Mitogen Activation Induces the Enhanced Synthesis of Two Heat-Shock Proteins in Human Lymphocytes

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Abstract. We have used mitogenic lectin (PHA) and a monoclonal antibody (OKT3) to stimulate human peripheral blood (G_0) lymphocytes, in the presence of monocytes, and have found two major preferentially synthesized proteins, 73 and 95 kD, which are induced by the mitogens. The elevated synthesis of both proteins begins ∿4–6 h after mitogen addition (early to mid G_0/G_1) before entry into first S phase. Maximum synthesis of both proteins is reached by 12 h after mitogen addition when P95 synthesis represents ∿4%, and P73 ∿2%, of the total protein synthesis, compared with less than 0.5% for each protein in cells cultured without mitogen. Thus, the proteins appear to be major components of activated cells. We find that both P73 and P95 are induced by heat stress as well as mitogenic stimulation. The induction of the proteins is not affected by either deleting glucose from the cul-

ture media or, alternatively, by supplementing it. Using polyclonal antibodies prepared to each of the proteins isolated from mitogen activated cells and monoclonal antibodies that were raised to heat shock proteins, we are able to show that P95 is electrophoretically and immunologically identical to the HSP 90 induced by heat stress. P73 is one of the 70 kD HSPs, (termed HSC 70; Pelham, H. R. B. 1986. *Cell.* 46: 959–961), but is different from the most strongly heat inducible form of HSP 70 (72 kD). The distribution of both proteins in subcellular fractions of mitogen activated lymphocytes is similar to the reported localization of the respective HSP's in other cell types. The results suggest that HSP 90 and HSC 70 may have functional roles in stress response and growth processes of human lymphocytes.

THE heat-shock proteins, or HSPs,[1] are a highly conserved group of proteins inducible in all of the many species of organisms and cultured cell types that have been tested. The rapid onset of transcription and translation of this specific and limited subset of cell proteins is an apparently universal response to heat shock and other stressful conditions and is thought to aid recovery and confer tolerance. (For review, see reference 24.)

Two of the major HSPs, or closely related proteins (termed "HSP cognates"), with molecular masses of 90 and 70 kD are found in normal, unstressed cells where they may play a role in growth control mechanisms. Recent reports have demonstrated the association of HSP 90 with both PP60[c-src] (31) and with a component of the steroid receptor (5, 38). Similarly, members of the HSP 70 family have been reported to associate with P53, a nuclear protein expressed in the G_0/G_1 transition (34).

The relationship between the level of expression of HSP 70 in (unstressed) proliferating cells vs. their level in quiescent cells is controversial. Wu and Morimoto (51) found increased expression of an HSP 70 after serum stimulation of quiescent fibroblasts. The level of expression of an HSP 70 was also found to be increased in tumor derived cell lines

(13). On the other hand, Kaczmarek et al. (16) and Iida and Yahara (12) have correlated increased expression of an HSP 70 with cell quiescence, i.e., arrest in G_0. While there are no systematic studies of HSP 90 expression during the cell cycle, HSP 90 expression does change during *Drosophila* development and yeast sporulation (19).

Lymphocytes in the peripheral circulation are, for the most part, in a true (naturally occurring) G_0 state. The biochemistry of activation after in vitro stimulation of these quiescent cells has been extensively investigated. Mitogen induces departure from G_0 and transition through G_1, a 26–30-h period that is termed the lag phase, before DNA synthesis begins (10). Many of the initial stimulatory events at the cell surface in terms of mitogen binding and subsequent secondary messenger involvement are fairly well understood (47). It is known that during the lag phase, extracellular signals such as mitogens, growth factors and interleukins (9, 44) induce sequential gene expression (15, 35) culminating in DNA synthesis and cell division. The function and identity of many of the gene products appearing in the lag phase are not known. However, it is reasonable to propose that proteins induced by the stimulatory signals in lag phase play an essential role in the onset of DNA synthesis and mitosis (8, 11, 21). In this paper we present evidence that induction of synthesis of two heat-shock proteins, HSP 90 and HSC 70, occurs in

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1. *Abbreviation used in this paper*: HSP, heat-shock proteins.

human peripheral blood mononuclear cells during the lag phase after activation by mitogen.

Materials and Methods

Cell Isolation and Culture

Human peripheral blood mononuclear cells were obtained by separation of whole blood on Ficoll-Hypaque discontinuous density gradients, yielding ~90% lymphocytes (29). The cell suspension medium contained RPMI 1640 (Gibco, Grand Island, NY), supplemented with penicillin, streptomycin, and L-glutamine. A Selectamine kit (Gibco) was used to prepare RPMI 1640 without glucose. Pooled human serum prepared from 10 male donors was used for routine cultures, but FCS was used for the experiments using OKT3. Cells were cultured at 0.5-5 × 10⁶/ml in culture plates for radioactive amino acid labeling. [³H]leucine (Research Products International, Mt. Prospect, IL) at a specific activity of 50 Ci/mmole and at a dose of 100 μCi/ml and [³⁵S]methionine (Amersham, Arlington Heights, Il.) at a specific activity of 344 mCi/mmole and a dose of 40 μCi/ml were used to metabolically label cellular proteins when cells were pulse labeled for 6 h (also used in the 4-h experiments). For longer label periods the dose was proportionally reduced to maintain a similar level of radioactive incorporation (label times are indicated in each figure legend).

Subcellular Fractionation

After culture the flasks were cooled to 4°C and scraped with a rubber policeman to remove the cells. The cells were centrifuged, washed twice in cold media, and lysed in a low concentration detergent solution (0.1% Triton X-100, 150 mM KCl, 8 mM MgCl, 20 mM Tris 2 mM PMSF, pH 7.5) at a ratio of 100 μl/20 × 10⁶ cells. Insoluble (predominantly nuclear) components were rapidly separated from the 0.1% detergent soluble ("cytoplasmic") material in a microcentrifuge. The insoluble nuclear pellet was washed once (150 mM NaCl, 10 mM MgCl₂, 20 mM Tris, 2 mM PMSF, pH 7.5) to remove components soluble at low salt concentration (nuclear wash), resuspended in DNase buffer (100 mM NaCH₃COO-, 5 mM MgSO₄, 20 mM Tris 2 mM PMSF, pH 6.0) and treated with 100 μg/ml DNase I (protease free, Cooper Biomedical, Malvern, PA) to disperse the material. The procedure yields three fractions, based on solubility, termed the cytoplasmic fraction, the nuclear wash fraction, and the nuclear fraction. The method allows rapid, conservative isolation of all cell proteins while producing relatively pure nuclei as determined by phase contrast microscopy and the presence of relatively low amounts of intermediate filament material when analyzed on SDS gels. Residual nuclear matrix was prepared by a modification of the method of Berezney and Coffey (3). Nuclei were briefly (3 s) microcentrifuged to concentrate them, then resuspended and extracted with 2.0 M NaCl, 1% Triton X-100, 100 mM Tris, 1 mM PMSF, pH 7.4 for 1 h on ice. The residue was washed, then suspended (using sonication if necessary) in DNase buffer and treated with DNase I (200 μg/ml) and RNase (100 μg/ml) for 1 h. The extracted "matrix", or high salt/detergent insoluble fraction, was then dissolved in SDS-PAGE sample buffer.

Electrophoresis and Protein Blotting

Proteins of the subcellular fractions were separated by the method of Laemmli (20). For two-dimensional electrophoresis, by the method of O'Farrell (28), the cells were removed from culture plates and washed as above, then lysed in 2-D lysis buffer at 100 μl/20 × 10⁶ cells. Protein transfer to nitrocellulose was done with a 1-h blot in a plate electrode blotter (Idea Scientific, Corvallis, OR). After reaction with the rabbit antiserum (Fig. 4) or with the mouse monoclonal AC88 (Fig. 5) the blots were developed with appropriate Vectastain ABC kits (Vector Laboratories, Burlingame, CA). The blots reacted with rat monoclonal antibody 7.1 (Fig. 6) were developed in Dr. S. Lindquist's laboratory with a horseradish peroxidase conjugated secondary antibody. Optical density scans of the 1-D fluorograms were obtained using a Kratos Spectrodensitometer Model SD 3000 (Schoeffel Inst.) and an HP 3390 A integrator (Hewlitt Packard, Palo Alto, CA).

Antiserum Production

To produce antisera, cytoplasmic samples of cells cultured with PHA were applied to 8-14% gradient SDS-PAGE gels (30 cm gel). The gels were not stained, but treated with Enlightening (NEN, Boston, MA), then dried on cellophane, and exposed for fluorography to locate the bands at 73 and 95 kD. The bands were excised, rehydrated, and finely ground in a Dounce homogenizer, then eluted by diffusion into SDS-glycine buffer until few counts remained in the gel residue. The protein was concentrated in Centricon filters (30-kD cutoff; Amicon, Danvers, MA) and the procedure repeated until roughly 100-μg protein was available for injection into rabbits (New Zealand White). The initial injection was prepared with complete Freund's adjuvant. After 8 wk a boost with ~50 μg protein, in incomplete Freund's adjuvant, was injected. The rabbits were first bled 4 wk after the boost. 40-ml ear bleeds were routinely done until antibody titers dropped noticeably.

Results

Preferential Synthesis of P73 and P95 by PHA

Fig. 1 demonstrates the preferential induction of two major synthesized proteins, with M_r of 73 (P73) and 95 kD (P95) in the 0.1% Triton X-100 soluble "cytoplasmic" fractions of cells cultured with 1 μg/ml PHA and 10% pooled human serum. In our hands this serum concentration and 0.5-1.0 μg/ml of PHA result in maximal rate of entry into the first S phase with an average lag period of 26 h (10). As shown in the figure, induction of P73 and P95 is evident by 6 h and peaks at 12-18 h. For comparison, cytoplasmic fractions of cells incubated for 18 h with the mitogenic monoclonal antibody, OKT3 (anti-CD3) are also shown in Fig. 1. There are other proteins that are induced by the mitogens, among them a 66- and a 55-kD protein described by Hall et al. (11) and bands at 60, 51, and 39 kD. These proteins have not been identified but their kinetics of induction by PHA and OKT3,
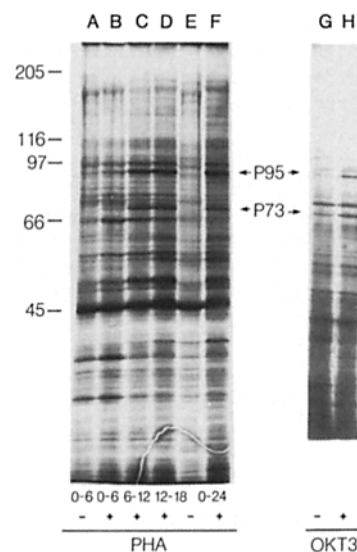

Figure 1. Cytoplasmic proteins (See Materials and Methods) synthesized in PHA-stimulated cells at various times in the lag phase, shown together with cells cultured in the presence of a mitogenic antibody. Cells were cultured and labeled with both [³H]leucine and [³⁵S]methionine, for 0-6 h (*A*) and 0-24 h (*E*) in pooled human serum without PHA and labeled 0-6 h (*B*), 6-12 h (*C*), 12-18 h (*D*), 0-24 h (*F*) after addition of PHA and serum. Also shown are cells cultured for 18 h (continuous label) in the presence of 0.1 μg/ml OKT3 (anti-CD3), lane *H*, adjacent to cells cultured for 18 h in FCS alone, lane *G*. The fluorograms of 8-14% gradient gels are shown. The positions of molecular weight standards are indicated at the left side of the figure with their molecular weights.
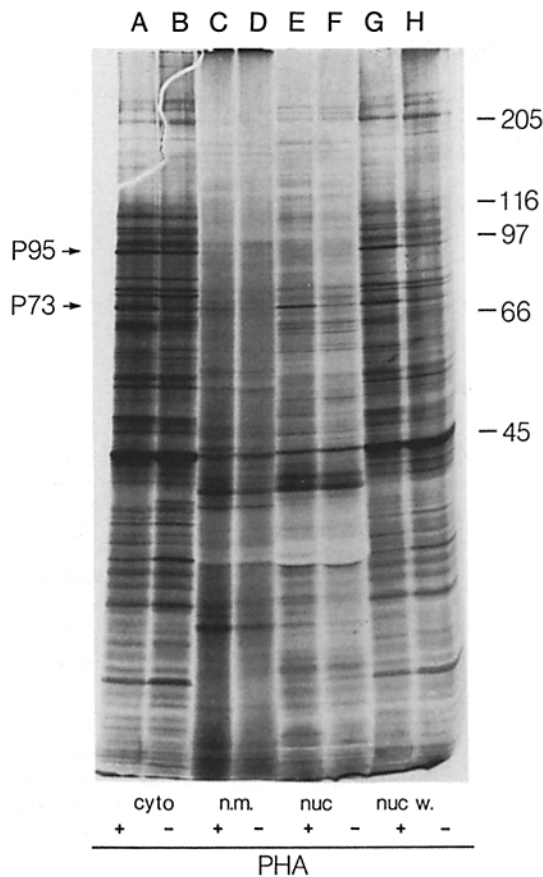
A B C D E F G H

P95 →
P73 →

— 205

— 116
— 97

— 66

— 45

cyto   n.m.   nuc   nuc w.
 +   -   +   -   +   -   +   -

PHA

Figure 2. Labeled proteins of four subcellular fractions of lymphocytes after 12 h of culture. Cells were cultured in PHA (0.75 μg/ml) plus 10% pooled human serum (lanes *A, C, E,* and *G*) or in pooled human serum alone (lanes *B, D, F,* and *H*). As described in Materials and Methods, the fractionation procedure yielded solubility fractions termed the cytoplasmic fraction, (*A* and *B*); the residual nuclear matrix, (*C* and *D*); the (whole) nuclear fraction, (*E* and *F*); and the nuclear wash fraction, (*G* and *H*). The proteins from the subcellular fractions were separated by SDS-PAGE on an 8–14% gradient gel, and the fluorogram is shown. Label was present from the start of the culture and an amount of protein containing equivalent cpm was added to both the serum and PHA lanes of a given fraction. The bars indicate 205, 116, 97, 66, and 45 kD molecular mass standards.

and 2-D gel analysis are the subject of ongoing investigation (Haire, R. N., and J. J. O'Leary. Manuscript submitted for publication).

Fig. 2 shows the preferential induction of synthesis of P73 and P95 within subcellular fractions at 12 h of culture. Synthesized P73 is present in all fractions, including a weakly labeled band visible in the residual nuclear matrix fraction. Synthesized P95 is visible only in the detergent soluble "cytoplasmic" fraction and low salt soluble nuclear fraction, consistent with a cytoplasmic location.

In Fig. 3, the level of induction of synthesized proteins by PHA was estimated by densitometry. Cells were pulse labeled for 4 h in late lag phase (16–20 h after PHA) and the lanes of a lightly exposed 1-D gel fluorogram were scanned to determine the relative level of radiolabel incorporation into cytoplasmic fractions. The fraction of label incorporated into the band at 95 kD, expressed as percent total integrated

area increases from 1.3% in serum to 11.2% in the PHA lane and the area of the 73-kD band increases from 1.4% to 5.5%. Because the cytoplasmic fraction contains ∼60% of total cellular incorporation and some bands at one end of the gel were omitted from the scan, we estimate that at maximal stimulation the P95 synthesis comprises up to 6% of total cell label incorporation, compared with ∼0.6% in unstimulated cells. Similarly, synthesis of P73 is enhanced from 0.6% in resting cells to almost 3% of total label incorporation in activated cells. When cells are cultured with varying PHA concentrations, the level of P73 and P95 synthesis, at 12–18 h, rises with PHA dose (data not shown) and reaches a maximum at 0.5 to 1 μg/ml PHA. The rate of entry of lymphocytes into first S phase, as previously shown by Hall et al. (11), shows a similar PHA dose response.

Serum is not Required for PHA Induction of P73 and P95 Synthesis

Growth factors in serum are required for the proliferative response of lymphocytes activated by PHA (39). Lymphocytes stimulated in media without serum will not proliferate and have a low overall rate of protein synthesis. If an appropriate amount of albumin is substituted for serum, cell motility, and protein synthesis are enhanced (our unpublished observations), but the cells are only marginally stimulated to proliferate by PHA, as measured by the thymidine uptake assay (29). As shown in Fig. 4, the PHA induction of synthesis of P73 and P95 in serum-free or albumin-supplemented medium is similar to that occurring in the presence of serum. The basal level of the proteins in the lanes cultured without PHA is similar, but this fact is partially obscured by the failure to apply equal counts to all lanes, especially, lane *E*. Thus, induction of the proteins is apparently largely independent of the serum factors required for optimal lymphocyte proliferation.

P73 and P95 are Mammalian Heat-Shock Proteins

Human mononuclear cells, exposed to heat shock, then allowed to recover at 37°C, show reduced overall protein synthesis but enhanced synthesis of a small number of specific proteins, Fig. 5. Labeled proteins from heat-shocked and PHA-activated cells were run on an SDS gel, and transferred to nitrocellulose. After autoradiography (right side of Fig. 5) the blot was incubated with a mixture of the polyclonal rabbit antisera raised to the 95- and 73-kD bands isolated from gels of PHA activated cells (left side of Fig. 5). The major synthesized band at ∼95 kD induced by heat stress in the cytoplasmic fraction in the autoradiogram aligns with the band identified as P95 in the PHA-activated cells. As shown in the blot, the anti-P95 antiserum in the mixture reacts with corresponding bands and P95 cannot be detected in the nuclei of the heat-shocked cells.

In both the nuclear and cytoplasmic fractions of the heat-shocked cells in Fig. 5, two bands are preferentially induced in the 70-kD region. The major heat-shock–inducible band at 72 kD is not induced by PHA activation, but shows some cross-reactivity with the anti-P73 antiserum in the blot. A less heavily synthesized band in the heat-shocked cells corresponds to the position of P73 in the fluorogram of PHA activated cells and appears to be the same band recognized by the anti-P73 antiserum in both cases. This band is enhanced
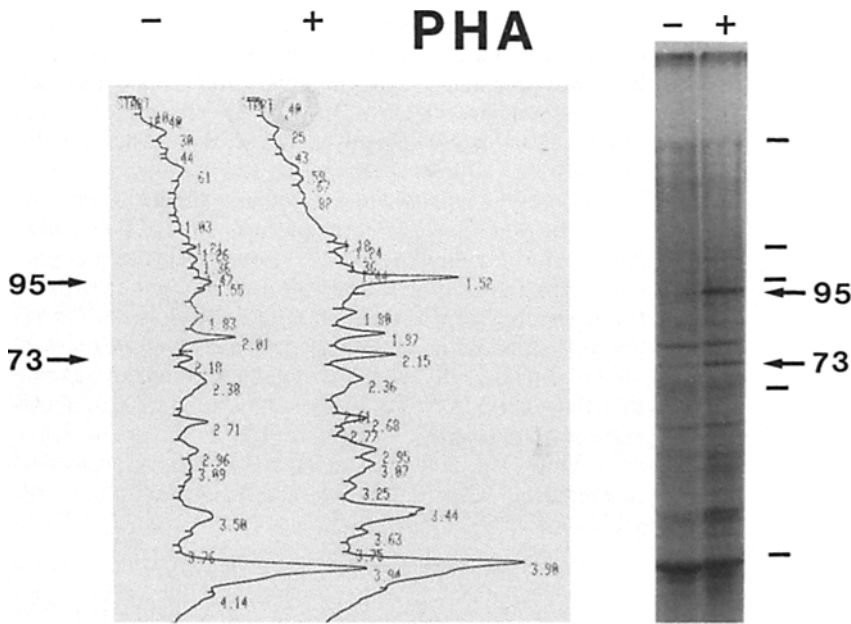
Figure 3. Densitometric determination of level of synthesis of P73 and P95 in resting and stimulated cells. Cytoplasmic fractions of cells cultured for 20 h were pulse labeled the last 4 h of culture and run on a 10–16% gradient gel. A lightly exposed autoradiogram was made and scanned with a spectrodensitometer to determine the fractional level of incorporation in the bands. The densitometer tracings are shown on the left with the peaks aligned with the respective fluorogram bands. The right tracing and right gel lane are PHA cultured cells. The bars indicate 205, 116, 97, 66, and 45 kD molecular mass standards.
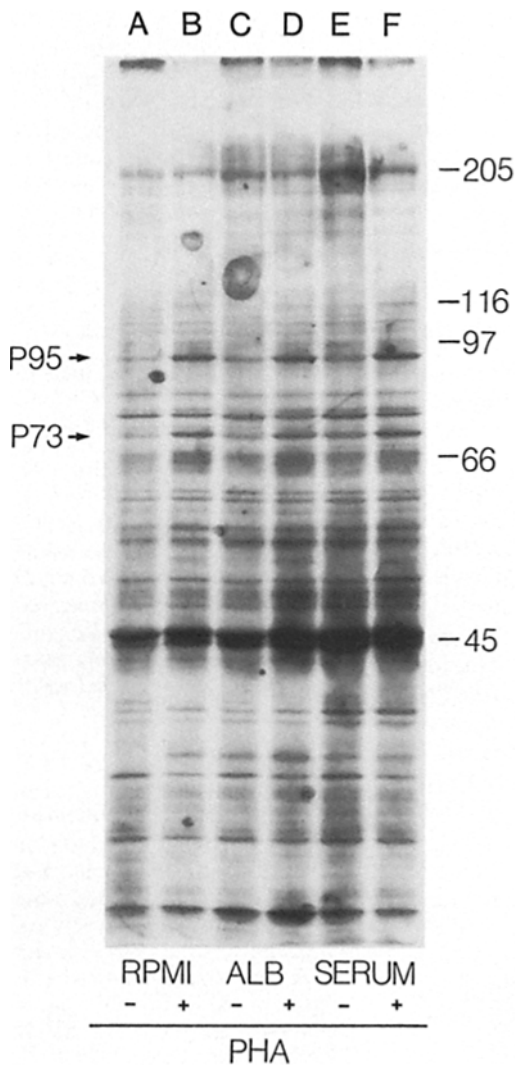
in the heat-shock cells, relative to synthesis of other (non-heat-shock) proteins. Heat shock at lower temperature results in stronger induction of the 73 and 95-kD bands (see Fig. 6). Both P73 and P95 are detectable, by antibody reactivity, in unstimulated cells cultured in serum for 2 and 18 h. Development of similar blots with only one of the two polyclonals (not shown) shows no cross-reactivity between the bands at 73 and 95 kD.

Fig. 6 shows the induction of synthesis of proteins, by both heat shock (42°C for 90 min) and PHA, examined by 2-D electrophoresis. The 73-kD spot induced by PHA and heat shock is labeled "1" and the main heat induced form (72 kD) is labeled "2" in 6A. The pI of P73 is ∼5.6 and the 72-kD protein is slightly more basic. As indicated by the large arrows in Fig. 6, A–D, both spots are induced by heat-shock, but only spot 1 is induced by PHA. In panels *B* and *D* P95 appears as a streak, indicated by the brackets, inducible by both heat-shock and PHA.

A blot of the gels from Fig. 6, *B* and *D* with the monoclonal antibody, 7.1, is shown in the lower part of Fig. 6. This antibody was raised to HSP 70 from *Drosophila* and reacts with most members of the HSP 70 family in other eukaryotic cells (19). Both spot 1 (P73) and spot 2 (the major heat inducible 70-kD protein) show positive reactivity. The polyclonal rabbit antiserum raised to PHA induced P73 shows a very similar pattern on 2-D blots, but reacts with two small spots of 72 kD seen on protein blots but not detectably synthesized on the fluorograms.

Figure 4. Dependence of P73 and P95 induction on the presence of PHA. Shown are the labeled proteins (continuous label) of the cytoplasmic fractions from cells cultured for 18 h in media alone, (*A* and *B*); in human serum albumin (5 mg/ml), (*C* and *D*); and in serum, (*E* and *F*). Lanes *A*, *C*, and *E* have no PHA, while lanes *B*, *D*, and *F* have 1 mg/ml PHA. Only the cells in lane *F* will proliferate. A fluorogram of an 8–14% gradient gel is shown. The bars indicate 205, 116, 97, 66, and 45 kD molecular mass standards.
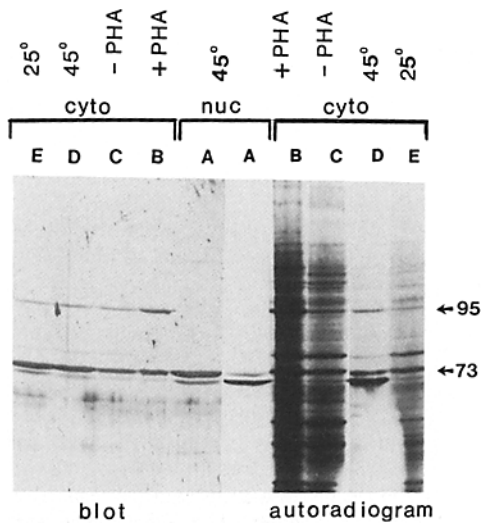
Figure 5. Recognition of major preferentially synthesized proteins in heat shocked and PHA stimulated cells by antibodies to 95 and 73 kD proteins purified from PHA activated cells. The Western blot, left side, and an autoradiogram made from the blot, right side, are presented as mirror images. The Western blot was developed with a 1:1 mixture of P95 and P73 antisera. Lane A shows the nuclear fraction of heat-shocked cells cultured for 2 h (at 37°C) after heat shock for 10 min at 45°C. The cytoplasmic proteins synthesized by the heat-shocked cells (D) and the control (E) are compared with the labeled proteins of cytoplasmic samples of cells cultured with PHA (B) and in serum (C) for 18 h. The arrows indicate P95 and P73 positions. The major synthesized band in lanes A and D just below P73 is presumed HSP 70 (72 kD). The fluorogram lanes D and E were light and a second exposure, made from the blot, has been superimposed to augment the image.

In Fig. 7, the monoclonal antibody AC 88 was used to probe a blot of a 2D gel from PHA-activated cells. This antibody was prepared to a fungal protein and is known to react with mammalian HSP 90 (37). P95 appears a discrete spot in this gel and is the only spot reactive with AC88. In some 2-D gels P95/HSP 90 is resolved into a doublet in the SDS dimension gels. Such doublet spots have identical isoelectric points and both spots are induced by mitogen and heat shock. The pI of P95, estimated from pH measurement of the gels, is ~5.1–5.2, in good agreement with the PI reported for HSP 90 by Welch and Feramisco (48). The polyclonal rabbit antiserum prepared to P95 reacts with the synthesized spot identified as HSP 90 and with a non-synthesized 94-kD protein abundant in platelets, which contaminate the mononuclear cell preparations. The nonsynthesized antigen is absent from Molt 4 transformed T cells, cultured non-transformed fibroblasts (ATCC CCL 210) and epithelial carcinoma cells (ATCC CRL 1555), all of which contain 95-, 72-, and 73-kD bands recognized by the polyclonal antiserum (data not shown).

As shown in Fig. 8, the induction of P95 and P73 by PHA is independent of initial cell concentration and not affected by the glucose concentration of the culture media. In Fig. 8, the induction of P73 and P95 by PHA and general pattern of protein synthesis in mononuclear cells cultured for 15 h in glucose free medium, and conversely in medium supplemented with glucose, or at initial cell concentrations of 0.5 × 10^6 and 3.0 × 10^6 cells/ml are all nearly identical. Thus, it seems unlikely that induction of P73 and P95 is a
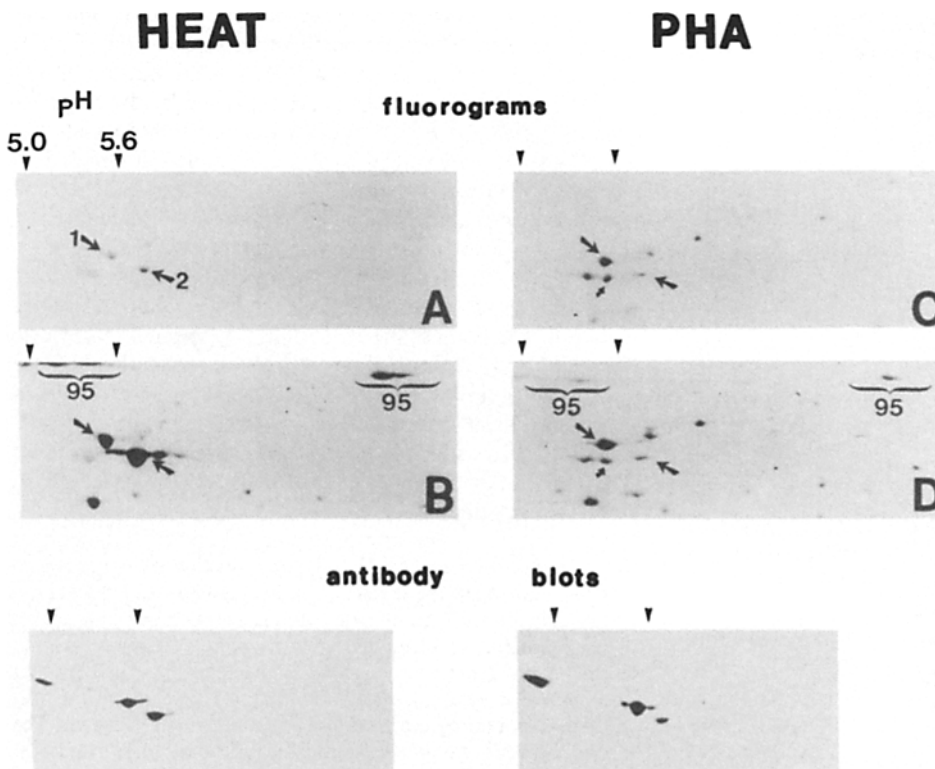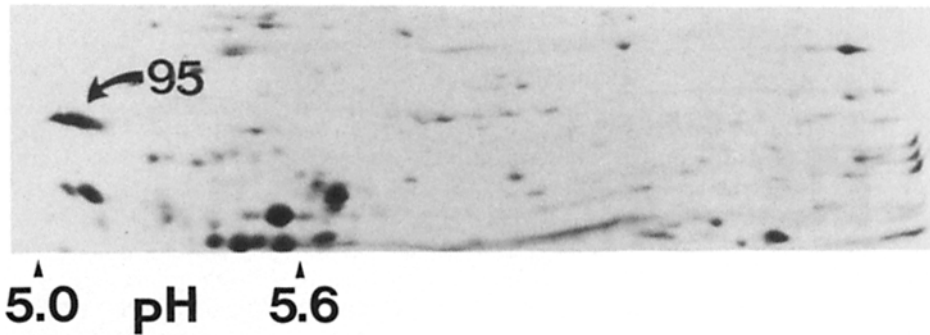

Figure 6. 2-D electrophoresis of the 95- and 73-kD protein(s) induced by PHA and heat shock. In the upper panels the induction of synthesis is shown by the fluorograms. (A) 2-h culture, no heat shock vs.; (B) cells heat shocked at 42°C for 90 min; (C) Cells cultured 18 h in serum and (D) in serum with PHA. The two large arrows throughout the fluorogram panels indicate the positions of the major heat-induced 73- (spot 1) and 72- (spot 2) kD proteins. P95 (HSP 90) appears in these gels as the streak seen in panels B and D indicated by the double brackets, all of which reacts with the polyclonal anti-P95 (antibody reaction not shown). The small arrow in panel C indicates spot 3, induced in cells cultured (in media or serum) for 18 h, but not at 2 h, and uninfluenced by PHA. The lower panels show the reactivity with the monoclonal antibody 7.1 in similar gels of heat shocked (left) and PHA stimulated cells (right). The two-dimensional electrophoresis was done by the O'Farrell method, presented with the acid end to the left. Only a portion of the gel (60–100 kD) is shown in the figure.

fluorogram

5.0 pH 5.6

antibody AC88 blot

Figure 7. Reaction of P95 in PHA-activated cells with the monoclonal antibody AC 88 which is specific for HSP 90. The upper panel shows the autoradiogram prepared from the 2-D protein blot shown in the lower panel. Cells were cultured for 18 h. The portion of the gel shown is similar to that in Fig. 5. The position of P95 is indicated on both panels.

stress response due to nutrient deprivation and enhanced metabolic activity of the activated cells.

Discussion

The results show that two major synthesized proteins, preferentially enhanced in mid to late lag phase in mitogen stimulated human lymphocytes, are HSP 90 (P95) and an HSP 70 family member (P73). The evidence for this conclusion is based primarily on the observed induction of electrophoretically and immunologically identical proteins by both heat stress and mitogen activation. Both P95 and P73 are induced by heat shock, and the labeled bands in heat-shocked cells cross-react with polyclonal antibodies raised to P73 and P95 from PHA-activated cells (Fig. 5). The pI of P73 in 2-D gels is consistent with the pI of members of the HSP70 family (33) and the pI estimated for P95 is in good agreement with the pI for HSP 90 reported by Welch and Feramisco (48). Perhaps the most convincing evidence is the reaction of P73 and P95 with the monoclonal antibodies (Figs. 6 and 7). P73 is recognized by the monoclonal antibody 7.1, which reacts with both the cognate "HSC 70" and major heat–induced "HSP 70" (after the designation of Pelham, 33) forms of the eukaryote HSP 70 family, which are usually reported as bands of 72 and 73 kD in human cells (23). P95 is recognized by the antibody AC 88 which cross-reacts with eukaryote HSP 90 (37).

In addition, the subfractionation solubility behavior (Fig. 2) of the PHA-induced proteins in lymphocytes is in agreement with that of the respective HSPs in other cell types. Mitogen-induced P95 is a detergent soluble, "cytoplasmic", protein, as is HSP 90 (24). P73 is found in cytoplasmic and

nuclear fractions and a portion of it is resistant to high salt and detergent extraction (residual matrix associated). HSP 70 family members have been shown to exhibit similar behavior (22), though the nuclear proportion markedly increases following heat shock (42, 49).
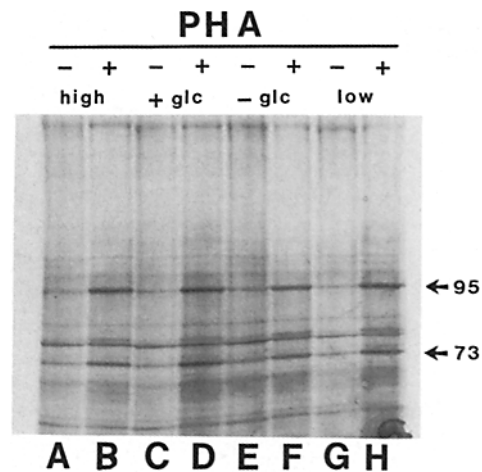

PHA

| | − | + | − | + | − | + | − | + |
| high | | + glc | | − glc | | low | |

←95

←73

A B C D E F G H

Figure 8. Induction of cytoplasmic P73 and P95 is not affected by glucose and cell concentration. Lymphocytes were cultured for 15 h, and pulse labeled for 11–15 h, at either 3 × 10⁶ cells/ml (lanes *A–F*) or 0.5 × 10⁶ cells/ml (lanes *G* and *H*). The cells in *A*, *B*, *G*, and *F* were cultured in standard RPMI 1640, containing 11 mM glucose. In lanes *C* and *D* the medium was supplemented at initiation of culture with an additional 11 mM glucose and again at 6 h with 11 mM glucose. In lanes *E* and *F* cells were cultured in glucose free RPMI 1640. Lanes *B*, *D*, *F*, and *H* were stimulated with 1 μg/ml PHA. A portion of the fluorogram from an 8–14% gradient gel is shown.

It is known that the HSP 70 family in mammalian cells is composed of multiple forms of closely related proteins, some of which are stress induced while others are present in unstressed cells. As shown in Figs. 5 and 6, P73 is synthesized in unstressed and unstimulated human lymphocytes, and although not shown in the figures, corresponds to a Coomassie Blue–stained band in 1-D gels. One of the two major forms of the HSP 70 family is abundant in unstressed cells with M_r 73 kD, (HSC 70) slightly higher than the strictly heat-inducible form (72 kD, HSP 70; reference 49). Clathrin uncoating ATPase was shown to be an HSP 70 cognate in mammalian cells (7, 40). Watowich and Morimoto (46) have recently described a constitutively expressed HSP 70–related protein from HeLa cells having identical electrophoretic mobility on 2-D gels. PHA-induced P73 has a slightly more acidic pI and higher M_r than the major heat–induced form. Thus, it appears that P73 is identical to the protein designated "P72" by Watowich and Morimoto and is probably HSC 70 using the functional designation of Pelham.

Although stress proteins can be induced by many factors, including experimental manipulations (43), we show the induction of HSP 90 and HSC 70 appears to be unrelated to metabolic stress per se, in mitogen activated cells. When the cell concentration is varied over a wide range, and glucose is either deleted from or supplemented to the culture media, we observe no influence on the level of induction of either protein (Fig. 8). The preferential induction of both proteins in stimulated cells is a PHA or OKT3 specific event. Further, while PHA binds to many glycolipids and glycoproteins including the T cell antigen receptor (17), OKT3, which induces both proteins, binds specifically to the T cell antigen receptor complex. OKT3 has a narrower range of metabolic effects than PHA (45), and its use as a mitogen reduces the possibility that a stress might be caused by PHA binding. The induction of HSC 70 and HSP 90 by OKT3 also strongly implicates T lymphocytes as the likely source of these proteins. Experiments using the fluorescence activated cell sorter to isolate pure T lymphocytes, based on recognition by OKT3 have confirmed this conclusion (Haire, R. N., and J. J. O'Leary. Manuscript submitted for publication).

The results presented here and elsewhere indicate there may be a shared requirement for the functional capabilities of HSC 70 and HSP 90 in both heat shock and mitogen stimulation. Both HSP 90 and HSP 70 related proteins appear to be involved in organization of cellular components. The induction of HSC 70 in mitogen stimulated lymphocytes, known to be a clathrin uncoating ATPase (40), may reflect in part, an increase in receptor mediated endocytosis activity. However, HSC 70 is a major cell component distributed in both the cytoplasm and nucleus, and it is doubtful that this is its only role. HSP 70 and HSC 70 are ATP-binding proteins and it has been proposed that HSP 70 binds to exposed hydrophobic regions of heat denatured proteins, preventing aggregate formation and possibly contributing to protein renaturation (23). HSP 90 is found associated with a number of cellular proteins (33) and has recently been shown to be an actin binding protein (18).

Evidence that these functional properties of HSP 70–related proteins and HSP 90 are important in cell growth has been accumulating for some time. Cellular translocation of the src oncogene kinase, PP60, from cytoplasm to membrane

and dissociation from HSP 90 are co-incident (4). Similarly, translocation of the steroid receptor coincides with its dissociation from HSP 90 (5, 38). Dissociation from HSP 90 is possibly linked to activation of the ligands and HSP 90 might serve as a shuttle protein that regulates the activity of its ligands (38).

The co-precipitation of both major forms of HSP 70 of 68 and 70 kD in rodents (HSP 70 and HSC 70) with the tumor antigen P53 in a transformed rat embryo fibroblast cell line (34) may be evidence of a shuttle/regulatory function. HSP 70 is specifically induced by the adenovirus transformation associated gene E1A (27) and induction of HSP 70 occurs in transformed cells (13). HSP 70 family regulation during early stages of mouse embryo development (2) and the transcription of HSP 84 (the 90 equivalent) and HSP 70 cognates during Drosophila oogenesis (19) further supports the possible association of growth and heat-shock protein function.

In contrast to our findings, two recent reports indicate that forms of the mammalian HSPs are preferentially synthesized in the quiescent G_0 phase of the cell cycle. Kaczmarek et al. (16) used a probe for an undefined hsp70 mRNA species in human lymphocytes, and found reduced expression in cultured cells relative to freshly isolated cells. Iida and Yahara (12) compared mouse spleen lymphocytes, cultured with Con A for several days, with freshly isolated cells. They identified proteins in the lymphocytes, electrophoretically related to the HSPs of yeast, and conclude that synthesis of HSP 70 forms are elevated in quiescent G_0 cells compared with 48 h after mitogen. The authors point out, however, that heat did not induce expression of the protein spots and they may have observed members of the HSP family other than HSC 70 (P73). It is likely that the different forms of HSP 70 have different functions and that the genes may be differentially regulated by growth factors, as well as stress (1, 14). Our observations are based on cells in the first 24 h of stimulation, before entry into first S phase, while Iida and Yahara deal with cells at a considerably later stage of culture. It is possible that induction of a given member of the HSP 70 family following mitogen stimulation may be accompanied by suppression of another.

In this paper we have presented evidence that HSP 90 and an HSP 70 family member (HSC 70) are induced in lymphocytes by heat stress and are components of the mechanism of growth activation in mitogen stimulated normal human lymphocytes. Both proteins are preferentially synthesized in mid G_1 phase, the portion of the cell cycle responsible for the variability in cell cycle duration and growth rate (32) and account for a significant proportion of the protein synthesis (at 20 h into lag phase) in mitogen-activated cells. Our results support the conclusion that an HSP 70 cognate, P73, identical to the P72 identified in HeLa cells (46) and the clathrin uncoating ATPase of unstressed human cells (40), is preferentially induced during the transition from quiescence to growth in lymphocytes. To our knowledge, induction of HSP 90 synthesis has not been previously reported during the transition from quiescence to growth in other cell types. The level of synthesis of both proteins is responsive to mitogen concentration and is maximal under conditions that are optimal for rapid entry of cells into S phase of the cell cycle. The proteins are induced by the mitogenic signal per se, apparently independent of other serum signals. This evidence

indicates that HSP 90 and HSC 70 are proteins intimately involved in the growth processes of normal human lymphocytes.

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