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NOTES

Ca^{2+} Is Essential for Multistep Activation of the Heat Shock Factor in Permeabilized Cells

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We have developed a novel permeabilized-cell system to study transcription mechanisms. In permeabilized cells, heat-induced activation of the heat shock factor and transcription of the hsp70 gene require Ca^{2+}. Activation involves at least two steps: Ca^{2+}- and heat-dependent activation of heat shock factor binding and a second step, prior to transcription of hsp70, that requires ATP and is sensitive to genistein, a protein kinase inhibitor.

Exposure of cells to elevated temperature induces expression of the heat shock proteins (13). Expression is controlled by the heat shock factor (HSF), a sequence-specific DNA-binding protein which interacts with the heat shock element (HSE) in the promoter region of the heat shock proteins (3). Mammalian cells contain an inactive HSF which is converted to a DNA-binding form by heat shock (10, 15). In yeasts, the HSF binds constitutively to the HSE and transcriptional activation may occur by phosphorylation of the HSF (16, 17). However, the precise mechanism of activation of the HSF in vivo is unclear. To address this question, we have developed a novel permeabilized-cell system using digitonin-treated NIH 3T3 cells. Digitonin permeabilization of the plasma membrane has been widely used to study metabolic activity in the context of unperturbed cell architecture (5) and offers an intermediary between in vivo and in vitro systems. In our system, both activation of the HSF and transcription of the hsp70 gene occur after heat shock. This novel system allows us to study the requirements for DNA binding by the HSF and for initiation of hsp70 transcription.

Methods. NIH 3T3 cells were incubated in buffer containing KCl (137 mM), NaCl (5.4 mM), $MgCl_2$ (0.75 mM), $MgSO_4$ (0.75 mM), Na_2HPO_4 (0.5 mM), glucose (10 mM), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (10 mM, pH 7.4), bovine serum albumin (0.5 mg/ml), and digitonin (40 μM). In 40 μM digitonin, >95% of the cells took up trypan blue and approximately 70% of the soluble lactate dehydrogenase activity was released. Cells were harvested and then frozen at −80°C for 1 h, and extracts containing the HSF were prepared as described previously (21). Gel mobility shift assays contained 10 μg of extract, 0.5 ng of ^{32}P-HSE (derived from positions 452 to 483 of a mouse hsp70 clone [9]), and 0.5 μg of poly(dI-dC) in 10 mM Tris (pH 7.8)–50 mM NaCl–1 mM EDTA–0.5 mM dithiothreitol–5% glycerol. Binding reactions were incubated at 25°C for 20 min and separated on 4% nondenaturing acrylamide gels in 15 mM Tris–35 mM borate–1 mM EDTA at 140 V until the unlabeled probe was just run off the gel. Free Ca^{2+} was set by using Ca^{2+}–ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA) buffers (2). For Northern (RNA) hybridization analysis, cells were incubated in 3 ml of medium with digitonin (40 μM), Ca^{2+} (1 mM), and ATP (1 mM). After heat shock, cells were returned to 37°C, the medium was supplemented with 1 mM each (final concentration) UTP, GTP, CTP, ATP, *S*-adenosylmethionine, dithiothreitol, and $MnCl_2$ and 5 mM $MgCl_2$, and the cells were allowed to make RNA for 35 min. RNA was isolated by the guanidinium isothiocyanate method, and 15 μg of RNA was separated on 1% agarose–2.2 M formaldehyde gels (4). Equal loading was confirmed by using a β-actin probe. After transfer to GeneScreen Plus (DuPont), blots were probed with an *Xba*I-*Bgl*II fragment from the 3′ end of the hsp70 gene (bases 2762 to 3517 [9]) or a *Cla*I-*Eco*RI fragment from the 3′ end of the hsc70 gene (6) and washed as described previously (9).

Results. Figure 1a shows gel mobility shift assays using extracts from control NIH 3T3 cells (lanes 1 and 2) or from cells treated with 40 μM digitonin (lanes 3 to 10). Cell extracts contained a number of HSE-binding activities (lanes 1 and 2). Heat-shocked cell extracts (lane 2) contained a new, more slowly migrating band that was absent from non-heat-shocked cells (lane 1) and that corresponds to the HSF. The band labeled sp (lane 1) was greatly decreased after heat shock (lane 2), but the significance of this finding is unclear. Addition of excess, unlabeled, non-specific oligonucleotides released all the binding activities except the sp band (lane 1) and the HSF band (lane 2). The HSF and sp bands were specifically inhibited only by unlabeled HSE (data not shown). Cells permeabilized in medium containing digitonin, Ca^{2+}, and ATP (lanes 3 and 4) showed a binding pattern similar to that in intact cells (lanes 1 and 2); this pattern similarity demonstrates that digitonin does not interfere with the heat-induced binding of the HSF. Medium containing Ca^{2+} but lacking ATP still permitted induction of DNA binding (lanes 5 and 6). However, in the absence of Ca^{2+} (but with ATP [lanes 7 and 8]), the HSF was no longer activated by heat shock. Figure 1b shows the concentration dependence of this effect in digitonin-treated cells. Incubation of cells at 37°C with 0 to 1,000 μM Ca^{2+} (lanes 1 to 3) failed to induce binding of the HSF. When cells were heat shocked with increasing levels of Ca^{2+}, activation of the

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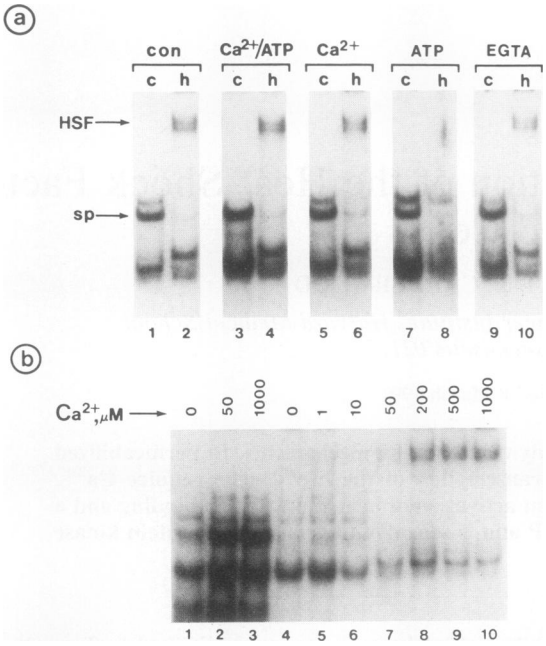

FIG. 1. Activation of the HSF in permeabilized cells requires Ca^{2+} and heat. Cells were heat shocked for 11 min at 45°C as described in the text, and extracts were prepared for gel mobility shift assays. (a) Lanes 1 and 2 (con), no digitonin; lanes 3 to 10, 40 μM digitonin. Ca^{2+} (1 mM) and ATP (1 mM) were added as indicated. EGTA (4 mM) was added to extracts 5 min before the ^{32}P-HSE. c, 37°C; h, 45°C. (b) Ca^{2+} dependence of HSF activation in digitonin-permeabilized cells. Lanes 1 to 3, 37°C; lanes 4 to 10, 45°C.

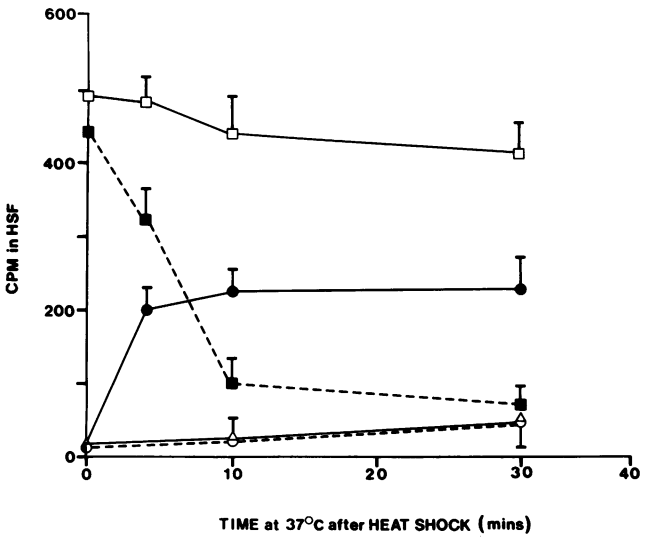

FIG. 2. Inactivation of the HSF after heat shock. Cells were heat shocked as described in the legend to Fig. 1 in the presence of digitonin (40 μM) and allowed to recover at 37°C for 0, 4, 10, or 30 min before preparation of extracts. After separation on 4% acrylamide gels, the HSF bands were cut out and the associated ^{32}P was measured by liquid scintillation counting. Results are an average of three experiments plus or minus the standard error of the mean. Cells were heat shocked in the presence (□ and ■) or the absence (○ and ●) of 1 mM Ca^{2+} or incubated at 37°C with 1 mM Ca^{2+} (△). After heat shock, cells were allowed to recover at 37°C with no further additions (□, ○, and △) or with addition of 4 mM EGTA (■) or 1 mM Ca^{2+} (●) at t = 1 min.

HSF occurred above 50 to 200 μM Ca^{2+}. However, the activated HSF in cell extracts does not require Ca^{2+} to bind, since incubation of extracts with EGTA prior to binding did not affect binding activity (Fig. 1a, lanes 9 and 10). Neither Mg^{2+}, Zn^{2+}, La^{3+}, Mn^{2+}, Cd^{2+}, nor Sr^{2+} could substitute for Ca^{2+} (data not shown). Figure 1 clearly shows that activation of the HSF requires both heat and Ca^{2+}, since either factor individually was insufficient to activate the HSF.

In whole cells, the HSF remains activated for 70 to 100 min after heat shock before being turned off (e.g., see reference 15). We investigated this inactivation in permeabilized cells. Cells were heat shocked and then returned to 37°C for 0 to 30 min before extracts were made. HSF bands were cut from the gel, and the associated ^{32}P activity was determined to provide a quantitative measure of binding activity. Cells heat shocked without Ca^{2+} (open circles, Fig. 2) or kept at 37°C in the presence of Ca^{2+} (open triangles, Fig. 2) contained no HSE-binding activity. Cells heat shocked with Ca^{2+} and then returned to 37°C retained HSF-binding activity for at least 30 min (open squares, Fig. 2). When cells heat shocked with Ca^{2+} were returned to 37°C and EGTA was added 1 min later, the binding activity was rapidly lost (closed squares, Fig. 2). Ca^{2+} is, therefore, required to convert the HSF to a DNA-binding form and to maintain the HSF in this activated state in the cell, although, as noted in Fig. 1, the binding of the HSF to the HSE in cell extracts does not require Ca^{2+}. Conversely, when cells were heat shocked without Ca^{2+} and returned to 37°C, addition of Ca^{2+} to the cells 1 min later rapidly induced HSF binding, up to 60% of control levels (closed circles, Fig. 2). Heat-shocked cells therefore turn on the mechanism which converts the HSF to a DNA-binding form, but this activity remains latent if no Ca^{2+} is available, even after the cells are returned to 37°C. Activation of the HSF appears to be a dynamic process; when Ca^{2+} is present, the HSF is maintained in a DNA-binding form. Removal of Ca^{2+} causes loss of HSF binding. Whether Ca^{2+} acts to block the off mechanism or to activate the on mechanism is unclear.

To determine whether activation of the HSF, in the presence of Ca^{2+}, correlates with production of hsp70 mRNA, Northern blot analysis of digitonin-permeabilized cells was carried out as described above. Northern blots (Fig. 3) show that digitonin-permeabilized cells heat shocked with Ca^{2+} and ATP accumulated the 3.2-kb hsp70 mRNA after heat shock (lanes 1 and 2). Lane 6 shows that failure to provide exogenous nucleotides (−XTPs) after the heat shock prevents mRNA synthesis, indicating that the cells are permeabilized and dependent on external nucleotide triphosphates. When Ca^{2+} was omitted during the heat shock, no hsp70 mRNA was detected (lanes 3 and 4), presumably because the HSF is unable to bind to the promoter and activate transcription. Figures 1 and 3 indicate that binding of the HSF is required for transcription of the hsp70 gene. Surprisingly, when ATP was omitted during the heat shock (but was present during the 35-min recovery at 37°C), Ca^{2+} failed to significantly increase the level of hsp70 mRNA (lane 5), even though the HSF binds under these conditions (Fig. 1). The requirement for ATP during heat shock for accumulation of hsp70 mRNA suggests that there is an ATP-dependent step or a phosphorylation involved in activation of the HSF and assembly of the transcription complex. This

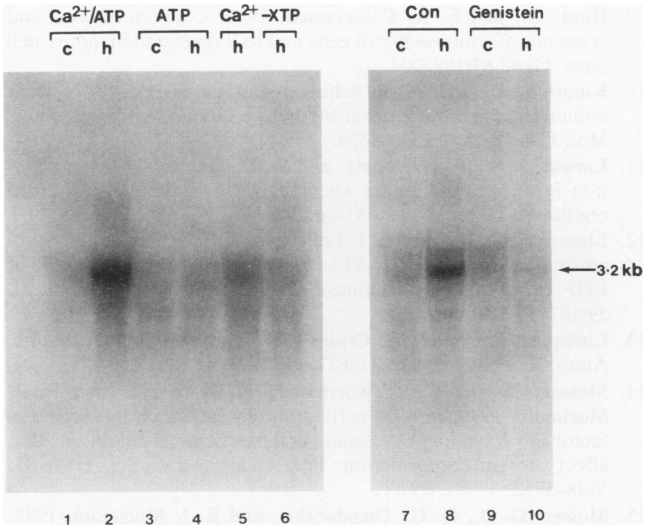

FIG. 3. Induction of hsp70 mRNA in digitonin-permeabilized cells. Cells were incubated in medium containing digitonin (40 μM), Ca^{2+} (1 mM), ATP (1 mM), or genistein (100 μM). After heat shock (11 min at 45°C), cells were returned to 37°C, exogenous nucleotides were added as described in the text, and RNA was separated and probed with an hsp70-specific probe. c, 37°C; h, 45°C. Lanes 6 to 10 contained 1 mM Ca^{2+} and 1 mM ATP. −XTP, no nucleotides added.

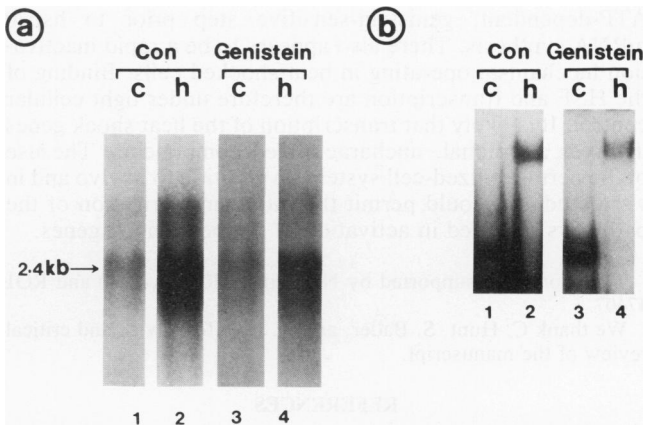

FIG. 4. Effect of genistein on transcription of hsc70 and binding of the HSF. (a) Cells were incubated in medium (minus digitonin) as described in the text and preincubated for 5 min with genistein (100 μM) as indicated. Cells were heat shocked and allowed to make RNA for 2 h as described in the legend to Fig. 3. RNA was prepared and analyzed by Northern blotting with an hsc70-specific probe. (b) Gel mobility shift assay. Cells were preincubated in dimethyl sulfoxide (lanes 1 and 2, con) or 100 μM genistein (lanes 3 and 4) for 5 min and then incubated for 10 min at 37°C (c) or 45°C (h). Extracts were prepared as described in the legend to Fig. 1.

is supported by the finding that in myeloid erythroleukemic (MEL) cells, heat shock results in binding of the HSF but the factor fails to initiate transcription of the hsp70 gene, possibly because the HSF is not phosphorylated (7). To test this hypothesis, genistein, an inhibitor of protein kinases, was used (1, 8, 12). In Fig. 3, cells were either untreated (lanes 7 and 8) or incubated for 10 min in genistein (100 μM) prior to heat shock (lanes 9 and 10). Ca^{2+} and ATP were present throughout. Genistein inhibits the heat-induced increase in hsp70 mRNA in permeabilized cells (Fig. 3, lanes 7 to 10). Data from nuclear run-off assays show that this inhibition is at the level of transcription rather then message stability (B. Price, unpublished observation). As a control, we examined expression of hsc70, a member of the hsp70 gene family present at low levels in cells but increased three- to fivefold by heat shock (6, 9). Northern blot analysis was performed exactly as described in the legend to Fig. 3 except that blots were probed with an hsc70 fragment (6). Figure 4 shows that heat shock increases hsc70 mRNA levels (lanes 1 and 2) and that genistein has only a small effect on this increase (lanes 3 and 4). Genistein, therefore, exhibits some specificity for hsp70, although it does inhibit transcription of a number of genes (12). In gel mobility shift assays, genistein did not block the activation of the HSF (Fig. 4b, lanes 1 to 4). This indicates that genistein can block a step which occurs after the binding of the HSF but before the initiation of transcription.

In conclusion, our experiments using a novel permeabilized-cell system indicate that there are multiple steps in the activation of transcription of hsp70. Other groups have reported that exposure of nuclear extracts to heat, detergents, Ca^{2+}, etc. can activate the HSF (11, 14). Whether in vitro systems replicate the physiological activation of HSF is unclear, since nuclear extracts apparently lack the endogenous inactivation machinery (Fig. 2) and the in vitro-activated HSF is not phosphorylated (11). In permeabilized cells, Ca^{2+} activates HSF binding only in combination with heat shock. The situation in intact cells appears to be similar, since heat shock elevates intracellular Ca^{2+} (18) but raising intracellular Ca^{2+} fails to activate the heat shock response (19, 20), indicating that Ca^{2+} alone is insufficient to activate expression of the heat shock genes. How Ca^{2+} interacts with the HSF activation machinery is not clear, since the HSF is not a Ca^{2+}-binding protein. Ca^{2+} may be required for an accessory protein involved in the activation process, or it may be involved in the inactivation of an inhibitor.

Genistein is an inhibitor of a number of tyrosine kinases, including the epidermal growth factor and platelet-derived growth factor receptors and v-*src* (1, 8, 12), and blocks transcription of the hsp70 gene but not HSF binding or accumulation of hsc70 mRNA. Whether genistein blocks tyrosine phosphorylation of the HSF or acts elsewhere is not clear. In yeasts, the HSF is apparently phosphorylated on serine or threonine residues (16), which should not be blocked by a tyrosine kinase inhibitor. The phosphorylation of the HSF alters its electrophoretic mobility (7, 11, 17), but treatment with genistein does not block this shift (data not shown). However, the residues phosphorylated in the mammalian HSF are unknown and may consist of both serine or threonine phosphorylation and tyrosine phosphorylation. If only tyrosine phosphorylation were inhibited by genistein, then genistein would have little effect on the phosphorylation-induced electrophoretic mobility shift described above. Although we have no direct evidence that genistein is specifically inhibiting a kinase or that it is inhibiting HSF phosphorylation, its effect suggests that there is an important phosphorylation event which occurs after Ca^{2+} activation of the HSF. This is supported by data showing that activation of hsp70 transcription required both ATP and Ca^{2+}, while induction of DNA binding required only Ca^{2+} (Fig. 1 and 3).

The data indicate that heat-induced transcription of the mammalian hsp70 gene may involve at least two steps. These steps include an initial heat-dependent step which, in the presence of Ca^{2+}, causes activation of the HSF, resulting in high-affinity binding of the HSF to the HSE, and an

ATP-dependent, genistein-sensitive step prior to hsp70 mRNA synthesis. There also appears to be a rapid inactivation mechanism operating in heat-shocked cells. Binding of the HSF and transcription are therefore under tight cellular control. It is likely that transcription of the heat shock genes involves additional, uncharacterized components. The use of the permeabilized-cell system, in addition to in vivo and in vitro studies, should permit the full characterization of the pathways involved in activation of the heat shock genes.

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