DETECTION OF G1 PROTEINS IN CHINESE HAMSTER
CELLS SYNCHRONIZED BY
ISOLEUCINE DEPRIVATION OR MITOTIC SELECTION

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ABSTRACT

Examination of labeling patterns of proteins in Chinese hamster cells (line CHO)
revealed the presence of a class of protein(s) that is synthesized during G1 phase of
the cell cycle. Cells arrested in G1 by isoleucine (Ile) deprivation were prelabeled
with [14C]Ile, induced to traverse G1 by addition of unlabeled Ile, and labeled with
[3H]Ile at hourly intervals. Cells were fractionated into nuclear and cytoplasmic
portions, and proteins were separated by sodium dodecyl sulfate-polyacrylamide
gel electrophoresis. Gel profiles of proteins in the 45,000–160,000 mol wt range
from the cytoplasm of cells in G1 were similar to those from cells arrested in G1
except for the presence of a major peak of [3H]Ile incorporated into a protein(s) of
approximately 80,000 mol wt. Peaks of net [3H]Ile incorporation were not detected
in nuclear preparations. Cellular fractionation by differential centrifugation
showed that peak I protein was located in the soluble supernatant fraction of the
cytoplasm. Time-course studies showed that synthesis of this protein began 1–2 h
after initiation of G1 traverse; the protein reached maximum levels in 4–6 h and was
reduced to undetectable levels by 9 h. A cytoplasmic protein with similar
electrophoretic mobility was found in G1 phase of cells synchronized by mitotic
selection. This class of proteins is synthesized by cells before entry into S phase and
may be involved in initiation of DNA synthesis.

Elucidation of biochemical events which occur
during the G0 → G1 transition and subsequent
traverse of the G1 phase of the cell cycle may
counter to our understanding of how initiation
of DNA synthesis is regulated. The isoleucine
(Ile) deprivation technique for synchronizing Chi-
nese hamster cells in G1 phase (13, 24) has
provided one means of determining some of the
sequential biochemical events that occur during G1
progression (22).

Abbreviations used in this paper: Ile, isoleucine; SDS,
sodium dodecyl sulfate; TdR, thymidine.
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The cells were resuspended in lie- F-10 medium with Ile- F-10 that contained twice the normal concentration of glutamine and 10% dialyzed calf-fetal sera. In our hands the CHO grew with a doubling time of 17 h. Pneumoneumonia-like organism contamination was not detected when cells were routinely cultured on medium described by Chanock et al. (2). Exponentially growing CHO cells at 3.4 x 10^5/ml were labeled with 3 µCi or 10 µCi L-[4,5-^3H]leucine (73.5 Ci/mmol, New England Nuclear, Boston, Mass.) per ml of medium at hourly intervals.

**Cell Fractionation**

Cells were fractionated into crude cytoplasmic and nuclear portions as described by Becker and Stanners (1). This procedure involved treating cells with a mixture of Tween 40 and sodium deoxycholate (16), rupturing the cells by twice forcing the suspension through a 26-gauge needle, and pelleting the nuclei by centrifugation at 800 g for 10 min. The nuclei were washed twice and dissolved in 1% SDS buffer at a concentration of 5 x 10^9 nuclei/0.5 ml. The cytoplasmic portion was treated with an equal volume of 2% SDS buffer. Preparations were made 4% in mercaptoethanol, boiled for 2-3 min, and chilled on ice. Protein was determined by the method of Lowry et al. (14) with bovine serum albumin as the standard.

An alternate method of cell fractionation was used to determine the subcellular component with which the peak 1 protein was associated. Cells were washed three times with phosphate-buffered saline and allowed to swell in a hypotonic buffer (0.01 M NaCl, 0.0015 M MgCl2, 0.01 M Tris, pH 7.4) for 10 min, and cells were then lysed with a Dounce homogenizer until about 90% of the cells were broken, as judged by phase-contrast microscopy. Nuclear pellets were pelleted by centrifugation at 800 g for 10 min at 4°C, and the cytoplasmic fraction was spun at 100,000 g for 1 h at 4°C to yield the microsomal pellet and soluble supernatant. Each of these fractions was dissolved in SDS buffer, and the polypeptides were separated by gel electrophoresis.

**Gel Electrophoresis**

Electrophoresis was carried out on 7% SDS-polyacrylamide gels as described by Becker and Stanners (1) after the method of Laemmli (10). Approximately 100 µg of protein contained in 50 µl of reservoir buffer plus 0.25 M sucrose were layered on 5 x 58-mm gels and electrophoresis was run at 1.7 mA/gel for 2 h. Bromophenol blue was used to mark the buffer front. Gels were then fixed overnight in 7% acetic acid and sliced into 0.75-mm slices with an automatic slicing devise (11). The slices were treated with 0.5 ml of NCS (Amersham/Searle, Arlington Heights, III.) which contained 10% H2O and heated at 50°C for 3 h. After the samples were chilled, 10 ml of Omnifluor (New England Nuclear) liquid scintillation cocktail were added per scintillation vial.

Radioactivity was measured with a Packard Tri-Carb 3330 liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Discriminators were set such that counts attributable to tritium were not detected in the ^3H channel and about 25% of counts from ^4C spillover over into the tritium channel. Tritium counts were corrected for ^4C spillover, ^3H or ^14C was summed.

**Materials and Methods**

**Propagation of Cells**

Chinese hamster cells (line CHO) were grown as suspension cultures in Ham's F-10 without CaCl2 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 5% fetal calf and 10% calf sera (Flow Laboratories, Rockville, Md.) and penicillin-streptomycin. In our hands the CHO grew with a doubling rate of 1.7 x 10^5 cells/ml medium.

**Production of Synchronized Cells**

G1 Cells

Exponentially growing CHO cells at 3.4 x 10^5/ml were pelleted by centrifugation at 500 g and washed once with Ile^- F-10 that contained twice the normal concentration of glutamine and 10% dialyzed calf-fetal sera (20). The cells were resuspended in Ile^- F-10 medium at an initial concentration of 1.7 x 10^6 cells/ml medium. Cell concentration of these cultures after G1 arrest was about 2.5 x 10^6 cells/ml.

Populations of G1 cells were also produced by the mitotic selection technique as originally described by Terasima and Tolmach (19) and modified by Tobey et al. (21) Metaphase inhibitors were not used. The initial mitotic index of these populations was 0.90-0.95.

**Conditions for Labeling with Radioactive Precursors**

24 h after cells were deprived of Ile, 0.25 µCi of uniformly labeled [14C]Ile (312 µCi/mmol; Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.) was added per ml of cell suspension. 32 h after deprivation, the cells were spun out of the medium containing [14C]Ile and resuspended in "conditioned" Ile^- F-10 which had been used to grow CHO cells to a state of G1 arrest. After a wait of 4 h for incorporation of residual [14C]Ile, cells were released from G1 by the addition of 2 x 10^-4 M Ile, and 25-ml aliquots containing 2.4 x 10^4 cells/ml were labeled with 3 µCi or 10 µCi L-[4,5-^3H]isoleucine (73.5 Ci/mmol, New England Nuclear, Boston, Mass.) per ml of medium at hourly intervals.

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for the gel slices, and the percentage of total $^3$H or $^{14}$C was determined for each slice.

Appropriate mol wt markers (Worthington Biochemical Corp., Freehold, N. J.) were dissolved in 1% SDS buffer and separated by electrophoresis. Gels were fixed in 5% TCA for 30 min at 56°C, stained for 30 min at 56°C with a solution of Coomassie brilliant blue R (Sigma Chemical Co., St. Louis, Mo.), and destained by frequently changing the destaining solution. These solutions were prepared according to the method of Weber and Osborn (25).

RESULTS

Labeling Patterns of Proteins in G$_1$-Arrested Cells

Since our objective was to prelabel with $[^{14}$C]Ile those proteins that are synthesized in cells arrested in G$_1$ by Ile deprivation, it was first necessary to determine the maximum amount of $[^{14}$C]Ile that could be added to the G$_1$ cells without inducing traverse of G$_1$. This was accomplished by adding varying amounts of Ile to arrested cells and measuring the relative amount of DNA synthesis by incorporation of $[^3$H]thymidine ([3H]TdR). An increase in DNA synthesis would indicate that cells had escaped the G$_1$ block and entered S phase. Data presented in Fig. 1 illustrate the dose-response curve for incorporation of [3H]TdR as a function of Ile concentration. DNA synthesis did not occur at concentrations below $1.25 \times 10^{-6}$ M, whereas a fourfold increase to $5 \times 10^{-8}$ M stimulated about 90% of maximum [3H]TdR incorporation. The amount of $[^{14}$C]Ile added for prelabeling in the following experiments did not exceed a total Ile concentration of $8 \times 10^{-7}$ M. At this concentration of $[^{14}$C]Ile, proteins in the mol wt range of interest were labeled such that about 4–6 $\times 10^{4}$ cpm were separated on each gel.

Labeling Patterns of Proteins in Cells Traversing G$_1$

Cells prelabeled with $[^{14}$C]Ile were induced to traverse G$_1$ by addition of Ile, labeled with $[^3$H]Ile after 4–5 h, and fractionated into crude cytoplasmic and nuclear portions. Polypeptides were subjected to electrophoresis on SDS-polyacrylamide gels as described in Materials and Methods. A typical separation profile of the distribution of radioactively labeled cytoplasmic proteins is presented in Fig. 2. Distribution of polypeptides less than 45,000 mol wt were not included because they were too small to be properly resolved at this concentration of acrylamide. Gel profiles of polypeptides in the 45,000–160,000 mol wt range from cells traversing G$_1$ were similar to those from cells arrested in G$_1$ except for the presence of a major peak of [3H]Ile in gel slices that corresponds to a mol wt of approximately 80,000. A lesser and perhaps insignificant amount of [3H]Ile was present in peak II. Peaks of net [3H]Ile incorporation were not detected in nuclear preparations. Further cell fractionation by Dounce homogenization and differential centrifugation revealed that peak I protein is located in the soluble supernatant fraction of the cytoplasm.

Time-Course Pattern of Peak I Synthesis

The rate of appearance of peak I protein during G$_1$ traverse was measured by labeling cells during early, middle, or late G$_1$ phase (Fig. 3). As seen from these profiles, net incorporation of [3H]Ile into peak I was greatest during mid-G$_1$. This
FIGURE 2 SDS-polyacrylamide gel electrophoretic patterns of proteins prelabeled with $^{14}$C]Ile (---) before release of cells in G$_1$ arrest and proteins labeled with $[3H]$Ile (---) 4-5 h after reversal of G$_1$ arrest. Total radioisotope associated with proteins separated on each gel was $6.5 \times 10^6$ cpm $^{14}$C]Ile and $2.5 \times 10^6$ cpm $[3H]$Ile. Mol wt markers used were: human gamma globulin, 160,000 daltons; bovine serum albumin, 67,000 daltons; and ovalbumin, 45,000 daltons. Electrophoresis was from left to right.

indicated that the rate of synthesis of peak I protein was highly variable during G$_1$. The kinetics of peak I synthesis during G$_1$ progression were established by labeling traversing cells at hourly intervals for 9 h after release. Percentage net $[3H]$Ile in peak I was calculated by determining the distribution of label as described in Materials and Methods, except that, instead of whole gels, only 10-15 gel slices in the area of peak I were included in the calculations. Net percentage of Ile in peak I was equal to $\Sigma$ %$[3H]$Ile - %$^{14}$C]Ile for each slice under peak I curve, and this value was plotted as a function of time after release from G$_1$ arrest. Net synthesis began during the 1-2-h labeling interval, was maximal in the 5- and 6-h samples, and was greatly reduced by 9 h (Fig. 4). Maximum synthesis of peak I protein occurred at the time when the fraction of cells initiating DNA synthesis was about 0.15.

**Synthesis of Peak I Protein in G$_1$ Cells Synchronized by Mitotic Selection**

Since the above data indicated that a class of proteins could be detected with isotopically labeled Ile in cells synchronized by Ile deprivation, we then sought a similar protein in G$_1$ cells synchronized by an alternate method. Mitotic selection was used to produce G$_1$ cells that are relatively unperturbed biochemically and possess time-course patterns of DNA synthesis and cell division that are similar if not identical to those of cells synchronized by Ile.
DISCUSSION

The results presented here show that cells synchronized by Ile deprivation or mitotic selection synthesize a class of soluble cytoplasmic proteins of approximately 80,000 mol wt during the G1 phase of the cell cycle.

Peak I protein was detected as a class of labeled polypeptides that migrated in SDS-polyacrylamide gels with an apparent mol wt of 80,000. Although a more definite characterization should be forthcoming, unpublished results by Darby and Ley indicate that the protein is composed of a single polypeptide.

The protocol used for prelabeling with [14C]Ile those cytoplasmic proteins which are synthesized during G1 arrest was based on the following characteristics of our system: By 24 h after CHO cells were initially deprived of Ile, cells in S, G2, and M at the time of deprivation continued to traverse and are now arrested in G1 (13, 5). Proteins being synthesized during G1 arrest were labeled with [14C]Ile. The fact that there is a threshold concentration of Ile above which cells begin to escape the Ile block (as determined by initiation of DNA synthesis) allowed us to prelabel deprivation (20). The profiles in Fig. 5 represent separation of cytoplasmic proteins from cells labeled in late interphase and collected at mitosis (Fig. 5 A), and G1 cells synchronized by mitotic selection and then labeled 4 h after cells entered G1 (Fig. 5 B). These data show a peak of radioisotope incorporation in G1 cells produced by mitotic selection that corresponds to peak I protein in G1 cells synchronized by Ile deprivation. Peak I in the separation patterns of proteins from cells in G2 is very greatly reduced, which suggests that this protein may be specific for G1 phase of the cell cycle.

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with concentrations of $[^{14}\text{C}]\text{Ile}$ that did not exceed $10^{-8}\text{ M}$ (see Fig. 1) without inadvertently triggering $G_1$ traverse. This minimum concentration of Ile compares well with the minimum concentrations previously reported (13) which allowed a few mitotically selected cells to traverse $G_1$ and enter $S$ phase ($6.0 \times 10^{-8}\text{ M}$) or some cells of an Ile-deprived culture to complete mitosis ($1 \times 10^{-8}\text{ M}$).

Evidence that depriving CHO cells of Ile did not cause abnormal labeling patterns is presented in Fig. 6 which illustrates the similarity between polyacrylamide gel profiles of $G_1$ proteins synthesized in cells which were synchronized by Ile deprivation or by mitotic selection. Cells synchronized by mitotic selection are selected out of a random population of cells and thus are less likely to be biochemically perturbed than cells synchronized by induction techniques (for discussion on methods of cell synchronization, see Mitchison [15]).

Other investigators have compared the patterns of proteins synthesized in resting and proliferating cells. Becker and Stanners (1) examined newly synthesized proteins of 30,000–150,000 mol wt in hamster cells growing in synchrony and cells in stationary phase by double-labeling techniques and SDS-polyacrylamide gel electrophoresis. These workers did not detect significant differences between gel profiles of cytoplasmic proteins synthesized in different phases of the cell cycle; however, they did detect differences when profiles from stationary phase cells were compared with any phase of proliferating cells. This is in contrast to our findings where we detected differences in the pattern of cytoplasmic proteins from different phases of the cell cycle, notably the peak $I$ protein, but the patterns between traversing and $G_1$-arrested cells are similar. One explanation for the similarity of patterns for noncycling vs. cycling cells in our system may be the possibility that Ile-deficient $G_1$ arrest is not analogous to stationary phase. Indeed, the results of Enger and Tobey (4) indicate that the rates of macromolecular synthesis in high density, stationary phase cells are much lower than in cells arrested in $G_1$ by Ile deprivation. Kolodny and Gross (9) also reported differences in patterns of proteins synthesized during $G_2$ phase and patterns obtained in other phases of the cell cycle.

Numerous studies suggest that factors required for initiation of DNA synthesis are made in the cytoplasm (3, 6, 7, 17, 18, and others). Our results showed that the peak $I$ protein was synthesized in the cytoplasm of cells traversing $G_1$ and that synthesis declined as the cells entered $S$ phase. Because synthesis of peak $I$ protein precedes initiation of DNA synthesis, we propose that this protein is required for initiation of DNA synthesis. Studies in our laboratory are currently directed at testing this hypothesis by attempting to clarify the role of this protein in $G_1$ progression and its relationship to DNA synthesis.

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