Rapid hormonal regulation of N-acetylglucosamine transferase I

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ABSTRACT
Previous studies have shown that, in unstimulated mammary epithelial cells from virgin mice, prolactin receptors are retained intracellularly because of their incomplete N-glycosylation. Activation of the nitric oxide/cGMP pathway stimulates N-acetylglucosamine (NAG) transferase I activity, completion of terminal glycosylation, and redistribution of the receptors to the cell surface. In this study, it was shown that nitric oxide could stimulate the phosphorylation of NAG transferase I in intact cells and that the cGMP-dependent protein kinase (PKG) could directly phosphorylate the purified enzyme. Furthermore, this modification was associated with enhanced enzymatic activity. Conversely, this stimulation of activity was blocked in intact cells by coincubation with a PKG inhibitor and reversed in the immunoprecipitated enzyme by alkaline phosphatase treatment. Kinetic analysis revealed that this effect on enzyme activity was due to an increase in $V_{\text{max}}$ without any change in $K_m$. Therefore, it appears that the nitric oxide/cGMP pathway activates NAG transferase I via direct phosphorylation by PKG.

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INTRODUCTION
In this laboratory it has previously been shown that, in resting mouse mammary epithelium, the majority of prolactin receptors are retained intracellularly in a partially glycosylated state (Bolander 1999). In particular, this post-translational modification appears to be arrested at the N-acetylglucosamine (NAG) transferase I step. However, the nitric oxide/cGMP signaling pathway rapidly shifts the prolactin receptor to the cell surface by stimulating the activity of this enzyme, which then completes terminal glycosylation. It was the purpose of this study to investigate the mechanism responsible for NAG transferase I regulation by nitric oxide/cGMP.

cGMP has three major targets: a protein kinase (protein kinase G, PKG), phosphodiesterases and ion channels (Schmidt et al. 1993). The stimulatory effect of cGMP on some phosphodiesterases is simply a mechanism for feedback inhibition and/or cross-talk with the cAMP pathway. The effect on cation channels could produce an increase in intracellular calcium, a known activator of nitric oxide synthase activity; this represents a positive feedback loop that would amplify the cGMP signal, rather than a primary effect. Therefore the most likely primary output of cGMP in the mammary gland is PKG. As there is a PKG consensus phosphorylation site in the sequence of mouse NAG transferase I (Pownall et al. 1992), it was of interest to determine if nitric oxide/cGMP could induce the acute phosphorylation of this enzyme in cell culture, if PKG could directly phosphorylate this enzyme, and if this modification had any effect on enzyme activity.

MATERIALS AND METHODS

Materials
Hepes, Tris, phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), EDTA, sodium pyrophosphate, Tween 20, Triton X-100, 4-chloronaphthol, phosphotungstic acid, ovalbumin, bovine serum albumin (BSA), goat antirabbit antibody, adenosine 5’ triphosphate (ATP), guanosine-3’,5’-cyclic monophosphate (cGMP), PKG (10 200 U/µg), alkaline phosphatase (2400 U/mg), UDP-N-acetylglucosamine (UDP-NAG) and UDP-N-acetyl-[6-3H(N)]glucosamine (60 Ci/mmol) were purchased from Sigma Chemical Company (St Louis, MO, USA). Nitrocellulose paper, goat antirabbit antibody conjugated...
to horseradish peroxidase, acrylamide, bisacryl-

amine, pre-stained low-range molecular weight

standards, and all other reagents for sodium dodecyl

sulfate (SDS) electrophoresis were obtained from

Bio-Rad Laboratories (Hercules, CA, USA). Phos-

phate buffered saline (PBS) and Medium 199 with

Earle’s salts were from Grand Island Biological

Company (Grand Island, NY, USA); collagen-

tase type I (179 U/mg) was purchased from

Worthington Biochemicals (Freehold, NJ, USA).

Sodium nitroprusside was from Alexis Corporation

(San Diego, CA, USA) and 8-bromoguanosine-

3’,5’-cyclic monophosphorothioate, Rp-isomer (a

PKG inhibitor, PKGI) was purchased from BioLog

Life Science (Bremen, Germany). X-Omat AR

X-ray film was obtained from Eastman Kodak

(Rochester, NY, USA). [32P]Orthophosphoric acid

(850 Ci/mmol) and [γ32P]-adenosine 5’ triphos-

phate ([32P]ATP; 3000 Ci/mmol) were from New

England Nuclear (Boston, MA, USA).

Cell culture

Virgin mice (C3H/HeN) were obtained from the

Frederick Cancer Research Facility (Frederick,

MD, USA). The mice were killed by cervical

dislocation and an epithelial cell-enriched fraction

was isolated from mammary glands as previously

described (Vonderhaar et al. 1973). Cells were

cultured for 30 min at 37 °C in Medium 199

containing 20 mM Hepes (pH 7.6) and combi-
nations of the following reagents as required by

the individual experiment: sodium nitroprusside

(100 µg/ml) and PKGI (250 µM). When proteins

were to be labeled in intact cells, cells were

equilibrated overnight with [32P]orthophosphoric

acid (50 μCi/ml) before the 30-min incubation.

N-Acetylglucosamine transferase I activity

NAG transferase I was measured by the method of

Vischer & Hughes (1981). Briefly, mammary

epithelial cells were homogenized or immuno-

precipitates suspended in 50 mM sodium phosphate

buffer (pH 6.9) containing 10 mM KCl, 5 mM

MgCl2, 5 mM MnCl2, 5 mM pyrophosphate, 0.1% Triton

X-100 and 2 µM PMSF. UDP-N-acetyl-

[3H]glucosamine and ovalbumin were added to the

reaction mixture to give a final concentration of

1 mM and 4 mg/ml, respectively, in a final volume

of 0.5 ml. After 2 h at 37 °C, the labeled ovalbumin

was precipitated by 1 ml 1% phosphotungstic acid in

0.5 M HCl, washed with 1 ml ice-cold 95% ethanol

and dissolved in 0.5 ml 0.5 M NaOH. After

neutralization, scintillation fluid was added and the

samples counted. When enzyme kinetics were

performed the ovalbumin substrate concentration

was varied, the incubation period shortened to

30 min in order to obtain initial reaction rates,

and the specific activity of the UDP-N-acetyl-

[3H]glucosamine doubled in order to obtain

measurable radioactive incorporation during the

shorter incubation period. Eadie–Hofstee plots

were performed in quadruplicate.

Antibody production and characterization

A peptide with a sequence corresponding to the

linker region of mouse NAG transferase I (residues

31–50; Pownall et al. 1992) was synthesized by

Genemed Synthesis (South San Francisco, CA, USA).

This peptide was then coupled to limpet

hemocyanin and used as an antigen to generate

polyclonal antibodies in New Zealand albino

rabbits by the Monoclonal Antibody Laboratory

at the University of South Carolina (Columbia,

SC, USA). The resulting antiserum was immuno-

adsorbed with hemocyanin before use.

Samples for western blots were subjected to SDS-

PAGE according to the method of Laemmli (1970) and

immunoblotted using the method of Hawkes et al.

(1982). Briefly, samples were separated on a 13 cm

slab gel containing 12.5% acrylamide after being con-

centrated on a stacking gel of 5% acrylamide. After the

proteins were transferred to nitrocellulose, the paper

was deblocked in PBS containing 3% BSA, rinsed in

PBS and incubated overnight with the NAG trans-

ferase I antibody (1:1000 in PBS with 1% BSA).

After rinsing in PBS, the nitrocellulose was incubated

with goat antirabbit antibody conjugated to horse-

radish peroxidase (1:1000 in PBS with 1% BSA) for

1 h, rinsed twice in PBS with 0.05% Tween 20 and

once in PBS. Bands were visualized by placing the

paper in a solution of 4-chloronaphthol (2.4 mg/ml)

and hydrogen peroxide (60 µl 30% solution in 100 ml

PBS). All of these procedures were performed at room

temperature. Densitometry readings were used to

adjust samples to identical enzyme concentrations for

subsequent experiments.

Radiolabeling of proteins

Proteins in intact cells were labeled as described

above (Cell culture). The labeling of purified

proteins was performed on NAG transferase I

immunoprecipitated by the method of Ono & Oka

(1980) except that the buffers contained 5 mM

pyrophosphate, a general phosphatase inhibitor.

Half of the immunoprecipitated enzyme was

incubated with unlabeled ATP, as described below,

and assayed for enzyme activity. The other half was

incubated with [32P]ATP and used to determine the

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incorporation of phosphorus-32. Briefly, the immunoprecipitate was suspended in 0·1 ml 50 mM Tris–HCl (pH 7·5), 10 mM MgCl2, 2 mM DTT, 0·2 mM EDTA, 5 U PKG, 0·5 µmol cGMP, and 0·2 mM ATP or [32P]ATP (1000 c.p.m./pmol). Parallel samples were incubated without PKG and cGMP and used as controls for contaminating kinases. All samples were incubated at 37 °C for 30 min, after which the immunoprecipitates were washed.

Dephosphorylation of the labeled enzyme was accomplished by alkaline phosphatase. Briefly, NAG transferase I immunoprecipitated from nitric oxide-stimulated cells was suspended in 0·1 ml 75 mM Tris–HCl (pH 9·0). To half of each sample, 5 U alkaline phosphatase were added; the other half also included 5 mM pyrophosphate as a control for specificity. Both halves were incubated at 37 °C for 30 min, after which the reaction was stopped by the addition of 1 µl 1 M DTT and the immunoprecipitates were washed. Enzyme activity was then measured as described above.

Incorporation of phosphorus-32 was assessed by SDS gel electrophoresis as described for western blots, except that the immunoprecipitates were dissociated in 4 M urea before loading onto the gels and after electrophoresis the gels were dried and exposed to Kodak X-Omat AR film for 1–3 days at −70 °C. Western blots were performed in quadruplicate.

A summary of these experiments and their rationale is given in Fig. 1.

RESULTS

The specificity and efficacy of the antiserum were determined by SDS gel electrophoresis and enzyme assays. The rabbit antimouse NAG transferase I antibody detected a single band with a molecular mass of 52·3 kDa (Fig. 2), which compares favorably with the theoretical value of 50·5 kDa (Pownall et al. 1992). In addition, the antiserum
precipitated more than 95% of the enzymatic activity from homogenates (data not shown).

NAG transferase I appeared to be basally phosphorylated (Fig. 2, lane 1) and this modification increased dramatically after the cells had been exposed to nitric oxide (lane 2). This effect appears to be mediated by PKG, as it was blocked by a specific PKGI (lane 3). In addition, the label could be partially removed from the immunoprecipitated enzyme by alkaline phosphatase (lane 4) and this dephosphorylation was blocked by a phosphatase inhibitor (lane 5).

Although these experiments implicate PKG in the regulation of NAG transferase I, they do not provide evidence for a direct modification of the enzyme. Although this glycosylating enzyme does have a phosphorylation consensus site for PKG, anchoring proteins and molecular scaffolds can compartmentalize kinases, thereby limiting their access to potential substrates (Schillace & Scott 1999). For example, it is possible that the observed phosphorylation was the result of a protein kinase cascade initiated by PKG; such a cascade leading from cGMP/PKG to mitogen-activated protein kinase has been demonstrated in several tissues (Callisen et al. 1998, Ho et al. 1999). Therefore the ability of PKG to label the purified enzyme was investigated. No labeling occurred in the presence of [32P]ATP when PKG was omitted from the reaction mixture, indicating that no contaminating kinases were coprecipitated with NAG transferase I (Fig. 2, lane 6). PKG was able to phosphorylate the enzyme directly (lane 7), and this labeling was markedly reduced by the simultaneous presence of a PKGI (lane 8), demonstrating the specificity of the phosphorylation.

To determine if this modification had any effect on NAG transferase I activity, split samples were subjected to enzyme assays (Fig. 3). There was a good correlation between enzyme phosphorylation and activity under all circumstances. In particular, NAG transferase I activities were increased in enzymes labeled either in intact cells or in purified proteins and were reduced in enzymes the phosphorylation of which was either blocked by a PKGI or removed by alkaline phosphatase.

In order to analyse the mechanism for this enhanced enzyme activity, Eadie–Hofstee plots were generated (Fig. 4). In these graphs, \( V_{\text{max}} \) is obtained from the \( X \)-intercept and \( K_m \) from the slope \( (m = -1/K_m) \). \( K_m \) for ovalbumin did not differ significantly between the control and nitric oxide-treated cells: \( 31 \pm 7 \mu \text{M} \) and \( 38 \pm 7 \mu \text{M} \) respectively (means ± s.e. for six determinations). However, \( V_{\text{max}} \) of the NAG transferase I immunoprecipitated from the nitric oxide-treated cells was twice that for the enzyme from control cells: \( 4.3 \pm 0.4 \mu \text{mol/min \cdot mg enzyme} \) and \( 2.1 \pm 0.2 \mu \text{mol/min \cdot mg enzyme} \) respectively (\( P<0.001 \)).

**DISCUSSION**

Members of this laboratory are primarily concerned with investigating the upregulation of prolactin receptors in mammary epithelium. Previous studies have shown that long-term upregulation is a result of increased transcription of the receptor gene (Bolander et al. 1997); however, there is also a rapid component that is independent of gene expression. This acute phase is caused by a redistribution of prolactin receptors from a substantial intracellular reservoir to the cell surface. This migration is induced by cGMP (Bolander 1998).

Cyclic nucleotides have been shown to be involved in the translocation of several receptors and transducing molecules; for example, cAMP has
been implicated in the nerve growth factor receptor (Meyer-Franke et al. 1998), the water channel aquaporin-2 (Fushimi et al. 1997), an epithelial chloride channel (Shintani & Marunaka 1996) and the cystic fibrosis transmembrane conductance regulator (Lehrich et al. 1998). Furthermore, cGMP is involved in the membrane recruitment of the dopamine receptor (Holtbäck et al. 1999).

Aquaporin-2 is a direct substrate of the cAMP-dependent protein kinase, and its phosphorylation is required for the redistribution of the water channel. However, the prolactin receptor has no consensus sites for PKG within its sequence (Davis & Linzer 1989, Clarke & Linzer 1993), therefore the effect of cGMP had to be indirect.

Subsequent studies demonstrated that the prolactin receptors from the internal pool were hypoglycosylated and that cGMP stimulated the maturation of this modification concomitant with translocation. Finally, it was shown that the receptors were arrested at the NAG transferase I step and that cGMP stimulated the activity of this enzyme (Bolander 1999). The present work completes the missing link in this pathway by demonstrating that cGMP increases NAG transferase I activity via PKG phosphorylation.

This method of receptor regulation is rather novel. The requirement for core glycosylation in the constitutive targeting of receptors to the plasmalemma has been well documented (Collier et al. 1993, Rodriguez et al. 1995, Couvineau et al. 1996, Hall et al. 1997, Buteau et al. 1998). However, the role of terminal glycosylation in the physiological regulation of receptor shuttling is less well studied. In fibroblasts, platelet-derived growth factor increases the receptors for insulin-like growth factor (IGF) by inducing 3-hydroxy-3-methylglutaryl-coenzyme A reductase, an enzyme required for the synthesis of the lipid carrier used in core glycosylation. The subsequent availability of this carrier stimulates the N-linked glycosylation of the IGF receptor and its appearance at the cell surface (Carlberg & Larsson 1996, Carlberg et al. 1996). Although this is an example of the transcriptional regulation of core glycosylation, as opposed to the acute regulation of terminal glycosylation, it does demonstrate that physiological systems can use receptor glycosylation as a molecular switch to shuttle proteins to different cellular locations. A potentially more relevant example is found in the intestinal guanylate cyclase C (GC-C) receptor, which is predominantly found intracellularly in a hypoglycosylated state during fasting. Feeding leads to both the maturation of glycosylation and the redistribution of GC-C to the cell surface (Scheving et al. 1996). Although the responsible second messenger has not been identified, the fact that the output of GC-C is cGMP and that many hormones can recruit their own receptors to the plasmalemma (Holtbäck et al. 1999) suggests that cGMP may be involved.

In summary, this study has shown that an enzyme of terminal glycosylation, NAG transferase I, can be acutely regulated by PKG phosphorylation. Furthermore, this increased activity enhances the capacity of the system to handle proteins such as the prolactin receptor, which are stalled in a hypoglycosylated state under basal conditions.

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