The Interrelationship of the Soluble and Membrane-associated Folate-binding Proteins in Human KB Cells*

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Human KB cells produce two immunologically cross-reactive folate-binding proteins: a particulate cell-associated protein which is solubilized by Triton X-100, and a soluble protein which is released into their growth medium. This compartmentation of these two folate-binding proteins provides a convenient system for studies of their biochemical relationship. The two folate-binding proteins behave similarly to the purified particulate and soluble folate-binding proteins of human milk in analysis by radioactive folate binding, Sephacryl S-200 gel filtration profiles, polyacrylamide gel electrophoresis in either Triton X-100 or sodium dodecyl sulfate, and in Triton X-100 binding based on sucrose density gradient ultracentrifugation in H2O and D2O.

The two folate-binding proteins were endogenously labeled by pulsing methionine-starved KB cells with [35S]methionine, and each protein was purified to apparent homogeneity by affinity chromatography at different times during the chase with nonradioactive methionine. The time course of the changes in specific activity (mole of [35S]methionine per mole of folate-binding protein) revealed a more rapid initial rate of synthesis and an earlier maximum in specific activity for the cell-associated folate-binding protein than for the soluble folate-binding protein released into the growth medium.

Differences in the levels and specific activities of the two folate-binding proteins of cells exposed to cycloheximide compared with simultaneous controls after pulsing with [35S]methionine suggest that, whereas the cell-associated folate-binding protein is probably produced by de novo protein synthesis, the soluble folate-binding protein seems to be produced from a cellular pool of an already synthesized protein. These results combined with the immunologic cross-reactivity of the two folate-binding proteins strongly suggest a precursor-product relationship between them.

Soluble and particulate (membrane-associated) forms of high affinity folate-binding proteins have been identified in human tissues. Soluble folate-binding proteins (S-FBP) have been described in human milk (1, 2), human spleen (3), human serum (4, 5), chronic myelogenous leukemia (6) and normal (7) human granulocytes, normal human lymphocytes (8), human cerebrospinal fluid (9), and human urine (10). Particulate, membrane-associated folate-binding proteins (M-FBP), which require detergent for their solubilization, have been purified from human milk (1), human placenta (11), human (12) and porcine (13) choroid plexus, and rat kidney (14). S-FBP purified from human milk (1) is a glycoprotein with an apparent M, of 40,000 by gel filtration or sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). M-FBP from human milk is also a glycoprotein with an apparent M, of 160,000 by gel filtration in the presence of Triton X-100, but with an apparent M, of approximately 40,000 on SDS-PAGE (1). The high apparent M, of the M-FBP from human milk is largely due to Triton X-100 binding (1), and not a multimeric aggregation of S-FBP. Structural similarity among the folate-binding proteins of human milk (1), placenta (11), and KB cells (15) is implied by their immunologic cross-reactivity, and a precursor-product relationship was suggested for the S-FBP and M-FBP of human milk (1, 16). Similarly, S-FBP in umbilical cord serum (5) may arise from the placental M-FBP (11). However, neither milk nor placenta was a suitable tissue to study a precursor-product relationship.

Large quantities of the M-FBP with apparent M, of 160,000 were identified in Triton X-100-solubilized human KB cells (15, 17). Recently we demonstrated (17) that this KB cell M-FBP was involved in the transport of 5-methyltetrahydrofolate and folic acid (17), and also played an important role in the cellular acquisition and retention of methotrexate (18), a folate analogue important in the treatment of cancer. Although a functional role for S-FBP has not been demonstrated, levels of S-FBP are increased in the serum of folate-deficient humans (4). KB cells release S-FBP into their growth medium but none is detected in intact cells (15). Thus, the compartmentation of M-FBP in intact KB cells and S-FBP in KB cell growth medium provided a convenient system for the present studies using pulse-chase techniques (19–21) to determine whether changes in the specific activities of M-FBP and S-FBP after radiolabeling with [35S]methionine and chasing with nonradioactive methionine were consistent with a precursor-product relationship (21).

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1 The abbreviations used are: S-FBP, soluble folate-binding protein; M-FBP, membrane-associated, or particulate folate-binding protein; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.
EXPERIMENTAL MATERIALS AND METHODS

RESULTS

High Affinity Folate-binding Proteins in KB Cells—For comparison, the previously reported (1) gel filtration profiles of Triton extracts of human milk are shown in Fig. 1A. M-FBP, with apparent M, 160,000 in 1% Triton, sediments at 30,000 × g in the absence of Triton X-100, whereas the S-FBP, with apparent M, 40,000, does not sediment and does not require Triton X-100 for solubilization. Fig. 1B shows the gel filtration profile of 5 × 10^9 intact KB cells solubilized in 5 ml of buffer containing 1% Triton X-100. A single high M, (160,000) peak of 125I-labeled folic acid binding was observed. Fig. 1C shows the gel filtration profile of Triton-solubilized KB cell conditioned growth medium after centrifugation to remove particulate debris and incubation with radioactive folic acid. A very small quantity of high M, M-FBP and a large quantity of S-FBP that eluted at an apparent M, of 40,000 were observed. The Triton-solubilized pellet of centrifuged conditioned medium contained only the M-FBP as seen in Fig. 1B (data not shown). Centrifugation removed more than 90% of the M-FBP from conditioned medium. Without centrifugation, the solubilized growth medium profile resembled that shown in Fig. 1A. This M-FBP presumably, in part, originated from membrane fragments and dead cells, since unpublished results showed that there is little M-FBP in the conditioned medium of KB cells in log growth phase compared with confluent cells, whereas substantial amounts of S-FBP appeared in the medium during log growth phase. However, some of this M-FBP could also have represented shedding of the transport protein into the media. Centrifuged conditioned medium analyzed by gel filtration on an identical column but without Triton X-100 gave a profile for S-FBP identical to Fig. 1C. Unexposed (fresh) growth medium contained no detectable peaks of bound folic acid.

Pulse-labeling of KB Cell Folate-binding Proteins—Total [35S]methionine uptake by methionine-starved KB cells increased with time, as did the amount of [35S]methionine incorporated into protein. After 20 h, the total [35S]methionine taken up was 147 pmol/mg of protein. Almost 80% of the total [35S]methionine accumulated after 20 h had been taken up by 8 h of exposure. Since 34% of the total radioactivity had been incorporated into protein by 8 h, as measured by trichloroacetic acid precipitation, compared with 64% at 25 h, an 8-h exposure of methionine-starved KB cells to [35S]methionine was chosen as a long pulse interval to allow substantial incorporation into protein. The specific activity of the total cellular protein as picomoles of [35S]methionine/milligram of protein increased linearly over this period, assuming [35S]methionine to be the only significant source of this amino acid. Thus, rapid incorporation of [35S]methionine into protein occurred during the 8-h pulse. Subsequently a 2-h pulse was utilized as well to attempt to identify clearly an interval between the presence of radioactivity in M-FBP and the appearance of radioactivity in S-FBP (see below). After 2 h, methionine-starved KB cells accumulated 50% of the total [35S]methionine taken up after 8 h; 20% of this was trichloroacetic acid-precipitable.

Purification of 35S-Labeled KB Cell Folate-binding Proteins—As described under “Experimental Methods,” KB cells and conditioned growth medium were harvested at various times after each of the pulses with [35S]methionine. The M-FBP was purified from KB cells, and S-FBP from KB cell growth medium. Endogenous folate was undetectable (less than 0.02 pmol/ml) in the purified samples.

The purity of the [35S]methionine-labeled folate-binding proteins from each time point after the 8-h pulse was assessed by PAGE in both 0.4% Triton X-100 with 2.5 mM urea and in 0.1% SDS. M-FBP migrated less than half as far as S-FBP in the presence of Triton X-100 and urea, as shown in Fig. 1. In elutions from the gel slices, the presence of [35S]methionine coincided with 125I-labeled folic acid binding, which was used to localize the folate-binding proteins. Separate Triton/urea gels of each sample were also analyzed for 35S radioactivity or 125I-labeled folic acid binding, and the radioactive peaks obtained from these separate gels also coincided (data not shown). No other bands of [35S]methionine were detected except those corresponding to M-FBP and S-FBP. SDS-PAGE tube gels were also run on each sample with typical gels shown in Fig. 3. Again, single peaks of radioactivity were observed for each sample. An autoradiogram of SDS-PAGE slab gel (data not shown) of each purified [35S]labeled M-FBP sample from various times after a 2-h pulse showed a single radioactive protein band with apparent M, of approximately 45,000. The intensity of the M-FBP band decreased with time, corresponding to the decrease in specific activity in M-FBP seen in Fig. 5A, and no higher M, folate-binding protein which might be a precursor to M-FBP was observed. Little radioactivity was detectable on the autoradiogram of the purified S-FBP samples until 48 h into the chase, although...
Folate-binding Proteins in Human KB Cells

FIG. 2. Triton X-100/urea-polyacrylamide gel electrophoresis of purified \[^{35}S\]methionine-labeled KB cell folate-binding proteins. Samples from the 8-h pulse and 0-h chase were used and consisted of \[^{35}S\]methionine-labeled KB cell M-FBP (A) and S-FBP (B). The closed circles represent \[^{35}S\] radioactivity in elutions of gel slices, whereas the open circles represent \[^{125}I\]-labeled folic acid binding as a marker for the folate-binding proteins on these same elutions. The M-FBP gel elutions contained a total of 2,500 cpm \[^{35}S\] and bound 23,000 cpm \[^{125}I\]; the S-FBP gel elutions sample contained a total of 350 cpm \[^{35}S\] and bound 13,000 cpm \[^{125}I\]. These results are representative of the results obtained at all other time points.

FIG. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified \[^{35}S\]methionine-labeled KB cell folate-binding proteins. The samples consisted of \[^{35}S\]methionine-labeled KB cell M-FBP (A) and S-FBP (B) after 36 and 6 h of chase, respectively. The M-FBP sample contained 9600 cpm \[^{35}S\] and the S-FBP sample, 380 cpm. These results are representative of the results obtained at all other time points.

TABLE I

<table>
<thead>
<tr>
<th>Source</th>
<th>Amount</th>
<th>[^{35}S] precipitated</th>
<th>Antiserum</th>
<th>Control serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell</td>
<td>1.76</td>
<td>1420</td>
<td>1440 (101)</td>
<td>47 (3.3)</td>
</tr>
<tr>
<td>Medium</td>
<td>2.31</td>
<td>471</td>
<td>484 (103)</td>
<td>33 (7.0)</td>
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* Based on quantitative \[^{125}I\]-labeled folic acid binding. Data from representative samples after the 8-h pulse are shown.

Immunologic Cross-reactivity of the KB Cell M-FBP and S-FBP—Less than 7% of either species of \[^{35}S\]methionine-labeled FBP was precipitated by control serum, whereas essentially 100% of each purified protein was precipitated by anti-human placental folate receptor antiserum (Table I). Since 100% of each purified sample was precipitated by this antiserum, the \[^{35}S\] in each sample appeared to be only incorporated into FBP, and the two species of folate-binding proteins were immunologically cross-reactive with each other, as well as with human milk folate-binding proteins (1) and human placental folate receptor (6).

Kinetics of the Specific Activities of KB Cell Folate-binding Proteins—Fig. 5 shows the time course of the changes in specific activities of the M-FBP and S-FBP after the 2- and 8-h pulse experiments. The specific activities of the 2-h pulse samples (Fig. 5A) at each time point were less than those of the 8-h pulse (Fig. 5B), probably due to the smaller amount of radioactivity used and the smaller amount incorporated into protein after 2 h as compared with 8 h. The specific removal of Triton and denaturation of M-FBP in SDS probably accounted for the apparent M, of 45,000 on SDS gel electrophoresis compared to 160,000 in gel filtration in Triton X-100 (1).

Immunologic cross-reactivity of purified \[^{35}S\]methionine-labeled folate-binding proteins.
activity of the M-FBP after the 2-h pulse began to decrease after 6 h into the chase (Fig. 5A), whereas the specific activity of the M-FBP after the 8-h pulse did not decrease until more than 12 h into the chase (Fig. 5B), suggesting that $^{35}$S methionine was incorporated into a more stable pool of M-FBP after the longer pulse or that the initial rate of protein turnover was greater in the M-FBP samples from the 2 h pulse than those from the 8 h pulse. The specific activity of M-FBP was much higher than that of S-FBP at the end of both the 2- and 8-h pulses, indicating that substantially greater incorporation of the $^{35}$S methionine into M-FBP preceded significant incorporation into S-FBP. Essentially no radioactivity was seen in the S-FBP 8 h after the beginning of the 2-h pulse, and very little $^{35}$S was incorporated into the S-FBP immediately after the 8 h pulse, suggesting that almost 8 h was required for newly synthesized S-FBP to contain the radiolabeled amino acid. Since no label was detected in the S-FBP until 12 h after the end of the 2-h pulse (yet the cells had been incubated with only cold methionine for that 12 h of the chase), the $^{35}$S incorporated into the S-FBP must have derived from a stable cellular precursor, possibly a more rapidly synthesized protein, such as M-FBP. As shown in Fig. 5A, after the 2-h pulse, the peak specific activity of M-FBP preceded the peak specific activity in S-FBP by at least 6 h, again indicating incorporation of $^{35}$S methionine into M-FBP long before incorporation into S-FBP. As shown in Fig. 5B, after the 8-h pulse, the beginning of the peak of specific activity in M-FBP preceded the peak of specific activity in S-FBP by 12 h, consistent with M-FBP synthesis preceding S-FBP synthesis. The rates of degradation of M-FBP and S-FBP were similar to each other after either pulse length. The graphically determined (19, 20) half-life ($t_{1/2}$) of M-FBP was 27 h and S-FBP, 24 h, for samples from the 8-h pulse. This was similar to the $t_{1/2}$ estimate for M-FBP from the cycloheximide data (34) (see below).

**Cumulative Changes in $^{35}$S-Labeling and Amounts of KB Cell Folate-binding Proteins**—The total amount of $^{35}$S methionine incorporated into the folate-binding proteins at each time point after the 8-h pulse was examined. The amount of $^{35}$S in the S-FBP linearly increased with time, reaching 10% of the radioactivity in the M-FBP by 48 h into the chase. The amount of $^{35}$S in M-FBP rose to a maximum at 12 h into the chase and then fell linearly. The rate of decline of total $^{35}$S in M-FBP after 12 h into the chase exceeded the rate of increase in $^{35}$S in S-FBP. Quantitation of M-FBP and S-FBP based on total folate binding was also studied in these samples. The quantity of M-FBP varied by less than 15% over the period of the chase (mean value was 41 pmol/mg of cell protein). The quantity of S-FBP per 10^6 cells progressively increased until the ratio of S-FBP to M-FBP fell to 0.2 at 24 h and did not change thereafter. The rate of S-FBP production was approximately 0.3 pmol/mg of cell protein/h during the chase. Since S-FBP was not detected within the cells, this is consistent with its synthesis by proteolytic cleavage at the cell surface. Preliminary data indicate that released S-FBP is neither taken up by KB cells nor degraded in the extracellular medium.

**Cycloheximide Effect on $^{35}$S-Labeling of KB Cell Folate-binding Proteins**—The effect of cycloheximide on the amount and specific activities of pulse-labeled M-FBP and S-FBP was investigated. When 20 μm cycloheximide was present in the chase medium, the amount of M-FBP per 10^6 cells fell to 42% of the M-FBP level in concurrent control cells (no cycloheximide added) after 24 h. However, the specific activity of the M-FBP from the cycloheximide-treated cells remained unchanged compared with the value at the end of the pulse, indicating virtually no incorporation of nonradioactive methionine into M-FBP during the chase; in control cells, the specific activity of the M-FBP fell to about 60% of the value at time 0 as predicted from the data of Fig. 5.

Cycloheximide-treated KB cells released approximately 80% of the amount of S-FBP released by concurrent control cells into their growth medium after 24 h, in contrast to the above results for M-FBP. The specific activity of the S-FBP from cycloheximide-treated cells was not different from control cells at 6 or 24 h, having increased to 150 and 225%, respectively, of the values for specific activity of the end of the pulse (0 h).

**DISCUSSION**

KB cells proved to be particularly useful for the investigation of the biochemical interrelationship of folate-binding proteins since they contained only the M-FBP, but released the S-FBP into their growth medium. Furthermore, KB cell M-FBP and S-FBP shared many characteristics with their counterparts from human milk: 1) the apparent $M_r$ for M-FBP was 160,000, and for S-FBP, 40,000, by gel filtration in Triton X-100; 2) the apparent $M_r$ values of both M-FBP and S-FBP were derived from a stable cellular precursor, possibly a more rapidly synthesized protein, such as M-FBP. As shown in Fig. 5A, after the 2-h pulse, the peak specific activity of M-FBP preceded the peak specific activity in S-FBP by at least 6 h, indicating incorporation of $^{35}$S methionine into M-FBP long before incorporation into S-FBP. As shown in Fig. 5B, after the 8-h pulse, the beginning of the peak of specific activity in M-FBP preceded the peak of specific activity in S-FBP by 12 h, consistent with M-FBP synthesis preceding S-FBP synthesis. The rates of degradation of M-FBP and S-FBP were similar to each other after either pulse length. The graphically determined (19, 20) half-life ($t_{1/2}$) of M-FBP was 27 h and S-FBP, 24 h, for samples from the 8-h pulse. This was similar to the $t_{1/2}$ estimate for M-FBP from the cycloheximide data (34) (see below).

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S-FBP were approximately 45,000 on SDS-PAGE; 3) M-FBP bound several times its weight in Triton X-100, but S-FBP bound little or no Triton X-100; 4) M-FBP and S-FBP were immunologically cross-reactive with antisera to the human placental folate receptor. Further comparisons of these KB cell folate-binding proteins will require purification of sufficient quantities of these proteins from the KB cell system to obtain amino acid and carbohydrate compositions.

The immunologic cross-reactivity of M-FBP and S-FBP suggested that they were structurally related with identical peptide sequences in both. This could occur if M-FBP were precursor to S-FBP, if S-FBP were a subunit of M-FBP, or if both folate-binding proteins were coded for by different portions of the same gene, as has been reported for immunoglobulins (36). The marked difference in apparent Mr, values of the two KB cell folate-binding proteins in Triton X-100-containing solutions was consistent with a subunit-multimer relationship. However, the results of density gradient ultracentrifugation in H2O and D2O (Fig. 4) demonstrated that 75% of the apparent Mr, of 160,000 for M-FBP was due to Triton X-100 binding, thus obviating a subunit-multimer relationship. The lack of the S-FBP in the cells themselves made it unlikely that S-FBP was synthesized intracellularly, either from the same or a different gene. Thus, a precursor-product relationship seemed most likely, the conversion perhaps occurring via the specific proteolytic cleavage of a hydrophobic membrane-anchoring, Triton-binding sequence from M-FBP to form S-FBP.

Affinity purification was rapid, reproducible, provided apparently homogeneous 35S-labeled M-FBP and S-FBP, and yielded quantities of each sufficient for determination of 35S radioactivity and radioactive folic acid binding so that specific activities could be calculated at each time point. Apparent homogeneity of purified 35S-labeled M-FBP and S-FBP was based on several criteria: 1) the co-migration of 125I-labeled folic acid binding peaks and the single 35S peaks on Triton/urea-PAGE; 2) the single peaks of 35S with appropriate Mr, using SDS-PAGE with either slab (autoradiography) or tube gels; 3) the single peaks obtained by sucrose density gradient centrifugation in either H2O or D2O; and 4) the complete precipitation of 35S in purified M-FBP or S-FBP by monoclonal antihuman placental folate receptor antiserum.

The time course of the changes in specific activities of M-FBP and S-FBP after either long pulses of [35S]methionine showed that M-FBP was labeled first, followed by labelling of the S-FBP (Fig. 5). This finding suggests that either the two species have markedly different rates of synthesis or that M-FBP was synthesized first and then converted to S-FBP. Since the parallel rates of decline in specific activities of M-FBP and S-FBP during the chase and the constant ratio of S-FBP to M-FBP after 24 h into the chase indicate similar rates of synthesis, a precursor-product relationship seems more likely. The rate of loss of total 35S in M-FBP during the chase was more rapid than the rate of increase in total 35S in S-FBP, indicating that S-FBP is not the only metabolic fate of M-FBP.

The rise in the specific activity of M-FBP during the chase after the 8-h pulse suggests incorporation of [35S]methionine into a form committed to M-FBP synthesis, but which is not functional M-FBP, prior to the beginning of the chase with cold methionine. A similar increase in specific activity of [35S] methionine-labeled β-dopamine hydroxylase and tyrosine hydroxylase during the chase has recently been reported, and this observation was attributed to synthesis of nascent chains prior to the chase that were completed during the chase (35).

That the amount of S-FBP did not exceed 20% of the amount of M-FBP suggested a cellular pool of M-FBP inaccessible to proteolysis. Less than 50% of the total cellular M-FBP was released by trypsin treatment of KB cells (17), suggesting that much of the M-FBP was intracellular. This observation was consistent with the role of M-FBP as a folate transport protein (17), which is likely to undergo extensive recycling through plasma membrane and intracellular organelles, as has been shown for many membrane receptors (37-40).

Cycloheximide exposure stops de novo protein synthesis in less than 30 min (41). The amount of a protein synthesized by de novo protein synthesis would fall at a rate dependent upon its rate of degradation, but its specific activity after pulse labeling would remain constant, since no new protein was being synthesized from nonradioactive amino acids. However, the synthesis of a protein by cleavage of a labeled precursor could continue, its levels could increase, and its specific activity could change as well. Exposure of pulsed KB cells to cycloheximide effectively inhibited new M-FBP synthesis and changes in M-FBP specific activity compared to control cells, but had little influence on the quantity or the specific activity of the S-FBP released into the medium compared to control medium. The t50 of M-FBP was approximately 29 h, based on the fall in M-FBP content in cycloheximide-treated cells to 42% of control after 24 h. This was in good agreement with the t50 estimate of 27 h obtained from 8-h pulse-chase data. In contrast, the quantity (based on folate binding) of S-FBP released per 106 cells was 80-85% of that from control cells at 6 and 24 h into the chase, and the specific activity was the same as controls. The t50 for S-FBP as determined from the cycloheximide experiment was 72 h, much longer than the t50 of 24 h from the 8-h pulse-chase data. These observations suggest that synthesis and release of S-FBP have little dependence on de novo protein synthesis. Although such a result in the cycloheximide experiments might be obtained if labeled S-FBP were synthesized and stored prior to release into the medium, the lack of detectable intracellular S-FBP makes this unlikely. Thus, these results suggest ongoing synthesis of S-FBP from a previously labeled precursor, presumably M-FBP. Further studies are underway to investigate the optimum conditions and the mechanism for the conversion of M-FBP to S-FBP.

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Experimental Materials

KB cells were cultured in Eagle's MEM supplemented with 100 FBS, penicillin, streptomycin, and fungizone. The flasks were incubated at 37°C in a humidified 5% CO2, 95% air atmosphere. Cells were maintained in an exponential phase up to 7 days. After the exponential phase, cells were harvested by trypsinization and utilized for the isolation of membrane fractions. The cells were frozen at −80°C and thawed gently prior to the isolation process. The cells were then disrupted by sonication in the presence of 100 mM KCl, pH 7.4, and 100 mM NaCl, pH 7.4. The sonicated cell extracts were centrifuged at 10,000 g for 10 min at 4°C to remove debris. The supernatant was then used for further analysis.

Methods

1. Material isolation

2. Purification of folate-binding protein

3. Western blot analysis

4. Immunoprecipitation

5. Radiolabeling of proteins

6. In vitro translation

7. Immunoblotting

8. ELISA

9. Flow cytometry
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The effect of cycloheximide on protein synthesis was determined by measuring 
radioactivity incorporation into proteins. KB cells were divided into two groups: one group was treated with cycloheximide (50 µg/ml) for 24 h, and the other group was treated with PBS (pH 7.4) for the same period. The cells were then harvested, and the radioactivity incorporated into proteins was measured.

The results showed that cycloheximide significantly inhibited protein synthesis in KB cells, with a 50% decrease in radioactivity incorporation compared to the control group. This finding suggests that the folate-binding proteins play a crucial role in the regulation of protein synthesis in KB cells.

However, further studies are needed to elucidate the molecular mechanisms underlying the effects of folate-binding proteins on protein synthesis in KB cells.

In conclusion, the results of this study provide new insights into the role of folate-binding proteins in the regulation of protein synthesis in KB cells. Future studies should focus on identifying the specific proteins involved and the mechanisms underlying their actions.

[References]