The EMBO Journal vol.4 no.1 pp.139–145, 1985

Double-stranded DNA induces the phosphorylation of several proteins including the 90 000 mol. wt. heat-shock protein in animal cell extracts

Anthony I.Walker[1], Tim Hunt, Richard J.Jackson and Carl W.Anderson[2]

Department of Biochemistry, Cambridge University, Tennis Court Road, Cambridge CB2 1QW, UK

[1]Present address: ICI Plant Protection Division, Jealott's Hill Research Station, Bracknell, Berkshire RG12 6EY, UK
[2]On sabbatical leave from: The Biology Department, Brookhaven National Laboratory, Upton, NY 11973, USA
Communicated by T.Hunt

Double-stranded DNA (dsDNA) induces the transfer of phosphate from ATP to several proteins in extracts of widely divergent eukaryotic cells. Extracts of HeLa cells, rabbit reticulocytes, *Xenopus* eggs and *Arbacia* eggs all show dsDNA-dependent protein phosphorylation. The mechanism is specific for dsDNA and will not respond to either RNA or single-stranded DNA. One of the proteins which is phosphorylated in response to dsDNA has a subunit mol. wt. of 90 000 and has been identified as a heat-shock protein (hsp90). Although mouse cell extracts were shown to contain hsp90, they failed to show a dsDNA-dependent protein phosphorylation. The observation that dsDNA can modulate the phosphorylation of a set of proteins raises the possibility that dsDNA may play a role as a cellular regulatory signal.
Key words: DNA/heat-shock protein/HeLa cells/phosphorylation/reticulocyte lysate

Introduction

This paper describes the discovery that double-stranded DNA (dsDNA) causes the phosphorylation of a set of proteins in extracts of certain eukaryotic cells. We first observed this type of protein phosphorylation during studies on the initiation of protein synthesis; the discovery was made as a consequence of the fact that an RNA preparation used in these studies contained a small amount of dsDNA. Cytoplasmic extracts of cultured human HeLa cells, rabbit reticulocyte lysate, extracts of frog eggs and extracts of sea urchin eggs all show dsDNA-dependent phosphorylation.

One of the substrates of this dsDNA-dependent protein phosphorylation has been identified. It is an abundant cytoplasmic protein, composed of 90 000 mol. wt. subunits, the synthesis of which is enhanced in response to cellular stresses including heat shock. A similar 90 000 mol. wt. heat-shock protein (hsp90) is found in many kinds of cells (Kelley and Schlesinger, 1982). HeLa cell hsp90 has been purified and partially characterised by Welch and Feramisco (1982); the nucleotide sequence of yeast hsp90 was recently reported by Farrelly and Finkelstein (1984). Although the function of hsp90 is unknown, the homologous chick cell protein has been found in a complex with $pp60^{src}$ and a 50 000 mol. wt. cellular protein (Oppermann *et al.*, 1981; Brugge *et al.*, 1981, 1983).

Kinases have been described which are activated by a variety of agents including cyclic nucleotides, phorbol esters, phospholipids, calcium ions, heme and double-stranded RNA (dsRNA) (see Krebs, 1983). Phosphoprotein phosphatase activators and inhibitors have also been described (see Li, 1982). To our knowledge, a phosphorylation-dephosphorylation system that responds to dsDNA has not been described previously.

Results

Double-stranded DNA induces protein phosphorylation

The addition of calf thymus DNA to a cytoplasmic extract of HeLa cells stimulated the phosphorylation of at least 12 polypeptides (Figure 1a,b) with approximate mol. wts. of 200 000, 120 000, 91 000, 90 000, 37 000, 65 000, 56 000, 48 000, 45 000, 34 000, 30 000 and 15 000. Rabbit reticulocyte lysates show a similar response to DNA; two polypeptides, having mol. wts. of 90 000 and 45 000, are the major components phosphorylated in response to DNA (see Figure 5a,b). These two polypeptides were also the major substrates of DNA-dependent phosphorylation in HeLa cell extracts.

Phosphorylation of the 90 000 and 45 000 mol. wt. polypeptides was observed in response to the addition of any dsDNA, including chicken erythrocyte DNA, bacteriophage lambda DNA, plasmid pBR322 DNA, the replicative (double-stranded) form of bacteriophage M13 DNA and poly d(A-T). The minimum concentration of DNA needed to cause this effect was found to be ~1 μg/ml; concentrations of dsDNA up to 1 mg/ml were active and did not inhibit the phosphorylation reaction. Digestion of the dsDNA with either DNase I or DNase II before addition to the HeLa extract abolished the phosphorylation inducing activity (Figure 1c,d), whereas pre-incubation with RNase A had no effect (Figure 1e). Furthermore, addition of pure single- or double-stranded RNA did not induce phosphorylation of this set of proteins; however, as expected (see Hunt, 1983), low doses of dsRNA did induce the phosphorylation of eukaryotic initiation factor 2 (eIF-2) alpha subunit (mol. wt. 38 000) in the reticulocyte lysate (data not shown). Some RNA preparations were found to induce phosphorylation of the 90 000 and 45 000 mol. wt. polypeptides, but pre-treatment with DNase and fractionation of the RNA preparations showed that this phosphorylation-inducing activity was due to DNA contaminating these RNA preparations (data not shown).

The addition of pure single-stranded M13 virion DNA to a HeLa cell extract did not induce phosphorylation even at 1 mg/ml. Heating native calf thymus DNA destroyed its activity as a phosphorylation inducer in a temperature- and salt-dependent manner, as would be expected if the inducer was dsDNA. Thus, heating the DNA in 10 mM Tris-Cl, pH 7.5, to 70°C or in 10 mM Tris-Cl, 40 mM NaCl to 90°C for 5 min inactivated the inducer; however, in 400 mM NaCl it was stable above 90°C (data not shown). Addition of poly(dA) or poly(dT) separately to the HeLa extract failed to induce phosphorylation. If, however, the poly(dA) and poly(dT) were first mixed, heated to 30°C for 10 min and then added to the system, phosphorylation was observed

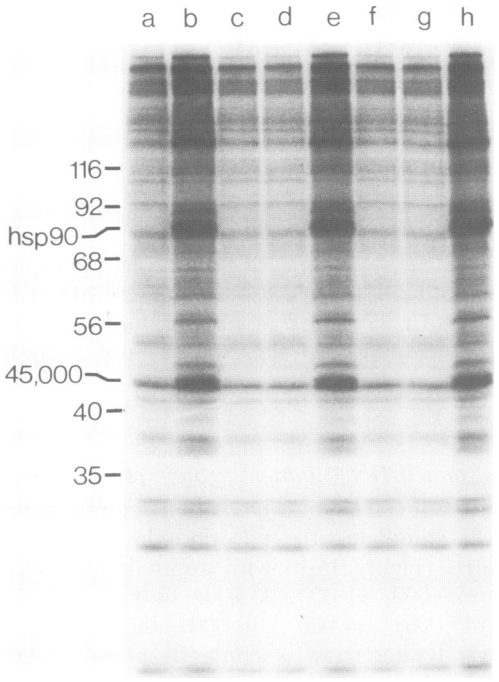

Fig. 1. dsDNA-dependent phosphorylation in a HeLa cell extract. An extract of HeLa cells was assayed for phosphorylation as described in Materials and methods. The autoradiograph shows HeLa extract incubated with **(a)** no addition, **(b)** calf thymus DNA (20 μg/ml), **(c)** DNase I-treated DNA, **(d)** DNase II-treated DNA, **(e)** RNase A-treated DNA, **(f)** poly(dA) (100 μg/ml), **(g)** poly(dT) (100 μg/ml) and **(h)** a mixture of poly(dA) and poly(dT) (both at final concentrations of 50 μg/ml) pre-annealed as described in the text. DNase I, DNase II and RNase A treatment of DNA is described in Materials and methods. In this photograph, only some of the polypeptides phosphorylated in response to dsDNA (see text) are visible. The positions of mol. wt. markers and of the 90 000 (hsp90) and 45 000 mol. wt. substrates for dsDNA-dependent phosphorylation are indicated at the left of the figure.

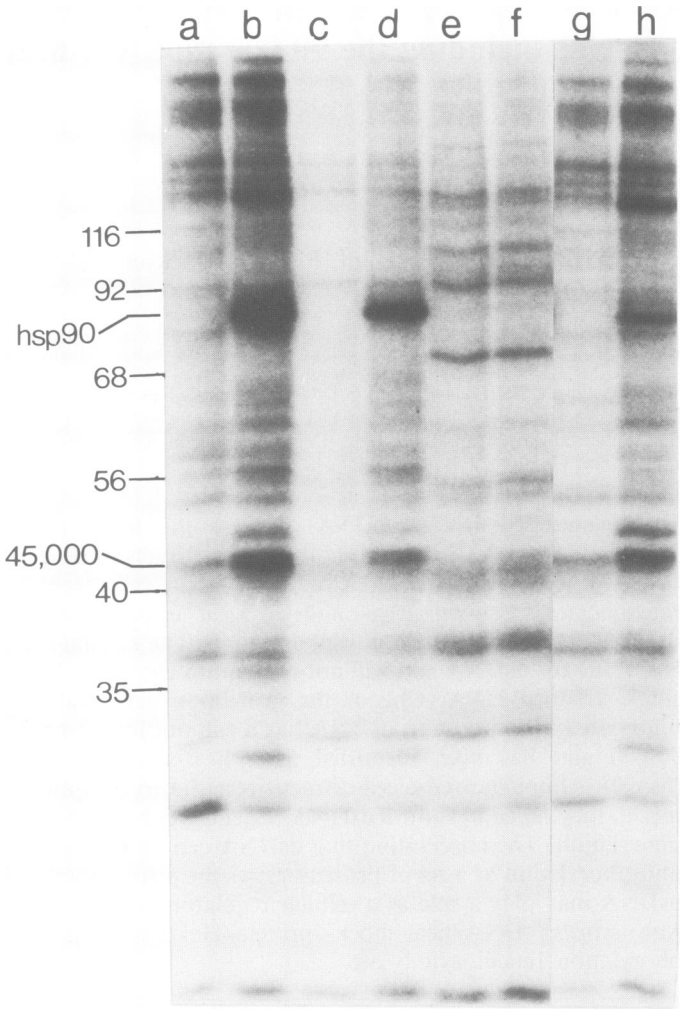

Fig. 2. Phosphorylation in HeLa extracts prepared by different methods. Extracts of HeLa cells were prepared by homogenization **(a,b)**, a single freeze-thaw cycle **(c,d)**, or lysis by NP-40 **(e,f)**. Tracks **(g)** and **(h)** show an assay in the presence of 1% NP-40 of an extract produced by homogenization. Phosphorylation assays in the absence **(a, c, e** and **g)** or presence **(b, d, f** and **h)** of calf thymus DNA (20 μg/ml) were performed as described in Materials and methods. The stained, dried polyacrylamide gel was autoradiographed with a phosphotungstate intensifying screen (DuPont). The positions of mol. wt. markers are shown at the left of the figure.

(Figure 1f–h). Taken together, the above data show that the inducer of phosphorylation is double-stranded DNA and that the inducing activity is sequence independent, since both simple and complex dsDNAs are equally effective.

Characteristics of dsDNA-dependent protein phosphorylation in HeLa cell extracts and reticulocyte lysates

Neither cAMP nor cGMP were found to affect dsDNA-dependent protein phosphorylation and [γ-^{32}P]GTP could not function as a phosphate donor in the reaction (data not shown).

Although the conditions under which the dsDNA-dependent phosphorylations occur have not been thoroughly investigated, several points of practical significance have emerged. The inclusion of 1 mM EGTA in phosphorylation assays suppressed the phosphorylation of a 92 000 mol. wt. polypeptide and increased the visibility of the effects of dsDNA on protein phosphorylation in some extracts. Increasing the concentration of KCl stimulated the dephosphorylation of several phosphoproteins including those labelled in the presence of dsDNA. Dephosphorylation of the 90 000 mol. wt. phosphoprotein at elevated KCl was somewhat inhibited by NaF, but not by phenylmethanesulfonyl fluoride (PMSF), EDTA or β-glycerol phosphate. The dsDNA-dependent phosphorylation reaction was not itself inhibited by 50 mM NaF or 80 mM β-glycerol phosphate. The dsDNA-dependent phosphorylation response could be observed at Mg^{2+} concentrations from 0.5 to >5.0 mM (data not shown). Extracts could be pre-incubated in the presence of dsDNA for up to 1 h before the addition of [γ-^{32}P]ATP without affecting the labelling of the 90 000 and 45 000 mol. wt. polypeptides. The response was observed using labelling times between 2 and 60 min; however, little increase in labelling was observed beyond 10 min.

Extracts of HeLa cells produced by a single cycle of freezing and thawing after hypotonic swelling were almost identical with respect to their protein content and phosphorylation response to extracts produced by homogenization (Figure 2a–d). However, extracts produced by Nonidet P-40 (NP-40) lysis showed no dsDNA-dependent phosphorylation, and exhibited a different pattern of phosphorylated polypeptides (Figure 2e,f). The addition of NP-40 to 1% final concentration to an extract of HeLa cells made by homogenization resulted in only a modest inhibition of the dsDNA-dependent phosphorylation and no change in the phosphoprotein profile was observed (Figure 2g,h).

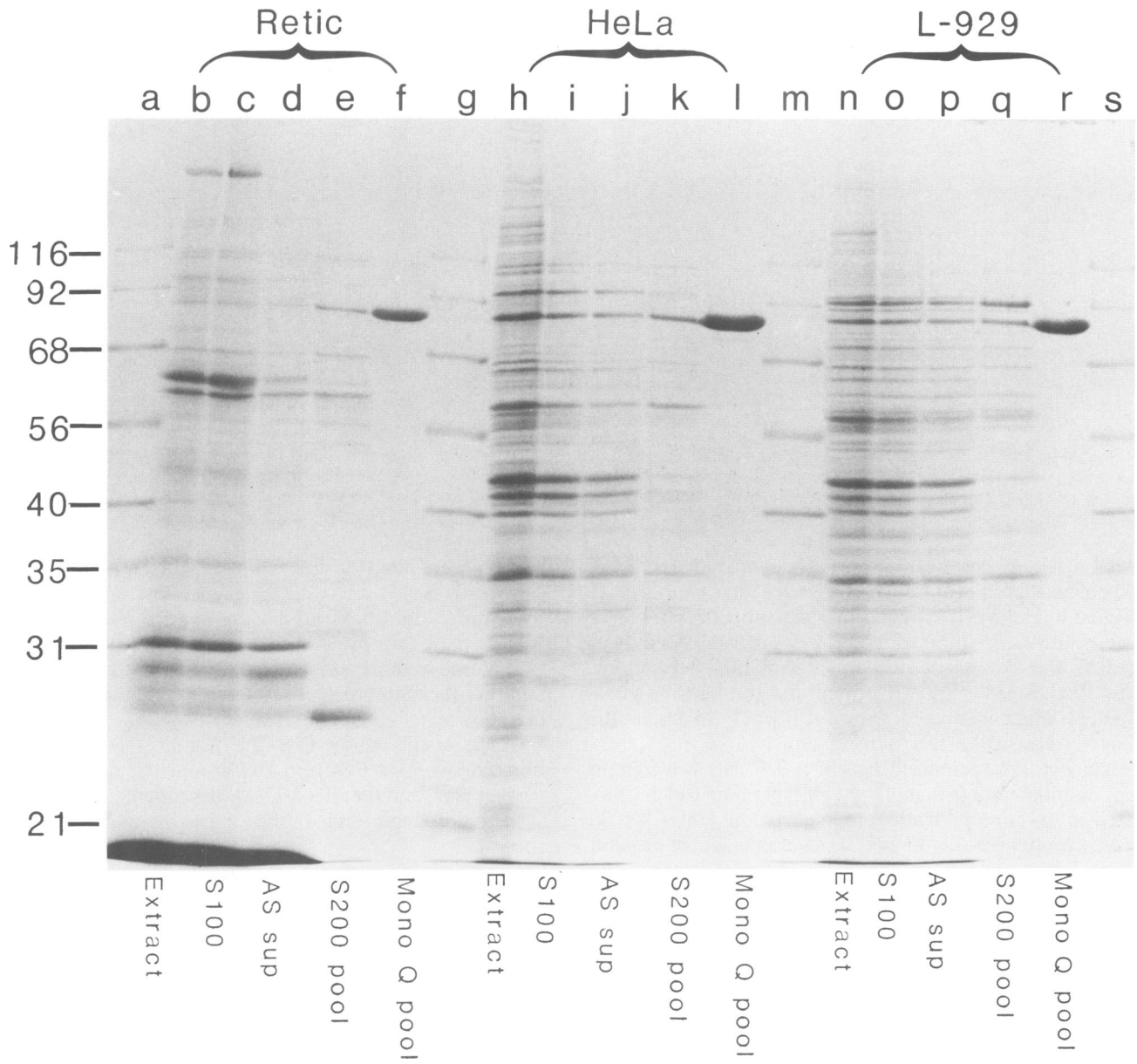

Fig. 3. Purification of hsp90 from rabbit reticulocyte lysate, HeLa cell extract and L-929 cell extract. The purification procedure is described in Materials and methods. The figure shows a photograph of a Coomassie brilliant blue stained gel after different stages of purification of 5 ml of **(b)**–**(f)** rabbit reticulocyte lysate, **(h)**–**(l)** HeLa cytoplasmic extract and **(n)**–**(r)** L-929 cytoplasmic extract. Tracks **(b)**, **(h)** and **(n)** are of the original extract; **(c)**, **(i)** and **(o)** are S100 supernatants; **(d)**, **(j)** and **(p)** are the 35% ammonium sulphate supernatants; **(e)**, **(k)** and **(q)** are the pool of fractions containing hsp90 after S200 gel filtration; **(f)**, **(l)** and **(r)** are the pool of fractions containing hsp90 after anion exchange chromatography. Tracks **(a)**, **(g)**, **(m)** and **(s)** show mol. wt. markers.

dsDNA does not affect protein synthesis in reticulocyte lysates

Since we first observed dsDNA-dependent phosphorylation in cell-free protein synthesis systems, the effects of dsDNA on translation were examined. Under conditions where the 90 000 and 45 000 mol. wt. components were phosphorylated, no effect was observed on the rate of initiation or elongation of protein synthesis in reticulocyte lysates translating endogenous message, or in nuclease-treated lysates programmed with cytoplasmic RNA isolated from uninfected or adenovirus-infected HeLa cells. Nor did we observe any effect of dsDNA on the pattern of products translated from these messages (data not shown).

The 90 000 mol. wt. substrate for phosphorylation is a heat-shock protein

When a HeLa cell extract was labelled with [γ-^{32}P]ATP in the presence of dsDNA and then passed through a Sepharose CL-6B gel filtration column equilibrated with low salt buffer (LSB – see Materials and methods), the 90 000 mol. wt. component retained its ^{32}P label and behaved as expected of a globular protein of ~200 000 mol. wt. When fractions containing this component were applied to an anion exchange column (Mono Q, Pharmacia) and eluted with a linear gradient of KCl (in LSB), polyacrylamide gel electrophoresis of the fractions showed that the labelled 90 000 mol. wt. component eluted at ~400 mM KCl, well after most of the proteins

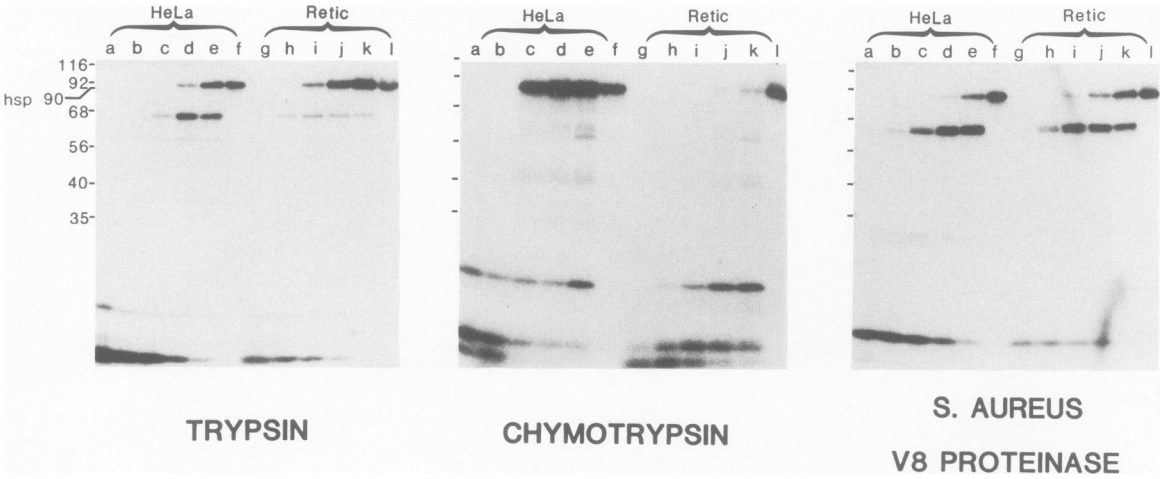

Fig. 4. Partial proteolytic digests of HeLa and reticulocyte ^{32}P-labelled hsp90. ^{32}P-labelled hsp90 was prepared and digested with trypsin **(left panel)**, chymotrypsin **(middle panel)** or *S. aureus* protease V8 **(right panel)** as described in Materials and methods. Tracks **(f)** and **(l)** from each panel contain samples of HeLa or reticulocyte hsp90 respectively that were incubated in the absence of added protease. The ^{32}P-containing digestion products were resolved on a 17.5% SDS-polyacrylamide gel and detected by autoradiography. The positions of mol. wt. markers are shown at the left of the figure.

in a HeLa cell extract. Furthermore, the radioactive material eluted coincidentally with the major 90 000 mol. wt. polypeptide found in HeLa cell extracts (data not shown). After these two steps, the 90 000 mol. wt. component was >95% pure as judged by the Coomassie brilliant blue stained gel pattern. The purified 90 000 mol. wt. protein did not become phosphorylated when incubated with [γ-^{32}P]ATP in either the presence or absence of DNA.

Reticulocyte lysates and extracts of L-929 cells also contain a very abundant 90 000 mol. wt. polypeptide which fractionated in an almost identical manner to the HeLa 90 000 mol. wt. component (see Figure 3). The reticulocyte protein, however, differed from the HeLa protein in that on Sepharose CL-6B gel filtration chromatography, the reticulocyte protein was found to behave as expected for a globular protein of ~500 000 mol. wt. (data not shown). The HeLa and reticulocyte components also behaved identically during hydroxylapatite chromatography (data not shown).

Examination of the published literature revealed that the purification properties of the 90 000 mol. wt. polypeptide phosphorylated in response to dsDNA in extracts of HeLa cells precisely match those reported for the 90 000 mol. wt. heat-shock protein (hsp90) (Welch and Feramisco, 1982). This protein is the major 90 000 mol. wt. polypeptide in HeLa cells, accounting for at least 1% of the total cellular protein even under non-stress conditions; it fractionates on gel filtration, anion exchange chromatography and hydroxylapatite chromatography in precisely the same way as the 90 000 mol. wt. substrate for phosphorylation.

Analysis of HeLa and reticulocyte hsp90 and 45 000 mol. wt. phosphoproteins

To compare the dsDNA-dependent phosphorylations in the HeLa and rabbit reticulocyte extracts, each was incubated with dsDNA and [γ-^{32}P]ATP, the polypeptides were resolved by SDS-polyacrylamide gel electrophoresis, the hsp90 and 45 000 mol. wt. polypeptides were located by autoradiography of the wet gels, and then eluted electrophoretically. SDS-polyacrylamide gel electrophoresis of the protease products obtained wtih trypsin, chymotrypsin or *Staphylococcus aureus* protease V8 revealed that the two hsp90s had virtually identically sized ^{32}P-labelled partial digestion products (Figure 4). The 45 000 mol. wt. phosphoproteins from the two sources were likewise similar by the same criterion (data not shown).

We have also examined the amino acids phosphorylated in each of these four proteins. dsDNA induced the phosphorylation of one or more serine and threonine residues in each protein; no phosphotyrosine was detected in either protein (data not shown).

These results show that the primary sequences of the homologous proteins from HeLa and reticulocytes must be very similar, and that the dsDNA-dependent phosphorylation systems of man and rabbit must have nearly identical specificities.

Phosphorylation induced by dsDNA in extracts from other animal cells

We have examined extracts from a limited number of other cell types to determine the extent to which different cells are capable of phosphorylating proteins in response to dsDNA. We have observed dsDNA-induced phosphorylation in *Xenopus* egg extracts and *Arbacia* (sea urchin) egg extracts. In *Xenopus* egg extracts 200 000, 110 000, 90 000, 45 000, 43 000, 37 000, 33 000, 25 000, 18 000, 17 000 and 15 000 mol. wt. polypeptides were phosphorylated in response to dsDNA (Figure 5e,f). In *Arbacia* egg extracts, at least 15 polypeptides having approximate mol. wts. of 200 000, 150 000, 112 000, 98 000, 90 000, 60 000, 50 000, 43 000, 38 000, 33 000, 28 000, 22 000, 21 000, 20 000 and 15 000 became phosphorylated in response to dsDNA (Figure 5g,h). Several of these substrates were relatively minor phosphoproteins and were not always readily observed.

Phosphorylation induced by dsDNA was not observed in extracts of mouse L-929 cells (Figure 5i,j), chick embryo fibroblasts or baby hamster kidney cells (data not shown). However, no systematic study of these cells has been made. Mixtures of L-cell extracts with HeLa cell extracts failed to reveal the presence of a dominant inhibitor of the reaction in the L-cell extract (data not shown).

Discussion

This paper reports a novel observation: dsDNA induces the transfer of phosphate from the gamma position of ATP to a set of proteins found in several different animal cells. DNA is

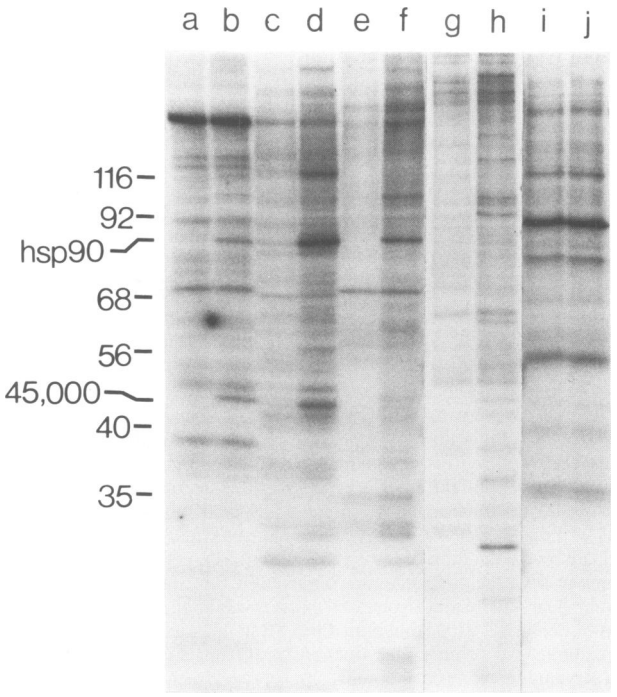

Fig. 5. Phosphorylation in various animal cell extracts. Extracts of HeLa cells, L-929 cells, rabbit reticulocytes and *Arbacia* eggs were prepared as described in Materials and methods. Extract of *Xenopus* eggs was the generous gift of Professor R.A.Laskey, Department of Zoology, University of Cambridge. Phosphorylation assays were performed as described in Materials and methods in the absence (**a, c, e, g** and **i**) or presence (**b, d, f, h** and **j**) of calf thymus DNA (25 μg/ml). Tracks (**a**) and (**b**) show a rabbit reticulocyte lysate; (**c**) and (**d**) show a HeLa cell extract; (**e**) and (**f**) show a *Xenopus* egg extract; (**g**) and (**h**) show an *Arbacia* egg extract; (**i**) and (**j**) show an extract of L-929 cells. The samples were loaded to approximately equalize the amount of phosphoprotein in each pair of tracks; to keep the total amount of protein per track constant, some samples were diluted with unlabelled extracts. In this photograph, only some of the polypeptides phosphorylated in response to dsDNA (see text) are visible.

often considered only as a template or a substrate, although it is an activator of several enzymes which act directly on DNA (e.g., Mace and Alberts, 1984). The role of DNA in the DNA-dependent phosphorylation reaction described in this paper is to regulate an enzymatic activity; we are not aware of other examples where DNA acts to control the activity of an enzyme not directly involved in DNA metabolism. This pattern of protein phosphorylation is induced specifically by dsDNA and does not depend upon a specific nucleotide sequence. This mechanism is reminiscent of, but clearly distinct from, the dsRNA-activated kinase which phosphorylates the alpha subunit of initiation factor eIF-2, and which is found in rabbit reticulocytes and other mammalian cells (see Lengyel, 1982; Hunt, 1983).

We have studied the dsDNA-dependent phosphorylation reaction only in crude cell-free extracts. These contain a multitude of ATPases, nucleases, kinases and phosphatases, thus we cannot yet say whether the increased labelling of certain proteins in the presence of dsDNA represents a net increase in the phosphorylation of these polypeptides or merely a turnover of phosphate at sites previously phosphorylated. Nor have we determined whether dsDNA exerts this effect by modulating the activity of a protein kinase or phosphatase, or by altering the conformation of the substrate polypeptides. However, preliminary studies indicate that these extracts do contain protein kinase activity activated by dsDNA.

Although dsDNA-dependent phosphorylation was observed in cells from a wide range of eukaryotic organisms, the response was absent from several cultured cell extracts including L-929 cell and chick embryo fibroblast cell extracts. L-929 cell extracts were found to possess a high concentration of hsp90 (Figure 3), and chick embryo fibroblasts are known to possess an homologous protein (Kelley and Schlesinger, 1982). The fact that extracts of HeLa cells produced by lysis in NP-40 lack the dsDNA-dependent response seen in extracts produced by mechanical disruption suggests that the response may depend on some, as yet undefined, feature of the method of cell breakage and fractionation. Therefore, negative results such as were obtained with L-929 cells and chick embryo fibroblasts need to be interpreted with caution.

The purification properties of the 90 000 mol. wt. polypeptide phosphorylated in response to dsDNA in extracts of HeLa cells are identical to those reported for hsp90 (Welch and Feramisco, 1982). The homologous protein in rabbit reticulocyte lysates has a native mol. wt. that is two to three times larger than that of the HeLa protein (data not shown), and in this respect resembles the chick hsp90, which is reported to have a mol. wt. of 500 000 (Kelley and Schlesinger, 1982). We found both serine and threonine residues to be major sites for the phosphorylation of HeLa and reticulocyte hsp90 *in vitro*, whereas phosphoserine, but not phosphothreonine or phosphotyrosine, was found in previous studies of chick hsp90 (Brugge *et al.*, 1981; Kelley and Schlesinger, 1982). The results of two-dimensional polyacrylamide gel analyses imply that hsp90s from other cell types are also phosphorylated at multiple sites (Welch and Feramisco, 1982). Brugge *et al.* (1981) have reported that eight of the tryptic peptides derived from chick hsp90 were found to contain phosphate residues.

Although interest in hsp90 has stemmed from its enhanced synthesis in stressed cells, it is an abundant protein in unstressed cells, representing at least 1% of total cellular protein in HeLa cells grown under normal conditions. Proteins immunologically related to chicken hsp90 have been found as a major component of many cell types including *Drosophila melanogaster, Xenopus* kidney cells and human fibroblasts (Kelley and Schlesinger, 1982). These findings indicate that hsp90 may have an important function during the normal cell life cycle, quite independent of the response to heat shock and other stresses. Whilst any such function of hsp90 remains a matter for speculation, the transient association of chick hsp90 with the avian tumour virus $pp60^{src}$ and with a 50 000 mol. wt. cellular protein has led to the suggestion that it may have a role in protein transport (Brugge *et al.*, 1981, 1983).

To our knowledge, no change in the phosphorylation state of hsp90 has ever been observed during heat shock or other stress responses, although changes in the phosphorylation state of several other proteins have been reported (Caizergues-Ferrer *et al.*, 1980; Ernst *et al.*, 1982; Scharf and Nover, 1982; Kennedy *et al.*, 1984). None of the other polypeptides which become phosphorylated in a HeLa cell extract in response to dsDNA show an obvious correlation with previously described heat shock or stress response products. Thus we have no reason to suppose that the dsDNA-dependent phosphorylation of hsp90 and other substrates is related to the cellular response to stress.

Although we have not yet observed changes in the phosphorylation of hsp90 and the 45 000 mol. wt. polypeptide in whole cells under any naturally occurring cir-

cumstances, we have recently observed (manuscript in preparation) that in *Xenopus laevis* oocytes, at least one protein of 15 000 mol. wt. becomes phosphorylated when calf thymus DNA is injected into the germinal vesicle. Furthermore, extracts prepared from *Xenopus* germinal vesicles contain an activity that is capable of phosphorylating purified HeLa hsp90 in a DNA-dependent manner. While these results perhaps indicate a nuclear location for the enzyme system, we have not yet attempted subcellular fractionation of any other cells; and of course, reticulocytes lack a nucleus.

While the physiological significance of dsDNA-dependent protein phosphorylation is unknown, we can suggest three possible roles of the dsDNA-dependent phosphorylation system: (i) detection of foreign (e.g., viral) DNA in a manner analogous to the dsRNA-activated kinase system; (ii) detection of intracellular damage to chromatin or mitochondrial DNA; or (iii) monitoring the state of nuclear DNA during the cell cycle. We are hopeful that the identification of additional substrates for dsDNA-dependent phosphorylation together with the purification of the enzyme(s) that catalyze DNA-dependent phosphorylation in different cells, will provide insight into the function of this novel phosphorylation system. The fact that distantly related cell types possess this sensitive mechanism for detecting dsDNA suggests that it may be of fundamental importance.

Materials and methods

Materials

Calf thymus DNA (type I), DNase I (DN-EP, 2400 U/mg), RNase A, poly(dA) and poly(dT) were obtained from Sigma. DNase II was obtained from Worthington Biochemicals. Chick embryo fibroblasts were kindly provided by Dr. T.Barrett, Department of Pathology, University of Cambridge; baby hamster kidney cells were provided by the laboratory of Dr. R.Johnson, Department of Zoology, University of Cambridge. *Xenopus* egg extracts were generously provided by Professor R.A.Laskey, Department of Zoology, Cambridge University.

Preparation of cell extracts

Cytoplasmic extracts of HeLa and mouse cells were prepared from cells grown in suspension culture to $\sim 5 \times 10^5$ cells/ml. Cells were harvested by centrifugation at 4°C. After three washes in ice-cold phosphate buffered saline (PBS), the cell pellet was resuspended in one packed cell volume of ice-cold low salt buffer (LSB: 25 mM KCl, 10 mM NaCl, 1.1 mM $MgCl_2$, 0.1 mM EDTA, 10 mM Hepes, pH 7.2 with KOH at 20°C), and allowed to swell for 10 min. Cells were broken by 20 strokes in a tight fitting stainless steel homogenizer (Wheaton Instruments). An S-10 supernatant was prepared from the homogenate by centrifugation at 10 000 *g* for 30 min at 4°C. Supernatants were stored frozen in liquid nitrogen in small aliquots. Extracts had an absorbance at 260 nm of between 50 and 100. To prepare extracts from small numbers of cells (e.g., $\sim 5 \times 10^7$) the homogenization step was replaced by a single freeze-thaw cycle.

Rabbit reticulocyte lysates were prepared as described by Jackson and Hunt (1983). Extracts of *Arbacia* eggs were prepared as described by Ballinger *et al.* (1984).

Phosphorylation assay

The standard assay mixture contained: 100 μl cytoplasmic extract, 6 μl KM (2 M KCl, 10 mM $MgCl_2$), 8 μl 0.2 M creatine phosphate, 2 μl 5 mg/ml creatine kinase, 2 μl 0.2 M EGTA, and 20 μl water. For *Xenopus* and *Arbacia* extracts, the mix was composed of 100 μl extract and 10 μl of 10 x TMA (200 mM Tris-Cl, pH 8.0, 20 mM $MgCl_2$, 10 mM ATP). 10 μl reactions were performed in 0.5 ml plastic tubes at 30°C; *Xenopus* and *Arbacia* egg extracts which were incubated at 20°C and 15°C, respectively. To each 10 μl reaction was added 1 μl [γ-^{32}P]ATP (10–40 mCi/ml, 40–80 Ci/mmol). Reactions were stopped by the addition of five volumes of SDS-sample buffer (Anderson *et al.*, 1973) and heating to 90°C for 3 min. Samples were analyzed by SDS-polyacrylamide gel electrophoresis (Anderson *et al.*, 1973). The pattern of ^{32}P-labelled proteins was visualised by autoradiography of the dried gel. ^{14}C-Labelled marker proteins were prepared as described in Evans *et al.* (1983). The marker mixture contained: β-galactosidase (*Escherichia coli*), mol. wt. 116 000; phosphorylase a, mol. wt. 92 000; bovine serum albumin, mol. wt. 68 000; glutamate dehydrogenase, mol. wt. 56 000; creatine kinase, mol. wt. 40 000; glyceraldehyde-3-phosphate dehydrogenase, mol. wt. 35 000; carbonic anhydrase (unlabelled), mol. wt. 30 000; soybean trypsin inhibitor, mol. wt. 21 000; RNase A, mol. wt. 14 000.

[γ-^{32}P]ATP was prepared from carrier-free phosphoric acid (Amersham PBS.41) by a minor modification of the method of Glynn and Chappell (1964).

Nuclease treatment of calf thymus DNA

Calf thymus DNA (700 μg/ml) was incubated with DNase I (1.0 U/ml), DNase II (1.5 U/ml) or with RNase A (70 μg/ml) for 30 min at 30°C. These reactions were then placed on ice pending a phosphorylation assay in which 1 μl of each reaction was assayed in 10 μl of cell extract. The buffer for reactions containing DNase I and RNase A was 7 mM Hepes, pH 7.2, 0.7 mM EDTA and 3.5 mM $MgCl_2$; the buffer for the reaction containing DNase II was 70 mM sodium acetate, pH 5.2 and 3 mM $MgCl_2$.

Purification of hsp90

Our original purification was very similar to that described by Welch and Feramisco (1982) for hsp90 from HeLa cells, and involved gel filtration on Sepharose CL-6B followed by anion exchange chromatography utilizing a Pharmacia Mono Q Fast Protein Liquid Chromatography column. For examining extracts of different cells for hsp90, the following procedure was employed. An S100 fraction was prepared by centrifugation of the extracts at 100 000 *g* for 4 h at 4°C. This supernatant was made 35% in ammonium sulphate by the addition of 0.97 g solid $(NH_4)_2SO_4$ per 5 ml of extract. After 30 min at 4°C, the precipitate was removed by centrifugation at 10 000 *g* for 30 min. The supernatant (from 5 ml extract) was applied to a 20 x 2.5 cm column of Sephacryl S200 equilibrated in LSB at 4°C; hsp90 appears in or near the column void volume. The fractions containing hsp90 were identified by polyacrylamide gel electrophoresis, appropriate fractions were pooled, and these were applied to a Pharmacia Mono Q anion exchange column equilibrated with LSB at room temperature. This column was eluted with a linear gradient of KCl (in LSB); hsp90 eluted at ~400 mM KCl in LSB. Fractions could be pooled to give hsp90 that was >95% pure as judged by the Coomassie brilliant blue staining pattern of an SDS-polyacrylamide gel. HeLa cells yielded ~5 mg of purified hsp90 per 5 ml of cytoplasmic extract; the same volume of reticulocyte lysate yielded ~1 mg hsp90.

Partial protease maps and phosphoamino acid analysis

HeLa extract and reticulocyte lysate were incubated at 30°C for 10 min with [γ-^{32}P]ATP in the presence of calf thymus DNA (100 μg/ml). After separation by preparative SDS-polyacrylamide gel electrophoresis, the positions of the radioactive polypeptides were determined by autoradiography of the wet, unfixed gel. Proteins were recovered by electroelution as previously described (Anderson, 1982) except that the elution buffer was 0.05 M ammonium bicarbonate, 0.1% SDS. Partial protease digestions were carried out essentially as described by Cleveland (1983). A portion of the sample was mixed with bovine serum albumin (to provide a known protein concentration), adjusted to 0.1% SDS, 0.05 M sodium bicarbonate, and the desired protease was added to give a series of reactions that decreased in protease concentrations in 2-fold steps. Reactions were incubated for 20 min at 30°C and stopped by the addition of SDS sample buffer. The ^{32}P-labelled proteolytic products were detected by autoradiography after SDS-polyacrylamide gel electrophoresis.

For phosphoamino acid analysis, SDS was removed by TCA precipitation followed by extraction of the samples with acetone:triethylamine:acetic acid (90:5:5). The dried protein precipitates were sealed in glass tubes with 0.25 ml 6 N HCl. Hydrolysis was at 105°C for 2–6 h. After HCl removal, the residue was dissolved in a small volume of water for application to 20 cm cellulose thin layer plates. Electrophoresis at 1000 V was for 45 min at pH 3.5 [pyridine:acetic acid:water (5:50:945)] and 30 min at pH 6.5 [pyridine:acetic acid:water (100:5:895)].

Acknowledgements

We would like to thank our colleagues in the Department of Biochemistry for their help and advice. We are particularly indebted to Dr. T.Hunter (Salk Institute, La Jolla, California) for drawing our attention to the work of Welch and Feramisco, and to Professor R.A.Laskey (Department of Zoology, University of Cambridge) for his generous assistance and valuable advice. This work was supported by research grants from the Medical Research Council of Great Britain and by the US Department of Energy. C.W.A. was the recipient of a senior research fellowship from the Institute of Allergy and Infectious Diseases (US); A.I.W. was supported by a Science and Engineering Research Council studentship.

References

Anderson,C.W. (1982) in Setlow,J.K. and Hollaender,A. (eds.), *Genetic Engineering, Principles and Methods,* Vol. **4**, Plenum Publishing Corp., NY, pp. 147-167.

Anderson,C.W., Baum,P.R. and Gesteland,R.F. (1973) *J. Virol.*, **12**, 241-252.
Ballinger,D.G., Bray,S.J. and Hunt,T. (1984) *Dev. Biol.*, **101**, 192-200.
Brugge,J.S., Erikson,E. and Erikson,R.L. (1981) *Cell*, **25**, 363-372.
Brugge,J.S., Yonemoto,W. and Darrow,D. (1983) *Mol. Cell. Biol.*, **3**, 9-19.
Caizergues-Ferrer,M., Bouche,G., Amalric,F. and Zalta,J.-P. (1980) *Eur. J. Biochem.*, **108**, 399-404.
Cleveland,D.W. (1983) *Methods Enzymol.*, **96**, 222-229.
Ernst,V., Baum,E.Z. and Reddy,P. (1982) in Schlesinger,M.J., Ashburner, M.A. and Tissieres,A. (eds.), *Heat Shock: From Bacteria to Man*, Cold Spring Harbor Laboratory Press, NY, pp. 215-225.
Evans,T., Rosenthal,E.T., Youngblom,J., Distel,D. and Hunt,T. (1983) *Cell*, **33**, 389-396.
Farrelly,F.W. and Finkelstein,D.B. (1984) *J. Biol. Chem.*, **259**, 5745-5751.
Glynn,I.M. and Chappell,J.B. (1964) *Biochem. J.*, **90**, 147-149.
Hunt,T. (1983) *Phil. Trans. R. Soc. Lond. B*, **302**, 127-134.
Jackson,R.J. and Hunt,T. (1983) *Methods Enzymol.*, **96**, 50-74.
Kelley,P.M. and Schlesinger,M.J. (1982) *Mol. Cell. Biol.*, **2**, 267-274.
Kennedy,I.M., Burdon,R.H. and Leader,D.P. (1984) *FEBS Lett.*, **169**, 267-273.
Krebs,E.G. (1983) *Phil. Trans. R. Soc. Lond. B*, **302**, 3-11.
Lengyel,P. (1982) *Annu. Rev. Biochem.*, **51**, 251-282.
Li,H.-C. (1982) *Curr. Top. Cell. Regul.*, **21**, 129-174.
Mace,D.C. and Alberts,B.M. (1984) *J. Mol. Biol.*, **177**, 279-293.
Oppermann,H., Levinson,W. and Bishop,J.M. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 1067-1071.
Scharf,K.-D. and Nover,L. (1982) *Cell*, **30**, 427-437.
Welch,W.J. and Feramisco,J.R. (1982) *J. Biol. Chem.*, **257**, 14949-14959.

Received on 14 September 1984; revised on 30 October 1984