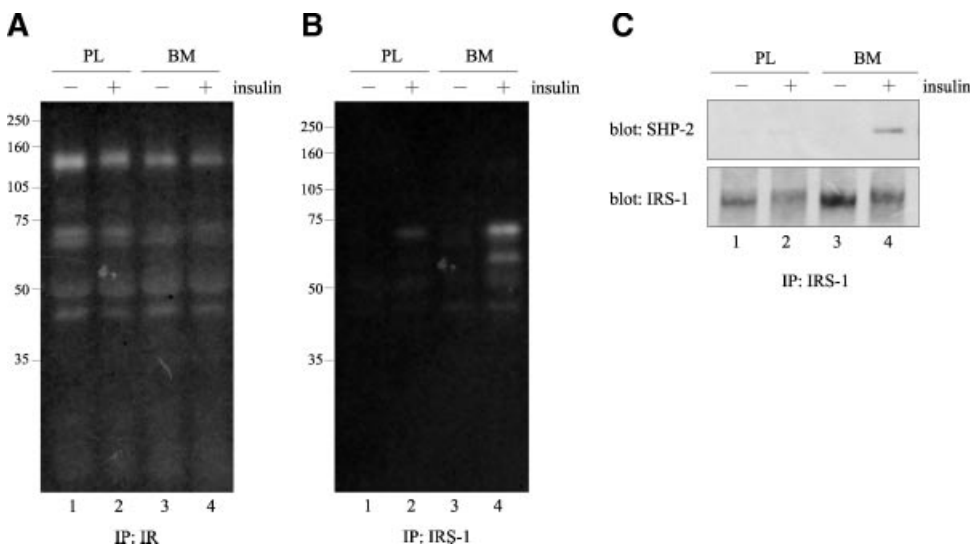

Fig. 7. PTP association with IR and IRS-1. MECs cultured on plastic (PL) or BM were incubated in the absence or presence of 100 nM insulin for 15 min. A,B: Total cell lysates were immunoprecipitated (IP) with antibody to IR (A) or IRS-1 (B), followed by in-gel PTP assay. C: Total cell lysates were immunoprecipitated with anti-IRS-1 antibody followed by immunoblotting with antibody to SHP-2. The blot was then stripped and reprobed with antibody to IRS-1.

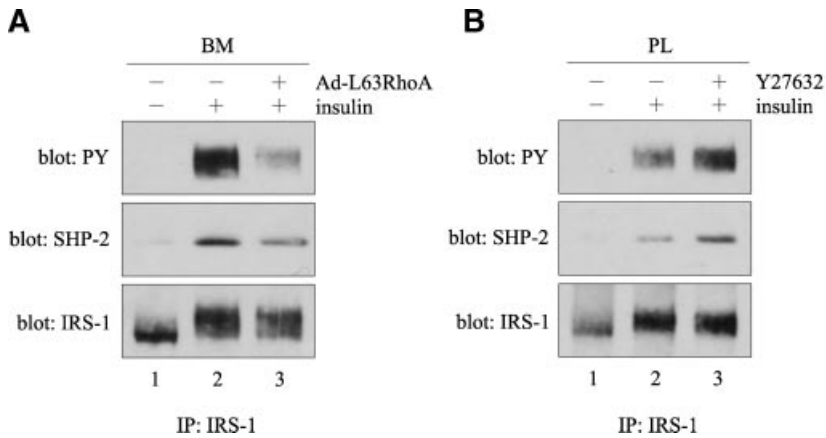

Fig. 8. Insulin-induced recruitment of SHP-2 to IRS-1 is repressed upon overexpressing constitutively active RhoA, but is augmented by Y27632 treatment. A: MECs cultured on BM were trypsinized, mock-infected or infected with adenovirus carrying constitutively active RhoA (Ad-L63RhoA), and replated on BM. After 24 h, cells were serum-starved for 8 h and stimulated with 100 nM insulin for 15 min. B: MECs cultured on plastic (PL) were serum-starved for 8 h, pretreated without or with 15 μM Y27632 for 1 h, and then incubated with 100 nM insulin for 15 min. Total cell lysates were immunoprecipitated (IP) with antibody to IRS-1, followed by immunoblotting with anti-phosphotyrosine antibody (PY) and SHP-2. Blots were then stripped and reprobed with antibody to IRS-1.

developmental importance because if MECs, which normally contact BM in vivo, migrate into another ECM environment such as the subtending stroma, they undergo apoptosis. This is a key mechanism to maintain epithelial positioning within mature tissues and provides a strong selection to prevent inappropriate migration in malignancy (Streuli, 2006).

RhoA is activated in culls cultured on plastic

In our study, all experiments were performed in cells cultured on plastic or BM for at least 3 days. A striking difference between these cells is their morphology. MECs on plastic form a monolayer, whereas those on BM adopt an alveolar structure. Thus, it is conceivable that biochemical signals elicited by cell adhesion to these substrata are different. Indeed, our results indicate that RhoA is predominantly activated in cells cultured on plastic. Since MECs on BM still maintain the differentiated phenotype and survive but cells cultured on plastic do not, our result is consistent with previous observations that laminin-induced cell differentiation is accompanied by downregulation of RhoA activity (Gout et al., 2001; Beqaj et al., 2002).

Another possibility for the differential activation of RhoA is the rigidity of the substratum. T47D breast epithelial cells cultured on plastic or 2D collagen exhibit higher level of RhoA than those on a 3D floating collagen gel (Wozniak et al., 2003). BM, like the 3D floating collagen gel, is pliable compared to the rigid surface of plastic dishes, and it has been proposed that cells exposed to malleable conditions do not need much RhoA activity to exert internal contractility because the opposing tension generated from cell adhesion is weaker (Olson, 2004). In addition to the composition and flexibility of the substratum, cell–cell interactions also influence RhoA activity, and vice versa. Cadherin engagement inhibits RhoA via tyrosine phosphorylation of p190RhoGAP (Noren et al., 2003). Conversely, constitutive activation of RhoA disrupts tight junction and adherens junction in rat mammary epithelial tumor cells (Rubenstein et al., 2003). Therefore, cells cultured on BM have lower degree of RhoA activity since MECs require proper cell–cell junctions to achieve alveolar morphology and differentiation.

Thus a unifying explanation for the inefficient insulin signaling in MECs cultured in the conventional way (i.e., on tissue culture plastic) is that increased tension generated by adhesion to a rigid surface, leads to RhoA activation and the consequent Rok-induced inhibition of insulin signaling. Although such conditions do promote stress fiber formation and motility, which are important mediators of the wound-healing response, the same environment is not conducive for cellular differentiation and not ideal for epithelial cell survival.

Rok is activated in cells cultured on plastic and causes serine phosphorylation of IRS-1

Serine phosphorylation of IRS plays an important role in the control of insulin signaling. IRS phosphorylation mediated by PKB and 5′-AMP-activated protein kinase (AMPK) is beneficial to insulin signaling, whereas the effect of PKC, Erk, JNK, IKKβ, GSK-3, mTOR, p70S6K, and Rok is detrimental (reviewed by Zick, 2001; Johnston et al., 2003; Taniguchi et al., 2006). Regarding the latter, several mechanisms have been elucidated. Serine phosphorylation of IRS can cause dissociation of IRS from IR, suppression of IRS-1 tyrosine phosphorylation, alteration of the subcellular location of IRS-1, augmentation of IRS degradation and inhibition of IR tyrosine kinase activity. All of these events lead to diminishment of insulin signaling. Here we found that MECs cultured on plastic exhibited higher extent of IRS-1 serine phosphorylation (Fig. 4A). This might hinder the association of IRS-1 with IR since we have observed that IR and IGF-IR form hybrid receptors in MECs, but the recruitment of IRS-1 to the receptor complex in response to insulin is impaired in cells cultured on plastic (data not shown). Conceivably, downregulation of IRS-1 tyrosine phosphorylation would thus take place (Lee and Streuli, 1999).

Among the serine/threonine kinases involved in IRS-1 serine phosphorylation, Rok, a downstream effector of Rho (Riento and Ridley, 2003), is emerging as an important regulator of insulin/IGF-1 signaling. A *Xenopus* homolog of Rok binds to the PTB domain of IRS-1, and microinjection of the mRNA corresponding to the C-terminus of Rok inhibits insulin-induced oocyte maturation (Farah et al., 1998). In vascular smooth muscle cells, Rok activation in response to thrombin results in an increase in its association with IRS-1, parallel with the enhancement of IRS-1 serine phosphorylation and inhibition of insulin signaling (Begum et al., 2002). The role

of Rok in insulin resistance has also been documented in several reports. Compared to the lean rats, RhoA/Rok activity and level of IRS-1 seine phosphorylation are elevated in obese rats, coinciding with the reduction of insulin-induced signaling and responses (Kanda et al., 2006). Similar effects of Rok were observed in vascular endothelial cells because C-reactive protein, a molecule related to insulin resistance, activates RhoA/Rok/JNK via spleen tyrosine kinase (Syk), rendering serine phosphorylation of IRS-1 and suppression of insulin responses (Xu et al., 2007). During muscle differentiation and adipogenesis mediated by insulin and IGF-I, a decline of RhoA/Rok activity occurs and inhibition of Rok facilitates differentiation. Likewise, upregulation of IRS-1 serine phosphorylation and downregulation of insulin/IGF-1 signaling are associated with higher Rok activity (Lim et al., 2007; Noguchi et al., 2007).

In concordance with these studies, we demonstrate here that RhoA/Rok is involved in substratum-mediated regulation of insulin signaling in MECs, and it exerts an adverse effect in cells that lack adherence to BM.

The Rho pathway and mammary differentiation

RhoGAP negatively regulates Rho activity. In p190-B RhoGAP-deficient mice, elevated RhoA activity results in Rok-mediated IRS-1 serine phosphorylation and the interruption of IGF-I signaling, thereby reducing cell and organ size as well as promoting adipogenesis rather than myogenesis from mesenchymal stem cells (Sordella et al., 2002, 2003). Defective embryonic mammary bud development and adult ductal morphogenesis, caused by decreased cell proliferation, have been observed in these mice, and these features are phenocopied in IGF-IR and IRS-1/2 knockout mice (Chakravarty et al., 2003; Heckman et al., 2007). However, in the mammary glands of p190-B overexpressing mice, ductal morphogenesis is impaired and hyperplastic lesions is induced, suggesting that a fine tuning of Rho activity is required for proper mammary gland development (Vargo-Gogola et al., 2006).

Collectively, these studies demonstrate the role of RhoGAP in development and differentiation through a control on IGF-I/insulin signaling. This is consistent with our and other observations that RhoA/Rok inhibits IGF-I/insulin signaling (Farah et al., 1998; Begum et al., 2002; Kanda et al., 2006; Lim et al., 2007; Noguchi et al., 2007; Xu et al., 2007).

One function of insulin is to cooperate with other lactogenic hormones, prolactin and hydrocortisone, to promote milk synthesis. In further studies, we have examined the effect of RhoA/Rok on prolactin signaling and the expression a milk protein gene, β-casein. As is the case with insulin signaling, we found that RhoA/Rok had an inhibitory effect on prolactin signaling (manuscript in preparation). We therefore suggest that, in the absence of proper cell–BM interactions, an over-activated RhoA/Rok pathway suppresses both insulin and prolactin signaling, culminating in a milk deficiency and reduced differentiation.

IRS-1 associates with SHP-2

A further mechanism for regulating insulin signaling is through PTPs (Lee and Streuli, 1999). Here we examined PTPs targeting to IR and IRS-1, particularly those whose effect was regulated by ECM, initially with the aim of identifying PTPs that might suppress insulin signaling in cells cultured on plastic. However, we were unable to identify the association of specific PTPs with IRS-1 under these conditions. This suggests that the low levels of tyrosine phosphorylation on IRS-1 are caused via a different mechanism, which is likely to be the elevated serine phosphorylation caused by Rok.

Using an in-gel assay, we found that several PTPs were associated with IR but the extent of association was comparable in cells cultured on plastic and BM (Fig. 7A). This is in agreement with our previous result that insulin stimulates tyrosine phosphorylation of IR irrespective of substratum (Lee and Streuli, 1999). On the other hand, coprecipitation of SHP-2 and IRS-1 in response to insulin was more prominent in cells cultured on BM (Fig. 7B,C). This might be as a result of elevated IRS-1 tyrosine phosphorylation (Lee and Streuli, 1999). Furthermore, we found that the effect of RhoA/Rok on IRS-1/SHP-2 association was similar to that on IRS-1 tyrosine phosphorylation (Fig. 8), indicating that SHP-2 is not involved in RhoA/Rok-mediated inhibition of insulin signaling. Here we suggest that SHP-2 might have a positive role in insulin signaling. This agrees with previous studies where IRS-1 tyrosine phosphorylation and downstream signaling are attenuated in transgenic mice expressing dominant negative SHP-2 (Maegawa et al., 1999). The underlying mechanism for the positive effect of SHP-2 on signaling likely occurs through dephosphorylating docking sites for negative regulators (Ali et al., 2003; Neel et al., 2003). Although our data suggest that SHP-2 does not play a role in matrix-mediated control of insulin signaling, the possibility that other PTPs are involved cannot be excluded.

Summary

We have dissected some aspects of the mechanism by which cell–ECM interactions control insulin signaling in mammary gland. We find that cell adhesion to different substrata differentially activates the RhoA/Rok pathway, which then controls signal propagation downstream of IR by a mechanism involving serine phosphorylation of IRS-1. Thus, the ECM exerts an intricate control on the ability of MECs to receive and respond to insulin signals, so that adhesion to a permissive BM promotes signaling while it is prevented on stromal matrix. It is likely that the tension of cytoskeleton, organization of cell interior, compartmentalization of signaling molecules, and even programming of gene expression are altered by different microenvironmental constraints on cells. Perhaps the integration of all these effects confers the ultimate selectivity in insulin signaling.

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