

My journey in the discovery of nucleotide sugar transporters of the Golgi apparatus

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Defects in protein glycosylation can have a dramatic impact on eukaryotic cells and is associated with mental and developmental pathologies in humans. The studies outlined below illustrate how a basic biochemical problem in the mechanisms of protein glycosylation, specifically substrate transporters of nucleotide sugars, including ATP and 3'-phosphoadenyl-5'-phosphosulfate (PAPS), in the membrane of the Golgi apparatus and endoplasmic reticulum, expanded into diverse biological systems from mammals, including humans, to yeast, roundworms, and protozoa. Using these diverse model systems allowed my colleagues and me to answer fundamental biological questions that enabled us to formulate far-reaching hypotheses and expanded our knowledge of human diseases caused by malfunctions in the metabolic processes involved.

My early years

As you can see, I have a Spanish first name and German-Jewish last name. The origin of my name is rooted in simple history. My parents were born in Silesia, when it was part of Germany. My father was a judge, which at that time in Germany was an academic career, separate from becoming an attorney. My mother was among the first students to study social work, which then had a strong nursing component. In 1933, with Hitler's ascent to power, my father, along with other Jewish judges, was thrown out of the German court system. This was an unjust act, as Germany had agreed after World War I, because parts of Silesia had been in Poland, not to discriminate in any way toward minorities in that region until 1938. Legal action was taken, and someone appealed to an international court, claiming that minorities also included Jews, and surprisingly, Hitler abided by this.

My father went back to work, but everyone knew that by 1938 this would end. Toward the end of that year, my parents moved to Berlin to prepare for their departure to Chile, where fortunately, my father's brother and his family had earlier immigrated. A few days after Kristallnacht (November 8, 1938), my father was detained and sent to a concentration camp. Although the stay was short, it was a life-changing experience for him. Fortunately, at that time, if one had an exit visa to leave Germany, one was freed from the camp and allowed to leave

Germany. Thus, my parents and my sister left Germany for Chile in March 1939. A German judge could not really find an adequate job in Chile, so my father worked odd jobs as an accountant while my mother's nursing training helped the family survive. A few years later, I was born in a country which, as stated in its national anthem state, "is an asylum against oppression."

My parents were eking out a living when my father died in a skiing accident. My mother was left with my sister, age 16, and me, age 4. When the time came for me to attend kindergarten, I was sent to a Swiss school in Santiago where I stayed until graduating from high school. My mother thought that the discipline and languages offered at the school would be good for me. In addition to Spanish, we learned German, English, and French. These languages would be of great help to me when I worked on my Ph. D. degree because, as part of the requirements for the degree, we had to translate scientific papers from German and French into English. Did they help me later in my scientific career? Not really!

University in Chile

In high school, I found humanities very easy, while sciences required more effort. In Chile, at that time, most university studies were free, but very competitive to get into. One first had to take a general exam, called "Bachillerato," to attend a university, and then another exam to enter the specific school within the university you wanted to attend. I chose to study biochemistry because I did not like pure chemistry or pure biology. However, I did not even know what biochemistry really was! In Chile at that time, a student entered directly from high school into a professional school, with the risk that if you did not like the particular choice, you could not transfer to another school without starting from scratch. For the first 3 years working toward my professional degree as a "Biochemist," the seven other students and I studied with students in the Chemistry and Pharmacy programs, while for the last 2 years, we studied in separate programs.

One could not take a final exam in each course without a "pass," an average C grade or 12 points. This scale was absolute with God receiving the top grade, 21 points, the professor, 18 points, and the rest of us mortals scoring between 12 and 18 points. Once you were allowed to take the final exam in a course, you could, of course, fail again. If you failed a course, you had one chance to retake the exam, and if you failed again or had more than one course without a pass for the final, you had to

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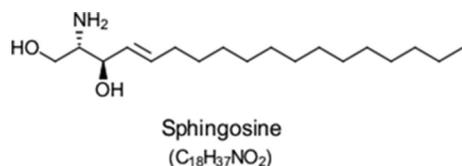


Figure 1. Structure of sphingosine.

retake these courses again the next year and could not enroll in any subject for the following year curriculum. This was clearly a total waste of taxpayer money and contributed to the fact that only eight students, myself included, of the initial 100 finished the program in the minimum of 5 years! Unfortunately, the school wouldn't rank us, so our transcripts weren't particularly impressive.

Graduate school in the U.S.A.

At that time, there were no graduate programs in sciences in Chile. For family reasons, I then enrolled at Rutgers in an MS program in Nutrition with a minor in Biochemistry. The graduate Biochemistry courses were easy compared with the courses I had in Chile. For my MS thesis, I worked on lipids of the avian brain with few interesting and publishable results. This experience taught me a life lesson about the importance of choosing a good thesis project and would serve me well as an advisor for my students.

Thereafter, I entered the Ph. D. program in the Biochemistry Division of the Chemistry Department at the University of Illinois-Urbana. The department was then ranked as one of the highest in the country, and during the first year I took courses in physical chemistry, biophysical chemistry, and organic chemistry. The courses were so difficult for me that at times I thought I wouldn't survive and that I would have to return to Chile with a Master's degree, which I rationalized wouldn't be that bad. I distinctly remember thinking twice about whether or not to renew a subscription to a magazine as there was a good chance I would not be around to receive new issues! In the end, I did just fine. I hadn't taken into account that I would be graded on a curve. In one case, my organic chemistry professor gave back seven exams (one of which was mine) out of approximately ~60, saying that there was no point in returning the rest of the exams! I looked at mine, and I had received a score of 4/20. I folded my exam and kept very quiet. Only much later did I find out, to my surprise, that the exams he had not returned had scored 0/20.

As mentioned before, during my MS thesis at Rutgers, I had become interested in lipids and sought out Professor Herb Carter for my Ph. D. thesis. His area of research was the chemistry of sphingolipids, which are lipids containing the long-chain base sphingosine (Fig. 1). He was the head of the Chemistry Department (with 60 faculty members), and I was told that he wasn't taking any more students. After listening to the other faculty members' projects, I became rather discouraged and told one of the younger professors that I was thinking of perhaps leaving the department. He told me to talk to Herb again, and he was sure Herb would take me as a student. I prepared a long speech of why I wanted to work with him, and as soon as I saw him and started my reasoning, he told me, "Ok, I'll tell 'so and so' to give you a desk in my laboratory." I had over-prepared

my speech, but in retrospect, working in Herb's lab was one of the best decisions in my scientific career.

My first project was to describe which sphingosine bases occurred in the beef kidney. GLC was one of the preferred methods to identify long-chain bases based on their relative mobility on GLC columns. One would also routinely reduce the long-chain bases' double bonds with hydrogen and palladium and analyze the corresponding changes in mobility on the GLC columns. After some routine analyses, I found that before and after catalytic hydrogenation it appeared that one peak still migrated in the position of C18 sphingosine, which, of course, has a double bond and should have been completely reduced to C18 sphinganine. At some point, I decided to do a control to see whether, in my hands, standard C18 sphingosine was completely reduced: alas it was! The only conclusion left was that the peak, which co-migrated with C18 sphingosine *after* catalytic hydrogenation, was not C18 sphingosine, but another long-chain base. This hypothesis led to further analyses and the discovery of branched-chain long-chain bases in the kidney and to my first paper published in *Biochemistry* (1). I then had a pivotal conversation with Herb: he told me that I could either look for more of these long-chain bases in other tissues or pursue something totally different. It was my choice, and I chose to pursue something different. I never forgot this conversation because there is always a dilemma of where to go next in research, especially after making a new discovery.

While I was engaged in my Ph. D. research and contemplating my future research, Herb became Vice Chancellor for academic affairs at Urbana and George Schroepfer kindly allowed me to transfer to his group to work on my own project. I thought that figuring out the *in vitro* mechanism by which the double bond in sphingosine arose, which was unknown at that time, would be a nice and important project. After many months of trying everything I could think of and getting nowhere, I was desperate. In my desperation, I did a somewhat irrational experiment, where I added ATP to a reaction mixture with tritiated sphinganine (a saturated sphingosine base) on the assumption that energy might be required for the double bond formation. It was not, but I noticed that the radiolabel became soluble in the aqueous layer of a water/chloroform/methanol mixture and not as sphinganine in chloroform/methanol. This led to the discovery of a crude sphinganine kinase, a *Journal of Biological Chemistry* paper, and the conclusion of my Ph. D. thesis (2).

Postdoctoral studies

With these results, I got the courage to tell Herb Carter and George Schroepfer that I wanted to go to Gene Kennedy's lab at Harvard Medical School to work as a postdoc on phospholipids. Fortunately for me, they were supportive, and after an interview with Gene, I was accepted in his lab, provided I could get a fellowship. I was in the U. S. on a student visa and could not apply to NIH or NSF for fellowships. I ended up looking at small foundations that would allow foreigners to apply. I discovered the Jane Coffin Childs Memorial Fund for Medical Research and applied, having no idea that it was rather prestigious! I was asked for a final interview to visit Charles Huggins in Chicago. Huggins was on the Board of the Fund and had received the

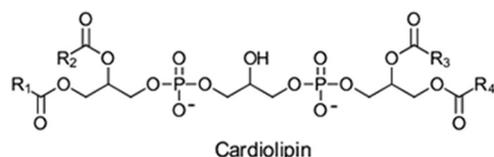


Figure 2. Structure of cardiolipin.

Nobel Prize for his work on prostate cancer. I did not know this and George Schroepfer later told me he did not want to scare me, but Huggins had a reputation of intimidating scientists, both young and old. The interview took place in Huggins' lab: he was standing on one side of a lab bench, and I was sitting on a lab stool on the other side. He asked his first question: What is science? Without waiting for an answer, he asked the second question: What is research? At some point, during one of my answers, I said that I sometimes got emotional about my lab work. He stopped me and asked me sternly: "You get emotional about your lab work?" I started to backtrack and clarified by saying yes, but that I was also aware that this could have some limitations. He then asked whether I had met Gene Kennedy. I answered yes, and Huggins started his elegy about Kennedy and at the end said that Gene also gets emotional about his work! Lucky me, I had said the right words! I left the interview exhausted but got the fellowship, which ended up being more than generous (more about this later).

Gene Kennedy's lab was small, around seven people. While there, I made lifelong friends with Bill Dowhan (who has made very important and novel contributions to the role of phospholipids in membranes and wrote a Reflections article in this journal recently), Chris Raetz (whose untimely passing was tragic and whose friendship is thoroughly missed), Bill Wickner (a world expert in protein translocation across membranes), and toward the end of my stay, Jim Rothman (who received the 2013 Nobel Prize in Physiology and Medicine).

During those times, postdoctoral fellowships lasted for 2 years. At the end of my first year as a postdoctoral fellow, I had accomplished absolutely nothing. My project was to purify *Escherichia coli* cardiolipin synthase using a radiolabeling activity assay with CDP-diglyceride and radiolabeled phosphatidylglycerol. This was then thought to be the pathway for biosynthesis of cardiolipin (Fig. 2). I was unable to solubilize the enzyme and do assays while the enzyme remained active. I felt Gene thought my lack of ability was the cause for the slow progress of the project. Having once seen a full list of the postdocs who had been in his lab, half of which we had never heard of, I feared that I was destined to join them, and I was pretty discouraged. Fortunately, Gene let me test an alternative hypothesis, namely that cardiolipin was synthesized by the condensation of two phosphatidylglycerols, yielding cardiolipin and free glycerol. I would have done this on my own, but I could not order radioactive substrates without Gene knowing. I synthesized tritiated phosphatidyl glycerol and [³²P]CDP-diglyceride, showed that the latter was not a substrate, and showed that both free glycerol and cardiolipin were reaction products. It turns out that Joan Lusk in Gene Kennedy's lab had previously obtained *in vivo* evidence supporting this pathway: she had found that, upon feeding radiolabeled glycerol to *E. coli*, some of the starting product was converted to phosphatidylglycerol,

and after a lag, additional free glycerol and cardiolipin appeared within the cell, consistent with the *in vitro* pathway we had described. Fortunately for me, Gene decided to publish our results in the *Proceedings of the National Academy of Sciences of the U.S.A. (PNAS)* (3). At that time, if a National Academy member (Gene) wanted to publish in *PNAS*, there was no further required review of the manuscript!

While in Boston, I spent some of my time outside of lab attending many, somewhat esoteric, classical music concerts. During one such concert at the Old West Church in Boston, which celebrated the 300-year anniversary of Heinrich Schütz's death, I met a wonderful woman. Her name was Lois. She later became my wife, and we have been married ever since.

I decided to do a second, 2-year postdoc in Phil Robbins' lab at MIT. I applied for a 1-year extension of my Jane Coffin Childs Memorial Fund for Medical Research Fellowship and was advised by the director that while this was not unprecedented, I had to convince the Board of Scientific Advisors that I deserved this extra year more than new applicants who had never had a fellowship. I still wince at this, but as luck would have it, I had an ally in Joan Lusk. Joan, as mentioned previously, had been a former student in Kennedy's lab and had moved on to a postdoc position in Salvador (Salva for short) Luria's lab at MIT. Luria happened to be on the Board of the Jane Coffin Childs Memorial Fund for Medical Research, and Joan had told him about our cardiolipin synthase results. I was able to get a third year of funding!

During my stay in Phil Robbins' lab at MIT, I studied the relation of lipids of Sindbis virus, and those of the host cell plasma membrane, and found them to be very similar (4). The major emphasis of Phil's labs was glycosylation of proteins, and my stay made two major long-term impacts on my life: I decided to work on glycosylation in my future, independent scientific career, and I developed a lifelong friendship and professional relationship with Phil, which lasted up to our retirement.

Real job

St. Louis, MO

In 1973, there were few academic job openings. I started to worry whether Gene was recommending me for these positions and whether I would be able to obtain one of them. Fortunately, his secretary, Phyllis Elfman, who really ran Gene's lab, told me that Gene wasn't getting many letters looking for job applicants.

Eventually, there was an opening in the Biochemistry Department at St. Louis University School of Medicine, and when Bob Olson, the Chairman, asked Gene for a letter of recommendation, Gene called him by phone. This made an enormous difference, as it got me an interview. I later heard that for three job openings, they got 400 applications! I got the job, but before leaving MIT, I received a phone call from Salva Luria. My blood pressure immediately went up!

I had received money, for what amounted to approximately half an NIH grant, from the Jane Coffin Childs Memorial Fund for Medical Research to start up my independent laboratory. Salva wanted to know whether I would return the unspent money to the Fund if I received an NIH grant during the first

year. With this money and the startup money from St. Louis University, I was in good shape for some time, so I was happy to tell Salva “yes.” It’s easy to be generous if you have money!

At St. Louis University, I was fortunate enough to get two 3-year RO1 grants within a short period of time: one on glycosylation and one on sphingolipids. The former grant would last for over 30 years without interruption, while I let the latter expire after 6 years! I then started a second grant on sulfation and phosphorylation of sugars in secreted proteins such as casein, which lasted 20 years without interruption. My first grant on glycosylation was focused on the existence of cell-surface glycosyltransferases. Saul Roseman had postulated that cell-surface glycosyltransferases, in conjunction with extracellular nucleotide sugars, led to glycoconjugates being synthesized on cell surfaces, where they were enriched in the cell and thought to play important roles in cell–cell interactions. While at MIT, my lab bench had been behind Bart Sefton, who was also a postdoc and who knew a lot about cell biology and cells in tissue culture. Bart always mentioned to me that removing cells that had been grown in tissue culture from a monolayer for biochemical experiments most likely caused severe injury to these cells, if not death. If broken or dying cells were incubated with nucleotide sugar, it would be difficult, if not impossible, to do experiments with cultured cells, where one could ascribe glycosylation to the cell surface, and not to other internal organelles. Most, if not all, cell-surface glycosylation studies at the time had removed cells grown in monolayer with trypsin, EDTA, or even scraping. We determined that under all these conditions of cell removal followed by mock incubations, the great majority of cells had most likely died as they would not re-adhere to tissue culture plates and grow. The control consisted of removing cells from the monolayer with trypsin and resuspending in tissue culture medium with serum, in which case ~80% of cells adhered to the plates and grew. So, clearly one could not remove cells from monolayers for the experiments we wanted to do. When we incubated cells grown in monolayers with nucleotide sugars, in the presence of incubation buffers, we found that virtually all nucleotide sugars were degraded, and free radioactive sugar was found in the incubation medium. A small amount of radioactive sugar was covalently bound to cell surfaces, but a large amount of soluble radioactive sugar was within the cells. Thus, we could not rule out that free sugar, from nucleotide sugars, was entering cells, subsequently being converted to nucleotide sugars, and then acting as donors to glycans within cells in reactions catalyzed by glycosyltransferases within cells. C. P. Leblond and his group had recently obtained electron microscopic–autoradiographic evidence for this internal (in the Golgi apparatus) glycosylation.

The problem for us was that many of the leading laboratories in glycosylation were publishing papers in top journals on the existence of cell-surface glycosyltransferases. We were newcomers to the field! Fortunately for us, Ed Heath (then chair of the Biochemistry Department at the University of Iowa) came to the department to give a seminar, and when I showed him our results, he gave me two important pieces of advice. Ed had been a postdoc in Saul Roseman’s laboratory. He told me that if Saul isn’t working on this anymore, it means he is skeptical about the whole theory. In addition, he suggested we not say

that surface glycosyltransferases do not exist, but instead say the current evidence does not support the hypothesis. With this advice, we submitted a paper to *Biochemistry* (5). One reviewer thought it was fine, the second stated that so many papers could not be wrong and recommended rejection, and fortunately, the final referee recommended publication! With this I was able to renew my RO1 grant.

Soon after that, I unexpectedly got a phone call from Saul Roseman. I had seen Saul tear a speaker (who later became a National Academy of Sciences member) to shreds at a FASEB meeting in Atlantic City by asking him to summarize the theory of Arrhenius plots, which many, at the time, were using to study lipid membrane fluidity! The first thing Saul said to me over the phone was that he did not want to discuss cell integrity, but “your results contradict your own hypothesis,” he said. “How?” I asked, and he reiterated the finding that we had found some radiolabeled sugar covalently attached to cell surfaces, which he suggested supported his theory. When I replied that this was most likely due to the free sugars within cells, which had been converted to nucleotide sugars within cells, making them substrates for internal glycosylation, he told me to forget about the soluble radioactivity within cells. My answer was that I wanted to be quantitative and to do so we had to take this into account. Obviously, he was testing me and we later became good friends! According to my Chair, he even wrote me a positive letter of recommendation for my promotion to Associate Professor with tenure!

In the early 1970s, thanks to the pioneering studies by George Palade, Günter Blobel, and David Sabatini, it was established that membrane and secreted proteins were synthesized on membrane-bound polysomes, were then translocated into the lumen of the endoplasmic reticulum, and thereafter, migrate via vesicles to the Golgi apparatus. From there they go to the plasma membrane or are secreted. It is in the lumen of the endoplasmic reticulum where many proteins are initially glycosylated by sugars, covalently linked to the lipid dolichol. Through the work of C. P. Leblond’s group and others, it had also been shown that in the Golgi apparatus lumen, many additional sugars are added to these proteins, not by dolichol, but instead directly by nucleotide sugars (Fig. 3). The latter are synthesized in the cytosol and must, therefore, reach the lumen of the Golgi apparatus by what was then an unknown mechanism. We postulated that there were transporters for these nucleotide derivatives in the Golgi apparatus membrane.

Together with David J. Carey, we decided to test whether we could demonstrate *in vitro* transport of nucleotide sugars into Golgi vesicles from rat liver. Before such experiments could be done, we had to demonstrate that the vesicles we used were sealed and of the same *in vivo* membrane topography. Fortunately, we were able to prove both points. The first substrate we tested was CMP-sialic acid, and we compared the behavior of this nucleotide sugar with that of standard “penetrators” such as deoxyglucose, which enters the Golgi apparatus passively, and that of nonpenetrators such as inulin. We observed that radioactivity from CMP-³H]sialic acid entered the lumen of the vesicles at a much faster rate than deoxyglucose and free sialic acid. Additional results also further supported our hypothesis: CMP-sialic acid did not enter vesicles derived from

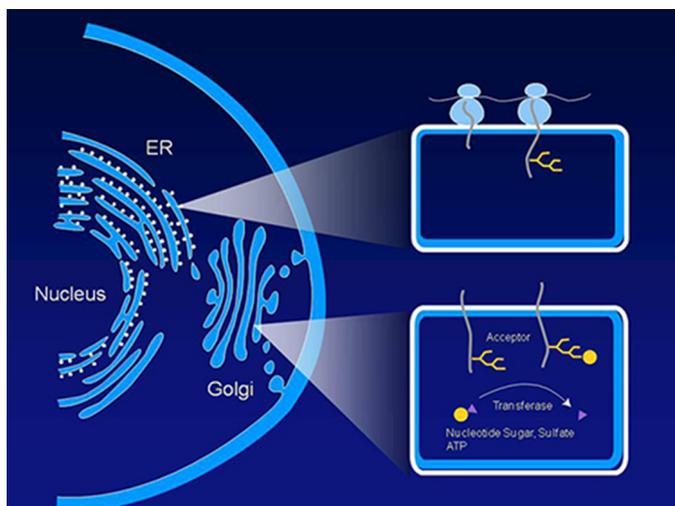


Figure 3. Schematic of a eukaryotic cell. Membrane and secreted proteins are made on membrane-bound polysomes and translocated into the lumen of the endoplasmic reticulum (ER). Many proteins undergo initial glycosylation in the lumen via dolichol sugars. Proteins then migrate via vesicles to the Golgi apparatus. In the lumen of the Golgi apparatus, additional posttranslational modifications occur catalyzed by different transferases and kinases. Donors are nucleotide sugars, ATP, and PAPS, which are synthesized outside the Golgi apparatus lumen and are transported via transporters shown in Fig. 4.

the endoplasmic reticulum, transport was saturable with a K_m in the 1–2 μM range, and when we mixed commercially available CMP-[^3H]sialic acid with [^{14}C]CMP-sialic acid, synthesized in the lab, the entire molecule appeared to enter as a unit. We submitted this evidence to *Cell* (6), where it was accepted.

We were still worried about adsorption of nucleotide sugars to vesicles, rather than transport into vesicles, although the evidence argued strongly against adsorption. While no reviewer of subsequent papers brought this up, it eventually came up during a seminar I gave at Merck. During the seminar, Chris Raetz (who was at Merck at that time), my good friend from Gene Kennedy's lab, promptly raised the question of adsorption. By then, Juan Capasso, in our lab, in collaboration with Tom Keenan, had demonstrated transport of ATP into the Golgi apparatus, of rat mammary glands and showed that although 4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS) (a stilbene derivative and anion transport inhibitor) inhibited transport of ATP, it did not inhibit transfer of phosphate from ATP onto endogenous luminal Golgi acceptors such as casein. On the other hand, EDTA, which inhibited transfer of phosphate from ATP to casein and did not enter Golgi vesicles, did not inhibit transport of ATP onto the lumen of Golgi vesicles and subsequent transfer to casein. This was unequivocal proof that transport of ATP into the Golgi lumen was followed by transfer of phosphate to luminal casein, and by extrapolation, that transfer of nucleotide sugars was followed by transfer of sugars to endogenous protein and lipid acceptors (7). Luigi Puglielli, in my lab, later purified this transporter to homogeneity. Subsequently Caroline Clairmont, also in my lab, demonstrated transport of ATP into the endoplasmic reticulum and Eduardo Guillen partially purified this transporter. Transport of nucleotide sugars such UDP-glucose and UDP-GlcNAc into the endoplasmic reticulum of rats was shown by Mary Perez (8).

In earlier *in vitro* nucleotide sugar transport studies, we had measured the internal volume within vesicles and concluded that nucleotide sugars were concentrated within the lumen of the vesicles 10–30-fold, compared with their concentration in the reaction medium. If this was correct, then energy would be required, but we knew that the assay did not require ATP or another energy source in the reaction medium. The fact that several ionophores had no effect on transport led us to hypothesize that an antiport mechanism was responsible for the uphill transport of nucleotide sugars into the Golgi lumen. Together with Juan Capasso (9), we showed that incubating vesicles with [^3H]GDP-fucose resulted in tritiated GMP accumulation in the lumen. If we followed the above incubation with GDP-[^{14}C]fucose, we saw an exit of tritiated GMP from the lumen and covalent attachment of ^{14}C -radiolabeled fucose within the Golgi apparatus lumen. This suggested that entry of nucleotide sugar into the Golgi lumen, against a concentration gradient, was coupled to the exit of the corresponding nucleoside monophosphate. As a control, we used GDP-mannose, which did not enter the lumen of Golgi vesicles, and determined that this exit did not occur without entry of a structurally similar nucleotide sugar. Previous studies had shown that the lumen of the endoplasmic reticulum and Golgi apparatus contained nucleoside phosphatases of unknown function. Now, with our studies, it became clear what they were for.

Could one obtain genetic evidence to support the existence of Golgi nucleotide sugar transporters? Pamela Stanley, at the Albert Einstein College of Medicine, and independently, Eve Barak in Stu Kornfeld's lab (at Washington University in St. Louis), had characterized two Chinese hamster ovary cell lines that were thought to be defective in the above transport system. Both cell lines were defective in glycosylation: one lacked sialic acid, and the other lacked galactose and sialic acid. Neither cell line had a defect in glycosyltransferases, nor in nucleotide sugar biosynthesis. Sue Deutscher, in my lab, grew liters of these mutant and WT cells in tissue culture and isolated a crude Golgi fraction with which she was able to measure transport of CMP-sialic acid (10) and UDP-galactose (11). Both cell lines were defective in transport of the above two substrates, and importantly, the latter was *not* defective in transport of other uridine nucleotide sugars, suggesting that there were individual transporters for the different uridine-containing nucleotide sugars and not a common one (9). As will be shown later, we were very fortunate because this story became much more complex in other biological systems.

We also knew that if we were going to eventually purify these transporter proteins, we had to develop a reconstitution assay with liposomes, which would enable us to measure transport *in vitro*. While in my lab, Marcos Milla (12) developed such a system and was able to show that the reconstituted system with Golgi membrane proteins retained the transport characteristics of the original Golgi vesicle system. Using this assay, Elisabet Mandon and Luigi Puglielli, in a collaborative work with Anant Menon's group, later successfully purified several rat liver Golgi nucleotide sugar transporters to homogeneity. Because such transporters are in such low abundance, final purity had to be confirmed by radioiodination of the proteins prior to SDS gel electrophoresis (13, 14).

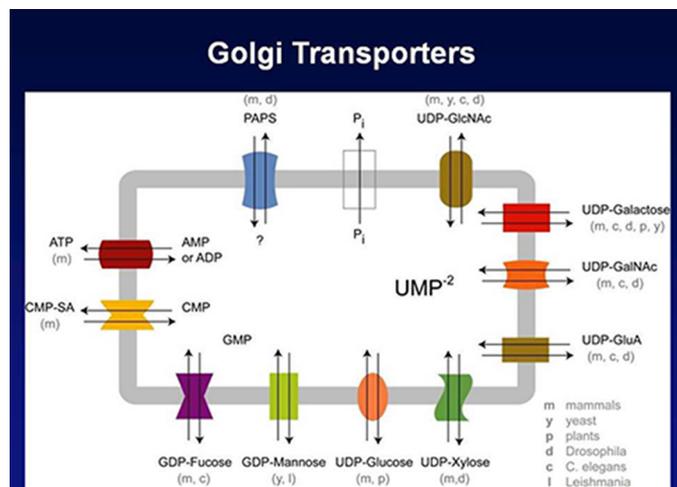


Figure 4. Transport activities of nucleotide sugars, ATP, and PAPS in the Golgi apparatus membrane of different organisms. SA, sialic acid.

Our lab also spent considerable effort in the field of proteoglycans. These are glycosylated proteins with covalently linked amino sugars, such as uronic or iduronic acid. I never completely understood why this was a different field from glycoproteins and glycolipids. Yes, the functions of proteoglycans are very different from glycoproteins, but then again they share major biosynthetic pathways. Through the work of Enrique Brandan, Ariel Orellana, and Yasuhiro Hashimoto in my lab, the pivotal enzyme heparan sulfate *N*-deacetylase *N*-sulfotransferase was purified, cloned, and functionally expressed (15, 16) in an era when this was very challenging for intrinsic membrane proteins. Later on, in collaboration with Stu Swiedler's group, we showed that the enzyme had the above dual activity, that it functioned as a monomer in the Golgi membrane, and that its active site was on the C terminus. Leny Toma demonstrated that transport of nucleotide sugars regulates the biosynthesis of proteoglycans (17).

The sulfate donor of proteoglycans is PAPS,² which had been discovered by Phil Robbins while he was a postdoc in Fritz Lipmann's laboratory. I remember after a talk I gave at a Gordon Conference, Phil suggested I look into transport of the above nucleotide into the Golgi apparatus. I had not heard of PAPS before, but we determined that transport of such a molecule into the Golgi lumen occurred, and later Elisabet Mandon purified this transporter (8).

Transport of other nucleotide sugars that are building blocks for proteoglycans such as UDP-GlcA, UDP-xylose, and UDP-glucose into the Golgi apparatus was demonstrated by Mary Perez, Elisabet Mandon, and Naziha Nuwayhid, the latter in collaboration with Ed Conrad's group (8). A summary of nucleotide sugar transporters in different organisms is shown in Fig. 4.

Worcester, MA

In 1987, I moved my lab to the University of Massachusetts Medical School in Worcester, a city 40 miles west of Boston. Claudia Abeijon, who had been a graduate student of mine and

stayed on as a postdoc, went to Phil Robbins' lab at MIT to learn yeast genetics, a subject my lab was not familiar with, but Phil's was. The addition of yeast as a model system proved to have major advantages for us. With its "awesome" power of genetics, it allowed us to unequivocally prove the above described antiport system. As will be discussed below, we were also able to clone the first nucleotide sugar transporter and develop a simple assay for putative transporters in non-yeast model systems.

Shortly after arriving at UMass, one of my NIH grants was awarded MERIT status. What a wonderful feeling! 10 years of not having to go through study section! While this made me more than happy, I found out that when I moved to UMass, their fringe benefits rate, charged to NIH grants, were much higher than those in St. Louis. The new NIH grant budget on my MERIT award did not reflect this increase in fringe benefits. Even with the help of my NIGMS program director, Lee van Lenten, I could not get the substantial extra money for the new grant because it was considered a supplement. When I received the MERIT award, Lee suggested I write to then NIGMS director Ruth Kirschstein, who I had met earlier in my career. How do I get Kirschstein's attention knowing that she was busy and received many letters every day? I started my letter by stating that I was both honored and disappointed to receive my MERIT award and then explained my situation regarding the fringe benefits! Voila! In a few days, I received the adjustment of my grant. The lesson, which I shared from then on with my students and postdocs, was that if the first three lines in a petition letter do not have an impact on the receiver, you have lost! The same applied in certain sections of grant writing, and it never failed!

We had previously obtained *in vitro* evidence that transport of nucleotide sugars into the Golgi apparatus lumen was energetically uphill, and had postulated that the entry was coupled with exit from the lumen into the cytosol, down a concentration gradient of the corresponding nucleoside monophosphate (7). This compound, in turn, would arise from Golgi luminal nucleoside diphosphatases acting upon nucleoside diphosphates, which are the reaction products following transfer of sugars from nucleotide sugars to endogenous acceptors in reactions catalyzed by glycosyltransferases. Because in *Saccharomyces cerevisiae* the major sugar donor is GDP-mannose, we postulated the existence of a Golgi luminal GDPase. In a collaborative study with Phil Robbins' group, we characterized, purified, and cloned a *S. cerevisiae* Golgi luminal GDPase and showed that deletion of this gene resulted in shorter mannan chains on Golgi proteins and lipids (8). This provided unequivocal evidence for the antiporter system.

I remember the day Claudia Abeijon showed me a paper from Clinton Ballou's lab in which they described a mutant of *Kluyveromyces lactis*, which lacked terminal GlcNAc in its mannan chains, but was not defective in its *N*-acetylglucosaminyltransferase, nor in synthesis of UDP-GlcNAc. The authors postulated a possible compartmentalization defect. Claudia went on to show that indeed the mutation caused a defect in transport of UDP-GlcNAc into the Golgi lumen of *K. lactis*. She then was successful in cloning the gene that corrected the mutant phenotype. I distinctly remember the day she came and showed me the putative protein profile of the gene product. It

² The abbreviations used are: PAPS, 3'-phosphoadenylyl-5'-phosphosulfate; LAD II, leukocyte adhesion deficiency II; GlcA, glucuronic acid.

was a polytopic protein that traverses the membrane many times (18). We were elated! As usually happens with important discoveries, a short time later Rita Gerardy-Schahn's lab was successful in cloning the mammalian CMP-sialic acid transporter, and thereafter, several groups cloned other nucleotide sugar transporters.

Eduardo Guillen, in my lab, undertook an ambitious project to determine whether one could correct the *K. lactis* mutation with the corresponding mammalian gene. There were many potential pitfalls with this project, including the poor quality of the mammalian cDNA library, lack of mammalian transporter gene expression in yeast, improper protein targeting to the Golgi membrane, and/or inability to correct cell-surface expression of GlcNAc in *K. lactis*. Fortunately, *all* of this worked, and we had not only cloned the corresponding mammalian transporter gene in a heterologous system, but it made us optimistic that this approach would work in other heterologous systems (19). This would prove invaluable in our next major enterprise in which we studied the role of nucleotide sugar transporters in multicellular systems such as *Caenorhabditis elegans*.

Boston, MA

In 1998, I became Professor and Founding Chair of the Department of Molecular and Cell Biology at the Henry M. Goldman School of Dental Medicine of Boston University. Spencer Frankl, the Dean, wanted to diversify the NIH research portfolio of the School. I accepted the offer with two caveats: that I could hire diverse faculty in basic sciences and that Phil Robbins would take early retirement from MIT and join me in establishing this new department. We were fortunate in hiring several outstanding faculty members who are world experts in their respective fields: Miklos Sahin-Toth in pancreatitis, John Samuelson in protozoan parasites, and David Levin in stress signaling and cell wall biogenesis in fungi. We also started two seminar series: the first invited outside experts in diverse areas of biology, and the second invited scientists and research clinicians from Boston University. While our aim with the latter series was to become acquainted with ongoing research at Boston University, we also became aware that this gave an opportunity for the faculty of Boston University to get to know our department. Thus, we invited faculty from the Dental and Medical Schools, the College of Engineering, and the College of Arts and Sciences. These seminars became the department's window to the university and a chance for the university faculty to get to know us. Our faculty also became participants in the Bioinformatics program, administered by the College of Engineering, and developed a course in molecular genetics for first-year dental students.

My lab's expansion into *C. elegans* as a model system to study nucleotide sugar transporters was fortuitous and proved to be a gold mine. Tory Herman in Bob Horvitz's (Nobel Prize in Physiology and Medicine in 2002) lab at MIT had found eight complementation groups that corrected the squashed vulva phenotype of *C. elegans* mutants she had generated. Mutant SQV-7 appeared to encode a nucleotide sugar transporter. The literature was full of putative transporters for these substrates, yet they had no function that had been established experimentally!

We had previously found that the primary amino acid sequence of these transporter proteins could *not* predict their substrate specificity and that two transporters for UDP-GlcNAc, one from *K. lactis* and one from canines, had very different amino acid sequences, and yet, had the same substrate specificity. This was true even when the latter was expressed in a heterologous system such as *K. lactis*, where we had shown it complemented the *K. lactis* mutant phenotype!

The only way to establish the function of a putative nucleotide sugar transporter was to directly measure transport! Patricia Berninsone, in my lab, first attempted to express SQV-7 in *S. cerevisiae* without success. We wondered whether any person had ever attempted this (a *C. elegans* gene in yeast). Fortunately, it proved to be a technical problem, and she eventually succeeded in expression. She then isolated Golgi vesicles from the transformed yeast and assayed every radiolabeled nucleotide sugar we could get our hands on for transport. As stated previously, *S. cerevisiae* has only two endogenous transporters, those for GDP-mannose and UDP-glucose. Initially, after transformation, we were shocked to find transport signals for UDP-Gal, UDP-GlcNAc, and UDP-GlcA! We had never seen a transporter for more than one substrate, and this specificity had been one of our landmark biochemical signals, which had given us confidence in the reliability of the *in vitro* transport assay! Fortunately, SQV-7 did not appear to transport UDP-GalNAc or other nucleotide sugars, again suggesting substrate specificity, including carbohydrate anomers. The two mutants isolated by Tory Herman were defective in transport of the three above substrates (UDP-Gal, UDP-GlcNAc, and UDP-GlcA) to different extents, but transport of the other nucleotide sugars appeared to be normal. This reassured us a bit. If the transporter could transport three substrates, then the simplest model would be for transport among substrates to be competitive. Fortunately, this turned out to be the case (20).

Our studies of nucleotide sugar transporters in *C. elegans* also led to the discoveries of two additional important novel concepts. In one case, Carolina Caffaro and Patricia Berninsone, determined that a transporter for UDP-GlcNAc and UDP-Gal transported both substrates in a simultaneous and independent manner. Furthermore, Carolina constructed deletion mutants and found that they transported one substrate, but not the other, strongly suggesting that the transporter protein has two different substrate-binding sites. This was a distinct transport model from that found in studies with the SQV-7 mutants, where transport of substrates was competitive (21).

We also found, in *C. elegans*, that individual nucleotide sugar transporters were tissue-specific. This represented an additional and important new concept regarding the genetic redundancy of nucleotide sugar transporters and tissue development. This discovery was made by Carolina Caffaro and Patricia Berninsone. A *C. elegans* double mutant in two nucleotide sugar transporters, both of which shared one substrate and partial overlapping tissue specificity, showed a different phenotype than each individual mutant (22). Important studies on the structure of glycoconjugates in *C. elegans* were done by John Cipollo, in collaboration with Cathy Costello's group (23). In retrospect, we had been extremely fortunate in our earlier biochemical studies on transporters by initially finding that trans-

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porters were highly substrate-specific, something that the biochemical community found easy to accept.

With the characterization of nucleotide sugar transporter mutants in yeast and Chinese hamster ovary cells, we began to think early on about the possibility of human genetic diseases involving nucleotide sugar transporters. However, we also thought that such diseases might be very pleiotropic, as many glycoproteins might be affected, and we also suspected the diseases to have a high likelihood of being lethal. Thus, we never gave too much thought to finding such diseases, even if they were nonlethal.

Major credit for discovering such diseases goes to Amos Etzioni and colleagues at the Medical Center in Haifa, Israel. In 1992, two unrelated Arab boys living in Israel, then 3 and 5 years old, each the offspring of a consanguineous mating, came to Etzioni's clinic. Both showed severe psychomotor and mental retardation together with a flat face and depressed nasal bridge. They also presented recurrent infections with marked neutrophilia and periodontitis. The children had a rare blood phenotype called Bombay, wherein the red blood cells express a non-fucosylated variant of the H antigen. Also missing from the red blood cells were Lewis blood group antigens such as sialyl Lewis X, an important ligand for selectins on leukocyte surfaces. Fucosyltransferases, catalyzing the addition of fucose via different linkages to other sugars, were normal in the two boys, as was the biosynthesis of GDP-fucose, the universal fucose donor to macromolecules. The disease was named leukocyte adhesion deficiency II (LAD II). LAD II was distinct from LAD I, which arises from mutations in the gene encoding the integrin $\beta 2$ subunit. In addition, the infections in LAD I are more serious, but patients do not suffer other symptoms, including mental or growth retardation.

Etzioni, in collaboration with Michaela Tonetti's group at the University of Genoa, Italy, immortalized lymphoblasts from one of the above children, as well as those from both parents and a control. Laura Sturla, from Tonetti's lab, came to our lab, and together, we isolated Golgi vesicles from the child's immortalized lymphoblasts, the mother, the father, and a control. We then performed transport assays using GDP-fucose. We found that the child had approximately one-third of the V_{\max} of that of the parents and normal control. Transport of other nucleotide sugars was normal in all samples (24).

Later, von Figura's group cloned the GDP-fucose transporter gene and established that the above child had a point mutation in the coding region. When Carolina Caffaro expressed the mutated and WT protein transporters and reconstituted them into liposomes, she found that the mutation caused a partial loss of GDP-fucose transport. At the same time, Thorsten Marquardt at the University of Muenster had also identified another child, of Turkish origin, with a defect in GDP-fucose transport. Since then, other examples of defects in nucleotide sugar transport in children have also been described.

Meeting two of the above named children proved to be a memorable occasion in my scientific life career as a basic research scientist. At a meeting in Germany, I met the Turkish child and the above named Arab-Israeli child at his home in Israel. At that time, the latter was about 17 years old. While mentally challenged, he went to a specialized school and could



Figure 5. Photo of the author, 2013 ASBMB archives.

understand the love of his family and several siblings, who fortunately were all normal. Although the mutation was recessive, each sibling had a 25% chance of having the disease. Before meeting these two children, I talked to one of our human geneticists at Boston Medical Center. He gave me two pieces of advice: do not mention prenatal diagnosis unless asked, and do not mention gene therapy because for these children, it is unrealistic. I will never forget meeting these children and their loving parents as it brought our basic science into "real world."

Some of the students and postdocs in the lab had prior experience with protozoan parasites. We thought that studying nucleotide sugar transporter in these organisms might give us novel biological and biochemical insights. Studies that showed the occurrence of nucleotide sugar transporters in protozoan parasites established additional new concepts. In collaboration with John Samuelson's group, Luis Bredeston of my lab found endoplasmic reticulum and Golgi apparatus functions in these organisms (25), and in collaboration with Jay Bangs and Barbara Burleigh's groups, Li Liu and Yu Xin Xu of my lab described a nucleotide sugar transporter that transports both purine and pyrimidine substrates in *Trypanosoma brucei*, something we had never seen in any organism before (26). Together with John Boothroyd's group, Carolina Caffaro showed that a deletion of a transporter gene in *Toxoplasma gondii* contributed to changes in infectivity (27).

In 2013, I received the Rosalind Kornfeld Lifetime Achievement Award from the Society for Glycobiology (Fig. 5). This was a particular honor for me because Rosalind had been the vice chair of the 1989 Gordon Research Conference on Glycoproteins and Glycolipids, which I had chaired. Rosalind was not only an outstanding scientist, but she was an incredibly kind

and caring human being. She, together with Stuart Kornfeld and Jacques Baenziger, had been instrumental in helping me arrange the scientific program for that conference.

One year later, I decided to close the lab, retire, and move with my wife Lois to Southern California. I still travel, once a year, for 2 weeks, to the Universidad Andres Bello in Santiago, Chile, where I am a professor. While there, I teach a rigorous seminar course for Ph. D. students on critical thinking. It deals with classical experiments in biochemistry, based on the *Journal of Biological Chemistry* Classics series, that led to key concepts such as DNA replication, discoveries of cAMP, growth factors, tRNA, phospholipid pathways, and feedback inhibition, among others (28). I tell the students that if they learn to think in science as these great scientists did, then they are assured to become first-rate scientists. I also help friends and friends of friends with their grant proposals.

In looking back, I feel most fortunate to have had very talented graduate students and postdocs who enabled me to have a wonderful scientific career. Fortunately, most of them are still in science, and many have made very important contributions to their own fields of study.

Unfulfilled dreams

We would have liked to do some structural studies on nucleotide sugar transporters. Unfortunately, we never succeeded in obtaining sufficient amounts of protein for such studies. While I was working on this article, however, an excellent paper was recently published in *Nature* by Parker and Newstead (29) in which they obtained the first crystal structure of a Golgi nucleotide sugar transporter from *S. cerevisiae* that transports GDP-mannose. This outstanding study also includes biochemical studies confirming the antiporter hypothesis and is hopefully only the first of many structural studies of these transporters.

Important and interesting structural studies on these proteins should include those of multisubstrate transporters, including those with multiple competitive substrates and those of independent and simultaneous substrates.

Cloning and deletion studies of the ATP transporters of the Golgi apparatus and endoplasmic reticulum, which from reconstitution studies must have structural differences, should yield important results of additional functions of these proteins.

There should be diseases affecting the luminal Golgi and endoplasmic reticulum nucleoside diphosphatases, although their phenotypes will most likely be pleiotropic, such as those of nucleotide sugar transporters.

Obviously there are many important and exciting projects to be done! I wish I had more time, but I am sure younger scientists will explore them.

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