Biochem. J. (1989) 261, 389–393 (Printed in Great Britain)

Studies on the effect of lysosomotropic agents on the release of Galβ1-4GlcNAc α-2,6-sialyltransferase from rat liver slices during the acute-phase response

Gwen LAMMERS and James C. JAMIESON*
Department of Chemistry, University of Manitobia, Winnipeg, Manitoba, Canada R3T 2N2

The mechanism of release of Galβ1-4GlcNAc α-2,6-sialyltransferase (CMP-*N*-acetylneuraminate:β-galactoside α-2,6-sialyltransferase, EC 2.4.99.1) from rat liver during the acute-phase response is due to the action of a cathepsin D-like proteinase that cleaves the *trans*-Golgi membrane-bound enzyme from a membrane anchor; this allows a major portion of the enzyme containing the catalytic site to escape into the extracellular space [Lammers & Jamieson (1988) Biochem. J. **256**, 623–631]. The release of sialyltransferase was most effective at pH 5.6, suggesting that release of sialyltransferase from the Golgi in whole cells is dependent on maintaining an acidic environment in the *trans*-Golgi compartment of the hepatocyte. Golgi membranes contain a proton pump that maintains the acidic pH in these compartments [Glickman, Croen, Kelly & Al-Awquati (1983) J. Cell Biol. **97**, 1303–1308; Yamashiro, Tycko & Maxfield (1984) Cell (Cambridge, Mass.) **37**, 789–800; Zhang & Schneider (1983) Biochem. Biophys. Res. Commun. **114**, 620–625; Anderson & Pathak (1985) Cell (Cambridge, Mass.) **40**, 635–643]. Lysosomotropic agents, such as NH_4Cl, chloroquine and methylamine can penetrate acidic compartments of the cell, such as the Golgi complex, raise the pH, and thus affect proteolytic cleavage events. The present paper describes the effect of lysosomotropic agents on the release of sialyltransferase from the hepatocyte using liver slices as a whole-cell system. Slices were prepared from control rats and rats suffering from the acute-phase response, where release of sialyltransferase is increased substantially [Lammers & Jamieson (1988) Biochem. J. **256**, 623–631; Kaplan, Woloski, Hellman & Jamieson (1983) J. Biol. Chem. **258**, 11505–11509]. Release of sialyltransferase was almost abolished in presence of 50 mM-NH_4Cl, 50 mM-methylamine or 1 mM-chloroquine. Inhibition of release of sialyltransferase was reversed when the lysosomotropic agents were removed from the medium, showing that these agents are not cytotoxic to the cells under the conditions used. The secretion of rat α_1-acid glycoprotein, which is not subject to proteolytic processing in the Golgi complex, was not found to be substantially affected by the presence of lysosomotropic agents. The results suggest that proteolytic cleavage of the catalytic site of sialyltransferase is a process that is significantly affected by the intra-Golgi pH

INTRODUCTION

Inflammation in humans and experimental animals results in a variety of biochemical and physiological changes referred to as the 'acute-phase response' [5–10]. A major change is an increase in the circulating levels of a variety of serum glycoproteins referred to as the 'acute-phase reactants' [7]. The acute-phase reactants are mainly liver-synthesized secretable glycoproteins such as α_1-acid glycoprotein and fibrinogen [8,9]. Recently, we have characterized Galβ1-4GlcNAc α-2,6-sialyltransferase (CMP-*N*-acetylneuraminate:β-galactoside α-2,6-sialyltransferase, EC 2.4.99.1) as a new type of acute-phase reactant [1]. This enzyme is responsible for attaching NeuAc in α-2,6 linkage to terminal positions of *N*-linked chains of glycoproteins and is located in a membrane-bound form at the luminal face of the *trans*-Golgi compartment [10]. In a recent study we showed that sialyltransferase was released from the rat Golgi membrane by the specific action of a cathepsin D-like proteinase, which acted at the luminal face and which showed the greatest activity at pH 5.6 [1]. This proteinase cleaved a major portion of the enzyme containing the catalytic site from a membrane anchor and allowed the enzyme to pass into the extracellular space; proteolytic cleavage was most effective during the acute-phase response, thus explaining why sialyltransferase is an acute-phase reactant [1].

Weakly basic amines, such as NH_4Cl, chloroquine and methylamine penetrate acidic compartments of the cell and accumulate as protonated forms, resulting in an increase in the intravesicular pH [12–16]. These agents have been recently shown to be particularly effective in blocking proteolytic events that occur in the Golgi [12–14]. In the present study we show that lysosomotropic agents are potent inhibitors of the release of sialyltransferase from liver slices and the effect is most pronounced with slices from inflamed rats. The secretion of α_1-acid glycoprotein was studied concurrently as a control. This protein also behaves as an acute-phase reactant in rats [17], but it is not subject to proteolytic processing in the Golgi. The secretion of this acute-phase reactant was not substantially affected by the presence of lysosomotropic agents under the conditions used.

The results provide further evidence to support the idea that the release of sialyltransferase from the Golgi

* To whom correspondence and reprint requests should be sent.

complex is an event that is dependent on maintaining acidic conditions in the lumen of the Golgi complex to allow the cathepsin D-like proteinase that releases sialyltransferase during the acute-phase response to act.

MATERIALS AND METHODS

Materials

CMP-*N*-[4,5,6,7,8,9-^{14}C]acetylneuraminate (247 mCi/mmol) was from New England Nuclear Corp., Lachine, Quebec, Canada. CMP-NeuAc, CDP-choline, penicillin G (sodium salt), streptomycin, chloroquine and methylamine were from Sigma Chemical Co., St. Louis, MO, U.S.A. Dextran T70 was from Pharmacia, Montreal, Quebec, Canada. Rat α_1-acid glycoprotein, asialo-α_1-acid glycoprotein and antiserum containing antibodies against rat α_1-acid glycoprotein were prepared as described previously [6,18].

Liver-slice system

Inflammation was induced in 150–200 g male Long–Evans hooded rats by subcutaneous injection of 0.5 ml of oil of turpentine per 100 g body weight as described previously [19]; controls received injections of sterile 0.15 M-NaCl. Animals were killed at 36 h after injection, and livers were perfused *in situ* with 0.15 M-NaCl via the portal vein. The procedure used for preparing and incubating liver slices is described in detail elsewhere [20] and used 10 ml of medium and 1 g of liver slices; incubation was under O_2/CO_2 (19:1) for up to 4 h at 37 °C [20]. Lysosomotropic agents at concentrations up to 50 mM-NH_4Cl, 50 mM-methylamine or 1 mM-chloroquine were added as appropriate. The effect of removing lysosomotropic agents on the release of sialyltransferase into medium was also studied. In these reversal experiments, flasks containing slices from 36 h-inflamed rats were divided into three sets. In the first set, slices were preincubated for 1 h, and medium was changed to fresh medium and incubation continued up to a further 70 min (control samples). The second set contained 50 mM-NH_4Cl, 50 mM-methylamine or 0.5 mM-chloroquine as appropriate, medium was changed as above, but the second medium also contained lysosomotropic agents (inhibited sample). In the third set the initial incubation was in presence of lysosomotropic agents, but the second medium contained no lysosomotropic agents (reversal samples). The presence of lysosomotropic agents had no effect on the pH of the incubation medium.

Assay for sialyltransferase and α_1-acid glycoprotein

The assay for sialyltransferase was exactly as described in detail elsewhere [1,6], except that enzyme was added in the form of 120 μl of medium from slice experiments; the final assay volume was 150 μl. One unit of sialyltransferase activity is defined as equal to the transfer of 1 pmol of acetylneuraminate to acceptor protein/h as described previously [1,6]. The presence of lysosomotropic agents did not interfere with the assay for sialyltransferase.

Rat α_1-acid glycoprotein in medium from slice experiments was determined immunologically on the basis of previous methods [21,22]. The procedure was scaled up to 0.5 ml of medium and 0.1 ml of rabbit antiserum against rat α_1-acid glycoprotein; 0.15 M-NaCl and 4% (w/v) Dextran T70 were present as described previously [21,22]. The mixtures were incubated at 37 °C for 45 min and were left at 4 °C for 48 h. Immune precipitates were collected and assayed for protein as described by Lowry *et al.* [23], as modified by Miller [24]; α_1-acid glycoprotein was determined by reference to a standard curve [21,22]. Immune precipitation was not affected by the presence of NH_4Cl, methylamine or chloroquine.

RESULTS

Effect of lysosomotropic agents on the release of sialyltransferase from liver slices

Fig. 1 shows the effect of the presence of up to 50 mM-

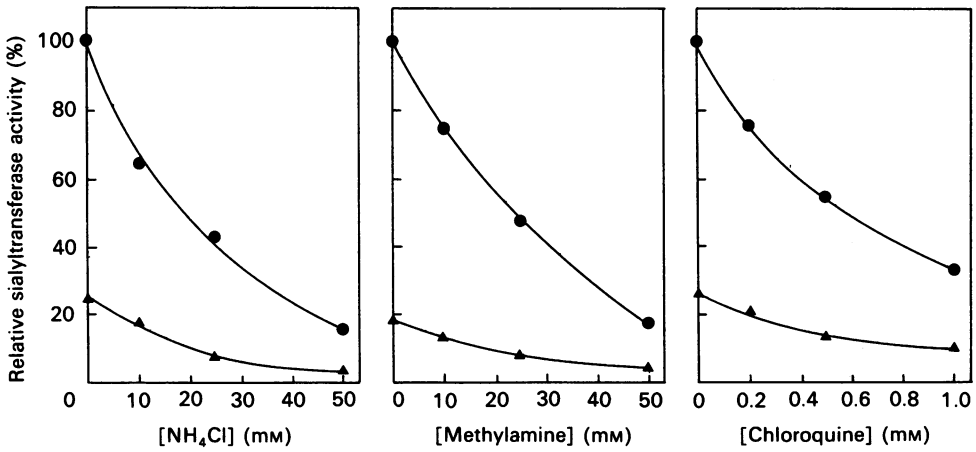

Fig. 1. Effect of concentration of lysosomotropic agents on the release of sialyltransferase from liver slices from control and 36 h-inflamed rats

Samples (1 g) of liver slices from control and 36 h-inflamed rats were incubated with 10 ml of medium for 4 h either alone or in the presence of up to 50 mM-NH_4Cl, 50 mM-methylamine or 1 mM-chloroquine. ▲, ●, Sialyltransferase activity present in medium from slices from control (▲) and 36 h-inflamed (●) rat liver. The amount of sialyltransferase released from slices from inflamed rats for 4 h in the absence of lysosomotropic agents was taken as 100%. This value represents 400, 308 and 255 units of sialyltransferase activity for the NH_4Cl, methylamine or chloroquine experiments respectively. Each point represents the mean from three experiments; reproducibility was within ±15%.

NH_4Cl, 50 mM-methylamine or 1 mM-chloroquine on the release of sialyltransferase from liver slices into medium using slices from control and 36 h-inflamed rats after incubation for 4 h. All three agents were potent inhibitors of the release of sialyltransferase from slices from control and inflamed rats, with NH_4Cl and methylamine being the most effective (Fig. 1). The inhibitory effect of lysosomotropic agents on the release of sialyltransferase was most dramatic with slices from inflamed livers because of their increased capacity to release sialyltransferase into medium (Fig. 1) [6].

The effect of time of incubation on the release of sialyltransferase in the presence of the lysosomotropic agents used in Fig. 1 was also studied. Typical results are shown in Fig. 2 for 25 mM-NH_4Cl, 25 mM-methylamine and 0.5 mM-chloroquine for slices from 36 h-inflamed rats. The release of sialyltransferase from liver slices was fairly linear as a function of time, with values from

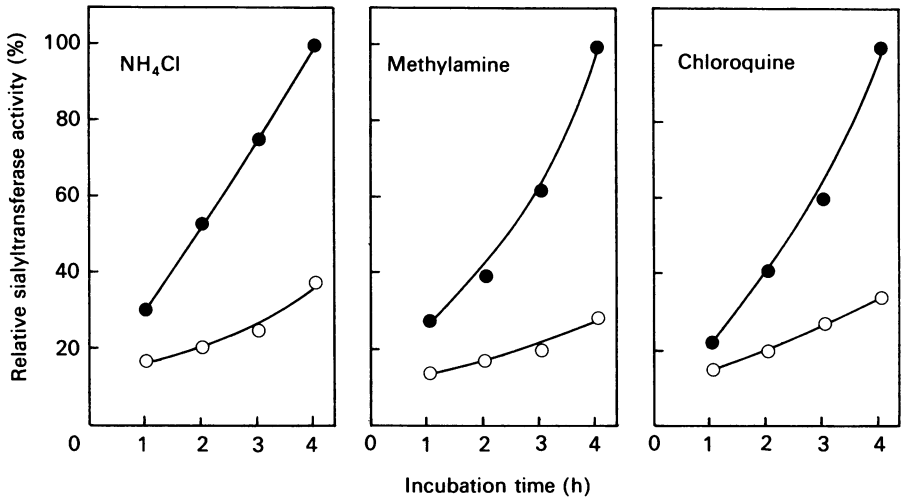

Fig. 2. Effect of incubation time on the release of sialyltransferase from liver slices from control and 36 h-inflamed rats in the presence or absence of lysosomotropic agents

Slices were incubated as described in the legend to Fig. 1 in the presence of 25 mM-NH_4Cl, 25 mM-methylamine or 0.5 mM-chloroquine, and the release of sialyltransferase into the medium was monitored as a function of the period of incubation. ●, ○, Sialyltransferase activity present in medium in absence (●) or presence (○) of lysosomotropic agents. The 100% values were those described in the legend to Fig. 1. Each point represents the mean from three experiments; reproducibility was within ±15%.

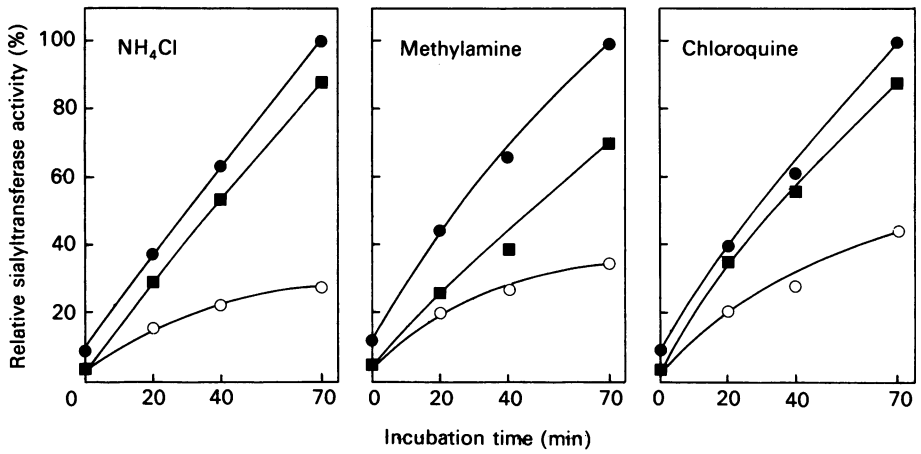

Fig. 3. Effect of removal of lysosomotropic agents from the incubation medium on the release of sialyltransferase from liver slices from 36 h-inflamed rats

Slices prepared from 36 h-inflamed rat liver were incubated as described in the legend to Fig. 1. In these experiments one set of flasks was preincubated for 1 h in the absence of lysosomotropic agents (controls) and the other two sets of flasks were incubated with 25 mM-NH_4Cl, 25 mM-methylamine or 0.5 mM-chloroquine. At the end of the 1 h incubation period, medium was removed from all flasks and fresh medium was added as described for the reversal experiments (see the Materials and methods section). All flasks were then incubated for up to 70 min, and sialyltransferase in the medium was monitored as a function of time. ●, ■, ○, Sialyltransferase activity present in control samples (●), reversal samples (■) and inhibited samples (○). The 100% value for these experiments was determined as described in the legend to Fig. 1 for the 70 min control samples; this represented 144 units of sialyltransferase activity. Each point represents the mean from three experiments; reproducibility was within ±15%.

Table 1. Effect of lysosomotropic agents on the secretion of α_1-acid glycoprotein from liver slices from control and 36 h-inflamed rats

Liver slices from control and 36 h-inflamed rats were incubated for 4 h either alone, or in presence of NH_4Cl, chloroquine and methylamine at the concentrations listed below. α_1-Acid glycoprotein was determined in the medium by using an immunological method. Results are means ± S.E.M. from four assays from a typical experiment.

Additions	α_1-Acid glycoprotein in medium (μg/g wet weight of liver)	
	Control-rat liver slices	Inflamed-rat liver slices
None	69±6	162±12
NH_4Cl		
10 mM	73±9	180±15
25 mM	69±7	179±12
50 mM	77±7	170±12
Chloroquine		
250 μM	67±6	150±10
500 μM	65±7	149±9
1 mM	71±6	145±10
Methylamine		
10 mM	64±6	142±10
25 mM	60±6	129±8
50 mM	58±5	125±9

samples containing lysosomotropic agents being much lower than the uninhibited values; similar results were obtained with control slices, except that sialyltransferase activities were much lower (see Fig. 1).

Fig. 3 shows that the inhibitory effect of lysosomotropic agents on the release of sialyltransferase from liver slices were reversible. Slices quickly recovered from the inhibitory effects of lysosomotropic agents when these agents were removed from the medium. These results suggest that the lysosomotropic agents were not cytotoxic for the cells under the experimental conditions used.

Effect of lysosomotropic agents on the release of α_1-acid glycoprotein from liver slices

Rat α_1-acid glycoprotein is not subject to proteolytic processing beyond the stage of removal of the signal in the rough endoplasmic reticulum [25,26], but it is secreted via the channels of the Golgi complex [27,28]. Secretion of this protein was studied concurrently in order to determine if a protein that is not subject to proteolytic processing in the Golgi is affected by the presence of lysosomotropic agents in the incubation mixture. Table 1 shows typical results from these experiments for 4 h incubations of liver slices from control and 36 h-inflamed rats. The presence of NH_4Cl had little effect on the secretion of α_1-acid glycoprotein into the medium, whereas there was a slight inhibition of secretion of α_1-acid glycoprotein in presence of the higher concentrations of methylamine and chloroquine, but the inhibition of secretion was much less than that found for the release of sialyltransferase (Fig. 1).

DISCUSSION

Lysosomotropic agents have been shown to perturb lysosomes and endosomes [29,30], but recently these agents have been shown to enter the *trans*-Golgi compartment and cause an increase in the intraluminal pH [14,31]. Increased Golgi pH has been shown to inhibit the processing of pro-forms of secretable proteins in the Golgi which depend on the action of proteinases that act at acidic pH. For example, albumin and complement component C3 accumulate in the Golgi complex as their pro-forms, and these forms are eventually secreted into the medium [12–14]. The conclusion from these studies was that lysosomotropic agents impeded proteolytic events in the Golgi complex mainly because of an upward movement in pH in the intraluminal Golgi compartment [12–14].

Sialyltransferase is a *trans*-Golgi membrane-bound enzyme which is subject to proteolytic cleavage by an endogenous cathepsin D-like proteinase which is most active at pH 5.6 [1]. The proteinase removes a major portion of the enzyme containing the catalytic site from a membrane anchor and allows the enzyme to escape into the extracellular space. The release of sialyltransferase was very dependent on maintaining an acidic environment in the Golgi lumen to allow the proteinase to act [1]. In order to examine further the importance of intra-Golgi pH in the release of sialyltransferase, we have used lysosomotropic agents to raise the Golgi pH and we have monitored the release of sialyltransferase. The whole-cell slice system developed in our previous work was used in the present study, since it allows the release of sialyltransferase to be monitored over longer time periods [20]. The results provide strong evidence that release of sialyltransferase is substantially reduced by the presence of lysosomotropic agents, most likely due to the elevation of intra-Golgi pH, which has an inhibiting effect on the proteinase that cleaves the enzyme from the membrane anchor. The effect of lysosomotropic agents on the release of sialyltransferase could be reversed, suggesting that the agents were not cytotoxic to the cells over the range of concentrations used. The observation that reversal is rapid tends to rule out the possibility that lysosomotropic agents are substantially affecting the transit times from the rough endoplasmic reticulum to Golgi of sialyltransferase or the cathepsin D-like proteinase that is involved in the release.

The secretion of α_1-acid glycoprotein was monitored concurrently as a control to determine if there were any substantial changes in the release of a typical secretory protein which is not subject to proteolytic processing in the Golgi. Secretion of α_1-acid glycoprotein was only slightly affected by lysosomotropic agents, suggesting that release of sialyltransferase is probably not due to modifications in the secondary machinery of the Golgi under the experimental conditions used.

It is known that proteinases are subject to proteolytic processing during transit to the lysosome [32–34]. Assuming that the cathepsin D-like proteinase that cleaves sialyltransferase is the lysosomal enzyme, as was suggested previously [1], then lysosomotropic agents could affect the release of sialyltransferase by indirectly preventing the processing of this proteinase. However, since this would also be a pH-dependent event, it does not invalidate the idea that Golgi pH is an important controlling factor in the release of sialyltransferase into the extracellular space. Further studies are required to determine whether intra-Golgi pH is significantly lower during the acute-phase state, since this could affect the physiological and biochemical response of liver during

the acute-phase response to injury, particularly as it relates to the release of sialyltransferase.

This work was supported by the Natural Sciences and Engineering Research Council of Canada (grant no. A5394); G.L. is supported by a U.S. Navy Veterans Benefits Scholarship and a University of Manitoba Graduate Fellowship. We thank Elzbieta Gospodarek for valuable technical assistance.

REFERENCES

1. Lammers, G. & Jamieson, J. C. (1988) Biochem. J. **256**, 623–631
2. Glickman, J., Croen, K., Kelly, S. & Al-Awquati, Q. (1983) J. Cell Biol. **97**, 1303–1308
3. Yamashiro, D. J., Tycko, B. & Maxfield, F. R. (1984) Cell (Cambridge Mass.) **37**, 789–800
4. Zhang, F. & Schneider, D. L. (1983) Biochem. Biophys. Res. Commun. **114**, 620–625
5. Anderson, R. G. W. & Pathak, R. K. (1985) Cell (Cambridge, Mass.) **40**, 635–643
6. Kaplan, H., Woloski, B. M. N. R. N. J., Hellman, M. & Jamieson, J. C. (1983) J. Biol. Chem. **258**, 11505–11509
7. Koj, A. (1974) in Structure and Function of Plasmsa Proteins (Allison, A. C., ed.), pp. 74–131, Plenum Press, New York
8. Jamieson, J. C., Kaplan, H. A., Woloski, B. M. R. N. J., Hellman, M. A. & Ham, K. (1983) Can. J. Biochem. Cell Biol. **61**, 1041–1048
9. Jamieson, J. C., Lammers, G., Janzen, R. & Woloski, B. M. R. N. J. (1987) Comp. Biochem. Physiol. **87B**, 11–15
10. Beisel, W. R. (1980) Fed. Proc. Fed. Am. Soc. Exp. Biol. **39**, 3105–3108
11. Roth, J., Taatjes, D. J., Lucocq, J. M. & Paulson, J. C. (1985) Cell (Cambridge, Mass.) **43**, 287–295
12. Oda, K., Koriyama, Y., Yamada, E. & Ikehara, Y. (1986) Biochem. J. **240**, 739–745
13. Oda, K. & Ikehara, Y. (1985) Eur. J. Biochem. **152**, 605–609
14. Strous, G. J., DuMaine, A., Zijderhand-Bleekemolen, J. E., Slot, J. W. & Schwartz, A. L. (1985) J. Cell Biol. **101**, 531–539
15. Maxfield, F. R. (1982) J. Cell Biol. **95**, 676–681
16. Caplan, M. J., Stow, J. L., Newman, A. P., Madri, J., Anderson, H. C., Farquhar, M. G., Palade, G. & Jamieson, J. C. (1987) Nature (London) **329**, 632–635
17. Jamieson, J. C., Friesen, A. D., Ashton, F. E. & Chou, B. (1972) Can. J. Biochem. **50**, 856–870
18. Jamieson, J. C., Ashton, F. E., Friesen, A. D. & Chou, B. (1972) Can J. Biochem. **50**, 871–880
19. Ashton, F. E., Jamieson, J. C. & Friesen, A. D. (1970) Can. J. Biochem. **48**, 841–850
20. Jamieson, J. C., Morrison, K. E., Molasky, D. & Turchen, B. (1975) Can. J. Biochem. **53**, 401–414
21. Jamieson, J. C. & Ashton, F. E. (1973) Can. J. Biochem. **51**, 1034–1045
22. Simkin, J. L. & Jamieson, J. C. (1967) Biochem. J. **103**, 153–164
23. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
24. Miller, G. L. (1959) Anal. Chem. **31**, 964
25. Ricca, G. A. & Taylor, J. M. (1981) J. Biol. Chem. **256**, 11199–12202
26. Reinke, R. & Fiegelson, P. (1985) J. Biol. Chem. **260**, 4397–4403
27. Jamieson, J. C. & Ashton, F. E. (1973) Can. J. Biochem. **51**, 1281–1291
28. Jamieson, J. C., Kaplan, H., Woloski, B. M. R. N. J., Hellman, M. & Ham, K. (1983) Can. J. Biochem. **61**, 1041–1048
29. Dean, R. J., Jessup, W. & Roberts, C. R. (1984) Biochem. J. **217**, 27–40
30. DeDuve, C. (1983) Eur. J. Biochem. **137**, 391–397
31. Schwartz, A. L., Strous, G. J. A. M., Slot, J. W. & Geuze, H. J. (1985) EMBO J. **4**, 899–904
32. Erickson, A. H., Conner, G. E. & Blobel, G. (1981) J. Biol. Chem. **256**, 11224–11231
33. Hasilik, A., von Figura, K., Conzellmann, E., Nehrkorn, H. & Sandhoff, K. (1982) Eur. J. Biochem. **125**, 317–321
34. Yonezawa, S., Takahashi, T., Wang, X.-J., Wong, R. N. S., Harstuck, J. A. & Tang, J. (1988) J. Biol. Chem. **263**, 16504–16511

Received 13 March 1989/25 April 1989; accepted 3 May 1989