BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **220,** 153–159 (1996)
ARTICLE NO. 0373

HSP70 Overexpression Mediates the Escape of a Doxorubicin-Induced G2 Cell Cycle Arrest

Jan Karlseder, Dorte Wissing,* Gerhard Holzer, Lukas Orel, Gerhard Sliutz, Herbert Auer,† Marja Jäättelä,* and Manuel M. Simon[1]

*Center of Applied Genetics, Univ. BOKU Vienna, Peter-Jordanstrasse 82, A-1190 Vienna, Austria; †Dept. of Occupational Medicine, Universitätsklinik für Innere Medizin IV, AKH, Universität Wien, 1090 Vienna, Austria; *Div. Cancer Biology, Dept of Tumor Cell Biology, Danish Cancer Society Research Center, 2100 Copenhagen, Denmark*

Received January 8, 1996

The stress inducible heat shock protein 70 (hsp70) confers resistance to a variety of adverse environmental conditions including certain anticancer drugs. In the present study we explored cellular consequences of hsp70 overexpression with respect to genotoxic stress. Employing an isogenic set of stable transfectant cells, one overexpressing hsp 70 and the other serving as control, we found that a high hsp70 expression level is sufficient to provide protection against the cytotoxicity of doxorubicin. In addition, hsp70-protected cells showed the capability to restart cell proliferation at concentrations where control cells arrested. However, the DNA lesion density was comparable in the lines used. Recording the cell cycle control revealed a dramatic shortening of the doxorubicin-mediated cell cycle arrest in the G2 phase upon hsp70 overexpression. Our data suggest an involvement of hsp70 in the regulation of the cell cycle.

All species studied so far have a versatile cytoprotective machinery at their disposal comprising of a set of highly conserved proteins entiteled heat shock proteins (hsps). The expression of these proteins confers tolerance to a markedly broad variety of adverse environmental conditions like UV-light or ionizing radiation, heavy metals, or certain metabolic poisons (for review see(1)). It has also been reported that hyperthermic treatment and subsequent recovery (2) or overexpression of certain heat shock proteins (3) confer resistance to several anticancer drugs. In the past decade these proteins as well as their constitutively expressed relatives emerged to support a variety of cellular functions not linked to the response to environmental stress. The presumably best studied heat shock protein family is a set of highly homologous proteins of 70 kDa (hsp70). Members of that family were found to be engaged in as diverse functions as the secretory pathway (4), the glucocorticoid response (5) or also differentiation and cell cycle regulation (6,7). The anticancer drug doxorubicin is an anthracyclin type antibiotic exerting its activity by multiple mechanisms including the inhibition of DNA topoisomerase II (8) and the generation of reactive oxygen intermediates (ROI) (9). In addition, the DNA damage introduced by doxorubicin causes target cells to accumulate in the G2/M phase of the cell cycle (10,11). In the present study we sought to investigate the consequences of constitutive hsp70 overexpression on cell viability, doxorubicin mediated formation of ROI, cell cycle distribution and DNA-damage upon doxorubicin treatment.

MATERIAL AND METHODS

Cells. An isogenic set of stably transfected WEHI-S cells, a murine fibrosarcoma cell line, was used throughout the study. WEHI-S cells were stably transfected to overexpress the human hsp70 cDNA driven by the SV40 promoter along with plasmid SV2neo as described (12) and were named WN113. A combined culture of single clones stably transfected to

[1] To whom correspondence should be addressed. Fax: +431-47654-2292. E-mail: SIMON@MAIL.BOKU.AC.AT.

Abbreviations used: BHA, butylated hydroxyanisole; hsp, heat shock protein; Dox, doxorubicin; FACS, fluorescence-activated cell sorting; ROI, reactive oxygen intermedietes.

0006-291X/96 $18.00

express the neomycin resistance alone were used as control cells (WN10x). Cells were cultured in RPMI-1640 with 10% FCS and 200 μg/ml G418 (all GIBCO, Grand Island, NY).

Western blot analysis. Hsp70 protein expression in WN113 cells was determined by Western blot analysis. Briefly, cells were lysed by two freeze/thaw cycles in PBS containing 0,5% Triton X-100 (Sigma, St. Louis, MO), electrophoresed by denaturating polyacrylamide/SDS gel electrophoresis and blotted onto nicrocellulose membranes. For detection an antibody specific for the inducible form of hsp70 (SPA810, StressGen, Victoria, BC, Canada) was used. In addition, hsp70 over-expression was repeatedly tested by a functional assay basing on the hsp70 induced resistance to hydrogenperoxide as described (13,14).

Chemotherapeutic drugs and cytotoxicity assay. Doxorubicin (Sigma, St. Louis, MO) was dissolved in DMSO and stored at −20°C. Cell survival was determined as described (14). Briefly, cells were seeded in 96 well plates in RPMI 1640 containing 10% of FCS and 200 μg/ml G418 (GIBCO, Grand Island, NY). After growth over night cells were treated with the drug at concentrations as indicated. In parallel, a "zero survival" sample was prepared by complete lysis of cells by addition of 5% of sodium-N-lauroylsarcosin (Sigma, St. Louis, MO). Cells were incubated for a period of 48 h at 37°C. To determine the metabolic activity MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) to a final concentration of 300 μg/ml was added and incubated at 37°C for exactly 6 hours. Finally cells were incubated with the same volume lysis buffer (10%SDS, 50% formamide adjusted to pH 4.7 with acetic acid) and incubated overnight at room temperature. The clear solution was measured at 595 nm against the "zero survival" control. The growth rates of untreated WN10x and WN113 cells were not significantly different (not shown). Survival rates were calculated as percent OD595 of the untreated cells normalized to the zero "survival" value.

For determination of the capability of cells to re-start growth after doxorubicin treatment the metabolic activity of cells at the time point of withdrawal of the drug was determined and compared with metabolic activities after 24 h later of recovery. The capability to start cell growth was expressed as percentual change of metabolic activity within 24 h after withdrawal of the drug, 100% represent the metabolic activity at the time point of drug removal.

Membrane disintegration assay. Membrane integrity was measured by a peroxidase exclusion assay. To block the uptake of peroxidase by pinocytosis cells were treated and washed on ice. Cells were seeded into 6 well plates and grown overnight. Then doxorubicin was added at concentrations as indicated and incubated for 1 h. Supernatant medium was removed and replaced by ice cold PBS containing 1% FCS. The well plates were placed on ice for 15 min. Then horseradish peroxidase (Sigma, St. Louis, MO) to a final concentration of 20 μg/ml was added and incubated on ice for 1 h. After two washing cycles with PBS/1%FCS, cells were lysed by two freeze-thaw cycles in 10mM Tris/HCl pH 7.5, 10 mM KCl, 1 mM EDTA, 1mM phenylmethyl-sulfonylfluoride. Peroxidase activity contained in the lysates was assayed by standard procedures. Assays were performed in doublicates, peroxidase assays were performed in triplicates. Data are expressed in the percentage peroxidase activity +/− standard deviation.

Measurement of doxorubicin-induced intracellular ROI formation by flow cytometry. Intracellular ROI formation was determined using the specific probe dihydrorhodamine 123 (DHR123, Molecular Probes) essentially as described previously (15). DHR123 was prepared as a 5 mM stock solution in DMSO and used at a final concentration of 1 μM. An antioxidant, butylated hydroxyanisole (BHA), was dissolved in ethanol at a concentration of 10 mM. Cell grown to subconfluency were trypsinized and resuspended in normal media at a concentration of 10^6 cells/ml. Following a 30 min incubation at 37°C in humified 5% CO_2, DHR123, doxorubicin and BHA at a final concentration of 10 μM was added where indicated. Cell samples were drawn at regular time intervals and following resuspension in PBS cells were analyzed using a Becton Dickinson flow cytometer (San Jose, CA). Green fluorescence resulting from DHR123 oxidation was excited at 488 nm and detected between 515 and 550 nm. 3000 cells were measured per sample. Data represent the median of the frequency distribution as representative of the DHR123 level of the sample.

Measurement of DNA single strand breaks by in situ nick translation. In situ nick translation was performed essentially as described (16). Briefly, cells were treated with doxorubicin for 16 h. Then, they were washed twice with PBS and fixed by addition of 66% ethanol. Ethanol was evaporated and cells were washed with 0.9% NaCl. Incorporation of [^{32}P] labelled dCTP was performed in a reaction buffer containing 50mM Tris/HCl pH 7.9, 50 mM $MgCl_2$, 0, 1% BSA and 0.5% DMSO. dATP, dTTP and dGTP at a final concentration of 40 μM, unlabelled dCTP at a concentration of 5 nM and 10 μCi per ml of alpha [^{32}P]dCTP (3000 Ci/mmol; New England Nuclear/DuPont, Boston, MA) were added. The reaction buffer was finally supplemented with 10 U/ml DNA-Polymerase I (Boehringer Mannheim, Mannheim, FRG). Cells were incubated with the labelling mix for 20 min at room temperature. Cells were washed 2 times with 5 mM EDTA, 100 mM sodium pyrophosphate, 0,4 μM unlabelled dATP), and digested by addition of 5 mg/ml proteinase K in 50 mM Tris/HCl pH 8.5/l mM EDTA. The lysate was ethanol precipitated onto 0,22 μm membranes in filtration plates using the Millipore Multi-ScreenAssay System vacuum manifold (Millipore, Vienna, Austria) in which the precipitate was washed twice with 70% ethanol. The incorporation of ^{32}P dCTP was determined by autoradiography of the membranes. Autoradiographs were scanned and analyzed using the Phoretix quantification software.

Determination of the DNA content. The DNA content of cells was determined by the fluorescence-activated cell sorting (FACS) analysis essentially as described (17). Cells were treated with doxorubicin. At different time points cells were trypsinized, washed twice with ice cold PBS and resuspended in 1 ml of PBS. Then the cell suspension was dropwise added to 8 ml of ice cold 85% ethanol. After centrifugation the pellet was resuspended in 200 μl of 0,05% pepsin and 1 ml of

2μg/ml DAPI (4,6-diamidino-2-phenylindol-dihydrochlorid) was added. DNA profiles were determined in a Partek PAS-2 FACS. Cell number contained within the G0/G1 and G2/M fraction was determined by peak analysis. From these values a percentual G1 fraction index was calculated representing the G0/G1 peak.

RESULTS AND DISCUSSION

It was previously demonstrated that high expression of hsps induce tolerance to anticancer drug treatment (2,3). This finding can be of particular impact since the overexpression of hsp27 as well as hsp70 was observed in certain tumor tissues and is therefore regarded as a tumor marker of prognostic value (18,19). In the present study we investigated the cellular consequences of hsp70 overexpression upon anticancer drug treatment. In order to evaluate the hsp70 expression characteristic of the cell used throughout the study a Western blot analysis was performed. WN113 cells expressed high amounts of hsp70(fig. 1; lane 2) whereas in control cells (WN10x) hsp70 was not detectable (fig. 1; lane 3). To compare the constitutive hsp70 expression of WN113 cells with expression levels obtained upon heavy metal stress which triggers the genuine heat shock response, WN10x cells were induced with $ZnCl_2$. This treatment resulted in a slightly smaller hsp70 signal as compared to the constitutive expression in WN113 cells (fig.1, lane 1). Having this isogenic set we first explored whether hsp70 expressing cells are tolerant to enhanced doses of doxorubicin, which was clearly the case (fig.2a). Experiments using $ZnCl_2$ induced WN10x cells are not informative, since the multiple drug resistance gene (mdr-1) is under the same transcriptional control as the heat shock proteins (20). Membrane damage is a known consequence of treatment with doxorubicin. Therefore, membrane integrity was determined in WN10x versus WN113 cells by a peroxidase exclusion assay. As depicted in fig. 2b WN113 cells were protected from damage by doxorubicin as compared to WN10x cells.

Next, we asked whether the obvious cytoprotection provided by hsp70 overexpression also enables protected cells to re-start proliferation after withdrawal of the drug. We monitored the growth behavior of the cells by measuring metabolic activity at the timepoint where the drug was removed and after additional 24 h of growth in RPMI/10% FCS. As depicted in fig. 3 the improved survival of hsp70 overexpressing cells was associated with an enhanced capability of these cells to restart cell growth.

Doxorubicin is reported to induce a ROI burst, which is thought to contribute to the toxic activity of the drug (9,15). To evaluate the efficacy of hsp70 overexpression on this contribution we applied the drug with or without the antioxidant butylated hydroxyanisole (BHA). As also shown in fig. 2 hsp70 overexpression provided protection to doxorubucin. Co-administration of the antioxidant BHA markedly increased cell survival of WN10x cells, however, BHA had only a minor influence, if any, on survival rates of WN113 (table 1). This finding reflects the known feature of hsp70 to confer resistance to oxidative stress. We next asked whether hsp70 overexpression in WN113 cells interferes with the formation of ROI. Both, WN10x and WN113 cells induced doxorubicin mediated DHR123 flourescence, by a similar factor (table 1). The signal was specific since addition of BHA reduced the flourescence to the control levels. From these results we conclude, that the

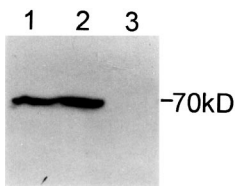

FIG. 1. Expression level of hsp70 in WN10× and WN113 cells. Equal amounts of total protein extract obtained from WN10x cells treated for 3 h with 100 μM $ZnCl_2$ (lane 1), WN113 cells (lane 2) and untreated control cells WN10× (lane 3) were analysed by Western blot. Hsp70 was identified using an antibody specific for stress induced hsp70 (hsp72, SPA-810).

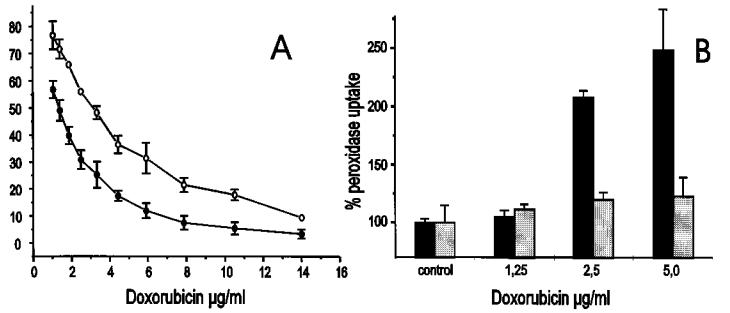

FIG. 2. Cell survival and membrane integrity of WN10× and WN113 cells after treatment with doxorubicin. (A) Cells were treated with doxorubicin at dosages as indicated. After 24 h of continuous doxorubicin treatment an MTT survival assay was performed. Closed circles, WN10×; open circles WN113. Values are expressed as percentage metabolic activity. Experimental series were performed in triplicates. Error bars represent the standard deviation. (B) Membrane integrity was assayed after treatment with doxorubicin for 1 h. Peroxidase activity contained within the cells was measured and normalized by the protein content of the samples. Samples were prepared in duplicate and peroxidase measurements were performed in triplicate. Black bars, WN10×; grey bars, WN113. Data were pooled and represent the percentage of peroxidase taken up compared to the untreated control. Error bars represent the standard deviation.

actual ROI generation is not influenced by hsp70 overexpression. The resistance seen in cells overexpressing hsp70 is due to an increased tolerance of WN113 cells to ROI rather than to a reduced intracellular ROI concentration.

According to the common hypothesis the mechanism of hsp70 protective activity consists in its binding of misfolded or damaged proteins. The subsequent release from the complex is thought to enable these proteins to acquire a proper folding (4). Hsp70 from other organisms was able to restore enzyme activity of damaged proteins (21,22). With respect to the result of fig. 2 and 3 this hypothesis suggests that essential protein functions, damaged during the drug treatment are protected. We therefore asked what the cellular consequences of hsp70 overexpression regarding the genome integrity would be. DNA damage, as mediated by most DNA damaging agents like gamma irradiation, DNA alkylation or DNA lesions e.g. introduced by a ROI burst induce cells to arrest in the G2/M phase of the cell cycle (23). Refering to our observation that hsp70 overexpressing

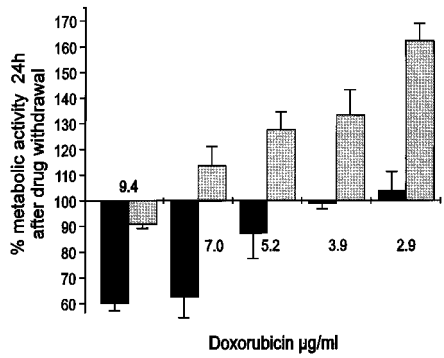

FIG. 3. Ability of restart of cell growth after drug withdrawal. Two sets of 96-well plates with WN10× or WN113 cells were prepared, of which all were treated with doxorubicin as indicated. After 24 h of continuous doxorubicin treatment a MTT cell survival assay was performed with the first set of plates. The second set of plates as washed and incubated for an additional 24 h followed by a MTT assay. For a given doxorubicin dosage the percentage of the metabolic activity contained in the second set of plates compared to the activity measured in the first was calculated. Values represent the percentage of change in metabolic activity representing the capability to restart cell growth after withdrawal of the drug. The 100% value represents the metabolic activity at the time point of drug removal. Black bars, WN10× cells; grey bars, WN113 cells. Experimental series was performed in triplicate. Error bars represent the standard deviation.

TABLE 1
Determination of Doxorubicin-Mediated ROI formation

	WN10x	WN113
Cell survival (percentage of control)		
+Dox	39	91
+Dox + BHA	61	91
Formation of ROI (percentage of control)		
+Dox	127	141
+Dox + BHA	89	93

Cell survival was determined by an MTT assay after treatment with 1 μg/ml doxorubicin for 21 h. Standard deviations were smaller than 10%. Values represent the percentage of metabolic activity as compared to untreated control cells. The generation of ROI was measured by FACS analysis. Cells were incubated with 1.5 μg/ml doxorubicin for 21 h with or without 10 μM BHA. Then they were harvested and the DHR 123 fluorescence was determined. Experimental series were performed three times. Data represent the median of the frequency distribution as a measure for the ROI level within the cells.

cells are tolerant to the cytotoxic and growth inhibiting effects of doxorubicin (fig. 2 and 3) we investigated, whether cells exhibit an altered DNA lesion density when hsp70 is overexpressed. Our results depicted in fig. 4 clearly show that this is not the case. In order to elucidate the mechanism of hsp70 mediated tolerance with respect to the inability of WN113 cells to arrest cell growth upon DNA damage we sought to study the cell cycle regulation during continuous doxorubicin treatment in a time course. We employed two doxorubicin dosages: first, a dosage where WN113 as well as WN10x cells were able to enter the cell cycle (fig. 5, panel A) and second, a higher concentration, where WN10x cells remained arrested (fig. 5, panel B) according to our results of fig. 3. Untreated WN10x and WN113 cells exhibit a G1 fraction of some 75%. After doxorubicin addition, as expected, cells accumulated in the late S and predominantly in the G2/M phase (data not shown). Regarding early time points both concentrations of doxorubicin induced a depletion of cells in the G1 fraction regardless of the hsp70 expression level. These data indicate

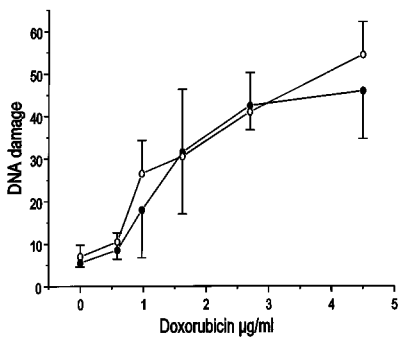

FIG. 4. *In situ* nick translation after treatment with doxorubicin. Cells were incubated with doxorubicin at dosages as indicated for 16 h. Then, the *in situ* nick translation assay was performed, [^{32}P] incorporation was detected by autoradiography. The signals were evaluated by scanning the autoradiographs using a photometric linear scanner. Values represent the arbitrary peak area of the spots which reflect the *in situ* [^{32}P] incorporation, normalized to the film background value. Assays were performed in triplicate. Closed circles, WN10× cells; open circles, WN113 cells. Error bars represent the standard deviation.

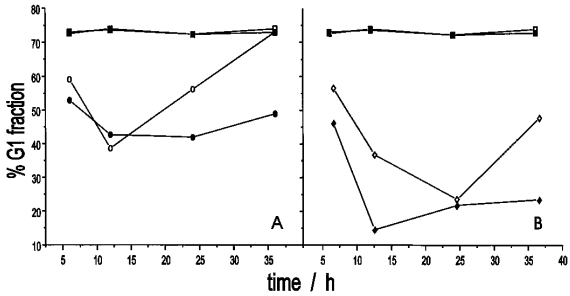

FIG. 5. Analysis of the DNA content during doxorubicin treatment. Cells were grown in the presence of 2.5 μg/ml (circles, panel A) or 5 μg/ml (rhombes, panel B) doxorubicin or without the drug (boxes, both panels). At the time points indicated cells were prepared for determination of DNA content as described. Values represent the fraction of cells in the G1 phase of the cell cycle. Closed symbols, WN10× cells; open symbols, WN113 cells.

that the G2/M checkpoint for entry into mitosis is functional in the cells used and appears not to be influenced by hsp70 overexpression. At later time points, however, hsp70 overexpressing cells exhibit a marked increase in cells of the G1 fraction, whereas control cells remained in their cell cycle arrest state. These results are in accordance with our observation that hsp70 overexpression enhances the capability to re-start proliferation after drug treatment demonstrated in fig. 3 and strongly suggest an involvement of hsp70 in controling the duration of the G2 cell cycle arrest during the doxorubicin treatment. The mechanisms of the cell cycle arrest in the G2/M phase turned out to be highly complex involving a variety of effector molecules (review in ref (24)). The identification of the molecular targets of a hsp70 interaction will remain a topic for further studies. A second conclusion can be drawn from the present results. Hsp70 protected cells are viable and do not arrest their growth having received a considerably higher DNA lesion density whereas wild type cells will not survive with a similar DNA damage. Consequently, tumor cells constitutively protected by hsp70 overexpression bear obviously a higher risk of developing viable cells carrying a damaged genome after the treatment with genotoxic anticancer drugs as compared to unprotected cells.

ACKNOWLEDGMENTS

We gratefully thank Dr. J. Glössl for critical reading of the manuscript and Dr. S. Slobodinsky for fruitful discussion.

REFERENCES

1. Parsell, D. A., and Lindquist, S. (1994) *in* The Biology of Heat Shock Proteins and Molecular Chaperones (R. I. Morimoto, A. Tissiere, C. Georgopoulos, Eds.), pp. 457–494. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
2. Ciocca, D. R., Fuqua, S. A. W., Lock-Lim, S., Toft, D. O., and Welch, W. J. (1992) *Cancer Res.* **52,** 3648–3654.
3. Österreich, S., Weng, C. N., Qiu, M., Hilsenbeck, S. G., Osborne, C. K., and Fuqua, S. A. W. (1993) *Cancer Res.* **53,** 4443–4448.
4. Gething, M. J., and Sambrook, J. (1992) *Nature* **355,** 33–45.
5. Bohen, S. P., and Yamamoto, K. R. (1994) *in* The Biology of Heat Shock Proteins and Molecular Chaperonines (R. I. Morimoto, A. Tissiere, and C. Georgopoulos, Eds.), pp. 313–334. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
6. Hainaut, P., and Milner, J. (1992) *EMBO J.* **11,** 3513–3520.
7. Nihei, T., Takahashi, S., Sagae, S., Sato, N., and Kikuchi, K. (1993) *Cancer Res.* **53,** 1702–1705.
8. Tewey, K. M., Bowe, T. C., Yang, L., Halligan, B. D., and Liu, L. F. (1984) *Science* **226,** 466–468.
9. Weiner, L. M. (1994) *Meth. Enzymol.* **233,** 92–105.
10. Gamba-Vitalo, C., Blair, O. C., Tritton, T. R., Lane, P. A., Carbone, R., and Sartorelli, A. C. (1987) *Leukemia* **11,** 188–198.
11. Barlogie, B., Drewinko, B., Johnston, D. A., and Freireich, E. J. (1976) *Cancer Res.* **36,** 1975–1979.
12. Jäättelä, M., Wissing, D., Bauer, P. A., and Li, G. C. (1992) *EMBO J.* **11,** 3507–3512.
13. Jäättelä, M., and Wissing, D. (1993) *J. Exp. Med.* **177,** 231–236.

14. Simon, M. M., Reikerstorfer, A., Schwarz, A., Krone, C., Luger, T. A., Jäättelä, M., and Schwarz, T. (1995) *J. Clin. Invest.* **95,** 926–933.
15. Ubezino, P., and Civoli, F. (1994) *Free. Rad. Biol. Med* **16,** 509–516.
16. Einhaus, M., Holz, O., Meissner, R., Krause, T., Warncke, K., Held, I., Scherer, G., Tricker, A. R., Adlhofer, F., and Rüdiger, H. W. (1994) *Clin. Invest.* **72,** 930–936.
17. Parks, D. R., and Herzenberg, L. A. (1984) *in* Methods in Enzymology (G. Di Sabato, J. J. Lagone, and H. Van Vunakis, Eds.), pp. 197–241. Academic Press, London.
18. Fuqua, S. A., Österreich, S., Hilsenbeck, S. G., von Hoff, D. D., Eckardt, J., and Osborne, C. K. (1994) *Breast Cancer Res. Treat.* 32**,** 67–71.
19. Takahashi, S., Mikami, T., Watanabe, Y., Okazaki, M., Okazaki, Y., Okazaki, A., Sato, T., Asaishi, K., Hirata, K., and Narimatsu, E., and et al (1994) *Am. J. Clin. Pathol.* **101,** 519–525.
20. Gottesman, M. M., and Pastan, I. (1988) *J. Biol. Chem.* **263,** 12163–12166.
21. Skowyra, D., Georgopoulos, C., and Zylicz, M. (1990) *Cell* **62,** 939–944.
22. Schroder, H. T., Langer, T., Hartl, F. U., and Bukau, B. (1993) *EMBO J.* 4137–4144.
23. Rao, P. N. (1980) *Mol. Cell Biochem.* **29,** 47–57.
24. Li, J. J., and Deshaies, R. J. (1993) *Cell* **74,** 223–226.