Interaction of Filamin A with the Insulin Receptor Alters Insulin-dependent Activation of the Mitogen-activated Protein Kinase Pathway*

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Hua-Jun He‡, Sutapa Kole‡, Yong-Kook Kwon‡, Michael T. Crow§, and Michel Bernier¶
From the ‡Diabetes Section, Laboratory of Clinical Investigation and the §Vascular Studies Unit, Laboratory of Cardiovascular Science, NIA, National Institutes of Health, Baltimore, Maryland 21224

The biological actions of insulin are associated with a rapid reorganization of the actin cytoskeleton within cells in culture. Even though this event requires the participation of actin-binding proteins, the effect of filamin A (FLNa) on insulin-mediated signaling events is still unknown. We report here that human melanoma M2 cells lacking FLNa expression exhibited normal insulin receptor (IR) signaling, whereas FLNa-expressing A7 cells were unable to elicit insulin-dependent Shc tyrosine phosphorylation and p42/44 MAPK activation despite no significant defect in IR-stimulated phosphorylation of insulin receptor substrate-1 or activation of the phosphatidylinositol 3-kinase/AKT cascade. Insulin-dependent translocation of Shc, SOS1, and MAPK to lipid raft microdomains was markedly attenuated by FLNa expression. Coimmunoprecipitation experiments and in vitro binding assays demonstrated that FLNAs binds constitutively to IR and that neither insulin nor depolymerization of actin by cytochalasin D affected this interaction. The colocalization of endogenous FLNa with IR was detected at the surface of HepG2 cells. Ectopic expression of a C-terminal fragment of FLNa (FLNaCT) in HepG2 cells blocked the endogenous IR-FLNa interaction and potentiated insulin-stimulated MAPK phosphorylation and transactivation of Elk-1 compared with vector-transfected cells. Expression of FLNaCT had no major effect on insulin-induced phosphorylation of the IR, insulin receptor substrate-1, or AKT, but it elicited changes in actin cytoskeletal structure and ruffle formation in HepG2 cells. Taken together, these results indicate that FLNa interacts constitutively with the IR to exert an inhibitory tone along the MAPK activation pathway.

Insulin is a pleiotropic hormone with multiple integrated metabolic and mitogenic signaling pathways. Upon binding of insulin, the cell surface insulin receptor (IR) undergoes auto-phosphorylation on several tyrosine residues located in the cytoplasmic portion of the β-subunit, with subsequent increase in its intrinsic tyrosine kinase activity. A number of adaptor proteins, including insulin receptor substrate (IRS) 1/2, the Src and collagen homologous (Shc) molecules, Cbl, Gab1, p60 Dok, and APS, are recruited to the activated IR through their Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains and become tyrosine-phosphorylated, thus allowing formation of signaling competent complexes subjacent to the inner wall of the plasma membrane (1). Specificity in signaling is achieved through differences in the ability of IR to interact with these adaptor molecules.

The submembranous actin microfilament network links various signaling proteins (e.g. phosphatidylinositol (PI) 3-kinase and Rho family of proteins) that play an important role in membrane trafficking, cellular integrity, and homeostasis (2–4). Incidentally, insulin is known to induce rapid dynamic reorganization of the actin cytoskeleton to generate the forces necessary for plasma membrane ruffling formation and a host of other cellular processes, including proper insertion of insulin-regulatable glucose transporter 4 in the cell surface (5). The stabilization of actin network at the periphery of the cytoplasm and its attachment to cellular membranes are orchestrated by actin-binding proteins. The filamin family of actin-binding proteins bind to actin filaments and to a number of macromolecules (reviewed by Stossel et al. (6)), notably small GTPases (7) and p21-activated kinase 1 (Pak1) (8). Filamins are rod-shaped proteins of 280 kDa that contain an N-terminal actin-binding domain followed by 24 repeats each of 96 amino acids. Repeat and submembranous actin microfilament network links various signaling proteins (e.g. phosphatidylinositol (PI) 3-kinase and Rho family of proteins) that play an important role in membrane trafficking, cellular integrity, and homeostasis (2–4). Incidentally, insulin is known to induce rapid dynamic reorganization of the actin cytoskeleton to generate the forces necessary for plasma membrane ruffling formation and a host of other cellular processes, including proper insertion of insulin-regulatable glucose transporter 4 in the cell surface (5). The stabilization of actin network at the periphery of the cytoplasm and its attachment to cellular membranes are orchestrated by actin-binding proteins. The filamin family of actin-binding proteins bind to actin filaments and to a number of macromolecules (reviewed by Stossel et al. (6)), notably small GTPases (7) and p21-activated kinase 1 (Pak1) (8). Filamins are rod-shaped proteins of 280 kDa that contain an N-terminal actin-binding domain followed by 24 repeats each of 96 amino acids. Repeat 24 contains the dimerization domain of filamin. Significantly, most of the interactions between filamin and its binding partners occur through the C-terminal end of filamins, thus allowing the interwebbing of actin scaffolds with membrane-bound proteins (6).

Several proteins involved in signal transduction events are partitioned in lipid rafts, a process that allows proper compartmentalization and spatial/temporal organization of functionally competent signaling complexes (9). The IR segregates to glycolipid-enriched raft domains of the plasma membrane in a variety of cell types (10, 11). Of interest, inducible association of signaling proteins with lipid rafts has been shown to depend on the actin cytoskeleton through a mechanism involving raft coalescence (12, 13). Therefore, colocalization of filamin and resident raft proteins, including the scaffolding protein caveolin-1 (14), is likely to be of physiological importance in the
clustering of lipid rafts and organization of multiple signaling pathways by the actin cytoskeleton. Until now, however, little is known about a role that filamins would play in the trans-mission of the diverse effects of insulin.

In this study, we have investigated the relative contribution of filamin A (FLNa) to the regulation of insulin signaling in human melanoma cell lines (M2 cells) that have spontaneously lost expression of FLNa, and a subline with stable expression of recombinant FLNa (A7 cells) (15). By using this cell model, we have found that FLNa expression attenuated insulin mitogenic signaling by selectively inhibiting the recruitment and tyrosine phosphorylation of Shc and subsequent activation of p42/44 MAPK. Of interest, neither early steps in insulin signaling (e.g. IR and IRS-1 tyrosine phosphorylation) nor the activation of the IRS-1/PI3 kinase/akt pathway were affected by FLNa. MAPks transduce a mitogenic signal by phosphorylating transcription factors such as Elk-1, which leads to regulation of critical genes (16). Our results have indicated also that FLNa binds directly to IR and that ectopic expression of a C-terminal fragment of FLNa disrupts constitutive IR-FLNa interaction in HepG2 cells, thereby inducing a marked increase in insulin-stimulated MAPK phosphorylation and Elk-1 transactivation. These results indicate that FLNa has a negative role in MAPK-mediated Elk-1 transcriptional activation in response to insulin, in part, by interacting directly with the IR.

EXPERIMENTAL PROCEDURES

Materials—The anti-human IR antibodies for immunoprecipitation (clones 29B4 and CII 25.3) were purchased from Calbiochem. The anti-IR β-subunit antibody as well as heroseradish peroxidase-linked phosphotyrosine (clone RC20) antibody for Western blot were from Transduction Laboratories. The anti-IR β-subunit antibody (60-46-92) for immunofluorescence studies, and the anti-Shc, phosphotyrosine, SOS1, IRS-1, p85 subunit of PI3-kinase, and p42/44 MAP kinase (ERK1 and 2) antibodies were from Upstate Biotechnology. Inc. The monoclonal anti-FLNa antibody for immunoprecipitation and immunofluorescence studies was purchased from Chemicon International, and that used for Western blot analysis was from Research Diagnostics Inc. The anti-IR α-subunit and c-Src antibodies were from Santa Cruz Biotechnology. The phospho-ERK and phospho-AKT (Ser473) antibodies were purchased from Promega and Cell Signaling Technology, respectively. The Alexa Fluor secondary antibodies, Alexa Fluor-conjugated phallidins, and Topro-3 were from Molecular Probes. The recombinant human insulin and EGF were from Calbiochem and Upstate Biotechnology, Inc., respectively. FuGENE 6 and LipofectAMINE 2000 were from Roche Applied Science and Invitrogen, respectively. Recombinant human insulin-like growth factor 1 (IGF-1), cytochalasin D, 2-mercaptoethanol, sodium orthovanadate, and Me2SO were from Sigma. The commercial sources for electrophoresis reagents, culture media, sera, filaris, heroseradish peroxidase-linked secondary antibodies, and the enhanced chemiluminescence detection system for immunoblot detection have been described previously (17).

Plasmid Construction—cDNA encoding the FLNa C-terminal fragment (amino acids 2357-2687) was amplified by PCR using full-length human filamin A cDNA (kindly provided by Dr. Yasutaka Ohta (Harvard Medical School, Boston)) as the template. The following primer pairs were used: forward primer, 5'-TAGGAGCTCATGCGCGATCATGATGATCTGCTTCCAGAGGCGGAAC-3'; reverse primer, 5'-CGACTAGTCTAGGGCGACACAAAC-3'. The underlined nucleotides indicate the KpnI and SpeI sites in the forward and reverse primers, respectively, and the italic nucleotides indicate the HA epitope. The PCR product was subcloned into the pcDNA3.1 vector (Amersham Biosciences). GST and GST-IR fusion protein were expressed in BL21, induced by 0.5 mM isopropylthio-β-D-galactopyranoside, and purified by affinity chromatography with glutathione-Sepha-rose (Amersham Biosciences) according to the manufacturer's protocols. The resulting eluates were concentrated by ultrafiltration and stored at -20° C. Translation and product size were verified by analyzing an aliquot of the samples by SDS-PAGE and Colloidal blue staining of the gel, as well as by immunoblot analysis. The integrity of the GST-IR construct was verified by automated sequencing.

Cell Treatment—M2 and A7 cells were cultured in minimal essential medium (MEM) supplemented with 10 mM Hepes, pH 7.4, 0.25% bovine serum albumin, 2 mM l-glutamine, 100 μg/ml streptomycin, 8% newborn calf serum, and 2% fetal calf serum and maintained in a humidified atmosphere of 5% CO2 in air at 37 °C. Before treatment, cells were serum-starved for 18 h in MEM supplemented with 0.1% FBS, washed with phosphate-buffered saline (PBS), and then incubated in Krebs-Ringer phosphate (KRP) buffer. Cells were stimulated with the absence or the presence of 200 μM of orthovanadate for 30 min followed by the addition of 100 nM insulin for periods up to 10 min. In some experiments, cells were stimulated with either 25 nM insulin, 20 nM EGF, 14 nM IGF-1, or 20% FBS for 15 min. These cells were washed in PBS and immersed in liquid nitrogen. The human HepG2 hepatoma cells and HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) and McCoy's 5A medium, respectively, supplemented with 10% FBS, 2 mM l-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin.

Transient Transfection Assays—HepG2 cells were cultured for 24 h until 60–80% confluence was reached. Transient transfection was performed according to the manufacturer's protocol for the use of LipofectAMINE 2000. In brief, HepG2 cells were transfected with the expression vector (pcDNA3.1) or expression plasmid encoding HA-FLNa-C1 was mixed with the transfection reagent and directly added to the culture plates at a ratio of 6 μg/80-mm dish. Twenty four to 48 h later, cells were used for various experiments. Expression of HA-FLNa-C1 was analyzed in total cell lysates by immuno blotting with anti-HA antibody (Clontech, Palo Alto, CA). Cells were serum-starved for 18 h and incubated for 30 min with 200 μM orthovanadate prior to 100 nM insulin treatment for 10 min.

Elk-1 Transactivation Assay—Transactivation of Elk-1 was examined by the PathDetect Elk-1 trans-Reporting System (Stratagene). In brief, HepG2 cells were cotransfected with pFR-Luc, pEA-Elk-1 and 0.2 μg of pCMV-β-galactosidase and either 5 μg of pcDNA3.1 or 3 μg of HA-FLNa-C1 expression plasmid encoding HA-FLNa-C1 were stimulated with 100 nM insulin for 24 h. Elk-1 luciferase and β-galactosidase activities were measured using assay system kits from Promega according to the manufacturer’s instructions, and the luciferase values were normalized to β-galactosidase.

Detergent-free Isolation of Lipid Rafts—Isolation of lipid rafts was accomplished using a detergent-free sucrose gradient centrifugation method as described previously (19). In brief, cells from 150-mm culture dishes were resuspended in 1 ml of 0.5 M sodium carbonate, pH 11, supplemented with a protease inhibitor mixture (Calbiochem), and homogenized by passing cells 15 times through a 23-gauge needle and two 10-s bursts of a sonicator probe on ice. The sucrose concentration in centimeters (w/w) (w) by the addition of 1.8 volume of 70% (w/w) sucrose prepared in MBS containing 0.25M sodium carbonate. At the bottom of an ultracentrifuge tube, 2 ml of the extracts were placed, followed by the addition of 5 ml of 35% (w/w) and 4 ml of 5% (w/w) sucrose prepared in MBS containing 0.25 M sodium carbonate. After centrifugation at 200,000 × g for 16 h at 4° C in a Beckman SW-41 rotor, a total of 11 fractions (1 ml each) were collected from the top of each gradient and used for immunoblotting.

Immunoprecipitation and Immunoblotting—Unless otherwise indicated, cells were scraped in a lysis buffer (20 mM Hepes, pH 7.4, 137 mM NaCl, 100 mM NaF, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 0.02% Na3VO4, and 1 mM sodium orthovanadate) supplemented with 10% glycerol, 1 mM EDTA, 2 mM dithiothreitol, and protease inhibitor mixture, and the clarified supernatants were incubated with 1 μg of each clone, IRS-1 (5 μg), Shc (5 μg), or phosphotyrosine (5 μg) antibody at 4° C overnight. Alternatively, to detect FLNa-IR association, cells were solubilized in TBL buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.02% sodium deoxycholate, 1% methyl cellulose, 1% glycerol, 1 mM EDTA, 2 mM dithiothreitol, and protease inhibitor mixture), and the clarified supernatants were incubated with IR antibodies. Then, protein A/G-agarose (Oncogene Science) beads were added, and the incubation was continued at 4° C for 4 h. The beads were pelleted by centrifugation and washed twice in lysis buffer and twice in 50 mM Hepes, pH 7.4, supplemented with 0.1% Triton X-100. Cell lysates were incubated in Laemmli sample buffer (20) supplemented with 5% 2-mercaptoethanol. In some experiments, cells were lysed directly in Laemmli sample buffer containing 5% 2-mercaptoethanol and 1 mM orthovanadate. After heating at 70° C for 10 min, proteins were separated by
The effect of FLNa expression on insulin-mediated tyrosine phosphorylation of the IR, IRS-1, and Shc in human melanoma cells. FLNa-deficient M2 cells and FLNa-repleted A7 cells were serum-starved for 18 h and then left alone (A) or incubated with 200 μM orthovanadate for 30 min (B and C) followed by the addition of 100 nM insulin for 10 min. Cell extracts were immunoprecipitated (Ip) with anti-IR (A), IRS-1 (B), or phosphotyrosine (PY) antibody (C). The immuno pellets were resolved by SDS-PAGE and immunoblotted with RC20 (PY) and anti-IR α-subunit (A), RC20, IRS-1, and p85 subunit of PI3-kinase (B), and anti-Shc antibodies (C). Shown is a representative experiment that was repeated four times with similar results.

Measurement of Thymidine Incorporation—M2 and A7 cells were grown to 75% confluence in 6-well cluster plates. The growth medium was replaced with serum-free MEM for 18 h. Cells were stimulated with insulin at the indicated concentrations (0, 1, 10, and 100 nM) for 18 h and then pulsed with [3H]thymidine (PerkinElmer Life Sciences), 2 μCi/ml, for 2 h at 37 °C. The cells were washed three times in ice-cold PBS, and DNA was precipitated with 10% trichloroacetic acid for 30 min on ice. After a rapid wash, trichloroacetic acid-precipitable material was dissolved in 0.5 ml of 1 M NaOH and then neutralized with HCl.

RESULTS

Selective Impairment in Insulin Signaling in FLNa-expressing Cells—Insulin elicits rapid autophosphorylation of the IR and tyrosine phosphorylation of substrates, including IRS-1 and Shc proteins, in a number of cell lines. We examined FLNa-deficient M2 cells and FLNa-expressing subline A7 cells for their insulin responsiveness, and we found that both cell lines exhibited a comparable increase in insulin-induced IR autophosphorylation (Fig. 1A). Under basal conditions, cells that were pretreated with the protein-tyrosine phosphatase inhibitor orthovanadate had very low levels of IRS-1 and Shc tyrosine phosphorylation (Fig. 1, B and C). Addition of insulin elicited a significant increase in IRS-1 phosphorylation in vanadate-pretreated M2 and A7 cells (Fig. 1B). In contrast, the combination of insulin and vanadate strongly stimulated the levels of Shc tyrosine phosphorylation in M2 cells, while being rather ineffective in the FLNa-expressing A7 cells (Fig. 1C).
PI3-kinase is an important enzyme implicated in insulin signal transduction through its interaction with tyrosine-phosphorylated IRS proteins (21). It is a heterodimeric enzyme encompassing a p85 regulatory subunit and a catalytic subunit (p110). We therefore examined the cosedimentation of p85 in IRS-1 immunoprecipitates to determine whether p85 association to IRS-1 might be affected by FLNa expression. As shown in Fig. 1B, insulin was able to stimulate recruitment of PI3-kinase to IRS-1 both in vanadate-pretreated M2 and A7 cells. Two of the major insulin signaling events initiated downstream of the IR is activation of the PI3-kinase/AKT pathway and Ras/MAPK cascade. To evaluate the involvement of FLNa expression on the regulation of these pathways, we examined the ability of insulin to activate AKT and p42/44 MAPK (also known as extracellular signal-regulated kinase, ERK 1/2) by immunoblotting cell lysates with phospho-specific antibodies (Fig. 2). The levels of phosphorylation of AKT at Ser-473, a modification required for its activation (22), were increased upon stimulation of M2 and A7 cells with insulin. In contrast to AKT, there was a marked attenuation in insulin-induced ERK phosphorylation in FLNa-expressing A7 cells (Fig. 2 upper panel, 9th versus 2nd lane). We then investigated whether the lack of FLNa plays a role in the phosphorylation of AKT and ERK in response to other stimuli. Both cell lines were equally responsive to EGF and serum; however, the increase in ERK phosphorylation levels induced by IGF-1 in M2 cells was blocked in FLNa-expressing A7 cells (Fig. 2, upper panel). Similar amounts of ERK protein were present in either cell line (Fig. 2, lower panel). Thus, expression of FLNa differentially affects ERK regulation in response to various stimuli. These results are consistent with a selective effect of FLNa in the signaling pathway used by insulin and IGF-1 to regulate p42/44 MAPK cascade.

It is well recognized that phosphorylation of Shc by IR is necessary for activation of the Ras/MAPK pathway and mitogenesis (23). In order to investigate the ability of FLNa to modulate mitogenic responses of insulin, we incubated M2 and A7 cells with increasing concentrations of the hormone for 18 h and measured DNA synthesis. FLNa-depleted M2 cells displayed an enhanced sensitivity to insulin when compared with FLNa-expressing A7 cells (Fig. 3). Both cell lines responded to serum (10%) stimulation by increasing thymidine incorporation to the same levels. The sensitivity of insulin-dependent activation of the Shc/MAPK cascade and the subsequent mito-

![Figure 3](http://www.jbc.org/)

**Fig. 3.** The role of FLNa on insulin-induced thymidine incorporation. Subconfluent M2 and A7 cells were serum-starved for 18 h and then incubated overnight with insulin at the indicated concentrations or with 10% FBS. Cells were subsequently labeled with [3H]thymidine for 2 h, and its incorporation into DNA was measured as described under "Experimental Procedures." Results show the mean ± S.E. from a representative experiment performed in quadruplicate wells. Comparable results were obtained in three other independent experiments. *, **, p < 0.05 and 0.01, respectively.

![Figure 4](http://www.jbc.org/)

**Fig. 4.** Effects of FLNa on insulin-stimulated association of signaling intermediates to membrane rafts. A, M2 and A7 cells were serum-starved overnight and then lysed directly in sample buffer. Equivalent amounts of proteins were subjected to SDS-PAGE and immunoblotted with the indicated antibodies. B, serum-starved M2 (panels I and II) and A7 cells (panels III and IV) were left untreated (panels I and III) or treated with 100 nM insulin (panels II and IV) for 10 min. Cells were lysed and fractionated using a detergent-free sucrose density gradient centrifugation method as described under "Experimental Procedures." Fractions were collected from the top of the gradient, separated by SDS-PAGE, and immunoblotted with anti-SOS1 antibody. C, lipid raft fractions (fractions 4–6) were prepared from insulin-stimulated M2 and A7 cells and immunoblotted with anti-Shc, phosphoactive ERK, total ERK, and c-Src antibodies. Shown is a representative experiment that was repeated twice with similar results.
Fig. 5. Identification of IR as an FLNa-interacting protein. A, interaction of endogenous FLNa with endogenous IR. HEK293, HepG2, M2, and A7 cells were serum-starved for 16–18 h and then left untreated or stimulated with 10 nM insulin for 5 min. Cells were lysed and subjected to immunoprecipitation (IP) with anti-IR antibody. The immune pellets were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies. B, serum-starved HepG2 cells were treated with 2 μM cytochalasin D (Cyto D) or vehicle for 30 min. Left panels, cells were lysed, and immunoprecipitation was performed with non-immune IgG (NI), anti-IR, or anti-FLNa antibodies followed by Western blot analysis for the detection of IR β-subunit and FLNa; right panels, confocal microscopy analysis of actin cytoskeleton in HepG2 cells treated or not with cytochalasin D. Actin filaments are detected with AlexaFluor 568-conjugated phalloidin. C, in situ colocalization of endogenous FLNa and IR. Untreated HepG2 cells were fixed, permeabilized, and stained for FLNa (red) and IR β-subunit (green). Bound primary antibodies were detected by Alexafluor-conjugated secondary antibodies. Cell nuclei were then counterstained with Topro-3 (blue). Yellow depicted in the merged images indicates colocalization. Bar, 10 μm.

genic responses to FLNa expression suggest specific perturbation of the signaling pathway proximal to the IR.

Effect of FLNa Expression on Cellular Redistribution of Signaling Intermediates—Translocation of the Shc adaptor from the cytosol to lipid raft microdomains leads to MAPK activation (24). Moreover, the guanine nucleotide exchange factor SOS1 is prevalently cytosolic and must be brought to the cytosolic side of the plasma membrane in close juxtaposition to Ras to allow GDP/GTP exchange and Ras activation (25). To establish whether expression of FLNa could influence the ability of signaling intermediates to be redistributed upon insulin stimulation, lipid rafts were isolated by sucrose gradient centrifugation from lysates of M2 and A7 cells and immunoblotted with specific antibodies. Preliminary studies showed that both cell types contained similar levels of signaling molecules, such as Shc, SOS1, ERK, and c-Src (Fig. 4A). Light fractions, enriched in rafts, and heavy fractions, containing most soluble proteins, were first tested by immunoblot with anti-SOS1 antibodies. As shown in Fig. 4B (panels I and III), endogenous SOS1 was found only in the heavy fractions of unstimulated cells, demonstrating that SOS1 is predominantly in the cytosol. However, addition of insulin resulted in inducible localization of SOS1 in the light fractions of FLNa-depleted M2 cells but not from FLNa-expressing A7 cells (Fig. 4B, panel II versus IV). The latter cells were found to have also impaired translocation of Shc, ERK, and phosphoactive ERK to the membrane rafts following insulin treatment (Fig. 4C). Of importance, FLNa expression did not abrogate the levels of c-Src, and presumably, of other raft-associated proteins, in these specialized membrane microdomains (Fig. 4C, bottom panel). These results are consistent with the hypothesis that FLNa plays an important role in insulin-dependent Shc/MAPK cascade signaling at a step proximal to the IR.

The IR Is an FLNa-interacting Protein—FLNa interacts with a number of cell surface proteins and intracellular signaling molecules (6). We examined whether IR could form a complex with FLNa in intact cells. Our data demonstrated that IR was associated constitutively with FLNa in A7 cells but not in M2 cells that lack FLNa (Fig. 5A, right panels). Insulin treatment did not cause a significant change in the formation of the IR-FLNa complex in A7 cells. Similar results were obtained in HEK293 cells and HepG2 hepatoma whereby IR-FLNa association was detected under basal and insulin-stimulated conditions (Fig. 5A, left panels). In light of previous evidence supporting the actin-binding properties of FLNa, we sought to examine the role of cytoskeletal organization in the regulation of IR-FLNa association. To this end, HepG2 hepatoma were treated with cytochalasin D, an agent that causes depolymerization of actin (26), followed by reciprocal immunoprecipitation/immunoblotting assays as well as fluorescence microscopy. The inhibitor failed to block FLNa association to the IR, although it blocked the filamentous pattern of actin staining along the longitudinal axis of the cell (Fig. 5B, left panels). Thus, it would appear that the functional pattern of filamentous actin does not regulate IR-FLNa interaction.

To verify independently that a population of the IR is colocalized with FLNa, HepG2 hepatoma were stained with antibodies to FLNa and IR β-subunit. As shown in Fig. 5C, both FLNa and IR in the basal state exhibited a prominent plasma membrane staining.
HepG2 cells were treated with or without insulin, and the also to cosediment with recombinant IR (Fig. 6, association of IR with endogenous FLNa (Fig. 7 analyzed by immunoblotting for the presence of FLNa. GST-IR tagged cytoplasmic domain of the IR (Fig. 7 is the quantitative data of four independent observations. We then explored the role of FLNaCT in the activation of IR-mediated downstream signaling events. HepG2 cells that were transfected with either pcDNA or FLNaCT displayed no detectable difference in basal and insulin-induced AKT phosphorylation, whereas the phosphorylation levels of ERK were significantly increased in the FLNaCT-transfected cells (Fig. 7D). Similarly, expression of FLNaCT in HEK293 cells consistently led to far greater insulin-induced phosphorylation of ERK, but not AKT, compared with the corresponding control vector-expressing cells (data not shown). It is therefore unlikely that FLNa is involved in regulating the activation of the PI3-kinase/AKT pathway by insulin.

Next, to determine whether IR could be a direct FLNa-interacting partner, lysates from HepG2 cells were incubated with a GST fusion protein containing the cytoplasmic domain of the human IR (amino acids 941–1343) (GST-IRβ) and analyzed by immunoblotting for the presence of FLNa. GST-IRβ associated with FLNa, whereas GST protein alone was unable to interact (Fig. 6, lane 6). Incubation with GST-tagged SH2SH2SH3 domain of PLCγ1 did not result in the recruitment of FLNa (data not shown). Purified chicken gizzard FLNa was then tested for its ability to bind GST-IRβ and was found also to cosediment with recombinant IR (Fig. 6, lane 8). Taken together, these data indicate that IR interacts with FLNa and may be physiologically relevant.

Expression of the FLNa C-terminal Region Potentiates Insulin-mediated MAPK Activation—In order to determine whether the C-terminal region of FLNa is required for the interaction with IR and the regulation of insulin downstream signaling, an FLNa construct comprising the C-terminal repeat regions Arg22–Arg24 (amino acids 2357–2647) was fused to the HA epitope (HA-FLNaCT) and transiently expressed in HepG2 cells (Fig. 7A). Although ectopic expression of FLNaCT blocked the association of IR with endogenous FLNa (Fig. 7B, upper panel), it did not affect the extent of IR autophosphorylation elicited by insulin (Fig. 7B, middle panel). Shown in Fig. 7C is the quantitative data of four independent observations. We then explored the role of FLNaCT in the activation of IR-mediated downstream signaling events. HepG2 cells that were transfected with either pcDNA or FLNaCT displayed no detectable difference in basal and insulin-induced AKT phosphorylation, whereas the phosphorylation levels of ERK were significantly increased in the FLNaCT-transfected cells (Fig. 7D).

The present data provide evidence that expression of the actin-binding protein FLNa promotes selective deactivation of insulin signaling events that regulate MAPK phosphorylation and activation. The inhibition of insulin-stimulated MAPK phosphorylation was deemed to be specific because FLNa expression did not interfere with other insulin-induced signals, such as those that lead to IR and IRS-1 tyrosine phosphorylation and activation of the PI3-kinase/AKT cascade. Various growth factors such as IGF-1, EGF, and serum have been used to demonstrate the relative selectivity of FLNa expression in insulin signaling. Consistent with its role as a negative regulator of insulin-mediated activation of MAPK, FLNa expression potently inhibited the ability of insulin to induce Shc tyrosine phosphorylation and translocation of various signaling intermediates (e.g., Shc, SOS, and MAPK) to membrane raft-like A7 cells. Physical association between FLNa and the IR was demonstrated but was found to be blocked by overexpression of a C-terminal fragment of FLNa (FLNaCT) in the human liver-derived HepG2 cells. Finally, our results show a selective potentiation of insulin-mediated phosphorylation of MAPK and Elk-1 transcriptional activity in HepG2 cells expressing FLNaCT. Increase in Elk-1 transcriptional activity is thought to be an essential event for the initiation of numerous physiological responses (28, 29). These observations and those showing that FLNaCT expression leads to alteration in cell morphology and increased membrane ruffle formation would suggest that FLNa is a possible modulator of insulin action.
the FLNa effects toward the insulin/IGF-1 responses and support our observations indicating that FLNa expression selectively impairs insulin-dependent thymidine incorporation, while having no effect on serum-stimulated DNA synthesis. There is evidence to suggest that TNF-stimulated MAPK activation is potently inhibited in M2 cells (30), whereas heregulin efficiently stimulates MAPK in both M2 and A7 cells (8). On the other hand, the G protein-coupled extracellular calcium-sensing receptor can activate MAPK very efficiently as a result of specific interaction between FLNa and the calcium-sensing receptor (31, 32). These observations raise the interesting possibility that although FLNa is dispensable for MAPK activation in response to EGF or serum, it might be required at some regulatory step proximal to the highly homologous receptors for insulin and IGF-1, possibly in cooperation with a subset of signal transducing molecules converging to MAPK (33).

FIG. 7. Expression of FLNa C-terminal fragment leads to specific increase in insulin-stimulated ERK activation in HepG2 cells. Cells were transiently transfected with empty vector or HA-tagged FLNaCT for 24 h, serum-starved for 18 h, and then left untreated or stimulated with 100 nM insulin for 10 min. Cell lysates were directly analyzed by SDS-PAGE and immunoblotted with anti-HA antibody (A) or immunoprecipitated (Ip) with control IgG (NI) or anti-IR antibody followed by immunoblot analysis using anti-FLNa, RC20 and IR β-subunit antibodies (B). C, bands corresponding to FLNa were quantitated by laser densitometry, and a value of 1.0 was assigned to pcDNA-transfected, untreated cells. Open bars, no insulin; filled bars, with insulin. Data are mean ± S.E. from 4 independent experiments. D, control and HA-tagged FLNaCT-transfected HepG2 cells were serum-starved overnight, treated with 200 μM orthovanadate for 30 min, and then stimulated or not with 100 nM insulin for 10 min. Cell lysates were separated by SDS-PAGE and immunoblotted with phospho-AKT and phospho-ERK (upper panel), HA (middle panel), and total ERK 1/2 antibodies (bottom panel). Shown is a representative experiment that was repeated three times.

The role of Shc in insulin-induced mitogenic signaling follows a path that has been well characterized. The adaptor Shc binds via its PTB domain to the juxtamembrane region of the activated IR (34), which then leads to Shc tyrosine phosphorylation and subsequent association with preformed Grb2-SOS complex to the plasma membrane where Ras is known to reside (35). Because of the role of Shc in linking the activated IR to the Ras/MAPK pathway via the guanine nucleotide exchange factor SOS, elucidation of the mechanisms by which FLNa impairs Shc tyrosine phosphorylation is key for understanding the mitogenic signaling of insulin. Localization to membrane rafts is required for Shc activity (36). Indeed, addition of the membrane localization sequence for Ras leads to constitutive Shc targeting to the plasma membrane, thus resulting in ligand-independent activation of the Ras/MAPK cascade (24). This is somewhat consistent with our observation that FLNa-dependent interactions might inhibit insulin-induced Shc tyrosine phosphorylation due to impaired translocation of Shc to membrane rafts where the IR is known to reside. It is noteworthy that the ability of insulin to promote redistribution of SOS and MAPK to lipid rafts is also blocked as a consequence of FLNa expression in A7 cells. This argues well for the role of FLNa in governing insulin-dependent signaling events via regulation of protein trafficking.

FLNa is an actin-binding protein expressing a number of repeats that have been shown to associate with receptors or signaling proteins (reviewed by Stossel et al. (6)). For instance, FLNa binds Smads, a group of proteins necessary for the regulation of TGF-β signaling (37). Many proteins known to bind FLNa appear to be functionally linked to downstream signaling components via the actin cytoskeleton. As such, FLNa is a determinant of the submembranous cytoskeletal architecture of cells, and consistent with this role, it has an important function in the endocytic sorting and recycling pathways (38, 39). The presence of filamin has been reported to be necessary for translocation of the 5-phosphatase SHIP-2 at the membrane ruffles and the cortical actin rim at the periphery of the COS-7 cells after EGF stimulation (40). Similarly, FLNa may
be required for proper cell surface expression of the D2 dopamine receptors (41). On the other hand, ligand-induced movement of the human androgen receptor from the cytoplasm to the nucleus is a process that requires FLNa (42). Unlike these FLNa-binding proteins, the association between the IR to FLNa does not appear to be obviously related to intact actin cytoskeletal assembly as actin depolymerization with cytochalasin D failed to disrupt the constitutive IR-FLNa interaction.

Immunofluorescence studies revealed that transiently expressed FLNa CT colocalizes with the IR at the surface of HepG2 cells. Whereas FLNa CT expression blocks the constitutive association between endogenous FLNa and the IR, it potentiates insulin-induced MAPK phosphorylation and Elk-1 transcriptional activity when compared with pcDNA-transfected cells treated with insulin. However, FLNa CT had no effect on insulin-induced IR or IRS-1 tyrosine phosphorylation and an increase in AKT phosphorylation elicited by insulin. It is therefore likely that overexpressed FLNa CT titrates inhibitory proteins out of endogenous signaling complexes involved in MAPK cascade activation, thereby potentiating insulin signal transduction. In light of these results, what role might FLNa play in constitutive and IR-mediated activation of MAPK? With the exception perhaps of a weak binding of MEK-1 to FLNa in two-hybrid assays (30), no component of MAPK-activating pathways other than the IR, as demonstrated here, has been shown to bind to FLNa to date. Thus, it is possible that alteration in FLNa expression might affect the signaling potential of the IR at a step proximal to the receptor, namely the tyrosine phosphorylation of a subset of protein substrates and their recruitment to appropriate location. Alternatively, a subpopulation of the IR could potentially couple to the MAPK pathway, at least through its interaction with FLNa, whereas the receptor in other subcellular locations might interact with additional scaffold proteins that enable it to modulate the IRS-1/PI3-kinase/AKT pathway.

The localization of FLNa CT to membrane ruffles in HepG2 hepatoma may enable it to function as a scaffold upon which reorganization of membrane actin cytoskeleton and cell morphology can take place. Activation of the small GTPases of the Rho family and their downstream targets, Pak1s, produces phenotypic changes consistent with actin cytoskeletal rearrangements. Notably, FLNa interacts directly with Trio, a unique protein that contains three guanine nucleotide exchange factors (Fig. 9).

**Fig. 8.** Insulin-induced Elk-1 transactivation in HepG2 cells. HepG2 cells were transiently transfected with empty vector or HA-tagged FLNa CT together with pFR-Luc, pFA-Elk-1, and pCMV-β-galactosidase and then rendered quiescent by serum deprivation. Following stimulation with 100 nM insulin for 24 h, the lysates were prepared from these cells, and the activities of luciferase and β-galactosidase were determined. Normalized transactivation value (luciferase/β-galactosidase activities) for the unstimulated vector control was assigned a value of 1. Data represent the means ± S.E. from a representative experiment performed in triplicate dishes. Similar results were obtained in a second separate experiment. *, p < 0.05 versus unstimulated control; **, p < 0.01 versus insulin-stimulated pcDNA control.

**Fig. 9.** Subcellular distribution of FLNa CT in HepG2 cells. Cells were transiently transfected with empty vector or HA-tagged FLNa CT and then rendered quiescent by serum starvation. Cells were fixed, permeabilized, and stained for F-actin with Alexafluor-488 conjugated phalloidin (B, D, and F) or anti-IR β-subunit (H). Transfected HA-tagged FLNa CT was stained with anti-HA (A, C, E, and G). Areas of colocalization at the cell surface are depicted in the merged image (I). Arrow, localization of FLNa CT in the ruffles of transfected cells. Data are representative of two experiments. Bar, 15 μm.
Rho GEF (43), and Pak1 (8). It remains to be determined if FLNa regulates the assembly of signaling complexes after activation of the IR. Although insulin treatment did not appear to change the binding of endogenous and transfected FLNa to the IR in HepG2 hepatoma and A7 cells, respectively, these experiments do not rule out a signal-dependent posttranslational modification of FLNa. In this regard, p56lck has been proposed to phosphorylate filamin, thus controlling its association with cell surface receptors such as β2 integrins and actin filament cross-linking (44). Moreover, Pak1 controls the actin cytoskeletal and ruffle formation, in part by phosphorylating FLNa (8). FLNa has also been found to undergo phosphorylation in situ in response to serum, lysophosphatidic acid, and other stimuli (45). Thus, it is likely that functional alterations in FLNa might occur as a consequence of phosphorylation events. Incidentally, phosphorylation of IRS-1 on tyrosine residues allows only one of many possible pathways leading to the control of MAPK activation by insulin.

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Interaction of Filamin A with the Insulin Receptor Alters Insulin-dependent Activation of the Mitogen-activated Protein Kinase Pathway
Hua-Jun He, Sutapa Kole, Yong-Kook Kwon, Michael T. Crow and Michel Bernier

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