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Glucose is a key substrate for lactating and neoplastic mammary epithelial cells. Our purpose is to understand how lactating breast meets its need for glucose transport into Golgi, the site of lactose synthesis, and whether this mechanism has physiological, pathophysiological, or potentially therapeutic relevance in breast cancer. The scope of the research includes established cell lines, primary cells, and mammary glands, from both mice and humans. Major findings are: 1) in mouse and human mammary epithelial cells in culture and also in mouse mammary gland, the hormonal milieu of lactation induces GLUT1 and causes it to be targeted to Golgi, 2) GLUT1 targeting to Golgi is rapidly reversible during forced weaning, and 3) GLUT1 is virtually absent from the plasma membrane in MCF7 and MDA231 cancer cells even in the absence of lactogenic hormones, and is targeted to a non-Golgi compartment. The results suggest that GLUT1 is sequestered within the cell and is not responsible for plasma membrane glucose transport activity in breast cancer. A unique mechanism, possibly with effects on targeting and activity of other proteins, must underlie the sequestration of GLUT1 in breast cancer cells. These cells may express a novel glucose transporter, potentially an attractive therapeutic target.

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Peter M. Honey, MD PhD 10/7/99
PI - Signature FOR Date
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Table of Contents

1. Front Cover.....	1
2. Report Documentation Page.....	2
3. Foreword.....	3
4. Table of Contents.....	4
5. Introduction.....	5
6. Body.....	6
7. Key Research Accomplishments	14
8. Reportable Outcomes.....	15
9. Conclusions.....	17
10. References.....	19
11. Appendices.....	20
1. Figures 1-16 (8 pages)	
2. Manuscript 1: Localization of ... (32 pages)	
3. Manuscript 2: Golgi targeting of... (29 pages)	
4. Manuscript 3: Liposome-mediated...(13 pages)	

Introduction

As of July 1, 1997, I became an Assistant Professor of Pediatrics at the Baylor College of Medicine in Houston Texas. I am affiliated with the Section of Neonatology and with Texas Children's Hospital and my lab is in the U.S.D.A./A.R.S. Children's Nutrition Research Center. Note that, since DOD policy did not allow the grant to be transferred from Washington University to Baylor College of Medicine, F. Sessions Cole, M.D. graciously agreed to serve as Principal Investigator and to subcontract the work to me at Baylor College of Medicine. Therefore, I am technically submitting this report on his behalf. Note also that this submission is in the form of an Annual Report, although a Final Report was requested. This is because a 12 month extension of the project was granted, as acknowledged by Grants Officer Patricia McAllister on August 27, 1999. The extension will allow me to complete the research. A Final Report will be filed in October, 2000.

Glucose is critical to mammary epithelial cells because it serves as a fuel and as a building block for glycoproteins and glycolipids. Furthermore, it is the major substrate for the synthesis of lactose and lipid, which together contribute 80-90% of the calories in human milk. Lactose is the major carbohydrate constituent of human milk and the major determinant of its osmolarity, and therefore, of milk production. Synthesis of lactose is carried out exclusively within the Golgi apparatus of mammary epithelial cells, in a reaction catalyzed by galactosyltransferase complexed to the tissue-specific protein alpha-lactalbumin. Lipid components of human milk, which are also primarily derived from glucose, provide about one-half of the caloric content of milk. Thus, regulation of glucose uptake in mammary epithelia must account for two very different states, the quiescent state, with a relatively small demand for glucose, and the lactating state, with an extraordinary demand for glucose to fuel the high metabolic rate of the epithelial cells themselves as well as to provide substrate for the synthesis of milk to sustain the young. Breast cancer cells also exhibit an increased demand for glucose. The molecular mechanisms by which the enhanced transport of this vital nutrient into tumor cells is accomplished require further investigation. Elucidation of the molecular mechanisms by which the mammary epithelia achieves the adaptations in glucose transport needed for lactation, and the examination of their possible dysregulation in neoplastic mammary epithelium, form the central goals of this proposal. The specific hypotheses to be tested are: 1) glucose transport into mammary epithelial cells is subject to a high degree of regulation, and 2) abnormal glucose transport in mammary epithelial cells (i) is associated with abnormal cell growth, and (ii) may facilitate abnormal cell growth. In order to test these hypotheses, the following specific aims were chosen: 1) description of the developmental and hormonal regulation of glucose transport in mammary gland, 2) identification of novel proteins involved in glucose transport in lactating mammary epithelia, and 3) examination of a possible association between abnormal glucose transport and the neoplastic phenotype. The scope of the work includes primary mammary epithelial cells, established mammary epithelial cell lines in culture, and intact mammary glands from mice and humans.

BodyResults and Discussion of Research Accomplishments to Date*Task 1: Description of the developmental and hormonal regulation of glucose transport and lactose biosynthesis in CIT3 cells and in mammary gland*

Previous Annual Reports have described the developmental and hormonal regulation of glucose transport in mouse mammary epithelial cells in culture and in mouse mammary gland. During the past year two manuscripts based on this have been submitted for publication.

The work regarding mouse mammary epithelial cells in culture has been judged as accepted for publication in Cell Biology International subject to minor revision. The manuscript is appended in its current form as Appendix 1. The abstract reads:

The synthesis of lactose, the major carbohydrate and osmotic constituent of human milk, takes place in the Golgi. To provide substrate for lactose synthesis in mammary epithelial cells, free glucose must be transported into Golgi. The GLUT1 glucose transporter is the only isoform of the facilitated diffusion family of glucose transporters expressed in mammary gland. The hypothesis that lactogenic hormones induce GLUT1 and cause its localization to the Golgi of mammary epithelial cells was tested. CIT₃ mouse mammary epithelial cells treated with prolactin and hydrocortisone showed a 15-fold induction of GLUT1 by Western blotting. In contrast, 2-deoxyglucose uptake, which reflects the uptake of glucose across the plasma membrane, decreased in response to this treatment. Subcellular fractionation and iodixanol density gradient centrifugation demonstrated enrichment of Golgi fractions with GLUT1. Lactogenic hormones enhanced GLUT1 glycosylation, but did not determine whether GLUT1 was targeted to plasma membrane or to Golgi. Confocal immunofluorescent microscopy revealed that lactogenic hormones alter GLUT1 targeting from a plasma membrane pattern to a perinuclear distribution with a punctate pattern scattered through the cytoplasm, characteristic of Golgi. However, GLUT1 colocalized neither with BODIPY-TR ceramide, a trans-Golgi marker, nor with α -mannosidase II or β -COP, markers of medial- and cis-Golgi, respectively. Brefeldin A (used at a concentration that disrupted trans-Golgi but not cis- or medial-Golgi of lactating rabbit mammary epithelial cells) caused disruption of the trans-Golgi but did not grossly affect targeting of GLUT1, α -mannosidase II, or β -COP. However, Brefeldin A did cause colocalization of GLUT1 with α -mannosidase II and β -COP, suggesting a subtle effect on membrane trafficking in cis- and medial-Golgi. Therefore, in mammary epithelial cells, prolactin and hydrocortisone induce GLUT1, enhance GLUT1 glycosylation, and cause glycosylation-independent targeting of GLUT1 to a subcompartment of cis- and/or medial-Golgi, but not to trans-Golgi. These results suggest a tissue- and developmental stage-specific mechanism for Golgi targeting of GLUT1, and indicate an important role for GLUT1 in the provision of substrate for lactose synthesis.

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The work regarding mouse mammary gland has been judged as possibly acceptable for publication in *Pediatric Research* subject to revision. The manuscript is appended in its current form as Appendix 2. The abstract reads:

Lactose, the major carbohydrate of human milk, is synthesized in the Golgi from glucose and UDP-galactose. The lactating mammary gland is unique in its requirement for the transport of glucose into Golgi. GLUT1 is the only isoform of the glucose transporter family expressed in mammary gland. In most cells, GLUT1 is localized to the plasma membrane and is responsible for basal glucose uptake; in no other cell type is GLUT1 a Golgi resident. To test the hypothesis that GLUT1 can account for Golgi glucose transport, the amount and subcellular distribution of GLUT1 were examined in mouse mammary gland at different developmental stages. Methods, including immunohistochemistry, immunofluorescence, and subcellular fractionation, density gradient centrifugation, and Western blotting, yielded consistent results. In virgins, GLUT 1 expression was limited to plasma membrane of epithelial cells. In late pregnant mice, GLUT1 expression was increased, with targeting primarily to basolateral plasma membrane, but also with some intracellular signal. By 8 d after delivery, GLUT1 expression was further increased and targeting to intracellular compartments predominated. Removal of pups 18 d after delivery resulted in retargeting of GLUT1 from Golgi to plasma membrane and a decline in total cellular GLUT1 within 3 h. In mice undergoing natural weaning, GLUT1 expression declined. Changes in the amount and targeting of GLUT1 during mammary gland development indicate a key role for GLUT1 in supplying substrate for lactose synthesis and milk production.

Thus, task one has been accomplished regarding the mouse. A model involving the induction of GLUT1 and its retargeting from the plasma membrane to the Golgi under the influence of the hormonal milieu of lactation is confirmed by the data. This model serves well for further experiments to understand the mechanism of the retargeting of GLUT1.

During the past year, we have turned our attention to the human mammary epithelial cell, intending to accomplish the same understanding in cells in culture. It is not possible to carry out studies of the lactating human mammary gland in a parallel fashion. However, mammary epithelial cells can be isolated from milk during the first week of lactation, and from surgical specimens. We carried out our studies in commercially available cells (Clonetics, Walkersville, MD) derived from normal surgical specimens.

Our first goal was to establish the usefulness of ECFP-Golgi as a Golgi marker in these cells. This is expressed in cells transfected with the commercially available plasmid pECFP-Golgi, and consists of enhanced cyan fluorescent protein fused to an 85 amino acid sequence of galactosyltransferase which contains its Golgi targeting determinant. This is a most attractive possibility for our use as a marker, since galactosyltransferase is one of the components of lactose synthetase and should actually mark the compartment in which lactose is synthesized. We were able to obtain very high quality images using a deconvolution microscope (Figure 1), and we are working to optimize the use of this marker with standard epifluorescence. Human mammary epithelial cells were maintained in growth medium, and in secretion medium which also contained prolactin. We found differences in GLUT1

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targeting after four days in secretion medium compared to growth medium (Figure 2). In growth medium (Figure 2A), staining was predominantly localized to the plasma and nuclear membranes. In secretion medium (Figure 2B), there was very little plasma membrane staining, but abundant staining of the nuclear membrane and perinuclear region consistent with Golgi. This impression was confirmed by staining cells transiently transfected with pECFP-Golgi and grown in secretion medium (Figure 3). In these cells, GLUT1 is targeted to a perinuclear subcompartment of the ECFP-Golgi staining. We also undertook conventional colocalization studies (Figure 4) of GLUT1 with beta-COP, a cis-Golgi marker (Figure 4B), alpha-mannosidase, a medial Golgi marker (Figure 4E), and Bodipy-TR ceramide, a trans-Golgi marker (Figure 4H). Colocalization was most evident for alpha-mannosidase (Figure 4F), and also beta-COP (Figure 4C), and was not observed for Bodipy TR-ceramide (Figure 4I). In summary, the immunofluorescence studies confirm that, just as we demonstrated for mouse, human GLUT1 is targeted to Golgi in response to the hormonal milieu of lactation. Preliminary results indicate that we can obtain useful information regarding hormonal regulation of 2-deoxyglucose uptake and lactose biosynthesis in these cells. These results will be the first report of glucose transporter biology in normal human breast.

We have also begun to develop a ribonuclease protection assay for detection of all glucose transporters simultaneously. The principle for this is that a unique region of each glucose transporter mRNA, of between 150 and 400 nucleotides, differing in size by at least 20 bases, have been selected. PCR primers for each sequence have been made and used to synthesize PCR products with a T7 promoter at the 3' end of the antisense sequence. These PCR products served as substrate for T7 RNA polymerase, which made antisense RNA species for each sequence. These will be used as riboprobes after labeling with psoralen-biotin and used in the ribonuclease protection assay. If the corresponding mRNA is present, the labeled probe will be detected, and a signal of the appropriate size will be seen. If not, not. The strength of the signal will be proportional to the amount of glucose transporter RNA present. Since each transporter will give a signal at a unique size, we will be able to determine the amount of each transporter simultaneously. This will be important not only in eliminating the possibility that other glucose transporters are expressed in normal mammary gland, but also in efficiently evaluating multiple tumors under Task 3.

Thus, I conclude that Task 1 has been essentially accomplished. The formality of excluding other glucose transporters using ribonuclease protection assay is all that remains. We are preparing a manuscript on the hormonal regulation of glucose transport in human mammary epithelial cells for submission to the Journal of Cell Science.

Task 2. Identification of novel proteins involved in glucose transport in lactating mammary epithelia

We had concentrated on differential display as the method of choice for pursuit of this task. One gene previously identified, LDH-A, has been further studied, and evidence regarding its induction by lactogenic hormones was provided in previous reports. We also showed a 2-3 fold induction of LDH activity in last year's report. In order to determine

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whether the presumed induction of LDH-A mRNA by prolactin and hydrocortisone was also associated with an increase in LDH-A protein, LDH isoform analysis was conducted. The results demonstrated no significant change in LDH-A protein (Figure 5), and lead to a new interpretation of the positive differential display result and the apparent difference in enzyme activity. The results suggest that the specific activity of LDH may be increased by lactogenic hormones, since the amount of protein did not change. While interesting, this observation is not relevant to our specific aims, and we have not yet pursued LDH-A induction further.

Previous Annual Reports included progress with several novel expressed sequence tags identified by this process. These ESTs correspond to genes expressed in response to lactogenic hormones but are actually unlikely to be specifically related to regulation of glucose transport. I periodically search the rapidly accumulating gene databases for homologies to these sequences. During the past year, one of the novel sequences we identified showed an extremely high degree of homology with the newly deposited sequence of human sec24, a protein required for vesicular transport from endoplasmic reticulum to Golgi. Our sequence appears to be mouse sec24. It is not surprising that a protein required for vesicular transport along the secretory pathway would be induced during lactation, and this is presumably a true positive, but irrelevant to our current specific questions regarding glucose transport. Nevertheless, it does point out an interesting area for future investigation.

As I pointed out in last year's Annual Report, because a number of recent papers have appeared pointing out the difficulties and large amount of efforts and resources required to bring differential display analyses to a conclusion, and because of rapid progress of the human genome project information which will facilitate identification of the current sequences, and because of rapid development of DNA microchip arrays, which offer many advantages over differential display, I have deferred further pursuit of the ESTs at this time. I do continue to search the databases routinely for homology to the currently identified sequences.

Because of a report of GLUT1-C-terminal binding proteins that associate with GLUT1 and alter its targeting (Bunn et al., 1999), we undertook PCR-based screening to attempt to identify a GLUT1-CBP related protein in lactating mouse mammary gland. Four sets of primers were chosen from the coding region, and all possible permutations were used with cDNA from a lactating mouse mammary gland cDNA library. No PCR products were obtained, suggesting that neither GLUT1CBP nor any other related protein is expressed, or that all areas chosen diverge sufficiently in structure such that no PCR product was obtained. I will follow the literature in this area closely and continue to screen for other GLUT1CBPs, if any others are identified. The GLUT1CBP acts by binding to the 4 amino acid PDZ domain at the C-terminus of GLUT1. We are planning to use site-directed mutagenesis of the GLUT1-EGFP fusion proteins described below to eliminate these 4 amino acids and test the effect on targeting of the fusion protein. The results will guide the aggressiveness of our search for a homologous mammary gland glucose transporter binding protein that might account for the unique characteristics of GLUT1 targeting in this gland.

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I described in last year's Annual Report a fascinating and potentially very direct approach involving the use of dodecyl maltoside and Blue Native protein electrophoresis. This approach is designed to not disrupt associations between proteins; thus, the signal on the gel reflects the sum of the molecular weight of the protein being detected and the molecular weight of other protein(s) associated with it. When this procedure is carried out on iodixanol density gradient purified Golgi fractions from lactating mammary gland or from CIT3 cells grown in secretion medium (data not shown), a single broad band running at about 130 kD was identified. The simplest interpretation of this would be that GLUT1 is associated with a single protein of about 80-90 kD. This would presumably be a Golgi resident protein responsible for retaining GLUT1 in the Golgi. Note that virtually no signal is identified at about 45 kD, where monomeric GLUT1 would be seen; thus, under these conditions, virtually no Golgi GLUT1 exists as a free integral membrane protein. The next steps I described were to further purify this protein by denaturing gel electrophoresis and by 2-D gel electrophoresis, to obtain peptide sequence information, and to identify the associated protein as a known protein or to clone it. The most efficient purification scheme would use GLUT1 antibody to immunoaffinity purify GLUT1 and its associated proteins. This requires a large amount of antibody, and necessitated that we raise our own antibody to GLUT1 rather than rely on kind gifts. This has been accomplished during the past year. Rabbit antibody was raised against the sequence SGFRQGGASQSDKT, amino acids 464-477, and also to PEELFHPLGADSQV, amino acids 478-491. The second sequence, at the extreme C-terminus, is the traditionally chosen antigenic site for generation of glucose transporter antibodies. The first sequence was chosen because analysis of antigenicity suggested that this sequence might be more highly antigenic. Interestingly, the antibody against amino acids 464-477 of GLUT1 yields a purely intracellular signal on immunocytochemistry of human mammary epithelial cells, with no staining of the nuclear membrane (Figure 6). The significance of this is still under investigation. The traditionally chosen site did prove highly antigenic, and at least 50 cc of high-titer rabbit antiserum has been raised. This antibody has been purified and characterized, and reacts highly specifically with mouse brain GLUT1 (Figure 7). Immunocytochemistry results are consistent with previous GLUT1 antibodies (data not shown). This antibody will be used as described above to help identify GLUT1 associated proteins.

Task 3. Examination of a possible association between abnormal glucose transport and the neoplastic phenotype

I chose to begin our studies of neoplastic cells with well established cultured cell lines, so we could develop an understanding of the mechanisms of glucose transport in neoplastic mammary epithelia before utilizing human tissue. These experiments were generally designed to test the hypothesis that neoplastic mammary epithelial cells would demonstrate high levels of glucose transport activity and GLUT1 protein to support a high rate of glucose utilization, and that GLUT1 in these cells would be targeted primarily to the plasma membrane, again in order to support a high rate of glucose utilization. The hope I set forth in my proposal was that activation of the Golgi targeting mechanism for GLUT1 in a neoplastic cell might serve to deprive the cell of substrate, limit its growth, and make it more vulnerable to chemotherapy.

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In last year's Annual Report I described our results with MCF7 cells, a non-metastatic line, and MDA231 cells, a metastatic line. In brief, MCF7 cells showed very low glucose uptake and were not responsive to prolactin and hydrocortisone. In contrast, MDA231 appeared to demonstrate an exaggeration of the pattern seen in CIT3 cells, with a very high glucose uptake under basal condition, and 73% inhibition by prolactin and hydrocortisone, consistent with very high total cellular levels of GLUT1 with highly regulated changes in subcellular distribution paralleling those seen in CIT3 cells. However, hormonal treatment affected neither the amount nor the subcellular targeting of GLUT1 in MDA231 cells, and there was very little GLUT1 detected in the homogenate and plasma membrane fractions, suggesting that GLUT1 was not the glucose transporter responsible for the very high rates of glucose transport activity.

We have concentrated during the past year on understanding the intracellular compartment to which GLUT1 is targeted and the mechanisms involved. The need to do so is the reason an extension was requested to complete the proposal, since this is a necessary prerequisite to our studies of human tumors and genetically altered and transplanted mouse mammary glands.

These studies have involved two major areas, immunocytochemistry in fixed cells, and immunofluorescence due to the expression of GLUT1-EGFP fusion proteins in living cells. The use of fluorescent fusion proteins of GLUT1 is attractive because their use avoids potential artifacts of fixation, allows the study of the same cells over time, permits studies of exocytosis and endocytosis, not just steady state distributions, facilitates studies of organized cells in extracellular matrix, permits Fluorescence Resonance Energy Transfer (FRET) studies of molecular interactions, permits targeting of chimeric proteins to be evaluated in an antibody-independent fashion, and confirms we are studying GLUT1, not a novel, lactation-specific Golgi glucose transporter isoform that shares the GLUT1 epitope. In last year's Annual Report, the successful construction of an expression vector for a GLUT1-EBFP (enhanced blue fluorescent protein) fusion protein, and the expression of this fusion protein by liposome-mediated transfection of CIT3 cells, was discussed. We initially chose blue fluorescent protein constructs so that green signal could be reserved for other proteins or markers. However, work during the past year led us to conclude that the blue fluorescent protein constructs were plagued by rapid photobleaching and such a weak blue fluorescent signal that their use was limited to static images, and therefore not useful for studies of trafficking kinetics. This is demonstrated in Figure 8, which shows that the sensitivity of the green fluorescent protein is at least an order of magnitude superior to blue fluorescent protein, and that the image quality using blue fluorescent protein is inadequate for our purposes.

We therefore set aside our EBFP-glucose transporter constructs, and proceeded to construct two plasmids, one for expression of GLUT1-EGFP, and the other for expression of EGFP-GLUT1. For EGFP-GLUT1, EGFP is fused to the N-terminus of GLUT1; for GLUT1-EGFP, EGFP is fused to the C-terminus of GLUT1. We considered it important to make both fusion proteins and observe targeting from both constructs so as not to be misled

by artifact. Each construct was checked for appropriate size and right orientation of GLUT1 insert by restriction digest, followed by DNA sequencing. An important initial goal was to validate appropriate plasma membrane and Golgi targeting of GLUT1-GFP fusion proteins in living mammary epithelial cells. Studies were carried out using both transient transfection, with analysis at 24-96 h, and by stable transfection, using 1) CaPO₄, 2) DEAE-Dextran, 3) Polycationic transfection reagent (SuperFect), and 4) Liposome-mediated transfection (LipoFectAmine). These studies were done systematically to determine the optimal transfection system. For further details, please refer to the Appendix for the draft manuscript "Liposome-mediated stable transfection of CIT3 mouse mammary epithelial cells," which is to be submitted shortly to Biological Chemistry. The abstract reads:

Transfection of mammary epithelial cells can provide important insights into the molecular biology of milk secretion and breast cancer. However, investigators have acknowledged difficulties transfecting these cells. The objective of this work was to determine an efficient method for achieving stable transfections in these cells. The relative transfection efficiency of CaPO₄, DEAE-Dextran, cationic polymers and cationic liposomes was compared. CIT₃ mouse mammary epithelial cells were transfected with green and blue fluorescent protein plasmid vectors. Transfection efficiency, expressed as the number of stably transfected colonies selected by geneticin, was highest for cationic liposomes compared to all other transfection methods ($p < 0.05$).

Having established the usefulness of liposome-mediated transfection, this method was used for both transient and stable transfection with pEGFP-GLUT1 and with pGLUT1-EGFP. As demonstrated in Figure 9, both constructs target as native GLUT1 does, in both growth medium and secretion medium, in CIT3 mouse mammary epithelial cells. Having established this, we proceeded to label transfected cells with Bodipy-TR ceramide and to study the living cell. Figure 10 demonstrates both plasma membrane and intracellular staining for GLUT1 in mouse mammary epithelial cells in growth medium; there is only slight colocalization of GLUT1-EGFP with the trans-Golgi marker, consistent with our results in fixed cells that the trans-Golgi are not a significant targeting compartment for GLUT1. Figure 11 again demonstrates the transition to intracellular GLUT1 targeting and loss of plasma membrane GLUT1 upon exposure to prolactin and dexamethasone. However, the very slight degree of colocalization again indicates that the trans-Golgi are not a significant targeting destination of GLUT1. Certain cells responded to secretion medium by accumulating a large number of intracellular vesicles. The limiting membrane of these vesicles is the major intracellular localization of GLUT1 in these cells (Figure 12), suggesting that the vesicles represent lactose-containing vesicles. This is the first time GLUT1 has been visualized in the membrane of large intracellular vesicles.

We have begun studies of expressed GLUT1-EGFP fusion proteins in cancer cells. We continue to concentrate on the MCF7 and MDA231 cells at this point. Initial studies using transient transfection are shown. Figure 13 shows the unique intracellular targeting of GLUT1-EGFP to intermediate-size, GLUT1-dense vesicles in MCF7 cells in growth medium. Little colocalization with Bodipy-TR ceramide was seen. This is particularly interesting, since we were unable to detect the cell's own GLUT1 using our standard immunofluorescence techniques. Strikingly similar results were obtained in MDA231 cells

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(Figure 14). The cis-Golgi marker beta-COP showed no colocalization with the GLUT1-EGFP fusion protein in these cells (Figure 15).

The major potential of the GLUT1-EGFP fusion protein lies not in recapitulating what we have already shown in fixed cells, but in taking advantage of the opportunity to observe changes in one cell over time. This will not only help identify the intracellular compartment containing GLUT1 in neoplastic cells, it will also help us establish the cellular processes underlying the targeting, the kinetics of the targeting, the relative importance of exocytosis and endocytosis, and the effects of exogenous agents. An example of this is shown for CIT3 mouse mammary cells in Figure 16, which demonstrates : a) vesicle transfer within Golgi, b) vesicle uptake into Golgi, c) endocytic vesicle uptake into Golgi, d) endocytosis from plasma membrane, and e) exocytic pathway vesicles trafficking toward plasma membrane. These results demonstrate that GLUT1 targeting is a dynamic process with the potential for regulation both at the level of exocytosis and the level of endocytosis. Preliminary results show a similar dynamic process in MCF7 and MDA231 cells as well.

We have invested considerable effort to isolate and expand stably transfected clones of cells expressing the fusion proteins, and we have several unique colonies for each cell type at this point. Multiple independent clones must be studied in order to conclude that results are not a function of clonal variation or of inappropriate levels of fusion protein expression.

In summary, with respect to Task 3, at this point we have demonstrated that the neoplastic phenotype of mammary epithelial cells, but not the normal phenotype, includes an absence of plasma membrane targeting of GLUT1, which is instead targeted to an intracellular compartment which does not appear to be Golgi. The results therefore indicate that the intracellular targeting of GLUT1 is not caused by an abnormally timed triggering of the GLUT1 targeting mechanism operative during lactation. Rather, it represents a mechanism specific to the neoplastic cell. We continue to define the intracellular compartment in which GLUT1 resides, focusing on the aggresome and the endosome rather than the Golgi. We will commence our studies of human tumors and transplanted mouse mammary glands when we have identified this compartment and useful markers for it. The results also suggest that neoplastic cancer cells do not rely on GLUT1 for transport of glucose across the plasma membrane.

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Key Research Accomplishments

- In normal mouse and human mammary epithelial cells in culture, the GLUT1 glucose transporter is induced by prolactin and hydrocortisone and undergoes a change in intracellular trafficking from a plasma membrane distribution to an intracellular pattern consistent with targeting to the Golgi.
- In mouse mammary gland, the GLUT1 glucose transporter is induced during pregnancy and lactation and undergoes the same change in intracellular trafficking described above.
- Golgi targeting of GLUT1 in mouse mammary gland during lactation is reversible and is influenced by suckling and weaning.
- In MCF-7 and MDA-231 human cancer cells, GLUT1 is targeted not to the plasma membrane, but to an intracellular compartment, and is not subject to hormonal regulation.
- Liposome-mediated transfection can be used efficiently in both normal and neoplastic mammary epithelial cells.
- In cancer cells, GLUT1 levels and subcellular targeting are not correlated with glucose transport activity. The regulation of glucose transport activity by prolactin and hydrocortisone in these cells suggests the possibility of another glucose transporter playing an important role.
- Fusion proteins of GLUT1 with green fluorescent protein reveal dynamic intracellular trafficking of GLUT1 in both normal and neoplastic cells and will be an important tool in understanding aberrant glucose transporter targeting in cancer cells.

Reportable Outcomes

1. Manuscripts, Abstracts, Presentations

Manuscripts

1. Haney, PM. Localization of the GLUT1 glucose transporter to the Golgi complex of differentiated CIT3 mouse mammary epithelial cells, *Cell Biology International*, accepted subject to minor revision
2. Nemeth, BN, Tsang, ST, Geske, RS, Haney, PM. Golgi targeting of the GLUT1 glucose transporter in lactating mouse mammary gland, *Pediatric Research*, submitted 6/4/99
3. Riskin, A, Nannegari, V, and Haney, PM. Liposome-mediated stable transfection of CIT3 mouse mammary epithelial cells, *Biological Chemistry*, in preparation

Abstracts and Presentations

1. Haney PM. Structural determinants of glucose transporter targeting. Tenth Annual Mead Johnson Neonatal Nutrition Symposium, Marco Island, FL, December 1995.
2. Haney PM. Exposure of mammary epithelia to lactogenic hormones causes Golgi sequestration and plasma membrane polarization of glucose transport activity. *Ped Res* 1996; 39:89A (SPR platform presentation).
3. Nemeth BA, Haney PM. Preferential targeting of GLUT1 to Golgi of mouse mammary epithelial cells during lactation is rapidly but reversibly lost during premature weaning. *Ped Res* 1997; 41:70A (SPR poster symposium presentation).
4. Haney, PM. Regulation of glucose transport in quiescent and lactating mammary epithelia. *Proceedings of the Department of Defense Breast Cancer Research Program Meeting* 1997; vol. 1: 269-270.
5. Haney, PM. Premature weaning alters glucose transporter targeting in lactating mouse mammary gland. *Mol. Biol. Cell* 1997; 8, 307a (ASCB poster presentation).
6. Geske, S, Haney, PM. Developmental regulation of the amount and subcellular targeting of the GLUT1 glucose transporter in mouse mammary gland. *Ped Res* 1998; 43:75A (SPR poster symposium presentation).
7. Haney, PM. Hormonal regulation of the amount, activity, and subcellular targeting of GLUT1 in normal and neoplastic mammary epithelial cells. *Ped Res* 1998; 43:77A (SPR poster symposium presentation).
8. Riskin, A, Nannegari, V, Haney, PM. Efficient transfection of CIT3 mammary epithelial cells using liposomes. *FASEB J.* 1999; 13:A432 (FASEB poster presentation).
9. Haney, PM. Lactogenic Hormones Cause Glycosylation-Independent Targeting of the GLUT1 Glucose Transporter of Mammary Epithelial Cells to a Subcompartment of cis- and/or medial-Golgi. *Ped Res* 1999; 45:90A (SPR poster symposium presentation).

2. Patents and licenses- none

3. Degrees- none

4. Development of cell lines, tissue or serum repositories- none

5. Informatics- none

6. Funding applied for based on work supported by the award

1. Lactose synthesis regulation: role of glucose transport, National Institutes of Health, FIRST Award, Principal Investigator, 9/20/98-9/19/03, \$70,000/yr., \$350,000, 1R29HD/DK34701.

7. Employment or research opportunities

Assistant Professor of Pediatrics, Baylor College of Medicine, 7/1/97-present

Conclusions

1. In normal CIT3 mammary epithelial cells, GLUT1 colocalizes with Golgi markers b-COP and a-mannosidase but not with the trans-Golgi marker Bodipy TR-ceramide.
2. GLUT1 targeting to Golgi is sensitive to Brefeldin A.
3. There are no higher molecular weight isoforms of GLUT1.
4. Glycosylation plays no role in GLUT1 targeting to Golgi.
5. There is no evidence that lactogenic hormones stimulate expression of a novel glucose transporter.
6. Forced weaning disrupts targeting of GLUT1 to Golgi even more rapidly than reported last year.
7. Golgi markers demonstrate heterogeneity in subcellular distribution in mammary gland.
8. Changes in Golgi markers with forced weaning suggest that changes in GLUT1 targeting during that time may reflect a dynamic reorganization process affecting all Golgi constituents.
9. Sec24 is differentially expressed in response to prolactin and hydrocortisone.
10. GLUT1-EBFP fusion protein offers the opportunity to study transporter targeting in living cells.
11. Golgi GLUT1 purified under non-denaturing conditions has an apparent molecular weight of 130 kD, suggesting that it may be associated with a protein of 70-90 kD.
12. MCF7 cancer cells exhibit very low rates of glucose transport.
13. MDA231 cells exhibit very high rates of glucose transport but do not appear to utilize GLUT1 for this purpose, suggesting expression of a novel transporter or "oncotransporter."
14. MDA231 cells sequester GLUT1 in an atypical-appearing intracellular compartment whether or not prolactin and hydrocortisone are present.
15. GLUT1 targeting in human mammary epithelial cells isolated from milk parallels findings in mouse cells.
16. Liposome-mediated transfection is highly efficient in mammary epithelial cells.
17. There is currently no evidence that a GLUT1-C-terminal binding protein regulates GLUT1 targeting in mammary gland.
18. GLUT1-EBFP fusion proteins are insufficiently sensitive to be useful.
19. GLUT1-EGFP fusion proteins are valuable tools for understanding the dynamics of glucose transporter targeting in mammary epithelial cells.
20. Glucose transporter targeting is a dynamic process in both normal and neoplastic mammary epithelial cells.
21. Intracellular targeting of GLUT1 in neoplastic mammary epithelial cells is unrelated to intracellular targeting of GLUT1 to Golgi during lactation.

So what? The results provide insight into the normal function of the mammary gland, the production of milk. Glucose transport into the Golgi may determine the rate of lactose synthesis and therefore milk production. Now that the mechanism of glucose transport into Golgi has been explained, it may be possible to develop ways to facilitate lactation and increase the prevalence and duration of breastfeeding. This will improve the

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health and neurodevelopment of the nation's children. The results also have important implications for breast cancer. We appear to be identifying targeting machinery unique to the neoplastic mammary epithelial cell which affects GLUT1, and may very well affect the targeting and activity of other cellular proteins in cancer cells. Furthermore, the results show that the only glucose transporter expressed in normal mammary gland, GLUT1, can not account for the glucose uptake of breast cancer cells, suggesting that a unique glucose transporter may be present, and may be an attractive therapeutic target.

Reference was made to the Statement of Work throughout the Body section of this report. I propose no changes in the SOW. I anticipate completing all tasks on the SOW during the no-cost extension year of my New Investigator Award.

References

Bunn RC, Jensen MA, Reed BC, Protein interactions with the glucose transporter binding protein GLUT1CBP that provide a link between GLUT1 and the cytoskeleton. *Mol Biol Cell* 1999 Apr;10(4):819-32

Appendices

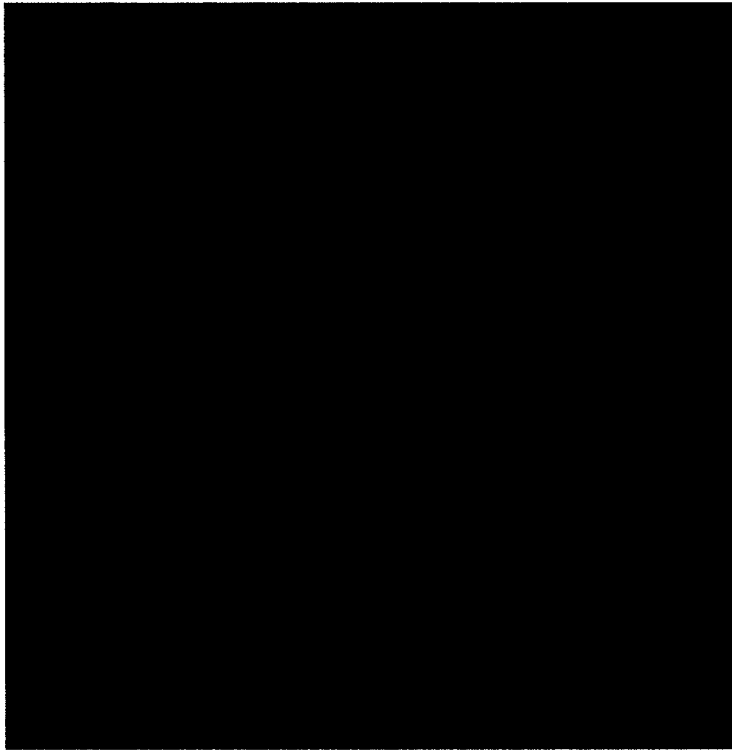


Figure 1. Expression of ECFP-Golgi (blue) in cells labeled with Bodipy-TR ceramide (red). ECFP-Golgi is a fusion of enhanced cyan fluorescent protein and an 85 amino acid sequence of galactosyltransferase that carries its Golgi targeting determinant. ECFP-Golgi specifically labels medial Golgi, while Bodipy-TR ceramide specifically labels trans-Golgi. Signal detected with deconvolution microscopy.

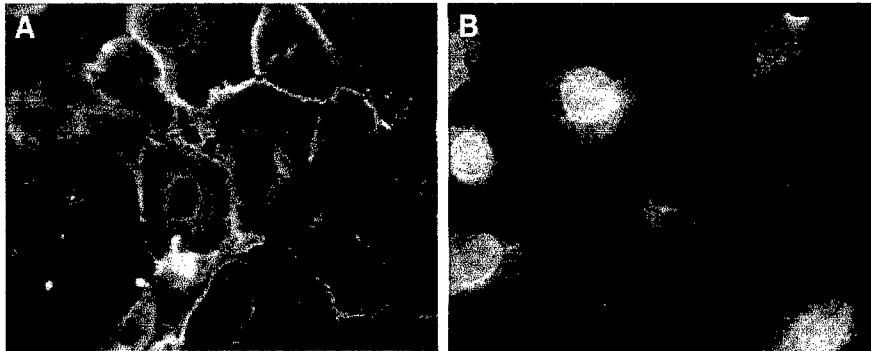


Figure 2. Human mammary epithelial cells stained for GLUT1. A, growth medium; B, secretion medium. In growth medium, staining is predominantly in plasma and nuclear membranes, while in secretion medium, staining is in nuclear membrane and the perinuclear region.

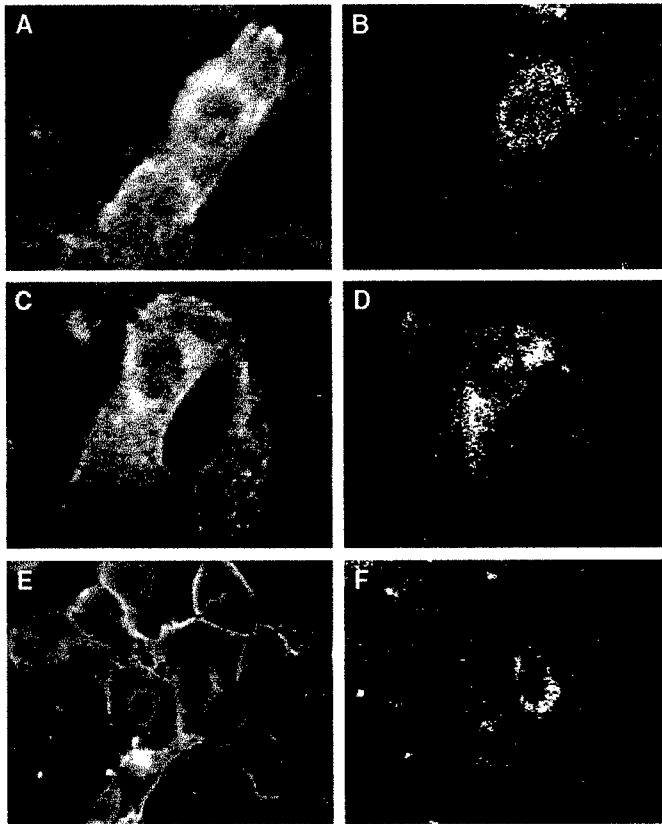


Figure 3. Human mammary epithelial cells stained with antibody to GLUT1 (A, C, E), and expressing ECFP-Golgi detected with conventional epifluorescence (B, D, F). Note that intracellular GLUT1 colocalizes with the medial Golgi marker ECFP-Golgi.

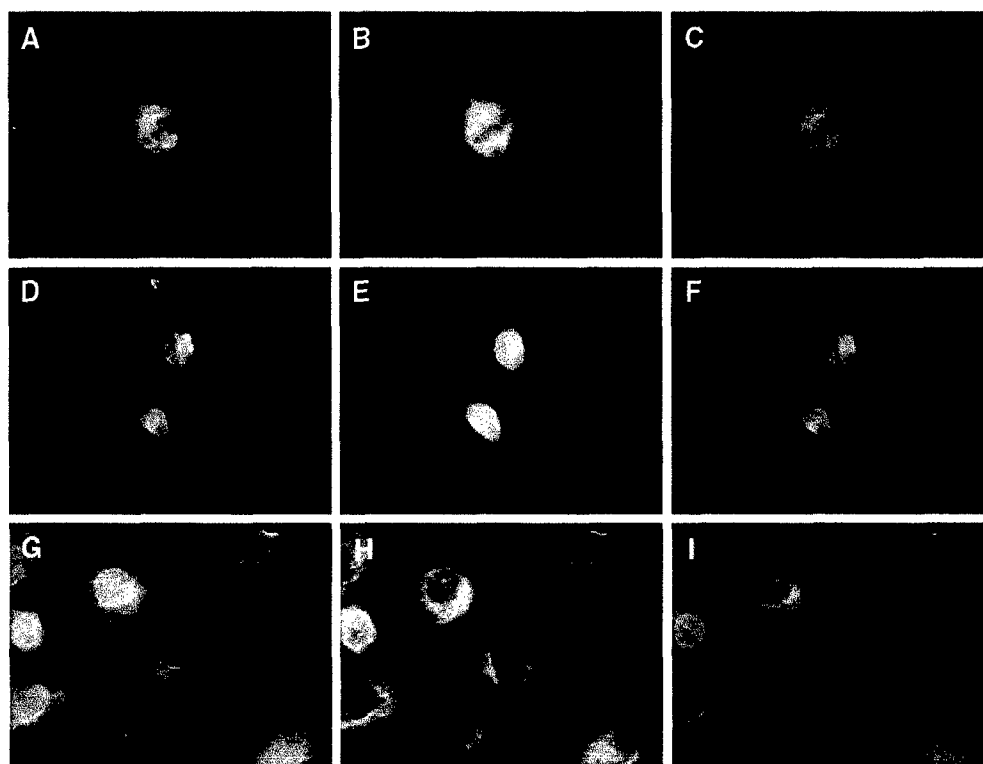


Figure 4. Human mammary epithelial cells stained for GLUT1 (A, D, G), beta-COP (B), alpha-mannosidase (E), and Bodipy-TR ceramide (H). Note colocalization (yellow) of GLUT1 with beta-COP (C), a cis-Golgi marker, and particularly for alpha-mannosidase (F), a medial Golgi marker. There was no significant colocalization with Bodipy-TR ceramide, a trans-Golgi marker.

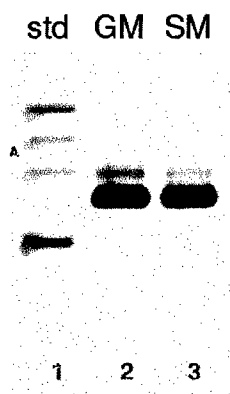


Figure 5. Isoform analysis of LDH from CIT3 mouse mammary epithelial cells. Standard is human LDH. There was no significant difference in LDH signal from growth medium (GM) and secretion medium (SM).

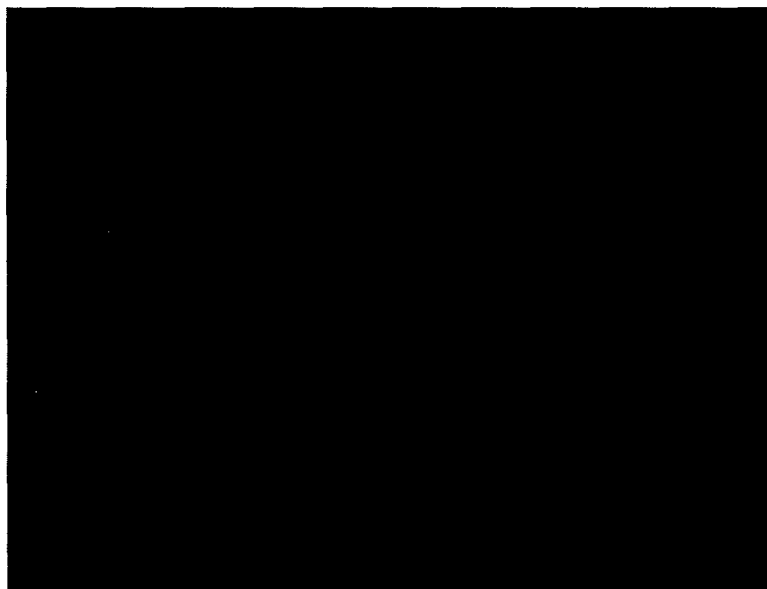


Figure 6. Human mammary epithelial cells grown in secretion medium and stained with an antibody to amino acids 464-477, the mid C-terminal cytoplasmic tail of GLUT1, SGFRQGGASQSDKT. Staining of the perinuclear region is consistent with Golgi targeting, but no staining of the nuclear membrane was observed.



Figure 7. Western blot of 20 micrograms of mouse brain homogenate with peptide affinity purified antibody to GLUT1. Antibody was prepared in rabbits using a peptide containing PEELFHPLGADSQV, amino acids 478-491 of GLUT1 as antigen. The single band at a molecular weight of approximately 60,000 is appropriate for mouse brain GLUT1 and demonstrates the very high degree of specificity of the antibody.



Figure 8. Relative usefulness of EBFP and EGFP. Cells were transiently transfected and studied at the peak of expression of fluorescent protein, 24-48 hours later. Although minimal signal required at least $\frac{1}{4}$ sec for EBFP, EGFP could be far more easily seen at $\frac{1}{30}$ sec, indicating that EGFP has at least an order of magnitude more sensitivity than EBFP.

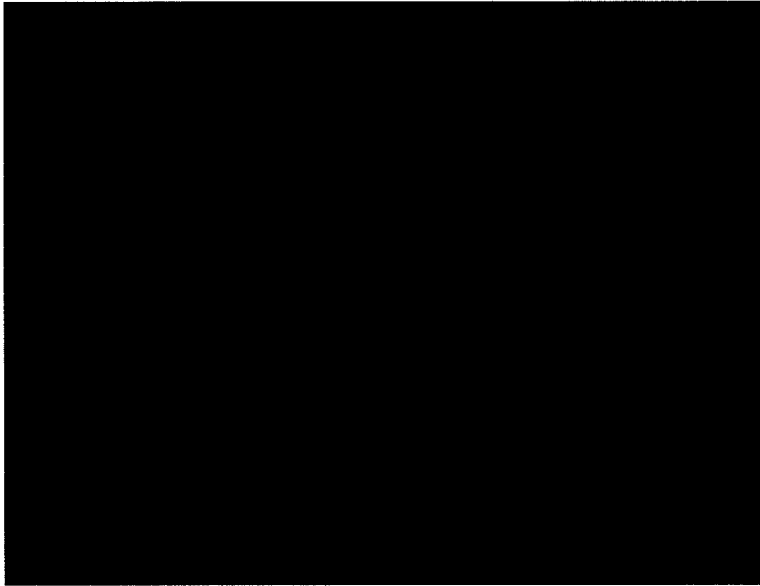


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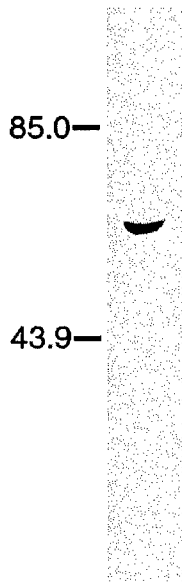


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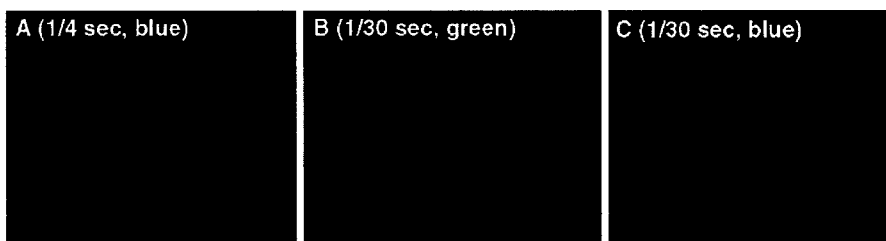


Figure 8. Relative usefulness of EBFP and EGFP. Cells were transiently transfected and studied at the peak of expression of fluorescent protein, 24-48 hours later. Although minimal signal required at least sec for EBFP, EGFP could be far more easily seen at 1/30 sec, indicating that EGFP has at least an order of magnitude more sensitivity than EBFP.

EGFP-GLUT1	GM	GLUT1-EGFP	GM
EGFP-GLUT1	SM	GLUT1-EGFP	SM

Figure 9. Expression of fluorescent fusion proteins of GLUT1 in transiently transfected living CIT3 mouse mammary epithelial cells. The construct and medium type (growth medium, secretion medium) are indicated. For EGFP-GLUT1, EGFP is fused to the N-terminus of GLUT1; for GLUT1-EGFP, EGFP is fused to the C-terminus of GLUT1.

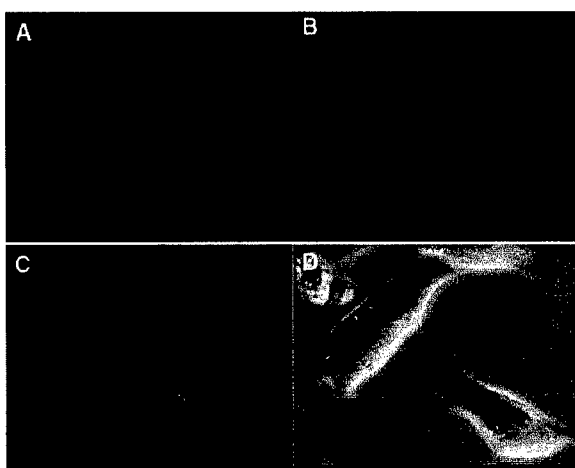


Figure 10. Expression of GLUT1-EGFP (A) in transiently transfected living CIT3 mouse mammary epithelial cells in growth medium. Trans-Golgi were stained red with Bodipy-TR ceramide (B). A slight degree of colocalization was seen (yellow, C). Cells are also shown in phase contrast (D).

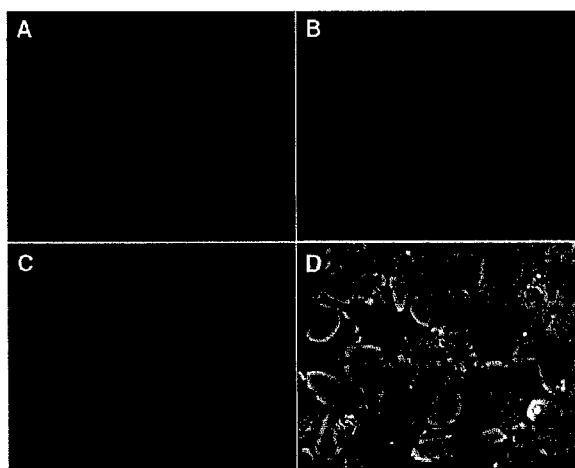


Figure 11. Expression of GLUT1-EGFP (A) in transiently transfected living CIT3 mouse mammary epithelial cells in secretion medium. Trans-Golgi were stained red with Bodipy-TR ceramide (B). A slight degree of colocalization was again seen (yellow, C). Cells are also shown in phase contrast (D).

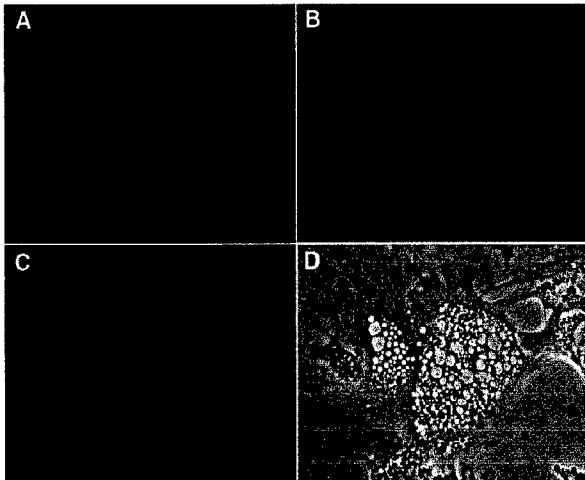


Figure 12. Expression of GLUT1-EGFP (A) in transiently transfected living CIT3 mouse mammary epithelial cells in secretion medium. These cells were atypical in their very high degree of differentiation as distinguished by intracellular vesicle content. These vesicles contain GLUT1 in their membrane, and presumably are lactose containing vesicles. Trans-Golgi were stained red with Bodipy-TR ceramide (B). No colocalization was seen (note absence of yellow, C). Cells are also shown in phase contrast (D).

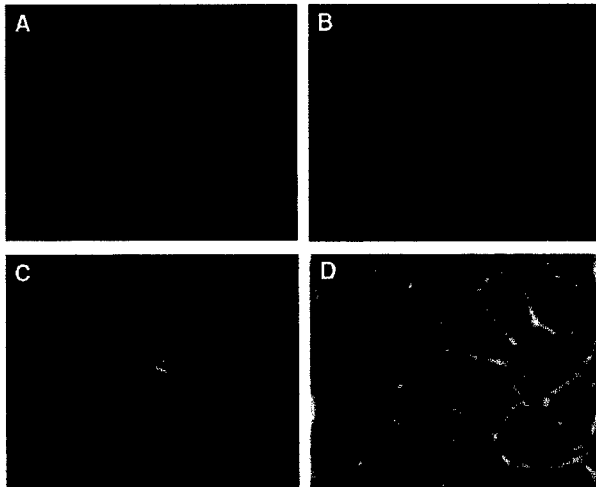


Figure 13. Expression of GLUT1-EGFP (A) in transiently transfected living MCF7 neoplastic mouse mammary epithelial cells in growth medium. Trans-Golgi were stained red with Bodipy-TR ceramide (B). A slight degree of colocalization was seen (yellow, C). Cells are also shown in phase contrast (D).

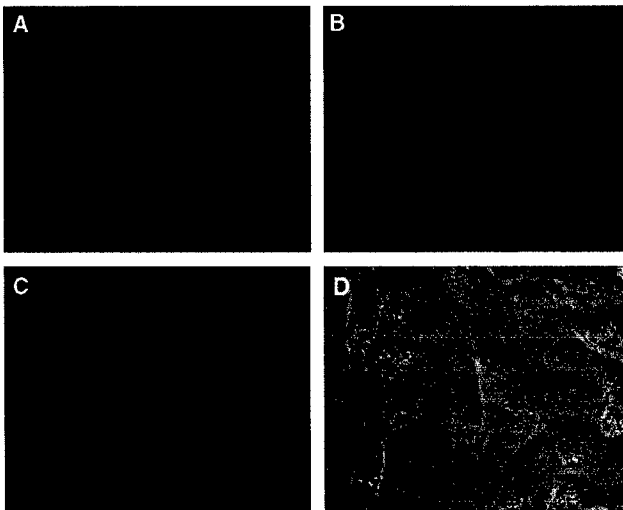


Figure 14. Expression of GLUT1-EGFP (A) in transiently transfected living MDA231 neoplastic mouse mammary epithelial cells in growth medium. Trans-Golgi were stained red with Bodipy-TR ceramide (B). A slight degree of colocalization was seen (yellow, C). Cells are also shown in phase contrast (D).



Figure 15. Expression of GLUT1-EGFP in transiently transfected living MDA231 neoplastic mouse mammary epithelial cells in growth medium. Medial-Golgi was stained red with antibody to beta-COP. A slight degree of colocalization was seen in certain cells (yellow), but in most cells there was no colocalization of GLUT1 and beta-COP.

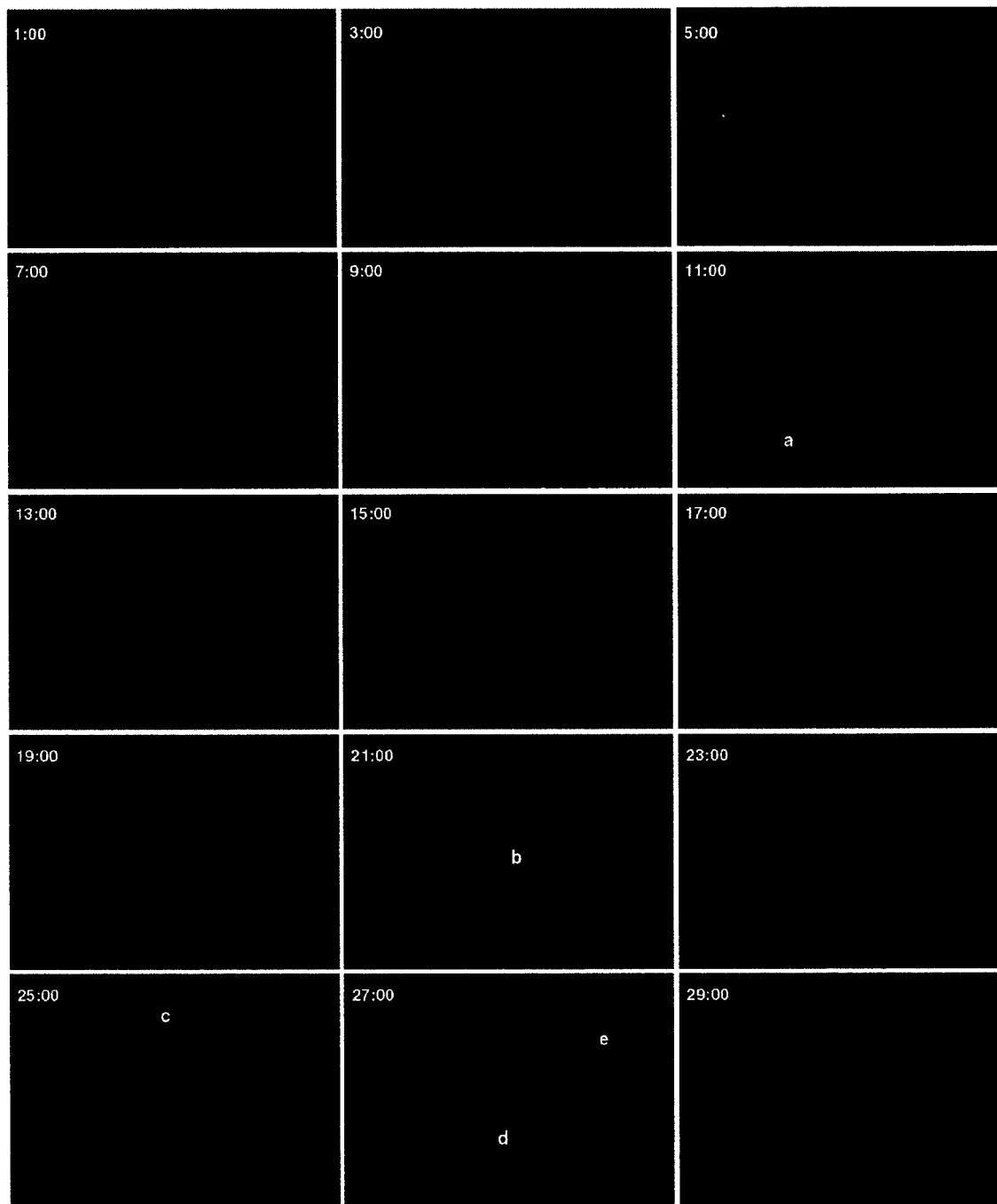


Figure 16. Video microscopy of GLUT1-EGFP in living CIT3 mouse mammary epithelial cells. Cells were maintained at 37°C and photographs taken every two minutes are shown as indicated. Note: a) vesicle transfer within Golgi, b) vesicle uptake into Golgi, c) endocytic vesicle uptake into Golgi, d) endocytosis from plasma membrane, and e) exocytic pathway vesicles trafficking toward plasma membrane.

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LOCALIZATION OF THE GLUT1 GLUCOSE TRANSPORTER TO
THE GOLGI COMPLEX OF DIFFERENTIATED CIT₃ MOUSE
MAMMARY EPITHELIAL CELLS

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Running title: Localization of GLUT1 to Golgi

Keywords: GLUT1, glucose transport, protein targeting, Golgi, mammary gland

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Abstract

The lactating mammary epithelial cell has a unique requirement for the transport of free glucose across the Golgi membrane to serve as substrate for lactose synthesis. The hypothesis that lactogenic hormones induce the GLUT1 glucose transporter and cause its localization to the Golgi of mammary epithelial cells was tested. CIT₃ mouse mammary epithelial cells treated with prolactin and hydrocortisone showed a 15-fold induction of GLUT1, associated with a paradoxical decrease in 2-deoxyglucose uptake across the plasma membrane. Subcellular fractionation and iodixanol density gradient centrifugation demonstrated enrichment of Golgi fractions with GLUT1. Enhanced GLUT1 glycosylation was observed but did not determine whether GLUT1 was targeted to plasma membrane or to Golgi. Confocal immunofluorescent microscopy confirmed Golgi targeting of GLUT1, and Brefeldin A effects further indicated that GLUT1 is targeted to a subcompartment of cis- or medial Golgi. Therefore, in mouse mammary epithelial cells, prolactin and hydrocortisone induce GLUT1, enhance GLUT1 glycosylation, and cause glycosylation-independent targeting of GLUT1 to a subcompartment of cis- and/or medial-Golgi, but not to trans-Golgi. These results suggest a tissue- and developmental stage-specific mechanism for Golgi targeting of GLUT1 and indicate an important role for GLUT1 in the provision of substrate to the Golgi for lactose synthesis.

Introduction

Lactose is the major carbohydrate and the major osmotic constituent of human milk. Therefore, the amount of lactose synthesized determines the volume of milk

produced by the lactating human mammary gland. The substrates for lactose synthesis are UDP-galactose and free glucose. Direct measurement of intracellular glucose concentration demonstrated that glucose transport into the mammary epithelial cell must be rate-limiting for lactose synthesis (Wilde and Kuhn, 1981). Lactose synthesis takes place within the Golgi (Keenan *et al.*, 1970) and is catalyzed by lactose synthetase, a complex of galactosyltransferase and the mammary gland specific protein α -lactalbumin. The mammary epithelial cell not only must transport glucose from the blood across the basal membrane into the cell, it must also deliver this glucose to the Golgi. The lactating mammary gland is unique in its requirement for free glucose within the Golgi. A Golgi specific glucose carrier protein accounting for Golgi glucose uptake during lactation was proposed 5 years before the cloning of GLUT1, the first member of the family of facilitated diffusion glucose transporter isoforms (White *et al.*, 1980).

The only isoform of the facilitated diffusion family of glucose transporters known to be expressed in mammary gland is GLUT1. In most cell types, GLUT1 is targeted to the plasma membrane. Neither GLUT1 nor any other glucose transporter isoform has been proven to target to the Golgi. In one study, subcellular fractionation and Western blotting of day-10 lactating rat mammary glands suggested that during lactation, GLUT1 may also be found in the Golgi (Madon *et al.*, 1990). However, in other studies (Camps *et al.*, 1994; Takata *et al.*, 1997) using microscopy, Golgi targeting of GLUT1 was not observed in day 10-12 lactating rat mammary gland, although high and polarized expression of plasma membrane GLUT1 during lactation was seen.

The purpose of this study was to test the hypothesis that prolactin and hydrocortisone cause the targeting of GLUT1 to Golgi in cultured mouse mammary epithelial cells. To provide multiple lines of evidence, methods included 2-deoxyglucose uptake assays, subcellular fractionation and density gradient centrifugation, and confocal immunofluorescent microscopy. The results indicate that the GLUT1 glucose transporter is indeed induced and targeted to the Golgi under the influence of lactogenic hormones. This supports an important role for GLUT1 in the delivery of glucose to the Golgi, the regulation of lactose synthesis, and the control of milk production.

Materials and Methods

Antisera and reagents

A well characterized, highly specific rabbit polyclonal antiserum to human GLUT1, raised against synthetic peptide made up of the 16 C-terminal amino acids, was a kind gift of Dr. M. Mueckler (Washington University School of Medicine, St. Louis, MO). This antibody was affinity purified using the same synthetic peptide bound to thiopropyl sepharose (Parekh *et al.*, 1989) prior to use for immunoblotting or immunocytochemistry. Mouse monoclonal antibodies to rat β -COP were obtained from Sigma (St. Louis, MO); mouse monoclonal antibodies to rat α -mannosidase II were from Babco (Richmond, CA). Fluorescein-labeled goat anti-rabbit and Texas-red sheep anti-mouse antibodies were from ICN (Aurora, OH). Reagents were from Sigma unless otherwise specified.

Cell culture

CIT₃ cells are an established mouse mammary epithelial cell line (Toddywalla *et al.*, 1997), kindly supplied by Dr. M.C. Neville (University of Colorado School of Medicine, Denver, CO). Cells were maintained in growth medium, consisting of Dulbecco's Modified Eagle's Medium (DMEM) with Ham's F12 (50:50), supplemented with 2% heat-inactivated fetal bovine serum (FBS), 10 µg/ml insulin and 5 ng/ml epidermal growth factor (EGF). To differentiate the cells, growth medium lacking EGF but containing 3 µg/ml prolactin and 3 µg/ml hydrocortisone was used; this medium was designated "secretion medium". In certain experiments, cells were treated with 50 nM Brefeldin A (Pauloin *et al.*, 1997) for 30 minutes at 37°C.

2-Deoxyglucose uptake

[³H]2-Deoxyglucose uptake was measured as previously described (Tordjman *et al.*, 1989). Non-carrier-mediated uptake was measured in the presence of 20 µM cytochalasin B and was subtracted from total 2-deoxyglucose uptake so that results are expressed as specific carrier-mediated uptake. Non-carrier-mediated uptake was less than 21% of total 2-deoxyglucose uptake under all circumstances. Statistical significance of the difference between the groups was calculated using Student's t-test for independent samples.

Subcellular fractionation and density gradient centrifugation

CIT₃ cells were rinsed twice with ice-cold PBS and once with ice-cold homogenization buffer. Cells were harvested using a rubber policeman. The pellet was resuspended in a small volume of homogenization buffer and homogenized with five

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strokes in a tight-fitting Dounce homogenizer. After one centrifugation at 3,000 g for 10 minutes at 4°C, the supernatant was centrifuged at 17,000 g for 10 minutes at 4°C. This supernatant was centrifuged at 100,000 g for 30 minutes at 4°C. The 17,000 g pellet was resuspended and subjected to density gradient centrifugation in a self-generating iodixanol density gradient (10%-37%) at 180,000 g for 3 hours at 4°C. Twenty fractions were collected from the top (Labconco Auto-densi Flow, Kansas City, MO) and analyzed. Alkaline phosphatase (Langridge-Smith *et al.*, 1998) and galactosyl transferase (Graham, 1993) were assayed as described.

Western blotting

Samples were prepared and subjected to standard SDS-PAGE on 10% gels as previously described (Haney *et al.*, 1991). Proteins were immobilized on nitrocellulose by wet transfer. The peptide affinity purified GLUT1 antibody described above (1 µg/ml in 5% nonfat dry milk in PBS) was used as primary antibody. Secondary antibody was HRP-linked donkey anti-rabbit antibody (Amersham, Piscataway, NJ), and signal was detected using ECL-PlusTM (Amersham). Quantitative differences in signal strength were measured using a STORM analyzer (Molecular Dynamics, Sunnyvale, CA).

Immunocytochemistry

Cells were grown on glass coverslips, washed with PBS, and fixed in 4% paraformaldehyde in serum-free medium for 20 minutes at room temperature. Cells were then treated with 100 mM glycine in PBS for 10 minutes and permeabilized with 0.1% Triton X-100 for 15 minutes. Cells were blocked with 2% horse serum in PBS for 15 minutes. Treatment with primary antibody in 0.1% horse serum in PBS was overnight at

4°C. GLUT1 antibody was at 6 µg/ml, α-mannosidase II antibody was at 1:100, and β-COP antibody was at 1:80. After three 10-minute washes with PBS, secondary antibody, diluted 1:100 in 0.1% horse serum, was added for 30 minutes at room temperature. FITC-conjugated goat anti-rabbit antibody and Texas Red conjugated rabbit anti-mouse antibody were used. Coverslips were then washed three times with PBS and mounted in ProLong anti-fade medium (Molecular Probes, Eugene, OR) on glass slides for examination using a Molecular Dynamics Multiprobe™ 2010 inverted confocal laser scanning microscope. The images are shown as acquired; no enhancement or intensification was performed.

Results

Lactogenic hormones cause intracellular targeting of GLUT1

CIT₃ cells are an established mouse mammary epithelial cell line selected from Comma-1-D cells for their ability to express β-casein, form tight junctions, and exhibit polarized transport in response to treatment with prolactin and hydrocortisone (Toddywalla *et al.*, 1997). The total cellular content of GLUT1 in CIT₃ cells rose 15-fold in response to prolactin and hydrocortisone (Fig. 1A). Paradoxically, the 2-deoxyglucose uptake of these cells fell 70% in response to these hormones (Fig. 1B). In other words, cells maintained in secretion medium exhibited only a small fraction of the plasma membrane glucose transport activity predicted from their GLUT1 content. This suggested a major effect of lactogenic hormones either on the specific activity of the transporter or on the amount of transporter in the plasma membrane.

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To distinguish between these possibilities, subcellular fractionation was carried out. Pellets from the 17,000 g and 100,000 g centrifugations were enriched in Golgi and plasma membrane, respectively, as indicated by assays of galactosyltransferase and alkaline phosphatase. Each marker was enriched 2.3-fold (for galactosyl transferase in 17,000 g pellet vs. homogenate, and for alkaline phosphatase in the 100,000 g pellet vs. homogenate) in the corresponding fraction. Compared to the enrichment observed for these markers, the enrichment of GLUT1 was much greater. GLUT1 was enriched 19.0-fold in the 100,000 g pellet, compared to homogenate, from cells maintained in growth medium, and was enriched 11.0-fold in the 17,000 g pellet, compared to homogenate, from cells grown in secretion medium (Fig. 2). Cells grown in growth medium showed predominance of GLUT1 in the plasma membrane-enriched fraction, while secretion medium caused predominance of GLUT1 targeting in the Golgi-enriched fraction (Fig. 2). The relative molecular mass of GLUT1 in cells grown in secretion medium was slightly higher than in growth medium, 53 kDa compared to 50 kDa, suggesting the possibility of a second glucose transporter closely related to GLUT1. Therefore, homogenate, 17,000 g pellets, and 100,000 g pellets from cells maintained in both growth medium and secretion medium were subjected to enzymatic deglycosylation. In all cases, the deglycosylated protein had an identical relative molecular mass of 37 kDa, consistent with GLUT1 (Fig. 2).

To confirm that the GLUT1 contained in the 17,000 g pellet was targeted to Golgi, iodixanol density gradient centrifugation was carried out. GLUT1 was concentrated in the low-density fractions (1.05-1.08 g/cm³), consistent with Golgi (Fig. 3). The difference in the absolute amount of GLUT1 targeting to Golgi between cells

grown in growth medium and in secretion medium was consistent with that seen in Fig.

2.

Intracellular GLUT1 is in a subcompartment of the Golgi

Confocal immunofluorescent microscopy of cells maintained in growth medium demonstrated a plasma membrane pattern for GLUT1 (Fig. 4A). In contrast, consistent with the results of subcellular fractionation and density gradient centrifugation, cells maintained in secretion medium exhibited much less plasma membrane signal. Rather, these cells showed a significant intracellular signal for GLUT1, distributed in a perinuclear pattern and in an intense punctate pattern throughout the cytoplasm (Fig. 4D), characteristic of Golgi staining. The trans-Golgi marker BODIPY-TR ceramide (Pagano *et al.*, 1991) demonstrated the expected punctate staining both in cells grown in growth medium (Fig. 4B) and in secretion medium (Fig. 4E). There was no colocalization of GLUT1 and the trans-Golgi marker in either growth medium (Fig. 4C) or secretion medium (Fig. 4F). Neither β -COP, which resides in cis-Golgi as well as certain transport vesicles (Fig. 5B), nor α -mannosidase II, a medial-Golgi constituent (Fig. 5E), demonstrated colocalization with GLUT1 in cells grown in secretion medium. Since GLUT1 demonstrated a plasma membrane targeting pattern in cells maintained in growth medium, there was no colocalization of GLUT1 with these markers of intracellular compartments (data not shown).

Brefeldin A was used to further dissect Golgi targeting. Cells maintained in growth medium and subjected to Brefeldin A showed distribution of the trans-Golgi marker BODIPY-TR ceramide throughout the cytoplasm (Fig. 6B). The plasma

membrane targeting of GLUT1 was not affected (Fig. 6A), and there was no specific colocalization of GLUT1 and BODIPY-TR ceramide (Fig. 6C). BODIPY-TR ceramide was also distributed throughout the cytoplasm in cells maintained in secretion medium and exposed to Brefeldin A (Fig. 6E), and, again, there was no specific colocalization of GLUT1 and BODIPY-TR ceramide (Fig. 6F).

The apparent subcellular distributions of β -COP and α -mannosidase II were not significantly affected by Brefeldin A (Figs 7B,E; compare to Figs 5B,E); both continue to demonstrate a perinuclear distribution. GLUT1 was again found in a perinuclear distribution and in a punctate pattern throughout the cytoplasm (Figs 6D, 7A,D). Brefeldin A enhanced colocalization of GLUT1 both with β -COP (Fig. 7C; compare to 5C) and with α -mannosidase II (Fig. 7F; compare to 5F). This indicates that in the absence of Brefeldin A, lactogenic hormones present in secretion medium cause substantial GLUT1 targeting to a Golgi subcompartment distinct from those subcompartments marked by BODIPY-TR ceramide, β -COP, and α -mannosidase II. Brefeldin A appeared to cause mixing of the GLUT1 subcompartment with the β -COP and α -mannosidase II subcompartments, but not the subcompartment marked by BODIPY-TR ceramide.

Discussion

GLUT1 is the only member of the facilitated diffusion glucose transporter family known to be expressed in mammary gland. However, in the many cell types in which it is expressed, GLUT1 is a plasma membrane protein, and is responsible for the basal

uptake of glucose into most cells. None of the isoforms of the glucose transporter family is considered to be a Golgi resident, although a six-amino acid portion of GLUT4 does confer targeting to the trans-Golgi network and insulin-sensitive translocation to the plasma membrane in fat and muscle cells (Haney *et al.*, 1995). Furthermore, lactose synthesis within the Golgi is a unique function of differentiated mammary epithelial cells; no other cell type requires free glucose within the Golgi.

Several investigators have studied GLUT1 expression in the lactating mammary gland, with conflicting results described above. The purpose of the experiments reported here was to test, in mammary epithelial cells in culture, whether the subcellular trafficking of GLUT1 is hormonally regulated in a manner consistent with an important role for GLUT1 in the provision of substrate for lactose synthesis.

Prolactin and hydrocortisone trigger differentiation of mammary epithelial cells. Total cellular GLUT1 levels increased 15-fold in response to this treatment. Importantly, consistent results from two independent methods, subcellular fractionation followed by density gradient centrifugation, and confocal immunofluorescent microscopy, indicate that prolactin and hydrocortisone cause GLUT1 to be targeted not to the plasma membrane, as it is in most cells, but to a subcompartment of the Golgi. The subcellular fractionation and density gradient characterization include expected degrees of enrichment in appropriate marker enzymes and demonstrate that the GLUT1-enriched fractions exhibit a very low density ($1.05\text{-}1.08\text{ g/cm}^3$) fraction of the light mitochondrial (17,000 g) pellet, consistent with Golgi localization. The specificity of the confocal immunofluorescent signal for the Golgi markers is confirmed by the colocalization of GLUT1 with these markers brought about by Brefeldin A; were the Golgi marker staining

nonspecific, this could not occur. Thus, under the influence of prolactin and hydrocortisone, GLUT1 is sequestered within the cell, accounting for the paradoxical decrease in plasma membrane glucose transport activity despite increased GLUT1 levels caused by these hormones. This leads to the conclusion that GLUT1 is diverted from normal sorting pathways to the Golgi in order to provide glucose within the Golgi for lactose synthesis. We have not yet examined whether the turnover of GLUT1 is altered under these conditions, but adequate time to achieve a new steady state was insured by allowing four days of exposure to prolactin and hydrocortisone before assessing changes in steady-state distributions. This eliminates the possibility that altered turnover alone is responsible for changes in GLUT1 targeting.

Brefeldin A causes the rapid and reversible retrograde movement of Golgi resident proteins back to the endoplasmic reticulum (Klausner *et al.*, 1992). In most cells, trans-Golgi network proteins respond to Brefeldin A by fusing with early endosomes; the hybrid organelles are stable and tubulovesicular in contrast to the Golgi, which disappears (Lippincott-Schwartz *et al.*, 1991). However, polarized epithelial cells respond differently to Brefeldin A. In MDCK cells, BFA promoted extensive tubulation of the trans-Golgi network while the medial Golgi marker alpha-mannosidase II was not affected (Wagner *et al.*, 1994). As was recently demonstrated in mammary epithelial cells from lactating rabbit, Brefeldin A (50 nM) caused dissociation of trans-Golgi but not medial Golgi marker enzymes (Pauloin *et al.*, 1997). Failure of Brefeldin A to dissociate GLUT1 in CIT₃ cells provides further evidence that GLUT1 is a cis- or medial-Golgi protein.

The results suggest a tissue- and developmental stage-specific Golgi targeting mechanism for GLUT1. The structural determinants of targeting of resident proteins to Golgi are controversial. For certain glycosyltransferases, the transmembrane spanning domain or specific amino acid motifs within it have seemed important (Nilsson and Warren, 1994). However, examination of a large number of cloned glycosyltransferases does not reveal a common retention signal (Keenan, 1998). Alternative hypotheses include the formation of non-mobile protein oligomers and the influence of the high cholesterol content of Golgi membranes on mobility of resident Golgi enzymes (Munro, 1998). Although glycosylation serves to direct protein targeting to apical membrane of polarized epithelial cells under certain circumstances (Gut *et al.*, 1998), it has not been linked with targeting of proteins to Golgi. In fact, a mutant form of GLUT1 lacking the N-glycosylation site shows intracellular targeting (Asano *et al.*, 1993). Although we observed enhanced glycosylation of GLUT1 in response to prolactin and hydrocortisone, deglycosylation experiments reported here found no evidence that differential glycosylation is responsible for Golgi targeting. In the context of our primitive general understanding of the determinants of protein targeting to Golgi, it is not surprising that the molecular basis of the hormonally regulated Golgi targeting of GLUT1 is unclear. Hormonal regulation of the process of Golgi targeting of GLUT1 suggests a flexibility of the Golgi targeting machinery that has not previously been appreciated. The results suggest a tissue-specific GLUT1 sorter is active during lactation. Further study may reveal mechanisms regarding GLUT1 targeting that are relevant to other Golgi proteins as well.

Deglycosylation experiments (Fig. 2) showed that the differences in relative molecular mass of GLUT1 between cells maintained in growth medium and in secretion medium need not be attributed to expression of a distinct glucose transporter isoform containing the GLUT1 antigenic determinant. However, the results do not exclude the previously suggested (Madon *et al.*, 1990) possibility that a novel transporter, yet to be identified, resides in the Golgi.

The data were developed in an established mammary epithelial cell line grown on tissue culture plastic, in the absence of reconstituted basement membrane. The subcellular fractionation and iodixanol density gradient centrifugation procedures resulted in enriched, not purified, fractions, which nevertheless demonstrated altered GLUT1 targeting, confirmed by confocal immunofluorescent microscopy. Taken together, the data demonstrate specific changes in glucose transporter targeting, and support the conclusion that important cellular mechanisms must underlie them, in this simplest possible, but artificial, system. Studies in more physiologically relevant model systems are under way. Future work will focus on understanding the mechanism and regulation of GLUT1 targeting to Golgi during lactation.

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Figure legends

Figure 1. Total cellular GLUT1 content and 2-deoxyglucose uptake of CIT₃ cells maintained for 4 days in growth medium and in secretion medium. Secretion medium, which contains prolactin and hydrocortisone, caused a 15-fold increase in total cellular GLUT1 (A); this was associated with a paradoxical decrease in 2-deoxyglucose uptake (B). Samples from cells grown in growth medium (60 µg protein, lane 1) and secretion medium (20 µg protein, lane 2) were analyzed by SDS-PAGE and Western blotting. 2-Deoxyglucose uptake reflects glucose transport across the plasma membrane and was measured as described in Materials and Methods. Results are means $\pm$ S.E.M. for six determinations. Student's t-test for independent samples resulted in a p value of 0.003 for the difference between groups in 2-deoxyglucose uptake.

Figure 2. Subcellular distribution and PNGase F digestion of GLUT1 in CIT₃ cells maintained for 4 days in growth medium and in secretion medium. Subcellular fractions were prepared and 10 µg aliquots were analyzed by SDS-PAGE and Western blotting as described in Materials and Methods. In growth medium, GLUT1 was most highly enriched in the 100,000 g pellet (lane 5), but in secretion medium GLUT1 was most highly enriched in the 17,000 g pellet (lane 9). The increase in signal in the homogenate between growth medium (lane 1) and secretion medium (lane 7) is consistent with the result in Figure 1A. The identical relative molecular mass of GLUT1 from all PNGase F treated samples, 37 kDa, indicates that the slightly higher apparent M_r of GLUT1 in

secretion medium, 53 kDa, compared to growth medium, 50 kDa, is due to differential glycosylation. Note also that targeting to subcellular fractions is not a function of the degree of glycosylation. Growth medium, lanes 1-6; secretion medium, lanes 7-12.

Figure 3. Iodixanol density gradient centrifugation of the 17,000 g pellet from CIT₃ cells maintained for 4 days in growth medium and in secretion medium. Signal was found in only the least dense fractions, indicating that, under both conditions, the GLUT1 content of the 17,000 g pellet reflected its presence in the Golgi. Samples of 10 µg protein from iodixanol density gradient fractions of cells grown in growth medium (upper panel) and secretion medium (lower panel) were analyzed by SDS-PAGE and Western blotting as described in Materials and Methods.

Figure 4. Confocal images of GLUT1 (A,D) and the trans-Golgi marker BODIPY-TR ceramide (B,E) in CIT₃ cells maintained for 4 days in growth medium (A-C) and in secretion medium (D-F). Cells were fixed and stained, and confocal microscopy was performed as described in Materials and Methods. Right panels (C, F) are superimpositions of the left and middle panels; GLUT1 is shown in green, BODIPY-TR ceramide is shown in red, and areas of coincident staining appear yellow. Bar, 2 µm. Little overlap is observed. GLUT1 displayed a predominant plasma membrane pattern in growth medium, but in secretion medium, GLUT1 was distributed in a perinuclear pattern and in a punctate pattern throughout the cytoplasm.

Figure 5. Confocal images of GLUT1 (A, D), β -COP (B), and α -mannosidase II (E) in CIT₃ cells maintained for 4 days in secretion medium. Cells were fixed and stained and confocal microscopy was performed as described in Materials and Methods. Right panels (C, F) are superimpositions of the left and middle panels. GLUT1 is shown in green; β -COP and α -mannosidase II are shown in red, and areas of coincident staining would appear yellow. Bar, 2 μ m. Note that there is little overlap of GLUT1 with these Golgi marker proteins.

Figure 6. Confocal images of GLUT1 (A, D) and BODIPY-TR ceramide (B, E) in CIT₃ cells maintained for 4 days in secretion medium. Cells were treated for 30 minutes with 50 nM Brefeldin A. Cells were fixed and stained and confocal microscopy was performed as described in Materials and Methods. Right panels (C, F) are superimpositions of the left and middle panels. GLUT1 is shown in green; BODIPY-TR ceramide is shown in red, and areas of coincident staining appear yellow. Bar, 2 μ m. Brefeldin A completely disrupted BODIPY-TR ceramide targeting, but did not cause colocalization of GLUT1 and BODIPY-TR ceramide.

Figure 7. Confocal images of GLUT1 (A, D), β -COP (B), and α -mannosidase II (E) in CIT₃ cells maintained for 4 days in secretion medium. Cells were treated for 30 minutes with 50 nM Brefeldin A. Cells were fixed and stained and confocal microscopy was performed as described in Materials and Methods. Right panels (C, F) are superimpositions of the left and middle panels. GLUT1 is shown in green; β -COP and

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α -mannosidase II are shown in red, and areas of coincident staining appear yellow. Bar, 2 μ m. Brefeldin A did not substantially alter the targeting of GLUT1, β -COP or α -mannosidase II. However, yellow signal (C, F) indicates that Brefeldin A did cause colocalization of GLUT1 with α -mannosidase II and β -COP, revealing a subtle effect of Brefeldin A on membrane trafficking in the cis- and medial-Golgi of mammary epithelial cells.

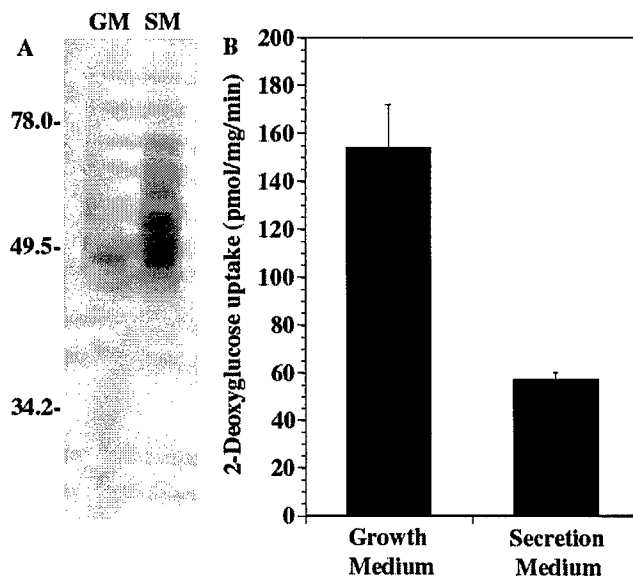


Figure 1

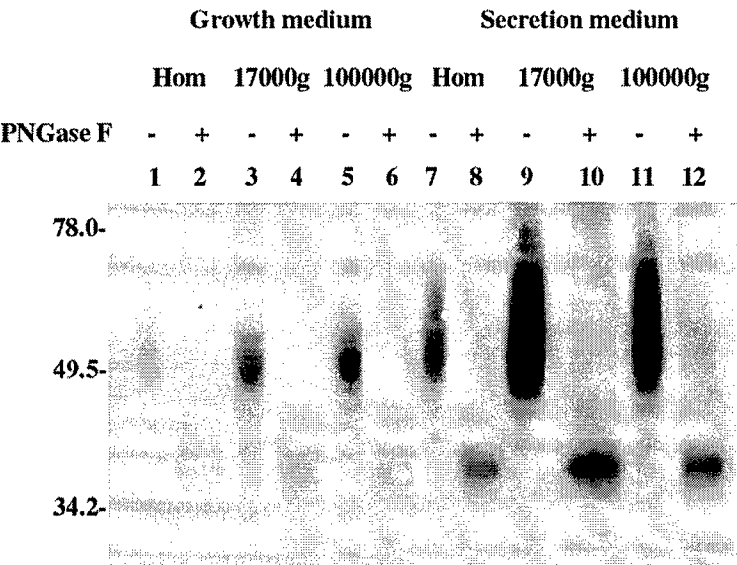


Figure 2

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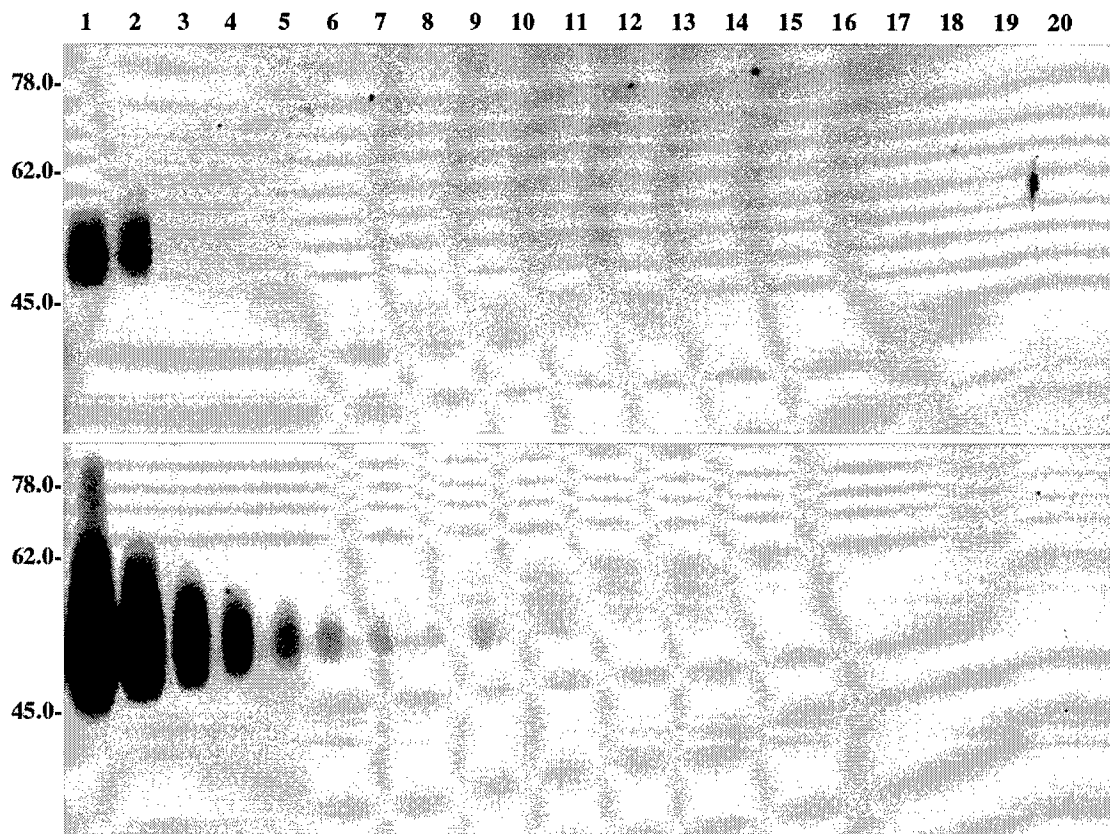


Figure 3

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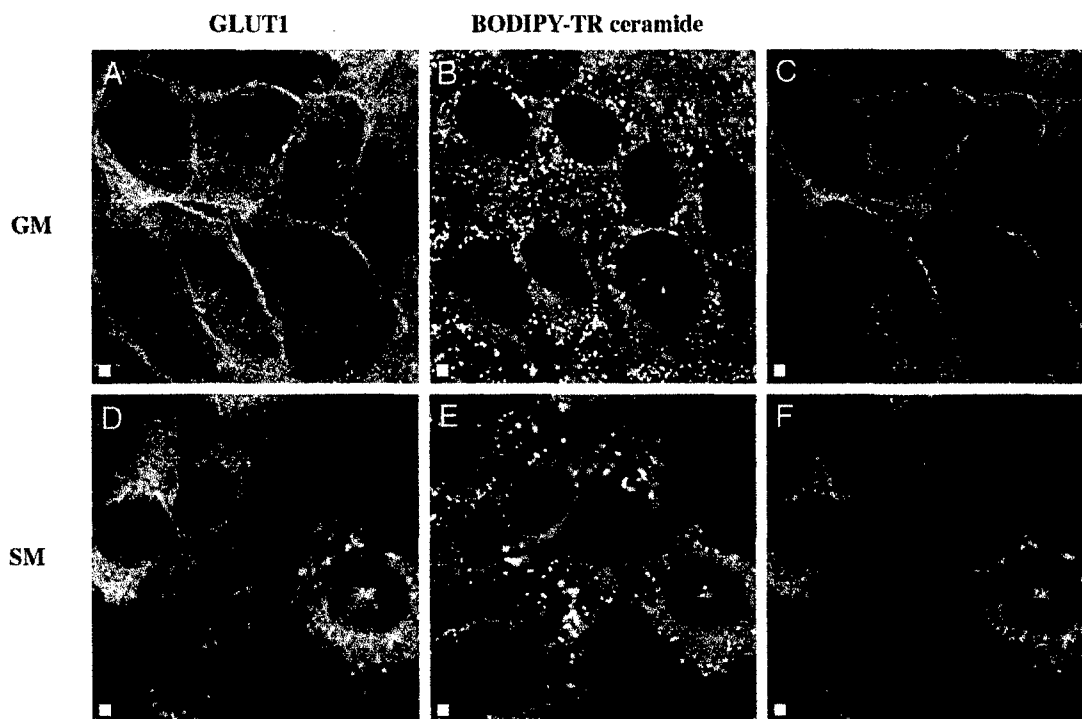


Figure 4

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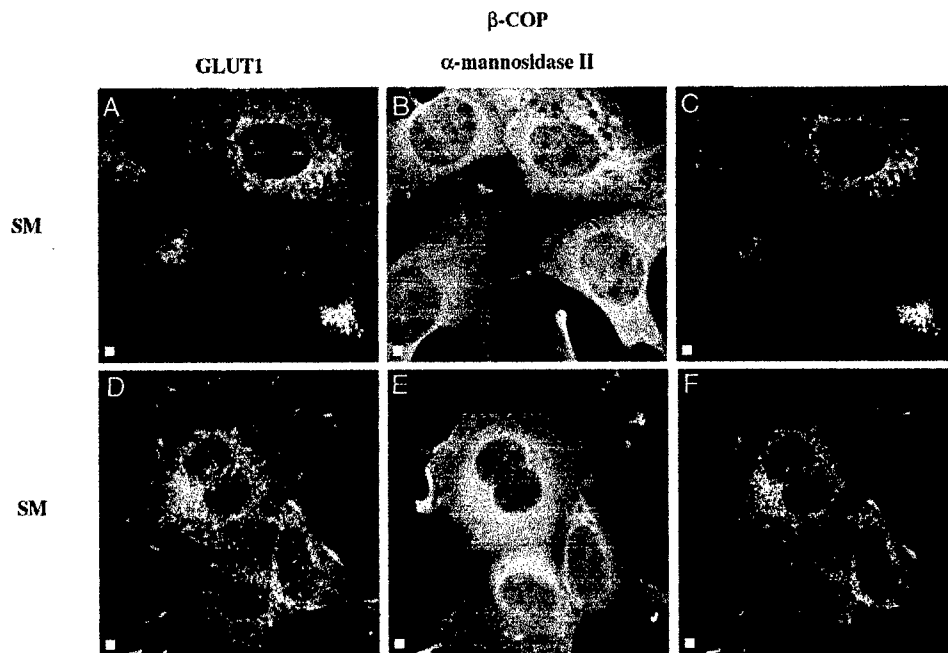


Figure 5

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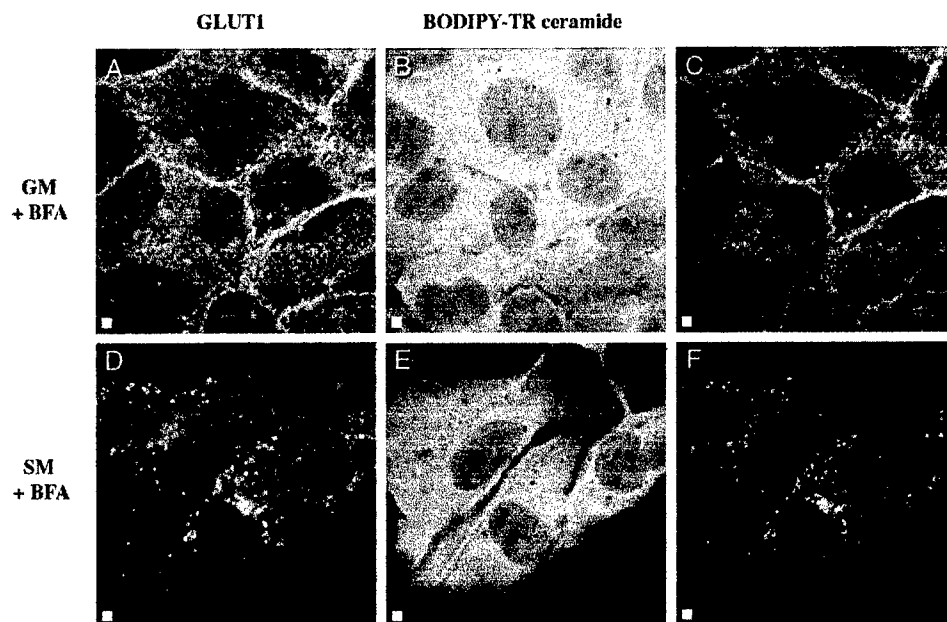


Figure 6

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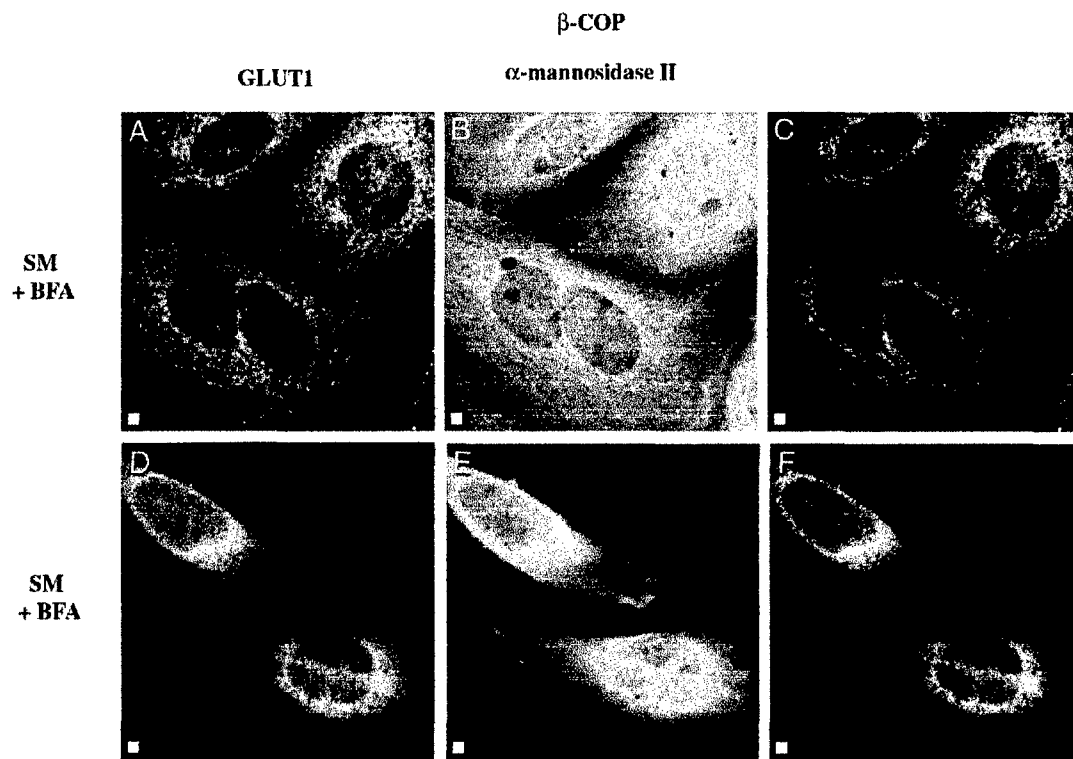


Figure 7

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Running title: Golgi targeting of GLUT1 in lactation

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GOLGI TARGETING OF THE GLUT1 GLUCOSE TRANSPORTER
IN LACTATING MOUSE MAMMARY GLAND

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Abstract

Lactose, the major carbohydrate of human milk, is synthesized in the Golgi from glucose and UDP-galactose. The lactating mammary gland is unique in its requirement for the transport of glucose into Golgi. GLUT1 is the only isoform of the glucose transporter family expressed in mammary gland. In most cells, GLUT1 is localized to the plasma membrane and is responsible for basal glucose uptake; in no other cell type is GLUT1 a Golgi resident. To test the hypothesis that GLUT1 can account for Golgi glucose transport, the amount and subcellular distribution of GLUT1 were examined in mouse mammary gland at different developmental stages. Methods, including immunohistochemistry, immunofluorescence, and subcellular fractionation, density gradient centrifugation, and Western blotting, yielded consistent results. In virgins, GLUT 1 expression was limited to plasma membrane of epithelial cells. In late pregnant mice, GLUT1 expression was increased, with targeting primarily to basolateral plasma membrane, but also with some intracellular signal. By 8 d after delivery, GLUT1 expression was further increased and targeting to intracellular compartments predominated. Removal of pups 18 d after delivery resulted in retargeting of GLUT1 from Golgi to plasma membrane and a decline in total cellular GLUT1 within 3 h. In mice undergoing natural weaning, GLUT1 expression declined. Changes in the amount and targeting of GLUT1 during mammary gland development indicate a key role for GLUT1 in supplying substrate for lactose synthesis and milk production.

Keywords: Monosaccharide transport proteins, mammae, milk, cell fractionation, lactation

Introduction

The recommendation by pediatricians that mothers breast-feed (1) is based on the “unique superiority” of human milk and on the nutritional, neurodevelopmental, immunological, and psychological advantages it confers. Yet our understanding of the elaborate machinery responsible for milk synthesis and secretion, and the manner of its regulation in health and disease, is rudimentary. Advances in knowledge of the cellular physiology of the mammary gland may provide avenues to influence the quantity and quality of milk produced by nursing mothers and increase the rate and average duration of breastfeeding.

The most common explanation for premature cessation of breastfeeding is the mother's perception that her milk production is inadequate (2,3). Since lactose is the major carbohydrate and osmotic constituent of human milk, the volume of milk produced is a function of the rate of lactose synthesis. Lactose synthesis takes place within the Golgi (4) and is catalyzed by lactose synthetase, a complex of galactosyltransferase and the mammary gland-specific protein α -lactalbumin. The substrates for lactose synthesis are UDP-galactose and free glucose. Direct measurement of intracellular glucose concentration demonstrated that glucose transport into the mammary epithelial cell may be rate-limiting for lactose synthesis (5). The mammary epithelial cell not only must transport glucose from the blood across the basal membrane into the cell, it must also deliver this glucose to the Golgi. The requirement for free glucose within the Golgi is unique to the lactating mammary gland. A Golgi-specific glucose carrier protein

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accounting for Golgi glucose uptake during lactation was proposed (6) 5 y before the cloning of GLUT1 (7), the first member of the family of facilitated diffusion glucose transporter isoforms.

GLUT1 is the only isoform of the facilitated diffusion family of glucose transporters known to be expressed in mammary gland. GLUT1 is targeted to the plasma membrane in most cell types. No glucose transporter isoform has been proven to target to the Golgi. Subcellular fractionation and Western blotting of d 10 lactating rat mammary glands suggested that during lactation, GLUT1 may also be found in the Golgi (8), but no other timepoints were examined. In other studies (9,10) relying only upon microscopy, Golgi targeting of GLUT1 was not observed in d 10-12 lactating rat mammary gland. High and polarized expression of plasma membrane GLUT1 during lactation was seen. GLUT1 (9) after weaning pups for 24 h.

The purpose of this study was to test the hypothesis that the activity and subcellular targeting of GLUT1 in lactating mouse mammary gland are regulated such that GLUT1 plays a key role in supplying free glucose to Golgi as a substrate for lactose synthesis. Specifically, we hypothesized that during lactation, the amount of GLUT1 would increase, and a transition from plasma membrane targeting to Golgi targeting would occur. Furthermore, we tested the hypothesis that under conditions of forced weaning, abruptly curtailing milk synthesis, Golgi targeting of GLUT1 would diminish. In order to provide multiple lines of evidence, methods included subcellular fractionation and density gradient centrifugation, immunofluorescent microscopy, and immunohistochemistry.

Methods

Antisera and reagents

GLUT1 antibody was a well characterized, highly specific rabbit polyclonal antiserum to human GLUT1, raised against synthetic peptide made up of the 16 C-terminal amino acids, a kind gift of Dr. M. Mueckler (Washington University School of Medicine, St. Louis, MO). This antibody was affinity purified using the same synthetic peptide bound to thiopropyl sepharose (Pharmacia, Piscataway, NJ) (11) prior to use for immunoblotting or immunocytochemistry. Fluorescein-labeled goat anti-rabbit antibodies were from ICN (Aurora, OH). Reagents were from Sigma unless otherwise specified.

Animals

Nulliparous female CD-1 mice (Harlan Sprague-Dawley, Indianapolis, IN) were mated, and at parturition (d 0 of lactation) the litters were adjusted to 10 pups. Animals were fed on Purina lab chow (Ralston Purina, St. Louis, MO) and had access to water ad libitum and a daily photoperiod of 12 h. Experiments were carried out in duplicate or triplicate and representative results are shown. Studies were reviewed and approved by the Animal Studies Committees of the Washington University School of Medicine and the Baylor College of Medicine.

Subcellular fractionation and density gradient centrifugation

Mammary glands were removed and rinsed twice with ice-cold PBS and once with sucrose solution (0.25M sucrose, 10mM triethanolmine, 10mM acetic acid, pH 7.8), resuspended in a small volume of homogenization buffer (PBS, 1 mM EDTA), and homogenized with five strokes in a tight-fitting Dounce homogenizer. After

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centrifugation at 3,000 g for 10 min at 4°C, the supernatant was centrifuged at 17,000 g for 10 min at 4°C. This supernatant was centrifuged at 100,000 g for 30 min at 4°C. In certain experiments, the 17,000 g pellet was resuspended and subjected to density gradient centrifugation in a self-generating iodixanol density gradient (10%-37%) at 180,000 g for 3 h at 4°C. Fractions were collected from the top (Labconco Auto-densi Flow, Kansas City, MO) and the lowest density fraction (1.05-1.08 g/cm³) was analyzed. Alkaline phosphatase (12) and galactosyl transferase (13) were assayed as described.

Western blotting

Samples were prepared and subjected to standard SDS-PAGE on 10% gels as previously described (14). Proteins were immobilized on nitrocellulose by wet transfer. Primary antibody was the peptide affinity purified GLUT1 antibody described above (1 µg/ml in 5% nonfat dry milk in PBS). HRP-linked donkey anti-rabbit antibody (Amersham, Piscataway, NJ) served as secondary antibody, and signal was detected using ECL-PlusTM (Amersham). Quantitative differences in signal strength were measured using a laser densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Immunohistochemical staining

Mammary tissue was fixed in 10% neutral buffered formalin supplemented with zinc chloride (Anatech, Ltd., Battle Creek, MI) and processed for paraffin sections using standard techniques. Tissues were embedded in paraffin and sectioned at 4µm on a rotary microtome (Leitz 1512), collected on standard glass microscope slides, and stained with hematoxylin and eosin (H&E) using a routine Harris hematoxylin solution and alcoholic

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eosin counter-stain. Sections were affixed to capillary gap glass microscope slides (Ventana Medical Systems, Inc., Tucson, AZ), dried at 60°C for 1 h and deparaffinized. The tissue sections were incubated for 20 min in a 1:10 citrate buffer solution (Dako Corporation, Carpinteria, CA) in a steam environment to enhance antigen availability, then incubated in the same solution for an additional 20 min at room temperature. The sections were rinsed in phosphate buffered saline, 0.2% Tween-20, pH 7.3 (PBS/T) to enhance flow in the capillary gap; PBS/T was used to rinse the sections between all steps of the protocol. All reagents used in the immunohistochemical procedure were made in PBS/T supplemented with 0.5% crystalline grade BSA (Sigma Chemical Co., St. Louis, MO) as a protein carrier. GLUT1 immunohistochemistry was performed using a Tek-Mate 500 automated system (Ventana Medical Systems, Inc., Tucson, AZ). Sections were incubated in a 1:75 solution of normal goat serum (Vector Laboratories, Burlingame, CA) for 20 min at room temperature. The serum was removed, anti-GLUT1 antibody was applied at a concentration of 2.5 µg/mL, and the sections were incubated in a humid chamber at room temperature overnight. Negative control sections were incubated in diluent rather than the GLUT1 antibody. Following incubation, sections were rinsed and a biotin-conjugated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) was applied at 2.25 µg/ml for 45 min at room temperature. This was followed by endogenous peroxidase exhaustion using 3% H₂O₂ in absolute methanol, three changes of 5 min each. The sections were treated with a peroxidase tagged avidin-biotin complex (Vector laboratories, Burlingame CA) for 45 min at room temperature. Antigenic sites were visualized using diaminobenzidine enhanced with 1% nickel chloride as the chromagen (Sigma Chemicals, St. Louis, MO). The tissue sections were counterstained with eosin,

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then processed through ascending grades of ethyl alcohol and xylene, and mounted on coverslips using a synthetic mountant.

Immunofluorescent staining

Sections were prepared as described above. Following incubation with primary antibody against GLUT1, sections were rinsed and an FITC conjugated antibody directed against rabbit IgG (Dako Corporation, Carpinteria, CA) was applied at a concentration of 1:30 for 45 min in the dark. Sections were washed well, mounted in a non-fluorescent aqueous medium and viewed with a Zeiss Axiophot epifluorescent microscope at a wavelength of 490 nm. The images are shown as equivalent exposures acquired by a Cohu (San Diego, CA) 4910 uncooled CCD camera; no enhancement or intensification was performed.

Results

Developmental changes in GLUT1 expression

GLUT1 expression in the mouse mammary gland (Figure 1) was studied in virgins, in late pregnancy (d 20), on the day of delivery (d 0), at mid-lactation (d 8), and at the peak of lactation (d 18), and then at different timepoints during weaning (d 21, 23, and 29). Expression gradually rose from an extremely low level in virgins, increased on the day of delivery to a peak during lactation, then declined to very low levels as weaning progressed (Figure 1).

To demonstrate whether there are changes in the subcellular targeting of GLUT1 as it is induced during pregnancy and lactation, subcellular fractionation was used to prepare a 17,000 g pellet enriched in Golgi and a 100,000 g pellet enriched in plasma membrane. Enrichment was demonstrated by assays of marker enzymes. Relative to total cellular levels of each marker, the activity of galactosyltransferase, a Golgi marker, was enriched 3.5- to 4.3-fold in the Golgi-enriched fraction, while alkaline phosphatase, a marker of plasma membrane, was enriched 4.5- to 8.6-fold in the plasma membrane-enriched fraction. Virgins demonstrated predominance of GLUT1 in the plasma membrane-enriched fraction, while mammary gland differentiation during pregnancy and lactation was associated with an increase in GLUT1 targeting to the Golgi-enriched fractions (Figure 2). GLUT1 was preferentially targeted to the Golgi-enriched fraction, as indicated by 3.9-fold enrichment on d 8 (Figure 2), compared to an enrichment of 1.9-fold in the plasma membrane-enriched fraction. As expected, there was no significant targeting of GLUT1 to the 3,000 g pellet, which is enriched in nuclei, or to the 100,000 g supernatant, which is enriched in cytosol. Immunohistochemistry, a method well-suited

for evaluation of plasma membrane staining, demonstrated labeling of plasma membrane in mammary gland of virgin, pregnant, and lactating mice (Figure 3). The increase in GLUT1 expression during pregnancy is partially accounted for by the proliferation of mammary epithelial cells. Staining for GLUT1 was not observed during weaning, consistent with results of Western blotting. Virgin gland demonstrated a predominance of fat cells, but the mammary epithelial cells showed significant plasma membrane targeting to both the basolateral and apical membrane. In contrast, during lactation there was intense staining of the basolateral membrane and no staining of the apical plasma membrane, indicating a polarization of membrane targeting. Immunofluorescent microscopy, which is suitable for evaluation of intracellular as well as plasma membrane targeting, confirmed nonpolarized targeting of GLUT1 in the virgin gland, and an increase in expression during pregnancy and lactation (Figure 4). In the lactating gland, intracellular staining as well as basolateral plasma membrane staining were seen. No specific staining was seen in mammary gland during weaning.

Reversible changes in GLUT1 content and subcellular targeting during forced weaning

The removal of nursing 18-d-old pups from their dams resulted in changes in the amount and subcellular targeting of mammary gland GLUT1 within 3 h (Figure 5). A decline in total GLUT1 content, and a change from predominance of GLUT1 targeting in the Golgi-enriched fraction to predominance in the plasma membrane-enriched fraction, were observed. Iodixanol density gradient centrifugation of the 17,000 g pellet was used to provide further enrichment. Since Golgi membranes have the lowest buoyant density of any subcellular membrane fraction, the lowest density fraction, corresponding to a density of 1.05-1.08 g/cm³, was analyzed. A fall in Golgi enrichment of GLUT1 occurred

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within 3 h, and there was no Golgi enrichment of GLUT1 by 5 h (Figure 6). However, when pups were then returned to the dam for 5 h, Golgi enrichment of GLUT1 was again observed. When pups were returned for 15 h, Golgi enrichment of GLUT1 was fully restored.

Discussion

The production of an adequate volume of milk by the nursing mother is a prerequisite for successful lactation. Because lactose is the major osmotic constituent of human milk, the synthesis of lactose determines the volume of milk produced. Since changes in lactose synthetase activity do not correlate with changes in milk production (15), the process may be regulated at the level of substrate availability. The lactating mammary gland is unique in its requirement for the transport of free glucose across the Golgi membrane. GLUT1 is the only member of the facilitated diffusion glucose transporter family expressed in mammary gland. Neither GLUT1 nor any other isoform of the glucose transporter family is considered to be a Golgi resident, although a six-amino acid portion of GLUT4 does confer targeting to the trans-Golgi network and insulin-sensitive translocation to the plasma membrane in fat and muscle cells (16).

Several investigators have studied GLUT1 expression in the lactating mammary gland, with conflicting results described above. The purpose of the experiments reported here was to systematically test whether the developmental regulation of the amount and subcellular trafficking of GLUT1 in mouse mammary gland indicates an important role for GLUT1 in the provision of substrate for lactose synthesis. In contrast to previous studies, independent methods were utilized, and multiple timepoints during the normal developmental cycle and the forced weaning-refeeding cycle were examined.

Importantly, consistent results from independent methods, subcellular fractionation followed by density gradient centrifugation, and immunofluorescent microscopy indicate that during lactation, GLUT1 is targeted not to the plasma membrane, as it is in most cells, but to the Golgi. This leads to the conclusion that

GLUT1 is diverted from normal sorting pathways to the Golgi in order to provide glucose within the Golgi for lactose synthesis. The results do not exclude the previously suggested (8) possibility that a novel transporter, yet to be identified, resides in the Golgi.

The results suggest a tissue- and developmental stage-specific Golgi targeting mechanism for GLUT1. The structural determinants of targeting of proteins to Golgi are controversial. The transmembrane-spanning domain or specific amino acid motifs within it have seemed important (17). However, no common retention signal is apparent on examination of a large number of cloned glycosyltransferases (18). Other proposed explanations include the formation of non-mobile protein oligomers or the influence of the high cholesterol content of Golgi membranes on mobility of resident Golgi enzymes (19). Glycosylation serves to direct protein targeting to the apical membrane of polarized epithelial cells under certain circumstances (20), but has not been linked with targeting of proteins to Golgi. In fact, a mutant form of GLUT1 lacking the N-glycosylation site shows intracellular targeting (21). Since our general understanding of the determinants of protein targeting to Golgi is limited, it is not surprising that the molecular basis of the hormonally regulated Golgi targeting of GLUT1 is unclear. Further study may reveal mechanisms regarding GLUT1 targeting that are relevant to other Golgi proteins as well. Hormonal regulation of GLUT1 subcellular targeting suggests a flexibility of the Golgi targeting machinery that has not previously been appreciated.

The reversible nature of the targeting of GLUT1 to Golgi, and the time course over which changes in GLUT1 targeting are observed, suggest a dynamic process which requires frequent suckling to maintain substrate supply for lactose synthesis. Future work

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will explore the relevance of this mechanism to the phenomenon of decreased production of human milk when nursing intervals are prolonged.

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Figure legends

Figure 1. GLUT1 induction during pregnancy and lactation. Homogenate fractions from mouse mammary gland were subjected to SDS-PAGE, Western blotting, ECL, and laser densitometry as described in Materials and Methods. Samples containing 10 μ g of total cellular protein were run on the same gel and ECL exposure time was one minute. Results are expressed relative to peak GLUT1 expression, which was observed 8 d after delivery. GLUT1 rises from very low levels in virgin gland during pregnancy, increases further during lactation, then rapidly declines upon weaning.

Figure 2. Golgi targeting of GLUT1 during lactation. Subcellular fractionation of mammary gland was carried out as described in Materials and Methods. Lane 1, homogenate; lane 2, 3,000g (nuclear) pellet; lane 3, 3,000g supernatant; lane 4, 17,000g (Golgi-enriched) pellet; lane 5, 100,000g (plasma membrane-enriched) pellet; lane 6, 100,000g supernatant (cytosol). Samples contained 60 μ g of protein, except for the virgin samples, which contained 25 μ g. ECL exposure times were adjusted as needed to assess relative targeting to Golgi-enriched and plasma membrane-enriched fractions while avoiding saturation of signal from any one fraction, and ranged from overnight for the virgin samples to 30 s for the samples from d 8. Total GLUT1 expression at these timepoints was directly compared in Figure 1. GLUT1 targeting shifts from predominance in the plasma membrane-enriched fraction in the virgin, to approximately

equivalent plasma membrane and Golgi targeting in late pregnancy and on the day of delivery, to predominance in the Golgi-enriched fraction by d 8.

Figure 3. Basolateral plasma membrane targeting of GLUT1 during lactation. A, virgin; B, pregnant d 20; C, lactating (d 18 after delivery); and D, weaning (d 21 after delivery). Bar, 60 μ m. Immunocytochemistry was carried out as described in Materials and Methods. Positive staining for GLUT1 is indicated by brown. Control slides showed no signal. The virgin gland is predominantly fat, but non-polarized plasma membrane targeting of GLUT1 is seen in islands of mammary epithelial cells. During lactation, GLUT1 staining is intense, but is observed only in basolateral plasma membrane and not in apical plasma membrane.

Figure 4. Intracellular targeting of GLUT1 during lactation. A, virgin; B, pregnant d 20; C, lactating (d 18 after delivery); and D, weaning (d 21 after delivery). Bar, 60 μ m. Immunofluorescent staining and microscopy was carried out as described in Materials and Methods. Control slides showed a level of signal equivalent to that seen in weaning gland. Non-polarized plasma membrane targeting of GLUT1 in virgin gland and polarized targeting of GLUT1 in the lactating gland are seen. In addition, strong intracellular signal is observed in the lactating but not the virgin gland. The weaning gland shows only non-specific staining. Strong signal is also observed in red blood cells, which contain very high levels of GLUT1.

Figure 5. Decline in GLUT1 expression and in Golgi targeting of GLUT1 during forced weaning. 18-d-old pups were removed from lactating dams for the specified time.

Subcellular fractionation of mammary gland was carried out as described in Materials and Methods. Lane 1, homogenate; lane 2, 3,000g (nuclear) pellet; lane 3, 3,000g supernatant; lane 4, 17,000g (Golgi-enriched) pellet; lane 5, 100,000g (plasma membrane-enriched) pellet; lane 6, 100,000g supernatant (cytosol). Samples contained 60 μ g of protein, and ECL exposure times were identical. Declines in the amount and Golgi targeting of GLUT1 are apparent 3 h after weaning and are pronounced by 5 h after weaning.

Figure 6. Reversibility of the effect of forced weaning on GLUT1 targeting. Pups were removed from lactating dams for the specified time. In certain experiments, indicated in crosshatch, pups were returned to their dams after a 5-h weaning period. Subcellular fractionation and iodixanol density gradient centrifugation of mammary gland was carried out as described in Materials and Methods. Results are expressed as enrichment of the purified Golgi fraction relative to total cellular levels of GLUT1. Golgi targeting of GLUT1 was lost by 5 h and was not observed after 10 or 20 h of weaning. However, when pups were returned to the dam after a 5-h absence, Golgi targeting of GLUT1 was observed within the next 5 h, and was fully restored 15 h after lactation was resumed.

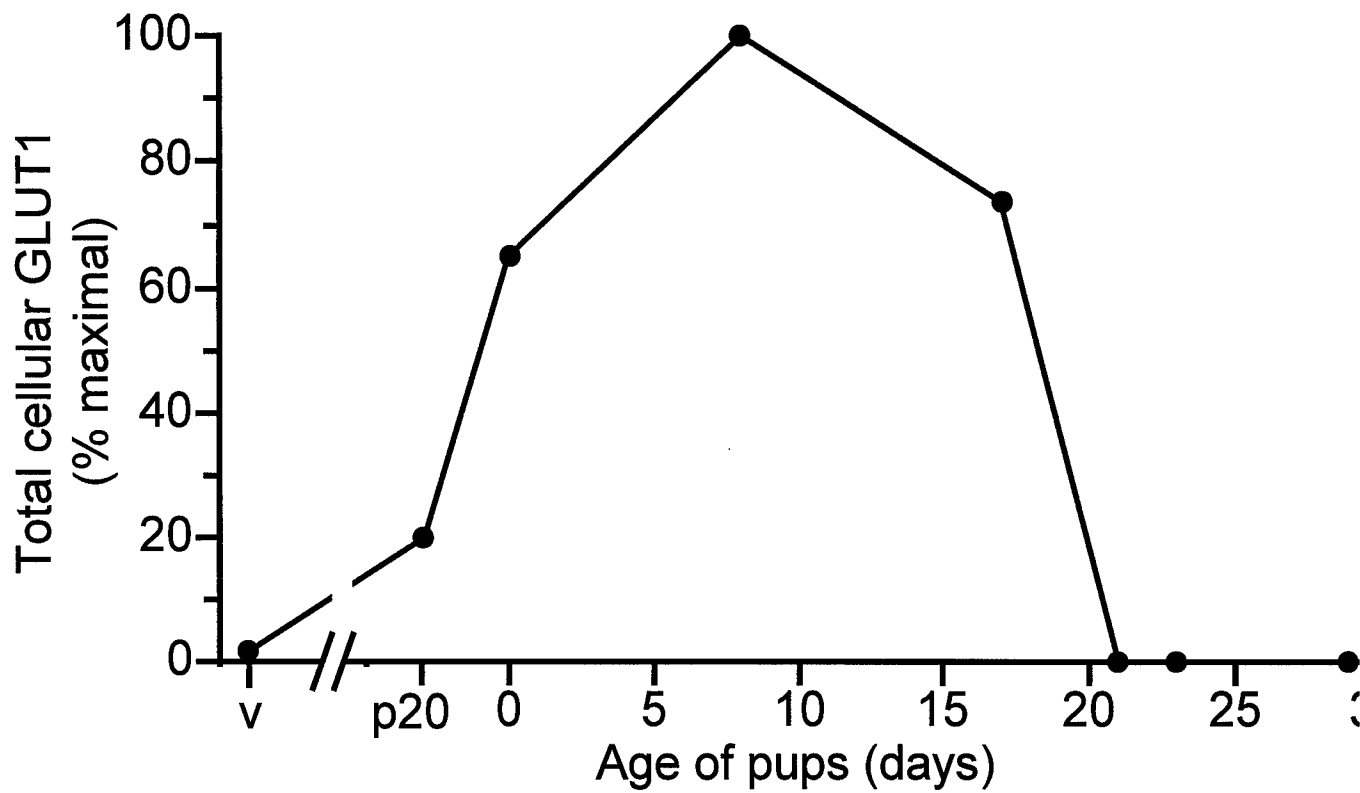


Figure 1

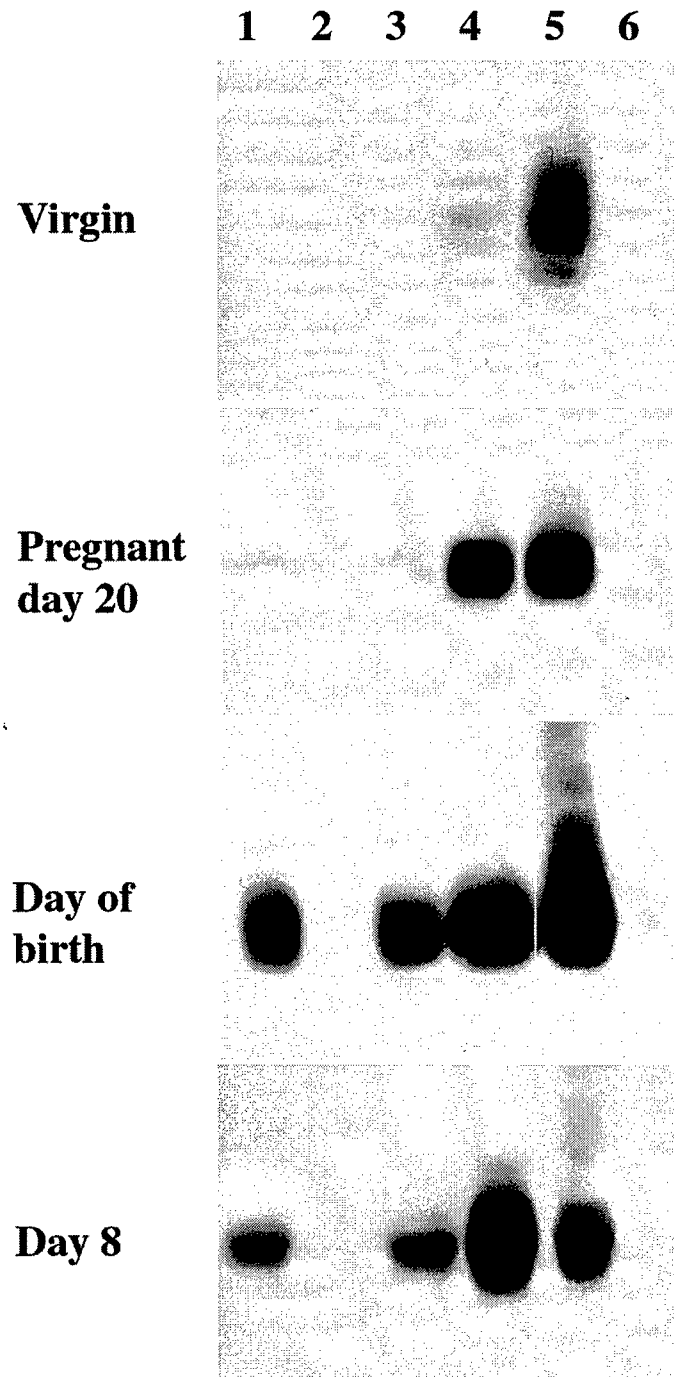


Figure 2

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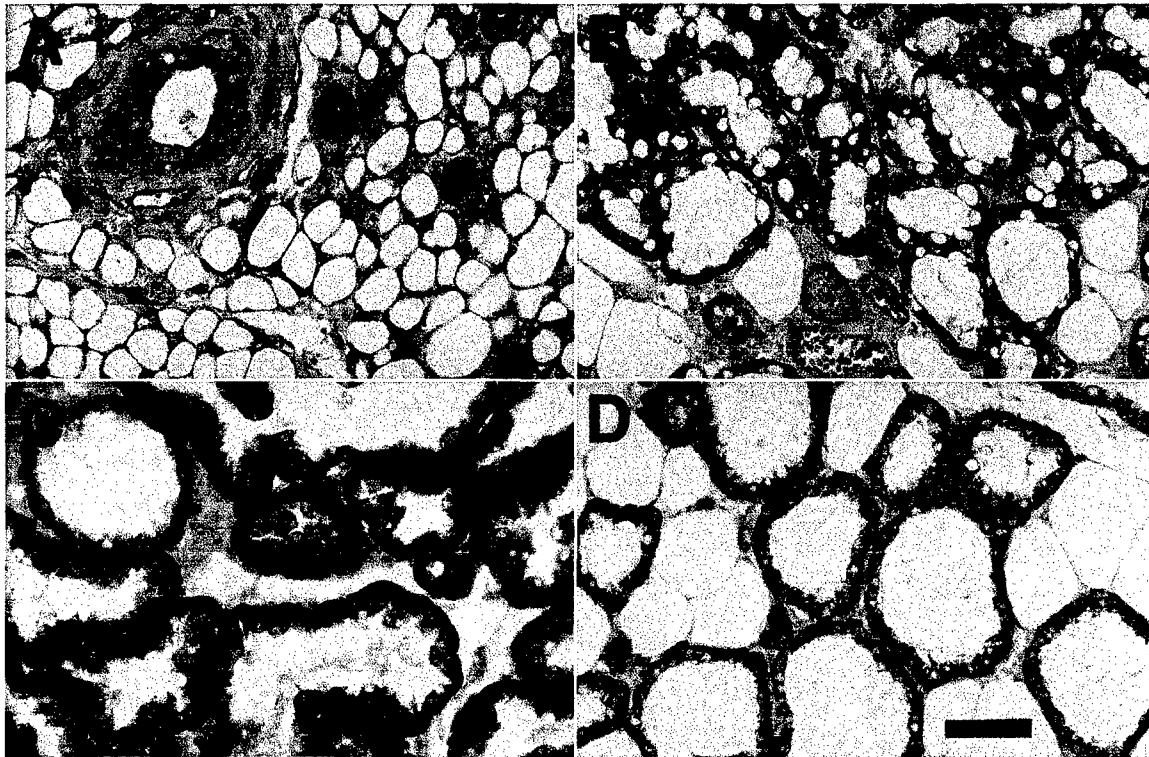


Figure 3

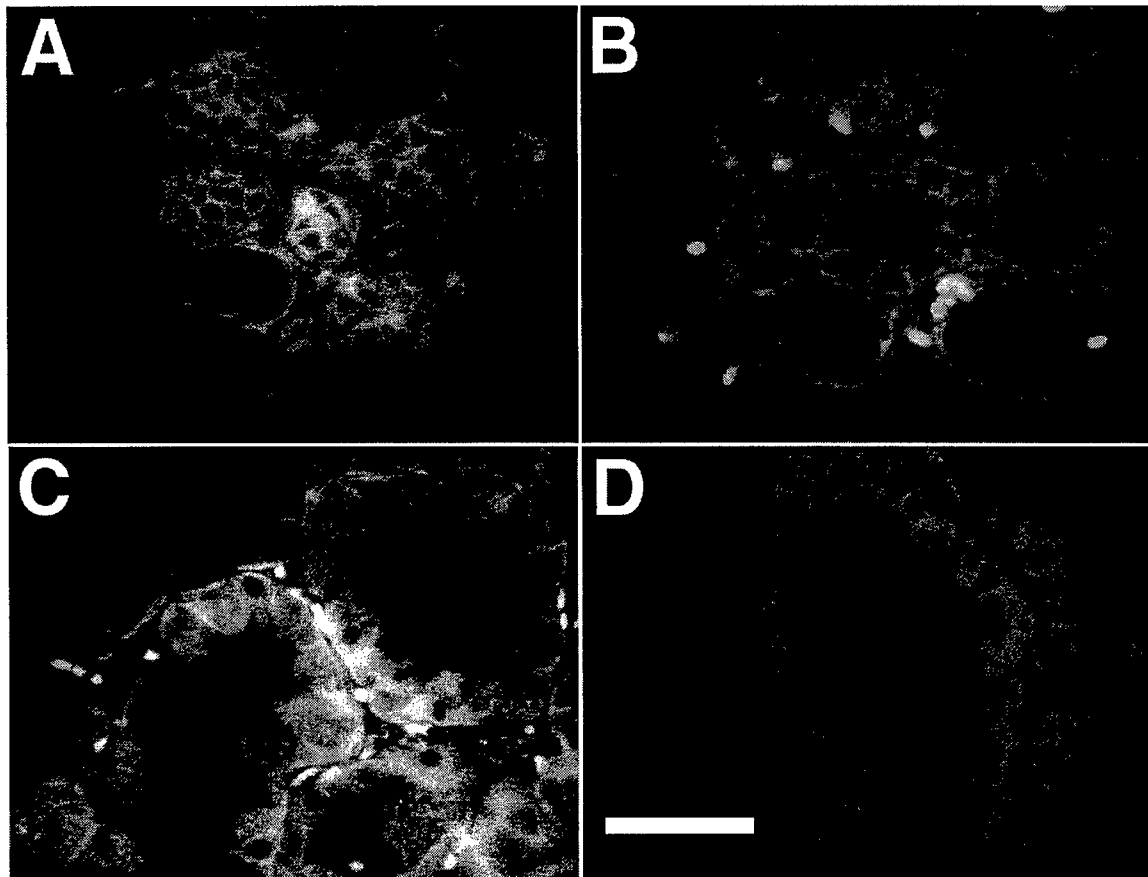


Figure 4

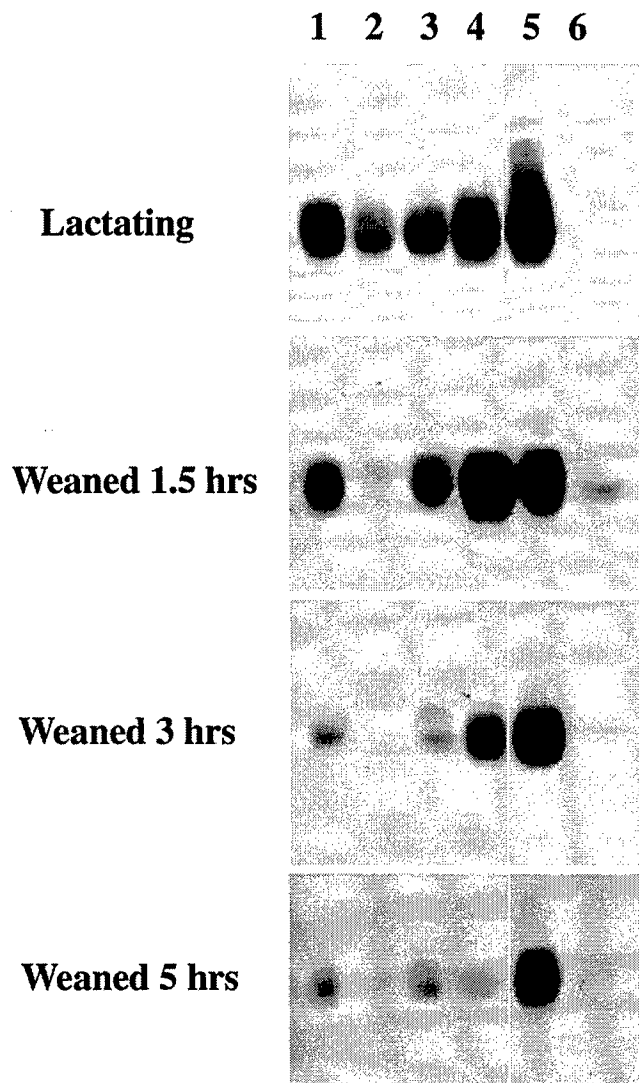


Figure 5

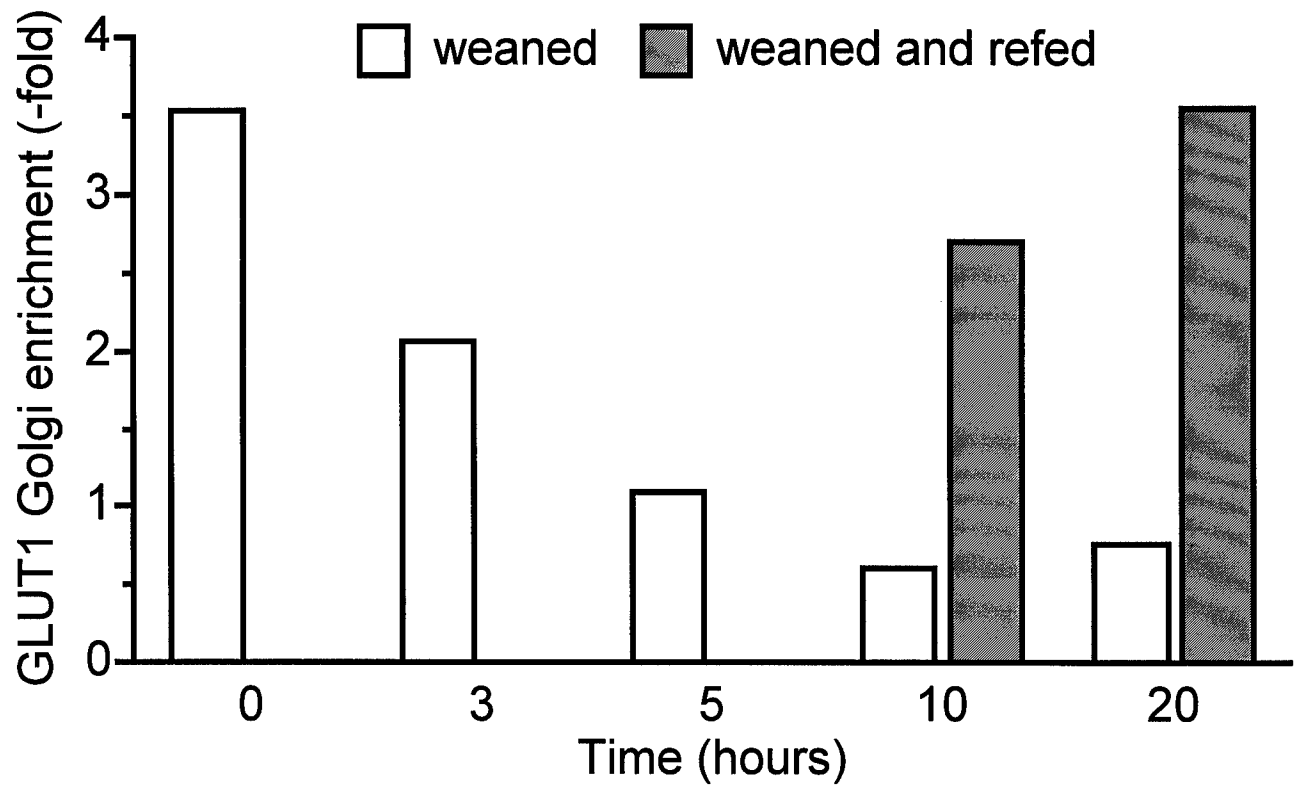


Figure 6

**LIPOSOME-MEDIATED STABLE TRANSFECTION OF CIT₃ MOUSE MAMMARY
EPITHELIAL CELLS.**

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SUMMARY

Transfection of mammary epithelial cells can provide important insights into the molecular biology of milk secretion and breast cancer. However, investigators have acknowledged difficulties transfecting these cells. The objective of this work was to determine an efficient method for achieving stable transfections in these cells. The relative transfection efficiency of CaPO_4 , DEAE-Dextran, cationic polymers and cationic liposomes was compared. CIT₃ mouse mammary epithelial cells were transfected with green and blue fluorescent protein plasmid vectors. Transfection efficiency, expressed as the number of stably transfected colonies selected by geneticin, was highest for cationic liposomes compared to all other transfection methods ($p < 0.05$).

Key words: Mammary epithelial cells / Transfection / Liposomes

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Mammary epithelial cell differentiation and the regulation of secretion of milk components are important for understanding mechanisms of milk production. The mammary epithelial cell is also a useful general model of secretory processes (Burgoyne *et al.*, 1994, Shennan, 1998). Abnormalities in mammary cell differentiation may be related to the development of breast cancer (Petersen *et al.*, 1998, Rudland *et al.*, 1988, Russo *et al.*, 1997).

Transfection introduces foreign DNA into eukaryotic cells in order to study regulation of gene expression. Efficiency of transient and stable transfection varies widely as a function of different cell types, cell lines and promoters (Kane, 1991, Kucherlapati *et al.*, 1984, Ray *et al.*, 1992). Recently, Bischof *et al.* (Bischof *et al.*, 1999) reported successful transient transfection of mouse mammary epithelial cells, but acknowledged long-recognized difficulties encountered in the transfection of mammary epithelial cells. For many applications, including our intended studies of protein targeting in mouse mammary epithelial cells, stably transfected cells are advantageous. Colonies with uniform, appropriate level of exogenous gene expression can be studied. Our objective was to determine the most efficient chemical method for stable transfection of mouse mammary epithelial cells.

CIT₃ cells are a non-neoplastic cell line derived from mouse mammary epithelial cells. They were selected from Comma-1-D cells for their ability to grow well on filters, form tight junctions and exhibit polarized transport (Toddywalla *et al.*, 1997). CIT₃ cells were provided by M.C. Neville, Ph.D., University of Colorado School of Medicine. Cells were maintained in growth medium (GM), which is a nutrient-defined basal medium (DMEM/F12, GibcoBRL, Life Technologies Inc., Rockville, MD), containing 10µg/ml insulin (Sigma, St. Louis, MO) and 5ng/ml EGF (Epithelial Growth Factor, GibcoBRL, Life Technologies Inc., Rockville, MD).

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pEGFP and pEBFP-C1 (Green and Blue Fluorescent Protein plasmid vectors, Clontech Laboratories Inc., Palo Alto, CA, # 6077-1 and # 6070-1, respectively) have a neomycin resistance (Neo^r) gene for selection (using Geneticin) in eukaryotic cells. pEBFP has the human CMV immediate early promoter for high level expression and SV40 origin of replication. Glucose transporter cDNA (Mueckler *et al.*, 1985) was subcloned to pEBFP to create a GLUT1-EBFP fusion protein. Expression of pEGFP was driven by the β -casein promoter, provided by D. Hadsell, Ph.D., Baylor College of Medicine, Houston, TX.

The transfection methods employed were:

1. Liposomes (LipoFectAmine Reagent, which is a 3:1 (w/w) liposome formulation of the polycationic lipid 2,3-dioleoyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA) and the neutral lipid dioleoyl phosphatidylethanolamine (DOPE), GibcoBRL, Life Technologies Inc., Rockville, MD, #18324-012),
2. Cationic polymers (SuperFect Reagent, which consists of activated-dendrimer molecules, that have a defined spherical architecture, with branches that radiate from a central core and terminate at charged amino groups, Qiagen Inc., Valencia, CA, #301305),
3. CaPO₄ (Mammalian Transfection Kit, Stratagene, La Jolla, CA, #200285), and
4. DEAE-Dextran (Mammalian Transfection Kit, Stratagene, La Jolla, CA, #200285).

All transfection reagents were used according to the manufacturer's instructions.

Initial toxicity was estimated based on the percentage of cell detachment within 24 hours after transfection (Table 1). Direct initial toxicity from the transfection procedure was highest for Liposomes and SuperFect reagent and lowest for CaPO₄ and DEAE-Dextran.

Transfection efficiency was expressed as number of colonies ≥ 1 mm in diameter per 100 mm plate. This threshold was selected because colonies of this size can consistently be transferred and expanded for further study. Liposome-mediated transfection was 40-60% more efficient in mouse mammary epithelial cells compared to all other transfection methods ($p < 0.05$) (Figure 1). Cationic polymers were also consistently useful, although less efficient. CaPO_4 , although efficient sometimes, was not consistent. DEAE-Dextran was not suitable for transfecting mammary epithelial cells, yielding no colonies at all. Cost per transfection and convenience of use was similar for all methods. The mean area of the individual colonies was similar and independent of the transfection method employed, implying that cell growth is independent of transfection method (Figure 2). This finding supports the use of number of colonies as a comparison measure for efficiency in stable transfections done by different methods.

The majority of previous studies compared transient transfection efficiency, using measures of gene expression (e.g. β -galactosidase, luciferase or chloramphenicol acetyltransferase activity, fluorescent in situ hybridization or GFP transient expression)(Bischof, et al., 1999, Budker *et al.*, 1997, Cheng *et al.*, 1996, Verma *et al.*, 1998). We judged transfection efficiency by measure of stably transfected colonies, which incorporated the foreign gene, and thus became resistant to the selective medium. This is a more direct measure of transfection efficiency, and is not related to other factors which may influence gene expression. This approach for assessing transfection efficiency in mammary epithelial cells was described previously by Basolo et al.(Basolo *et al.*, 1990), who demonstrated the best transfection efficiency with CaPO_4 , especially when combined with glycerol shock. However, the results of Basolo et.al. were obtained using an immortalized cell line, and were not compared to liposomes

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or cationic polymers transfection reagents, which became more widely available since that report. Our results for stable transfections, demonstrating highest transfection efficiency in mammary epithelial cells using cationic liposomes complement those of Bischof et al. (Bischof, et al., 1999), who utilized also PEI/DNA adenovirus system, but demonstrated the usefulness of liposomes in these cells, using GFP fluorescence as measure of gene expression in transient transfections.

In summary, cationic liposomes provide a consistently efficient, cost-effective, and convenient method for transfection of mammary epithelial cells. Expression of heterologous proteins in these cells using this reliable transfection method is expected to yield important insight into milk secretion and breast cancer.

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Table 1. Estimated initial toxicity based on the percentage of cell detachment within 24 hours after transfection. Transfection of 2 μ g DNA was performed in 35mm dishes containing 5 X 10⁵ cells per plate. All transfection reagents were used according to the manufacturer's instructions. Initial toxicity was estimated based on the percentage of cell detachment within 24 hours after transfection.

	Cationic Liposomes	Cationic Polymers	CaPO ₄	DEAE-Dextran
Initial toxicity	40-50%	35-45%	10-20%	0-10%

Figure 1. Transfection efficiency expressed as number of colonies ≥ 1 mm in diameter per 100 mm plate as counted 17 days after transfection. Transfection of 2 μ g DNA was performed in 35mm dishes containing 5×10^5 cells per plate. All transfection reagents were used according to the manufacturer's instructions. After 48 hours, cells were split 1:8. Selective medium containing 0.5 mg/ml Geneticin (GibcoBRL, Life Technologies Inc., Rockville, MD) was added 24 hours later. Cells were maintained in selective media containing Geneticin until the colonies were counted 17 days after transfection. Stable transfection efficiency was determined by the number of surviving colonies with diameter of 1mm or greater per 100mm plate. Dishes were photographed using a Cohu 4910 uncooled CCD camera and quantitated with the aid of image processing software (Image Tool, UTHSCSA, San Antonio, TX). Statistical analysis was by one way ANOVA (SigmaStat, version 2.03). Liposome-mediated transfection is consistently the most efficient compared to other methods ($p < 0.05$, one way ANOVA). DEAE-Dextran yielded no colonies in any experiment. In expt. 1, transfection of pEBFP-GLUT1 with CaPO_4 yielded no colonies.

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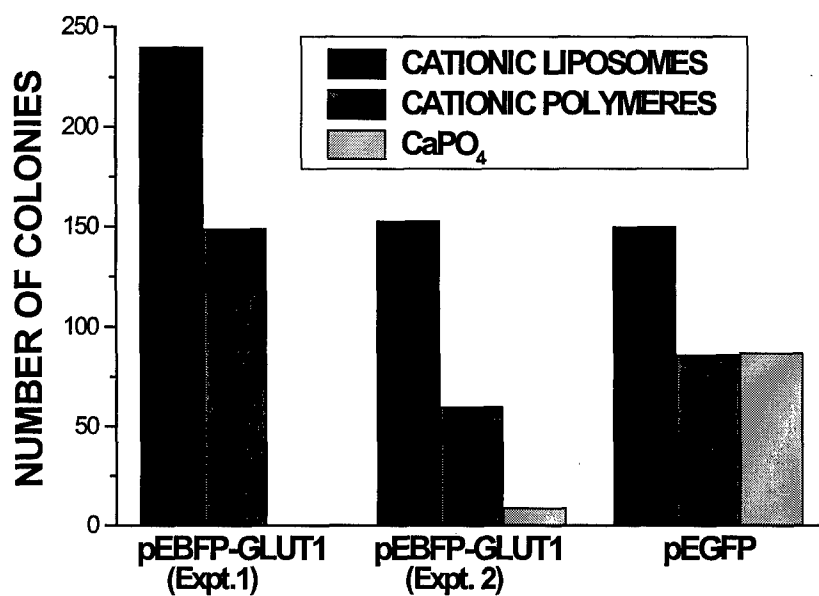
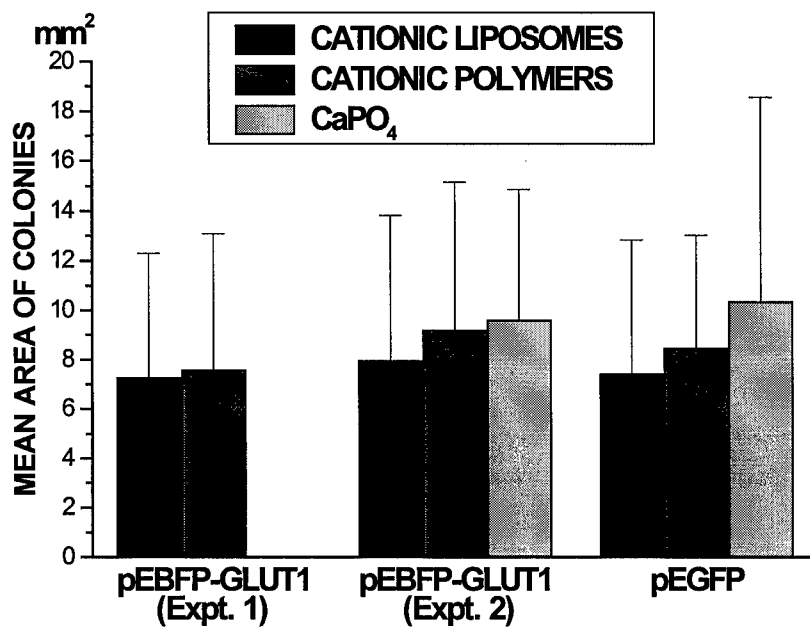


Figure 2. The area of the individual colonies was similar and independent of the transfection method employed: Transfection of 2 μ g DNA was performed in 35mm dishes containing 5 X 10⁵ cells per plate. All transfection reagents were used according to the manufacturer's instructions. After 48 hours, cells were split 1:8. Selective medium containing 0.5 mg/ml Geneticin (GibcoBRL, Life Technologies Inc., Rockville, MD) was added 24 hours later. Cells were maintained in selective media containing Geneticin until the colonies were counted 17 days after transfection. Dishes were photographed using a Cohu 4910 uncooled CCD camera and quantitated with the aid of image processing software (Image Tool, UTHSCSA, San Antonio, TX). The mean area of the colonies counted on day 17 post-transfection is shown. Statistical analysis was by one way ANOVA (SigmaStat, version 2.03). There is no statistically significant difference between the areas of the colonies under different transfection methods. In expt. 1, transfection of pEBFP-GLUT1 with CaPO₄ yielded no colonies.

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DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

23 Aug 01

MEMORANDUM FOR Administrator, Defense Technical Information
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,
VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to the technical reports listed at enclosure. Request the limited distribution statement for these reports be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl


PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

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