Early Synthesis of Specific Cytoplasm Proteins Is Correlated with the Rate of Exit of Lymphocytes from the Resting State

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ABSTRACT   We investigated the initiation of synthesis of proteins in human lymphocytes exposed to the mitogen phytohemagglutinin (PHA) for 6 h. Radiolabeled proteins in three subcellular fractions, cytoplasmic, nuclear salt wash, and nuclear, were separated on polyacrylamide gels. Compared with cells incubated for the same time in the absence of PHA only two cytoplasmic proteins of M_r 51 and 60 kd showed increased synthesis in a dose-dependent fashion. Synthesis of the 60-kd protein shows the strongest correlation with rate of entry into the first S phase and with rate of cellular aggregation. Thus, the 60-kd protein appears to be a major early response-associated protein for entry of lymphocytes into the first S phase after PHA stimulation.

Animal cells in the G_0/G_1 phase of the cell cycle can be stimulated in vitro to enter S phase and eventually mitosis by the addition of exogenous growth factors or mitogens. Since control of overall proliferation is expressed primarily within the G_0/G_1 phase it is expected that certain biochemical events occurring within this phase are responsible for this control and that the rate at which cells enter first S phase should be a function of these events (22). In this regard it has been shown that human lymphocytes stimulated by the mitogens concanavalin A or phytohemagglutinin (PHA)[1], in conjunction with serum factors, enter S phase after a lag duration of ~26 h (2, 19). Entry into S phase occurs in an exponential fashion yielding an apparent first order entry rate constant (k). The entry rate constant is a function of the concentration of concanavalin A and PHA (2, 19; and the results of this paper) while the lag duration and responding cell number are much less sensitive. Thus the entry rate constant is the most characteristic feature of the mitogenic response and is assumed to reflect the events within the lag period that control entry into S phase.

Following mitogen stimulation a number of complex biological processes are activated throughout the lag period, such as ion flux, protein synthesis, cyclic nucleotide metabolism, specific gene expression, etc., that are necessary for ultimate entry of the cells into S phase (7). In particular, events occurring within the first 5–6 h (early events) have attracted

interest due to the implication that they have an important role in initiating the proliferative response. For example, it has been shown that the rate of protein synthesis increases significantly 1 h after PHA stimulation and doubles by 4 h (1, 8). This early increase in protein synthesis is accompanied by an accumulation of polyribosomes (10). It is of interest then to identify the proteins synthesized during this early period because they may provide direct information about early regulatory events. Along this line, Lester et al. (13) found that the synthesis of a 25,000-dalton protein (pI 5.2) from whole cell extracts is prominently enhanced by 4 h of PHA stimulation. Yet Degen et al. (1) have shown that for a majority of lymphocyte proteins there is a quantitative rather than a qualitative difference in the level of synthesis following concanavalin A stimulation.

To separate the general effects of enhanced metabolic activity from synthesis of gene products associated only with the proliferative response we have asked the following questions. Do one or more proteins show increased synthesis that correlates with an increase in entry rate constant over increasing doses of PHA within 6 h of stimulation? If so, is this increased synthesis due to signals generated by cell-cell contacts formed during mitogen driven aggregation? We have found that a cytoplasmic protein of 60,000 daltons (P60) shows increased synthesis in response to increasing concentrations of PHA. This increase in synthesis correlates well with an increase in entry rate constant and rate constant for cell aggregation with increasing dose of PHA. Further, P60 is not appreciably synthesized in the presence of PHA and serum albumin where there is significant aggregation but no entry into S phase. The

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[1] *Abbreviations used in this paper:* [³H]TdR, [³H]thymidine; P51, 51-kd protein; P60, 60-kd protein; PDGF, platelet-derived growth factor; PHA, purified phytohemaglutinin.

molecular weight and inductive behavior points to similarities between P60 and a platelet-derived growth factor (PDGF)-induced protein, possibly the c-myc gene product.

MATERIALS AND METHODS

Cell Isolation, Culture Conditions, and Radiolabeling: Human peripheral blood lymphocytes were obtained from young healthy donors by separation of whole blood on Ficoll-Hypaque® discontinuous density gradients, giving ~90% lymphocytes and 10% monocytes (18). Approximately 60–70% of the lymphocyte population are T-cells that are responsive to PHA while the remainder are unresponsive B-cells (16). The heterogeneous mononuclear cell population is necessary for optimal stimulation (20, 26). The final suspension medium contained RPMI 1640 (Gibco Laboratories, Grand Island, NY), supplemented with penicillin, streptomycin, and L-glutamine. Lymphocytes in RPMI 1640 with or without 10% pooled human serum (PHS) and with or without purified phytohemagglutinin (PHA, Wellcome, Beckenham, England) were cultured at 0.5 × 10⁶/ml in Lindbro tissue culture plates (24 well, polystyrene, Lindbro, Hamden, CT) or polypropylene Eppendorf® tubes. Colchicine (Sigma Chemical Co., St. Louis, MO) added at 24 h of culture and at a concentration of 0.5 µg/ml was used to inhibit mitosis in the [³H]thymidine (³H-TdR) uptake experiments. This dose and time of addition has no effect on either the entry rate constant or on the transport of ³H-TdR into the cell (3). For the nonmitogenic induction of aggregation with PHA, human serum albumin (Sigma Chemical Co.) was used at a concentration of 5 mg/ml, approximating that found in 10% serum. For determinations of the synthesis of specific proteins, cells were cultured at 2–3 × 10⁶/ml in Falcon tissue culture flasks (75 cm², polystyrene, Falcon Labware, Oxnard, CA). For the studies on the inhibition of RNA synthesis, actinomycin D (Sigma Chemical Co.) or α-amanitin were added to a concentration of 5 or 50 µg/ml, respectively. This was found to inhibit the uptake of [³H]uridine into total trichloroacetic acid precipitable material by 80% for actinomycin D additions or by 50% for α-amanitin additions (data not shown).

The rate of aggregation of PHA-stimulated cells was measured by determining the decrease in numbers of single cells over time (4). This was done by gently inverting the tubes three times and then withdrawing a small amount of cell suspension into a capillary tube. A drop of this cell suspension was placed onto a hemocytometer counting chamber and the concentration of single cells was determined. Since lymphocytes do not strongly adhere to the polypropylene tube surface this gentle inversion of the tubes appeared enough, by microscopic examination, to dislodge the cells from the tube surface.

For assay of proliferative response [³H]thymidine (³H-TdR, Research Products International, Mount Prospect, IL) was used at a specific activity of 6.7 Ci/mmol and at a dose of 10 µCi/ml for uptake into cells. The cells were pulsed for 3 h. ³H-TdR uptake for this pulse duration is linear with respect to time and proportional to the numbers of cells in S phase by KN_s = counts per minute (18). Following the pulse the cells were harvested from the tubes onto glass fiber filters that were placed in glass vials with a toluene-based cocktail and counted in a scintillation counter.

For assay of protein synthesis [³H]leucine ([³H]Leu, RPI) at a specific activity of 50 Ci/mmol and at a dose of 100 µCi/ml and [³⁵S]methionine ([³⁵S]Met, Amersham Corp., Arlington Heights, IL) at a specific activity of 344 mCi/mmol and dose of 42 µCi/ml were used to label cellular proteins. Following the appropriate stimulation cells were allowed to settle in the tissue culture flasks for 30 min then pulsed for 5.5 h with both [³H]Leu and [³⁵S]Met. The flasks were then cooled to 4°C and prepared for subcellular fractionation as described below.

Subcellular Fractionation and Polyacrylamide Gel Electrophoresis: Following label incorporation into cellular protein the flasks, cooled to 4°C, were scraped with a rubber policeman to remove all of the cells. The cells were pelleted, washed twice in cold RPMI 1640, and then lysed in 150 mM KCl, 8 mM MgCl₂, 20 mM Tris, 20 mM phenylmethylsulfonylfluoride, and 0.1% Triton X-100 at pH 7.5. The nuclei were separated from cytoplasmic materials by centrifugation at 900 g for 10 min in a Sorvall GLC® centrifuge. The nuclei were then resuspended in a "low salt wash" of 150 mM NaCl, 2 mM phenylmethylsulfonyl fluoride, 20 mM Tris, and 10 mM MgCl₂ at pH 7.5 for 5–10 min and then pelleted at 1,100 g for 15 min. The pelleted nuclei were then resuspended in 100 mM NaAcetate, 5 mM MgSO₄, 20 mM Tris, 2 mM phenylmethylsulfonyl fluoride at pH 6.0, and 50 µg/ml DNAse I. All operations were carried out on ice. This procedure yielded a cytoplasmic fraction, a nuclear salt wash fraction, and a nuclear fraction. By this method 85% of the DNA was recovered in the nuclear fraction as determined by the diphenyl amine reaction (data not shown). The nuclear fraction was treated with DNAse I to disperse the nuclei allowing equal withdrawal of aliquots for gel electrophoresis. Relative protein concentrations were determined by the Bio-Rad Protein Assay® (Bio-Rad Laboratories, Richmond, CA).

The radiolabeled proteins from the three subcellular fractions were separated by SDS PAGE according to the method of Laemmli (12). The same amounts of protein was added to each lane of the gels. For all samples containing serum, with or without PHA, equal amounts of protein produced equal CPM. The exception was the samples containing medium only. In this case equal amounts of protein added produced lower CPM. Molecular weight standards were carbonic anhydrase, actin, ovalbumin, albumin, phosphorylase b, β-galactosidase, and myosin (Sigma Chemical Co.). The gels were then stained with Coomassie Blue R250, soaked in Enlightening (Kodak), dried, and then put on X-ray film for autoradiography.

RESULTS

Aggregation and Entry into S Phase as a Function of PHA Concentration

Purified phytohemagglutinin (PHA) in conjunction with 10% serum induces resting lymphocytes to both aggregate and enter first S phase. The process of aggregation as shown by Peters (23) is a requirement for entry of lymphocytes into S phase. To correlate aggregation with entry into S phase it is necessary to measure the rate of aggregation. By measuring the number of unaggregated cells over time the rate of aggregation can be determined (4). As seen in Fig. 1 (left) the decline in the concentration of single cells appears log-linear over the first 6 h, reaching a plateau thereafter. It is apparent from the figure that the rate constant for aggregation, determined from the slopes of the fitted lines, varies as a function of PHA concentration (seen in Fig. 4). Fig. 2 shows the time-dependent formation of aggregates in Linbro® plates at two doses of PHA when viewed microscopically. It is apparent from this figure that the formation of aggregates differs for the two doses of PHA and correlates well with the aggregation data of Fig. 1.

The entry of PHA stimulated lymphocytes into first S phase, as measured by consecutive ³H-TdR pulses (18) when colchicine is added at the end of the lag period, is characterized by
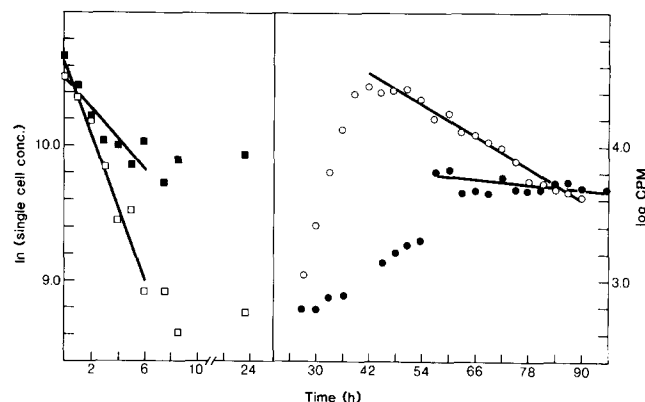

FIGURE 1   PHA dose dependence of the kinetics of aggregation and entry into S phase. Lymphocytes were stimulated with 0.5 µg/ml (□, ○) or 0.1 µg/ml PHA (■, ●) at zime zero in either Eppendorf® tubes (1.5 ml) for determination of aggregation rates (left) or in microtiter plates for determination of rates of entry into S phase (right). The single cell concentration was determined by gently inverting the tubes and placing a drop of cell suspension on a hemocytometer counting chamber. Entry into S phase was measured by treating cells with colchicine at 24 h to inhibit re-entry into S phase and then pulsing replicate plates with 20 µCi/ml ³H-TdR for 3 h in a consecutive fashion. The lines in the Figure are linear regression estimates of the data within that range of points. The rate constant for aggregation is given by the slope of the lines in the left while the entry rate constant k equals the slope (multiplied by 2.303) of the lines in the right.
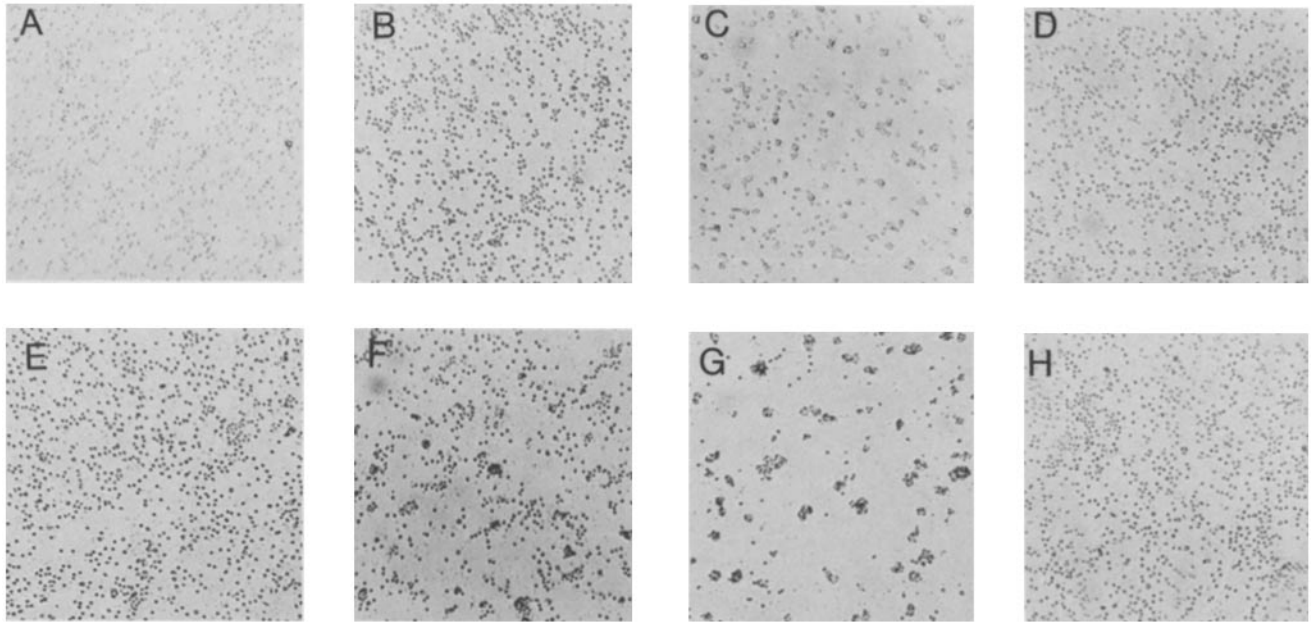
FIGURE 2 The PHA dose dependence of lymphocyte aggregation. Lymphocytes were stimulated with 10% serum plus PHA at 0.1 µg/ml (A and E), 0.5 µg/ml (B and F), 5 µg/ml (C and G), or treated with 10% serum only (D and H). The cells were cultured in Lindbro 24-well plates. The micrographs represent culture times of 3 h (A-D) or 7 h (E-H).

an apparent first order rate constant, k (2, 19). As seen in Fig. 1 (right) the slope of the lines, representing least squares fit of the data, reflect the magnitude of k (19). It is apparent that this constant also varies as a function of PHA concentration in a similar fashion to that seen for aggregation (see Fig. 4), yet the lag duration (i.e., the time until the first cells enter S phase) does not.

PHA Modulated Early Synthesized Proteins

To determine if specific early proteins were synthesized as a direct response to PHA stimulation, lymphocytes stimulated with varying doses of PHA were double labeled with [³H]-leucine and [³⁵S]methionine for 5–6 h following PHA addition (during the period of rapid aggregation). Labeled proteins from three subcellular fractions (i.e., cytoplasmic, nuclear salt wash, and nuclear) were analyzed by SDS PAGE. Fig. 3 shows a fluorogram of the cytoplasmic proteins analyzed on a 10% gel. Equal amounts of protein were added to each lane. Total amount of label incorporated per milligram protein is about the same in lanes a–e where 10% serum was present in the cultures and is reflected in the nearly equal intensity of most labeled bands. In the absence of serum (lane f) label incorporation is significantly reduced even with PHA addition, although cell viability after 6 h was >95% by Trypan Blue exclusion.

Two proteins in Fig. 3 show increased synthesis with respect to increasing dose of PHA. A protein of M_r 60,000 (P60) shows dramatically increased synthesis while another protein, M_r 51,000 (P51), shows only moderate synthesis, when compared with the control with serum, or PHA in media only. The increased synthesis of P60, estimated by scanning the fluorogram with a densitometer is plotted as a function of PHA concentration in Fig. 4. The dose response of rate constant of aggregation and entry into S phase correlates well with the synthesis of P60. P60 could not be detected on the Coomassie Blue stained gel.

To determine if P60 was formed from newly synthesized mRNA, actinomycin D (5 µg/ml) was added to the cultures to inhibit transcription. This dose inhibits [³H]uridine uptake into trichloroacetic acid-insoluble material by 85% (data not shown). Fig. 5 shows a fluorogram of a gel separating proteins from cells stimulated with PHA and serum or in serum only in the presence and absence of actinomycin D. The arrows indicate the position of P60 and P51. Although the doublet at P60 (lane a) is not as clearly resolved as in Fig. 3, synthesis of both proteins is inhibited by actinomycin D treatment. When α-amanitin (50 µg/ml) was used to block mRNA synthesis the synthesis of P60 and P51 was again significantly diminished (data not shown). Despite the presence of these inhibitors aggregation could be observed microscopically with PHA and serum, although aggregation rate was not determined in these cultures.

The nuclear salt wash fractions were analyzed on an 8% gel as seen in the fluorogram of Fig. 6. P60 shows increasing intensity with respect to increasing dose of PHA. P51 was not detectable. Upon close inspection of the fluorogram it is also apparent that there is a slight increase in the intensity of a number of bands in the PHA-treated samples vs. the serum control (compare lanes d and e). This is indicated by the arrows to the right. The constant intensity of the doublet at 42,000 daltons, indicates that an overall increase in synthesized proteins was not occurring in this fraction. Again reduced overall synthesis is seen in the absence of serum (lane f).

Analysis of the proteins in the third or nuclear fraction on a 14% gel showed no dramatic changes in the intensity of any bands with increasing dose of PHA, as shown in the fluorogram in Fig. 7A. However, a number of bands (arrows on (arrow, left) appear to show a slight increase in intensity at high PHA concentration compared with the serum control (compare lanes d and e, see arrows, right). Analysis of the corresponding Coomassie Blue-stained gel in Fig. 7B shows
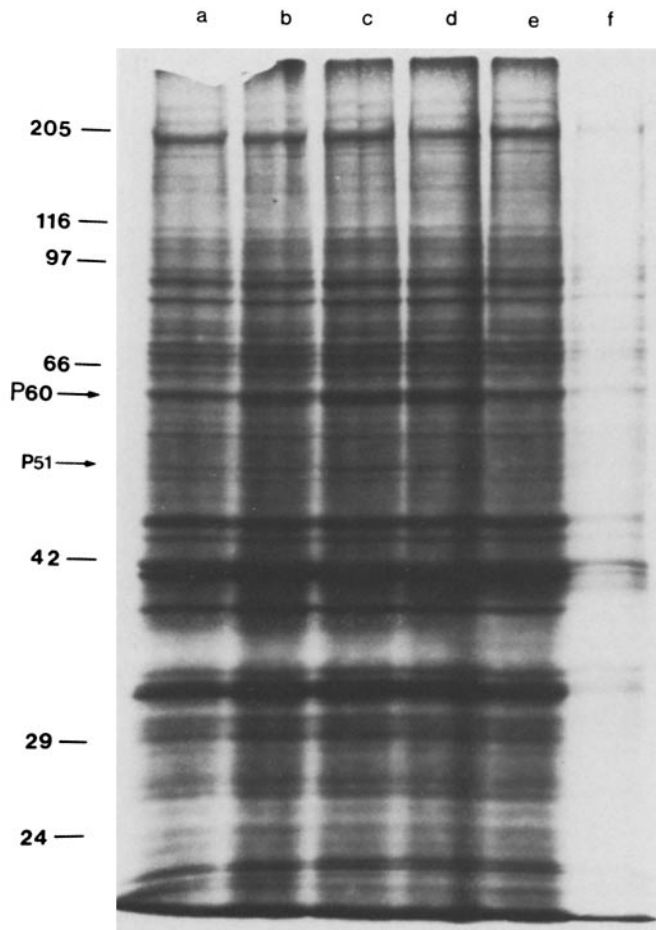
FIGURE 3   Analysis of the labeled proteins from the cytoplasmic fractions of lymphocytes stimulated with varying doses of PHA. Lymphocytes in tissue culture flasks were labeled with [³H]leucine (100 μCi/ml) and [³⁵S]methionine (42 μCi/ml) for 6 h following PHA addition. Cells were cultured in 10% serum plus PHA at 0.1 μg/ml (a), 0.2 μg/ml (b), 0.35 μg/ml (c), 0.75 μg/ml (d), 10% serum only (e), or in PHA (0.75 μg/ml) in media only (f). The cells were lysed and the cytoplasmic fractions were separated from the nuclei. Equal amounts of protein were added to each lane of a 10% SDS polyacrylamide gel. A fluorogram of the dried gel is shown. P60 and P51 are indicated along with the molecular weight markers.

on the contrary, an increase in a 205,000-dalton protein. This protein co-migrates with cytoplasmic myosin and is immunoprecipitated by myosin antibody (data not shown). The increase in this 205-kd protein in Fig. 7B is not accompanied by an increase in labeling intensity on the autoradiogram in Fig. 7A.

Induction of P60 and Aggregation under Nonmitogenic Conditions

From the results of Fig. 4 P60 shows increased synthesis with respect to PHA concentration, correlating well with increases in the rate constant for aggregation and entry into S phase. Yet P60 is induced only in the presence of PHA plus serum and not in PHA and medium or serum alone. Thus we wanted to know if the process of aggregation in the presence of some specific serum factor regulated the expression of P60. As seen in Fig. 8 incubation of the cells with human serum albumin at 5 mg/ml (the concentration found in 10% serum) plus PHA for 6 h produced rapid aggregation

similar to that found for PHA and 10% serum, incubation of cells with albumin and PHA is not mitogenic, and no significant aggregation is seen when cells are cultured in PHA and media only. When the cytoplasmic proteins were analyzed by gel electrophoresis, seen in Fig. 9, P60 was only weakly induced in the presence of PHA and albumin and not induced in either serum or albumin alone. The reduced intensity of many of the bands in cells cultured in albumin reflects significant reduction in label incorporation, similar to that seen in media alone with or without PHA. These observations provide for a further test for the potential role assigned to P60. Because treatment with PHA and albumin, albumin alone, and serum alone leads to changes in cell aggregation and protein synthesis but not to accumulation of P60 any pretreatment of cells under such conditions should not lead to increased entry rate. Table I shows this to be the case further supporting the correlation between rate of synthesis of P60 and the rate of entry into proliferative cycle. In this experiment the lag duration with PHA and serum added initially was ~26 h. For all three pretreatments significant numbers of cells appeared in S phase starting at ~32 h, i.e., 6-h pretreatment plus 26-h lag duration. Thus, pretreatment does not appear to significantly affect the lag duration.

DISCUSSION

In this paper we have attempted to determine the nature of the early molecular events that control the rate of entry of PHA-stimulated lymphocytes into first S phase from a resting or G_0 state. The rate of entry into first S phase, reflected by the first order entry rate constant k, is highly dependent on the concentration of PHA (Figs. 1 and 4). If entry into S phase is dependent on the early synthesis of key regulatory proteins then these proteins should also show increasing levels of synthesis with respect to increasing dose of PHA. From Fig. 3 it is evident that a protein of M_r 60 kd (P60) shows dramatically increased synthesis with increasing dose of PHA,
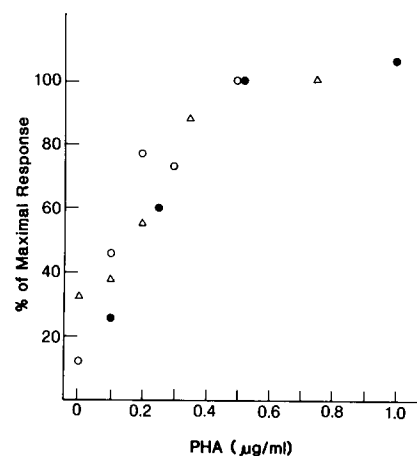

FIGURE 4   The effect of PHA dose on the production of P60, the rate constant for aggregation and the rate constant for entry into S phase. The values of the rate constant for aggregation (O), and entry into S phase (●) were determined from the data of Fig. 1 over a range of PHA concentrations. These values were normalized to the value at 0.5 μg/ml PHA as 100%. The levels of P60 synthesis (△) were determined by scanning the fluorogram of Fig. 3 with a densitometer. These values were normalized to the value at 0.75 μg/ml PHA as 100%. Cells in 10% serum only were taken as zero PHA concentration.
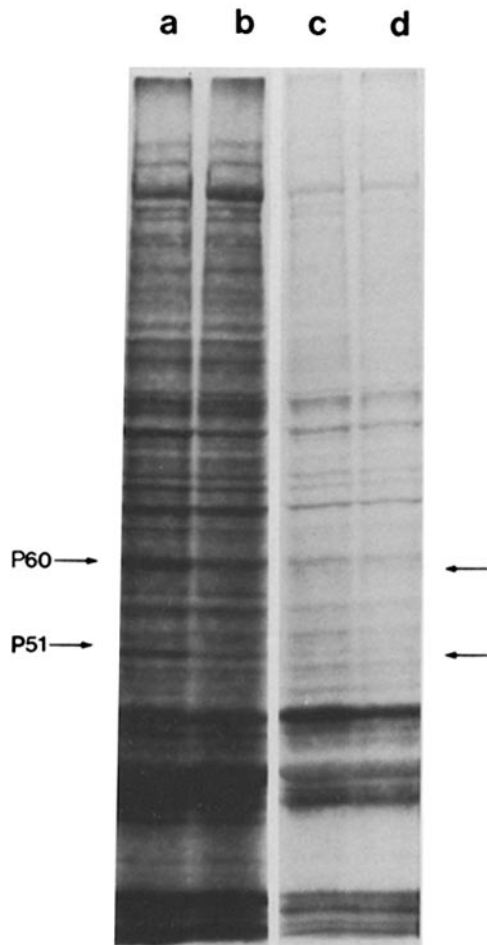
FIGURE 5 Inhibition of cytoplasmic P60 production by actinomycin D treatment. Cells were stimulated with 0.75 µg/ml PHA and 10% serum (a and d) or just 10% serum (b and c), as in Fig. 3, in the presence (c and d) or absence (a and b) of 5 µg/ml actinomycin D. The cells were labeled with [³H]leucine and [³⁵S]methionine as in Fig. 3, lysed, and the cytoplasmic fractions were run on a 10% SDS polyacrylamide gel. A fluorogram of the dried gel is shown. Equal amounts of protein were added to each lane. The arrow indicates the locations of P60 and P51.

3T3 cells is synthesized within 2–4 h by concanavalin A-stimulated lymphocytes. This protein complexes with the large T antigen in SV40 transformed cells and is dependent on de novo transcription. This 53-kd protein could be related to P51 or P60, but it is not clear that it can be detected without sensitive immunologic probes. Lester et al. (13) found that after 24 h of PHA stimulation of purified T cells, a 50- and 60-kd protein show increased synthesis in response to PHA stimulation above all other proteins. In contrast to P51 and P60 they could not detect these proteins by 4 h stimulation and found increase only in a 25-kd protein. Our one-dimensional gels do not resolve such a band. The purified T cell population used by Lester et al. (13) shows in our hands only marginal responses to PHA; thus early appearance of P60 and P51 is unikely.

Early proteins of similar molecular weight have been identified from the cytoplasmic fractions of PDGF stimulated BALBc/3T3 cells. Pledger et al. (25) have shown that following 4.5 h of PDGF treatment, five cytoplasmic proteins showed increased synthesis compared with controls with no PDGF. Two of these proteins had M_r of 45 and 60 kd. The relationship of the PDGF-modulated proteins to those presented here remains to be seen. It is interesting to note that while a protein of M_r 51 kd showed moderate increases in synthesis. When the level of P60 was plotted as a function of PHA dose (Fig. 4) it correlated well with the rate constant for entry into S phase. Thus P60 appears to be a major early response-associated protein in the cytoplasmic fraction. The synthesis of P60 and P51 was significantly inhibited by either actinomycin D or α-amanitin treatment indicating that these proteins are synthesized from newly transcribed genes.

P60 is present in the low salt wash, while P51 is not. Also, both P51 and P60 were not detectable in the remaining nuclear material. Detergent lysis, followed by nuclear isolation, may solubilize some loosely associated nuclear proteins, as illustrated by the recent report of Olashaw and Pledger (17). Although the detergent concentration used here is quite low (0.1% Triton X-100), we can only conclude that P60 and P51 are not tightly associated with nuclear structures, e.g., nuclear matrix.

P60 and P51 have not been previously identified as early synthesized proteins in PHA-stimulated lymphocytes. However, Milner and Milner (14) have shown that a 53-kd protein that is normally synthesized in SV40 transformed BALBc/
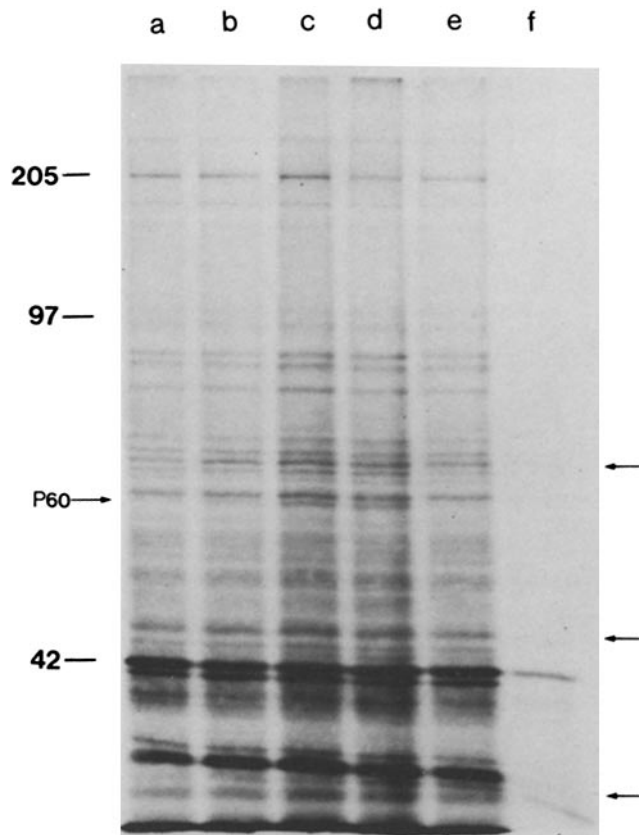

FIGURE 6 Analysis of the labeled proteins from the nuclear salt wash fraction of cells stimulated with varying doses of PHA. Nuclei from the cells in Fig. 3, treated with 10% serum and PHA at 0.1 µg/ml (a), 0.2 µg/ml (b), 0.35 µg/ml (c), 0.75 µg/ml (d), 10% serum only (e), or in PHA (0.75 µg/ml) in media alone (f) were washed once in a low salt (0.15 M NaCl) solution. The labeled proteins retained in this salt solution were run on a 10% SDS polyacrylamide gel. The fluorogram of the dried gel is shown and P60 is indicated. Equal amounts of protein were added to each lane. The arrows on the right indicate those proteins showing slight increases in synthesis with PHA dose.
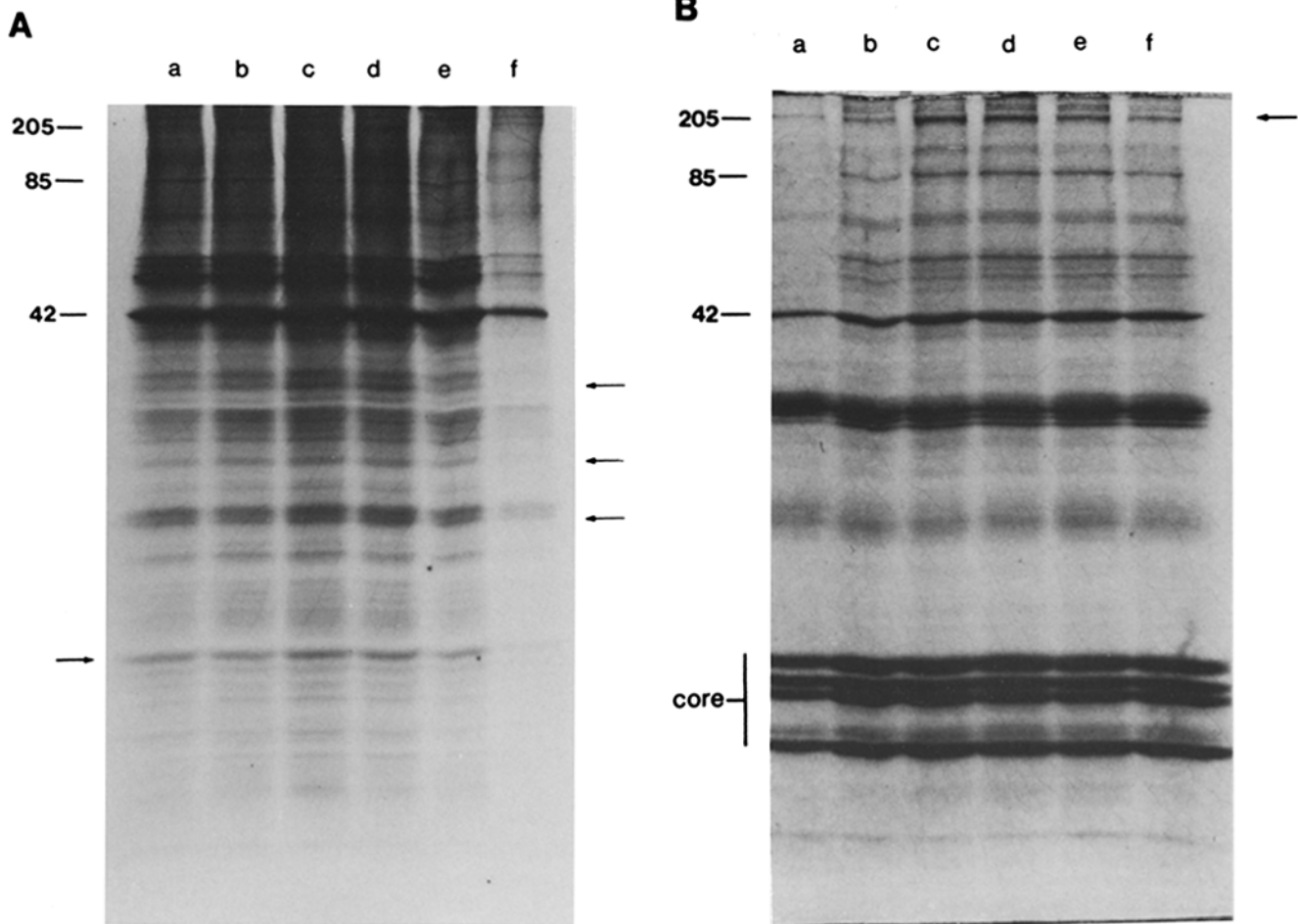
FIGURE 7 Analysis of the labeled proteins from the nuclear fraction of lymphocytes stimulated with varying doses of PHA. (A) The nuclei remaining after the low salt wash described in Fig. 6 were resuspended in a DNAse I buffer and then digested with 50 μg/ml of DNAse I for 30 min at 4°C. The lanes in A correspond to cells cultured in 10% serum plus PHA at 0.1 μg/ml (a), 0.2 μg/ml (b), 0.35 μg/ml (c), 0.75 μg/ml (d), 10% serum only (e), and PHA (0.75 μg/ml), and media alone (f). A fluorogram of the dried gel is shown. The proteins were run on a 14% gel. The band corresponding to histone H3 is shown at the left. The arrows on the right indicate those proteins showing slight increases in synthesis with PHA dose. B shows the corresponding Coomassie Blue stained gel, where the lanes correspond to cells cultured in 0.75 μg/ml PHA and media alone (a), 10% serum only (b), and 10% serum plus PHA at 0.75 μg/ml (c), 0.35 μg/ml (d), 0.2 μg/ml (e), and 0.1 μg/ml (f). The core histones are indicated. The arrow to the right indicates the myosin band.

Kelly et al. (11) have shown that c-*myc* mRNA is enhanced by 3 h of PDGF treatment of BALBc/3T3 cells as well as in concanavalin A-stimulated lymphocytes. Further, Hann et al. (5) have found that antisera to v-*myc* protein recognizes a 62-kd protein from bursal lymphoma cells and a 60-kd protein from normal cells. The 62-kd protein was localized in the nucleus in the transformed cells. The relationship of the 60-kd proteins in both PHA-stimulated lymphocytes and in PDGF-stimulated fibroblasts to the c-*myc* gene product is not known at this time but is under investigation.

As mentioned above rate of entry into S phase increases with increasing PHA concentration. This also correlates well with the dose dependence of the rate of aggregate formation (from Figs. 1 and 4). Aggregate formation is a required event for lymphocyte stimulation and will progress to a point where cells within an aggregate communicate through a gap junction-like structure (6, 21, 22). However, rapid aggregation can be stimulated by treatment of cells with PHA and albumin only (Fig. 8), a condition that is nonmitogenic. Despite rapid aggregation with PHA and high cell viability with or without

PHA label incorporation per cell or per milligram protein is lower with albumin than with serum addition, and P60 synthesis is only weakly stimulated in albumin plus PHA (Fig. 9). As noted in Results the cells also appear to aggregate in the presence of actinomycin D or α-amanitin where P60 synthesis is inhibited. Thus, aggregation per se does not appear to induce P60 expression or depend on new gene expression. Specific serum factors strongly enhance general protein synthesis and in conjunction with PHA appear necessary for optimal P60 synthesis and ultimate proliferation. As shown in Table I, 6 h of preincubation in PHA plus albumin followed by serum addition leads to only a small increase in entry rate constant over cells with serum plus PHA from the beginning. This result strengthens the association between P60 accumulation and control of entry and indicates that aggregation and the proteins produced in this process are not rate limiting for entry into S phase. However, cells pretreated with just serum or albumin alone have a significantly decreased entry rate constant when compared with cells pretreated with PHA plus albumin (Table I). This may be due to a number of complex
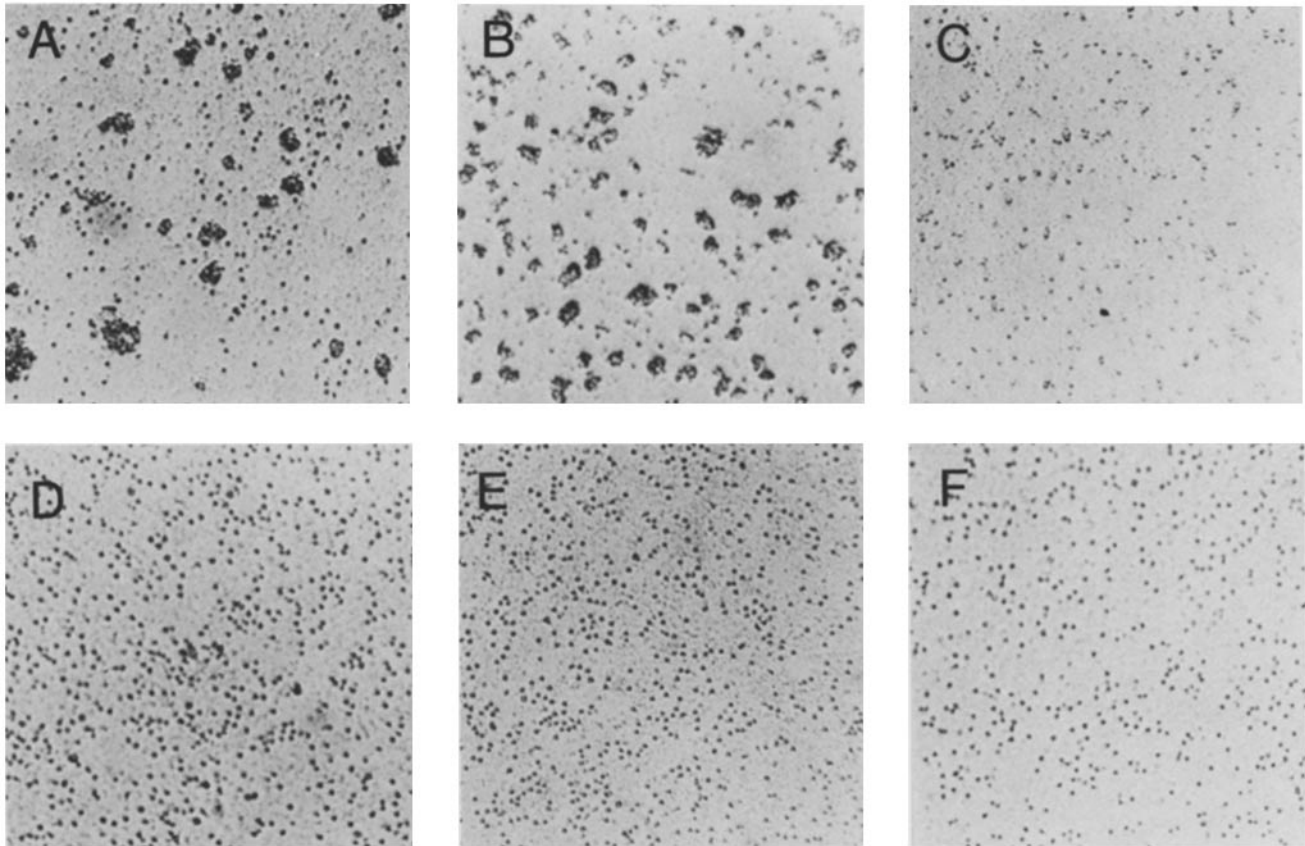
FIGURE 8 Lymphocyte aggregation induced by PHA and albumin. Lymphocytes were cultured in Lindbro plates (24 well) with (A, B, and C) or without (D, E, and F) PHA at 0.75 μg/ml. In addition cells were cultured in either 10% serum (A and E), 5 mg/ml albumin (B and E), or in media only (C and F).

TABLE I

Effects of Pretreatment of Lymphocytes in Culture on the Subsequent Entry Rate Constant, k

Initial conditions	Treatment at 6 H	k
Albumin + PHA	Plus serum	0.035 ± 0.0012
Serum	Plus PHA	0.016 ± 0.004
Albumin	Plus PHA and serum	0.026 ± 0.0026
Serum + PHA added initially	None	0.031 ± 0.0012

k, given in hours^{-1}, was calculated from a least squares fit to the points in the log-linear decay phase from a plot of ^{3}H-TdR uptake vs. time as in Fig. 1 (in the presence of colchicine). Cells were cultured in Lindbro plates (12 well) under the various conditions listed above in parenthesis and treated with serum, PHA, or PHA plus serum after 6 h. Replicate plates were then given consecutive ^{3}H-TdR pulses over a 96-h period. The cells were harvested and counted as in Fig. 1.

effects these protein solutions have on the cells and is currently under investigation.

In the fluorogram of the nuclear fraction (Fig. 7A) we see a slight increase in the intensity of a number of bands in the PHA-stimulated cells compared with the serum control. This is consistent with the flux of certain proteins into the nucleus following stimulation reported for PHA stimulated lymphocytes by Johnson et al. (9). This flux is most clearly seen by the increase in the Coomassie Blue-staining intensity of the myosin band with increasing doses of PHA. This protein does not show extensive labeling on the fluorogram. The role of myosin in the nucleus is unclear but it has been shown to be associated with the nuclear matrix (15, 22). A band on the

fluorogram of Fig. 7A (arrow, left) corresponds to histone H3 on the Coomassie-blue stained gel (Fig. 7B). If this is H3 it shows significant synthesis compared with the other histones, in contradiction to the results of Waithe et al. (27).

In conclusion we have observed a dose dependent increase in the synthesis of two cytoplasmic proteins (P51 and P60) during the first 6 h of PHA stimulation. Besides the described changes in synthesis, a dose-dependent redistribution in form of flux to the nucleus is seen for the heavy chain of myosin (205 kd). P60 shows the most extensive increase in synthesis with dose of PHA. By 6 h the bulk of synthesized P60 is seen in cytoplasmic fractions although P60 may be loosely associated with the nucleus. The increases in the rate of synthesis of P60 with dose of PHA correlates well with increases in the rate of cellular aggregation and the rate of entry of cells into first S phase. Thus, P60 may be the major response associated protein to be synthesized during the first 6 h of stimulation. Initiation of P60 requires the presence of both PHA and serum and although the rate of cellular aggregation shows good correlation with P60 synthesis the latter is not a simple consequence of aggregation per se.
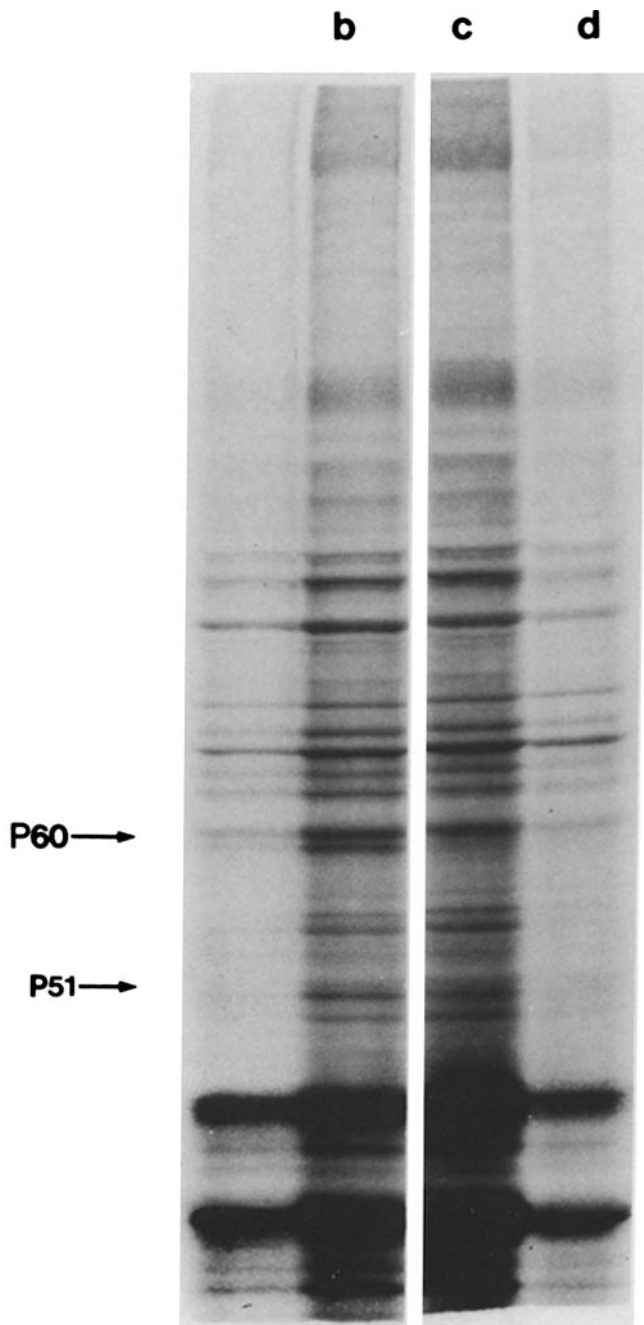
FIGURE 9 Analysis of the labeled proteins from the cytoplasmic fractions following induction of aggregation in PHA and albumin. Lymphocytes in tissue culture flasks were labeled with [³H]leucine and [³⁵S]methionine as in Fig. 3. The cells were cultured in 5 mg/ml albumin plus 0.75 μg/ml PHA (a), 10% serum plus 0.75 μg/ml PHA (b), 10% serum (c), or in 5 mg/ml albumin (d). A fluorogram of the dried gel is shown. P60 and P51 are indicated. The proteins were run on an 8–14% gradient gel.

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