Evidence for a Role of the Exocyst in Insulin-stimulated Glut4 Trafficking in 3T3-L1 Adipocytes*

Received for publication, August 30, 2004, and in revised form, October 6, 2004
Published, JBC Papers in Press, November 17, 2004, DOI 10.1074/jbc.M409928200

Marie-Ann Ewart, Mairi Clarke, Susan Kane‡, Luke H. Chamberlain, and Gwyn W. Gould§

From the Henry Wellcome Laboratory of Cell Biology, Division of Biochemistry and Molecular Biology, Davidson Building, Faculty of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, Scotland, United Kingdom

Insulin stimulates glucose transport in adipocytes and muscle by inducing the redistribution of Glut4 from intracellular locations to the plasma membrane. The fusion of Glut4-containing vesicles at the plasma membrane is known to involve the target SNAREs syntaxin 4 and SNAP-23 and the vesicle SNARE VAMP2. Little is known about the initial docking of Glut4 vesicles with the plasma membrane. A recent report has implicated Exo70, a component of the mammalian exocyst complex, in the initial interaction of Glut4 vesicles with the adipocyte plasma membrane. Here, we have examined the role of two other exocyst components, rsec6 and rsec8. We show that insulin promotes a redistribution of rsec6 and rsec8 to the plasma membrane and to cytoskeletal fractions within 3T3-L1 adipocytes but does not modulate levels of these proteins co-localized with Glut4. We further show that adenosinergic-mediated overexpression of either rsec6 or rsec8 increases the magnitude of insulin-stimulated glucose transport in 3T3-L1 adipocytes. By contrast, overexpression of rsec6 or rsec8 did not increase the extent of the secretion of adipsin or ACRP30 from adipocytes and had no discernible effect on transferrin receptor traffic. Collectively, our data support a role for the exocyst in insulin-stimulated glucose transport and suggest a model by which insulin-dependent relocation of the exocyst to the plasma membrane may contribute to the specificity of Glut4 vesicle docking and fusion with the adipocyte plasma membrane.

Insulin-stimulated glucose transport in adipose cells is known to be mediated by the mobilization of intracellular glucose transporter (Glut4)-containing vesicles to the plasma membrane, where they dock and fuse, resulting in increased levels of Glut4 at the cell surface (reviewed in Ref. 1). This process is impaired in peripheral tissues from individuals with insulin resistance or type 2 diabetes (2, 3). The exocytic delivery of intracellular Glut4-containing vesicles to the plasma membrane can be conceptually resolved into distinct stages: the trafficking of Glut4 vesicles to the plasma membrane, their initial association with the plasma membrane ("docking"), and the subsequent fusion of the two lipid bilayers. Some of the molecules that are required for these distinct stages have been identified, including the SNARE proteins that mediate the terminal fusion step (see for example Refs. 1, 4, and 5). However, the machinery involved in the initial docking step has, until recently, been elusive.

Studies of the late-acting Sec mutants in yeast have identified a 19.5 S complex containing sec3p, sec5p, sec6p, sec8p, sec10p, sec15p, sec4p, and Exo70 (6–8). This complex, subsequently termed the exocyst, is localized to the tip of the yeast bud, the main site of exocytosis, and was proposed as a candidate for the initial docking/targeting interactions of exocytic vesicles with the plasma membrane (9). Subsequently, mammalian homologues of many of the proteins in this complex have been identified and shown to function in exocytic events, for example targeted exocytosis such as the delivery of transport containers to the basolateral membrane of polarized cells, and in neuronal cell exocytosis (9–15). Interestingly, a recent report identified Exo70 as a candidate molecule involved in Glut4 vesicle exocytosis in adipocytes (16). Overexpression of Exo70 was found to potentiate insulin-stimulated glucose transport. By contrast, an Exo70 mutant inhibited insulin-stimulated glucose transport but not Glut4 translocation to the plasma membrane, presumably by interfering with an initial docking stage or preventing the Glut4 vesicle from accessing the correct fusion site within the plane of the plasma membrane (16). Although compelling, these data did not address the generality of the role of Exo70 in adipocyte exocytosis, and the role of other exocyst components was not addressed.

In this study, we have examined the role of two other exocyst components, rsec6 and rsec8, in adipocyte exocytosis. We show that insulin promotes a redistribution of rsec6 and rsec8 to the plasma membrane and to cytoskeletal fractions within 3T3-L1 adipocytes but does not modulate levels of these proteins co-localized with Glut4. We further show that adenosinergic-mediated overexpression of either rsec6 or rsec8 increases the magnitude of insulin-stimulated glucose transport in 3T3-L1 adipocytes. Overexpression of rsec6 or rsec8 was without effect on the secretion of adipin or ACRP30 from adipocytes or on transferrin receptor traffic. Taken together, these data support a role for the exocyst selectively in insulin-stimulated glucose transport and is consistent with the notion of an important role for this complex in regulated exocytosis. We suggest a model by which insulin-dependent relocation of the exocyst to the plasma membrane and/or sites of insulin-signaling molecules may contribute to the specificity of Glut4 vesicle docking and fusion with the adipocyte plasma membrane.

* This work was supported by Grants 17/C12621 and 17/RE118423 from the Biotechnology and Biological Sciences Research Council (to G. W. G.), grants from The Wellcome Trust (Grant 060629 and a Research Leave Award to G. W. G. and a Fellowship to L. H. C.), and a grant from the Diabetes Research and Wellness Foundation (to G. W. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ Present address: Department of Biochemistry, Dartmouth Medical School, Hanover NH 03755, United States of America.
§ To whom correspondence should be addressed. Tel.: 44-141-330-5263; Fax: 44-141-330-4620; E-mail: G.Gould@bio.gla.ac.uk.

The abbreviations used are: SNARE, soluble NSF attachment protein receptors; NSF, N-ethylmaleimide-sensitive factor; LDM, light density microsomes; deGlc, 2-deoxy-D-glucose; IRS, insulin receptor substrate; IRAP, insulin responsive aminopeptidase.

This paper is available on line at http://www.jbc.org

Published, JBC Papers in Press, November 17, 2004, DOI 10.1074/jbc.M409928200

Printed in U.S.A.
EXPERIMENTAL PROCEDURES

Materials—Antibodies specific for Glut4 were those described in Ref. 17, and monoclonal anti-IRAP serum was provided by Professor Morris J. Birnbaum (University of Pennsylvania) (18). Porcine insulin was from Drs. Svend Harelund and Jens Dangaard (Novo Nordisk, Bagsvaerd, Denmark). All other reagents were as described in Refs. 18 and 20. Rsc6 and rsec8 cDNAs were from Dr Richard Scheller (Genentech Inc.). Antibodies against rsec6 or sec8 were from Calbiochem.

Cell Culture—3T3-L1 fibroblasts were grown and differentiated into adipocytes as described (19, 20). Cells were used between passages 2 and 12 and at days 7–12 after differentiation. Prior to experiments, cells were incubated in serum-free medium for 2 h.

Adenovirus Production and Infection—cDNAs encoding full-length rsec6 or rsec8 were amplified by PCR, subcloned into pCMV (Invitrogen), and sequenced on both strands. The constructs were subcloned into the pShuttle cytomegalovirus vector, linearized with PmeI, and co-transformed with pAdEasy into Escherichia coli strain B5183 by electroporation. Recombinants were selected and amplified in E. coli DH5α. The chosen clones were linearized with PacI to expose the inverted terminal repeat and allow viral packaging when transfected in HEK293 cells. Large scale amplification and titer of viral stocks was performed as outlined in Ref. 21.

For infection of 3T3-L1 adipocytes, cells were washed in serum-free Dulbecco’s modified Eagle’s medium containing 5 mg/ml bovine serum albumin and then incubated in the same medium containing virus overnight. Half of a 12-well plate was incubated in 0.5 ml of medium containing 20 × 10⁶ infectious particles; multiplicity of infection ~40:1). The next day, the viral-containing medium was aspirated and replaced with normal medium (Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum), and the cells were used 24 h later. In our results, this procedure routinely resulted in over 90% of the cells on a dish being infected. For most of the assays described here, we infected cells at day 6 after differentiation and thus assayed at day 8 (unless otherwise stated).

SDS-PAGE and Immunoblotting—SDS-PAGE and immunoblotting were performed exactly as described in Refs. 19 and 20. Quantification of immunoblot signals was performed as outlined by using a Bio-Rad scanner and associated software. In all cases, multiple exposures of x-ray film were analyzed to be certain of studying immunoreactive signals within the linear range of both the detection antibodies and the film.

Subcellular Fractionation and Sucrose Gradient Centrifugation—3T3-L1 adipocytes (either untreated or stimulated with 1 μM insulin for 30 min) were washed twice in ice-cold HES buffer (20 mM Hepes, 1 mM EDTA, 225 mM sucrose, pH 7.4) and then scraped into HES supplement containing a protease-inhibitor mixture (Roche Applied Science). The cells were homogenized and subjected to a differential centrifugation procedure to prepare plasma membrane and heavy and light microsome fractions, as outlined in Ref. 19. Light microsomes (LDM) contain the majority of the Glut4 that traffics to the cell surface in response to insulin. Heavy microsomes contain dense material such as the endoplasmic reticulum (see Ref. 22). Further fractionation of the LDM using velocity gradient sedimentation was performed essentially as outlined in Refs. 23 and 24. Briefly, the LDM fraction (which is known to be comprised of membranes and a cytoskeletal network, which have been resolved using this and other approaches) was resuspended in 5% sucrose in 20 mM Hepes, 1 mM EDTA, pH 7.4, and layered on top of a 5–30% sucrose gradient. The tubes were then centrifuged at 90,000 g for 90 min, and fractions were collected from the bottom of the tube by gravity. This approach separates material on the basis of size and has been shown to effectively resolve Glut4-containing membranes from the majority of proteins in the LDM fraction (including IRS proteins and cytoskeletal elements) (23, 24).

Deoxyglucose Transport—2-Deoxy-D-glucose (dGlc) transport was assayed in 12-well plates of cells. Briefly, cells were rinsed in Kreb’s Ringer phosphate buffer at 37 °C three times and then covered with 0.5 ml of the same with or without 10 μM cytochalasin B. Insulin was added at the concentrations and times shown in the figures. Transport was initiated by the addition of an aliquot of dGlc such that the final concentration was 50 μM, with 0.25 μCi/well. Transport was assayed for 2 min at 37 °C, after which cells were rapidly washed in ice-cold PBS, air-dried, and then solubilized in 1% Triton X-100. The radioactivity associated with the cells was determined by liquid scintillation spectroscopy. Nonspecific association of dGlc with the cells was determined by performing parallel assays with 10 μM cytochalasin B.

Other Assays—Adipsin secretion was assayed as outlined previously (20) using an antibody against murine adipin kindly supplied by Dr. Jess Miner (University of Nebraska). ACRP30 secretion was assayed in a similar fashion using an anti-peptide antibody raised against the C-terminal 15 amino acids of murine protein (Zymed Laboratories Inc., South San Francisco, CA). Cell surface transferrin receptor levels were determined exactly as outlined in Ref. 20.

RESULTS

We examined the subcellular distribution of rsec6 and rsec8 in membrane fractions of 3T3-L1 adipocytes treated with or without insulin (Fig. 1A). In agreement with published data (16), we consistently observed an increase in the plasma membrane content of rsec6 and rsec8 in response to acute insulin treatment; however, we also reproducibly observed increased levels of rsec6 and rsec8 associated with the LDM fraction (Fig. 1, A and B). Since the LDM fraction of 3T3-L1 adipocytes is known to comprise both membrane and cytoskeletal elements (23, 24), we further fractionated LDMs by velocity sedimentation analysis. This approach has previously been shown to effectively separate Glut4-containing membranes from the cytoskeletal and signaling elements known to populate the LDM fraction (23, 24). Fig. 1C shows that the amount of rsec6 or rsec8 associated with Glut4- or IRAP-positive membranes (fractions 6–12 in the experiment shown) is modest. Most of the immunoreactive rsec6 and rsec8 is located within fractions 11–14, which are enriched in signaling molecules such as IRS-1 and also in high levels of cytoskeletal elements such as actin and vimentin (data not shown). Although the amount of rsec6 or rsec8 associated with the membranous component of the LDMs did not change in response to insulin, we consistently observed an increase in the rsec6 and rsec8 content of the cytoskeletal fraction (compare lanes 11–14 with or without insulin). Immunoadsorption of Glut4-containing vesicles did not reveal the presence of either rsec6 or rsec8 in these structures (data not shown).

To probe the functional role of the exocyst, we developed recombinant adenoviruses to overexpress FLAG-tagged rsec6 or rsec8 in these cells. Fig. 2 shows that these viruses are capable of driving high level overexpression of these proteins in 3T3-L1 adipocytes. We calculate that our typical infection conditions overexpress rsec6 or rsec8 between 3- and 7-fold. Neither the commercial anti-sec6 or anti-sec8 antibodies nor anti-FLAG gave good staining for immunofluorescence; thus, we have been unable to localize these proteins in cells by this means.

We next assayed membrane trafficking events in cells overexpressing rsec6 or rsec8. Fig. 3 shows that neither the rate of the secretion of ACRP30 or adipsin nor cell surface levels of transferrin receptors were modulated by overexpression of these proteins. We assayed the secretion of both ACRP30 and adipsin over a range of time courses from 2 to 30 min and found no discernible differences in the extent of secretion upon overexpression of either rsec6 or rsec8 (Fig. 3A). Similarly, neither the levels of transferrin receptors at the surface of adipocytes nor the ability of insulin to promote increased cell surface transferrin receptor levels were altered by overexpression of any of the exocyst components (Fig. 3B).

We next examined the effect of overexpression of rsec6 or rsec8 on insulin-stimulated dGlc transport. As shown in Fig. 4, overexpression of rsec6 or rsec8 significantly enhanced the maximum rate of insulin-stimulated dGlc uptake. Although there was a tendency toward an increase in the rate by which insulin stimulated glucose transport, this did not reach significance in every experiment. The dose dependence of insulin-

2 M.-A. Ewart, M. Clarke, S. Kane, L. H. Chamberlain, and G. W. Gould, unpublished results.
stimulated deGlc uptake was not enhanced by overexpression of these proteins (data not shown). In control experiments, we have shown that the total cellular levels of Glut4 are unchanged after overexpression of rsec6 or rsec8.2

**DISCUSSION**

The mechanisms that govern Glut4-containing vesicle targeting, docking, and fusion at the adipocyte plasma membrane remain only partially understood. A significant advance in this regard was recently provided by Saltiel and co-workers (16), who identified Exo70 as a candidate molecule involved in Glut4 vesicle exocytosis. They observed that overexpression of Exo70 increased insulin-stimulated glucose transport. By contrast, an Exo70 mutant inhibited insulin-stimulated glucose transport but not Glut4 translocation to the plasma membrane, presumably by interfering with an initial docking stage or preventing the Glut4 vesicle from accessing the correct fusion site within the plane of the plasma membrane (16). Although compelling, these data did not address the generality of the role of Exo70 in adipocyte exocytosis, and the role of other exocyst components was not addressed. Here, we have sought to establish whether other exocyst components are involved in Glut4 translocation, in particular, sec6 and sec8.

In agreement with the data of Inoue et al. (16), we observed...
insulin-stimulated rsec6 and rsec8 translocation to the plasma membrane; such data argue for an important role in insulin action in these cells. Interestingly, we found little rsec6 or rsec8 associated with Glut4-containing membranes, but rather, the majority was present in a cytosolic pool or associated with structures enriched in cytoskeletal elements and components of the insulin signaling cascade. Our data differ somewhat from those of Inoue et al. (16) in that in our hands, the level of rsec6 and rsec8 associated with the LDM fraction is increased upon insulin stimulation, whereas Inoue et al. report a decrease.

There are several potential explanations for this. Firstly, it should be noted that we have used longer times of incubation with insulin (30 min as compared with 2–10 min in Inoue et al. (16)), and the association/dissociation of the complex may vary between such times. Secondly, we have used subtly different fractionation conditions that may explain these differences. This is particularly important since the LDM fraction has been shown to contain significant levels of cytoskeletal elements (23,
Exocyst and Glut4 Translocation

24. Here, we show that upon detailed analysis of the LDM fraction, the cytoskeletal-enriched fractions exhibit an insulin-dependent increase in rsec6 and rsec8 content. Thus, subtly different fractionation conditions or buffers could alter the levels of these fractions in the LDM and may give rise to the differences between our data and those of Inoue et al (16). This distinction apart, the observation that insulin promotes increased rsec6 and rsec8 levels at the plasma membrane with decreased levels in the cytosolic pool is consistent between both studies. This supports the notion developed in other experimental systems that the exocyst is a dynamic complex, the assembly and disassembly of which may be acutely regulated, and supports the hypothesis that the exocyst is important in the membrane trafficking events stimulated by insulin. However, insulin is known to promote a range of trafficking events, including Glut4 translocation, increased endosomal traffic (such as elevations in cell surface transferrin receptors (19, 25–27)), and increased rates of the secretion of molecules such as adipsin and ACRP30 (20, 28). Thus, we sought to determine whether the exocyst functioned in one or all of these pathways. To achieve this, we overexpressed wild-type rsec6 or rsec8 using adenoviruses (Fig. 2). Fig. 3 reveals that the rate of the secretion of ACRP30/adiponectin and adipsin was unaffected by overexpression of these exocyst components. Similarly, the levels of cell surface transferrin receptors were largely unchanged. By contrast, we consistently observed that overexpression of rsec6 or rsec8 markedly potentiates insulin-stimulated deGlc uptake (Fig. 4). Expression of either rsec6 or rsec8 led to a significant rise in the maximal rate of insulin-stimulated deGlc uptake. The modest increase in the rate by which insulin stimulated deGlc uptake in rsec6- or rsec8-expressing cells was not evident in every experiment. We did not observe any alterations in the dose dependence of insulin-stimulated deGlc uptake upon overexpression of rsec6 or rsec8; thus, overexpression of exocyst components facilitates increased levels of Glut4 translocation but does not enhance the insulin-sensitivity of the cells.

The fact that overexpression of individual components of the exocyst complex (Exo70, rsec6, or rsec8) each can promote increased insulin-stimulated Glut4 translocation or elevations in deGlc uptake is consistent with the notion that this complex is a dynamic structure, forming and disassociating as trafficking takes place. Such a hypothesis has been advanced by studies in other cell systems (15) but is likely to be applicable in the specific example of the fat cell. Thus, overexpression of individual components (which may otherwise be rate-limiting) would result in increased levels of functional exocyst complex and thus increased levels of Glut4-vesicle docking with the plasma membrane, presumably resulting in an increased rate of vesicle fusion (and thus increased glucose transport). We attempted to overexpress both rsec6 and rsec8 simultaneously in cells with the rationale that this would be expected to produce a synergistic increase in deGlc uptake. However, this was found not to be the case. Further analysis of this situation suggested that the levels of expression of both proteins expressed in the same cell population did not reach the levels achieved when one virus alone was used.

Whatever the mechanism, our data clearly demonstrate that additional exocyst components, other than just Exo70, are involved in insulin-stimulated glucose transport and are consistent with models in which the exocyst is proposed to function in regulated exocytosis (see for example Refs. 15, 29, and 30). Moreover, we have shown that under conditions in which insulin-stimulated glucose transport is increased by overexpression of rsec6 or rsec8, neither secretion nor endosomal traffic appears to be modulated. Collectively, these data lend further support to an important and selective role for the exocyst in regulated exocytosis.

Acknowledgment—We thank Dr Richard Scheller for the rsec6 and rsec8 cDNA.

REFERENCES

1. Bryant, N. J., Govers, R., and James, D. E. (2002) Nat. Rev. Mol. Cell. Biol. 3, 287–297
2. Reaven, G. M. (1988) Diabetes 37, 1595–1607
3. Reaven, G. M., Chang, H., Hoffman, B. B., and Azhar, S. (1989) Diabetes 38, 1155–1159
4. Rea, S., and James, D. E. (1997) Diabetes 46, 1667–1677
5. Pessin, J. E., Thurmond, D. C., Elmendorf, J. S., Coker, K. J., and Okada, S. (1999) J. Biol. Chem. 274, 2593–2596
6. Bowser, R., Muller, H., Govindan, B., and Novick, P. (1992) J. Cell Biol. 118, 1041–1056
7. TerBush, D. R., and Novick, P. (1995) J. Cell Biol. 130, 299–312
8. TerBush, D. R., Maurice, T., Roth, D., and Novick, P. (1996) EMBO J. 15, 6485–6494
9. Hsu, S.-C., Hazuka, C. D., Feletti, D. L., and Scheller, R. H. (1999) Trends Cell Biol. 9, 150–153
10. Hsu, C.-C., Ting, A. E., Hazuka, C. D., Davanger, S., Kenny, J. W., Kee, Y., and Scheller, R. H. (1996) Neuron 17, 1209–1219
11. Grindstaff, K. K., Yeaman, C., Anandadasabapathy, N., Hsu, S.-C., Rodriguez-Boulan, E., Scheller, R. H., and Nelson, W. J. (1996) Cell 83, 731–740
12. Matern, H. T., Yeaman, C., Nelson, W. J., and Cheller, R. H. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 9648–9653
13. Shin, D. M., Zhao, X.-S., Zeng, W., Mootha, M., and Mualem, S. (2000) J. Cell Biol. 150, 1101–1112
14. Ting, A. E., Hazuka, C. D., Hsu, S.-C., Kirk, M. D., Bean, A. J., and Scheller, R. H. (1995) Proc. Natl. Acad. Sci. (U. S. A.) 92, 9613–9617
15. Yeaman, C., Grindstaff, K. K., Wright, J. R., and Nelson, W. J. (2001) J. Cell Biol. 155, 593–604
16. Inoue, M., Chang, L., Hwang, J., Chiang, S.-H., and Saltiel, A. R. (2003) Nature 422, 629–633
17. Beant, A. M., Jee, T. J., Milligan, G., Brown, C. M., and Gould, W. G. (1993) Biochem. Biophys. Res. Commun. 192, 1297–1302
18. Garza, L. A., and Birnbaum, M. J. (2000) J. Biol. Chem. 275, 2560–2567
19. Millar, C. A., Sherwan, A., Hickson, G. R. X., James, D. E., and Gould, G. W. (1999) Mol. Biol. Cell 10, 3675–3688
20. Millar, C. A., Meerloo, T., Martin, S., Hickson, G. R. X., Shimwell, N. J., Wakelam, M. J. O., James, D. E., and Gould, G. W. (2000) Proc. Natl. Acad. Sci. U. S. A. 95, 2529–2534
21. Peipert, R., Hess, L. J., and James, D. E. (1991) Annu. Rev. Physiol. 53, 579–598
22. Clark, S. F., Martin, S., Carozzi, A. J., Hill, M. M., and James, D. E. (1998) J. Biol. Chem. 140, 1211–1225
23. Clark, S. F., Molero, J. C., and James, D. E. (2000) J. Biol. Chem. 275, 3819–3826
24. Tannen, L. I., and Lienhard, G. E. (1987) J. Biol. Chem. 262, 8975–8980
25. Tannen, L. I., and Lienhard, G. E. (1989) J. Cell Biol. 106, 1537–1545
26. Oka, Y., Motola, C., Oppenheimer, C. L., and Ozbek, M. F. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 4028–4032
27. Kitagawa, K., Rosen, B. S., Spiegelman, B. M., Lienhard, G. E., and Tanner, L. I. (1989) Biochim. Biophys. Acta 1014, 83–89
28. EauClaire, S., and Guo, W. (2003) Neuron 37, 369–374
29. Murphy, M., Garza, D., Scheller, R. H., and Schwartz, T. L. (2003) Neuron 37, 433–447
Evidence for a Role of the Exocyst in Insulin-stimulated Glut4 Trafficking in 3T3-L1 Adipocytes
Marie-Ann Ewart, Mairi Clarke, Susan Kane, Luke H. Chamberlain and Gwyn W. Gould

*J.* Biol. Chem. 2005, 280:3812-3816.
doi: 10.1074/jbc.M409928200 originally published online November 17, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M409928200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 30 references, 17 of which can be accessed free at http://www.jbc.org/content/280/5/3812.full.html#ref-list-1