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NCS-1 Inhibits Insulin-stimulated GLUT4 Translocation in 3T3L1 Adipocytes through a Phosphatidylinositol 4-Kinase-dependent Pathway*

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Expression of NCS-1 (neuronal calcium sensor-1, also termed frequenin) in 3T3L1 adipocytes strongly inhibited insulin-stimulated translocation of GLUT4 and insulin-responsive aminopeptidase. The effect of NCS-1 was specific for GLUT4 and the insulin-responsive aminopeptidase translocation as there was no effect on the trafficking of the cation-independent mannose 6-phosphate receptor or the GLUT1 glucose transporter isoform. Moreover, NCS-1 showed partial colocalization with GLUT4-EGFP in the perinuclear region. The inhibitory action of NCS-1 was independent of calcium sequestration since neither treatment with ionomycin nor endothelin-1, both of which elevated the intracellular calcium concentration, restored insulin-stimulated GLUT4 translocation. Furthermore, NCS-1 did not alter the insulin-stimulated protein kinase B (PKB/Akt) phosphorylation or the recruitment of Cbl to the plasma membrane. In contrast, expression of the NCS-1 effector phosphatidylinositol 4-kinase (PI 4-kinase) inhibited insulin-stimulated GLUT4 translocation, whereas cotransfection with an inactive PI 4-kinase mutant prevented the NCS-1-induced inhibition. These data demonstrate that PI 4-kinase functions to negatively regulate GLUT4 translocation through its interaction with NCS-1.

Neuronal calcium sensor 1 (NCS-1)[1] is one member of a large family of calcium-binding proteins that includes frequenin, recoverin, guanylate cyclase-activating proteins, neurocalcin, and visinin (1–4). This family of proteins has been linked to the regulation of various intracellular events including phototransduction, neurotransmitter release, control of cyclic nucleotide metabolism, gene expression, ion channels function, and phosphoinositide metabolism. For example, *Drosophila* mutants overexpressing frequenin display a frequency-dependent facilitation of neurotransmitter release at the neuromuscular junction (5). Similarly, overexpression of frequenin in PC12 cells potentiates agonist (ATP)-stimulated secretion of growth hormone (6). These data suggest that NCS-1/frequenin may function as a positive regulator of vesicle exocytosis. However, a negative role for NCS-1 was observed in Madin-Darby canine kidney cells, where overexpression of NCS-1 inhibited apical membrane transport without affecting trafficking to the basolateral membrane (7).

Recently the yeast homologue of frequenin (Frq1p) was reported to function as a calcium-sensing subunit of Pik1p, a phosphatidylinositol 4-kinase (8). Pik1p is an essential enzyme necessary for normal secretion, Golgi membrane traffic, and vacuole membrane dynamics and endocytosis in yeast (9, 10). In these cells, Pik1p mutants exhibit severe protein trafficking defects and accumulate morphologically aberrant Golgi membranes (9, 11). In mammalian cells, NCS-1 directly interacts with the phosphatidylinositol 4-kinase β isoform, the human homologue of yeast Pik1p (12). Furthermore, the binding of NCS-1 stimulates the lipid kinase activity of phosphatidylinositol (PI) 4-kinase β (13).

Previously, it was observed that highly purified GLUT4-containing vesicles from both rat adipocytes and skeletal muscle were highly enriched for PI 4-kinase activity (14, 15). Because NCS-1 appears to function as a regulatory subunit of the PI 4-kinase and has been implicated in the control of several membrane transport processes, we have examined the function of NCS-1 in the regulation of insulin-stimulated GLUT4 translocation. In this study, we demonstrate that overexpression of NCS-1 in 3T3L1 adipocytes inhibits GLUT4 translocation through its interaction with PI 4-kinase β.

EXPERIMENTAL PROCEDURES

Materials—Insulin, endothelin-1, dexamethasone, and 3-isobutyl-2-methylxantine were obtained from Sigma. Ionomycin (free acid) was obtained from Calbiochem. The PI 4-kinase β antibody was purchased from Upstate Biotechnology, and the NCS-1 antibody was prepared as previously described (16). Akt and the phosphoserine 473-specific Akt antibodies were purchased from New England Biolabs. The HA antibody (SC-7392) was obtained from Santa Cruz Biotechnology, and fluorescent secondary antibodies were purchased from Jackson Immunoresearch Laboratories. Horseradish peroxidase-conjugated antibodies and enhanced chemiluminescence reagents were obtained from Pierce.

Tissue Culture and Transient Transfection of 3T3L1 Adipocytes—Murine 3T3L1 preadipocytes were purchased from American Type Tissue Culture repository. Cells were cultured at 37 °C and 8% CO_2 in Dulbecco's modified Eagle's medium supplemented with 25 mM glucose, 10% bovine calf serum containing penicillin and streptomycin. Cells were differentiated into adipocytes with 1 μg/ml insulin, 1 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine as described previously (17). Fully differentiated 3T3L1 adipocytes were transfected by low voltage (0.15 V at 960 μF) as previously described (18). After electroporation, the cells were plated on collagen IV-coated glass cov-

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[1] The abbreviations used are: NCS-1, neuronal calcium sensor 1; EGFP, enhanced green fluorescent protein; PI 4-kinase, phosphatidylinositol 4-kinase; HA, hemagglutinin.

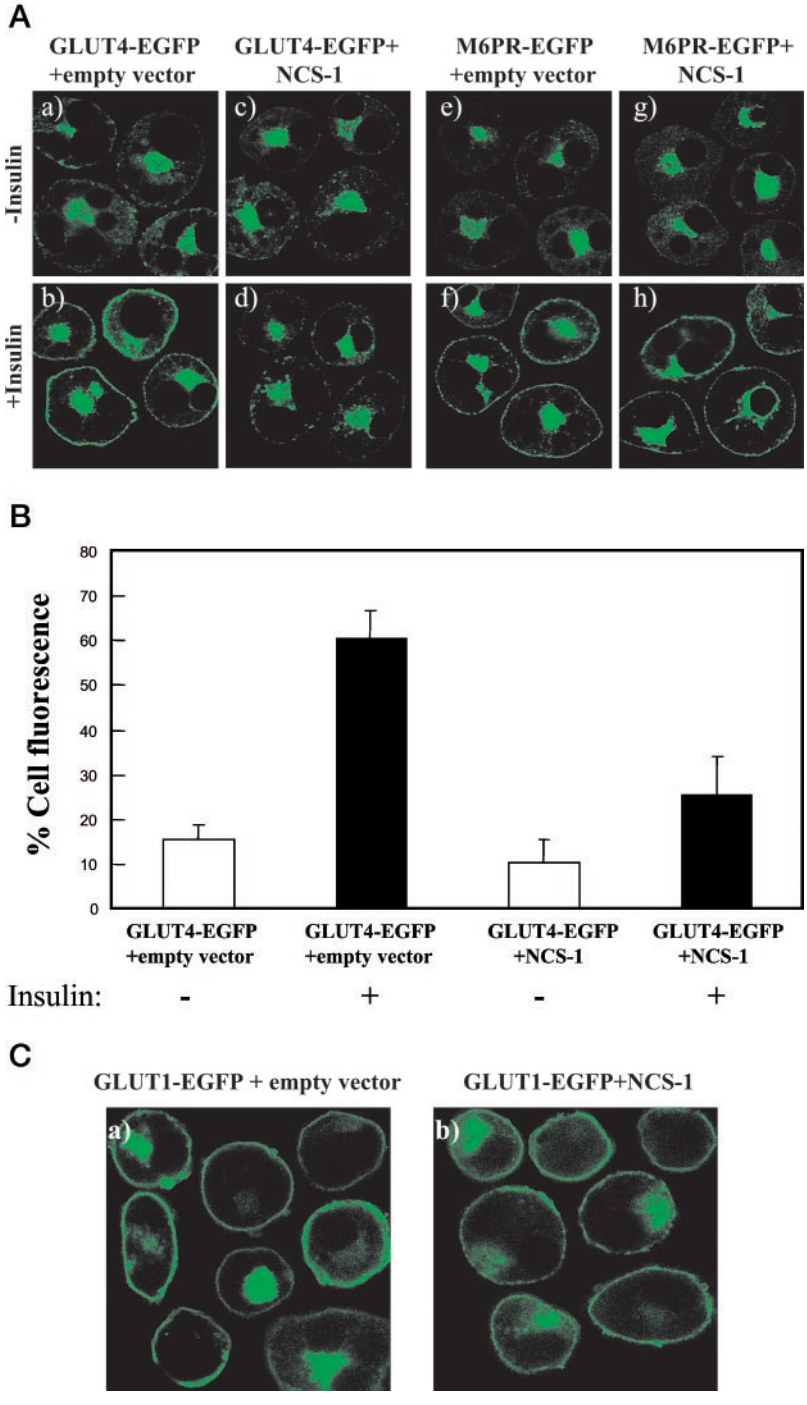

FIG. 1. **NCS-1 inhibits GLUT4-EGFP insulin-induced translocation to the plasma membrane but not mannose 6-phosphate-EGFP.** *A*, cells were electroporated with 50 μg of GLUT4-EGFP and 200 μg of NCS-1 plasmid or 50 μg of mannose 6-phosphate receptor (*M6PR*)-EGFP with 200 μg of NCS-1 plasmid as described under "Experimental Procedures." Twenty-four h after transfection, cells were serum-starved and stimulated with 100 nM insulin at 37 °C for 30 min, washed, and fixed as described under "Experimental Procedures." The images shown are representative of several experiments in cells co-expressing NCS-1 and GLUT4-EGFP. *B*, quantification of cell surface fluorescence after insulin stimulation of cells expressing GLUT4-EGFP and empty vector or GLUT4-EGFP with NCS-1. These data represent the average values ± S.D. from at least three independent experiments. *C*, cells were electroporated with 50 μg of GLUT1-EGFP and 200 μg of either empty vector or plasmid coding for NCS-1, as described under "Experimental Procedures." Twenty-four hours after transfection, cells were serum-starved and stimulated with 100 nM insulin at 37 °C for 30 min, washed, and fixed as described under "Experimental Procedures."

erslips and allowed to recover for 20–32 h in complete medium before analysis.

Immunofluorescence—The cells were incubated at 37 °C for 2 h in Dulbecco's modified Eagle's medium without serum and then either left untreated or stimulated with 100 nM insulin for 30 min. The cells were washed once with ice-cold phosphate-buffered saline and fixed/permeabilized with a solution containing 4% paraformaldehyde and 0.2% Triton X-100 for 15 min at room temperature. The samples were then washed with phosphate-buffered saline and blocked with a solution containing 1% bovine serum albumin and 5% donkey serum for 1 h at room temperature, and primary antibodies (1:50 for NCS-1 and 1:100 dilution for PI 4-kinase β) were added for 1 h at room temperature. The samples were again washed with phosphate-buffered saline, incubated with a secondary antibody conjugated to Texas Red (1:100 dilution) for 1 h, and washed, and the coverslips were mounted on Vectashield for visualization using a Zeiss LSM510 confocal microscope.

Calcium Measurements—Intracellular Ca^{+2} levels were measured using a video microscope digital image analysis system (Photon Technology International, Inc., South Brunswick, NJ) as described previously (19). Briefly, differentiated 3T3L1 cells were placed in serum-free Dulbecco's modified Eagle's medium supplemented with 30 mM Hepes, pH 7.0, and 0.2% bovine serum albumin for 2 h. FURA02 (Molecular Probes Inc, Eugene, OR) was added to the cells (1 μM) for 30 min at 37 °C, and the cells were then washed 3 times with Dulbecco's modified Eagle's medium and incubated an additional 20–30 min before measurement of Ca^{+2} levels. Basal readings were determined before stimulation with either insulin (100 nM), endothelin (100 nM), or ionomycin (1 μM) and recorded at 37 °C every 10 s over a 15–20-min time range.

Immunoprecipitation and Western Blot Analysis—After transfection of the differentiated 3T3L1 adipocytes, whole cell extracts were prepared by scraping the cells in lysis buffer (25 mM Hepes, pH 7.5, 1% Nonidet P-40, 5% glycerol, 130 mM NaCl, 50 mM NaF, 10 mM $Na_2HP_2O_4$, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin). Protein content in the extracts was assayed by the BCA method (Pierce), and for immunoprecipitation, 400 μg of protein were incubated with 6 μg of the HA

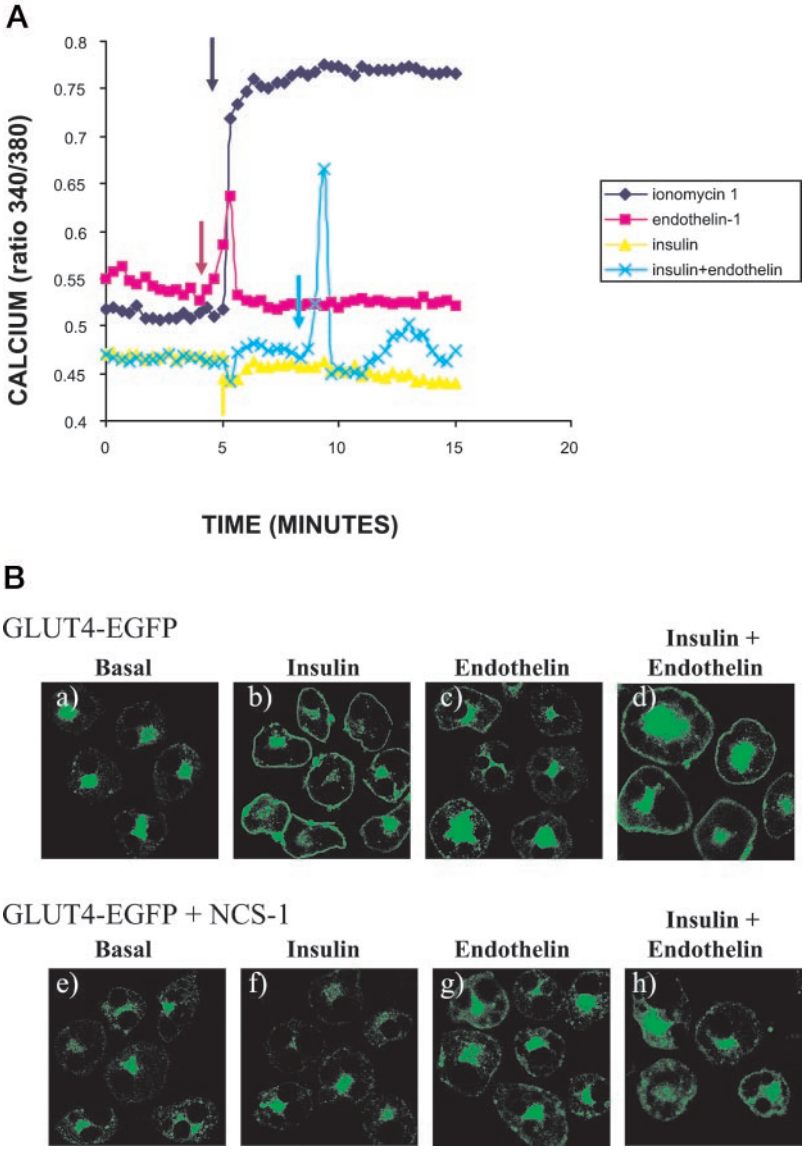

FIG. 2. **Ionomycin and endothelin-1 but not insulin increase intracellular calcium in 3T3L1 adipocytes.** *A*, cells were loaded with FURA02 as described under "Experimental Procedures." Basal calcium content was taken and averaged for several cells, then cells were stimulated with 100 nM endothelin-1, 100 nM insulin, or 1 μM ionomycin, and calcium measurements were obtained at different times. The *arrows* indicate the time of the stimulation. *B*, cells were transfected with either GLUT4-EGFP (50 μg) and empty vector (200 μg) or GLUT4-EGFP (50 μg) and NCS-1 (200 μg) as described under "Experimental Procedures." Cells were serum-starved for 2 h before stimulation with 100 nM insulin alone or insulin and endothelin-1 (100 nM). Cells were subsequently washed and fixed as described under "Experimental Procedures." Only cells positive for both GLUT4-EGFP and NCS-1 were examined for their responsiveness to hormone treatment.

antibody coupled to goat anti-mouse IgG-agarose beads or with 8 μg of the NCS-1 polyclonal antibody coupled to protein A beads. For Western blotting 6 μg of protein were separated by SDS-PAGE. After immunoprecipitation and SDS-polyacrylamide gel electrophoresis, the samples were then transferred to nitrocellulose membrane and immunoblotted for NCS-1, PI 4-kinase, GLUT4, caveolin-1, and flotillin.

RESULTS

Overexpression of NCS-1 Inhibits Insulin-stimulated GLUT4 Translocation in 3T3L1 Adipocytes—To examine the potential effect of NCS-1 on insulin-stimulated GLUT4 translocation, we co-transfected 3T3L1 adipocytes with NCS-1 and a GLUT4-EGFP fusion reporter protein or with GLUT4-EGFP plus an empty vector (Fig. 1*A*). As typically observed, cells that were transfected with GLUT4-EGFP and an empty vector exhibited a robust translocation of GLUT4-EGFP from intracellular storage sites to the plasma membrane upon insulin stimulation (Fig. 1*A*, *panels a* and *b*). Although expression of NCS-1 had no significant affect on the intracellular localization of GLUT4-EGFP in the basal state, there was a marked inhibition of insulin-stimulated GLUT4-EGFP translocation to the plasma membrane (Fig. 1*A*, *panels c* and *d*). Quantitation of the number of cells displaying a plasma membrane rim fluorescence indicated that in the empty vector transfected cells, insulin-stimulated GLUT4 translocation from ~15% to greater than 60% (Fig. 1*B*). In contrast, NCS-1 expression had no significant affect in the basal state but reduced the number of cells displaying insulin-stimulated GLUT4 translocation to 25% (Fig. 1*B*).

The insulin-responsive aminopeptidase is another marker for insulin-responsive vesicular compartments, as this protein co-localizes with GLUT4 and undergoes a nearly identical pattern of insulin-stimulated plasma membrane translocation (20–22). Similar to GLUT4, expression of NCS-1 also inhibited the insulin-stimulated translocation of insulin-responsive aminopeptidase to the plasma membrane (data not shown). The cation-independent mannose 6-phosphate/insulin-like growth factor-2 receptor is localized to compartments distinct from GLUT4 and insulin-responsive aminopeptidase but can also undergo an insulin-stimulated translocation to the plasma membrane (23, 24) (Fig. 1*A*, *panels e* and *f*). The ability of insulin to simulate cation-independent mannose 6-phosphate receptor plasma membrane translocation was also not significantly affected by the co-expression of NCS-1 (Fig. 1*A*, *panels g* and *h*). Furthermore, the co-expression of NCS-1 with GLUT1-EGFP did not prevent its normal basal state (data not shown) or insulin-stimulated trafficking to the plasma membrane (Fig. 1*C*). Together, these data demonstrate that NCS-1 overexpression inhibits the insulin-stimulated translocation of GLUT4-

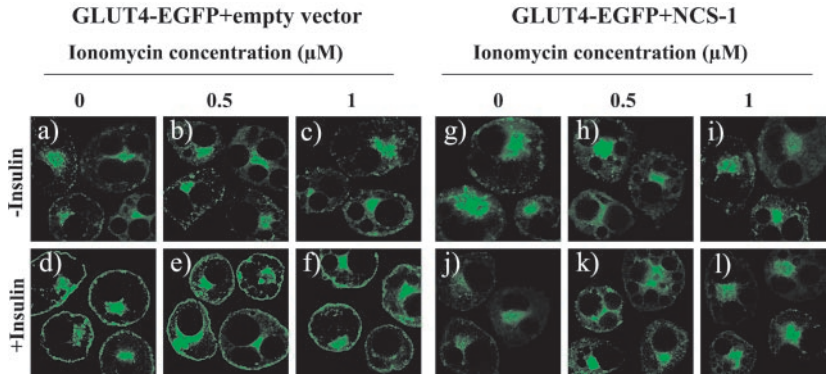

FIG. 3. **Ionomycin does not rescue insulin-induced GLUT4-EGFP translocation in cells expressing NCS-1.** Cells were transfected with either GLUT4-EGFP (50 μg) and empty vector (200 μg) or GLUT4-EGFP (50 μg) and NCS-1 (200 μg) as described under "Experimental Procedures." Cells were serum-starved for 2 h before stimulation with 100 nM insulin or insulin plus ionomycin at 0.5 or 1 μM. Pictures shown are representatives of several cells obtained from at least two independent experiments. Only cells positive for both GLUT4-EGFP and NCS-1 were examined for their responsiveness to insulin.

containing vesicles but not other insulin-responsive intracellular compartments or constitutive plasma membrane protein transport. Thus, we conclude that the effect of NCS-1 was relatively specific for GLUT4/insulin-responsive aminopeptidase compartments.

NCS-1 Inhibition of GLUT4 Translocation Is Specific for Insulin and Is Not Mediated through Chelation of Intracellular Calcium—Although calcium is not a mediator of insulin-stimulated GLUT4 translocation or glucose uptake, intracellular calcium levels are permissive because very low levels or very high levels prevent insulin action (25, 26). More recently, calmodulin inhibitors and chelation of intracellular calcium have been shown to block insulin-stimulated GLUT4 translocation and glucose uptake in 3T3L1 adipocytes (27–29). Because NCS-1 contains four EF-hand domains that can bind calcium, it was possible that the inhibition of insulin-stimulated GLUT4 translocation was due to the chelation intracellular calcium. To test this possibility, we first examined the ability of insulin, endothelin-1, and the calcium ionophore ionomycin to increase calcium levels in 3T3L1 adipocytes. As expected, insulin treatment had no significant effect on the steady-state calcium levels in adipocytes (Fig. 2*A*). In contrast, endothelin-1 stimulation resulted in a transient increase in intracellular calcium that also occurred in the presence of insulin. Treatment with ionomycin not only induced a larger increase in intracellular calcium levels but was persistent throughout the entire time course examined.

Having established that both endothelin-1 and ionomycin treatment result in increased cytosolic calcium in 3T3L1 adipocytes, we next examined the effect of NCS-1 under these conditions (Fig. 2*B*). As previously observed, insulin treatment resulted in the translocation of the GLUT4-EGFP reporter protein that was markedly inhibited upon co-expression with NCS-1 (Fig. 2*B*, *panels a*, *b*, *e*, and *f*). Endothelin-1 also has been reported to induce the translocation of GLUT4, but to a substantially reduced extent compared with insulin (30, 31). Similarly, we observed a small effect of endothelin-1 on the subcellular redistribution of GLUT4-EGFP that was unaffected by expression of NCS-1 (Fig. 2*B*, *panels c* and *g*). Furthermore, pretreatment of the adipocytes with endothelin-1 followed by insulin also induced GLUT4-EGFP translocation to a similar extent as insulin stimulation alone (Fig. 2*B*, *panel d*). However, in the presence of NCS-1, pretreatment with endothelin-1 followed by insulin was unable to rescue the insulin-stimulated extent of GLUT4-EGFP translocation, whereas the extent of endothelin-1 stimulation remained intact (Fig. 2*B*, *panel h*).

It remained formally possible that the inability of endothelin-1 to rescue insulin-stimulated GLUT4 translocation could have been due to the transient nature of intracellular calcium increase under these conditions. To address this issue, we utilized ionomycin to induce a persistent increase in intracellular calcium levels (Fig. 3). Pretreatment with ionomycin had no significant effect on either the basal or insulin-stimulated translocation of GLUT4-EGFP (Fig. 3, *panels a–f*). As was the case for endothelin-1, ionomycin was unable to restore insulin-stimulated GLUT4-EGFP translocation in cells expressing NCS-1 despite the persistent elevation in intracellular calcium (Fig. 3, *panels g–l*). Thus, these data indicate that the inhibitory actions of NCS-1 are probably not due to the chelation of intracellular calcium.

NCS-1 Does Not Alter Insulin-stimulated Akt Activation or Plasma Membrane Recruitment of Cbl—To determine whether NCS-1 blocked GLUT4 translocation by interfering with insulin signaling pathways, we first examined the effect of NCS-1 expression on the insulin-stimulated phosphorylation of Akt (Fig. 4). HA-Akt-transfected adipocytes displayed Akt expression, as seen by an Akt immunoblot in total cell extracts (Fig. 4*A*, *lanes 1* and *2*). Similarly, cells cotransfected with HA-Akt and NCS-1 also displayed expression of both HA-Akt and NCS-1 (Fig. 4*A*, *lanes 3* and *4*). The reduced expression of HA-Akt in cells co-expressing NCS-1 was consistently observed and probably reflects competition of exogenous gene expression between these two plasmids. In any case, normalized cell extracts were immunoprecipitated with the HA antibody and examined for serine 473 phosphorylation as an indicator of Akt activation (Fig. 4*B*). As is readily apparent, insulin was equally effective in stimulating the serine phosphorylation of Akt both in the absence and presence of NCS-1 overexpression (Fig. 4*B*, *lanes 1–4*). Furthermore, NCS-1 had no significant effect on the insulin stimulation of phosphatidylinositol 3,4,5-trisphosphate formation as assessed by the plasma membrane recruitment of a EGFP-PH/Grp-1 fusion protein (data not shown). These data demonstrate that NCS-1 does not affect the insulin stimulation of insulin receptor substrate/phosphatidylinositol 3-kinase/Akt pathway.

It has been recently reported that CAP, Cbl, and the small GTPase TC10 define a second insulin-signaling pathway required for GLUT4 translocation to the plasma membrane (32–34). We next tested the effect of NCS-1 expression on this signaling pathway by monitoring Cbl recruitment to the cell surface in response to insulin. Twenty-four hours after cotransfection with HA-Cbl and NCS-1, cells were serum-starved for 2 h, insulin-stimulated, then washed and fixed as described under "Experimental Procedures." As expected in HA-Cbl and vector-transfected cells, insulin stimulated the redistribution of HA-Cbl to the plasma membrane (Fig. 5, *panels a* and *b*). Similarly, insulin stimulation of adipocytes expressing NCS-1 had a similar extent of Cbl recruitment to the plasma membrane (Fig. 5, *panels c* and *d*). Together these data indicate that the known insulin-signaling effectors thought to mediate insulin-stimulated GLUT4 translocation were not altered by the expression of NCS-1.

Expression of PI 4-Kinase Inhibits Insulin-stimulated

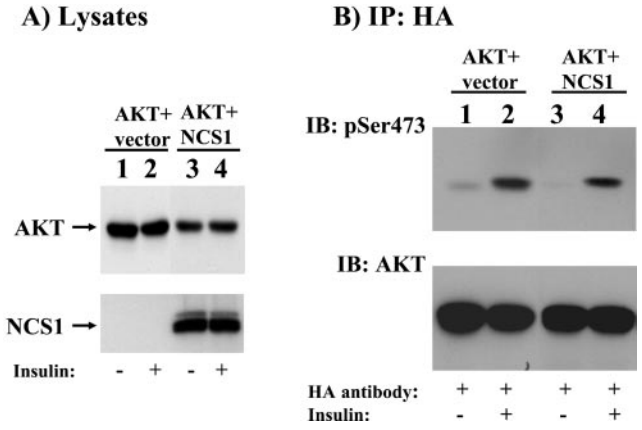

FIG. 4. **NCS-1 does not prevent activation of Akt by insulin.** Cells were electroporated with 50 μg of HA-Akt and 200 μg of either an empty vector or a plasmid encoding NCS-1 as described under "Experimental Procedures." Twenty-four hours after electroporation cells were serum-starved for 2 h, and whole cells lysates were obtained as described under "Experimental Procedures." *A*, whole cell lysates (50 μg) were loaded as a control for expression of both AKT and NCS-1. *B*, whole cell lysates (150 μg) were immunoprecipitated (*IP*) with an HA antibody, and 1/2 volume of the pellets were separated by SDS-PAGE, transferred to a nitrocellulose filter, and immunoblotted (*IB*) with specific antibodies against Akt or phosphoserine 473-Akt.

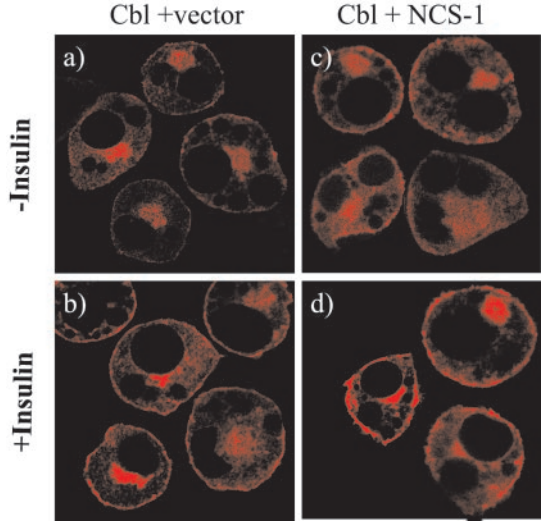

FIG. 5. **NCS-1 does not inhibit recruitment of Cbl to the plasma membrane in response to insulin.** Cells were electroporated with 50 μg of Cbl cDNA and 200 μg of either an empty vector or a plasmid encoding NCS-1 as described under "Experimental Procedures." Twenty-four hours after transfection cells were serum-starved and then stimulated with 100 nM insulin, washed, and fixed as described under "Experimental Procedures." The photographs shown are representative cells of two independent experiments.

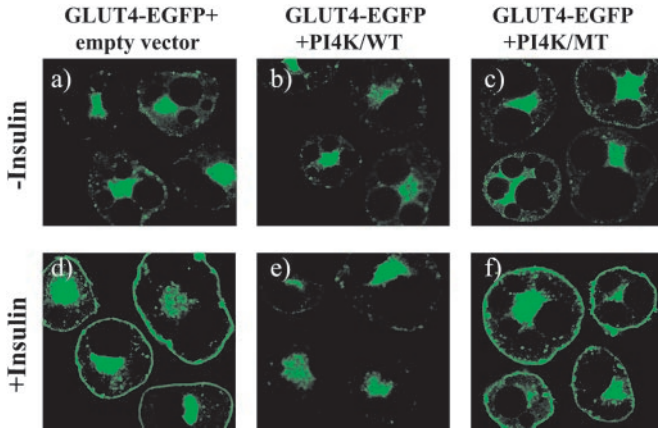

FIG. 6. **Overexpression of wild type phosphatidylinositol 4-kinase but not a kinase-deficient mutant inhibits insulin-stimulated GLUT4-EGFP translocation to the plasma membrane.** Cells were electroporated with 50 μg of GLUT4-EGFP and 200 μg of either an empty vector or a plasmid coding for wild type PI 4-kinase (*PI4K/WT*) or a plasmid encoding for a PI 4-kinase activity-deficient mutant (*PI4K/MT*) as described under "Experimental Procedures." Twenty-four hours after transfection, the cells were serum-starved and then stimulated with 100 nM insulin at 37 °C for 30 min, washed, and fixed as described under "Experimental Procedures." The photographs shown are representative of two independent experiments from cells co-expressing GLUT4-EGFP with either wild type PI 4-kinase or PI 4-kinase activity-deficient mutant.

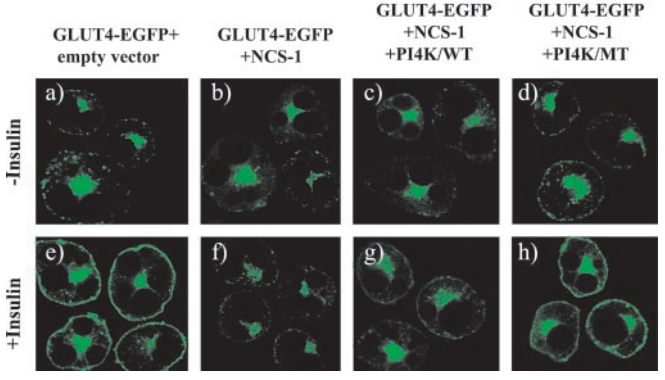

FIG. 7. **Overexpression of the PI 4-kinase mutant but not the wild type isoform rescues insulin-stimulated GLUT4-EGFP translocation to the plasma membrane.** Cells were electroporated with 25 μg of GLUT4-EGFP and 200 μg of empty vector (*panels a* and *e*), 25 μg of GLUT4-EGFP and 75 μg of NCS-1 (*panels b* and *f*), 25 μg of GLUT4-EGFP, 75 μg of NCS-1, and 150 μg of wild type PI 4-kinase (*PI4K/WT*) (*panels c* and *g*), or 25 μg of GLUT4-EGFP, 75 μg of NCS-1, and 150 μg of PI 4-kinase activity-deficient mutant (*PI4K/MT*) (*panels d* and *h*) as described under "Experimental Procedures." Twenty-four hours after transfection cells were serum-starved and then stimulated with 100 nM insulin at 37 °C for 30 min, washed, and fixed as described under "Experimental Procedures." The photographs shown are representative of two independent experiments.

GLUT4 Translocation—Recently, the yeast homologue of NCS-1 was shown to activate the lipid kinase activity of pik1p, the yeast homologue of PI 4-kinase (8). As typically observed, insulin stimulated the plasma membrane translocation of GLUT4-EGFP-expressing cells (Fig. 6, *panels a* and *d*). Expression of wild type PI 4-kinase (*PI4K/WT*) markedly inhibited insulin-stimulated GLUT4 translocation similar to that of NCS-1 overexpression (Fig. 6, *panels b* and *e*). In contrast, expression of a kinase-defective PI 4-kinase mutant (*PI4K/MT*) had no significant effect on insulin-stimulated GLUT4 translocation (Fig. 6, *panels c* and *f*). These results suggest that the interaction of NCS-1 with the PI 4-kinase is responsible for the inhibition of insulin-stimulated GLUT4 translocation.

To test this hypothesis, we next co-expressed NCS-1 with either the wild type or kinase-defective PI 4-kinase (Fig. 7). As previously observed, co-expression of NCS-1 substantially reduced the extent of insulin-stimulated GLUT4 translocation compared with cells transfected with GLUT4-EGFP alone (Fig. 7, *panels b* and *f*). Similarly, co-expression of NCS-1 with the wild type PI 4-kinase (*PI4K/WT*) also displayed a reduction in insulin-stimulated GLUT4-EGFP translocation (Fig. 7, *panels c* and *g*). In any case, co-expression of the kinase-defective PI 4-kinase (*PI4K/MT*) completely protected the cells from the NCS-1-induced inhibition of insulin-stimulated GLUT4 translocation (Fig. 7, *panels d* and *h*). Thus, these data demonstrate a functional interaction of NCS-1 with PI 4-kinase in 3T3L1 adipocytes.

Localization of NCS-1 and PI 4-Kinase and Expression during Adipogenesis—Because increased expression of NCS-1 and

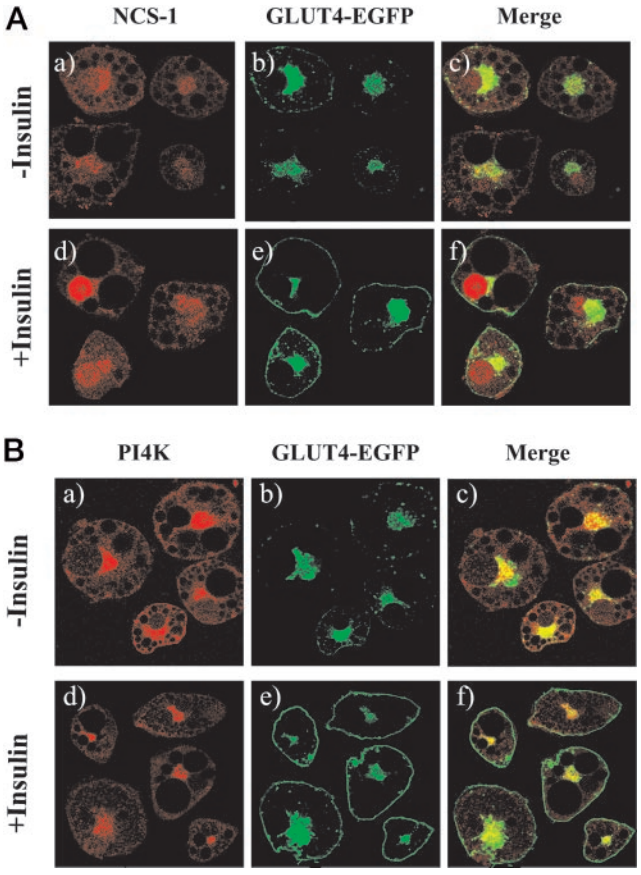

FIG. 8. **Colocalization of the endogenous PI 4-kinase and NCS-1 with GLUT4-EGFP.** Cells were electroporated with 50 μg of GLUT4-EGFP and 24 h later serum-starved and stimulated with 100 nM insulin at 37 °C for 30 min. The cells were then washed, fixed, and subjected to confocal fluorescent microscopy. *A*, immunofluorescence of endogenous NCS-1 (*panels a* and *d*) compared with GLUT4-EGFP (*panels b* and *e*). The merged images are presented in *panels c* and *f*). *B*, immunofluorescence of endogenous PI 4-kinase (*PI4K*; *panels a* and *d*) compared with GLUT4-EGFP (*panels b* and *e*). The merged images are presented in *panels c* and *f*).

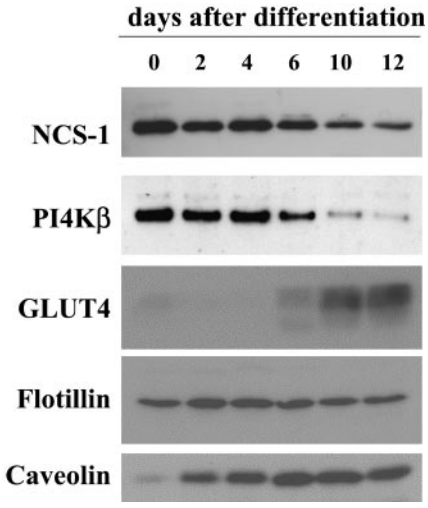

FIG. 9. **Expression of the endogenous NCS-1 and PI 4-kinase during 3T3L1 adipocyte differentiation.** Whole cell lysates were obtained from 3T3L1 cells after different stages of differentiation as indicated in the figure. Six μg of protein were separated by SDS-PAGE immunoblotted with specific antibodies for NCS-1, PI 4-kinase β (*PI4K*β), GLUT4, flotillin, and caveolin. The results shown here are representative of two independent experiments.

the PI 4-kinase are functionally inhibitory to insulin-stimulated GLUT4 translocation, we next compared the subcellular localization of the endogenous proteins with GLUT4-EGFP (Fig. 8). NCS-1 was distributed in small vesicular structures throughout the cell and in most cells was concentrated in the perinuclear region that partially colocalized with GLUT4-EGFP (Fig. 8*A*, *panels a–c*). In some cells, NCS-1 also resulted in a nuclear localization (Fig. 8*A*, *panel d*), but this is likely due to nonspecific labeling by the NCS-1 antibody, as it was not detected when NCS-1 was overexpressed (data not shown). Nevertheless, insulin treatment had no significant effect on the distribution of the endogenous NCS-1 protein, whereas GLUT4-EGFP displayed the typical plasma membrane translocation (Fig. 8*A*, *panels d–f*). Similarly, the endogenous PI 4-kinase was also distributed throughout the cell interior in small vesicular structures as well as in the perinuclear region with a more pronounced colocalization with GLUT4-EGFP (Fig. 8*B*, *panels a–c*). In addition, insulin had no significant effect on the localization of the endogenous PI 4-kinase (Fig. 8*B*, *panels d–f*).

The colocalization of NCS-1 and the PI 4-kinase with intracellular compartmentalized GLUT4 suggests that adipocytes have a mechanism to reduce their inhibitory action. We therefore examined the relative expression levels of NCS-1 and PI 4-kinase during 3T3L1 adipocyte differentiation (Fig. 9). As expected, caveolin and GLUT4 expression were markedly up-regulated during the conversion from pre-adipocytes (fibroblasts) to adipocytes, albeit with caveolin induction preceding GLUT4. On the other hand, the levels of flotillin expression were not significantly affected during the differentiation process. In contrast, both NCS-1 and PI 4-kinase were significantly down-regulated in parallel with the up-regulation of GLUT4 expression and the acquisition of insulin-responsive GLUT4 translocation. Thus, these data indicate that the reduction of NCS-1 and PI 4-kinase expression is necessary for adipocytes to acquire insulin-sensitive GLUT4 trafficking.

DISCUSSION

Recent studies demonstrate a role for NCS-1 in the potentiation of neurotransmitter release and in promoting exocytosis in adrenal chromaffin cells and PC12 cells (6). These secretory events are calcium-dependent; however, a role for NCS-1 in non-calcium-dependent secretion has not yet been examined. One of the best examples of regulated membrane transport is the insulin-stimulated translocation of the GLUT4 glucose transporter in adipocytes (36–39). This transport pathway is not regulated by changes in intracellular calcium levels but is dependent upon the insulin-stimulated formation of phosphatidylinositol 3,4,5-trisphosphate (22, 40–42).

Our data demonstrated that overexpression of NCS-1 in differentiated 3T3L1 adipocytes markedly inhibited the insulin-stimulated translocation of GLUT4 from its intracellular storage site to the plasma membrane. This inhibitory function appears specific for GLUT4 because the constitutive trafficking of GLUT1 was unaffected. In addition, insulin can also induce the plasma membrane translocation of the MP6R from compartments distinct from GLUT4 (24, 43). Consistent with a specific GLUT4 inhibitory function, NCS-1 overexpression had no effect on the insulin-stimulated translocation of MP6R.

Although calcium does not function as a mediator of insulin-stimulated GLUT4 translocation, chelation of intracellular calcium with BAPTA or blocking of calmodulin function is inhibitory (27, 28). BAPTA appeared to function by preventing the insulin stimulation of Akt activation that was reversed by elevation of intracellular calcium by treatment with the calcium ionophore A23187. Because NCS-1 can efficiently bind calcium, it was formally possible that NCS-1 was functioning similar to BAPTA. However, the inhibitory effect of NCS-1 was

not reversed by increasing intracellular calcium by either endothelin-1 or ionomycin treatment. Furthermore, NCS-1 expression had no significant effect on insulin-stimulated Akt phosphorylation. These results suggested that NCS-1 was not exerting its effects though a calcium chelation mechanism. In addition, insulin-stimulated plasma membrane recruitment of Cbl was unaffected, suggesting that both the phosphatidylinositol 3-kinase and CAP/Cbl insulin-signaling pathways were functional.

More recently, NCS-1 appears to directly interact with PI 4-kinase β and to activate its lipid kinase activity (13). In this regard, it has been reported that highly purified intracellular membrane compartments containing GLUT4 also contain PI 4-kinase activity (14, 15). Consistent with these data, both NCS-1 and PI 4-kinase partially colocalized with GLUT4. Although a specific role for PI 4-kinase activity in regulating GLUT4 trafficking has not been established, our data indicate that overexpression of wild type but not a kinase-defective PI 4-kinase β prevented insulin-stimulated GLUT4 translocation. These data are consistent with PI 4-kinase activity functioning as a negative regulatory signal in this process. Moreover, expression of the kinase-defective PI 4-kinase β restored the insulin-induced translocation of GLUT4 to the plasma membrane in cells overexpressing NCS-1. Because NCS-1 is a known activator of PI 4-kinase activity, these data strongly support a model whereby NCS-1 activation of PI 4-kinase generates an inhibitory signal specific for the insulin-stimulated GLUT4 translocation process. This conclusion is supported by the down-regulation of NCS-1 and PI 4-kinase expression that accompanies adipocyte differentiation. It remains to be determined whether or not PI-4 kinase activity prevents sorting of GLUT4 to its insulin-responsive compartment, exit from this compartment, or in the translocation process itself.

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