Inhibition of Initiation of Protein Synthesis in Mammalian Tissue Culture Cells by L-1-Tosylamido-2-phenylethyl Chloromethyl Ketone

Sheng-Shung Pong,* Donald L. Nuss, and Gerdhard Koch
From the Roche Institute of Molecular Biology, Department of Cell Biology, Nutley, New Jersey 07110

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SUMMARY

Incorporation of amino acids into proteins in HeLa cells, virus-transformed 3T3 mouse fibroblastic, and mouse plasmacytoma cells is inhibited after the addition of L-1-tosylamido-2-phenylethyl chloromethyl ketone, an alkylating agent and chymotrypsin-specific protease inhibitor. Addition of this drug to tissue culture cells at concentrations of 20 to 30 μg per ml results in an irreversible inhibition of the incorporation of amino acids into cellular proteins, and a rapid and complete breakdown of polyribosomes. A comparative study examining the effects of L-1-tosylamido-2-phenylethyl chloromethyl ketone and several known inhibitors of in vivo protein synthesis, with known mechanisms of action, revealed that an optimal concentration of L-1-tosylamido-2-phenylethyl chloromethyl ketone: (a) immediately and selectively inhibits initiation of protein synthesis, (b) does not significantly affect normal elongation rates, and (c) does not promote a premature release of nascent peptides. L-1-Tosylamido-2-phenylethyl chloromethyl ketone may prove to be a useful tool in investigating the initiation of protein synthesis in eukaryotic cells.

Tissue culture cells contain proteolytic enzymes which play a key role in the maturation of several DNA and RNA viruses by specific cleavage of viral precursor proteins (1, 2). Extracts from uninfected cells will convert the isolated picornavirus “polyproteins” (2) into their primary cleavage products (3). This cleavage process can be inhibited by a number of agents in vivo and in vitro but most effectively by L-1-tosylamido-2-phenylethyl chloromethyl ketone, a chymotrypsin-specific protease inhibitor (4). Therefore, TPCK has recently been used in studies of possible post-translational cleavage processing in several in vivo systems (3, 5–7). TPCK also has been used to inhibit proteolytic activities associated with virus-transformed cells and phytohemagglutinin-stimulated and unstimulated lymphocytes (8–10). It had previously been reported that treatment of intact cells with TPCK was accompanied by a reduction in the incorporation of labeled amino acids into proteins (6, 7). In a study designed to investigate the possible role of post-translational cleavage in uninfected HeLa cells, we observed that TPCK is a potent and specific inhibitor of initiation of protein synthesis. Further investigation revealed that protein synthesis in other tissue culture cells, including virus-transformed 3T3 mouse fibroblasts and mouse plasmacytoma cells was also inhibited by TPCK treatment. This property of TPCK severely restricts the use of this drug in the investigation of the post-translational processing of newly synthesized proteins and in studies examining the role of proteolytic activities in the expression of the transformed state (8, 9). However, the results reported here suggest that TPCK may be useful in studying the process of peptide chain initiation in eukaryotic cells.

MATERIALS AND METHODS

Chemicals—Spectral quality sucrose was purchased from Mann Biochemical Co. ¹⁸⁵S]Methionine with a specific activity of 100 Ci per mmol was obtained from Amersham-Searle. Dimethylsulfoxide was a product of Matheson Coleman and Bell. TPCK, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid and tris(hydroxymethyl)aminomethane were obtained from Calbiochem. Cycloheximide was supplied by Sigma Chemical Co. Spectrofluorinor was obtained from Yorktown Research Co. and Aquasol from New England Nuclear. Joklik-modified MEM (F-13) and Dulbecco’s modified MEM (H-16) were supplied by Gibco. Nonidet P-40 was provided by Shell Chemical Co.

Growth of Cells—HeLa S1 cells were grown at 37°C in suspension in Joklik-modified MEM supplemented with 5% fetal calf serum. Cultures were maintained at cell densities of 2 to 4 x 10⁵ cells per ml.

Plasmacytoma cells MPC-11, kindly furnished by Dr. Matthew Scharff, Albert Einstein College of Medicine, New York, were grown in suspension in Dulbecco’s modified MEM supplemented with 20% horse serum. Myeloma cell cultures were maintained at a cell density of 5 to 9 x 10⁵ cells per ml.

Kirsten virus-transformed 3T3 mouse fibroblast cells, a gift from Dr. T. T. August, Albert Einstein College of Medicine, New York, were grown in Dulbecco’s modified MEM supplemented with 10% fetal calf serum.

Amino Acid Incorporation Studies—Cell cultures were harvested by centrifugation for 5 min at 300 x g and resuspended at a density of 4 x 10⁶ cells per ml (HeLa) or 2.5 x 10⁶ cells per ml (myeloma) in serum-supplemented or serum-free MEM plus 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.2.
Inhibition of Amino Acid Incorporation by TPCK—The effect of several concentrations of TPCK on the incorporation of $^{35}$S methionine into HeLa cell proteins is presented in Fig. 1. HeLa cells were incubated in serum-free medium containing $^{35}$S methionine for 7.5 min prior to the addition of TPCK to the final concentrations of 5, 10, 20, and 30 µg per ml (1.4 × 10^{-2} to 8.5 × 10^{-1} M). Since the TPCK added was dissolved in dimethylsulfoxide at a concentration of 10 mg per ml, 0.3% dimethylsulfoxide was added to the control cells in this and subsequent experiments. It was shown previously that dimethylsulfoxide, in concentrations up to 4%, does not affect protein synthesis in HeLa cells (14). Only a 5 to 10% inhibition of methionine incorporation is observed at a concentration of 5 µg of TPCK per ml. At a concentration of 10 µg of TPCK per ml, a progressive inhibition of amino acid incorporation is observed: 75% inhibition from 10 to 20 min and 100% inhibition from 20 to 30 min. Inhibition is 75% within 5 min and complete from 10 to 20 min after the addition of TPCK to 20 µg per ml. A further increase in the concentration of TPCK (30 µg per ml) produces a complete inhibition of methionine incorporation within 3 to 7 min.

Since TPCK has been used as a protease inhibitor in several in vivo studies utilizing serum-supplemented medium, we performed an experiment to examine the effect of serum on the inhibition of amino acid incorporation by TPCK. Fig. 2 shows the effect of TPCK on amino acid incorporation in the mouse plasmacytoma cell (MPC-11) in the absence (A) and presence
addition of TPCK to tissue culture cells has an immediate effect on protein synthesis.  

The disappearance of polyribosomes after TPCK addition could, however, be attributed to factors other than the inhibition of protein synthesis, such as increased levels of RNase or residual effects of TPCK after cell lysis. To exclude these possibilities, the effect of TPCK on polyribosome distribution was investigated and compared with the effect of hypertonicity resulting from an elevated NaCl concentration. Cycloheximide inhibits the translocation of ribosomes on mRNA, resulting in stabilization of polyribosomes (15). Proper elevation of the NaCl concentration selectively inhibits the initiation of polypeptide synthesis, but has no effect on elongation and termination processes (16). HeLa cell culture suspensions at a density of 4 x 10^6 cells per ml were treated with 0.3% dimethylsulfoxide (control), 30 μg of TPCK per ml, 100 μM additional NaCl, 100 μg of cycloheximide per ml, or a combination of cycloheximide and TPCK. Fig. 5a shows the characteristic disappearance of polyribosomes following treatment with 30 μg of TPCK per ml. Fig. 5b shows that the addition of NaCl to a final concentration of 210 mM also results in a complete conversion of polyribosomes to 80 S ribosomes. The similarity in the kinetics of polyribosome disappearance following treatment of cells with either TPCK or hypertonic medium (data not shown) suggested to us that these inhibitors of protein synthesis may have similar mechanisms of action. Also shown in Fig. 5b is
the stabilization of polyribosomes resulting from treatment of cells with 100 μg of cycloheximide per ml. Fig. 5c reveals that preincubation of cells with cycloheximide, or the addition of cycloheximide at the same time as TPCK, prevents TPCK-induced polyribosome breakdown. These results seem to exclude the possibilities mentioned above that the observed effect of TPCK on polyribosomes is not directly related to the inhibition of protein synthesis.

Although the results of the experiments just described allow us to conclude that TPCK-induced polyribosome breakdown is a direct result of inhibition of protein synthesis, they do not permit us to make any definite statements with respect to the mechanism of inhibition resulting from TPCK treatment. On the basis of the kinetics of inhibition of amino acid incorporation following TPCK addition and the similarity between TPCK and NaCl-induced polyribosome breakdown, it was thought that TPCK inhibited protein synthesis, most probably at the level of initiation. However, the possibility that TPCK might act by promoting premature release of nascent peptides could not be excluded.

Elongation and Release of Nascent Polypeptides after TPCK Addition—To investigate the effect of TPCK on elongation and release of nascent polypeptides, pulse-chase experiments were performed to compare the effect of TPCK and other inhibitors of protein synthesis on peptide chain completion. HeLa cells were pulse-labeled for 45 s with [35S]methionine, poured onto crushed, frozen MEM with an excess of unlabeled methionine, centrifuged, and washed twice with the same medium. Following the final resuspension, the cell suspension was divided into five equal portions. One portion was lysed immediately, and the other portions were chased for 12 min in the presence of 0.3% dimethylsulfoxide (control cells), 30 μg of TPCK per ml, 12% dimethylsulfoxide, or 210 mM NaCl. Following the chase period, the cells were poured over 10 volumes of crushed, frozen MEM containing a 1 × 10^6 excess of unlabeled methionine, centrifuged, and washed twice with the same medium. Following the final resuspension, the cell suspension was divided into five equal portions. One portion was lysed immediately, and the other portions were chased at 37° for 12 min in the presence of 30 μg of TPCK per ml. Control cells were chased in the presence of 0.3% dimethylsulfoxide; ○ — ○ , control cells lysed immediately; ● — ● , cells chased in hypotonic medium; ○ — ○ , cells chased in the presence of 12% dimethylsulfoxide; - - - , cells lysed immediately.

Elongation and Release of Nascent Polypeptides under Hypertonic Conditions—To investigate the effect of hypertonicity on polyribosome distribution, cytoplasmic extracts from HeLa cell cultures suspended at a density of 4 × 10^6 cells per ml, treated as described below, were prepared and fractionated as described in Fig. 4. a — — , control cells incubated for 15 min with 0.3% dimethylsulfoxide; - - - , cells incubated for 15 min with 100 μg of cycloheximide per ml; - - - , cells incubated for 15 min in the presence of 210 mM NaCl. c — — , cells incubated for 15 min with 100 μg of cycloheximide per ml and 30 μg of TPCK per ml; - - - , cells incubated for 15 min with 30 μg of TPCK per ml. Note the 6-fold shift in the ordinate scale at the beginning of the polyribosome region in a, b, and c.

Fig. 5. Comparative effect of TPCK, cycloheximide, and hypertonicity on polyribosome distribution. Cytoplasmic extracts from HeLa cell cultures suspended at a density of 4 × 10^6 cells per ml, treated as described below, were prepared and fractionated as described in Fig. 4. a — — , control cells incubated for 15 min with 0.3% dimethylsulfoxide; - - - , cells incubated for 15 min with 30 μg of TPCK per ml. b — — , cells incubated for 15 min with 100 μg of cycloheximide per ml; - - - , cells incubated for 15 min in the presence of 210 mM NaCl. c — — , cells incubated for 15 min with 100 μg of cycloheximide per ml and then for 15 min with 30 μg of TPCK per ml. Fig. 6. Elongation of nascent polypeptides in the presence of TPCK, NaCl, and dimethylsulfoxide. HeLa cells suspended at a density of 4 × 10^6 cells per ml in MEM lacking methionine were pulse-labeled for 45 s with [35S]methionine (7.5 μCi per ml). The cells were then poured over 10 volumes of crushed, frozen MEM containing a 1 × 10^6 excess of unlabeled methionine, centrifuged, and washed twice with the same medium. Following the final resuspension, the cell suspension was divided into five equal portions. One portion was lysed immediately, and the other portions were chased at 37° for 12 min in the presence of 0.3% dimethylsulfoxide (control cells), 30 μg of TPCK per ml, 12% dimethylsulfoxide, or 210 mM NaCl. Following the chase period, the cells were poured over crushed, frozen NaCl solution, centrifuged, washed twice with the cold NaCl solution, and lysed. Analysis of the labeled polypeptides by sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out as described under "Materials and Methods." The direction of electrophoresis is from left to right. A : O - - - O , cells lysed immediately; ● - - - ● , control cells chased in the presence of 0.3% dimethylsulfoxide; Δ - - Δ , cells chased in the presence of 30 μg of TPCK per ml. B : ● - - - ● , cells chased in hypotonic medium; ○ - - - ○ , cells chased in the presence of 12% dimethylsulfoxide; - - - , cells lysed immediately.

Without a 12-min chase in the presence or absence of 30 μg of TPCK per ml, comparison of the size distribution of labeled polypeptides from cells lysed immediately after pulse labeling and cells chased for 12 min clearly shows the shift of 35S counts into higher molecular weight peptides. Comparison of the polypeptide size distribution from cells chased in the presence or absence of 30 μg of TPCK per ml reveals no differences. Thus, elongation and termination of nascent polypeptides appear to proceed normally in the presence of inhibiting concentrations of TPCK, and there is no evidence of premature release of nascent polypeptides. For comparison, Fig. 6B shows the size distribution of labeled polypeptides from cells chased for 12 min at an elevated NaCl concentration and cells chased in the presence of 12% dimethylsulfoxide. Normal completion of nascent pulse-labeled polypeptides occurs in cells chased in hypertonic medium. However, on the basis of the differences in size distribution of labeled polypeptides from control and dimethylsulfoxide-treated cells, it is evident that premature release of nas-
control cells pulse-labeled for 2 min; Sample b (×--×) cells treated with TPCK (30 μg per ml) for 1 min and pulse-labeled for 2 min; Sample c (×), cells treated with TPCK (30 μg per ml) for 4 min and pulse-labeled for 2 min. Following pulse labeling, all samples were poured onto 10 volumes of crushed, frozen MEM containing a 1 X 10^{4}-fold excess of unlabeled methionine and centrifuged. After one wash with the same medium, the cells were resuspended and incubated at 37° for 11 min for Sample a, 9 min for Sample b, and 6 min for Sample c. Cytoplasmic extracts were prepared and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described under “Materials and Methods.” Noncapsid and capsid polypeptides derived from poliovirus-infected HeLa cells were used as molecular weight markers: NCVPI (125,000); NCVP2 (90,000); VP0 (40,000); VP1 (35,000); VP3 (23,000).

Fig. 7. Sequential inhibition of HeLa cell peptides with time after TPCK addition. HeLa cell suspensions at a density of 4 X 10^9 cells per ml in MEM lacking methionine were pulse labeled with [35S]methionine (5 μCi per ml) as follows: Sample a (○--○), control cells pulse-labeled for 2 min; Sample b (●--●), cells treated with TPCK (30 μg per ml) for 1 min and pulse-labeled for 2 min; Sample c (×), cells treated with TPCK (30 μg per ml) for 4 min and pulse-labeled for 2 min. Following pulse labeling, all samples were poured onto 10 volumes of crushed, frozen MEM containing a 1 X 10^{4}-fold excess of unlabeled methionine and centrifuged. After one wash with the same medium, the cells were resuspended and incubated at 37° for 11 min for Sample a, 9 min for Sample b, and 6 min for Sample c. Cytoplasmic extracts were prepared and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described under “Materials and Methods.” Noncapsid and capsid polypeptides derived from poliovirus-infected HeLa cells were used as molecular weight markers: NCVPI (125,000); NCVP2 (90,000); VP0 (40,000); VP1 (35,000); VP3 (23,000).

Fig. 8. Correlation between the molecular weight and percentage of incorporation of [35S]methionine into HeLa peptides with time after TPCK addition. The data plotted in this figure were obtained from the experiments described in Fig. 7, by the following operations. The counts per min value in each fraction of the polyacrylamide gel profile of cytoplasmic extracts from cells pulse-labeled for 2 min, 1 min after TPCK addition, was divided by the counts per min value in the corresponding fraction of the gel profile of cytoplasmic extracts from control cells. This value, multiplied by 100, yielded the percentage of incorporation in that fraction. The polypeptide molecular weight corresponding to each gel fraction was calculated by using the poliovirus peptides as reference. The percentage of incorporation for each gel fraction was then plotted against the reciprocal of the peptide molecular weight corresponding to that gel fraction.

DISCUSSION

The results described in this paper show that the addition of 20 to 30 μg of the chymotrypsin-specific protease inhibitor per ml (TPCK) to cultures of HeLa, virus-transformed C3A, or myeloma MPC-11 cells, in the presence or absence of serum, results in a rapid and irreversible inhibition of protein synthesis.

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At optimal concentrations of TPCK, inhibition of amino acid incorporation (Fig. 1) and polyribosome breakdown (Fig. 4) is complete within 5 to 7 min after TPCK addition. Prevention of TPCK-induced polyribosome breakdown by the addition of cycloheximide (Fig. 5) clearly rules out the possibility that the disappearance of polyribosomes after TPCK treatment is due to increased RNase levels or physical effects following cell lysis. Comparative pulse-chase experiments (Fig. 6) indicate that premature release of nascent peptides does not occur in TPCK-treated cells. Finally, size distribution analysis of peptides pulse-labeled at different times after TPCK addition, and properly chased, clearly shows that peptide chain initiation is inhibited immediately, but that incorporation of amino acids continues into preinitiated peptides at the normal rate until they are completed (Figs. 7 and 8).

TPCK has been used as an inhibitor of proteolytic activities associated with virus-transformed mammalian cells (6, 9) and with mitogen-stimulated lymphocytes (10). Although TPCK is specific in its ability to inhibit proteolytic activities, it is a strong alkylating agent especially reactive with certain sulfhydryl groups (18). It is, therefore, likely that this reagent would have other effects on cell activities. It has recently been reported that TPCK inhibits RNA synthesis (19) and that tosyl-lysyl chloromethyl ketone, a trypsin-specific protease inhibitor, inhibits DNA synthesis in eukaryotic cells (20). Since TPCK inhibits protein synthesis in a matter of minutes and RNA synthesis only after 30 min, under the conditions described in this report, we suggest that the effects of this protease inhibitor on the other cellular processes may well be a consequence of the inhibition of protein synthesis. Tosyl-lysyl chloromethyl ketone also affects protein synthesis in HeLa cells under the conditions in this report, but 5 to 10 times higher concentrations are required for a comparable degree of inhibition of amino acid incorporation (data not shown).

A reduction in amino acid incorporation and a preferential inhibition in the labeling of small molecular weight peptides following TPCK treatment of virus-infected and uninfected cells has previously been reported (5–7). Since TPCK was reported to have no effect on in vitro protein synthesis in mammalian cell-free extracts (6, 21), it was suggested that the observed relative increase in labeling of large molecular weight peptides, after the addition of TPCK, was a result of inhibition of post-translational processing and not due to inhibition of peptide chain initiation (6). Our data show that TPCK inhibits peptide chain initiation in vivo and would, therefore, preferentially inhibit the synthesis of small molecular weight peptides very early after addition to the culture medium. Thus, the apparent preferential labeling of large molecular weight peptides in cells treated with TPCK cannot be taken as unequivocal evidence for the existence of post-translational cleavage mechanism.

TPCK has been reported by several investigators to inhibit irreversibly peptide chain elongation in bacterial extracts (22, 23). However, several observations show that peptide chain initiation and not elongation is affected by TPCK in tissue culture cells. First, inhibition of amino acid incorporation and polyribosome disappearance is completed within 5 to 7 min after addition of an optimal concentration of TPCK. Second, chasing of pulse-labeled peptides proceeds normally in the presence of TPCK, with no evidence of premature release of nascent peptides. Principally, very good agreement was obtained between experimental and predicted values for sequential inhibition of peptide labeling with respect to their molecular weights at times after TPCK addition. The combined data compel us to conclude that TPCK can be used to block the initiation of protein synthesis selectively in a number of tissue culture cells.

Since peptide chain initiation is a multistep process involving many specific cellular components, one might speculate that TPCK interacts with certain factors required for in vitro peptide chain initiation. Alternatively, since TPCK effectively inhibits in vitro protein synthesis, but inhibits in vivo protein synthesis only to a limited extent, it is suggested that the inhibition of protein synthesis elicited by this reagent is membrane-mediated. This possibility is currently being investigated. In any event, TPCK, like other inhibitors of initiation of protein synthesis (14, 16, 24), may prove to be very useful in studying the mechanism of peptide chain initiation in vivo.

REFERENCES


Inhibition of initiation of protein synthesis in mammalian tissue culture cells by L-1-tosylamido-2-phenylethyl chloromethyl ketone.
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