Heat shock protein 70 (Hsp70)-stimulated deoxycytidine deaminases from a human lymphoma cell but not the activation-induced cytidine deaminase (AID) from Ramos 6.4 human Burkitt's lymphoma cells

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Abstract Deoxycytidine deaminase enzyme activity was reduced in lysates of human leukemic THP1 cells 24 h after transfection with siRNA designed to inhibit cell synthesis of heat shock protein 70 (Hsp70)1a and Hsp701b. The cytidine deaminase enzyme activity from the cell lysates was purified from an affinity column which contained bound single-stranded oligodeoxycytidylic acid. Deficient enzyme activity in certain elution fractions from the siRNA-transfected cells was restored by including recombinant HSP 70 in the assays. Enzyme activity in some other fractions was increased after siRNA transfection. Activation-induced cytidine deaminase (AID) is a central factor in the immune response. A more specific assay for AID was used to study the influence of Hsp70 on AID activity. Unlike Hsp70's ability to stimulate certain enzymes of DNA base excision repair and other cytidine deaminases, it had little effect on AID activity in vitro, or was weakly inhibitory.

Keywords Hsp70 · AID · Cytidine deaminases

Introduction

Roles for heat shock protein 70 (Hsp70) in the immune response have regularly been proposed. For example, receptors for Hsp70 in antigen presenting cells have been described (Liu and DeFillippo 2002; Binder et al. 2004). Furthermore, Hsp70-peptide complexes strengthen peptide immunogenicity (Moroi et al. 2000; Enomoto et al. 1999;

Calderwood and Gong 2006), perhaps by chaperoning immunogenic peptides to enter cell nuclei. Hsp70 enters cells of hemopoietic origin and proceeds from the cell cytoplasm to enter cell nuclei. Hsp70 is able to chaperone associated functional protein constituents, such as NF- κ B, into cell nuclei (Fujihara and Nadler 1999).

Accumulating evidence indicates that enzymes of DNA base excision repair are stimulated by Hsp70 (Bases 2005, 2006; Kenny et al. 2001; Mendez et al. 2003a, b). Cell DNA, which is a substrate for cytidine deaminase, contains uridine after deamination of cytidine (Bransteitter et al. 2003). The next repair steps are removal of uridine by the ubiquitous uracil DNA glycosylase (UDG) (Wu and Stavnezer 2007), followed by DNA strand cleavage by AP endonuclease (HAP1) or by heat and alkali, followed by repair by DNA polymerase β (Hagen et al. 2006). All these enzyme activities are stimulated by Hsp70. As expected, UDG and HAP1 activities were depressed in THP1 leukemic cells 24 h after transfection with an siRNA designed to inhibit Hsp70 (Bases 2005, 2006). Rohde et al. showed that Hsp70 and its mRNA were depressed in such siRNA-transfected cells but only after a 36 h lag (Rohde et al. 2006 and personal communication). Our recent experiments showed inhibition of Hsp70 24 and 48 h after transfection with the siRNA.

Here, we show that Hsp70 did not stimulate AID in leukemic Burkitt lymphoma cell line Ramos 6.4, in contrast to its ability to stimulate other cytidine deaminases and other enzymes of DNA repair.

The steps in the immune response after AID are the subject of intense interest because of its influence upon class switch recombination and somatic hypermutation. A previous report (Son et al. 2008) described disruptions of RAG/DNA complexes by Hsp70, but there has been no previous information on effects of Hsp70 chaperones on AID activity.

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Materials and methods

Cells

THP1 human leukemic cells, and CRL 2337, human lymphoblastoid B cells, continuous lines of human lymphoma cells, were purchased from American Type Culture Collection, Manassas, VA 20108. They were grown in suspension at 37°C in Roswell Park Memorial Institute 1640 medium with 10% fetal bovine serum in a 5% CO₂ humidified atmosphere incubator. For THP1 and CRL 2337 cultures, the cell culture population doubling times were 36 and 24 h, respectively. Media and sera were from ATCC. Ramos 6.4 cells were grown in Iscove's modification of DMEM (Fisher Scientific, Pittsburgh, PA 15276-9952) with 10% fetal bovine serum. The cell culture population doubling time was 22 h.

Cell lysates

Cells were sedimented from phosphate buffered saline without Ca⁺⁺ or Mg⁺⁺, resuspended in lysis buffer (Bases 2006) and sonicated with 20 one-second strokes, leaving 1–2% unbroken cells. Lysates that were clarified by centrifugation at 15,500×g for 12 min contained 1 µg/µl of protein (Bradford 1976). In some experiments, cell lysates were concentrated by centrifugation through Centricon 3 filters (Millipore Corporation, Bedford, MA 01730).

Deoxycytidine deaminase assay

Thin layer chromatography procedures (Mac Ginnitie et al. 1995) were followed with minor modifications. Cell lysates were assayed in 10-µl aliquots containing: 3.3 µl deoxy (5-³H) cytidine at 21.0 Ci/mmol (777 G Bq/mmol where 1 µl=1 µCi), 0.25 µl 2 M Tris, pH 7.4, 1.45 µl H₂O, and 5 µl of cell lysate. Unlabeled cytidine or uridine was not added to the assay mixtures.

Incubation at 37°C for 2 h was stopped by addition of unlabeled deoxycytidine, 20 µg and deoxyuridine, 20 µg. After centrifugation at 15,500×g for 2 min, 4 µl samples were spotted on to polyethyleneimine (PEI) cellulose thin layer chromatography plates (VWR International, West Chester, PA 19380). The chromatogram was developed in 7:2 (v/v) isopropanol: 10% HCl for 14 to 16 h. The faster migrating uridine was 6 cm ahead of the cytidine spot, as determined by illumination with 254 nm UV light. The radiolabelled species in each UV light reactive spot were collected by scraping the PEI into scintillation vials which were counted in 10 ml of scintillation fluid in a liquid scintillation spectrometer. The fraction of deoxycytidine converted varied from 0.5% to 12%, i.e., 200 CPM to 6,000 CPM out of the total 50,000 CPM. Substrate was therefore in excess. PEI quenching of the harvested ³H-labeled uridine and cytidine consistently yielded

5% of the ³H activity expected. Quenching was standardized in all the TLC derived samples; they each contained 2 µg of unlabeled pyrimidine. The spot sizes determined under UV light illumination were uniform. Therefore, equal amounts of PEI were scraped into the sample vials along with comigrating radiolabelled pyrimidines. In six experiments with unincubated eluate mixtures, the mean radiolabel (CPM) recovered from ³H cytidine-labeled spots in the chromatogram was 38,374±2300 standard error of the mean.

Oligodeoxycytidylic affinity column purification of AID

One-milliliter HiTrap affinity column (GE Health Care, Biosciences, Uppsala, Sweden SE 75184) were first prepared by binding with 185 µg of oligodeoxy cytidine (American Biosciences, Piscataway, NJ 08855) according to the manufacturer's directions. We expected that few of the primary amino groups of the polydeoxycytidylic would be eliminated by the binding step at 4°C; 137 µg of oligodeoxycytidylic were bound to the column. Cell lysates (600 µg) were introduced for binding at 4°C and after 150 min, the column was washed at 4°C to eliminate unbound AID and other proteins. The column was kept in phosphate buffered saline (PBS) at 4°C to prevent cytidine deaminase digestion of the column bound cytidylic amino groups.

To release cytidine deaminases from the column, elutions were made with 0.5 ml of 0.2 N NaCl in 0.04 M Glycine at pH 3.0 and then 0.5 ml of 0.5 N NaCl in 0.1 M Glycine at pH 3.0, followed by PBS. A 5.0-µl aliquots of each 500 µl column fraction were assayed for cytidine deaminases by thin layer chromatography. Samples were neutralized, as needed.

Transfection A 5 nm of RNA (siRNA or scrambled RNA) was transfected in 6×10⁷ cells as recommended by the manufacturer (Bases 2005, 2006).

Western blots

For most experiments, 7 cm minigels, purchased from BioRad Laboratories, Los Angeles, CA were used. Buffer without SDS or methanol containing 25 mM Tris, pH 8.3, and 192 mM glycine were used for gel electrophoresis and Western blot transfer.

Detection of Hsp70 in cell lysates and in elution fractions from affinity columns was made in Western blots, using mouse anti-human Hsp70 monoclonal antibodies (Stress Gen SPA 820) in 20 µg/20 ml of phosphate buffered saline in 5% milk proteins with 0.05% polyoxyethylene sorbitan monolaurate (Tween 20, Sigma, St. Louis, MO 63178) in the first layer, incubated overnight at 4°C. The second antibody layer was goat anti-mouse IgG precoupled to horseradish peroxidase, Stress Gen SAB 100, 1:5,000 dilutions for 2 h before ECL

Fig. 1 Determination of deoxycytidine deaminase activity in affinity column fractions of lysates of THP 1 leukemic cells. After 24 h of incubation at 37°C, the protein content in 500 µl eluates from cells after transfection with *filled circles* scrambled RNA or *filled triangles* siRNA was determined. Six hundred micrograms of crude lysates from the experiment shown in this figure were fractionated off the affinity column. Twenty 500-µl fractions were collected and 5 µl of each were assayed. ³H uridine released in assays of 5 µl of the indicated fractions from *empty circles*, scrambled or *empty triangles*, siRNA-transfected cells. *Filled squares*, one micrograms of Hsp70 was included in the assay mixture in a separate study of the indicated elution fractions. Note the change in scale. With 1 µg of BSA instead of Hsp70 in fraction 9, 803 CPM were released. Without cell lysates in the assay mixture, but including 1 µg of Hsp70, 575 CPM were released

determination. Recombinant Hsp70 was purchased from Stress Gen, now Assay Designs, Ann Arbor, MI 48108. To standardize sample recovery, actin was determined in cell isolates.

Nucleic acid gel electrophoresis assay for AID

A single-stranded substrate DNA, TTT TTT TTT TTT TTA GCA TTT TTT TTT TTT, was purchased from INVITRO-GEN, CARLSBAD, CA 920081. 5' ³²P end-labeling was accomplished with T4 polynucleotide kinase from INVITRO-GEN according to the manufacturer's instructions, using 5 pmol of substrate and 25 µCi of [³²P] ATP, 3,000 Ci/mmol, Perkin Elmer, Shelton, CT 06484. After labeling, the reaction was stopped by addition of EDTA to 5 mM and incubation at 65°C for 5 min. The 30-nt long labeled product was purified by centrifugation through a Micro Bio-Spin 6 Column, BioRad, to remove smaller species (Brar et al. 2008).

Enzyme activity was measured by cleavage at the single cytidine. Usually, 10 µl reaction mixtures containing 0.5 pmol of ³²P-labeled substrate in 10 mM Tris-HCl pH 8.0, 1 mM EDTA, and 1 mM DTT were incubated with 5 µl of sample at 37°C for 15 min. Cell lysates or purified AID enzyme (Enzymax, Lexington, KY 40503) were pretreated by

digestion with 1 mg/ml of DNase free pancreatic RNase for 30 min at 37°C.

Uracil DNA glycosylase, 1 µl (1 unit/µl), Invitrogen, was added, followed by 15 min of incubation at 37°C to remove uracil products of AID digestion. The resulting abasic sites were cleaved by adding 1 µl of 2 N NaOH to the reaction mixture and heating for 5 min at 95°C. Then 10 µl of formamide were added. In some experiments concentration of cell lysate fractions 60-fold from polydeoxycytidylic sepharose columns was achieved by centrifugation through Amicon Ultra Columns (Millipore Corp., MA 01821)

After these additions, 10 to 15 µl of TBE sample buffer (BioRad, 161-0768)s, were added and the samples were placed in the 30 µL sample wells of 15% acrylamide TBE urea gels (Biorad 161_1117). After electrophoresis for 60–75 min, the gels were exposed to X-ray film for 30 min or more. The radioautograms were scanned by quantitative densitometry. Densities of faster migrating 16 nucleotide (nt) DNA enzyme lysis products were quantitated and expressed as fractions of the sum of residual 30 nt substrate and 16 nt product.

Results

Preliminary studies showed that overall cytidine deaminase activity in THP1 cell lysates was depressed by siRNA. ³H uridine which was released from the ³H cytidine substrate in lysates from cells that had been transfected with scrambled RNA 24 h before was readily detected in the time-course experiments using thin layer chromatography (not shown). Less activity was apparent in lysates from THP1 cells which had been transfected 24 h before with siRNA designed to inhibit Hsp70 mRNA. Supporting studies showed that their Hsp70 was 31% of control

Fig. 2 In this experiment, Hsp70s effect on AID release of 16 nt ³²P-labeled fragments from the ³²P-labeled 30 nt substrate was measured. Lanes 3, 4, 5 show results of gel electrophoresis after digestion of substrate by 0.2 µg of AID in the presence of 1.7 µg of Hsp70 in the standard assay; 41% of substrate was released. Lanes 6, 7, 8 show results when Hsp70 was omitted; 47% of substrate was released, not a significant difference. Lanes 1, 2, 9, 10 show the release of small amounts of polydisperse small fragments released by digestion without AID or Hsp70

Fig. 3 Binding and release of AID from an affinity column containing ~300 μg of bound 50-nt long polydeoxycytidine oligomers. The column was charged with 4.5 μg of purified AID (Enzymax), equivalent to 45 units of enzyme activity at 4°C for 2.5 h. Thirty-seven units of AID were eluted and recovered in the fractions shown, as calculated from the release of 16 nt ^{32}P -labeled product by AID in each column fraction, comparing their activities with the AID

standard. The left ordinate indicates the fractions of 5^{32}P -labeled 30-nt long substrate released as 16-nt long ^{32}P -labeled product. For this experiment, the standard one unit of AID activity was equivalent to that in 0.1 μg of an aliquot of the purified AID. One unit of AID released 19% of the substrate in 15 min at 37°C, in the assay described in “Materials and methods”. A similar elution pattern was obtained in a second study, *filled triangles*

Fig. 4 Assay of AID from Ramos 6.4 cells. AID activity was recovered by elution of cell lysates from a poly dC affinity column described before (Figs. 2, 3). 7×10^8 Ramos 6.4 Burkitt lymphoma cells were lysed by sonication and in 2 ml of lysis buffer, clarified by sedimentation at $12,000 \times g$ for 16 min at 4°C; 0.5 ml of the clarified lysate was prepared by adding 0.5 ml of pancreatic ribonuclease A (1.0 mg/ml) for 30 min at 37°C. The lysate (now 1 ml) was applied to the column for 150 min at 4°C and 0.5 ml fractions were eluted. Effluents 8, 9, 10 were combined and 11, 12, 13 were combined. Each of the 1.5 ml effluent fractions was concentrated 60 fold by centrifugation at $7,500 \times g$ at 4°C for 40 min in Amicon membrane filters (Millipore). The 25 μL 60-fold concentrates were assayed in 5 μL aliquots to determine release of 16 nt enzyme products from 5^{32}P -labeled 30 nt substrate. Densitometry was applied to films exposed for 25 min. The fractions of substrate released by digestion to 16 nt products shown in *lane 1* (effluent 8–10) and *lane 2* (11–13) were 0.25 and 0.28. For comparison, 142 ng of bonafide AID (Enzymax) released 0.17 of the substrate in this assay (*lane 3*). Little or no 30 nt substrate was converted to smaller species when incubated without cell lysate or AID. *Lane 4* shows the pattern obtained with this substrate alone (25 min exposure) in the experiment. *Lane 5* is from a 2.5-h exposure of the same film. The longer incubation was applied to develop a 30 nt species autoradiograph equivalent in density to that of *lanes 1, 2, and 3*, since the original gel lane was underloaded. The densities in *lane 5* below 30 nt are polydisperse, with no specific density at the region of 16 nt

values. Their time-course kinetic was flat. By contrast, after scrambled RNA transfection the uridine release was uninterrupted for several hours. Interference from other factors and other cytidine deaminases in the crude lysates had to be considered, as well as off-target effects of siRNA (Jackson et al. 2006). Therefore, the lysates from siRNA-treated cells and scrambled RNA-treated cells were further purified and their cytidine deaminase activities were determined by thin layer chromatography.

Cytidine deaminase activity purified by elution from an affinity column

Oligodeoxycytidine was covalently bound to a sepharose column as described in “Materials and methods”. Since not all the primary amino groups of oligodeoxycytidine would likely be blocked or removed at 4°C in the binding reaction, cytidine deaminases were expected to bind to other free amino groups still available for enzyme-substrate binding. At 4°C, little enzyme digestion of these groups was expected and the proteins could subsequently be released by high salt and low pH.

Results of Fig. 1 indicate that cytidine deaminase activity was largely recovered in three eluate fractions. Firstly, unbound deaminases were removed with other proteins in the first PBS eluates. When 0.2 N NaCl and 0.04 M glycine pH 3.0, and then 0.5 N NaCl and 0.1 M glycine pH 3.0 were applied, more cytidine deaminase was recovered. For the purpose of estimating recovery here, 1 unit of cytidine deaminase activity is defined as the release of 100 CPM of ^3H uridine in 2 h at 37°C. The 600 μg in 600 μL of crude lysate protein applied to the column contained an estimated

3,600 units. Little enzyme activity appeared to be lost in this purification.

With lysates from siRNA-transfected cells, cytidine deaminase in the first NaCl/glycine eluates (fractions 7–10) were less active than with lysates from control cells, $p < 0.001$. Cytidine deaminase activity in the second glycine eluates was actually greater than with control eluates (fractions 15–17). Column elution of siRNA-transfected cells' lysates was estimated to be 4,000 units. Hsp70 supplement in the assay of eluates once off the column clearly showed massive stimulation of cytidine deaminase with eluates from fractions 9–10. Note the change of scale in Fig. 1. By contrast, cytidine deaminase activity in fractions 11–14 was increased after siRNA treatment and exogenous Hsp70 failed to stimulate their enzyme activity.

Western blot studies with antibodies to Hsp70 and authentic Hsp70 standards determined the immuno-reactivity content in fractions 9–10 of both sets of eluates; 0.030 μg of Hsp70/50 μl fraction was found in the lysate 24 h after siRNA, as compared with 0.085 μg after scrambled RNA (not shown); i.e., siRNA transfection reduced Hsp70 content to 31% of control values.

The relationship between Hsp70 seen here and the numerous Hsp70 congeners described by Daugaard et al. (2005) is not known.

In summary, siRNA treatment depressed the Hsp70 content of THP 1 cells which in turn depressed cytidine deaminase activity in the major elution fraction. The Hsp70 content of this fraction (9–10 of Fig. 1) from siRNA-treated cells was about one third of control values. Furthermore, addition of Hsp70 to the cytidine deaminase assay of fractions 7–10 restored and exceeded control values, implicating Hsp70 as an important adjuvant to overall cytidine deaminase activity.

Determination of AID in cell lysates

Since AID is one of many cellular cytidine deaminases, results of Fig. 1 might not be relevant to AID. Therefore, AID was determined in enzyme assays using 5' ^{32}P -labeled 30 nt substrate containing a single cytidine sequence, AGCA, a site known to be sensitive substrate for AID (Brar et al. 2008), as in the "Materials and methods".

Release of the 16-nt long 5' ^{32}P -labeled product was determined by gel electrophoresis and densitometer scans of gel autoradiograms as described in "Materials and methods". Figure 2 compares the release of 16 nt ^{32}P -labeled fragments by purified AID with or without supplementation by Hsp70. In contrast to results in Fig. 1, Hsp70 did not stimulate AID in vitro and may have slightly depressed AID activity.

Figure 3 demonstrates binding and release of purified AID from a polydeoxycytidine bound column similar to those of Fig. 1, providing comparable elution patterns which were then applied to Ramos 6.4 cell lysates. Figure 4 shows the release of 16 nt fragments by activity in column

purified Ramos 6.4 lysates. Polydisperse fragments interfere somewhat with the determinations of 16-nt long AID products and may indicate the activity of associated enzymes.

Discussion

Results of these studies provide a way to assay for AID enzyme activity in cells of hemopoietic origin. Results presented here demonstrate that Hsp70 stimulated the activity of some of the cellular cytidine deaminases in THP 1 cells (Fig. 1).

By contrast, siRNA designed to inhibit Hsp70 synthesis depressed the cell content of Hsp70 and depressed the activity of certain of the other cytidine deaminases in cells 24 h after transfection. Rohde and others observed deficits in cellular Hsp70 and its mRNA after 36–72 h following siRNA transfection (Rohde et al. 2006 and personal communication). Reduction in cell Hsp70-stimulated cytidine deaminases in certain THP1 cell lysates (fractions 12–18, Fig. 1). Levels of AID in Ramos 6.4 cells after transfection with siRNA were determined after elution from the poly dc column. They were also slightly elevated, i.e., 2.0-, 1.5-, 1.3-, 1.2-, 5.0-fold in experiments not shown.

The small increase in AID activity might be due to reduction in HSP70 by siRNA but off-target effects could account for this.

AID has been the center of much effort in establishing its role in the immune response (Muramatsu et al. 2000; Revy et al. 2000; Martin et al. 2002; Martin and Scharff 2002; Li et al. 2006). Previous studies had not implicated Hsp70 as a modifier of the activity of AID or other cytidine deaminases. We speculate that Hsp70 may be a factor in the immune response by reducing AID activity. If so, high fever induced Hsp70 might actually be expected to interfere with AID and immune responses.

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