THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 19, pp. 16775–16782, May 13, 2011
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# Differential Signaling by Adaptor Molecules LRP1 and ShcA Regulates Adipogenesis by the Insulin-like Growth Factor-1 Receptor*

Received for publication, December 16, 2010, and in revised form, March 21, 2011 Published, JBC Papers in Press, March 22, 2011, DOI 10.1074/jbc.M110.212878

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**The low density lipoprotein receptor-related protein (LRP1) is a transmembrane receptor that integrates multiple signaling pathways. Its cytoplasmic domain serves as docking sites for several adaptor proteins such as the Src homology 2/α-collagen (ShcA), which also binds to several tyrosine kinase receptors such as the insulin-like growth factor 1 (IGF-1) receptor. However, the physiological significance of the physical interaction between LRP1 and ShcA, and whether this interaction modifies tyrosine kinase receptor signaling, are still unknown. Here we report that LRP1 forms a complex with the IGF-1 receptor, and that LRP1 is required for ShcA to become sensitive to IGF-1 stimulation. Upon IGF-1 treatment, ShcA is tyrosine phosphorylated and translocates to the plasma membrane only in the presence of LRP1. This leads to the recruitment of the growth factor receptor-bound protein 2 (Grb2) to ShcA, and activation of the Ras/MAP kinase pathway. Conversely, in the absence of ShcA, IGF-1 signaling bifurcates toward the Akt/mammalian target of rapamycin pathway and accelerates adipocyte differentiation when cells are stimulated for adipogenesis. These results establish the LRP1-ShcA complex as an essential component in the IGF-1-regulated pathway for MAP kinase and Akt/mammalian target of rapamycin activation, and may help to understand the IGF-1 signaling shift from clonal expansion to growth-arrested cells and differentiation during adipogenesis.**

The insulin-like growth factor 1 (IGF-1)[5] biological actions are mediated by the IGF-1 receptor, a member of the tyrosine kinase family of growth factor receptors, composed of two extracellular α-subunits and two membrane-spanning β-subunits, encoding an intracellular tyrosine kinase (1). Upon IGF-1 binding, the activated IGF-1 receptor is generally thought to proceed through phosphorylation of specific cytosolic substrates, such as the adaptor proteins Src homology 2/α-collagen (Shc). The IGF-1 signal is then transmitted to two downstream pathways: ERK of the MAPK family and phosphatidylinositol 3-kinase (PI3K), which potentially leads to two distinct events: cell proliferation or cell differentiation (1). How IGF-1 signaling bifurcates at some point from proliferation to differentiation is not known. IGF-I is a critical regulator of adipose tissue mass through its regulation of adipogenesis (2). During adipogenesis, dividing preadipocytes shift to growth-arrested cells allowing full differentiation of adipocytes. IGF-1 mediates both proliferation and differentiation of preadipocytes *in vivo* (3) and *in vitro*, including 3T3-L1 cells (2) and primary cultures of mouse (4) and human cells (5). IGF-1 stimulation of mitogen-activated protein kinase (MAPK) activity is lost as preadipocytes differentiate, and this is paralleled by a loss in Shc tyrosine phosphorylation. Inhibition of Shc tyrosine phosphorylation inhibits DNA synthesis and increases expression of the master regulator of adipogenesis, the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ) and its target gene *aP2* (6, 7).

The Shc protein family of molecular adaptors is composed of four *Shc* gene products, the ubiquitous *ShcA*, and *ShcB*, *ShcC*, and *ShcD*, which are predominantly expressed in neuronal cells. *ShcA* knock-out mice are embryonic lethal. *ShcA* is expressed as three isoforms of 46, 52, and 66 kDa (8). These isoforms are composed of two distinct domains that bind phosphotyrosine-containing sequences, an amino-terminal phosphotyrosine binding domain and a carboxyl-terminal Src homology 2 (SH2) domain, and of a central collagen homology region that harbors tyrosine phosphorylation sites (8, 9). The 66-kDa isoform contains an additional amino-terminal domain (collagen homology region 2) including a serine phosphorylation site (10, 11). ShcA binds multiple membrane receptors. For instance, through its phosphotyrosine binding domain, ShcA binds to the phosphorylated IGF-1 receptor (12, 13), whereas the SH2 domain of ShcA allows ShcA binding to the PDGF receptor (14).

The tyrosine-phosphorylated low-density lipoprotein receptor related protein 1 (LRP1) also associates with ShcA in a tyrosine phosphorylation-dependent manner through the ShcA

* This work was supported, in whole or in part, by grants from the National Institutes of Health (to J. H.), the Centre National Pour la Recherche Scientifique (CNRS), Nouvelle Société Française d'athérosclérose (NSFA), Fondation pour la Recherche Médicale (FRM), Fondation de France, and Agence Nationale de la Recherche Grant ANR-06-Physio-032.

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[3] Supported by grants from the American Health Assistance Foundation, the Humboldt Foundation, SFB780, and the Consortium for Frontotemporal Dementia.

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[5] The abbreviations used are: IGF-1, insulin-like growth factor 1; SH2, Src homology domain 2; MEF, mouse embryonic fibroblast; mTOR, mammalian target of rapamycin; LRP1, lipoprotein receptor-related protein 1; PPAR, peroxisome proliferator-activated receptor; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; ShcA, Src homology 2/α-collagen.

phosphotyrosine binding domain (15). This phosphorylation occurs on a tyrosine residue located within the second NP*XY* motif in the cytoplasmic domain of LRP1 (16), which then provides a docking site for adaptor proteins such as ShcA. However, the physiological significance of this association is unknown. LRP1 is a member of the low density lipoprotein receptor gene family (17). It is implicated in the endocytosis of numerous ligands including lipoproteins, cytokines, and growth factors (18), and integrates multiple signaling pathways at the surface of the plasma membrane. LRP1 is required for cholesterol homeostasis in adipocytes and vascular smooth muscle cells through the canonical Wnt5a pathway (19) and integrates PDGF-BB (20) and TGF$\beta$ signaling (21) in the arterial wall. It co-immunoprecipitates with the PDGF-BB receptor in vascular smooth muscle cells (16, 20, 22) and through this physical interaction protects against atherosclerosis by regulating PDGFR$\beta$ activity. The present studies were undertaken to investigate the role of the ShcA-LRP1 complex in controlling the IGF-1-mediated molecular switch between proliferation and differentiation of fibroblasts, and its implication during adipogenesis.

## EXPERIMENTAL PROCEDURES

*Materials*—Recombinant human IGF-1 and protein A-agarose beads were from Sigma. Anti-IGFR polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-ShcA, anti-Grb2, anti-Crk2, and anti-phosphotyrosine (4G10) antibodies were from Upstate Cell Signaling Solutions (Lake Placid, NY). Anti-phospho-p44/42 MAP kinase ($Thr^{202}/Tyr^{204}$), anti-p44/42, anti-phospho-AKT ($Ser^{473}$), anti-AKT, anti-phospho-mammalian target of rapamycin (mTOR) ($Ser^{2448}$), and anti-mTOR antibodies were from Cell Signaling Technology (Beverly, MA).

*Cell Culture and Treatments*—LRP1-deficient MEF (MEF $LRP1^{-/-}$ cells) and LRP1-expressing MEF (MEF $LRP1^{+/+}$ cells) (19) were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum and antibiotics. Prior to stimulation cells were cultured for 18 h in DMEM containing 0.3% BSA and then stimulated with IGF-1 (20 nM) at 37 °C in 5% $CO_2$. Src/Yes/Fyn-deficient ($SYF^{-/-}$) and control ($SYF^{+/+}$) fibroblasts were kindly provided by Dr. Richard W. Anderson. ShcA-deficient mouse embryonic fibroblasts were isolated from $ShcA^{lox/lox}$ mice generated by homologous recombination. These cells were cultured in 10% New-Born Calf Serum DMEM and infected with adenovirus expressing Cre recombinase as described previously (19). Whole cell lysates were prepared and analyzed by immunoblot as described previously (19). For siRNA transfection, cells were transfected with control or *ShcA* siRNA for 24 h in DMEM supplemented with 10% FBS. Cells were then cultured for 18 h in DMEM with 0.3% BSA and treated with 20 nM IGF-1 for the indicated times.

Adipocyte differentiation was induced using the adipogenic mixture containing IGF-1 (7 nM) or insulin (10 $\mu$g/ml), dexamethasone, isobutylmethylxanthine, and the PPAR$\gamma$ agonist rosiglitazone. Oil Red O staining was performed as described (19).

*Cell Fractionation*—For cell fractionation (23), cells were washed twice with chilled PBS then scraped in PBS and centrifuged at 1,000 × *g* for 5 min at 4 °C. Cell pellets were resuspended in buffer A (0.5 M sucrose, 2 mM EDTA, 40 mM Tricine, pH 7.8, 50 mM NaF, 2 mM $Na_2VO_4$, 25 mM $\beta$-glycerophosphate) supplemented with protease and phosphatase inhibitors, and Dounce homogenized 20 times on ice. For crude membrane preparation, lysates were centrifuged at 1,000 × *g* for 10 min at 4 °C and the supernatants were centrifuged at 29,000 × *g* for 30 min at 4 °C to separate membranes from cytosol. For plasma membrane preparation, lysates were centrifuged at 1,000 × *g* for 10 min at 4 °C, the supernatants were loaded on a 30% Percoll gradient (Sigma) and centrifuged at 29,000 × *g* for 30 min at 4 °C to separate plasma membrane from cytosol. Plasma membranes were then sonicated on ice and plasma membrane and cytosol were analyzed by immunoblot.

*Immunoprecipitation*—Cells were washed with ice-cold PBS and lysed for 20 min on ice in lysis buffer supplemented with protease and phosphatase inhibitors as described (24). Lysates were precleared then incubated overnight at 4 °C with the indicated antibody and protein A-Sepharose CL-4B beads (500 $\mu$g of protein, 5 $\mu$g of IgG, 50 $\mu$l of beads). Immunoprecipitates were washed twice with lysis buffer and lysis buffer containing 2 M NaCl, and twice in 10 mM Tris, pH 8, 50 mM NaCl. Proteins were eluted from beads with SDS sample buffer, separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and blotted with the indicated antibody.

*Ras Activation Assay*—Ras activity was assessed with an Ras activation assay kit from Cell Biolabs. After 20 nM IGF-1 stimulation cells were washed twice with ice-cold PBS and then lysed in 700 $\mu$l of assay/lysis buffer containing protease inhibitors (10 $\mu$g/ml of aprotinin, 10 $\mu$g/ml of leupeptin, 1 mM phenylmethylsulfonyl fluoride). After centrifugation at 14,000 × *g* for 10 min at 4 °C, cell lysates were incubated at 4 °C for 1 h with 40 $\mu$l of the Raf1 Ras binding domain-agarose bead. Lysates were then washed with assay/lysis buffer containing protease inhibitors, and the presence of activated Ras (Ras-GTP) was determined by Western blotting with anti-RAS monoclonal antibody.

*Real Time PCR Analysis*—RNA was isolated using TRIzol reagent (Sigma) according to the manufacturer's instructions and analyzed for the indicated gene as described previously (19).

*Statistical Analysis*—Results are from three to four separate experiments. Student's *t* test was used to compare the differences between control and treatment groups. $p < 0.05$ was considered statistically significant.

## RESULTS

*LRP1 Co-immunoprecipitates with the IGF-1 Receptor*—To analyze the potential role of LRP1 in controlling the IGF-1 signaling pathway, we first tested whether LRP1 and the IGF-1 receptor are present in the same microcompartment of cellular membrane domains. Fig. 1*A* shows that, in the cultured bovine vascular smooth muscle cell line CRL 20-18, in which LRP1 is known to co-immunoprecipitate with PDGFR$\beta$ (20, 22), LRP1 and the IGF-1 receptor are present in the same complex. Formation of this complex is increased when cells are treated with 20 nM IGF-1 for 10 min (Fig. 1*A*). Caveolae/rafts are cholesterol-rich membrane microdomains that compartmentalize a

variety of signaling molecules, including the IGF-1 receptor (25) and LRP1 (22). Caveolae are enriched in caveolin 1 oligomers, the major protein constituent of the structure, which is a distinct characteristic from lipid rafts that lack caveolin (26). Because caveolin 1 was not present within the complex, the data suggest that LRP1 and the IGF-1 receptor interact directly and that their biochemical association was not merely due to immunoprecipitation of the entire caveolae fraction (Fig. 1*A*). The formation of this complex is not limited to vascular smooth muscle cells because co-immunoprecipitation between LRP1 and the IGF-1 receptor was also observed in mouse embryonic fibroblasts (MEFs) (Fig. 1*B*).

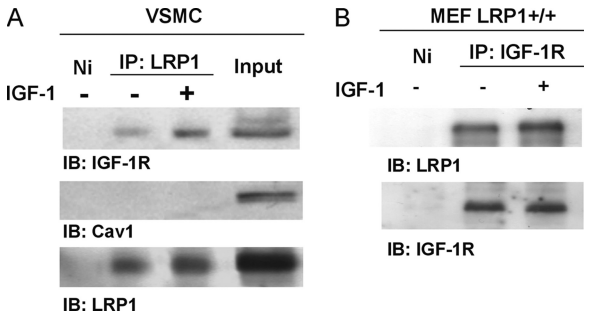


FIGURE 1. **LRP1 co-immunoprecipitates with the IGF-1 receptor.** *A*, purified plasma membrane fractions from starved vascular smooth muscle cells (*VSMC*) treated with 20 nM IGF-1 for 10 min were immunoprecipitated (*IP*) with an anti-LRP1 and immunoblotted (*IB*) with an antibody directed against the IGF-1 receptor. Western blot analysis of caveolin 1 (*Cav1*) ensures the specificity of the immune complex. *B*, whole cell lysates from starved LRP1$^{+/+}$ MEFs treated with 20 nM IGF-1 for 10 min were immunoprecipitated with an anti-IGF-1 receptor antibody and immunoblotted with an anti-LRP1 antibody. *Ni*, non-immune control.

*IGF-1-dependent Recruitment of ShcA to the Plasma Membrane in the Presence of LRP1*—Because ShcA binds to both the IGF-1 receptor and LRP1, we tested whether the recruitment of adaptor protein ShcA to the plasma membrane upon IGF-I stimulation is affected in the absence of LRP1. Immunoblot analysis of purified plasma membrane from LRP1$^{+/+}$ MEFs showed that expression of ShcA isoforms is almost undetectable in unstimulated cells, and increased upon stimulation with 20 nM IGF-I to reach a maximum expression after 30 min of treatment (Fig. 2*A*). This increase of ShcA protein expression levels at the plasma membrane was paralleled by a decrease of its expression in the cytosol (Fig. 2*A*). Conversely, in the absence of LRP1, translocation of ShcA to the plasma membrane failed to respond to IGF-1 stimulation (Fig. 2*A*). The three isoforms of ShcA exhibited high plasma membrane protein levels in untreated, as well as in IGF-1-treated cells (Fig. 2*A*). Increased ShcA protein levels were also observed in the crude membrane preparation of LRP1$^{+/+}$ MEFs stimulated with 100 nM insulin, which is known to partly signal through the IGF-1 receptor (Fig. 2*B*). These data show that the recruitment of ShcA at the plasma membrane is sensitive to IGF-1 stimulation only in the presence of LRP1.

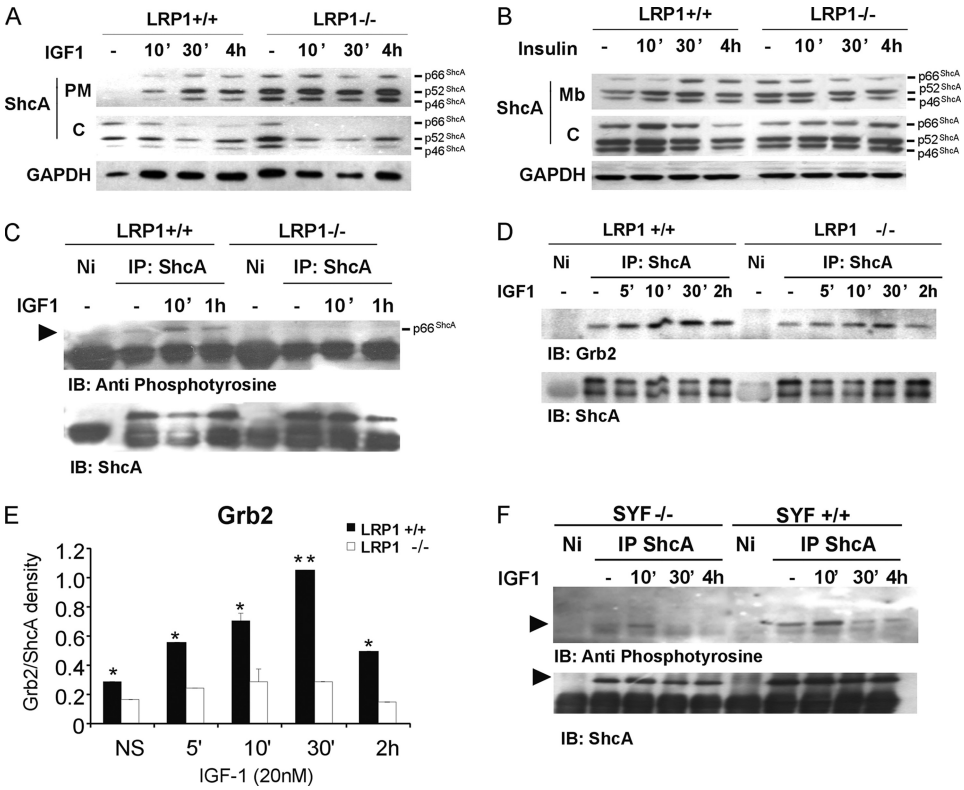


FIGURE 2. **The recruitment and activation of ShcA at the plasma membrane are sensitive to IGF-1 treatment only in the presence of LRP1.** *A*, Western blot analysis of ShcA in purified plasma membrane (*PM*), crude membrane preparation (*Mb*), and cytosol (*C*) from LRP1$^{-/-}$ and LRP1$^{+/+}$ MEFs treated with 20 nM IGF-1 or (*B*) treated with 100 nM insulin for the indicated times. *C*, whole cell lysates were immunoprecipitated (*IP*) with anti-ShcA and immunoblotted (*IB*) with antibody 4G10 directed against phosphotyrosine or (*D*) with anti-Grb2 antibodies. *E*, quantification of the coimmunoprecipitation between ShcA and Grb2 by densitometry. *F*, immunoprecipitation of ShcA upon IGF-1 treatment in Src/Yes/Fyn deficient (SYF$^{-/-}$) and control (SYF$^{+/+}$) fibroblasts, and immunoblot with antibody 4G10 directed against phosphotyrosine. Results are expressed as mean ± S.E. *, $p < 0.05$, and **, $p < 0.01$ compared with controls. *Ni*, non-immune.

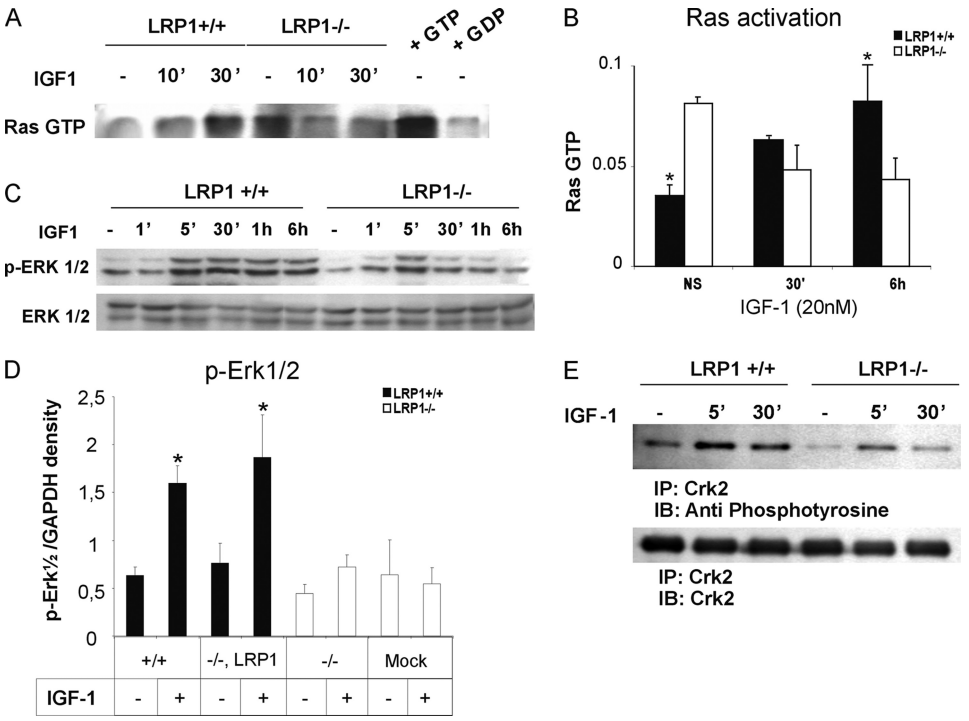


FIGURE 3. **LRP1 is required for the IGF-1-induced MAPK cascade activation.** $LRP1^{-/-}$ and $LRP1^{+/+}$ cells were treated with or without 20 nM of IGF-1 for the indicated times. *A*, whole cell lysates were incubated with agarose conjugated with the Ras-binding domain of Raf1, then the bound activated Ras (Ras-GTP) was analyzed by Western blotting with anti-Ras antibodies. *B*, quantification of the blots. *C*, Western blot analysis of phospho and total ERK1/2. *D*, densitometric scanning of the Western blot analysis of phospho and total ERK1/2 in $LRP1^{-/-}$, $LRP1^{+/+}$, $LRP1^{-/-}$ retransfected with LRP1 (−/−, LRP1), and mock retransfected (*Mock*) $LRP1^{-/-}$ MEFs treated with or without 20 nM IGF-1 for 10 min. Results are expressed as mean ± S.E. *, $p < 0.05$. *E*, Western blot analysis of phospho and total Crk2. Whole cell lysates were immunoprecipitated (*IP*) with anti-Crk2 and immunoblotted (*IB*) with antibody 4G10 directed against phosphotyrosine (*top panel*) or with anti-Crk2 antibodies (*bottom panel*).

We next analyzed whether LRP1 is required for the IGF-1-induced tyrosine phosphorylation of ShcA by immunoprecipitation. Fig. 2*C* shows that IGF-1 treatment induced tyrosine phosphorylation of the $p66^{ShcA}$ isoform in $LRP1^{+/+}$, but not in $LRP1^{-/-}$ MEFs (Fig. 2*C*). Because tyrosine phosphorylation of ShcA is strongly decreased in Src/Yes/Fyn-deficient cells (Fig. 2*F*), one or all of these kinases are involved in ShcA phosphorylation. This tyrosine phosphorylation of the $p66^{ShcA}$ isoform was accompanied by a marked IGF-1-induced recruitment of Grb2 to ShcA in $LRP1^{+/+}$, but not in $LRP1^{-/-}$ MEFs with maximum levels after 30 min of treatment (Fig. 2, *D* and *E*). Taken together, these data show that LRP1 is an important component of the IGF-1 signaling pathway, and suggest that LRP1 is required for the IGF-1-induced ShcA activation. It is required for tyrosine phosphorylation of the plasma membrane $p66^{ShcA}$ isoform, and subsequent binding of Grb2 to ShcA.

*LRP1 Is Required for the IGF-1-induced Ras and ERK1/2 Activation*—The recruitment of Grb2 to ShcA is known to activate the guanine nucleotide exchange factor Sos, which triggers the Ras/Raf/ERK signaling cascade (27, 28). To determine the consequence of the absence of LRP1 on the Ras/MAPK pathway, we measured Ras-GTP levels by Western blotting in $LRP1^{+/+}$ and $LRP1^{-/-}$ MEFs upon IGF-1 stimulation. Whereas Ras activation in $LRP1^{+/+}$ MEFs increased rapidly after the addition of 20 nM IGF-1 (Fig. 3, *A* and *B*), this activation is maximal in unstimulated LRP1-deficient cells and severely blunted upon treatment with the same amount of IGF-I over a time course of 10 min (Fig. 3, *A* and *B*). The decrease in Ras activation in $LRP1^{-/-}$ MEFs was accompanied by a decrease in ERK1/2 tyrosine phosphorylation (*p*-ERK1/2) starting after 10 min of IGF-1 treatment (Fig. 3, *C* and *D*). Upon 6 h of stimulation, IGF-1 decreased *p*-ERK1/2 by >95% (Fig. 3*C*). Conversely, IGF-1 stimulation induced a robust increase of *p*-ERK1/2 in $LRP1^{+/+}$ MEFs (Fig. 3, *C* and *D*) or in $LRP1^{-/-}$ MEFs re-transfected with LRP1 (Fig. 3*D*). Thus, LRP1 is required for activation of the IGF-1-induced ShcA/Ras/Raf/ERK signaling pathway.

One explanation for the high basal Ras activity associated with low levels of ERK activation in the $LRP1^{-/-}$ cells (Fig. 3, *A*–*D*) would be that in the absence of LRP1, PDGFR signaling is activated (20). Furthermore, several data already suggested that in the absence of LRP1 some small GTPases are activated (29, 30). For instance, LRP1 is a major activator of Rac1 and a reciprocal inhibitor of RhoA in Schwann cells (29). Crk2 is a small adapter protein comprised of SH2 and SH3 domains (31) and tyrosine phosphorylated by the insulin receptor in parallel to the phosphorylation of Shc (32). Stimulation of Crk2 induces the inactivation of the Ras antagonist Rap1 leading to the activation of the ERK kinase cascade (32). Conversely, when tyrosine phosphorylation of Crk2 is reduced, activation of Rap1 inhibits the ERK cascade, but Ras accumulates. We tested the hypothesis that the high basal Ras activity associated with low levels of ERK activation in the absence of LRP1 is due to a reduced Crk2 activity and observed a reduced level of *p*-Crk2 in LRP1-deficient cells (Fig. 3*E*). Thus, Ras is activated in the absence of LRP1 but uncoupled from the ERK kinase cascade.

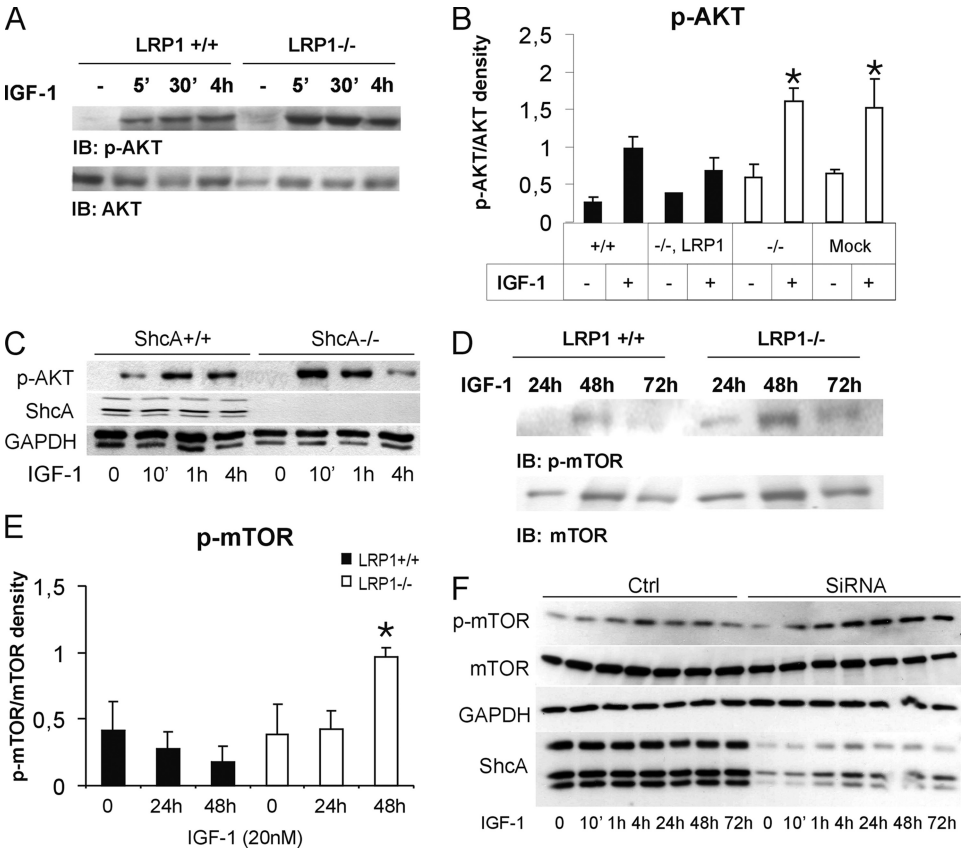


FIGURE 4. **LRP1 down-regulates IGF-1 activation of the Akt/mTOR pathway.** Western blot analysis of *p*-AKT in (*A*) LRP1$^{-/-}$ and LRP1+/+ MEFs or (*C*) ShcA$^{flox/flox}$ MEFs inactivated with a Cre/recombinase adenovirus, stimulated with IGF-1 (20 nM) for the indicated times. *B*, densitometry of the Western blot analysis of *p*-AKT in LRP1$^{-/-}$ and LRP1$^{+/+}$ MEFs treated or not with IGF-1 (20 nM) for 10 min. *D*, Western blot analysis of *p*-mTOR in LRP1$^{-/-}$ and LRP1$^{+/+}$ MEFs stimulated with IGF-1 (20 nM) for the indicated times. *E*, densitometric scanning of the Western blot analysis of *p*-mTOR in MEFs. *, $p < 0.05$ compared with control. *F*, Western blot analysis of *p*-mTOR and total mTOR in 3T3-L1 preadipocyte ShcA inactivated with siRNA or treated with small interfering control (*Ctrl*) and stimulated with IGF-1 (20 nM) for the indicated times.

*Negative Control of the Akt-mTOR Pathway by the LRP1-ShcA Complex*—The IGF-1 receptor signals through multiple signaling pathways including the phosphoinositide 3-kinase (PI3K)/Akt pathway (33, 34). In an attempt to determine whether the LRP1-ShcA complex could switch the IGF receptor coupling from one pathway, *i.e.* the Ras/Raf/MEK/ERK pathway to another, *i.e.* the PI3K/Akt pathway, we examined by Western blotting Akt activation in IGF-1-stimulated LRP1$^{-/-}$, ShcA$^{-/-}$, and control cells. Our data showed that the level of *p*-Akt was significantly higher in LRP1-deficient cells than in LRP1$^{+/+}$ cells (Fig. 4, *A* and *B*). Similarly, a complete deletion of ShcA in skin fibroblasts using the Cre/lox system leads to a marked induction of *p*-Akt upon IGF-1 stimulation, when compared with control cells (Fig. 4*C*). This indicates that LRP1 and ShcA are common mediators of the IGF-1 action that down-regulates Akt phosphorylation.

Akt has several substrates, and can activate the mTOR, a highly conserved protein kinase that controls cell growth and metabolism in response to nutrients and growth factors (35). This regulation occurs through phosphorylation of mTOR on serine 2448 (34). To determine whether the LRP1-ShcA complex can impact the IGF-1-induced Akt-dependent mTOR activation, we analyzed mTOR serine 2448 phosphorylation after IGF-1 stimulation in LRP1$^{+/+}$ and LRP1$^{-/-}$ MEFs, and in 3T3 L1 pre-adipocytes knocked down for ShcA using the siRNA technology. A 20 nM IGF-1 treatment induced a time-dependent increase in *p*-mTOR levels, which decreased after 30 min in LRP1$^{+/+}$ cells (Fig. 4, *D* and *E*). By contrast, in LRP1$^{-/-}$ MEFs, mTOR phosphorylation increased gradually, and high levels of its protein expression was observed even after 48 h of IGF-1 stimulation (Fig. 4, *D* and *E*). Similarly, upon IGF-1 stimulation, mTOR phosphorylation increased in 3T3 L1 pre-adipocytes knocked down for ShcA compared with control cells (Fig. 4*F*).

IGF-1 through activation of the PI3K/Akt pathway is involved in control of the adipogenic program (36–38). mTOR and its target the p70 S6 kinase (p70S6K) are also strongly activated in human mesenchymal stem cells under adipogenesis-inducing conditions. Treatments with LY294002 and rapamycin, which block the activation of PI3K/Akt and mTOR/p70S6K, respectively, result in pronounced inhibitory effects on adipogenesis (39). Furthermore, in pre-adipocytes, the IGF-1-induced switch from clonal expansion to differentiation correlates with loss of ShcA phosphorylation (6). To determine whether the IGF-1/PI3K/Akt/mTOR pathway could activate the adipocyte differentiation program through ShcA, we evaluated the IGF-1-induced activation of the adipocyte differentiation program in 3T3-L1 preadipocytes knocked down for ShcA. Fig. 5 shows that absence of ShcA strongly accelerated adipocyte differentiation as evaluated by Oil Red O staining

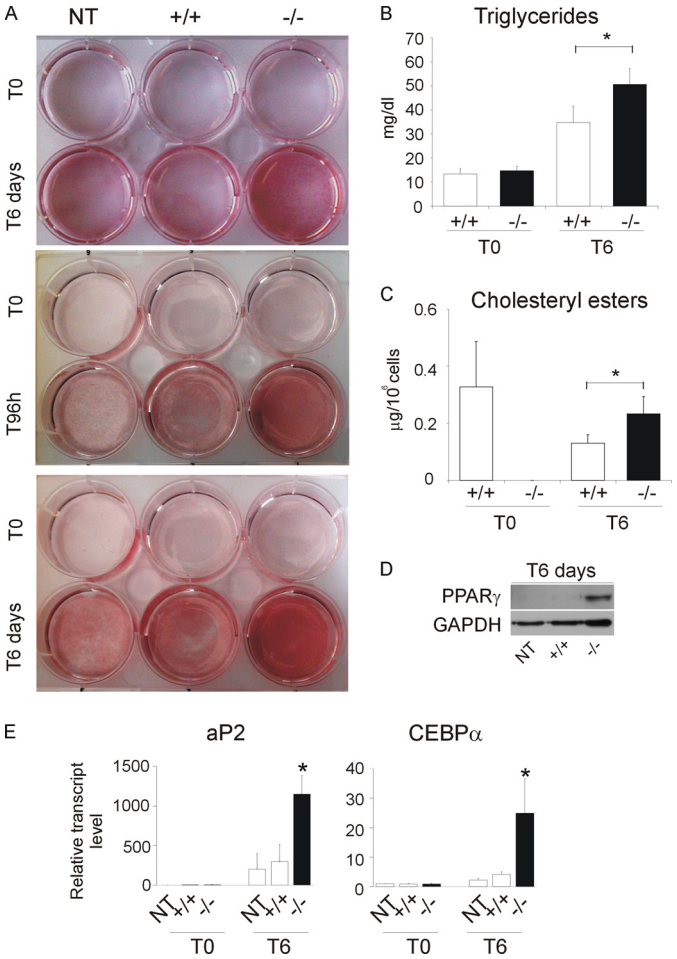


FIGURE 5. **Activation of the adipocyte differentiation program by IGF-1 in ShcA-deficient cells.** *A*, 3T3-L1 preadipocyte knockdown for ShcA (−/−), transfected with small interfering control (+/+) and non-transfected (*NT*) cells induced for adipocyte differentiation with a differentiation mixture containing IGF-1 (*top panel*) or insulin (*middle* and *bottom panels*) during the indicated times. Triglyceride (*B*) and cholesterol esters (*C*) quantification in 3T3-L1 preadipocytes knockdown for ShcA (*black*) and control cells (*white*) induced for adipogenesis during 6 days with a differentiation mixture containing IGF-1. *D*, Western blot analysis of PPARγ; *E*, quantitative RT-PCR analysis of the indicated genes in 3T3-L1 preadipocytes knockdown for ShcA (−/−), transfected with small interfering control (+/+) or non-transfected (*NT*) during adipogenesis.

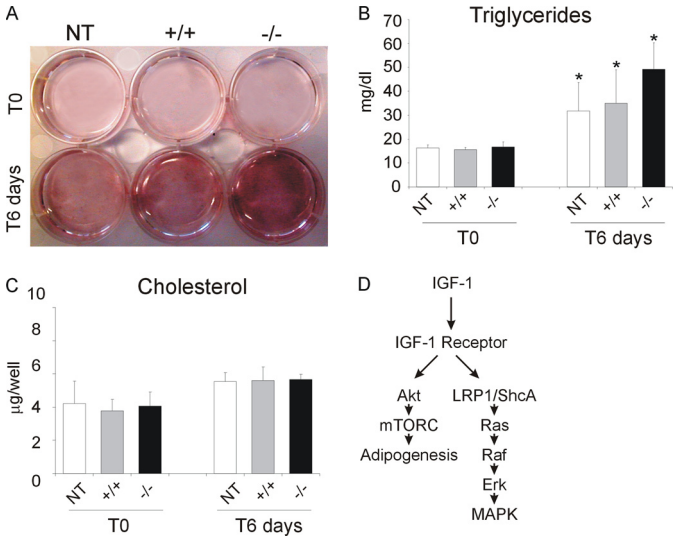


FIGURE 6. **Activation of the adipocyte differentiation program by IGF-1 in LRP1-deficient cells.** *A*, 3T3-L1 preadipocytes knockdown for LRP1 (−/−), transfected with small interfering control (+/+) and non-transfected (*NT*) cells induced for adipocyte differentiation with a differentiation mixture containing IGF-1 for 6 days. Intracellular triglycerides (*B*) and cholesterol (*C*) quantification in 3T3-L1 preadipocytes knockdown for LRP1 (*black*), control (*gray*), and non-transfected cells (*white*) induced for adipogenesis during the indicated time with a differentiation mixture containing IGF-1. *D*, the LRP1/ShcA switches IGF-1 signaling from the Akt/mTOR to the Grb2/Ras/ERK1/2 pathway. LRP1 is required for a full activation of the ShcA/Grb2/Ras/ERK1/2 pathway and progression in the cell cycle in response to IGF-1. When LRP1 is lacking, the Akt/mTOR pathway is activated. Results are expressed as mean ± S.E. *, $p < 0.05$.

when treated with a differentiation mixture containing IGF-1 (Fig. 5*A*, *top panel*) or insulin, which is also known to signal through the IGF-1 receptor (Fig. 5*A*, *middle* and *bottom panels*). When treated with an adipogenic mixture containing IGF-1, 3T3-L1 preadipocytes in which ShcA had been knocked down accumulated 1.5–2-fold more triglycerides and cholesteryl esters compared with control cells (Fig. 5, *B* and *C*). ShcA$^{-/-}$ cells also expressed higher levels of PPARγ (Fig. 5*D*) and PPARγ target genes such as *aP2* and *CEBPα* than controls (Fig. 5*E*), even after only 6 days of treatment. Similarly, decreased expression of LRP1 in 3T3-L1 preadipocytes accelerated neutral lipid accumulation as evaluated by Oil Red O staining when these cells were treated with a differentiation mixture containing IGF-1 for 6 days (Fig. 6*A*). They also accumulated more triglycerides than control cells, without reaching significance (Fig. 6*B*), but similar levels of cholesterol than control cells (Fig. 6*C*). Thus, decreased ShcA-LRP1 complex activity re-routes IGF-1 signaling from the Grb2/Ras/ERK1/2 to the Akt/mTOR pathway, an event that accelerates differentiation of 3T3-L1 preadipocytes committed to adipogenesis (Fig. 6*D*).

## DISCUSSION

In the present study we used adipocyte differentiation in fibroblasts as a model to investigate whether the LRP1-ShcA complex plays a role in the re-routing of IGF-1 action from increased MAP kinase signaling, which stimulates cell division, to activation of the Akt/mTOR pathway with leads to growth arrest and differentiation.

LRP1 integrates multiple signaling pathways at the surface of the membrane. It has been shown to form complexes with cell surface receptors such as the PDGFβ receptor (20, 24) or the urokinase-type plasminogen activator receptor (40, 41). LRP1 modulates PDGF signaling by controlling ubiquitination and endocytosis of the PDGF receptor β (24). It also appears to escort the urokinase-type plasminogen activator receptor from caveolae (42) to clathrin-coated pits (43). However, the molecular mechanisms by which the signaling molecules that bind to its external domain or its cytoplamic domain relay the information are not known. The results of the present study indicate that LRP1 interacts with the IGF-1 receptor. Co-immunoprecipitation between LRP1 and the IGF-1 receptor, which is already detected without any IGF-1 stimulation strongly suggests that LRP1 also modulates IGF-1 receptor-dependent signaling events.

Among the IGF-1 receptor downstream substrates lies the adaptor protein ShcA (28, 44). Here, we show that LRP1 promotes the IGF-1-dependent translocation of the three isoforms

of ShcA from the cytosolic to the plasma membrane compartment. Deletion of LRP1 alters this dynamic recruitment and impairs IGF-1 signaling.

Recruitment of ShcA to specific membrane compartments, *i.e.* caveolae, has been shown to be required for its subsequent phosphorylation upon IGF-I stimulation (44). Our data show that LRP1 is required for the IGF-1-induced tyrosine phosphorylation of the p66 isoform of ShcA. In the absence of LRP1, IGF-1-induced p66 ShcA tyrosine phosphorylation is drastically blunted. ShcA tyrosine phosphorylation leads to Grb2 recruitment to ShcA (28). Here, we also show that LRP1 deficiency leads to a blunted IGF-1-induced Grb2 recruitment to ShcA. Altogether, our data indicate that LRP1 is required for the optimal activation of ShcA upon IGF-1 stimulation.

The recruitment of Grb2 to ShcA leads to the activation of the guanine nucleotide exchange factor SoS, which activates Ras and triggers the Ras/Raf/MEK/ERK signaling cascade (27, 28). As expected, we show that upon IGF-1 stimulation, there is a time-dependent increase in Ras activation in the presence of LRP1, whereas in the absence of LRP1, IGF-1 induces a decrease in Ras activation. Also, upon IGF-1 stimulation, there is a sustained ERK1/2 activation in the presence of LRP1. However, in LRP1-deficient cells, IGF-1-induced ERK1/2 activation is impaired and transient, highlighting that full activation of the Ras-ERK1/2 pathway by IGF-1 requires LRP1.

Besides ShcA/Grb2/Ras/ERK1/2, the IGF-1 receptor, once activated by IGF-1, can also stimulate the Akt/PKB pathway (33). Akt has several substrates such as mTOR (34). Our results show that before and after IGF-1 stimulation *p*-mTOR levels follow the same activation kinetics as Akt, in $LRP1^{+/+}$ as well as $LRP1^{-/-}$ cells. Altogether our results show that the LRP1-ShcA complex induces a switch of IGF-1 receptor coupling away from Akt/mTOR and toward the Grb2/Ras/ERK1/2 pathway.

In our fibroblast experiments, in the absence of ShcA the IGF-1-induced activation of the Akt/mTOR pathway and the concomitant decrease in Ras and ERK1/2 activation accelerate the adipocyte differentiation program and facilitate intracellular storage of cholesteryl esters and triglycerides. This is consistent with previous findings by Hosooka *et al.* (45), who demonstrated that the protein downstream of tyrosine kinases-1 (Dok1) binds and activates Ras in response to insulin, thereby regulating mitogenic signaling mediated by Ras and the ERK pathway (45). Dok1-deficient fibroblasts exhibit impaired adipocyte differentiation, possibly due to an increase in ERK activity and a consequent increase in the phosphorylation, and thus inhibition, of PPARγ. Dok1-deficient mice are leaner and show improved glucose tolerance and insulin sensitivity (45). PPARγ plays a crucial role in adipogenesis and our data are also consistent with the fact that the ERK family is known to repress the transcriptional and biological functions of *PPARγ* and reduces its ability to promote adipogenesis (46). Conversely, PKB/Akt signaling promotes 3T3-L1 adipocyte differentiation upon IGF-I stimulation (47), whereas mTOR positively regulates adipocyte differentiation by controlling the activation of PPARγ (48). Thus, by decreasing PKB/Akt activation in response to IGF-I stimulation, ShcA protects against adipocyte hypertrophy by activating the inhibitory effect of ERK on PPARγ and may thus protect against diet-induced obesity.

In conclusion, we have shown in this study that the LRP1·ShcA·IGF-1 receptor complex acts as a molecular switch required for the full activation of the Ras/ERK1/2 pathway, whereas absence of ShcA leads to activation of the Akt/mTOR pathway and entry into the adipocyte differentiation program. This has important physiological consequences for the regulation of cell differentiation and proliferation in diverse cell types *in vitro* and *in vivo* and, specifically, for the process of adipose tissue formation, and obesity.

*Acknowledgments—We thank Dr. Daniel Metzger (University of Strasbourg, France) for critical reading of the manuscript and helpful suggestions, and Dr. Richard G. W. Anderson (University of Texas, Southwestern Medical Center, at Dallas) for providing the SYF cells.*

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