Heat-induced proteolysis of HSF causes premature deactivation of the heat shock response in Nb2 lymphoma cells

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Abstract Nb2-11 cells, a prolactin (PRL)-dependent T-lymphoma cell line, display an unusual response to heat stress characterized by the lack of expression of inducible *hsp70* mRNA transcripts and a reduction in the levels of constitutively expressed heat shock protein (HSP) genes. This aberrant heat shock response appears to result from heat-induced proteolytic fragmentation of heat shock factor (HSF). In this report, we have investigated processes that promote HSF fragmentation and identified characteristics of a protease that may be responsible for this effect. Cycloheximide did not affect HSF fragmentation of heat-shocked Nb2-11 cells suggesting that proteases responsible for this proteolysis are constitutively expressed and become activated by the heat shock conditions. PRL protected Nb2-11 cells from heat-induced fragmentation whereas sodium butyrate (NaBT) rendered a fragmentation-resistant cell line (Nb2-SFJCD1 cells) sensitive to HSF proteolysis. Heat-induced HSF fragmentation in Nb2-11 cells was not affected by pretreating cultures with several serine protease inhibitors. However, a dose-dependent decrease in HSF fragmentation was achieved by pretreating cultures with iodoacetamide, a cysteine protease inhibitor that is active in apoptosis. Apparently, the heat shock response in Nb2 cells is attenuated by a mechanism that involves the premature deactivation of HSF by its selective proteolysis. Attenuation of this critical cellular stress response may be an important contributor to the progression of hormone-dependent tumors possibly by influencing apoptotic processes known to regulate the activity of these cells.

INTRODUCTION

Most cells respond to heat stress by increased expression or induction of one or more of the highly conserved cellular stress response proteins, heat shock proteins (Hsps). However, a deficient Hsp response has been reported in a murine B-cell lymphoma cell line and in Y79 retinoblastoma cells (Mathur et al 1994; Davidson et al 1995). Likewise, we previously reported that rat Nb2-11 cells, a prolactin (PRL) dependent pre T-cell line, display reduced Hsp expression following exposure to elevated

temperatures (Blake et al 1995). After heat stressing these cells (41°C, 1 h), there was no evidence of inducible *hsp70* gene expression and expression of constitutive members of the Hsp70 and Hsp90 families were reduced. Gel mobility shift assays revealed that heat shock factor (HSF) was activated in spite of the lack of expression of inducible Hsp70 transcripts, although its DNA binding rapidly deteriorated. Most importantly, we found that heat shock caused fragmentation of HSF suggestive of proteolysis that may be responsible for the deterioration of heat shock element (HSE)-binding. Thus, the impaired Hsp response in Nb2 cells appears to occur as a result of the premature termination of HSF activity by its selective degradation rather than from an inhibition of its activation.

The molecular regulation of HSP expression has been studied extensively in cultured cells. Expression of the

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human constitutive *hsp70* gene(s) is cell cycle regulated (Milarski and Morimoto 1986) and is induced by both serum (Wu and Morimoto 1985) and the adenovirus E1a protein (Wu et al 1986). Constitutive Hsp70 transcription is regulated by a 'basal' promotor that is predominantly controlled by a CCAAT element at position -70 relative to the transcription initiation site (Wu et al 1986; Greene et al 1987) but also involves other *cis*-acting elements such as Sp1 and ATF (Morimoto et al 1992). The regulation of stress-inducible HSPs is controlled by pre-existing protein factors (HSF) that can be activated by heat shock or treatment with specific chemical agents (Pellham 1982; Holmgren et al 1986). In mammalian cells, the exact mechanism of this activation appears to involve the association of two or more HSF proteins. The multimerization of HSF confers DNA binding activity which allows the complex to bind to a conserved and repetitive DNA sequence (HSE) that has been identified in the promotor region of the *hsp70* gene (Morimoto et al 1994; Wu et al 1994). DNA-bound HSF then becomes activated, resulting in an increased transcription of the *hsp70* gene. Presumably, Hsp expression in all organisms is regulated by similar molecular response elements.

The PRL-dependent rat Nb2 lymphoma cell line was originally derived from a lymph node from an estrogen-treated Noble rat (Gout et al 1980) and is dependent upon lactogens (PRL, placental lactogens, or human growth hormone) for proliferation. A partially synchronous growth-arrest in the early G₁ phase of cell cycle can be induced in exponentially-growing Nb2-11 cell cultures by an 18–24 h incubation in lactogen-deficient medium. The addition of picogram quantities of PRL to such stationary cells reinitiates cell cycle progression (Buckley et al 1993). Thus, this line has proven invaluable for the study of PRL receptor coupling to a growth response.

In addition to the hormone-dependent lines (e.g. Nb2-11), several PRL-independent Nb2 sublines have been developed by lactogen starvation and cloning of surviving cells (Gout 1987). One such line, designated Nb2-SFJCD1, is completely autonomous but retains a partial sensitivity to the mitogenic effects of PRL (Buckley et al 1993). Compared to parent PRL-dependent Nb2 cultures, which are relatively benign when transplanted into Noble rats, Nb2-SFJCD1 cells are highly metastatic; they rapidly develop into lethal lung, kidney, liver and heart malignancies (Gout et al 1994). Therefore, the PRL-dependent Nb2 cells, together with the autonomous Nb2-SFJCD1 subline, provide a useful model system to investigate the molecular consequences of tumor progression from hormone (or growth factor)-dependency to tumor cell autonomy.

In this report, we employ the Nb2 cell model to investigate the molecular mechanisms responsible for the selective proteolysis of HSF that results in an impaired Hsp

response to heat shock. We determined that heat-induced HSF fragmentation is not dependent on new protein synthesis but rather may occur by the activation of an existing protease. This protease activity was blocked by pretreating Nb2 cells with PRL and could be activated in the hormone insensitive Nb2-SFJCD1 cells by treating them with a differentiating agent. Using several protease inhibitors, we determined that the protease responsible for HSF fragmentation is a cysteine protease that is selectively sensitive to iodoacetamide. HSF proteolysis may be an important general mechanism for the termination of the Hsp response that is activated prematurely in Nb2-11 cells which contributes to their hormone-dependent phenotype.

MATERIALS AND METHODS

Cell culturing

The PRL-dependent rat T lymphoma cell line, Nb2-11, originally cloned by Dr H. G. Freisen (Winnipeg, Canada) and the PRL-independent subline, Nb2-SFJCD1, developed from the parental line by lactogen starvation and cloning of surviving cells were obtained from Dr P. W. Gout (Vancouver, Canada). Nb2-11 cells were maintained at 37°C in Fischer's medium containing 10% fetal bovine serum (FBS; BioWhittaker, Walkersville, MD, USA) as a source of lactogen, 10% horse serum (HS; BioWhittaker), 2-mercaptoethanol (2-ME; 10⁻⁴ M), penicillin (50 U/ml), and streptomycin (50 µg/ml) as described by Gout et al (1980). For various experiments, cells were growth-arrested in the early G₁ phase of cell cycle by an 18–24 h preincubation in lactogen-free medium, i.e. Fischer's medium supplemented with 2-ME, antibiotics, and 10% non-mitogenic gelding serum (ICN, Irvine, CA, USA) (assay medium). The Nb2-SFJCD1 line was cultured in maintenance medium but without the addition of FBS. Under these conditions, the cells grow exponentially. In some experiments, Nb2-SFJCD1 cells were preincubated with 2 mM sodium butyrate for 72 h prior to additional treatments described.

Heat stress treatments

Growth-arrested Nb2-11 or similarly pretreated Nb2-SFJCD1 cells were suspended in pre-gassed assay medium (1–2 × 10⁶ cells/ml) in 50 ml conical culture tubes immersed in a water bath maintained at 41°C for 60 min. Subsequent to heat exposure, the cultures were returned to 37°C. The cultures were harvested at various timepoints for Northern and immunoblotting assays. Other chemical agents and a maximally mitogenic concentration of PRL (20 ng/ml) were added prior to heat shock in some experiments.

RNA isolation and northern hybridization analysis

Following exposure conditions, Nb2-11 and Nb2-SFJCD1 cells were disrupted in RNazol (Tel-Test, Inc.) using a Polytron tissue homogenizer (Brinkman Inst.) at high speed for 10 s. Total cellular RNA was isolated using the manufacturers protocol (Tel-Test, Inc.) and was quantitated spectrophotometrically. For Northern analysis, RNA samples were denatured in formaldehyde and fractionated in 1.0% agarose gels containing 2.2 M formaldehyde. RNA was transferred to Genescreen Plus membranes (DuPont) according to the manufacture's recommended conditions in $10 \times \text{SSC}$ ($1 \times \text{SSC} = 0.15 \text{ M}$ sodium chloride, 0.015 M sodium citrate) then baked for 2 h at 80°C . Filters were hybridized and washed at 65°C according to the procedure of Church and Gilbert (1984).

Protein extraction and western blotting

Cellular protein was extracted from Nb2-11 and Nb2-SFJCD1 cells by homogenization in ice-cold buffer containing 20 mM HEPES (pH 7.5), 1.5 mM MgCl_2 , 0.2 mM EDTA, 0.2 mM dithiothreitol, 0.4 M NaCl, 20% glycerol, 0.5 mM phenylmethylsulfonylfluoride (PMSF), 10–25 $\mu\text{g}/\text{ml}$ leupeptin and 10–25 $\mu\text{g}/\text{ml}$ aprotinin at 4°C . Insoluble material was removed by microcentrifugation (5 min) and protein in the supernatant was quantitated spectrophotometrically using a modified Lowry method (BIO-RAD). Individual protein samples (20 $\mu\text{g}/\text{lane}$) were fractionated on 12% SDS-polyacrylamide gels. Following electrophoresis, the proteins were transferred to Immuno-Lite Blotting Membrane (BIO-RAD). Membranes were blocked in 5% non-fat dry milk and immunoblotting was performed using rabbit polyclonal antibody that recognizes HSF1 (graciously provided by Dr R. I. Morimoto). A goat anti-rabbit IgG conjugated to alkaline phosphatase (1:3000) was used as a secondary antibody. Proteins were visualized utilizing a chemiluminescent reaction (Immun-Lite Assay Kit; BIO-RAD), followed by exposure to X-ray film.

DNA mobility shift assay

Nb2-11 or Nb2-SFJCD1 cells were homogenized on ice in a buffer containing 20 mM HEPES (pH 7.5), 1.5 mM MgCl_2 , 0.2 mM EDTA, 0.2 mM dithiothreitol, 0.4 M NaCl, 20% glycerol, 0.5 mM PMSF and 0.5 mM leupeptin. Insoluble cellular material was pelleted by microcentrifugation (4°C ; 5 min). Protein content in the resulting supernatant was determined using a modification of the Lowry assay (DC Protein Assay: BioRad, Richmond, CA, USA). Assays were performed using the GELSHIFT kit supplied by Stratagene (catalog no. 201001, Los Angeles, CA, USA). Ten micrograms of protein from cell lysates were incubated with 1 ng of ^{32}P -labeled oligonucleotide

corresponding to the HSE (5'-GCCTCGAATCTTCGC-GAACTTTTCG-3') on ice for 30 min in incubation buffer containing poly (dI/dC)•(dI/dC) (Sigma Chemical Co., St Louis, MO, USA). Specificity of the binding activity was determined by incubation of reaction lysates in the presence of competing unlabeled HSE (25 ng). Incubation reactions were fractionated on SDS-polyacrylamide gels, dried, then apposed to X-ray film for visualization.

Probes, labeling reactions and chemicals

A cDNA for Hsp70, isolated from a Chinese hamster ovary cell line (Fornace et al 1989), was provided by Dr, Albert J. Fornace. Purified inserts were labeled with ^{32}P -dCTP (DuPont-NEN, Boston, MA, USA) using the random primer method (Feinberg and Vogelstein 1983). Ovine PRL (NIDDK oPRL-20, AFP10677C) was obtained through the NIH Pituitary Hormone and Antisera Program (Rockville, MD, USA). The serine protease inhibitors TPCK (L-1-Chlor-3-(4-tosylamido)-4-phenyl-2-butanone), TLCK (L-1-Chlor-3-(4-tosylamido)-7-amino-2-heptanone-hydrochloride), Pefabloc (Pefabloc SCCAEBF hydrochloride) were purchased from Boehringer Mannheim (Indianapolis, IN, USA). The cysteine protease inhibitors, iodoacetamide (IOD) and N-ethylmaleimide (NEM), were purchased from Sigma Chemical Co. (St Louis, MO, USA). The ICE-like protease inhibitors ICE inhibitor I (reversible) and ICE inhibitor II (irreversible) were purchased from Calbiochem (La Jolla, CA, USA).

RESULTS

Fragmentation of HSF does not require synthesis of new proteins

Based on our previous work (Blake et al 1995), we hypothesized that the aberrant heat shock response of Nb2-11 cells is attributed to premature fragmentation of HSF. The fragmentation pattern elicited by heat shock suggested that specific proteases may mediate this response. To investigate whether de novo protein synthesis is required for this putative protease activity, stationary Nb-11 cells were treated with cycloheximide (CHX; 10 $\mu\text{g}/\text{ml}$) for 15 min, prior to exposure to heat shock (41°C , 1 h).

HSF fragmentation in control and CHX-treated Nb2-11 cells is displayed in Figure 1A. Characteristic of HSF activation, HSF migration was slightly retarded after 30 min of exposure in control and CHX-treated cells. This observed shift in mobility has been attributed to phosphorylation of HSF during the process of its activation (Cotto et al 1996; Liu and Thiele 1996). CHX pretreatment did not alter heat-induced HSF fragmentation in Nb2-11 cells. Fragmentation was apparent by 60 min and was maximal by 2 h in both control and CHX-treated

Fig. 1 Cycloheximide (CHX) pretreatment did not affect HSF fragmentation or HSP70 mRNA expression in Nb2-11 cells. Panel A: HSF fragmentation was assessed by immunoblotting in vehicle and CHX-treated Nb2-11 cells. Cells were untreated or treated with CHX (10 μ g/ml) 15 min prior to heat shock (1 h, 41°C). Cells were harvested 15 min after treatment (0 timepoints), at 30 and 60 min during heat exposure, and after 1 h of recovery from heat shock (R; 2 hr). Panel B: HSP70 mRNA expression was determined in CHX-treated Nb2-11 cultures by Northern blotting. Cells were treated with CHX as described in Panel A. HSP70 mRNA levels were determined in untreated control cultures (CTL), 15 min after CHX treatment, at 30 and 60 min during heat shock, and at 30 min, 1 h, and 2 h after removal from heat (Recovery). Panel C: HSP70 mRNA expression in untreated heat-shocked Nb2-11 cells. Cells were heat shocked for 1 hr beginning at the 0 timepoint. The 75 and 90 min timepoints represent samples taken after 15 and 30 min or recovery respectively.

cells. Additionally, the level of constitutive *hsp70* mRNA declined and no inducible Hsp70 transcripts were observed in CHX-treated cells (Fig. 1B). This mRNA response was similar to that observed in untreated Nb2-11 cells exposed to heat-shock (Fig. 1C). Collectively, these results indicate that protein synthesis is not a prerequisite for heat-induced HSF fragmentation in these cells. Apparently, the protease responsible for this fragmentation pre-exists in Nb2-11 cells and becomes activated by the heat shock conditions.

Prolactin blocks heat-induced HSF fragmentation and the decline of constitutive *hsp70* mRNA expression

Previously, we demonstrated that treatment of Nb2-11 cells with prolactin (20 ng/ml) for 6 h prior to heat shock (1 h, 41°C), prevented HSF fragmentation in Nb2-11 cells (Blake et al 1995). The effects of prolactin are thought to be mediated by activation of several cellular signal transduction pathways leading to increased transcription of a

Fig. 2 PRL prevents HSF fragmentation and the decline of HSP70 mRNA expression in heat shocked Nb2-11 cells. Growth-arrested Nb2-11 cells were treated with a mitogenic dose of PRL (20 ng/ml) for 0, 0.5, 1, 3, 4, 5 and 6 h then heat shocked for 1 h (41°C) followed by a 1 h recovery at 37°C. HSF fragmentation determined by immunoblotting is displayed in the upper panel. HSP70 mRNA expression in identically treated cultures is displayed in the lower panel.

variety of growth-related genes (Buckley et al 1995; Horseman 1995; Rao 1995). Apparently, these intracellular signalling pathways are coupled to the inhibition of the activity of a protease capable of degrading HSF. Thus, prolactin is potentially a useful tool for investigating the regulation of the protease activity responsible for the degradation of HSF.

To investigate the temporal relationship between prolactin treatment and the inhibition of HSF protease activity, stationary Nb2-11 cells were treated with prolactin (20 ng/ml) for 0, 0.5, 1, 2, 3, 4, 5, or 6 h before exposure to a 1-h heat shock and subsequent 30-min recovery period. Both HSF fragmentation and expression of constitutive *hsp70* mRNA expression was determined at each time point (Fig. 2). Reduced HSF fragmentation was observable in heat-shocked cells after just 1 h of preincubation with prolactin. Maximally protective effects of prolactin were apparent by 2 h. Thereafter, very little, if any, HSF fragmentation was observed at any timepoint to 6 h.

Expression of constitutive *hsp70* mRNA paralleled HSF fragmentation. Levels were markedly reduced in non-treated cells, but showed gradual improvement with continued exposure to prolactin. Although we originally believed that HSF fragmentation was not directly related to the decline in constitutive *hsp70* mRNA expression, these results suggest that they are not completely independent events.

To determine whether continuous exposure to prolactin was required for its protective effects, Nb2-11 cells were treated with prolactin for 2 h then washed free of the lactogen before the cells were exposed to the heat shock regimen. Results of this experiment showed that HSF fragmentation was reduced and constitutive *hsp70* mRNA expression was conserved as it was at the 2-h time point shown in Figure 2. Thus, prolactin protection requires a minimum 2 h treatment but is not needed in the media during heat shock to produce its effects.

The heat shock response of sodium butyrate-treated Nb2-SFJCD1 cells is similar to Nb2-11 cells

Nb2-SFJCD1 cells do not require prolactin for maintenance of growth and are highly metastatic *in vivo* (Gout et al 1994). Karyotypic analysis of this line indicates that it arose from the parental cells by clonal expansion. Unlike heat shocked Nb2-11 cells, HSF fragmentation in Nb2-SFJCD1 cells is not as prominent, constitutive *hsp70* mRNA expression does not decline with heat stress, and inducible *hsp70* mRNA transcripts can be detected (Blake et al 1995). Treatment of these cells with the differentiating agent, sodium butyrate (NaBT), causes them to revert to a phenotype where PRL is required for proliferation and where they display cell cycle characteristics and expression of genes associated with proliferation similar to Nb2-11 cells (Buckley et al 1996; Gout 1987). Thus, while prolactin appears to negatively regulate the activity of a heat sensitive HSF proteolytic factor, NaBT may be a positive regulator of it in the Nb2 cell model system.

To investigate the regulatory effects of NaBT on HSF fragmentation, exponentially growing Nb2-SFJCD1 cells were incubated with NaBT (2 mM) for 72 h, which induces growth arrest in the G₁ phase of the cell cycle (Gout 1987). The cells were then washed to remove NaBT, resuspended in assay medium lacking PRL, then heat shocked for 1 h at 41°C. NaBT-treated and untreated cells were harvested at 0.5, 1.0, 1.5, 2.0 and 4.0 h after heat shock then assayed for HSF fragmentation and *hsp70* mRNA expression. In untreated cells, slight HSF fragmentation was observed 90 min after heat shock that worsened somewhat by the 2- and 4-h timepoints (Fig. 3A). In NaBT-treated cells, fragmentation was detectable by 60 min and marked fragmentation was evident at 1.5, 2.0 and 4.0 h after heat shock. Apparently, NaBT increased the sensitivity of Nb2-SFJCD1 cells to heat-induced HSF fragmentation possibly through modification of a specific protease.

HSP70 mRNA expression in the same samples are presented in Figure 3B. Untreated Nb2-SFJCD1 cells displayed a characteristic increase in *hsp70* mRNA transcripts that included elevations in expression of both constitutive and inducible members of the *hsp70* gene family. In NaBT-treated cells, the response was qualitatively distinct from that observed in either untreated Nb2-SFJCD1 or Nb2-11 cells. No inducible *hsp70* mRNA transcripts were detected at any time after heat shock in cultures exposed to NaBT. Interestingly, levels of constitutive *hsc70* mRNA did not decline. Rather, levels of this transcript actually increased in response to the heat treatment. Although NaBT reverts Nb2-SFJCD1 cells to an Nb2-11-like phenotype in terms of HSF fragmentation, this does not appear to be directly coupled to expression of *hsp70* mRNA transcripts.

Fig. 3 Sodium butyrate (NaBT) augments HSF fragmentation in PRL-independent Nb2-SFJCD1 cells. NaBT-treated Nb2-SFJCD1 cells were incubated with NaBT (2 mM) for three days prior to heat shock. Treated and untreated cultures were heat shocked (1 h, 41°C) with cultures being harvested after 0, 30 and 60 min of heat stress and after 0.5, 1 and 3 h of recovery. Panel A: HSF fragmentation assayed by immunoblotting in control and NaBT-treated Nb2-SFJCD1 cells. Panel B: Northern blots displaying HSP70 mRNA expression in identically treated cultures. Molecular weight markers are indicated on the left.

Prolactin reversed the effects of NaBT on the heat shock response of Nb2-SFJCD1 cells

In the preceding experiments, prolactin prevented heat-induced HSF fragmentation in Nb2-11 cells whereas NaBT augmented fragmentation in Nb2-SFJCD1 cells. To further define the regulatory effects of PRL and NaBT on this putative protease activity, these agents were administered in combination to Nb2-SFJCD1 cells prior to heat shock.

NaBT-treated SFJCD1 cells were incubated with PRL (20 ng/ml) or vehicle 6 h prior to heat shock (41°C, 1 h). Cells were again harvested at 0.5, 1.0, 1.5, 2.0 and 4.0 h after heat shock and assayed for HSF fragmentation and *hsp70* mRNA expression. Prolactin partially reversed the effects of NaBT on both HSF fragmentation and expression of *hsp70* mRNA transcripts (Fig. 4). Inducible *hsp70* mRNA transcripts could be detected in PRL-treated cultures at 2.0 and 4.0 h after heat stress but not in cultures that received NaBT treatment alone (Fig. 4A). Correspondingly, HSF fragmentation was less in cultures receiving PRL and NaBT than in cultures that received only NaBT (Fig. 4B). These results are significant in that they identify agents that are either positive or negative regulators of the activity of the protease that degrades HSF in Nb2-11 cells. Since the result of HSF degradation is to inhibit *hsp70* gene transcription, these agents can effectively activate or inhibit the heat shock response in this model system.

Fig. 4 PRL reversed the effects of NaBT on the heat shock response of Nb2-SFJCD1 cells. Two cultures of Nb2-SFJCD1 cells were treated with NaBT (2 mM) for three days. One culture was then incubated with PRL (20 ng/ml) for 6 h prior to heat shock. Both cultures were heat shocked (41°C, 1h), then allowed to recover for up to 3 h at 37°C. Samples from both cultures were collected after 0, 30 and 60 min of heat stress and after 0.5, 1 or 3 h of recovery. Panel A: HSF fragmentation assayed by immunoblotting. Panel B: Northern blots displaying HSP70 mRNA expression in identically treated cultures. Molecular weight markers are indicated on the left.

A select cysteine protease inhibitor blocked HSF proteolysis in heat-shocked Nb2-11 cells

Our previous data established that HSF proteolytic activity was not regulated simply by increased synthesis of a protease. Moreover, this activity was negatively affected by PRL and positively affected by NaBT administered prior to heat shock. Irrespective of this regulation, it was evident that cellular events initiated by heat exposure were ultimately responsible for its activation. To further characterize this protease activity, Nb2-11 cells were treated with a series of protease inhibitors with differing specificities prior to exposure to heat shock in an attempt to block HSF fragmentation.

Nb2-11 cultures were first exposed to several serine protease inhibitors (Fig. 5). The effects of Pefabloc SC (PEFA) on HSF fragmentation induced by heat shock is shown in Figure 5A. PEFA did not inhibit fragmentation and may have even accelerated it since a greater degree of fragmentation was apparent at 30 min in the treated cells compared to controls. In addition to PEFA, two additional serine protease inhibitors, TLCK and TPCK, were screened for their ability to alter HSF fragmentation in response to heat shock. None of these compounds reversed the decline in constitutive *hsp70* mRNA expression characteristic of the response in these cells (Fig. 5B). Additionally, TCPK and TLCK did not alter HSF fragmentation patterns when administered prior to heat shock (data not shown).

The effect of several cysteine protease inhibitors on HSF fragmentation in Nb2-11 cultures was also determined. Duplicate results employing one such inhibitor, IOD, are displayed in Figure 6. Nb2-11 cells were treated

Fig. 5 Serine protease inhibitors had no effect on the heat shock response in Nb2-11 cells. Three separate cultures of Nb2-11 cells were treated with TLCK (100 μ M, 1 h), TPCK (25 μ M, 1 h), or Pefabloc[®] Sc (2 mM, 15 min) dissolved in culture medium. Untreated cultures served as controls. Immediately following treatment, the cultures were heat stressed (1 h, 41°C) then samples were harvested after 30 and 60 min of heat stress and after a 1 h recovery. Panel A: Immunoblots displaying HSF fragmentation in control and Pefabloc[®] Sc-treated cultures. Panel B: Northern blots displaying HSP70 mRNA expression in control (CTRL) cultures and cultures treated with Pefabloc Sc, TLCK or TPCK. Molecular weight markers are indicated on the left.

with increasing concentrations of IOD (10, 50 and 100 μ M) for 1 h, followed by a 1 h heat shock (41°C) and subsequent 30 min recovery at 37°C. Experimental controls included cells that were not exposed to heat shock or IOD (C) and cells that were heat shocked in the presence of vehicle only (Veh).

Characteristic HSF fragmentation was observed in vehicle-treated cultures. The lowest concentration of IOD (10 μ M) appeared to exacerbate heat-induced HSF fragmentation. In stark contrast, virtually no fragmentation was apparent in cells treated with either 50 or 100 μ M IOD. Interestingly, the level of constitutive *hsp70* mRNA expression displayed a close correlation with HSF fragmentation patterns. Constitutive *hsp70* mRNA was reduced in vehicle-treated cultures, almost non-existent in cells treated with 10 μ M IOD, but dramatically improved in cells treated with either 50 or 100 μ M IOD.

In addition to IOD, other cysteine protease inhibitors that were tested for their ability to prevent heat-induced HSF fragmentation in Nb2-11 cells included N-ethylmaleimide (NEM), and two ICE-like proteases (interleukin-1 β converting enzyme), one that is reversible (ICE-inhibitor I) and another that is irreversible (ICE-inhibitor II). None of these protease inhibitors blocked HSF fragmentation in heat-shocked Nb2-11 cells. The two inhibitors of ICE-like proteases had no effect at

Fig. 6 Iodoacetamide (IOD) blocks heat-induced proteolysis of HSF and the decline of HSP70 mRNA in Nb2-11 cells. Growth-arrested Nb2-11 cells were treated with vehicle or 0.01, 0.05 or 0.1 mM of IOD for 1 h prior to heat shock (41°C, 1 h) then allowed to recover for 30 min at 37°C. Upper panel: HSF fragmentation assayed by immunoblotting. Lower panel: Northern blots displaying HSP70 mRNA expression in identically treated cultures. Molecular weight markers are indicated on the left.

Fig. 7 Iodoacetamide does not prevent heat-induced fragmentation of the transcription factors STAT5B or STAT1. Growth-arrested Nb2-11 cells were exposed to heat shock conditions (41°C, 1 h) or IOD (0.05 mM) for 1 h prior to heat shock. Cells were harvested at 15, 30, 60 or 120 min from the initiation of the heat shock. Control cultures (C) received no treatment; – indicates IOD treatment but no heat shock. Panel A: Representative blot probed with an antibody that recognizes STAT5B. Panel B: Representative blot probed with an antibody that recognizes STAT1. Molecular weight is indicated on the left.

concentrations up to 20 μ M. NEM was also not effective. Moreover, concentrations of NEM greater than 100 μ M were cytotoxic. Collectively, these results suggest that the protease responsible for HSF fragmentation was a cysteine protease that is distinct from ICE that is selectively sensitive to IOD.

To assess the specificity of the protective effects of IOD on HSF fragmentation, the stability of STAT5B and STAT1 transcription factors following heat shock and IOD treatment was determined in Nb2-11 cells. These transcription factors were chosen because they have been implicated in the signal transduction cascade that supports the proliferative effects of prolactin and are thought to be regulated by specific proteases (Azam et al 1997). The level of both STAT5B and STAT1 declined after heat

treatment (Fig. 7). The loss of signal was near complete for both proteins after 60 min. Although no smaller fragments of STAT5B were detected, a smaller immunogenic fragment of STAT1 was observed suggesting both of these factors were sensitive to proteolysis induced by our heat shock conditions. In contrast to HSF, fragmentation of these proteins was not affected by prior administration of IOD.

Others have reported that IOD itself can activate HSF at concentrations similar to those used in this report in a porcine renal epithelial cell line (Liu et al 1996). If so, it is possible that IOD generates an activated form of HSF that is resistant to degradation. To address this possibility we exposed NB2-11 cells to IOD then assessed its effects on HSF migration and HSE binding activity. Although heat shock slowed HSF migration, indicative of HSF activation by phosphorylation, IOD was without effect (Fig. 8A). Furthermore, HSE binding activity was not different between control and IOD-treated cultures (Fig. 8B). These data indicate that HSF does not become activated by IOD in our Nb2 cell culture system.

DISCUSSION

An important aspect of *hsp* gene expression is that the response is transient. That is, the heat shock response is initiated by cell stress but will eventually abate even in the continued presence of the stressor. Although many reports have focused on the mechanisms whereby HSF initiates *hsp* gene expression, few studies have addressed the mechanisms involved in terminating the heat shock response. Phosphorylation of HSF has been suggested to serve as a regulatory mechanism to deactivate HSF in yeast (Hoj and Jakobsen 1994). In the Nb2 cell model, it appears that termination of the heat shock response is mediated by a rapid proteolysis of HSF. The deactivation of *hsp* gene expression is central to our investigations since HSF activation appears normal in Nb2 cells.

Results of this report show that HSF migration was retarded on polyacrylamide gels which has been used as an index for HSF phosphorylation and activation (Cotto et al 1996; Liu and Thiele 1996). In previous work, we showed that HSE-binding activity, assessed by gel mobility shift assay, was augmented by heat shock in Nb2-11 cells (Blake et al 1995). However, the binding activity quickly deteriorated coincident with HSF fragmentation. Thus, initiation of HSF activity does not appear to be impaired, rather, there seems to be a premature termination of its action. Apparently, normal mechanisms regulating the transient nature of the HSP response are accelerated to such an extent in Nb2 cells that stress-induced transcription is greatly reduced.

The degradation patterns of HSF that are generated by heat shock suggest that HSF is fragmented by protease

Fig. 8 Iodoacetamide does not activate HSF in Nb2-11 cells. Growth arrested Nb2-11 cells were exposed to heat shock (41 °C) then harvested at 15 min, 30 min or 60 min of exposure. Separate cultures were exposed to iodoacetamide (0.01, 0.05 or 0.1 mM) then harvested after 1 h. Control cultures (C) received neither heat shock nor iodoacetamide. Panel A: HSF migration in protein samples from each treatment group assessed by immunoblotting. Panel B: HSE binding in protein samples from treatment groups assessed by gel retardation assay. Lane 1 (—) is a protein sample taken from heat-stressed (41°C) Nb2-SFJCD1 cells, serving as a positive control. Lane 2 (HSE) is a sample that was preincubated with unlabeled HSE (10X), serving to define specific HSE binding activity. HSF: specific HSE binding activity; NSB: non-specific binding activity; F: free probe.

activity. The proteolysis of transcription factors was not unique to HSF as both STAT5B and STAT1 factors suffered a similar proteolytic demise. This protease activity appears to be present constitutively in Nb2-11 cells since cycloheximide did not alter HSF fragmentation (Fig. 1). HSF became fragmented following heat shock indicating that protease(s) responsible for the fragmentation become activated following exposure to heat. As is evident in all our Western blots, HSF is also phosphorylated prior to proteolysis in these cells suggesting the protease can recognize active forms of HSF. It seems unlikely that heat would activate a protease that attenuates the

response it normally initiates, unless the activity of this protease reflects a general mechanism used to terminate Hsp expression that has gone awry in Nb2 cells.

PRL is a pituitary hormone and cytokine that regulates diverse physiological processes, such as reproduction, immune function, fluid balance, and cellular growth and differentiation. Additionally, a direct mitogenic action of PRL has been well documented in the Nb2 cell model (Gout 1980). Addition of PRL to quiescent Nb2-11 cells initiates cell cycle progression and is associated with expression of growth-related genes. In this report, we demonstrated that PRL also prevented heat-induced fragmentation of HSF when administered at least 2 h prior to heat shock (Fig. 2). In unpublished experiments, we determined that CHX blocks this effect of PRL to prevent HSF degradation. Thus, PRL may regulate HSF fragmentation by inducing expression of a factor that inhibits the activity of the protease that degrades HSF. However, we have also found that incubation of Nb2-11 cells with CHX or actinomycin-D for extended periods will also cause HSF fragmentation without prior exposure to heat shock. A definitive answer to whether PRL's protective effects are mediated by synthesis of new proteins or modification of pre-existing proteins awaits further investigation.

Butyrate is a four-carbon, diet-derived fatty acid that inhibits proliferation and induces differentiation in numerous cellular systems (Tsao et al 1982; Herz and Halwer 1983; Gum et al 1987). Exposure of PRL-independent Nb2-SFJCD1 cultures to millimolar concentrations of NaBT has been shown to transiently revert these cells to a PRL-dependent phenotype for proliferation (Gout 1987). Nearly 90% of NaBT-treated Nb2-SFJCD1 cells were observed to be arrested early in the G₁ phase of cell cycle after a 72 h exposure. Addition of PRL to these growth-arrested cells reinitiated cell cycle progression characterized by the rapid expression of immediate early genes and proliferation characteristic of the PRL-dependent Nb2-11 cell line (Buckley et al 1995, 1996). Thus, prior exposure of Nb2-SFJCD1 cells to NaBT differentiates this line to one that is very similar to Nb2-11 cells with respect to growth factor requirements and associated gene expression.

The results presented in this report further support this interpretation and suggests a mechanism that may underlie the effect of NaBT in Nb2-SFJCD1 cells. Untreated Nb2-SFJCD1 cells exhibited an expected, albeit somewhat attenuated, heat shock response (Fig. 3). Inducible Hsp 70 transcripts were detectable and HSF remained relatively intact following heat exposure. In heat-stressed NaBT-treated cultures, no inducible Hsp70 transcripts were expressed and HSF was markedly fragmented. Thus, NaBT treatment appeared to have rendered Nb2-SFJCD1 cells sensitive to the proteolytic destruction of HSF similar to the Nb2-11 line.

Importantly, PRL reversed the effects of NaBT by restoring heat-induced *hsp70* mRNA transcription and attenuating HSF fragmentation. As with PRL, NaBT markedly affects selected gene transcription (Gupta et al 1994; Buckley et al 1996) as a component of its differentiating effects. Thus, addition of PRL to either Nb2-11 or NaBT-pretreated Nb2-SFJCD1 cells may induce expression of a protease inhibitor which, in turn, prevents heat-induced HSF fragmentation. Alternatively, NaBT may directly regulate expression of the protease that degrades HSF.

The cysteine protease inhibitor, IOD, displayed a dose-dependent ability to protect HSF from heat-induced fragmentation (Fig. 6). This effect of IOD appears to be very selective since several serine protease inhibitors and even other cysteine protease inhibitors were ineffective. Furthermore, IOD attenuated fragmentation of HSF by heat shock while having no effect on fragmentation of STAT5B or STAT1. Even though several transcription factors may become fragmented by our heat shock conditions, only HSF is protected by prior treatment with IOD.

IOD inhibits the activity of CPP32, an ICE-like protease that belongs to a family of cysteine proteases that are active in apoptosis (Darmon et al 1995; Lippke et al 1996). Nb2-11 cells are highly sensitive to the apoptotic effects of dexamethasone whereas Nb2-SFJCD1 cells are relatively resistant to apoptosis but become more sensitive if treated with NaBT. Although it is highly correlational at this point, there seems to be a close association between HSF fragmentation and the sensitivity of Nb2 cells to programmed cell death. Specifically, apoptosis occurs more readily in cells with a compromised Hsp response (i.e. Nb2-11 and NaBT-treated Nb2-SFJCD1 cells).

Both PRL and IOD preserved expression of constitutive *hsp70* mRNA transcripts in heat shocked Nb2-11 cells (Fig. 6). Expression of the human constitutive *hsp70* gene(s) is cell cycle regulated (Milarski and Morimoto 1986) and is predominantly controlled by a CCAAT element at position -70 relative to the transcription initiation site (Wu et al 1986; Greene et al 1987). However, we found that the decline of constitutive *hsp70* mRNA in heat-shocked Nb2-11 cells occurs primarily as a result of its reduced stability rather than from a defect in transcriptional regulation. The loss of constitutive Hsp70 transcripts coupled with the absence of inducible transcripts due to HSF fragmentation effectively eliminates the heat shock response in Nb2-11 cells. The co-regulation of these distinct but related events and their relationship to apoptosis are a major focus of our continued investigation.

The proposed functions of Hsps are diverse but center around their ability to complex with other proteins. As a result of this interaction, important cellular proteins may be transported to their appropriate intracellular

compartment, protected from the adverse effects of environmental and metabolic stressors, or allowed to achieve the conformation necessary for optimal cellular function. Given the fundamental nature of the action of Hsps, it is not surprising that the heat shock response is one of the most highly conserved cellular stress responses known. Although heat shock gene expression may be induced by several mechanisms, the activation of HSF has been consistently shown to be a key regulator of this response. In this report, we identified mechanisms that support premature proteolysis of HSF that contribute to the abnormal heat shock response of a pre T-lymphoma cell line. HSF proteolysis appears to be a mechanism that has emerged in Nb2 cells to reduce their normal HSP response. Attenuation of this critical cellular stress response may be an important contributor to the progression of hormone-dependent tumors possibly by influencing apoptotic processes known to regulate the activity of these cells.

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