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Effect of Hyperthermia on Activity of Three Glycosyltransferases in Chinese Hamster Ovary Cells[1]

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ABSTRACT

We measured activities of three glycosyltransferases at various times during heat-induced thermotolerance development. Glycosyltransferases are normally located in the Golgi apparatus and catalyze cellular glycosylation reactions. UDP-Gal:*N*-acetylglucosamine β1,4-galactosyltransferase (β1,4-GalT) is known to participate in the formation of *N*-linked glycoproteins; when compared to cell survival, β1,4-GalT activity was significantly more heat resistant (50% loss of activity: 80 min, 45°C) and showed little elevation at a time when thermotolerance was fully expressed. However, β1,4-GalT activity increased twofold by 24-h postheating when thermotolerance had begun to decay.

Activity of β1,4-GalT was compared with glycosyltransferase activities that are considered to be specific for *O*-linked glycoproteins: UDP-Gal:*N*-acetylgalactosamine-β1,3-galactosyltransferase (β1,3-GalT), and UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase (GalNAcT). Heat-inactivation experiments with heating times up to 60 min at 45°C failed to reduce either activity below that of unheated control cells. Instead both β1,3-GalT and GalNAcT activity increased approximately twofold immediately after 10 min at 45°C. Activity of β1,3-GalT rapidly decreased with time after heating and returned to control levels by 6-h postheating. In contrast, GalNAcT activity continued to increase with time after 10 min at 45°C, and was 4.5-fold above unheated controls by 6-h postheating. GalNAcT activity returned to control levels 24- to 48-h postheating. A comparison with the cellular survival response showed that GalNAcT activity preceded thermotolerance expression by 2–4 h and also decayed more rapidly than heat resistance in thermotolerant cells. These data, together with other published results, suggest that expression of thermotolerance may be associated with enhanced glycosylation of intracellular proteins.

INTRODUCTION

Hyperthermia is currently being evaluated as an adjunct to conventional cancer therapy; biochemical consequences of hyperthermia and the cellular response to heat, however, are not yet fully understood (1). In mammalian cells, elevated temperatures in the range of 40–45°C are known to affect virtually all cellular structures and functions, including chromatin conformation, macromolecular synthesis, the synthesis of heat shock proteins, and cellular morphology (1). One of the first observable morphological alterations upon heating is an enlargement of the Golgi apparatus, first reported by Overgaard (2) as a "rapid conspicuous hypertrophic" response. Subsequently other investigators (3, 4) have confirmed this observation in other cell lines and under various experimental conditions.

The altered morphology of the Golgi apparatus may reflect a functional change of these organelles; the above studies, however, included no functional endpoint. The Golgi apparatus is the location of numerous enzymes involved both in protein glycosylation, processing of oligosaccharides, and in lactose biosynthesis (5). We wanted to determine the effect of hyperthermia and the development of thermotolerance on Golgi function, specifically on glycosyltransferase activity involved in *N- versus O*-linked glycosylation. The latter has been proposed to play a role in the expression of thermotolerance (6).

Activity of UDP-Gal[3]:*N*-acetylglucosamine β1,4-galactosyltransferase (β1,4-GalT: E.C. 2.4.1.38) has been utilized as a marker enzyme and/or as a functional probe of the Golgi apparatus, where it is localized in the *trans* cisternae (7, 8). UDP-Gal:glucopyranose β1,4-galactosyltransferase (EC 2.4.1.22) and β1,4-GalT probably represent the same enzyme; by kinetic, isoelectric, and immunochemical criteria they are closely related and occur ubiquitously (9, 10). Thus, β1,4-GalT catalyzes the transfer of galactose from UDP-Gal to a variety of acceptor substrates, *i.e.*, to glucose in lactose synthesis or to GlcNAc in glycoprotein synthesis (7). Galactosyltransferases themselves are glycoproteins (9, 10), containing 10–15% carbohydrate (11).

Localization of another galactosyltransferase, UDP-Gal:*N*-acetylgalactosamine β1,3-galactosyltransferase (β1,3-GalT: EC 2.4.1.74), overlaps with that of β1,4-GalT, but whereas β1,4-GalT acts on asparagine-linked oligosaccharides, β1,3-GalT appears to be specific for oligosaccharides linked through serine and/or threonine residues (11, 12) and plays a major role in the synthesis of glycoconjugates, such as mucins (12). Competition studies have shown that β1,4-GalT and β1,3-GalT activity is attributable to different enzymes with distinct and separate functions (11).

Another glycosyltransferase, UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase (GalNAcT: EC 2.4.1.41) is normally located in the Golgi complex and transfers α-GalNAc residues from UDP-GalNAc to specific serine or threonine residues of polypeptide chains. Since GalNAc is generally the linkage sugar in *O*-linked glycoconjugates (11–13), GalNAcT activity is a key step in *O*-linked glycoprotein synthesis.

In an independent study, we have observed the appearance of a glycoprotein with a molecular weight of 50,000 that correlated with the development of cellular thermotolerance in CHO cells (14). We hypothesized that glycosylation of the M_r 50,000 band might be preceded by enhanced activity of the Golgi apparatus. This study, therefore, was designed to measure the activity of β1,4-GalT, β1,3-GalT and GalNAcT in the same cell line after hyperthermia and during thermotolerance development.

MATERIALS AND METHODS

Details of cell culture, measurement of cell survival, and heating of culture flasks have been reported previously (15). Measurements of β1,4-GalT activity were performed as described by Verdon and Berger (9), based on labeling of ovalbumin with [^{14}C]UDP-Gal, its retention and quantitation on glass fiber filters. Specifically, a CHO cell preparation was obtained by plating cells 48 h prior to the experiment at a density of 6×10^5/T-75 flask. At the beginning of an experiment, each

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[3] The abbreviations used are: Gal, D-galactose; CHO, Chinese hamster ovary; GalNAc, *N*-acetylgalactosamine; GalNAcT, *N*-acetylgalactosaminyltransferase; GalT, galactosyltransferase; GlcNAc, *N*-acetylglucosamine; HSP, heat shock proteins.

flask typically contained 6–8 × 10^6 cells in exponential growth; these were heated, cells were trypsinized, washed in buffer, and resuspended in 0.1% Triton X-100/distilled water, pH 7.2, generally at a concentration of 10^6 cells/10 μl. Cells were disrupted by three cycles of freeze-thawing, unless noted otherwise (Fig. 1, *inset*), and kept on ice until addition of a total-cell lysate sample to the reaction mixture (see below).

Activities of the three glycosyltransferases were measured essentially under identical conditions, using appropriate acceptors and substrates (see below). Reaction solutions were prepared fresh for each experiment and contained (final concentration in the assay mixture): 43.8 mM Na cacodylate, pH 7.35, 8.75 mM $MnCl_2$, 47.2 mM NaCl, and the acceptor substrate. The cell lysate sample (10 μl) was mixed on ice with 35 μl of the reaction solution, and the assay was started by adding 5 μl of the radioactively labeled sugar donor to the reaction mixture. After an incubation period of 1 h, 30°C, the reaction was stopped with the addition of 1 ml ice-cold phosphotungstic acid (5% w/v). The precipitate was filtered (Whatman 934-AM, 1827-024) and washed twice with 2 ml of ice-cold ethanol (1% v/v). Filters were dried for 15 min at room temperature and added to scintillation vials containing 4 ml of toluene-based scintillation fluid. Results were corrected for controls corresponding to zero time incubation at 30°C; in representative experiments we also measured endogenous acceptor binding during 1 h at 30°C when the exogenous acceptor (ovalbumin, asialomucin, or apomucin) was deleted from the reaction mixture. The addition of either 2 mM ATP or 0.3 mM UMP (final concentration) to selected samples, designed to inhibit potential breakdown of the UDP-sugar donor by nucleotide pyrophosphatase (9), had no measurable effect on the activity of any of the three enzymes (data not shown). ATP or UMP, therefore, were not routinely included in the reaction mixtures.

For β1,4-GalT measurements, the sugar donor was uridine 5′-diphospho-D-[*U*-^{14}C]Gal (155 mCi/mmol, Amersham) at a final concentration of 50 μM, 5 μCi/ml; the acceptor substrate was ovalbumin, 20 mg per sample, equivalent to 407 μM (9). The fraction of radioactive counts that bound to endogenous acceptors, measured in representative experiments with unheated control cells after 1 h at 30°C without exogenous acceptor, relative to samples with exogenous acceptor, was 11.4%. The zero-time incubation control with ovalbumin bound 5.8% of radioactivity. Heat effects of endogenous binding are described below (Table 1).

Addition of protease inhibitors (phenylmethylsulfonyl fluoride, 1.73 mg/ml; leupeptin, 0.5 mg/ml; pepstatin, 0.1 mg/ml) to the reaction mixture had no effect on β1,4-GalT activity: under identical conditions acid-precipitable activity was 698 ± 49 and 695 ± 68 (N = 4) cpm/10^6 cells in the absence and presence of protease inhibitors, respectively. Sonication of cell preparations, to obtain a uniform suspension of cell lysate (1 min, maximum setting for microtip, Fisher sonic dismembrator 300), decreased precipitable counts to 40% of controls. Therefore, we used neither protease inhibitors, nor sonication in routine assays of glycosyltransferase activity.

Activities of β1,3-GalT and GalNAcT were measured as described by Elhammer and Kornfeld (12). β1,3-GalT assays, like those for β1,4-GalT, used labeled UDP-Gal as sugar donor, but asialomucin (7.5 mg/sample, Sigma Chemical Co., or prepared as described in Reference 12) as acceptor. Endogenous acceptor binding in control cells without *versus* with exogenous acceptor after 1 h, 30°C was 13.5% of radioactivity; the zero time incubation control was 10.6% in the same experiment.

The sugar donor and the acceptor substrate for GalNAcT assays were uridine diphospho-*N*-acetyl-D-[1-^{14}C]galactosamine (60 mCi/mmol, Amersham) and apomucin (0.175 mg/sample, prepared as described in Reference 16), respectively. The zero-time incubation control with unheated cells was 22.6% and the incorporation of radioactivity into control cells during 1 h at 30°C without exogenous acceptor ranged up to 67% in control cells, where GalNAcT activity was low. In thermotolerant cells with high GalNAcT activity, however, this fraction was approximately five times lower (see below, Table 1). Measured activities in Figs. 1, 2, 4, and 5 represent the average of two independent experiments, where each measurement was made in triplicate. Indicated errors represent the standard error of the mean, unless indicated otherwise.

Immunoperoxidase staining for β1,4-GalT in control and thermotolerant cells was based on a rabbit polyclonal antibody to human β1,4-GalT, previously characterized by Chatterjee *et al.* (10). Equal numbers of control and thermotolerant CHO cells were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Replicate lanes were either stained with amido black or incubated overnight in the cold with the β1,4-GalT antibody, washed and developed with a goat-antirabbit antibody linked to peroxidase (BioRad Horseradish peroxidase substrate kit, 172-1064) following the manufacturer's specifications. Neither antibodies for β1,3-GalT nor for GalNAcT were available (see below).

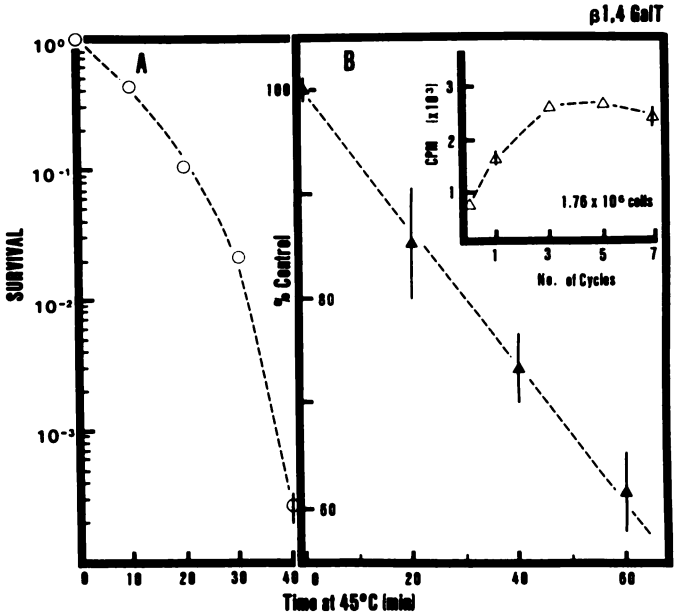

Fig. 1. Heat sensitivity of cell survival (*A*) *versus* activity of β1,4-GalT in CHO cells (*B*). *A*, semilogarithmic plot showing a typical cell survival curve with an exponential slope after an initial "shoulder," whereas *B* shows a linear decrease in enzyme activity (% *control*) with 45°C hyperthermia. The plot shown in the inset represents the amount of incorporated radioactivity, per 10^6 cells, as a function of the number of freeze-thaw cycles that were used to disrupt cells.

RESULTS

Cellular reproductive capacity was significantly more sensitive to 45°C hyperthermia than β1,4-GalT activity (Fig. 1). For example, cell survival after 40 min at 45°C was reduced to 2.6 × 10^{-4} (Fig. 1*A*), but the same heat treatment caused only a 27% loss of β1,4-GalT activity (Fig. 1*B*) in CHO cell lysates after three freeze-thaw cycles (Fig. 1*B*, *inset*). When cells were incubated at 37°C after hyperthermia of 10 min, 45°C, β1,4-GalT activity increased gradually and was twice that of unheated controls by 24-h postheating (Fig. 2). Careful measurements of β1,4-GalT activity at 6-h postheating, a time when thermotolerance is expressed (6), showed only a small (20–30%), but significant increase (Fig. 2, *inset*).

When thermotolerant and control cells were probed by immunoperoxidase staining on a Western plot, using a polyclonal antibody to human β1,4-GalT, thermotolerant cells showed only a small increase in staining over controls (Fig. 3). Fig. 3 shows replicate lanes of control and thermotolerant cells (10 min, 45°C + 6 h, 37°C) after visualization with amido black (Fig. 3, *left side*) or after immunoperoxidase staining (Fig. 3, *right side*). Protein bands stained with amido black are essentially identical; immunoperoxidase staining showed a band with M_r 61,000 both in lanes marked *C* and *h*. The M_r 61,000 bands are faint and appear to correspond to a major band on the *left side*. Densitometric scanning of the M_r 61,000 bands, using the photographic negative, showed that the area under the peak in

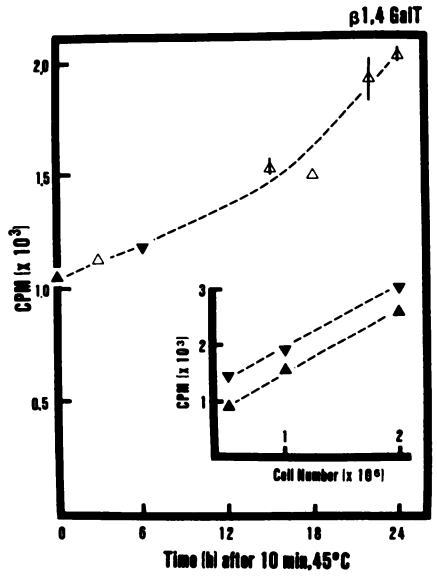

Fig. 2. Activity of β1,4-GalT at 0–24 h after 10 min, 45°C. Cells were heated and then incubated at 37°C, as indicated, and then disrupted with three freeze-thaw cycles and assayed for activity as described in the text. The ordinate shows cpm of radioactivity, per 10^6 cells, incorporated into the acid-precipitable fraction. The measurements represent the average of two independent experiments; *error bars*, SEM. *Solid symbols* for the 0- and 6-h points indicate identical treatment conditions to those in the *inset*. For the *inset*, enzyme activity was determined in separate experiments at three different cell concentrations for the 0- and 6-h posthyperthermia time point.

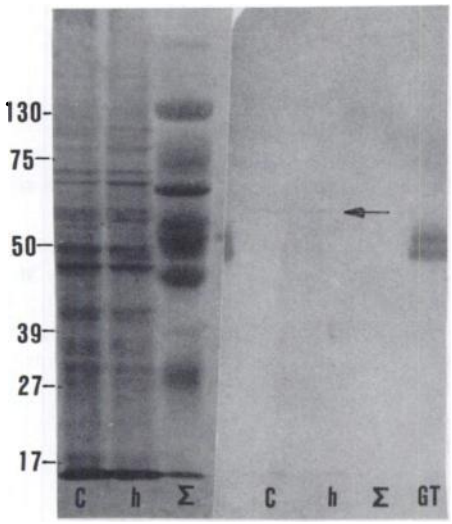

Fig. 3. Immunoperoxidase detection of β1,4-GalT in control *versus* thermotolerant cells. *Lanes on left*, portion of the nitrocellulose filter that was stained with amido black; *right*, immunoperoxidase-stained portion of the membrane. *Bands*, indicated by *arrow*, were quantitated separately by densitometry (see text). *Lane C*, control cells; *lane H*, equal number of cells that were heated 10 min at 45°C and then incubated 6 h, 37°C for thermotolerance development. *Lane GT*, enzyme purified from human milk; *lane* Σ, an enzyme preparation from bovine milk that was purchased from Sigma Chemical Co. The empty *lane* Σ, at *right*, shows lack of immunoreactivity between the polyclonal antibody and the bovine β1,4-GalT.

lane h was 25% larger than that in *lane C*. A replicate experiment included a 24-h time point when Fig. 2 shows β1,4-GalT activity to be twice that of control; in that experiment relative intensity of the M_r 61,000 band, determined by reflectance densitometry of the nitrocellulose filter, was 22 and 33% above that of controls at 6 and 24-h posthyperthermia, respectively.

Immunoperoxidase staining also included purified β1,4 GalT, isolated from human milk (*lane GT*) as a positive control. The soluble enzyme had a lower M_r of 49,000 and 53,000 compared to its membrane-bound homolog (11). Another sample of GalT derived from bovine milk (Sigma Chemical, No. G 5507; *lane* Σ), however, showed no detectable immunoperoxidase staining when lanes were loaded for equal amounts of (impure) protein (empty *lane* Σ, *right side*, Fig. 3).

Fig. 4 shows β1,3-GalT activity immediately after 45°C hyperthermia (Fig. 4*A*) and at various times after 10 min, 45°C (Fig. 4*B*). The 45°C dose response showed an approximate twofold increase in activity after 10 to 40 min, 45°C; only prolonged heat treatments of 50 to 60 min, 45°C reduced activity to near-control levels. When cells were incubated at 37°C following hyperthermia of 10 min at 45°C, β1,3-GalT activity decreased rapidly and returned to near control levels by 6 h when cells are known to be thermotolerant.

Fig. 5 shows similar data for GalNAcT activity: Fig. 5*A* describes the loss of activity as a function of heating time at 45°C, and Fig. 5*B* illustrates variation in activity as a function of time after 10 min, 45°C. As with β1,3-GalT, a mild heat treatment of 10 min at 45°C (survival = 45%, Fig. 1) increased GalNAcT activity approximately twofold. Prolonged heat treatments up to 60 min at 45°C failed to reduce GalNAcT activity below that of unheated controls (Fig. 5*A*). GalNAcT activity, measured at various times after 10 min at 45°C, progressively increased up to 4.5 times that of control cells (6 h). Between 24 and 48 h after hyperthermia, GalNAcT activity returned to that of unheated controls.

Measurements of glycosyltransferase activity could reflect both enzyme activity and/or binding of labeled donor to endogenous acceptors. The observed change in precipitable radioactivity following hyperthermia, therefore, could reflect either altered enzymatic activity or a heat-induced change in endogenous acceptor concentration. Heat-induced alterations in endogenous acceptor concentration were assessed by measuring radioactivity (UDP-Gal or UDP-GalNAc) that bound to cell lysate without addition of exogenous acceptor at various times

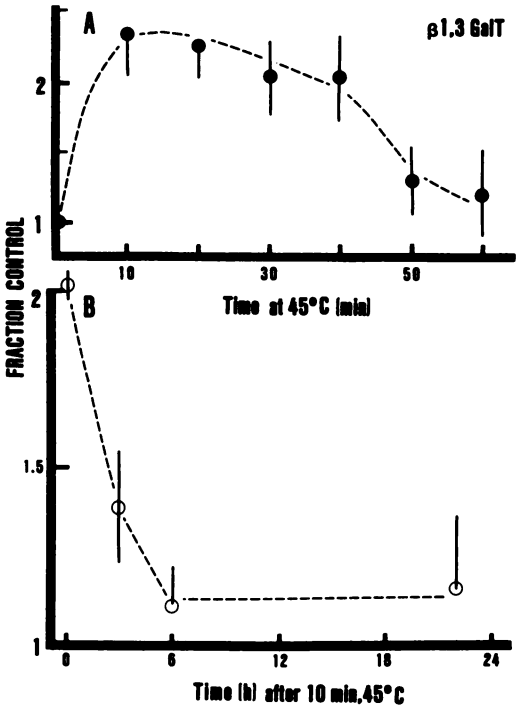

Fig. 4. Activity of β1,3-GalT after 45°C hyperthermia. *A*, enzyme activity measured immediately after hyperthermia lasting from 10 to 60 min at 45°C; *B*, activity measured at 0 to 24 h after a 10 min, 45°C treatment that was used to induce thermotolerance.

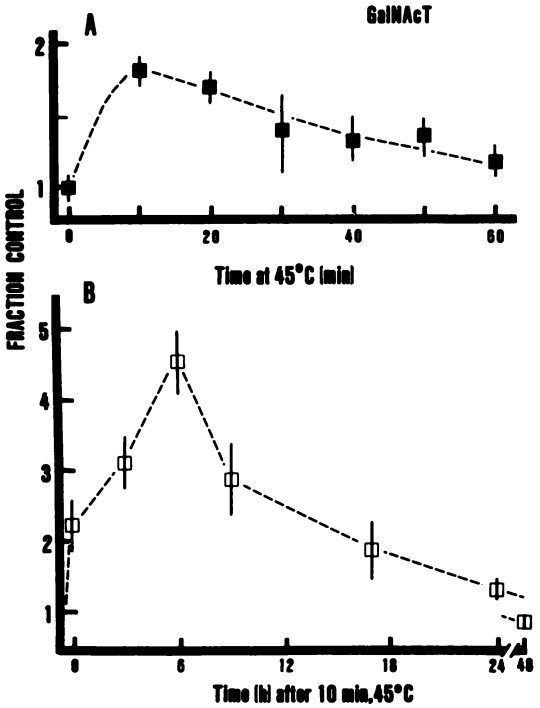

Fig. 5. Activity of GalNAcT after 45°C hyperthermia. *A*, heat response after 10–60 min, 45°C; *B*, activity at 0–48 h following hyperthermia of 10 min, 45°C.

Table 1 *Bound radioactivity[a] (cpm ± SD) of tritiated UDP-sugars to endogenous acceptors (2 × 10⁶ cells) at various times after 10 min, 45°C*

Treatment	UDP-Gal	UDP-GalNAc
Unheated control	522 ± 94	493 ± 42
10 min, 45°C + 0 h	575 ± 46	520 ± 43
10 min, 45°C + 6 h	528 ± 51	220 ± 47
10 min, 45°C + 24 h	530 ± 117	180 ± 31

[a] Bound radioactivity was determined after a 2-min, room temperature, incubation of the enzyme reaction mixture without exogenous acceptor and with cell samples representing 2 × 10⁶ cells.

after 10 min, 45°C. Binding was assayed after 2 min at room temperature (Table 1) and did not increase during thermotolerance development. Therefore, data in Figs. 1–4 reflect a true change in glycosyltransferase activity.

DISCUSSION

The results of this study may be related to the rapid, heat-induced "hypertrophy" of the Golgi apparatus, originally reported in 1976 by Overgaard (2). Glycosyltransferase activities, measured in this study, are normally localized in the Golgi apparatus, although probably not all within the same Golgi cisternae (12). We conclude that hyperthermia can selectively augment Golgi function and that altered function may be related to altered morphology.

The heat-induced increase in Golgi function showed a distinct time dependence for glycosyltransferase activities involved in *N- versus O*-linked glycosylation. Whereas β1,4-GalT increased slowly and steadily over 24-h postheating, both *O*-glycan-specific transferases showed a twofold increase in activity immediately after heating. The mechanism for these increased activities could not be addressed in this study, although the limited data obtained with the antibody to β1,4-GalT suggested that increased activity was partially accomplished by a modest increase in cellular enzyme concentration. Mechanistic studies of increased glycosyltransferase activity are generally complicated by the low immunogenicity of glycosyltransferases and considerable difficulties in purifying membrane-bound enzymes without loss of activity. The objective of our study was restricted to comparative measurements of enzyme activity under identical conditions at various stages of thermotolerance development. The unusual response of glycosyltransferases to heating *in situ* is all the more remarkable in light of their extreme susceptibility to heat denaturation upon isolation and purification (17).

The assay for β1,3-GalT activity is probably not as specific as that for β1,4-GalT or GalNAcT since the exogenous acceptor for β1,3-GalT, asialomucin, contains both GlcNAc and GalNAc residues (18). However, β1,3-GalT activity doubled at a time when activity of β1,4-GalT increased only slightly; we conclude, therefore, that our measurements of β1,3 GalT activity were not affected in a major way by the incorporation of radiolabeled Gal into *N*-glycans.

The data presented in this paper are raising numerous questions relating to the nature of the glycosylation substrates, their localization, and the regulation of glycosyltransferase activity after heating. Previous studies of factors controlling GalNAcT activity failed to identify specific amino acid sequence homologies for *O*-glycosylation (11). Instead, glycosylation of GalNAcT may involve enzyme recognition of three-dimensional acceptor conformations (11). This hypothesis could be relevant to heat shock conditions and putative function of HSPs in altering protein conformation (19, 20); these, in turn, could regulate intracellular GalNAcT activity.

The temporal relationship between the expression of thermotolerance and the activity of GalNAcT, following an identical heat treatment, is shown in Fig. 6. Both survival data, based on slopes of hyperthermia cell survival curves (21), and GalNAcT activity (from Fig. 5) are expressed as a fraction of control. Fig. 6 shows the development of thermotolerance lagging behind the increase in GalNAcT activity by 2–4 h, as hypothesized above (see "Introduction"). Similarly, GalNAcT activity returned more quickly to control levels than cellular heat sensitivity.

Thermotolerance expression generally correlates with HSP synthesis (22, 23) and the appearance of a glycosylation product with a molecular weight of 50,000 (14). One of the specific functions of the M_r 70,000 HSP (20, 21) involves the unfolding of proteins associated with transmembrane transport. This

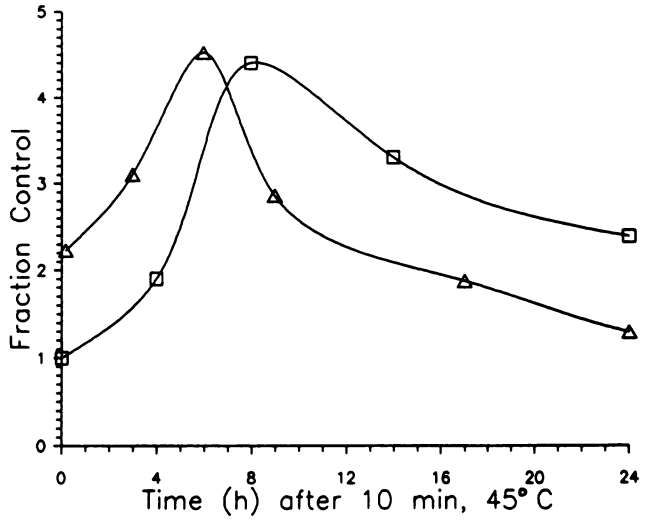

Fig. 6. Temporal relationship between GalNAcT activity (△) and thermotolerance (□) expression in CHO cells. Data for GalNAcT activity were taken from Fig. 4*B*, deleting error bars for clarity. Thermotolerance was also expressed as a fraction of control using the ratio of reciprocal slopes from survival curves, *i.e.*, D_0 values, from Reference 20.

function may facilitate cellular recovery from heat shock and contribute to enhanced cell survival of thermotolerant cells after hyperthermia. At the same time, enhanced protein glycosylation could protect specific intracellular sites against thermal denaturation and/or participate in protein redistribution. Increased activity of glycosyltransferase, *e.g.*, GalNAcT, preceding the expression of thermotolerance, would be consistent with this concept. Specific relationships between HSP function, elevated glycosyltransferase activity, the appearance of the M_r 50,000 glycosylation product and the phenomenon of thermotolerance, however, are still obscure and remain to be elucidated.

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