Dual Regulation of Rho and Rac by p120 Catenin Controls Adipocyte Plasma Membrane Trafficking*

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During 3T3L1 adipogenesis there is a marked reduction in β-catenin and N-cadherin expression with a relatively small decrease in p120 catenin protein levels. Cell fractionation demonstrated a predominant decrease in the particulate (membrane-bound) pool of p120 catenin with little effect on the soluble pool, resulting in a large redistribution from the plasma membrane to the cytosol. Reexpression of p120 catenin inhibited constitutive (transferrin receptor) and regulated mannose 6-phosphate receptor and GLUT4 trafficking to the plasma membrane. The inhibition of membrane trafficking was specific for p120 catenin function as this could be rescued by co-expression of N-cadherin. Moreover, overexpression of a p120 catenin deletion mutant (p120Δ622–628) or splice variant (p120-4A), neither of which could regulate Rho or Rac activity, showed no significant effect. The inhibition of GLUT4 translocation was also observed upon the simultaneous expression of a constitutively active Rac mutant (Rac1/Val12) in combination with a dominant-interfering Rho mutant (RhoA/Asn19). This was recapitulated by expression of the Rho ADP-ribosylation factor (C3ADP) in combination with constitutively active Rac1/Val12. Moreover, siRNA-mediated knockdown of p120 catenin resulted in increased basal state accumulation of GLUT4 at the plasma membrane. Together, these data demonstrate that p120 catenin serves an important role in maintaining the basal tone of membrane protein trafficking in adipocytes through the dual regulation of Rho and Rac function and accounts for reports implicating Rho or Rac in the control of GLUT4 translocation.

The mechanisms and intracellular signaling pathways that regulate intracellular membrane trafficking are quite complex. In the case of glucose uptake in adipose and striated muscle, the insulin-responsive glucose transporter protein-4 (GLUT4) is predominantly localized to intracellular membrane compartments and undergoes a dramatic redistribution to the cell surface following insulin stimulation through a process termed translocation (1–5). It has become increasingly apparent that the actin cytoskeleton and its dynamic rearrangement and remodeling are involved in the intracellular trafficking of many proteins including GLUT4 translocation (6–16). For example, treatment of adipocytes with actin-depolymerizing agents cytochalasin D and latrunculin A or B and the actin-stabilizing agent jasplakinolide inhibits insulin-stimulated GLUT4 translocation (6, 17–20). In addition, insulin-induced dynamic actin remodeling has been observed at the inner surface of the plasma membrane and in the perinuclear region in differentiated 3T3L1 adipocytes, which is prevented by the Rho-selective Clostridium difficile toxin B (6). Currently, Cdc42, Rac1, and RhoA have been most extensively characterized and shown to regulate filopodia, lamellipodia, and stress fiber/focal adhesion formation, respectively. Several studies have also implicated Cdc42, Rac1, and RhoA as critical components in mediating insulin-stimulated GLUT4 plasma membrane translocation (21–23).

In this regard, it has been recently observed that p120 catenin is an unusual Rho family GTPase regulator that activates Rac and simultaneously inactivates Rho (24–26). p120 catenin belongs to the armadillo (Arm) family proteins (27, 28) and was originally identified as a substrate for Src (27) and other tyrosine kinases (29, 30). p120 catenin directly interacts with the cadherin family of cell-cell adhesion receptors that may also serve as part of the junctional complexes linked to the actin cytoskeleton (31). More recently, studies have shown that p120 exists in three pools including the membrane-associated cadherin-bound pool, a cytoplasmic pool, and a nuclear pool (26, 31–34). Shuttle of p120 between cadherin-bound and cytoplasmic pools has been suggested to regulate the functional role of p120 catenin (31). Thus, sequestration by the membrane-bound cadherins may provide a mechanism to buffer p120 catenin action on Rho GTPases in the cytosol.

Based upon these considerations, we have found that during adipogenesis p120 catenin redistributes from a membrane-bound to a cytosolic pool concomitant with a reduction in cadherin expression. Moreover, the cytoplasmic pool of p120 catenin sets the basal state of membrane trafficking through the dual regulation of Rho and Rac function.

**EXPERIMENTAL PROCEDURES**

**Materials**—p120 catenin and N-cadherin cDNA were cloned from 3T3L1 cDNA library by RT-PCR. p120Δ622–628 and the p120-4A splice variant were obtained as described previously (32). Murine 3T3L1 pre-adipocytes were purchased from the American Type Culture Collection repository and differenti-
ated as described previously (35). p120 and N-cadherin monoclonal antibodies were purchased from BD Transduction Laboratories. Fluorescent secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, and horseradish peroxidase-conjugated secondary antibodies were from Sigma. p120 siRNA oligonucleotides were purchased from Dharmacon.

Cell Culture and Transient Transfection of 3T3L1 Adipocytes—Murine 3T3L1 pre-adipocytes were cultured in Dulbecco’s modified Eagle’s medium supplemented with 25 mM glucose and 10% calf serum at 37 °C with 8% CO2. Cells were differentiated into adipocytes with 1 μg/ml insulin, 1 μM dexamethasone, and 0.5 mM isobutyl-1-methylxanthine as described previously (35). Differentiated adipocytes were electroporated using the Gene Pulser II (Bio-Rad) with settings of 0.16 kV and 950 microfarads (36). Following electroporation, cells were plated on glass coverslips and allowed to recover for about 16–18 h. Then the cells were starved with serum-free Dulbecco’s modified Eagle’s medium for 2–3 h and stimulated with 100 nM insulin for 30 min.

Immunofluorescence—Transfected adipocytes were fixed for 15 min in 4% paraformaldehyde containing 0.2% Triton X-100. Cells were then blocked in 5% donkey serum (Sigma) plus 1% bovine serum albumin (Sigma) for 1 h at room temperature. Primary and secondary antibodies were used at 1:100 dilutions in blocking solution, and samples were mounted on glass slides with Vectashield (Vector Labs). Fluorescent images were acquired by confocal fluorescent microscopy (Zeiss LSM 510).

siRNA-mediated p120 Knockdown in 3T3L1 Adipocytes—siRNA-mediated knockdown of p120 was performed using a double strand siRNA oligonucleotide as described previously (37). Briefly, 1 nmol of p120 siRNA oligonucleotide or random oligonucleotide was electroporated into 3T3L1 adipocytes (170 V, 950 microfarads). Under these conditions, more than 95% of the adipocyte population was positive of siRNA uptake, and cell lysates were collected at various times as indicated in the legend to Fig. 6.

Cell Fractionation and Immunoblotting—Differentiated 3T3L1 adipocytes were starved in serum-free Dulbecco’s modified Eagle’s medium for 2–3 h, and then treated with or without 100 nM insulin for 30 min. Cells were washed with ice-cold phosphate-buffered saline and collected in 1 ml of ice-cold phosphate-buffered saline containing protease inhibitors, and the cells were passed 10 times through a 22-gauge needle. The homogenized cells were centrifuged at 14,000 × g for 30 min at 4 °C. The protein content of the supernatant (cytosolic protein) and the pellet (membrane protein) was quantified using the BCA protein assay kit (Pierce). Equal protein amounts (50 μg) from each of the fractions were loaded onto 4–15% gradient SDS-polyacrylamide gels, subjected to electrophoresis, transferred to polyvinylidene difluoride membranes, and immunoblotted with monoclonal antibodies directed against p120, N-cadherin, and β-catenin, respectively.

RT-PCR—RNA was isolated, using the Micro-FastTrack 2.0 kit (Invitrogen), from murine 3T3L1 pre-adipocytes and differentiated adipocytes (4, 8, and 12 days after addition of differentiation medium). First strand cDNAs were synthesized from mRNAs with oligo(dT)18 primer using Moloney murine leukaemia virus reverse transcriptase according to the manufacturer’s instructions (BD Biosciences). The cDNAs were then used as templates for PCR with primers specific for N-cadherin (forward, 5′-AAGTGCCATTAGCTAAAGGCAATTCA-3′, and reverse, 5′-CTTTTAATGTCACTGGAGATAAAGG-3′), OB-cadherin (forward, 5′-ACATTGATCGGAGTTCACGCTC-3′, and reverse, 5′-GTTTGTTGGTTGTGCATGATTTCAGG-3′), and H/T-cadherin (forward, 5′-AGGACCCACTGG-TACCCGACGT-3′ and reverse, 5′-CTTTATCGGACTGTTCGTTATAGA-3′). The PCR products were analyzed by agarose gel electrophoresis for quantity and size determination.

RESULTS

Regulation of N-cadherin, β-Catenin, and p120 Catenin during Adipogenesis—It is well established that β-catenin is a suppressor of adipocyte differentiation, and consistent with previous studies (38–40), we also observed a marked reduction of β-catenin protein levels during 3T3L1 adipocyte differentiation by immunoblotting (Fig. 1C, lanes 1–4). In parallel, there was also a marked reduction in the expression of the cell surface adhesion receptor, N-cadherin (Fig. 1B, lanes 1–4). However, although p120 catenin could directly bind to N-cadherin, there was only a modest reduction in the expression of p120 catenin (Fig. 1A, lanes 1–4). Because p120 catenin can also bind to other cadherin family members, we examined the differentiation-dependent expression of several other cadherins by RT-PCR. PCR screening of 14 different cadherin family members demonstrated that 3T3L1 fibroblasts only expressed N-, OB-, and HT-cadherins (data not shown). Consistent with the immunoblotting data, N-cadherin mRNA was relatively abundant in predifferentiated adipocytes and was markedly down-regulated during the initiation of 3T3L1 adipogenesis (Fig. 2).

Despite the large reduction in cadherin mRNA and N-cadherin protein levels, p120 catenin protein expression was only marginally decreased in differentiated adipocytes compared

FIGURE 1. Expression of p120 catenin, N-cadherin, and β-catenin protein during 3T3L1 adipogenesis. 3T3L1 cells were induced to differentiate as described under “Experimental Procedures.” Total cell lysates were collected either right before differentiation (lane 1) or after 4 (lane 2), 8 (lane 3), or 12 (lane 4) days of differentiation. Equal protein amounts (50 μg) were loaded onto a 4–15% gradient SDS-polyacrylamide gels, subjected to electrophoresis, transferred to polyvinylidene difluoride membranes, and immunoblotted with monoclonal antibodies directed against p120 catenin (A), N-cadherin (B), and β-catenin (C), respectively.
with pre-adipocytes (Fig. 1). We therefore examined the membrane versus cytosolic distribution of p120 catenin, N-cadherin, and β-catenin during adipogenesis (Fig. 3). In pre-adipocytes, p120 catenin, N-cadherin, and β-catenin were primarily found in the particulate (membrane) fraction with substantially reduced amounts in the cytosolic fraction. As observed in the whole cell extracts, when the cells differentiated into adipocyte phenotypes, there was a dramatic reduction in N-cadherin and β-catenin protein levels in both the membrane and cytosolic fractions (Fig. 3). In contrast, p120 catenin was predominantly decreased in the membrane fraction with only a minor reduction in the cytosolic fraction (Fig. 3A). These results in a difference in the ratio of particulate:cytosolic p120 catenin from approximately 6:1 to 0.8:1. The developmental changes in p120 catenin distribution were unaffected by insulin stimulation.

p120 Catenin Expression Inhibits Plasma Membrane Trafficking in Adipocytes—Previous studies have suggested that p120 catenin may function to regulate intracellular trafficking pathways (24–26, 32, 41). To first assess the effect of p120 catenin on adipocyte membrane trafficking, we overexpressed p120 catenin with the constitutive recycling membrane receptor protein, the transferrin receptor (Fig. 4A). Following 24 h of expression, a substantial portion of the expressed transferrin receptor was localized to the plasma membrane. However, in the presence of p120 catenin, there was a dramatic reduction in the amount of cell surface localized transferrin receptor.

To determine whether the inhibitory effect was specific to the unbound form of p120 catenin, we next examined the ability of overexpressed N-cadherin to reverse the inhibitory property of overexpressed p120 catenin on the trafficking of the GLUT4 reporter (Fig. 5). As is apparent, co-expression of N-cadherin increased the basal trafficking of GLUT4 to the plasma membrane without any significant effect on the insulin-stimulated translocation of GLUT4. N-cadherin expression also prevented the inhibitory action of p120 catenin on insulin-stimulated GLUT4 translocation. Similarly, a p120 catenin deletion mutant (p120Δ622–628) and splice variant (p120-ΔA) that cannot regulate Rho or Rac activity, respectively, were completely devoid of any inhibitory function with respect to insulin-stimulated GLUT4 translocation.

To further confirm the functional role of the endogenous p120 catenin protein, we utilized siRNA to specifically reduce p120 catenin protein levels (Fig. 6A). Transfection of fully differentiated 3T3L1 adipocytes with a p120 catenin siRNA resulted in a time-dependent reduction in p120 catenin protein...
levels (Fig. 6A, lanes 1, 4, and 7) compared with mock-transfected (lanes 2, 5, and 8) and random (lanes 3, 6, and 9) siRNA-transfected cells. The total p120 catenin protein levels were reduced by 80 and 90% between 48 and 96 h post-siRNA transfection, respectively.

Having established an efficient protocol to reduce p120 catenin expression, we then co-transfected the adipocytes with GLUT4 and p120 catenin siRNA and determined the extent of basal and insulin-stimulated GLUT4 translocation (Fig. 6, B–D). Twenty-four hours after siRNA expression, p120 catenin protein levels were reduced by ~50% with no significant effect on GLUT4 trafficking to the plasma membrane (Fig. 6B). In contrast, by 48 and 72 h when p120 catenin levels were decreased ~80 and 90%, respectively, there was a concomitant increase in the basal state accumulation of GLUT4 at the plasma membrane. This occurred without any effect on insulin-stimulated GLUT4 translocation and was also not observed in the mock or random siRNA-transfected cells (Fig. 6, C and D). These data confirm that the level of p120 catenin functions as a basal state repressor of adipocyte membrane trafficking.

p120 Catenin Inhibits Membrane Trafficking through Dual Regulation of Rho and Rac—Rho and Rac family members of small GTP-binding proteins play central roles in the control of actin polymerization, and it has recently been reported that p120 catenin can function to activate Rac1 and inhibit RhoA (24). Because the p120Δ622–628 deletion mutant and p120-4A splice variant were unable to affect membrane GLUT4 translocation (Fig. 5), we next assessed the potential role of Rho and Rac proteins in adipocyte membrane trafficking (Fig. 7). As observed previously, expression of p120 catenin inhibited insulin-stimulated GLUT4 translocation. In comparison, expression of either the dominant-interfering Rac1 (Rac1/Asn17) or RhoA (RhoA/Asn19) mutants had no significant effect on GLUT4 translocation. Although expression of the constitutively active Rac1 (Rac1/Val12) and RhoA (RhoA/Val14) mutants partially increased basal GLUT4 translocation, there was no significant effect on insulin-stimulated translocation. In contrast, co-expression of active Rac1/Val12 with dominant-interfering RhoA/Asn19, a combination that simulates the function of p120, recapitulated the inhibition of insulin-stimulated GLUT4 translocation. The converse experiment, co-expression of inactive Rac1/Asn17 with active RhoA/Val14,
increased the basal state translocation of GLUT4 without affecting insulin-stimulated translocation. Moreover, although expression of the RhoA ADP-ribosylation inhibitor (C3ADP) alone had no effect, co-expression of C3ADP with active Rac1/Val12 also inhibited insulin-stimulated GLUT4 translocation. Together, these data are fully consistent with the soluble pool of p120 catenin functioning to activate Rac and inhibit Rho as the responsible targets mediating p120 catenin inhibition of adipocyte membrane trafficking.

**DISCUSSION**

In the basal state, the GLUT4 protein is predominantly localized to as yet undefined intracellular compartments probably through a dynamic retention mechanism that continually retrieves intracellular GLUT4 vesicles (45, 46). Insulin stimulation results in the tyrosine phosphorylation of various effectors and signaling proteins leading to an enhanced rate of GLUT4 exocytosis, thereby increasing the steady-state distribution in the plasma membrane (47–51). This is thought to occur through an insulin-stimulated trafficking of GLUT4 from a sequestered compartment into the constitutively recycling endosome system. Although the pathways utilized by insulin to stimulate GLUT4 exocytosis have been intensively investigated, the mechanisms responsible for basal retention have been poorly studied. In particular, another insulin-regulated protein, IRAP (insulin-responsive aminopeptidase), is expressed in pre-adipocytes and during adipogenesis becomes sequestered into the same intracellular compartments as GLUT4 (52–55). In addition to the specialized insulin-responsive compartment, adipocytes also display an insulin-responsive plasma membrane translocation of classical constitutive trafficking endosomal proteins such as the transferrin receptor and the MPR, albeit to a reduced extent (56–58). Thus, during adipogenesis, a mechanism must be initiated that results in a reduction of the basal plasma membrane trafficking for at least several basal recycling proteins.

Several studies have implicated the actin cytoskeleton in the regulation of membrane trafficking (6, 8, 9, 14–16). During adipogenesis, polymerized actin converts from primarily a stress fiber organization to a strong cortical actin network that appears to control both constitutive and insulin-regulated membrane trafficking (59). Rho and Rac family members of small GTP-binding proteins play central roles in the control of actin polymerization, and it has recently been reported that p120 catenin can function to activate Rac1 and inhibit RhoA (24). Based upon the essential requirement of filamentous actin in adipocyte trafficking events, we examined the effect of various agents known to regulate filamentous actin organization. In this regard, we observed that p120 catenin appears to play an important role in maintaining a low basal rate of membrane transport in adipocytes. This was based upon p120 overexpression that inhibited the plasma membrane trafficking of the transferrin receptor, MPR and GLUT4. Moreover, p120 catenin knockdown induced an increased distribution of GLUT4 to the cell surface consistent with p120 functioning as a critical component setting the basal rate of exocytosis.

During adipogenesis the amount of membrane-bound p120 catenin was markedly reduced, whereas the cytosolic p120 catenin levels remained relatively constant. Thus, the portion of cytosolic/membrane p120 catenin levels markedly increased in fully differentiated adipocytes compared with pre-adipocytes. This apparently resulted from a down-regulation of the membrane cadherin proteins directing p120 catenin to the plasma membrane and suggests that in adipocytes the relative redistribution of p120 was responsible for alteration in function. Consistent with this interpretation, reexpression of N-cadherin increased basal state plasma membrane trafficking consistent with a redistribution of p120 from the cytosol back to a membrane-bound form. Interestingly, in epithelial cells the loss of p120 catenin expression appears to be responsible for the stability of cadherin (37, 60). Because adipocytes are relatively nonadherent, it is not surprising that there is a marked decrease in the expression of cadherin cell surface adhesion proteins. Whether or not the decrease in cadherin expression during adipogenesis was a cause or consequence of changes in p120 catenin levels, only the membrane-bound form of p120 catenin was affected. Nevertheless, our data are fully consistent with the cytosolic pool of p120 catenin adipocyte reducing membrane trafficking as retargeting of the cytosolic p120 catenin to the plasma membrane prevented its inhibitory effect.

In any case, the ability of p120 catenin to modulate membrane trafficking is apparently because of its ability to confer a dual regulation of both Rho and Rac. Recently, the cytosolic form of p120 catenin was found to activate Rac1 concomitantly with an inhibition of RhoA (24). Although the specific molecular mechanism(s) accounting for this coordinated dual regulation is not established, we could fully recapitulate the inhibitory action of p120 catenin by the co-expression of an active Rac1 and inactive RhoA mutants. Importantly, neither the individual Rac1 and RhoA mutants nor co-expression of inactive Rac1 with active RhoA was inhibitory. Moreover, inhibition of endogenous RhoA with C3ADP was also without effect, however, in the presence of active Rac1 again resulted in an inhibition of membrane translocation. Thus, these data demonstrate that the underlying steady-state rate of adipocyte membrane trafficking is set by the extent of cytosolic p120 catenin protein through the dual regulation of Rac and Rho.

In this regard, numerous studies have established that both Rho and Rac play fundamental roles in the regulation of the actin cytoskeleton (6, 22, 61). In particular, Rho is generally thought to control stress fiber filamentous actin, whereas Rac appears to regulate actin-based membrane ruffling at the leading edge of cells (62, 63). Several Rho family members of small GTP-binding proteins have been implicated in the control of insulin-regulated membrane trafficking in muscle and adipocytes through modulation of actin polymerization (6, 7, 23, 47, 64). In pre-adipocytes, filamentous actin is primarily organized into stress fibers, whereas during adipogenesis it converts to a cortical form of filamentous actin beneath the plasma membrane (59). In addition, several studies have previously shown that insulin induces actin ruffling and that inhibition of actin polymerization has a dramatic effect on adipocyte GLUT4 trafficking (6, 42, 65). Thus, it is likely that p120 catenin exerts its effect on adipocyte membrane protein trafficking through modulation of actin organization via the dual regulation of Rho and Rac function.
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