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Transcription of thrombomodulin mRNA in mouse hemangioma cells is increased by cycloheximide and thrombin

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ABSTRACT **We have measured mRNA levels for thrombomodulin, an endothelial membrane cofactor for the activation of protein C by thrombin, in a mouse hemangioma cell line. Cycloheximide, an inhibitor of protein synthesis, increased levels of thrombomodulin mRNA, as measured in an S1 nuclease protection assay, to 2.5–4.0 times control levels. Thrombomodulin transcription in response to cycloheximide treatment, as determined by nuclear run-on analysis, was 3.9 ± 1.3 (mean ± SD) times that found in untreated cells. Thrombin also increased thrombomodulin mRNA levels to 151 ± 21% (mean ± SD) of control levels after 2 hr. Transcription increased in response to thrombin by 2.1- to 7.3-fold. The combination of thrombin and cycloheximide had no additive effect on thrombomodulin mRNA levels. Thrombin treatment of hemangioma cells also caused an increase in thrombomodulin protein synthesis to 142 ± 17% (mean ± SD) of control levels as determined by immunoprecipitation of [^{32}S]methionine-labeled thrombomodulin. We conclude that thrombomodulin expression is determined in part by the rate of transcription and that thrombomodulin mRNA levels in hemangioma cells are increased by treatment with cycloheximide or thrombin. The increased transcription in response to cycloheximide suggests the existence of a labile protein repressor of thrombomodulin transcription.**

Thrombomodulin is an endothelial cell integral membrane protein that acts as a cofactor for the activation of protein C by thrombin. When thrombin binds to thrombomodulin, the activation of protein C by thrombin is accelerated several thousandfold (1, 2). Activated protein C is a protease that inactivates activated factors V and VIII, thereby inhibiting thrombin formation (3–5). The activity of thrombomodulin on the cell surface, and therefore the anticoagulant effect of endothelial cells, is subject to regulation. In the absence of protein C, thrombin induces internalization of surface thrombin–thrombomodulin complexes, which decreases thrombomodulin activity on endothelial cells (6). Protein C, but not activated protein C, will inhibit the endocytosis (7). Phorbol 12-myristate 13-acetate, a tumor-promoting phorbol ester that activates protein kinase C, decreases cell surface thrombomodulin antigen and thrombomodulin activity and is associated with phosphorylation and endocytosis of thrombomodulin, as demonstrated in a murine hemangioma cell line (8). Tumor necrosis factor also decreases thrombomodulin activity on endothelial cells (9) due to endocytosis of cell surface thrombomodulin (10). Endotoxin (11) and interleukin 1 (12) also decrease cell surface thrombomodulin activity, although the mechanism by which they act has not been defined. A rapid decrease in endothelial thrombomodulin by inhibition of protein synthesis or transcription is unlikely since both the protein and the mRNA have relatively long half-lives (protein, $t_{1/2}$ = 19.8 hr; mRNA, $t_{1/2}$ = 8.9 hr) (8).

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Although a decrease in thrombomodulin by a variety of different agents has been described, increased activity of thrombomodulin in response to experimental manipulations has not yet been demonstrated. Such an increased cell surface activity of thrombomodulin and resultant protein C activation might play a role in reducing thrombotic events, if achievable *in vivo*. Injection of activated protein C in a baboon model of thrombosis has produced an antithrombotic effect (13). Activated protein C will also protect baboons from septic death associated with infusion of live *Escherichia coli* (14). We have previously shown that injection of solubilized thrombomodulin in a mouse thrombosis model will partially protect mice from the lethal effects of injected thrombin (15). Although the long half-life of thrombomodulin mRNA and protein precludes a rapid decrease in thrombomodulin activity by transcription changes, up-regulation with subsequent increased anticoagulant activity could occur by augmenting translation or transcription. Regulation of proteins by transcriptional control is common (16, 17). Thrombin causes increased transcription of the platelet-derived growth factor B chain but not the A chain in endothelial cells (18, 19). Cycloheximide, an inhibitor of protein synthesis, "superinduces" mRNA levels of a variety of genes—for example, c-*fos* (20–22), c-*myc* (23), interferon (24), the platelet-derived growth factor-inducible JE gene (25), and actin (26). For c-*fos*, c-*myc*, interferon, and JE, this is, in part, due to mRNA stabilization. In this study we have investigated the effects of cycloheximide and thrombin on transcription and mRNA levels of thrombomodulin.

EXPERIMENTAL PROCEDURES

Materials. [γ-^{32}P]ATP (>5000 Ci/mmol; 1 Ci = 37 GBq) and [^{32}S]methionine were from Amersham. [α-^{32}P]GTP (3000 Ci/mmol) was from Amersham or ICN Radiochemicals. Restriction enzymes, calf intestine alkaline phosphatase, T4 polynucleotide kinase, and cycloheximide were from United States Biochemical. Dithiothreitol, phenol, S1 nuclease, and DNA molecular size markers were from Bethesda Research Laboratories. Individual nucleotides were from Pharmacia LKB. Bluescript plasmids were from Stratagene. Creatine phosphate and creatine phosphokinase were from Sigma. Other chemicals were from Sigma or Fluka. Antibodies to rodent thrombomodulin and human thrombin were isolated as previously described (15, 27). Modified M199 medium (MM199, M199 with basal medium Eagle's vitamins and amino acids and Earle's salts) and Dulbecco's phosphate-buffered saline were from the Washington University Medical School Center for Basic Cancer Research (Saint Louis, MO). Plasmids used contained cDNAs for murine thrombomodulin [pMTM.2B2 (8) and pMTM. E1 (28)], human β-actin (29) (provided by Timothy Ley, Washington University, Saint Louis, MO), and β_2-microglobulin (23, 30). Mouse

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hemangioma cells were kindly provided by Jack Hoak (University of Iowa, Iowa City).

RNA Isolation and S1 Nuclease Assay. RNA was isolated from hemangioma cells in culture by the method of Chomczynski and Sacchi (31). S1 nuclease assays of β_2-microglobulin and thrombomodulin were performed as previously described (8), except that the cycloheximide and half-life studies where both β_2-microglobulin and thrombomodulin probes were allowed to hybridize overnight at 52°C and the DNA·RNA hybrids were treated with 50 units of S1 nuclease for 30 min at 37°C. The thrombomodulin probe protected a 128-nucleotide fragment starting at nucleotide 446 of mouse thrombomodulin cDNA. After electrophoresis and autoradiography, bands were cut from the gel, and radioactivity was measured by scintillation counting or by densitometry of autoradiographs with an LKB Ultroscan XL.

Nuclear Run-On Analysis. Nuclear run-on analysis was performed as described by Ley *et al.* (32) with modifications. Confluent hemangioma cell monolayers in 175-cm^2 tissue culture flasks were washed with sterile Dulbecco's phosphate-buffered saline (all procedures were performed at 4°C); then 25 ml of a solution containing 138 mM sodium chloride, 5 mM potassium chloride, 5.6 mM dextrose, 21 mM Hepes at pH 7.1 was added to each flask; and the cells were scraped and collected by centrifugation at 2000 × *g* for 15 min. The supernatant fraction was removed, and the cells were suspended in 5 ml of hypotonic lysis buffer (10 mM Hepes, pH 8.0/1.5 mM magnesium chloride/10 mM potassium chloride) and placed on ice for 15 min. The cells were disrupted (but nuclei remained intact) by rapidly expelling them through a 21-gauge needle four times (Timothy Ley, personal communication). The disrupted cells (<5% excluded trypan blue) were counted in a hemocytometer; nuclei were pelleted by centrifugation at 1000 × *g* for 10 min and were resuspended at a concentration of 5 × 10^6 nuclei per ml in transcription buffer [20 mM Tris·HCl, pH 8.0/6 mM magnesium acetate/84 mM potassium chloride/10 mM ammonium chloride/0.3 mM EDTA/1 mM dithiothreitol/10% (vol/vol) glycerol]. Creatine phosphokinase was added to 10 μg/ml; creatine phosphate was added to 2.5 mg/ml; ATP, CTP, and UTP were added to 125 μM; and [α-^{32}P]GTP (3000 Ci/mmol) was added to 0.275 μM (833 μCi/ml). The samples were incubated for 30 min at 30°C and then centrifuged at 1500 × *g* at 4°C for 3 min, and the supernatant fractions were removed. The pellets were dissolved in guanidine RNA isolation buffer (31), and the RNA was isolated as described above. The radiolabeled RNA was used as a probe for 1.25 μg of linearized plasmids containing the cDNAs for human actin, murine β_2-microglobulin, or thrombomodulin or a plasmid control, immobilized onto nitrocellulose, and prehybridized in 50% (vol/vol) formamide/600 mM sodium chloride/60 mM sodium citrate, pH 7.0/bovine serum albumin (0.4 mg/ml)/0.004% polyvinylpyrrolidone/0.004% Ficoll, *E. coli* tRNA (20 μg/ml)/50 mM sodium phosphate/0.1% sodium dodecyl sulfate at 42°C. Hybridization with labeled RNA was for 16 hr at 42°C in the same buffer. The nitrocellulose was washed twice in 300 mM sodium chloride/30 mM sodium citrate, pH 7.0/0.1% sodium dodecyl sulfate at room temperature for 15 min, then twice in 30 mM sodium chloride/3 mM sodium citrate, pH 7.0/0.1% sodium dodecyl sulfate at room temperature for 15 min, and once in 30 mM sodium chloride/3 mM sodium citrate, pH 7.0/0.1% sodium dodecyl sulfate at 42°C for 15 min. The washed sheet was exposed to Kodak X-Omat film, and the radioactivity was measured by densitometry.

Cell Culture. Mouse hemangioma cells were cultured as previously described (8). Sixteen hours prior to experiments, the medium was changed to serum-free medium (MM199 with bovine serum albumin at 500 μg/ml, insulin at 1 μg/ml, transferrin at 5 μg/ml). Cycloheximide was dissolved at 10 μg/ml in serum-free medium. Human thrombin was diluted in serum-free medium to 3 NIH units/ml.

Metabolic Labeling and Immunoprecipitation. Hemangioma cells were grown to confluence in 35-mm diameter tissue culture dishes as previously described (8). After a 4-hr incubation in serum-free medium, the medium was changed to labeled serum-free medium ([^{35}S]methionine, 200 μCi per dish), with or without thrombin at 1 unit/ml, and the cells were incubated for an additional 4 hr. The medium was removed, the cells were washed and lysed, and radiolabeled thrombomodulin was immunoprecipitated as previously described (8). Immunoprecipated thrombomodulin was measured by fluorography and densitometry.

RESULTS

Hemangioma cells exposed to cycloheximide (10 μg/ml) contain increased levels of thrombomodulin mRNA (Fig. 1). After 3 hr of cycloheximide treatment, thrombomodulin mRNA levels were 2.5–4.0 times untreated levels when normalized for β_2-microglobulin mRNA levels (two separate experiments). This increase was not a result of increased mRNA stability; the long half-life of thrombomodulin mRNA (8.9 hr) (8) precludes this degree of augmentation due to increased thrombomodulin mRNA stability alone. In fact, the half-life of thrombomodulin mRNA after actinomycin D treatment of hemangioma cells was not affected by cycloheximide (data not shown). Since mRNA stability is not changed, increased levels of mRNA reflect increased rates of mRNA synthesis. We found that cycloheximide increased thrombomodulin mRNA transcription measured by nuclear run-on assay in all experiments performed. Exposure to cycloheximide at 10 μg/ml for 30 min increased thrombomodulin mRNA transcription 3.9 ± 1.3-fold (mean ± SD; $P < 0.005$; $n = 4$ experiments) over untreated cells when compared to the relatively stable transcription of β_2-microglobulin mRNA (19). An autoradiograph demonstrating increased transcription of mRNA for both thrombomodulin and actin is shown in Fig. 2. Cycloheximide has been shown to increase actin mRNA transcription (26, 33). Transcription of thrombomodulin increases rapidly after cycloheximide treatment, wih a return to baseline by 2 hr (Fig. 3). A similar

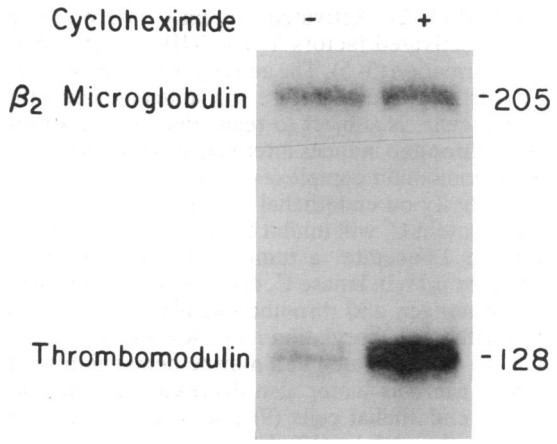

FIG. 1. S1 nuclease protection analysis of control and cycloheximide-treated hemangioma cells. Hemangioma cells were grown to confluence in 100-mm tissue culture dishes, and the medium was changed to serum-free MM199 for 16 hr. At time zero, the medium was again changed to serum-free MM199 with or without cycloheximide at 10 μg/ml and incubated for an additional 3 hr at 37°C. After incubation the cells were harvested, total RNA was extracted, and 10 μg was analyzed in an S1 nuclease protection assay. The probe for β_2-microglobulin protects a fragment of 205 nucleotides, and the thrombomodulin probe protects a 128-nucleotide fragment. The absence (−) or presence (+) of cycloheximide is indicated.

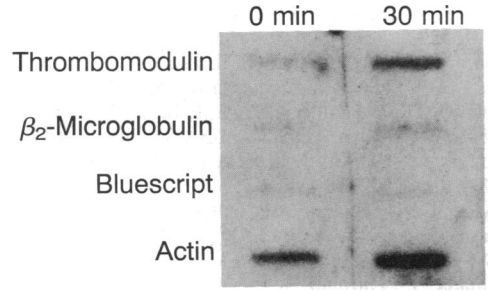

FIG. 2. Nuclear run-on analysis of control and cycloheximide-treated hemangioma cells. Hemangioma cells were grown to confluence in 175-cm^2 tissue culture flasks. The medium was changed to serum-free MM199 16 hr prior to the experiments and then was changed to fresh serum-free medium with or without cycloheximide at 10 μg/ml. After 1 hr, cells were collected, and nuclear RNA was labeled with [α-^{32}P]GTP and isolated. The radiolabeled RNA was used to probe linearized Bluescript plasmids containing, from top to bottom, cDNAs for thrombomodulin (full length, pMTM.E1), β_2-microglobulin, no insert, or β-actin, which had been immobilized onto nitrocellulose. Equal amounts of radioactive probe were added to each. On the left is DNA probed with RNA from cells before treatment with cycloheximide (0 min); on the right is DNA probed with RNA after 30 min of cycloheximide treatment.

pattern of response was seen in all experiments. The late decline may result from effects of cycloheximide on the machinery of transcription and is similar to the decline seen for transcription of other genes following cycloheximide treatment (26, 33).

We evaluated the effect of thrombin on thrombomodulin mRNA levels. Thrombomodulin mRNA levels, measured by the S1 nuclease assay in six experiments, were 121 ± 26% (mean ± SD; $P = 0.05$) of control levels by 1 hr and by 2 hr had risen to 151 ± 24% (mean ± SD; $P < 0.005$). A time course of thrombin-induced elevation thrombomodulin mRNA levels from one experiment is shown in Fig. 4. The half-life of thrombomodulin mRNA, determined by decay in RNA levels following actinomycin D treatment, was unchanged in the presence of thrombin (data not shown). The increased levels of mRNA cannot be explained by decreased degradation and therefore must be due to increased synthesis. Nuclear run-on assays of thrombomodulin mRNA showed enhanced transcription 1 hr after treatment of cells with thrombin (2.1- to 7.3-fold increase in two experiments when compared to β-actin mRNA transcription).

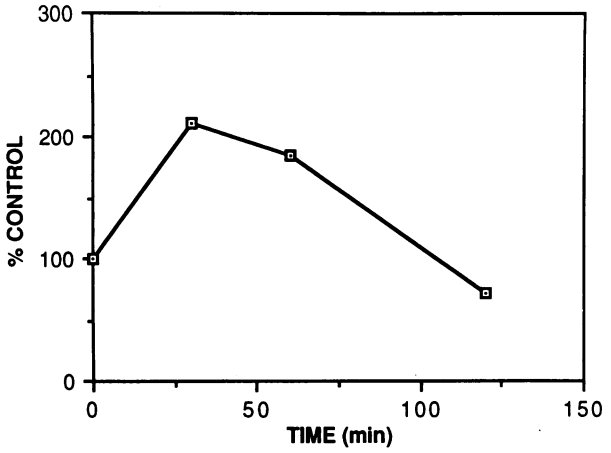

FIG. 3. Nuclear run-on analysis of thrombomodulin mRNA following cycloheximide treatment of hemangioma cells. Hemangioma cells were grown to confluence and treated as described in Fig. 2. At the indicated times after addition of cycloheximide at 10 μg/ml, cells were harvested, RNA was labeled with [α-^{32}P]GTP, and the RNA was used to probe immobilized plasmids. Levels of hybridization were determined by densitometry and were normalized for the level of hybridization to a plasmid without an insert. Values are presented as the percent of normalized hybridization at time zero.

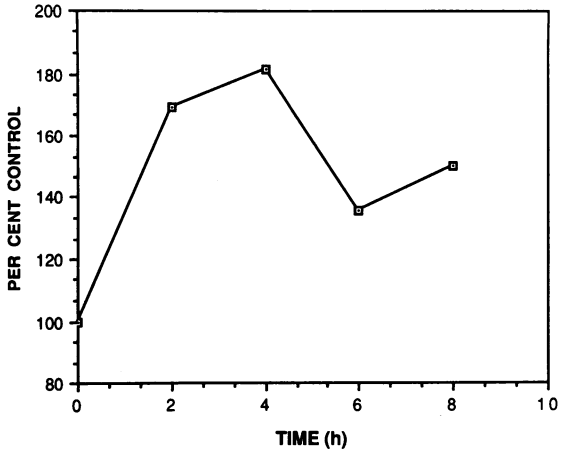

FIG. 4. Time course of thrombomodulin mRNA levels after thrombin treatment of hemangioma cells. Hemangioma cells were grown to confluence in 100-mm tissue culture dishes, the medium was changed to MM199 without serum for 16 hr, and then the medium was changed to fresh serum-free medium without (time zero) or with thrombin at 3 units/ml. At the indicated times, cells were harvested, and total RNA was isolated as described in Fig. 1. S1 nuclease protection assays were performed on 10 μg of total RNA and analyzed. Thrombomodulin levels were normalized for β_2-microglobulin levels and are presented as the percent of time zero controls.

The increase in thrombomodulin mRNA synthesis was accompanied by an increase in the synthesis of thrombomodulin protein determined by immunoprecipitation of metabolically labeled thrombomodulin. After 4 hr of exposure to thrombin, thrombomodulin synthesis was 142 ± 17% (mean ± SD; $P < 0.02$) of the control.

There was little difference in the normalized levels of thrombomodulin mRNA with thrombin or cycloheximide treatment alone or in combination (Fig. 5) (thrombin alone, 2.5-fold increase; cycloheximide alone, 2.7-fold increase; combination, 2.1-fold increase).

DISCUSSION

Cell surface expression of thrombomodulin can be reduced by endocytosis and subsequent degradation of thrombomod-

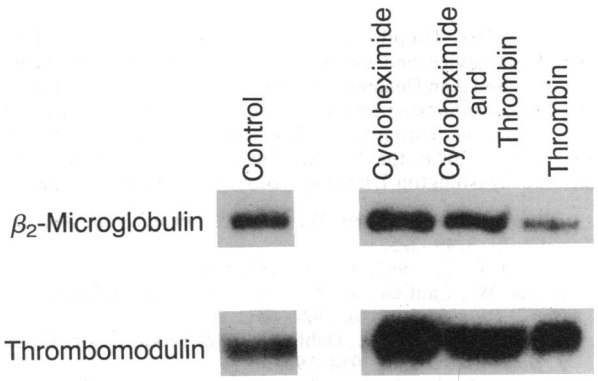

FIG. 5. S1 nuclease protection analysis of hemangioma cells treated with thrombin, cycloheximide, or both. Hemangioma cells were grown to confluence and treated as described in Fig. 1 with cycloheximide at 10 μg/ml for 3 hr, thrombin at 3 units/ml for 2 hr, or both. Cells were harvested, total RNA was extracted, and 10 μg of RNA was analyzed. The probe for thrombomodulin protects a 128-nucleotide fragment; the probe for β_2-microglobulin protects a 205-nucleotide fragment.

ulin (8). Increases in the cell surface expression of thrombomodulin could result from synthesis of thrombomodulin following increased transcription. An increase in thrombomodulin mRNA levels following 6 hr of cycloheximide exposure has been previously described (34). We have demonstrated a rapid increase in the level of thrombomodulin mRNA as early as 30 min after treatment with cycloheximide. This could not result from loss of a labile "RNase" and a subsequent prolongation of mRNA survival, as has been suggested for c-*myc*, (23) c-*fos*, (20–22), and interferon (24) mRNAs. Although thrombomodulin mRNAs have a 3′ untranslated A+T-rich motif common to those mRNAs, which is suggested to confer a short mRNA half-life (35–39), the half-life for thrombomodulin mRNA of 8.9 hr is too long to allow rapid increases in levels due to mRNA stabilization. We have shown that both cycloheximide and thrombin increase the transcription of thrombomodulin mRNA. This result suggests that a labile inhibitor of thrombomodulin transcription may control production of this molecule in endothelial cells. The other systems where cycloheximide causes increased transcription [for example, actin, c-*fos*, c-*myc* (33), and the epidermal growth factor receptor (40)] loss of a labile protein repressor of transcription has been postulated as the mechanism. The induction of transcription by either cycloheximide or thrombin does not fully account for the increased mRNA levels determined by S1 nuclease protection but provides a minimal *in vitro* estimation of transcriptional responses (41). Ligands for receptors have been demonstrated to increase the levels of their corresponding receptors in a number of systems. Interleukin 2 has been shown to increase transcription rates, protein synthesis, and surface expression of interleukin 2 receptors (42–44). Epidermal growth factor receptors are also increased in response to epidermal growth factor (40, 45–47), as are insulin (48) and prolactin receptors (49).

Increased thrombomodulin expression *in vivo* could produce a therapeutic antithrombotic effect. It is not clear, however, that transcriptional control by thrombin will allow for increased cellular thrombomodulin activity. Thrombin causes increased endocytosis of thrombomodulin, which, in the case of phorbol ester treatment, is associated with increased degradation of thrombomodulin. Thus, is it possible that transcription and subsequent translation induced by thrombin compensates for increased degradation induced by endocytosis and that no net increase in cellular thrombomodulin is achieved. An agent that is not an agonist for thrombomodulin itself but that increases transcription of thrombomodulin could selectively increase endothelial cofactor activity.

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