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MOLECULAR ACTIONS OF INSULIN ON GLUCOSE TRANSPORT

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ABSTRACT

Low basal glucose uptake by insulin-sensitive muscle and adipose cells reflects rapid endocytic retrieval of GLUT4 glucose transporters from the cell surface and their retention in intracellular membranes. Both GLUT4 endocytosis and its intracellular retention are governed by a dileucine motif in its COOH-terminal region. Acute stimulation of sugar uptake by insulin results from GLUT4 redistribution to the plasma membrane and may reflect disruption of dileucine

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motif function as well as enhanced bulk membrane exocytosis. Candidate signaling elements for these postulated actions of insulin are PI 3–kinase and $p21^{ras}$, both acutely activated by the hormone. Recent work in our laboratory and others demonstrates the localization of PI 3–kinase to intracellular membranes via its docking to the insulin receptor substrate–1 (IRS-1). An important hypothesis for future testing is that 3′ phosphoinositides generated in the endosomal tubulovesicular system in response to insulin cause budding or movements of GLUT4-containing membranes to the cell surface.

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INTRODUCTION

One of the most dramatic scientific breakthroughs in the history of medicine occurred on January 23, 1922, when Leonard Thompson, emaciated and dying of type I diabetes, was injected with a partially purified preparation of insulin (10). Continued therapy resulted in remarkable weight gain, restored muscle strength, and reprieve from certain death, results due in part to insulin's acute and selective stimulation of sugar uptake into muscle and adipose cells. This effect of insulin helps lower circulating glucose concentrations and promotes muscle glycogen storage and adipocyte triglyceride synthesis. The physiological importance of these functions and their impaired response to insulin in type II diabetes (41, 138) have challenged scores of laboratory groups to determine the molecular basis of glucose transport regulation by insulin. Decades of effort have yielded the unifying concept that insulin binding to its unique receptor heterotetramer (119, 192), a tyrosine kinase (89), initiates events that rapidly increase cell-surface localization (32, 186) of a specific glucose transporter protein isoform, GLUT4 (9, 18, 52, 77, 84, 214). Insulin-like growth factor (IGF)–I receptors, which are abundant in muscle, initiate this response as well (8).

One approach to unraveling the molecular details of the regulation of glucose transport exploits new findings indicating that GLUT4 continuously recycles (33, 79, 208) through membranes of the endosomal tubulovesicular system (27, 173, 174) and the plasma membrane. As first reported in 1980 (32, 186), insulin-mediated GLUT4 occupancy of the cell-surface membrane occurs by partial depletion of GLUT4 content in intracellular membranes. The largely intracellular steady-state distribution of GLUT4 in the basal state appears to be directed in large part by a dileucine motif (27, 194) located in its COOH-terminus (34, 117, 195). This motif appears to cause rapid endocytosis and retention of GLUT4 within intracellular membranes (27). Its mode of interaction with cellular components may provide important insight into the sites and mechanisms of insulin action on GLUT4 trafficking. Understanding the exact membrane-sorting pathway transited by GLUT4 also will be critical to future progress.

Another approach to understanding insulin action on glucose transport is to discover the earliest signaling events triggered by insulin receptor activation that specifically relate to regulation of glucose transport. Recent work has revealed that several insulin receptor–mediated signaling reactions resemble those initiated by other receptor tyrosine kinases (202), including receptors that do not regulate glucose transport (48, 152). Activations of $p21^{ras}$ (166), protein kinase cascades (31, 40), and phosphatidylinositol 3 (PI 3)–kinase (71, 159) are examples of such common receptor signaling events. Thus, an important question is whether another as yet unidentified signaling circuit unique to insulin action exists, or whether a variation of some mechanism common to other receptor tyrosine kinases is sufficient to regulate glucose transport. Elucidation of how the mechanisms of insulin receptor signaling merge with GLUT4 membrane trafficking should provide insights of central biological importance.

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INSULIN RECEPTOR SIGNALING MECHANISMS

Tyrosine Phosphorylation of Signaling Proteins

Point mutations affecting a single lysine critical for the ATP-binding and tyrosine kinase activities of the insulin receptor abolish its signaling capability (158). Some data suggested that ATP binding alone is sufficient for receptor function (113), but strong evidence now implicates receptor tyrosine kinase activity in this process. Thus, several cellular proteins with apparent signaling function are phosphorylated acutely on tyrosines in response to insulin (Table 1). Furthermore, two types of functional responses to such tyrosine phosphorylations have been characterized: a change in the enzyme activity (tyrosine kinase) of the receptor itself (157, 211) and recruitment of signaling proteins to tyrosine phosphate sites (141). An acute tyrosine phosphorylation event or events likely initiate mechanisms that mediate regulation of glucose transport by insulin in muscle and adipose tissue.

At least three of the known insulin-elicited tyrosine phosphorylations in intact cells listed in Table 1 appear to be catalyzed directly by the receptor itself. A β subunit in the insulin receptor heterotetrameric structure (119) catalyzes tyrosine phosphorylation of the neighboring β subunit in response to insulin (108, 190, 191), which in turn causes marked further stimulation (>10-fold) of the insulin receptor tyrosine kinase activity. High levels of tyrosine phosphorylation and elevated kinase activity apparently remain associated with insulin receptors during their endocytosis (96). Insulin receptor substrate-1 (IRS-1) is a 131-kDa protein (2, 93, 183) with multiple tyrosines that are readily phosphorylated in intact cells as well as by the insulin receptor in vitro. The apparent function of IRS-1 tyrosine phosphates is to recruit

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Table 1 Cellular proteins that are phosphorylated on tyrosines in response to insulin in intact cells

Proteins that are phosphorylated on tyrosines in intact cells	Insulin receptor substrate in vitro	Presumed function(s) of tyrosine phosphorylation	References
Insulin receptor	Yes	Receptor kinase activation	157, 211
Insulin receptor substrate–1 (IRS-1)	Yes	Binding of SH2-containing proteins Binding and activation of PI 3–kinase Binding and activation of SH-PTP2 Binding of adapter proteins Grb-2 (which can bind Sos and Dynamic) and Nck	188 4, 129 102, 181 6 107
4PS (IRS-2)	Not reported	Presumed homologue of IRS-1	198, 199
SHC	Not reported	Assembly of Grb-2/son-of-sevenless complex to activate p21 Ras	148 99, 147, 171
pp60	Not reported	Binding and activation of PI 3–kinase	105, 124
422 (aP2) fatty acid binding protein	Yes	Possible modulation of fatty acid binding	112
p62 GAP-associated (mRNA processing) protein	Not reported	Unknown	146, 184
pp120 ecto-ATPase	Not reported	Unknown	130

specific proteins (see Table 1) that bind these sites via specialized domains (denoted SH2, src homology 2) consisting of ~100 amino acids (141). SH2 domains occur in many signaling proteins, display significant sequence similarity, and exhibit specificity for binding to tyrosine phosphates based on amino acid sequences flanking the phosphorylated site (141, 176, 177). Autophosphorylation of the insulin receptor and tyrosine phosphorylation of IRS-1 [and of a related protein, 4PS (198, 199), in lymphoid cells] seem to account for much of the total tyrosine phosphorylation by insulin in various cells studied.

In rat adipocytes, insulin causes extensive tyrosine phosphorylation of a protein or proteins in the 60-kDa range (pp60) as well as of its receptor and IRS-1 (105, 124). A cDNA encoding this species has not yet been isolated. The pp60 may be most abundant in (or unique to) primary adipocytes because it is not detected in cultured mouse 3T3-L1 adipocytes. However, Shc proteins of 46 and 52 kDa are tyrosine phosphorylated in response to insulin in these cultured adipocytes and primary adipocytes (L Kozma & MP Czech, unpublished data) as well as in cultured cell types overexpressing human insulin receptors (99, 147, 148, 171). Like IRS-1, the pp60 and Shc proteins bind through their tyrosine phosphate sites to specific SH2-containing proteins potentially involved in cellular signaling. Consistent with this notion, IRS-1, pp60, and Shc proteins lack any known catalytic activity. It is not known whether the insulin receptor kinase directly phosphorylates pp60 or Shc in vitro. Tyrosine kinase activity in anti-Shc immunoprecipitates that appear to lack insulin receptors suggests that another tyrosine kinase may be involved in Shc phosphorylation (148). Insulin is reported to stimulate tyrosine phosphorylation of three other proteins—fatty acid–binding protein 422 (aP2) (112), an ecto-ATPase (130), and a $p21^{ras}$ GAP-associated 62-kDa protein with sequence similarity to mRNA processing proteins (184). The physiological relevance, if any, of the tyrosine phosphorylation of these three proteins is not understood.

The above considerations indicate that a primary function of the insulin receptor kinase is to place docking sites (tyrosine phosphates) on IRS-1, pp60, and Shc for recruitment of signaling proteins. This finding implies that docking might alter catalytic activity of the associated proteins or that cellular localization of these proteins is critical for their signaling function. Both of these mechanisms appear to operate in the insulin signaling pathway. Catalytic activities of two proteins that associate with IRS-1 tyrosine phosphates, PI 3-kinase (4, 129) and the tyrosine phosphatase SH-PTP2 (181), are significantly stimulated upon binding of their SH2 domains to tyrosine-phosphorylated peptides. Similarly, the GTPase activity of dynamin has been reported to be stimulated upon its association with IRS-1 via the adapter protein Grb-2 (1). On the other hand, recruitment to IRS-1 (6) and Shc (99, 147, 148, 171)

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of complexes of Grb-2 and the guanine nucleotide exchange factors Sos-1 and Sos-2 may colocalize the latter two with their substrate, $p21^{ras}$. Thus, GTP loading of p21Ras is stimulated by insulin (6, 121).

That the events described above are important in signal transduction by insulin is suggested by the reported ablation of mitogenesis upon microinjection of anti–IRS-1 (156), anti-p85 (80), or anti–SH-PTP2 (206) antibodies into cells. Dominant inhibitory SH-PTP2 also blocked insulin action (123). Consistent with these results, the *Drosophila* corkscrew tyrosine phosphatase, a homologue of SH-PTP2, is involved in conjunction with raf protein kinase in mediating the action of the tyrosine kinase torso (142). Anti-Shc antibodies also inhibit the action of insulin in stimulating mitogenesis in cultured cells (162). Conversely, overexpression or microinjection of IRS-1 into cells augments insulin action (22, 182). However, there is no direct evidence to indicate that Shc, IRS-1, or SH-PTP2 directly participates in or is necessary for the metabolic effects of insulin in primary adipocytes or muscle cells.

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PI 3–Kinase Regulation by Insulin

Insulin-regulated PI 3–kinase is a dimeric enzyme consisting of a regulatory 85-kDa subunit and a 110-kDa catalytic subunit that promotes the phosphorylation of phosphatidylinositol at position D3 of the inositol ring (15, 125). Binding of p85 to p110 is reportedly necessary for catalytic activity of the enzyme (97). Amino acid sequences of two isoforms of the p110 subunit (66, 72) and of two isoforms of p85 (46, 139, 172) have been deduced from full-length cDNA clones. The p85α and β subunits contain two SH2 domains, one SH3 domain, and two proline-rich, SH3-binding regions that can interact with the Abl, Lck, and Fyn tyrosine kinases as well as with the p85 SH3 itself (88). Interaction of the SH3 domain with one or both of its proline-rich regions may help regulate protein-protein interactions of p85. The proline-rich domains flank a region of p85 that exhibits sequence similarity to Rho-GAP, n-chimerin, and bcr, although no GTPase activity of p85 isoforms has been detected to date (88). However, functional Rho appears necessary for the activation of PI 3–kinase in response to thrombin in platelets (127) and can activate PI 3–kinase in the presence of GTP (212).

Acute insulin stimulation of glucose transport in intact rat adipocytes is accompanied by the accumulation of PI-3,4-P and PI-3,4,5-P, products of PI 3–kinase–catalyzed reactions (95). This accumulation apparently results from the association of SH2 domains within p85 of PI 3–kinase with tyrosine phosphorylated IRS-1 or pp60 or both, which causes activation of enzyme activity and colocalization with lipid substrates (7, 50, 55, 94, 159). Recent results indicate that PI 3–kinase activity associated with p85α but not p85β is activated by insulin, even though both p85 isoforms apparently associate with IRS-1 and the p110 catalytic subunit (7). Presumed disruption of these reac-

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tions by overexpression of p85 in cells is accompanied by blockade of transcriptional regulation by insulin (207).

Stimulation of PI 3–kinase activity by insulin may also be linked to regulation of glucose transport. The fungal product Wortmannin completely inhibits PI 3–kinase activity at 100 nM in vitro, apparently without affecting PI 4–kinase activity or protein kinases (209). At 100 nM, the drug completely inhibits stimulation of glucose transport by insulin (85, 137). Redistribution of glucose transporter proteins to the cell-surface membrane is also blocked by LY294002, another inhibitor of PI 3–kinase (19). These data suggest that cellular PI 3–kinase activity is necessary for insulin modulation of glucose transport. However, epidermal growth factor (EGF) also activates the PI 3–kinase reaction (71), although it fails to stimulate glucose uptake in 3T3-L1 adipocytes (48, 152). This observation suggests that PI 3–kinase activation alone may not be sufficient to stimulate glucose transport or that an insulin-specific cellular localization of PI 3–kinase may be necessary for regulation of glucose transport. Moreover, slightly higher concentrations of Wortmannin inhibit myosin light chain kinase (131). Thus, additional data are required to definitively implicate PI 3–kinase activation in the signaling circuit that results in regulation of glucose transport by insulin.

Insulin Signaling Through p21Ras

Trimeric GTP-binding proteins that cycle from the biologically inactive, GDP-bound state to the biologically active, GTP-bound state in response to receptor activation represent a major mode of hormone receptor–mediated signal transduction (13). The recognition that certain plasma membrane receptors, including the tyrosine kinases, can specifically stimulate GTP binding to $p21^{ras}$ proteins (14, 164, 165), long studied in mutant forms as oncogenic small GTP-binding proteins (160), has expanded this general paradigm. Four mammalian gene products, H-Ras, K-RasA, K-RasB, and N-Ras, can transform cells when expressed with mutations that increase their steady-state GTP binding levels (11). In general, such mutations inhibit the ability of Ras proteins to interact appropriately with GTPase-activating proteins (GAPs), which normally enhance the intrinsic ability of the Ras proteins to hydrolyze GTP to GDP. However, elevation of cellular GTP•$p21^{ras}$ levels by insulin seems not to involve modulation of Ras GAPs, but rather regulation of guanine nucleotide exchange factors (6, 121, 145, 166, 210). These latter proteins bind $p21^{ras}$ in the nucleotide-free state (103), thus promoting the binding of GTP, which is present at a higher concentration than GDP in intact cells. In primary adipocytes and cultured 3T3-L1 adipocytes labeled with ^{32}P, $p21^{ras}$ bound to [^{32}P]GTP apparently represents ~10% of the total $p21^{ras}$ GTP plus $p21^{ras}$ GDP concentration in the basal state and increases approximately two- to threefold within 5 min of addition of insulin (J Klarlund, L Kozma & MP Czech,

unpublished data). In addition to causing them to interact effectively with $p21^{ras}$, insulin may stimulate the catalytic activity of guanine nucleotide exchange factors (45). Localization of $p21^{ras}$ proteins to the plasma membrane appears to result from constitutive farnesylation and palmitoylation (except for K-RasB) at their COOH-termini (118).

Hypothetically, insulin can stimulate the levels of biologically active, GTP-bound $p21^{ras}$ proteins by two independent signaling circuits (Figure 1). Tyrosine phosphate sites on IRS-1 and Shc proteins exhibit high affinities for the SH2 domain of Grb-2, which in turn associates via its SH3 regions with proline-rich sequences in the COOH-terminus of guanine nucleotide exchange isoforms Sos-1 and Sos-2. Binding of Sos/Grb2 complexes to IRS-1 also may occur indirectly through other associated proteins (111). Insulin causes rapid association of Grb-2 with both IRS-1 and Shc proteins (see Table 1). In addition, Sos proteins associate with IRS-1 (6) and Shc (147) in response to the hormone. Plasma membrane localization of IRS-1 may occur through its association with the insulin receptor itself (5, 7), whereas Shc binding to the plasma membrane occurs via an undefined mechanism in primary insulin-sensitive cells. Perhaps Shc associates through its own SH2 domain with a tyrosine-phosphorylated membrane protein (as depicted in Figure 1). Although direct evidence links the Sos proteins to insulin action within this hypothetical scheme, other exchange proteins may also be involved. For example, the

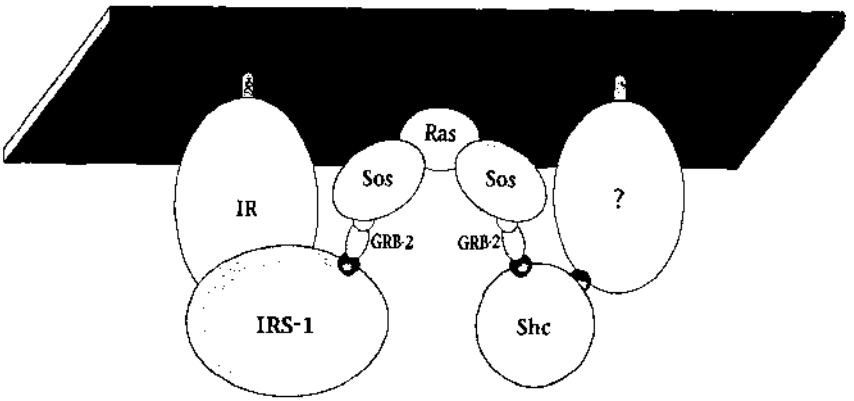

Figure 1 Two pathways by which insulin may mediate GTP loading of $p21^{ras}$. One pathway involves association of IRS-1 with insulin receptors, causing tyrosine phosphorylation of IRS-1 and its recruitment of complexes of Grb2 and guanine nucleotide exchange factor Sos. A second hypothetical pathway is initiated by the tyrosine phosphorylation of an unidentified component (?), causing recruitment of Shc via its SH2 domain. This in turn leads to tyrosine phosphorylation of Shc, catalyzed by the receptor, the unidentified component (?), or other tyrosine kinase, and recruitment of Grb2/Sos complexes via the SH2 domain of Grb2. Darkly shaded areas connecting Grb2 to other proteins indicate SH2 domains that bind to tyrosine phosphate, whereas lightly shaded areas represent proline-rich SH3 binding regions. See text for details.

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recently cloned C3G exchange factor may bind Grb-2 (187) and is a candidate for recruitment to insulin-mediated Shc or IRS-1 protein complexes. Interestingly, only very small fractions of the total cellular Grb-2 and Sos proteins are actually recruited to Shc and IRS-1 complexes by insulin. Current evidence seems to support the view that the Shc pathway may be the predominant or even sole circuit leading to $p21^{ras}$ activation in response to insulin (136, 161). Recent data also suggest that prolonged insulin treatment causes $p21^{ras}$ desensitization, possibly through disassembly of Sos from Grb2 (20).

At least one effector function of $p21^{ras}$ is to interact with the N-terminal domain of the Raf protein kinases, causing cellular events that lead to protein kinase activation (54, 56, 213). Phosphorylation and activation of mitogen-activated protein (MAP) kinase kinases by Raf kinases in turn result in phosphorylation and activation of MAP kinases (31, 40). MAP kinases phosphorylate downstream kinases (178, 179) that appear to catalyze phosphorylation of glycogen synthase kinase–3 (GSK-3), thereby inhibiting the latter (185, 201). In keeping with this complex scheme of protein serine kinase modulation by insulin through $p21^{ras}$, insulin action initiates Raf activation (100). Additionally, the p70 ribosomal S6 protein kinase, which is rapidly activated by insulin through an independent mechanism, also reportedly phosphorylates and inactivates GSK-3 (185). It has been hypothesized that phosphorylation of phosphatase I by a MAP kinase–sensitive kinase (43) and inhibition of GSK-3 (185) combine to cause the dephosphorylation and acute activation of glycogen synthase observed in response to insulin.

Several studies suggest that $p21^{ras}$ function is indeed necessary for cellular signaling by insulin. Microinjection of anti-Ras antibody blocked insulin action in frog oocytes (98). The dominant inhibitory mutant $p21^{ras}N17$ that disrupts endogenous $p21^{ras}$ function also blocks insulin regulation of transcription and DNA synthesis (78) when overexpressed or microinjected into cells. Recently, Manchester et al (114) reported that microinjection of anti-$p21^{ras}$ antibodies into cardiomyocytes inhibits stimulation of glucose uptake by insulin by more than 50%, although similar experiments in 3T3-L1 cells had no effect (62). Expression of an oncogenic $p21^{ras}$ mutant in 3T3-L1 adipocytes (101) or cardiomyocytes (114) resulted in increased glucose uptake and, in the cultured adipocytes, an apparent increased redistribution of both GLUT1 and GLUT4 to the plasma membrane fraction (101). However, other convincing evidence suggests that activation of the MAP kinase cascade by insulin is not sufficient to explain its effect on glucose transport. Thus, both EGF and insulin reportedly stimulate the MAP kinase cascade in cultured 3T3-L1 adipocytes, but only insulin stimulates glucose uptake (48, 152). Because stimulation of MAP kinase is presumably mediated by $p21^{ras}$ activation, the data imply that $p21^{ras}$ modulation alone is not sufficient to mediate insulin action on glucose transport. Similarly, in cells overexpressing an activated form of Raf kinase, GLUT4

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redistribution to the cell surface was not observed (47). However, $p21^{ras}$ interactions with other effectors may be involved in regulation of glucose transport. Inhibition of the p70 ribosomal S6 kinase by rapamycin also failed to blunt stimulation of glucose transport by insulin (49). Taken together, the data available do not indicate clearly whether intact $p21^{ras}$ function may be necessary for the full stimulatory effect of insulin on glucose transport. If $p21^{ras}$ proteins do play a role in this process, one or more additional signaling systems, such as the PI 3–kinase reaction, appear to be required.

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GLUCOSE UPTAKE IN INSULIN-SENSITIVE CELLS

Glucose Transporter Proteins

The most abundant glucose transporter protein present in insulin-sensitive adipocytes and muscle cells is GLUT4 (67, 116). The fact that GLUT4 is not expressed in other tissues underscores its importance for insulin action. This protein is one of five mammalian hexose transporters (GLUT1–5, which show 39–65% sequence identities in humans by paired comparison) known to operate at the plasma membrane of mammalian cells by a facilitative diffusion mechanism (for review see 128). The GLUT1 glucose transporter isoform is widely distributed among cell types and is the principal catalyst of glucose uptake in most cells. Low levels of the GLUT1 isoform are also present in adipose tissue and muscle. However, GLUT4 appears to mediate virtually all of the insulin-stimulated glucose uptake in model systems containing both isoforms, whereas GLUTI contributes significantly to basal hexose transport (57, 153). Thus, threefold overexpression of human GLUT1 in cultured 3T3-L1 adipocytes elevates basal glucose uptake to about the same extent without influencing the net increase resulting from insulin action (57). Conversely, overexpression of GLUT4 in cultured L6 myocytes significantly enhances the effect of insulin on glucose transport (153).

GLUT4 is one of more than 100 known biological transporter proteins for which deduced amino acid sequences from cDNA clones suggest the presence of 12 transmembrane α-helices (see 65 for review). For GLUT4, this structure is derived from hydropathic profile analyses of primary sequence, from the location of a single glycosylation site, and from extrapolation of data from other related proteins, most notably GLUT1. The 12-transmembrane structure for GLUT1 is supported, but not proven, by studies using (*a*) the nonpenetrating sulfhydryl reagent pCMBS with a Cys 429 mutant of GLUT1 showing predicted localization of this Cys (200); (*b*) the nonpenetrating reagent ASA-BMPA [2N-(4-azidosalicoyl)-1,3,-bis(D-mannose-4′-yloxy)propyl-2-amine], which labels the proposed exofacial loop 5 (68, 70); and (*c*) antibodies raised against a portion of predicted cytoplasmic loop 3 that bind only to the cyto-

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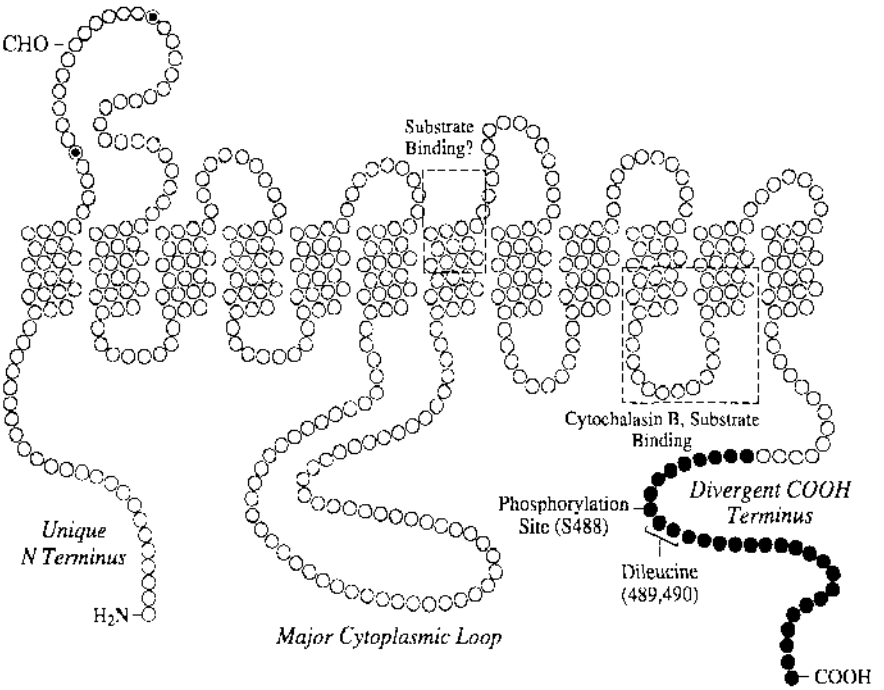

Figure 2 Structural features of the human GLUT4 glucose transporter protein. Unique N-terminus refers to 12 additional residues (*open circles*) not present in GLUT1 or other transporters. Residues in major exofacial loop (*black dot within circles*) are trypsin cleavage sites (17). Divergent COOH-terminus refers to 30 residues with low (~20%) sequence similarity to GLUT1. See text for additional details.

plasmic surface of red cell GLUT1 (36, 37). The predicted cytoplasmic binding region of GLUT4 for the potent transport inhibitor cytochalasin B (Figure 2) also derives from numerous studies on GLUT1 implicating a region within residues 369–412, especially tryptophan 412 and/or 388 (53, 196). Transporter activity is inhibited by mutation of tryptophan 412 or 388 (91). Asn 415 of GLUT1 also has been implicated in the structure of the cytoplasmic binding site for substrate (74). A significant role for transmembrane helices 9, 10, and 11 in transport function was suggested by pH dependence of intrinsic fluorescence changes in GLUT1 on glucose or cytochalasin binding (21). In GLUT1 Gln 282 (61) and Tyr 293 (126) in helix 7 are proposed to function in the exofacial binding of hexose. The COOH-terminal cytoplasmic amino acids may be important in defining the kinetic parameters of transport (90, 134). Interestingly, evidence from studies on the lactose-H^+ symporter of *Escherichia coli* indicates that helix 7 may interact with helix 10 or 11 (109).

Mechanism of Glucose Transport

The molecular details of hexose transport mediated by the transporter proteins have not been fully elucidated. Although several bacterial sugar transporters can be overexpressed to very high levels and prepared in large quantities (64), no three-dimensional structures are available for any of these transporters. Transfer of glucose by transporter proteins is thought to occur through an aqueous pore formed by the juxtaposition of several transporter helices containing sites that interact with hexose molecules (67). Much of our actual information about this process comes from kinetic measurements of sugar transport into intact cells or isolated membranes. Again, GLUT1 has been a useful model in this regard because it is so abundant in human red cells. Even with the GLUT1, however, kinetic data have led to conflicting models of transporter catalysis. On the one hand, the presence of an exofacial hexose binding site alternating with the appearance of a cytoplasmic binding site was proposed (204). On the other hand, the hypothesis that hexose binding sites on both sides of the membrane transporter are present simultaneously has also been suggested (69; see also 16, 197, and 205 for review).

Recent experiments have clarified some of the differences in previous kinetic data for GLUT1-mediated transport. Thus, native GLUT1 in detergent solution apparently exists as a tetramer that can be converted to a dimer by reductant (63). This conversion results from conformation changes induced by reduction of intramolecular disulfides within the GLUT1 monomers. GLUT1 also forms homooligomers, at least in detergent solution, as was demonstrated by coimmunoprecipitation of GLUT1/GLUT4 chimera proteins with native GLUT1 in Chinese hamster ovary (CHO) cells (142a). GLUT1 tetramers and dimers display cytochalasin B binding stoichiometry of 0.5 and 1 mole per mole monomer, respectively. Importantly, GLUT1 in native red cell membranes binds 0.5 mole cytochalasin B per mole monomer, indicating that it exists in the native membrane as a tetramer (63). These and other data support the novel idea that two hexose influx sites and two hexose efflux sites are present in each tetramer (63). These sites are proposed to be interactive in antiparallel fashion, such that conversion of the two influx sites to efflux sites is accompanied by obligatory conversion of the original efflux sites to influx sites. This model assures more rapid cycling of unoccupied efflux sites back to influx sites than would occur in monomeric transporter proteins. Moreover, a tetrameric structure with such characteristics has excellent potential to regulate catalytic activity. It will be important to test these concepts with respect to the mechanism of transport catalysis by GLUT4.

Kinetic analysis of glucose flux resulting from individual transporters can be used to compare the catalytic functions of GLUT4 and GLUT1. Because some catalytic properties of GLUT1 appear to differ among cell types, such

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comparisons should be made within the same model system. Two studies using frog oocytes heterologously expressing mammalian GLUT1 and GLUT4 proteins (92, 132) and one using 3T3-L1 adipocytes (140) rigorously demonstrated a higher apparent K_m for 3-O-methylglucose transport with GLUT1 (21–26 mM) than with GLUT4 (2–7 mM). This finding is consistent with results showing that the apparent K_m of glucose transport into adipocytes containing GLUT1 and GLUT4 falls upon addition of insulin, concomitant with the increased concentrations of GLUT4 at the cell surface (203). Careful analysis of the amount of GLUT1 and GLUT4 protein content at the cell-surface membrane in frog oocytes showed the turnover rate of both transporters to be ~20,000 min^{-1} (132). GLUT1 and GLUT4 also have similar turnover rates in 3T3-L1 adipocytes (140), although the absolute values differ somewhat from those seen in the study of frog oocytes. This result is consistent with the hypothesis that the molecular mechanisms of transport are similar for these two proteins.

The fact that GLUT1 and GLUT4 have similar turnover rates indicates that the catalytic efficiency of the latter is significantly higher at physiological glucose concentrations of ~5 mM. Thus, a greater number of glucose molecules pass through each GLUT4 transporter protein per unit of time than through each GLUT1 under these conditions. This outcome is due solely to the lower apparent K_m exhibited by GLUT4 and, in combination with the high levels of GLUT4 in muscle and adipose tissue, amplifies the contribution of this protein to insulin-stimulated glucose uptake in these tissues.

Regulation of the Catalytic Activity of Transporters

The kinetic values determined for GLUT1 and GLUT4 in frog oocytes and 3T3-L1 cells assume that all transporters in the membrane are catalytically active. This assumption appears to be correct in frog oocytes but may be erroneous in 3T3-L1 adipocytes owing to inadequate quantification of the amounts of transporter protein at the cell surface of intact insulin-sensitive adipocytes. The method often used relies on photoaffinity labeling of GLUT1 and GLUT4 with membrane-impermeable reagents that interact with the exofacial hexose binding sites of these proteins (79, 208). However, glucose transporter proteins reversibly inactivated by cellular regulatory mechanisms may not expose such sites to the external medium. Extensive evidence indicates that this is the case for GLUT1 in unstimulated 3T3-L1 adipocytes (for review see 35). Thus, cell-surface GLUT1 measured by an antibody to an antiexofacial domain of GLUT1 was 25-fold higher in 3T3-L1 adipocytes than in CHO-K1 fibroblasts, whereas basal hexose transport was 50% lower in the former (59). Holman and colleagues have proposed that even transporters that can be photolabeled at the cell surface may not fully participate in hexose transport (140). Taken together, these data suggest that basal hexose uptake catalyzed

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by GLUT1 in insulin-sensitive cells may be restrained by a mechanism that reduces or inactivates intrinsic catalytic activity as well as by intracellular sequestration of glucose transporters (35).

The hypothesis that intrinsic catalytic activity of GLUT1 is highly regulated in intact cells is supported by the observed stimulatory effects of several agents, including insulin, on cellular sugar uptake in the absence of detectable changes in the content of cell-surface transporters. Addition of cholera toxin (23) and cadmium (58) to 3T3-L1 adipocytes markedly stimulates glucose uptake by mechanisms additive to insulin. However, these agents do not enhance the ability of insulin to increase GLUT1 or GLUT4 levels at the cell surface. Insulin also increases hexose uptake in CHO-K1 cells without changing cell-surface GLUT1 (56a), although the effect on transport is minimal (~50%). Blockade of protein synthesis in 3T3-L1 adipocytes for several hours by anisomycin causes a marked enhancement of sugar uptake (by ~7.5-fold) without concomitant changes in the amount of either GLUT1 or GLUT4 in the plasma membranes (24). Interestingly, labeling of GLUT1 in 3T3-L1 adipocytes by the exofacial photolabeling reagent ATB-BMPA is markedly stimulated by anisomycin, but antiexofacial domain GLUT1 antibody binding to these cells is unaffected (60). These results suggest that a population of GLUT1 proteins resides in 3T3-L1 adipocytes in an inactive, inward-facing conformation (ATB-BMPA unreactive) and is activated (ATB-BMPA reactive) in response to prolonged inhibition of protein synthesis. GLUT4 transporters also may be subject to regulation of catalytic efficiency, which may contribute to the overall effect of insulin action on hexose uptake. The molecular mechanisms underlying such potential regulation of intrinsic catalytic activity are unknown at present.

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MEMBRANE TRAFFICKING OF GLUCOSE TRANSPORTERS

Cellular Localization of GLUT4

The contribution of GLUT4 to basal glucose uptake in adipose tissue and muscle is very small because GLUT4 is virtually absent from the plasma membrane (33, 51, 115, 174). Although only very low amounts of GLUT1 are present in these cells, the higher relative abundance of GLUT1 at the plasma membrane probably contributes significantly to basal sugar uptake. Insulin causes redistribution of about half of the GLUT4 in intracellular membranes to the cell-surface membrane within ~5 minutes at 37°C. This mechanism has been documented in intact cells by both biochemical and morphological approaches. Photoaffinity labeling of GLUT4 by membrane-impermeable reagents (79), selective cleavage of cell-surface GLUT4 by trypsin (33), and

binding of antibody to GLUT4 constructs tagged on the exofacial side (86, 149) formally demonstrate GLUT4 protein exposure to the media in response to insulin. Consistent with this conclusion are immunoelectron microscopic analyses of GLUT4 in both adipose tissue (151, 174, 175) and muscle (12, 51), which show the appearance of immunoreactive GLUT4 at the cell-surface membrane in response to insulin. The magnitude of this acute increase in GLUT4 on the plasma membrane is similar to that for glucose transport (~10–30-fold). Thus, membrane redistribution of transporters alone may account for insulin action in adipose tissue and muscle, assuming that all intracellular transporters are already fully active. Although this assumption may be correct, it has not been proved. Nonetheless, occupancy of the plasma membrane by GLUT4 is a key element of insulin action.

Investigators have been tempted to consider redistribution of GLUT4 to the plasma membrane in response to insulin to be analogous in mechanism to secretion in specialized endocrine cells or cells that release neurotransmitters (for review see 76). Accordingly, the GLUT4 protein has been expressed heterologously in neuroendocrine PC12 cells, and its partial localization to large dense-core vesicles has been noted (73). Furthermore, vesicles immunoisolated from low-density membranes of adipocytes (189) or muscle cells (154) with anti-GLUT4 antibody appear to have a unique protein pattern and consist of 5% or more GLUT4. This observation is consistent with the presence of specialized sequestered secretory vesicles in these cells. These vesicles from adipocytes contain relatively small amounts of GLUT1 and other proteins thought to recycle constitutively among cellular membranes.

However, careful analysis of both morphological and biochemical data indicate that this analogy may be incorrect. No unique system of secretory membranes containing GLUT4 in adipocytes or muscle cells has been identified consistently by electron microscopy. For the most part, electron microscopy of brown adipose tissue and muscle (174) shows localization of immunoreactive GLUT4 in endosomal–*trans* Golgi reticulum membranes containing proteins that continuously recycle. Furthermore, proteins found in isolated GLUT4-enriched vesicles of rat adipocytes are thought to regulate general membrane trafficking and recycling pathways common to all cells. Thus, the small GTP-binding protein Rab4 (26) as well as GDI-2, an isoform of the GDP dissociation inhibitor protein (169, 170), implicated in endosomal membrane dynamics, are present in GLUT4-enriched vesicles. Insulin treatment of adipocytes decreases the levels of both of these regulator proteins and of GLUT4 in such vesicles (26, 169). PI 4–kinase is also associated with GLUT4-enriched vesicles, suggesting the presence of phosphorylated derivatives of PI that might be involved in membrane budding or fusions (42). However, PI 4-kinase is also present in all other membrane fractions isolated from adipocytes except those of mitochondria and nuclei. In addition, proteins

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associated with GLUT4 vesicles, such as the synaptobrevins (vesicle-associated membrane proteins, VAMPs) (25), or secretory carrier membrane proteins (SCAMPs) (104), which were originally considered specific for synaptic vesicles or secretory granules, are now known to be widely distributed among cell types (for review see 180). The above considerations do not unequivocally support a role for static secretory vesicles or granules containing GLUT4 in insulin-sensitive cells and instead suggest that GLUT4 is associated with membranes of the recycling endosomal system.

Glucose Transporter Endocytosis

Consistent with the above conclusion are recent studies showing continuous and rapid membrane recycling of GLUT4 between intracellular and cell-surface membranes, even under basal conditions (33, 79, 208). Exploiting predicted trypsin cleavage sites in the major exofacial loop of GLUT4 (Figure 2), we found rapid movement of GLUT4 from the surface of adipocytes, identified by cleavage with the protease, to intracellular low-density microsomes (33). Endocytosis of affinity-photolabeled GLUT1 and GLUT4 also has been observed in untreated adipocytes, with similar rates obtained for both transporters (79, 208). Because the steady-state concentrations of GLUT4 in the plasma membrane remained unchanged during these measurements, continuous recycling must occur. More recently, experiments with CHO and COS-1 cells expressing native and chimeric glucose transporters engineered to contain an antibody binding site for a hemaglutinin epitope (HA) in the exofacial loop have confirmed rapid transporter endocytosis and recycling rates (27). Both biochemical and morphological analysis of the internalization of anit-HA antibodies directed by these transporter proteins revealed that a more rapid endocytosis rate was conferred to GLUT1 upon substitution of the COOH-terminal 30 amino acids of GLUT4 (about twofold). These data suggest that GLUT4 may be internalized more rapidly than GLUT1; this would partially account for the higher steady-state intracellular concentration of the former. Failure to observe differential endocytosis rates of photolabeled GLUT1 compared with GLUT4 in adipocytes (208) suggests that additional experiments are required in order to test this hypothesis in insulin-sensitive cell types using a different methodology.

The disappearance of a given population of cell-surface GLUT4 proteins undergoing endocytosis is characterized by a $t_{1/2}$ of only a few minutes at 37°C (33, 79, 208), indicating a relatively low residence time for these transporters in the plasma membrane during recycling. This low residence time assures rapid retrieval of GLUT4 from the cell surface under basal conditions and points to a possible site of regulation by insulin. Indeed, three studies have shown a decreased rate of GLUT4 proteins internalized in insulin-treated cells (30–70%) when normalized to the cell-surface GLUT4 concentration (33, 79,

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208). However, another study failed to detect this effect, perhaps because true basal rates of GLUT4 endocytosis were not measured (163). Instead, to determine the basal state, rates of GLUT4 internalization were assayed in stimulated cells immediately after removal of insulin, without assurance that regulation of endocytosis by insulin may decay quickly. As previously discussed (27), the apparent decrease in rate constant for GLUT4 endocytosis in cells incubated with insulin may not be a direct action of the hormone. Rather, the endocytosis rate constant for GLUT4 may change as a function of its cell-surface concentration, perhaps owing to saturation of one or more steps in the internalization pathway at the high levels observed in the presence of insulin. Interestingly, insulin and IGF-1 actions also are associated with a decrease in the relative rate of endocytosis of transferrin receptors (39). Whatever the mechanism, a relative decrease in GLUT4 internalization in insulin-treated cells contributes to the magnitude of the steady-state redistribution of this transporter in response to insulin.

Substantial evidence indicates that cellular internalization of GLUT4 occurs via clathrin-coated pits. GLUT4 is associated with these cellular structures upon their isolation (17) and based on immunoelectron microscopy of intact brown adipocytes (174) or 3T3-L1 adipocytes (151). The degree to which cell-surface GLUT4 is present in coated residues is decreased by insulin, a result consistent with inhibited endocytosis rates. Potassium depletion, which disrupts normal function of coated pits, also inhibits GLUT4 internalization (133). Recently, colocalization of newly internalized glucose transporter proteins and transferrin in apparent endosomal structures was visualized in reconstructed images using digital imaging microscopy (27). Taken together, these data indicate that GLUT4 is internalized through the coated-pit pathway, as are many receptors that carry nutrients or growth factors into cells, e.g. receptors for transferrin, low-density lipoproteins, and IGF-II.

Intracellular Retention

The steady-state concentration of GLUT1 at the cell surface is markedly higher than that of GLUT4, even when these transporters are heterologously expressed in various cell types (3, 27, 34, 117, 143, 194, 195). Other receptor proteins that recycle also have higher ratios of steady-state concentration at the cell surface to that in intracellular membranes than does GLUT4 under basal conditions. The twofold higher initial endocytosis rate conferred to GLUT1 by substitution of the COOH tail of GLUT4 does not appear to account fully for this discrepancy (27). Intracellular accumulation of this chimera construct after internalization from the cell surface exceeds that of GLUT1 to a greater extent as time of internalization increases (twofold vs fivefold after 2 and 10 min of endocytosis, respectively). These data are consistent with the concept that in unstimulated cells, GLUT4 protein movement through the intracellular

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membrane recycling pathway is significantly and selectively retarded relative to GLUT1 protein movement. It is also possible to calculate from the estimated rate constant for GLUT4 endocytosis that its inhibition in insulin-treated cells is not sufficient to account for the rapid time course of GLUT4 redistribution to the cell surface in response to the hormone (33, 79, 208). Thus, it is generally agreed that a major effect of insulin action is to modulate one or more steps during the sorting or exocytosis of GLUT4.

GLUT4 Sorting Motifs

In order to determine the structural elements within GLUT4 that enable this protein to achieve its unique membrane sorting pattern, investigators in several laboratories have studied the cellular localizations of expressed GLUT1/GLUT4 chimera proteins. Initial reports (144, 145) that the intracellular disposition of GLUT4 was conferred primarily by its unique N-terminal segment (see Figure 2) could not be confirmed by results from four other laboratories (3, 27, 34, 117, 194, 195). Three of these latter groups found that the COOH-terminal 30 residues of GLUT4 contained the structural elements necessary to restrict its cell-surface localization (27, 34, 117, 194, 195). The motif identified in the COOH-terminal region of rat GLUT4 responsible for this effect is a dileucine at positions 489 and 490 (27, 194). Internalization of anti-HA antibody directed by exofacial HA-tagged glucose transporter constructs containing native as opposed to mutated dileucines implicates this motif in directing both rapid endocytosis and intracellular retention mechanisms (27). Structural elements near the middle intracellular loop also have been implicated in the unique GLUT4 sorting pattern (3), especially at high levels of expression (34). Taken together, this dileucine and its surrounding residues form a structure that apparently plays a dominant role in GLUT4 trafficking.

Dileucines in the cytoplasmic domains of other receptors also appear to function in both internalization and lysosomal sorting pathways (81, 82, 110, 168, 193). Interestingly, these receptors include the IGF-II/Man-6-P receptor, which also assumes a greater cell-surface concentration in response to insulin (28, 29, 135). The function of the dileucine motif in the T-lymphocyte CD3 receptor requires an intact flanking serine, implying that phosphorylation is involved in the regulation of internalization (44). Serine 488 of GLUT4 is adjacent to the double leucine motif (Figure 2) and is phosphorylated in response to agents that elevate cAMP (75) and dephosphorylated in response to insulin (75, 106). The IGF-II receptor also exhibits apparent decreased phosphorylation in response to insulin, which suggests that phosphorylation is involved the endocytosis of this receptor (28, 29). It is tempting to speculate that phosphorylation of GLUT4 may help regulate its recycling, but a direct test of this hypothesis has not been reported.

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Models of GLUT4 Recycling

A simple model that accounts for the continuous recycling of GLUT4, for its rapid endocytosis through coated pits, and for its retention within one or more intracellular compartments is depicted in Figure 3*a*. In its simplest form (omitting material within the dashed enclosure in the figure), this hypothesis sug-

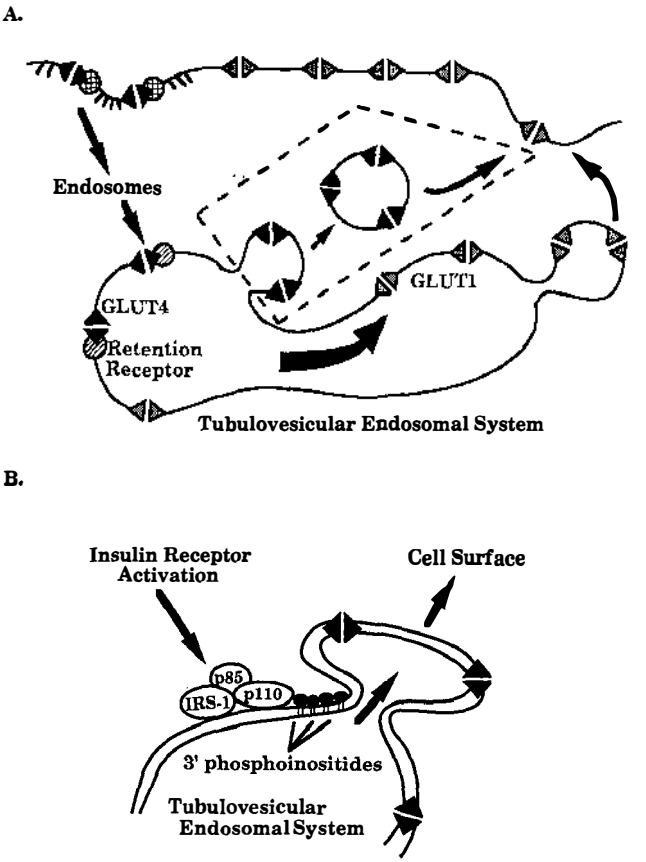

Figure 3 Models for membrane trafficking of the GLUT4 and GLUT1 glucose transporter proteins and for a role of PI 3–kinase in insulin action. (*A*) In one model of membrane trafficking (omit components within dashed line enclosure), GLUT4 and GLUT1 traverse the same endosomal recycling compartments, but GLUT4 movement is restricted by its association with a retention receptor that binds its double leucine motif. In a second model (include components within dashed line enclosure), interaction of GLUT4 with retention receptors or other elements directs it into a specialized vesicular compartment that excludes GLUT1. See text for details. (*B*) In this model, insulin receptor activation is hypothesized to lead to tyrosine phosphorylation of IRS-1 localized in the tubulovesicular endosomal membrane. The recruitment of PI 3–kinase to this pool of IRS-1 supposedly results in the accumulation of 3′ phosphorylated phosphoinositides that catalyze membrane budding or movement to the cell surface.

gests that GLUT4 recycles through the same endosomal tubulovesicular elements that carry many other proteins known to recycle between intracellular and plasma membrane compartments. According to this model, both GLUT1 and GLUT4 traffic through identical membrane compartments, but the predominantly intracellular distribution of GLUT4 and other proteins such as p165 (87, 120) is caused by unique retention in a compartment of this pathway. In keeping with this concept, GLUT4-enriched vesicles would result from the concentration of GLUT4 at this location in the endosomal tubulovesicular system and from the membrane shearing of this compartment during cell homogenization. This simple scheme is compatible with current data and explains why heterologously expressed GLUT4 assumes an intracellular distribution in all cells tested. Alternatively, (see within dashed enclosure in Figure 3*a*), GLUT4 recycling may involve delivery of this protein to a unique compartment (the regulated recycling model in 76) directed by its dileucine and perhaps other motifs. This latter hypothesis differs from the simpler scheme outlined above in that it suggests that GLUT1 and other recycling proteins bypass the putative unique, specialized membrane compartment that carries GLUT4. This conceptual difference provides a focus for future experiments.

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Potential Sites of Insulin Action

The effects of insulin receptor signaling logically might merge at two or more points in the overall pathway of GLUT4 recycling. Disruption of the GLUT4 dileucine motif function (and of the function of other potential retention motifs) is one such potential action. Mutation of the GLUT4 COOH terminal dileucine motif in chimera transporters (27, 194) mimics the ability of insulin to redistribute these proteins to the cell surface. Approximately 50% of total cellular GLUT4 distributes to the adipocyte plasma membrane in response to insulin in a process similar to the distribution of GLUT1 (which lacks the dileucine motif) expressed in cultured cells. The dileucine motif apparently directs a more rapid rate of endocytosis as well as intracellular retention of GLUT4 (27). Thus, inhibition of its function by insulin would contribute to both the inhibition of GLUT4 endocytosis and the release of its intracellular retention observed in response to the hormone.

Eliminating the unique sorting pattern of GLUT4 in the membrane by disrupting its dileucine function is not sufficient to explain the full effect of insulin on redistribution of GLUT4 in the membrane because it also increases cell-surface display of GLUT1 (23, 214). The decrease in GLUT1 content of intracellular low-density microsomes in response to the hormone is in fact as great or greater than the decrease in GLUT4 content. Like the IGF-II/Man-6-P receptor, membrane proteins such as the α_2 macroglobulin receptor (30) and the transferrin receptor (38, 39) are modulated by insulin action. Rab4 and GDI-2, which are thought to regulate membrane movements, also undergo

changes in cellular localization in response to insulin (26, 169). These observations suggest that insulin action can increase bulk membrane flow to the plasma membrane. This finding implies that insulin has an effect on membrane structure, dynamics, or motility within the recycling endosomal system in adipocyte and muscle cells. Such an action might occur via a mechanism that simultaneously disrupts GLUT4 retention, leading to the full effect of insulin.

What molecular signaling mechanisms might accomplish these hypothetical actions of insulin on GLUT4 recycling? Analysis of all the available data suggests that the insulin-stimulated PI 3–kinase reaction is the best candidate at present. However, p21Ras also may play an important role in this process. Recent evidence suggests that activation of $p21^{ras}$ promotes the PI 3–kinase reaction (155), indicating a possible convergence of signaling by these two systems. The reported role of Rho both in PI 3–kinase activation (212) and in membrane events initiated by agents known to activate $p21^{ras}$ (150) is also consistent with a highly integrated signaling network that is responsive to insulin.

New experimental results suggesting a role of the 3′ phosphorylated products of PI in membrane trafficking of receptor proteins in yeast (167) and mammalian cells (83) reinforce the hypothesis that such lipids might mediate insulin-regulated membrane movements directly. Thus, activation of PI 3–kinase activity by insulin might selectively generate elevated concentrations of these highly acidic lipid products at key sites of the endosomal membrane system, which in turn may modulate a rate-limiting step in sorting or membrane flow (Figure 3*b*). Specificity of this response to insulin and IGF-I may be directed by the unique cellular localization of tyrosine phosphorylated IRS-1, pp60, and other specific substrates of these receptors that bind and activate PI 3–kinase. Recent experiments in our laboratory using anti–IRS-1 antibody we raised in rabbits revealed significant amounts of IRS-1 protein bound to PI 3–kinase in low-density microsomes of 3T3-L1 adipocytes. Furthermore, we and others (95) found that PI 3–kinase associated with this endosomal IRS-1 is increased in response to insulin. This observation is consistent with the notion of an insulin-directed endosomal localization of 3′ phosphorylated phosphoinositides that could catalyze membrane movements to the cell surface (Figure 3*b*).

The recent results described above indicate a critical need to learn more about the specific membrane compartments through which GLUT4 recycles in adipose and muscle cells. Moreover, the molecular components that regulate and coordinate the dynamics of these membrane systems represent an important target for future investigations. Much more information about these processes will be required in order to rigorously test whether 3′ phosphoinositides or other candidate signaling elements directly interact with the cellular machinery that regulated GLUT4 recycling. It is particularly exciting that a

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general framework for such future investigations appears to have been established.

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