

DIMINISHED PHOSPHORYLATION OF A HEAT SHOCK PROTEIN (HSP 27)
IN INFANT ACUTE LYMPHOBLASTIC LEUKEMIA

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SUMMARY: We have previously reported lack of expression of a polypeptide designated L3 in infant acute lymphoblastic leukemia (ALL). Expression of L3 occurred predominantly in older children with pre-B ALL. We have recently reported the expression during B cell ontogeny of two other polypeptides, designated L2 and L4 with a similar Mr as L3, which were identified as phosphorylated and non-phosphorylated forms respectively of the low Mr heat shock protein, hsp27. In this study we have characterized L3 and identified it as another phosphorylated form of hsp27. The two phosphorylated forms appear to be differentially expressed in acute leukemia. L3 levels in infants who expressed hsp27 isoforms L2 and L4 were significantly diminished compared to levels in older children with an equivalent amount of hsp27. We conclude that leukemic cells in infant ALL exhibit a unique pattern of phosphorylation of hsp27 expressed at a pre-B cell stage of differentiation. © 1991 Academic Press, Inc.

We have identified, using two-dimensional polyacrylamide gel electrophoresis (2-D PAGE), a 27 kD cytosolic polypeptide, termed L3, which was restricted in its expression to leukemic cells from patients with pre B acute lymphoblastic leukemia (ALL) (1). Quantitative analysis of L3 expression showed that infants expressed little or no L3 compared to older children with an otherwise similar cell surface marker phenotype. In a separate study, we have detected two 27 kD cytosolic polypeptides, designated L2 and L4, predominantly in cells from patients with pre B ALL (2,3). Structural studies indicated that both polypeptides have an amino acid sequence identical to hsp27 (4), not known previously to be constitutively expressed in lymphoid cells. L2 was found to be a phosphorylated form of hsp27 and L4 the non-phosphorylated form (3). Since hsp27 is known to have multiple phosphorylated forms in a variety of cell types (5-8) and L3 has a pI more acidic than L2, L3 might represent another phosphorylated form of hsp27.

Considering that in ALL infants have a generally poor prognosis relative to older children (9,10), characterization of L3 could provide important clues as to the underlying basis for variable outcome in ALL. In the present study we report that L3 is a phosphorylated form of hsp27. Quantitative studies relating expression of L3 in pre-B ALL patients to that of total hsp27 suggests that the phosphorylation pattern of hsp27 differs in infants compared to older children with ALL.

MATERIAL AND METHODS

Source of cells: Leukemic cells of four pre-B ALL patients (2 infants and 2 two year old) were used for phosphorylation, heat shock and structural studies. Informed consent was obtained for

subjects in accordance with institutional guidelines. For analysis of the quantitative relationship between L3 expression and expression of L2 and L4, we utilized a portion of our leukemia cell two-dimensional gel data base consisting of a group of 13 infants (≤ 12 months) and 79 children (2 to 7 years) with pre-B ALL studied at the time of initial diagnosis. Specific diagnoses were made as previously described (1). Leukemic cells were obtained by Ficoll-Hypaque gradient centrifugation and constituted 85% or more of the total mononuclear cells. Additional samples included peripheral blood lymphocytes from four normal individuals, four T cell clones derived from a single non-leukemic individual (3), the erythroleukemia cell line K562, and an EBV-transformed B lymphoblastoid cell line derived from a non-leukemic individual with Bloom syndrome.

Cell Culture and $^{32}\text{P}_i$ Labeling: Cell lines and T cell clones were cultured in RPMI 1640 supplemented with 10% fetal bovine serum. Normal peripheral blood lymphocytes or patient lymphoblasts were maintained in culture medium at 37° C overnight prior to labeling with $^{32}\text{P}_i$. Cells (5×10^6) were incubated with PO_4 -free RPMI 1640 for 30 min. [^{32}P] H_3PO_4 (Amersham, carrier free) was added at 0.25 mCi/ml for 2 h at 37° C. For heat shock treatment, cells were incubated at 42° C for 2 h and returned to 37° C overnight to allow newly synthesized protein to accumulate or $^{32}\text{P}_i$ labeling was done immediately following heat shock. Identical flasks maintained at 37° C served as controls. Cells were washed three times with phosphate buffered saline, pelleted at $3,000 \times g$ and stored as frozen pellets at -80° C.

Two-dimensional Electrophoresis: 2-D PAGE was done using a carrier ampholyte-based first dimension system as previously described (11). For the second-dimension separation, an acrylamide gradient of 11.4-14.0 g/dl was used. Protein spots in gels were visualized either with silver-staining (12) or by autoradiography. Autoradiographs were prepared using Kodak X-Omat AR film with intensifying screens at -80° C.

Purification and Peptide Mapping of L2 and L3: Polypeptides were isolated from K562 cells following heat shock treatment or from cells of a 2 year old patient with pre-B ALL. Solubilized proteins were separated by 2-D PAGE (15×10^6 cell equivalents per gel). Phosphorylated polypeptides were located on dried gels by autoradiography and excised. Gel pieces were rehydrated with distilled deionized water, separated from the paper backing, extensively washed with water and lyophilized. Greater than 10 fold excess of TPCK-trypsin (0.5 ml; 1 mg/ml in 0.2 M ammonium bicarbonate, pH 8.2) was added and digestion proceeded overnight at 37° C. Tryptic peptides were eluted with 0.1%, v/v, trifluoroacetic acid (TFA) and applied to a C_4 reverse phase column (4.2 x 100 mm, 10 μ , Vydac) equilibrated with solvent A (0.1%, v/v, TFA in water). Peptides were eluted with a gradient of solvent B (0.075% TFA in acetonitrile) from 0% to 40% B in 40 min, then to 80% B in 20 min and finally to 100% B in 5 min. Fractions (0.5 min) were collected and ^{32}P -peptides detected by liquid scintillation counting. As an internal control for reproducibility of peptide elution times, column eluant was monitored at 214 nm to detect trypsin, its autodigestion products and other uv absorbing components eluted from the *in situ* gel digests. Identical uv absorbance tracings were observed for these components from all samples analyzed. Retention times for each component varied by less than 0.3 min over the range of elution times within which ^{32}P -peptides were eluted.

Quantification of Polypeptides: Gels were scanned in a 1024 x 1024 pixel format and spot detection and quantitation were performed as previously described (3). Twenty reference spots were used to adjust the L3, L2 and L4 spot integrated intensities using the mean ratio adjustment method (13). Gels exhibiting L3, L2 and L4 spots that were readily detectable by visual inspection of the gel yielded an integrated intensity greater than 0.4 O.D. x mm^2 . Therefore, this value was chosen as a cutoff for categorizing cases with absence of L3, L2 or L4.

RESULTS

Identification of L3 as a Phosphorylated Polypeptide: Lymphoblasts from pre-B ALL patients which express L3, L2 and L4 were labeled in culture with $^{32}\text{P}_i$ for 2 h. Polypeptides were separated by 2D polyacrylamide gel electrophoresis. Gels were silver stained and autoradiograms

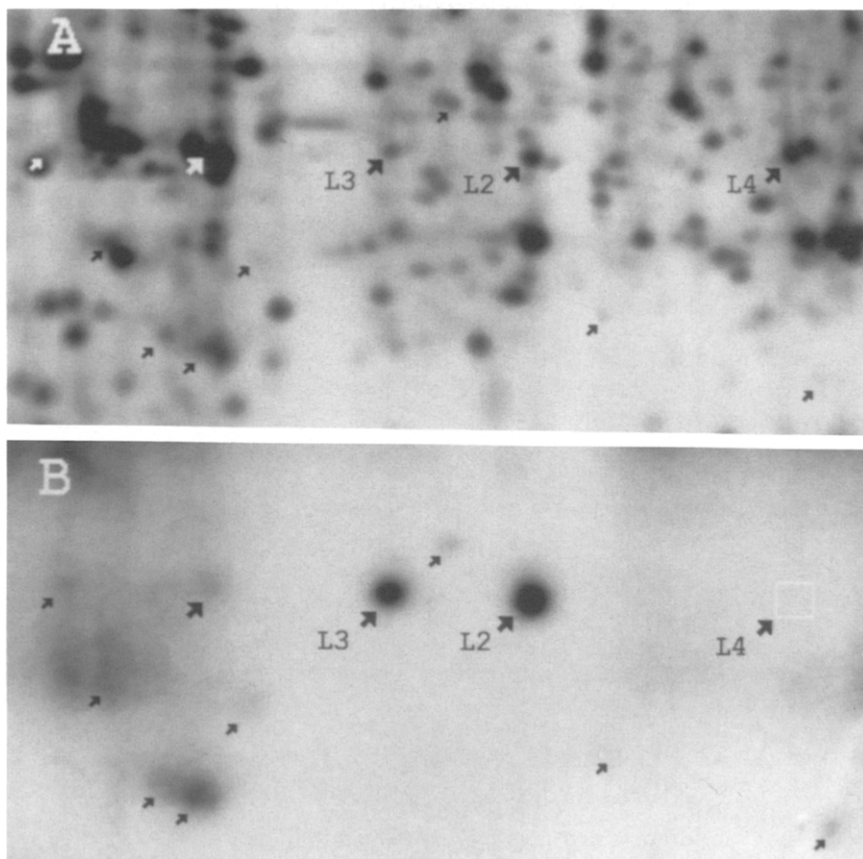


Figure 1. Identification of L3 as a phosphoprotein. Lymphoblasts of a 22 month old pre-B ALL patient which express L3, L2 and L4 were labeled in culture with $^{32}\text{P}_i$ for 2 h. Following silver staining, the gel was dried and an autoradiogram prepared. A: Close-up section of silver-stained gel. B: Autoradiograph of the same gel. Small arrows indicate phosphorylated polypeptides which were unambiguously identified by overlaying the autoradiograph on the dried, silver-stained gel. Large arrows indicate L3, L2 and L4. The white box shows the absence of radioactivity associated with L4 on the autoradiograph.

prepared. The apparent M_r of L3 is similar, and its isoelectric point more acidic, than that of the phosphorylated and non-phosphorylated forms of hsp27 designated L2 and L4 respectively (Fig. 1A). L3 visualized by silver-staining unambiguously coincides with a phosphorylated polypeptide on the autoradiograph of the same gel (Fig. 1B). The more basic prominent phosphorylated polypeptide coincides with L2 and no radioactivity is associated with L4, as was shown previously for a non-leukemic B cell line (3).

Differences are observed in the relative amount of several 27 kD phosphorylated polypeptides (pp a, pp b and pp c, Fig.2) among patient lymphoblasts and cell lines. Comparison of the abundance of pp a and pp b seen on autoradiographs agrees with the abundance of L2 and L3 seen on silver-stained gels. Lymphoblasts from an infant which express L2 but have no detectable L3, have a prominent pp a and pp b is faint (Fig. 2A). The intensity of pp b is increased in cells from a 2 year old patient expressing L3 (Fig. 2B). A prominent pp c is also seen. In K562 cells, L3 is a prominent polypeptide and in a non-leukemic B cell line it is faint and near the detection thresh-

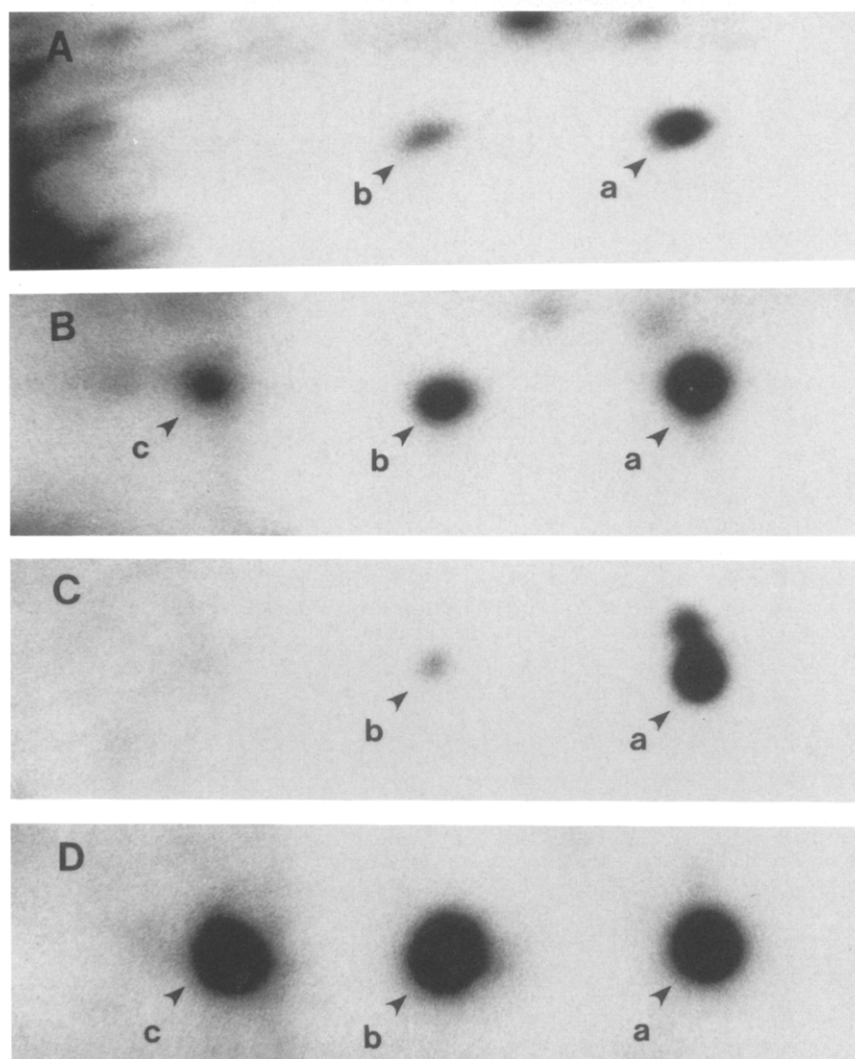


Figure 2. Close-up sections of autoradiographs of phosphorylated 27 kD polypeptides. A: Infant pre-B ALL lymphoblasts which express L2 and L4 but have no detectable L3 on silver-stained 2-D gels. B: Twenty month old pre-B common ALL whose lymphoblasts express L3, L2 and L4. C: Non-leukemic B lymphoblastoid cell line. D: K562.

Table 1. Incorporation of ^{32}P into L3 and L2 in response to heat shock

	n	Integrated Intensity, O.D. x mm ²			
		L3		L2	
		control	heat shock	control	heat shock
peripheral blood lymphocytes	4	0.32	1.00	0.60	2.70
pre-B ALL (4 month old)	7	0.11	0.34	0.45	1.86
pre-B ALL (20 month old)	2	1.86	2.96	2.63	4.74
non-leukemic B cell line	2	0.44	2.30	5.09	5.62

Cells were untreated or heat shocked for 2 h at 42°C and then labelled with $^{32}\text{P}_i$ for 2h at 37°C. Phosphorylated polypeptides were separated by 2D PAGE and quantitated from autoradiographs as described in "Methods".

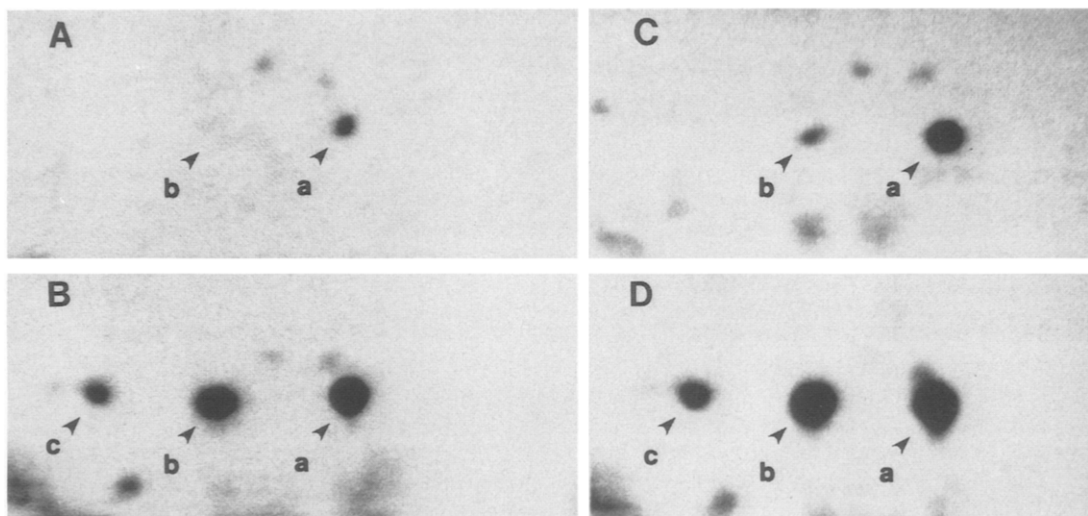


Figure 3. Autoradiograph of pre B ALL lymphoblast phosphorylated polypeptides in response to heat shock. Lymphoblasts were maintained at 37°C (control) or were heat shock treated at 42°C for 2h and returned to 37°C. Cells were labeled with $^{32}\text{P}_i$ for 2h at 37°C. Control (A) and heat shock treated (C) phosphorylated polypeptides from a four month old pre-B ALL patient. Control (B) and heat shock treated (D) pattern from a 20 month old pre-B ALL patient.

hold of silver-staining. The amount of pp b relative to pp a in K562 cells (Fig. 2C) and in the B cell line (Fig. 2D) agrees with the abundance of L3 and L2 respectively on silver stained gels. Pp a and pp b are also unambiguously associated with silver-stained spots identified as L2 and L3 in the B and K562 cell lines (data not shown). In lymphoblasts, radioactivity corresponding to pp c does not correspond to a discrete silver-stained spot (cf., large white arrow Fig. 1A) due to the close proximity of two large non-phosphorylated polypeptides. K562 cells do not express these polypeptides and a faint spot can be identified at the location of pp c on the silver-stained gel.

Expression of L3 in response to heat shock: Hsp27 polypeptides L2 and L4 were previously shown to be induced in T cell clones which do not normally express these polypeptides or to increase two to four fold in ALL 16 h after heat shock during which time newly synthesized protein could accumulate (3). Reexamination of those silver-stained gels revealed a small amount of L3 in response to heat shock. To assess the expression of phosphorylated polypeptides following heat shock, cells were heat shocked for 2 h at 42°C, incubated with $[^{32}\text{P}] \text{P}_i$ for 2 h and cellular polypeptides analyzed by 2-D PAGE. The results are summarized in Table 1. There are approximately four and three fold increases in incorporation of radioactivity into pp a and pp b respectively in normal peripheral blood lymphocytes and in leukemic cells from a four month old patient which do not have detectable L3 in silver-stained 2-D gels of non-stressed cells. Similar increases are seen in the B cell line. Sections of the autoradiographs of the leukemic cells are shown in Fig. 3A (untreated) and Fig. 3C (heat shock). Less of an increase in pp a and pp b is observed in a 20 month old patient (untreated, Fig. 3B, and heat shock, Fig. 3D) whose cells already express appreciable amounts of L2 and L3 as judged by analysis of silver-stained gels. We conclude that pp a is L2, that pp b is L3, and that L2 and L3 are phosphorylated forms of hsp27.

Comparative Tryptic Peptide Mapping of 27 kD Phosphorylated Polypeptides: The structural

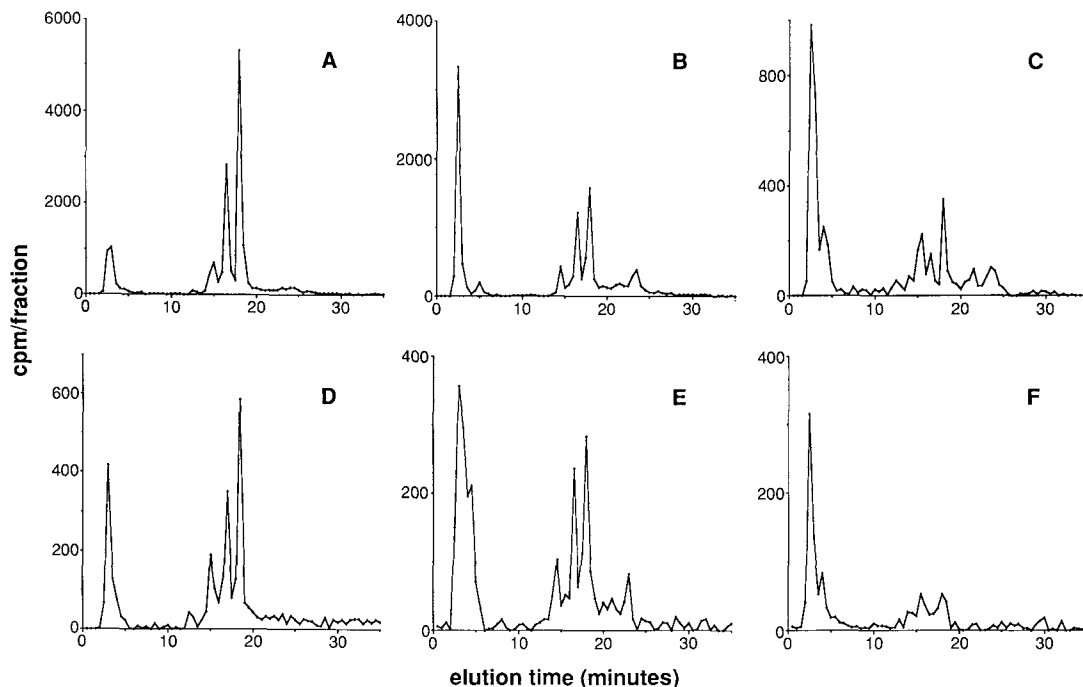


Figure 4. Reverse phase HPLC of ^{32}P -tryptic peptides of pp a (L2), pp b (L3) and pp c. K562 cells and lymphoblasts of the patient shown in Fig. 2B were labeled with $^{32}\text{P}_i$. L2, L3 and pp c were isolated from 2D gels and ^{32}P -tryptic peptides prepared. ^{32}P -tryptic peptide maps of L2 (A), L3 (B) and pp c (C) from K562 cells and L2 (D), L3 (E) and pp c (F) from lymphoblasts.

relationship of pp b, as well as pp c, to pp a was further characterized by comparative peptide mapping of their phosphorylated tryptic peptides using reverse phase HPLC. Phosphorylated polypeptides were isolated from cells of the patient shown in Fig. 2C and from K562 cells. The reverse phase HPLC comparative phosphopeptide maps are shown in Fig. 4 A-C for K562 cells and Fig. 4 D-F for the pre-B ALL patient. The elution profiles of pp a and of pp b from the leukemic patient and from K562 cells are virtually identical. Three retained peaks of radioactivity at 14.5, 16.5 and 18 min in the elution profile of pp b (Fig. 4B and 4E) have similar elution times and relative abundance as those of pp a (Fig. 4A and 4C). Two additional peaks of radioactivity at 5 and 23.5 min are seen for pp b suggesting utilization of additional phosphorylation sites in pp b not used in pp a. The phosphopeptide map of pp c from K562 (Fig. 4C) is similar to that of pp a and pp b, however additional peaks of radioactivity and the amount of radioactivity in similarly eluting peaks are found suggesting that pp c is a further phosphorylated form of hsp27. No conclusion could be made for pp c from the patient (Fig. 4F). Control digests of three unrelated phospho-rylated polypeptides having molecular weights in the range 18-19 kD are dissimilar to those of pp a and pp b (data not shown).

Diminished Expression of Phosphorylated hsp27 Form L3 in Infant ALL: The identification in this study of L3 as a phosphorylated form of hsp27 led us to investigate the quantitative relationships between L3 expression and that of L2 and L4 as a function of patient age at the time of initial diagnosis. Without regard to age differences, for all patients in the study there is a high correlation between L2 and L4 expression ($r=0.80$, $p<0.001$) similar to that reported previously (3). There is

a similar high correlation between amounts of L3 and L2 ($r=0.73$, $p<0.001$) and less correlation between L3 and L4 ($r=0.43$, $p<0.001$). Total hsp27 (L4+L2+L3) is greater in children aged 2-7 years ($4.83 \text{ O.D.} \times \text{mm}^2 \pm 0.31 \text{ S.E.}$, $n=79$) than in infants ≤ 12 months ($1.26 \pm 0.23 \text{ S.E.}$, $n=13$). However when expression of L3 in infants is compared with expression in children, for the group of patients that express comparable amounts of L2 and L4, there is a significantly lower amount of L3 in the infant group. In 9 of 13 infants where one or more forms are expressed, total hsp27 has a range of 1.08 to 2.64 O.D. $\times \text{mm}^2$ (mean $1.73 \pm 0.17 \text{ S.E.}$). Eleven children have total hsp27 within this range (mean $2.04 \pm 0.14 \text{ S.E.}$). Comparison of these two groups shows L3 to be significantly less in infants ($0.28 \pm 0.06 \text{ S.E.}$) than for children ($0.45 \pm 0.05 \text{ S.E.}$), $p=0.03$. Thus decreased total hsp27 alone can not completely account for lower L3 expression in infants.

DISCUSSION

The data presented in this study demonstrates the structural relatedness of L3 to L2 and L4. The findings of increased levels of L3 in response to heat shock and the similarities of the HPLC phosphopeptide chromatographs of L3 and L2 allow us to conclude that L3 is the product of progressive phosphorylation of hsp27.

It is becoming increasingly evident that the proteins associated with cellular heat shock response play an important role in the normal function of a variety of cell types outside of the heat shock response (14-16). While specific functions have been determined in non-stressed cells for several of the major heat shock protein families, such as hsp90 (16), hsp70 (15) and hsp58 (17), specific functions for members of the hsp27 family have not been established. Synthesis of hsp70 (18,19) and hsp27 (20,21) during development suggest that they serve a role in cell differentiation.

The results we have obtained lead us to conclude that phosphorylation of hsp27 to form L3, distinguishes between infant ALL and the majority of cases of pre-B ALL in older children. It has been previously shown that a large proportion of infant ALL represent leukemic cells that are arrested at a pre-B stage of differentiation prior to CALLA expression (15,16). We have previously demonstrated that expression of hsp27 substantially overlaps with CALLA expression. Our prior observation that some pre-B ALL cases that expressed CD19 also expressed hsp27 but failed to express CALLA led us to suggest that expression of hsp27 occurs after CD19 and precedes CALLA (3). The identification of L3 as a phosphorylated form of hsp27, delineates an additional subtype of pre-B ALL. Thus, the CD19 positive, hsp27 positive group can be divided into two subtypes, based on the occurrence of L3. The CD 19 positive, hsp27 positive (L2 and L4), L3 negative group predominates in infants and the CD19 positive, hsp27 and L3 positive group predominates in older children, most of whom are also CALLA positive.

The mechanism accounting for differentiation stage-specific difference in phosphorylation status of hsp27 is unknown. Studies of hsp27 phosphorylation in human mammary carcinoma cell lines (5) and other immature cell types (6-8,20) have implicated Ca^{++} /phospholipid dependent protein kinase C (PKC). In human leukemia and lymphoma cell lines, total PKC activity was found to be lower in B-cell and cell lines identified as non-T non-B, compared to T-cell lines (22). Decreased PKC activity may be responsible for the variability in expression of L2 which we have

observed (3), however decreased kinase activity alone may not account for the difference in hsp27 phosphorylation status between L2 and L3. Several possibilities exist including involvement of a) a protein kinase other than PKC similar to that reported for tumor necrosis factor-mediated phosphorylation of this heat shock protein (23), b) differential expression or activation of PKC subtypes, or their down-regulation due to chronic activation, c) presence of a protein kinase inhibitor and d) differential stability of L3 and L2 due to specific phosphatases or proteases.

The extent to which differential phosphorylation of hsp27 in leukemic cells contributes to treatment outcome remains to be determined. Preliminary analysis of relapse rates for patients lacking L3 is about twice as high as that for patients expressing L3, even after excluding the infant group (Hanash, unpublished). Differential phosphorylation of hsp27, and possibly other proteins, suggests key differences in regulation of metabolic activity between these patient groups which may be crucial to treatment outcome.

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