Biochimica et Biophysica Acta, 777 (1984) 133–139
Elsevier

BBA 72268

EFFECT OF NUCLEOTIDES ON TRANSLOCATION OF SUGAR NUCLEOTIDES AND ADENOSINE 3′-PHOSPHATE 5′-PHOSPHOSULFATE INTO GOLGI APPARATUS VESICLES

JUAN M. CAPASSO * and CARLOS B. HIRSCHBERG **

E.A. Doisy Department of Biochemistry, Saint Louis University School of Medicine, Saint Louis, MO 63104 (U.S.A.)

(Received April 6th, 1984)

Key words: Sugar nucleotide; Adenosine 3′-phosphate 5′-phosphosulfate; Nucleotide translocation; (Golgi apparatus)

Recent studies from this laboratory have suggested that rat-liver Golgi apparatus derived membranes contain different proteins which can translocate in vitro CMP-*N*-acetylneuraminic acid, GDP-fucose and adenosine 3′-phosphate 5′-phosphosulfate from an external compartment into a lumenal one. The aim of this study was to define the role of the nucleotide, sugar and sulfate moieties of sugar nucleotides and adenosine 3′-phosphate 5′-phosphosulfate in translocation of these latter compounds across Golgi vesicle membranes. Indirect evidence was obtained suggesting that the nucleotide (but not sugar or sulfate) is a necessary recognition feature for binding to the Golgi membrane (measured as inhibition of translocation) but is not sufficient for overall translocation; this latter event also depends on the type of sugar. Important recognition features for inhibition of translocation of the above sugar nucleotides and adenosine 3′-phosphate 5′-phosphosulfate were found to be the type of nucleotide base (purine or pyrimidine) and the position of the phosphate group in the ribose. Thus, UMP and CMP were found to be competitive inhibitors of translocation of CMP-*N*-acetylneuraminic acid, while AMP did not inhibit. Structural features of the nucleotides which were less important in inhibition of translocation (and thus presumably in binding) of the above sugar nucleotides and adenosine 3′-phosphate 5′-phosphosulfate were the number of phosphate groups in the nucleotide (CDP and CMP inhibited to a similar extent), the presence of ribose or deoxyribose in the nucleotide, a replacement of hydrogen in positions 5 of pyrimidines or 8 in purines by halogens or an azido group. The sugar or sulfate did not inhibit translocation of the above sugar nucleotides and adenosine 3′-phosphate 5′-phosphosulfate into Golgi vesicles and therefore appear not to be involved in their binding to the Golgi membrane.

Introduction

Recent work from this and other laboratories has shown that rat-liver Golgi-derived vesicles translocate in vitro CMP-NeuAc [1,2], GDP-fucose [1] and adenosine 3′-phosphate 5′-phosphosulfate [3] from an external compartment into a lumenal one. Translocation of these sugar nucleotides and adenosine 3′-phosphate 5′-phosphosulfate was (a) saturable at high concentrations (b) temperature-dependent and (c) inhibited by treatment of the Golgi-derived vesicles with proteinases under conditions where lumenal Golgi marker enzymes were not inactivated. This behavior was consistent with the existence of a translocation protein in the Golgi apparatus membrane, portions of which faced the cytoplasmic side of the Golgi membrane [1,3]. Since the above sugar nucleotides and

* On leave from the Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina.
** To whom correspondence should be addressed.

adenosine 3′-phosphate 5′-phosphosulfate did not inhibit translocation of each other [1,3], it was hypothesized that there were different translocators in the Golgi apparatus membrane. Indirect evidence for translocation of UDP-galactose into Golgi vesicles from mammary gland and liver has also been obtained [4,5].

The aim of the present study was to examine the structural requirements of the sugar nucleotides and adenosine 3′-phosphate 5′-phosphosulfate for the different putative protein translocators. For this purpose, the role of different nucleotides on translocation into Golgi vesicles of CMP-NeuAc, GDP-fucose and adenosine 3′-phosphate 5′-phosphosulfate was determined. It was also hoped that these studies would be of value in allowing one to choose appropriate nucleotide derivatives for use as affinity probes in the isolation and characterization of putative Golgi translocators of sugar nucleotides and adenosine 3′-phosphate 5′-phosphosulfate.

Materials and Methods

Radioactive and nonradioactive substrates. The following radioactive compounds were used: CMP-[9-^{3}H]NeuAc (18.9 Ci/mmol), New England Nuclear; GDP-L-[1^{14}C]fucose (18 Ci/mol), Amersham; GDP-L-[1-^{14}C]fucose (192 Ci/mol) New England Nuclear; adenosine 3′-phosphate 5′-phospho[^{35}S]sulfate (1.9 Ci/mmol) New England Nuclear. CMP-NeuAc and adenosine 3′-phosphate 5′-phosphosulfate were from Sigma (St. Louis, MO). GDP-fucose was a generous gift of Drs. M. Hayes and R. Barker, Cornell University, (Ithaca, NY).

Isolation, integrity and topography of Golgi vesicles. Golgi vesicles were isolated from rat liver according to Leelavathi et al. [6]. Vesicles were enriched 36-fold in sialyltransferase activity (22% yield of total homogenate activity). 92% of the vesicles were sealed and of the same orientation as in vivo, as determined by latency of neuraminidase-catalyzed removal of radiolabeled sialic acid from vesicles prelabeled with radioactive CMP-NeuAc [1,3].

Translocation assays. The theoretical basis for the assays of translocation of the different nucleotide derivatives into Golgi vesicles has been previously described [1,3]. Briefly, it consists of (1) determining the total radioactive solutes associated with the Golgi pellet, following incubation with radioactive substrates and centrifugation of the Golgi vesicles (see below) and (2) subtracting from this amount the total radioactive solutes outside (between) the vesicles in the Golgi pellet. This latter value is the product of multiplying the volume outside the vesicles in the Golgi pellet, as determined by [^{3}H]methoxyinulin which does not penetrate the Golgi vesicles, and the concentration of radioactive solutes in the incubation medium.

To measure translocation of CMP-[^{3}H]NeuAc, (at 2.0 μM final concentration), the sugar nucleotide (120 Ci/mol) was dried under a stream of nitrogen and dissolved in 0.9 ml buffer 1 (0.25 M sucrose/10 mM Tris-HCl/1 mM $MgCl_2$, pH 7.5). To this solution, 0.1 ml of a Golgi vesicle suspension (4–6 mg protein/ml) in the same buffer was added. Following incubation for 10 min at 25°C, the mixture was centrifuged at 100 000 × *g* for 30 min at 4°C. The supernatant solution was removed and an aliquot was taken to calculate the concentration of radioactive solutes in the incubation medium [1,3]. The surface of the pellet (P_1) was washed three times, each with 1.5 ml ice-cold buffer 2 (10 mM Tris-HCl/150 mM KCl/1 mM $MgCl_2$, pH 7.5). Water (0.5 ml) was added to the pellet and the mixture was frozen. The pellet was then sonicated for 30 min at 4°C in a Heat Systems Ultrasonic sonicator. The suspension was then transferred with a Pasteur pipet to a 1.5 ml conical centrifuge tube. Perchloric acid (8%, 0.5 ml) was added and the mixture was centrifuged a 11 000 × *g* for 5 min at room temperature. A 0.5-ml aliquot of the supernatant solution was taken to determine the total amount of solutes in the pellet. For the determination of acid-insoluble radioactivity in the pellet, the surface of the pellet (P_1) was washed three times, each with 1.5 ml 4% perchloric acid. Following sonication in a bath sonicator for 1 h at 4°C in 1 ml 4% perchloric acid, the samples were centrifuged for 5 min at 11 000 × *g* at room temperature. The supernatant solution was removed and the surface of the pellet was washed once with 1.5 ml water. 1 ml 1 M NaOH was added to dissolve the pellet. Following neutralization, the samples were counted in Aquasol-2 (New England Nuclear). GDP-[^{14}C]fucose (100 Ci/mol)

translocation (at 2.0 μM final concentration) was assayed as described for CMP-NeuAc, except that the initial incubation mixture comprised 50 mM Tris-HCl/0.5 mM 2,3-dimercaptopropanol/10 mM NaF. The amount of solutes within the vesicles was determined exactly as previously described using [^{3}H]methoxyinulin as standard nonpenetrator [1]. Translocation of adenosine 3′-phosphate 5′-phosphosulfate was determined exactly as described [3].

Results

Previous studies had shown that translocation of CMP-NeuAc, GDP-fucose and adenosine 3′-phosphate 5′-phosphosulfate into Golgi vesicles was inhibited by the corresponding nucleotide [1,3]. The sugar and sulfate of the previous compounds did not inhibit translocation, suggesting that the putative translocation protein(s) recognized solely the nucleotide for initial binding to the Golgi membrane. It was important to determine which structural features of the nucleotide of CMP-NeuAc, GDP-fucose and adenosine 3′-phosphate 5′-phosphosulfate were required for the initial binding to the Golgi membrane. For this purpose, a series of studies were performed in which the effect of the following modifications of the nucleotide on the rate of translocation into Golgi vesicles of the above sugar nucleotides and adenosine 3′-phosphate 5′-phosphosulfate was determined: (a) type of nucleotide base (purine and pyrimidine), (b) the site and number of phosphates in the nucleotide, (c) the presence or absence of hydroxyl groups in the ribose moiety of nucleotides and (d) the substitution of hydrogen by halogens or nitrogen in the bases.

The effect of different nucleotides on the translocation of CMP-NeuAc into Golgi vesicles is shown in Table I. 5′-CMP and several of its derivatives were very effective inhibitors of CMP-NeuAc translocation. Inhibition by 5′-CMP was competitive (Fig. 1) with K_i of 4.5 μM (at 10–25 μM 5′-CMP). The presence and the position of the monophosphate group in the ribose appeared to be of major importance for inhibitory activity, since 3′-CMP, 2′-CMP, cytidine, deoxycytidine and 3′,5′-cCMP, when present in a 10-fold excess over CMP-NeuAc, inhibited translocation of the sugar nucleotide by less than 5% (Table I). 5′-dCMP was an effective inhibitor of translocation (Table I), suggesting that substitution in the sugar moiety of a hydroxyl group with hydrogen is not an important recognition feature for binding of the sugar nucleotide to the Golgi membrane. Substitution of hydrogen with halogens in position 5 of the pyrimidine ring increased the inhibitory activity of the 5′-pyrimidine monophosphates (Table I). Inhibition of CMP-NeuAc translocation by 5-Br-dCMP was competitive (Fig. 2) with a K_i of 1.7 μM (at 2–10 μM 5-Br-dCMP). Other pyrimidine 5′-monophosphates such as UMP were also effective inhibitors of CMP-NeuAc translocation, but at higher concentrations than the corresponding

TABLE I

EFFECT OF NUCLEOTIDES AND NUCLEOTIDE-SUGARS ON CMP-NeuAc TRANSLOCATION INTO GOLGI VESICLES

Translocation of CMP-NeuAc (at 2 μM) was measured as described in Materials and Methods. Translocation of controls was 141 pmol/mg protein per 10 min. Results are average of two separate determinations.

Addition	Inhibition of translocation (% of control)			
	Concn. added (μM): 2	10	20	210
5′-CTP				55
5′-CDP				83
5′-CMP	17		53	93
3′-CMP			< 5	
2′-CMP			< 5	
3′,5′-cCMP			< 5	
dCMP		56	63	
Cytidine			< 5	
dCytidine			< 5	
5-I-CMP	19		89	
5-I-dCMP	50	88		
5-Br-CMP		50		
5-Br-dCMP	37	68		
5′-UTP				56
5-Br-dUTP		36		
5′-UDP		10	24	66
5′-UMP		16	21	93
dUMP			47	
5-Br-dUMP	10	31		
5-TMP			32	
UDP-galactose				61
UDP-*N*-acetyl-glucosamine				41

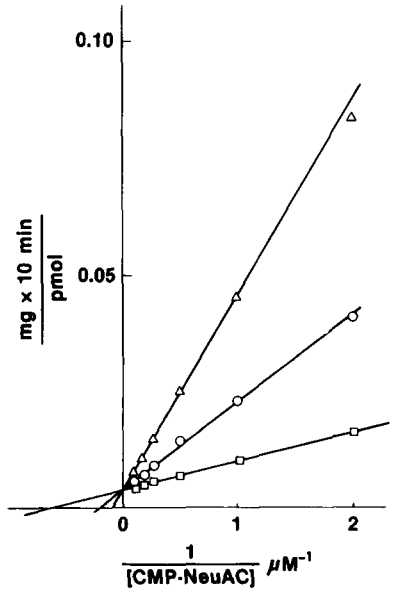

Fig. 1. Inhibition of CMP-NeuAc translocation into Golgi vesicles by CMP. Translocation was measured as described in Materials and Methods and was 97 pmol/mg protein per 10 min at 1 μM CMP-NeuAc. Results are average of two separate determinations. CMP concentration (μM): □, none; ○, 10; △, 25.

cytidine derivatives (Table I). The inhibition of 5′-UMP was competitive, although not simple (Fig. 3) as in the previously shown cases (Figs. 1 and 2). The K_i was 18 μM at 25 μM UMP. Purine nucleotides and derivatives such as 5′-ATP, 3′-ADP, 2′-ADP, 5′-GTP, 5′-GDP, 5′-GMP, GDP-mannose and adenosine 3′-phosphate 5′-phosphosulfate, all when present in a 100-fold excess over CMP-NeuAc, inhibited translocation of the sugar nucleotide by less than 25% (not shown); 5′-ADP and 5′-AMP inhibited translocation under similar conditions by 41 and 55%, respectively (not shown).

The effect of nucleotides on translocation of GDP-fucose is shown in Table II. Several guanosine- and adenosine-containing nucleotide derivatives, when present in a 5-fold excess (10 μM), were effective inhibitors of GDP-fucose translocation (Table II). The most effective inhibitors, when present in a 2-fold excess over GDP-fucose were 8-Br-GMP, 8-azido-GTP and GDP (Table II). Substitution of a hydrogen in position 8 of the purine ring with bromine appeared to increase the inhibition of translocation of GDP-fucose by

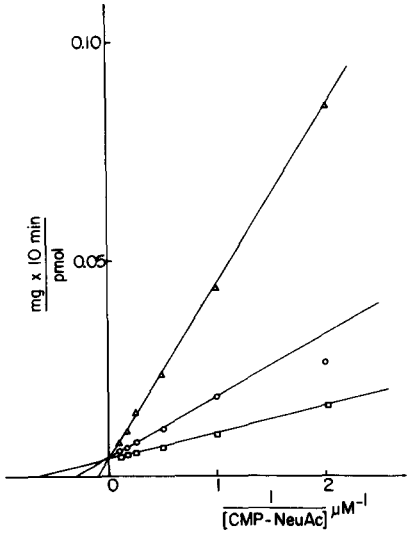

Fig. 2. Inhibition of CMP-NeuAc translocation into Golgi vesicles by 5-Br-dCMP. Translocation was measured as described in Materials and Methods and was 97 pmol/mg protein per 10 min at 1 μM CMP-NeuAc. Results are average of two separate determinations. 5-Br-dCMP concentration (μM): □, none; ○, 2; △, 10.

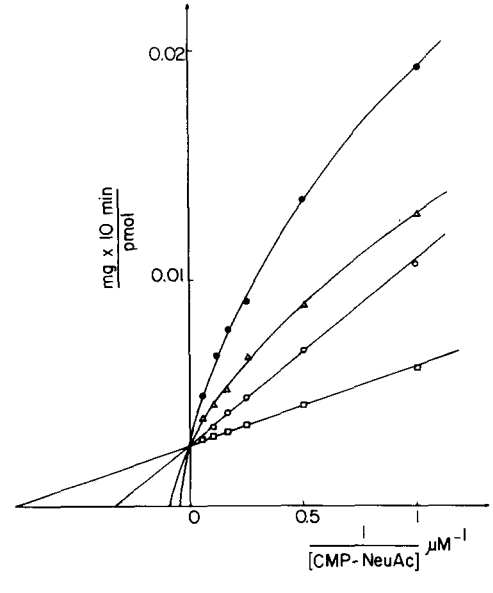

Fig. 3. Inhibition of CMP-NeuAc translocation into Golgi vesicles by UMP. Translocation was measured as described in Materials and Methods and was 138 pmol/mg protein per 10 min at 1 μM CMP-NeuAc. Results are average of two separate determinations. UMP concentration (μM): □, none; ○, 25; △, 50; ●, 100.

TABLE II

EFFECT OF NUCLEOTIDES ON GDP-FUCOSE TRANSLOCATION INTO GOLGI VESICLES

Translocation of GDP-fucose (at 2 μM) was measured as described in Materials and Methods. The rate of translocation of controls was 31 pmol/mg protein per 10 min. Results are average of two separate determinations.

Addition	Inhibition of translocation (% of control)	
	4 μM nucleotide	10 μM nucleotide
GMP	5	65
8-Br-GMP	75	86
GDP	28	71
GDP-Mannose	21	49
GTP	7	69
8-Azido-GTP	53	82
IDP	5	43
8-Br-IDP	2	17
AMP	2	9
8-Br-AMP	19	46
ADP	7	20
8-Br-ADP	16	43
3',5'-ADP	13	18
ATP	5	21
UDP	4	16

GMP, GTP, AMP and ADP, when used at low concentrations (Table II). Inhibition by 5'-GDP and 8-azido-GTP was competitive (Figs. 4 and 5). The K_i for GDP was 3.9 μM (at 20 μM GDP) and for 8-azido-GTP was 1.4 μM (at 4 and 16 μM 8-azido-GTP). Pyrimidine-containing nucleotides such as UDP appeared to have low inhibitory activity on GDP-fucose translocation (Table II). IDP also had low inhibitory activity (Table II), suggesting that the presence of an amino group in the purine ring may play a role in binding of GDP-fucose to the Golgi membrane.

The effect of nucleotides on translocation of adenosine 3'-phosphate 5'-phosphosulfate can be seen in Table III. At low concentrations (3-fold excess over adenosine 3'-phosphate 5'-phosphosulfate) 3',5'-ADP was the most effective inhibitor of translocation of adenosine 3'-phosphate 5'-phosphosulfate, while 2',5'-ADP did not inhibit. Other nucleotides such as adenosine 5'-phosphosulfate, 5'-ATP, 5'-ADP and 8-azido-AMP were considerably less effective inhibitors (Table III). These results suggest that the carrier protein for adenosine 3'-phosphate 5'-phosphosulfate may

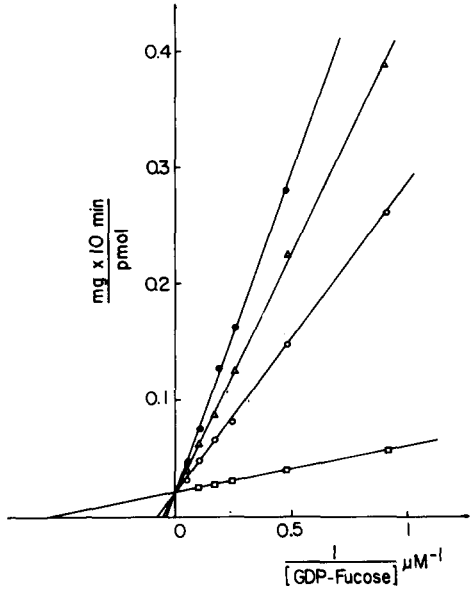

Fig. 4. Inhibition of GDP-fucose translocation into Golgi vesicles by GDP. Translocation was measured as described in Materials and Methods and was 17 pmol/mg protein per 10 min at 1 μM GDP-fucose. Results are average of two separate determinations. GDP concentration (μm): □, none; ○, 20; △, 40; ●, 80.

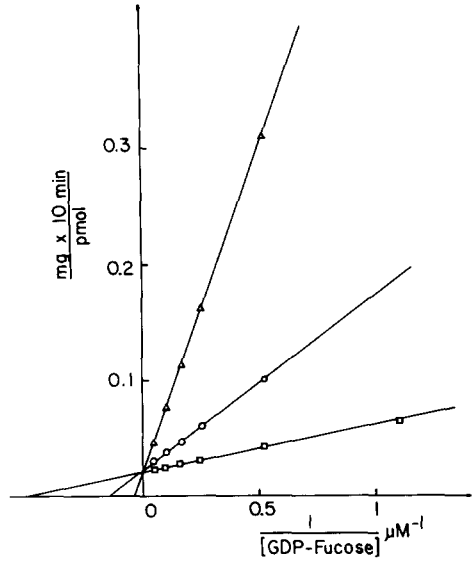

Fig. 5. Inhibition of GDP-fucose translocation into Golgi vesicles by 8-azido-GTP. Translocation was measured as described in Materials and Methods and was 16 pmol/mg protein per 10 min at 1 μM GDP-fucose. Results are average of two separate determinations. 8-azido-GTP concentration (μM): □, none; ○, 4; △, 16.

TABLE III

EFFECT OF NUCLEOTIDES ON ADENOSINE 3′-PHOSPHATE 5′-PHOSPHOSULFATE TRANSLOCATION INTO GOLGI VESICLES

Translocation of adenosine 3′-phosphate 5′-phosphosulfate (at 0.8 μM) was measured as described in Materials and Methods. The rate of translocation of controls was 46 pmol/mg protein per 10 min. Results are average of two separate determinations.

Addition	Inhibition of translocation (% of control)	
	2.5 μM nucleotide	10 μM nucleotide
Adenosine-5′ phosphosulfate	21	64
3′,5′-ADP	76	97
2′,5′-ATP	9	12
5′-ATP	13	56
5′-ADP	10	60
5′-AMP	6	60
3′-AMP	0	4
8-Azido-AMP	19	55
5′-GTP	–	10
5′-GMP	–	37
cAMP	–	0
CMP-NeuAc	–	0

have some affinity (although low) for 5′-adenosine phosphate-containing nucleosides but not for those containing 2′-phosphates.

Discussion

The purpose of this work was to obtain information regarding the structural requirements for the different putative sugar nucleotide and adenosine 3′-phosphate 5′-phosphosulfate translocation proteins in the Golgi apparatus membrane. The principal hypothesis underlying these studies is that inhibition of translocation of sugar nucleotides and adenosine 3′-phosphate 5′-phosphosulfate may occur either by prevention of binding of these compounds to the Golgi membrane or by inhibition of subsequent translocation across the Golgi membrane. Whether or not these two events are mediated by one or more proteins is not known. In those instances where inhibition of translocation by a given nucleotide, sugar or sulfate was competitive, one may postulate that the binding or translocation protein(s) domains recognize similar features in sugar nucleotides and adenosine 3′-phosphate 5′-phosphosulfate. Absence of inhibition suggests that those features do not play an important recognition role in the overall translocation process.

The observation that the nucleotide of sugar nucleotides and adenosine 3′-phosphate 5′-phosphosulfate, but not the sugars or sulfate, inhibit translocation suggests that the nucleotide is the component of sugar nucleotides and adenosine 3′-phosphate 5′-phosphosulfate which is recognized by the putative binding and/or translocation protein(s) in the Golgi membrane. Since the inhibition of translocation of sugar nucleotides and adenosine 3′-phosphate 5′-phosphosulfate by the corresponding nucleotide was competitive, one may infer that the nucleotide occupies the same active site as the sugar nucleotide and adenosine 3′-phosphate 5′-phosphosulfate in the putative binding and/or translocation protein(s). These results provide yet additional support for the hypothesis that translocation of sugar nucleotides and adenosine 3′-phosphate 5′-phosphosulfate across Golgi vesicle membranes occurs via protein carriers. We have obtained evidence suggesting that the nucleotide moiety of a sugar nucleotide and adenosine 3′-phosphate 5′-phosphosulfate contain necessary but not sufficient information for overall translocation into the Golgi lumen. This was inferred from experiments in which it was found the GDP-fucose but not GDP-mannose can be translocated into Golgi vesicles; GDP is a competitive inhibitor of GDP-fucose translocation, but neither fucose nor mannose inhibited translocation of the sugar nucleotide.

From the results shown above we draw the following conclusions regarding structural requirements of nucleotide sugars and adenosine 3′-phosphate 5′-phosphosulfate for their overall translocation across Golgi vesicle membranes:

1. The type of nucleotide base, purine or pyrimidine, is recognized in binding of nucleotide sugars and adenosine 3′-phosphate 5′-phosphosulfate to the Golgi membrane, i.e., UMP is a competitive inhibitor of CMP-NeuAc translocation while AMP does not inhibit.

2. The position of the phosphate groups appears to be critical for binding. The presence of a

5′-phosphate group in nucleotides results in high inhibitory activity, while the 3′- or 2′-phosphate derivatives have virtually none.

3. The number of phosphate groups in the critical position of the purine or pyrimidine base appears to be of less importance; thus, the inhibitory activities of 5′-CTP, 5′-CDP and 5′-CMP are similar.

4. The presence of a hydroxyl group in the sugar moiety of the nucleotide does not constitute an important recognition feature; thus, dCMP appears to be as good an inhibitor as CMP.

5. Replacement of hydrogen in position 5 of pyrimidines and position 8 of purines with halogens or an azido group is not an important recognition feature for binding. We are not certain whether the slightly higher inhibitory activity of Br-CMP compared to CMP is due to a higher stability towards phosphatases or to the presence of the bromine group per se.

6. The sugar or sulfate moieties do not appear to be recognition features for binding to the Golgi membrane; however, they appear to be critical for subsequent translocation into the Golgi lumen.

We are currently using the above information to synthesize nucleotide sugar analogues with photoactivatable groups as probes to identify and purify the putative nucleotide-derivative carrier proteins of the Golgi apparatus membrane.

Acknowledgements

We thank Dr. Martin Snider for reading the manuscript and Janet Kaufhold for expert typing. This work was supported by NIH grant GM 30365 and a Faculty Research Award of the American Cancer Society to C.B.H.

References

1 Sommers, L.W. and Hirschberg, C.B. (1982) J. Biol. Chem. 257, 10811–10817
2 Creek, K. and Morré, D. (1981) Biochim. Biophys. Acta 643, 292–305
3 Schwarz, J.K., Capasso, J.M. and Hirschberg, C.B. (1984) J. Biol. Chem. 259, 3554–3559
4 Kuhn, N.J. and White, A. (1976) Biochem. J. 154, 243–244
5 Brandan, E. and Fleischer, B. (1982) Biochemistry 21, 4640–4645
6 Leelavathi, D.E., Estes, L.W., Feingold, D.S. and Lombardi, B. (1970) Biochim. Biophys. Acta 211, 124–138