Volume 259, number 1, 91-94 FEB 07928 December 1989

Inhibition by glucosamine of myristoylation in human H9 lymphocytes and rat liver cells

C. Jacobsen, H. Andreassen and T. Saermark

Laboratory for Protein Chemistry, University of Copenhagen, Sigurdsgade 34, DK-2200 N Denmark

Received 27 October 1989

N-Myristoyl transferase (NMT) activity was measured in rat liver and H9 cells using an in vitro assay based on acylation of synthetic peptides. Glucosamine was found to inhibit the NMT activity. Using a synthetic peptide mimicking the N-terminus of HIV p27*nef* a K_m value of 2.4 μM and a V_{max} of 240 pmol/mg per h was found. In the presence of glucosamine the V_{max} was lowered indicating that glucosamine acted as a non-competitive inhibitor. Glucosamine also inhibited incorporation of radiolabelled myristic acid into H9 cell proteins in vivo. In liver cells using a peptide from the N-terminus of p60 SRC only the V_{max} was affected.

Fatty acid acylation; Myristoylation; Glucosamine; *N*-Myristoyl transferase

1. INTRODUCTION

Glucosamine has been shown to inhibit or interfere with glycosylation of a number of viruses [1]. The consequences of this may be dependent on the virus and host cell in question. Semliki forest virus release is inhibited by glucosamine but this is not the case for Newcastle disease virus [2]. It was assumed that the altered glycosylation affected different functions in the viruses tested depending on the functions of glycosylations in each case. However, as pointed out by Hayman et al. [3], it was very surprising that deletion mutants of avian RNA tumor virus that lack glycoproteins are able to form virus particles [4,5], whereas glucosamine inhibits glycosylation as well as release of the normal avian RNA tumor virus [6]. These results suggest that glucosamine inhibits release of avian RNA tumor virus by interfering with a process different from glycosylation (see also [3]).

Studies on N-terminal acylation of viral proteins have suggested that acylation of viral proteins is necessary for processing of precursors and release of intact particles [7-10]. If glucosamine inhibited not only glycosylation but also acylation, it would be expected that virus production would be inhibited, even in such cases where glycoproteins were absent. Alternatively, acylation of host cell proteins may be important for virus release in certain cases.

Using N-terminal octapeptides from HIV p27*nef* and p17*gag* as well as p60*src*, all known to be acylated proteins in vivo [11-13], we have determined the effects of glucosamine on *N*-myristoyl transferase (NMT) activity in vitro. Both the in vitro assay as well as myristic acid incorporation into H9 cells in culture was affected by glucosamine.

Correspondence address: C. Jacobsen, Laboratory for Protein Chemistry, University of Copenhagen, Sigurdsgade 34, DK-2200 N Denmark

2. MATERIALS AND METHODS

N-Myristoyl transferase (NMT) activity was assayed by the transfer of [^{14}C]myristic acid from myristoyl-coenzyme A (myr-CoA, Amersham, England; 54 mCi/mmol) to synthetic peptides based on the N-terminal octapeptide sequence of HIV p27*nef* and p60*src*. The assay was carried out at pH 7.5 in a buffer containing 20 mM Tris-hydroxymethylaminomethane (Tris), 10 mM $MgCl_2$, 2 mM dithiothreitol, 1% Triton X-100, 0.2 mM EGTA and 0.25 μCi myr-CoA. 10 μg of cell lysate and synthetic peptide was included in the assay, the synthetic peptide concentration being 10 μmol/l unless otherwise stated. The incubation volume was 200 μl and the incubation temperature was 37°C. After 1 h of incubation the assay was stopped by adding 110 μl of 8% trichloroacetic acid in methanol followed by centrifugation (8000 × g_{av}, 5 min) at 4°C. The radiolabelled peptides were isolated by reverse-phase HPLC on 5 C4 columns (Machery-Nagel, FRG). Their elution was identified by synthetic myristoylated peptides. The buffer system used was an isocratic elution by 32% acetonitrile (ACN) in 0.1% trifluoroacetic acid (TFA) followed by a gradient to 80% ACN in 0.1% TFA in 15 min to elute the remaining radiolabelled myr-CoA. The radiolabel was measured by scintillation counting.

The procedure described above was changed slightly to measure myristoylation of the N-terminal fragment of HIV p17*gag*. In this case the peptide corresponded to the first 11 amino acids of HIV p17 and had a Tyr in position 12 to allow iodination. Using iodinated peptide and unlabelled myr-CoA, myristoylation could be measured by the large change in retention time caused by the acylation. In this case the acylated product was isolated on a C18 column (Machery-Nagel, FRG) using a linear gradient from 32% to 80% ACN in 0.1% TFA over 20 min. The peptide was iodinated using the iodogen procedure [14].

The enzyme preparations used were from rat liver or human H9 lymphoma cells. The liver preparation was obtained from male Wistar rats (200 g) by homogenization of the minced liver in a 20 mM Tris, 250 mM sucrose, 0.5 mM EGTA buffer (pH 7.5) followed by centrifugation at 20000 × g_{av} for 10 min at 4°C. The H9 cells were

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00145793/89/$3.50

lysed by nitrogen cavitation and homogenization followed by fractionation using the same procedure as the liver cells. The material was frozen in aliquots at −20°C.

Synthetic peptides were prepared on an LKB Biolynx peptide synthesizer according to the instruction manual (LKB Biochrome, England). Coupling time was 60 min and the peptides were synthesized with a carboxyl terminal group. The decoupling and deprotection was carried out in 95% TFA, 2.5% anisole and 2.5% ethanedithiol for 2 h. The peptides were precipitated 5 times in diethyl ether and desalted on a Sephadex G-10 column (Pharmacia, Sweden). The synthetic myristoyl-octapeptides used as standards, were prepared by using symmetrical anhydrides of myristic acid in coupling to the synthetic peptides. The peptides were purified to homogeneity on an LKB TSK120T C18 column using linear gradients from 32% to 80% ACN in 0.1% TFA over 120 min.

The effect of glucosamine on fatty acid and amino acid incorporation was quantitated by drying a 100 μl sample of either a [^{3}H]-myristate or a [^{14}C]amino acid labelled cell suspension on a 5 × 5 cm Whatman 3 mm filter paper followed by precipitation with 10% trichloroacetic acid. The filter paper was extracted five times with chloroform/methanol (2:1, v/v) and dried. Radioactivity on the filter was determined by scintillation counting.

3. RESULTS

Rat liver cells contained NMT activity able to catalyze the incorporation of radioactive myristic acid from myristoyl-CoA into synthetic octapeptides. In fig.1 the distribution of radiolabel incorporated into N-terminal p27*nef* octapeptide is shown on a C4 reverse-phase HPLC gradient. The radiolabel coeluted with synthetic myristoylated N-terminal p27*nef* octapeptide. A similar gradient was also used to determine radiolabel incorporation into an N-terminal p60*src* octapeptide. This peptide eluted 2 min earlier on the same gradient as indicated by a synthetic myristoylated standard. The incorporation of label into both of these peptides was time and protein dependent and had a pH optimum of 7.5. Acylation of these two peptides and p17*gag* was used to determine NMT activity in liver and H9 cells.

In fig.2A is shown the NMT activity in rat liver membrane as a function of increasing concentration of substrate (p60*src* octapeptide). The K_m was found to be 5.9 μM and the V_{max} was 43 pmol/mg per h. Also shown in fig.2A is the effect of glucosamine (20 mM)

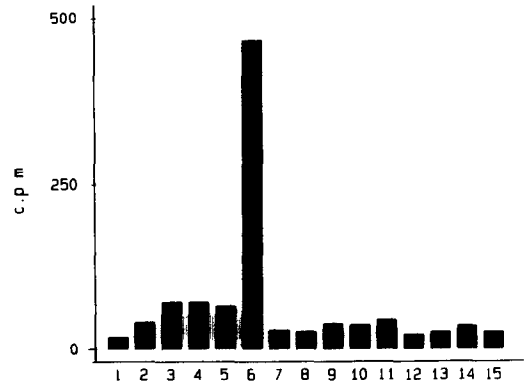

Fig.1. Distribution of radiolabel incorporated into N-terminal p27*nef* on a C4 reverse-phase column using an isocratic gradient at 32% ACN.

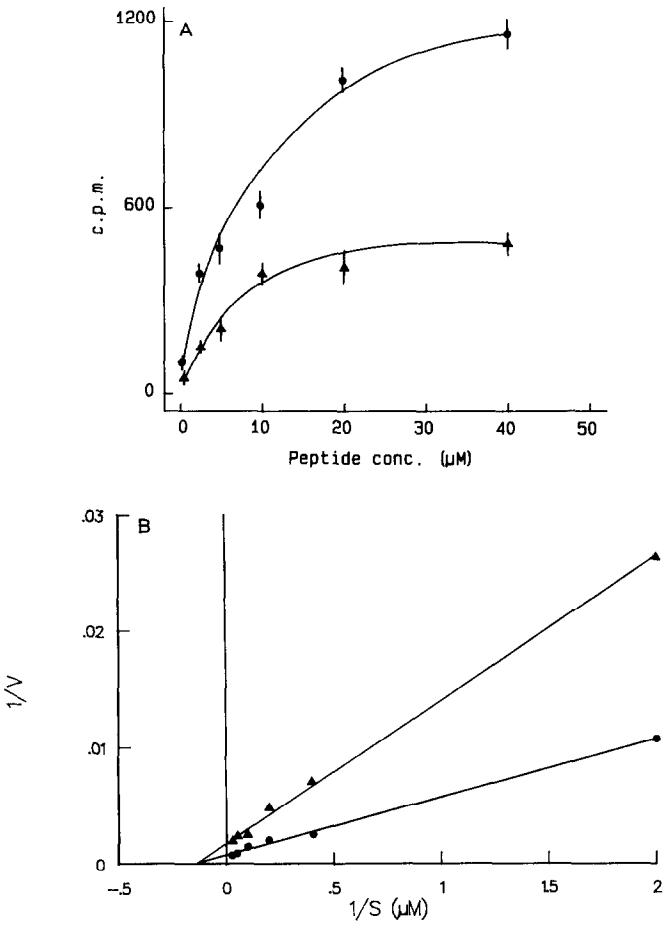

Fig.2. (A) NMT activity in rat liver membranes as a function of substrate concentration. The NMT activity was assayed using N-terminal p60*src* octapeptide as a substrate in the absence (●) or presence (▲) of glucosamine (20 mM). Values are means ± SD, n = 3. (B) Lineweaver-Burk plots of NMT activity in rat liver membranes. NMT activity was measured with (▲) or without (●) the presence of glucosamine (20 mM) using N-terminal p60*src* octapeptide as a substrate. The K_m value was 5.9 μM and V_{max} 43 pmol/mg per h in the absence of inhibitor. In the presence of glucosamine the V_{max} was lowered to 21 pmol/mg per h whereas the K_m is unchanged.

on the NMT activity as a function of substrate concentration. It was found that glucosamine inhibited by reducing the V_{max}. A Lineweaver-Burk plot of the results is shown in fig.2B.

The effect of glucosamine was tested not only on rat liver cells but also on H9 cell NMT activity. In table 1 is shown a comparison of the effect of glucosamine on both liver and H9 cells. Also included is the effect of glucose at the same concentrations. Only glucosamine affected the NMT activity.

The concentration dependency of the effect of glucosamine on NMT activity in both liver and H9 cells was tested using N-terminal p17*gag* octapeptide as substrate (fig.3). The effect was found to be half-maximal at 0.8 mM glucosamine in H9 cells and 1.2 mM in liver cells.

Table 1

Cell type	H9	r	Liver	r
K_m (μM)	2.4	0.98	5.9	1.0
V_{max} (relative)	1.0		1.0	
K_m + glucosamine (20 mM)	3.1	0.95	6.1	1.0
V_{max} + glucosamine (20 mM)	0.8		0.5	
V_{max} + glucose (20 mM)	1		1	

H9 cell or liver cell NMT activity was assayed in the presence or absence of glucosamine (mM) as a function of substrate concentration (μM). The V_{max} and K_m values were determined. Also included is the effect of glucose on NMT activity (V_{max}). r is the correlation coefficient

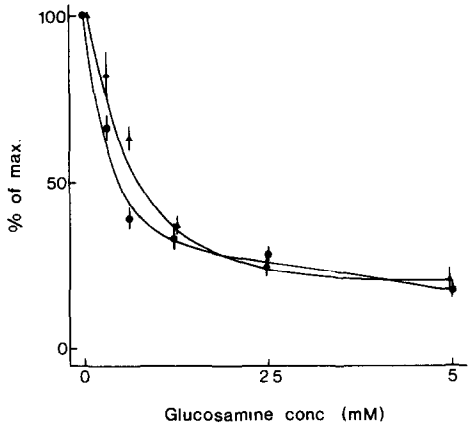

Fig.3. NMT activity in rat liver and H9 membranes as a function of glucosamine concentration. The NMT activity was assayed using p60*src* (●) or p17*gag* (▲) peptide as substrate and rat liver or H9 membranes as enzyme source. Values are means ± SD, n = 3.

The effect of glucosamine on acylation in tissue culture cells was illustrated using an assay of total fatty acid incorporation into cells. In fig.4 is shown the incorporation of [^{3}H]myristic acid into H9 cell proteins and its inhibition by glucosamine. The effect of glucosamine was found to be half-maximal at concentrations between 0.5 and 1 mM. This effect was not due to inhibition of protein synthesis (fig.4). Using a filtration assay for incorporation of radiolabel into total cell protein the half-maximal inhibition by glucosamine was found to be 0.8 mM.

4. DISCUSSION

NMT activity was found in both human H9 lymphoma cells and rat liver cells. Similar activity has been described previously in both liver, brain [15] and yeast [16]. The enzyme activity in the liver cell lysate used here had the same order of affinity for the N-terminal *src* octapeptide as was found in a previous study [15]. The enzyme activity in H9 cells was very similar to the activity found in the rat liver cells in terms of pH optimum and affinity for different peptides. The affinity for these two peptides was very similar in both H9 and rat liver cells.

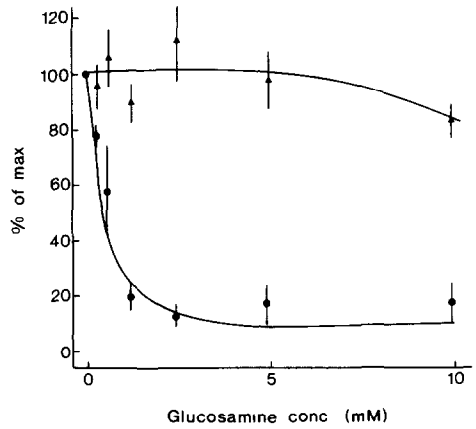

Fig.4. [^{3}H]Myristic acid and [^{14}C]amino acid incorporation in the presence of various concentrations of glucosamine. [^{3}H]Myristic acid (●) and [^{14}C]amino acid (▲). Values are means ± SD, n = 3.

Previous studies on the effect of glucosamine on virus assembly and glycosylation have shown that although glucosamine inhibits glycosylation, this may not explain why it also inhibits virus release [3]. Non-glycosylated avian RNA sarcoma viruses have been found that are released normally, although not glycosylated and not infectious since binding of the virus to target cells was dependent on glycosylation [4,5]. However, these viruses have been found to be released in the presence of glucosamine [6]. This was partly due to inhibition of protein synthesis by glucosamine, but also due to inhibition of processing of the synthesized precursors for viral proteins [3]. These results point to an effect by glucosamine on a process necessary for virus assembly but different from glycosylation. It was also observed that the virus protein precursors were synthesized although they were not glycosylated [3]. Thus a process necessary for transport of the proteins to the cell surface may have been affected by glucosamine.

The results presented here show that acylation is inhibited by glucosamine both in rat liver cells and H9 cells. We found that this was due to a direct effect on the NMT activity in these cells. Glucosamine not only inhibited acylation in an in vitro assay for NMT activity but also inhibited acylation in tissue cultures of H9 cells. Acylation of precursor proteins has been shown to be necessary for normal processing in the case of Molony MLV *gag* precursor proteins [9]. Acylation was also found to be necessary for transport of the SIV *gag* precursor to the plasma membrane [10]. We found an inhibition by glucosamine on acylation but not on amino acid incorporation in H9 cells at a concentration range (0.5–5 mM) that did not affect protein synthesis. The observed inhibition by glucosamine on acylating activity in H9 cells may thus explain why glucosamine was found to inhibit assembly of certain viruses, since inhibition of acylation would prevent transport of the actylated viral proteins to the cell surface.

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