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Tumor Necrosis Factor Induces the Rapid Phosphorylation of the Mammalian Heat Shock Protein hsp28

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Tumor necrosis factor α was found to rapidly phosphorylate the unique mammalian small heat shock protein hsp28 without impairing its cytoplasmic localization and without inducing the synthesis of the heat shock proteins. In contrast to the C-kinase-dependent phosphorylation of hsp28 in response to the tumor promoter phorbol-12-myristate-13-acetate, the heat- and tumor necrosis factor-mediated phosphorylation of this heat shock protein appears to occur independently of C kinase. These observations suggest that a C-kinase-independent phosphorylation of hsp28 may be an early event in the cellular action of tumor necrosis factor α.

Tumor necrosis factor α/cachectin (TNF) is a 17-kilodalton (kDa) monocyte/macrophage-derived protein which induces a variety of effects on cell function (16). Among these are the necrosis of solid tumors in vivo (10, 12) and the inhibition of the growth of several normal and tumor cell lines in vitro (33, 36). In addition, TNF is one of the principal endogenous mediators of endotoxic shock (37). TNF also stimulates extracellular matrix degradation, plays a role in both degenerative joint and inflammatory diseases (15), and stimulates prostaglandin E_2 expression (13). This monokine is part of the released factors of activated monocytes and macrophages responsible for stimulating the hepatic production of acute-phase reactants (8, 23). Interestingly, TNF also activates the expression of human immunodeficiency virus type 1 (14). The sequence of events responsible for these effects of TNF is, as yet, unknown. However, the TNF-mediated cytotoxicity may involve ADP ribosylation (1), and results from several laboratories indicate that this monokine is internalized by receptor-mediated endocytosis (6, 18, 38) and degraded in lysosomes (24, 38).

Though TNF is a mediator of inflammation and displays pyrogenic activity when injected into animals (16), this monokine does not appear to mimic the cellular effects generated by environmental stress. Indeed, no induction of the heat shock (or stress) proteins and no drastic impairment of the level of protein synthesis was observed in response to TNF treatment (33). I made a similar observation for HeLa cells treated for 30 min with TNF (2,000 U/ml; human recombinant TNF, ICN Biochemical, GmbH, Federal Republic of Germany) (not shown), in spite of the cytostatic and cytotoxic effect of this monokine in those cells. In order to gain more information on the cellular action of TNF, I analyzed whether this monokine, similarly to the heat shock treatment (4, 5, 22, 39), was able to rapidly alter the pattern of protein phosphorylation in HeLa cells. Labeling with $^{32}P_i$ (carrier free; Amersham Corp., United Kingdom) was for 1 h, in the presence or absence of 2,000 U of TNF per ml added during the last 30 min of the labeling period. Labeling was also performed immediately after a heat shock treatment at 44.5°C for 30 min. Total cellular proteins were then analyzed by two-dimensional gel electrophoresis (pH range, 5 to 8) as previously described (4, 5). In response to both TNF and heat shock, the increased phosphorylation of a preexisting 28-kDa phosphopolypeptide (Fig. 1, arrowhead b) was seen as well as the appearance of new 28-kDa phosphopolypeptides (arrowheads c and d). In both cases, the more acidic 28-kDa polypeptide d was weakly labeled and was not always observed. Consequently, this polypeptide was not further analyzed in this study. Moreover, the 28-kDa polypeptides showing an increased phosphorylation after heat shock are known to be isoforms of the heat shock protein hsp28 (4, 5, 22, 39). Therefore, TNF may stimulate the phosphorylation of hsp28. Interestingly, several authors also reported the increased phosphorylation of 27- to 28-kDa polypeptides in TNF-treated cells, but they did not demonstrate that these proteins were indeed hsp28 isoforms (19, 21, 31, 34).

In addition to the 28-kDa polypeptide, several other polypeptides also increased their levels of phosphorylation after TNF treatment (Fig. 1). Among them were acidic polypeptides (indicated by open arrowheads) which, to a lesser degree, also exhibited an increased phosphorylation after heat shock. Usually, the proteins showing a decreased phosphorylation at elevated temperatures were not affected by TNF (indicated by open circles in Fig. 1). Heat shock also increased the phosphorylation of several polypeptides which were unaffected by TNF (indicated by asterisks in Fig. 1, i.e., actin). In addition, a decreased phosphorylation of the actin isoforms was observed in response to TNF treatment.

Immunoblots of two-dimensional gels probed with anti-hsp28 antiserum (4, 5) and revealed with goat anti-rabbit antiserum conjugated to horseradish peroxidase (Amersham) (4) were performed to provide evidence that the 28-kDa polypeptides phosphorylated in response to TNF are hsp28 isoforms. Indeed, in cells treated with TNF, anti-hsp28 antiserum recognized the 28-kDa b and c polypeptides (Fig. 2A). In addition, the antiserum recognized a more basic isoform a which is the unphosphorylated form of hsp28 (4). This analysis also indicates that a large fraction of hsp28 (>50%) is phosphorylated by TNF. A similar conclusion was reached in the case of heat stress (4). The origin of the well-defined phosphorylated isoforms of hsp28 is unknown, but they may be a consequence of conformational changes of this polypeptide.

Immunoprecipitation was used as another approach to demonstrate that the 28-kDa phosphoprotein detected after TNF treatment is in fact hsp28. After being labeled with ^{32}P as described above, the cells were lysed and hsp28 was immunoprecipitated by using anti-hsp28 antiserum and protein A-Sepharose as already described (2, 5). This experi-

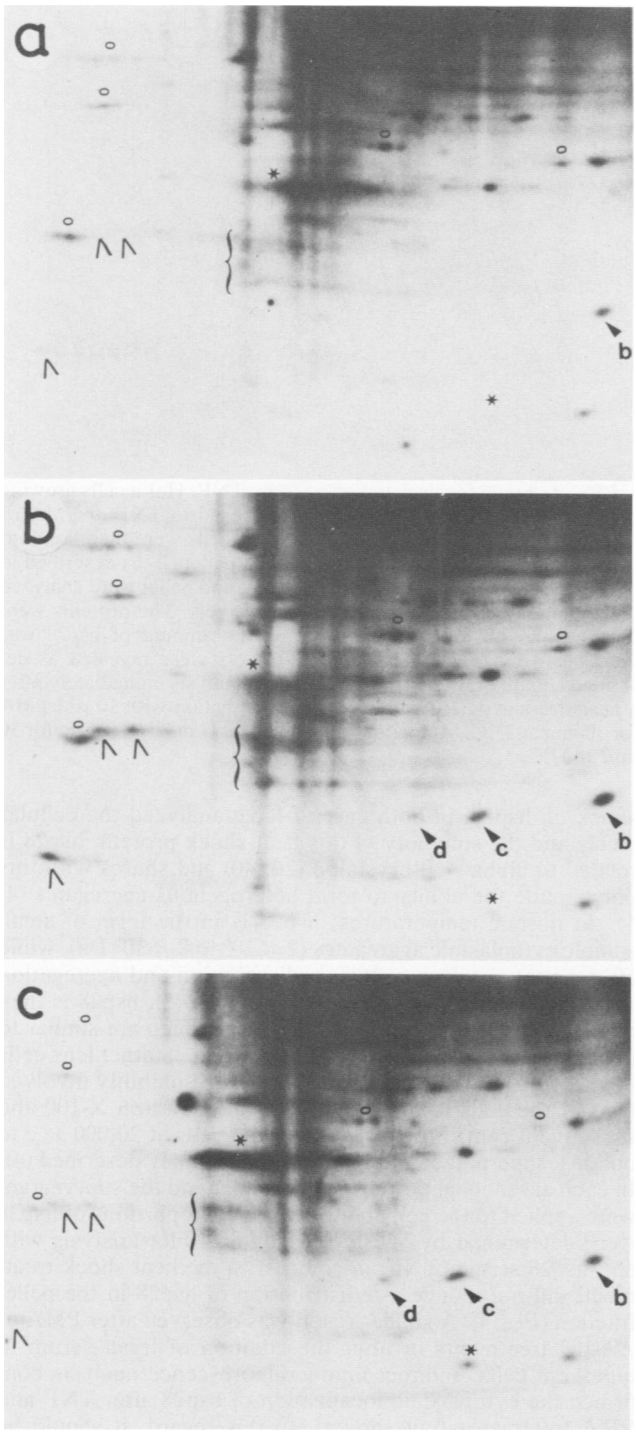

FIG. 1. TNF and heat shock induce the phosphorylation of similar 28-kDa polypeptides. HeLa cells growing on 35-mm Falcon dishes (Becton Dickinson Labware, Oxnard, Calif.) either were kept at 37°C and treated with 2,000 U of TNF per ml for 30 min or were heat treated at 44.5°C for the same time. Labeling was for 1 h at 37°C with $^{32}P_i$. TNF was added during the last 30 min of the incubation. In the case of heat stress, labeling was performed immediately after the heat shock treatment. The labeled proteins were analyzed in the first dimension by isoelectric focusing (pH 5 to 8) followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 15% polyacrylamide gel. The acidic end is to the left. (a) Control cells. (b) Cells treated with TNF. (c) Cells labeled after a heat stress of 30 min at 44.5°C. The arrowheads labeled b, c, and d indicate the positions of the 28-kDa polypeptides. The open arrowheads indicate the positions of acidic proteins also exhibiting an increased phosphorylation after TNF and heat treatments. Positions of proteins which increased their phosphorylation status only after TNF treatment are indicated ({), as are the positions of proteins showing a decreased phosphorylation after heat shock but which were unaffected by TNF (○) and the positions of proteins exhibiting an increased phosphorylation only after the heat shock treatment (*).

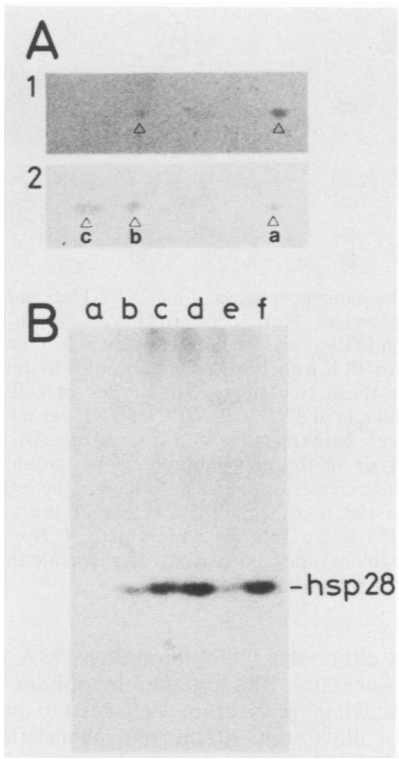

FIG. 2. Evidence that TNF induces the phosphorylation of the heat shock protein hsp28. (A) Immunoblots of two-dimensional gels revealed the 28-kDa polypeptides phosphorylated by TNF to be hsp28 isoforms. Anti-hsp28 antiserum was used to probe protein blots of two-dimensional gels from either control cells (gel 1) or cells incubated for 30 min with 2,000 U of TNF per ml (gel 2) as described in the text. The fraction of the blot showing hsp28 isoforms is presented. The blot was revealed by goat anti-rabbit serum coupled to horseradish peroxidase. The acidic end is to the left. The arrowheads labeled a, b, and c indicate the positions of hsp28 isoforms. The more basic isoform of hsp28 (a) does not contain phosphate. (B) Immunoprecipitation of cell lysates. HeLa cells growing on 35-mm Falcon dishes either were kept at 37°C or were heat treated at 44.5°C for 30 min or were treated with 2,000 U of TNF per ml or 10 μM PMA for 30 min. Labeling was for 1 h at 37°C with $^{32}P_i$. TNF and PMA were added during the last 30 min of incubation. In the case of heat stress, labeling was performed immediately after heat shock treatment. Cell lysates were used to immunoprecipitate hsp28 by using anti-hsp28 serum as previously described (5). An autoradiograph of the sodium dodecyl sulfate gel is presented. Lanes: a, control experiment using preimmune serum, cells kept at 37°C; b through f, immune serum plus control cells kept at 37°C (b), cells treated with PMA (c), heat-treated cells (d), control cells kept at 37°C (e), and cells treated with TNF (f).

ment confirmed that TNF stimulated the phosphorylation of hsp28 (Fig. 2B). The phosphorylation observed in the presence of 2,000 U of TNF per ml was as intense as that observed after a heat stress at 44.5°C for 30 min. It has been reported that hsp28 phosphorylation rapidly increased in

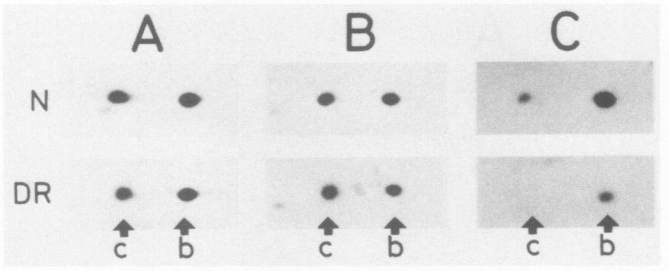

FIG. 3. The phosphorylation of hsp28 by TNF and heat shock is C kinase independent. HeLa cells growing on 35-mm Falcon dishes were divided into two sets. One set was kept at 37°C, while the other was treated for 48 h with 10 μM PMA in order to down regulate C kinase. After these treatments, both types of cells either were exposed for 30 min at 37°C to 2,000 U of TNF per ml (A) or 10 μM PMA (C) or were heat treated at 44.5°C for 30 min (B). Labeling with $^{32}P_i$ and analysis of the phosphoproteins by two-dimensional gel electrophoresis was performed as described in the legends to Fig. 1 and 2 and in the text. Only the fraction of the autoradiograph showing hsp28 b and c isoforms is presented. N, Normal cells, DR, cells down regulated for C kinase. Arrows indicate the positions of the b and c phosphorylated isoforms.

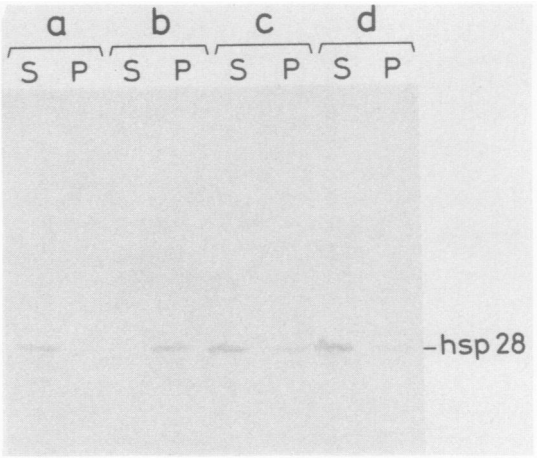

FIG. 4. hsp28 locale is not affected by TNF. HeLa cells growing on 35-mm Falcon dishes either were exposed to TNF or PMA or were heat treated as described above. The cells were then fractionated into a low-speed supernatant (S) and pellet (P) as described in the text. Equal portions of the supernatant and pellet were analyzed on sodium dodecyl sulfate-polyacrylamide gels. The proteins were then transferred to nitrocellulose, and the amount of hsp28 was determined by Western blotting. The blots were revealed as described above. (a) Cells kept at 37°C. (b) Analysis immediately after a heat stress at 44.5°C for 30 min. (c) Incubation with 10 μM PMA for 30 min at 37°C. (d) Incubation with 2,000 U of TNF per ml for 30 min at 37°C.

response to either the tumor promoter PMA (phorbol-12-myristate-13-acetate), the calcium ionophore A23187, or mitogens and when fresh serum was added to quiescent cells (30, 39). An illustration of the phosphorylation of hsp28 induced by PMA (10 nM for 30 min; Sigma) is shown in Fig. 2. In addition, when the incubation in the presence of TNF was reduced to 10 min, an intense phosphorylation of hsp28 was still observed (not shown). Therefore, the phosphorylation of hsp28 is one of the earliest cellular effects of TNF which has been reported in the literature.

The phosphorylation of hsp28 by PMA was shown to be dependent on C kinase (30). To address this issue in regard to TNF and heat shock, HeLa cells were incubated for 24 h at 37°C with 10 nM PMA to induce the translocation of protein kinase C and its subsequent down regulation (30). The level of phosphorylation of hsp28 after TNF, heat, or PMA treatment was then compared with that observed in cells which were not down regulated for C kinase. After TNF or heat shock, the increased phosphorylation of the hsp28 b isoform and the appearance of the c phosphorylated isoform were not impaired in cells down regulated for C kinase (Fig. 3). In contrast, the increased phosphorylation of hsp28 isoforms in response to PMA was not observed in cells down regulated for C kinase (30). A dependence on C kinase was also observed for the serum- and A23187-mediated phosphorylation of hsp28 (not shown). Therefore, the phosphorylation of hsp28 in response to TNF and heat shock is probably not C kinase dependent. This result implies that at least two different kinases are involved in hsp28 phosphorylation. It is also interesting that the level of phosphorylation of the b and c isoforms was almost identical in response to TNF and heat shock. This observation contrasts with the effect of PMA, which even at high concentrations induced a more intense phosphorylation of the b isoform. In addition, the phosphorylation by TNF of the other polypeptides described in Fig. 1B was also C kinase independent (not shown). Taken together, these results suggest that TNF may mimic the effect of a heat shock treatment at the level of hsp28 phosphorylation. However, more work is needed to confirm that the same sites of hsp28 are phosphorylated by the same kinase in response to TNF and heat shock.

In order to further compare the effects of TNF and heat shock on hsp28, in both cases I have analyzed the cellular locale and the solubility of this heat shock protein. hsp28 is related to alpha-A,B-crystallin (20, 40) and shares with this polypeptide the ability to form heterogenous aggregates (4, 5). At normal temperatures, hsp28 is in the form of small soluble cytoplasmic aggregates (2×10^6 to 8×10^6 Da), while after a heat shock, a cellular redistribution and aggregation of the small heat shock proteins occurs (2–5). hsp28 is then in the form of large nuclear aggregates, which are similar to the alpha-crystallin structures observed in cataract lens cells ($>15 \times 10^6$ Da) (4, 35). Analysis of hsp28 solubility involved lysis of the cells in the presence of 0.5% Triton X-100 and subsequent centrifugation of the lysed cells at 20,000 × *g* to obtain a supernatant and a pellet as previously described (4). In each case, equal portions of the pellet and the supernatant were applied to the gel, and the relative proportions of hsp28 were determined by Western (immuno-) blot analysis with anti-hsp28 serum. TNF, in contrast to the heat shock treatment, did not induce a redistribution of hsp28 in the pellet fraction (Fig. 4). A similar result was observed after PMA or A23187 treatments or after the addition of fresh serum to quiescent cells. Indirect immunofluorescence analysis confirmed the cytoplasmic localization of hsp28 after TNF and PMA treatments (not shown). In this regard, it should be noted that factors other than phosphorylation appear to regulate the cellular redistribution of the small heat shock proteins at elevated temperatures (4, 32). These results indicate that the TNF-mediated phosphorylation of hsp28 is a cytoplasmic event.

Despite reports suggesting that hsp28 is involved in the development of cellular resistance to stress (9, 11, 27), the exact function of this protein is not yet clear. One hypothesis is that hsp28 phosphorylation in response to TNF reflects the cellular resistance toward the cytostatic and cytotoxic action of this monokine. Interestingly, TNF and heat shock induce common cellular perturbations. Among them are the

impairment of mitochondrial activity, leading to an inhibition of respiration (17, 26), the alteration of the intracellular level of cyclic AMP, which may result in the activation of A-kinase activity (7, 25), and the production of reactive oxygen, which in turn causes cell damage by oxidative stress (16, 28, 29). The phosphorylation of hsp28 may also be a consequence of the signal transduction, via intracellular second messengers, of the binding of TNF to its receptor. Whether the C-kinase-independent phosphorylation of hsp28 by TNF or heat shock is simply a biochemical marker of these cellular perturbations or is in fact a regulator of these processes remains to be shown. Furthermore, Kaur and Salklatvala (21) have shown that interleukin-1, which shares pleiotropic effects with TNF, rapidly phosphorylates a 27-kDa polypeptide which is also resolved in multiple isoforms in two-dimensional gels. Taken together, these observations suggest that the phosphorylation of hsp28 may be an early event in the regulatory cascade leading to the cellular actions of TNF and interleukin-1.

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LITERATURE CITED

1. **Aggawal, S., B.-E. Drysdale, and H. S. Shin.** 1988. Tumor necrosis factor-mediated cytotoxicity involves ADP-ribosylation. J. Immunol. **140**:4187–4192.
2. **Arrigo, A.-P., J.-D. Darlix, E. W. Khandjian, M. Simon, and P.-F. Spahr.** 1985. Characterization of the prosome from Drosophila and its similarity to the cytoplasmic structure formed by the low molecular weight heat shock proteins. EMBO J. **4**: 399–406.
3. **Arrigo, A.-P., S. Fakan, and A. Tissières.** 1980. Localization of the heat shock induced proteins in Drosophila melanogaster tissue culture cells. Dev. Biol. **78**:86–103.
4. **Arrigo, A.-P., J. Suhan, and W. J. Welch.** 1988. Dynamic changes in the structure and locale of the mammalian low-molecular-weight heat shock protein. Mol. Cell. Biol. **8**:5059–5071.
5. **Arrigo, A.-P., and W. J. Welch.** 1987. Characterization and purification of the mammalian 28,000 dalton heat shock protein. J. Biol. Chem. **262**:15359–15369.
6. **Baglioni, C., S. McCandless, J. Tavernier, and W. Fiers.** 1985. Binding of human tumor necrosis factor to high affinity receptors on Hela and lymphoblastoid cells sensitive to growth inhibition. J. Biol. Chem. **260**:13395–13397.
7. **Baud, L., J. Perez, G. Friedlander, and R. Ardailloud.** 1988. Tumor necrosis factor stimulates prostaglandin production and cyclic AMP levels in rat cultured mesangial cells. FEBS Lett. **239**:50–54.
8. **Baumann, H., V. Onorato, J. Gauldie, and J. P. Jahreis.** 1987. Distinct sets of acute phase plasma proteins are stimulated by separate human hepatocyte-stimulating factors and monokines in rat hepatoma cells. J. Biol. Chem. **262**:9756–9768.
9. **Berger, E. M., and M. P. Woodward.** 1983. Small heat shock proteins of Drosophila may confer thermal tolerance. Exp. Cell Res. **147**:437–442.
10. **Carswell, E., L. Old, R. Kassel, S. Green, N. Fiore, and B. Williamson.** 1975. An endotoxin-induced serum factor that causes necrosis of tumors. Proc. Natl. Acad. Sci. USA **72**: 3666–3670.
11. **Chretien, P., and J. Landry.** 1988. Enhanced constitutive expression of the 27-KDa heat shock protein in heat resistant variant from chinese hamster cells. J. Cell. Physiol. **137**:157–166.
12. **Creasey, A. A., M. T. Reynolds, and W. Laird.** 1986. Cures and partial regression of murine and human tumors by recombinant human tumor necrosis factor. Cancer Res. **46**:5687–5690.
13. **Dayer, J.-M., B. Beutler, and A. Cerami.** 1985. Cachectin/tumor necrosis factor stimulates collagenase and prostaglandin E_2 production by human synovial cells and dermal fibroblasts. J. Exp. Med. **162**:2163–2168.
14. **Duh, E. J., W. J. Maury, T. M. Folks, A. S. Farci, and A. B. Robson.** 1989. Tumor necrosis factor-alpha activates human immunodeficiency virus type 1 through induction of nuclear factor binding to the NF-kB site in long terminal repeat. Proc. Natl. Acad. Sci. USA **86**:5974–5978.
15. **Fiers, W., P. Brouckaert, Y. Guisez, E. Remaut, F. Van Roy, R. Devos, L. Fransen, G. Leroux-Roels, A. Marmenout, J. Tavernier, and J. Van der Heyden.** 1986. Recombinant interferon gamma and its synergism with tumor necrosis factor in the human and mouse systems, p. 241–255. *In* W. E. Stewart and E. Schellekens (ed.), Biology of the interferon system. Elsevier Scientific, Amsterdam.
16. **Fiers, W., P. Brouckaert, R. Devos, L. Franzen, G. Leroux-Roel, E. Remaut, P. Suffys, J. Tavernier, J. Van der Heyden, and F. Van Roy.** 1986. Lymphokines and monokines in anticancer therapy. Cold Spring Harbor Symp. Quant. Biol. **51**: 587–595.
17. **Findly, R. C., R. J. Gillies, and R. G. Shuman.** 1983. In vivo phosphorus-31 nuclear magnetic resonance reveals lowered ATP during heat shock of Tetrahymena. Science **219**:1223–1225.
18. **Hass, P. E., A. Horchkiss, M. Mohler, and B. B. Aggarwal.** 1985. Characterization of specific high affinity receptors for tumor necrosis factors on mouse fibroblasts. J. Biol. Chem. **260**: 12214–12218.
19. **Helburn, A., D. Demolle, J.-M. Boeynaems, W. Fiers, and J. E. Dumont.** 1988. Rapid phosphorylation of a 27 Kd protein induced by tumor necrosis factor. FEBS Lett. **227**:175–178.
20. **Ingolia, T. D., and E. Craig.** 1982. Four small Drosophila heat shock proteins are related to each other and to mammalian alpha-crystallin. Proc. Natl. Acad. Sci. USA **79**:2360–2364.
21. **Kaur, P., and J. Salklatvala.** 1988. Interleukin-1 and tumor necrosis factor increase the phosphorylation of fibroblast proteins. FEBS Lett. **241**:6–10.
22. **Kim, Y.-J., J. Shuman, M. Sette, and A. Przybyla.** 1984. Nuclear localization and phosphorylation of three 25-kilodalton rat stress proteins. Mol. Cell. Biol. **4**:468–474.
23. **Koj, A., J. Gauldie, E. Regoeczi, D. N. Sauder, and G. D. Sweeney.** 1984. The acute-phase response of cultured rat hepatocytes. Biochem. J. **224**:505–514.
24. **Kull, F. C., Jr., and P. Cuatrecasas.** 1981. Possible recruitment of internalization in the mechanism of in vitro cytotoxicity in tumor necrosis serum. Cancer Res. **41**:4885–4890.
25. **Lamb, J., A. Fernandez, J. Feramisco, and W. J. Welch.** 1989. Modulation of vimentin containing intermediate filament distribution and phosphorylation in living fibroblasts by the cAMP-dependent protein kinase. J. Cell Biol. **108**:2409–2422.
26. **Lancaster, J. R., Jr., S. M. Laster, and L. R. Gooding.** 1989. Inhibition of target cell mitochondrial electron transfer by tumor necrosis factor. FEBS Lett. **248**:169–174.
27. **Landry, J., P. Chretien, H. Lambert, E. Hickey, and L. A. Weber.** 1989. Heat shock resistance conferred by expression of the human HSP27 gene in rodent cells. J. Cell Biol. **109**:7–15.
28. **Lindquist, S.** 1986. The heat shock response. Annu. Rev. Biochem. **55**:1151–1191.
29. **Nover, L.** 1984. Heat shock response of eukaryotic cells. Springer-Verlag KG, Berlin.
30. **Regazzi, R., U. Eppenberger, and D. Fabbro.** 1988. The 27,000 dalton stress proteins are phosphorylated by protein kinase C during the tumor promoter-mediated growth inhibition of human mammary carcinoma cells. Biochem. Biophys. Res. Commun. **152**:62–68.
31. **Robaye, B., A. Hepburn, R. Lecocq, W. Fiers, J.-M. Boeynaems, and J. E. Dumont.** 1989. Tumor necrosis factor-α induces the

phosphorylation of 28-kDa stress proteins in endothelial cells: possible role in protection against cytotoxicity. Biochem. Biophys. Res. Commun. **163**:301–308.

32. **Rossi, J., and S. Lindquist.** 1989. The intracellular location of yeast heat shock protein 26 varies with metabolism. J. Cell Biol. **108**:425–439.
33. **Ruggiero, V., K. Latham, and C. Baglioni.** 1987. Cytostatic and cytotoxic activity of tumor necrosis factor on human cancer cells. J. Immunol. **138**:2711–2717.
34. **Schütze, S., P. Scheurich, K. Pfizenmayer, and M. Kronke.** 1989. Tumor necrosis factor signal transduction. J. Biol. Chem. **264**: 3562–3567.
35. **Seizen, R. J., J. G. Bindel, and H. J. Hoenders.** 1978. The quaternary structure of bovine alpha-crystallin. Eur. J. Biochem. **91**:387–396.
36. **Sugarman, B. J., B. B. Aggarwal, P. E. Hass, I. S. Figari, M. A. Palladino, and H. M. Shepard.** 1985. Recombinant human tumor necrosis factor-alpha: effect on proliferation of normal and transformed cells in vivo. Science **230**:943–945.
37. **Tracey, K. J., B. Beutley, S. F. Lowry, J. Merryweather, S. Wolpe, I. W. Milsark, R. J. Hariri, T. J. Fahey, A. Zentela, J. D. Alberts, G. T. Shires, and A. Cerami.** 1986. Shock and tissue injury induced by recombinant human cachectin. Science **234**: 470–474.
38. **Tsujimoto, M., Y. K. Yip, and J. Vilcek.** 1985. Tumor necrosis factor: specific binding and internalization in sensitive and resistant cells. Proc. Natl. Acad. Sci. USA **82**:7626–7630.
39. **Welch, W. J.** 1985. Phorbol ester, calcium ionophore, or serum added to quiescent rat embryo fibroblast cells result in the elevated phosphorylation of two 28,000 dalton mammalian stress proteins. J. Biol. Chem. **260**:3058–3062.
40. **Wistow, G.** 1985. Domain structure and evolution in alpha-crystallins and small heat shock proteins. FEBS Lett. **181**:1–6.