# Kinetic Analysis of Glucose Transporter Trafficking in Fibroblasts and Adipocytes†

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*Received August 10, 1995; Revised Manuscript Received September 27, 1995*⊗

ABSTRACT: Insulin regulates hexose uptake by the redistribution of glucose transport proteins from intracellular compartments to the cell surface. We have submitted the trafficking of GLUT1, GLUT4, and GLUT1/GLUT4 chimeras to a mathematical analysis in the context of different models. Our data suggest that a model with one intracellular and one cell surface compartment can describe the glucose transporter-trafficking kinetics in fibroblasts. Moreover, the difference in cellular distribution between GLUT1 and GLUT4 overexpressed in fibroblasts is best explained by a slower rate of movement of GLUT4 to the plasma membrane. In 3T3-L1 adipocytes, glucose transporter-trafficking kinetics is adequately described by a three-pool model which includes flow of transporters from the endosomal compartment to the cell surface. The kinetic roles of previously identified motifs in GLUT4 trafficking were defined in the proposed fibroblast and adipocyte glucose transporter-trafficking models. The C-terminus is important in reducing the exocytosis rate from the endosomal compartment to the cell surface in both fibroblasts and adipocytes, and the N-terminus behaves similarly in adipocytes. The C-terminus has an additional signal(s) that allows GLUT4 to be sequestered more efficiently into the insulin responsive vesicle compartment. Mutation of the dileucine motif in the C-terminus significantly reduces the endocytosis of GLUT4 in both fibroblasts and adipocytes, but these amino acids appear not to be primarily responsible for the different kinetics of wild-type GLUT1 and GLUT4.

The insulin responsive tissues, muscle and adipose, express two isoforms of the facilitative glucose transporter family, GLUT1 and GLUT4. Insulin increases the amount of GLUT1 and GLUT4 protein on the plasma membrane (PM)[1] due to translocation of the transporters from an intracellular storage site(s) to the cell surface (Birnbaum, 1992). GLUT4, which is expressed virtually exclusively in muscle and adipose cells, increases at the cell surface 10−30-fold, from essentially nondetectable to ~40% of the total GLUT4 (Calderhead et al., 1990; Holman et al., 1990; Clark et al., 1991; Piper et al., 1991; Slot et al., 1991a,b; Smith et al., 1991; Yang & Holman, 1993). This increase in GLUT4 is similar to the increase in the rate of glucose transport after insulin stimulation, for which the relocation of GLUT4 to the plasma membrane is believed to be primarily responsible (Birnbaum, 1992). The more ubiquitously expressed GLUT1 increases 2−5-fold at the cell surface in response to insulin, from 20−30% to 50−70% of the total GLUT1 protein. For both GLUT1 and GLUT4, insulin stimulation causes a translocation of 30−40% of the total transporter to the cell surface; however, the difference between GLUT1 and GLUT4 in increase is a result of the distinct amount of each isoform at the plasma membrane in the basal state (Clark et al., 1991; Yang et al., 1992a; Satoh et al., 1993; Yang & Holman, 1993). Although these isoforms are 65% identical at the amino acid level, GLUT4 is virtually excluded from the cell surface in the absence of hormone. In addition, although there is a significant intracellular pool of both isoforms in the basal state, studies utilizing electron and immunofluorescence microscopy, as well as immunoadsorption of GLUT4-containing vesicles, have suggested that these intracellular transporters reside in different compartments (Zorzano et al., 1989; Piper et al., 1991; Slot et al., 1991).

The construction of GLUT1/GLUT4 chimeric transporters has allowed for the identification of several motifs important in the subcellular trafficking of GLUT4. Sequences present in the cytosolic $NH_2$ and COOH termini of GLUT4, particularly Phe5 and Leu489Leu490, have been implicated in the intracellular sequestration of this isoform (Piper et al., 1992, 1993; Corvera et al., 1993; Marshall et al., 1993; Verhey et al., 1993; Czech et al., 1994; Verhey & Birnbaum, 1994). However, these studies utilized the overexpression of GLUT1, GLUT4, and chimeric transporters in non-insulin responsive, heterologous cell types which do not normally express the GLUT4 isoform and thus may not contain the trafficking machinery necessary for the correct intracellular sequestration of this isoform. Until recently, it was not clear whether the conclusions drawn from these studies would be physiologically relevant. Indeed, the expression of chimeric transporters in insulin responsive cell lines, 3T3-L1 adipocytes and L6 myoblasts, has shown that the previously identified sequences are important for the trafficking of GLUT4 between the cell surface and endosomes but that sequences distinct from those previously identified are important for the targeting of GLUT4 to the insulin responsive vesicles (Haney et al., 1995; Verhey et al., 1995).

Several kinetic analyses of the subcellular trafficking of GLUT1 and GLUT4 in isolated rat and 3T3-L1 adipocytes have been reported (Clark et al., 1991; Jhun et al., 1992; Yang et al., 1992a,b; Yang & Holman, 1993; Holman et al.,

† This work was supported by NIH Grant DK39519.

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⊗ Abstract published in *Advance ACS Abstracts,* November 1, 1995.

[1] Abbreviations: PM, plasma membrane; IRV, insulin responsive vesicles; HA, hemaglutinin antigen; M6P/IGF-II, mannose-6-phosphate/insulin-like growth factor II.

0006-2960/95/0434-15523$09.00/0 

1994). Experiments utilizing surface labeling of the transporters with impermeant hexose analogues have led to the conclusion that GLUT1 and GLUT4 have similar endocytosis rates which decrease modestly in the presence of insulin and that GLUT4 is excluded from the cell surface in the basal state due to a lower exocytosis rate constant. This implies that either GLUT1 contains a signal(s) for exocytosis and/or GLUT4 contains a signal(s) for intracellular retention. In contrast, Czech et al. have concluded, on the basis of the overexpression of chimeric transporters in the non-insulin responsive cell types COS-7 and CHO cells, that a greater rate of endocytosis of GLUT4 contributes to its intracellular location (Czech et al., 1994). This exemplifies the difficulty in reconciling disparate conclusions drawn from two different systems and approaches. The elegant mathematical analysis of Holman and co-workers predicts that GLUT4 localizes to at least three distinct functional compartments in fat cells; however, such a detailed analysis has not been undertaken for GLUT1 or the chimeric transporters (Holman et al., 1994). A detailed discussion of the kinetic analysis of glucose transporter trafficking in various proposed models should be helpful in designing experiments to fully account for the kinetics involved.

In this report, we discuss several models to explain the subcellular trafficking of both GLUT1 and GLUT4 and several chimeric transporters in fibroblasts and adipocytes. We have found that the subcellular trafficking of glucose transporters in fibroblasts can be explained by a two-pool model and have attempted to explain trafficking of transporters in these cells solely on the basis of the absence of a storage compartment. In addition, the kinetic roles of three previously identified sorting signals in GLUT4, the amino and carboxy termini and the dileucine motif, are discussed for each model.

## MATERIALS AND METHODS

The theoretical predictions of the time course of glucose transporter trafficking are based on analytical solutions of the rate equations, assuming steady-state kinetics. The rate of movement of transporters out of a compartment equals the product of the rate constant times the amount of transporter in that compartment (nonsaturable first order kinetics). We assume that any rate constant of a GLUT1/GLUT4 chimera has a value equal to that of GLUT1 or GLUT4, as determined by the presence of a specific sorting signal. We also assume that insulin affects the subcellular distribution of glucose transporters by changing only the rate constants for movement to and from the plasma membrane and that all changes in rate constants occur instantaneously.

*Two-Pool Model.* The two-pool model, consisting of one compartment at the plasma membrane and one in the interior of the cell, is illustrated in Figure 1. The fraction of glucose transporter at the plasma membrane at time $t$ is $x_p(t)$. The rate constant for movement from the plasma membrane to the interior compartment is $k_{en}$, whereas the rate constant for movement in the opposite direction is $k_{ex}$. The change in the amount of transporter at the plasma membrane over time is defined by

$$dx_p(t)/dt = -k_{en}x_p(t) + k_{ex}[1 - x_p(t)] \quad (1)$$

which is solved as

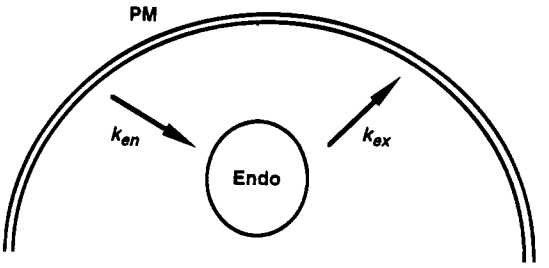


FIGURE 1: Two-pool model. Glucose transporters cycle between two compartments: one on the plasma membrane (PM) and one in the cytoplasm (Endo). $k_{en}$ is the rate constant for movement from the plasma membrane to the cytoplasmic compartment, and $k_{ex}$ is the rate constant for moving in the opposite direction. The fraction of the glucose transporter on the plasma membrane at any time $t$ is denoted by $x_p(t)$.

$$x_p(t) = c_0 + c_1e^{-(k_{en}+k_{ex})t} \quad (2)$$

where $c_0$ and $c_1$ are constants determined by the boundary conditions, that is, the known conditions at any time point. In practice, three parameters have been measured in experiments studying the kinetics of GLUT1 and GLUT4: the fraction of glucose transporters on the cell surface, the half-time of transition from one state to another, and the initial rate of endocytosis after the cell surface transporters were labeled. An experimental strategy frequently utilized is labeling the plasma membrane transporters followed by an incubation of the cells at 37 °C for varying time periods; for such a protocol, mathematical estimates of $c_0$ and $c_1$ are derived as follows. At time 0, all of the labeled transporters are on the plasma membrane so

$$x_p(0) = 1 \quad (3)$$

From eq 2, we have

$$x(\infty) = c_0 = k_{ex}/(k_{en} + k_{ex}) \quad (4)$$

and

$$c_1 = x_p(0) - c_0 = k_{en}/(k_{en} + k_{ex})$$

so

$$x_p(t) = k_{ex}/(k_{en} + k_{ex}) + [k_{en}/(k_{en} + k_{ex})]e^{-(k_{en}+k_{ex})t} \quad (5)$$

At steady state, the fraction of glucose transporters in the interior compartment is

$$1 - x_p(\infty) = k_{en}/k_{en} + k_{ex} = c_1 \quad (6)$$

and the ratio of transporters on the plasma membrane to that in the interior compartment is

$$4/6 = k_{ex}/k_{en} \quad (7)$$

Following eq 5, the half-time of equilibration ($t_{1/2}$) after surface labeling is determined by the sum of $k_{en} + k_{ex}$ according to the equation

$$t_{1/2} = 0.693/(k_{en} + k_{ex}) \quad (8)$$

The initial rate of endocytosis after the transporters on the

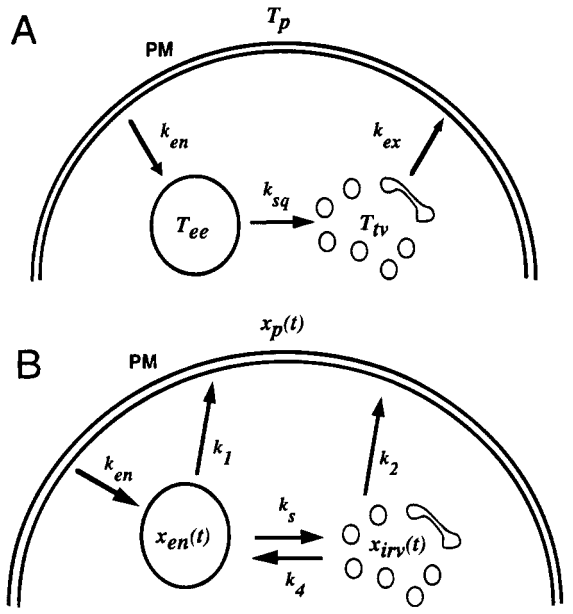


FIGURE 2: (A) Holman's three-pool model. The nomenclature and meaning of each compartment and rate constant are defined in Holman et al. (1994). (B) Modified three-pool model. There are two intracellular compartments, endosomes and insulin responsive vesicles (IRV), and one plasma membrane (PM) compartment. In this model, there is no direct flow of glucose transporters from the plasma membrane to the IRV. The rate constants are as follows: $k_{en}$, from the PM to endosome; $k_1$, from endosome to PM; $k_2$, from IRV to PM; $k_s$, from endosome to IRV; and $k_4$, from IRV to endosome. Insulin stimulation increases $k_1$ to $k_{1i}$ and $k_2$ to $k_{2i}$.

PM are labeled is $k_{en}$, since

$$dx_p(0)/dt = -k_{en}x_p(0) + k_{ex}[1 - x_p(0)] = -k_{en} \quad (9)$$

Thus, each of the three commonly measured parameters is a simple function of the two rate constants in the two-pool model. The surface/intracellular transporter fraction reflects the $k_{ex}/k_{en}$ ratio (shown in eq 7), the half-time for reaching a new steady state is determined by an inverse of the sum of $k_{ex}$ and $k_{en}$ (shown in eq 8), and the true initial rate of internalization represents a direct measurement of $k_{en}$ (shown in eq 9). Any two of the three experimentally derived values can determine $k_{ex}$ and $k_{en}$ in the model. Though it is not necessary to do all three measurements, however, their availability serves as a good check of the validity and coherence of the model. According to eqs 7−9, the three parameters must satisfy the equation

$$t_{1/2} = 0.693[1 - x_p(\infty)]/[dx_p(0)/dt] \quad (10)$$

If the three measurements do not conform to eq 10, further investigation is warranted.

*Modified Three-Pool Model.* Figure 2 presents two alternative three-pool models: that proposed by Holman (Figure 2A) and a slightly modified form (Figure 2B). We considered the three-pool model shown in Figure 2B. There is one compartment on the plasma membrane; the fraction of glucose transporters in this compartment at any time $t$ is denoted by $x_p(t)$. There are two intracellular compartments; the fractions of the glucose transporter in the endosomes or insulin responsive vesicles[2] (IRV) at any time $t$ are denoted by $x_{en}(t)$ and $x_{irv}(t)$, respectively. $k_{en}$ is the rate constant of movement from the PM to the endosomal compartment. $k_1$ is the rate constant of movement from endosomes back to the PM. $k_s$ is the rate constant of movement from endosomes to the IRV. $k_2$ is the rate constant of movement from IRV back to endosomes. $k_2$ is the rate constant of movement from IRV to the PM. Furthermore, we will denote rate constants for cells exposed to insulin by an additional subscript $i$, e.g., $k_2$ in the presence of insulin is $k_{2i}$. We define the insulin-dependent activation in movement to the plasma membrane by the terms $a = k_{1i}/k_1$ and $b = k_{2i}/k_2$.

In this analysis, we did not consider the possibility that transporters can move directly from the PM to the IRV. It can be demonstrated that this possibility will not change the overall argument and mathematical expression of the system (data not shown). In addition, the evidence to date suggests that GLUT4 does not move directly to IRV but instead follows the clathrin-mediated route of internalization (Slot et al., 1991b; Robinson et al., 1992; Nishimura et al., 1993b).

The rate equations describing trafficking of transporters in cells which have been previously exposed to insulin but from which hormone has been removed, i.e., the transition from the stimulated to the basal state, are

$$dx_p(t)/dt = -k_{en}x_p(t) + k_1x_{en}(t) + k_2x_{irv}(t) \quad (11)$$

$$dx_{en}(t)/dt = k_{en}x_p(t) - k_1x_{en}(t) - k_sx_{en}(t) + k_4x_{irv}(t) \quad (12)$$

$$dx_{irv}(t)/dt = k_sx_{en}(t) - k_4x_{irv}(t) - k_2x_{irv}(t) \quad (13)$$

$$x_p(t) + x_{en}(t) + x_{irv}(t) = 1$$

which is solved as

$$x_p(t) = m_0 + m_1e^{n_1t} + m_2e^{n_2t} \quad (14)$$

where

$$n_1 = -(k_{en} + k_1 + k_2 + k_s + k_4)/2 + \sqrt{(k_{en} + k_1 + k_2 + k_s + k_4)^2 - 4[(k_{en} + k_1)(k_2 + k_4) + k_s(k_{en} + k_2)]}/2$$

$$n_2 = -(k_{en} + k_1 + k_2 + k_s + k_4)/2 - \sqrt{(k_{en} + k_1 + k_2 + k_s + k_4)^2 - 4[(k_{en} + k_1)(k_2 + k_4) + k_s(k_{en} + k_2)]}/2$$

$$m_1 = f - m_0 - m_2$$

$$m_2 = [-(k_{en} + k_2)f + (k_1 - k_2)g + k_2 - n_1(f - m_0)]/(n_2 - n_1)$$

$$f = (k_{1i}k_{2i} + k_{1i}k_4 + k_{2i}k_s)/[k_{2i}(k_{en} + k_s + k_4) + k_{en}(k_s + k_4) + k_{1i}k_4]$$

$$g = f(k_{en} + k_{2i})/(k_{1i} - k_{2i}) - k_{2i}/(k_{1i} - k_{2i})$$

In case the term inside the square root sign for $n_1$ and $n_2$ is negative, $x_{p(t)}$ then becomes

$$x_p(t) = m_0 + m_1e^{ut}\cos(ut) + m_2e^{ut}\sin(ut) \quad (15)$$

where

[2] Insulin responsive vesicles has been chosen for the name of the GLUT4-containing compartment for ease of presentation. It should be emphasized that this compartment is not uniquely insulin responsive; i.e., protein located in endosomes also redistributes to the cell surface in response to hormones. Moreover, the term vesicle should not be construed as indicative of the true morphology of this compartment.

$$u = \sqrt{-(k_{en} + k_1 + k_2 + k_s + k_4)^2 + 4[(k_{en} + k_1)(k_2 + k_4) + k_s(k_{en} + k_2)]}/2$$

$$v = -(k_{en} + k_1 + k_2 + k_s + k_4)/2$$

if $n_1 = n_2$, then

$$x_p(t) = m_0 + m_1 e^{vt} + m_2 t e^{vt}$$

$$m_0 = (k_1k_2 + k_1k_4 + k_2k_s)/[k_2(k_{en} + k_s + k_1) + k_{en}(k_s + k_4) + k_1k_4]$$

$$v = -(k_{en} + k_1 + k_2 + k_s + k_4)/2$$

The solutions for $m_1$ and $m_2$ can be determined in a similar way.

The mathematical expressions describing $x_{en}(t)$ and $x_{irv}(t)$ are of the same form as eq 14, but with each term having different coefficients. The latter are determined by the boundary conditions, that is, the known states of the system. Each term in eq 14 is decaying at a different rate. Depending on the coefficients, the largest one will dominate the overall rate of decay for eq 14 and therefore will also be the major determinant of the half-time of the transition from one state to a different one. Thus, eq 14 mathematically states that only in a model with three or more pools of cellular transporter can there be a different $t_{1/2}$ transition from one state to another, depending on the initial and final states; observation of more than one $t_{1/2}$ excludes a two-compartment model.

*Holman's Three-Pool Model.* The three-compartment model proposed by Holman et al. (Figure 2A) can be viewed as a special case of the model just analyzed in which transporters do not travel from IRV to the endosomes nor from endosomes to the plasma membrane, i.e., $k_1 = 0$ and $k_4 = 0$ (Holman et al., 1994). The nomenclature used by Holman et al. translates to that of the modified three-pool model as $k_{en} = k_{en}$, $k_{sq} = k_s$, $k_{ex} = k_2$, $T_p(t) = x_p t$, $T_{ee}(t) = x_{en} t$, and $T_{tv}(t) = x_{irv} t$ (compare Figure 2A to Figure 2B).

*General Solutions to Models with Four or More Pools.* The mathematical equations for models with four or more transporter pools can be derived as follows. We first analyzed Holman's version of a multiple compartment model, shown in Figure 3. The rate equations are

$$dT_p(t)/dt = k_o T_{po}(t) - k_c T_p(t)$$

$$dx_0(t)/dt = k_n x_n(t) - k_0 x_0(t)$$

$$dx_i(t)/dt = k_{i-1}x_{i-1} - k_i x_i \quad \text{for} \quad i = 1, 2, ..., n \quad (16)$$

$$\sum_{i=0}^{n} x_i(t) = 1 \quad (17)$$

By rearranging and substituting each term, as shown above for the modified three-pool model, we can write the equations as

$$\sum_{j=0}^{n} [c_i + a_j x_i^{(j)}(t)] = 0 \quad \text{for} \quad i = 0, 1, 2, ..., n \quad (18)$$

where $x_i^{(j)}(t)$ is the $n$th derivative of $x_i(t)$ and $x_i^{(0)}(t)$ is $x_i(t)$.

The general solution for eq 21 is

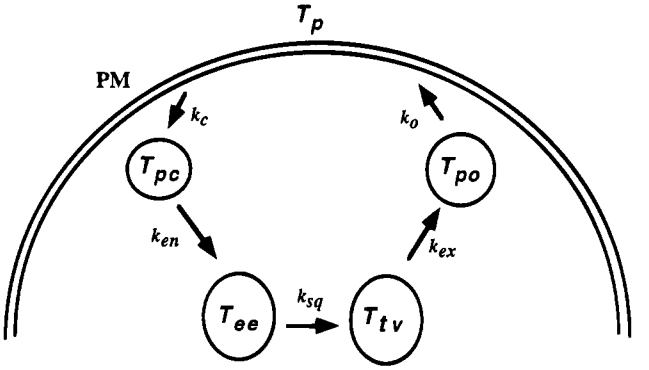


FIGURE 3: Holman's five-compartment model from Holman et al. (1994). Arrows indicate the movements allowed in the models.

$$x_i(t) = c_{i0} + \sum_{j=1}^{n} c_{ij} t e^{a_j t} \quad (19)$$

where $a_1, a_2, ..., a_n$ are the roots of the polynomial equation

$$y^n + \sum_{i=1}^{n} [\sum_{i1 \neq i2 \; dq... \neq ii}^{ij=0,1,...,i} (a_{i1}, ..., a_{ii}) y^{n-i}] = 0 \quad (20)$$

The fundamental theorem of algebra guarantees that there are $n$ complex roots to eq 20, although no simple formulas are available to solve the equations in general. Equation 19 is similar to eqs 14 and 15 in form.

Now consider the situation where multiple trafficking pathways are allowed to occur between the compartments. The rate equations can be written as

$$x_i'(t) = c_{i0} + \sum_{j=0}^{n} c_{ij} x_j(t) \quad i = 0, 1, ..., n \quad (21)$$

By operations similar to the derivation of eq 18, it can be shown that

$$\sum_{i=0}^{n} d_i x^{n-i} = 0 \quad (22)$$

where $d_i$ is a constant. This equation is identical in form to that of eq 18, and the solutions for eq 22 are identical in form to those for eqs 14 and 15.

*Assignment of Rate Constants in Different Models.* In the two-pool model, there are two rate constants, $k_{en}$ and $k_{ex}$, which are related by two equations, eqs 7 and 8, so the rate constants can be uniquely determined. This is not the case in models with three or more cellular pools of transporter. Because there are more rate constants than the number of equations relating them, there are many sets of rate constants that can satisfy the equations. Due to technical limitations, only the rate constant $k_{en}$ can be reliably determined in the three-pool model, and the accuracy of this measurement depends on $k_1$ being relatively low. Even if the transitions and the time course of the fraction of glucose transporters on the PM are also known with precision, the other rate constants cannot be unambiguously determined. In their analysis, Holman and co-workers borrowed from the literature constants measured in other systems and set the unknown values by fitting computer-simulated transition curves to those determined experimentally for each condition (Holman et al., 1994).

In the modified three-pool model, the rate constants for GLUT1 and GLUT4 trafficking were based on experimental data in rat and 3T3-L1 adipocytes and the estimated rate constants of Holman and co-workers. In addition, the following conditions were set. First, direct measurements in rat and 3T3-L1 adipocytes have indicated that $k_{en}$ is virtually identical for GLUT1 and GLUT4 in the absence and presence of hormone and that insulin stimulation causes a modest decrease in the endocytosis rate constant (Jhun et al., 1993; Yang & Holman, 1993). Second, there is no evidence to suggest significant flow of transporters from the IRV back to the endosomes, so $k_4$ is set very small in numerical comparisons. Third, the rate of movement of IRV to the PM ($k_2$) is identical regardless of the transporter cargo. Fourth, insulin stimulation affects the translocation of proteins from both intracellular pools to the cell surface, although not equally. Thus, the rate of translocation from endosomes ($k_1$) increases 2−5-fold upon insulin stimulation (constant $a$, Figure 2 legend), consistent with the behavior of GLUT1 in adipocytes and GLUT4 in fibroblasts, whereas the rate of translocation from IRV ($k_2$) increases 10−100-fold (Kanai et al., 1993; Holman et al., 1994).

Despite the difficulty in estimation of rate constants, the relationship between different rate constants can still be derived. An illustration of this is shown below for the trafficking of GLUT1 in Holman's three-pool model (Figure 2A), given the following measurements for GLUT1 trafficking in 3T3-L1 adipocytes: $T_p = 23\%$, $T_{pi} = 51\%$, $k_{en} = 0.121$, and $k_{eni} = 0.093$ (Yang & Holman, 1993).

At steady state ($t = \infty$), the rate of change of GLUT1 in each compartment is 0.

$$T_{ee}/T_p = k_{en}/k_{sq} \quad \text{so} \quad T_{ee} = T_p k_{en}/k_{sq}$$

$$T_{tv}/T_p = k_{en}/k_{ex} \quad \text{so} \quad T_{tv} = T_p k_{en}/k_{ex}$$

because

$$T_p + T_{ee} + T_{tv} = 1$$

then

$$T_p = 1/[1 + k_{en}/k_{sq} + k_{en}/k_{ex}]$$

Since $T_p = 0.23$, $k_{en} = 0.121$, and the ratio $k_{en}/k_{sq} > 0$, then

$$0 \leq k_{en}/k_{ex} \leq 3.35$$

$$k_{ex} \geq 0.036$$

After insulin stimulation

$$T_{pi} = 1/[1 + k_{eni}/k_{sq} + k_{eni}/k_{exi}]$$

Since $T_{pi} = 0.51$, $k_{eni} = 0.093$, and the ratio $k_{eni}/k_{sq} > 0$, then

$$0 \leq k_{eni}/k_{exi} \leq 0.96$$

$$k_{exi} \geq 0.097$$

This analysis shows that insulin must increase the exocytosis rate constant for GLUT1 by at least 2.7-fold. Assuming the fraction of GLUT4 on the plasma membrane rises from 1 to 40% after insulin stimulation, similar analysis shows that insulin increases the exocytosis rate constant at least 67-fold (data not shown).

## RESULTS

*Glucose Transporter Trafficking in Fibroblasts.* The targeting of GLUT1 and GLUT4 to distinct subcellular locales occurs in non-insulin responsive cells. These isoforms have been introduced by gene transfer of their cloned cDNAs into heterologous cell types such as mouse 3T3-L1 and NIH 3T3 fibroblasts, hamster CHO cells, human HepG2 cells, and rat PC12 cells (Haney et al., 1991; Hudson et al., 1992, 1993; Piper et al., 1992; Shibasaki et al., 1992). Even in such diverse cell lines, the transporters show characteristic localization patterns: perinuclear staining for GLUT4 and predominant cell surface labeling for GLUT1. Chimeric transporters in which reciprocal portions of the GLUT1 and GLUT4 proteins were exchanged have also been expressed in fibroblast cell lines and their subcellular distributions determined by immunofluorescence and electron microscopy, by the PM sheet assay, and by subcellular fractionation (Piper et al., 1992, 1993; Czech et al., 1993; Marshall et al., 1993; Verhey et al., 1993; Corvera et al., 1994; Verhey & Birnbaum, 1994).

In a two-pool model, the distribution of GLUT4 could be accounted for either by a $k_{en}$ greater or by a $k_{ex}$ less than that for GLUT1 (Figure 1). The trafficking in CHO or COS-7 cells for chimeric transporters containing the COOH-terminus of GLUT4, as well as a hemaglutinin antigen (HA) epitope tag in the major exofacial loop, has been studied by Corvera et al. (1994). Recombinant, cell surface transporter was labeled with anti-HA antibody and the ratio of surface to internal transporters determined over time. Corvera and co-workers reported that the GLUT4 COOH-terminal chimera shows a faster initial rate of endocytosis ($k_{en}$) than that of GLUT1. However, the data also indicate that the chimeric transporter has a much longer $t_{1/2}$ of equilibration than GLUT1 [see Figure 6 in Corvera et al. (1994)]. As the steady-state distribution of the GLUT4 COOH-terminal chimera is similar to that of intact GLUT4, the chimera must have a lower $k_{ex}/k_{en}$ ratio (eq 7) than GLUT1. In addition, the increased $t_{1/2}$ of equilibration indicates that the chimera must have a lower value of $k_{en} + k_{ex}$ compared to GLUT1 (eq 8). These parameters cannot both be satisfied if the GLUT4 COOH-terminus confers only an increase in $k_{en}$; they could, however, be satisfied by only a decrease in $k_{ex}$ or by both an increase in $k_{en}$ and a coincident larger decrease in $k_{ex}$. Alternatively, the rate of internalization measured was not a true initial velocity and, thus, the estimate of $k_{en}$ unreliable. This argument is illustrated graphically in Figure 4, assuming, for this example, that substituting the GLUT4 COOH-terminus onto this region of GLUT1 causes the transporter distribution to change from 67% on the PM to 33%. The graphs demonstrate that, if the major effect of the GLUT4 COOH-terminus is to confer an increase in $k_{en}$, the $t_{1/2}$ of equilibration will be short. In contrast, if the major effect of this region is to confer a decrease in $k_{ex}$, then the $t_{1/2}$ of equilibration will be long. If both rate constants are affected, then the $t_{1/2}$ of equilibration will be somewhere in between. Thus, the major contribution of the GLUT4 COOH-terminus to the trafficking of chimeric transporters in fibroblasts is likely a decrease in $k_{ex}$.

Mutation of the dileucine motif in the COOH-terminal cytosolic tail of GLUT4 causes a predominant cell surface

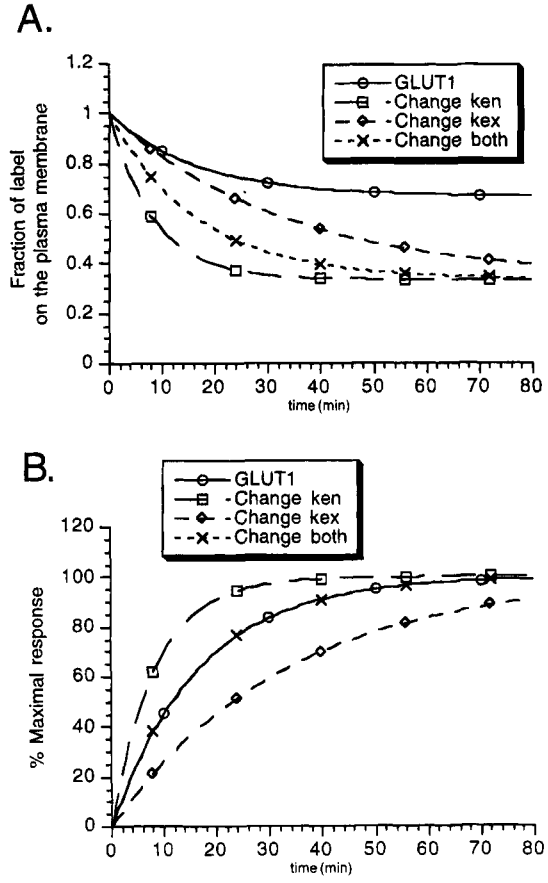


FIGURE 4: Predicted time course of equilibration after the glucose transporters on the plasma membrane at time 0 in fibroblasts were labeled on the basis of computer simulation. This is a hypothetical situation where the $k_{en}/k_{ex}$ ratio of GLUT1 is changed from $^1/_2$ (GLUT1, $k_{en} = 0.02$, $k_{ex} = 0.04$) to 2 by changing $k_{en}$ ($k_{en} = 0.08$, $k_{ex} = 0.04$), $k_{ex}$ ($k_{en} = 0.02$, $k_{ex} = 0.01$) or both ($k_{en} = 0.04$, $k_{ex} = 0.02$). Panel A shows the fraction of labeled transporter that remained on the plasma membrane vs time. Panel B shows the percent of maximal response vs time (the actual decrease expressed as a percentage of the maximal decrease).

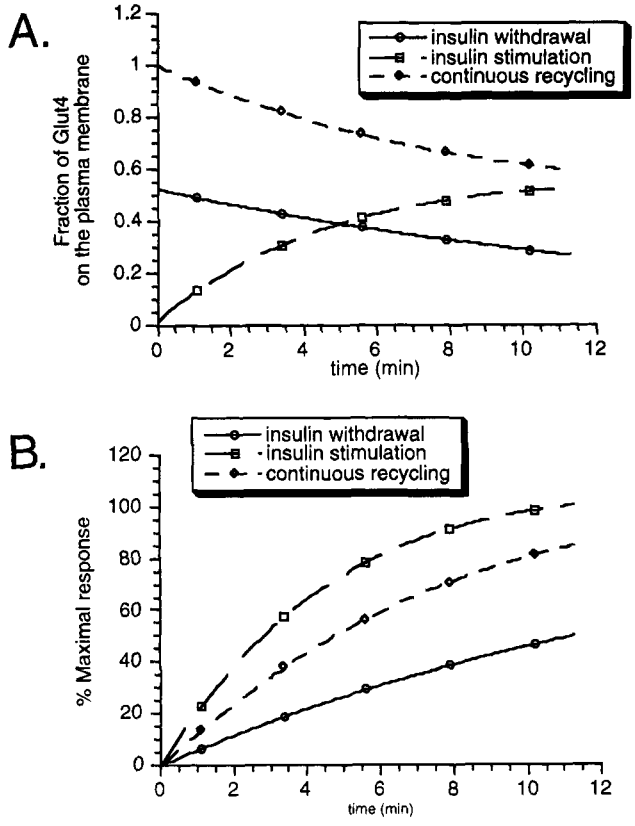


FIGURE 5: Computer simulation of the plasma membrane GLUT4 change under three different conditions in adipocytes: insulin stimulation, insulin withdrawal, and recycling in the continuous presence of insulin. In an actual experiment, GLUT4 on the plasma membrane would be quantified by Western blotting for the first two conditions. For the third condition, plasma membrane GLUT4 would be labeled at time 0 and the fraction of label remaining on the plasma membrane as a function of time measured. The analysis is based on the modified three-pool model. Panel A shows the percentage of GLUT4 on the cell surface vs time. Panel B shows the amount of change expressed as a percentage of the maximal change vs time.

localization of chimeric transporters (Czech et al., 1994; Verhey & Birnbaum, 1994). In the system of Czech et al., the HA-tagged mutant transporters showed an intermediate $t_{1/2}$ between that of the construct containing the wild-type GLUT4 carboxyl-terminus and GLUT1. Thus, the data in Corvera et al. are not sufficient for determining whether the dileucine mutation produces a greater effect on $k_{en}$ or $k_{ex}$.

*Glucose Transporter Trafficking in Adipocytes.* The two-pool model adequately describes the kinetics of trafficking of GLUT1, GLUT4, and chimeric glucose transporters in fibroblasts. However, as detailed by Holman and co-workers, this model is insufficient for describing the kinetics of glucose transporter trafficking in adipocytes [see Holman et al. (1994) and Holman and Cushman (1994)]. Briefly, two main arguments support the existence of at least three pools of cellular transporter. First, the $t_{1/2}$ of transition of GLUT4 from the basal to the insulin-stimulated state is much less than the $t_{1/2}$ of GLUT4 recycling in the continuous presence of insulin. The dependence of the transition half-time on the initial conditions is a characteristic of models with three or more pools. To relate our analysis of the modified three-pool model to that of Holman et al., eqs 14 and 15 can be directly solved using the rate constants of Holman et al. [$k_{en} = 0.06$, $k_1 = 0$, $k_{sq} = 0.15$, $k_4 = 0$, and $k_2 = 0.001$ (basal), and 0.12 (insulin)] (Holman et al., 1994).

Thus, the fractions of GLUT4 on the PM, $T_p(t)$, are as follows.

For the transition from the basal to the insulin-stimulated state,

$$T_p(t) = 0.53 - 0.51e^{-0.165t}\cos(0.0835t) + 0.38e^{-0.165t}\sin(0.0835t) \quad (23)$$

from the insulin-stimulated to the basal state,

$$T_p(t) = 0.016 + 0.51e^{-01.65t} - 0.00095e^{-0.149t} \quad (24)$$

and in the continuous presence of insulin, when surface GLUT4 is completely labeled at time 0,

$$T_p(t) = 0.53 + 0.47e^{-0.165t}\cos(0.0835t) + 0.22e^{-0.165t}\sin(0.0835t) \quad (25)$$

The graphs of eqs 16−18 show that the $t_{1/2}$ of transition for each condition is clearly different (Figure 5). The predicted $t_{1/2}$ values for eqs 23−25 are 2.9, 11.3, and 4.7 min, respectively. These approximate quite closely both those values predicted by Holman et al. using computer simulations as well as the available experimental data [see Holman et al. (1994)].

The three-pool model proposed by Holman et al. (Figure 2A) can account for the kinetic data available on the

subcellular trafficking of GLUT4 in insulin responsive cells. While Holman et al. briefly considered GLUT1 trafficking, they did not submit it to a detailed mathematical analysis. The subcellular trafficking of any transporter in Holman's three-pool model at steady state can be described as

$$dT_{ee}(t)/dt = k_{en}T_p(t) - k_{sq}T_{ee}(t) = 0$$

$$T_{ee} = T[(1/k_{sq})/(1/k_{en}) + 1/k_{sq} + 1/k_{ex}] \quad (26)$$

$$dT_{tv}(t)/dt = k_{sq}T_{ee}(t) - k_{ex}T_{tv}(t) = 0$$

$$T_{tv} = T[(1/k_{ex})/(1/k_{en}) + 1/k_{sq} + 1/k_{ex}] \quad (27)$$

$$dT_p(t)/dt = -k_{en}T_p(t) + k_{ex}T_{tv}(t) = 0$$

$$T_{tp} = T[(1/k_{en})/(1/k_{en}) + 1/k_{sq} + 1/k_{ex}] \quad (28)$$

Since in the basal state $k_{ex}$ is less than the other rate constants, most of the transporters, whether GLUT1, GLUT4, or chimeric, will be localized to the $T_{tv}$. Experimental data indicate that GLUT1 and the GLUT4 amino terminal chimera (see below) do not localize to this compartment in the basal state (Zorzano et al., 1989; Piper et al., 1991; Slot et al., 1991; Verhey et al., 1995). Thus, this model is insufficient for explaining trafficking of all transporters in insulin responsive cells.

We propose a modification of Holman's three-pool model (Figure 2A) which includes direct movement of transporters from the endosomal compartment to the PM (Figure 2B) and thus can account for all of the kinetic data available on the subcellular trafficking of glucose transporters in insulin responsive cells. In adipocytes, more than half of the total GLUT1 protein is located in the interior of the cell in the basal state but is likely in a pool biochemically and kinetically distinct from that in which GLUT4 is located. In the basal state, the rate constant for the exocytosis of GLUT4 ($k_{ex}$) is much lower than that of GLUT1 (Jhun et al., 1992; Yang & Holman, 1993; Holman et al., 1994). Insulin elicits the exocytosis of both transporters to the PM, albeit not equally. The amount of GLUT1 protein at the PM increases 2−5-fold, whereas GLUT4 increases 10−20-fold. Surface labeling of glucose transporters in rat and 3T3-L1 adipocytes has shown that GLUT1 and GLUT4 have similar rates of endocytosis ($k_{en}$) in the absence and presence of insulin (Jhun et al., 1992; Yang & Holman, 1993; Holman et al., 1994). In addition, since $k_2$ is identical for all transporters and $k_4$ is very small in numerical comparisons (see Materials and Methods), the major kinetic difference between GLUT1 and GLUT4 must lie in the $k_s/k_1$ ratio. The three-pool model proposed by Holman et al. also fails to explain the subcellular trafficking of chimeric transporters in insulin responsive cells. Chimeric transporters which contain the $NH_2$-terminus of GLUT4 substituted onto GLUT1 show a pattern similar to that of GLUT1 itself when heterologously expressed in L6 myoblasts (approximately 60% intracellular), a hormone responsive cell line (Haney et al., 1995). When stably expressed in 3T3-L1 adipocytes, the chimeras are located intracellularly in the basal state and recruit minimally to the cell surface in response to insulin (Verhey et al., 1995). This suggests that the GLUT4 amino terminal chimera is in either the endosomal pool or the IRV but, if in the latter, is not participating in the full translocation response to insulin. As discussed above, since we have set $k_2$ identical for all transporters (see Materials and Methods), the former explanation is the simplest and most plausible.

In the modified three-pool model at steady state,

$$dx_{irv}(t)/dt = k_s x_{en}(t) - k_4 x_{irv}(t) - k_2 x_{irv}(t) = 0$$

$$x_{en}/x_{irv} = (k_2 + k_4)/k_s \quad (29)$$

Thus, if the GLUT4 amino terminal chimera is predominantly in endosomes, the ratio in eq 29 must be higher for this chimera than for GLUT4. Since, according to our model, both $k_2$ and $k_4$ are identical for GLUT1 and GLUT4 and presumably all chimerae, transporters containing the GLUT4 amino terminus and the GLUT1 carboxy terminus must have a decreased rate of sequestration in the IRV ($k_s$) with respect to that of GLUT4; i.e., this chimera has the same $k_s$ as GLUT1. In addition, the subcellular distributions indicate that the GLUT4 amino terminus must contribute a decreased rate of exocytosis from endosomes back to the PM ($k_1$) with respect to that of GLUT1, as all transporters have an equal $k_{en}$. This analysis suggests that sequences within the GLUT4 amino-terminus are capable of preventing the transporter from efficiently returning to the cell surface but are not sufficient to allow sorting to the IRV.

Chimeric transporters containing the carboxy terminal cytosolic tail of GLUT4 substituted onto GLUT1 display a subcellular distribution similar to that of GLUT4 in the basal and the insulin-stimulated states, as well as similar kinetics of transition between these states (Verhey et al., 1995). This suggests that the GLUT4 COOH-terminus can confer the full kinetic characteristics of GLUT4 onto GLUT1, that is, a low $k_{ex}$ in the basal state due to sequestration in a maximally insulin responsive pool ($k_s$).

Mutation of the dileucine motif in the COOH-terminal cytosolic tail of GLUT4 redistributes chimeric transporters to a predominant cell surface localization in fibroblasts (above). In contrast, when stably expressed in 3T3-L1 adipocytes, the mutant transporters are absent from the cell surface in the basal state and undergo a marked translocation to the PM in response to insulin, similar to that of GLUT4 itself. Under the conditions of insulin withdrawal, however, the mutant transporters remain at the cell surface, resulting in a greater $t_{1/2}$ of equilibration than that of GLUT4 (Verhey et al., 1995). At steady state in the presence of insulin

$$dx_p(t)/dt = -k_{en}x_{pi} + ak_1x_{eni} + bk_2x_{irvi} = 0 \quad (30)$$

When insulin is withdrawn, the rate of the glucose transporters disappearing from the PM pool is

$$dx_p(t)/dt = -k_{en}x_{pi} + k_1x_{eni} + k_2x_{irvi} = -(a-1)k_1x_{eni} - (b-1)k_2x_{irvi} \quad (31)$$

and

$$-(b-1)k_{en}x_{pi}/b < dx_p(t)/dt < -(a-1)k_{en}x_{pi}/a \quad (32)$$

if $a = 3$ and $b = 100$ [even if $b \ll 100$, it will not change the conclusion (data not shown)], and if $x_{pi} = 0.4$ for GLUT4, the GLUT4 carboxy terminal chimera and the LL489AS mutant, then

$$-0.99k_{en}x_{pi} < dx_p(t)/dt < -1/2k_{en}x_{pi} \quad (33)$$

Thus, the initial rate of glucose transporters disappearing from the PM is primarily a reflection of the endocytosis rate constant ($k_{en}$), indicating that the major effect of the dileucine mutation in the GLUT4 carboxy terminal chimera is a reduction in this rate constant.

## DISCUSSION

In this report, we have derived mathematical equations to analyze the subcellular trafficking of glucose transporters in fibroblasts and adipocytes. Our results demonstrate that a two-pool model, with one pool at the PM and one in the interior of the cell, is sufficient to describe the trafficking of GLUT1, GLUT4, and chimeric transporters in fibroblasts. In agreement with Holman and co-workers, our results suggest that a three-pool model, in which there are two interior compartments of cellular transporter, is necessary to describe transporter trafficking in adipocytes. However, we suggest that a modification of Holman's three-pool model to allow the movement of transporters from the endosomal compartment back to the PM is necessary to fully account for the kinetics of GLUT1 and GLUT1/GLUT4 chimeric transporters.

In regard to the trafficking of glucose transporters in fibroblasts, several important conclusions can be drawn on the basis of the analyses presented above. The first relates to the intracellular sequestration of GLUT4 in non-insulin responsive cell types, which could be the result of either a lower $k_{ex}$ or a greater $k_{en}$ compared to those of GLUT1. Analysis of the data of Corvera et al. according to eq 8 and as illustrated in Figure 4 clearly demonstrates that the predominant difference between GLUT1 and GLUT4 is $k_{ex}$. The effect of mutation of the dileucine motif is not clear from the data of Corvera et al. However, extrapolation from the analysis of such a mutant in 3T3-L1 adipocytes and summarized quantitatively in eq 33 suggests that the major effect of ablating dileucine is reduction of $k_{en}$. This raises the interesting possibility that the dileucine amino acid pair does not form part of the GLUT4 sequestration motif but that mutation of these amino acids decreases endocytosis of GLUT4 to a degree which fortuitously compensates for its delayed exocytosis.

Another point which becomes clear from the analysis presented above is that the absence of an insulin responsive compartment, IRV, in fibroblasts, i.e., $k_s = 0$, is adequate to explain some of the discordant targeting of chimerae in fibroblasts vs adipocytes. In other words, any mutation or chimera which affects only $k_s$ would be expected to be without effect in nondifferentiated cells. A more subtle prediction of this model is that alternations in $k_{en}$ might be obscured in adipocytes in the steady state by a high $k_s$. Thus, the LeuLeu mutation in GLUT4 has minimal effect in adipocytes since, even with a decrease in the rate of endocytosis, the transporter is effectively sequestered in IRV. The decreased $k_{en}$ becomes quite apparent, however, when the mutant is expressed in fibroblasts which lack a compartment for sequestration or in adipocytes during nonequilibrium conditions.

A major difference between the analysis of Holman et al. and the current one is our assumption that the major route of return to the plasma membrane for GLUT1 is directly from endosomes and not via IRV. Stated in terms of rate constants, we have set for GLUT1 a finite $k_1 > 0$ and a GLUT1 $k_s \ll k_s$ for GLUT4. Several lines of evidence favor this model. The existence of distinct pathways for the movement of GLUT1 and GLUT4 to the cell surface is supported by biochemical and morphological data (Zorzano et al., 1989; Piper et al., 1991; Slot et al., 1991), as well as the observation that drugs can dissociate translocation of the two isoforms. For example, phenylarsine oxide blocks insulin-dependent GLUT4, but not GLUT1, translocation (Yang et al., 1992). Other agents can partially mimic the actions of insulin; for example, phorbol 12-myristate 13-acetate (PMA) stimulates GLUT1 translocation to the same degree as insulin, whereas GLUT4 is recruited to only 20−25% of the levels achieved by insulin (Holman et al., 1990). Perhaps one of the more compelling pieces of evidence derives from the behavior in 3T3-L1 adipocytes of 4HB1, a chimeric transporter containing the amino terminus of GLUT4 and the carboxyl terminus of GLUT1 (Verhey et al., 1995). This transporter is sequestered within the cell quite effectively but does not translocate to surface in response to insulin. The most plausible explanation is that there exists within the cell a compartment normally inhabited by GLUT1 which translocates minimally in response to insulin. Such a compartment is likely to be what we have designated endosomes.

An important assumption for this analysis is that all cargo proteins in IRV are moved with identical rates. Clearly, without this restriction, the observations discussed in the preceding paragraph could be interpreted quite differently. However, the similarities between GLUT4 translocation and regulated secretion, in which the secretory vesicles move as a unit, and the prevailing view of various forms of constitutive vesicular transport support our assumption (Bennett & Scheller, 1994; Rothman & Orci, 1992). Moreover, the recent identification of another IRV resident protein, a 165 kDa glycoprotein, which translocates to the cell surface in a manner indistinguishable from that of GLUT4, is consistent with this view (Mastick et al., 1994; Kandror & Pilch, 1994).

Lastly, the relationship of kinetic compartment to true cellular entity deserves comment. In this analysis, we have assigned intracellular compartments the names endosomes and IRV because (1) these seemed plausible postulates and (2) the use of concrete terms simplifies the discussion. However, the data supporting the localization of the transporters in these compartments in insulin responsive cells are inconclusive, and the relevance of adipocyte organelles to endosomal structures best characterized in nondifferentiated cells is unclear. While we have elected to discuss the movement of transporter in terms of distinctive subcellular compartments, it should be recognized that identical mathematical treatments pertain to other cellular mechanisms, for example, sequestration of GLUT4 in a specialized domain of an organelle common to GLUT1 following passage through the common domain. In addition, kinetic treatments such as these are useful only in specifying minimal numbers of functional compartments. In this analysis, we have not considered the potential "occluded" plasma membrane discussed in detail by Holman et al. (1994).

## ACKNOWLEDGMENT

We thank Sharon F. Hausdorff for many helpful discussions and Cass Lutz for the assistance in the preparation of the manuscript.

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BI951872U