Cellular levels of thymosin immunoreactive peptides are linked to proliferative events: Evidence for a nuclear site of action

(Thymosin α/Prothymosin α/Immunoelectron microscopy/Nuclear peptide)

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ABSTRACT Thymosin α1 (Tα1), the N-terminal 28-amino acid fragment of prothymosin α (ProTα), and ProTα, although originally isolated from whole thymus extracts, are also present in nonthymic cells and tissues. We used an ELISA with an antibody raised against Tα1 to investigate the relationship between intracellular levels of thymosin immunoreactive peptide(s) (TIP) and cell proliferation in a rat small intestinal IEC-6 cell line. Increasing TIP levels were observed during cell proliferation, which decreased when proliferation was halted by cellular contact inhibition. Serum feeding of cells previously rendered quiescent by serum starvation resulted in a significant increase in TIP within 1 hr. Conversely, serum starvation decreased TIP levels within 1 hr. Peak TIP levels appeared after 3 hr of serum incubation, while maximum [3H]thymidine incorporation was noted after 9 hr, suggesting maximum TIP concentrations in the G1 phase of the proliferative cycle. Immunoelectron microscopy demonstrated an association of TIP with condensed nuclear chromatin. These results support a relation of intracellular TIP levels to IEC-6 cell proliferation and also a nuclear site of action. HPLC analysis of cellular homogenates from proliferating IEC-6 cells revealed a peak of immunoreactivity that elutes in the position of Tα1.

Prothymosin α (ProTα) and thymosin α1 (Tα1) are polypeptides originally isolated from thymus or extracts of thymic tissue and having molecular weights of 12,600 and 3108, respectively. The sequence of Tα1 (1) (28 amino acids) is identical to the N terminus of ProTα, from which it is considered to be derived (2). Thymosin immunoreactive peptide(s) (TIP) has been localized by immunohistochemical staining techniques in the cytoplasm of medullary thymic epithelial cells and in cultured thymus epithelial cells (3). TIP has also been reported to be secreted by thymic epithelial cells in culture, consistent with a potential role in thymic differentiation (4). Most recently, secretion and synthesis of ProTα has been reported in different subpopulations of calf and rat thymocytes (5).

The presence of TIP in other nonthymic tissues (6), however, and a structural similarity between ProTα and nuclear proteins (7) have raised the possibility that these polypeptides may also participate in intracellular events. A possible relation of ProTα to cell proliferation was suggested by Eschenfeldt and Berger (8), who observed a significant increase in ProTα mRNA in an NIH 3T3 cell line within the first 12 hr after initiation of proliferation by serum refeeding and a decrease when proliferative quiescence was induced by serum starvation. In addition, covalent binding of ProTα to a cytoplasmic mRNA was described in mouse ascites carcinoma cells (9) and higher levels of human ProTα mRNA were found in proliferating immature T lymphocytes from patients with acute lymphocytic leukemia than in lymphocytes from healthy controls (10). Oates et al. (11) have described a Tα1-induced growth factor-like effect on cell proliferation in the breast carcinoma cell line MCF-7, suggesting a potential autocrine growth factor activity.

With a polyclonal antiserum for Tα1 that cross-reacts with ProTα, TIP was detected in the mucosa of the rat small intestine and in a rat small intestinal crypt cell line (IEC-6) (12). A demonstrable increase in TIP was seen in these cells during the proliferative phase as compared to cells in quiescence.

Utilizing an ELISA with an antibody raised against Tα1, we report here the relationship of TIP to cell proliferation in the IEC-6 cell line. This line retains a structural similarity to the differentiated cells of the small intestinal crypt (13). IEC-6 cells proliferate in response to several growth factors and hormones considered trophic for the growth of the small intestinal mucosa (14, 15). The proliferation of these cells is inhibited by cellular contact and by fractions isolated from small intestinal villus cell homogenates (15). We find that TIP is present in IEC-6 cells with negligible levels of cross-reacting materials present in the medium. Total intracellular concentrations of TIP are increased when IEC-6 cell proliferation is promoted by serum feeding. Furthermore, immunoelectron microscopy revealed a tendency for localization of TIP in the nuclei of the proliferating cells.

MATERIALS AND METHODS

IEC-6 Cell Line. IEC-6 cells (a generous gift from John Adams, Orthopedic Hospital, University of Southern California, Los Angeles) from passage 11 were grown in 75-cm² culture flasks (GIBCO) in the presence of Dulbecco’s modified Eagles’ medium (DMEM) with 5.0% fetal calf serum (PH 7.3) (FCS; GIBCO). This is a continuous cultured cell line originally isolated from the small intestine of germ-free neonatal Sprague-Dawley rats by Quaroni et al. (13), who have characterized this cell line in detail.

Experimental Procedures. IEC-6 cells (1.5 × 10⁶ cells) harvested from actively proliferating cultures grown in 0.0% FCS were seeded into 75-cm² flasks (Costar) and grown under the conditions outlined. Harvesting of the cells was performed by exposure to a trypsin/EDTA solution (0.5% trypsin/5.3 mM EDTA; GIBCO) for 11 min. Culture media obtained immediately prior to harvesting were saved for TIP measurements. The harvested cells were washed with cold

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3269
phosphate-buffered saline (PBS) after centrifugation for 6 min at 1200 x g at 4°C and counted with a hemocytometer. After a final washing in PBS, the cells were resuspended in 1.0 ml of PBS in Eppendorf tubes. These tubes were then frozen in liquid nitrogen and thawed in water at room temperature three times. The suspension was checked microscopically for completeness of lysis and centrifuged in a Beckman Microfuge for 30 min at 15,000 x g. The supernatants were aspirated from the pelleted debris and maintained at -60°C for later analysis. TIP concentrations were measured by ELISA (16) and expressed as Tα1 picoequivalents (peq) per 10⁶ IEC-6 cells. The polyclonal antibody to Tα1 used in the ELISA shows no significant cross-reactivity with common serum proteins and other thymus-derived peptides such as thymic humoral factor, facteur thymini serique, or thymopoietin (16, 17). ProTα at concentrations up to 500 ng/ml does not interfere with Tα1 measurements in the ELISA (F.E.W. and M.G.M., unpublished data).

RIA for Tα1 and ProTα in HPLC Fractions. Tα1 and ProTα (18) concentrations in the HPLC fractions collected were measured with a modification (19) of the original Tα1 RIA (17). The HPLC fractions from the IEC-6 cell extracts were assayed in duplicate.

Statistics. Data are presented as means ± SD and comparisons were made by Student’s t test.

RESULTS

Correlation of TIP with Cell Proliferation. Proliferation of IEC-6 cells promoted by DMEM/5% FCS was associated with a concomitant increase in total TIP (Fig. 1 A and B), which increased with time until the entire culture reached confluence on day 4. Cellular TIP (per 10⁶ cells) decreased continuously with time as the ratio of proliferating to nonproliferating cells decreased (20) and until the entire culture achieved confluence on day 4 (Fig. 1C). When stimulated by refeeding (i.e., changing to fresh DMEM/5% FCS on day 6), cell numbers and total as well as cellular TIP increased significantly (P < 0.001; Fig. 1). After a single and final cell doubling from day 6 to day 8 (24 ± 2 to 53 ± 3 x 10⁶ cells), no additional proliferation was noted and no further increase in TIP was observed (Fig. 1). At no time did we observe significant quantities of TIP (>0.17 Tα1 peq/ml) in the incubation medium, although total TIP increased >70-fold (Fig. 1A). DMEM/5% FCS without cells contained 0.16 Tα1 peq/ml.

Relationship of TIP to the Proliferative Cycle. There was a correlation between TIP levels and the proliferative cycle. Refeeding rendered quiescent by 24-hr incubation in DMEM/0.5% FCS produced a doubling in cell number that terminated with cellular confluence (Fig. 2). A >3.3-fold increase in cellular TIP occurred within 1 hr of changing the medium to DMEM/5% FCS (0.04 ± 0.01 to 0.14 ± 0.01 Tα1 peq per 10⁶ cells; P < 0.001) with maximum TIP levels observed at 3 hr after refeeding (0.16 ± 0.02 Tα1 peq per 10⁶ cells; Fig. 2). A continuous decrease in TIP was observed from the 3rd hour to the 12th hour after refeeding (0.16 ± 0.02 to 0.07 ± 0.01 Tα1 peq per 10⁶ cells; P < 0.001). From this minimum, TIP increased to 0.14 ± 0.02 Tα1 peq per 10⁶ cells at 24 hr after refeeding. This time point (24 hr) is ~3 hr into the following proliferative cycle as determined by the original investigation of this cell line by Quaroni et al. (13).

To correlate changes in TIP levels with the phases of the cellular proliferative cycle a series of experiments was performed parallel to those illustrated in Fig. 2. The time at which maximum [¹³H]thymidine incorporation was observed

![Graph](image-url)

**Fig. 1.** Correlation of TIP levels and cell proliferation in a 9-day culture system. IEC-6 cells (1.5 x 10⁶ cells) were seeded into parallel flasks and grown in DMEM/5% FCS. After visual cell confluence (day 4) and an absence of significant cell proliferation over the ensuing 48 hr, the medium was replaced on day 6 with fresh DMEM/5% FCS (refeeding) and the cultures were maintained until no evidence of further cell proliferation was seen (day 9). Cells were harvested at 24-hr intervals over the 9-day culture and the supernatants of cell lysates were measured for TIP. (A) Increasing total TIP is shown with time. (B) Growth curve of the IEC-6 cells. Note the quiescence between days 4 and 6 with subsequent resumption of proliferation with addition of fresh medium on day 6. (C) Change in cellular TIP over the 9-day incubation period. The decrease in cellular TIP seen during the first 4 days reflects the decreasing ratio of absolute number of proliferating cells to the absolute number of confluent, nonproliferating cells (20). Thus, while there are more cells present by day 4, proportionately fewer are replicating. TIP values are expressed as mean peq of Tα1 ± SD.

![Graph](image-url)

**Fig. 2.** Relationship of cellular TIP concentration to cell proliferation. IEC-6 cells were seeded into parallel flasks and incubated for 48 hr in DMEM/5% FCS. The medium was changed to DMEM/0.5% FCS and incubation continued for 24 hr, during which time cell quiescence was achieved. Medium was changed once again (at time 0) to DMEM/5% FCS (refeeding), which resulted in stimulation of cell proliferation. Flasks were harvested, cells were counted, and supernatants of cell lysates were measured for Tα1 peq at the time intervals indicated. All values are means ± SD.
FIG. 3. Correlation of cellular TIP levels with \[^{3}H\]thymidine incorporation by IEC-6 cells. Parallel flasks were seeded with IEC-6 cells and grown as described in Fig. 2. After refeeding and at 1 hr prior to the time intervals for harvesting indicated in Fig. 2, 50 μl of \[^{3}H\]thymidine (134 μCi/ml; 1 Ci = 37 GBq; New England Nuclear) per ml of culture medium was added to each flask. The harvested cells were disrupted with 2 ml of M NaOH, passed through sintered glass filters (Whatman), and washed with cold PBS. The filtrates were assayed for \[^{3}H\]thymidine activity by liquid scintillation spectrometry. A sample at each time interval was measured for protein concentration by the Bradford microassay (Bio-Rad). Results are expressed as μM \[^{3}H\]thymidine incorporation per mg of cell protein per hr (solid line). TIP was measured in supernatants of cell lysates and is expressed as T\(_{T1}\) peq (dashed line). All values are means ± SD.

was used to define the S phase of the proliferative cycle (Fig. 3). Cell counts \((\times 10^4)\) at selected time points \((t = 0\) hr, 10.6 ± 0.8\); \(t = 18\) hr, 19.6 ± 0.09; \(t = 24\) hr, 20.5 ± 0.7) were used to confirm the presence of a normal proliferative cycle. Maximum cellular TIP levels were seen 3 hr after the proliferative cycle was reestablished by refeeding. Concomitant measurement of \[^{3}H\]thymidine incorporation revealed that maximum incorporation increased from 1.0 ± 0.5 μM \[^{3}H\]thymidine per mg of cell protein per hr at time 0 to 6.5 ± 0.5 μM \[^{3}H\]thymidine per mg of cell protein per hr at 9 hr \((P < 0.001)\), thereafter declining to a nadir at 18 hr (Fig. 3). Thus, separate and discrete peaks were observed for cellular TIP levels (at 3 hr) and \[^{3}H\]thymidine incorporation (at 9 hr).

**Influence of the Removal of the Proliferative Stimulus on Cellular TIP Levels.** One hour after removal of the proliferative stimulus, cellular TIP levels fell to less than one-half with maximum decline at 6–9 hr (Fig. 4). Noted during this experiment was the continuation of cellular proliferation during the period of falling cellular TIP. None of the previously described peak TIP level prior to cell proliferation (Fig. 2) was seen during this terminal proliferative cycle.

**Immunoelectron Microscopic Localization of TIP in Proliferating Cells.** In IEC-6 cells grown in DMEM/5% FCS, much of the immunogold label was present in the nucleus, predominately over condensed heterochromatin (Fig. 5). Cytoplasmic labeling was present to a lesser degree and in a somewhat more random distribution. This is more clearly observed at higher magnification (Fig. 5 insert). Control cells treated with antisera to T\(_{T1}\) absorbed with immobilized synthetic T\(_{T1}\) revealed only background immunogold deposition in the nucleus and cytoplasm.

**Characterization of TIP in IEC-6 Cells.** HPLC analysis of extracts prepared from IEC-6 cells and immediately exposed to boiling yielded a major peak that eluted in the position of

**FIG. 4.** Influence of the removal of the proliferative stimulus (DMEM/5% FCS) on IEC-6 cell growth and cellular TIP concentrations. IEC-6 cells were seeded into parallel flasks and incubated in DMEM/5% FCS. Removal of the proliferative stimulus was achieved by changing the medium to DMEM/0.5% FCS at 48 hr. During the subsequent 24-hr period, cells were harvested and counted. TIP, expressed as T\(_{T1}\) peq, was measured in cell lysate supernatants at the time intervals indicated. All values are means ± SD.

T\(_{T1}\) (Fig. 6). Only a trace of immunoreactivity was found in the region corresponding to ProTa (12–13 min). The antibody used would have detected ProTa with reduced (50%) sensitivity corresponding to T\(_{T1}\) (P.H.N., M.R.E., and A.L.G., unpublished data).

**DISCUSSION**

This investigation provides evidence relating thymosin-like peptides to proliferation in rat small intestinal crypt cells. The correlation of cell proliferation in a nonlymphoid cell to increasing cellular content of TIP is in agreement with the earlier observation of an increase in ProTa mRNA content in proliferating NIH 3T3 fibroblasts as opposed to quiescent NIH 3T3 cells (8). A nuclear site of action for ProTa has been proposed based on structural similarities of this peptide to such nuclear-related agents as nucleoplasm, cyclin, nucleolin, N038, N1/N2, and several of the high mobility group proteins (7). Evidence for this hypothesis was provided by Watts et al. (22), with the demonstration that ProTa injected into the cytoplasm of oocytes migrated to the nucleus. Contes et al. (12) have also proposed a nuclear localization. TIP was identified by immunoelectron microscopy on the heterochromatin of the nuclei in crypt cells of the small intestine of the Sprague–Dawley rat and in the IEC-6 cell line with smaller quantities of TIP detected in the cytoplasm of these cells. The results of the present study provide important insights into the relationship of TIP to cell proliferation. Total TIP increased with active cell proliferation but decreased as the cells reached confluence. Initiation of proliferation by refeeding the cells also produced an increase in total TIP, which ceased as cell proliferation halted (Fig. 1A and B). There was a tendency for cellular TIP concentrations to reach similar levels after refeeding (Fig. 1C).

During the 9-day cell culture period there was no evidence of significant release of TIP into the incubation medium over each 24-hr period. Thus, the function of TIP would appear to be related to intracellular events. The apparent absence of TIP secretion by this cell line suggests that an endocrine-paracrine role for TIP in cell proliferation is unlikely in this nonlymphoid cell line. A similar conclusion was reached earlier by Goodall et al. (23) and by Eschenfeldt and Berger (8) based on the lack of coding for a signal peptide in the cloned ProTa cDNA. More recently, Eschenfeldt et al. (24) described the localization of ProTa mRNA on free polyosomes.
Fig. 5. Ultrastructural immunolocalization of TIP in IEC-6 cells. IEC-6 cells were incubated for 48 hr in DMEM/5% FCS. The medium was changed to fresh DMEM/5% FCS and, 24 hr later, the cells were harvested, washed twice in PBS, and fixed in periodate/lysine/parafomaldehyde fixative (21) for 2 hr at room temperature. Postfixation with osmium tetroxide was omitted, and the cells were dehydrated and then embedded by a low denaturation method in Lowicryl K4M polar embedding medium (Polysciences). Ultrathin sections were cut and collected on 300-mesh nickel grids, which were washed in two changes of PBS for 2 min by constant dipping and then transferred directly to a drop of normal goat serum (diluted 1:50 in 1% bovine serum albumin in PBS) for 5 min. The “blocked” grids were transferred, without washing, to drops of primary antibody (polyvalent rabbit anti-Tal diluted 1:500 in 1% bovine serum albumin in PBS) and incubated for 2 hr at room temperature in a moist chamber. After three washes with PBS, the sections were blocked again, as described above, and incubated with colloidal gold complexed to the second antibody (goat anti-rabbit IgG; particle diameter, 10 nm; Jansen Pharmaceutica, diluted 1:25 with PBS) for 1 hr. Grids were then washed twice in PBS and twice in deionized water. The reacted sections were contrasted with 5% aqueous uranyl acetate for 3 min and photographed in a Phillips EM 400 electron microscope. Cytochemical controls, which included (i) omission of anti-Tal antibody, (ii) diluted nonimmune rabbit serum in place of the primary antibody, or (iii) Tal antiserum absorbed with synthetic Tal (Alpha 1 Biomedicals, Foster City, CA) followed by gold-conjugated second antibody, produced essentially negative immunostaining. Gold particles are prominent over condensed chromatin in the nucleus (N). The cytoplasm (Cy) also contains gold particles, but to a lesser extent and more randomly distributed. (x23,400.) (Inset) Boxed area shown at higher magnification. (x53,700.)

Our present findings suggest that changes in TIP concentration precede cell proliferation and are not so much associated with cellular DNA synthesis (S phase) as with events in the preproliferation (G1) phase. When cells were allowed to complete a terminal proliferation after DMEM/5% FCS was replaced with DMEM/0.5% FCS, cell proliferation continued for one cycle in the presence of falling levels of cellular TIP (Fig. 4), indicating that there is not only a rapid clearance of TIP from the cell after removal of the proliferative stimulus but, in addition, a probable lack of a requirement for elevated levels of TIP for completion of the cycle of cell division. The role for TIP as suggested by this study implies an association with preproliferative rather than proliferative events.

Correlation of TIP and cellular proliferation (Figs. 1–4)
In any event, the results obtained in the HPLC studies confirm that a peptide related to ProTa was measured in the cell proliferation studies. The observations in this study support the hypothesis that ProTa and/or Tα₁ may play an important intracellular and intranuclear role linked to cell proliferation.

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